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Synthesis and biological evaluation of cinnamyl compounds as potent antitumor agents

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Abstract—A series of cinnamyl compounds related to 2′-hydroxycinnamaldehyde were synthesized and their antitumor effects against human cancer cells evaluated. Hydroxylamine derivative **6** inhibited the growth of human cancer cells and human colon tumor xenograft in nude mice. Its antitumor effects belong to the induction of apoptosis and arresting cell cycle at G_2/M phase, which is confirmed by detection of apoptosis markers and cell cycle analysis.

Natural products are still major sources of new drug development, for example, between 1981 and 2002, 5% of the 1031 new chemical entities approved as drugs by the US Food and Drug Administration (FDA) were natural products, and another 23% were natural-product-derived molecules.[1] Antitumor drugs such as taxol, topotecan, irinotecan, and vinblastine, isolated from natural sources, were clinically used.

Cinnamomum cassia Blume (Lauracea) has been traditionally used to treat dyspepsia, gastritis, blood circulation disturbances, and inflammatory disease in both Eastern and Western countries.[2–4] 2′-Hydroxycinnamaldehyde was isolated from the stem bark of *Cinnamomum cassia* and reported to have an inhibitory effect on farnesyl protein transferase activity and inhibited the proliferation of several human cancer cell lines including breast, leukemia, ovarian, lung, and colon tumor cells.[5,6] The cinnamaldehyde has also been shown to have various activities such as anti-angiogenic activity, immunomodulation, inhibition of CDK4/cyclinD1 kinase activity, and anti-inflammatory activity through the inhibition of NO production.[7–10] Antitumor effects of 2′-hydroxylcinnamaldehyde and its derivatives belong to the induction of apoptosis and arresting cell cycle at G_2/M phase.[11,12]

Antitumor agents inhibited the growth of tumor cell and induced cell death by activating key elements of the apoptosis program. Proteolytic enzymes (caspases) are main effectors of apoptosis. Apoptosis was initially described by its morphological characteristics, including cell shrinking, chromatin condensation, and nuclear fragmentation.[13] Defects in apoptotic pathways are considered to contribute to a number of human diseases, ranging from neurodegenerative disorders to malignancy. Subsequent studies reveal high frequency of apoptosis in spontaneously regressing tumors and in tumor treated with anticancer agents.

Cinnamaldehydes are very unstable in serum, because they have an aldehyde group.[14] However, 2′-hydroxycinnamyl alcohol and acid did not inhibit the growth of human tumor cells,[15] which means that the aldehyde group is a key functional group for the antitumor effects. Therefore, we modified the aldehyde group with hydroxylamines and synthesized the cinnamyl compounds **4**–**7** without aldehyde group. The growth inhibitory activity of the compounds against tumor cells was evaluated.

In this report, we described the antitumoric activity of synthesized cinnamyl compounds against human cancer

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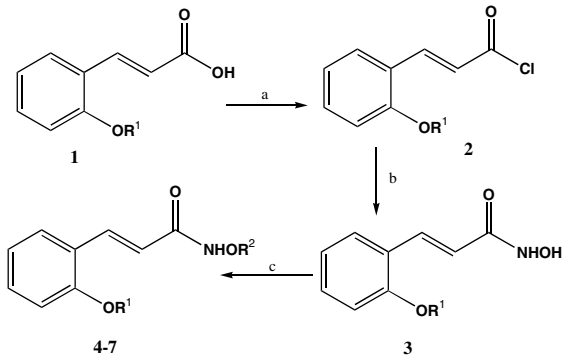


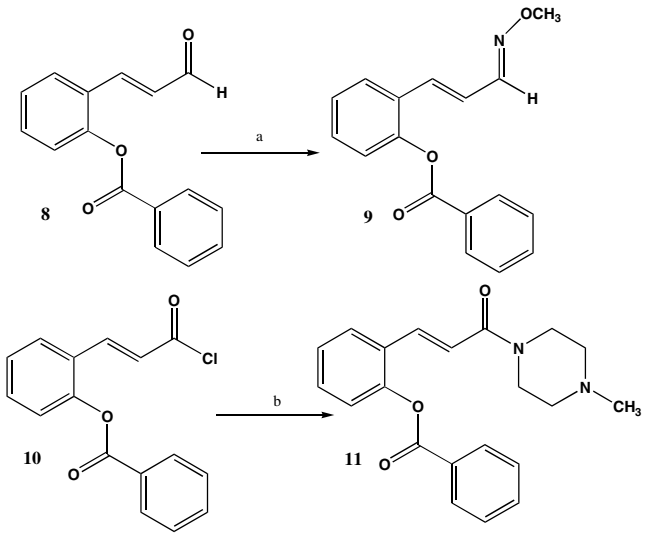
Scheme 1. Synthesis of *N*-hydroxylamine derivatives. Reagents and conditions: (a) thionyl chloride in chloroform, reflux, 5 h; (b) $NH_2OH{\cdot}HCl$ and $NaHCO_3$ in water, 0 °C, 2 h; (c) carbonyl chlorides, CH_3CN, 25 °C, 5 h, K_2CO_3.

cells. We also investigated biologically relevant aspects such as apoptotic markers to identify the mechanism of the antitumor effect and tumor xenografted in nude for in vivo activity of hydroxylamine derivative **6**.

As shown in Scheme 1, the hydroxylamine derivatives were synthesized from the 2′-substituted cinnamic acid **1**. The compound **3** was prepared by the reaction of cinnammic carbonyl chloride **2** with hydoxylamine in presence of base and *N*-hydroxylamine derivatives **4**–**7** were obtained from acylation of compound **3** with commercially available carbonyl chlorides.[16]

We also synthesized imino-type compounds **9** from cinnamaldehyde **8** for examining antitumor effects against human tumor cells. Amide **11** was also prepared from the acylation of *N*-methylpiperidine with compound **10** in presence of base (see Scheme 2).

In order to investigate whether the synthesized compounds inhibit cancer cell proliferation, HCT116



Scheme 2. Reagents and conditions: (a) $NH_2OCH{\cdot}HCl$ and $NaHCO_3$ in CH_3OH, 25 °C, 2 h; (b) *N*-methylpiperazine for **11** and K_2CO_3 in CH_3CN, 25 °C, 3 h.

(human colon tumor cell), NCI-H23 (human lung tumor cell), SK-OV3 (human ovarian tumor cell), A549 (human lung tumor cell), and HEK-239 (human kidney tumor cell) were treated at different concentrations from 0.1 to 100 μM for 24 h (Table 1). Growth of human tumor cells was strongly inhibited by the compounds **3**–**6** with GI_{50} value of 3–50 μM and mildly inhibited the cells by compound **7** in comparison with the other hydoxylamines. However, imino-compound **9** and amide **11** did not inhibit growth of the tumor cells.[17]

Antitumor agents are considered to mediate cell death by activating key elements of the apoptosis program. To characterize the mechanism of synthetic compound-induced apoptosis, activation of casepase-3 and degradation of its substrates were monitored in a dose-dependent manner. To determine the effect of compound **6** on the cell cycle, after treatment of the compound in SW620 and HCT116 (human colon tumor cell), the cells were harvested and analyzed with a FACScalibur. As shown in Table 2 and Figure 1, compound **6** induced a cell cycle arrest at G_2/M phase. When the compound **6** was treated in SW620 and HCT116 cells, we found apoptosis signals on FACS diagram (see Fig. 1).[18] Even though the cells are human colon tumor cells, SW620 is less sensitive than HCT116 against compound **6**.

In order to study of the antitumor effects of compound **6** in detail, we monitored dose-dependent response of colon tumor cells after compound **6** treatment. It was found that compound **6**-treated cells were inhibited in the cell spreading and becoming of round morphology of the cells. To confirm the effects of **6** on apoptosis, we investigated the cleavage of apoptosis marker protein such as PARP (poly ADP-ribose polymerase), which is involved in DNA repair in response to environmental stress and one of the apoptosis markers.[19] As shown in Figure 2, PARP degradation was strongly induced by the compound **6** at 20 μM and, in addition, cleavage of caspase-3 was also observed by Western analysis using specific caspase-3 antibody in HCT116.[20] As already mentioned, when 30 μM of compound **6** was treated, we could observe the apoptosis signals in SW620 cells. These results strongly support that compound **6** exerts its antitumor activity against colon tumor cells through cell cycle arrest at G_2/M and apoptosis (Table 3).

Even though SW620 is less sensitive than HCT116 against compound **6**, SW620 tumor xenograft model of nude mouse was well established in our laboratory, we tested the antitumor effects of the compound **6** in vivo using SW620 human tumor cells. SW620 cells were implanted subcutaneously into the right flank of nude mice on day 0 and compound was intraperitoneously administered at a concentration of 50 mg/kg per day for 14 days. The mice were sacrificed and the tumors were weighed on day 14. Tumor volume was decreased by 33.6% compared to control mice and the loss of body weight was not observed in the nude mice at the doses of 50 mg/kg.[21]

Table 1. Inhibitory activity of cinnamyl compounds against human tumor cells

Compound	R_1	R_2	HCT-116[a]	NCI-H23	SK-OV3	A549	HEK-239
3a	H	H	16.8	27.8	50.4	55.2	49.8
3b	Benzoyl	H	11.2	17.5	35.3	44.9	20.2
3c	CH_3	H	15.3	26.9	48.8	53.5	16.8
4	Benzoyl	Acetyl	21.3	26.3	16.8	30.2	18.6
5	Benzoyl	Benzoyl	13.6	18.2	12.9	8.8	3.2
6	Benzoyl	4-Morpholine carbonyl	12.5	12.9	12.7	25.2	5.8
7	Benzoyl	4-Methyl-1-piperazinecarbonyl	40.4	45.2	>100	>100	89.3
9	Benzoyl	CH_3	>100	>100	>100	>100	>100
10	Benzoyl	CH_3	>100	>100	>100	>100	>100
11	Benzoyl	4-Methyl-1-piperazine	>100	>100	>100	>100	>100

[a] Selective cytotoxicity against human tumor cell line; GI_{50} (μM).

Table 2. Effects of compound **6** on cell cycle progression[a]

Cell line	Concn (μM)	Cell distribution (%)			
		Sub G_1	G_0/G_1	S	G_2/M
HCT116	0	6.35 ± 0.12	42.23 ± 1.54	27.35 ± 1.09	30.42 ± 2.77
	20	32.36 ± 1.36	42.72 ± 2.23	22.05 ± 0.77	35.23 ± 1.98
	30	39.14 ± 1.72	41.40 ± 1.58	20.39 ± 1.05	38.22 ± 2.32
	40	36.83 ± 1.34	31.06 ± 1.76	26.88 ± 1.56	42.06 ± 2.56
SW620	0	0.92 ± 0.03	56.01 ± 2.01	23.13 ± 0.77	20.66 ± 1.64
	20	3.03 ± 0.04	48.88 ± 1.32	27.79 ± 1.43	22.33 ± 1.89
	30	3.50 ± 0.09	46.46 ± 2.31	26.12 ± 1.78	26.41 ± 1.33
	40	12.7 ± 0.07	33.34 ± 1.22	26.52 ± 1.33	40.14 ± 1.81

[a] All experiments were independently performed three times.

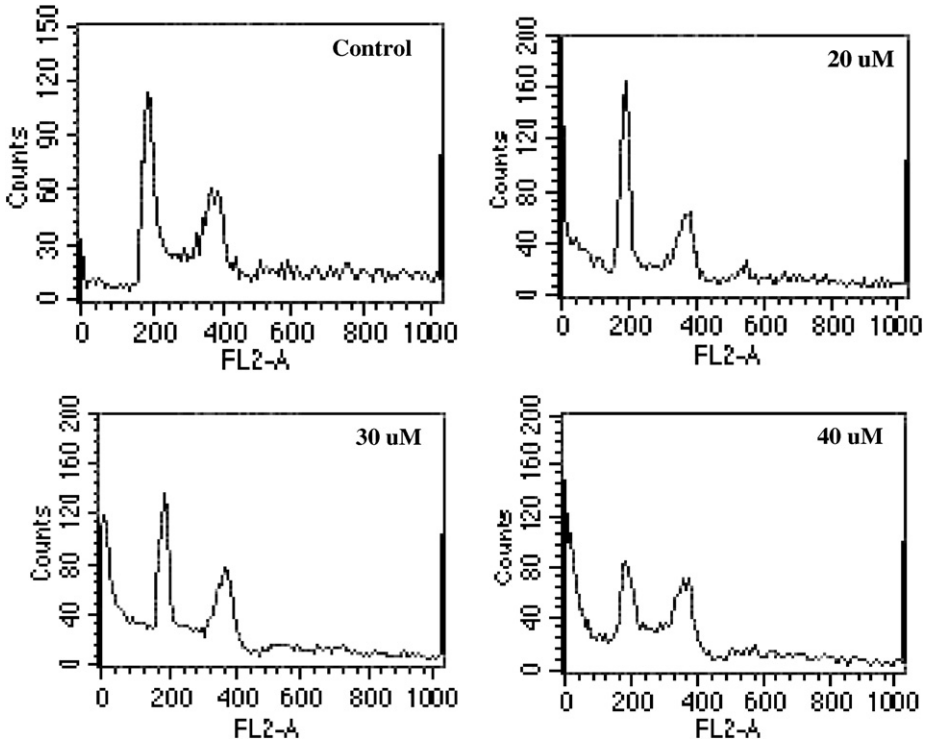


Figure 1. Effects of compound **6** on cell cycle. HCT116 cells were treated with compound **6**. Cells were harvested after 24 h and were subjected to FACScalibur analysis to determine the distribution of cells through the G_0/G_1, S, and G_2/M phase.

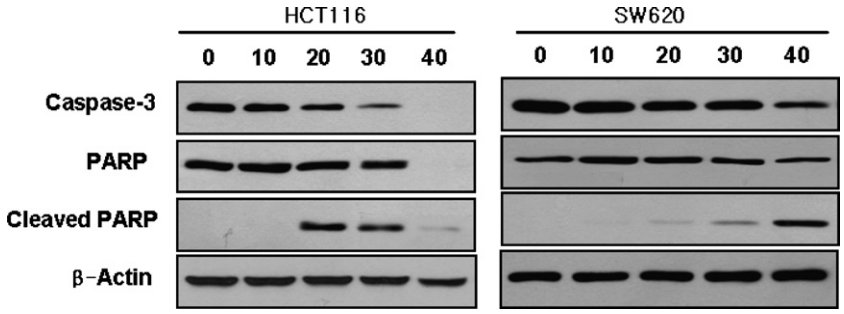


Figure 2. Induction of cleavage of PARP and caspase-3 by compound **6** which was treated in SW620 and HCT 116 cells. Cell lysates were prepared with RIPA buffer after 24 h and 40 μg of lysates was resolved by SDS–PAGE.

Table 3. Antitumoric effects of compound **6** in nude mice

Treatment	Dose (mg/kg)	Number of mice		Tumor volume (mm^3)				
		Start	End	Day 5	Day 7	Day 9	Day 12	Day 14
Control (0.5% Tween 80)	0	8	8	8.0 ± 3.9	18.0 ± 9.8	48.0 ± 14.2	85.4 ± 18.7	134.1 ± 34.3
Compound **6**	50	8	8	1.4 ± 1.3	4.3 ± 3.5	17.3 ± 7.1	45.0 ± 22.3	89.0 ± 32.6
Doxifluridine	50	8	8	3.8 ± 3.0	15.0 ± 6.2	41.4 ± 19.6	77.9 ± 33.0	120.7 ± 32.3

It can be concluded that the hydroxylamine derivatives of 2′-hydroxycinnamaldehyde will be a new class of antitumor agents inducing apoptosis and arresting cell cycle. Even though the compound **6** showed relatively weak activity in tumor cells and nude mice experiment in comparison with 2′-hydroxycinnamaldehyde,[5,6] these results suggest that hydroxylamine derivatives could be a good lead molecule for the design of new antitumor agents.

Acknowledgments

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16. Typical procedure for the synthesis of compound **6**. To a solution of 1 (10 mmol of 2′-hydroxylcinnamic acid or 2′-benzoylcinnamic acid) in chloroform (100 mL), thionyl chloride (15 mmol) was added and the solution was refluxed for 2 h. The reaction mixture was evaporated and then dried in vacuo to give **2**. To a solution of $NH_2OH{\cdot}HCl$ (10 mmol) in saturated $NaHCO_3$ water (100 mL), a solution of **2** in chloroform (50 mL) was added at 0 °C. After stirring for 30 min, the mixture was extracted two times with EtOAc. The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered, and evaporated to give crude product **3**. The crude compound **3** was used for synthesis of compounds **4**–**7** without further purification, because the compound **3** was not very stable at room temperature. 4-Morpholinecarbonyl chloride (10 mmol) was added to a solution of **3** in CH_3CN with K_2CO_3. After stirring 5 h at room temperature, the mixture was filtered and the filtrate was concentrated in vacuo. The residue was chromatographed over silica gel eluting with 30% EtOAc in hexane to give **6** as a pale yellow solid (1.2 g, 30.3%): mp 154–156 °C; $C_{21}H_{20}N_2O_6$, MS (EI): 396.1325 (Calcd 396.1342), mp: 154–156 °C, ^1H NMR ($CDCl_3$/TMS) (ppm); δ 3.52

(broad, 4H), 3.70 (t, 4H, $J = 4.8$ Hz), 6.47 (d. 1H, $J = 15.9$ Hz), 7.22–7.68 (m, 7H), 7.88 (d, 1H, $J = 15.9$ Hz), 8.20 (d, 2H, $J = 7.5$ Hz), 9.16 (broad, 1H).

17. Growth inhibitory assay. Cells were seeded at a density of 5000 cells/well in a 96-well microtiter plate. Cells were counted with a hemocytometer. After 24 h, cells were replenished with fresh complete medium containing compounds or 0.1% DMSO. After incubation for 48 h, cell proliferation reagent WST-1 (Roche) was added to each well. The amount of WST-1-formazan produced was measured at 450 nm by ELISA Reader (Bio-Rad).
18. Cell cycle analysis. To analyze the DNA content by flow cytometry, cells were trypsinized from the culture flask. After centrifugation at 300*g* for 5 min at room temperature, the supernatant was removed. The cells were then washed twice with PBS solution and fixed with 3 mL of ice-cold 70% EtOH overnight. Fixed cells were harvested by centrifugation at 300*g* for 3 min at room temperature and washed twice with PBS containing 1% FBS. Collected cells were resuspended in PBS (100 μl/1×10^5 cells) and treated with 100 μg/mL of RNase A at 37 °C for 30 min. Propidium iodide was then added to a final concentration of 50 μg/mL for DNA staining and 20,000 fixed cells were analyzed on a FACScalibur (Becton–Dickinson, San Jose, CA). Cell cycle distribution was analyzed using the Modifit's program (Becton–Dickinson).
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20. SW620 and HCT 116 cells were treated with 10–40 μM of **6** for 24 h and a 20 μg protein isolated from cell lysates was resolved by 15% SDS–PAGE and transferred to the PVDF membrane (Roche, Germany). The membrane was blocked with 5% nonfat dried milk in TBS-T (50 mM Tris–HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20). The primary antibodies used were from Cell Signaling. The secondary antibodies used were horseradish peroxidase-conjugated goat anti-rabbit IgG (1:3000) from Jackson immunology. The antibodies were used at dilution recommended by the manufacturers. Membrane was incubated with primary antibody for 2 h at room temperature, washed five times with TBS-T. And the proteins were developed using a chemiluminescence peroxidase reagent (Roche Applied Science) and exposed to X-ray films.
21. For the evaluation of in vivo antitumor activity, SW620 human colon adenocarcinoma cells (3×10^7 cells/mL) were implanted subcutaneously into the right flank of nude mice on day 0. Compounds were dissolved in 0.5% Tween 80 and were intraperitoneously administered at a concentration of 50 mg/kg per day for 20 days. Doxifluridine was used as a reference compound and its dosage was 50 mg/kg. Test substances were administrated in a volume of 0.2 mL per 20 g body weight of animals. On day 14, the mice were sacrificed and tumor volumes were estimated [length (mm) × width (mm) × height (mm)/2]. Animal experiments were performed under the permission according to 'Institutional Guideline of Animal Experiments' of Korea Research Institute of Bioscience & Biotechnology.