Akt up-regulation increases resistance to microtubule-directed chemotherapeutic agents through mammalian target of rapamycin

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Abstract

Chemotherapeutic agents induce apoptosis in cancer cells through effects on multiple intracellular targets. Recent observations suggest that a consistent cellular response to chemotherapeutic agents of disparate classes is down-regulation of glycolytic metabolism. Inhibition of glycolytic activity has been linked to apoptotic induction in several models. The serine/threonine kinase Akt (protein kinase B) promotes both glycolytic metabolism and survival, and these functions have been shown to be linked. Because of its key role in both glycolysis and survival, we examined the function of Akt in the cellular response to cytotoxic agents. Following exposure to any of several chemotherapeutic agents, an initial up-regulation in endogenous Akt activity is rapidly suppressed. Using cells containing constitutively active myristoylated Akt, dominant-negative kinase-dead Akt, or an empty vector control, we show here that Akt activation markedly increases resistance to microtubule-directed agents, including vincristine, colchicine, and paclitaxel. Akt also maintains increased glycolytic rate in response to antimicrotubule treatment. Rapamycin inhibits Akt-mediated maintenance of glycolysis and therapeutic resistance, indicating that these effects are dependent on mammalian target of rapamycin (mTOR). Furthermore, an activated mTOR mutant confers resistance to antimicrotubule agents. Taken together, these observations suggest that activation of the Akt-mTOR signaling pathway can augment glucose utilization and promote resistance to chemotherapeutic agents that do not directly target metabolic regulation. These data provide insight into potentially synergistic combinations of anticancer therapies. [Mol Cancer Ther 2004;3(12):1605–13]

Introduction

Chemotherapeutic agents kill cancer cells through interaction with several distinct intracellular targets, including factors affecting cell cycle progression and genomic integrity. A consistent and unifying feature of exposure to a broad spectrum of cytotoxic agents is apoptotic induction (1–3). For many commonly used cytotoxic agents, the molecular pathways from initial cellular damage to mitochondrial disruption and apoptotic commitment have not been fully defined. Better elucidation of these pathways will promote understanding of the mechanisms of action of these agents and will facilitate the rational incorporation of newer molecularly targeted therapeutics in the treatment of patients with cancer.

Several recent observations suggest that inhibition of glycolytic metabolism is a common trigger leading to loss of mitochondrial homeostasis and apoptosis. Growth factor withdrawal leads to marked down-regulation of glucose uptake and glycolytic activity, effects that precede apoptotic commitment (4). Enforced expression of glucose transporters and hexokinase can inhibit Bax activation and subsequent cell death following growth factor withdrawal (5). It has been hypothesized that maintenance of glycolytic metabolism inhibits apoptosis in part by maintaining the supply of bioenergetic substrates for oxidative metabolism by mitochondria, promoting their homeostasis (6).

Glycolytic metabolism is coordinately regulated in the cellular response to chemotherapeutic agents. We have shown previously that exposure to a variety of cytotoxics is associated with down-regulation of expression of glucose transporters and several key glycolytic enzymes and with a decrease in the rates of glycolytic metabolism and oxygen consumption (7). The changes in gene expression and glycolytic activity following cytotoxic exposure are similar to those noted following growth factor withdrawal. As with growth factor withdrawal, antiapoptotic Bcl-2 family members promote cell survival following cytotoxic drug exposure through mitochondrial stabilization but do not affect the preceding inhibition of glucose uptake and glycolytic activity.

In contrast, the serine/threonine kinase Akt is an antiapoptotic factor that does regulate glycolytic activity. Akt promotes cell survival through multiple mechanisms, both dependent and independent of its role in regulating glycolytic activity. Akt activation stimulates transcription of glucose transporter genes and translocation to the

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plasma membrane of glucose transporters and other nutrient transporters (4, 8). Akt also activates 6-phosphofructo-2-kinase, a positive regulator of glucose metabolism (9). Akt-mediated resistance to growth factor withdrawal is associated with maintenance of glucose transporter expression and glycolytic rate (4).

In addition to its role in metabolic regulation, Akt can inhibit apoptosis through regulation of Bcl-2 family member availability. Akt directly and indirectly phosphorylates Bad, promoting its sequestration in the cytoplasm by 14-3-3 proteins and preventing Bad-mediated apoptotic induction. In addition, Akt phosphorylates and inhibits members of the Forkhead family of transcription factors, which in turn regulate expression of other proapoptotic Bcl-2 family members such as Bim (10).

One of the critical downstream targets of Akt in metabolic signaling is the phosphoinositide 3-kinase–related factor mammalian target of rapamycin (mTOR). Akt phosphorylates tuberous sclerosis complex 2, leading to dissociation of tuberous sclerosis complexes 1 and 2. Together, tuberous sclerosis complexes 1 and 2 compose a GTPase complex for Rheb, which in turn activates mTOR (11). In addition, evidence suggests that Akt directly activates mTOR by phosphorylating an autoinhibitory region of mTOR (12). Thus, the overall effect of Akt activation is stimulation of mTOR through multiple mechanisms. mTOR integrates signals regarding nutrient and energy availability and mitogen activation and, in response, regulates growth and nutrient utilization. The kinase inhibitor rapamycin specifically inhibits mTOR. The ability of Akt to maintain nutrient uptake and to promote survival following growth factor withdrawal was sensitive to mTOR inhibition by rapamycin (8).

Constitutive activation of Akt-dependent signaling due to activating mutations of Akt or mutation of the upstream regulators PTEN or phosphoinositide 3-kinase has been a common feature of many solid tumors, including cancers of the lung, breast, liver, gastrointestinal tract, and ovary (13). Expression of a dominant-negative *Akt* allele suppressed tumor growth *in vivo* (14). In addition, constitutive activation of Akt has been associated with resistance to multiple chemotherapeutic agents (15–17). Deregulated activity of the phosphoinositide 3-kinase-Akt pathway may in part be responsible for the bioenergetic shift to dependence on glycolytic metabolism characteristic of most malignant cells, known as the Warburg effect (18). Several targeted therapeutics directed against Akt or mTOR are now in preclinical or early clinical development. Further characterization of the roles of Akt-dependent signaling in chemotherapeutic resistance may help to define the contexts in which these novel agents might have greatest clinical utility.

Materials and Methods

Cell Culture and Drug Exposure

FL5.12 is a nontransformed murine hematopoietic cell line that is dependent on the growth factor interleukin-3 for both survival and proliferation (19). Generation of stable FL5.12 derivatives transfected with pSSV-Bcl-x_L (FL5.Bcl-x_L) or with doxycycline-inducible pRevTRE-HA (empty vector control), pRevTRE-mAkt-HA [a myristoylated Akt (mAkt)], or pRevTRE-K179M Akt-HA [a dominant-negative kinase-dead Akt (KD-Akt) construct with a point mutation in the kinase domain] has been described previously (4, 20). The mutant mTOR (ΔTOR) FL5.12 cells have also been described previously (21). All FL5.12 derivative lines were cultured in 5% CO_2 at 37°C in RPMI 1640 containing 10% fetal bovine serum, 50 μmol/L β-mercaptoethanol, 2 mmol/L glutamine, 1 mg/mL geneticin, 100 IU/mL penicillin, 100 μg/mL streptomycin, 20 mmol/L HEPES, and 10% supernatant from WEHI-3B cells as a source of interleukin-3. Prior to treatment with chemotherapeutic agents, mAkt, KD-Akt, and empty vector control cells were treated with 1 μg/mL doxycycline for 20 hours to induce gene expression. Unless otherwise noted, cisplatin, etoposide, and vincristine treatments were given at 5, 10, or 0.1 μg/mL, respectively, based on previous cytotoxicity data for the parental line (22). Paclitaxel, colchicine, and topotecan treatments were given at 4 μg/mL, 0.2 μg/mL, and 1 μmol/L, respectively, to achieve similar cytotoxicity profiles with all chemotherapeutic agents. Unless otherwise noted, rapamycin (Calbiochem, La Jolla, CA) was used at 20 nmol/L. Statistical comparison was done with Student's *t* test. All *P*s are two tailed.

Western Blotting

Whole cell lysates were prepared in radioimmunoprecipitation assay buffer, and protein concentrations were determined by a protein assay (Bio-Rad Laboratories, Hercules, CA). Equal protein concentrations of each sample were run on 10% Tris-glycine gels and electrophoretically transferred to polyvinylidene difluoride membrane. Membranes were probed sequentially with antibodies against phosphorylated Akt (Ser^{473}), Akt (Cell Signaling Technology, Beverly, MA), and β-actin (AC-15, Sigma-Aldrich, St. Louis, MO) or against phosphorylated p70/S6K (Thr^{389}) and p70/S6K (Cell Signaling Technology). Blots were developed by using an enhanced chemiluminescence kit (Amersham Biosciences, Little Chalfont, United Kingdom) according to the manufacturer's instructions.

Cell Viability Assay

Cells were resuspended in buffer containing PBS (pH 7.4), 1% fetal bovine serum, 0.1% sodium azide, and 2 μg/mL propidium iodide. Cells were run on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) and analyzed using CellQuest Pro software (Becton Dickinson). Viable cells were distinguished from dead cells based on their ability to exclude propidium iodide.

Measurement of Glycolytic Rate

Determination of glycolytic rate was measured as conversion of [5-^{3}H]glucose to ^3H_2O as described previously (6, 23). Briefly, 10^6 cells were washed in PBS and resuspended in 500 μL Krebs buffer [25 mmol/L $NaHCO_3$, 115 mmol/L NaCl, 2 mmol/L KCl, 2 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, and 0.25% bovine serum albumin (pH 7.4)] containing 10 mmol/L glucose and 1 μCi [5-^{3}H]glucose and

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incubated for 60 minutes in 5% CO_2 at 37°C. Aliquots (100 μL) of each were added to 50 μL of 0.2 N HCl in open 500 μL centrifuge tubes, which were placed upright in scintillation vials containing 1 mL H_2O. The vials were sealed and the ^3H_2O produced was allowed to equilibrate with H_2O outside the centrifuge tube for 24 hours at room temperature. The amount of ^3H retained within the centrifuge tube and the amount that had diffused into the surrounding H_2O by evaporation and condensation were determined separately. [5-^{3}H]glucose and ^3H_2O standards were included in each experiment, allowing calculation of the rate of conversion of [5-^{3}H]glucose to ^3H_2O (23). Statistical comparison was done using the Student's *t* test. All *P*s are two tailed.

Measurement of Mitochondrial Membrane Potential

Mitochondrial membrane potential was measured 24 hours following exposure to chemotherapeutic agents. Cells were treated with 20 nmol/L tetramethylrhodamine ethyl ester and incubated 30 minutes at 37°C. Another aliquot of the same cells was treated in parallel with 20 nmol/L tetramethylrhodamine ethyl ester, 50 μmol/L carbonyl cyanide *m*-chlorophenylhydrazone, and 25 μmol/L chloroquine as a control for maximal mitochondrial membrane depolarization. Cells were analyzed on a FACScan flow cytometer using CellQuest software (Becton Dickinson).

Results

Initial Up-Regulation of Akt Activity Is Rapidly Suppressed following Exposure to Chemotherapeutic Agents

In a genetic screen for changes in gene expression induced by DNA damage, we found that exposure to a variety of cytotoxic agents was associated with rapid suppression of multiple key regulators of glucose uptake and glycolytic metabolism and with a concomitant inhibition of glycolytic activity (7). Given that Akt has been implicated in regulation of both glycolytic metabolism and cell death, we hypothesized that these cytotoxic exposures might lead to a decrease in Akt activity. We therefore evaluated the relative concentration of phosphorylated (active) Akt following exposure to the DNA cross-linking agent cisplatin, the topoisomerase II inhibitor etoposide, and the microtubule depolymerizing agent vincristine. Exposure to all the chemotherapeutic agents tested led to an initial up-regulation of phosphorylated Akt followed by rapid suppression to baseline levels or lower (Fig. 1).

Constitutive Akt Activation Promotes Resistance to Vincristine

Given the effect of Akt activation on glycolysis, deregulated Akt activation, as occurs in many tumor cells, might contribute to maintenance of an elevated glycolytic rate. Alternatively, Akt activation may not affect either glycolysis or induction of apoptosis in these cells. To directly test the involvement of Akt in these pathways, stable transfectants of FL5.12 were generated by using a doxycycline-inducible expression vector encoding constitutively active

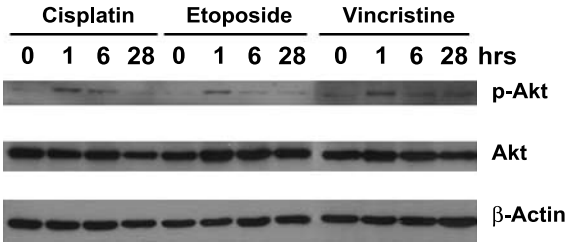

Figure 1. Akt phosphorylation is reduced following treatment with chemotherapeutic agents. Western blot of FL5.12 cell lysates following treatment for 0, 1, 6, and 28 hours with 5 μg/mL cisplatin, 10 μg/mL etoposide, or 0.1 μg/mL vincristine. The blot was sequentially probed for phosphorylated Akt (Ser^{473}), total Akt, and β-actin.

mAkt, dominant-negative *KD-Akt*, or neither factor (control). Many chemotherapeutic agents preferentially target proliferating cells. To ensure that the effects of Akt modulation observed were not due to a difference in population doubling time, we measured the proliferative rate of mAkt, KD-Akt, and control cells. The presence of either constitutively active or dominant-negative Akt did not have a significant effect on the rate of cell proliferation (data not shown).

To assess the contribution of Akt to chemotherapeutic resistance, mAkt, KD-Akt, and control cells were treated with cisplatin, etoposide, or vincristine. Expression of constitutively active mAkt markedly increased resistance to vincristine treatment (Fig. 2C). In contrast, constitutive activation or Akt inhibition seemed to have minor effects on cell sensitivity to either of two DNA-damaging agents, cisplatin and etoposide (Fig. 2A and B). These data suggest that activated Akt confers preferential resistance to vincristine.

To evaluate whether the mAkt-mediated selective resistance to vincristine treatment was a function of drug concentration, we compared the relative sensitivity of mAkt, KD-Akt, and control cells across a range of vincristine, cisplatin, and etoposide concentrations. Consistent with our initial observations, Akt activation seemed to have no effect on cisplatin cytotoxicity and a minimal protective effect against etoposide across the concentrations tested (Fig. 2D and E). In contrast, *mAkt* expression markedly increased vincristine resistance at all concentrations tested (Fig. 2F).

Akt-Mediated Vincristine Resistance Acts Downstream of Cell Cycle Arrest and Prevents Apoptotic Death

Akt-mediated vincristine resistance may be due to the ability of Akt to inhibit vincristine activity, either through direct inactivation or by increasing vincristine clearance from the cell. Alternatively, Akt may permit vincristine activity but suppress the cellular apoptotic response. To distinguish between these possibilities, we examined the cell cycle of mAkt, KD-Akt, and control cells by staining their DNA following exposure to vincristine. Vincristine binds tubulin and inhibits microtubule assembly, leading to mitotic arrest. As with vincristine-treated control and

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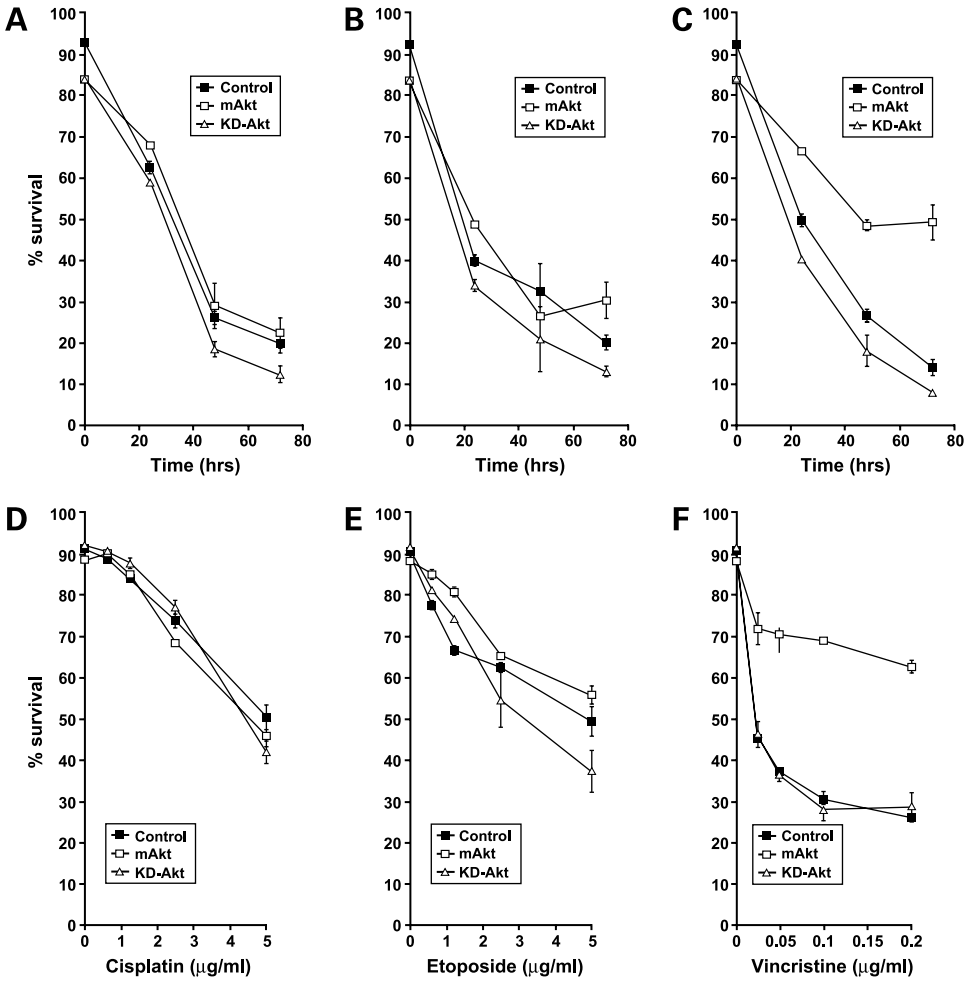

Figure 2. mAkt expression preferentially protects against treatment with vincristine. FL5.12 cells expressing mAkt, KD-Akt, or an empty vector control (*Control*) were treated with (**A**) 5 μg/mL cisplatin, (**B**) 10 μg/mL etoposide, or (**C**) 0.1 μg/mL vincristine. Cell viability was measured every 24 hours using propidium iodide exclusion. The same cells were treated with (**D**) cisplatin, (**E**) etoposide, or (**F**) vincristine at the doses indicated for 72 hours, at which time cell viability was measured using propidium iodide exclusion. *Bars,* SD.

KD-Akt cells, treated mAkt cells consistently arrested in G_2-M (Fig. 3), indicating that vincristine is active even in the presence of activated Akt and that Akt confers resistance downstream of vincristine action.

In addition, examination of cellular DNA content allowed us to evaluate the mode of death caused by vincristine. Following exposure to vincristine, a sub-G_1 peak appeared (Fig. 3), consistent with cell death via an apoptotic pathway. The sub-G_1 peak was suppressed by constitutively active Akt in the mAkt-transfected cells compared with the control or KD-Akt cells, consistent with the cell death observed by propidium iodide exclusion (Fig. 2). These data further support that Akt mediates resistance to vincristine-induced apoptosis.

Akt Activation Maintains Glycolytic Rate in a mTOR-Dependent Manner following Exposure to Vincristine

Akt activation leads to phosphorylation and activation of mTOR (24). mTOR, in turn, regulates several downstream pathways influencing metabolic rate. Given the tight link between the effects of Akt on metabolism and survival, we hypothesized that mTOR might be a critical component of the mechanism by which Akt augments survival following exposure to therapeutic agents. To measure the effectiveness of mTOR inhibition by rapamycin, we measured phosphorylation of p70/S6K, a direct target of mTOR, following treatment with rapamycin (25). As expected, exposure of mAkt, KD-Akt, or control cells to 20 nmol/L rapamycin completely inhibits p70/S6K phosphorylation (Fig. 4A).

The antiapoptotic activity of Akt is dependent on the availability of glucose, and the ability of Akt to increase nutrient uptake following growth factor withdrawal is dependent on mTOR (8). To determine whether mTOR-dependent glucose utilization is required for Akt-mediated survival following vincristine treatment, we measured the rate of glycolysis in cells treated with vincristine and rapamycin for 18 hours. Vincristine exposure markedly increased the rate of glucose utilization in cells constitutively expressing active *Akt* compared with untreated cells (Fig. 4B). This increase, observed in multiple independent experiments, did not occur in control cells or cells expressing the dominant-negative KD-Akt and was suppressed when mTOR was inhibited by treatment with rapamycin.

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Akt-Mediated Vincristine Resistance Is Dependent on mTOR

To determine if the selective protection by Akt against vincristine cytotoxicity is dependent on mTOR activation, we treated mAkt, KD-Akt, and control cells with cisplatin, etoposide, or vincristine, with or without rapamycin. Regardless of the cytotoxic agent used, rapamycin had little or no evident effect on the survival of control or KD-Akt cells (Fig. 5A–C), and rapamycin alone had no effect on survival of any of the cell lines (Fig. 5D). However, rapamycin exposure significantly reduced the protective effect of mAkt against vincristine (Fig. 5C). Thus, it seems that a major component of Akt-mediated vincristine resistance is mTOR dependent. The ability of rapamycin to suppress Akt-mediated vincristine resistance correlates with the elimination of the augmented glycolytic activity in *mAkt*-expressing cells exposed to vincristine (Fig. 4B).

In addition to being required for complete Akt-mediated vincristine resistance, mTOR may also be sufficient for resistance to antimicrotubule agents. One means by which Akt activates mTOR is through direct phosphorylation of a mTOR repressor domain (12). Deletion of a portion of this domain (amino acids 2430–2450) creates an activated mTOR mutant, ΔTOR (21). Using FL5.12 cells expressing this activated *mTOR*, we asked whether constitutive mTOR activation is sufficient for vincristine resistance. Two independent ΔTOR subclones were resistant to vincristine exposure relative to empty vector controls (Fig. 5E). Consistent with our finding that mTOR is required for a major component of Akt-mediated vincristine resistance, mTOR activation seems to be sufficient for substantial resistance to vincristine.

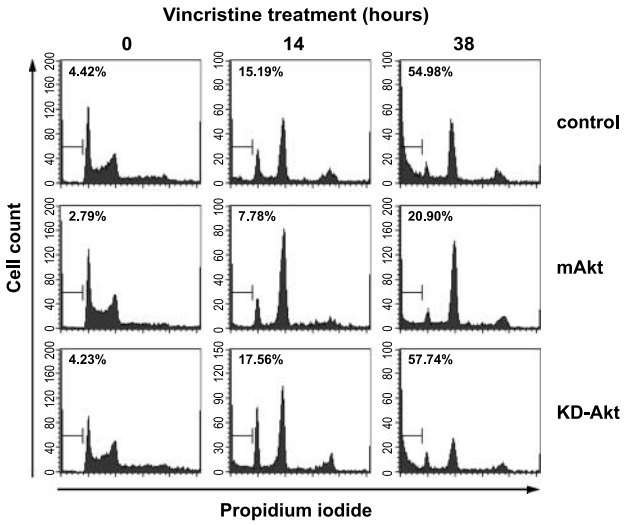

Figure 3. Akt-mediated resistance is downstream of vincristine-mediated cell cycle arrest and protects against apoptotic death. FL5.12 cells expressing mAkt, KD-Akt, or an empty vector control (*control*) were treated with 0.1 μg/mL vincristine for 0, 14, or 38 hours, fixed, and stained with propidium iodide. Cells were analyzed by flow cytometry. Percentage of cells in each sub-G_1 peak is indicated.

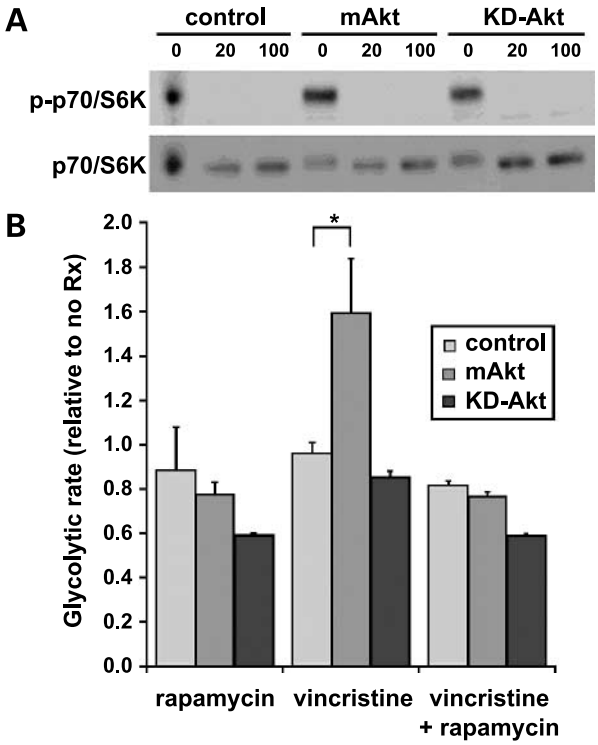

Figure 4. Vincristine treatment together with mAkt expression increases the rate of glucose consumption by a mTOR-dependent mechanism. **A,** Western blot of FL5.12 cells expressing mAkt, KD-Akt, or an empty vector control (*control*) following 18 hours of treatment with 0, 20, or 100 nmol/L rapamycin. The blot was sequentially probed for phosphorylated p70/S6K (Thr^{389}) and total p70/S6K. **B,** FL5.12 cells expressing mAkt, KD-Akt, or an empty vector control (*Control*) were treated for 18 hours with 20 nmol/L rapamycin, 0.1 μg/mL vincristine, or both rapamycin and vincristine or left untreated. After normalizing for number of viable cells, the rate of glucose utilization was measured as conversion of [5-^{3}H]glucose to ^3H_2O. *Bars,* SD. *, $P < 0.05$, mAkt + vincristine versus control + vincristine.

Akt Mediates Resistance to Several Antimicrotubule Agents

Vincristine does not directly damage DNA but rather prevents assembly of microtubule filaments. Paclitaxel and colchicine also interfere with normal microtubule function by blocking tubulin depolymerization and inhibiting microtubule assembly, respectively. In contrast, cisplatin and etoposide are both DNA-damaging agents acting either by direct cross-linking or by inhibition of topoisomerase II, resulting in both single- and double-strand breaks. Topotecan is another widely used DNA-damaging agent, which acts by inhibition of topoisomerase I. To determine if Akt confers resistance preferentially to vincristine alone or to microtubule inhibitors as a class, we treated mAkt, KD-Akt, and control cells with paclitaxel, colchicine, and topotecan (Fig. 6). Similar to results with etoposide, mAkt conferred minimal resistance to topotecan. *Akt* overexpression conferred much greater protection to the antimicrotubule agents paclitaxel and colchicine, consistent with the results seen with vincristine. Thus, Akt seems to confer selective and broad-spectrum resistance to microtubule-directed cytotoxics.

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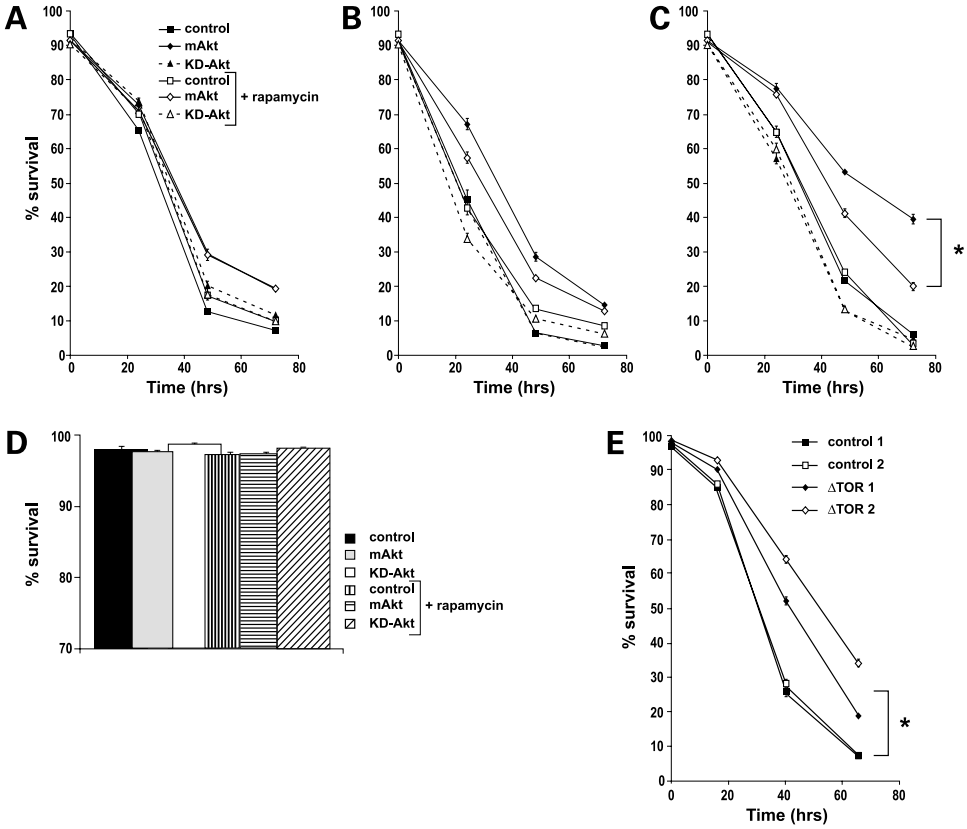

Figure 5. Rapamycin suppresses the resistance of mAkt cells to vincristine, and activated mTOR protects against treatment with vincristine. FL5.12 cells expressing mAkt, KD-Akt, or an empty vector control (*control*) were treated with (**A**) 5 μg/mL cisplatin, (**B**) 10 μg/mL etoposide, or (**C**) 0.1 μg/mL vincristine with or without 20 nmol/L rapamycin, and cell viability was measured every 24 hours. *, $P < 0.05$, mAkt versus mAkt + rapamycin at the final time point. **D,** the same cells were treated with 20 nmol/L rapamycin for 48 hours. **E,** FL5.12 cells expressing an activated mTOR mutant (ΔTOR) or an empty vector control were treated with 0.1 μg/mL vincristine for 48 hours. Cell viability was measured using propidium iodide exclusion. *Bars,* SD. *, $P < 0.05$, control 1 or 2 versus ΔTOR 1 or 2 at the final time point.

Akt-Mediated Resistance Does Not Correlate with Maintenance of Mitochondrial Membrane Potential

Maintenance of glycolytic activity may be a key determinant of the ability of Akt to protect against microtubule-directed agents as suggested by the sensitivity of this effect to rapamycin. Alternatively, Akt could inhibit the cytotoxicity of these agents through direct or indirect effects on mitochondrial membrane homeostasis. As noted above, Akt has been reported to down-regulate the activity of proapoptotic Bcl-2 family members through multiple mechanisms and thus could promote survival through effects on mitochondrial membrane integrity independent of glycolytic regulation. We used the fluorescent dye tetramethylrhodamine ethyl ester to monitor mitochondrial membrane potential in mAkt, KD-Akt, and control transfectants treated with etoposide, topotecan, vincristine, paclitaxel, and colchicine. Cells overexpressing *mAkt* showed relative stabilization of mitochondrial membrane potential as compared with control and KD-Akt cells. However, this effect was seen following exposure to genotoxic agents

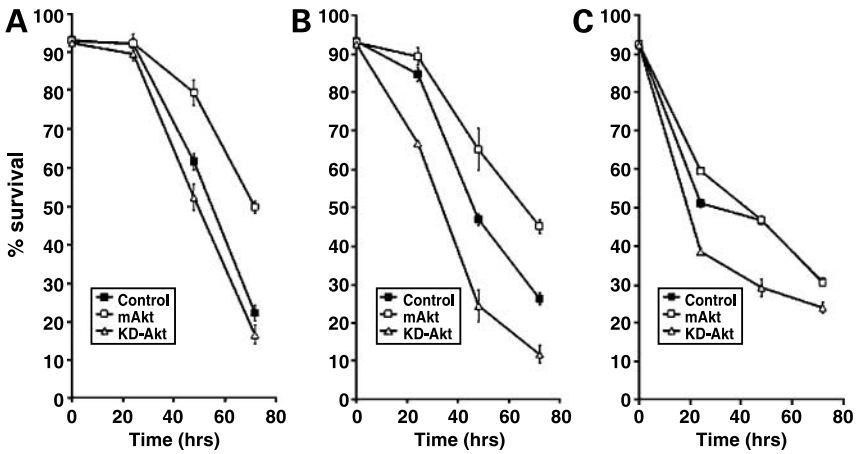

Figure 6. mAkt expression preferentially protects against treatment with antimicrotubule agents. FL5.12 cells expressing mAkt, KD-Akt, or an empty vector control (*Control*) were treated with (**A**) 4 μg/mL paclitaxel, (**B**) 0.2 μg/mL colchicine, or (**C**) 1 μmol/L topotecan. Cell viability was measured every 24 hours by propidium iodide exclusion. *Bars,* SD.

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(etoposide and topotecan) as well as microtubule-directed agents (vincristine, paclitaxel, and colchicine) and thus did not correlate with the ability of mAkt to protect against cell death (Fig. 7). These data suggest that although *mAkt* overexpression does affect mitochondrial homeostasis, perhaps through effects on Bcl-2 family member activity, this observation does not explain the preferential resistance to antimicrotubule agents conferred by Akt activation.

Discussion

Tumor cells show consistent alterations in metabolic regulation and commonly have defects in apoptotic control. Recent evidence points to an emerging association between these two aberrant phenotypes. Akt seems to play key roles in maintaining glycolytic activity and promoting cell survival. These two Akt-mediated activities are tightly linked: the ability of Akt to promote survival is dependent on glucose availability (4). In some but not all contexts, Akt-mediated survival seems to be dependent on the first step of glycolysis because it is blocked by a hexokinase inhibitor but not by other glycolytic inhibitors (26). Interestingly, mitochondrial hexokinase II binds the voltage-dependent anion channel, and this interaction has been shown to inhibit the association between the voltage-dependent anion channel and the proapoptotic factor Bax (27). Furthermore, hexokinase IV, or glucokinase, is found at the mitochondria in a complex that includes the BH3-only protein Bad (28). Bad is required for assembly of this complex, and phosphorylated Bad is required for normal glucose regulation. Together, these data suggest a mechanistic link between glycolysis, hexokinase activity in particular, and regulation of apoptosis.

We have expressed constitutively active *Akt* in an immortalized, nontransformed cell line to examine the role Akt specifically plays in resistance to chemotherapeutic agents. We found that Akt has a marginal effect on survival following exposure to genotoxic agents. This supports data from others showing that a phosphoinositide 3-kinase inhibitor sensitizes Baf-3 cells to growth factor withdrawal but not γ-irradiation (29). In contrast, activated Akt promotes significant resistance to antimicrotubule agents. The Akt-mediated resistance we describe here does not correlate with effects on mitochondrial membrane potential, suggesting it is not dependent on Bcl-2 family members, which act at the level of the mitochondria. A large component of Akt-mediated resistance is reversed by the specific mTOR inhibitor rapamycin. Furthermore, activated mTOR alone confers resistance to vincristine. Together, these data indicate the importance of mTOR in Akt-mediated therapeutic resistance.

Like Akt, mTOR function seems to link glycolytic and survival pathways. mTOR integrates signals regarding availability of nutrients, ATP, and growth stimuli. mTOR promotes mRNA translation of *cyclin D1* and the oncogene c-*myc* (30), which leads to proliferation and increased metabolic demand. mTOR also has more direct effects on metabolism. Akt-mediated nutrient uptake following growth factor withdrawal is sensitive to rapamycin (8). In addition, the activated mTOR truncation mutant used in this study has been shown to maintain nutrient uptake, glycolytic rate, and cell size following growth factor withdrawal (21). We show that activated Akt allows cells exposed to vincristine to up-regulate glucose consumption and that this up-regulation is suppressed by rapamycin. Our data contribute to the growing body of research showing that mTOR not only responds to nutrient availability but also is involved in determining the ability of the cell to use available nutrients.

In addition to its role in cell metabolism, increasing evidence links mTOR with survival and transformation. In many tumor cell lines, exposure to rapamycin causes cell cycle arrest and, in combination with serum deprivation, can cause cell death (31). Cells are sensitized to rapamycin by mutations in p53 or PTEN, and rapamycin has been reported to reverse transformation mediated by epidermal growth factor, phosphoinositide 3-kinase, or Akt (31–33). Recent data from an *in vivo* model suggest that rapamycin can sensitize lymphoma cells to chemotherapeutic treatment (34).

Our data show that a significant component of Akt-mediated protection against antimicrotubule agents is mTOR dependent. Although not affecting the viability of

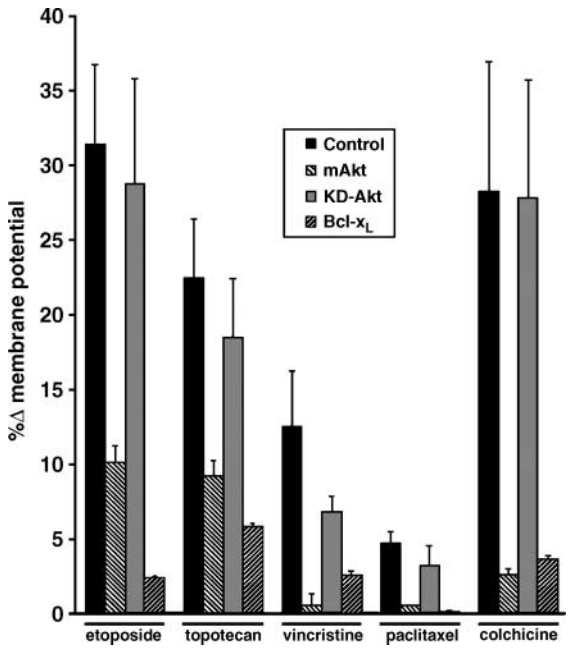

Figure 7. mAkt expression stabilizes mitochondrial membrane potential independently of protection against cell death. FL5.12 cells expressing Bcl-x_L, mAkt, KD-Akt, or an empty vector control (*Control*) were treated with etoposide, topotecan, vincristine, paclitaxel, or colchicine. Following 24 hours of drug treatment, cells were stained with 20 nmol/L tetramethylrhodamine ethyl ester and analyzed by flow cytometry. Only live cells were gated and analyzed. Percentage of Δ membrane potential is expressed relative to control staining with tetramethylrhodamine ethyl ester in the presence of 50 μmol/L carbonyl cyanide *m*-chlorophenylhydrazone and 25 μmol/L chloroquine. *Bars,* SD.

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control cells, mTOR inhibition decreased the resistance of cells expressing activated *Akt*. mTOR is one of several targets downstream of Akt. Although the ΔTOR mutant does not confer vincristine resistance to the same degree as mAkt, it indicates that mTOR supports therapeutic resistance independent of other targets downstream of Akt and highlights the important role for mTOR in therapeutic resistance. The sensitivity of mAkt cells, but not KD-Akt or control cells, to rapamycin supports the role of rapamycin in targeted therapy for cancers dependent on activated Akt-mTOR signaling.

Together, our data suggest that the ability of Akt to promote survival in this context is dependent not only on glucose availability but also on the ability to activate mTOR and use the available glucose. This metabolic dependence applies not only to growth factor withdrawal but also to chemotherapeutic agents that do not directly target metabolic regulation. We have shown a correlation between Akt-mediated vincristine resistance and Akt-dependent upregulation of glycolysis following exposure to vincristine. Moreover, both of these Akt-mediated activities are dependent on mTOR. Although the mechanism whereby increased glycolytic rate promotes cell survival remains to be fully defined, it seems to involve modulating apoptotic sensitivity and not vincristine action because all three cell lines underwent G_2-M arrest regardless of Akt status.

Beyond expanding our understanding of the connection between metabolism and cell survival, these data provide insight into potentially synergistic combinations of targeted anticancer therapies. The cytotoxic activities of many agents targeting growth factor signaling receptors, including gefitinib (directed against epidermal growth factor receptor 1), trastuzumab (directed against HER-2/*neu*), or CI-1033 (directed against multiple epidermal growth factor receptor family members), are critically dependent on suppression of Akt activity (35–37). Several agents targeting Akt and/or mTOR directly are in preclinical and early clinical development (31). The rapamycin analogues RAD001 and CCI-779 have shown efficacy in treating patients with advanced stage renal cell carcinoma and are currently in phase I and III testing, respectively. Results from other kinase inhibitors argue that these agents may exhibit potent effects if selected patient populations and appropriate drug combinations are chosen for clinical evaluation (38). Our data suggest that specific inhibitors of the Akt-dependent signaling pathway may have greatest clinical efficacy in combination with microtubule-directed agents. In particular, such agents may augment the cytotoxicity of *Vinca* alkaloids and taxanes.

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