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Carboxyamidotriazole synergizes with sorafenib to combat non-small cell lung cancer through inhibition of NANOG and aggravation of apoptosis

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Running title page

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CAI, carboxyamidotriazole; DMSO, Dimethyl Sulphoxide; FCM, flow cytometry; FGF, fibroblast growth factor; LLC, lewis lung carcinoma; MDA, malondialdehyde; NSCLC, non-smal cell lung cancer; PEG 400, polyethylene glycol 400; ROS, reactive- oxygen species;

SFB, sorafenib.
Abstract

Lung cancer is the leading cause of cancer-related deaths in the world. In this study, we investigated the combination of carboxyamidotriazole (CAI) and sorafenib in 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 co-administration of CAI and sorafenib synergistically inhibited the proliferation of NSCLC cells (Lewis lung carcinoma, A549, NCI-H1975). The cell death led by the treatment of combination drugs was attributed to apoptosis which was accompanied with activation of caspase 3 and poly ADP-ribose polymerase. In addition, combined therapy induced accumulation of mitochondrial associated reactive oxygen species (ROS), depolarization of mitochondrial and reduced NANOG mRNA and protein expressions. The bFGF as a stimulator of NANOG was applied to identify the possible mechanism. Adding bFGF can stimulate NANOG expression and synchronously rescue the accumulation of ROS followed by combined therapy treatment. C57BL/6J mice bearing Lewis lung carcinoma were randomized to receive vehicle (PEG400), CAI (30 mg kg\(^{-1}\)), Sorafenib-low dose (SFB-L, 10 mg kg\(^{-1}\)), combination (CAI+SFB-L) and Sorafenib-high dose (SFB-H, 30 mg kg\(^{-1}\)). The combination group significantly suppressed tumor growth, and the efficacy of combination treatment was equivalent to sorafenib-high dose monotherapy group. Furthermore, combination group showed a reduced side effect than SFB-H group as indicated by the preservation of mice weight. Our study illustrates that CAI enhances the antitumor activity of sorafenib in NSCLC and provides a novel strategy to the treatment of NSCLC.
Introduction

Lung cancer is the leading cause of cancer-related deaths in the world and 5-year survival of lung cancer patients is approximate 4-17% along with differences in stage and region (Hirsch et al., 2016). Non-small cell lung cancer (NSCLC) accounts for 80% of all lung cancers in worldwide (Jemal et al., 2011). The most frequently used treatments for NSCLC are surgical, chemotherapy and radiotherapy. Unfortunately, when diagnosed, 2/3 of the patients are on an advanced and inoperable stage. Many of platinum-based regimens are first-line chemotherapies; Single-agent docetaxel, pemetrexed, and erlotinib are considered as second-line therapies. However, if patients fail to respond to conventional chemotherapies, the treatment options are limited (Langer et al., 2013). Presented therapies have shown to be inadequate and novel strategies are urgently required.

Sorafenib is a multikinase inhibitor that blocks Raf kinase, platelet-derived growth factor receptor (PDGFR) and vascular endothelial growth factor receptors (VEGFR). It was approved by FDA for the treatment of advanced renal cell carcinoma (RCC) and unresectable hepatocellular carcinoma (HCC) (Wilhelm et al., 2006). Sorafenib has been confirmed activity in preclinical models of NSCLC (Wilhelm et al., 2004; Gridelli et al., 2007), and it has also been under evaluation in several clinical trials (I-III phases) in advanced NSCLC patients (Clark et al., 2005; Moore et al., 2005; Blumenschein et al., 2009; Wakelee et al., 2012; Paz-Ares et al., 2015). One limitation that hampers the use of sorafenib as a monotherapy is side effects, such as hand-foot syndrome, rash, diarrhea, hypertension and fatigue, which has been harassing 80% of patients receiving sorafenib treatment (Batchelor et al., 2007; Strumberg et al., 2007). Most of the aforementioned side effects of sorafenib are dose-dependent, therefore reducing its dosage while
keeping same therapeutic effect may need further investigation.

Carboxyamidotriazole (CAI) is a blocker of non-voltage dependent calcium channel, which shows anti-angiogenic, anti-proliferative and anti-metastatic properties in preclinical research. CAI has been investigated under 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 exhibits mild anti-cancer properties in some of those 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 CAI may synergize with other anti-cancer drug through limiting the weight loss induced by chemotherapy. In chronic myeloid leukemia cells, CAI was shown to reduce cell viability and induce apoptosis by a redox-mediated way (Alessandro et al., 2008; Corrado et al., 2011). Our previous work showed that CAI synergized with 2-DG, a glycolysis inhibitor, in respect that CAI 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 transcription factor NANOG confers self-renewal and differentiation to cancer stem cells (CSCs), and it is often considered as a hallmarker of CSCs. Recently accumulated data show that the expression of NANOG in cancer cells can make it possess certain CSC properties such as
self-renewal, tumorigenicity, metastasis and resistance to chemotherapy (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 lung cancer patient, both NANOG mRNA and protein expressions in lung cancer tissues were much higher than that in patient- matched normal tissues (Du et al., 2013). As to the signaling that NANOG perturbed within cells, Chen et al. show that in tumor- initiating stem-like cells (TICs), NANOG prevented mitochondrial ROS production and NANOG- silenced TICs produced much more ROS than control (Chen et al., 2016).

Objectives of our study were firstly to probe whether a combined therapy of CAI and sorafenib may allow a dose reduction of sorafenib and avoid of lessening effectiveness, then to investigate the mechanism by which the combination was exerting its action.

**Materials and methods**

**Cell lines and reagents**

Lewis lung carcinoma (LLC) was purchased from Shanghai Institute for Biological Sciences, CAS. A549 and NCI-H1975 (H1975) cells were purchased from Institute of Basic Medical Sciences, CAMS&PUMC. LLC cell was cultured in DMEM (high glucose). A549 cell was maintained in F-12K. H1975 was grown in RPMI 1640. All mediums were with supplement of 10% Fetal Bovine Serum (Gibco), 50 mg/mL penicillin, 100mg/mL streptomycin and L-glutamine. Cells were maintained in a humidified atmosphere with 5% CO$_2$ at 37°C.

Carboxyamidotriazole was synthesized by the Institute of Materia Medica, Chinese Academy
of Medical Sciences (Beijing, China). Sorafenib was purchased from MedChem Express (NJ, USA). Glutathione-reduced ethyl ester (GSH, Sigma) was solved in sterile PBS as a 0.25 M stock solution. Z-VAD-FMK (Beyotime Biotechnology) was prepared in DMSO as a 20 mM stock solution. For in vitro studies, CAI and Sorafenib were dissolved in dimethyl sulfoxide (DMSO) and diluted with corresponding medium to the final concentration with 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, combination index

Proliferations of cells for different concentrations after indicated time of treatment were assessed through Sulforhodamine B (SRB) method (Vichai et al., 2006). DMSO was used as vehicle. Following treatment with CAI and/or sorafenib for indicated time period (24 h, 48 h and 72 h), cells in 96-well plates were fixed with 10% (wt/vol) trichloroacetic acid at 4°C for 1h, then the plates were washed by slow-running water for 5 times and dried at room temperature. Next, the intracellular proteins were stained by 0.4% SRB for 20min and then the plates were washed five times with 1% (vol/vol) acetic acid. After adding 200 µL of 10 mM Tris base solution in each well, the absorbance of SRB can be detected at 515nm in a microplate reader. Combination indices (CIs) were calculated using CompuSyn software (ComboSyn Inc, Paramus, NJ, USA) (Chou et al., 1984; Chou, 2006). According to Chou-Talalay, a CI <1 or >1 indicated synergism or antagonism, respectively.

Apoptosis and cell cycle analysis by flow cytometry
Cells were seeded and treated with CAI and/or Sorafenib for 24 h, then they were detached from the plates with Trypsin-EDTA, and washed with PBS for two times. The apoptosis induction was measured by FCM using Annexin V- FITC/PI kit (Dojindo, Kyushu, Japan). The approximate fluorescence excitation/emission wavelengths of FITC-Annexin V and Propidium iodide are 494/518 nm and 488/617 nm, respectively. Both Annexin V and PI negative subpopulation indicates intact cells, while Annexin V positive and PI negative subpopulation represents cells that are in early apoptosis. Cells that are in late apoptosis or already dead are both Annexin V and PI positive and necrotic cells are Annexin V negative and PI positive. Apoptotic cell rate was calculated by adding the portion of cells in Annexin V+/PI- and in Annexin V+/PI+.

Following treatment with CAI and/or sorafenib for 48 h, cells were harvested and fixed in 75% ethanol at 4°C for 18 h. After washing with cold PBS twice, DNA staining was performed using propidium iodide (0.05 mg/ml) and RNase (2 mg/ml) (Beyotime Biotechnology) at room temperature for 30 min. The cell cycle distributions were measured by FCM (excitation/ emission wavelength: 488 nm/617 nm). The distribution of cell cycle phases were calculated using ModFit LT software (Verity Software House, Topsham, ME, USA).

Determination of intracellular ROS level and mitochondrial membrane potential

Cells were seeded in 6-well plates and treated with indicated drugs. For FCM analysis: after 24 h treatment cells were detached from the plates with Trypsin-EDTA, washed with PBS buffer twice, and incubated with 20 µM DCFH-DA (Sigma) for 20min at 37°C. Then cells were washed gently three times with PBS buffer and finally tested cells using FCM. FCM was performed using excitation/emission of 488/525 nm for DCFH-DA.
For fluorescent microscope detection: after 24 h treatment cells were washed twice with PBS buffer, and incubated with 20 µM DCFH-DA for 20 min. Then cells were washed gently three times with PBS buffer and finally detected cells using fluorescent microscope.

**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 6-well plates and treated with indicated drugs. After 24 h treatment cells were detached from the plates with Trypsin-EDTA, washed with PBS buffer twice, and incubated with 5 µM MitoSOX for 10 min at 37°C. Then washed cells gently three times with PBS buffer and finally tested cells using FCM. FCM was performed using excitation/emission of 488/625 nm for MitoSOX. In negative control group, DMSO (solvent of MitoSOX) instead of MitoSOX was used to define the background and delimit the negative from positive regions.

**Analysis of mitochondrial membrane potential**

To measure the depolarization of the mitochondrial membrane potential, JC-1 staining (Mitochondrial membrane potential assay kit, Beyotimes, Nantong, China) 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 at 6-well plates and treated with CAI and/or Sorafenib. After 24 h, cells were detached from the plates with Trypsin-EDTA, washed by PBS, resuspended with 500µL of JC-1 working solution, and subsequently incubated at 37°C for 20 min. Finally, after washed by the assay buffer twice, cells were proceeded the FCM analysis. Flow
Cytometry was performed using excitation/emission of 485/529 nm for monomeric form and 535/590 nm for J-aggregates.

LLC xenograft model

Six-week-old male C57BL/6 mice (Institute of Laboratory Animal Sciences, CAMS&PUMC, China; weight 18-22g) were injected subcutaneously in right axillary fossa with 1×10^6 LLC cells re-suspended in 100μl PBS (Day 0). Mice were randomly divided into 4 different groups, with 10 animals in each group. The animals were treated by gavage once daily, with either vehicle control (PEG400), CAI (30mg kg^-1 day^-1), sorafenib-lower dosage (SFB-L, 10mg kg^-1 day^-1), sorafenib-higher dosage (SFB-H, 30mg kg^-1 day^-1), combination group (CAI: 30mg kg^-1 day^-1, SFB: 10mg kg^-1 day^-1). The changes of tumor length and width were measured with a caliper every other day from Day 10 to 29, and the tumor volumes (in mm^3) were calculated using the formula V=length×width^2×0.5. After 29 days of treatment mice were sacrificed. 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 (Thermo Fisher Scientific Inc., Pittsburgh, USA) with Phosphatase and Protease inhibitor cocktail (Roche, Mannheim, Germany), incubated for 15min, and then centrifuged at 16,100 × g at 4°C for 30 min. The protein-containing supernatant was collected and protein concentration was quantified using BCA protein Assay Kit (Biotek 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 PVDF membrane. After being blocked with 5% non-fat dry milk in Tris-buffered saline containing Tween-20, the membrane was incubated with the desired antibodies. The following primary antibodies were used: NANOG, PARP, cleaved-caspase 3, GAPDH (all from Cell Signaling Technology Inc, Danvers, MA), β-actin (Sigma-Aldrich). Subsequently, the membrane was incubated with appropriate secondary antibody and the immunoreactive protein bands were visualized using a chemiluminescence kit (Millipore, MA, USA) followed by ECL-based autoradiography (GE healthcare, UK). Western blots are representative of at least three independent experiments.

Immunohistochemistry staining

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

Total RNA extraction and RT (reverse transcription)-qPCR(quantitative PCR)

RNA was isolated from LLC cell using Pure RNA Extraction Kit (Biotek corp., Beijing, China) and 2 μg of total RNA was used to synthesize cDNA with the TransScript First-Strand
cDNA Synthesis supermix (TransGen Biotech Co., Beijing, China), as per the manufacturer’s instructions. qPCR was performed using the IQ5 Real-Time system (Bio-Rad, USA). Each RT-qPCR reaction (25μl) contained 2μl of diluted cDNA, 12.5μl of 2×Maxima SYBR Green (Thermo Fisher Scientific Inc., Pittsburgh, USA). All reactions were performed using the following thermal cycle conditions: 94°C for 2 min followed by 40 cycles of a two-step reaction, denaturation at 94°C for 10s, annealing at 60°C for 30s, followed by a melting curve from 55 to 95°C in 1 s increments of 0.5°C to ensure amplification specificity. Transcript levels of the target genes were normalized to GAPDH.

Malondialdehyde (MDA) assay

Tumor tissues were homogenized and prepared for MDA assay according to the manufacturer’s instructions in the Lipid Peroxidation MDA assay kit (Beyotimes, Nantong, China). The MDA concentration of each sample was detected at 532 nm (450 nm as a control), by microplate reader (Synergy 4, Biotek).

Statistical analysis

All data were expressed as the mean±S.D. of three independent set of experiments unless otherwise indicated and analysed by Student’s t-test or ANOVA followed by the Tukey's multiple comparison test. P-Values<0.05 were considered as 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 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, 10 μM and CAI at concentrations of 1, 2, 5, 10, 15, 20 μM and their combination were all applied. As shown in Fig 1, CAI, sorafenib and their combination led to a dramatically reduce cell proliferation, in a dose-dependent manner, in three cell lines investigated. It is noteworthy that the cytotoxicity effect of sorafenib as low as 1 μM in combination with CAI, was comparable to 10-15 μM sorafenib in single use in three cell lines and three time points. In order to do mathematical analysis, three cell lines were treated with different concentrations of CAI and sorafenib at a fixed ratio (2:1), using median effect analysis (Chou et al., 1984). As indicated in Fig 1, the combination index (CI) values for synergism evaluation were less than 1 in LLC cell and mostly less than 1 in A549 and H1975 cells. That means in LLC cell, the combination of sorafenib and CAI led to synergistically inhibit cell proliferation, and in A549 and H1975 cell lines the combination of both drugs especially in lower dose, generated synergistic effect.

Combination of sorafenib and CAI induce apoptosis in NSCLC

As we have showed the synergistic inhibitory effect of the combination treatment on NSCLC, specific mechanisms on cell death need to be further explored. To clarify the effect of combining these two agents, LLC cells were treated with the drugs individually or in combination and investigated by Annexin V/propidium iodide staining to evaluate apoptosis. After 48 h sorafenib (5 μM)-treated and CAI (10 μM)-treated LLC cells elicited 24.1% and 25.1% apoptosis, respectively and the combination induced more proportion of cell apoptosis up to 47.6%. In A549
cell, after the same treatments as that in LLC, the apoptotic cell percentages were 14.8% (CAI), 10.6% (Sorafenib) and 42.1% (Combination), respectively. Besides apoptosis, there was a portion of necrotic cells (Annexin V-/PI+) were observed (Fig 2A).

To further confirm the induction of apoptosis by this combination, we analyzed cell extracts for expression of poly (ADP-ribose) polymerase (PARP) (an endogenous substrate of caspase-3 and -7), and cleaved caspase-3 which is associated in programmed cell death (Fig 2B, 2C). The combination group elevated both cleaved PARP and cleaved caspase-3, indicating that cell was executed by programmed cell death. To testify if the increase of late apoptosis cells treated with CAI and/or sorafenib was associated with caspase signaling, cells were co-treated with 20 µM Z-VAD-FMK, a pan-caspase inhibitor. The apoptotic percentage of cells co-treated with Z-VAD-FMK was significantly decreased in comparison with treatment of indicated drugs alone (Fig 2A). These results show that the combination of CAI and sorafenib synergistically induces apoptosis in vitro. The cell cycle analysis showed LLC and A549 cells treated with the CAI were arrested in G2/M phase and cells treated with sorafenib were arrested in G0/G1 phase. In addition, the combination group showed a significantly decrease in S phase and increase in both 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 lead 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 on apoptosis of sorafenib and CAI might result in enhanced the abnormal level of ROS. We first utilized DCFH-DA as a probe and observed DCF fluorescence under a fluorescence microscope. In LLC and H1975 cell lines, ROS production were increased slightly in the presence of sorafenib or CAI, while it was provoked significantly in the combination group (Fig 3A). Then we further observed enhanced fluorescence intensity upon increased MitoSOX oxidation after CAI, sorafenib or the combination treatment, suggesting that these interventions could increase mitochondrial ROS or superoxide at various degrees in LLC cell (Fig 3B).

Since we found that mitochondrial released ROS, at least in part, contributed to the accumulation of the overall ROS in cells and high ROS level normally disrupted mitochondrial membrane potential ($\Delta \Psi_m$). In addition, mitochondrial depolarization is a distinctive feature of early stages of programmed cell death. Thus, we determined the changes in $\Delta \Psi_m$ by staining cells with dye JC-1. The treatment of CAI (10 μM) or sorafenib (5 μM) in LLC as monotherapy was slightly decreased $\Delta \Psi_m$, while the combination group was dramatically damaged mitochondrial function as decreased 50% the $\Delta \Psi_m$ compared with DMSO control. Thus, these data revealed 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 endowing cancer cells with certain stem cells characters 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 anti-cancer activity, we measured mRNA and protein levels of NANOG after exposing LLC cells with indicated treatment. As shown in Fig 4A, after 24 h treatment of CAI or sorafenib, NANOG mRNA level dropped slightly in single-agent groups compared with control, whereas the combination of CAI and sorafenib was more effective in restraining NANOG expression than as monotherapy (Fig 4A). Similarly, the 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 utilized basic fibroblast growth factor (bFGF), which is reported to stimulate NANOG expression in cancer cells (Shen et al., 2016). In the present study, bFGF stimulated NANOG expression in LLC cell and promoted both LLC and A549 cells proliferation (Fig 4C, 4D). In A549 and LLC cell lines, co-treatment of bFGF significantly reduced CAI and/or sorafenib mediated increase in intracellular ROS levels (Fig 4E, 4F). Strikingly, in A549 cells, co-treatment of bFGF almost strangled 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 5A, 5C, 40 ng ml\(^{-1}\) bFGF and 4 mM GSH partially reverted the effect of sorafenib and/or CAI on cell apoptosis. These data showed the production of ROS and the inhibition of NANOG are important upstream pathways of sorafenib and/or CAI induced cell apoptosis. In addition, in experiments with combination treatment of sorafenib and CAI increased amounts of necrotic cells (Annexin V-/PI+) were observed (Fig 5A, Fig 5C).

Combination of sorafenib and CAI exhibited synergistic antitumor activity \textit{in vivo}

Those data from \textit{in vitro} experiments incited us to evaluate the efficiency of this combination into \textit{in vivo} model. Thus, C57BL/6J mice bearing LLC tumor model were established. Mice were gavaged with vehicle (PEG400), sorafenib-low dose (SFB-L, 10mg kg\(^{-1}\) body weight), CAI (30mg kg\(^{-1}\) body weight), combination (sorafenib 10mg kg\(^{-1}\) body weight and CAI 30mg kg\(^{-1}\) body weight), or sorafenib-high dose (SFB-H, 30mg kg\(^{-1}\) body weight) every day for 29 days. 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, combination and SFB-H therapy were 4.77, 2.56, 2.14, 1.59 and 1.68 g, respectively. It is worthwhile noting that the dosage of sorafenib in combination group is 10mg kg\(^{-1}\) and in SFB-H group is 30mg kg\(^{-1}\), and there is no statistical significance in the tumor weight between two groups (Fig 6B).

To evaluate the side effect of mice in general, we monitored the weight of mice every day. The average body weights arranging from high to low were PEG400 (29.58 g), CAI (29.14 g), combination (27.64 g), SFB-L (27.18 g) and SFB-H (26.66 g) (Fig 6D). Although PEG400 group
was seemed to possess the highest body weight compared with other groups, the tumor weight in PEG400 group was much higher than others as mentioned before. So we calculated the carcass weight of each group to exclude the difference of tumor weights. As shown in Fig 6E, the carcass weight exhibited a little bit different tendency: CAI (26.59 g), combination (26.05 g), SFB-L (25.04 g), PEG400 (24.82 g), SFB-H (24.80 g). In consistency with our previous observation that CAI could ameliorate cancer associate cachexia (Chen et al., 2017), CAI group as well as combination group showed the potential to maintain the body weight of tumor-bearing mice compared with PEG400 group. It is noteworthy that the average carcass weight in combination group was significantly heavier than that in SFB-H group (Fig 6E). Taken together, these data suggested that CAI not only synergized with sorafenib in the \textit{in vivo} antitumor activity but also attenuated weight loss during cancer progression.

Sorafenib and CAI inhibit NANOG \textit{in vivo}

As \textit{in vitro} studies indicate that NANOG is a key mediator of CAI and sorafenib-induced cell death, we also investigated the expression of NANOG \textit{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 combination group as compared with vehicle or single agents. Using western blot analysis to test tumor lysis in each group, we further confirmed NANOG expression was decreased in combination group (Fig 7B). In addition, H&E staining revealed that greater necrosis was observed in tumors following sorafenib and CAI treatment than in vehicle or single agents (Fig 7A). The quantification of MDA is commonly applied as a marker for lipid
peroxidation and is often seen as the level of ROS-mediated injury. The level of MDA was increased in combination group compared with the other groups (Fig 7C). Taken together, these data confirmed NANOG was downregulated after CAI and sorafenib treatment in vivo.

Discussion

We report here the synergistic anti-tumor effect of sorafenib and CAI in NSCLC both in vivo and in vitro. The underlying mechanisms of this effect are associated with down-regulation of NANOG and induction of apoptosis. The combination therapies induced apoptosis was mostly associated with mitochondrial related cell death, because the accumulation of mitochondrial ROS, upregulation of cleaved-caspase 3, and depolarization of mitochondrial.

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

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

It is well-known that the excessive generation of ROS by various chemotherapies leads to the loss of mitochondrial membrane potential and induce 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 the decreased levels of ROS, even with the treatment of CAI and/or sorafenib. At the same time, with the decreased ROS production, the proliferation of cell was also promoted by the co-treatment of bFGF. It is implying that accumulation of ROS by CAI and/or sorafenib is partially through downregulation of NANOG. In addition, the augment of ROS by these two agents is possibly in an OXPHOS-dependent manner.

Interestingly, the CI values of A549 were mostly below 1, which meant the combination of CAI and sorafenib at different concentrations were synergistic. However, in H1975 cell the 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 cell possess wild type of EGFR and mutations of KRAS (Exon2), whereas H1975 cell harbors the L858R/T790M EGFR mutation and wild type of KRAS. The absence of synergistic effect of CAI and sorafenib in H1975 might due to the mutation of EGFR, since L858R/T790M double mutants
display a considerable increase in tyrosine phosphorylation level than 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 found in 10-30% of lung adenocarcinomas. These mutations lead to abnormal activation of KRAS signaling pathway that results in continuous cell proliferation and indicates poor prognostic of NSCLC. The combination of CAI and sorafenib conducts synergistic effect in cells regardless of KRAS mutations. These data imply that patients with KRAS mutations and without the $L858R/T790M$ EGFR mutations will benefit from synergistic effect of CAI and sorafenib.

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

**Authorship contributions**

Participated in research design: Chen, Guo, Ye and Zhang.

Conducted experiments: Chen, Shi, Ju and Chen.

Contributed new reagents or analytic tools: Li and Zhu.

Performed data analysis: Chen and Sun.

Wrote or contributed to the writing of the manuscript: Chen, Guo, and Ye.
References


Footnotes

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Competing financial interest

Authors declare no competing financial interest.
Legends for figures

Figure 1: Effects of sorafenib and CAI on LLC, A549 and NCI-H1975 NSCLC cell lines. NSCLC cells were treated with CAI or sorafenib alone, or in combination in a fixed concentration ratio for 24 h, 48 h, 72 h in LLC (A), A549 (B), NCI-H1975 (C) cell lines (n=5). The CI values were showed at corresponding right panel.

Figure 2: Combination of sorafenib and CAI induce apoptosis in NSCLC. (A) LLC cells were treated with 10μM CAI and/or 5 μM sorafenib and DMSO group were treated with or without 20 μM Z-VAD-FMK for 48 h, after which cells were stained with Annexin V/ PI and analyzed for apoptosis by flow cytometry. Statistical quantification of apoptotic cells were shown in right panel. Values are presented as means±SD. Statistical significances between co-treated with/without Z-VAD-FMK groups are indicated by * (P<0.05), ** (P<0.01). (B) Effect of 10 μM CAI and/or 5 μM sorafenib on the expression of cleaved-PARP, and (C) cleaved caspase-3. Protein levels of cleaved-PARP and cleaved caspase-3 from treated cell lysates were normalized against GAPDH levels. Band density were evaluated using Image J. Values are presented as means±SD.* P<0.05 vs. 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 h. The results shown are representative of 3 independent experiments (n=5).

Figure 3: Sorafenib and CAI increase DCFH-DA and MitoSOX oxidation suggesting increased intracellular ROS and provoke mitochondrial depolarization. (A) LLC and H1975 cells were incubated with DMSO (0.1%), sorafenib (5 μM ) and/or CAI (10 μM) for 24 hours, and cellular ROS was evaluated by DCFH-DA oxidation. (B) LLC cells were treated with DMSO (0.1%),
sorafenib (5 μM) and/or CAI (10 μM) for 24 hours, and MitoSOX was added before flow cytometric analysis. (C) Mitochondrial membrane potential was determined by JC-1 dye and measured by flow cytometric analysis (n=5). Statistical significance compared with DMSO is indicated by ** (P<0.01), *** (P<0.001); Statistical significance between monotherapy groups and combination group is indicated by ## (P<0.01).

Figure 4: Sorafenib and CAI decreased the expression of NANOG in vitro. LLC cells were incubated with DMSO (0.1%), sorafenib (5 μM) and/or CAI (10 μM) for 24 hours, then NANOG expression was measured by (A) qPCR and (B) western blot. Protein levels of NANOG from treated cell lysates were normalized against GAPDH levels. Band density were evaluated using Image J. Values are presented as means±SD. Statistical significance compared with DMSO is indicated by * (P<0.05), *** (P<0.001); Statistical significance between monotherapy groups and combination group is indicated by # (P<0.05), ## (P<0.01). Under the co-treatment of bFGF (40 ng ml⁻¹), (C) NANOG mRNA expression was elevated in LLC cell and (D) cell proliferation was stimulated in LLC and A549 cells. Statistical significance between adding bFGF groups and non-bFGF groups is indicated by * (P<0.05), ** (P<0.01). (E) LLC and A549 cells were incubated with DMSO (0.1%), sorafenib (5 μM) and/or CAI (10 μM) for 24 hours with or without bFGF (40 ng ml⁻¹), intracellular ROS was determined by DCFH-DA dye and evaluated by flow cytometric analysis. Mean fluorescence intensities of LLC and A549 were shown in F and G, respectively (n=5). Statistical significance between adding bFGF groups and non-bFGF groups is indicated by ** (P<0.01), *** (P<0.001). Statistical significance between monotherapy groups and combination group is indicated by # (P<0.05), ## (P<0.01).
Figure 5: Sorafenib and CAI induced apoptosis in a ROS and NANOG dependent manner in vitro. LLC cell (A) and A549 (C) cell were co-treated with 40 ng ml^{-1} bFGF or 4 mM GSH with sorafenib (5 μM) and/or CAI (10 μM) for 48 h, after which cells were stained with Annexin V/ PI and analyzed for apoptosis by flow cytometry. Statistical quantification of apoptotic cells were shown in (B) and (D) respectively. Values are presented as means±SD. *** P<0.001 indicates comparison between control groups and in co-treatment with bFGF groups. # P<0.05, ##P<0.01, ###P<0.001 indicate comparison between control groups and in co-treatment with GSH groups.

Figure 6: Combination of sorafenib and CAI exhibited 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^{-1}), SFB-L (10 mg kg^{-1}), combination (CAI 30 mg kg^{-1} and SFB 10 mg kg^{-1}) and SFB-H (30 mg kg^{-1}). (A) Tumor volumes were measured with calipers every other day. Mean tumor volume ±S.E.M. of each group was shown; n=6-10. Statistical significance between combination group with CAI group is indicated by * (P<0.05); Statistical significance between combination group with SFB-L group is indicated by # (P<0.05). (B) On day 29, tumors were exteriorized and weighed. Due to the progression of tumor, 4 mice died in PEG400 group, 4 mice died in CAI group, 1 mouse died in SFB-L group and 1 mouse died in SFB-H group before the end of experiment. The weights of tumors are presented as scatter dots. Statistical significance compared with PEG400 group is indicated by ** (P<0.01), *** (P<0.001). (C) Photograph of tumors in PEG400, CAI, SFB-L and combination group. (D) Body weight of mice in each group was measured everyday to roughly evaluate the side effect of different treatment. Mean body weight ±S.E.M. of each group was 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...
corresponding tumor weight. Statistical significance between CAI group with PEG400 group is indicated by * (P<0.05); Statistical significance between combination group with SFB-H group is indicated by # (P<0.05).

Figure 7: Sorafenib and CAI inhibit NANOG in vivo. (A) Immunohistochemistry of NANOG and H&E staining were performed on tumor sections in PEG400, CAI, SFB-L and Combination groups. (B) Tumor lysis was prepared using tumors that 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 were evaluated using Image J. Values are presented as means±SD. * P<0.05 vs. PEG400 group. (C) Tumor lysates were subjected to MDA assay. * P<0.05.
Fig. 2

A

B

C

D

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Fig. 3

A

LLC

NCI-H1975

CAI: - + - +
SFB: - +

B

Counts

DMSO CAI

SFB Combination

MitoSOX fluorescence intensity

C

JC-1 polymer/monomer fluorescence ratio

DMSO CAI SFB Combination

*** ** ** **

## ##

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Fig. 5

A

LLC

CAI: 

+ 

SFB: 

- 

+ 

+ 

Pl 

Annexin V

B

LLC

Control 

+ bFGF

+ GSH

Apoptotic cell% 

DMSO  

CAI  

SFB  

Combination

C

A549

CAI: 

+ 

SFB: 

- 

+ 

+ 

Pl 

Annexin V

D

A549

Control 

+ bFGF

+ GSH

Apoptotic cell% 

DMSO  

CAI  

SFB  

Combination
Fig. 6

A

Tumor volume (cm³)

PEG400
CAI
SFB-L
Combination
SFB-H

Days after inoculation

B

Tumor weight (g)

PEG400
CAI
SFB-L
Combination
SFB-H

C

PEG 400
CAI
Sorafenib
Combination

D

Body weight (g)

PEG 400
CAI
SFB-L
Combination
SFB-H

Days after inoculation

E

Carcass weight (g)

PEG 400
CAI
SFB-L
Combination
SFB-H

Days after inoculation
Fig. 7

A

NANOG

H&E

PEG400  CAI  SFB-L  Combination

B

NANOG

GAPDH

PEG400  CAI  SFB-L  Combination

C

Relative level of NANOG/GAPDH

PEG400  CAI  SFB-L  Combination

MDA (soluble tumor tissue protein)

PEG400  CAI  SFB-L  Combination

*