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2-Hydroxycurcuminoid induces apoptosis of human tumor cells through the reactive oxygen species–mitochondria pathway

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ABSTRACT

2-Hydroxycinnamaldehyde (HCA) and curcumin have been reported to have antitumor effects against various human tumor cells in vitro and in vivo by generation of ROS. Aldehyde-free HCA analogs were synthesized based on the structure of curcumin, which we have called 2-hydroxycurcuminoids. The hydroxyl group of curcuminoids enhances the ability to generate ROS. 2-Hydroxycurcuminoid (HCC-7) strongly inhibited the growth of SW620 colon tumor cells with a GI_{50} value of 7 μM, while the parent compounds, HCA and curcumin, displayed GI_{50} values of 12 and 30 μM, respectively. HCC-7 was found to induce apoptosis through the reactive oxygen species–mitochondria pathway and cell cycle arrest at G2/M phase.

Many natural products or synthetic compounds with antitumor activity have been studied in the pursuit of novel therapeutic agent. However, drug resistance and the genomic instability of cancer cells remain significant problems in the advancement of new cancer chemotherapies. Compared with normal cells, tumor cells seem to have higher levels of endogenous oxidative stress in culture and in vivo.[1,2] For example, lymphocytes isolated from the blood of patients with chronic lymphocytic leukemia produced more reactive oxygen species (ROS) than normal lymphocytes.[3] In solid tumors, products of oxidative damage, such as the oxidized DNA base 8-hydroxy-2′-deoxyguanosine (8-OHdG), and lipid peroxidation products, were detected in patient tumor specimens and cancer cells.[4] It has also been reported that levels of antioxidant enzymes, such as SOD and catalase, were decreased in some primary cancer cells.[5] Cancer cells that produce excessive levels of ROS are vulnerable to oxidative stress by treatment with an exogenous agent. Additionally, inhibition of ROS scavenging systems could be a promising strategy to selectively target cancer cells without toxicity to normal cells. Therefore, several redox-modulating agents, ROS-generating agents and ROS elimination inhibitors, are currently in clinical trials as single agents or in combination therapy.[6]

2-Hydroxycinnamaldehyde (HCA), isolated from the stem bark of *Cinnamomum cassia*, has been shown to inhibit on farnesyl protein transferase in vitro, as well as angiogenesis, and tumor cell growth.[7–9] Although HCA has strong anti-proliferative activity,[9] cinnamaldehyde (CA) has relatively weak cytotoxicity against human tumor cells lines grown in vitro and model murine xenografts.[10,11] A previous study showed that HCA and its synthetic analog BCA (2-benzoyloxycinnamaldehyde) induced apoptosis in SW620 human colorectal cancer cells via ROS generation.[12] Therefore, we hypothesized that the 2-hydroxy functional group of HCA critically enhances the antitumor effects of HCA derivatives.

Pharmacokinetic experiment showed that BCA was rapidly hydrolyzed to HCA, followed by rapid oxidation of the aldehyde functional group by aldehyde dehydrogenase to form coumaric acid.[13] We also reported that the oxidized HCA derivative 2-hydroxy cinnamic acid, as well as the reduced HCA derivative 2-hydroxycinnamyl alcohol, did not show any cytotoxicity against various human tumor cell lines.[14] To overcome the metabolic instability of HCA in vivo, we first thought to replace the aldehyde functional group of HCA with an enol or enone.

The rhizome of *Curcuma longa*, commonly known as turmeric, is used worldwide as a spice (e.g., curry), food preservative and coloring agent.[15] Curcumin, the main medicinal component of *Curcuma* species, has a variety of biological activities including suppression of inflammation, angiogenesis, tumorigenesis, diabetes, and neurological systems.[16] Perhaps the most important aspect of curcumin

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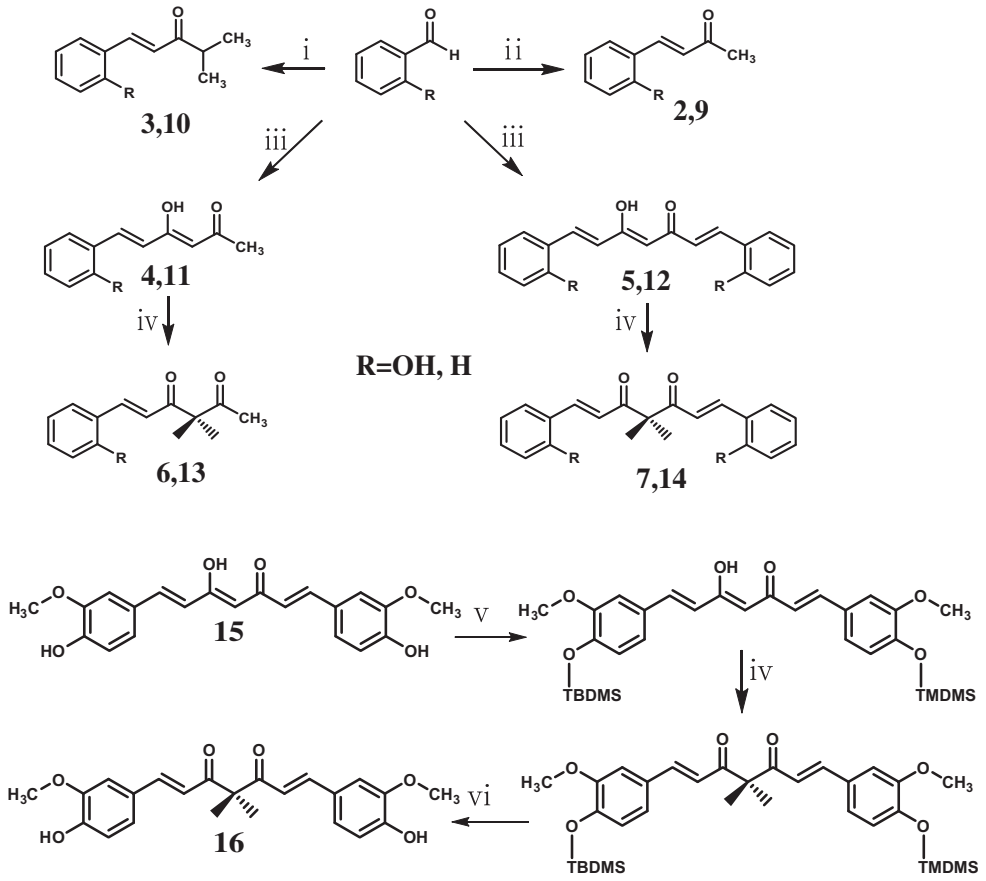

Scheme 1. Synthesis of curcuminoid derivatives. Reagents: (i) $Ba(OH)_2{\cdot}8H_2O$, 3,3-dimethylpentane-2,4-dione, MeOH; (ii) KOH, acetone, EtOH; (iii) B_2O_3, acetylacetone, tributyl borate, HCl, DMF; (iv) iodomethane, K_2CO_3, acetone; (v) *tert*-butyldimethylchlorosilane, imidazole, THF; (vi) tetrabutylammonium fluoride, THF.

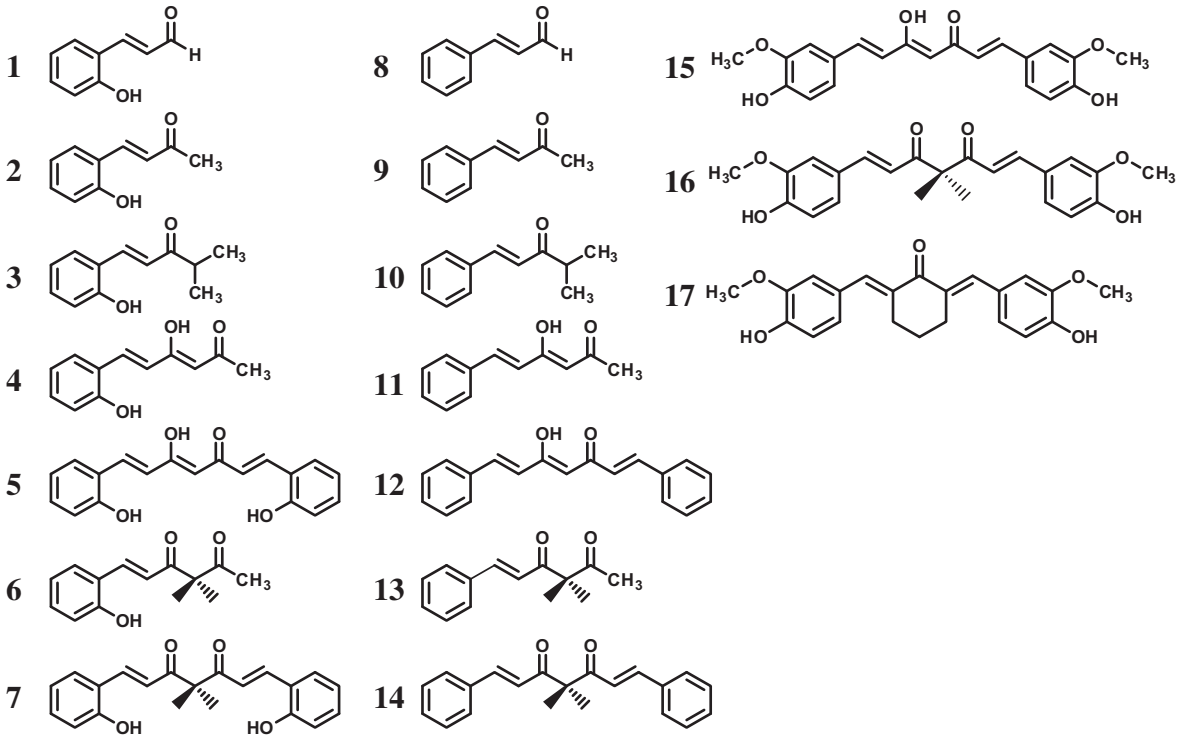

Figure 1. Structures of HCA and curcuminoids.

is its effectiveness against various types of cancer, having both chemopreventive and chemotherapeutic properties. In this context, curcumin is currently in human clinical trials against a number of cancers, including multiple myeloma, pancreatic cancer, myelodysplastic syndromes, and colon cancer.[17] Several in vitro studies suggest that curcuminoid-induced apoptosis is associated with ROS production and/or oxidative stress in transformed cells.[18,19] Curcumin predominantly exists in its enol-tautomer form, and it exhibits poor solubility in water and only modest solubility in MeOH. These properties may be responsible for its low bioavailability.[20] To improve upon its pharmacological properties, numerous efforts have been made to study curcumin's chemistry and biology.[19,21–23] To date, however, direct chemical connections between curcumin's structure and its mode of action remain incompletely understood.

In this present study, we designed a series of curcuminoids based on the structures of HCA and curcumin, and synthesized a panel of analogs with or without a 2-hydroxy group and cucuminoids eliminated the unstable β-diketone moiety.

To improve the metabolic stability and antitumor activity of these compounds, HCA and curcuminoid analogs (compound **1**–**14**, **16**) were synthesized as shown in Scheme 1.

As shown in Scheme 1, compounds **1**–**14** were synthesized by using benzaldehyde or 2-hydroxybenzaldehyde as starting materials and compound **16** was prepared from curcumin **15**. In order to replace the reactive aldehyde group with a more stable enone, HCA derivatives **2**, **3**, **9**, and **10** were prepared by acylation in the presence of potassium or barium hydroxide.[24] The enolic compounds **4**, **5**, **11**, and **12** were prepared through a one-pot reaction sequence.[25] The central β-dicarbonyl moiety of curcumin is subject to keto–enol tautomerization, which is hypothesized to influence the stability and bioavailability of curcumin.[16,17] To overcome this limitation, compounds **16** and **17** were prepared, in which the two hydrogen atoms on the central carbon of curcumin were replaced with geminal dimethyl substituents or a cyclohexyl ring compound, respectively (Fig. 1).[16] We synthesized the **6**, **7**, **13**, and **14** by the reaction of enol-containing derivatives with iodomethane in presence of acetone and potassium carbonate and cucuminoid **16**, eliminated the unstable β-diketone moiety, also prepared. The structures of these curcuminoids were confirmed by the presence of methyl protons and the absence of enolic protons in the ^{1}H NMR spectra.

ROS are required by both malignant and non-malignant cells for proliferation. However, the irregular elevation of ROS can selectively reduce proliferation and induce apoptosis of cancer cells. Therefore, we determined the degree to which ROS production increased following treatment with HCA and curcumin derivatives. ROS was measured by using DCF-DA, a specific oxidation-sensitive fluorescent probe of total intracellular ROS. As shown in Figure 2A, those compounds with a 2-hydroxy group (**1**–**7**) enhanced ROS generation by 1.5- to 2-fold in SW620 cells treated for 2 h. However, compounds lacking the hydroxyl group (**8**–**14**) weakly enhanced ROS generation in comparison with DMSO-treated cells. Especially HCC-7 (compound **7**) induced ROS on time dependent manner (Fig. 2B). To evaluate the effect of the β-diketone moiety of curcuminoids on ROS generation, we measured the ROS level after treatment of curcumin **15** or cucuminoid **16** in SW620 cells. As expected, it was found that cucuminoid **16** strongly increased the ROS level in comparison with that of curcumin **15** (Fig. 2A). These results suggest that hydroxyl group works as an enhancer and β-diketone moiety inhibits ROS generation. Therefore hydroxyl group and β-diketone moiety in these compounds play critical roles in ROS production.

To determine the biological activity of compounds **1**–**16** in human cancer cells, we investigated whether the compounds could inhibit the growth of SW620 cells (a human colorectal tumor cell

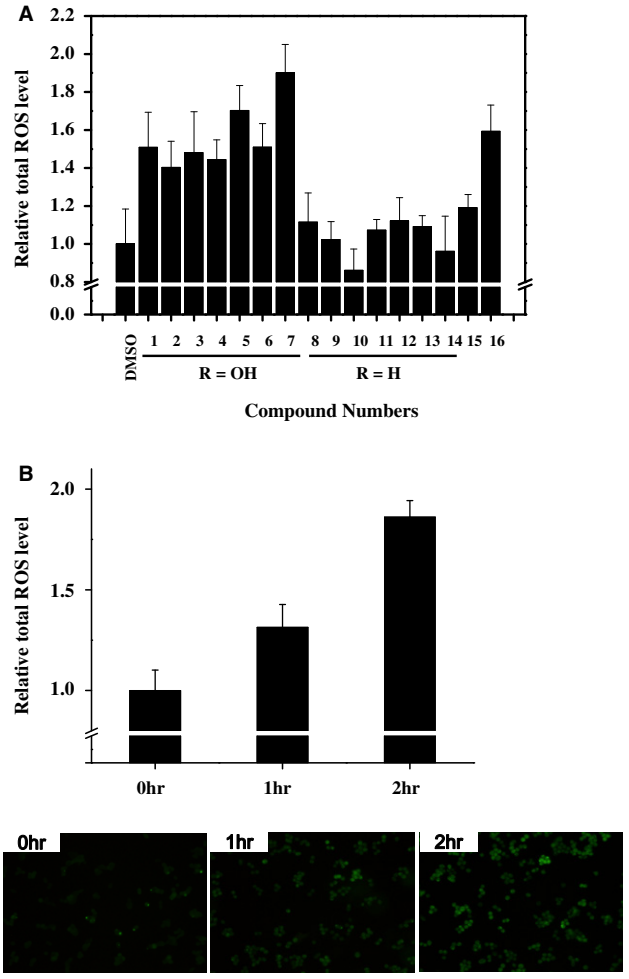

Figure 2. ROS production by treatment with compounds **1**–**16**. (A) SW620 cells were treated with compounds **1**–**16** (20 μM) and ROS were measured fluorimetrically with DCF-DA, using an excitation wavelength of 485 nm and an emission wavelength of 535 nm. (B) Time-dependence of ROS generation by SW620 cells following treatment with HCC-7 (20 μM). Images were captured by a Nikon fluorescence microscope.

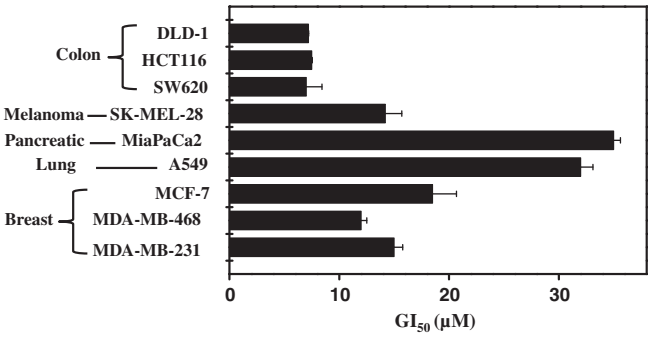

Figure 3. HCC-7 strongly inhibited human tumor cell growth. Proliferation of human tumor cells was measured by WST-1 assay kit at 24 h after the treatment of different concentrations of in HCC-7.

line). Cells were counted after treatment with different concentrations of each compound for 48 h. The GI_{50} value of compound **1**, **5**, **6**, **7**, **15**, and **16** is 15, 35, 40, 7, 32, and 7 μM, respectively. HCC-7 (compound **7**) and cucuminoid **16** showed stronger cytotoxicity against SW620 cells than HCA and curcumin. To further understand its biological activity in cancer cells, we investigated whether the growth of other human tumor cells could be inhibited by

incubation with HCC-7 for 48 h (Fig. 3). In particular, colon (HCT116, DLD-1, and SW620), lung (A549), breast (MDA-MB-231, -468, MCF-7), melanoma (SK-MEL-28), and pancreatic (MiaPaCa2) cell lines were examined. As shown in Figure 3B, colon cell lines including HCT116, SW620, and DLD-1 were more sensitive than other cell lines to HCC-7. Even though the actual mechanism of curcuminoid antitumor activity is still far from understood, a hydroxyl group greatly enhances the anti-proliferative effects of curcuminoid and the ability to generate ROS.

We have reported that the production of ROS was elevated in tumor cells after treatment of HCA, and that this effect was abrogated by pretreatment of cells with *N*-acetyl-cysteine (NAC) or GSH.[13] We observed by morphology recovery experiments with MDA-MB-231 cells that ROS were quenched when HCC-7 was co-administered with GSH or NAC, antioxidant molecules containing a thiol group (Fig. 4A). This is consistent with previous reports that ROS production was elevated in tumor cells following treatment of HCA, but not if the cells were pretreated with glutathione.[13]

PARP participates in DNA repair in response to environmental stress, and its cleavage by caspase-3 is an established marker of apoptosis. We found that HCC-7 induced PARP degradation and caspase-3 activation in SW620 cells. Apoptotic cell death induced by HCC-7 was also confirmed by FACS analysis (Fig. 4B). Cell morphology (Fig. 4A), PARP degradation, and activation of caspase-3 induced by HCC-7 were recovered by co-treatment with NAC or GSH.[13] It means that oxidative stress may contribute to HCC-7-induced apoptosis.

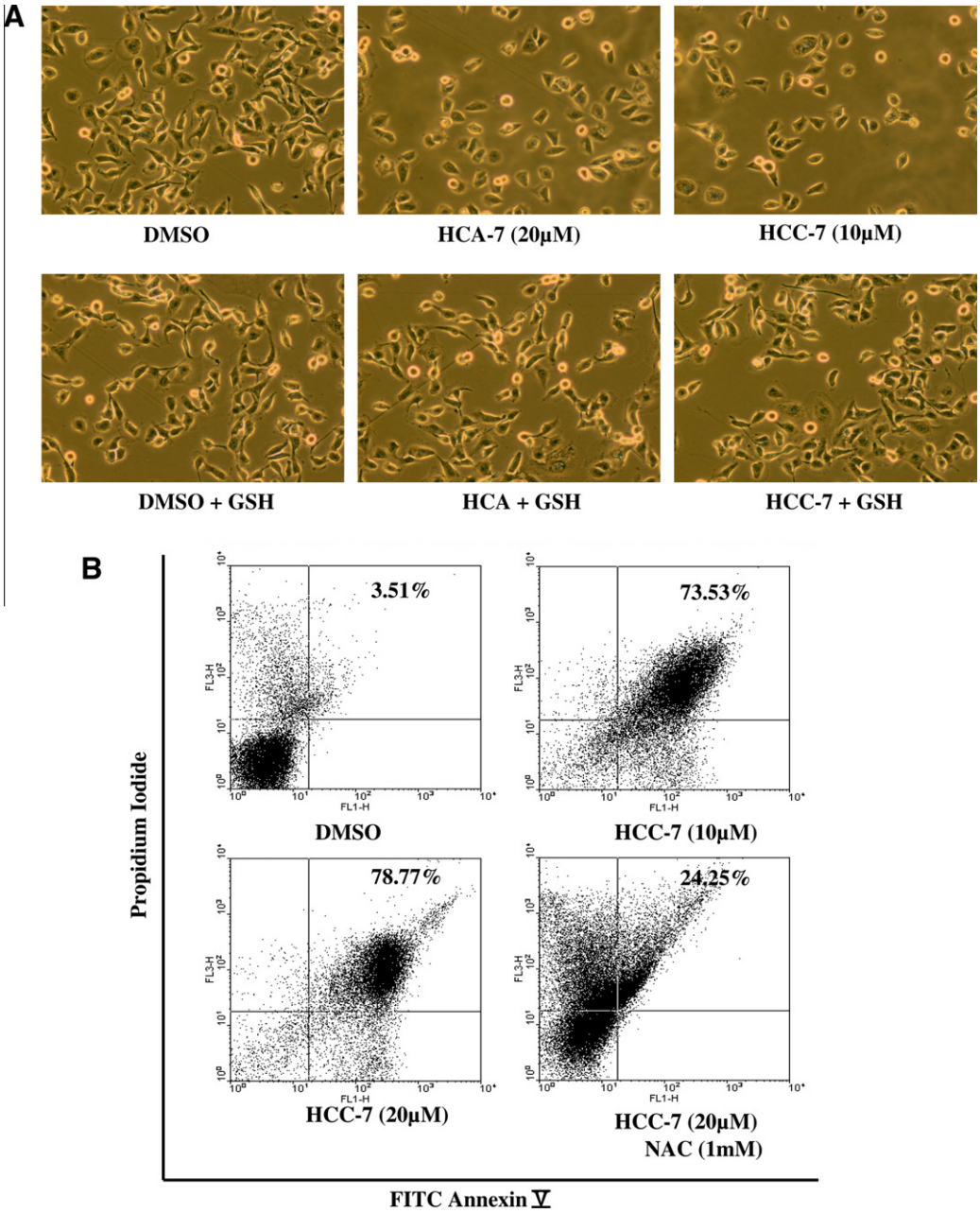

Figure 4. Apoptosis was induced by HCC-7 through morphology changes, cleavage of PARP, and activation of caspase-3. (A) MDA-MB-231 cells were treated with HCA (20 μM) or HCC-7 (10 μM) in the presence or absence of 1 mM GSH. Cell morphology was imaged by Nikon fluorescence microscope. (B) SW620 cells were treated with HCC-7 in the presence or absence of 1 mM NAC for 48 h and stained with annexin V and propidium iodide. Stained cells then were subjected to FACScalibur analysis to determine the distribution of cells.

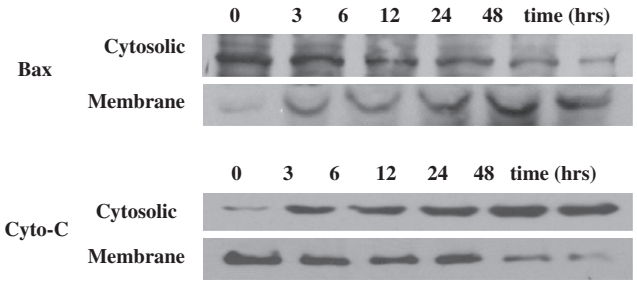

Figure 5. The effect of HCC-7 on the localization of apoptosis regulatory proteins Bax and cytochrome *c* (Cyto-*c*). SW620 cells treated with 10 μM of HCC-7 for the indicated time intervals and then the Bax and Cyto-*c* protein expression level were investigated in the cytosolic and membrane fractions by Subcellular fractionation followed by Western blot analyses.

As shown in Figure 4, the antioxidants NAC and GSH significantly inhibited HCC-7-induced apoptosis. It is well known that ROS accumulation is critical for apoptosis induction through mediation of mitochondrial perturbation. Therefore, we analyzed the decrease of the mitochondrial membrane potential by the reduction in the ratio of A590 (red):A520 (green). We observed that HCC-7 treatment caused a gradual decrease of membrane potential after 6 h, and reached to 60% of the ratio after 12 h, indicating a significant reduction in mitochondrial membrane potential (data not shown). And also the release of mitochondrial cytochrome *c* to cytosol, and in contrast, translocation of Bax from the cytosol to the membrane is representative features during mitochondrial apoptotic pathway.[26] Therefore it was investigated whether cytochrome *c* and/or Bax translocate between the membrane and cytosolic fractions upon treatment with HCC-7 using subcellular fractionation and Western blot analysis. When 10 μM of HCC-7 was treated in SW620 cells, the level of cytochrome *c* was markedly increased in the cytosolic fraction. Bax, on the contrary to cytochrome *c*, is remarkably increased at the membrane fraction with the concurrent decrease at cytosolic fraction (Fig. 5).

Previous studies have shown that HCA and its analogs induced cell cycle arrest at the G2/M phase.[13,27] Therefore, we chose to examine the mechanism of action of HCC-7 by cytofluorimetric analysis, using propidium iodide to label DNA. After treatment of SW620 and MCF-10A cells with HCC-7 (10 μM), the cells were harvested and analyzed with a FACScalibur flow cytometer. Treatment of SW620 cells with HCC-7 for 15 h increases DNA content and chromosome count to a tetraploid state, indicating cell cycle arrest at the G2/M phase. However, when MCF-10A (an immortalized non-tumorigenic cell line) was treated with HCC-7 (10 μM), DNA content at the G0/G1 phase was increased. It means that the compound exhibits a different cell cycle effect in a non-tumorigenic human cell line. It has been reported that immortalized human umbilical vein endothelial (ECV304) cells undergo arrest at the G_0 phase by curcumin (a structural relative of HCC-7), and that curcumin induced G2/M phase arrest in tumor cells.[28] These results suggest that HCC-7 selectively arrests the cell cycle at the G2/M phase in tumor-derived cells and retains mother compound's original mechanism of action in tumor cells.

In summary, HCC-7 and compound **16** is a relatively strong generator of ROS generator in comparison to the other compounds and has the strongest antitumor activity. These results suggest that ROS production is an optional source of curcuminoid bioactivity, and that the presence of a specific hydroxyl group critically enhances the anti-proliferative effects of curcuminoids and the ability to generate ROS. It is also found that the β-diketone moiety of curcuminoids affected the stability and antitumor effects of the compounds. And our results demonstrate that HCC-7-induced apoptosis in human colorectal tumor cell is mediated by the mitochondria perturbation that require ROS accumulation. This study thus provides a rationale for the development of curcuminoid as chemotherapeutic agent against human tumors.

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