Optimal Classes of Chemotherapeutic Agents Sensitized by Specific Small-Molecule Inhibitors of Akt *In Vitro* and *In Vivo*

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Abstract

Akt is a serine/threonine kinase that transduces survival signals from survival/growth factors. Deregulation and signal imbalance in cancer cells make them prone to apoptosis. Upregulation or activation of Akt to aid the survival of cancer cells is a common theme in human malignancies. We have developed small-molecule Akt inhibitors that are potent and specific. These Akt inhibitors can inhibit Akt activity and block phosphorylation by Akt on multiple downstream targets in cells. Synergy in apoptosis induction was observed when Akt inhibitors were combined with doxorubicin or camptothecin. Akt inhibitor–induced enhancement of topoisomerase inhibitor cytotoxicity was also evident in long-term cell survival assay. Synergy with paclitaxel in apoptosis induction was evident in cells pretreated with paclitaxel, and enhancement of tumor delay by paclitaxel was demonstrated through cotreatment with Akt inhibitor Compound A (A-443654). Combination with other classes of chemotherapeutic agents did not yield any enhancement of cytotoxicity. These findings provide important guidance in selecting appropriate classes of chemotherapeutic agents for combination with Akt inhibitors in cancer treatment.

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Keywords: Akt, inhibitors, chemosensitization, apoptosis, synergy.

Introduction

Akt is a serine–threonine kinase activated by growth factors or survival factors through phosphatidyl inositol 3′ kinase (PI3K)–3-phosphoinositide–dependent kinase 1 to promote cell growth and survival [1–3]. PTEN (phosphatase and tensin homolog deleted in chromosome 10), a lipid phosphatase, reverts the phosphorylation of phosphoinositol-3,4,5-triphosphate by PI3K and thus prevents Akt activation [2,4,5]. Akt promotes cell survival through phosphorylation and inactivation of key components in apoptotic cascade, such as Bad [6–8], caspase 9 [9], and ASK1 [10]. Akt also downregulates the expression of proapoptotic proteins, such as Fas ligand, through the phosphorylation and inactivation of forkhead transcription factors FOXO3 (FKHRL1) and FOXO4 (AFX) [11–13]. Another important downstream target of Akt is glycogen synthase kinase 3 (GSK3). Inactivation of GSK3 on phosphorylation by Akt leads to protection from apoptosis [2]. Furthermore, Akt has been shown to enhance the function of transcription factor NFκB, thereby upregulating the expression of antiapoptotic proteins, such as cIAP1 and cIAP2. Recently, Akt was also reported to phosphorylate and stabilize PED/PEA-15, an antiapoptotic protein [14].

Akt is either overexpressed or activated in a variety of human cancers, including lung, breast, ovarian, gastric, and pancreatic carcinomas [15–23]. Increased Akt expression also correlates with disease progression [17,24]. Furthermore, *PTEN* mutations that result in increased Akt activity have been reported in a wide variety of malignancies, including breast cancer, prostate cancer, melanoma, glioblastoma multiforme, and endometrial cancer [25–36].

Akt activation and overexpression are often associated with resistance to chemotherapy or radiotherapy [37–40]. Reversal of drug resistance has been demonstrated in both cell-based studies and animal models by PI3K inhibitors and PTEN overexpression in PTEN-null cells [41–47]. Dominant-negative mutants of Akt were also shown to enhance cytotoxicity by chemotherapeutic agents [48], suggesting an important role of Akt in drug resistance. Furthermore, inhibition of receptor tyrosine kinases, such as epidermal growth factor receptor, sensitizes cells to chemotherapy or radiotherapy through downregulation of the PI3K–Akt pathway [38,49–53]. Thus, clinically suitable small-molecule inhibitors of Akt have great potential in cancer treatment. In addition, identifying suitable classes of chemotherapeutic agents that could be sensitized by Akt inhibition is highly desired to guide the clinical application of Akt inhibitors.

Abbreviations: PI3K, phosphatidyl inositol 3′ kinase; PTEN, phosphatase and tensin homolog deleted in chromosome 10; GSK3, glycogen synthase kinase 3

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We have developed specific small-molecule inhibitors against Akt [54]. In this study, we have shown that Akt activity was modulated by various classes of chemotherapeutic agents. Akt inhibitors demonstrated synergy only with topoisomerase I inhibitors, topoisomerase II inhibitors, and paclitaxel in apoptosis induction in human cancer cell lines. Combination with other classes of chemotherapeutic agents did not enhance apoptosis induction. Akt inhibitors were also shown to enhance tumor growth delay by paclitaxel in a PC-3 xenograft model. Thus, we identified optimal classes of chemotherapeutic agents for combination with Akt inhibitors in cancer treatment.

Materials and Methods

Cell Lines and Materials

MiaPaCa, H460, 786-0, and MDA-MB468 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA). Cells were cultured according to instructions from the ATCC. Akt inhibitors were synthesized as described [54].

Western Blot Analysis

Cells were harvested and lysed in an insect cell lysis buffer (10 mM Tris, pH 7.5, 130 mM NaCl, 1% Triton X-100, 10 mM NaF, 10 mM NaPi, and 10 mM NaPPi) supplemented with 50× protease inhibitor cocktail (BD Pharmingen, Bedford, MA) and 1 μM microcystin LR (Sigma Chemical Co., St. Louis, MO). Fifty micrograms of total protein was loaded and resolved under reducing conditions on a 4% to 12% Tris-glycine gel (Invitrogen, Carlsbad, CA). Western blot analysis was performed with antibodies, as indicated. All antibodies were purchased from Cell Signaling, Inc. (Beverly, MA).

Caspase Assay

The assay was carried out as described [55]. Caspase activity is presented as units of fluorescence change per hour (dFU/hr). Each data point is the average of three values. Error bars represent standard deviation.

Soft Agar Assay

One milliliter of a 0.5% agar was first placed in each well of six-well plates to form the bottom layer of the agar. Then 2 ml of a 0.3% top agar containing 1×10^4 cells and complete medium was layered on top of the solidified bottom layer of the agar. After 2 weeks in culture, colonies were stained with *p*-iodonitrotetrazolium violet, and the numbers and sizes of colonies were quantified using Image-Pro Plus (Media Cybernetics, Silver Spring, MD). Each value is the average of two values. Error bars represent the range of values.

Tumor Efficacy Study

Animal studies were conducted following the guidelines of the Institutional Animal Care and Use Committee. Immunocompromised male *scid* mice (C.B-17-*Prkdc*^{scid}), at 6 to 8 weeks of age, were obtained from Charles River Laboratories (Wilmington, MA). PC-3 cells were obtained from the ATCC. Two million PC-3 cells in 50% Matrigel (BD Biosciences, Bedford, MA) were inoculated subcutaneously into the flank. Sixteen days after inoculation, tumors were assigned to treatment groups, with an average size of 185 mm^3 per group, and therapy was started on the same day (n = 10 mice per group). Tumor size was evaluated by twice-weekly measurements with digital calipers. Tumor volume was estimated using the formula: $V = LW^2$. Compound A was administered subcutaneously in a vehicle of 0.2% HPMC. Paclitaxel was obtained from Bristol-Myers Squibb (Princeton, NJ) and administered in three doses at 4-day intervals, according to the manufacturer's instructions.

Results

Properties of Akt Inhibitors

We developed small-molecule inhibitors against Akt1 [54]. The structure, potency, and selectivity of three Akt

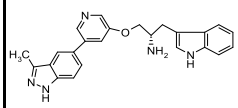
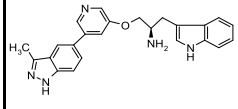
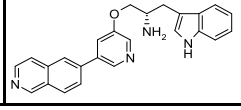
		K_i (nM)						
Compound	Structure	AKT1	CDK2	ERK2	PKA	PKC DELTA	PKC GAMMA	Src
A (A-443654)		0.16	23.8	340.0	6.3	32.6	23.7	3461.0
B		28.30	37.6	434.5	23.2	17.7	99.1	9533.2
C		0.7	81.7	524.5	2.1	224.7	314.2	2180.5

Figure 1. *Potency and selectivity of Akt inhibitors. The potency and selectivity of Akt inhibitors were measured as described [54]. Each value is the average of several determinations. Compound A is a very selective inhibitor; Compound B is the enantiomer of Compound A; Compound C is another selective Akt inhibitor.*

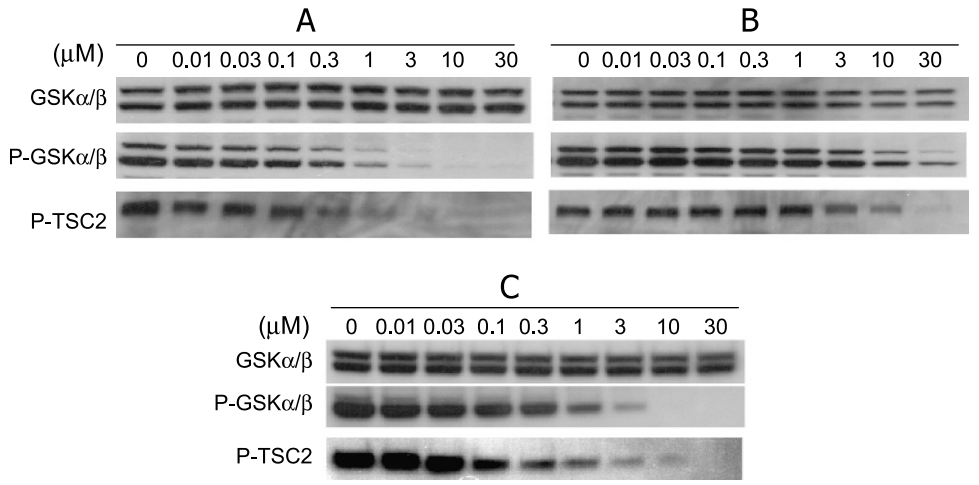


Figure 2. *Inhibition of cellular Akt activity by Akt inhibitors. MDA-MB468 cells were treated with Akt inhibitors for 2 hours. The total cell lysate was extracted and subjected to Western blot analysis. A corresponds to Compound A in Figure 1, while B corresponds to Compound B, and C corresponds to Compound C.*

inhibitors are shown in Figure 1. Compound A, which is a very selective inhibitor, inhibits Akt1, with a K_i = 160 pM, and it inhibits Akt2 and Akt3 with similar potency [54]. We have tested over 40 different kinases that represent different family members of the human kinome; the kinases that were inhibited significantly by Akt inhibitors are listed in Figure 1. PKA is the least selective kinase for Compound A, and the selectivity between Akt1 and PKA is 39-fold. Compound B is the enantiomer of Compound A. Its potency against Akt1 is 177-fold lower than that of Compound A, whereas the potencies against other kinases were very similar to that of Compound A [54]. Therefore, the pair of these two compounds provides an ideal tool to study Akt functions in cells. In addition, another selective Akt inhibitor, Compound C, inhibits Akt, with a K_i = 0.7 nM (Figure 1).

Akt inhibition by these compounds was measured in cells. The phosphorylation of multiple Akt downstream targets is shown in Figure 2. Consistent with Akt potencies in test tubes, Compound A inhibited the phosphorylation of these Akt substrates with an EC_{50} = 0.3 μM, whereas EC_{50} = 3 to 10 μM for Compound B. Compound C inhibited Akt in cells with an EC_{50} = 1 μM (Figure 2).

Synergy between Akt Inhibitors and Certain Classes of Chemotherapeutic Agents in Killing Human Cancer Cells

The ability of Akt inhibitors to potentiate apoptosis induction in human cancer cell lines was assessed in combination with various classes of chemotherapeutic agents. H460 cells were pretreated with Akt inhibitors, followed by cotreatment with camptothecin, doxorubicin, cisplatin, paclitaxel, 5-fluorouracil, and gemcitabine. Caspase activation was compared between cells on single treatment and cells on combination treatment. Caspase activation was minimal in cells treated with either Akt inhibitors alone or chemotherapeutic agents alone within the concentration range tested. Significant synergy was detected between Compound A and certain classes of chemotherapeutic agents, such as camptothecin and doxorubicin, whereas no synergy was observed with other classes of chemotherapeutic agents (Figure 3). Nearly all the cells underwent apoptosis with 1 μM Compound A and the highest concentration of doxorubicin or camptothecin (Figure 3*B*). Within the same concentration range wherein synergy was demonstrated with cytotoxic agents in apoptosis induction, Compound A inhibited the phosphorylation of GSK3 and tuberous sclerosis 2 by Akt in a dose-dependent manner (Figures 2 and 3). All of these are important Akt targets that are involved in apoptosis induction or cell growth/survival. Combination with paclitaxel in the same regimen did not exhibit any synergy between the two agents (data not shown). However, when the cells were pretreated with paclitaxel for 24 hours before the addition of Compound A, synergy was detected (Figure 3*D*). Treatment with another Akt inhibitor, Compound C, displayed a similar potentiation profile (Figure 3, *A*, *C*, and *D*).

We evaluated the enantiomer of Compound A (Compound B) in these combination experiments in the same concentration range. At these concentrations, Compound B did not inhibit Akt (Figure 1*B*). There was no evidence of synergy in caspase activation detected in combination with any chemotherapeutic agent (Figure 3). Therefore, synergy in apoptosis induction was demonstrated between Akt inhibitors and camptothecin, doxorubicin, and paclitaxel. The same set of combination treatments was also performed in the human colon carcinoma cell line DLD-1, and similar results were observed (data not shown). In contrast, no synergy was observed in any combination treatment in normal human fibroblasts (Figure 3*F*).

We examined Akt activation by different classes of chemotherapeutic agents. H460 lung carcinoma cells were treated with various chemotherapeutic agents for different lengths of time, and phosphorylation activation at serine 473 was used as an indicator of Akt activation [3]. Almost all classes of chemotherapeutic agents modulated Akt activity during the first few hours of treatment. Initial suppression of Akt was observed during the first hour of treatment. However, Akt phosphorylation returned to the basal level after

3 hours of treatment (Figure 4*A*). One can hypothesize that, immediately after DNA damage, cells may respond by decreasing signaling through mitogenic pathways, such as the PI3K–Akt pathway, to stop cell cycle progression. Subsequently, however, the cells struggle to survive during the DNA repair process; thus, upregulation of Akt for its apoptosis-protecting function may become critical for cell survival.

We also measured the levels of several antiapoptotic proteins in cells subjected to combination treatment. The levels of Bcl2, BclXl, and XIAP did not change in any combination treatment. However, although Mcl-1 levels were reduced by Compound A itself, more dramatic reductions in Mcl-1 levels were observed with the combination of Com pound A and camptothecin, doxorubicin, paclitaxel, and cisplatin (Figure 4). These may explain the synergy observed in caspase activation, except in the case of combination with cisplatin.

The impact of Akt inhibitors on cell survival after chemotherapy was investigated. We found that cells were more sensitive to either chemotherapeutic agents (doxorubicin and camptothecin) or Akt inhibitors alone in soft agar assay (data not shown). This may be a result of a much lower cell density at the beginning of drug treatments. Therefore, combination treatment was carried out with lower concentrations of both Akt inhibitors and chemotherapeutic agents. Both Compound A and Compound C significantly enhanced cytotoxicity by doxorubicin or camptothecin in the long-term soft agar assay (Figure 5).

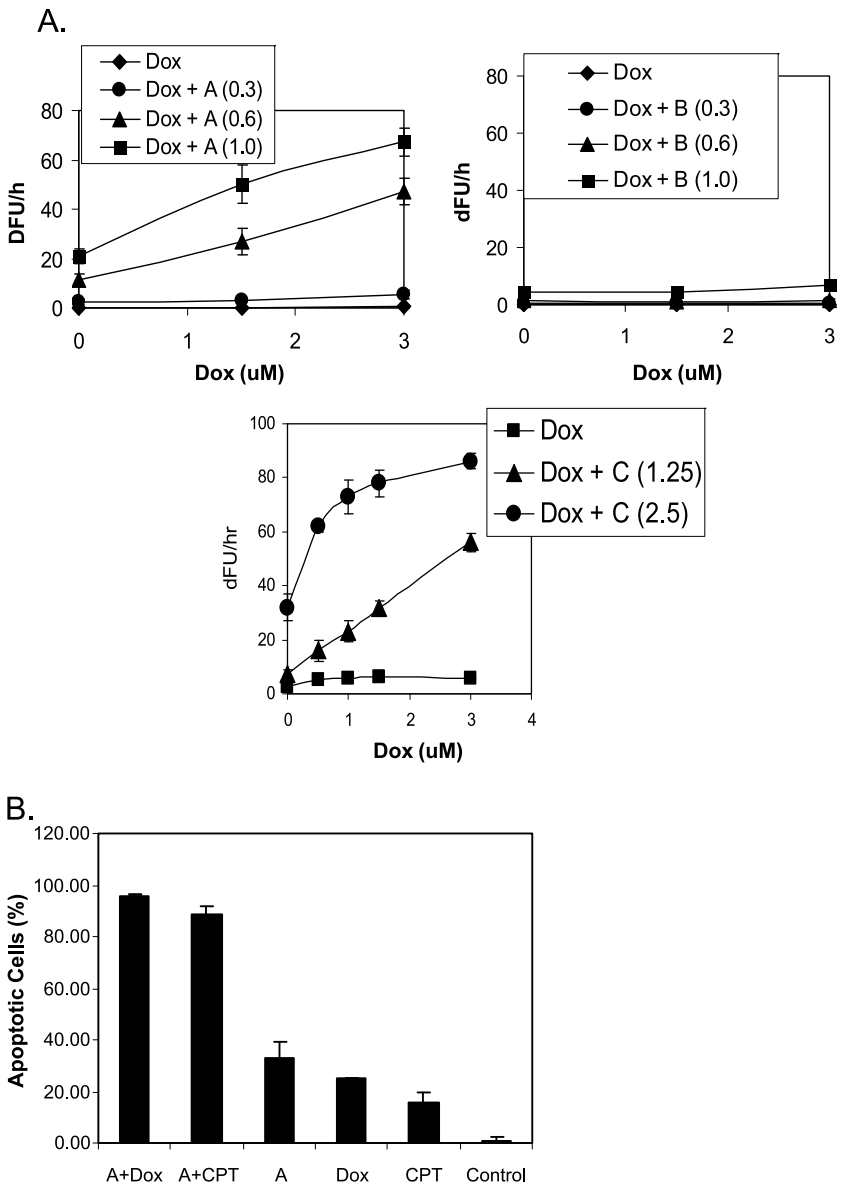


Figure 3. *Synergy between Akt inhibitors and certain classes of chemotherapeutic agents in apoptosis induction. (A, C, and E) H460 cells were treated with Akt inhibitors for 17 hours, followed by cotreatment with different chemotherapeutic agents for an additional 7 hours. Caspase assay was performed. (B) H460 cells were treated with 1 μM Compound A for 17 hours, followed by cotreatment with either 3 μM doxorubicin or 1 μM camptothecin for an additional 24 hours. Cells were stained with DAPI for DNA staining. The cells that contained nuclear condensation and fragmentation were scored as apoptotic cells. At least 700 cells were counted in each sample. (D) H460 cells were pretreated with paclitaxel for 24 hours followed by cotreatment with Akt inhibitors for an additional 17 hours. Caspase assay was performed. Representative data from several experiments are shown. (F) NHF cells were treated as in (C) and (E). Caspase assay was performed.*

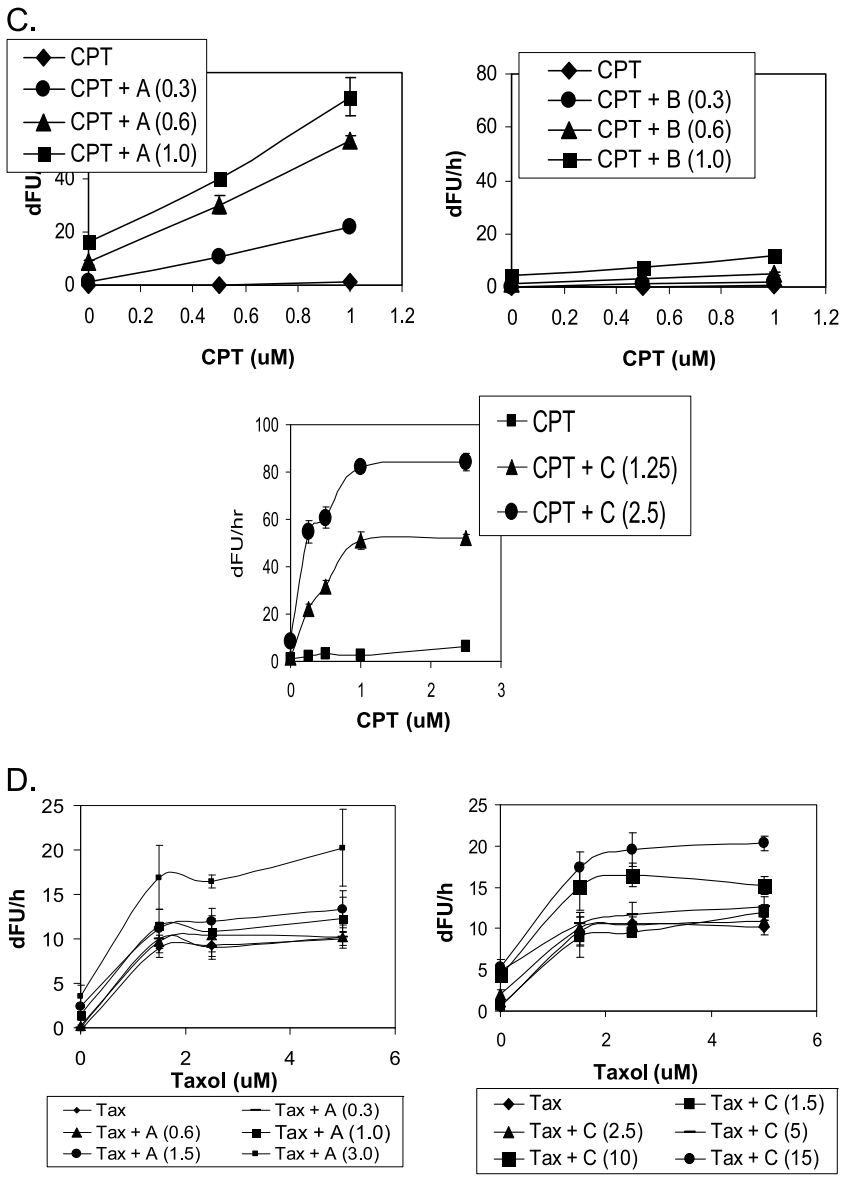


Figure 3. *(continued)*

Akt Inhibitor Sensitizes Tumors to Paclitaxel in the PC-3 Xenograft Model

To examine the ability of Akt inhibitors to potentiate the activity of cytotoxic agents *in vivo*, we studied a PC-3 prostate xenograft model utilizing Compound A in combination with paclitaxel as a test case. The treatment was initiated on established flank tumors. Compound A was efficacious at 7.5 mg/kg per day as monotherapy in a variety of tumor models [54]. When given as monotherapy at 2.5 mg/kg per day (from days 16 to 32), Compound A did not yield any statistically significant efficacy. Similarly, paclitaxel given at half of the maximally tolerated dose of 15 mg/kg per day (on days 16, 20, and 24) only generated modest, transiently significant efficacy. However, the combination of Compound A plus paclitaxel resulted in inhibition of tumor growth that was significantly improved compared to paclitaxel monotherapy ($P < .05$), consistent with the results we obtained in tissue-cultured cells (Figure 6). This result demonstrates the ability of Akt inhibitors to sensitize tumors to chemotherapy *in vivo*.

Discussion

The PI3K–Akt pathway plays a pivotal role in promoting cell survival, and it has been implicated in drug resistance. The inhibition of the pathway through either PI3K inhibitors or PTEN expression has been demonstrated to sensitize cancer cells to chemotherapy [39,41–44,46,47]. The role of Akt in the pathway for drug resistance was also suggested by demonstrating that Akt dominant-negative mutants sensitize cells to drug treatment [48]. Recently, several inhibitors that prevent Akt activation have been reported to induce cytotoxicity or to sensitize cancer cells to apoptosis [56–61]. To date, clinically relevant, pharmacologic

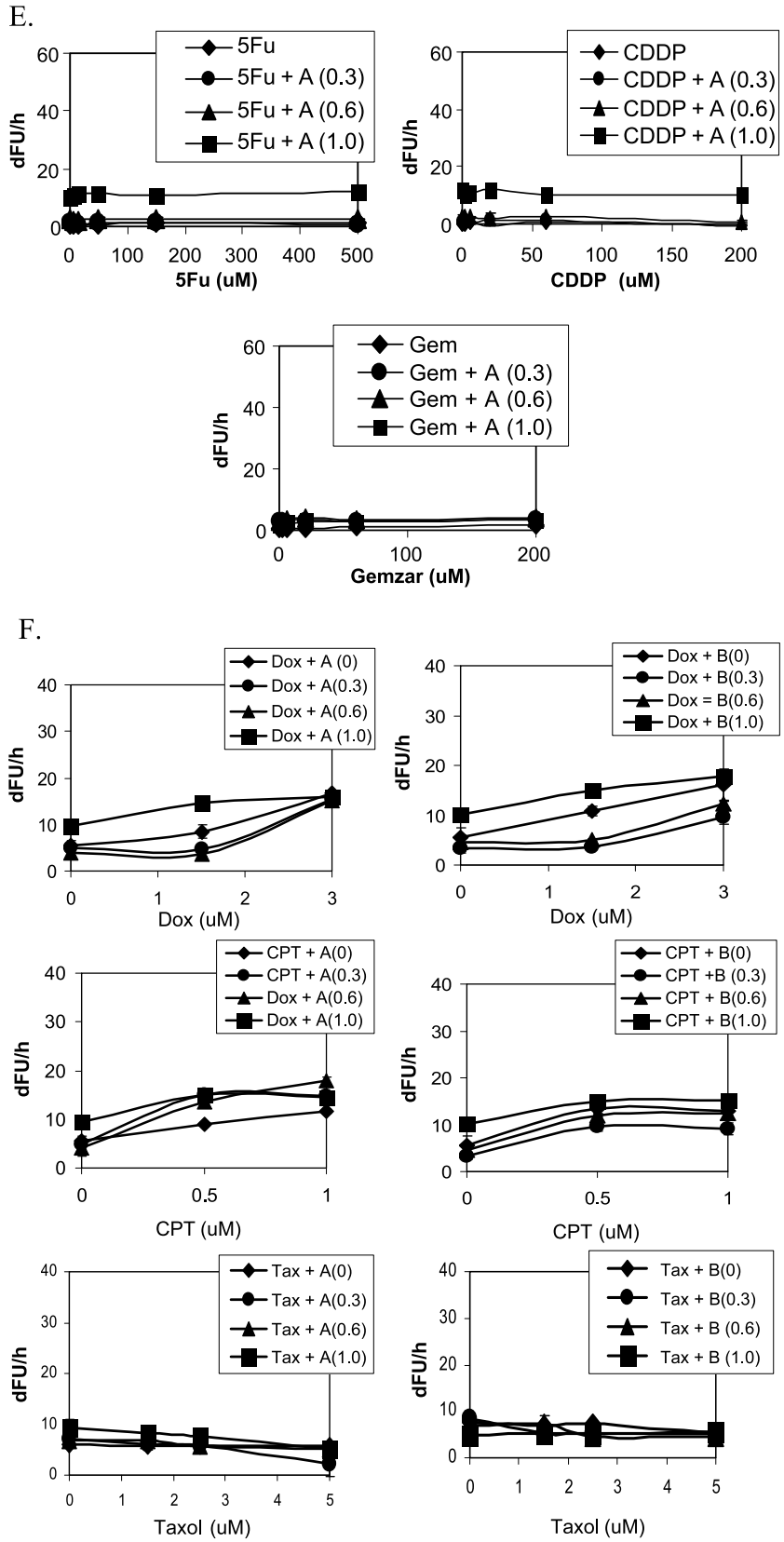


Figure 3. *(continued)*

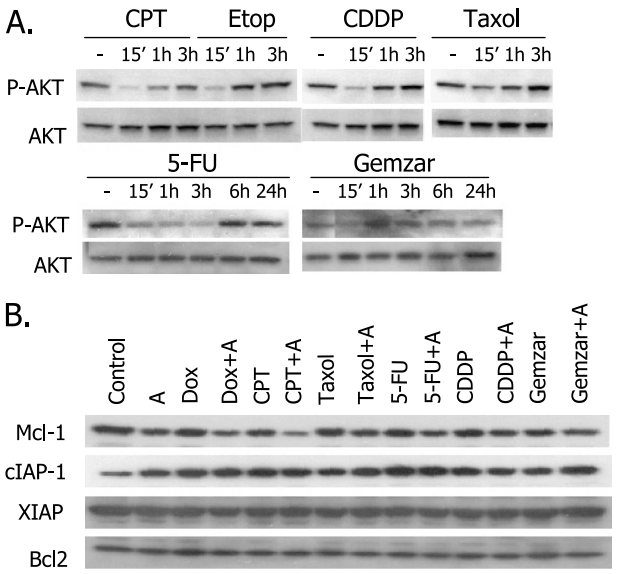


Figure 4. *(A) Akt phosphorylation is modulated by chemotherapy. Cells were treated with various chemotherapeutic agents and subjected to Western blot analysis as indicated. (B) Antiapoptotic protein levels in cells subjected to combination treatments. H460 cells were treated as in Figure 3, and lysates were made and subjected to Western blot analysis as indicated.*

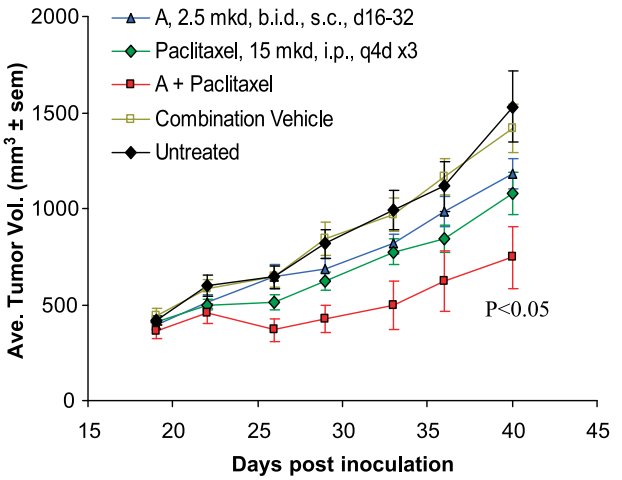


Figure 6. *Combination treatment of Compound A plus paclitaxel in the PC-3 xenograft flank tumor model. Sixteen days after inoculation, tumors were size-matched to approximately 185 mm^3, and therapy was initiated on the same day. Tumor volume* versus *days after inoculation is plotted.*

inhibition of Akt has not been examined in combination therapy *in vivo*, and the best class of chemotherapy that could be used in combination with Akt inhibitors has not been systematically investigated.

In this study, we report very potent and selective Akt inhibitors with K_i values as low as 160 pM. We have tested these Akt inhibitors in combination with representative classes of chemotherapeutic agents, and we have demonstrated that Akt inhibition sensitizes tumor cells only to certain chemotherapeutic agents, but not to others. Both Compound A and Compound C inhibited Akt pharmacologically and sensitized tumor cells only to camptothecin, doxorubicin, and paclitaxel (Figure 3). The enantiomeric isomer of Compound A (Compound B) was 177-fold less active against Akt, whereas it inhibited other kinases with similar potency. Compound B did not enhance cytotoxicity to any of these classes of chemotherapeutic agents, suggesting that Akt inhibition underlies the mechanism of chemosensitization. Some of these cytotoxic agents have been shown to be sensitized by PTEN overexpression [41,42] and Akt inhibition [61]. In contrast, no synergy in apoptosis induction was observed in the combination treatment with other classes of chemotherapeutic agents (Figure 3). PI3K inhibitors were shown to enhance the antitumor activity of gemcitabine

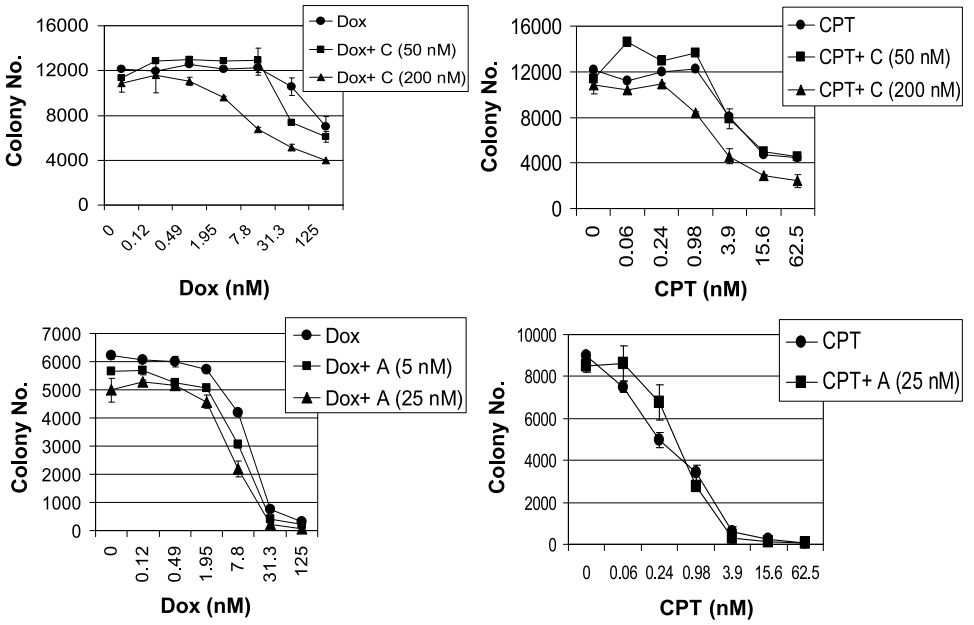


Figure 5. *Akt inhibition enhances the cytotoxicity of chemotherapeutic agents. Cells were pretreated with Akt inhibitors together with chemotherapeutic agents for 48 hours. Soft agar assay was performed to assess long-term cell survival after treatment. Representative data from several experiments were shown.*

[47,62], although we did not observe any enhancement of apoptosis induction when AKT inhibitors were combined with gemcitabine. This may be due to an Akt-independent activity of PI3K.

More importantly, we examined combination effects in the PC-3 xenograft model. Codosing of Compound A with paclitaxel enhanced tumor growth delay compared to that by paclitaxel alone, demonstrating the achievable benefits of combination therapy with Akt inhibitors *in vivo* (Figure 6). These findings have significant clinical value in guiding the selection of chemotherapeutic agents for optimal combination therapy with Akt inhibitors.

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