Carboxyamidotriazole Synergizes with Sorafenib to Combat Non–Small Cell Lung Cancer through Inhibition of NANOG and Aggravation of Apoptosis

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ABSTRACT

Lung cancer is currently the leading cause of cancer-related deaths worldwide. In this study, we investigated the combination of carboxyamidotriazole (CAI) and sorafenib in non–small cell lung cancer (NSCLC) in vitro and in vivo to test whether CAI enhances the antitumor effects of sorafenib and reduces its side effects. The combination index (CI) showed that coadministration of CAI and sorafenib synergistically inhibited the proliferation of NSCLC cells (Lewis lung carcinoma, A549, and NCI-H1975 cells). Cell death as a result of the combination treatment was attributed to apoptosis, which was accompanied by activation of caspase-3 and poly(ADP-ribose) polymerase. In addition, combination therapy induced the accumulation of mitochondrial-associated reactive oxygen species, as well as depolarization of mitochondrial and reduced NANOG (homeobox protein NANOG) mRNA and protein expression. Basic fibroblast growth factor, a stimulator of NANOG, was applied to identify the possible mechanism. The addition of basic fibroblast growth factor followed by combined treatment may stimulate NANOG expression and synchronously rescue the accumulation of reactive oxygen species. C57BL/6J mice bearing Lewis lung carcinoma were randomized to receive vehicle (polyethylene glycol 400), CAI (30 mg/kg), low-dose sorafenib (SFB-L; 10 mg/kg), high-dose sorafenib (SFB-H; 30 mg/kg), or a CAI and SFB-L combination. Tumor growth was significantly suppressed in the combination group, and the efficacy of combination treatment was equivalent to that of the SFB-H monotherapy group. Furthermore, the combination group had reduced side effects compared with the SFB-H group, as indicated by weight preservation in mice. Our study illustrates that CAI enhances the antitumor activity of sorafenib in NSCLC and provides a novel strategy for NSCLC treatment.

Introduction

Lung cancer is currently the leading cause of cancer-related death worldwide, and the 5-year survival of patients with lung cancer is approximately 4%–17%, with variations in disease stage and region (Hirsch et al., 2017). Non–small cell lung cancer (NSCLC) accounts for 80% of all lung cancers worldwide (Jemal et al., 2011). The most frequently used treatments for NSCLC are surgery, chemotherapy, and radiotherapy. Unfortunately, approximately two-thirds of patients have advanced or inoperable disease at diagnosis. Many platinum-based regimens are first-line chemotherapies; single-agent docetaxel, pemetrexed, and erlotinib are considered second-line therapies. However, if patients fail to respond to conventional chemotherapies, treatment options are limited (Langer et al., 2013). The currently available therapies are shown to be inadequate, and novel strategies are urgently required.

Sorafenib is a multikinase inhibitor that blocks the Raf kinase, platelet-derived growth factor receptors, and vascular endothelial growth factor receptors (EGFRs). The U.S. Food and Drug Administration approved sorafenib for the treatment of advanced renal cell carcinoma and unresectable hepatocellular carcinoma (Wilhelm et al., 2006). Sorafenib has confirmed activity in preclinical models of NSCLC (Wilhelm et al., 2004; Gridelli et al., 2007) and has also been evaluated in several clinical trials (phases I–III) in patients with advanced NSCLC (Clark et al., 2005; Moore et al., 2005; Blumenschine et al., 2009; Wakelee et al., 2012; Paz-Ares et al., 2015). One limitation that hampers the use of sorafenib as a monotherapy is its side effects (e.g., hand-foot syndrome, rash, diarrhea, hypertension, and fatigue), which affect approximately 80% of patients receiving
sorafenib treatment (Batchelor et al., 2007; Strumberg et al., 2007). Most of the aforementioned side effects are dose dependent; therefore, reducing the sorafenib dose while maintaining the same therapeutic effect may require further investigation.

Carboxymidotriazole (CAI) blocks nonvoltage-dependent calcium channels and has shown antiangiogenic, antiproliferative, and antimetastatic properties in preclinical research. CAI has been investigated in phase I–III clinical trials in various solid tumors (Berlin et al., 1997; Kohn et al., 1997; Hussain et al., 2003; Dutcher et al., 2005; Johnson et al., 2008). Although CAI exhibited mild anticancer properties in some clinical trials, the majority of toxicities noted were grade I, which means CAI was generally well tolerated (Hussain et al., 2003; Desai et al., 2004; Dutcher et al., 2005; Mikkelsen et al., 2007). In addition, CAI exhibited a protective role in treating cancer-associated cachexia by inhibiting muscle proteolysis and restraining inflammatory responses (Chen et al., 2017), which implies that CAI may syneritize with other anticancer drugs through limiting chemotherapy-induced weight loss. In chronic myeloid leukemia cells, CAI was shown to reduce cell viability and induce apoptosis in a redox-mediated way (Alessandro et al., 2008; Corrado et al., 2011). Our previous work showed that CAI synergized with 2-deoxy-d-glucose, a glycolysis inhibitor, and inhibited oxidative phosphorylation (OXPHOS) in cancer cells (Ju et al., 2016). These data imply that CAI may perturb cell metabolism, damage mitochondrial function, elevate reactive oxygen species (ROS) production, and, finally, induce cell death.

The NANOG transcription factor confers self-renewal and differentiation to cancer stem cells (CSCs) and is often considered a hallmark of CSCs. Recently accumulated data show that the expression of NANOG in cancer cells can make confer them certain CSC properties such as self-renewal, tumorigenicity, metastasis, and chemotherapy resistance (Gong et al., 2015). Overexpression of NANOG in benign 293 cells contributed to malignant transformation, along with tumorigenesis, dramatically increasing clonogenicity and promoting tumor growth in vivo (Lin et al., 2011). In patients with lung cancer, expression of both NANOG mRNA and protein in lung cancer tissues was much higher than in patient-matched normal tissues (Du et al., 2013). Regarding the signaling perturbed by NANOG within cells, Chen et al. (2016) showed that NANOG prevented mitochondrial ROS production in tumor-initiating stem-like cells (TICs), and NANOG-silenced TICs produced much more ROS than controls (Chen et al., 2016).

Our objectives were first to probe whether combination treatment with CAI and sorafenib may allow a dose reduction of sorafenib and avoid decreased effectiveness and then to investigate the mechanism by which the combination exerts its action.

**Materials and Methods**

**Cell Lines and Reagents.** Lewis lung carcinoma (LLC) cells were purchased from the Shanghai Institute for Biologic Sciences (Chemical Abstracts Service no. A549; Shanghai, China) and NCI-H1975 (H1975) cells were purchased from the Institute of Basic Medical Sciences at the Chinese Academy of Medical Sciences and School of Basic Medicine, Peking Union Medical College (Beijing, China). LLC cells were cultured in Dulbecco's modified Eagle's medium (high glucose). A549 cells were maintained in F-12K medium. H1975 was grown in RPMI 1640 medium. All media were supplemented with 10% fetal bovine serum ( Gibco, Grand Island, NY), 50 mg/ml penicillin, and 100 mg/ml streptomycin and l-glutamine. Cells were maintained in a humidified atmosphere with 5% CO₂ at 37°C.

CAI was synthesized by the Institute of Materia Medica at the Chinese Academy of Medical Sciences (Beijing, China). Sorafenib was purchased from MedChem Express (Mommouth Junction, NJ). Glutathione (GSH)-reduced ethyl ester (Sigma-Aldrich, St. Louis, MO) was dissolved in sterile phosphate-buffered saline (PBS) as a 0.25-M stock solution. Benzoyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (Z-VAD-FMK) (Beyotime Biotechnology, Nantong, China) was prepared in dimethylsulfoxide (DMSO) as a 20-mM stock solution. For in vitro studies, CAI and sorafenib were dissolved in DMSO and diluted with the corresponding medium to the final concentration with a DMSO concentration of 0.1%. For in vivo studies, compounds were dissolved in polyethylene glycol 400 (PEG400) (Sinopharm Chemical Reagent, Beijing, China).

**Cell Proliferation Assay and Combination Index.** The sulforhodamine B (SRB) method was used to assess cell proliferations for different concentrations after the indicated treatment times (Vichai and Kirtikara, 2006). DMSO was used as the vehicle. After treatment with CAI and/or sorafenib for the indicated time period (24 hours, 48 hours, and 72 hours), cells were fixed in 96-well plates with 10% (w/v) trichloroacetic acid at 4°C for 1 hour, and the plates were then washed with slow-running water five times and dried at room temperature. Next, the intracellular proteins were stained with 0.4% SRB for 20 minutes, and the plates were then washed five times with 1% (v/v) acetic acid. After adding 200 μl of 10 mM Tris base solution to each well, the absorbance of SRB was detected at 515 nm in a microplate reader. Combination indices (CIs) were calculated using CompuSyn software (ComboSyn Inc., Paramus, NJ) (Chou and Talalay, 1984; Chou, 2006). According to Chou and Talalay (1984), a CI less than 1 or greater than >1 indicates synergism or antagonism, respectively.

**Apoptosis and Cell Cycle Analysis by Flow Cytometry.** Cells were seeded and treated with CAI and/or sorafenib for 24 hours, and they were then detached from the plates with Trypsin-EDTA and washed with PBS two times. The induction of apoptosis was measured by flow cytometry (FCM) using an Annexin V–fluorescein isothiocyanate/propidium iodide (PI) kit (Dojindo, Kyushu, Japan). The approximate fluorescent excitation/emission wavelengths of fluorescein isothiocyanate–Annexin V and propidium iodide were 494/518 nm and 488/617 nm, respectively. Both the Annexin V– and PI-negative subpopulation indicated intact cells, whereas the Annexin V–positive and PI-negative subpopulation represented cells that were in early apoptosis. Cells that were in late apoptosis or already dead were both Annexin V and PI positive, and necrotic cells were Annexin V negative and PI positive. The apoptotic cell rate was calculated by adding the portion of Annexin V–positive/PI-negative cells and the portion of Annexin V–positive/PI-positive cells.

**After treated with CAI and/or sorafenib for 48 hours, cells were harvested and fixed in 75% ethanol at 4°C for 18 hours. After cells were washed twice with cold PBS, DNA staining was performed using PI (0.05 mg/ml) and RNase (2 mg/ml) (Beyotime Biotechnology) at room temperature for 30 minutes. Cell cycle distributions were measured by FCM (with an excitation/emission wavelength of 488 nm/617 nm). Distributions of cell cycle phases were calculated using ModFit LT software (Verity Software House, Topsham, ME).

**Determination of Intracellular ROS Level and Mitochondrial Membrane Potential.** Cells were seeded in six-well plates and treated with the indicated drugs. For FCM analysis, cells were detached from the plates with Trypsin-EDTA after 24-hour treatment, washed twice with PBS buffer, and incubated with 20 μM 2-,7-dichlorofluorescin diacetate (DCFH-DA) (Sigma-Aldrich) for 20 minutes at 37°C. Cells were then washed gently three times with PBS buffer and were tested using FCM. FCM was performed using an excitation/emission wavelength of 485/525 nm for DCFH-DA.

For fluorescence microscopy detection, cells were washed twice with PBS buffer after 24-hour treatment and incubated with 20 μM DCFH-DA for 20 minutes. Cells were then washed gently three times with PBS buffer and were detected using fluorescence microscopy.
MitoSOX Red is a fluorogenic dye, and higher fluorescence upon oxidation of the probe indicates increased mitochondrial ROS or superoxide (Invitrogen, Carlsbad, CA). Cells were seeded in six-well plates and treated with the indicated drugs. After 24-hour treatment, cells were detached from the plates with Trypsin-EDTA, washed twice with PBS buffer, and incubated with 5 μM MitoSOX for 10 minutes at 37°C. Cells were then washed gently three times with PBS buffer and were tested using FCM. FCM was performed using an excitation/emission wavelength of 488/625 nm for MitoSOX. In the negative control group, DMSO (solvent of MitoSOX) was used instead of MitoSOX to define the background and delimit the negative regions from the positive regions.

Analysis of Mitochondrial Membrane Potential. To measure the depolarization of mitochondrial membrane potential, JC-1 staining (mitochondrial membrane potential assay kit; Beyotime Biotechnology) was applied. In normal cells (high ΔΨm), JC-1 forms as J-aggregates with red fluorescence in the mitochondrial matrix. However, in apoptotic and necrotic cells (low ΔΨm), JC-1 remains in monomeric form with green fluorescence. Thus, mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio. The cells were seeded in six-well plates and treated with CAI and/or sorafenib. After 24 hours, cells were detached from the plates with Trypsin-EDTA, washed with PBS, resuspended with 500 μL JC-1 working solution, and subsequently incubated at 37°C for 20 minutes. Finally, cells were washed twice with the assay buffer and FCM analysis was performed. Flow cytometry was performed using the plates with Trypsin-EDTA, washed with PBS, resuspended with 500 μL JC-1 working solution, and subsequently incubated at 37°C for 20 minutes.

LLC Xenograft Model. On day 0, 1 × 10^6 LLC cells resuspended in 100 μL PBS were injected subcutaneously into the right axillary fossa of 6-week-old male C57BL/6 mice (Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences and School of Basic Medicine, Peking Union Medical College) weighing 18–22 g. Mice were randomly divided into four groups, with 10 animals in each group. The animals were treated by gavage once daily, with either vehicle control (PEG400), CAI (30 mg/kg per day), low-dose sorafenib (SFB-L; 10 mg/kg per day), high-dose sorafenib (SFB-H; 30 mg/kg per day), or a combination of CAI (30 mg/kg per day) plus SFB (10 mg/kg per day). Changes in tumor length and width were measured with a caliper every other day from days 10 to 29, and the tumor volumes (in cubic millimeters) were calculated using the following formula: Volume = length × width^2 × 0.5. Mice were euthanized after 29 days of treatment. Tumors were collected and weighed. All animal studies and procedures were approved by the Institutional Animal Care and Use Committee of Peking Union Medical College.

Tumor Sample Preparation. Tumor tissues were homogenized in ice-cold lysis buffer containing T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific Inc., Pittsburgh, PA) with Protease and Phosphatase Inhibitor Cocktail (Roche, Mannheim, Germany), incubated for 15 minutes, and then centrifuged at 16,100 × g at 4°C for 30 minutes. The protein-containing supernatant was collected and the protein concentration was quantified using the BCA Protein Assay Kit (BioTeke Corporation, Beijing, China).

Western Blot Assay. LLC cell lysates or tumor tissue homogenate containing 30 μg proteins was subjected to SDS/PAGE and separated proteins were transferred onto polyvinylidene fluoride membrane. After the membrane was blocked with 5% nonfat dry milk in Tris-buffered saline containing Tween-20, it was incubated with the desired antibodies. The following primary antibodies were used: NANO9, poly(ADP-ribose) polymerase (PARP), cleaved caspase-3, and glyceraldehyde 3-phosphate dehydrogenase (all from Cell Signaling Technology Inc., Danvers, MA), and β-actin (Sigma-Aldrich). Subsequently, the membrane was incubated with the appropriate secondary antibody and the immunoreactive protein bands were visualized using a chemiluminescence kit (Millipore, Billerica, MA) followed by ECL-based autoradiography (GE Healthcare, Hertfordshire, UK). Western blots are representative of at least three independent experiments.

Immunohistochemistry Staining. Tumor sections from a C57-bearing LLC transplant model were baked at 60°C for 20 minutes, deparaffinized with xylene, and rehydrated in graded ethanol series. After antigen retrieval and endogenous peroxidase activity blocking, the slides were stained for NANO9 (ab80892, dilution 1:1000; Abcam, Cambridge, UK). Localization of specific reactivity was detected using a secondary antibody conjugated to peroxidase followed by observation with 3,3′-diaminobenzidine (DAB) substrate (Zhongshan Golden Bridge Biotechnology, Beijing, China). Slides were counterstained with hematoxylin.

Total RNA Extraction and Reverse Transcription Quantitative Polymerase Chain Reaction. RNA was isolated from LLC cells using the Pure RNA Extraction Kit (BioTeke Corporation) and 2 μg total RNA was used to synthesize cDNA with TransScript First-Strand cDNA Synthesis Supermix (TransGen Biotech Co., Beijing, China), per the manufacturer’s instructions. Quantitative polymerase

![Image](https://example.com/image.png)

**Fig. 1.** Effects of sorafenib and CAI on LLC, A549, and NCI-H1975 NSCLC cell lines. (A–C) NSCLC cells were treated with CAI or sorafenib alone or in combination in a fixed concentration ratio for 24 hours, 48 hours, and 72 hours in LLC (A), A549 (B), NCI-H1975 (C) cell lines (n = 5). Corresponding CI values are shown to the right of each graph.
chain reaction was performed using the IQ5 Real-Time System (Bio-Rad, Hercules, CA). Each reverse transcription quantitative polymerase chain reaction (25 μl) contained 2 μl diluted cDNA and 12.5 μl 2 × Maxima SYBR Green (Thermo Fisher Scientific Inc.). All reactions were performed using the following thermal cycling conditions: 94°C for 2 minutes followed by 40 cycles of a two-step reaction, denaturation at 94°C for 10 seconds, and annealing at 60°C for 30 seconds followed by a melting curve from 55 to 95°C in 1-second increments of 0.5°C to ensure amplification specificity. Transcript levels of the target genes were normalized to glyceraldehyde 3-phosphate dehydrogenase.

**Malondialdehyde Assay.** Tumor tissues were homogenized and prepared for malondialdehyde (MDA) assay according to the manufacturer’s instructions in the Lipid Peroxidation MDA assay kit (Beyotime Biotechnology). The MDA concentration of each sample was detected at 532 nm (450 nm as a control) by a microplate reader (Synergy 4; BioTek Corporation).

**Statistical Analysis.** All data are expressed as means ± S.D. of three independent experiments unless otherwise indicated. All data were analyzed by the t test or analysis of variance followed by the Tukey multiple-comparisons test. *P* values <0.05 were considered statistically significant.

**Results**

**Effects of Sorafenib and CAI on LLC, A549, and H1975 NSCLC Cell Lines.** To measure the response of NSCLC cells to sorafenib and/or CAI, we first performed cell proliferation assays. We incubated the LLC, A549, and H1975 cell lines with the respective drugs and their combinations. Sorafenib at concentrations of 0.5, 1, 2.5, 5, 7.5, and 10 μM and CAI at concentrations of 1, 2, 5, 10, 15, and 20 μM and their combination were all applied. As shown in Fig. 1, CAI, sorafenib, and their combination showed significant growth inhibitory effects on LLC, A549, and H1975 cell lines.

**Fig. 2.** The combination of sorafenib and CAI induces apoptosis in NSCLC. (A) LLC cells were treated with 10 μM CAI and/or 5 μM sorafenib, and the DMSO group was treated with or without 20 μM Z-VAD-FMK for 48 hours. The cells were then stained with Annexin V/PI and analyzed for apoptosis by flow cytometry. Statistical quantifications of apoptotic cells are shown in the graphs on the right. Values are presented as means ± S.D. *P < 0.05; **P < 0.01 (significant between groups cotreated with or without Z-VAD-FMK). (B and C) Effect of 10 μM CAI and/or 5 μM sorafenib on the expression of cleaved PARP (B) and cleaved caspase-3 (C). Protein levels of cleaved PARP and cleaved caspase-3 from treated cell lysates were normalized against GAPDH levels. Band density was evaluated using ImageJ software (National Institutes of Health, Bethesda, MD). Values are presented as means ± S.D. *P < 0.05, **P < 0.01 (significant compared with DMSO). (D) Cell cycle analysis was conducted in LLC and A549 cells using flow cytometry. Cells were treated with CAI (10 μM) and/or sorafenib (5 μM) for 24 hours. The results are representative of three independent experiments (n = 5). GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
sorafenib, and their combination led to dramatically reduced cell proliferation, in a dose-dependent manner, in all three cell lines investigated. It is noteworthy that the cytotoxic effect of a sorafenib dose as low as 1 μM in combination with CAI was comparable to 10–15 μM sorafenib alone in all three cell lines and at three time points. The three cell lines were treated with different concentrations of CAI and sorafenib at a fixed ratio (2:1), using median effect analysis (Chou and Talalay, 1984). As indicated in Fig. 1, the CI values for all 5 combination groups at 3 indicated time points were less than 1 in LLC cells (Fig. 1A). And in A549 cells CI < 1 was observed for all 5 combination groups when cells were incubated with the combinations for 24h and 48h. The CI values for 3 lower-dose combination groups were less than 1 at 72h (Fig. 1B). And similar situation that lower-dose combination showed smaller CI appeared in H1975 cells treated with 5 combinations of CAI and sorafenib for various time (Fig. 1C). This means that the combination of sorafenib and CAI led to synergistic inhibition of cell proliferation in LLC cells, and the combination of both drugs, especially in a lower dose, generated a synergistic effect in the A549 and H1975 cell lines.

**The Combination of Sorafenib and CAI Induces Apoptosis in NSCLC.** Since the synergistic inhibitory effect of sorafenib and CAI on NSCLC specific mechanisms of cell death must be further explored. To clarify the effects of combining these two agents, LLC cells were treated with the drugs individually or in combination, and Annexin V/propidium iodide staining was used to evaluate apoptosis. After 48 hours, sorafenib (5 μM)−treated and CAI (10 μM)−treated LLC cells elicited 24.1% and 25.1% apoptosis, respectively, and the combination induced a larger proportion of cell apoptosis (up to 47.6%). In A549 cells, CAI-, sorafenib- or the combination-induced apoptotic cell percentage was 14.8%, 10.6%, and 42.1%, respectively. In addition, an increased portion of necrotic cells (Annexin V negative/PI positive) were observed in the combination group (Fig. 2A).

To further confirm the induction of apoptosis by this combination, we analyzed cell extracts for expression of PARP (an endogenous substrate of caspase-3 and caspase-7) and cleaved caspase-3 (which is associated with programmed cell death) (Fig. 2, B and C). The levels of cleaved PARP and cleaved caspase 3 were elevated in the combination group, indicating that cell was executed by programmed cell death. To verify whether the increase in late apoptotic cells treated with CAI and/or sorafenib was associated with caspase signaling, cells were cotreated with 20 μM Z-VAD-FMK, a pan-caspase inhibitor. The apoptotic percentage of cells cotreated with Z-VAD-FMK was significantly decreased compared with treatment with the indicated drugs alone (Fig. 2A). These results show that the combination of CAI and sorafenib synergistically induced apoptosis in vitro. Cell cycle analysis showed that CAI-treated LLC and A549 cells were arrested in the G2/M phase and sorafenib-treated cells were arrested in the G0/G1 phase. In addition, the combination group showed a significant decrease in the S phase and an increase in both the G0/G1 and G2/M phases (Fig. 2D).

**Sorafenib and CAI Increase DCFH-DA and MitoSOX Oxidation Suggesting Increased Intracellular ROS and Provoke Mitochondrial Depolarization.** Abnormal accumulation of intracellular ROS could induce oxidative stress in cancer cells, and excessive ROS could directly
attack DNA, protein, lipids, and other cellular components, eventually leading to cell apoptosis (Simon et al., 2000). Previous studies showed that sorafenib or CAI alone could induce the generation of intracellular ROS both in vivo and in vitro (Corrado et al., 2011; Coriat et al., 2012). Therefore, we speculated that the synergistic effect of sorafenib and CAI on apoptosis might enhance the abnormal level of ROS. We first used DCFH-DA as a probe and observed dichlorofluorescein fluorescence under a fluorescence microscope. In LLC and H1975 cell lines, ROS production increased slightly in the presence of sorafenib or CAI, whereas it was provoked significantly in the combination group (Fig. 3A). We further observed enhanced fluorescence intensity upon increased MitoSOX oxidation after CAI, sorafenib, or combination treatment, suggesting that these interventions could increase mitochondrial ROS or superoxide at varying degrees in LLC cells (Fig. 3B).

Since we found that mitochondrial released ROS contributed to the accumulation of the overall ROS in cells. Furthermore, high ROS levels normally disrupted mitochondrial membrane potential ($\Delta \psi_m$). In addition, mitochondrial depolarization is a distinctive feature of the early stages of programmed cell death. Thus, we determined changes in $\Delta \psi_m$ by staining cells with JC-1 dye. Compared with DMSO group, CAI (10 $\mu$M) or sorafenib (5 $\mu$M) in LLC cells as monotherapy slightly decreased $\Delta \psi_m$, whereas the combination group was dramatically damaged mitochondrial function as decreased 50% the $\Delta \psi_m$ compared with DMSO control. Thus, these data revealed that CAI and
Sorafenib induced ROS accumulation and sabotaged mitochondrial potential.

**Sorafenib and CAI Decreased the Expression of NANOG In Vitro.** Several studies reveal that NANOG is not only a marker of stem cells but also a key factor in endowing cancer cells with certain stem cell characteristics such as metastasis, tumorigenicity, self-renewal, and drug resistance (Gong et al., 2015). To investigate the role of NANOG in CAI- and/or sorafenib-induced anticancer activity, we measured mRNA and protein levels of NANOG after exposing LLC cells to the indicated treatment. As shown in Fig. 4A, after 24-hour treatment with CAI or sorafenib, NANOG mRNA levels decreased slightly in single-agent groups compared with the control, whereas the combination of CAI and sorafenib was more effective in restraining NANOG expression than the drugs used as monotherapy (Fig. 4A). Similarly, this tendency was confirmed by Western blot experiments (Fig. 4B).

To explore whether NANOG is involved in the accumulation of ROS after CAI and/or sorafenib treatment, we used basic fibroblast growth factor (bFGF), which is reported to stimulate NANOG expression in cancer cells (Shen et al., 2016). In this study, bFGF stimulated NANOG expression in LLC cells and promoted both LLC and A549 cell proliferation (Fig. 4, C and D). In A549 and LLC cell lines, cotreatment with bFGF significantly reduced the CAI- and/or sorafenib-mediated increase in intracellular ROS levels (Fig. 4, E and F). Strikingly, in A549 cells, cotreatment with bFGF almost inhibited the ability in increasing ROS of CAI or sorafenib as monotherapy (Fig. 4G). These results implied that NANOG expression was impaired by CAI in combination with sorafenib and the decrease of its expression was, at least partially, responsible for the accumulation of ROS.

**Sorafenib and CAI Induced Apoptosis in a ROS and NANOG-Dependent Manner In Vitro.** Sorafenib and CAI instigated aberrant accumulation of ROS, which were associated with the downregulation of NANOG expression. To further verify the role of NANOG and ROS in CAI- and/or sorafenib-induced cell apoptosis, bFGF and GSH were used concomitantly with the indicated drugs. As shown in Fig. 5, A and C, 40 ng/ml bFGF and 4 mM GSH partially reverted the effect of sorafenib and/or CAI on cell apoptosis. These data show that ROS production and NANOG inhibition are important upstream pathways of sorafenib- and/or CAI-induced cell apoptosis. In addition, increased amounts of necrotic cells (Annexin V negative/PI positive) were observed in experiments with the sorafenib and CAI combination treatment (Fig. 5, A and C).

**The Combination of Sorafenib and CAI Exhibited Synergistic Antitumor Activity In Vivo.** The data from our in vitro experiments incited us to evaluate the efficiency of this combination in an in vivo model. Thus, C57BL/6J mice bearing the LLC tumor model were established. Mice were gavaged with vehicle (PEG400), SFB-L (10 mg/kg body weight), CAI (30 mg/kg body weight), SFB-H (30 mg/kg body weight), or combination treatment with sorafenib (10 mg/kg body weight) and CAI (30 mg/kg body weight) every day for 29 days.

![Fig. 5. Sorafenib and CAI induced apoptosis in a ROS- and NANOG-dependent manner in vitro. (A and C) LLC cells (A) and A549 cells (C) were cotreated with 40 ng/ml bFGF or 4 mM GSH with sorafenib (5 μM) and/or CAI (10 μM) for 48 hours, after which cells were stained with Annexin V/PI and analyzed for apoptosis by flow cytometry. (B and D) Statistical quantification of apoptotic LLC cells (B) and A549 cells (D), respectively. Values are presented as means ± SD. **P < 0.05; ***P < 0.01; ****P < 0.001 (significant between control groups and in cotreatment with bFGF groups); #P < 0.05; ##P < 0.01; ###P < 0.001 (significant between control groups and in cotreatment with GSH groups).]


Combination treatment led to less tumor growth compared with single-drug treatment (Fig. 6A). We then assessed the tumor weight to further verify the efficiency of this combination (Fig. 6B). In accordance with tumor volumes, the average tumor weights associated with PEG400, CAI, SFB-L, SFB-H, and combination treatment were 4.77, 2.56, 2.14, 1.68 g, and 1.59, respectively. It is worth noting that the dose of sorafenib was 10 mg/kg in the combination group and 30 mg/kg in the SFB-H group; there was no statistically significant difference in tumor weight between two groups (Fig. 6B).

To evaluate side effects on mice in general, we monitored their weight daily. The average body weights (arranged from high to low) were 29.58 g (PEG400), 29.14 g (CAI), 27.64 g (combination), 27.18 g (SFB-L), and 26.66 g (SFB-H) (Fig. 6D).

**Fig. 6.** The combination of sorafenib and CAI exhibits synergistic antitumor activity in vivo. Male C57BL/6J mice bearing LLC tumors were randomly distributed into five groups (n = 10) and treated with PEG400, CAI (30 mg/kg), SFB-L (10 mg/kg), SFB-H (30 mg/kg), or the combination of CAI (30 mg/kg) and SFB (10 mg/kg). (A) Tumor volumes were measured with calipers every other day. Mean tumor volumes ± S.E.M. of each group are shown (n = 6–10). *P < 0.05 (significant between combination group with the CAI group); #P < 0.05 (significant between the combination group with SFB-L group). (B) On day 29, tumors were excised and weighed. As a result of tumor progression, 10 mice died before the end of the experiment (4 in the PEG400 group, 4 in the CAI group, 1 in the SFB-L group, and 1 in the SFB-H group). Tumor weights are presented as scatter dots. **P < 0.01; ***P < 0.001 (significant compared with the PEG400 group). (C) Photograph of tumors in the PEG400, CAI, SFB-L, and combination groups. (D) Body weights of mice in each group were measured daily to roughly evaluate the side effects of different treatments. Mean body weights ± S.E.M. of each group are shown (n = 6–10). (E) To exclude the effect of differences in tumor weights in body weight, we calculated carcass weights by using body weight minus the corresponding tumor weight. *P < 0.05 (significant between CAI group with PEG400 group); #P < 0.05 (significant between combination group with SFB-H group). ns, not significant.
Although the PEG400 group seemed to have the highest body weight compared with other groups, the tumor weight in the PEG400 group was much higher than the others (as mentioned earlier). Therefore, we calculated the carcass weight of each group to exclude the difference in tumor weights. As shown in Fig. 6E, the carcass weight exhibited a slightly different tendency: 26.59 g (CAI), 26.05 g (combination), 25.04 g (SFB-L), 24.82 g (PEG400), and 24.80 g (SFB-H). Consistent with our previous observation that CAI could ameliorate cancer-associated cachexia (Chen et al., 2017), the CAI and combination treatment groups showed potential to maintain the body weight of tumor-bearing mice compared with the PEG400 group. It is noteworthy that the average carcass weight in the combination group was significantly heavier than that in the SFB-H group (Fig. 6E). Taken together, these data suggest that CAI not only synergized in vivo antitumor activity with sorafenib but also attenuated weight loss during cancer progression.

**Sorafenib and CAI Inhibit NANOG In Vivo.** Because in vitro studies indicate that NANOG is a key mediator of CAI and sorafenib-induced cell death, we also investigated the expression of NANOG in vivo. We performed immunohistochemistry for NANOG expression in tumor sections after 29 days of treatment with sorafenib and/or CAI (Fig. 7A). The percentage of NANOG-stained tumor sections was significantly decreased in the combination group compared with groups treated with vehicle or single agents. We used Western blot analysis to test tumor lysis in each group, and we further confirmed that NANOG expression was decreased in the combination group (Fig. 7B). In addition, hematoxylin and eosin staining revealed that greater necrosis was observed in tumors after treatment with sorafenib and CAI compared with vehicle or single agents (Fig. 7A). The quantification of MDA is commonly applied as a marker for lipid peroxidation and is often seen at the level of ROS-mediated injury. The MDA level was increased in the combination group compared with the other groups (Fig. 7C). Taken together, these data confirmed that NANOG was downregulated after CAI and sorafenib treatment in vivo.

**Discussion**

We report here the synergistic antitumor effect of sorafenib and CAI in NSCLC both in vivo and in vitro. The underlying mechanisms of this effect are associated with downregulation of NANOG and induction of apoptosis. Combination

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**Fig. 7.** Sorafenib and CAI inhibit NANOG in vivo. (A) Immunohistochemistry of NANOG and H&E staining were performed on tumor sections in the PEG400, CAI, SFB-L, and combination groups. (B) Tumor lysis was prepared using tumors excised from mice at the end of the experiment. Levels of NANOG in tumor lysates of PEG400, CAI, SFB-L, and combination groups were measured using Western blot analysis. Protein levels of NANOG from tumor lysates were normalized against GAPDH levels. Band density was evaluated using ImageJ software. Values are presented as means ± SD. *P < 0.05 (versus PEG400 group). (C) Tumor lysates were subjected to the MDA assay. *P < 0.05. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; H&E, hematoxylin and eosin.
therapy–induced apoptosis was mostly associated with mitochondrial-related cell death, owing to the accumulation of mitochondrial ROS, upregulation of cleaved caspase-3, and depolarization of mitochondria.

NANOG has an important role in tumorigenesis, chemoresistance, relapse, and metastasis. A previous study showed that NANOG-positive CSCs were much more resistant to sorafenib than NANOG-negative CSCs (Shan et al., 2012). Here, we found that the combination of CAI and sorafenib could dramatically decrease mRNA and protein levels of NANOG, which to some extent transferred NANOG-positive cells to a state of NANOG-negative cells. This might be a reason why, NSCLC cells were more sensitive to sorafenib in combination with CAI both in vitro and in vitro. To better understand the exact mechanism by which the combination treatment dramatically inhibits NANOG, the upstream signaling of NANOG perturbed by these two agents must be further explored.

It was recently shown that in TICs, NANOG combines with the promoter of OXPHOS genes and restrains their expression, quenching ROS production (Chen et al., 2016). OXPHOS defects are a well known pivotal reason for the reduction in apoptosis in cancer cells (Yadav et al., 2015). The downregulation of NANOG may result in restored expression of OXPHOS-encoding genes. Our previous study showed that CAI, like rotenone, could inhibit the enzyme activity of one OXPHOS components (complex I) (Ju et al., 2016). It is clear that rotenone can block the electron transport chain, resulting in OXPHOS blockade and ROS accumulation (Radad et al., 2006). Similarly, it is possible that CAI may induce ROS production in a similar manner; thus, OXPHOS gene expression may necessitate exposing I to CAI and may trigger downstream signaling.

It is well known that the excessive generation of ROS by various chemotherapies leads to the loss of mitochondrial membrane potential and induces apoptosis in cancer cells. In addition, ROS are also key factors for chemoresistance of cancer. In LLC and A549 cells, using bFGF to upregulate NANOG is associated with decreased levels of ROS, even in CAI, sorafenib or combination groups. At the same time, with decreased ROS production, cell proliferation was also promoted by the cotreatment of bFGF. This implies that ROS accumulation by CAI and/or sorafenib occurs partially through downregulation of NANOG. In addition, the augment of ROS by these two agents is possibly associated with OXPHOS expressions.

Interestingly, CI values of A549 cells were mostly less than 1, which means that the combination of CAI and sorafenib at different concentrations was synergistic. However, in H1975 cells, CI values were less than 1 in a lower dosage of both drugs and more than 1 in higher dosages, which indicated that the combination lost efficacy in the high dosages. It is well known that A549 cells possess wild-type endothelial growth factor receptor (EGFR) and KRAS mutations (exon 2), whereas H1975 cell harbors the L858R/T790M EGFR mutations and wild-type KRAS. The absence of the synergistic effect of CAI and sorafenib in H1975 cells might be due to the mutation of EGFR, since L858R/T790M double mutants display a considerable increase in tyrosine phosphorylation levels compared with only one mutation in either L858R or T790M (Mulloy et al., 2007). The combination regimen may not be able to counteract the aberrant activation of EGFR signaling. Mutations in the KRAS proto-oncogene are common aberrations and are found in 10%–30% of lung adenocarcinomas. These mutations lead to abnormal activation of the KRAS signaling pathway that results in continuous cell proliferation and indicates poor NSCLC prognosis. The combination of CAI and sorafenib has a synergistic effect in cells regardless of KRAS mutations. These data imply that patients with KRAS mutations and without L858R/T790M EGFR mutations will benefit from the synergistic effect of CAI and sorafenib.

In conclusion, combined CAI and sorafenib therapy has a synergistic effect in resisting NSCLC in vivo and in vitro, which may contribute to the induction of apoptosis and inhibition of NANOG. The combination in vivo may allow a dose reduction of sorafenib and may avoid decreasing effectiveness. Thus, our findings provide a novel strategy to expand the application of sorafenib and may raise a new choice for treatment of NSCLC.

Authorship Contributions

Participants in research design: C. Chen, Zhang, Ye, Guo.

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References


CAI Synergizes with Sorafenib in NSCLC


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