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Dehydrozingerone, a Structural Analogue of Curcumin, Induces Cell-Cycle Arrest at the G2/M Phase and Accumulates Intracellular ROS in HT-29 Human Colon Cancer Cells

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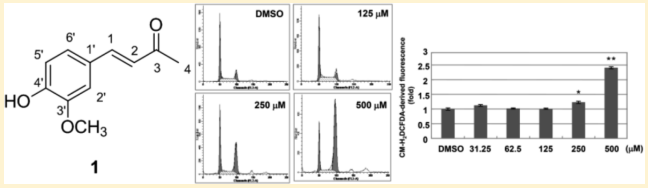
Supporting Information

ABSTRACT: Dehydrozingerone (**1**) is a pungent constituent present in the rhizomes of ginger (*Zingiber officinale*) and belongs structurally to the vanillyl ketone class. It is a representative of half the chemical structure of curcumin (**2**), which is an antioxidative yellow pigment obtained from the rhizomes of turmeric (*Curcuma longa*). Numerous studies have suggested that **2** is a promising phytochemical for the inhibition of malignant tumors, including colon cancer. On the other hand, there have been few studies on the potential antineoplastic properties of **1**, and its mode of action based on a molecular mechanism is little known. Therefore, the antiproliferative effects of **1** were evaluated against HT-29 human colon cancer cells, and it was found that **1** dose-dependently inhibited growth at the G2/M phase with up-regulation of p21. Dehydrozingerone additionally led to the accumulation of intracellular ROS, although most radical scavengers could not clearly repress the cell-cycle arrest at the G2/M phase. Furthermore, two synthetic isomers of **1** (iso-dehydrozingerone, **3**, and *ortho*-dehydrozingerone, **4**) were also examined. On comparing of their activities, accumulation of intracellular ROS was found to be interrelated with growth-inhibitory effects. These results suggest that analogues of **1** may be potential chemotherapeutic agents for colon cancer.



Colon cancer is a leading cause of death in many countries despite some clarification of its carcinogenic mechanisms. Novel approaches are thus required for more effective prevention or treatment. Many epidemiological studies have shown that consumption of fruits and vegetables decreases the risk of malignant tumors.[1] Therefore, several dietary polyphenolic phytochemicals that can prevent carcinogenesis and inhibit the growth of colon cancer cells have been subjected to clinical investigation.[2]

Curcumin (**2**), an active component of turmeric present in the rhizomes of *Curcuma longa* L. (Zingiberaceae), is a well-known dietary phytochemical and is currently being evaluated as a potential agent for chemoprevention and/or chemotherapy.[3−6] Many animal studies have shown that **2** prevents carcinogenesis in various organs including the colon.[7,8] Curcumin also exhibits antiproliferative and/or apoptosis-inducing activities in many human cancer cell lines, including colon cancer cell lines.[9−14] Interestingly, there are many reports suggesting that the growth-inhibitory effect of **2** is accompanied by an increase of reactive oxygen species (ROS).[9−13] Although **2** has been reported to have free-radical-scavenging activity,[15] this compound increases intracellular ROS and induces cell death.[16] These studies strongly suggest that accumulation of intracellular ROS is essential for the growth-inhibitory effect of **2**.

On the other hand, dehydrozingerone (**1**), isolated from *Zingiber officinale* Roscoe (Zingiberaceae), is a biosynthetic intermediate of **2**.[17] Structurally, **1** is a half an analogue of **2** and has also been found to possess antioxidant[17−21] and antimutagenic effects.[22,23] In addition, **1** was found to inhibit tumor promotion in an in vitro short-term assay measuring TPA-induced Epstein−Barr virus early antigen activation.[23−25] Although there are a few preliminary reports about the antiproliferative effect of **1** against cancer cells,[26,27] no molecular mechanisms of the action of **1** on cell growth have been clarified.

Therefore, the growth-inhibitory mechanisms of **1** were investigated using HT-29 human colon cancer cells. While **2** caused cell death as previously reported,[13,14] **1** predominantly induced cell-cycle arrest at the G2/M phase accompanied by accumulation of intracellular ROS and up-regulation of p21. Further analysis using compounds related in structure to **1** suggested that the growth-inhibitory effect was closely related to intracellular ROS accumulation. These results suggest that **1** might be a promising candidate for development of novel anticancer drugs.

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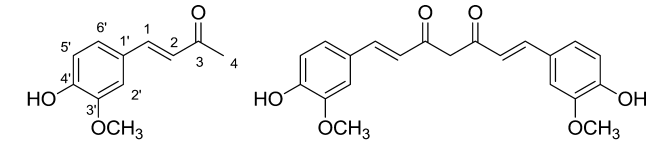

1 2

3 4

RESULTS AND DISCUSSION

Dehydrozingerone (1) Inhibits the Growth of HT-29 Human Colon Cancer Cells. As previously reported,[13,14,28−30] curcumin (**2**) induces apoptosis in HT-29 human colon cancer cells. To confirm the cytotoxicicity of **2**, its effects were examined on the cell viability of HT-29 cells using a Guava VIACount system. As shown in Figure 1A, the percentage of dead cells was

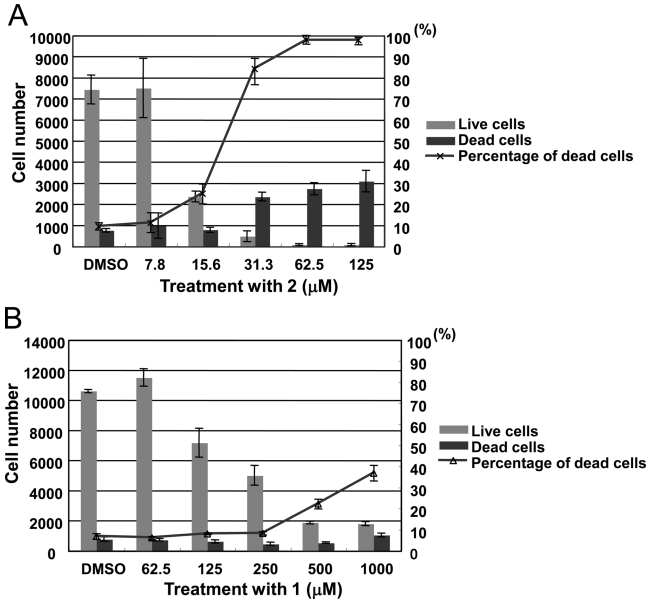

Figure 1. Induction of cell death by dehydrozingerone (**1**) and curcumin (**2**) in HT-29 human colon cancer cells. Cells were treated with **2** (A) or **1** (B) at the indicated concentrations or vehicle (0.1% DMSO) for 72 h, and live and dead cells were counted using a VIAcount kit with Guava EasyCyte Plus, as described in the Experimental Section. Then, the percentage of dead cells was calculated. The data are shown as means ± SD (n = 3).

increased markedly to 84−98% by treatment with **2** at 31.3 μM or more. The cytotoxic effect of **1** was then evaluated on the same cells. As shown in Figure 1B, the percentage of dead cells was increased to only 22% or 37% by treatment with 500 or 1000 μM **1**. Furthermore, at the concentration causing 70% inhibition of cell growth, **2** induced the death of 45% of the cells, whereas **1** induced the death of only 11% of the cells. These results suggest that the growth-inhibitory effect on HT-29 cells by **2** is mainly caused by induction of cell death, whereas that by **1** is due to repression of proliferation.

Dehydrozingerone (1) Causes Cell-Cycle Arrest at the G2/M Phase. To investigate further the inhibitory effect of **1** on cell growth, the cell-cycle distribution of HT-29 cells was examined by flow cytometric analysis. As shown in Figure 2, the

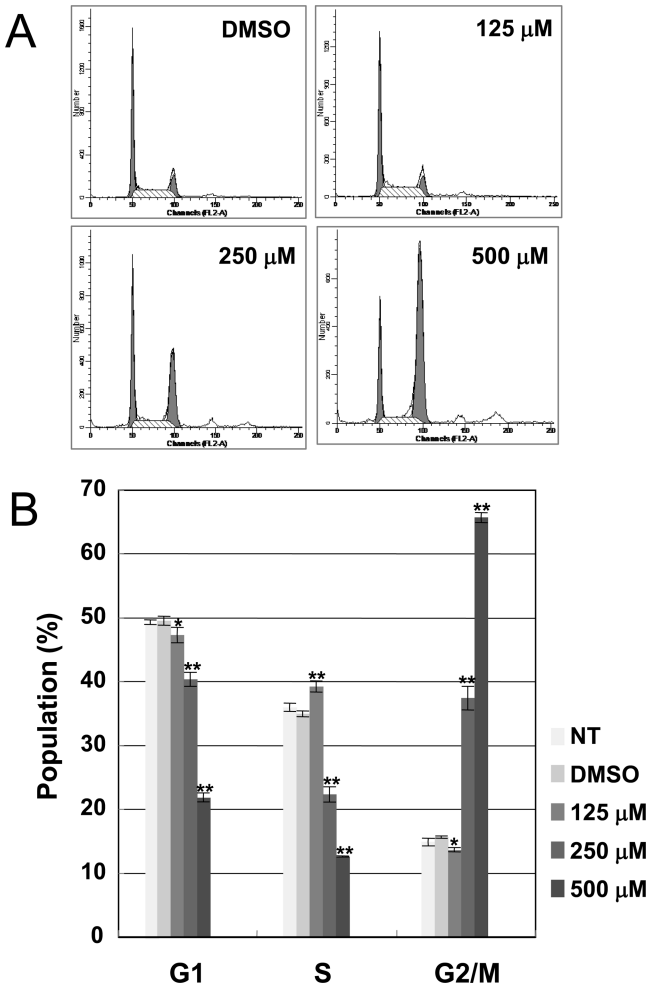

Figure 2. Dehydrozingerone (**1**) induces cell-cycle arrest at the G2/M phase in HT-29 cells. Cells were treated with **1** at 125, 250, and 500 μM for 24 h and stained with PI. Cells were then subjected to cell-cycle analysis using FACSCalibur. (A) Representative histogram patterns of the cell-cycle analysis of the HT-29 cells treated with 0.1% DMSO alone and **1** at 125, 250, and 500 μM for 24 h. (B) Analytical data of the percentages in the G1, S, and G2/M phases. The data are shown as means ± SD (n = 3); *p < 0.05, **p < 0.01 compared with the control (0.1% DMSO).

percentage of cells at the G2/M phase was increased about 2- to 4-fold by treatment with **1** at 250 μM or more. Cyclin-dependent kinase 1 (CDK1) and cyclin B complexes play important roles in the G2/M transition, so the expression of these proteins was examined by Western blotting. As shown in Figure 3A, the expression of CDK1 and cyclin B1 did not appear to have been changed. On the other hand, the expression of p21 protein increased by treatment with **1** at 250 μM or more. p21 is known to induce cell-cycle arrest at the G2/M phase[31] accompanied by dephosphorylation of Thr161 on CDK1.[32] Consistent with this report, phosphorylated CDK1 at Thr161 was decreased by treatment with **1** at 500 μM.

Next, the effects of knockdown of p21 by siRNA were examined on the induction of cell-cycle arrest by **1**. As expected,

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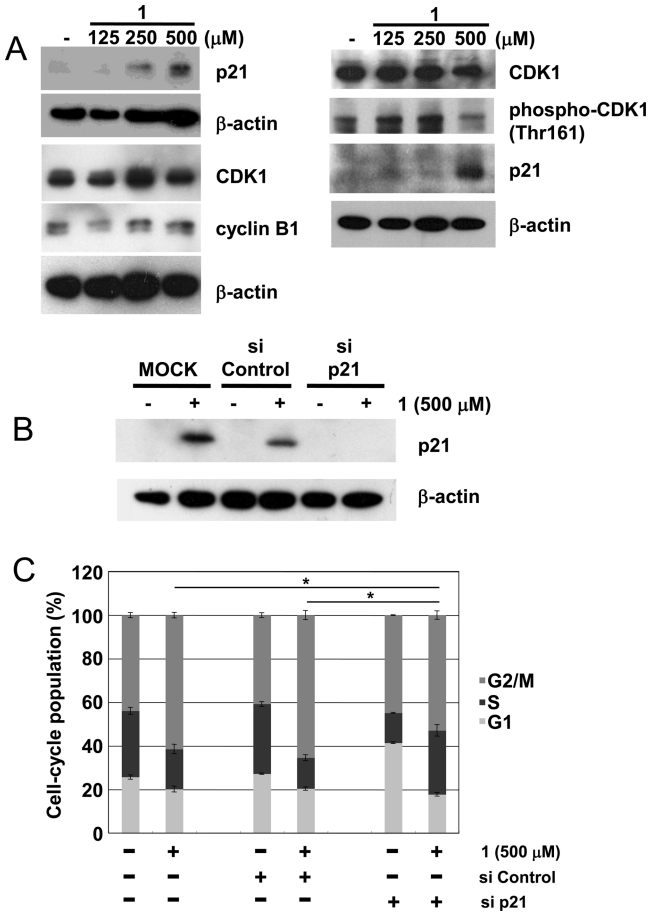

Figure 3. Dehydrozingerone (**1**) increases p21 levels in HT-29 cells. (A) Cells were treated with **1** at the indicated concentrations or 0.1% DMSO alone (−) for 24 h, and its effects on the expressions of each protein were analyzed by Western blotting, with β-actin used as loading control. (B) Effect of knockdown of p21 on protein expression by Western blotting. HT-29 cells were transiently transfected with siRNA as indicated for 24 h after incubation with 0.1% DMSO alone or **1** at 500 μM for 24 h, with β-actin used as loading control. (C) Effect of knockdown of p21 on cell-cycle arrest by treatment with **1**. Cells were transiently transfected with siRNAs as indicated for 24 h after the treatment with 0.1% DMSO alone or **1** at 500 μM for 24 h and then stained with PI. After staining, the populations in different phases of the cell cycle were analyzed by flow cytometry as described in the Experimental Section. The data are shown as means ± SD (n = 3); *p < 0.01, compared with the DMSO-treated control.

treatment with p21 siRNA resulted in a decrease of the protein level of p21 (Figure 3B). As shown in Figure 3C, the induction of cell-cycle arrest at the G2/M phase by **1** was repressed significantly by p21 siRNA compared with that by control siRNA. These results suggest that an increase of p21 at least partially contributes to the G2/M phase arrest induced by **1** in HT-29 cells.

Dehydrozingerone (1) Treatment Leads to Accumulation of Intracellular ROS in HT-29 Cells. It has been reported that **2** induces cell-cycle arrest[9,33,34] or apoptosis[9−11,13,15,16,34−37] via ROS generation. Therefore, it was examined whether **1** similarly induces intracellular ROS. However, it has been pointed out that fluorescence-based approaches are not suitable for investigating ROS production in cancer cells treated with curcumin (**2**), since this compound itself is strongly fluorescent, leading to false positives.[38,39] When compared with **2**, the natural fluorescence of **1** is weak, reducing the possibility of false positives (Figure S1, Supporting Information). Accordingly, CM-H_2DCFDA was used as a ROS indicator, and it was found that **1** at 250 μM or more increased intracellular ROS (Figure 4).

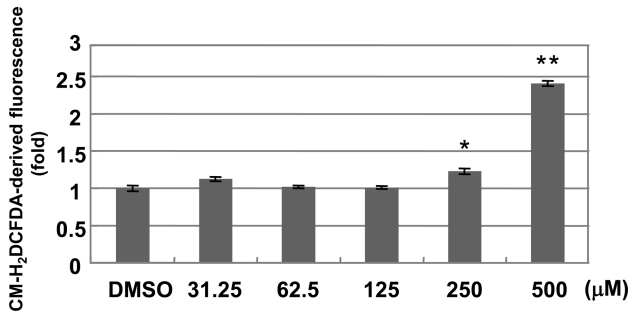

Figure 4. Intracellular ROS accumulation induced by dehydrozingerone (**1**). Cells were treated with **1** at the indicated concentrations for 24 h, and intracellular ROS was measured using a ROS indicator, CMH_2-DCFDA, as described in the Experimental Section. The data are shown as means ± SD (n = 3); *p < 0.05, **p < 0.01 compared with the control (0.1% DMSO).

Effects on Growth Inhibition and Accumulation of Intracellular ROS by Dehydrozingerone (1), Iso-Ddehydrozingerone (3), and *ortho*-Dehydrozingerone (4). Dehydrozingerone (**1**)-related compounds (isomers) were synthesized to examine the structure−activity relationship. Their growth inhibitory effects against HT-29 cells were examined by WST-8 assay as shown in Figure 5A. Among them, **4** showed the most potent growth-inhibitory effect against HT-29 cells when compared with **1** and **3**. The effects of the three isomers were examined on accumulation of intracellular ROS in HT-29 cells. As shown in Figure 5B−D, **4** drastically induced ROS with apoptosis, while **1** and **3** only slightly induced ROS with G2/M arrest.

In this study, it was found that **1**, **3**, and **4** increased intracellular ROS and repressed growth of HT-29 human colon cancer cells. The contribution of ROS to the induction of cell-cycle arrest at the G2/M phase by **1** was confirmed using *N*-acetyl-L-cysteine, known widely as a ROS scavenger. As shown in Figure S2A and B in the Supporting Information, *N*-acetyl-L-cysteine repressed both the G2/M phase arrest and the increase of p21 induced by **1**. On the other hand, however, it has been reported that **2** forms conjugates with GSH, which is a direct metabolite of *N*-acetyl-L-cysteine, in Caco-2 colon cancer cells.[40] Efflux of these conjugates depletes intracellular GSH and eliminates **2** from the cell, thereby decreasing its potency.[38] As **1** is structurally similar to **2**, other antioxidants, such as L-ascorbic acid, α-tocopherol, manganese(III) tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP), PEG-penetrated catalase, or PEG-penetrated superoxide dismutase (SOD), were examined to determine if they would repress the cell-cycle arrest at G2/M phase by **1**. The antioxidants other than *N*-acetyl-L-cysteine did not clearly suppress this effect (Figure S2C, D, Supporting Information), whereas MnTBAP showed only partial repression (Figure S2E, Supporting Information). From the results above, it could not be concluded that ROS is responsible for the G2/M phase arrest induced by **1**. On the other hand, L-ascorbic acid and α-tocopherol have been reported not to attenuate but to enhance the cytotoxic effects of **2**,[41] similar to the present results of **1**. These results might be explained by pro-oxidant property of

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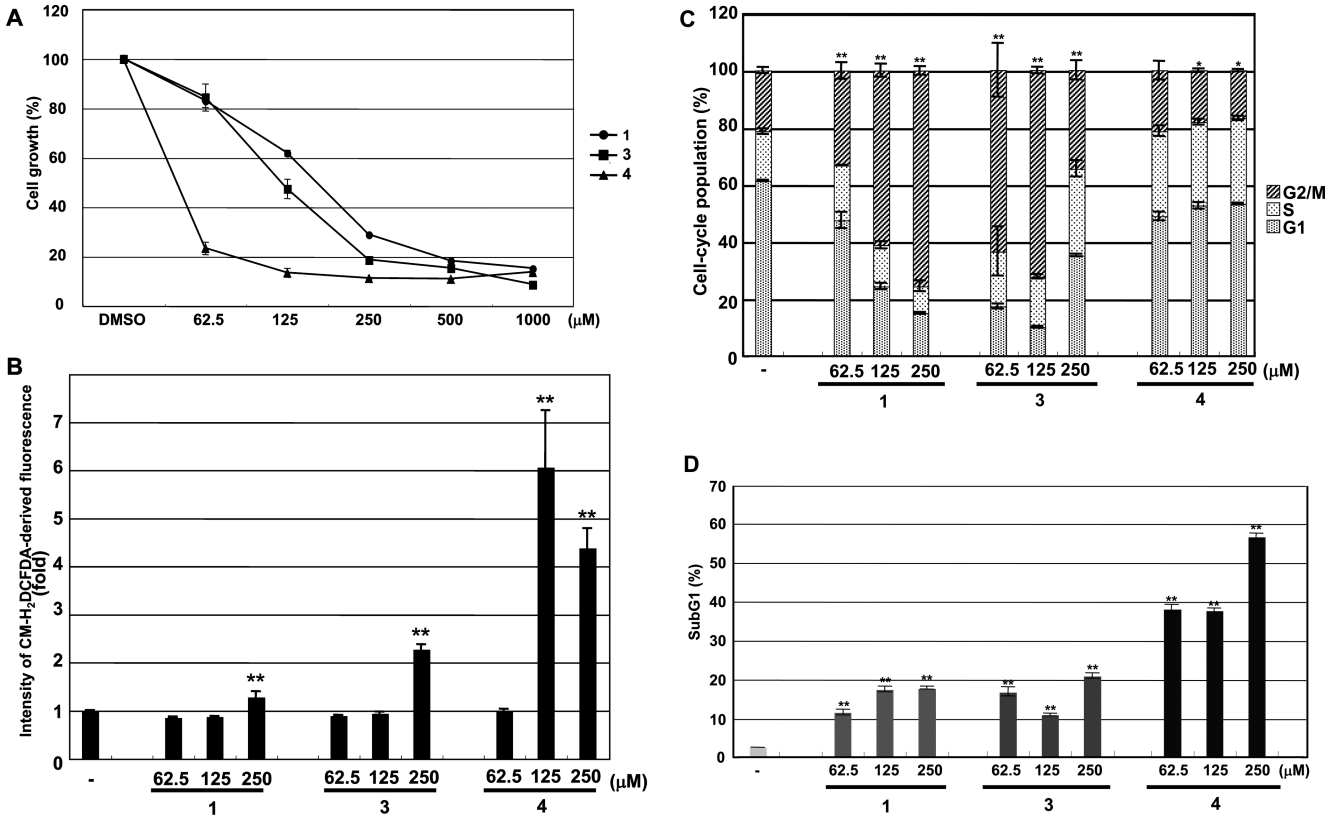

Figure 5. Effects on growth inhibition and accumulation of intracellular ROS by treatment with compounds related in structure to dehydrozingerone (**1**). (A) Growth-inhibitory effects of **1** (circles), **3** (squares), and **4** (triangles) on HT-29 cells. Cells were treated with each compound at the indicated concentrations for 72 h, and the percentage of viable cells was evaluated by a WST-8 assay. The data are shown as means ± SD ($n = 3$). (B) Accumulation of intracellular ROS by **1**-related compounds. Cells were treated with or without each compound for 24 h, and intracellular ROS was measured by flow cytometry using a ROS indicator, CMH_2−DCFDA, as described in the Experimental Section. The data are shown as means ± SD ($n = 3$); $^{**}p < 0.01$ compared with the control (0.1% DMSO). (C) Cells were treated with or without each **1**-related compound for 24 h and then stained with PI. After staining, the populations in different phases of the cell cycle were analyzed by flow cytometry as described in the Experimental Section. The data are shown as means ± SD ($n = 3$); $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the G2/M population of the cells treated with 0.1% DMSO. (D) Cells were treated with or without each compound related in structure to dehydrozingerone (**1**) for 72 h and then stained with PI. After staining, populations of sub-G1 were analyzed by flow cytometry as described in the Experimental Section. The data are shown as means ± SD ($n = 3$); $^{**}p < 0.01$ compared with the control (0.1% DMSO).

antioxidants under certain circumstances. Taken together, the combined effects of redox-active compounds are quite complex, so further studies are required to confirm the contribution of ROS to the cell-cycle arrest by **1**.

The effect of **1** on the growth of other colon cancer cell lines, HCT-116 and HCT-15, was also examined (Figure S3, Supporting Information). Similar to HT-29 cells, **1** inhibited the growth of HCT-116 and HCT-15 cells with G2/M phase arrest, which was inhibited by *N*-acetyl-L-cysteine, but not by L-ascorbic acid or α-tocopherol (Figure S4, Supporting Information). These results suggest that the G2/M phase arrest by **1** is not specific for HT-29 cells. In human fibroblast WI-38 cells, **1** also weakly inhibited cell growth and induced G2/M arrest (Figure S5, Supporting Information).

Although it was not elucidated if the accumulation of intracellular ROS is essential for **1**-induced cell-cycle arrest, oxidative stress is known to play an important role in mediating this phenomenon.[42] In human nasopharyngeal carcinoma cells, **2** has been reported to induce G2/M arrest by ROS generation accompanied by the reduction of CDK1 and cyclin B1.[43] In the present study, however, **1** did not clearly decrease CDK1 or cyclin B1, but increased p21 with accumulation of ROS. There are many reports stating that p21 is closely related to oxidative stress.[44−48] Treatment with compounds inducing oxidative stress has increased p21 through a p53-independent but ERK-dependent pathway.[44,45] The induction of p21 by **1** may be due to the same mechanism because HT-29 is known to have mutated p53 and B-Raf. In addition, it has also been reported that cell-cycle arrest is caused by p21 under moderate levels of oxidative stress, whereas apoptosis can be induced under higher oxidative stress conditions by destruction of the Nrf2-dependent antioxidant system.[46,47] Consistent with these reports, it was found that **1** or **3** caused cell-cycle arrest with a slight increase of intracellular ROS, whereas **4** induced apoptosis with an obvious increase of intracellular ROS. In contrast, **1** at 250 μM did not induce ROS or p21, while it clearly caused G2/M arrest. Therefore, the significance of ROS and p21 has not been shown clearly in the present study.

This is the first report on the interaction of **1** in relation to ROS, cell-cycle arrest, and apoptosis. On one hand, ROS have been shown to induce mutations of genes, possibly causing carcinogenesis. In contrast, the antioxidant system has been reported to promote carcinogenesis.[49] If this is true, the development of compounds related in structure to **1** that more potently induce intracellular ROS may be a promising new chemoprevention approach.

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EXPERIMENTAL SECTION

Reagents and Antibodies. The syntheses of compounds **1** (dehydrozingerone), **3** (iso-dehydrozingerone), and **4** (*ortho*-dehydrozingerone) and the spectroscopic data obtained are described in the Supporting Information. The purities of **1**, **3**, and **4** were estimated to be 100%, 93.1%, and 100%, respectively, by HPLC analyses. Compound **2** was purchased from Nacalai Tesque, Inc. (Kyoto, Japan), and its purity was 78.1%. Propidium iodide (PI) and anti-β-actin antibody were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Antibodies against cyclin B1 and phospho-CDK1 were purchased from Cell Signaling Technology (Beverly, MA, USA). An antibody against CDK1 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). 5-(and-6)-Chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) was purchased from Life Technologies (Carlsbad, CA, USA). ECL anti-mouse IgG and ECL anti-rabbit IgG were purchased from GE Healthcare (Piscataway, NJ, USA).

Cell Culture. HT-29 human colon cancer cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine, 50 U/mL penicillin G, and 100 μg/mL streptomycin at 37 °C in 5% CO_2.

Cell Growth Assay. Cells were incubated with or without reagents as indicated and then harvested. The cells were treated with a ViaCount kit (Merck, Darmstadt, Germany), and viability was measured using a Guava EasyCyte Plus flow cytometer (Merck), according to the manufacturer's instructions. After incubation with or without reagents as indicated for 72 h, the WST-8 assay was performed using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan), according to the manufacturer's instructions.

Cell-Cycle Analysis. Cells were incubated with or without **1** at the indicated concentrations and were harvested. Then, the cells were permeabilized using 0.1% Triton X-100 and stained with 100 μg/mL PI. Flow cytometry was carried out using FACSCalibur (Becton-Dickinson, Franklin Lakes, NJ, USA) and analyzed using CellQuest and ModFit LT software (Becton-Dickinson and Verity software house, Topsham, ME, USA).

Western Blotting. Cells were treated with or without **1** at the indicated concentrations and were harvested. The cells were then resuspended in lysis buffer (50 mM Tris-HCl, 1% SDS, 2 μg/mL leupeptin, 2 μg/mL aprotinin, 0.1% 2-mercaptoethanol, and 1 mM phenylmethyl sulfonyl fluoride). The blots were blocked in blocking buffer (5% skim milk/TBST) for 1 h at room temperature and incubated with an appropriate primary antibody in blocking buffer for 1 h at room temperature. The signal was detected with an ECL Western blot analysis system (GE Healthcare).

siRNA Transfection. p21 siRNA (s415) and negative control siRNA were purchased from Ambion. Cells were transfected with 10 nM siRNA using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA).

Measurement of ROS. The cells were plated and treated at the indicated concentrations. CM-H_2DCFDA (10 μM, Life Technologies) was added to the treated cells for 30 min prior to harvesting. The cells were collected by trypsinization, washed with PBS, and then analyzed by flow cytometry using FACSCalibur and CellQuest software (Becton-Dickinson).

ASSOCIATED CONTENT

Supporting Information

The spectroscopic data of compounds **1**, **3**, and **4** and Figures S1−S5 are available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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