Combretastatin A4-Induced Differential Cytotoxicity and Reduced Metastatic Ability by Inhibition of AKT Function in Human Gastric Cancer Cells

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ABSTRACT

Combretastatin A4 (CA4) is a drug that targets tumor vasculature to inhibit angiogenesis. Whether CA4 has a direct effect on gastric cancer is not known. We herein investigated the effect of CA4 on growth and metastasis of gastric cancer cells at clinically achievable concentration and explored the associated antitumor mechanisms. Nine human gastric cancer cell lines, including two metastatic gastric cancer cell lines (AGS-GFPM1/2), constitutively expressing green fluorescence protein (GFP) were used. These metastatic AGS-GFPM1/2 cells expressed a higher level of phosphorylated serine 473 on AKT (p-AKT). Our results showed that CA4 (0.02–20 μM) has significant in vitro effects on reducing cell attachment, migration, invasiveness, as well as cell cycle G2/M disturbance on p-AKT-positive gastric cancer cells. In addition, a phosphoinositide 3-kinase inhibitor, LY294002 [2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride], a specific AKT inhibitor, and 0.2 to 20 μM CA4 displayed a similar response profile on p-AKT-positive cells, suggesting that CA4-induced effect was mediated by inhibition of the PI3 kinase/AKT pathway. The results from in vivo GFP monitoring system indicated that CA4 phosphate (CA4-P; 200 mg/kg) significantly inhibited the s.c. and intra-abdominal growth of xenotransplanted AGS-GFPM2 cells in nude mice. Furthermore, CA4-P treatment showed a remarkable ability to inhibit gastric tumor metastasis as well as attenuate p-AKT expression. In conclusion, our study is the first to find that CA4 inhibited AKT activity in human gastric cancer cells. The decreased AKT activity correlated well with the CA4 antitumor growth response and decrease of metastasis. Further investigation on drugs targeting the PI3 kinase-AKT pathway may provide a new approach for the treatment of human gastric cancer.

Gastric cancer is one of the prevalent cancers in the Asia Pacific region, particularly in Korea, Japan, and Taiwan. It is the second most common cancer in the world (Ferlay et al., 2001). According to statistics, early detection of stage I gastric cancer patients have a 5-year survival rate over 88%. The overall incidences of lymph node metastasis, liver metastasis, and peritoneal metastasis are 47.8, 4.5, and 11.5%, respectively (Thompson et al., 1993; Ferlay et al., 2001). Peritoneal dissemination is often noted in patients at recurrence after primary treatment of the gastric cancer (Soga, 2005). Meanwhile, tumor resection could also induce increased expression of cytokines, such as hepatocyte growth factor, leading to tissue repair and reorganization. Hepatocyte growth factor facilitates migration of tumor cells to distant sites by AKT activation in gastric cancer cells (Trusolino et al., 2001). AKT functions as a signaling transducer of another important metastasis-related receptor protein (HER2) in breast cancer (Benovic and Marchese, 2004). Thus, an increasing

ABBREVIATIONS: CA4, combretastatin A4; p-AKT, phosphorylated serine 473 on AKT; GFP, green fluorescence protein; FBS, fetal bovine serum; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride; PVDF, polyvinylidene difluoride; PI3 kinase, phosphoinositide 3-kinase; CA4-P, combretastatin A4-phosphate; PHA-665752, (3S)-5-[[2,6-dichlorobenzyl][sulfonyl]-3-[[3,5-dimethyl-4-[[2(R)-2-(pyrrolidin-1-yl)methyl][pyrrolidin-1-yl]carbonyl]-1H-pyrrol-2-yl][methylen]-1,3-dihydro-2H-indol-2-one.

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number of studies is focusing on targeting AKT-related pathways to restrain tumor dissemination (Larue and Bellacosa, 2005; Yoeli-Lerner and Toker, 2006).

Combretastatin A4 (CA4), isolated from the African tree Combretum caffrum, demonstrated to inhibit tubulin polymerization at colchicines-binding site of β-tubulin (Woods et al., 1995; Liou et al., 2004). Using CA4 alone or combined with radiation or chemotherapeutic agents is the remedy for a variety of tumors (Tozer et al., 1999; Boehe et al., 2001; Young and Chaplin, 2004; Badn et al., 2006). The proposed action mechanism of CA4 is focused on tumor vasculature shrinkage and reducing tumor perfusion after treatment for 30 min to 6 h (Dziba et al., 2002; Anderson et al., 2003). Recent study demonstrated that CA4 inhibits endothelial cell migration and capillary tube formation through disruption of vascular endothelial-cadherin, β-catenin, and AKT signaling pathway, thereby leading to rapid vascular collapse and tumor necrosis (Vincent et al., 2005). However, whether the treatment of CA4 can directly inhibit AKT activity in gastric tumor cells and subsequently lead to decreased tumor cell growth and reduce tumor cell dissemination was still unclear.

In this study, we attempted to elucidate the possible action mechanism of CA4 on gastric cancer cell metastasis rather than vascular endothelial cells. Our results demonstrated that CA4 inhibited the growth of nine human gastric cancer cell lines with submicromolar IC_{50}. Interestingly, a trend that gastric cancer cells with the phosphorylated serine 473 on AKT (p-AKT) expression tend to be more sensitive to CA4 treatment in terms of cell viability is emerging. We found that CA4 inhibited AKT activation, and the differential cytotoxicity correlated well with p-AKT in positive and negative cell lines. Furthermore, inhibition of p-AKT by CA4 resulted in decreased cell proliferation, cell cycle arrest, and reduced in vitro migration/invasiveness and in vivo metastatic ability. These results suggest that activation of p-AKT is an important molecular event in the metastasis of gastric cancers, and inhibition of this oncogenic pathway by CA4 reduces metastasis.

Materials and Methods

Chemicals and Human Cancer Cell Culture. Gentamycin, G418, and Lipofectamine Plus were from Invitrogen (Carlsbad, CA). BCA protein assay and chemiluminescence detection kits were from Pierce Biotechnology (Rockford, IL). 3-(4,5-Dimethylthiazolo-1-2-y)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (Promega, Madison, WI), AKT (catalog no. 124005; Calbiochem, San Diego, CA), and CA4 and CA4 phosphate were synthesized as described previously (Tozer et al., 1999). Human gastric cancer cell lines SC-M1 (from Dr. C. L. Meng), NUGC-3 (from Dr. M. Sekiguchi), MKN1, MKN45, and MKN74 (Japanese Collection of Research Bioresources, Tokyo, Japan), and AGS (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 (Invitrogen) containing 10% fetal bovine serum (Invitrogen) and 10 μg/ml gentamycin in a CO_{2} incubator at 37°C, with 5% CO_{2} and 95% filtered air.

Establishment of Metastatic Human Gastric Cancer Cells with Stable Green Fluorescence Protein Expression in Nude Mice. AGS cells were transfected with the cDNA plasmid of green fluorescence protein (GFP) and selected for stably expressing GFP cells. Briefly, AGS cells were cultured in RPMI 1640 medium for 24 h at a cell density of 1 × 10⁶ in a six-well plate. GFP DNA plasmid (2 μg; pEGFP-C1 vector; BD Biosciences Clontech, Palo Alto, CA) formulated with Lipofectamine 2000 was delivered to the AGS cells. G418 sulfate was applied (400–2000 μg/ml) to select GFP-positive AGS cells. Two metastatic AGS-GFP (AGS-GFP1 and AGS-GFP2) cell clones were selected from the liver metastatic foci of AGS-GFP cells i.v. injected in nude mice.

Gastric Cancer Cell Viability and Mobility Evaluation. Cells were cultured in a 96-well cell culture cluster at a density of 3 × 10⁵ cells/well in 100 μl of medium. After the drug treatment for 24 h, the medium was discarded and replaced with an equal volume (100 μl) of fresh medium containing 0.2 mg/ml 3(4,5-dimethylthiazolo-1-2-y)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium and incubated for an additional 1.5 h. Cell growth was proportional to optical density (490 nm) value that was measured by colorimetric assay. The IC_{50} of each compound that inhibits 50% of cell growth activity was then determined. To evaluate the mobility of cancer cells, cells were seeded on a 24-well plate with 2 × 10⁵ cells/well for 24 h, and then a sterile plastic scraper was used. To quantitatively analyze the fluorescence intensity of the scratched area, the percentage of fluorescence signal per photographed field was analyzed by Image process software (Image-Pro Plus; Media Cybernetics, Inc., Silver Spring, MD).

In Vitro Cell Invasion Analysis. The 24-well plate Transwell system with a polycarbonate filter membrane of 8-μm pore size was used. The filter membrane was coated with Matrigel (BD Biosciences, San Jose, CA) and 400 μg/ml serum-free RPMI 1640 medium 100 μl/well and incubated overnight at 37°C. Cells were seeded to the upper compartment of the Transwell chamber at a cell density of 2 × 10⁵ in 100 μl of serum-free RPMI 1640 medium. The lower chamber was filled with 10% PBS-containing RPMI 1640 medium with or without LY294002 (Sigma-Aldrich, St. Louis, MO). After a 24-h incubation period, the cells remaining on the upper surface of the filter membrane were removed, and the cells on the opposite surface of the filter membrane were stained with hematoxylin for 1 h. The migrated cells were then visualized and counted from five different viewing areas of 100-fold magnification under an inverted microscope.

Cell Cycle Analysis. Both floating and adhesive cells were collected. Around 1 × 10⁶ cells were added with 500 μl of lysing buffer (0.5% Triton X-100, 0.2 μg/ml Na_{2}EDTA 2H_{2}O, and 1% bovine serum albumin in phosphate-buffered saline), and cells were stained with a DNA staining solution (50 μg/ml propidium iodide and 5 μ/ml RNase A). The DNA content of the stained cells was measured using a FACSCalibur flow cytometer (BD Biosciences). Cell cycle data from flow cytometry were obtained and analyzed by CellQuest and Modfit LT software, respectively.

Western Blot Analysis. The cells were lysed in the lysis buffer (20 mM Tris buffer, pH 7.5, 1 mM EDTA, 100 μM phenylmethylsulfonyl fluoride, 2 μg/ml aprotonin, 2 μg/ml leupeptin, and 2 μg/ml leupeptin) for 30 min at 4°C. The protein level was quantified using the BCA protein assay kit. Cell lysates containing an equal amount (25 μg) of total protein were separated by 10 or 12.5% SDS-polyacrylamide gel electrophoresis. The loaded protein samples were then transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, Billerica, MA). The PVDF membrane with transferred proteins was blocked with 5% nonfat dry milk in Tris-buffered saline for 1 h and incubated overnight with anti-AKT (1:2500, SKB1; Upstate Biotechnology, Lake Placid, NY) and p-AKT (1:500; Cell Signaling Technology, Beverly, MA). After washing, anti-mouse or anti-rabbit secondary antibody conjugated with horseradish peroxidase was added (1:5000; Pierce Biotechnology) for incubation for 1 h. The horseradish peroxidase activity was detected by incubating the membrane with enhanced chemiluminescence reagent (Pierce ECL WB). The image was visualized by developing on BioMax X-ray film. Cellular β-actin (1:5000, AC-15; Sigma-Aldrich) was also immunodetected on PVDF membrane and served as an internal standard of each sample.

In Vivo Analysis of the Effect of Combretastatin A4 on Tumor Metastasis. All the animal practices were in accordance with the institutional animal welfare guideline of Taipei Veterans
General Hospital. AGS-GFPM2 cells were injected into the s.c. and gastric sites of BALB/c nude mice aged 8 weeks. Each nude mouse received $5 \times 10^6$ and $1 \times 10^7$ cells on s.c. and abdominal gastric sites, respectively. After the s.c. tumors grew to around $3 \times 3 \text{mm}$ (in diameter) in size and were examined by GFP signals, CA4 phosphate (CA4-P) was administered by i.p. injection (100 or 200 mg/kg) at day 0 in a volume of 300 ml. The CA4-P treatment was then given to the mice on days 3 and 6 for a total of three times. In vivo GFP imaging was visualized and measured by an illuminating device (LT-9500 Illumatool TLS equipped with excitation illuminating source (470 nm) and filter plate (515 nm)). The tumor size was measured by a caliper, and the volume was calculated according to the formula: $(\text{length} \times \text{width}^2)/2$. The integrated optical density of green fluorescence intensity was captured and then analyzed by Image-Pro Plus software. Phosphorylated AKTs were analyzed in the tumor from control and CA4-P-treated groups using immunohistochemical assay. The anti-phosphorylated AKT (Cell Signaling Technology, Inc.) antibody was used. Procedures of deparaffinization, rehydration, antigen retrieval, and immunohistochemistry were performed as described previously (Chiou et al., 2006).

**Statistical Analysis.** The results were reported as mean $\pm$ S.D. Statistical analysis was performed using Student’s $t$ test or the one- or two-way analysis of variance test followed by Tukey’s test, as appropriate. $p < 0.05$ was considered to be statistically significant.

**Results**

**Combretastatin A4 Has a Preferential Antiproliferation Effect on p-AKT-Positive Gastric Cancer Cells.** In this study, six human gastric cancer cell lines (MKN1, MKN45, MKN74, AGS, SC-M1, and NUGC-3) and four human gastric cancer cell lines (MKN45-GFP, AGS-GFP, AGS-GFPM1, and AGS-GFPM2) with stable expression of GFP were investigated for AKT and p-AKT expression. It has been reported that AKT expression correlated with tumor cell migration on glioblastoma cells including a human U87MG cell line (Koul et al., 2005). Figure 1, A and B, shows a significant level of p-AKT expression in U87MG cells. Among the 10 gastric cancer cell lines, it was found that MKN45, MKN45-GFP, AGS, AGS-GFP, and SC-M1 had low or non-detectable p-AKT expression, whereas MKN1, MKN74, NUGC-3, AGS-GFPM1, and AGS-GFPM2 were found to have relatively higher p-AKT expression. We further evaluated the cytotoxic and antiproliferation activities of CA4 on nine human gastric cancer cell lines. Given the results of p-AKT expression (Fig. 1, A and B), the nine cell lines were separated into p-AKT-positive and p-AKT-negative groups. From the analysis of dose response of CA4 treatments on different p-AKT expression cell lines, it was observed that the most sensitive p-AKT-positive cell lines were NUGC-3 and AGS-GFPM1/2, with as low as a 0.02 $\mu$M growth-inhibitory concentration after 24-h CA4 treatment (Fig. 1C). In contrast to the p-AKT-positive group, there was no significant inhibitory effect on the growth of p-AKT-negative cells at 0.02 $\mu$M of 24-h CA4 treatment (Fig. 1D). It was found that all cell lines

**Fig. 1.** Detection of phosphorylated AKT expressions on human gastric cancer cell lines. The protein expression of p-AKT and total AKT were examined by Western blot assay. Human glioblastoma cell line (U87MG) was a positive control of p-AKT expression (A and B). The cell viability of human gastric cancer cell lines treated with CA4. The 24-h treatment effects of CA4 in five human gastric cancer cell lines with high p-AKT expression are shown (C). The same experimental condition was applied on low or nondetectable p-AKT expression gastric cancer cell lines (D). The attachment activity of AGS-GFP, AGS-GFPM1, and AGS-GFPM2 cells treated with chemotherapeutic agents. The cell suspension was incubated with different compounds in test tubes and then plated on cultured dishes for 6 h. Nonattached cells were aspirated, and adherent cells were visualized by microscopy. The concentration of each compound used was 0.02 $\mu$M (E).
with p-AKT-positive expression appeared significantly more sensitive to 0.02 μM CA4 than p-AKT-negative cell lines (Fig. 1, C and D).

**Combretastatin A4 Affected Human Gastric Cancer Cell Attachment and Mobility.** To examine the status of tumor cells attachment in vitro, a cultured system of cell attachment was employed. We have found that the morphology of AGS-GFP, AGS-GFPM1, and AGS-GFPM2 cells was rounded up as the CA4 dose increased to 0.02 μM at 24-h after CA4 treatment, but a low dose (0.002 μM) had no effect compared with the spread-out and well attached vehicle-treated cell lines (data not shown). At the same concentration (0.02 μM) treatment, less morphological changes were observed in 5-fluorouracil-, cisplatin-, and cyclophosphamide-treated cells (Fig. 1E). To further quantitatively evaluate the effect of CA4 on the gastric cancer cell mobility, GFP-expressed AGS-GFPM1/2 and other cell lines were employed on the scratch experiments (Fig. 2). Cell migration ability was

**Fig. 2.** Migration analysis on AGS-GFPM1 and AGS-GFPM2 cells treated with different chemotherapeutic compounds. AGS-GFPM2 cells migrated from scratch (A, bright field; B, fluorescence field) in the presence of CA4 treatment (E–H) or vehicle alone (C, bright field; D, fluorescence field) for 24 h. The migration activity of AGS-GFPM1 and AGS-GFPM2 cells were evaluated by scratched assay after 6- and 24-h CA4 treatment (I). After the treatment of different chemotherapeutic compounds, the migration abilities of AGS-GFPM1 and AGS-GFPM2 cells were determined (I–K). The effect of CA4 on migration ability of four cell lines is shown in L.
calculated as the ratio of integrated optical area over the dark field of the scratch site. The ratio of fluorescent signal was 0.2 at 0 h (Fig. 2, A and B) at the generation of scratch. After 6 h of incubation with 10% FBS medium, GFP-positive cells began to migrate from the scratch (Fig. 2I). At 24 h, the gap was closed at control group (Fig. 2D), but the gap junction at the CA4-treated cells had a dose-dependent inhibitory effect (Fig. 2, E–H). Our results demonstrated that CA4 could effectively inhibit AGS-GFPM1 and AGS-GFPM2 migration as compared with cisplatin, 5-fluorouracil, or cyclophosphamide treatment at a dose range of 0.02 to 2 μM. However, there was no significant difference for all compounds at the 0.002 μM treatment dosage (Fig. 2, J and K). The other four wild-type cell lines were also investigated for CA4-induced antimigration effect. Figure 2L shows that CA4 reduced AGS and NUGC-3 cells migration at the concentration of 0.02 μM, but CA4 had only a minor effect on the less migratory MKN45 and SC-M1 cells.

**Combretastatin A4 Inhibited Human Gastric Cancer Cell Invasion in Vitro.** As shown in Fig. 3, the data revealed that 24-h CA4 treatment significantly decreased the invasion of the p-AKT-positive cell lines (AGS-GFPM1, AGS-GFPM2, and MKN1) on the Transwell with a dose-responsive effect (Fig. 3, A and B). Our results also showed that LY294002 at 0.2 to 20 μM concentration significantly decreased tumor invasiveness of both AGS-GFPM1 and AGS-GFPM2 cells (Fig. 3C). To determine whether blocking AKT expression or reducing p-AKT levels would affect the activity of CA4 treatment, the specific AKT inhibitor (catalog no. 124005; Calbiochem) was used to inhibit AKT activity. The results showed that similar treatment effects were observed between CA4 and AKT inhibitor on AGS-GFPM1, AGS-GFPM2, and MKN1 cells (Fig. 3, B and D). More importantly, our results demonstrated that CA4 combined with the AKT inhibitor did not have significant or additive effects (Fig. 3E). These findings supported that CA4 inhibited the invasion ability of human gastric cells mainly through inhibition of the activation of AKT pathway.

**Combretastatin A4 Inhibited p-AKT Expression in Human Gastric Cancer Cells.** Figure 4A shows that CA4 inhibited p-AKT expression in a dose-dependent manner on AGS-GFPM1 and AGS-GFPM2 cells (Fig. 4A). To investigate the time sequence of CA4 effects on the reduction of p-AKT expression, two different doses (1 nM and 1 μM) of CA4 were applied on AGS-GFPM1 and AGS-GFPM2 cells. It was observed that the high dose (1 μM) inhibited p-AKT expression initially after 2 h of incubation and sustained its effect for 24 h (Fig. 4B). The low dose (1 nM) of CA4 decreased the p-AKT expression on AGS-GFPM2 cells after a 24-h treatment (Fig. 4B). Similar results were observed in AGS-GFPM1 cells (data not shown). When the PI3 kinase inhibitor, LY294002, was applied on AGS-GFPM2 cells, it was observed that 1 to 20 μM LY294002 effectively reduced the level of p-AKT (Fig. 4C). Moreover, in an attempt to examine whether the observation of p-AKT inhibition by CA4 could also be found on other p-AKT-positive human gastric cancer

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**Fig. 3.** The effects of CA4, LY294002, AKT inhibitor, or CA4 with AKT inhibitor on cell invasiveness. A, AGS-GFPM1, AGS-GFPM2, and MKN1 cells were seeded on the upper chamber of Transwell system with serum-free medium, and the lower chamber was filled with 10% FBS medium. B to E, CA4, LY294002, AKT inhibitor, or CA4 with AKT inhibitor was added in the lower chamber with 10% FBS medium for 24 h. The migrated cells in the lower surface of the upper chamber were stained by hematoxylin. Quantitative analyses of the effect of CA4, LY294002, AKT inhibitor, or CA4 with AKT inhibitor on cell invasiveness are indicated in B, C, D, and E, respectively.
cell lines, MKN74 and NUGC-3 were employed. As shown in Fig. 4D, it was apparent that CA4 was equally effective in reducing the level of p-AKT in MKN74 and NUGC-3 cells. In contrast, one p-AKT-negative human gastric cancer cell line (AGS), which showed no signal of p-AKT in different concentrations of CA4 treatments, was included as negative control (Fig. 4D).

**Disturbance of Cell Cycle Profile after Combretastatin A4 Treatment.** Considering the well known molecular target (colchicines binding site of microtubule) of CA4, cell cycle analysis of AGS-GFPM1 and AGS-GFPM2 cells was conducted to evaluate G2/M phase perturbation. Our data showed that there was no significant G2/M phase arrest after the treatment of CA4 (0.02–20 μM) on the two cell lines 6 h after the CA4 treatment (Fig. 5). Nevertheless, an obvious G2/M phase arrest was found when the concentration of CA4 increased from 0.02 to 20 μM for 24 h on AGS-GFPM1 cells. As for AGS-GFPM2, less G2/M phase arrest cells (30.25%) were observed after 0.02 μM CA4 treatment for 24 h, compared with the CA4 treatment on AGS-GFPM1 cells (58.57%). Generally, the concentrations (from 0.02–20 μM) of CA4-induced cell cycle G2/M phase were comparable with their effect on reducing the p-AKT expression.

**CA4-P Reduced s.c. and Abdominal Tumor Growth on Nude Mice.** Since abdominal metastasis of human gastric cancer is the most frequent event for tumor recurrence, the AGS-GFPM2 cells were directly injected to the gastric site of nude mice. AGS-GFPM2 cells were inoculated simultaneously at s.c. site to monitor green fluorescent tumor growth in animals. Figure 6 and Table 1 show that CA4-P attenuated s.c. AGS-GFPM2 tumor growth on nude mice (regressed 44.23 and 86.41% tumor volume for 100 and 200 mg/kg CA4-P treatment, respectively). After examining abdominal metastasis of AGS-GFPM2 cells by green fluorescence detection, it was observed that CA4-P treatment (100 and 200 mg/kg) reduced the foci formation from 9.16 of control to 4.0 and 2.6, respectively, and size of abdominal tumor from 2.26 mm³ of control to 1.41 and 1.28 mm³, respectively, in the nude mice (Table 1). We further performed an immunohistochemistry analysis of p-AKT to elucidate the relationship of p-AKT in gastric cancer growth and the antiproliferation effect of CA4-P through attenuation of p-AKT activity in vivo. Consistent with the treatment effects of CA4-P in tumor growth, we observed that the expression levels of p-AKT in CA4-P-treated groups were significantly lower than those in control groups (p < 0.05; Fig. 7, A–C). Moreover, the expression levels of p-AKT in the abdominal metastasis foci of the non-CA4-treated group (control; Figs. 6C and 7B) were significantly higher than those in the s.c. lesion of the non-CA4-treated group (control; Figs. 6A, 7A, and 7C; p < 0.01). Moreover, the mean survival rate of CA4-P-treated group was significantly higher than that of the control group (p < 0.05; Fig. 7D). These results supported that the treatment of CA4-P not only exhibited an ability to inhibit gastric tumor growth, but also showed considerable antitumor effects against abdominal metastasis.

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**Fig. 4.** The effect of CA4 on the level of phosphorylated AKT (p-AKT) and total AKT (AKT) in human gastric cancer cell lines. A, AGS-GFPM1 and AGS-GFPM2 cells were treated with CA4 for 24 h, and p-AKT and AKT expression were determined by Western blot. B, time course response of CA4 (1 nM and 1 μM) on the level of p-AKT and total AKT in AGS-GFPM2 cells. C, effect of PI3 kinase inhibitor (LY294002) on p-AKT and AKT expression in AGS-GFPM2 cells. D, dose-dependent effect of CA4 on p-AKT expressions in MKN74, NUGC-3, and AGS human gastric cancer cells.
growth but also showed a remarkable attenuation of p-AKT as well as gastric cancer metastasis.

**Discussion**

In this study, our results showed that CA4 (0.02–20 μM) has significant effects on reducing gastric cancer cell attachment, migration, and invasiveness in vitro (Figs. 1–4). Using the two metastatic clones (AGS-GFPM1 and AGS-GFPM2) with high AKT activities for in vitro assays, we found that CA4 not only effectively inhibited cell growth but also specifically down-regulated the AKT activities in these two clones. Furthermore, we demonstrated that CA4 significantly suppressed gastric cancer (AGS-GFPM2) tumor formation and metastatic ability on both s.c. and gastric injected sites (Fig. 6; Table 1). p-AKT expression correlated well with cytotoxic response to CA4 as well as LY294002 treatment. This effect was significantly in CA4-treated p-AKT-positive gastric cancer cells (Figs. 1 and 3). This higher toxic response on p-AKT-positive gastric cancer cell lines was also accompanied with inhibition of p-AKT expression (Fig. 4). Importantly, the in vivo study further demonstrated that the treatment of CA4 exhibited a remarkable ability to inhibit gastric tumor growth and metastasis as well as attenuate p-AKT expression (Fig. 7). To our knowledge, at present, this is the first study regarding the competency of CA4 treatment on the inhibition of AKT activity as well as antitumor metastasis in gastric cancer therapy.

CA4 has been reported to be more effective with a nanomolar IC₅₀ on cultured and primary tumor cell lines (Young and Chaplin, 2004). However, there are still some conflicting results regarding the extent of tumor vessel shrinkage in animal models and clinical trials using the different concentration of CA4 treatment (Badn et al., 2006). With respect to administering safe dosages of 52 and 68 mg/m² in clinical trials, it has been revealed that peak plasma concentrations (Cₘₐₓ) of CA4 are 1.89 and 2.26 μM, respectively (Dowlati et al., 2002). In this study, our data showed that a 24-h treatment of CA4 on p-AKT-positive gastric cancer cells resulted in a dose-dependent (0.02–20 μM) inhibition effect on cells growth, migration, and invasiveness as well as G₂/M phase accumulation. In addition, these effects corresponded well with the level of p-AKT in CA4-treated p-AKT-positive cells.

**Table 1**

<table>
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<tr>
<th>Dose (μM)</th>
<th>AGS-GFPM1</th>
<th>6 Hours</th>
<th>24 Hours</th>
<th>AGS-GFPM2</th>
<th>6 Hours</th>
<th>24 Hours</th>
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<tbody>
<tr>
<td>0</td>
<td>G0/G1: 22.56%</td>
<td>60.91%</td>
<td>35.97%</td>
<td>G0/G1: 32.82%</td>
<td>64.10%</td>
<td>31.49%</td>
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<tr>
<td>0.002</td>
<td>G0/G1: 23.27%</td>
<td>60.91%</td>
<td>35.97%</td>
<td>G0/G1: 31.49%</td>
<td>64.10%</td>
<td>31.49%</td>
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<tr>
<td>0.2</td>
<td>G0/G1: 24.08%</td>
<td>60.91%</td>
<td>35.97%</td>
<td>G0/G1: 31.49%</td>
<td>64.10%</td>
<td>31.49%</td>
</tr>
<tr>
<td>2</td>
<td>G0/G1: 24.08%</td>
<td>60.91%</td>
<td>35.97%</td>
<td>G0/G1: 31.49%</td>
<td>64.10%</td>
<td>31.49%</td>
</tr>
<tr>
<td>20</td>
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<td>60.91%</td>
<td>35.97%</td>
<td>G0/G1: 31.49%</td>
<td>64.10%</td>
<td>31.49%</td>
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*Fig. 5. The effect of CA4 treatment on cell cycle progression of human gastric cancer cells. AGS-GFPM1 and AGS-GFPM2 were used to evaluate the cell cycle disturbance with the treatment of CA4 for 6 or 24 h. Each phase of cell cycle percentage was indicated in the graph of DNA histogram.*
Fig. 6. The effect of CA4-P on nude mice bearing s.c. and gastric tumors. AGS-GFPM2 cells were injected on s.c. and gastric sites of mice. After the tumor on s.c. site grew to $3 \times 3$ mm, CA4-P was administered by i.p. injection (day 0) at the concentration of 200 mg/kg. The mice received another two CA4-P administrations on days 3 and 6. The vehicle control for mice bearing tumors was phosphate-buffered saline. The GFP signals of s.c. (A and B) or intra-abdominal (C and D) tumors was detected after 35 days of xenotransplantation, and the image was visualized by GFP imaging system. White arrows, green fluorescent tumor foci distribution in the mice.

TABLE 1

The effect of CA4-P on s.c. and peritoneal AGS-GFPM2 tumor growth

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Animal No.</th>
<th>s.c. Tumor Volume</th>
<th>Abdominal (Gastric Site)</th>
<th>Size of Foci</th>
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</thead>
<tbody>
<tr>
<td>Vehicle (PBS)</td>
<td>6</td>
<td>4953.6 ± 1791.6</td>
<td>9.16 ± 1.24</td>
<td>2.26 ± 0.03</td>
</tr>
<tr>
<td>CA4-P (100 mg/kg)</td>
<td>6</td>
<td>2762.2 ± 1469.1</td>
<td>4.00 ± 0.63</td>
<td>1.41 ± 0.03</td>
</tr>
<tr>
<td>CA4-P (200 mg/kg)</td>
<td>5</td>
<td>673.4 ± 184.7</td>
<td>2.60 ± 0.50</td>
<td>1.28 ± 0.08</td>
</tr>
</tbody>
</table>

Fig. 7. Detection of p-AKT expressions in the tumor formation of AGS-GFPM2 cells in nude mice with or without the treatment of CA4-P. Representative results of immunohistochemistry staining of p-AKT expressions in the s.c. foci (A) and the multiple foci (B) of intra-abdominal metastatic tumor of nude mice without CA4-P treatment (arrows, positive for p-AKT; bar, 100 μm). C, expression levels of p-AKT were detected in the s.c. and intra-abdominal metastatic tumor of nude mice with or without CA4-P-treatment groups (*, $p < 0.05$; **, $p < 0.01$). D, survival analysis of tumor-bearing (AGS-GFPM2 cells) nude mice with or without CA4-P treatment ($n = 12$ for each).
positive cell lines are more sensitive to CA4 (0.2–20 μM) after a 48-h treatment, and a dose-dependent increase of cell detachment and apoptosis was observed (data not shown). This finding is consistent with the previous report that a 24-h incubation with CA4P did not induce endothelial cell death but significantly decreased the cell viability after a 48-h incubation with CA4P (Vincent et al., 2006). These results together suggest that CA4-inhibitory activities on AKT phosphorylation in human gastric cancer cells (0.2–2 μM) may be pharmacologically accessible clinically.

PI3 kinase/AKT pathway is well known to play many critical functions on tumorigenesis and tumor cell dissemination (Bader et al., 2005; Koul et al., 2005). PI3 kinase and AKT protein molecules have been drawing attention in targeting therapy (Brazil et al., 2004). Moreover, the mutations on PI3 kinase resulted in differential cytotoxicity toward LY294002 on human colorectal tumor cell lines (HCT116 and DLD1), suggesting cell lines with higher PI3 kinase/AKT activity were more susceptible to compounds targeting on this pathway (Kang et al., 2005). In this study, 10 human gastric cancer cell lines with different expression levels of p-AKT were correlated with the treatment effect of CA4 in the modulation of the expression level of p-AKT (Figs. 1–4). Using the phosphoinositide 3-kinase inhibitor (LY294002) and specific AKT inhibitor as control treatment, CA4 displayed a similar toxic response on p-AKT-positive cells (Fig. 3, B and D). These results supported that CA4 can be an effective anticancer drug as mediating by targeting on the PI3 kinase/AKT pathway. In addition, the upstream tyrosine kinase receptors of PI3 kinase/AKT pathway included epidermal growth factor receptor and hepatocyte growth factor receptor. It has been reported that attenuated hepatocyte growth factor receptor function by a small molecule-specific inhibitor (PHA-665752) led to reduced gastric cancer cell migration through abolishing AKT activity in gastric cancer cells (GTL-16) (Christensen et al., 2003). Thus, whether the p-AKT expression pattern could be considered as another patient recruitment criteria or surrogate marker of therapeutic response needs further investigation.

In conclusion, our study is the first to reveal a novel mechanism of CA4 on targeting and inhibiting AKT activity and improving the treatment in human gastric cancer cells. Our results implicated that this AKT activity correlated with antitumor activities and reduced cell metastatic functions in vitro and in vivo under CA4 treatment. Given the more selective inhibition of the PI3 kinase-AKT pathway by new agents, it is promising to investigate other compounds with improved solubility and/or oral bioavailability in terms of chemical scaffold of CA4.

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