RESEARCH ARTICLE

Cucurbitacin B induces G_2 arrest and apoptosis *via* a reactive oxygen species-dependent mechanism in human colon adenocarcinoma SW480 cells

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Cucurbitacin B (cucB) is a triterpenoid constituent of Cucurbitaceae vegetables and a promising phytochemical for cancer prevention. However, the mechanism of anti-tumor activity of cucB remains unknown, especially in colon cancers. Here, we demonstrate for the first time that cucB inhibited growth of human colon cancer SW480 cells through a reactive oxygen species (ROS)-dependent mechanism. CucB induced G_2 phase arrest and apoptosis in a dose-dependent manner. At the molecular level, cucB reduced the expression of cyclin B1 and cdc25C proteins and activated caspases in SW480 cells. On the other hand, the state of phosphorylation of signaling transducer and activator of transcription 3 (STAT3) was unchanged. We found that cucB increased intracellular ROS levels, and *N*-acetylcysteine, a well-known antioxidant, reduced the changes in expression of the molecules, and suppressed both G_2 arrest and apoptosis. These results suggested that cucB induced G_2 arrest and apoptosis through a STAT3-independent but ROS-dependent mechanism in SW480 cells.

Keywords:
Apoptosis / Cancer / Cucurbitacin B / G_2 arrest / Reactive oxygen species

1 Introduction

Chemoprevention is promising for colon cancer. Accumulating evidence suggests that foods contain cancer preventive agents. Epidemiological studies have indicated that fruits and vegetables have a protective effect against colon cancer [1, 2]. Phytochemicals that are bioactive plant compounds play a significant role in reducing the incidence of colon cancer [3, 4].

Cucurbitacins, a group of phytochemicals, are noted for their cancer-preventing properties. Cucurbitacins constitute a group of triterpenoid substances, which are well known for their bitterness, and have been found in many Cucurbitaceae species [5]. Cucurbitacins have been reported to inhibit the growth of several types of cancers [6–13]. Cucurbitacin B (cucB) is one of the most expected agents as several reports have shown its anti-tumor effects [9, 11–13]. The anti-tumor activity of cucB has been ascribed mainly to its ability to inhibit signaling transducer and activator of transcription 3 (STAT3) signaling pathways. The constitutive activation of STAT3 is observed in many tumor cells, and inactivation of STAT3 is expected to be effective for cancer prevention [14].

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Abbreviations: **CM-H_2DCFDA,** 5-(and-6)-Chloromethyl-2′ 7′-dichlorodihydrofluorescein diacetate acetyl ester; **cucB,** cucurbitacin B; **DAPI,** 4′ 6-diamidino-2-phenylindole; **NAC,** N-acetylcysteine; **ROS,** reactive oxygen species; **STAT3,** signaling transducer and activator of transcription 3

CucB has been shown to down-regulate phosphorylation of STAT3 and to induce apoptosis in human laryngeal cancer Hep-2 cells and human hepatic cancer HepG2 cells [9, 12], raising the possibility that cucB exerts anti-tumor activity in cancer cells with an activated STAT3. However, cucB can also exert anti-tumor activity in the absence of activated STAT3 [10]. Therefore, the dependency on the STAT3 pathway of the anti-tumor activity of cucB has been controversial.

In this paper, we report that cucB induces G_2 arrest due to the down-regulation of multiple G_2/M regulating proteins and also induces caspase-dependent apoptosis mediated by the accumulation of reactive oxygen species (ROS) through a STAT3-independent mechanism in human colon cancer cells.

2 Materials and methods

2.1 Reagents and antibodies

CucB was purchased from ChromaDex, Santa Ana, CA, USA. Propidium iodide and anti-β-actin antibody were purchased from Sigma, St. Louis, MO. Antibodies against cyclin B1, cdc25C, caspase-3 and cleaved caspase-3 were obtained from Cell Signaling Technology, Beverly, MA, USA. Antibodies against cdk1, Bcl-2 and Bcl-xL were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Antibodies against caspase-7 and survivin were obtained from R&D Systems, Minneapolis, MN, USA. Antibodies against caspase-8 and caspase-9 were acquired from MBL, Nagoya, Japan. Antibodies against p-STAT3 and STAT3 were purchased from Abnova, Taipei, Taiwan. 5-(and-6)-Chloromethyl-2′, 7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) was obtained from Invitrogen, Carlsbad, CA, USA. ECL^{TM} anti-mouse IgG and ECL^{TM} anti-rabbit IgG were purchased from GE Healthcare, Piscataway, NJ, USA.

2.2 Cell culture

Human colon adenocarcinoma SW480 cells were grown in DMEM supplemented with 10% v/v fetal bovine serum, 2 mM glutamine, 50 units/mL of penicillin G and 100 μg/mL of streptomycin at 37°C in a 5% CO_2.

2.3 Cell viability assay

CucB stock solution was prepared at a concentration of 100 μM in DMSO(purchased from Wako, Japan) and mixed with fresh medium to achieve the desired final concentration for treatment of cells. Cells were incubated with or without cucB as indicated, and harvested. The cells were treated with a ViaCount kit (Guava Technologies, Hayward, CA, USA), and viability was measured with a Guava EasyCyte plus flow cytometer (Guava Technologies Inc.) according to the manufacturer's instructions.

2.4 Cell cycle analysis

Cells were incubated with or without cucB as indicated, and harvested. The cells were then fixed in 70% cold ethanol and stained with 100 μg/mL of propidium iodide. Flow cytometry was carried out with a FACScalibur (Becton-Dickinson, Franklin Lakes, NJ, USA) and CellQuest software.

2.5 Western blotting

Cells were incubated with or without cucB as indicated, and harvested. The cells were then re-suspended in lysis buffer (50 mM Tris-HCl, 1% SDS, 2 μg/mL leupeptin, 2 μg/mL aprotinin, 0.1% 2-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride). The lysate was sonicated and centrifuged at 14 000 *g* for 20 min at 4°C, and the supernatant was collected. Equal amounts of lysate were analyzed by SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, MA, USA). The blots were blocked in blocking buffer (5% skim milk/TBST) for 1 h at room temperature, and incubated with the appropriate primary antibody in blocking buffer for 1 h at room temperature. The blots were then washed and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h, and signals were detected with the ECL system (GE Healthcare).

2.6 DAPI staining

Cells were washed twice with PBS and fixed in 4% paraformaldehyde for 30 min. After being washed with PBS, the cells were incubated in 100 μg/mL of 4′, 6-diamidino-2-phenylindole (DAPI) for 30 min in the dark. The nuclei of the cells stained with DAPI were observed using a fluorescence microscope (Zeiss, Jena, Germany).

2.7 Measurement of ROS

Intracellular ROS was detected with FACScalibur (Becton-Dickson) after incubation of the cells with CM-H_2DCFDA for 30 min at 37°C.

3 Results

3.1 CucB inhibits the growth of human colon cancer cells

To test the anti-tumor activity of cucB in human colon adenocarcinoma SW480 cells, we first examined its effect on cell viability using the Guava ViaCount system. As shown in Fig. 1, the mean of cell viability was markedly decreased to $65.3 \pm 5.1\%$ by treatment with 40 nM cucB for 24 h. Maximum inhibition was observed with cucB at 40 nM or more.

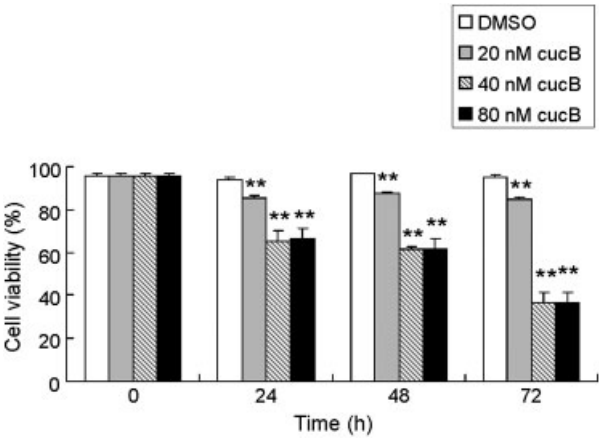

Figure 1. Effect of cucB on growth inhibition of human colon cancer SW480 cells. Cells were cultured with or without 20–80 nM cucB for 0–72 h, and cell viability was determined using Guava EasyCyte plus, as described in Section 2. Data are presented as mean percentages of viable cells ± SD; $n = 3$, $^{**}p < 0.01$.

3.2 CucB causes cell cycle arrest at the G_2 phase and apoptosis

To investigate further the inhibitory effect of cucB on cell growth, we examined the cell cycle distribution in SW480 cells by flow cytometry. As shown in Fig. 2A and B, the percentage of cells in the G_2/M phase was increased about threefold after 24 h of exposure to cucB at 40 nM or more. We then investigated the mechanism of G_2/M arrest. Histone H3 is known to be phosphorylated at the M phase. We then performed Western blotting to determine whether histone H3 was phosphorylated. The amount of phosphorylated histone H3 was decreased by treatment with cucB at 40 nM or more for 24 h (Fig. 2C), and this suggests that cucB causes G_2 arrest in SW480 cells. We then examined the expression of proteins regulating the G_2/M transition by Western blotting. As shown in Fig. 2D, cucB at 40 nM or more reduced the levels of cyclin B1 and cdc25C proteins. On the other hand, the expression of cdk1 was not reduced significantly in SW480 cells.

Furthermore, cucB increased the proportion of cells in the subG_1 phase as shown in Fig. 3A and B. The subG_1 population made up 12.1 ± 2.8, 39.7 ± 1.7 and 32.9 ± 4.8% of cells after 24, 48 and 72 h of treatment with 40 nM cucB, respectively. To determine whether cucB-induced cell death is apoptotic or not, cells were exposed to 40 nM cucB for 48 h and observed using DAPI staining (Fig. 3C). SW480 cells treated with cucB displayed the typical morphological hallmarks of apoptosis, including intense shrinkage, chromatin condensation and nuclear fragmentation. We further found that z-VAD-fmk, a caspase inhibitor, partially inhibited the apoptosis induced by treatment with cucB for 48 h (Fig. 3C and D). These results suggested that cucB induced apoptosis in SW480 cells. At the molecular level, the treatment with 40 and 80 nM cucB induced cleavage of caspase-3, -7, -8, and -9 (Fig. 3E). However, anti-apoptotic proteins such as Bcl-2 and Bcl-xL were not changed significantly (Fig. 3E). It is reported that cucB induces apoptosis by reducing levels of Bcl-2 and Bcl-xL expression

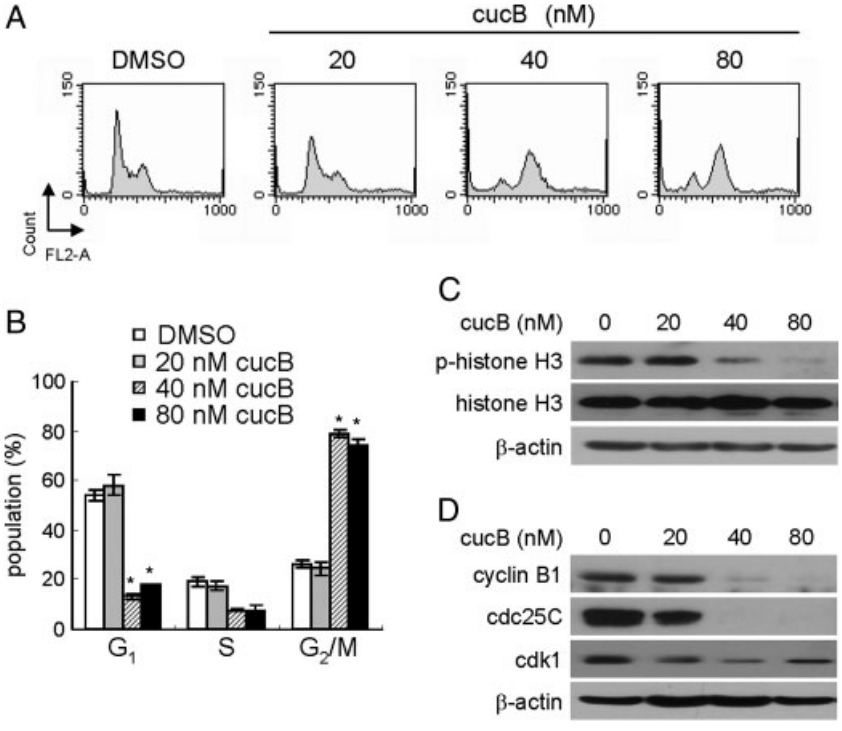

Figure 2. CucB induced cell cycle arrest in SW480 cells. (A, B) Cells were cultured with or without 20–80 nM cucB for 24 h. After fixation and staining with propidium iodide, cells were subjected to a cell cycle analysis using FACSCalibur. Representative histogram patterns are shown in panel A. Data are presented as the mean ± SD; $n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$. (C, D) Proteins were prepared from cells cultured with or without 20–80 nM cucB for 24 h, and then subjected to Western blotting using antibodies as indicated. The blots were re-probed with anti-β-actin antibody as a loading control.

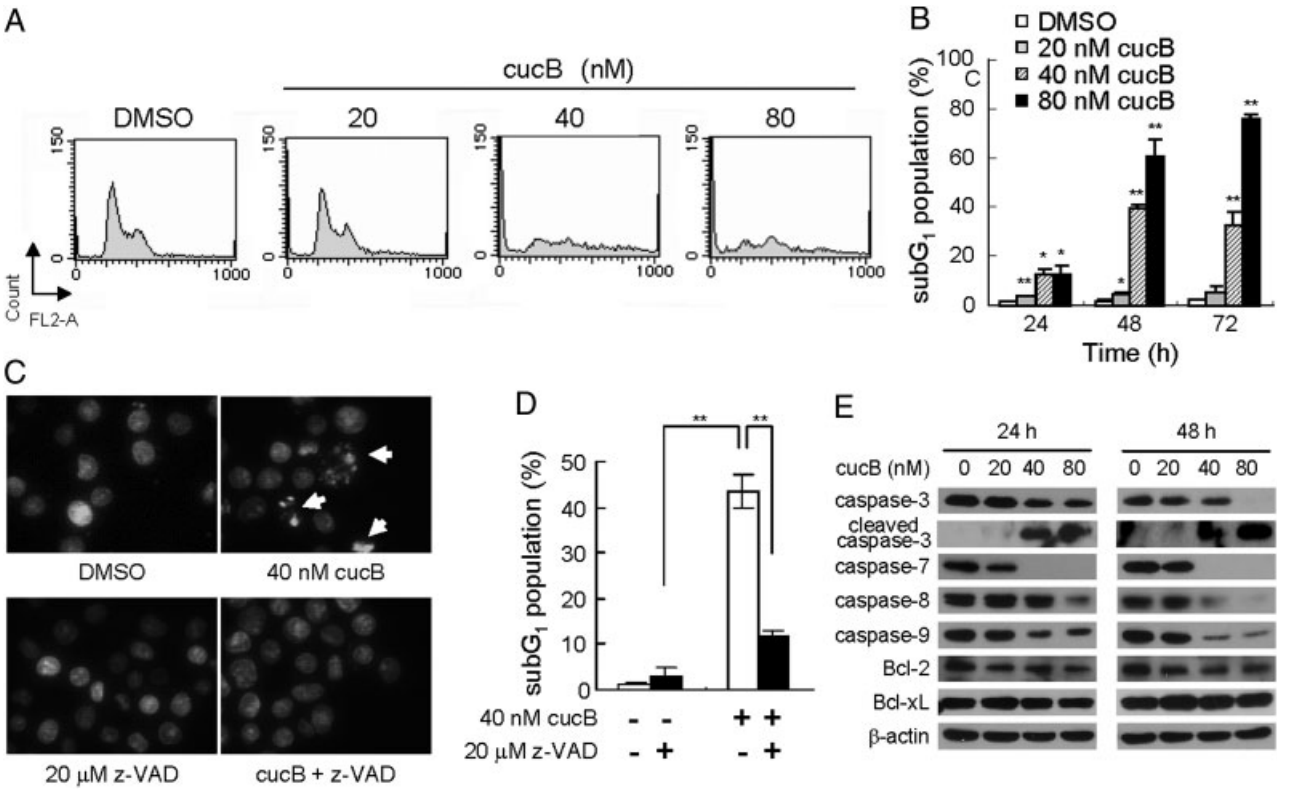

Figure 3. CucB induced apoptosis in SW480 cells. (A, B) Cells were cultured with or without 20–80 nM cucB. After fixation and staining with propidium iodide, cells were subjected to a cell cycle analysis using FACSCalibur. Representative histogram patterns are shown in panel A. Data are presented as the mean ± SD; $n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$. (C) Cells were cultured with or without 40 nM cucB and/or 20 μM z-VAD-fmk for 48 h. The nuclei of the cells stained with DAPI were observed as described in Section 2. (D) Cells were cultured with or without 20–80 nM cucB and/or 20 μM z-VAD-fmk for 48 h. $SubG_1$ populations were analyzed using FACSCalibur. Data are presented as the mean ± SD; $n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$. (E) Proteins were prepared from cells cultured with or without 20–80 nM cucB for 24 and 48 h, and then subjected to Western blotting using antibodies as indicated. The blots were re-probed with anti-β-actin antibody as a loading control.

through the inactivation of STAT3 [9, 12]. In this cell line, dephosphorylation of STAT3 after the treatment with cucB was not observed (Fig. 5E), raising the possibility that the apoptosis induced by cucB was mediated by other mechanisms.

3.3 CucB treatment causes intracellular ROS to accumulate in SW480 cells

The generation of ROS has been reported to be implicated in the induction of cell cycle arrest and apoptosis. Several phytochemicals reportedly induce apoptosis *via* the generation of ROS [15–17]. To examine whether ROS are related to the apoptosis induced by cucB, we measured changes of CM-H_2DCFDA fluorescence in SW480 cells exposed to cucB for 24 h by using flow cytometry. Treatment with cucB increased the proportion of cells with higher fluorescence intensity in a dose-dependent manner, indicating that cucB induced the accumulation of ROS (Fig. 4A). The increase in intracellular ROS levels was completely reduced by co-treatment with cucB and *N*-acetylcysteine (NAC), a widely used ROS scavenger (Fig. 4B).

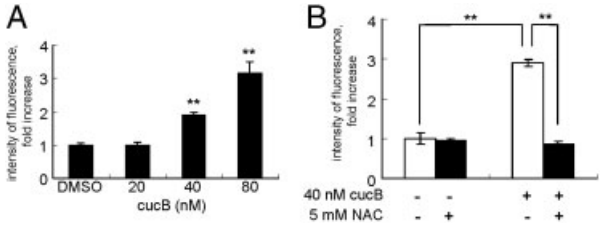

Figure 4. Effect of cucB on ROS accumulation in SW480 cells. (A) Cells were cultured with or without 20–80 nM cucB for 24 h, and the intracellular ROS were measured as described in Section 2. (B) Cells were cultured with or without 40 nM cucB and/or 5 mM NAC for 24 h, and the intracellular ROS were measured as described in Section 2. Data are presented as the geometric mean ± SD; $n = 3$, $^{**}p < 0.01$.

3.4 Inhibition of ROS generation suppresses G_2 arrest and apoptosis

To test whether the increase in ROS played a role in the anti-tumor effects of cucB, we investigated the effect of NAC on G_2 arrest and apoptosis induced by cucB. NAC inhibited both

the G_2 arrest and apoptosis induced by cucB (Fig 5A–D). At the molecular level, the decrease in the expression of cyclin B1 and cdc25C on treatment with cucB was reversed by the NAC treatment (Fig. 5E). In addition, the activation of caspases by cucB was attenuated by the NAC treatment (Fig. 5F). The phosphorylation of STAT3 and expression of targets of STAT3, such as Bcl-xL and survivin, were not affected significantly by treatment with cucB and/or NAC (Fig. 5G). These results suggest that the accumulation of ROS is required for both the G_2 arrest and apoptosis induced by cucB without inactivation of STAT3 in SW480 cells.

4 Discussion

It has been reported that cucB has the antiproliferative activity *in vivo* and *in vitro* [9, 12, 13]. Using xenograft models, 27.5–110 μg/kg/day of cucB (intravenous) and 1 mg/kg/day of cucB (intraperitoneal) inhibited tumor growth *in vivo* [9, 12, 13]. Cell cycle arrest and/or apoptosis are known to be induced in various tumor cells by treatment with cucB *in vitro* [9, 11–13]. We also tested growth inhibitory effect by cucB in several colon cancer cells (SW480, HT-29 and HCT-116) and normal fibroblast (WI-38). As shown

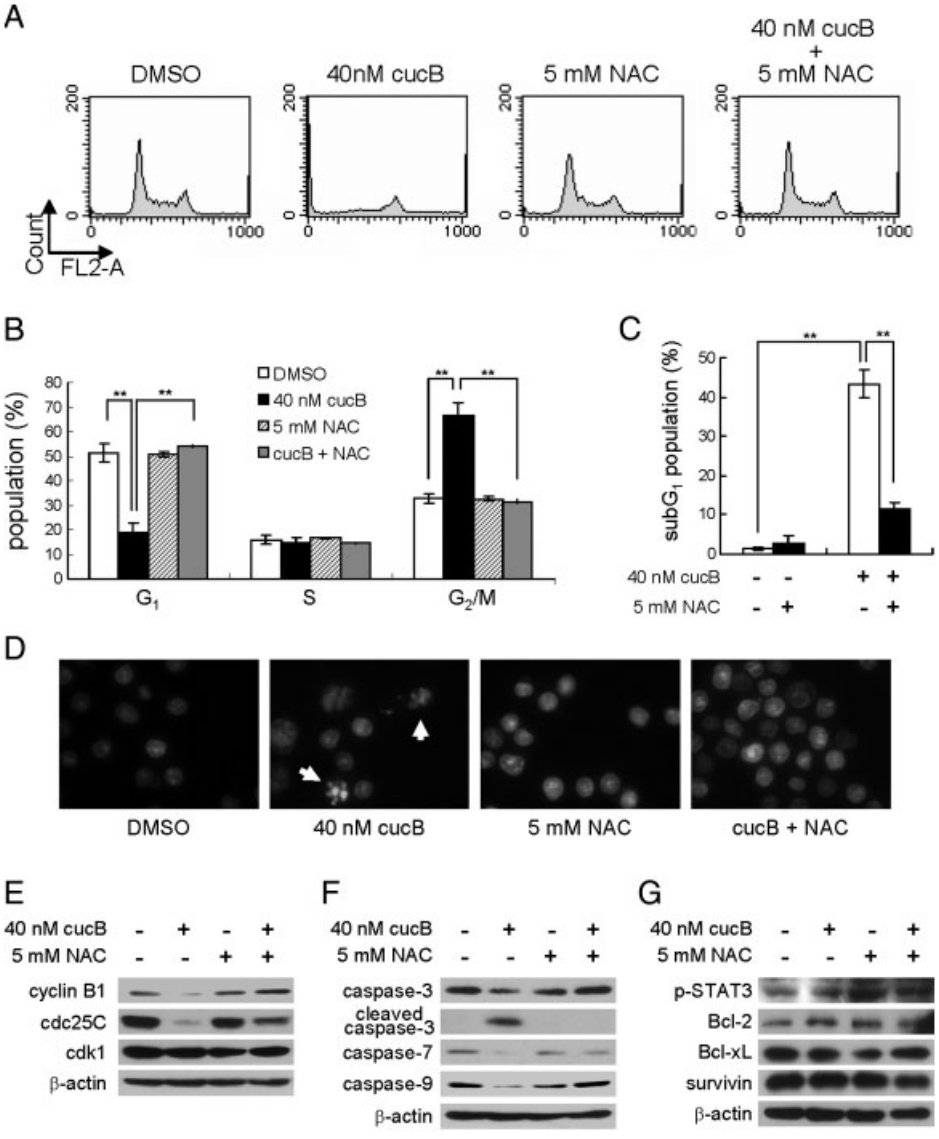

Figure 5. CucB caused G_2 arrest and apoptosis *via* a ROS-dependent pathway, not *via* the STAT3 pathway, in SW480 cells. (A, B) Cells were cultured with or without 40 nM cucB and/or 5 mM NAC for 24 h, and analyzed using FACSCalibur as described in Section 2. Representative histogram patterns are shown in panel A. Data are presented as the mean ± SD; $n = 3$, $^{**}p < 0.01$. (C, D) Cells were cultured with or without 40 nM cucB and/or 5 mM NAC for 48 h. After fixation and staining with propidium iodide, cells were subjected to a cell cycle analysis using FACSCalibur (C). The nuclei of the cells stained with DAPI were observed as described in Section 2 (D). (E–G) Proteins were prepared from cells cultured with or without 40 nM cucB and/or 5 mM NAC for 24 h (E) or 48 h (F, G), and then subjected to Western blotting using antibodies as indicated. The blots were re-probed with anti-β-actin antibody as a loading control.

in Supporting information Fig. S1, cucB inhibited growth of not only all the colon cancer cells but also normal fibroblast WI-38 cells. Therefore, we speculate that cucB might cause side effects if used for chemotherapy. We found that intracellular ROS levels were increased by cucB in SW480 cells (Fig. 4). Jayaprakasam *et al.* showed that micromolar concentrations of cucB had the antioxidant activity [6]. In contrast, our data showed that nanomolar concentrations of cucB induced intracellular ROS accumulation in SW480 cells (Fig. 4). Several phytochemicals having the anti-tumor activity are known to accumulate intracellular ROS. For example, curcumin also has been shown to scavenge ROS in the micro or millimolar range [18], but low micromolar ($<50\,\mu M$) concentrations of curcumin have been shown to promote oxidative stress followed by the altering of cellular redox homeostasis, and/or the disruption of mitochondrial function [17].

We showed that the induction of G_2 arrest and apoptosis by cucB was dependent of the accumulation of ROS (Fig. 5). Oxidative stress plays an important role in cell cycle arrest [19]. For example, a sublethal concentration of *t*-butyl hydroperoxide, a ROS inducer, caused G_2 arrest in human bladder cancer cells [20]. Organosulfur compounds and curcumin are phytochemicals inducing G_2/M arrest through the generation of ROS [15–17]. Diallyl trisulfide, an organosulfur compound, decreased the expression of cdc25C [15]. In addition, curcumin reduced the expression of cdc25C, cdk1 and cyclin B1 [17]. In the present study, we found that the expression of cyclin B1 and cdc25C decreased in SW480 cells treated with cucB in a ROS-dependent manner (Fig. 5). Collectively, these findings [15–17] may suggest the existence of a common pathway to ROS-dependent cell cycle arrest. The precise mechanism of cell cycle arrest *via* the generation of ROS is unclear [19], and further studies are needed.

CucB also induces apoptosis in tumor cells [7–12]. A decrease of Bcl-2 has been reported as a mechanism of induction of apoptosis by treatment with cucB in laryngeal squamous cell carcinoma [9], and hepatocellular carcinoma cells [12]. However, our study did not reveal any significant change in the expression of Bcl-2 protein with the cucB treatment (Fig. 3E). These results raise the possibility that the mechanisms of apoptosis induced by cucB differ among cell lines. In SW480 cells, intracellular ROS markedly accumulated on treatment with cucB, and NAC completely repressed the apoptosis induced by cucB (Fig. 5C). These results suggest that the accumulation of intracellular ROS is necessary for the apoptosis induced by treatment with cucB in SW480 cells, mediated by a Bcl-2-independent mechanism.

The constitutive activation of STAT3 has been reported in a large number of tumors, and STAT3 is closely linked with tumorigenesis [14]. Therefore, inhibitors of STAT3's activation are expected to be useful for cancer chemoprevention. CucB is considered a potential anti-tumor dietary factor because of its ability to suppress the STAT3 signaling pathway, directly inducing Bcl-2 expression [9, 12]. However, our data did not show any significant changes in the phosphorylation of STAT3 or the expression of its targets, such as Bcl-2, Bcl-xL and survivin (Figs. 5G and 3E). Our results are also consistent with the report that cucB exerted anti-tumor activity in several human colon cancer cell lines in a STAT3-independent manner [10]. Taken together, these results suggest that inactivation of STAT3 is not essential for cucB to induce apoptosis at least in a part of colon cancer cell lines.

In summary, we found that cucB causes G_2 arrest and apoptosis *via* a ROS-dependent pathway, and not *via* the STAT3 pathway. The molecular targets of cucB remain to be elucidated in the ROS-dependent mechanism of anti-tumor effect. In addition, studies *in vivo* are required to evaluate the biological efficacy of treatment with cucB, for cancer with or without activated STAT3.

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