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

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Running title: Combretastatin A4 inhibited AKT in gastric cancer cells

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Abbreviations: CA4, combretastatin A4; GFP, green fluorescence protein; EGFR, epidermal growth factor receptor; HGF, hepatocyte growth factor; HGFR, hepatocyte growth factor receptor; CA4-P, combretastatin A4-phosphate; MTS, 3-(4, 5-Dimethylthiazol 1-2-yl)-5-(3-carboxy methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PVDF, polyvinylidene fluoride; SDS-PAGE, sodium dodecyl sulfate
polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; IC_{50}, 50%

inhibitory concentration; IHC, immunohistochemistry

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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 anti-tumor mechanisms. Nine human gastric cancer cell lines including two metastatic gastric cancer cell lines (AGS-GFPM1/2) constitutively expressed green fluorescence protein (GFP) were used. These metastatic AGS-GFPM1/2 cells expressed a higher level of 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, PI3K inhibitor (LY294002), specific AKT inhibitor and 0.2-20 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 subcutaneous 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 anti-tumor growth response and decrease of metastasis. Further investigation on drugs targeting PI3 kinase-AKT pathway may provide a new approach for the treatment of human gastric cancer.
Introduction

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 incidence of lymph node metastasis, liver metastasis and peritoneal metastasis are 47.8%, 4.5% and 11.5%, respectively (Ferlay et al., 2001; Thompson et al., 1993). 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 (HGF), leading to tissue repair and re-organization. HGF 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 metastatic-related receptor protein (HER2) in breast cancer (Benovic and Marchese, 2004). Thus, an increasing number of studies is focusing on targeting AKT-related pathways to restrain tumor dissemination (Yoeli-Lerner and Toker, 2006; Larue and Bellacosa, 2005).

Combretastatin A4 (CA4), isolated from the African tree Combretum caffrum, demonstrated to inhibit tubulin polymerization at colchicines-binding site of \( \beta \)-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; Boehle 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 minutes to 6 hours (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 9 human gastric cancer cell lines with sub-micro molar IC₅₀. 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 (Life Technologies Inc). BCA protein assay & chemiluminescence detection kits (Pierce Biotechnology, Rockford, IL). 3-(4, 5-Dimethylthiazol 1-2-yl)-5-(3-carboxy methoxyphenyl)-2-(4-sulfophenyl)-2H- tetrazolium (MTS; Promega Corp). AKT (Cat. No. 124005; Calbiochem EMD Biosciences Inc). Combretastatin A4 (CA4) and CA4 phosphate were synthesized as described previously (Tozer et al, 1999). Human gastric cancer cell lines SC-M1 (from Dr. Meng CL), NUGC-3 (from Dr. Sekiguchi M), MKN1, MKN45, and MKN74 (the Japanese Collection of Research Bioresources, Japan) and AGS (American Type Culture Collection, Manassas, VA) were cultured in RPMI-1640 (GIBCO) containing 10% fetal bovine serum (GIBCO) and 10 μg/ml gentamycin in a CO2 incubator at 37°C, with 5% CO2 and 95% filtered air.

Establishment of Metastatic Human Gastric Cancer Cells with Stable GFP 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 hours at a cell density of 1x 10^5 in 6-well plate. GFP DNA plasmid 2 μg (pEGFP-C1 vector; CLONTECH Laboratories) 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-GFPM1 and AGS-GFPM2) cell clones were selected from the liver metastatic foci of AGS-GFP cells intravenously 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 3×10³ cells/well in 100 µl medium. After the drug treatment for 24 hours, the medium was discarded and replaced with an equal volume (100 µl) of fresh medium containing 0.2 mg/ml of MTS and incubated for an additional 1.5 hours. Cell growth was proportional to optical density (490 nm) value which was measured by colorimetric assay. The IC₅₀ 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 hours 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).

**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® (Becton Dickinson) 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 \(2 \times 10^5\) in 100 µl serum-free RPMI-1640 medium. The lower chamber was filled with 10% FBS-containing RPMI-1640 medium with or without LY294002 (Sigma). After a 24-hour incubation period, the cells remained on the upper surface of the filter membrane were removed and the cells on the opposite surface of the filter membrane was stained with hematoxylin for 1 hour. The migrated cells were then visualized and counted from 5 different viewing areas of 100 fold magnification under an inverted microscope.

**Cell Cycle Analysis.** Both floating and adhesive cells were collected. Around \(1 \times 10^6\) cells were added with 500 µl lysing buffer (0.5% Triton X-100, 0.2 µg /ml Na$_2$EDTA.2H$_2$O, and 1% bovine serum albumin in PBS) and cells were stained with a DNA staining solution (50 µg/ml propidium iodide, and 5 kunit/ml of RNase A). The DNA content of the stained cells was measured using a FACS Calibur flow cytometer (BD Biosciences). Cell cycle data from flowcytometry were obtained and analyzed by CellQuest™ and Modfit LT™ softwares, 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, aprotonin (2 µg/ml),
pepstatin (2 µg/ml) and leupeptin (2 µg/ml)] for 30 mins at 4°C. The protein level was quantified using the BCA protein assay kit. Cell lysates containing equal amount (25 µg) of total protein were separated by 10% or 12.5% SDS-PAGE. The loaded protein samples were then transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore). The PVDF membrane with transferred proteins was blocked with 5% nonfat dry milk in Tris-buffered saline for 1 hour and incubated overnight with anti-AKT (1:2500, SKB1, Upstate), phosphorylated serine 473 AKT (p-AKT; 1:500, Cell Signaling Technology). After washing, anti-mouse or anti-rabbit secondary antibody conjugated with horseradish peroxidase was added (1:5000, Pierce Biotechnology) for incubation for 1 hour. The horseradish peroxidase activity was detected by incubating the membrane with enhanced chemiluminescence reagent (Pierce ECL WBS). The image was visualized by developing on BioMax X-ray film. Cellular β-actin (1:5000, AC-15, Sigma) was also immuno-detected 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 subcutaneous 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 subcutaneous and abdominal gastric sites, respectively. After the subcutaneous tumors grew to around $3 \times 3$ mm (in diameters) in size and were examined by GFP signals, CA4 phosphate (CA4-P) was administered by intraperitoneal injection (100 or 200 mg/kg) at day 0 in a volume of 300 µl. 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 AKT were analyzed in the tumor from control and CA4-P treated groups using immunohistochemical assay. The anti-phosphorylated AKT (Cell Signaling Technology, Inc., Beverly, MA) antibody was used. Procedures of deparaffinization, rehydration, antigen retrieval and IHC procedure was performed as described previously (Chiou et al., 2006).

**Statistical Analysis.** The results were reported as mean ± SD. Statistical analysis was performed using Student’s-t test or the one-way or two-way ANOVA test followed by Tukey’s test, as appropriate. A $p<0.05$ was considered to be statistically significant.
Results

Combretastatin A4 has a Preferential Anti-proliferation Effect on p-AKT Positive Gastric Cancer Cells. In this study, 6 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 phosphorylated serine 473 AKT (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). Figures 1A and 1B show 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, while MKN1, MKN74, NUGC-3, AGS-GFPM1 and AGS-GFPM2 were found to have relatively higher p-AKT expression. We further evaluated the cytotoxic and anti-proliferation activities of CA4 on 9 human gastric cancer cell lines. Given the results of p-AKT expression (Figures 1A & B), the 9 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 µM growth inhibitory concentration after 24-hour CA4 treatment.
In contrast to the p-AKT positive group, there was no significant inhibitory effect on growth of p-AKT negative cells at 0.02 µM of 24-hour CA4 treatment (Figure 1D). It was found that all cell lines with p-AKT positive expression appeared significantly more sensitive to 0.02 µM CA4 than p-AKT negative cell lines (Figures 1C & D).

**Combretastatin A4 Affected Human Gastric Cancer Cells Attachment and Mobility.** In order 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 round-up as the CA4 dose increased to 0.02 µM at 24-hour after CA4 treatment, but low dose (0.002 µM) had no effect as compared to the spread-out and well attached vehicle-treated cell lines (data not shown). At the same concentration (0.02 µM) treatment, less morphological change were observed in 5-fluorouracil-, cisplatin- and cyclophosphamide-treated cells (Figure 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 (Figure 2). Cell migration ability was 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 hour (Figures 2A & B) at the generation of scratch.
After 6 hours of incubation with 10% FBS medium, GFP positive cells began to migrate from the scratch (Figure 2I). At 24 hours, the gap was closed at control group (Figure 2D) but the gap junction at the CA4 treated cells had a dose dependent inhibitory effect (Figures 2E-2H). Our results demonstrated that CA4 could effectively inhibit AGS-GFPM1 and AGS-GFPM2 migration as compared with cisplatin, 5-floururacil or cyclophosphamide treatment at dose range of 0.02-2 µM. However, there was no significant difference for all compounds at the 0.002 µM treatment dosage (Figures 2J, 2K). The other 4 wild type cell lines were also investigated for CA4-induced anti-migration 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 Cells Invasion In Vitro.** As shown in Figure 3, the data revealed that 24-hour 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 (Figures 3A & B). Our results also showed that LY294002 at 0.2 µM to 20 µM concentration significantly decreased tumor invasiveness of both AGS-GFPM1 and AGS-GFPM2 cells (Figure 3C). In order to determine whether block AKT expression or reduce p-AKT levels
will affect the activity of CA4 treatment, the specific AKT inhibitor (Cat 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 (Figures 3B & D). More importantly, our results demonstrated that CA4 combined with AKT inhibitor did not have significant nor additive effects (Figure 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 (Figure 4A). In order 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 hours of incubation and sustained its effect for 24 hours (Figure 4B). The low dose (1 nM) of CA4 decreased the p-AKT expression on AGS-GFPM2 cells after a 24-hour treatment (Figure 4B). Similar results were observed in AGS-GFPM1 cells (data not shown). When PI3 kinase inhibitor, LY294002, was applied on AGS-GFPM2 cells, it
was observed that 1 µM to 20 µM LY294002 effectively reduced the level of p-AKT (Figure 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 cell lines, MKN74 and NUGC-3 were employed. As shown in Figure 4D, it was apparent that CA4 was equally effective on reducing the level of p-AKT in MKN74 and NUGC-3 cells. In contrast, one p-AKT negative human gastric cancer cell line (AGS) showed no signal of p-AKT in different concentrations of CA4 treatments was included as negative control (Figure 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 in order 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 µM - 20 µM) on the two cell lines 6 hours after the CA4 treatment (Figure 5). Nevertheless, an obvious G2/M phase arrest was found when the concentration of CA4 increased from 0.02 µM to 20 µM for 24 hours 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 hours, as 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 to their effect on reducing the p-AKT expression.

**Combretastatin A4 Phosphate (CA4-P) Reduced Subcutaneous 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 subcutaneous site to monitor green fluorescent tumor growth in animals. Figure 6 and Table 1 show that CA4-P attenuated subcutaneous 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 (No.: 4.0 and 2.6 V.S. 9.16) and size of abdominal tumor (1.41 and 1.28 V.S. 2.26 mm³) in the nude mice (Table 1). We further performed an IHC analysis of p-AKT to elucidate the relationship of p-AKT in gastric cancer growth and the anti-proliferation 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;
Figures 7A-C). Moreover, the expression levels of p-AKT in the abdominal metastasis foci of non-CA4-treated group (control; Figures 6C & 7B) were significantly higher than those in the subcutaneous lesion of non-CA4-treated group (control; Figure 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; Figure 7D). These results supported that the treatment of CA4-P not only exhibited an ability to inhibit gastric tumor 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, invasiveness in vitro (Figures 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 of subcutaneous and gastric injected sites (Figure 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 (Figures 1 & 3). This higher toxic response on p-AKT positive gastric cancer cell lines was also accompanied with inhibition of p-AKT expression (Figure 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 (Figure 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 anti-tumor 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 mg/m² and 68 mg/m² in clinical trials, it has been revealed that peak plasma concentrations (C_max) of CA4 are 1.89 µmol.l⁻¹ and 2.26 µmol.l⁻¹, respectively (Dowlati et al. 2002). In this study, our data showed that a 24-hour 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 (Fig. 1-5). Meanwhile, we further found that the p-AKT positive cell lines are more sensitive to CA4 (0.2-20 µM) after a 48-hour treatment, a dose-dependant increase of cell detachment and apoptosis was observed (data not shown). This finding is consistent with the previous report that a 24-hour incubation with CA4P did not induce endothelial cell death but significantly decrease the cell viability after a 48-hour 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 (Koul et al., 2005; Bader 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, 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, ten 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 (Figures 1-4). Using PI3K inhibitor (LY294002) and specific AKT inhibitor as control treatment, CA4 displayed a similar toxic response on p-AKT-positive cells (Figures 3B-D). These results supported that CA4 can be an effective anti-tumor drug in mediated by targeting on the PI3 kinase/AKT pathway. In addition, the up-stream tyrosine kinase receptors of PI3 kinase/AKT pathway included epidermal growth factor receptor (EGFR) and hepatocyte growth factor receptor (HGFR). It has been reported that attenuated HGFR 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 anti-tumor activities and reduced cell metastatic functions in vitro and in vivo under CA4 treatment. Given the more selective inhibition of PI3 kinase-AKT pathway by new agents, it is promising to investigate other compounds with improved solubility and/or oral bio-availability in terms of chemical scaffold of CA4.
References


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Footnotes

Heng-Liang Lin and Shih-Hwa Chiou contributed equally in this study. Jing-Ping Liou and Chin-Wen Chi also contributed equally to this paper.

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Legends for Figures

FIGURE 1. Detection of phosphorylated AKT expressions on human gastric cancer cell lines. The protein expression of phosphorylated serine 473 of AKT (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, B). The cell viability of human gastric cancer cell lines treated with combretastatin A4 (CA4). The 24–hour treatment effects of CA4 in 5 human gastric cancer cell lines with high p-AKT expression are shown (C). The same experimental condition was applied on low or non-detectable 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 hours. Non-attached cells were aspirated and adherent cells were visualized by microscopy. The concentration of each compound used was 0.02 µM. (E)

FIGURE 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 hours. The migration activity of AGS-GFPM1 and AGS-GFPM2 cells were evaluated by scratched assay after 6- and 24-hour CA4 treatment (I). After the treatment of different chemotherapeutic compounds, the migration ability of AGS-GFPM1 and AGS-GFPM2 cells were determined (I-K). The effect of CA4 on migration ability of 4 cell lines is shown on (L).

**FIGURE 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-E) CA4, LY294002, AKT inhibitor, or CA4 with AKT inhibitor was added in the lower chamber with 10% FBS medium for 24 hours. 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 on B, C, D, E, respectively.

**FIGURE 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 hours and p-AKT and AKT expression were determined by Western blot. (B) The time-course response of CA4 (1 nM and 1 µM) on the level of p-AKT and total AKT in AGS-GFPM2 cells. (C) The effect of PI3 kinase inhibitor (LY294002) on p-AKT and AKT expression in AGS-GFPM2 cells. (D) The dose-dependent effect of CA4 on p-AKT expressions in MNK74, NUGC-3, and AGS human gastric cancer cells.

**FIGURE 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 hours or 24 hours. Each phase of cell cycle percentage was indicated in the graph of DNA histogram.

**FIGURE 6.** The effect of combretastatin A4 phosphate (CA4-P) on nude mice bearing subcutaneous and gastric tumors. AGS-GFPM2 cells were injected on subcutaneous and gastric sites of mice. After the tumor on subcutaneous site grew to 3×3 mm, CA4-P was administered by intraperitoneal injection (day 0) at the concentration of 200 mg/kg. The mice received another two CA4-P administrations on Day 3 and Day 6. The vehicle control for mice bearing tumors was phosphate buffered saline (PBS). The GFP signals of subcutaneous (A, B) or intra-abdominal (C,
D) tumors was detected after 35 days xenotransplantion and the image was visualized by GFP imaging system. The white arrows indicate green fluorescent tumor foci distribution in the mice.

**FIGURE 7.** Detection of p-AKT expressions in the tumor formation of AGS-GFPM2 cells in nude mice with or without the treatment of combretastatin A4 phosphate (CA4-P). Representative results of immunohistochemistry staining of p-AKT expressions in (A) the subcutaneous foci and (B) the multiple foci of intra-abdominal metastatic tumor of nude mice without CA4-P treatment (arrows: positive for p-AKT; Bar: 100 µm). (C) The expression levels of p-AKT were detected in the subcutaneous and intra-abdominal metastatic tumor of nude mice with or without CA4-P-treatment groups (*p<0.05; **p<0.01). (D) The survival analysis of tumor-bearing (AGS-GFPM2 cells) nude mice with or without CA4-P treatment (n=12 for each group).
Table 1: The effect of combretastatin A4 phosphate (CA4-P) on subcutaneous and peritoneal AGS-GFPM2 tumor growth

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Animal no.</th>
<th>Subcutaneous tumor volume ($\text{mm}^3$)</th>
<th>Abdominal (gastric site) Tumor foci (no.)</th>
<th>Size of foci (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (PBS)</td>
<td>6</td>
<td>$4953.6 \pm 1791.6$</td>
<td>$9.16 \pm 1.24$</td>
<td>$2.26 \pm 0.03$</td>
</tr>
<tr>
<td>CA4-P (100 mg/kg)</td>
<td>6</td>
<td>$2762.2 \pm 1469.1$</td>
<td>$4.00 \pm 0.63$</td>
<td>$1.41 \pm 0.03$</td>
</tr>
<tr>
<td>CA4-P (200 mg/kg)</td>
<td>5</td>
<td>$673.4 \pm 184.7$</td>
<td>$2.60 \pm 0.50$</td>
<td>$1.28 \pm 0.08$</td>
</tr>
</tbody>
</table>
Figure 1

(A) Western blot analysis of p-AKT and AKT in different cell lines.

(B) Western blot analysis of p-AKT and AKT in different cell lines.

(C) Cell growth (% of control) vs. concentration (μM) for various cell lines with p-AKT (+).

(D) Cell growth (% of control) vs. concentration (μM) for various cell lines with p-AKT (-).

(E) Vehicle (DMSO) and S-FU treatment effects on cell morphology for AGS-GFP, AGS-GFPM1, and AGS-GFPM2 cell lines.
Figure 2

(A) Control
(B) Control
(C) Control 24 hours
(D) Control 24 hours
(E) 0.002 μM CA4
(F) 0.02 μM CA4
(G) 0.2 μM CA4
(H) 2 μM CA4

Bar indicated 100 μm
AGS-GFPM2

(i) Mobilized cells on scratch area (Ratio of optical area)

0 Hour
6 Hours
24 Hours

AGS-GFPM1
AGS-GFPM2

(J) Mobility (% of control)

Dose (μM)

AGS-GFPM1

AGS-GFPM2

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Figure 2

Bar indicated 100 μm
Figure 3

(A) Bar indicated 100 μm

(B) CA4

(D) AKT inhibitor

(C) LY294002

(E) AKT inhibitor + CA4
Figure 4

(A) CA4

(B) 1 nM CA4 1 μM CA4

(C) DMSO 0.001 0.01 0.1 1 10 20 (μM) LY294002 (μM)

(D) DMSO 0.001 0.01 0.1 1 10 20 (μM) CA4
Figure 5

<table>
<thead>
<tr>
<th>Dose (μM)</th>
<th>AGS-GFPM1</th>
<th>AGS-GFPM2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>G0/G1: 22.56%</td>
<td>G0/G1: 32.82%</td>
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<tr>
<td></td>
<td>S: 55.99%</td>
<td>S: 52.11%</td>
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<tr>
<td></td>
<td>G2/M: 21.46%</td>
<td>G2/M: 15.07%</td>
</tr>
<tr>
<td>0.002</td>
<td>G0/G1: 23.27%</td>
<td>G0/G1: 31.49%</td>
</tr>
<tr>
<td></td>
<td>S: 58.2%</td>
<td>S: 53.38%</td>
</tr>
<tr>
<td></td>
<td>G2/M: 18.53%</td>
<td>G2/M: 15.13%</td>
</tr>
<tr>
<td>0.02</td>
<td>G0/G1: 20.07%</td>
<td>G0/G1: 28.23%</td>
</tr>
<tr>
<td></td>
<td>S: 60.91%</td>
<td>S: 64.10%</td>
</tr>
<tr>
<td></td>
<td>G2/M: 19.03%</td>
<td>G2/M: 7.67%</td>
</tr>
<tr>
<td>0.2</td>
<td>G0/G1: 20.08%</td>
<td>G0/G1: 33.96%</td>
</tr>
<tr>
<td></td>
<td>S: 57.71%</td>
<td>S: 53.17%</td>
</tr>
<tr>
<td></td>
<td>G2/M: 22.21%</td>
<td>G2/M: 12.87%</td>
</tr>
<tr>
<td>2</td>
<td>G0/G1: 19.95%</td>
<td>G0/G1: 24.83%</td>
</tr>
<tr>
<td></td>
<td>S: 58.69%</td>
<td>S: 53.17%</td>
</tr>
<tr>
<td></td>
<td>G2/M: 21.36%</td>
<td>G2/M: 12.87%</td>
</tr>
<tr>
<td>20</td>
<td>G0/G1: 18.41%</td>
<td>G0/G1: 24.61%</td>
</tr>
<tr>
<td></td>
<td>S: 53.92%</td>
<td>S: 58.58%</td>
</tr>
<tr>
<td></td>
<td>G2/M: 27.67%</td>
<td>G2/M: 9.89%</td>
</tr>
</tbody>
</table>
Figure 6

(A) Vehicle (PBS)

(B) CA4-P (200 mg/kg)

(C) Vehicle (PBS)

(D) CA4-P (200 mg/kg)
Figure 7

A

B

C

D

Protein expression (optical density)

Control CA4-P Control CA4-P

Skin lesion Metastasis

** *

The percentage (%) of survival

non CA4-P CA4-P

Weeks