1,4-Diamino-2,3-dicyano-1,4-bis(methylthio)butadiene (U0126) Enhances the Cytotoxicity of Combretastatin A4 Independently of Mitogen-Activated Protein Kinase Kinase

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ABSTRACT

Combretastatin A4 (CA4) is a novel vascular-disrupting agent that has shown promising anticancer effects through its inhibition of microtubule assembly and subsequent disruption of tumor blood flow. In this report, we demonstrate that 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene (U0126), a selective inhibitor of mitogen-activated protein kinase kinase (MEK), significantly enhances the cytotoxicity of CA4 in BEL-7402 cells, independently of MEK inhibition. This independence is evidenced by the fact that another, more specific MEK inhibitor, PD0325901, [N-((R)-2,3-dihydroxy-propoxy)-3,4-difluoro-2-[2-fluoro-4-iodo-phenylamino]-benzamide], does not have the same effect as U0126. The disassembled microtubules are able to reassemble in the later stages of CA4 treatment, because of the inactivating glucuronidation of CA4. U0126, but not PD0325901, inhibits CA4 glucuronidation, thereby blocking microtubule reassembly and enhancing CA4-induced G2/M cell-cycle arrest. Consistent with this, U0126 significantly enhances CA4-induced cytotoxicity for cells in which CA4 glucuronidation occurs, but not for cells in which such glucuronidation does not occur. These results suggest that great caution should be exercised when interpreting data obtained using U0126 or when CA4 is combined with inhibitors of glucuronidation in clinical practice. It is most important to note that these findings indicate that the combination of CA4 with inhibitors of glucuronidation may be a novel and rational strategy for cancer therapy.

Combretastatin A4 (CA4), originally isolated from the South African Combretum caffrum tree, is a microtubule-distabilizing agent that inhibits microtubule assembly by binding to tubulin at the same site as colchicine (Pettit et al., 1989). In vitro, CA4 produces a dramatic cytotoxicity toward actively proliferating vascular endothelial cells and human cancer cells (Nabha et al., 2002; Kanthou et al., 2004; Lin et al., 2007). In vivo, treatment with CA4P, a water-soluble prodrug of CA4, significantly reduces blood flow within tumors, causing them to undergo massive hemorrhagic necrosis (Dark et al., 1997; Grosios et al., 1999). However, a single dose of CA4P induces little tumor growth retardation; this may be because of the survival of a narrow rim of peripheral tumor cells (Chaplin et al., 1999) or the short half-life of CA4 in tumors. In the former case, a viable region of cells would be sufficient to rapidly repopulate the tumor, leading to minimal delay in tumor growth. To overcome this, various studies have examined the combination of CA4P with radiation or chemotherapeutic agents (e.g., 5-fluorouracil, cisplatin, and carboplatin) and have found that such combination treatments exhibit synergistic antitumor activity in rodent tumor models (Grosios et al., 2000; Pedley et al., 2001; Siemann et al., 2002; Bilenker et al., 2005).

U0126 is a non-ATP competitive inhibitor of mitogen-activated protein kinase kinase (MEK). It exerts its effect by inhibiting the ability of MEK to phosphorylate downstream mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) (Favata et al., 1998). The MEK-ERK1/2 pathway represents one of the major signaling pathways.

ABBREVIATIONS: CA4, combretastatin A4; CA4P, prodrug of combretastatin A4; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene; MEK, mitogen-activated protein kinase kinase; MAPK, mitogen-activate protein kinase; ERK, extracellular signal-regulated kinase; MMK, mitogen-activated protein kinase kinase; UGT, UDP-glucuronosyltransferase; LY294002, 2-(4-morpholinyl)-8-phenyl-4-(4H)-1-benzopyran-4-one; PD025360, 4-amino-5-(4-chlorophenyl)-7-(4-butyl)pyrazolo[3,4-d]pyrimidine; Y27632, (+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl) cyclohexane carboxamide dihydrochloride; PI, propidium iodide; MES, 2-(4-morpholinyl)ethanesulfonic acid; PBS, phosphate-buffered saline; HPLC, high-performance liquid chromatography; MS, mass spectrometry; LC, liquid chromatography; SRB, sulforhodamine B; PD0325901, N-[(R)-2,3-dihydroxy-propoxy]-3,4-difluoro-2-[2-fluoro-4-iodo-phenylamino]-benzamide.
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UD-glucuronosyltransferase (UGT) may be another potential target for U0126 and over time. Our data suggest that UDP-glucuronosyltransferase effects by inhibiting CA4 glucuronidation (a major metabolic processes such as apoptosis. U0126 has been shown to significantly enhance apoptosis induced by microtubule-targeting agents (e.g., paclitaxel and vinblastine) in breast, ovarian, lung, and myeloid leukemia cancer cells. These findings have long been interpreted as resulting from U0126-induced inhibition of MEK (MacKeigan et al., 2000; Stadheim et al., 2001). Indeed, in a screening of its effect on a range of 24 different protein kinases, U0126 was found to be rather selective for MEK (Davies et al., 2000). However, U0126 exhibits other effects independently of MEK inhibition; these effects include inhibition of the MKK5-ERK5 pathway, acceleration of K+ channel inactivation, activation of AMP-activated protein kinase, and induction of aryl hydrocarbon receptor activation (Kamakura et al., 1999; Andrieux et al., 2004; Dokladka et al., 2005; Yuan et al., 2006). These findings suggest that some caution should be used when applying U0126 as an inhibitor of MEK and that U0126 may have other important pharmacological effects that are presently unknown.

Previous work in our laboratory showed that CA4 stimulates both ERK1/2 and p38 MAPK activation in human hepatocellular carcinoma BEL-7402 cells. However, only p38 MAPK seems to be critically involved in the cytotoxicity of CA4, because the MEK-specific inhibitor PD0325901 does not enhance the cytotoxicity of CA4 (Quan et al., 2008). Recently, we began screening for compounds capable of synergistically enhancing the antitumor activity of CA4. During this screening, we found that U0126 synergistically enhanced the antitumor activity of CA4 at both the molecular and cellular levels, potentiating CA4-induced microtubule depolymerization, G2/M cell-cycle arrest, and cytotoxicity. To our surprise, this effect of U0126 on CA4 was found to be MEK-independent, as shown by the inability of PD0325901, a more specific inhibitor of MEK, to mimic these effects. Further studies demonstrated that U0126 achieved these effects by inhibiting CA4 glucuronidation (a major metabolic pathway of CA4), thereby maintaining the activity of CA4 over time. Our data suggest that UDP-glucuronosyltransferase (UGT) may be another potential target for U0126 and that great caution should be taken when interpreting data obtained from U0126. It is most important to note that this study provides evidence that CA4 in combination with glucuronidation inhibition may be a novel and potential strategy for cancer therapy.

Materials and Methods

Materials. CA4 was a generous gift from Dr. Weiping Sheng (Shanghai University, Shanghai, China). U0126, LY294002, PD2, 4',6-diamidino-2-phenylindole dihydrochloride, and the monoclonal antibody specific for β-tubulin were purchased from Sigma-Aldrich (St. Louis, MO). Cetuximab was purchased from Merck (Darmstadt, Germany). Fetal bovine serum was purchased from HyClone Laboratories (Logan, UT). Y27632 and propidium iodide (PI) were purchased from Promega (Madison, WI). Monoclonal antibodies to phospho-ERK1/2 and ERK1/2 were purchased from Cell Signaling Technology Inc. (Danvers, MA). Goat anti-mouse IgG conjugated with Alexa Fluor 488 was purchased from Invitrogen (Carlsbad, CA).

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100% for 5 min. Finally, B was quickly changed back to 5% at 15.1 min and maintained from 15.1 to 20 min, whereupon the next sample could be analyzed. The effluent was delivered at 0.3 ml/min throughout the entire gradient program. The sample injection volume was 10 μl. The mass spectrometer was operated in the negative electrospray ionization mode. In the LC/MS/MS experiments, ions were activated in Q2 with a 25-eV collision energy, whereas the collision gas (argon) pressure was maintained at 1.0 mtorr.

**Cell-Cycle Analysis.** Adherent and detached cells were collected by trypsinization and centrifuged at 300g. Cells were washed twice with ice-cold PBS and fixed in ice-cold 70% ethanol overnight at −20°C. Fixed cells were centrifuged at 300g and stained with 50 μg/ml PI containing 50 μg/ml DNase-free RNase A, at 37°C for 30 min. The DNA content of cells (10,000 cells/experimental group) was analyzed using an FACScan flow cytometer (BD Biosciences, San Jose, CA) and the ModFit LT Mac version 3.0 program.

**Sulforhodamine B Cell Survival Assay.** Cells were seeded in 96-well plates and then treated with different concentrations of the tested drugs. After 72 h of incubation, the cells were fixed with 10% trichloroacetic acid for 1 h at 4°C, washed five times with tap water, and then air-dried. Surviving cells were stained with 0.4% (w/v) sulforhodamine B (SRB) for 20 min at room temperature and then washed five times with 1% acetic acid. Bound SRB was solubilized with 10 mM Tris, and absorbance was measured at 565 nm.

**Statistical Analysis.** The paired Student’s t test was used to test for significance where indicated.

**Results**

**U0126 Blocks Microtubule Reassembly after CA4 Exposure.** To test the effect of CA4 on microtubule disassembly, BEL-7402 cells were treated with different concentrations of CA4 for 1 h. B, BEL-7402 cells were pretreated with or without 10 μM U0126 for 1 h and then exposed to 1 μM CA4 for different times. C, BEL-7402 cells were pretreated with 10 μg/ml cetuximab, 10 μM PP2, 10 μM Y27632, 25 μM LY294002, or 10 μM U0126 for 1 h and then exposed to 1 μM CA4 for 8 h. For A to C, total cell lysates were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotted with antibodies specific for phospho-ERK1/2 and ERK1/2.

![Fig. 1](image_url) U0126 blocks microtubule reassembly after exposure of BEL-7402 cells to CA4. A, BEL-7402 cells were treated with different concentrations of CA4 for 1 h. B, BEL-7402 cells were pretreated with or without 10 μM U0126 for 1 h and then exposed to 1 μM CA4 for different times. C, BEL-7402 cells were pretreated with 10 μg/ml cetuximab, 10 μM PP2, 10 μM Y27632, 25 μM LY294002, or 10 μM U0126 for 1 h and then exposed to 1 μM CA4 for 8 h. Photographs were taken using a fluorescence microscope. Data shown are representative of three independent experiments. Cet, cetuximab; Y, Y27632; LY, LY294002; U, U0126.

![Fig. 2](image_url) PD0325901 inhibits CA4-induced ERK1/2 activation but not microtubule reassembly. A, BEL-7402 cells were treated with 1 μM CA4 for different times. B, BEL-7402 cells were pretreated with different concentrations of U0126 or PD0325901 and then exposed to 1 μM CA4 for 45 min. For A and B, total cell lysates were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotted with antibodies specific for phospho-ERK1/2 and ERK1/2. C, BEL-7402 cells were pretreated with 10 μM U0126 and 100 nM PD0325901 for 1 h and then exposed to 1 μM CA4 for 8 h. Polymeric tubulin was determined by Western blotting using an antibody specific for β-tubulin. Data shown are representative of three independent experiments. PD, PD0325901; U, U0126.

As shown in Fig. 1B, microtubules were fully disassembled after 0.5 h of treatment with 1 μM CA4, but they reassembled after approximately 4 h of CA4 exposure. To explore the potential mechanisms involved in the observed microtubule reassembly, we assessed the effects of several protein kinase inhibitors on this process. We found that only the MEK inhibitor U0126, but not the epidermal growth factor receptor antibody cetuximab, the Src kinase inhibitor PP2, the Rho kinase inhibitor Y27632, or the phosphatidylinositol 3-kinase inhibitor LY294002, blocked microtubule reassembly after CA4 exposure (Fig. 1, A and C). Consistent with these findings, the results from immunofluorescence assays confirmed that U0126 completely inhibited the reassembly of microtubules even after 8 h of CA4 exposure (Fig. 1D).

**The Blockage of Microtubule Reassembly by U0126 Is Independent of MEK Inhibition.** We reported previously that CA4 stimulates ERK1/2 activation in BEL-7402 cells (Quan et al., 2008). Consistent with this, we observed ERK1/2 activation as early as 15 min after initiating treatment with 1 μM CA4; this activation reached a maximal level at 45 min (Fig. 2A). Given that CA4 stimulates ERK1/2 and U0126 is a MEK inhibitor, we investigated whether the MEK-EKR pathway was involved in the observed microtubule reassembly. To test this, we assessed the effect of PD0325901, another specific MEK inhibitor, on microtubule reassembly in CA4-treated cells. Our results revealed that PD0325901 inhibited CA4-stimulated ERK1/2 activation...
more potently than U0126; however, unlike U0126, PD0325901 failed to block microtubule reassembly (Fig. 2C). These results sufficiently exclude the possibility that ERK1/2 activation leads to the observed microtubule reassembly and clearly demonstrate that U0126 blocks microtubule reassembly independently of MEK inhibition.

**U0126 Inhibits CA4 Inactivation.** It is quite surprising that disassembled microtubules are able to reassemble in the presence of CA4. Because it has been reported that withdrawal of microtubule-disrupting agents could trigger microtubule reassembly (Feijoo et al., 2005), it is possible that microtubule reassembly during CA4 treatment may result from CA4 inactivation.

To address this possibility, we first examined the concentration change of active CA4 in cell culture media. Bel-7402 cells were treated with 1 μM CA4 for different times, and the medium supernatants were removed and used to treat intact cells in other wells for 1 h. We found that increasing treatment time was associated with a gradual decrease in the ability of CA4-containing supernatants to induce microtubule disassembly (Fig. 3A), suggesting that CA4 gradually lost its activity upon incubation with BEL-7402 cells. Moreover, treatment with U0126, but not PD0325901, significantly inhibited this time-dependent inactivation of CA4 (Fig. 3, B and C). Even when the incubation time was increased up to 12 h, the U0126-containing supernatant could still induce microtubule dissociation in intact cells within 1 h (Fig. 3C).

Next, we investigated CA4 inactivation in a cytotoxicity assay, comparing the cytotoxicity of CA4-containing cell culture supernatants with that of freshly prepared CA4. If the CA4 in the cell culture supernatant is inactivated, its cytotoxicity should be reduced; therefore, this measure should be useful for assessing the extent of CA4 inactivation. These studies were performed in gastric SGC-7901 cells, which are sensitive to CA4 (IC₅₀ = 6.4 nM) but strongly resistant to U0126 and PD0325901. As shown in Fig. 3D, the cytotoxicity of CA4 decreased rapidly with increasing incubation time, until more than 90% of the CA4 became inactive after incubation with BEL-7402 cells for 4 h (Fig. 3D). U0126, but not PD0325901, significantly blocked this CA4 inactivation, with CA4-induced cytotoxicity increasing from 26 to 76% in the presence of U0126 (Fig. 3D). Consistent with this finding, the presence of U0126 decreased the minimal concentration of CA4 required to prevent microtubule assembly in BEL-7402 cells from 0.4 μM (Fig. 1A) to 0.025 μM (Fig. 3E). In contrast, U0126 failed to lower the minimal concentration of CA4 required to induce microtubule disassembly in NCI-H460 cells (Fig. 3F), in which CA4 is not readily inactivated (Fig. 5B).

Finally, we confirmed the inactivation of CA4 using HPLC/MS. Our results revealed that CA4 was metabolized to CA4 glucuronide in BEL-7402 cells and that U0126 inhibited this glucuronidation (Fig. 4A). For example, 96% of supernatant CA4 was converted to CA4 glucuronide after incubation with cells for 5 h, whereas this proportion was only 46% in the presence of U0126 (Fig. 4A). In addition, the LC-MS/MS experiments showed that CA4 glucuronide could fragment

![Figure 3](https://example.com/fig3.png)

**Fig. 3.** U0126 inhibits CA4 inactivation in BEL-7402 cells. A, BEL-7402 cells were treated with 1 μM CA4 for different times, and then the culture supernatants were used to treat intact cells in other wells for 1 h. B, BEL-7402 cells were treated with 1 μM CA4 in the presence of 100 nM PD0325901 for different times, and then the culture supernatants were used to treat intact cells in other wells for 1 h. C, BEL-7402 cells were treated with 1 μM CA4 in the presence of 10 μM U0126 for different times, and then the culture supernatants were used to treat intact cells in other wells for 1 h. D, BEL-7402 cells were treated with 1 μM CA4 in the presence of 10 μM U0126 and PD0325901 for different times, and a SRB assay was used to assess the cytotoxicity of the culture supernatants against SGC-7901 cells during a period of 72 h. The proportion of active CA4 was determined by the ratio of the IC₅₀ value obtained from supernatant to that obtained from freshly prepared CA4. BEL-7402 (E) or NCI-H460 (F) cells were exposed to different concentrations of CA4 in the presence of 10 μM U0126 for 1 h. For A, B, C, E, and F, cell lysates were extracted as described under Materials and Methods, and polymeric tubulin levels were determined by Western blotting using an antibody specific for β-tubulin. Data shown are representative of three independent experiments. PD, PD0325901.
into CA4, verifying that CA4 glucuronide is the metabolite of CA4 in BEL-7402 cells (Fig. 4B).

We next investigated whether CA4 inactivation is a general phenomenon in tumor cells. In a screening of 10 tumor cell lines, we found that CA4 was inactivated in human ovarian cancer 3AO cells, human hepatocellular carcinoma SMMC-7721 cells, and human epithelial carcinoma KB cells. It is notable that U0126 effectively inhibited CA4 inactivation in all of these cell lines (Fig. 5A). However, CA4 was not inactivated by several other tumor cell lines, retaining more than 90% activity after incubation for 4 h with human non-small-cell lung cancer NCI-H460 cells, human breast cancer MDA-MB-231 and SK-BR-3 cells, human colon cancer HCT116 and DLD-1 cells, and human gastric cancer SGC-7901 cells (Fig. 5B). This suggests that CA4 inactivation is cell line-specific. Consistent with this notion, we found that disassembled microtubules only reassembled in cells in which CA4 was inactivated, including 3AO, SMMC-7721, KB, and BEL-7402 cells, but not in cells in which CA4 kept active, such as in NCI-H460 and MDA-MB-231 cells (Fig. 5C). It is most important to note that both microtubule reassembly and CA4 inactivation were effectively blocked by U0126.
U0126 in the former cells (Fig. 5C). Taken together, these results suggest that CA4 inactivation by some cell lines results in microtubule reassembly and that U0126 blocks microtubule reassembly by inhibiting CA4 inactivation in these cells.

**U0126 Enhances CA4-Induced G2/M Cell-Cycle Arrest and Cytotoxicity in BEL-7402 Cells.** U0126 potentiates CA4-induced microtubule disassembly by inhibiting CA4 glucuronidation, which may have an impact on cell survival and proliferation. To test this possibility, we examined the effects of U0126 on CA4-induced cell-cycle arrest. As shown in Fig. 6, treatment of BEL-7402 cells with either 1 µM CA4 or 10 µM U0126 alone did not induce G2/M arrest; the percentages of G2/M cells were 14, 15, and 13% in control, CA4- and U0126-treated cells, respectively (Fig. 6). However, cells cotreated with both CA4 and U0126 significantly accumulated in G2/M phase (78% of cells; Fig. 6). In contrast, PD0325901 treatment did not enhance CA4-induced G2/M cell-cycle arrest (Fig. 6), nor did treatment with other protein kinase inhibitors, including the phosphatidylinositol 3-kinase inhibitor LY294002 and the Rho kinase inhibitor Y27632 (data not shown). Taken together, our results indicate that U0126, but not PD0325901, significantly enhances CA4-induced G2/M cell-cycle arrest.

As noted above, CA4 inactivation by glucuronidation is cell line-specific. Consistent with this observation, we further found that U0126 enhanced the cytotoxicity of CA4 only for cells in which CA4 glucuronidation occurs (Table 1). In contrast, the MEK inhibitor PD0325901 failed to enhance the cytotoxicity of CA4 in these cells (data not shown), reconfirming that this effect of U0126 is independent of MEK.

**Discussion**

At present, U0126 is widely used as a selective inhibitor of MEK for studies of MAPK/ERK signaling. Here, we provide evidence that U0126 significantly enhances CA4-induced cytotoxicity independently of MEK inhibition. These findings call into question the selectivity of U0126 as an MEK inhibitor and raise the possibility that U0126 may have another important target besides MEK. U0126 enhances CA4-induced cytotoxicity by inhibiting CA4 glucuronidation, suggesting that UDP-glucuronosyltransferase may be a potential target of U0126. Moreover, for the first time, we studied herein the metabolism of CA4 in cultured tumor cells. This may prove to be a useful model for in vitro studies on the relationship between pharmacodynamics and pharmacokinetics for anticancer agents similar to CA4. Our findings collectively demonstrate that great caution should be exercised when interpreting data obtained using U0126 as a specific MEK inhibitor or when CA4 is combined with inhibitors of glucuronidation in clinical practice. It is most important to note that our findings indicate that the combination of CA4 with inhibitors of glucuronidation may be a novel and rational strategy for cancer therapy.

It has been suggested that dephosphorylation of CA4P into its active form, CA4, occurs relatively faster (Tozer et al., 1999), whereas glucuronidation of CA4 is much slower in tumor tissues than in plasma (Rustin et al., 2003; Kirwan et al., 2004). This makes CA4P preferentially target tumors. However, we show here that CA4 glucuronidation can also occur rapidly in some cultured tumor cells, including BEL-7402, SMCC-7721, 3AO, and KB cells. These results suggest that CA4 may be rapidly metabolized to its inactive form in patients with certain tumor types, which could explain some failures of CA4 treatment. Therefore, we suggest that it may be useful to screen tumors for their CA4 glucuronidation potential, perhaps by testing the activity of UGT, which catalyzes CA4 glucuronidation. This information may be helpful for designing more rational treatment modalities. In addition, considering that U0126 inhibits CA4 glucuronidation and subsequently enhances CA4-induced cytotoxicity, we believe that combining CA4P with U0126 or UGT substrates may increase the treatment efficacy for certain tumors in which CA4 glucuronidation occurs. Indeed, our unpublished data show that quercetin, a substrate of UGT (Boersma et al., 2002), competitively inhibits CA4 glucuronidation and enhances the cytotoxicity of CA4 for cells in which CA4 glucuronidation occurs (data not shown). However, such combined therapies may also enhance the accumulation of active CA4 in normal tissues, causing additional side effects. Therefore, great caution should be taken when CA4 is combined in clinical practice with agents that can act as substrates of UGT, such as paracetamol, morphine, 4-methylumbelliferone, bilirubin, estradiol, and testosterone (Lohr et al., 1998; McGurk et al., 1998).

Glucuronidation, which is mainly catalyzed by UGT, is one of the key processes in human metabolic catabolism (Miners and Mackenzie, 1991). UGT catalyzes the conversion of lipophilic molecules into more polar and hydrophilic glucuronides, thereby facilitating the subsequent elimination of metabolites via bile, feces, and urine. In humans, CA4P is rapidly dephosphorylated to CA4, which is then metabolized, with CA4 glucuronide being the major biotransformation product (Rustin et al., 2003; Stevenson et al., 2003). In plasma, the area under the concentration-time curve for CA4 glucuronide is much higher than that for CA4, and its clearance is predominantly via urinary excretion. The precise mechanism by which U0126 inhibits CA4 glucuronidation was not known yet. Our observation that incubation of U0126 with BEL-7402 cells for 8 h does not attenuate the activity of U0126 excludes the possibility that U0126 competitively inhibits CA4 glucuronidation by acting as a substrate of UGT (data not shown). In addition, CA4 is rapidly glucuronidated in BEL-7402 cells and U0126 inhibits CA4 glucuronidation immediately, so it seems unlikely that U0126 inhibits CA4.
glucuronidation through down-regulating UGT within such a short time. Therefore, U0126-induced inhibition of CA4 glucuronidation may result from U0126-mediated inhibition of UGT activation, direct inhibition of UGT activity, or inhibition of other factors that are important to CA4 glucuronidation.

Drug resistance is a major obstacle to the efficacy of chemotherapeutic agents. Several resistance mechanisms have been described for microtubule-targeting agents. One important mechanism is P-glycoprotein-mediated efflux (Szakács et al., 2006). The majority of microtubule-targeting agents, such as paclitaxel, docetaxel, vinblastine, vinorelbine, and colchicine, are all substrates of P-glycoprotein and are easily transported from the cytoplasm to the extracellular media. Other mechanisms for resistance to microtubule-targeting agents include tubulin mutations and altered expression of tubulin isotypes (Shalli et al., 2005; Wang et al., 2006). For CA4, one previous report suggested that decreased expression of class III β-tubulin led to CA4 resistance in NCI-H460 cells (Welbe et al., 2005). Here, we describe another mechanism for CA4 resistance. CA4 is rapidly metabolized to CA4 glucuronide in BEL-7402, SMMC-7721, 3AO, and KB cells, significantly decreasing the accumulation of active CA4 in cells. Thus, cells with the ability to metabolize CA4 may readily acquire resistance to CA4. Because CA4 is not susceptible to P-glycoprotein-mediated efflux, glucuronidation-mediated resistance may be a major mechanism for CA4 resistance.

It has been suggested that combined treatment with MEK inhibitors plus microtubule-targeting agents may be a feasible approach for anticancer therapy. For example, MEK-specific inhibitors such as U0126 and PD98059 have been shown to enhance paclitaxel- or docetaxel-induced cell death in breast, ovarian, and lung cancer cell lines, as well as in prostate cancer cells (MacKeigan et al., 2000; Zelivianski et al., 2003). Similar effects have also been observed with vinblastine (Stadheim et al., 2001). Here, we further demonstrate that combined treatment with U0126 plus CA4 dramatically enhances the cytotoxicity of CA4. However, there are significant mechanistic differences between those noted in the prior papers and in the present report. MEK inhibitors enhance the anticancer efficacy of the microtubule-targeting agents discussed in the other reports through direct inhibition of the MAPK pathway. In contrast, the inhibitor examined in this report, U0126, enhances CA4-induced cytotoxicity independently of MEK inhibition. This is mainly shown in the fact that PD3235901, a more specific inhibitor of MEK, fails to mimic the effect of U0126. Although U0126 has been widely used as a selective MEK inhibitor in a variety of studies, it is also known to exhibit other functions unrelated to MEK inhibition (Kamakura et al., 1999; Andrieux et al., 2004; Dokladda et al., 2005; Yuan et al., 2006). Among them, the best studied is the ability of U0126 to inhibit MKK5, a protein kinase that is closely related to MEK, and is involved in the MKK5-ERK5 pathway (Kamakura et al., 1999). However, the MKK5-ERK5 pathway does not seem to be involved in the process of microtubule reassembly, as shown by the inability of the MEK inhibitor PD98059, which also inhibits the MKK5-ERK5 pathway (Kamakura et al., 1999), to affect microtubule reassembly (data not shown). Therefore, on the basis of our results, we suggest that U0126 may have another important target besides MEK and MKK5. Because PD3235901 is a more selective and potent inhibitor of MEK than U0126, we recommend that future MEK-focused studies should use PD3235901, not U0126, as a specific MEK inhibitor.

In summary, we show herein that U0126 significantly enhances CA4-induced cytotoxicity for tumor cells in which CA4 is metabolized to CA4 glucuronide and that it confers this effect by inhibiting CA4 glucuronidation. Our finding that the U0126-induced enhancement of CA4-induced cytotoxicity is independent of MEK inhibition suggests that U0126 may have another target besides MEK, indicating that great caution should be exercised in interpreting data obtained using U0126. Furthermore, our results indicate that the combination of U0126 with CA4 may represent a rational and promising strategy for cancer therapy.

References
U0126 Enhances the Cytotoxicity of CA4 Independently of MEK


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