

Full Length Research Paper

Sunitinib enhanced the effect of combretastatin A-4 by inducing apoptosis *in vitro*

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Sunitinib is an inhibitor of angiogenesis, and CA-4 is being studied in clinical trials as a vascular disrupting agent. Various combinations involving an anti-angiogenic agent and an anti-vascular agent could cause much more effective anti-tumor responses. The present study indicated that the combination of an anti-angiogenic agent (sunitinib) and an anti-vascular agent (CA-4) exerted synergistic anti-proliferative effect against human carcinoma cells including HO-8910, HepG2 and SMMC-7721 *in vitro*. The enhanced apoptosis induced by sunitinib plus CA-4 was accompanied by greater extent of mitochondrial depolarization, caspases-3 activation and poly ADP-ribose polymerase (PARP) cleavage in HO-8910 cells. Furthermore, the induction of γ -H2AX was observed in sunitinib plus CA-4 treatment group in HO-8910 cells. Together, these data suggested that the combination of sunitinib and CA-4 could produce synergistic anti-tumor effect via DNA damage-induced apoptosis *in vitro*, and this combination might be a novel and promising therapeutic approach to the treatment of cancer.

Key words: Combretastatin A-4, sunitinib, combination therapy, apoptosis.

INTRODUCTION

Combretastatin A-4 (CA-4), a natural product isolated from the bark of a South African tree *Combretum caffrum*, has shown promising strong anti-cancer effects through inhibition of microtubule assembly and subsequent disruption of tumor blood flow (Dark et al., 1997). CA-4 phosphate (CA-4P), a water soluble pro-drug of CA-4, is rapidly dephosphorylated to the active compound CA-4 and shows reversible binding kinetics to tubulin, leading to disruption of microtubular structures (Dowlati et al., 2002). Although CA-4P is being studied in clinical trials as a vascular disrupting agent, cardiovascular toxicity and neurotoxicity are dose limiting for CA-4P (Nagaiah and Remick, 2010; Cooney et al., 2004). These severe side effects currently represent the main obstacles to broad clinical application of CA-4P (Simoni et al., 2008). Thus, it

is important to develop new anti-tumor combination therapy with lower concentration of CA-4 and more specificity for tumor endothelial cells than normal endothelial cells to avoid cardiac toxicity from endothelial damage.

Tyrosine kinase inhibitors (TKIs) are rapidly being integrated into the management of a variety of malignant diseases. Sunitinib malate (Sutent) is an ATP-competitive multi-targeted TKI with efficacy against renal cell carcinoma and gastrointestinal stromal tumor (Escudier, 2010; Bang et al., 2011). Moreover, it also has anti-cancer activity in patients with metastatic breast, hepatocellular, and ovarian cancer (Biagi et al., 2011; Koeberle et al., 2010; Mundhenke et al., 2009). Sunitinib inhibits multiple tyrosine kinases including platelet-derived growth factor receptors (PDGFR), the vascular endothelial growth

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factor receptors (VEGFR), the stem cell factor receptor c-KIT, FMS-like TK-3 receptor (FLT3), and the glial cell-line derived neurotrophic factor receptor (RET), which play a key role in both tumor angiogenesis and tumor cell proliferation (Chow and Eckhardt, 2007; Tanaka et al., 2011). However, most patients develop sunitinib resistance and progressive disease after about 1 year of treatment. Furthermore, resistance to sunitinib can be reversed by combining it with chemotherapeutic agents including cisplatin, sorafenib, docetaxel, etc (Castillo-Avila et al., 2009; Porta et al., 2011; Bergh et al., 2012).

The tumor vasculature is critical to both the survival of a solid tumor mass and its continued growth (Dark et al., 1997). Angiogenesis is a complex process that is essential for growth, invasion and metastasis of tumors (Gay and Felding-Habermann, 2011).

Two new classes of anti-cancer agents specifically target the tumor blood vessels, namely anti-angiogenic agents, which interfere with the formation of new blood vessel, and anti-vascular agents, which target the existing tumor blood vessels (Assifi and Hines, 2011). Various combinations involving an anti-angiogenic agent and an anti-vascular agent could cause much more effective and durable anti-tumor responses in various preclinical models than either type of drug used alone (Pasquier et al., 2011). Thus, we hypothesized that combining anti-angiogenic drug sunitinib with anti-vascular agent CA-4 might potentially enhance the anti-cancer therapeutic effects.

In this study, we showed for the first time that sunitinib and CA-4 in combination had substantial synergistic anti-tumor efficacy against human cancer cells *in vitro*. In addition, sunitinib greatly enhanced CA-4-mediated apoptosis in HO-8910 cells, accompanied by increased extent of mitochondrial depolarization, cleavage of poly ADP-ribose polymerase (PARP) and activation of caspase cascades. Interestingly, our results demonstrated that sunitinib plus CA-4 lead to increased levels of γ -H2AX indicating increased DNA damage in HO-8910 cells. These data suggested that the combination of sunitinib and CA-4 might be an effective therapeutic strategy to achieve synergistic activities in patients with solid tumors.

MATERIALS AND METHODS

Combretastatin A-4 was synthesized (>99% purity) at the Department of Chemical and Biochemical Engineering, Zhejiang University. Sunitinib was supplied by LC Laboratories (Woburn, MA, USA). Chemical structures of the agents are shown in Figure 1A. The primary antibodies against PARP (H250), procaspase-3 (H-277), XIAP (A-7), γ -H2AX (Ser139), and HRP-labeled secondary anti-mouse and anti-rabbit antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); cleaved-caspase-3 (D-175) from Cell Signaling Technology (Danvers, MA, USA); β -actin from BD Biosciences (Franklin Lakes, NJ, USA).

Cell culture

Human ovarian cancer cell line (HO-8910) and human hepato-

cellular carcinoma cell lines (HepG2, SMMC-7721) were purchased from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China); they were tested and authenticated for genotypes by DNA fingerprinting. HO-8910 and HepG2 were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, SMMC-7721 was cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. All the cells were maintained in a humidified atmosphere of 95% air plus 5% CO₂ at 37°C.

Experimental

Cells were seeded onto plates overnight and were divided into four groups receiving DMSO, sunitinib (4 μ M), CA-4 (200 nM) or the combination for the indicated time periods. The cells were washed three times with phosphate buffered saline (PBS) and were ready for use.

Cytotoxicity assay

The anti-proliferative activity of combination treatment with sunitinib and combretastatin A-4 was measured by sulforhodamine blue (SRB) cytotoxicity assay. Briefly, cells were fixed with 10% trichloroacetic acid (TCA) solution for 1 h, wells were rinsed 5 times with tap water and then cells were stained with 0.4% SRB solution (100 μ l/well) for at least 20 min at room temperature; wells were rinsed with 1% acetic acid to remove unbound dye, and were then left to air dry; the SRB dye was then solubilized by placing 100 μ l of unbuffered Tris-based solution in each well, and the absorbance was measured at 515 nm using a multi-scan spectrum. The inhibition rate of cell proliferation was calculated for each well as (A515 control cells - A515 treated cells) / A515 control cells \times 100%.

Analysis of apoptosis by Annexin V and propidium iodide staining

Apoptosis was quantified using the Annexin V-FITC/PI Apoptosis Detection Kit (BD Biosciences) according to the manufacturer's instructions. Briefly, the cells were incubated with 5 μ l Annexin V at room temperature for 15 min in the dark. Before flow cytometric analysis, 5 μ l of 50 mg/ml propidium iodide (PI) stock solution was added to the samples. For each sample, 1 \times 10⁴ cells were collected and analyzed using a FACSCalibur cytometer (Becton Dickinson) and Annexin V-FITC positive cells were designed as apoptotic cells.

Analysis of apoptosis by 4'-6-Diamidino-2-phenylindole (DAPI) staining

Briefly, cells in 96-well plates treated with sunitinib, combretastatin A-4 or the combination were washed twice with PBS, and then incubated with 0.1% Triton and 0.1% DAPI. The morphology of the cells' nuclei was observed and captured using a fluorescence microscope at excitation wavelength of 350 nm and the emission data at 490 nm were collected.

Determination of mitochondrial membrane depolarization

Cells (4 \times 10⁵ well⁻¹) were exposed to sunitinib, combretastatin A-4 or the combination for 48 h, and were collected and resuspended in fresh medium containing 10 μ g/ml 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide (JC-1). After incubation at 37°C for 30 min, cells were analyzed by flow cytometry.

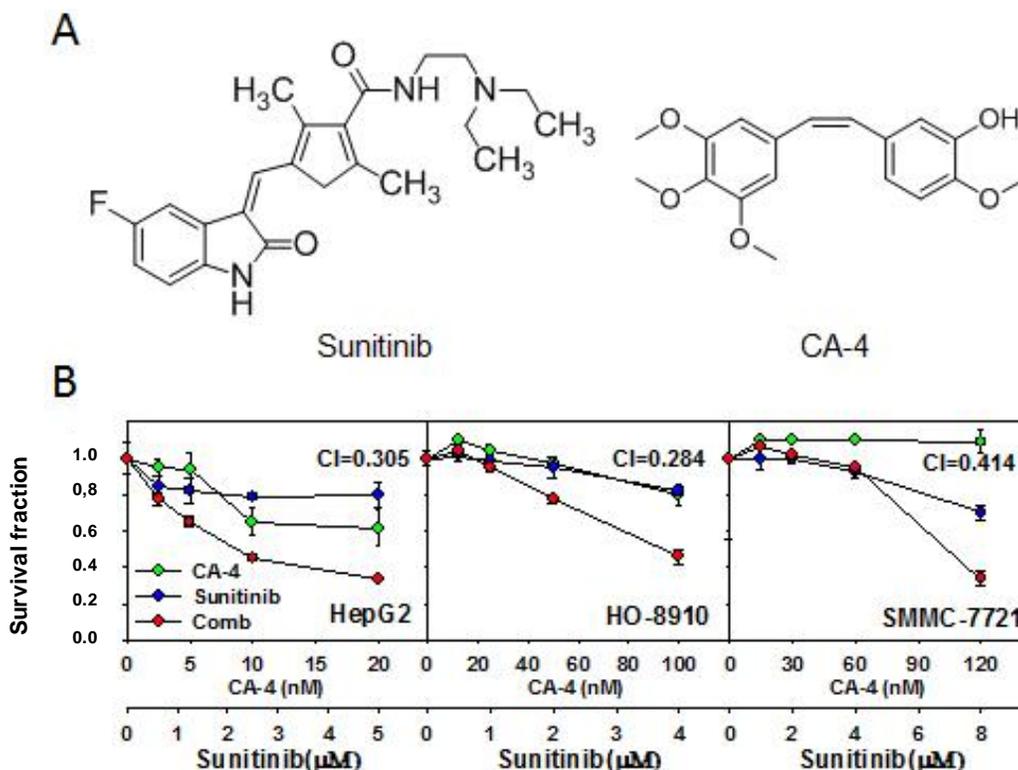


Figure 1. Combination cytotoxicity of sunitinib and CA-4. (A) Chemical structures of sunitinib and CA-4. (B) The cells were incubated with compounds for 72 h. Dose-response curves of human cancer cell lines to sunitinib, CA-4, or the combination. Each condition had 6 replicates, and error bars represent standard deviation.

Cell lysates and western blot analysis

Proteins were extracted with lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% sodium dodecyl sulfate (SDS), 0.5% deoxycholic acid, 0.02% sodium azide, 1% NP-40, 2.0 μ g/ml aprotinin, 1 mM phenylmethylsulfonylfluoride). The lysates were centrifuged at 10,000 g for 30 min at 4°C, the supernatants were transferred to a new tube and the protein concentration was determined. Proteins were fractionated on 8 to 15% Tris-glycine gels, and then they were transferred to PVDF membrane (Millipore, Bedford, MA, USA) and probed with primary antibodies (dilution range 1:500 to 1:1000) followed by horseradish peroxidase-labeled secondary antibodies at 1:5000 dilution. Antibody binding was then detected with the use of a chemiluminescent substrate and visualized on autoradiography film (Liu et al., 2010).

Immunofluorescence

The HO-8910 cells were seeded onto 35 mm dishes and exposed to sunitinib, combretastatin A-4 or the combination for 48 h. Thereafter, the cells were fixed with 4% formaldehyde for 15 min. After washing with PBS, the cells were blocked with 10% serum in PBS for 10 min and incubated at 37°C for 2 h with γ -H2AX (1:200, Santa Cruz, USA). Then, the cells were washed and incubated in the dark for 1 h at 37°C with goat anti-rabbit (FITC)-conjugated antibodies (1:200, Earthox, San Francisco, CA, USA). After washing, the nuclei were counterstained with DAPI, and then cells were washed in PBS and examined using a laser-scanning

confocal microscopy (Fluoview, Olympus, Tokyo).

Statistical analyses

Two tailed student's t test was used to determine the significance of differences between the various treatments. Differences were considered significant at $P < 0.05$. Combination index (CI) was well-accepted for quantifying drug synergism based on the multiple drug-effect equation of Chou and Talalay (1981, 1984). For *in vitro* experiments, CI values were calculated for each concentration of sunitinib, combretastatin A-4 and the combination in cell proliferation assays using CalcuSyn software (Biosoft, Cambridge, United Kingdom).

RESULTS

Cytotoxicity of the sunitinib and CA-4 combination in human cancer cell lines

The sensitivities of 3 human cancer cell lines to sunitinib, CA-4, or sunitinib + CA-4 were determined at clinically achievable concentrations using SRB cytotoxicity assay. Survival curves to sunitinib, CA-4, and sunitinib combined with CA-4 are shown in Figure 1B. CI values were calculated using CalcuSyn at the fixed-ratio

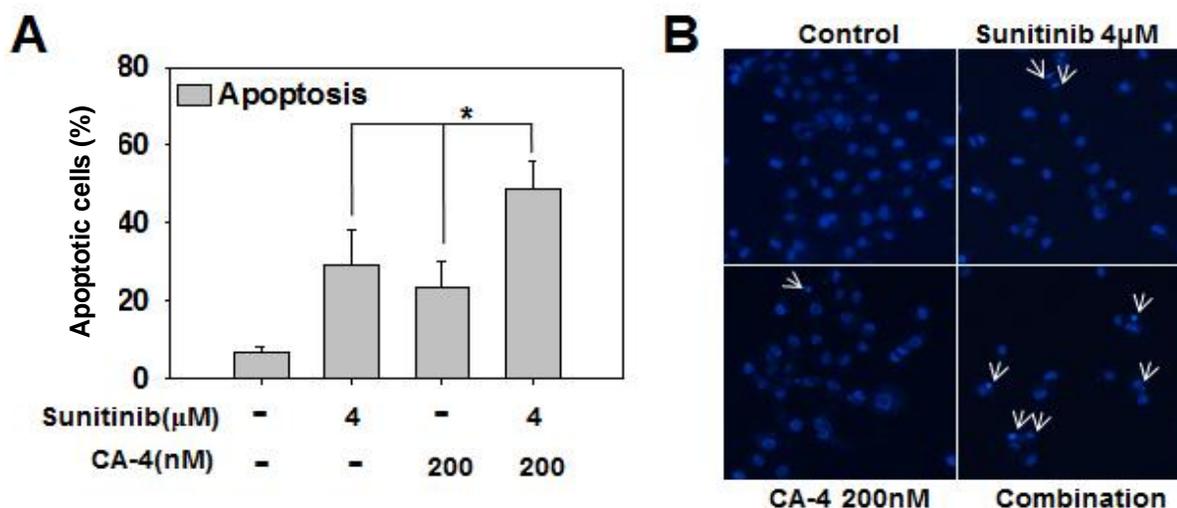


Figure 2. Sunitinib plus CA-4 caused enhanced apoptosis. (A) Annexin V-FITC/PI staining was used to evaluate the apoptosis. HO-8910 cells were treated with sunitinib (4 μM), CA-4 (200 nM) or the combination for 48 h. Total number of events analyzed for each condition was 10,000. The experiments were repeated three times, and error bars represent standard deviation. * $P < 0.05$ (two-sided Student t-test). (B) Sunitinib plus CA-4 induced apoptotic bodies in HO-8910 cells. Nuclear DNA was visualized by DAPI staining.

concentrations of sunitinib and CA-4 to assess combination activity. A CI less than 0.9 indicates synergism; 0.1, very strong synergism; 0.1 to 0.3, strong synergism; 0.3 to 0.7, synergism; 0.7 to 0.85, moderate synergism; 0.85 to 0.9, slight synergism; 0.9 to 1.10, additive; and more than 1.10, antagonism. CI values of three cell lines treated with sunitinib plus CA-4 were less than 0.7, indicating that sunitinib plus CA-4 showed synergy in HO-8910, HepG2 and SMMC-7721 cells (Figure 1B).

Sunitinib synergized with CA-4 to trigger apoptosis

To explore the mechanism of synergistic effects by combining sunitinib and CA-4, we first detected apoptosis by Annexin V-FITC/PI staining in HO-8910 cells that showed strongest synergistic effect in the cytotoxicity assay. As shown in Figure 2A, Annexin V-FITC/PI staining was used to characterize the apoptosis in HO-8910 cells treated with 4 μM sunitinib, 200 nM CA-4 or the combination for 48 h, the percentage of apoptotic cells was 5.91% in control cells, 19.45% with sunitinib, 15.61% with CA-4, and 46.10% in the combination treatment group. The apoptotic cells were significantly higher in the combination treatment when compared with single treatment ($P < 0.05$).

Furthermore, DAPI staining was also performed to visualize the apoptosis by assessing chromatin condensation. As shown in Figure 2B, 4 μM sunitinib plus 200 nM CA-4 triggered more apoptosis when compared with either treatment alone in HO-8910 cells, as indicated by the apoptotic bodies.

Sunitinib plus CA-4 induced apoptosis via mitochondrial pathway

To further confirm the combination effect of sunitinib and CA-4 on induction of the mitochondrial apoptosis pathway, we detected mitochondrial membrane potential by JC-1 staining in HO-8910 cells. HO-8910 cells were treated with 4 μM sunitinib, 200 nM CA-4 or the combination for 48 h. As demonstrated in Figure 3A, combined treatment with sunitinib and CA-4 resulted in an increased percentage of mitochondrial membrane depolarized HO-8910 cells than either single agent (42.67% in combination-treated cells, 32.34% in sunitinib-treated cells, 15.71% in CA-4-treated cells, and 4.99% in control group). In addition, sunitinib + CA-4 resulted in increased mitochondrial membrane potential than either drug alone (Figure 3B) in HO-8910 cells ($P < 0.01$ compared with CA-4 or $P < 0.05$ compared with sunitinib).

Combination therapy activated caspase cascades

Since caspase-3/PARP play a key role in initiation of cellular events during the apoptotic process, we next examined the effect of sunitinib, CA-4, and the combination on the activation of caspase-3, cleavage of PARP. As shown in Figure 4, we found that although sunitinib and CA-4 had little effect on caspase-3 and PARP, combination treatment was highly effective as it induced more significant cleavage of PARP and caspase-3 than either treatment alone. The X-linked inhibitor of apoptosis (XIAP) is the most potent caspase inhibitor in the IAP family and inhibits apoptotic cell death predominantly by preventing activation of initiator

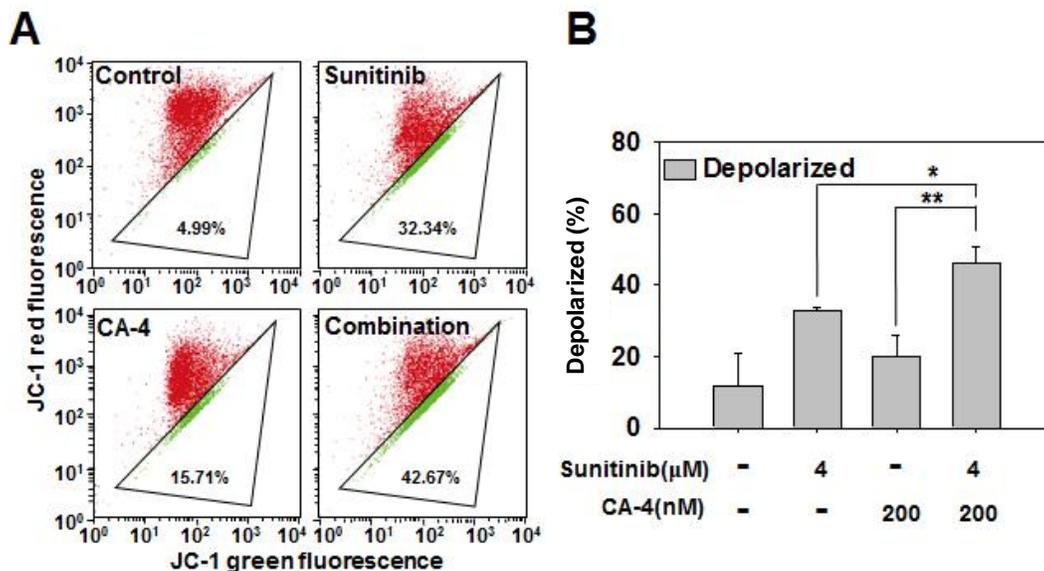


Figure 3. Sunitinib combined with CA-4 caused enhanced mitochondrial membrane depolarization. (A) and (B) The loss of $\Delta\Psi_m$ by sunitinib, CA-4, sunitinib + CA-4 was measured by flow cytometry using the JC-1 mitochondrial probe. HO-8910 cells in 6 well plates were exposed to sunitinib (4 μ M), CA-4 (200 nM) or the combination for 48 h, then, cells were incubated with JC-1 and analyzed by flow cytometry. Total number of events analyzed for each condition was 10,000. The experiments were repeated for three times, and error bars represent standard deviation. *P < 0.05, **P < 0.01 (two-sided Student t test).

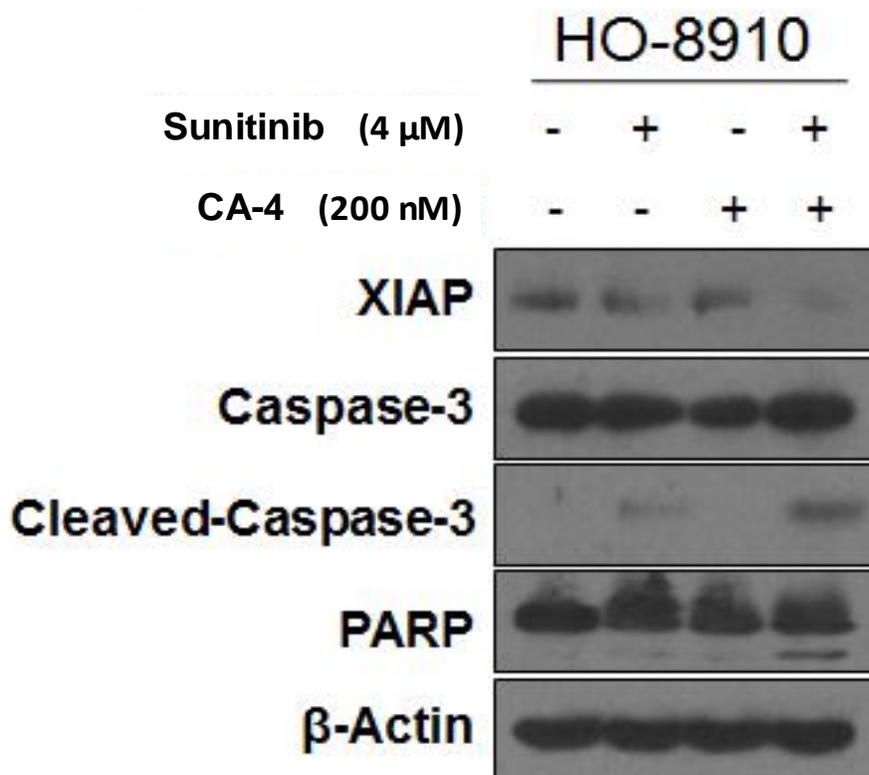


Figure 4. Sunitinib plus CA-4 caused activation of various apoptosis related proteins. HO-8910 cells were exposed to sunitinib (4 μ M), CA-4 (200 nM) or the combination for 48 h, after which protein extracts were immunoblotted with specified antibodies for PARP, XIAP, caspase-3 and cleaved-caspase-3.

caspase-9 as well as effector caspases-3 and-7 (Salvesen and Duckett, 2002). We further detected the expression of XIAP in sunitinib and CA-4-treated cells. Figure 4 shows that treatment of cells with sunitinib and CA-4 significantly reduced XIAP than the either treatment alone. Taken together, these results demonstrate that the caspase cascades were activated synergistically by sunitinib and CA-4 in HO-8910 cells as indicated by increased apoptosis.

Sunitinib in combination with CA-4 induced DNA damage

The induction of phosphorylated H2AX (γ -H2AX) is a marker of DNA damage. Therefore, our aim was to determine whether sunitinib in combination with CA-4 could induce DNA damage in HO-8910 cells. As shown in Figure 5A, the induction of γ -H2AX in HO-8910 cells was observed by Western blot, only in the combination setting after 6 h, indicating that DNA may be one of the key factors in inducing cell death by combination of sunitinib with CA-4. Furthermore, using immunofluorescence (Figure 5B), the ratio of γ -H2AX-positive HO-8910 cells was significantly increased in sunitinib plus CA-4 treatment group, when compared with either treatment alone.

DISCUSSION

The clinical cancer therapy now has the potential to explore combination regimens offering improved response rates when compared with mono-chemotherapeutics. Combination cancer therapies are frequently designed to target tumor growth with diverse mechanisms to better overcome resistance (Goudar et al., 2005). The therapeutic potential of the drugs that target the tumor vascular supply is now well recognized. Two distinct groups of vascular-targeted therapies have evolved: antiangiogenic agents and vascular-disrupting approaches (Siemann et al., 2005). Angiogenesis is a fundamental event in the process of tumor growth and metastatic dissemination (Hicklin and Ellis, 2005). Sunitinib, a multitargeted tyrosine-kinase inhibitor, extends survival of patients with metastatic renal-cell carcinoma and gastrointestinal stromal tumors and inhibits angiogenesis by diminishing signaling through VEGFR1, VEGFR2 and PDGFR β (Roskoski, 2007; Zhao et al., 2010). Inhibitors of angiogenesis interfere with new vessel formation and therefore have a preventative action, have little effect on the existing tumor blood vessels and are likely to be of particular benefit in early-stage cancer therapy (Osusky et al., 2004). Vascular-disrupting agents target the destruction of established tumor blood vessels, resulting in tumor ischemia and necrosis. CA-4 is the lead compound as vascular-

disrupting agents and is currently in Phase II/III clinical trials against a range of malignancies, in combination with conventional chemotherapeutic agents and radiotherapy (Tozer et al., 2008; Wu et al., 2009). Furthermore, CA-4 displays minimal toxicity profile at low dose, indicating its potential to be a candidate for drug combinations in the solid tumor therapy (Busk et al., 2011; Zhu et al., 2010). Since both the initiation of new vessel formation and the integrity of the existing blood vessel network are critical to a tumor's growth and survival, various combinations involving an anti-angiogenic agent and an anti-vascular agent could cause much more effective anti-tumor responses in various preclinical models (Siemann et al., 2004). Thus, combining sunitinib with CA-4 may be a logical way to potentially enhance response rates and prolong survival times for patients by targeting tumor blood vessels.

The results of the present report, for the first time, indicated that the synergistic anti-cancer effects *in vitro* achieved by sunitinib plus CA-4 were observed in human cancer cells. CI values and the significant decline in the survival of cells in combination group strongly demonstrated that sunitinib potentiated the CA-4-imposed cytotoxicity in HO-8910, HepG2 and SMMC-7721 cells. The anti-cancer efficacy by combining sunitinib and CA-4 *in vivo* may need to be further investigated. Our data showed that synergism *in vitro* achieved by the combination of sunitinib and CA-4 was accompanied with enhanced apoptosis. In addition, loss of mitochondrial membrane potential was significantly greater with sunitinib plus CA-4 than with either drug alone. Activation of caspase-3 leads to the cleavage of PARP, the presence of cleaved PARP is one of the most used diagnostic tools for the detection of apoptosis in many cell types (Narula et al., 1999). From western blot analysis, marked increases in cleaved-PARP and caspase-3 were observed in HO-8910 cells after sunitinib and CA-4 combination treatment. XIAP is a member of a novel family of inhibitor of apoptosis-inducing proteins (Porta et al., 2011). IAPs also play an important role during the apoptotic process, XIAP is the best known member of this family and correlates with resistance to chemotherapy (Kashkar, 2010). Our data showed that the synergistic effect on apoptosis obtained with the sunitinib/CA-4 co-treatment was accompanied by a large decrease in anti-apoptotic XIAP protein. The suppression of XIAP by the combination treatment resulted in a significant decrease in procaspase-3 levels, induced apoptosis, and ultimately resulted in the synergism of these two drugs.

Among the different forms of complex DNA damage, double-strand breaks (DSBs) are considered to be among the most lethal forms of DNA damage, severely compromising genomic stability (Kinner et al., 2008). H2AX moved to the center of cellular responses to DNA damage after the discovery that it becomes locally phosphorylated on Ser139, to generate γ -H2AX, in the

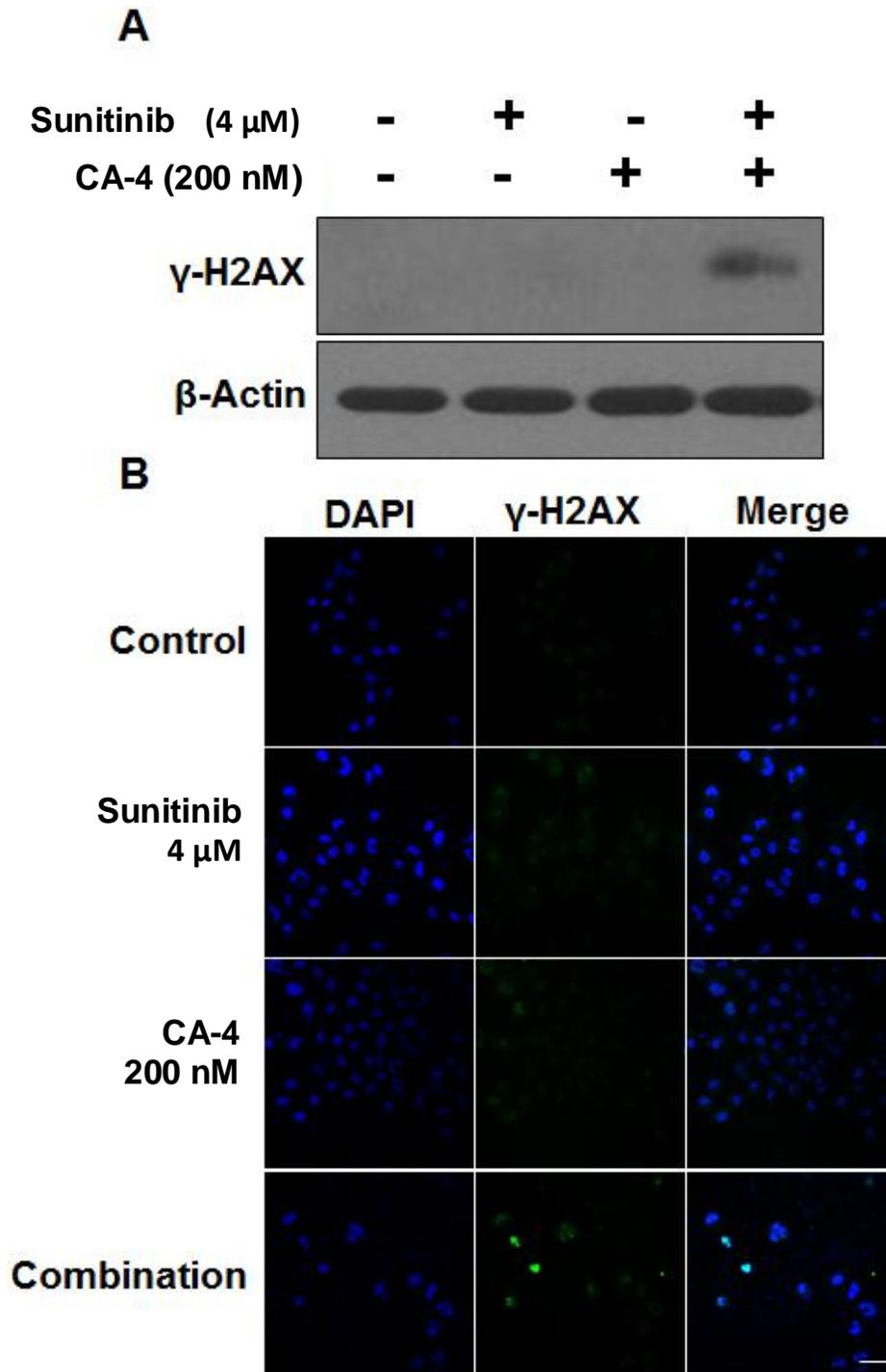


Figure 5. Sunitinib in combination with CA-4 induced DNA damage. (A) HO-8910 cells were exposed to sunitinib (4 μ M), CA-4 (200 nM) or the combination for 6 h, after which protein extracts were immunoblotted using γ -H2AX. (B) HO-8910 cells were treated with sunitinib (4 μ M), CA-4 (200 nM) or the combination for 6 h and imaged as in a stained with γ -H2AX immunofluorescence (green), DAPI (blue). γ -H2AX expression was indicated by a stippled appearance in the nucleus. Scale bar = 40 μ m.

vicinity of DSBs (Mah et al., 2010). Quantitation of γ -H2AX is one of the earliest events in the DNA damage

signaling and repair (Smart et al., 2011). To maintain genomic stability after DNA damage, multicellular organisms

activate checkpoints that induce cell cycle arrest or apoptosis (Gartner et al., 2000). A number of anti-cancer drugs exert their effect by causing DSBs and subsequent apoptosis induction, such as DNA replication inhibitors, crosslinking agents and topoisomerase inhibitors (Kawanishi and Hiraku, 2004). In our study, a clear increase in γ -H2AX expression was observed in the sunitinib + CA-4 group compared with mono-treatment groups. Furthermore, in HO-8910 cells, the number of γ -H2AX-positive cells increased in the combination treatment group. These results indicated that sunitinib plus CA-4 might induce DSBs in HO-8910 cells, thus activate apoptosis pathways, and finally result in the synergism of these two drugs.

In conclusion, we presented evidence showing better therapeutic activity of sunitinib when combined with CA-4 *in vitro*. The enhanced apoptosis induced by sunitinib plus CA-4 was accompanied by the greater extent of DNA damage, mitochondrial depolarization, caspases-3 activation and PARP cleavage in HO-8910 cells. Additional preclinical studies, such as anti-vascular and *in vivo* anti-cancer efficacy, are needed to systematically evaluate the applicability of this combination as a therapeutic strategy for cancer patients. As reported in this study, the combination of sunitinib and CA-4 might be an effective therapeutic strategy to achieve synergistic anti-tumor effects.

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