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Desacetyluvaricin Induces S Phase Arrest in SW480 Colorectal Cancer Cells Through Superoxide Overproduction

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ABSTRACT

Annonaceous acetogenins (ACGs) are a group of fatty acid-derivatives with potent anticancer effects. In the present study, we found desacetyluvaricin (Dau) exhibited notable in vitro antiproliferative effect on SW480 human colorectal carcinoma cells with IC_{50} value of 14 nM. The studies on the underlying mechanisms revealed that Dau inhibited the cancer cell growth through induction of S phase cell cycle arrest from 11.3% (control) to 33.2% (160 nM Dau), which was evidenced by the decreased protein expression of cyclin A Overproduction of superoxide, intracellular DNA damage, and inhibition of MEK/ERK signaling pathway, were also found involved in cells exposed to Dau. Moreover, pre-treatment of the cells with ascorbic acid significantly prevented the Dau-induced overproduction of superoxide, DNA damage and cell cycle arrest. Taken together, our results suggest that Dau induces S phase arrest in cancer cells by firstly superoxide overproduction and subsequently the involvement of various signaling pathways. J. Cell. Biochem. 115: 464–475, 2014.

KEY WORDS: CANCER CHEMOPREVENTION; DESACETYLUVARICIN; ANNONACEOUS ACETOGENIN; S PHASE ARREST; SUPEROXIDE

Cancer is still a major public health problem in the world, due to the increasing of population aging and growth, environmental change, as well as "westernized" diets [Jemal et al., 2011]. According to American Cancer Society (ACS), prostate, female breast, lung and bronchus, and colon and rectum were, at present, the four major cancer sites in men and women [Parkin et al., 2005; Jemal et al., 2009, 2011]. Chemotherapy is now considered as an extremely rational and appealing approach, and the most direct way to counteract the malignancy development of cancer. Chemotherapeutic agents enter the bloodstream and spread throughout the whole body, therefore attack cancer cells directly to intervene in the process of carcinogenesis [Tsao et al., 2004; Tsuda et al., 2004]. One promising candidate for chemotherapeutic and chemopreventive drugs is the Traditional Chinese medicines [Lee, 1999; Fabricant and Farnsworth, 2001]. A variety of novel natural compounds, such as paclitaxel, podophyllotoxin, vinblastine and camptothecin, have been isolated and used as agents for cancer chemotherapy and chemoprevention. *Annonaceae*, also called custard apple family or soursop family, with approximately 120 genera and 2,000–2,200 species, is the largest family in *Magnoliales*. The whole plants, fruits or seeds of Annonaceous plants were widely used in traditional and popular medicines for treating pain, fever, diarrhea, hypotension, internal, and external parasites [Alali et al., 1999]. Annonaceous acetogenins (ACGs), a group of fatty acid derivatives, are proprietarily isolated from *Annonaceae* plants [Bermejo et al., 2005]. When the first ACG compound, uvaricin, was isolated in 1982, its excellent in vivo antileukemia effect aroused widespread interest amongst natural product and medicinal chemists to isolate and identify this class of

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compounds [Liaw et al., 2010]. ACGs exhibited a broad spectrum of biological properties, such as cytotoxicity, antitumoral, antimalarial, antimicrobial, antiviral, antifeedant, anthelminitic, pesticidal, and immunosuppressive activities, suggesting many potentially useful applications [Alali et al., 1999; McLaughlin, 2008; Liaw et al., 2010]. The powerful cytotoxicity and antitumoral activity made ACGs as another "tomorrow anticancer star" after taxol [Yao and Wu, 1995; McLaughlin, 2008].

Desacetyluvaricin (Dau), also named squamocin-L, was first isolated from the roots of *Uvaria zeylanica* (*Annonaceae*) by Jolad et al. [1985]. In recent years, it was reported to be potently insecticidal and anticancer [Ohsawa et al., 1991; Chang et al., 2003; Fall et al., 2006; Yang et al., 2009]. Mechanism studies showed that Dau could induce G_1/G_0 phase arrest and apoptosis in HepG2.2.15 and HepG2 hepatoma cells [Chen et al., 2007; Ren et al., 2010]. Moreover, Dau was an effective inhibitor of mitochondrial complex I [Gallardo et al., 2000; Tormo et al., 2005], and had certain antioxidant capacity [Lima et al., 2010]. In the present study, the powerful in vitro anticancer activity of Dau was identified and the underlying mechanism was investigated. Our results showed in SW480 colorectal cancer cells, Dau triggered plenty of superoxide generation, which induced DNA damage and MAPK signaling pathway inhibition, subsequently the alteration of protein expressions of cell cycle regulators to arrest SW480 cells at S phase and eventually to necrotic death.

MATERIALS AND METHODS

MATERIALS

Ascorbic acid (AA), cisplatin, dihydroethidium (DHE), dimethylsulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 5-fluorouracil (5-FU), irinotecan, propidium iodide (PI) and the Bicinchoninic Acid Kit for protein determination were purchased from Sigma-Aldrich (St. Louis, MO). Annexin-V-FLUOS staining kit and cytotoxicity detection kit (LDH) were obtained from Hoffmann-La Roche (Basel, Switzerland). Single-cell gel electrophoresis assay (comet assay) reagent kit was obtained from Trevigen (Gaithersburg, MD). All of the antibodies, cell lysis buffer and LumiGLO® chemiluminescent substrate were purchased from Cell Signaling Technology (Beverly, MA). Dulbecco's modified Eagle's Medium (DMEM), RPMI-1640 Medium, Ham's F-12K Medium, Eagle's Minimum Essential Medium (EMEM), Leibovitz's L-15 Medium, fetal bovine serum (FBS), and antibiotic mixture (penicillin-streptomycin) were purchased from Invitrogen (Carlsbad, CA). All solvents used were of HPLC grade. The water used for all experiments was ultrapure, supplied by a Milli-Q water purification system (Millipore, Bedford, MA).

Ten ACG compounds were isolated from the root of *Uvarla calamistrata* (*Annonaceae*) as previously described [Zhou, 1999]. The structures were identified by ^{1}H-NMR, ^{13}C-NMR and LC-MS, and the purity was higher than 98%. The tested compounds were dissolved in DMSO at a concentration of 20 mg/ml as stock solutions. Stock solutions were diluted to the desired final concentration with phosphate buffered saline (PBS) just before use. The maximum final concentration of DMSO in medium was smaller than 0.5%.

CELL CULTURE

Human malignant melanoma A375 [CRL-1619], human prostate adenocarcinoma LNCaP [CRL-1740] and PC-3 [CRL-1435], human colorectal carcinoma Caco-2 [HTB-37], SW480 [CCL-228] and COLO 201 [CCL-224] cell lines and human normal skin fibroblast Hs68 [CRL-1635] cell line were purchased from American Type Culture Collection (ATCC, Manassas, VA). Cells were propagated in DMEM, or RPMI-1640 Medium, or Ham's F-12K Medium, or EMEM, or Leibovitz's L-15 Medium according to ATCC's recommendation. To complete the growth medium, the media were supplemented with 10% FBS, 1% penicillin (100 U/ml)-streptomycin (50 U/ml), and 0.1% fungizone. Generally, the cells were cultured at 37°C with 95% air and 5% CO_2. However, SW480 cells were cultured at 37°C without additional CO_2 in the humidified atmosphere. Cells were harvested with a mild solution of trypsin-EDTA when they were in a logarithmic phase of growth.

MTT ASSAY

Cell viability was determined by MTT assay in according to the manufacture's instruction. In brief, the growing cells were seeded in 96-well tissue culture plates at 2×10^4–10×10^4 cells per well for different cell types for 24 h at 37°C. Then the cells were exposed to ACG compounds with a series of concentrations in triplicate. Incubation was carried out at 37°C for 24, 48, or 72 h. After incubation, 20 μl MTT solution (5 mg/ml MTT in PBS) was added to each well and incubated for 4 h. MTT is reduced by mitochondrial succinic dehydrogenase of viable cells to an insoluble purple formazan product. The purple dye was subsequently dissolved in 150 μl DMSO. The color intensity of the colored solution was quantified by measuring absorbance at 570 nm wavelength by using a microplate spectrophotometer (SpectroAmax™ 250). The relative percentage of cell viability was determined by comparing the reading of compound-treated cells versus that of control cells. Each experiment was performed at least three times.

LDH ASSAY

The released lactate dehydrogenase (LDH) from damaged cells into extracellular medium is an indication of compromised cell membrane integrity and an index of cytotoxicity. Briefly, cells were incubated with Dau for 72 h. According to the manufacture's instruction, SW480 cells were not only exposed to culture medium as the low control, but also exposed to 2% Triton X-100 as the high control. After treatment, 96-well tissue culture plates were centrifuged (1,500*g*) for 10 min at room temperature to make cells precipitated. One hundred microliters supernatant of each well were transferred into new wells and incubated with 100 μl per well of the cytotoxicity detection kit for 30 min at room temperature. The absorbance of sample was measured at 492 nm using a microplate spectrophotometer (SpectroAmax™ 250). The LDH release was expressed as a proportion of the LDH released under each condition to the maximum LDH released. Each experiment was carried out at least three times.

FLOW CYTOMETRIC ASSAY

The flow cytometric assay was employed to evaluate the cell cycle perturbation of Dau-treated SW480 cells. Briefly, SW480 cells were seeded in six-well tissue culture plates and treated with indicated

doses of Dau. Thereafter, controlled and treated cells were harvested, washed with PBS, fixed in ice-cold 75% ethanol and stored at −20°C overnight. Cell pellets were re-collected and re-suspended in 50 μg/ml PI reagent (1.21 mg/ml Tris, 50.1 μg/ml PI, 700 U/ml RNase, pH 8.0) in dark at 4°C for 4 h. Flow cytometric determination of DNA content was conducted by Beckman Coulter Flow Cytometer (Miami, FL, US). The proportion of cells at each phase in cell cycle (G_0/G_1, S, G_2/M) can be scored by MultiCycle software (Phoenix Flow Systems, San Diego, CA, US). Apoptotic peak can also be confirmed from the cell cycle pattern. For each experiment, 10,000 nuclei per sample were collected. Each experiment was carried out at least three times.

ANNEXIN V/PI STAINING ASSAY

Cells were seeded in 6-well tissue culture plates with cover glasses. After treatment, the slides were washed with PBS and double stained with annexin V-FITC solution and PI solution for 20 min in dark. The slides were then photographed under a laser scanning confocal microscopy (Bio-Rad Radiance 2100 MP Scanning System), attached with 488 nm argon ion laser. Green fluorescence emitted from annexin V on apoptotic as well as necrotic cells was measured at 518 nm wavelength. Red fluorescence emitted from PI on necrotic cells was measured at 617 nm wavelength.

SUPEROXIDE GENERATION

The production of superoxide was determined by fluorometric DHE assay. Briefly, treated cells were harvested and suspended in PBS (1×10^6 cells/ml). The cell suspension was incubated with DHE at a final concentration of 10 μM at 37°C for 25 min. The labeled cells were re-collected and re-suspended in 1 ml PBS, and the fluorescence emission from ethidium was analyzed via flow cytometry (Beckman Coulter, Miami, FL).

WESTERN BLOTTING ANALYSIS

Controlled and treated cells were harvested and washed, then suspended in lysis buffer on ice for 1 h. The whole cellular proteins were extracted from the cell lysates. And the protein concentrations were determined by Bicinchoninic Acid (BCA) Protein Assay Kit. Equal amounts of proteins (40 μg) were electrophoresed by 10% tricine SDS–PAGE and electrotransferred onto nitrocellulose membranes. The blots were incubated with the dilutions of anti-cyclin A, -cyclin B1, -cyclin D1, -cyclin E, anti-Cdc2, -CDK2, -CDK4, anti-p21, anti-Rb (4H1), -p-Rb (Ser795), -p-Rb (Ser807/811), anti-E2F-1, anti-ERK1/2, -p-ERK1/2, -p38 MAPK, -p-p38 MAPK, -SAPK/JNK, -p-SAPK/JNK, MEK1/2, -p-MEK1/2, anti-PARP and anti-caspase-3 overnight at 4°C, and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 2 h at room temperature as followed. The bound antibodies were visualized using an Enhanced Chemiluminescence Reagent (ECL) and exposed to an X-ray film. To assess the presence of equal loading and transfer of proteins in each lane, the β-actin was detected. The protein expression rate was quantified by ImageJ software. Each experiment was carried out at least three times.

COMET ASSAY

The comet assay is single-cell gel electrophoresis to detect DNA damage. Briefly, treated and untreated cells were harvested and suspended in PBS to about 5×10^5 cells/ml. By using the Comet Assay Kit, cell suspensions were mixed with molten LMAgarose at a ratio of 1:10 (v/v) at 37°C. An aliquot (75 μl) was immediately and gently pipetted onto the slide (CometSlide™). After refrigeration for 40 min, the slides were sequentially immersed in pre-chilled lysis solution (containing 10% DMSO) on ice for 40 min, and incubated with freshly prepared alkaline solution (300 mM NaOH, 1 mM EDTA, pH > 13) in dark on ice for 40 min. After DNA denaturation, the slides were subjected to alkaline solution for electrophoresis. Electrophoresis was performed for 40 min in a Savant ps 250 system set at 300 mA and 1 V/cm in darkness. After electrophoresis, the slides were rinsed in distilled H_2O, fixed with 70% ethanol and air-dried overnight. DNA was stained with SYBR® Green I for epifluorescence microscopy. DNA damage was visualized at 200 × magnification under a fluorescence microscope (Nikon, Eclipse E-600 with an excitation filter of 510–560 nm) equipped with an image analysis system (Komet 3.1, Kinetics Imaging Ltd, Liverpool). A total of 50 cells per slide were measured at random. The tail length (measured from the middle of the head to the end of the tail), relative DNA content in the tail, and the tail moment (tail length × relative tail DNA content) were measured for analysis.

STATISTICAL ANALYSIS

The experimental results are expressed as the mean ± SD. Statistical analysis of two groups was performed using two-tailed student's *t*-test. Differences between more than two groups were analyzed by one-way ANOVA multiple comparisons. A value of $^{*}P < 0.05$ or $^{**}P < 0.01$ was considered statistically significant.

RESULTS

IN VITRO ANTICANCER ACTIVITIES OF ACGs

Following exposure to 10 ACGs (shown in Table I) for 72 h, cell viabilities of the tested human cancer cell lines were determined by measuring the absorbance of MTT dye for living cells. Table I showed the in vitro anticancer activities of these ACGs, which were represented as IC_{50} values. IC_{50} is the concentration of ACG compounds that necessarily inhibit 50% of cell population alive, determined by interpolation from dose–response curves. Among these compounds, Dau showed broad anti-cancer effect to 5 cancer cell lines out of 6. Moreover, Dau showed nanomolar level of cytotoxicity to SW480 cells and LNCaP cells. As far as the sensitivities of these cell lines were concerned, SW480 was found the most susceptible one. Five ACG compounds out of 10 exhibited nanomolar level of cytotoxicity to SW480 cells. In the further study, Dau exhibited a dose- and time-dependently antiproliferative effect on SW480 cells as shown in Figure 1B. For 72 h, Dau inhibited the growth of SW480 cells with IC_{50} value of 14.00 ± 1.50 nM. 5-FU, cisplatin and irinotecan (CPT-11) are regarded as backbones of colorectal cancer chemotherapeutic drugs in current clinical trials [Segal and Saltz, 2009]. The IC_{50} values of 5-FU, cisplatin and CPT-11 on SW480 cells were calculated to be 8.87 ± 3.16, 17.27 ± 3.55, and 32.10 ± 9.05 μM, respectively (Fig. 1C), which were 1,000-fold higher than IC_{50} value of Dau. Moreover, at 100 nM, the cell viabilities of SW480 cells treated with 5-FU, cisplatin and CPT-11 were higher than 100%, indicating no inhibitory effects of these commercial drugs. In

TABLE I. Growth Inhibition of Various ACGs Against Human Cancer Cell Lines

	IC_{50} (μM)					
ACGs	A375	LNCaP	PC-3	Caco-2	SW480	COLO 201
Cal A	1.5 ± 0.5	0.4 ± 0.1	>100	3.9 ± 1.4	0.07 ± 0.02	>100
Cal C	7.4 ± 1.7	1.5 ± 0.6	23.2 ± 4.9	6.6 ± 0.3	1.1 ± 0.2	>100
Cal D	>100	12.9 ± 4.9	>100	>100	2.9 ± 0.6	>100
Cal F	71.5 ± 4.5	1.7 ± 0.3	35.6 ± 8.2	64.4 ± 6.0	0.4 ± 0.07	>100
Cal G	5.5 ± 1.4	0.07 ± 0.003	46.0 ± 6.4	>100	0.02 ± 0.004	>100
Cal H	>100	>100	>100	60.4 ± 9.2	1.2 ± 0.4	>100
Cum I	12.3 ± 2.1	4.1 ± 1.8	>100	29.6 ± 9.1	2.8 ± 0.8	>100
Dau	2.9 ± 1.2	0.3 ± 0.2	26.8 ± 4.4	>100	0.01 ± 0.002	26.2 ± 8.5
Ret-1	4.5 ± 1.0	11.4 ± 4.2	>100	24.2 ± 4.7	13.2 ± 2.6	>100
Uvg	22.6 ± 8.9	0.1 ± 0.03	76.6 ± 5.6	6.3 ± 1.4	0.2 ± 0.1	>100

contrast, Dau at 80 nM showed 87% inhibitory effect and at 160 nM showed 96% inhibitory effect on SW480 cells, respectively (Fig. 1B). The cytotoxic effect of Dau on normal fibroblast Hs68 cells was evaluated by MTT assay and LDH assay (Fig. 1D). As shown in Figure 1D, Dau at 12.5 μg/ml inhibited 16% normal cells growth and induced 0.4% LDH release. The concentrations of Dau tested on HS68 normal cells were much higher than those on SW480 cancer cells. As aforementioned results, 160 nM Dau could inhibit 96% SW480 cancer cells growth as well as induce 98% LDH release (Fig. 1E). Taken together, Dau showed potent cytotoxicity on SW480 cancer cells as well as had greatly selective inhibitory effects between cancer cells and normal cells.

S PHASE ARREST INDUCED BY DAU

To gain insight into the mechanism responsible for Dau-mediated cell growth inhibition, cell cycle distribution of SW480 cells was evaluated by using flow cytometric assay. After treatment with Dau for 72 h, SW480 cells were accumulated in S phase of the cell cycle in a dose- and time-dependent pattern (Fig. 2). After treatment with 160 nM Dau, the percentage of cells in S phase increased from 11.3% to 33.2%, while cells in G_1/G_0 phase decreased from 70.9% to 50.6% ($P < 0.01$) concomitantly (Fig. 2A and B). Meanwhile, in the time-course study, cells were accumulated in S phase from 14.7% to 32.4% with the decrease in G_1/G_0 phase from 74.3% to 56.6% (Fig. 2C). These results suggested that Dau inhibited the cellular proliferation of SW480 cells via S phase arrest, which was accompanied by a decrease in G_1/G_0 phase cells. No significant sub-G_1 apoptotic peak was observed in cells exposed to Dau (Fig. 2A, C). Therefore, the increase in the S population was mostly at the expense of G_1/G_0-phase cells. These data clearly suggest that Dau inhibits the growth of SW480 cells by inducing S phase arrest.

EFFECTS OF DAU ON THE EXPRESSIONS OF CELL CYCLE REGULATORS INVOLVED IN S PHASE ARREST

Cell cycle progression is tightly regulated by cyclin/CDK complexes [Ford and Pardee, 1999]. To examine the mechanism of cell cycle arrest upon treatment with Dau, the protein levels of cyclins and CDKs from lysates of control and Dau-treated SW480 cells were determined by immunoblotting. As the representative blots shown in Figure 3A,B, Dau-treatment caused a marked reduction in the protein level of cyclin A from 1.0 to 0.1, while had no effect on the protein level of CDK2. The complex between cyclin A and CDK2 is important for S phase progression. Besides, Dau treatment resulted in an apparent down-regulation in the expressions of cyclin D1, CDK4 and cyclin B1from 1.0 to 0.0, respectively (Fig. 3A,B). An increased expression in cyclin E from 1.0 to 3.5 at time-dependent manner contributes to the cell cycle progression during G_1/S-phase transition (Fig. 3A,B).

In order to learn about the regulation of G_1/S transition in SW480 cells, the effects of Dau on the protein levels of Rb and E2F were examined. During cells passing through the restriction point in late G_1 phase, the wild-type Rb protein is phosphorylated by cyclin D1/CDK4 in G_1 phase to release E2F [Nevins, 2001]. In our study, the expression levels of wild-type Rb protein and phosphorylated Rb protein at serine 807/811 site were markedly down-regulated from 1.0 to 0.6 as early as 8 h after treatment (Fig. 3D). After treatment of Dau, increased E2F protein release has been found since 24 h (Fig. 3C). The activities of cyclin/CDK complexes are inhibited by CDK inhibitors (CDKIs) [Murray, 2004]. We examined the effect of Dau treatment on the expression levels of CDKI $p21^{waf1/cip1}$ in growth-arrested SW480 cells. The results of immunoblotting revealed that the expression level of $p21^{waf1/cip1}$ was up-regulated to 3.4 in the first 4 h after treatment, and then strikingly down-regulated to 0.0 as treatment time prolonged (Fig. 3C,D).

APOPTOSIS IS NOT INDUCED BY DAU

To determine the action mode of Dau, SW480 were combined stained with annexin V/PI and analyzed by confocal microscopy. As shown in Figure 4A, SW480 cells show positive binding with both annexin V and PI after treated with Dau for 72 h. It suggested that the cell death mode in SW480 cells induced by Dau was not apoptosis, which is consistent with the results of flow cytometric analysis (Fig. 2A). Moreover, the protein expression levels of caspase-3 (35 kDa) and PARP (116 kDa) did not change (Fig. 4B). Taken together, these results demonstrated that Dau did not induce apoptotic cell death in SW480 cells.

ACTIVATION OF MAPK PATHWAYS

The mitogen-activated protein (MAP) kinases are well known to play an important role in controlling cell growth, differentiation, development, and death [Pearson et al., 2001]. In order to determine the intracellular signaling mechanism involved in the anti-proliferative action of Dau, we investigated the state of activation of MAPK family members, including the extracellular signal-regulated kinase (ERK), the c-jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), and the p38 kinase. After treatment of Dau,

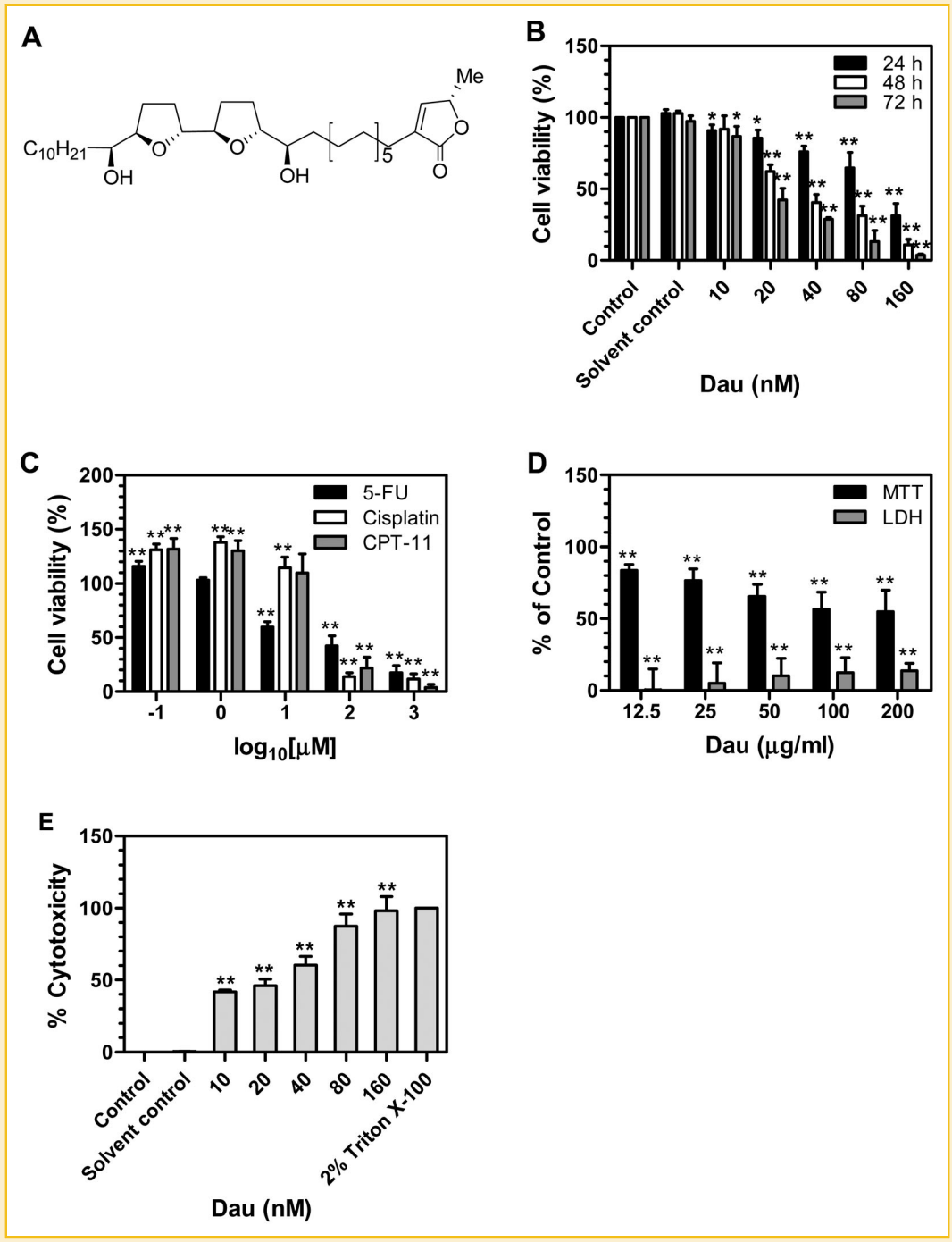

Fig. 1. Cytotoxic effects of Dau on SW480 colorectal cancer cells and Hs68 normal fibroblast cells. A: Chemical structure of Dau. B: Cell viability of SW480 cells after treatment with different concentrations of Dau for 24, 48, and 72 h as examined by MTT assay. The solvent controls were 0.5% DMSO. C: Cell viabilities of SW480 cells after treatment with 5-FU, cisplatin and irinotecan for 72 h as determined by MTT assay. D: Cell viability and LDH leakage of Hs68 cells after treatment with Dau for 72 h as determined by MTT assay and LDH assay, respectively. The percentage of viable cells was calculated as a ratio of treated to control cells (treated with medium). E: Dau-induced LDH leakage on SW480 cells. Control cells were normalized as 0% cytotoxicity and 2% Triton X-100-treatments as positive control were normalized as 100% cytotoxicity. The solvent control was 0.5% DMSO. Data represent the means ± SD ($n = 3$; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control).

phosphorylated-ERK1/2 (p-ERK1/2), which is the activated form of ERK1/2, has been totally inhibited since 24 h (Fig. 5A). And Dau also inhibited the activation of ERK1/2 in a dose-dependent manner (Fig. 5B). In contrast, no significant changes were observed in p-p38 MAPK. And no p-SAPK/JNK was expressed in SW480 cells. To further examine the effects of Dau on the ERK1/2 signaling pathway, the activation state of MEK1/2 was also observed. The expression of the active form of p-MEK1/2 was also decreased in a dose-dependent manner (Fig. 5B). Taken together, these results indicated that MEK/ERK signaling pathway in cancer cells was inactivated by Dau.

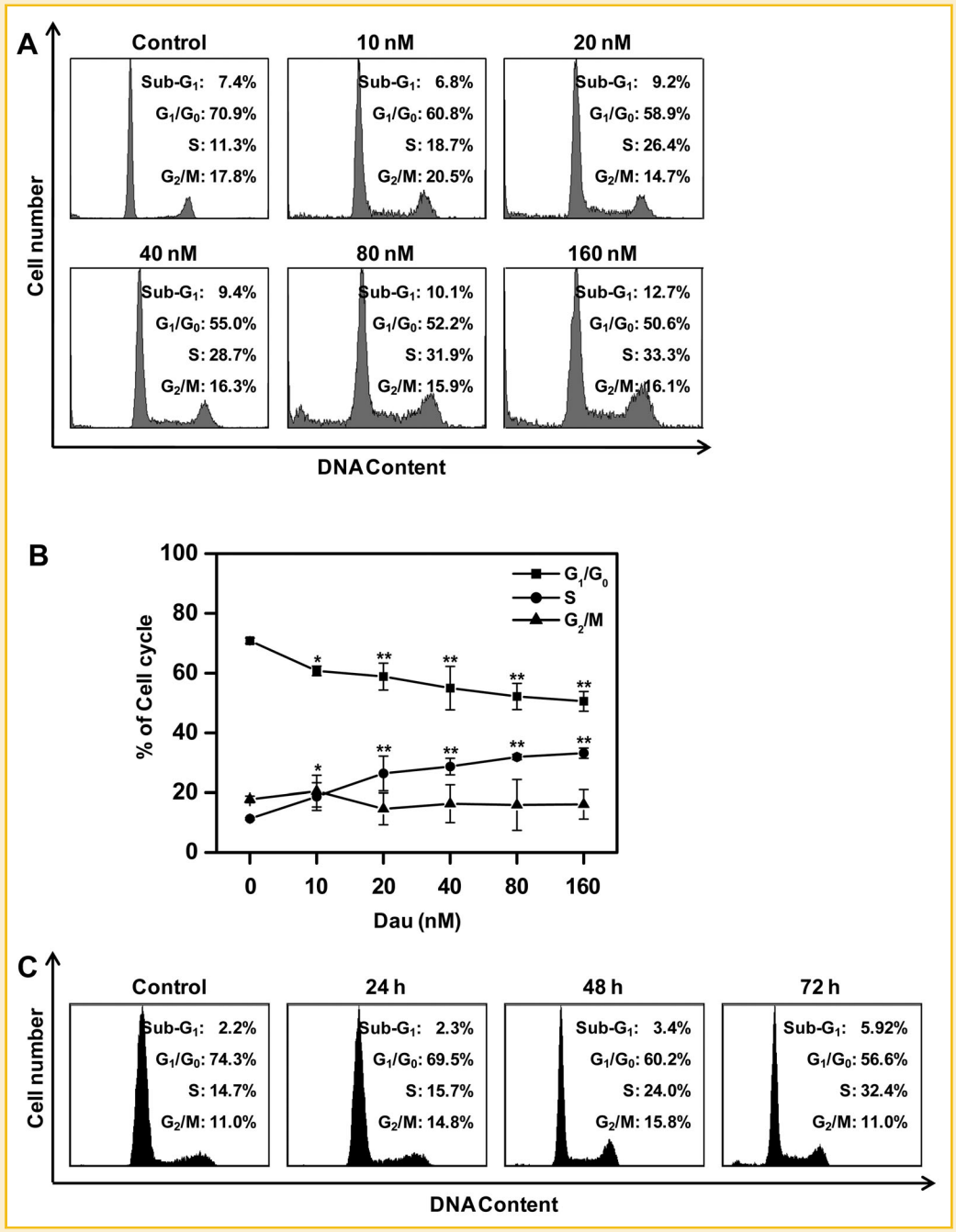

Fig. 2. Flow cytometric analysis of Dau-treated SW480 cells. A: The cell populations of SW480 cells in sub-G_1, G_1/G_0, S, and G_2/M phase were detected by flow cytometry after treatment with Dau at 72 h. Data were the representative graph from three independent experiments. B: Accumulation of S phase of cell cycle in Dau-treated SW480 cells. SW480 cells were treated with indicated concentrations of Dau for 72 h. Then cells were collected, stained with PI and subjected to flow cytometric analysis for cell distribution at each phase of the cell cycle, as described in Materials and Methods Section. Data represent the means ± SD ($n = 3$; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control).

SUPEROXIDE-MEDIATED DNA DAMAGE INDUCED BY DAU

We used the fluorescent probe dihydroethidium (DHE) to measure the production of Dau-induced intracellular superoxide. Basically, DHE can permeate cell membranes freely and then is oxidized by superoxide anions (O_2^-) producing red fluorescent product ethidium. Ethidium subsequently intercalates with DNA or RNA emitting red fluorescence. As shown in Figure 6A, treatment of SW480 cells with 80 nM Dau resulted in a time-dependent increase in intracellular superoxide. After 2 h treatment, the levels of superoxide started to significantly increase to 117% of control cells. To investigate possible oxidative damage as a result of intracellular superoxide production, chromosomal DNA damage in Dau-treated SW480 cells were studied by single-cell gel electrophoresis (comet assay). Based on the characteristic morphological changes of nucleus, we are able to readily identify the DNA damage caused by Dau (Fig. 6B). The nucleus of control cells remained intact when subjected to electrophoresis.

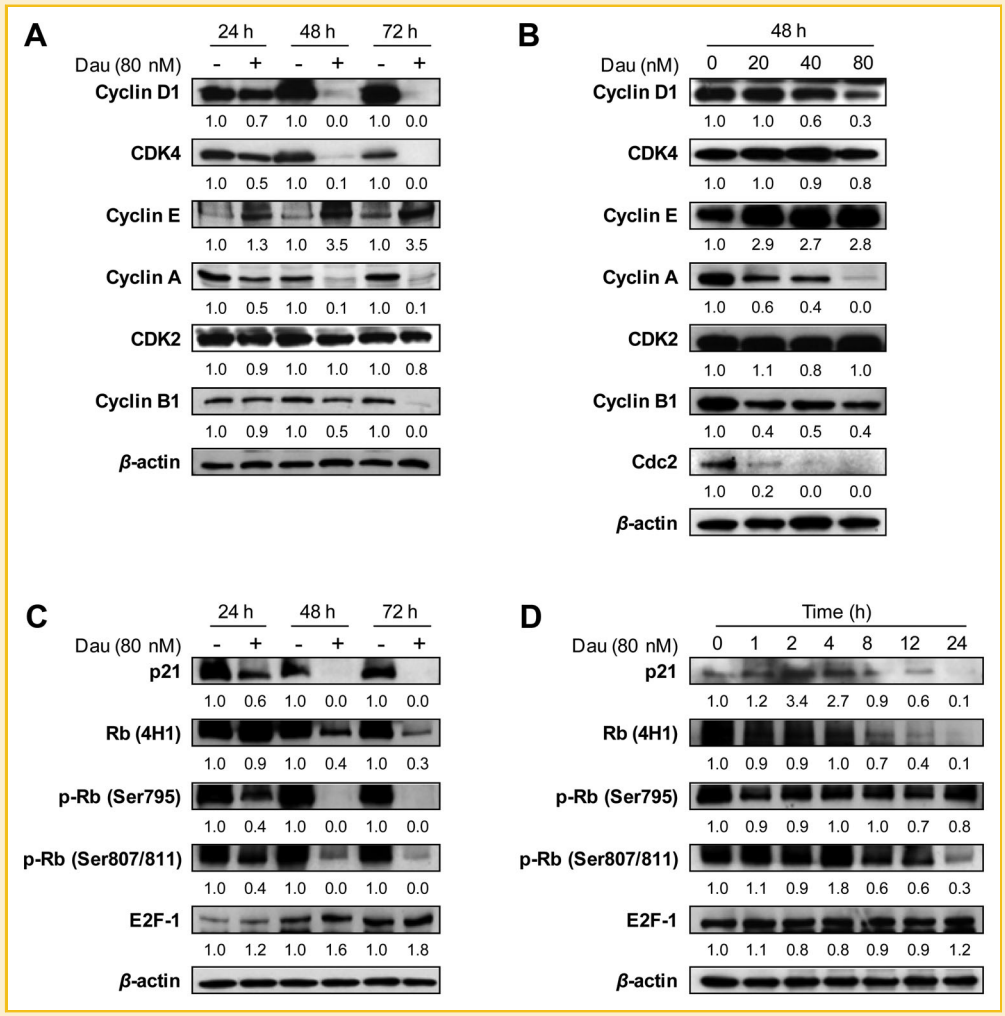

Fig. 3. Western blotting analyses for the levels of cell cycle regulator proteins. A&C: SW480 cells were treated with 80 nM of Dau for 24, 48, and 72 h. B: SW480 cells were treated with different concentrations of Dau for 48 h. D: SW480 cells were treated with 80 nM of Dau for different time points within 24 h.Total cell lysates were prepared and 40 μg/ml proteins was subjected to SDS–PAGE followed by electrophoresis and chemiluminescent detection. Each antigenic protein was detected by using the respective antibody against cyclin D1, CDK4, cyclin E, cyclin A, CDK2, cyclin B1, Cdc2, $p21^{waf1/cip1}$, Rb (4H1), p-Rb (Ser795), p-Rb (Ser807/811), E2F-1, or β-actin. Data represent similar results from three independent experiments.

The fragmented DNA in the nucleus of treated cells migrates highly away from the nuclear head. The broken chromosomal DNA strands can also be measured by the olive tail moment in the comet assay. As shown in Figure 6C, when SW480 cells were treated with Dau for 72 h, DNA damage, as indicated by increased olive tail moment, increased significantly ($P < 0.01$) from 5.8 to 44.7 in a dose-dependent manner. The olive tail moment has doubled (11.7) when cells treated with 40 nM Dau than control cells.

PREVENTION OF DAU-INDUCED SUPEROXIDE GENERATION AND DNA DAMAGE BY ANTIOXIDANTS

The protective effect of ascorbic acid (AA) against Dau-induced superoxide and cell death was studied. Figure 7A showed that 1 mM AA almost completely attenuated the intracellular Dau-induced superoxide generation. After 24-h treatment, both S and G2/M phase arrest was observed in cells treated with Dau. Interestingly, co-treatment of the cells with AA effectively inhibited the S phase arrest, but only slightly suppressed the G2/M arrest (Fig. 7B). To further evaluate the role of Dau-induced superoxide in DNA damages, we examined the effects of the superoxide scavenger AA on Dau-induced cell death. As shown in Figure 7C,D, AA was able to significantly protect SW480 cells from DNA damage caused by Dau. After co-treatment with AA, the morphology of Dau-treated SW480 cells and their nucleus returned back to normal shape, and the DNA had become concentrated. Taken together, these results suggest that, blocking ROS generation by antioxidants could prevent Dau-induced superoxide generation and DNA damage.

DISCUSSION

Colorectal cancer (CRC) is the second most prevalent cancers in females and the third in males worldwide. In 2008, it was estimated

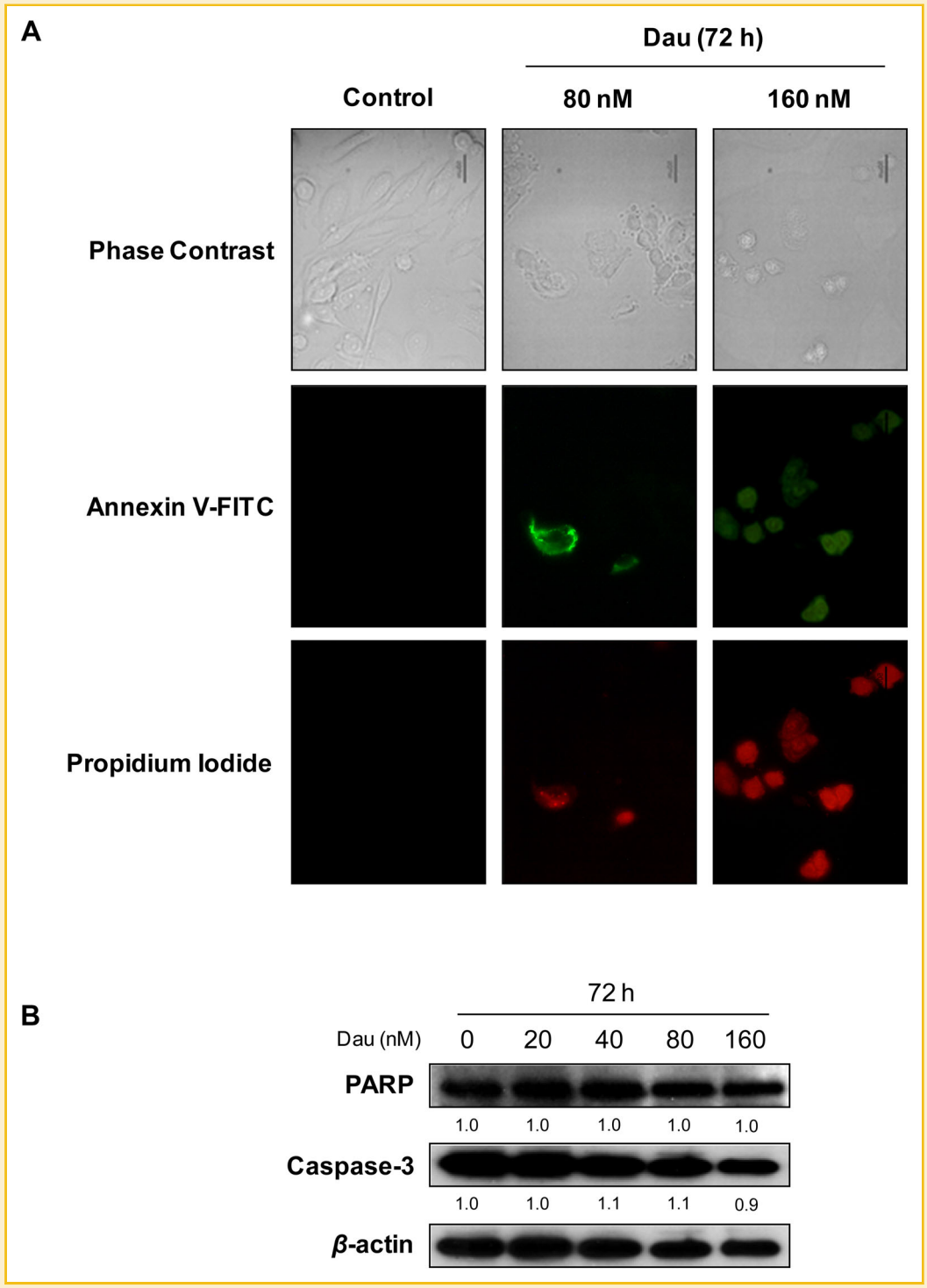

Fig. 4. Mode of cell death induced by Dau. A: Annexin V and propidium iodide (PI) combined staining of SW480 cells. Data were the representative graph (n = 3). B: Activation of caspase-3 and PARP cleavage by Dau-treatment. SW480 cells were treated with Dau for 72 h. Data represent similar results (n = 3).

that more than 1.2 million new cases occurred and 608,700 people died of CRC [Jemal et al., 2011]. Hence CRC is quite a major public health problem in the entire world. Although progress has been made in the treatment of this disease, great morbidity and mortality still happened. 5-FU in combination with oxaliplatin and followed by leucovorin (LV), is an effective chemotherapeutic agent to treat metastatic colorectal carcinoma [Segal and Saltz, 2009]. However, the introduction of new combination chemotherapy regimens, as well as the use of new cytotoxic agents, may lead to significant improvements in median survival rates of these patients. Therefore,

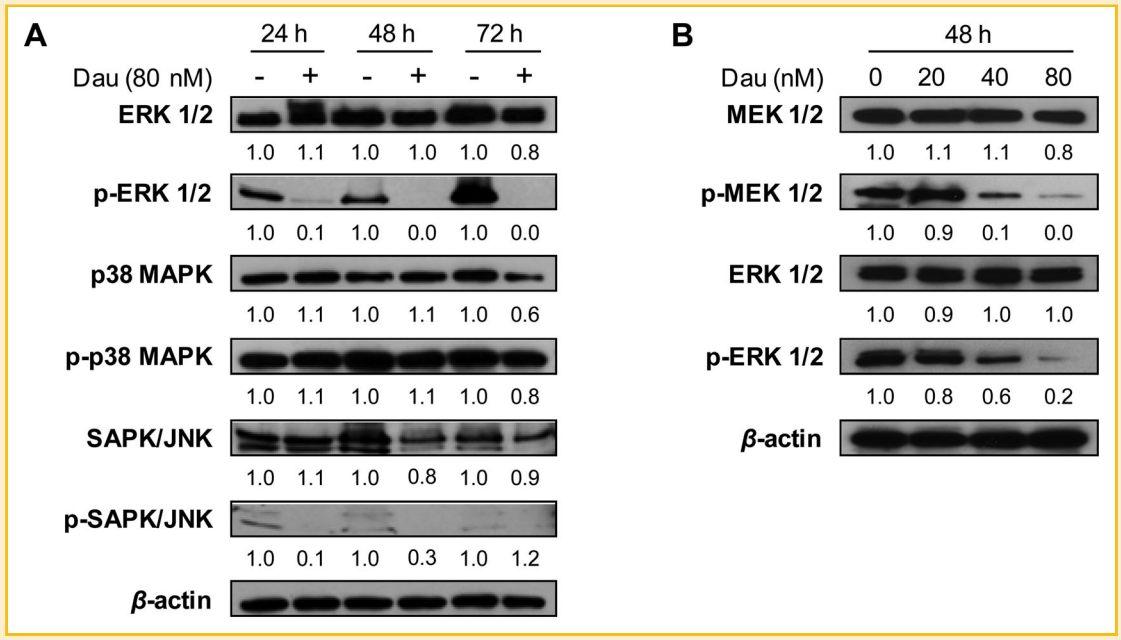

Fig. 5. Dau inhibited the activity of MEK/ERK signaling pathway in SW480 cells. A: Western blotting analysis of the MAPK signaling pathways in SW480 cells after treating with 80 nM Dau for various time spans. Data represent similar results from three independent experiments. B: Western blotting analysis of the MEK/ERK pathway in SW480 cells after treating with Dau for 48 h. The whole cell lysates were subjected to Western blotting analysis as described in Materials and Methods Section. Data represent similar results from three independent experiments.

it is quite meaningful for us to develop new effective anti-CRC drug.

Uvarla calamistrata (*Annonaceae*) is a climbing shrub that well distributed in Hainan, Guangdong and Guangxi provinces of China, and Vietnam. The local residents used its leaves and roots to treat diseases like lumbago and backache [Zhou et al., 1999]. In this study, we have screened several ACG compounds, which were isolated from the root of *U. calamistrata* by Prof. Zhou [Zhou et al., 1999, 2000, 2008; Chen et al., 2007], for the anticancer activity (Table I). In particular, Dau is the most cytotoxic compound to almost every cancer cell line tested except for Caco-2. And SW480 is the most sensitive cell line to all the ACGs tested. Therefore, Dau and SW480 human colorectal carcinoma cell line were chosen, and we investigated the in vitro effect of Dau on induction of cytotoxicity, cell cycle distribution and cell death in SW480 cells.

Dau, as showed in Figure 1A, exhibited great cytotoxicity on SW480 cells at nM level (Fig. 1B), which is about 1,000 times as powerful as that of 5-FU, cisplatin, and CPT-11 (Fig. 1C). Moreover, Dau also has great selectivity between normal cells and tumor cells (Fig. 1D). Collectedly, Dau is a potential compound with high anticancer efficiency and high selectivity for further mechanism study.

Although apoptosis is postulated as a critical strategy for cancer therapy, scientists kept finding out other forms of cell death caused by anticancer drugs, such as cell senescence, necrosis, autophagy, mitotic cell death, or a permanent cell arrest [Blank and Shiloh, 2007]. In our study, the combination of flow cytometric analysis for sub-G_1 populations, fluorescent staining for distinguishing apoptosis and necrosis, and Western blotting for cleavage of caspase3 and PARP analyses confirmed that Dau induce SW480 colorectal cancer cells necrotic cell death instead of apoptotic cell death.

Many anticancer agents can arrest the cell cycle at the G_1/G_0, S, or G_2/M phase and then induce cell death. Our cell cycle analysis revealed that Dau induced a dose- and time-dependent accumulation of SW480 cells in the S phase of the cell cycle (Fig. 2). To our knowledge, this is the first report describing the mechanism of S phase arrest of ACG compounds on colorectal cancer cells. To develop Dau as an anti-colorectal cancer drug, we also investigated the effect of Dau on other colorectal cancer cell strains. The mechanism study showed that Dau could arrest SW620, COLO201, Caco-2 colorectal cancer cells all at S phase (data not shown). Therefore, it is very convincing that the effect of Dau on colorectal cancer is S phase arrest. According to the Western blotting analyses, Dau can arrest SW480 cells at S phase directly through the down-regulation of cyclin A and the up-regulation of cyclin E. Lack of cyclin A to cyclin A/CDK2 complex formation may inhibit S phase prgression. Moreover, plenty of cyclin E may push the cells passing through the G_1/S boundary from G_1 phase into S phase. This phenomenon is also supported by flow cytometric analysis, which showed the increase in the S population was mostly at the expense of G_1/G_0 phase cells (Fig. 2). Other phase regulators of the cell cycle, such as cyclin D1, CDK4, cyclin B1 and Cdc2, were also found to be down-regulated (Fig. 3A,B). In the upper stream of cell cycle regulation, $p21^{waf1/cip1}$, a cyclin/CDK inhibitor in G1 phase and G2/M phase [Murray, 2004], showed slightly increased expression in the first few hours due to Dau-induced stress. Afterwards, $p21^{waf1/cip1}$ was significantly down-regulated due to binding with cyclin D1/CDK4 or cyclin B1/Cdc2 complexes to inhibit their activities [Han et al., 2008]. Dau also

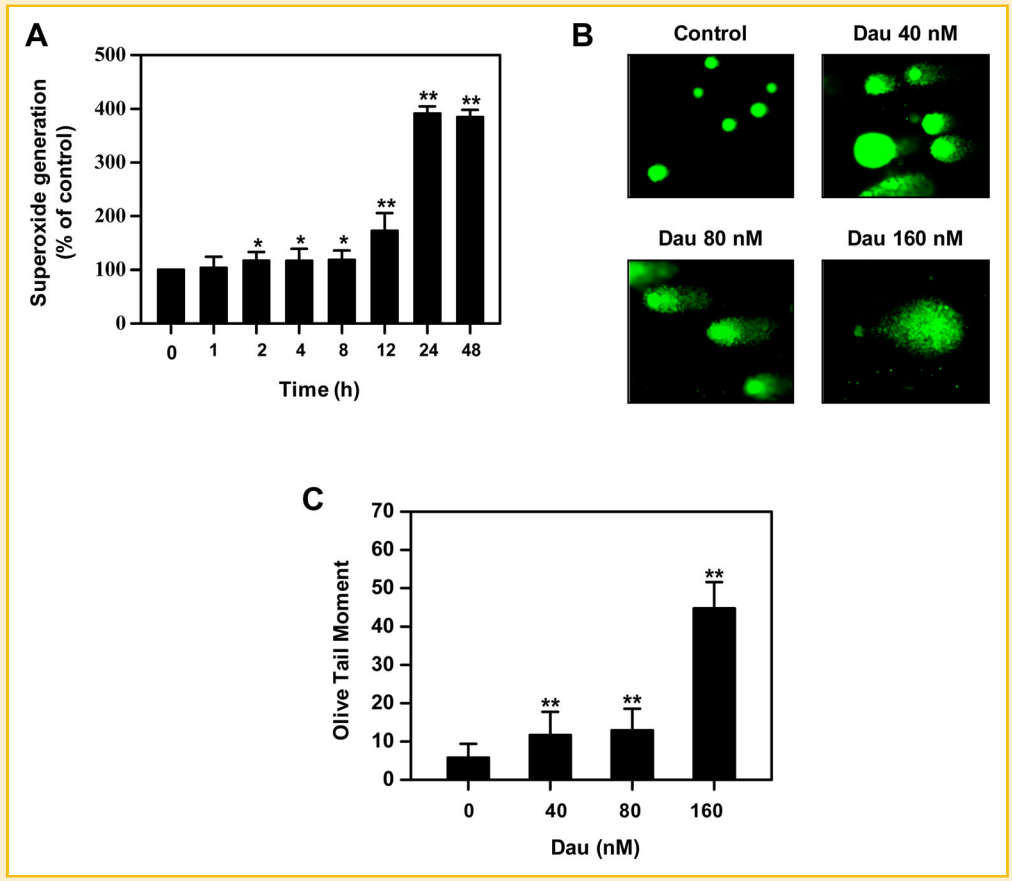

Fig. 6. Dau induced superoxide generation and DNA damage in SW480 cells. A: Induction of intracellular superoxide in SW480 cells by Dau. Dau-treated cells at various time points were loaded with 10 μM DHE for 25 min and then harvested and resuspended in PBS. Superoxide generation was determined by measuring DHE fluorescence intensity as described in Materials and Methods Section. B: Morphological results of comet assay. Data were the representative graph (n = 3). C: Induction of DNA strand breakage in SW480 cells by Dau based on tail moment analysis. SW480 cells were treated with different concentrations of Dau for 72 h, and subsequently analyzed by comet assay as described in Materials and Methods Section. $^{*}P < 0.05$, $^{**}P < 0.01$ versus controls.

inhibited both the synthesis of total Rb protein and its phosphorylation, and kept E2F persistently expressing, which may cause S phase delay. Collectively, Dau changed the expression levels of cell cycle regulators to arrest SW480 cells in S phase.

In mammalian cells, reactive oxygen species (ROS) is ubiquitous and generated constantly from many endogenous and exogenous sources. ROS encompass a variety of partially reduced oxygen species (e.g., superoxide anions O_2^-, hydroxyl radicals OH^-, and hydrogen peroxide H_2O_2). An excess of ROS is toxic and attacks cellular macromolecules, such as lipids, proteins, and especially the DNA, to cause cell death (apoptosis and necrosis). The most significant nuclear lesion caused by increasing ROS is DNA double strand broken [Martindale and Holbrook, 2002; Barzilai and Yamamoto, 2004]. In our study, Dau can cause a lot of superoxide production (Fig. 6A), which subsequently induced severe damages on DNA (Fig. 6B,C). AA, a ROS scavenger, effectively decreased the Dau-induced superoxide, overcame DNA damage and restored the cell cycle progression in SW480 cells (Fig. 7). It has been proved that increasing ROS can trigger checkpoint responses via the induced damage to DNA. The extent of the DNA damage determines cell fate: cell cycle arrest and DNA repair or the activation of apoptotic pathways [Baus et al., 2003]. These findings support that the notion that the cell cycle arrest caused by Dau was mediated by oxidative stress. Moreover, the MEK/ERK pathway, as a down-stream of ROS production, was found to be inhibited by Dau in SW480 cells. It has been reported that the ERK pathway is usually highly activated in melanomas, thyroid cancer, colorectal cancer and lung cancer to protect cells from death [Hoshino et al., 1999; Balmanno and Cook, 2009]. In this study, we showed that, Dau could inactivate ERK through regulation of intracellular ROS level, demonstrating the application potential of Dau in treatment of cancers with high expression levels of ERK.

In conclusion, in the present study, we demonstrated that Dau, an Annonaceous acetogenin, was a potent cytotoxic agent against SW480 human colorectal cancer cells with great selectivity between cancer and normal cells. Dau could trigger overproduction of superoxide in SW480 cells, and thus caused DNA strand break and inactivation of MEK/ERK pathway. Moreover, Dau could alter the expression levels of the cell cycle regulators, thus resulting in S-phase arrest. Taken together, our results showed that, Dau could inhibit cancer cell growth through regulation of ROS-mediated signaling

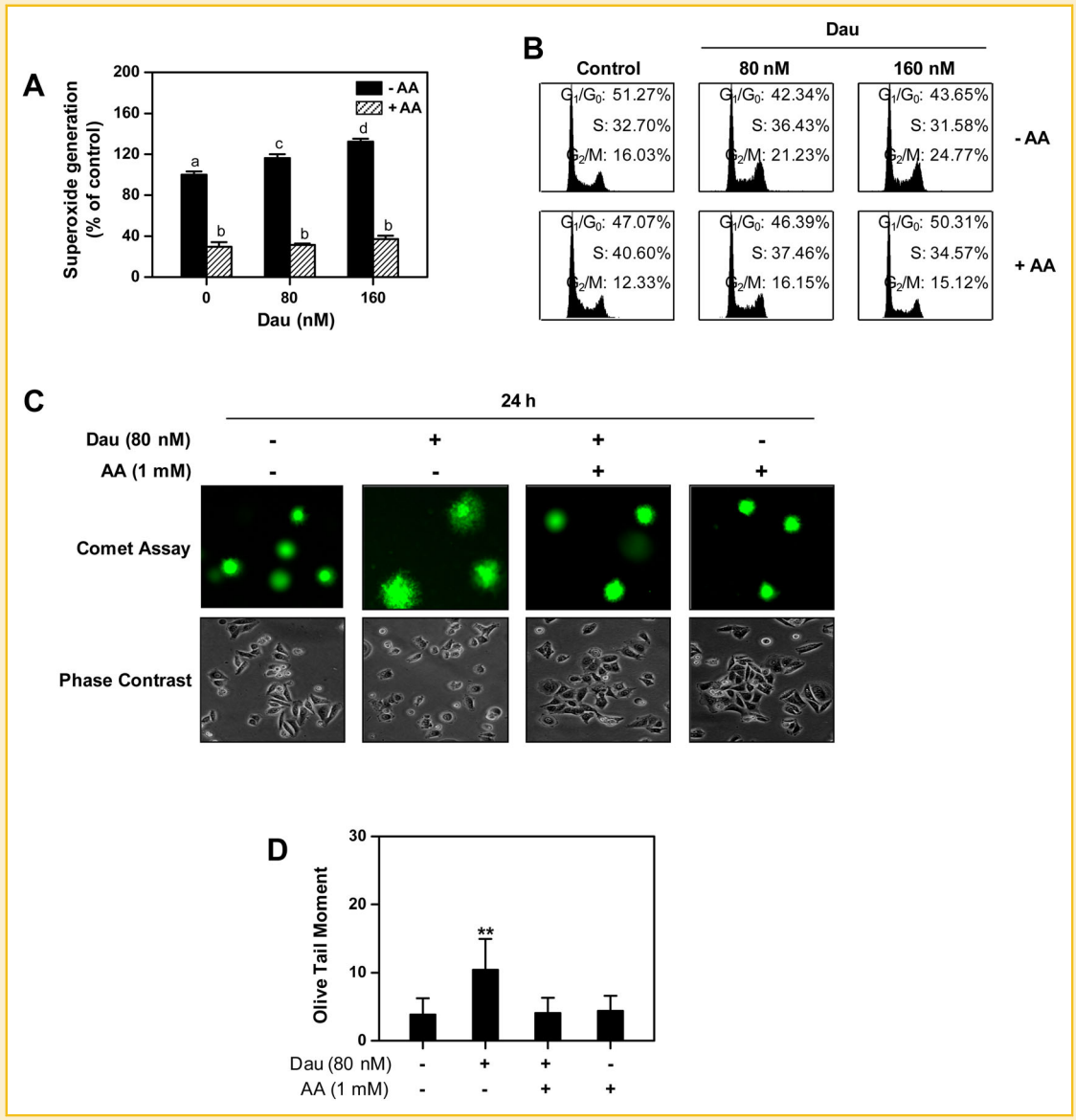

Fig. 7. Antioxidant protected SW480 cells from Dau-induced cell death. A: AA inhibited superoxide generation in SW480 cells. B: Protective effect of AA on the cell cycle phase distribution of Dau-treated SW480 cells. Flow cytometric analysis was conducted as described in Materials and Methods Section. C: Protection of AA on DNA damage in Dau-treated SW480 cells based on tail moment analysis. D: Morphological results of AA protecting Dau-treated SW480 cells from DNA strand breakage. Cells were pretreated with or without 1 mM AA for 2 h, and then treated with Dau for 24 h. $^{*}P < 0.05$, $^{**}P < 0.01$ versus controls.

pathways. Annonaceous acetogenins are novel cytostatic anticancer agents from natural sources, and may be candidates for further evaluation as chemotherapeutic agents.

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