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Sodium 5,6-Benzylidene-L-Ascorbate Induces Oxidative Stress, Autophagy, and Growth Arrest in Human Colon Cancer HT-29 Cells

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ABSTRACT

Our previous studies have demonstrated the oxidative stress properties of sodium ascorbate (SAA) and its benzaldehyde derivative (SBA) on cancer cell lines, but the molecular mechanisms mediating their cytotoxicity remain unclear. In this study, we treated human colon cancer HT-29 cells with SAA and SBA, and found a significant exposure time-dependent increase of cytotoxicity in both treatments, with a higher cytotoxicity for 24 h with SAA ($IC_{50} = 5$ mM) than SBA ($IC_{50} = 10$ mM). A short-term treatment of cells with 10 mM SAA for 2 h revealed a destabilization of the lysosomes and subsequent induction of cell death, whereas 10 mM SBA triggered a remarkable production of reactive oxidative species, phosphorylation of survival kinase AKT, expression of cyclin kinase-dependent inhibitor p21, and induction of transient growth arrest. The crucial role of p21 mediating this cytotoxicity was confirmed by isogenic derivatives of the human colon carcinoma HCT116 cell lines ($p21^{+/+}$ and $p21^{-/-}$), and immunoprecipitation studies with p21 antibody. The SAA cytotoxicity was blocked by co-incubation with catalase, whereas the SBA cytotoxicity and its subsequent growth arrest were abolished by *N*-acetyl-L-cysteine (NAC), but was not affected by PI3K phosphorylation inhibitor LY294002, or catalase, suggesting two separated oxidative stress pathways were mediated by these two ascorbates. In addition, neither active caspase 3 nor apoptotic bodies but autophagic vacuoles associated with increased LC3-II were found in SBA-treated HT-29 cells; implicating that SBA induced AKT phosphorylation-autophagy and p21-growth arrest in colon cancer HT-29 cells through an NAC-inhibitable oxidative stress pathway. J. Cell. Biochem. 111: 412–424, 2010.

KEY WORDS: OXIDATIVE STRESS; GROWTH ARREST; P21 INHIBITOR; AUTOPHAGY

Reactive oxidative species are not only metabolic by-products of normal aerobic cells, but also play essential roles as second messengers in intracellular signaling pathways. However, an overproduction of reactive species results in oxidative stress, which is associated with expression of growth inhibitor genes, disturbance of normal cell functions, growth arrest, and ultimately cell death. Although sodium ascorbate (SAA) is a well-documented reducing agent, it can induce lipid peroxidation [Song et al., 2001], methionine oxidation [Sakagami et al., 1997], protein misfolding [Banhegyi et al., 2003], DNA damage [Riviere et al., 2006], transient growth arrest [Thomas et al., 2005], and cell apoptosis [Lin et al., 2006]. A pharmacological dose of SAA selectively kills lymphomas and breast cancer cells [Chen et al., 2005], and these actions can be eliminated by co-incubation with catalase, a hydrogen peroxide scavenger, indicating that SAA mediates its cytotoxicity by generation of extracellular hydrogen peroxide [Chen et al., 2005].

Cell death is a result of unsuccessful cytoprotective mechanism against intracellular and extracellular stressors, and it is broadly divided into three forms: apoptosis, autophagy, and necrosis. Apoptosis (type I) is a programmed cell death characterized by cell shrinkage and blebbing, chromatin condensation, caspase activation, and DNA fragmentation [Lockshin and Zakeri, 2004]. Autophagy (type II) is a catabolic process for the degradation and recycling of long-lived proteins and defective organelles, and is

Additional Supporting Information may be found in the online version of this article.

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characterized by production of double membrane autophagic vesicles [Levine and Klionsky, 2004]. Autophagy is induced in response to nutritional starvation, and increasing evidence implicates an association of autophagy with an activation of PI3K/AkT/mTOR, and/or endoplasmic reticulum stress [Kouroku et al., 2007]. Necrosis (type III) is an uncontrolled cell death manifesting osmotic dispersion of cell and organelles.

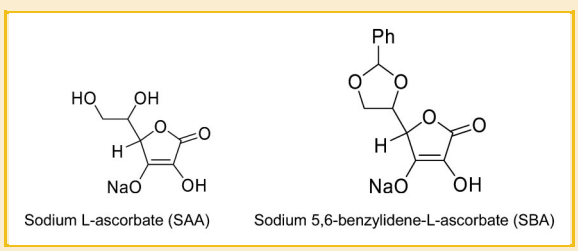
Sodium 5,6-benzylidene-L-ascorbate (SBA) is a benzaldehyde derivative of ascorbate, which has shown some anti-tumor activity against Ehrlich carcinoma, adenocarcinoma 755 and colon 38 in mice [Kochi et al., 1980]. Intravenous administration of SBA to patients with advanced, inoperable carcinoma [Kochi et al., 1988], or to rats with chemically induced hepatocellular carcinoma [Sakagami et al., 1991] induced remarkable tumor cell death by either apoptosis or necrosis. Previous comparative studies had demonstrated that both SAA and SBA shared some common biological activities, such as radical generation, methionine oxidation, and apoptosis-inducing activity, but they also responded differently to some metal ions (e.g., inhibition of cytotoxicity by iron and cysteine analog) and oxidation inhibitor, for example, catalase [Sakagami et al., 2000], implicating that the biological actions of these two compounds are mediated by two different pathways.

In order to investigate the molecular mechanisms inducing oxidative stress and subsequent cellular actions by SBA, a series of antioxidant enzymes and inhibitors were co-incubated with SBA for an elucidation of the oxidant production pathway(s) occurred in colon cancer HT-29 cells, and the short-term cytotoxic/cytostatic effects and the autophagic changes by SBA were studied by flow cytometry, immunoblotting analysis, fluorescence microscopy, and transmission electron microscopy. Combining our previous results on the SBA-mediated cytotoxicity and the findings in this study, we have demonstrated that SBA induced transient p21-mediated growth arrest and autophagy in HT-29 cells through the *N*-acetyl-L-cysteine-inhibitable oxidative stress pathway.

MATERIALS AND METHODS

TEST COMPOUNDS

Sodium 5,6-benzylidene-L-ascorbate (SBA, $C_6H_7NaO_6$, MW = 286.1; ChemiScience Ltd., Tokyo, Japan) and sodium L-ascorbic acid (SAA, $C_{13}H_{11}NaO_6$, MW = 198.1, A0539, TC1-gr; Kasei Kogyo Ltd., Tokyo, Japan; Fig. 1) were dissolved in medium to make a stock solution at a concentration of 40 mg/ml, which were then diluted to appropriate concentrations with culture medium before each experiment.



Sodium L-ascorbate (SAA) Sodium 5,6-benzylidene-L-ascorbate (SBA)

Fig. 1. Chemical structures of sodium L-ascorbate (SAA) and sodium 5,6-benzylidene-L-ascorbate (SBA).

CELL CULTURES

Human colon cancer HT-29 (HTB-38) cell line was obtained from American Type Culture Collection and was routinely cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; GIBCO/BRL, NY), 100 μg/ml penicillin G and 100 IU/ml streptomycin sulfate at 37°C in an atmosphere of 95% air and 5% CO_2. Human colon carcinoma HCT116 cell lines (wild type, $p21^{+/+}$, and p21-deficient cells, $p21^{-/-}$) were the generous gift of Dr. B. Vogelstein (The Johns Hopkins University), and the cells were maintained in McCoy 5A supplemented with 10% FBS and 100 μg/ml streptomycin and 100 IU/ml penicillin. For experiments, HT-29 cells were seeded at subconfluent densities for adherence overnight. Cells synchronized in G0/G1 phase of the cell cycle at 80% [Liu et al., 2005] were induced by serum starvation for 24 h and supplemented RPMI was then added to resume the cell cycle. Close monitoring of the HT-29 cells after resuming cell cycle showed 0–16, 17–20, and 21–28 h, for G1, S, and G2/M phases, respectively.

CYTOTOXICITY OF SBA AND SAA

HCT116 cells or HT-29 cells (2×10^4 cells/0.1 ml/well) were incubated with serial dilutions of SAA and SBA in 96-well culture plates (Costar, Cole-Parmer, IL) for the first 1, 2, or 24 h, washed with warm phosphate buffered saline (PBS), and incubated in fresh normal culture medium for a total of 24 h before subjected to MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) cytotoxicity assay. Data points represented the mean values and standard deviations of triplicate samples. Since SAA is a reducing agent that may interfere with MTT cytotoxicity assay, cell viability was determined by the trypan blue exclusion assay or the sulforhodamine B (SRB) colorimetric assay for the measurement of cellular protein [Tse et al., 2006].

The survival and proliferation of HT-29 cells after SAA or SBA treatment were studied using a soft agar clonogenic assay [Fiebig et al., 2004]. Briefly, synchronous cells (1×10^4/ml) in 6-well cultured plates were treated with 10 mM SAA or SBA for 2 h, washed with warm buffer, trypsinized, resuspended at 5×10^3 cells/ml fresh supplemented culture medium containing 0.3% Noble agar (Difco Laboratories, Detroit), and plated over the bottom layer that consisted 0.5% agar and 10% FBS in each well of a 6-well culture plate. The cells were allowed to culture for 6 and 10 days before 400 colonies were scored under an inverted microscope (Nikon, Chiyoda-ku, Tokyo, Japan). The percentage of positive colonies (≥50 μm in diameter) was calculated for evaluation of clonogenicity inhibition. At least three independent experiments, each using duplicate dishes, were performed.

MORPHOLOGICAL OBSERVATION

HT-29 cells were collected both immediately and 22 h after treatment with 10 mM SAA and SBA for 2 h (4 and 26 h of the cell cycle, respectively), washed briefly with PBS before they were fixed with 4% paraformaldehyde. The cells were differentially stained cellular DNA and RNA with 0.1 mg/ml acridine orange (01660; Fluka, Switzerland) and differentiated with 0.1 M calcium

chloride. Fluorescence micrographs were taken on a fluorescence microscope (Axioskop, Zeiss, Oberkochen, Germany) with a 450–490 nm excitation block filter and a 515 nm barrier filter [Liu et al., 2008]. Part of the fixed cells was immunostained with antibodies against cell survival proteins: p-AKT^{ser473} and p-AKT^{thr308} (4051 and 4056; Cell Signaling Tech., Denver, MA) for demonstration of their role(s) in mediating autophagic changes.

AUTOPHAGIC VESICULAR ORGANELLE FORMATION AND LYSOSOMAL DESTABILIZATION

To demonstrate if ascorbates induced autophagic vesicular organelle (AVO) formation, unfixed HT-29 cells were stained with acridine orange (2 μg/ml) for 30 min without further differentiation to manifest punctuate red cytosolic vesicles that are indicative of autophagic changes of the cell [Herman-Antosiewicz et al., 2006]. The AVO formation was analyzed by flow cytometry, and the structures were further confirmed by fluorescence microscopy [Paglin et al., 2001]. For ultrastructural observation by transmission electron microscopy, both SAA- or SBA-treated HT29 cells were fixed in 2% glutaraldehyde in 0.08 M cacodylic acid, embedded in Epon, sectioned and stained with lead citrate and uranyl-acetate for transmission electron microscopic analysis (Hitachi H600 Electron microscope, Japan) [Liu et al., 2005].

For analysis of lysosomal destabilization, both acridine orange stain using flow cytometry [Paglin et al., 2001] and LysoTracker DND-99 stain (L7528; Molecular Probes, Inc., OR) by fluorescence microscopy were performed. Briefly, 30 min before terminating experiment, 2 μg/ml acridine orange were loaded into the culture, and the cells were trypsinized for the red fluorescence analysis by a Beckman Coulter EPICS Altra Flow Cytometer (Miami, FL) with an argon laser. Integrated lysosomes were able to concentrate acridine orange in acidic environment to give out red fluorescence (>650 nm), representing the formation of AVO, which highly associated with the development of autophagy.

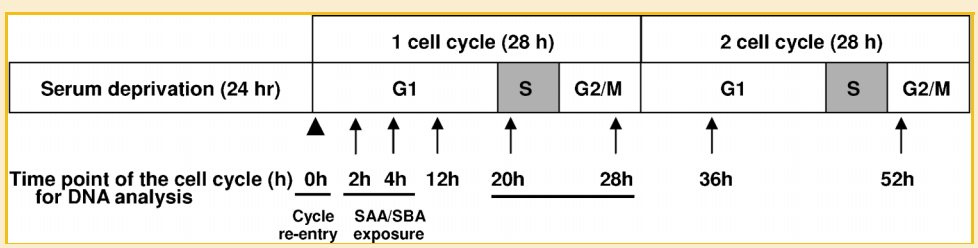
Cells pre-loaded with 50 nM LysoTracker Red DND-99 (Molecular Probes) for 30 min, washed with PBS to remove extracellular dye, and then cultured in the fresh culture medium in the presence of 10 mM SAA or 10 mM SBA for lysosomal integrity measurement using an Olympus confocal laser scanning microscope (FV1000; Olympus, Japan). LysoTracker Red DND-99 was excited at 577 nm, and the emission signal was detected at 590 nm. Punctuated bright red fluorescent pattern was observed in the healthy cells, but the fluorescence became dimmer after drug treatment, particularly in SAA-treated cells within 30 min. The real-time changing of the fluorescence in the cells was measured and analyzed using a FV-10 software (ASW ver.1.6, Olympus, Japan). Results of representative images between treatment and untreated control groups at 30 min were compared.

PRODUCTION OF REACTIVE OXIDATIVE SPECIES

Our previous studies have demonstrated strong oxidative activities of both ascorbates, and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA, D399 Molecular Probes) was used in this study to analyze reactive oxidative species induced by SAA and SBA. Briefly, HT-29 cells were plated at 3×10^5/ml supplemented RPMI medium in each well of a 6-well culture plate overnight, and the cultures were then incubated in PBS containing 10 μM H_2DCF-DA for 15 min at 37°C before being stimulated with various doses of SAA and SBA for 1, 2, and 4 h. The intensity of the fluorescence for reactive species was measured by flow cytometer at excitation/emission at 488/535 nm. Cells pre-incubated or co-incubated with catalase, DPI (1–10 μM), oxypurinol (10–50 μM), rotenone (1–10 μM), and NAC (10 mM) before ascorbate treatment were used to elucidate the sources of ROS production [Cheung et al., 2010].

CELL CYCLE ANALYSIS

For cell cycle analysis, HT-29 cells were synchronized at G0/G1 phase of the cell cycle by serum starvation for 24 h [Liu et al., 2005], and then released from arrest by re-introduction of serum in the culture medium for 2 h. Cells were then exposed to twofold serial dilutions of SAA and SBA for 2 h, washed with PBS, and then allowed to continue cell cycle progression in fresh supplemented cultured medium for 8, 16, 24, and 32 h (corresponding to 12, 20, 28, and 36 h of the cell cycle) which were approximately in the G1, S, and G2/M phases of the HT-29 cells (Scheme 1). The treated cells (1×10^6 cells), including adherent and floating cells in the culture, were harvested for cell cycle analysis using a Beckman Coulter EPICS Altra Flow Cytometer. Around 10,000 cells per sample were assessed using Expo32 software (Beckman Coulter, Inc.), and the percentage of cells in various phases of the cell cycle was determined by Modfit DNA analysis software (Beckman Coulter) [Liu et al., 2005]. To investigate if SBA specifically arrested cells at the G1 phase, cells at S and G2/M phases (20 and 28 h, respectively) were treated with 10 mM SAA or 10 mM SBA, and an increase of p21 protein and the associated delay of cell cycle were found in all



Scheme 1. Schematic presentation of the cell cycle analysis.

treatments, indicating a cell cycle-independent growth arrest was induced by SBA (data not shown).

To explore the roles of oxidative stress, and p-AKT and p21 expression in growth arrests induced by SAA or SBA, cells were pre-treated with catalase (a H_2O_2 scavenger), NAC (an oxidant scavenger), and LY294002 (a PI3K inhibitor) before treatments, and cells were collected at 20 h for cell cycle analysis by flow cytometry.

IMMUNOBLOTTING AND ELISA ANALYSES

HT-29 cells at 0, 12, 20, and 28 h of the cell cycle were collected, washed with PBS twice, resuspended in lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 0.2% Triton X-100, 10 μg/ml aprotinin, and 0.5 mM PMSF), and centrifuged at 10,000 rpm at 4°C for 10 min. Lysates were normalized for protein content using the protein assay reagent (500-0006; Bio-Rad Laboratories, CA), resolved on a 10% SDS–PAGE gel and transferred onto a PVDF membrane using a Semi-dry Transfer Cell (Bio-Rad). The blot was then rinsed with Tris-buffered saline containing 0.1% Tween 20 (TBS/T), pH 7.6 and soaked in blocking reagent to prevent non-specific binding before it was reacted overnight at room temperature with polyclonal antibodies (all antibodies except AKTs were from Santa Cruz Biotechnology, Inc.) against (1) apoptosis-related protein: caspase 3 (sc-7148); (2) cell cycle regulatory proteins: p-AKT^{ser473} and p-AKT^{thr308}, cyclin D (sc-8396), cyclin-kinase inhibitor p21 (sc-6246), and proliferating cell nuclear antigen (PCNA, sc-56); and (3) autophagic protein: LC3 (NB100-2220; Novus Biological, Littleton, CO), and followed by a secondary antibody IgG conjugated to horseradish peroxidase in TBS-T buffer. The signals were detected using the ECL Plus^{TM} Western Blotting Analysis System (Amersham Pharmacia Biotech, Piscataway, NJ), and followed by short exposure to Lumi-film Chemiluminescent Detection Film (1666657; Roche Diagnostics Corporation, Indianapolis, IN). Band intensities were quantified by the software PD Quest (Bio-Rad Laboratories) and normalized by β-actin [Liu et al., 2005]. Antibodies were stripped by the Re-Blot Plus Western Blot Recycling Kit (Chemicon International, Temecula, CA) and re-probed with other antibodies described as above. Data are presented as mean and standard deviation of three independent experiments. The kinetic analysis of p-AKT^{ser473} in SAA/SBA-treated cells was performed using an ELISA kit (Cell Signaling Tech).

IMMUNOPRECIPITATION–IMMUNOBLOTTING ANALYSES

To examine a direct involvement of p21 in growth arrest, 20 μg of the total cell lysate from ascorbate-treated HT-29 cells were incubated with 1/100 dilutions of antibody against p21 for overnight, followed by incubation with 10 μl of protein A-Sepharose CL-4B beads (Cat no. 17078001; Amersham Bio-Sciences, Uppsala, Sweden) for 1 h. The protein A beads-bound p21 protein complexes were collected, resolved by 12% SDS–PAGE before they were transferred onto a PVDF membrane by electro-blotting analysis with antibodies against cyclin D and p21 (at 1:2,000 dilutions), and detected by ECL blotting reagents (RPN2108; Amersham, UK) for 1 min and exposed to a Lumi film (Roche) [Liu et al., 2008].

STATISTICAL ANALYSIS

Data were analyzed by one-way analysis of variance (except comparison of oxidant production with SAA or SBA treatments with two-way analysis of variance), followed by Duncan's multiple range test to detect intragroup (intergroup for two-way analysis) differences using GraphPad Prism software 5.0 (USA). Significant difference was considered when $^{*}P < 0.05$ and $^{***}P < 0.001$.

RESULTS

CYTOTOXICITY OF SBA AND SAA

The MTT reduction is a common assay for evaluation of cell metabolism and viability, and was used to assess the cytotoxicity of SAA and SBA in colon cancer HT-29 cells. A slight increase of cell metabolism and viability was demonstrated at low doses of SAA (≤1.25 mM) for 24 h, followed by a prominent increase of cytotoxicity at 2.5 mM, and reached a peak at 10 mM, with the value of IC_{50} at around 5 mM (Fig. 2c). Unlike SAA, the potency of SBA cytotoxicity on HT-29 cells was less dramatic, with the value of IC_{50} at around 10 mM. However, a short exposure (1 h) of the cells to similar ranges of SBA showed a more cytotoxic effect than that of SAA after 24 h (Fig. 2a), while a 2-h-exposure exhibited similar level of cytotoxicity for both compounds (Fig. 2b).

To demonstrate that this cytotoxicity was not restricted to HT-29 cells, an isogenic, diploid human colon HCT116 cells ($p21^{+/+}$ and $p21^{-/-}$), and a human prostate cancer LNCaP cell line were treated with SAA and SBA for 24 h, and the results of SRB assays also show a stronger cytotoxicity of SAA on both cell lines (Fig. 2d, data for LNCaP cells not shown). In addition, this cytotoxicity up to a concentration of 10 mM SBA is cyclin kinase inhibitor p21-dependent, implicating a significant role ($P < 0.05$) of p21 in the SBA- but less significant in SAA-mediated cytotoxicity, because SAA exhibited similar growth kinetics in both $p21^{+/+}$ and $p21^{-/-}$ cell lines. About 20 and 25–35% dead cells, at 4 and 32 h respectively, were measured by the trypan blue exclusion assay (Fig. 2e), indicating that SBA and SAA killed about 25–35%, after 2-h-ascorbate exposure, and that the survivors continued to progress through the cell cycle around 32 h. To investigate the more imminent and short-term effects of both ascorbates, a 2-h-exposure system [Chen et al., 2005] was adopted for studies of molecular mechanisms underlying the short-term cytotoxicity of both SAA and SBA in HT-29 cells.

Treatment of cells at different phases of the cell cycle (i.e., G1, S, and G2) with 10 mM SAA or 10 mM SBA for 2 h showed similar growth arrests as measured by flow cytometry (data not shown), indicating this growth arrest was cell cycle-independent.

The clonogenic assay is an additional means to evaluate the long-term survival and colony formation of HT-29 cells after SAA or SBA treatment. About 54.4% of untreated control cells had replicated four times to form colonies (≥16 cells/colony), whereas SBA-treated cells displayed a significantly decreased clonogenicity (33.8%, $P < 0.05$) after 6 days (Fig. 2f). This decrease was minimized to only 10% ($P > 0.05$) at day 10 because most of the cells in both groups proliferated and formed ≥100-cell colonies (84.5% vs. 94.5% for SBA-treated and untreated control cells, respectively); these findings are in consistent with trypan blue exclusion assay and

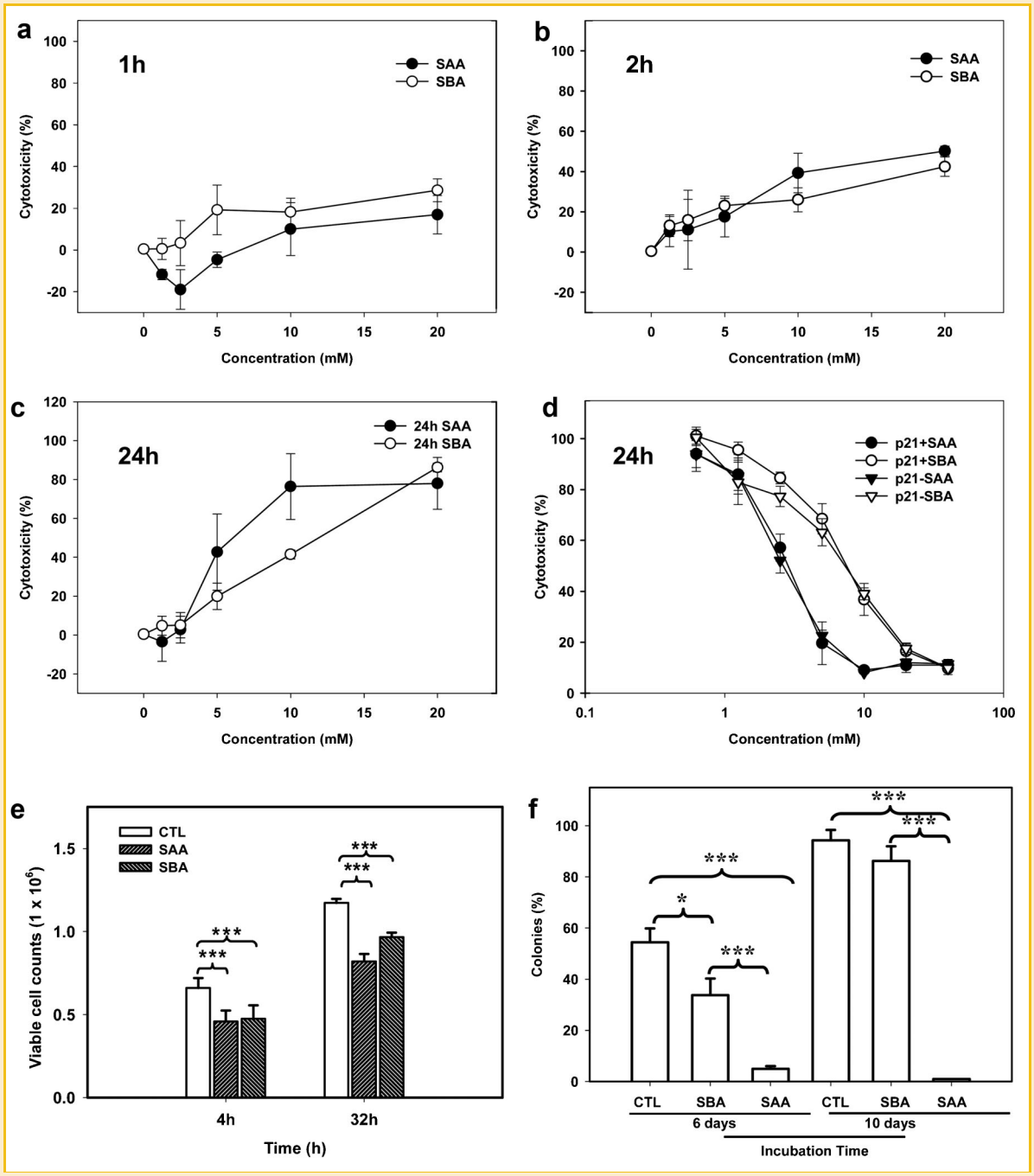


Fig. 2. Dose-cytotoxicity of ascorbates on colon cancer HT-29 cells (a–c, e,f) and HCT 116 cells (d) by MTT assay (a–c), SRB assay (d), trypan blue cell count (e), and clonogenic assay (f). HT-29 cells were exposed to varying concentrations of SAA and SBA for 1 (a), 2 (b), and 24 h (c), washed with warmed buffer, and cultured for a total of 24 h in fresh supplemented culture medium before subjected to MTT cytotoxicity assay. Error bars represent the standard deviation of three replicates at each treatment ($n = 3$). Wild type and isogenic $p21^{-/-}$ HCT116 human colon cancer cell lines were incubated with SBA or SAA for 24 h before the proteins of viable cells were fixed in cold 50% trichloroacetic acid and stained with SRB for cytotoxicity assay. The cell bound SRB was solubilized in 10 mM aqueous Tris base and the optical density was read at 540 nm using a BMG Fluostar microplate spectrophotometer (d). p21-deficient$^{(-/-)}$ HCT116 cells were more sensitive than $p21^{+/+}$ cells to SBA at doses $\leq$10 mM as analyzed by two-way analysis of variance followed by Duncan's test ($P < 0.05$), implicating a role of p21 in SBA-mediated cytotoxicity. Dose-viability studies in ascorbate-treated HT-29 cells by trypan blue exclusion cell count (e). Values represent the mean $\pm$ standard deviation of three separated experiments ($n = 3$). Synchronous HT-29 cells (1×10^4/ml) in six-well cultured plates were treated with 10 mM SAA or SBA for 2 h, washed with warm buffer, trypsinized, resuspended at 5×10^3 cells/ml fresh supplemented culture medium containing Noble agar in each well of a six-well culture plate. The cells were allowed to culture for 6 and 10 days before 400 colonies were scored under an inverted microscope (Nikon). The percentage of positive colonies ($\geq$50 μm in diameter) was calculated for evaluation of clonogenicity inhibition (f). All assays were performed in triplicates, and the mean $\pm$ standard deviations are presented. Significant difference was considered when $^{*}P < 0.05$ and $^{***}P < 0.001$.

flow cytometric cell cycle analysis that the inhibitory activity of SBA on HT-29 cells was transient and growth could be recovered over a period of time.

About one-third of the cells were found dead after treatment with SAA and SBA for 32 h, and SAA aggravated cell mortality to about 50% at 48 h as determined by the trypan blue exclusion assay, while the mortality of SBA-treated cells remained at around one-third (data not shown). The stronger cytotoxicity of SAA rendered only few colonies after 10 days (4.9% at Day 6 and $<1\%$ at Day 10; Fig. 2f), probably due to the death of these cells through a lysosomal destabilization pathway (Fig. S2c–e, Supporting Information).

MICROSCOPY OF ASCORBATE-TREATED CELLS

Normal HT-29 cells have a cell cycle of about 28 h, and thus mitotic figures were common at 26 h, which was in the M phase of the synchronous cultured cells (Fig. S1d, Supporting Information). Although SAA and SBA exhibited strong cytotoxicity on HT-29 cells in a dose-dependent manner (Fig. 2), and many studies have demonstrated the apoptotic activities of ascorbates in cultured cells [Sakagami et al., 2000; Lin et al., 2006], no apoptotic bodies were observed in HT-29 cells with ascorbate treatments at 4 and 26 h (Figs. S1a–f, Supporting Information). However, a significant elevation of acidic fluorescent red signal (1.28 vs. 0.6 for SBA-treated and untreated cells, respectively, $P<0.001$) was analyzed in SBA-treated cells by flow cytometry at 4 h (Fig. S2a, Supporting Information), which was consistent with results from fluorescence microscopy with acridine orange staining that a punctuate pattern of acidic vesicular organelles, a typical feature of autophagic changes, was present in the cytoplasm of SBA-treated cells (Fig. S1i, Supporting Information). The fluorescent signal was very weak to

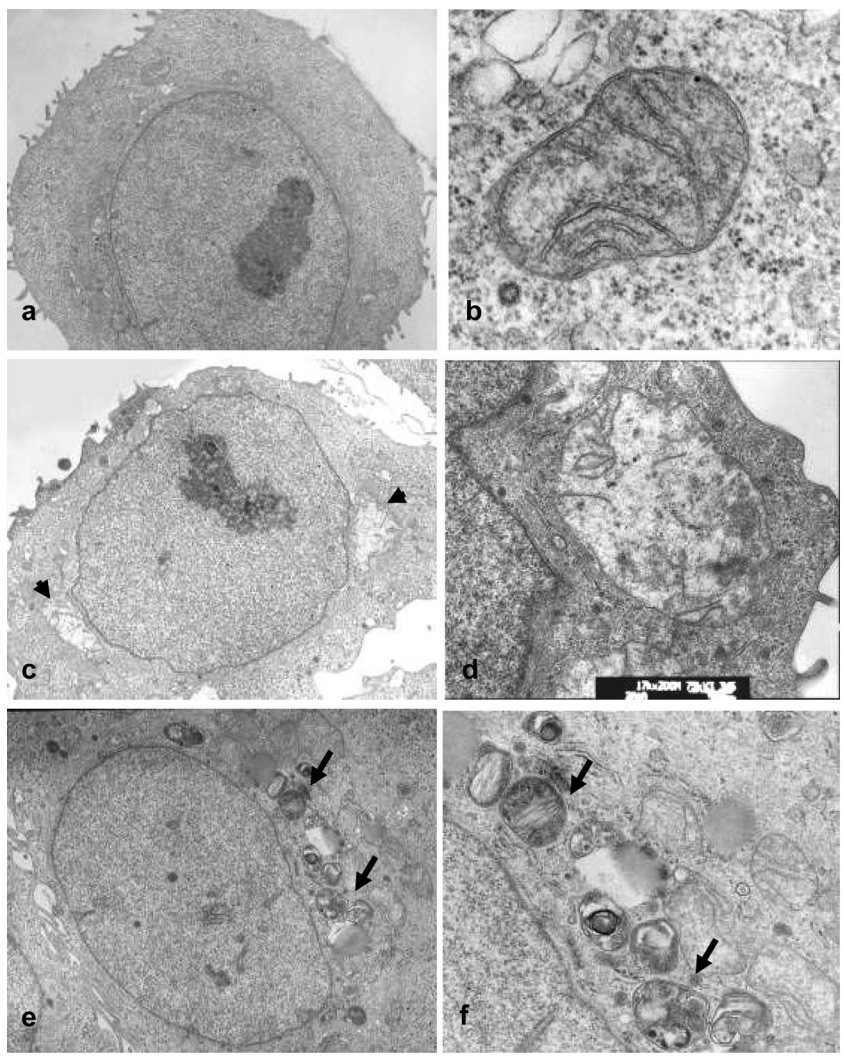


Fig. 3. Electron micrographs of ascorbate-treated HT-29 cells. Both SAA- and SBA-treated HT29 cells were fixed in 2% glutaraldehyde, embedded in Epon, sectioned and stained with lead citrate and uranyl-acetate for transmission electron microscopic analysis. Untreated HT-29 cells (a) have oval nucleus and mitochondria with well-defined inner and outer membrane, matrix, intermembranous space, and most importantly the tightly packed and shelf-like cristae (b). SAA-treated cells (c) looked similar to untreated cells except with distorted mitochondria and irregular cristae (arrow heads) (d). Autophagic vacuoles (arrows) were prominent in cells treated with 10 mM SBA (e,f) (a,c,e: 1,200×; b,d,f: 6,000×).

invisible level in untreated control and SAA-treated cells (Figs. S1g,h, Supporting Information), implicating autophagy instead of apoptosis is likely involved in cell death pathway by SBA. The expression of membrane bound LC3-II in SBA-treated cells further confirmed the autophagic changes (Fig. S2b, Supporting Information). However, the lysosomes destabilized as demonstrated by fluorescence staining with LysoTracker in SAA-treated cells but not in SBA-treated and untreated control cells, implicating SAA-cytotoxicity may be mediated by lysosomal destabilization (Fig. S2c–e, Supporting Information) and the subsequent proteolysis by lysosomal enzymes.

HT-29 cells are adherent cells with an oval nucleus and oval mitochondria as revealed by transmission electron microscopy (Fig. 3a,b). Mitochondria had well-defined inner and outer membrane, matrix, intermembranous space, and most importantly the tightly packed and shelf-like cristae (Fig. 3b). No condensed chromatin or apoptotic features but distorted mitochondria were found in SAA-treated cells (Fig. 3c,d), whereas numerous autophagic vesicles surrounded by double membranes, lysosomes, and distorted mitochondria were prominent in the cytoplasm of SBA-treated cells (Figs. 3e,f).

INDUCTION OF OXIDATIVE STRESS BY ASCORBATES

SAA is well-documented for its ability to activate extracellular production of H_2O_2 which can be eliminated by administration of catalase, an enzyme breaking H_2O_2 into oxygen and water, in the culture medium [Chen et al., 2005]. Our results agreed with these findings and further demonstrated by fluorescent DCF using flow cytometric analysis that (1) about fivefold higher level of oxidants were generated by HT-29 cells between 1 and 4 h exposure of 10 mM SBA than its SAA counterparts (10 mM; Fig. 4a), and (2) these oxidants induced by SBA could not be removed by 1,000 U catalase, but were partially inhibited by pharmacological oxidant inhibitors, including DPI, an inhibitor for flavoenzymes (20%), rotenone, a respiratory inhibitor (50%), and NAC, a general antioxidant and glutathione donor (85%; Fig. 4b), but very mild by oxypurinol, implicating different oxidant generation machineries were elicited by these two compounds.

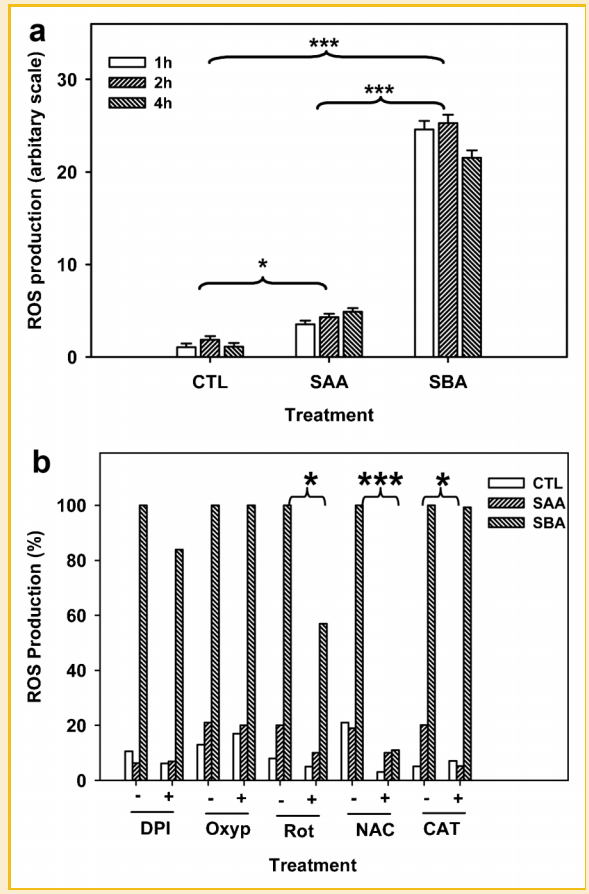


Fig. 4. Flow cytometric analysis of ROS production in ascorbates-treated HT-29 cells. HT-29 cells were pre-loaded with non-fluorescent probe H_2DCF-DA which became fluorescence before they were treated either with 10 mM SBA or 10 mM SAA for 1, 2, and 4 h. It was revealed that both ascorbates significantly [$^{*}P < 0.05$ and $^{***}P < 0.001$ for SAA and SBA, respectively, compared with the untreated controls (CTL)] stimulated ROS production by HT-29 cells; with SBA being fourfold more potent than SAA (a). Both ascorbates induced oxidative stress in HT-29 cells; SAA activity was abolished by catalase (CAT) whereas SBA by NAC ($^{***}P < 0.001$), partially by DPI and rotenone (Rot). Oxypurinol (Oxyp) failed to prevent cells from oxidant formation (b).

ASCORBATES ARREST GROWTH OF SYNCHRONOUS HT-29 CELLS

Flow cytometric analysis of DNA content was done on samples from synchronized HT-29 cells with 2-h-exposure of SAA (5 and 10 mM) or SBA (5 and 10 mM) followed by growth in normal media until for analysis at 12 (G1 phase), 20 (S phase), 28 (G2/M phase), and 36 h (second cell cycle). The results show an evident transient G1 arrest between 12 and 20 h (Fig. 5a) in all treatments in a dose-dependent manner, particularly a 8 h-growth delay in all cycle phases in 10 mM SBA group (Fig. 5a–c; Fig. S3, Supporting Information). A normal HT-29 cell required 28 h for one cell cycle, but about 36 h were required by HT-29 cells with 2-h-SBA exposure to finish one cell cycle, and it took even longer (about 52 h) for cells with 4-h-SBA exposure for one cell cycle (Fig. 5d), indicating the growth arrest activity of SBA increased with exposure time. The SAA-induced arrest (16.4%) was inhibited by 1,000 U/ml catalase (Fig. S4b, Supporting Information), whereas the SBA-induced G1 arrest (73.91% *vs.* 13.01% for SBA treatment and untreated control, respectively) was completely inhibited by co-incubating the cells with 10 mM NAC for 2 h (23.49% *vs.* 17.06% for SBA treatment and control, respectively; Fig. S4f Supporting Information), but not with various doses of DPI (1–10 μM), rotenone (1–10 μM), and oxypurinol (10–50 μM; data not shown). Although some of these inhibitors (rotenone and DPI) attenuated the production of oxidants, only co-incubation with NAC could relieve the oxidative stress and growth arrest by 10 mM SBA on HT-29 cells, further implicating the oxidative stress by SAA and SBA is elicited by two different oxidative pathways. A delay of cell cycle progression was also found in cells with SBA in S- and G2/M phases, indicating a cell cycle-independent growth arrest was induced by SBA (data not shown).

IMMUNOBLOTTING AND IMMUNOPRECIPITATION ANALYSES

Since HCT116 ($p21^{-/-}$) is more sensitive than HCT116 ($p21^{+/+}$) to the cytotoxicity of SBA at $\leq$10 mM (Fig. 2d), prompting us to

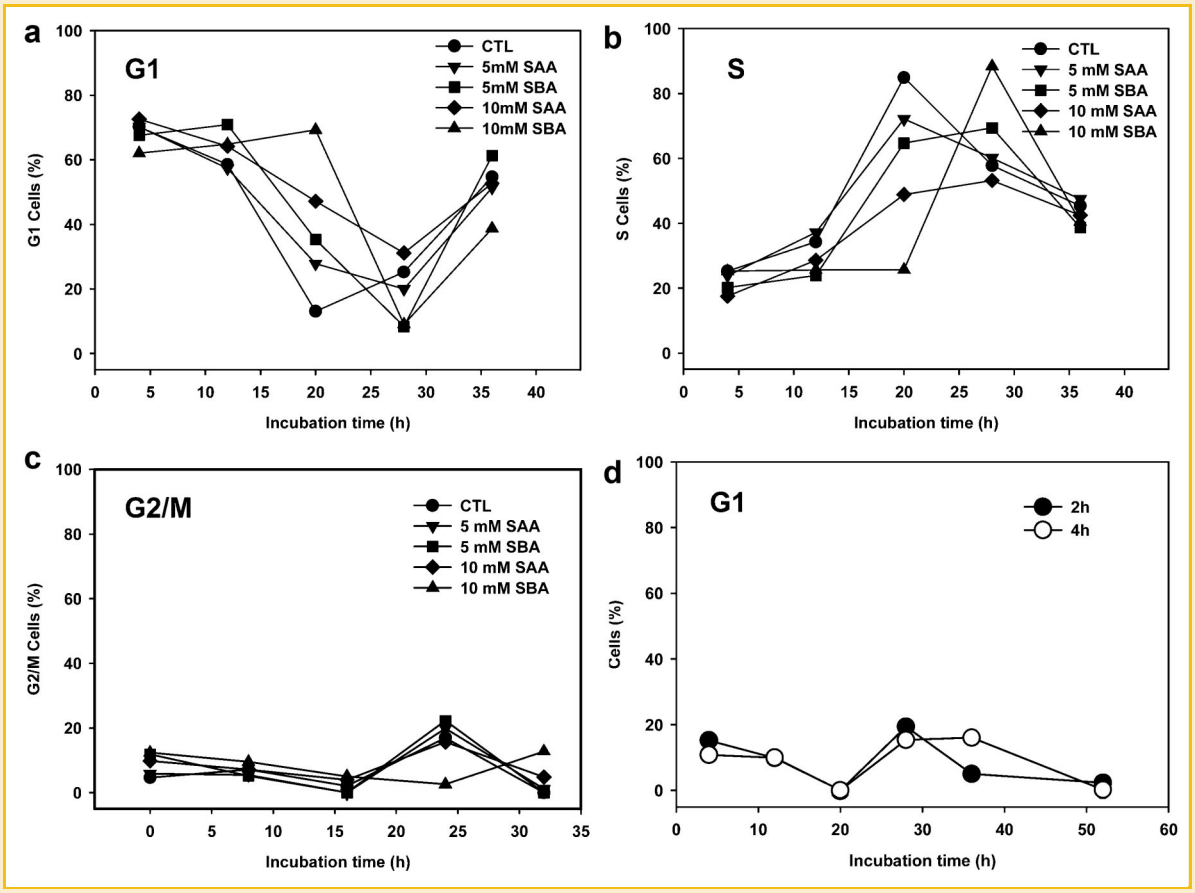


Fig. 5. Cell cycle analysis of synchronous HT-29 cells treated with 5 and 10 mM SAA or SBA for 2 h, harvested at 12, 20, 28, and 36 h of the cell cycle, fixed in cold 70% alcohol, and proceeded for DNA pattern analysis, as revealed by the propidium iodide staining using a flow cytometer. Cells with all treatments exhibited a similar cell cycle profile except 10 mM SBA which induced a prominent G1 arrest (69% vs. 15% for 10 mM SBA and untreated (CTL), respectively) (a), and an 8 h delay in S (b), and G2/M (c) phases. A further delay of the G1 phase to about 24 h was measured in cells treated with 10 mM SBA for 4 h (d). 10,000 cells were analyzed in each experiment and the data shown are representative of three independent experiments with similar results.

hypothesize a direct role of p21, the cyclin-dependent kinase inhibitor, in the regulation of cell cycle progression by SBA. Its induction normally resulted in a cell cycle arrest in either G1 or G2 phase, and apoptosis in other studies [Barnouin et al., 2002; Liu et al., 2005]. The findings of immunoblotting analysis demonstrated a robust induction of p21 protein by both ascorbates, sustaining for 4 and 8 h for SAA and SBA, respectively (Fig. 6a,b and Fig. S5, Supporting Information), with a temporal down-regulation of cyclin D and PCNA (Fig. 6a) that resulted in a delay of cell cycle G1-S progression, 4 and 8 h for respective SAA and SBA treatments (Fig. 6a,b). The findings were further confirmed in cells treated with SAA/SBA for 2 h by immunoprecipitation analysis using antibody against p21 and the immunoblotting analysis with antibodies against p21 and cyclin D. More than fourfold p21 protein was found in the cell lysate with 10 mM SBA treatment than with 10 mM SAA, but equal amount of cyclin D was bound by p21 protein (Fig. 6c). These results strongly indicated low level of cyclin D protein was available in the SBA-treated cells for formation of inhibitory complex (i.e., p21-cyclin D). The expression of p21 protein was inhibited by co-incubation of SAA with catalase, or SBA with NAC (Fig. 6d). In addition, no detectable active form of caspase 3 was found in all treatment groups (Fig. 6a); further confirming the SBA cytotoxicity was not mediated through an apoptosis pathway. These results agree with the findings demonstrated by fluorescence microscopy (Fig. S1, Supporting Information), and transmission electron microscopy (Fig. 3).

Peak levels of immunoreactivity for p21 and p-AKT were found in the nuclei of SBA-treated HT-29 cells at 4 and 6 h, respectively (Fig. 7c,f), indicating their possible intervention in cell cycle progression and growth arrest. The expression of both proteins could be blocked by pre-treatment with NAC, an oxidant scavenger (Figs. 6d and 7i), but only p-AKT expression was inhibited by the phosphorylation inhibitor LY294002 (Fig. 7j). This differential inhibition strongly suggested that p21 expression was a result of oxidative stress, and that p21 and p-AKT expressions were induced by two separated events.

A weak immunoreactivity for p21 was found predominantly in the cytoplasm of SAA-treated cells, which may underlie the weaker growth arrest ability elicited by SAA (Figs. 5 and 7b), and p-AKT was nearly invisible in both SAA and untreated controls (Fig. 7d,e).

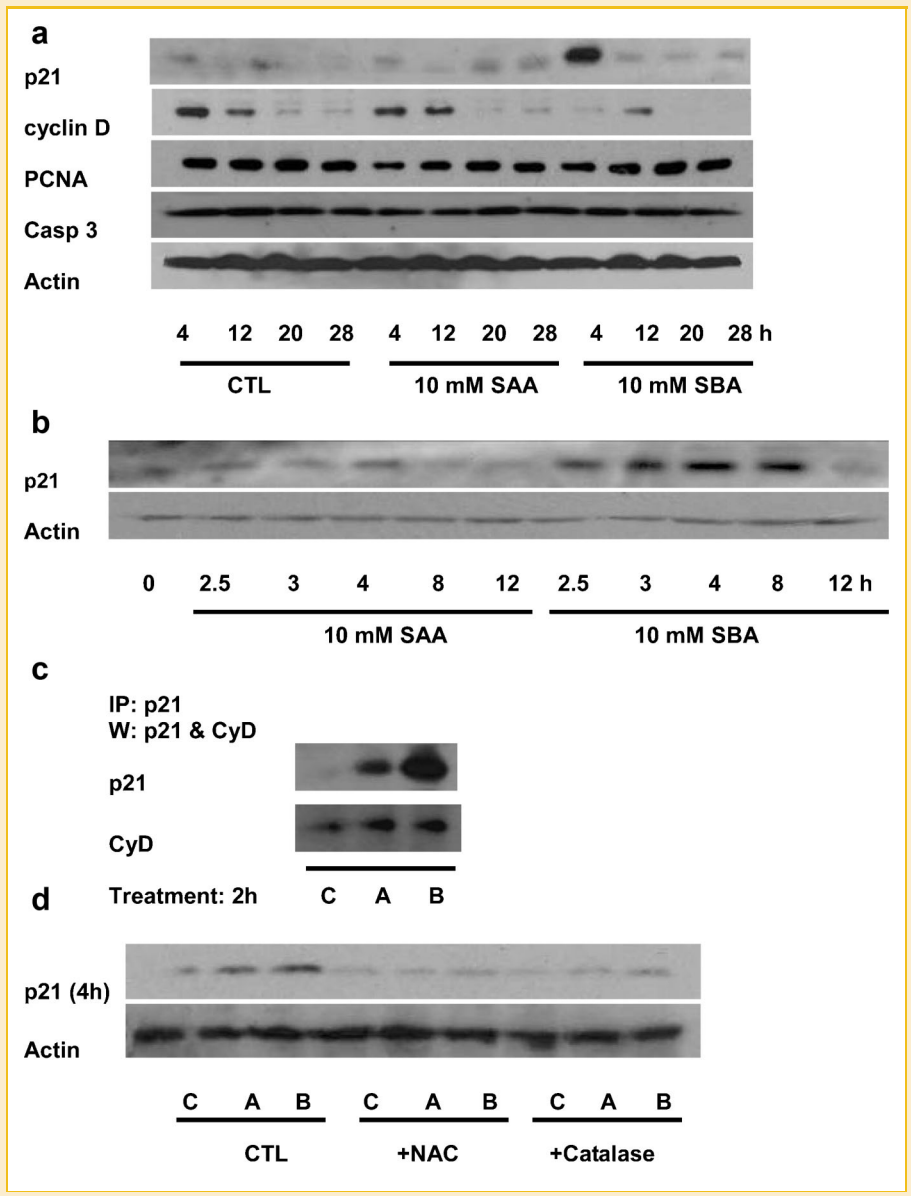


Fig. 6. Immunoblotting analysis of cell cycle proteins in ascorbate-treated HT-29 cells [untreated cells (C) and cells treated with SAA (A) and SBA (B)]. HT-29 cells treated either with SAA or SBA for 2 h and then further cultured in supplemented medium for 0, 8, 16, and 24 h (i.e., 4, 12, 20, and 28 h of the cell cycle) before subjected to immunoblotting analysis of p21, cyclin D, PCNA, and caspase 3 proteins (a). A robust induction of p21 protein sustained for 4 h and 8 h in HT-29 cells treated with SAA and particularly SBA, respectively (b). These findings were confirmed by a combination of immunoprecipitation and immunoblotting analysis that p21 expression is about fourfold stronger in SBA-treated cells than SAA-treated cells (Fig. S4, Supporting Information), but equal amount of cyclin D protein was bound by p21 protein for formation of inhibitor-cyclin D complex (c). The p21 expression could be inhibited by 1,000 U catalase in SAA-treated cells, or 10 mM NAC in SBA-treated cells (d). Cells did not receive ascorbate treatment served as control (CTL). The levels of protein expression were quantified by densitometry and normalized against those of actin. The data shown are representative of three independent experiments with similar results.

DISCUSSION

Sodium ascorbate is a dietary vitamin well-documented for its antioxidant property that prevents oxidative damages to DNA, proteins, and fatty acids in neuronal cells [Li et al., 2003], human endothelial cells [Serbecic and Beutelspacher, 2005], and human colon cancer HT-29 cells [Wenzel et al., 2004] at concentrations of $\leq$1.5 mM for 24–48 h. However, SAA at pharmacological concentrations (0.3–20 mM) inhibits growth of human malignant melanoma cells [Lin et al., 2006], glioblastoma cells [Thomas et al., 2005], and selectively killed some cancer cell types but not human colon HT-29 cells and normal cell types (e.g., breast cells, fibroblasts, lymphocytes, and monocytes) at clinical relevant conditions (i.e., 1-h-exposure of 20 mM SAA to mimic clinical intravenous use) [Chen et al., 2005]. This selective cancer killing property is mediated by SAA-induced production of extracellular H_2O_2 that can be abolished by oxidant scavenger enzyme, catalase, as reported by others [Chen et al., 2005; Cerella et al., 2009] and also

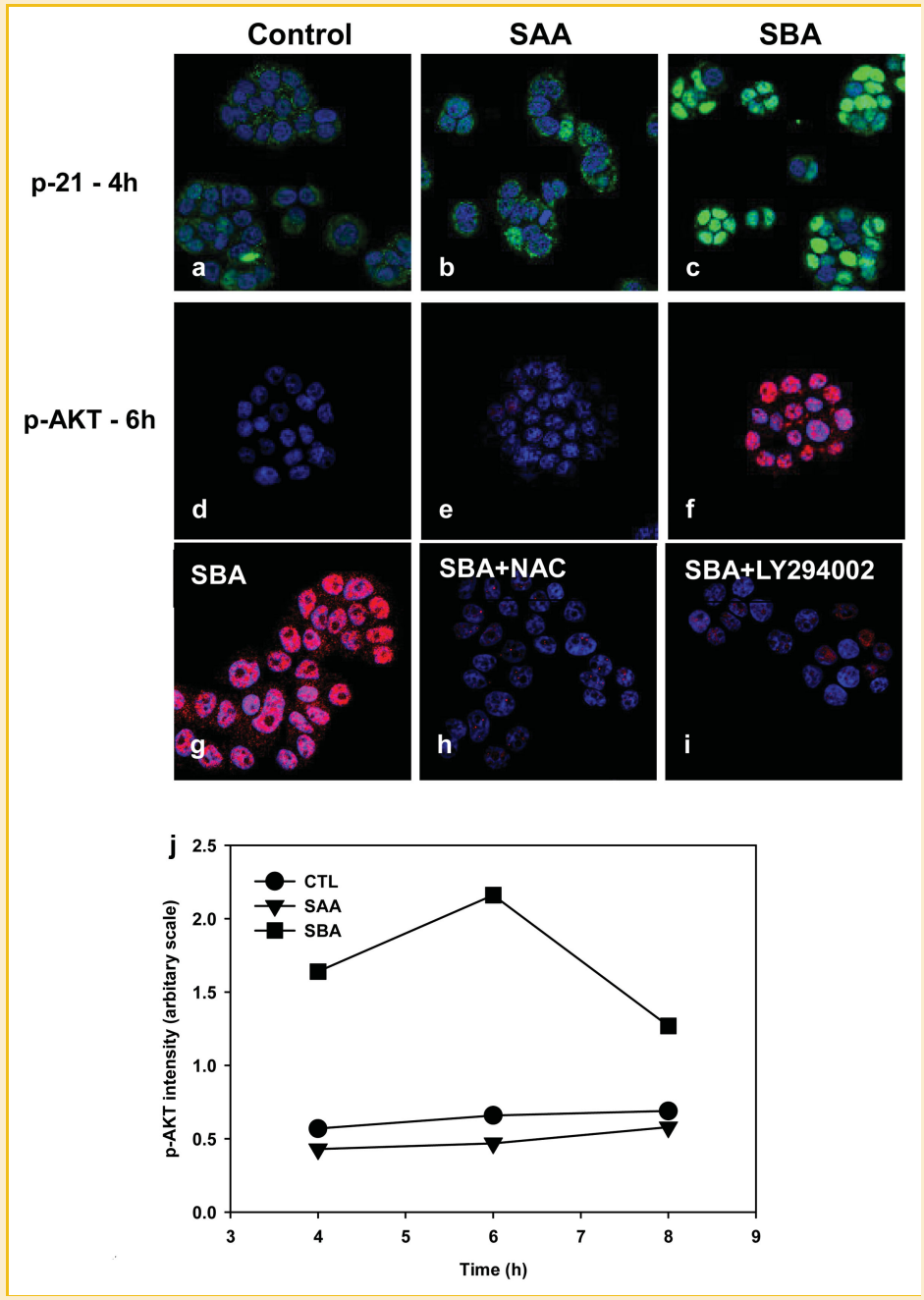


Fig. 7. Immunofluorescence analysis of p21 and p-AKT in ascorbate-treated HT-29 cells. HT-29 cells were treated with either 10 mM SAA or SBA for 2 and 4 h (4 and 6 h of the cell cycle, respectively), fixed with 4% paraformaldehyde and immunostained with antibodies against p21 and p-AKT. Strong immunofluorescent p21 (green fluorescence) and p-AKT (red fluorescence) proteins were localized to the nuclei of SBA-treated HT-29 cells at 4 and 6 h (c,f), but weak p21 signals and very low level of p-AKT in SAA-treated (b,e) and untreated control cells (a,d) which are in consistent with results from ELISA assay in which expression of p-AKT elevated after SBA treatment and reached a peak at 6 h and declined thereafter (g). Nuclei were counterstained with DAPI (blue fluorescence). Strong expression of p-AKT (red fluorescence) in SBA-treated cells (h) was inhibited by 10 mM NAC (i) or 200 μM Ly294002 for 2 h (j). Nuclei were counterstained with DAPI (blue fluorescence). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

in this study. Hydrogen peroxide is a potent oxidant which in the presence of redox-active copper and iron, and other factors can produce reactive oxygen species [Chandra et al., 2000] that rapidly modifies cellular components, and affects protein synthesis, cell growth, and proliferation, resulting in a biphasic pattern depending on the H_2O_2 concentrations; proliferative at low doses (<50 μM for 1 h); sublethal at medium doses (400 μM for 1 h) and apoptotic at high doses (1,000 μM for 1 h) [Cerella et al., 2009; Peterszegi et al., 2003]. To protect cells from oxidative damages, proliferating cells express p21 and enter a transient cell cycle arrest, during which the damaged DNA or oxidized proteins are repaired or removed [Barnouin et al., 2002], and thus p21 has a pro-survival role if it

is kept retaining in the cytosolic location [Keeshan et al., 2003]. Depending on the repairing conditions, cells either resume cell growth or enter a status of lysosomal destabilization [Broker et al., 2005], permanent growth arrest or cell death, by either apoptotic or necrotic pathways.

Different from the induction of extracellular H_2O_2 production by SAA, SBA produced about fourfold higher levels of ROS and p21 protein by HT-29 cells in 1–4 h, which could be eliminated by NAC, but not by catalase, and subsequently arrested synchronous cells at G1 phase. These findings implicate different molecular mechanisms underlying the oxidant-producing ability between SBA and SAA. In addition to its antioxidant and glutathione donor activities, NAC is also involved in a variety of cellular functions including inhibition of transcription factor activities through JNK-p38 MAPK pathway, PI3K/AKT/mTOR pathway, prevention of apoptosis or promotion of cell survival through extracellular signal-regulated kinase pathway [Zafarullaha et al., 2003].

$p21^{Waf1/Cip1}$ is a cyclin kinase inhibitor belonging to the Cip/Kip family (p21Waf1/Cip1, p27Kip1, and p57Kip2), which in a nuclear location exhibits both anti-proliferative and -apoptotic activities [Keeshan et al., 2003]. It controls cell cycle progression by binding to cyclin D, a G1 regulatory protein, and PCNA, an auxiliary protein required for replicative enzyme DNA polymerase δ during DNA synthesis and repair [Kontopidis et al., 2005]. In normal human diploid fibroblasts, p21 has been shown to be part of quaternary complexes that contain cyclin, CDK, and PCNA [Kontopidis et al., 2007]. Substantial evidences suggest that ROS production induces accumulation of p21 and p53 after exposure to various types of stimuli, leading to G1 and/or G2 arrests [Andreassen et al., 2001]. Treatment of HT-29 cells with sulforaphane, a chemopreventive agent [Shen et al., 2006]; tangutorine, an herbal metabolite [Liu et al., 2005]; and SAA [Olsson et al., 2004] also induced p21 expression and down-regulated expression of cyclin D and PCNA, culminating in G1 arrest. In this study, a similar 2-h-exposure cytotoxicity was observed in both SAA and SBA treatments ($IC_{50} \geq 10$ mM), and the experiments for comparison were conducted with 10 mM SAA or 10 mM SBA for 2 h. It was revealed that expression of p21 was restricted during the 2-h-SAA exposure (up to 4 h of the cell cycle), but was significantly activated and sustained for four more hours after SBA treatment (up to 8 h of the cell cycle) before it returned to the basal level. SBA induced about fourfold more p21 protein than SAA in HT-29 cells but only equal amount of cyclin D protein was bound by p21 protein as measured by a combination of immunoprecipitation and immunoblotting analyses. This attenuation of cyclin D synthesis is associated temporally with the up-regulation of p21 between 4 and 8 h, thereby blocking the G1-S progression, and resulting in a G1 arrest of the cells. Interestingly, a delay of 8 h was observed in the subsequent S and G2/M phases of the cell cycle, but the cell counts determined by the trypan blue exclusion assay showed a normal proliferation/duplication of the surviving cells after 32 h, and no significant difference in clonogenic assay after 10 days, indicating that the growth arrest was transient and reversible, and that the cell continued to progress through the cell cycle with a shift of 8 h in each time point of the experiment. Furthermore, similar transient arrests were found independent of cell cycle phases, implicating a crucial role of p21 over-expression after SBA treatment in the cell progression disturbance. P21 is one of the transcriptional targets of the tumor suppressor p53 whose expression is sensitive to oxidation [Xie et al., 2001]. However, the p21 accumulation in response to SBA in this study is independent of p53 because p53 protein is mutated in HT-29 cells.

SAA is a reducing agent that exhibits anti-oxidant activities at submillimolar levels but becomes potent apoptotic inducer at millimolar levels in rat neurons [Lee et al., 2003], human melanoma cells [Lin et al., 2006], and human skin fibroblasts [Peterszegi et al., 2002], which exhibited DNA fragmentation and expression of active form of caspase 3, typical features for apoptotic cells. Many ascorbate derivatives, for example, 6-*O*-palmitoyl-L-ascorbate [Kaap et al., 2004], acetylated ascorbate [Matsui-Yuasa et al., 1989], ascorbic acid 2-phosphate [Savini et al., 1999], and SBA [Sakagami et al., 1997], not only possess more potent cytotoxicity but mediate various cytotoxic pathways differing from their parental SAA. SBA is a benzaldehyde derivative of ascorbate, which is easily dissolved and stable in the culture medium [Sakagami et al., 1995]. It has exhibited anti-tumor activity in both patients with inoperable carcinoma [Kochi et al., 1988] and animals with chemically induced hepatocellular carcinoma without causing prominent side effects (such as loss of weight and hair) [Sakagami et al., 1991], but the molecular mechanisms by which it mediated is unclear. No active form of caspase 3 and apoptosis were found in HT-29 cells after SAA or SBA treatment, but autophagic changes were induced in SBA-treated cells at 4 h, in concomitant with the expression of the intrinsic autophagosomal membrane marker (the lipidated form of LC3-II), and the dramatic activation of AKT. AKT, a serine/threonine kinase, is a key mediator of cell survival, growth or apoptosis which is activated by phosphorylation at two residues, Thr308 and Ser473, in a phosphoinositide-3 kinase (PI3K)-dependent manner, but negatively regulated by PTEN phosphatase [Manning and Cantley, 2007] or PI3K inhibitor LY294002. In this study, the peak expression of both AKT^{thr308} and AKT^{ser473} appeared at 6 h, which lagged behind those of p21 protein and the appearance of autophagic vacuoles (AVO) at 4 h. The functional role of this p-AKT in SBA-treated cells is not clear, but its suppression by PI3K inhibitor LY294002 could not retain the normal cell progression; it is therefore unlikely acting as a growth arrest inducer. Relationship among ROS production, AVO production, AKT phosphorylation, and the level of PTEN are being studied to elucidate if p-AKT expression is a ''compensatory/feedback'' response of the cells to autophagic changes and growth arrest of SBA-treated cells [Manning and Cantley, 2007].

No evidence of similar vacuoles but a prominent lysosomal destabilization [Broker et al., 2005] was found in SAA-treated HT-29 cells in this study, although SAA had been implicated in enhancing protein degradation by lysosomal enzymes in autophagic vacuoles [Feng et al., 2005]. Autophagy is a process of self-digestion of long-lived proteins or defective organelles through a lysosomal degradation pathway. It is considered as the type II cell death and is controlled by PI3K/AKT pathways that impinge on the mammalian target of rapamycin (mTOR) [Feng et al., 2005]. mTOR is a protein kinase that integrates the cellular responses to regulate protein synthesis, promotes cell growth and proliferation, and inhibits autophagy. It is constitutively expressed and controls nuclear

translocalization of cyclin D in proliferating cells [Yamamoto et al., 2006]; its inhibition results in G1 arrest in ovarian cancer SKOV cells [Altomare et al., 2004] and prostate cancer cell lines [Gao et al., 2003]. All the above features, including appearance of autophagic vacuoles, transient G1 arrest between 12 and 20 h, and slightly down-regulation of mTOR expression (data not shown), are observed in the present study. Although SBA has been implicated a more potent oxidant inducer and stronger anti-tumor activity over SAA by induction of G1 arrest in a dose-dependent manner, a comparison between SAA and SBA, however, shows a stronger parabolic cytotoxicity of SAA than the linear SBA cytotoxicity in a longer incubation time, for example, 24 h, by four different cytotoxicity assays (MTT, SRB, trypan blue exclusion, and clonogenic assays). An understanding of the interaction of SBA with cell membrane, cellular uptake, and responses from short-term to long-term treatment will provide a better insight into the mechanisms of its antitumor activities.

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