Sub-toxic Cisplatin Mediates Anoikis Resistance through Hydrogen Peroxide-induced Caveolin-1 Up-regulation in Non-small Cell Lung Cancer Cells

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Abstract. *Background: Exposure to inadequate chemotherapy may alter cancer cell behavior including their metastatic potential. Because the molecular basis of such a phenomenon is largely unclear, we investigated the possible impact of cisplatin on anoikis response on human lung carcinoma cells. Materials and Methods: Using molecular and pharmacological tools, Caveolin-1 (CAV1) overexpressing and knock-down H460 cells were generated by stable transfection. The levels of CAV1 were determined by western blotting and reactive oxygen species (ROS) were detected by specific probes. Results: Sub-toxic concentrations of cisplatin suppressed anoikis response in H460 cells. The anoikis attenuation observed, was found to be caused by CAV1 up-regulation. Exposure to cisplatin induced superoxide anion and hydrogen peroxide generation; however, only hydrogen peroxide was found to be responsible for the CAV1 elevation. Conclusion: Exposure to cisplatin at sub-toxic concentrations induced hydrogen peroxide generation and the subsequent increase of ROS further regulated CAV1 levels and anoikis resistance. Our findings demonstrate a novel effect of cisplatin treatment on cancer cells which may lead to a better understanding of cancer biology and in the improvement of chemotherapy.*

Among various chemotherapeutic agents, cisplatin [cis-diamminechloroplatinum (II)] has been used and recognized as a potent effective drug for treatment of various solid tumors including ovarian, bladder, cervical, and lung cancer (1-4). Unfortunately, in some patients, the cancer relapses (5, 6) and acquires resistance to chemotherapy (7-10), or metastasis occurs (11). An effort has been made in determining the possible mechanisms regarding the control of tumor relapse and drug resistance. Studies have reported that cancer resists cisplatin through the induction of intracellular antioxidant activities, the attenuation of drug uptake, the alteration of drug detoxification and the increase of drug efflux (12-15). However, the mechanisms facilitating cancer metastasis are still largely unknown.

Cancer metastasis is a multi-step process involving cell invasion, dissemination and migration, by which the survival of the detached cells in circulation is an important step in determining the fate of metastatic cancer. Anoikis is a cellular mechanism of detachment-induced apoptosis, inhibiting cancer cells from successful metastasis; tolerance to this mechanism facilitates cancer cell survival during systemic circulation and the formation of secondary tumor distant sites (16-19). Several molecular signaling molecules have been found to regulate anoikis mechanism including caveolin-1 (CAV1) (20). CAV1, a 21-24 kDa structural protein in the plasma membrane, has been shown to be involved in cancer progression as a tumor promoter protein (21, 22). The elevation of CAV1 in several types of cancer, including lung, breast, prostate, and pancreatic cancer was strongly associated with the metastatic potential of cancer (23-27). It has been reported that CAV1 enhances anchorage-independent growth of non-small cell lung cancer cells, a crucial mechanism which provides survival of metastatic cancer cells (20).

Reactive oxygen species (ROS), namely superoxide anion, hydrogen peroxide, and hydroxyl radical, have been shown to regulate several cellular behaviors (28-31). The aggressiveness of cancer, such as invasion, migration and resistance to detachment-induced apoptosis has been reported to be tightly associated with the oxidative status of cancer cells (32-34). Recently, we showed that hydrogen peroxide produced during cell detachment plays a role in rendering lung carcinoma cells resistant to anoikis (20). Interestingly, the mechanism of hydrogen peroxide-mediated anoikis resistance was found to involve the up-regulation of the cellular protein CAV1.

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Key Words: Cisplatin, caveolin-1, hydrogen peroxide, anoikis, H460, lung cancer cells.

Cisplatin treatment resulted in an induction of cellular oxidative stress leading to cell apoptosis (35-38). However, whether or not the sub-toxic concentrations of this drug affect the cellular ROS production and the cell behavior is largely unknown. It remains to be further investigated whether the sub-toxic cisplatin treatment could have an effect in facilitating cancer cell metastasis by making cells resistant to anoikis. Using pharmacological approaches, we investigated the effect of cisplatin exposure on anoikis resistance in H460 non-small cell lung cancer cells.

Materials and Methods

Cells and reagents. H460 Human lung carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 2 mmol/l L-glutamine, and 100 units/ml penicillin/streptomycin in a 5% CO_2 environment at 37˚C. Cisplatin, *N*-acetylcysteine (NAC), reduced glutathione (GSH), sodium pyruvate, catalase, Mn(III)tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP), deferoxamine (DFO), hydrogen peroxide (H_2O_2), ferrous sulfate ($FeSO_4$), 2',7'-dichlorofluorescein diacetate ($DCFH_2$-DA), propidium iodide (PI) and Hoechst 33342 were obtained from Sigma Chemical, Inc. (St. Louis, MO, USA). Dihydroethidium (DHE) and 3'-(*p*-hydroxyphenyl) fluorescein (HPF) were purchased from Molecular Probes, Inc. (Eugene, OR, USA). Resazurin was purchased from Invitrogen (Carlsbad, CA, USA). A specific antibody for CAV1 and a peroxidase-conjugated secondary antibody were obtained from Abcam (Cambridge, MA, USA), and a specific antibody for β-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Plasmids and transfection. *CAV1* plasmids were obtained from the American Type Culture Collection (Manassas, VA, USA), and *CAV1* knockdown shRNACAV1 plasmids were purchased from Santa Cruz Biotechnology. *CAV1* overexpressing (H460/CAV1) and *CAV1* knockdown (H460/shCAV1) cells were established by transfection of the H460 cells with *CAV1* overexpressing or *CAV1* knockdown shRNACAV1, respectively, according to the manufacturer's instructions. Briefly, H460 cells at 60% confluence were incubated with 15 μl of Lipofectamine 2000 reagent and 2 μg of *CAV1* or shRNA-CAV1 plasmids. After 16 h, the medium was replaced with culture medium containing 10% fetal bovine serum. Approximately two days after the beginning of the transfection, the single-cell suspensions were plated onto 75-ml culture flasks and were cultured for 60 days with antibiotic selection. The expression of CAV1 protein in the transfectants was quantified by western blot analysis.

Cytotoxicity assay. Cells were seeded in 96-well plates and allowed to attach for 24 h. After specific treatments, cells were incubated with 1:50 resazurin for 1 h at 37˚C. Fluorescence intensity of resazurin product (resorufin) was measured at 530 nm (excitation wavelength) and at 590 nm (emission wavelength) using a microplate reader. Cell viability was calculated as a percentage relative to that of non-treated cells. All analyses were performed for at least three independent replicate experiments.

Apoptosis and necrosis assay. Apoptotic and necrotic cell death was evaluated by Hoechst 33342 and PI co-staining. After specific treatments, cells were incubated with 10 μM of Hoechst 33342 and 5 μg/ml PI dye for 30 min at 37˚C. Apoptotic cells having condensed chromatin, fragmented nuclei and PI-positive necrotic cells were visualized and scored under a fluorescence microscope (Nikon Inverted Microscope Eclipse Ti-U).

Anoikis assay. H460 cells were seeded in 6-well plates and treated with different concentrations (0-1 μM) of cisplatin for 24 h. Adherent cells were then detached and seeded in 6-well ultra low-attached plates in RPMI medium at a density of 40,000 cells/well. Suspended cells were incubated under normal culturing condition at 37˚C for different times up to 24 h. Cells were harvested, washed and incubated with 1:50 resazurin for 1 h at 37˚C, and the fluorescence intensity of the resazurin product (resorufin) was measured at 530 nm (excitation wavelength) and at 590 nm (emission wavelength) using a microplate reader. Relative cell viability was calculated as a percentage relative to that of non-treated cells. All analyses were performed for at least three independent replicate cultures.

ROS detection. Intracellular ROS were determined using the fluorescent probe DCFH2-DA, superoxide anion was determined by DHE, and hydroxyl radical was determined by HPF. Cells were incubated with 10 μM of $DCFH_2$-DA, HPF, or DHE for 30 min at 4˚C, after which they were washed and were immediately analyzed for fluorescence intensity by a fluorescence microplate reader (SpectraMax M5; Molecular Devices Crop., Sunnyvale, CA, USA) using a 480-nm excitation beam and a 530-nm bandpass filter for detecting DCF fluorescence, or a 490-nm excitation beam and a 515-nm band-pass filter for HPF, or by using a 488-nm excitation beam and a 610-nm band-pass filter for DHE, and were visualized under fluorescence microscopy (Nikon Inverted Microscopy Eclipse Ti-U).

Western blot analysis. After specific treatments, the cells were incubated in a lysis buffer containing 20 mM Tris·HCl (pH 7.5), 1% TritonX-100, 150 mM sodium chloride, 10% glycerol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 mM phenylmethylsulfonyl fluoride, and a commercial protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA) for 30 min on ice. Cell lysates were collected and determined for protein content using the Bradford method (Bio-Rad, Hercules, CA, USA). Equal amounts of proteins from each sample (40 μg) were denatured by heating at 95˚C for 5 min with Laemmli loading buffer and were subsequently loaded onto 10% SDS polyacrylamide gel for electrophoresis (PAGE). After separation, the proteins were transferred onto 0.45 μm nitrocellulose membranes (Bio-Rad). The then membranes were blocked for 1 h in 5% nonfat dry milk in TBST [25 mM Tris·HCl (pH 7.5), 125 mM NaCl, 0.05% Tween 20] and were incubated with the appropriate primary antibodies at 4˚C overnight. Membranes were washed twice with TBST for 10 min and were incubated with horseradish peroxidase-coupled isotype-specific secondary antibodies to anti-CAV1 antibodies for 1 h at room temperature. The immune complexes were detected by enhanced chemiluminescence substrate (Supersignal West Pico; Pierce, Rockford, IL, USA) and were quantified using analyst/PC densitometry software (Bio-Rad).

Statistical analysis. Mean data from at least three independent experiments were normalized to the results of the non-treated controls. Statistical differences between means were determined using an analysis of variance (ANOVA) and *post hoc* tests at a significance level of $p<0.05$, and results are presented as the mean±SD.

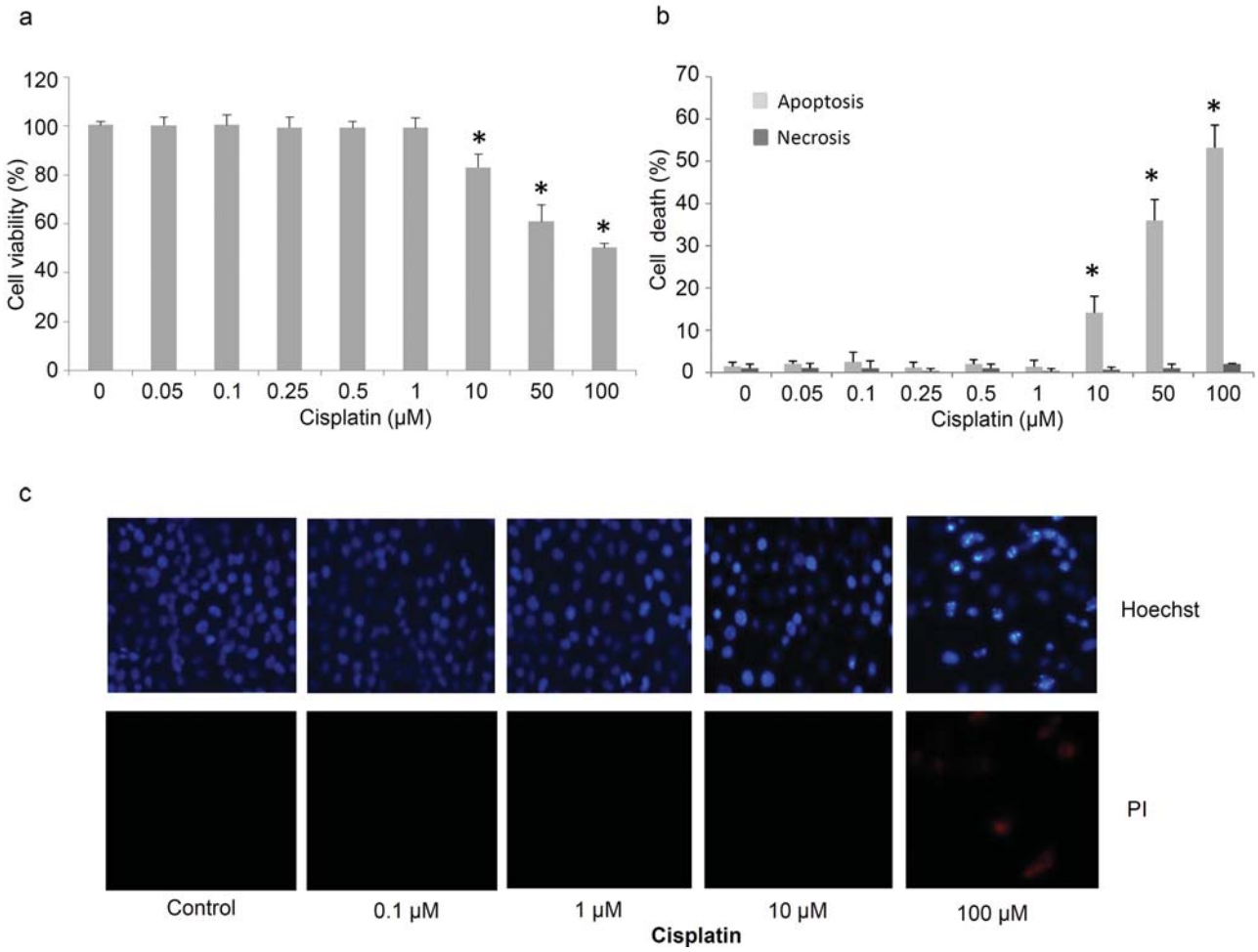

Figure 1. *Cisplatin mediates cytotoxicity in lung carcinoma H460 cells. a: Subconfluent (90%) monolayers of H460 cells were treated with doses (0-100 μM) of cisplatin for 24 h and the cell viability was determined by the resazurin cytotoxicity assay. b: Apoptotic and necrotic cell death was determined by Hoechst 33342/propidium iodide (PI) staining. Values are means±S.D. (n=3); *p<0.05 versus non-treated control. c: Morphology of apoptotic and necrotic cells was visualized under fluorescence microscopy.*

Results

Sub-toxic concentrations of cisplatin mediate anoikis resistance in lung carcinoma cells. In order to investigate whether sub-toxic concentrations of cisplatin could play a significant role on lung cancer cell anoikis resistance, we first characterized the cisplatin-mediated cytotoxicity in H460 cells and determined the sub-toxic doses of cisplatin. Cells were seeded in 96-well plates, were treated with various concentrations of cisplatin (0-100 μM) for 24 h, and were analyzed for cell viability by the resazurin cytotoxicity assay. Figure 1a shows that no significant decrease of cell viability was detected as a response to cisplatin, in concentrations ranging from 0.05 to 1 μM, while cisplatin at the concentrations of 10, 50 and 100 μM caused significant reduction in viable cells with approximately 83, 62 and 57% of the cells remaining alive, respectively. In addition, cells were similarly treated with cisplatin as above, and apoptotic and necrotic cell death was determined by staining with Hoechst 33342 and PI. Consistent with the above finding, Hoechst-positive cells containing condensed or fragmented nuclear fluorescence were not found in the cells treated with 0-1 μM cisplatin. As the dose of drug increased to 10, 50, and 100 μM, cisplatin-mediated apoptosis increased to 15%, 37%, and 52%, in a dose-dependent manner (Figure 1b and c). In the present study, necrotic PI-positive cells were barely detected at all cisplatin concentrations.

In order to determine the effect of sub-toxic cisplatin exposure on anoikis susceptibility of the cells, H460 cells were seeded in 6-well plates, and were treated with different sub-toxic doses of cisplatin (0-1 μM) for 24 h. Cells were then

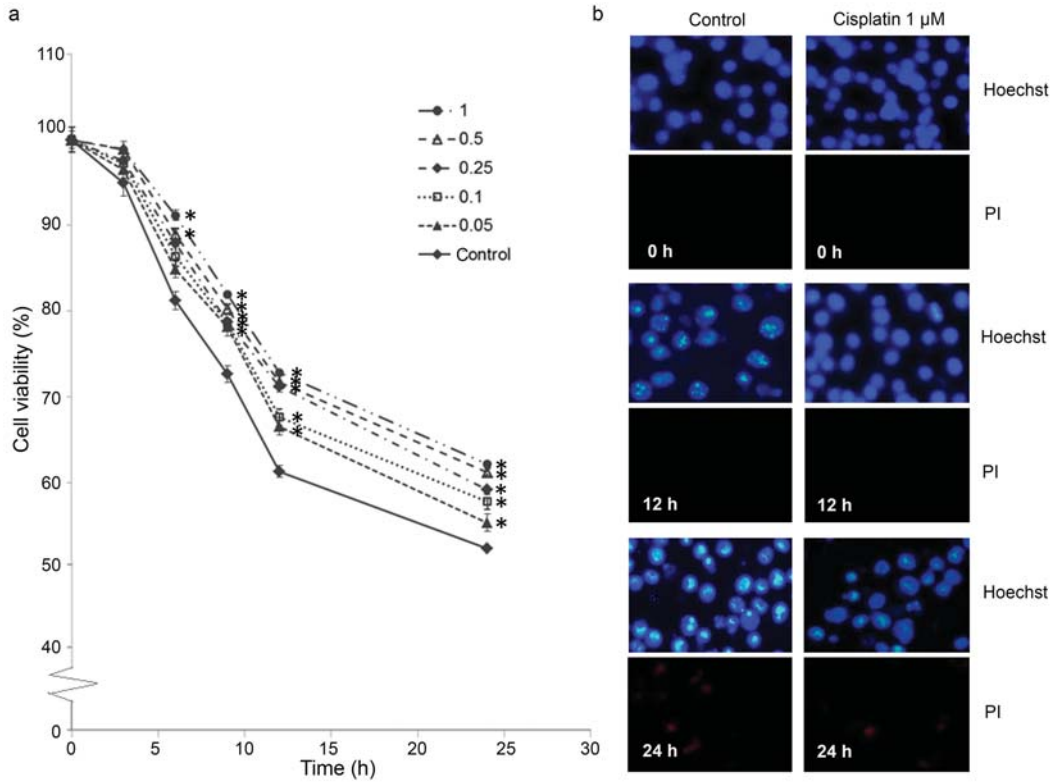

Figure 2. *Sub-toxic concentrations of cisplatin attenuate anoikis susceptibility. a: Sub-confluent (90%) monolayers of H460 cells were treated with (0-1 μM) of cisplatin for 24 h, and the cells were suspended in ultra low-attached plates for different times (0-24 h). The cell survival was determined at the indicated times by the resazurin cytotoxicity assay. The viability of cells is presented as cell viability relative to that of non-detached controls. Values are means±S.D; (n=3); *p<0.05 versus non-treated control. b: Cells' nuclei stain with Hoechst 33342 and propidium iodide (PI) was visualized under fluorescence microscopy.*

detached and incubated in ultra low-attached plates for various times (0-24 h) and cell viability was determined. Figure 2a shows that cisplatin treatment prior to cell detachment significantly rendered cells resistant to anoikis in a dose-dependent manner. Such alteration in anoikis sensitivity was apparent as early as 6 h after cell detachment, with approximately 89% and 91% of viable cells being detected in cells treated with 0.5 and 1 μM of cisplatin respectively, in comparison to 81% of viable cells in non-treated controls (Figure 2a). Figure 2b shows the characteristic morphology of detachment-induced apoptosis and further confirms the effect of cisplatin exposure on the inhibition of H460 cells anoikis. It is worth noting that nuclear PI fluorescence was not detectable in this experiment.

CAV1 mediates anoikis resistance. CAV1 has been demonstrated to regulate cancer cell aggressiveness and metastasis in various types of cancer (21, 22). In particular, our previous study indicated the role of CAV1 in the inhibition of cell anoikis resistance (20). Cells were stably transfected with *CAV1*, shRNACAV1, or control plasmids. After selection and propagation, CAV1- and shCAV1-transfected cells and control cells were subjected to anoikis assay and western blot analysis as described in the Materials and Methods. Western blotting with the use of anti-CAV1 antibodies showed substantial elevation of CAV1 levels in the cells stably transfected with *CAV1* plasmids, while significantly reduced CAV1 levels were detected in shCAV1-transfected cells (Figure 3a). In order to confirm the correlation of CAV1 levels and anoikis resistance, the cell viability after detachment was evaluated. Figure 3b shows that *CAV1*-transfected cells exhibited higher (~80%) of cell survival at 0-24 h after detachment compared with control cells (~40%), whereas cell viability of shRNACAV1-transfected cells was reduced to ~20% (Figure 3b). Hoechst33342 assay also showed an increase in nuclear fluorescence and chromatin condensation of the detached cells over time in all cells analyzed, with the highest proportion of anoikis being observed in shRNACAV1-transfected cells (Figure 3c). In contrast, no detectable change in nuclear PI fluorescence was identified during the period of detachment.

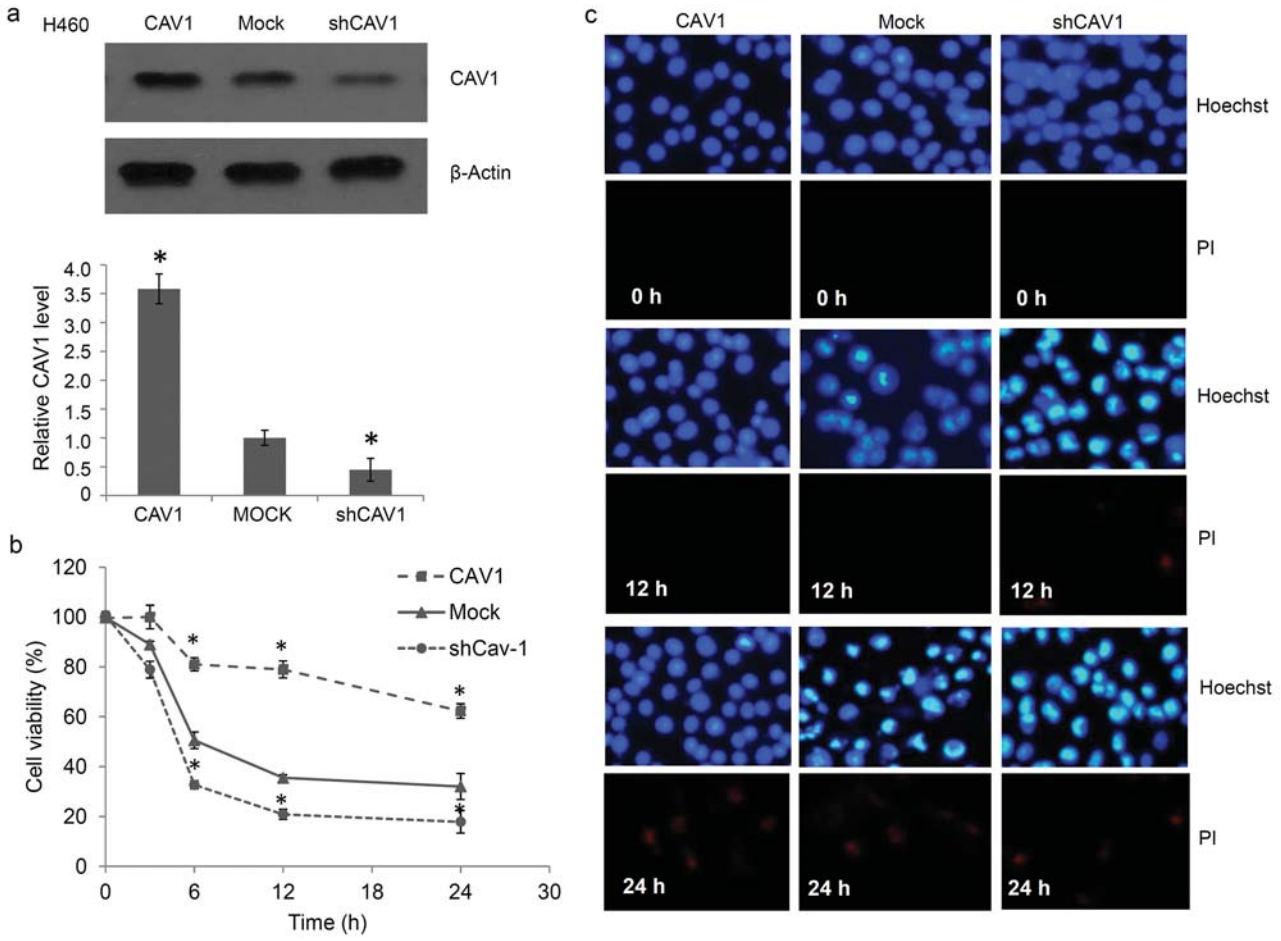

Figure 3. *Caveolin-1 (CAV1) promotes anoikis resistance in H460 cells. a: Cells were stably transfected with mock, CAV1, or shRNACAV1 plasmids, and were analyzed for CAV1 expression levels by western blotting. Values are means±S.D. (n=3); *p<0.05 versus control-transfected cells. b: Cells were detached and were suspended in ultra low-attached plates for different times (0-24 h). After the indicated times, cell survival was evaluated by the resazurin cytotoxicity assay. Values are means±S.D. (n=3); *p<0.05 versus control-transfected cells. c: Apoptotic and necrotic cells were determined by Hoechst 33342 and propidium iodide (PI) staining, and visualized under fluorescence microscopy.*

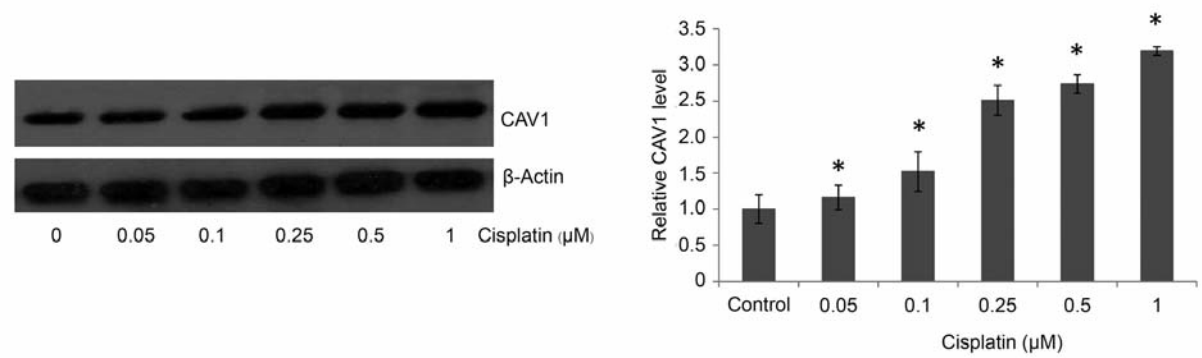

Figure 4. *Sub-toxic concentrations of cisplatin induce caveolin-1 (CAV1) up-regulation. H460 cells were treated with cisplatin (0-1 μM) for 24 h, and CAV1 expression levels were then determined by western blotting. Blots were reprobed with β-actin antibody to confirm equal sample loading. Immunoblot signals were quantified by densitometry and mean data from independent experiments were normalized to that of non-treated cells. Values are means±S.D. (n=3); *p<0.05 versus non-treated control cells.*

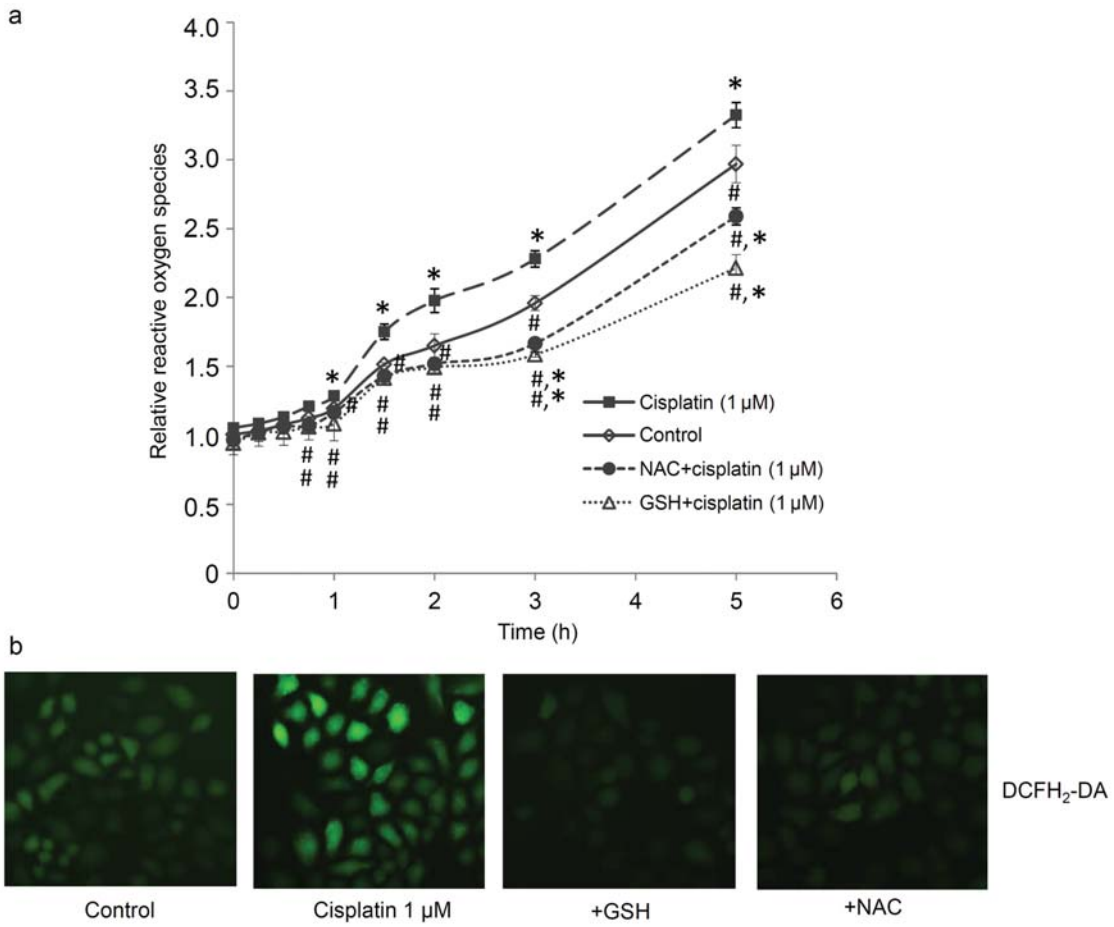

Figure 5. *Sub-toxic concentrations of cisplatin induced reactive oxygen species (ROS) generation. H460 cells were pre-treated with 1 mM reduced glutathione (GSH) or 1 mM N-acetyl cysteine (NAC) for 30 min, prior to cisplatin treatment (1 μM). After treatment with cisplatin, cells were incubated with 10 μM of 2',7'-dichlorofluorescein diacetate (DCFH2-DA) for 30 min. a: ROS generation was determined by a fluorescence microplate reader. Values are means±S.D. (n=3); *p<0.05 versus non-treated controls; #p<0.05 versus cisplatin-treated cells. b: Cellular ROS signal at 3 h after cisplatin treatment was visualized under fluorescence microscopy.*

Sub-toxic concentrations of cisplatin up-regulate CAV1 levels through a ROS-dependent mechanism. Having shown that CAV1 plays a crusial role in attenuating the anoikis response of H460 cells, we next determined the effect of sub-toxic cisplatin exposure on cellular CAV1 levels. Cells were treated with cisplatin (0-1 μM) for 24 h, and its effects on the CAV1 expression was evaluated by western blot analysis. The results clearly show that cisplatin at concentrations ranging from 0.05 to 1 μM significantly increased the CAV1 levels in a dose-dependent manner (Figure 4). These results revealed that exposure to sub-toxic cisplatin was able to elevate the CAV1 levels and subsequently the mediated anoikis resistance.

Cisplatin-induced cellular ROS generation has been widely accepted as a mechanism generated in the mode of action of cisplatin. It is very interesting to examine whether cisplatin at low concentrations, causing no cytotoxic effects, could modulate cellular ROS and affect, cell behavior. We, thus, incubated the cells with a sub-toxic dose of cisplatin (1 μM) and determined the intracellular ROS over time. Cells were pre-incubated with a specific ROS probe ($DCFH_2$-DA) for 30 min prior to cisplatin treatment. Accumulation of intracellular ROS inside these cells was evaluated by a fluorescence microplate reader and the results indicated that cisplatin did indeed cause a significant increase in ROS production in the cells as early as 1.5 h, when compared to non-treated control (Figure 5a).

In order to confirm the results of ROS induction by cisplatin, known antioxidants NAC and GSH were added to the cell cultures before cisplatin treatment. Administration of such antioxidants dramatically inhibited both base-line ROS production and cisplatin-mediated ROS up-regulation in these cells, thus confirming the effect of cisplatin on the cellular ROS profile (Figure 5b). Furthermore, we identified specific species of ROS induced by cisplatin using specific ROS probes and scavengers, and evaluated the fluorescence

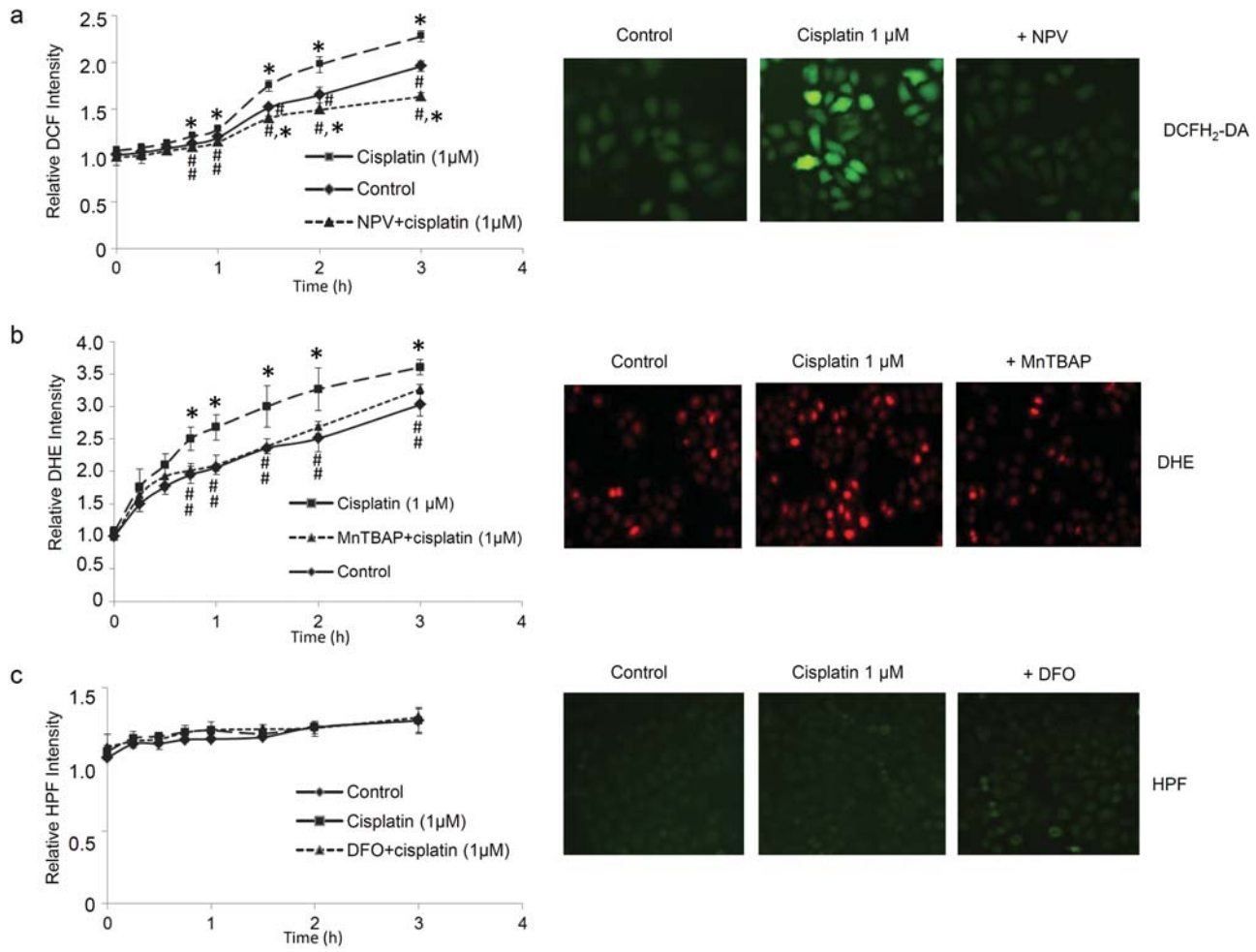

Figure 6. *Hydrogen peroxide and superoxide anion are key reactive oxygen species (ROS) induced by sub-toxic doses of cisplatin. a: H460 cells were left untreated or were pre-treated with 1 μM sodium pyruvate (NPV) for 30 min, prior to cisplatin treatment (1 μM); cellular ROS levels were determined by 2',7'-dichlorofluorescein diacetate (DCFH2-DA) probe. b: Cells were pre-treated with 50 μM Mn(III)tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP) for 30 min, were then treated with cisplatin, and then the superoxide anion levels were detected by dihydroethidium (DHE) probe. c: Cells were incubated with 1 μM deferoxamine (DFO) for 30 min prior to cisplatin treatment, and hydroxyl radical levels were detected using a 3'-(p-hydroxyphenyl) fluorescein (HPF) probe. All ROS signals were determined by a fluorescence microplate reader and were visualized under fluorescence microscopy. Values are means±S.D. (n=3); *p<0.05 versus non-treated control; #p<0.05 versus cisplatin-treated cells.*

signals by fluorescence microscopy and by a fluorescence microplate reader. Cells were similarly treated with cisplatin in the presence of specific ROS probes, namely DHE for superoxide detection and HPF for hydroxyl radical detection, as well as specific ROS scavengers, namely sodium pyruvate (hydrogen peroxide scavenger), MnTBAP (superoxide anion inhibitor), and DFO (hydroxyl radical inhibitor). Figure 6a and b indicate that treatment with 1 μM cisplatin induced hydrogen peroxide and superoxide anion production, as indicated by the increased cellular fluorescence intensity detected by $DCFH_2$-DA and DHE, respectively. Although the DCFH2-DA probe was not specific for hydrogen peroxide detection, sodium pyruvate abolished the cisplatin-mediated ROS induction, suggesting that hydrogen peroxide was the main ROS induced by cisplatin. An induction of DHE intensity clearly indicated the presence of superoxide anion in the treated cells. Furthermore, MnTBAP treatment blocked the DHE intensity, confirming the induction of superoxide anion production in response to cisplatin in these cells (Figure 6b). Regarding hydroxyl radical, cisplatin treatment caused no induction in the HPF signal compared to the non-treated control. Moreover, addition of DFO did not alter the HFP signal compared to the cisplatin- and non-treated cells (Figure 6c), suggesting that hydroxyl radical was not significantly induced in this sub-toxic cisplatin-mediated induction of ROS.

Hydrogen peroxide induced by cisplatin regulates anoikis resistance in lung cancer cells. Having shown that the sub-toxic dose of cisplatin increases cellular superoxide anion and hydrogen peroxide and the subsequent ROS inductions were concomitant with the CAV1 up-regulation, we next tested the role of ROS on the cisplatin-mediated induction of CAV1. Cells were treated with cisplatin (1 μM) in the presence or absence of pan-ROS scavengers, GSH (1 mM) and NAC (1 mM) for 24 h, and CAV1 expression was determined by western blotting. Figure 7a shows that cisplatin treatment for 24 h caused a significant increase in CAV1 levels and this protein induction was abolished in cells pre-incubated with GSH and NAC. Furthermore, we identified which ROS were involved in this process. H460 cells were pre-treated with specific ROS antioxidants including DFO (1 mM), MnTBAP (50 μM), sodium pyruvate (1 mM) or catalase (5,000 U/ml) prior to cisplatin treatment (1 μM), and the CAV1 expression levels were analyzed by western blotting. Figure 7b shows that cisplatin treatment increased the cellular CAV-1 levels and the addition of hydrogen peroxide scavengers, sodium pyruvate and catalase, completely inhibited the cisplatin-induced CAV1 up-regulation. On the contrary, pre-treatment with DFO and MnTBAP had only a minimal effect on cisplatin-induced induction of CAV1.

Since our results suggest that hydrogen peroxide induced by cisplatin was able to regulate CAV1 expression in these cells, the effect of exogenous hydrogen peroxide treatment on the CAV1 levels was evaluated in order to confirm the above findings. H460 cells were treated with hydrogen peroxide (100 μM) alone and the CAV1 levels were analyzed by western blotting. Figure 7c shows that hydrogen peroxide significantly elevated CAV1 expression as compared to non-treated controls. These results indicate that hydrogen peroxide is a major positive regulator of CAV1 expression in response to cisplatin treatment.

Discussion

The acquisition of cisplatin resistance, frequently found in human lung cancer, has been considered to be an important but complex obstacle of effective chemotherapy (39-41). Although the exact mechanism by which lung cancer cells tolerate cytotoxic drugs remains elusive, higher aggressiveness of surviving cancer cells after cisplatin-based regimens, existing as the principal problem in cancer treatment, has continuously been reported. The present study demonstrated for the first time that sub-lethal concentrations of cisplatin render human lung carcinoma cells resistant to detachment-induced apoptosis. Furthermore, we found that cisplatin-generated ROS were responsible for CAV1 up-regulation and, subsequently, anoikis resistance.

It is widely known that cisplatin treatment causes an induction of several ROS, namely superoxide anion, hydrogen peroxide, and hydroxyl radical, and such a ROS increase causes cytotoxic effects on cells (42-44). However, less is known regarding the ROS generated by sub-toxic concentrations of cisplatin. We report herein, for the first time, that a low concentration of cisplatin is capable to increase production of specific ROS, namely superoxide anion and hydrogen peroxide (Figure 6). Our results further revealed the effect of hydrogen peroxide in rendering cells resistant to anoikis. Most metastatic cancer cells resist detachment-induced apoptosis (anoikis). Anoikis plays a principal role in inhibition of cancer cells spreading from the original site to other sites. Several studies suggested that CAV1 expression was correlated with aggressive behaviors of lung cancer cells, including multidrug-resistance (45-47) and anoikis resistance (20). The present study demonstrated that after treatment with sub-toxic concentrations of cisplatin, CAV1 was up-regulated in a dose-dependent manner (Figure 4) and such up-regulation of CAV1 had an inhibitory effect on cell anoikis (Figure 2). Furthermore, we revealed that CAV1 expression in cisplatin-treated H460 cells was dependent on the oxidative stress induced by cisplatin. Addition of antioxidant GSH and NAC was able to attenuate the ROS induction and, subsequently, the CAV1 up-regulation (Figure 5). Previous studies reported that cisplatin-mediated death was related to the induction of cellular hydrogen peroxide (42, 48) and hydroxyl radical (49) production. However, in this study, a low concentration of cisplatin up-regulated hydrogen peroxide but did not alter the hydroxyl radical levels (Figure 6a and c). Since induction of cellular ROS by cisplatin has been previously shown to be dose dependent (43), it is possible that the production of cellular hydroxyl radical may be attenuated on low-dose cisplatin treatment and is overwhelmed by cellular antioxidants.

Various effects of specific ROS have been shown in many studies. We thus identified the specific ROS involved in the mechanism of cisplatin-mediated CAV1 up-regulation and anoikis resistance. Specific ROS scavengers, as well as specific ROS probes were used and the obtained results indicated that superoxide anion and hydrogen peroxide are two key ROS present in cisplatin-treated cells. The increase of CAV1 in response to cisplatin exposure was mainly due to hydrogen peroxide but not superoxide anion (Figure 7b). Our results demonstrated that the treatment of cells with exogenous hydrogen peroxide promoted the up-regulation of CAV1 (Figure 7c), while the superoxide anion generator 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) had no effect on the cultures (data not shown). In accordance with a previous study reporting the crucial role of hydrogen peroxide on CAV1 expression and cell anoikis (20), the present study indicates that hydrogen peroxide induced by sub-toxic concentrations of cisplatin can help cancer cells resist detachment-induced apoptosis and may facilitate the metastatic ability of cancer cells.

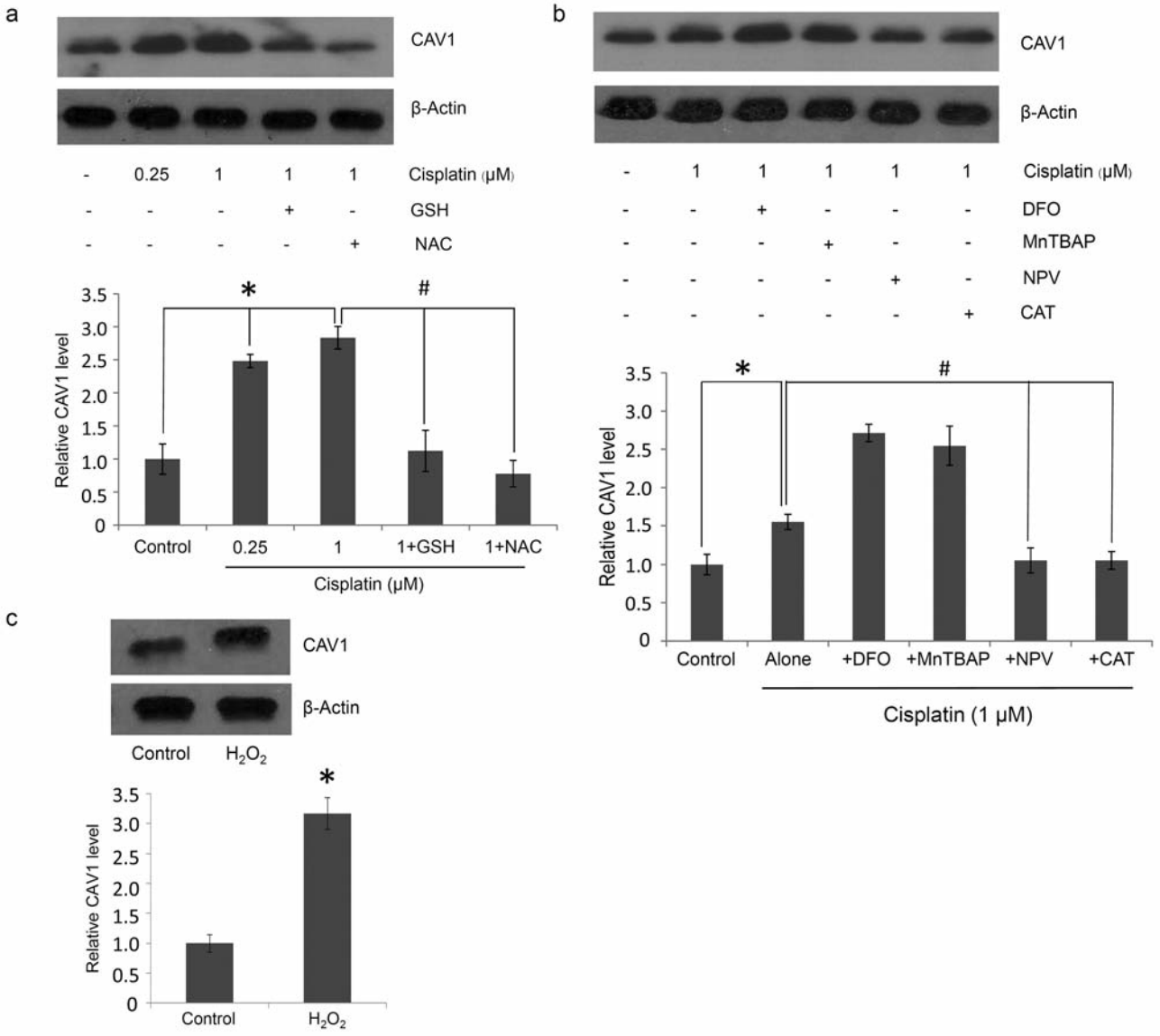

Figure 7. *Sub-toxic concentrations of cisplatin induce anoikis resistance in H460 cells through a hydrogen peroxide-dependent mechanism. a: H460 cells were left untreated or were pre-treated with 1 mM reduced glutathione (GSH) or 1 mM N-acetyl cysteine (NAC) for 30 min, and were then treated with 1 μM cisplatin for 24 h. The caveolin-1 (CAV1) level was evaluated by western blot analysis. b: Cells were left untreated or were pre-treated with 1 μM deferoxamine (DFO), 50 μM Mn(III)tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP), 1 μM sodium pyruvate (NPV) or 5,000 U/ml catalase (CAT) for 30 min and the cells were then treated with cisplatin for 24 h before analyses of CAV1 by western blotting. (c) H460 cells were treated with hydrogen peroxide (100 μM) for 24 h and CAV1 levels were determined by western blotting. Blots were reprobed with β-actin antibody to confirm equal sample loading. Immunoblot signals were quantified by densitometry and mean data from independent experiments were normalized to non-treated cells. Values are means±S.D. (n=3); *p<0.05 versus non-treated control cells; #p<0.05 versus cisplatin-treated cells.*

In summary, we report a novel effect and an underlying mechanism of sub-toxic concentrations of cisplatin in regulating anoikis resistance in H460 human lung carcinoma cells. Exposure to cisplatin at the sub-toxic concentrations induced ROS generation (mainly superoxide and hydrogen peroxide). Hydrogen peroxide induced by such cisplatin exposure mediated CAV1 up-regulation and anoikis resistance in these cells. Since the ability to up-regulate cellular ROS production, especially of hydrogen peroxide, is found in a number of chemotherapeutic agents and other drugs, this finding might lead to further investigations which will facilitate a better understanding regarding cancer cell biology and benefit the design of more effective treatment strategies for chemotherapy.

Acknowledgements

This research was supported by Thailand Research Fund (to P. Chanvorachote) and the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission (HR11XXA-55). The Authors wish to thank Mr. Krich Rajprasit (Srinakharinwirot University) for proofreading.

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Received March 8, 2012
Revised April 3, 2012
Accepted April 4, 2012