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Cyclopentenediones, inhibitors of farnesyl protein transferase and anti-tumor compounds, isolated from the fruit of *Lindera erythrocarpa* Makino

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Abstract—Four cyclopentenediones, farnesyl protein transferase inhibitors, and anti-tumor compounds were isolated from the methanolic extract of the fruits of *Lindera erythrocarpa* Makino (Lauraceae). The structure of the compounds was determined by spectral data including NMR and mass spectrometry, and cyclopentenediones such as methyllinderone (**1**), methyllucidone (**2**), lucidone (**3**), and linderone (**4**) were identified by comparing their reported spectral data with that of the literature values. Compounds **1**–**4** inhibited farnesyl protein transferase with IC_{50} value of 55.3 ± 4.1, 42 ± 1.9, 103 ± 5.1, and 40 ± 3.5 μM, respectively. Isolated compounds also inhibited the growth of various human cancer cell lines in a dose-dependent manner. Especially, Compounds **1** and **2** selectively inhibited the growth of H-*ras*-transformed rat-2 cell lines in comparison with normal rat-2 cells with a GI_{50} value of 0.3 and 0.85 μM, respectively. Methyllucidone strongly inhibited the growth of human cancer cells and colon tumor xenografted in nude mice. The anti-tumor effects of the compound were further confirmed with caspase-3 activation and degradation of PARP. The results suggest that methyllucidone can be a potential anti-cancer agent against H-*ras*-transformed tumor and will also be a good lead molecule for the development of anti-tumor drug.

1. Introduction

Ras proteins are small (21 kDa) GTPase which form part of several signaling pathways controlling notably gene expression and proliferation.[1] Farnesyl protein transferase (FPTase), a member of the prenyltransferase enzyme family, is a crucial enzyme which participates in the post-translational modification that the transfer of the farnesyl group from farnesyl pyrophosphate onto cysteine 186 at the C-terminal of the Ras proteins.[2,3] And this is a mandatory process for retention of transforming ability of Ras proteins.[4] When a farnesylation of these proteins is blocked, their oncogenic activity is abolished.[5,6] Therefore, the identification and synthesis of FPTase inhibitors has become an active area for the development of anti-tumor agents[3,4] and a couple of FPTase inhibitors are under clinical trials.[7,8]

In continued screening of plant extracts for potential FPTase inhibitors,[9–11] we have tested 3025 plant extracts including 342 herbal medicines against FPTase. We selected the extract of the fruits of *Lindera erythrocarpa* Makino (Lauraceae) as a good candidate for isolation of FPTase-specific inhibitors. *Lindera* species (Lauraceae), widespread throughout the world, are important medicinal plants including *L. lucida*, *L. strychnifolia*, *L. aggregate*, and *L. chunii*. *L. erythrocarpa* (Lauraceae) is also widely distributed in Korea, Japan, and the People's Republic of China, and its fruit is used as a traditional medicine as analgesic, digestive, diuretic, antidote, and antibacterial.

In this report, we described the activity-guided isolation from the fruits of *L. erythrocarpa* and their inhibitory activities on FPTase. It was examined to study the inhibitory activity of the isolated compounds against

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H-*ras*-transformed rat-2 cells and a variety of human cancer cell lines to confirm the anti-tumor effects of the isolated compounds. We also investigated biologically relevant aspects such as apoptotic markers to identify the mechanism of the anti-tumor effect and tumor xenografted in nude mice for in vivo activity of methyllucidone (**2**).

2. Results and discussion

2.1. Isolation and FPTase inhibition activity

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) was natural products, and another 23% was natural-product-derived molecules.[12] On the basis of the reports, we screened 3025 plant extracts including 342 herbal medicines against FPTase and the extract of the fruits of *L. erythrocarpa*. *L. erythrocarpa*, belongs to the family Lauraceae, was selected as a good natural source for the isolation of FPTase inhibitors, which consists of *L. lucida*, *L. strychnifolia*, *L. aggregate*, and *L. chunii*. Extract of the fruits of *L. erythrocarpa* was subjected to fractionation based on FPTase inhibitory activity. The major active fractions were collected and chromatographed over silica gel column. The combined active fractions were rechromatographed on silica gel column and then C-18 column to yield cyclopentenediones (Fig. 1). Structures of the isolated compounds were elucidated by spectroscopic method including mass spectral and NMR data and identified by comparison with the reported spectral data as methyllinderone (**1**), methyllucidone (**2**), lucidone (**3**), and linderone (**4**). Compound **2** was isolated as *cis*- and *trans*-**2** mixed forms.[13–15]

The isolated compounds methyllinderone (**1**), methyllucidone (**2**), lucidone (**3**), and linderone (**4**) inhibited FPTase activity with IC_{50} values of 55.3 ± 4.1, 42 ± 1.9, 103 ± 5.1, and 40 ± 3.5 μM, respectively (Fig. 2). Even though it was reported that methyllinderone inhibited a human chymase[13] and the extract of leaves of *L. erythrocarpa* inhibited HIV-1 protease,[16] there has been no report concerning the FPTase inhibition activity of the compounds.

2.2. In vitro anti-tumor activity

The FPTase inhibitors have been reported as a promising target for the development of anti-cancer agent in H-*ras*-activated cancer cell lines. We confirmed whether compounds **1** and **2**, the strong FPTase inhibitors of the isolated cyclopentenediones, were H-Ras- specific inhibitors or not through cell proliferation assay in H-*ras*-transformed rat-2 cells. When the compounds were treated in H-*ras*-transformed rat-2 cells for 48 h, the cell growth was significantly inhibited in a dose-dependent manner. Compounds **1** and **2** selectively

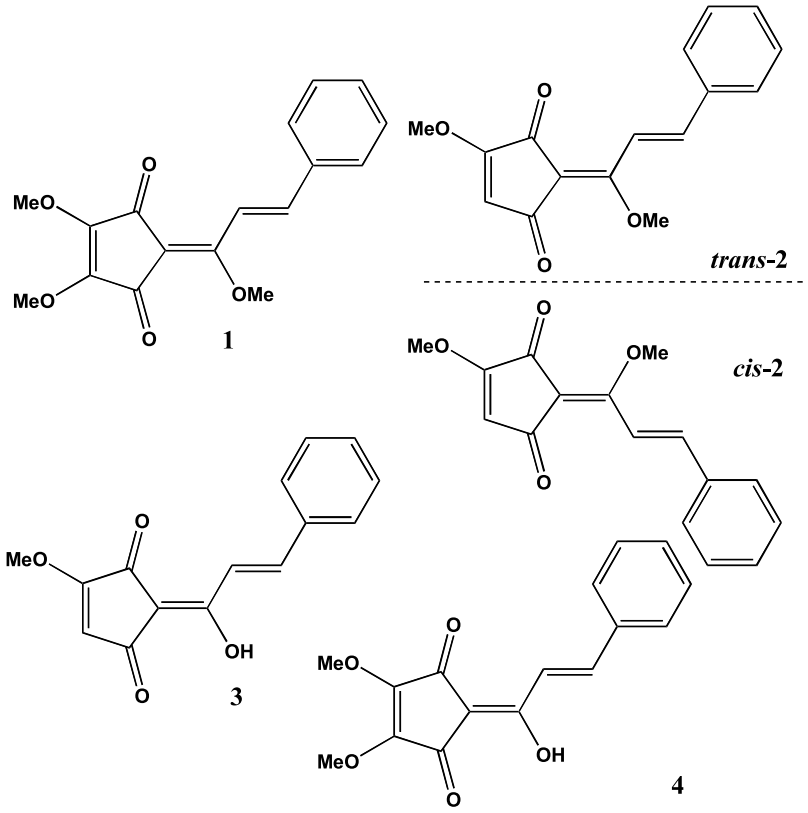

Figure 1. Structure of methyllinderone (**1**), methyllucidone (**2**), lucidone (**3**), and linderone (**4**) isolated from fruits of *L. erythrocarpa* Makino (Lauraceae).

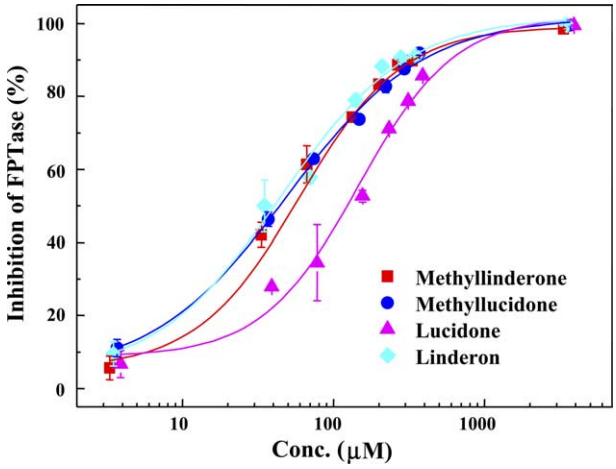

Figure 2. The isolated compounds methyllinderone (**1**), methyllucidone (**2**), lucidone (**3**), and linderone (**4**) inhibited FPTase activity with IC_{50} values of 55.3 ± 4.1, 42 ± 1.9, 103 ± 5.1, and 40 ± 3.5 μM, respectively.

inhibited the growth of H-*ras*-transformed rat-2 cells in comparison with that of normal rat-2 cells (Fig. 3, for compound **1**) with a GI_{50} value of 0.3 and 0.85 μM, respectively.

Compound **2**, which is a major component of the extracts of fruits of *L. erythrocarpa*, was treated in a variety of tumor cells including colon (HCT-116, HCA-7, and SW620), lung (A549 and NCI-H23), kidney (HEK293), breast (MDA-MB-231), and prostate (DU145) cancer cells. It was found that colon tumor cells were more sensitive than other cells against the compound **2**. Especially, the compound strongly inhibited the growth of HCT-116 tumor cells with a GI_{50} value of 1.0 μM and also blocked the growth of other colon tumor cells such as HCA-7 and SW620 with GI_{50} values of 1.4 and 3.0 μM, respectively (Fig. 4). To the best of our knowledge, this is the first report of an anti-tumor activity of cyclopentenediones through inducing apoptosis of human tumor cells and an inhibitory activity of FPTase of the compounds.

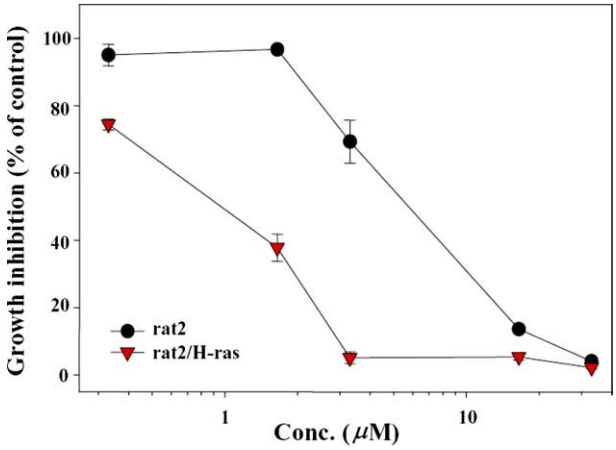

Figure 3. Effects of methyllinderone (**1**) on proliferation of rat-2 vector cells and H-*ras*-transformed rat-2 cells.

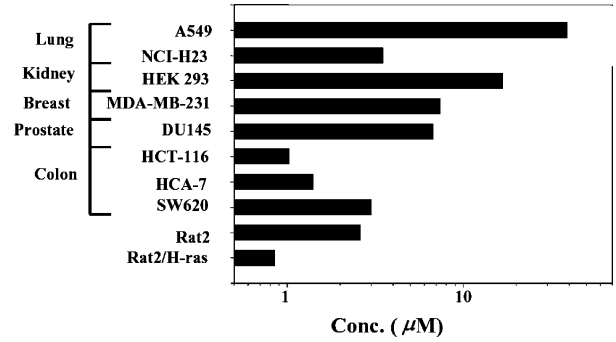

Figure 4. Anti-tumor activity of methyllucidone (**2**) against various human cancer cell lines (all experiments were repeated at least three times).

2.3. Methyllucidone (2) induces apoptosis in human colon cancer cell lines

Even though the compound **2** selectively inhibited the growth of H- *ras*-transformed rat-2 cells, we could not detect the blocking of Ras-processing in the compound **2**-treated H-*ras*-transformed rat-2 cells and human colon tumor HCT116 cells (data not shown; see Section 4 for the detailed experimental procedure). This is probably because the cytotoxicity of the compound is much stronger than the inhibition activity of FPTase of the compound. These results suggested that the growth inhibitory activity of compound **2** against tumor cells might be through another pathway such as apoptotic cell death.

To study the anti-tumor effects of compound **2** in detail, we monitored dose-dependent response of colon tumor cells after compound **2** treatment. It was found that compound **2**-treated cells were inhibited cell spreading and becoming round morphology of the cells. To examine the effects of **2** on apoptosis-related proteins, we investigated the expression of apoptosis marker protein such as PARP (poly-ADP-ribose polymerase), involved in DNA repair in response to environmental stress and one of the apoptosis markers, and activation of caspase-3.[17] As shown in Figure 5, PARP degradation was strongly induced by the compound at 7.5 μM and, in addition, activated caspase-3 was also detected by Western analysis using active-specific caspase-3 antibody. These results strongly support that methylluci-

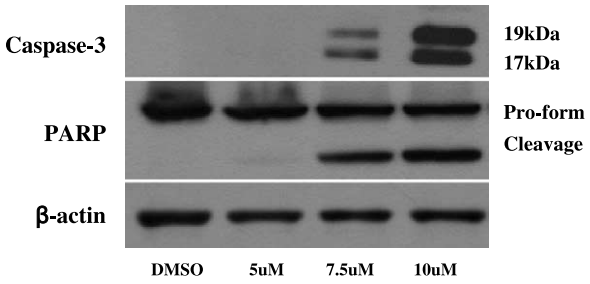

Figure 5. Methyllucidone (**2**) caused a cleavage of caspase-3 and PARP in human colon cancer cells SW620.

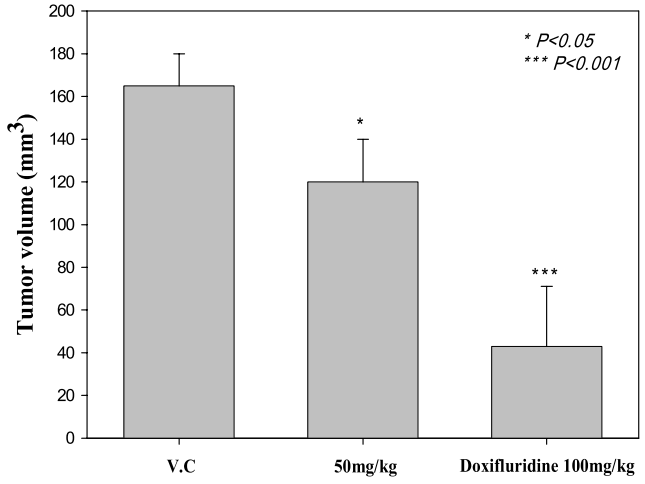

Figure 6. Methyllucidone (**2**) reduced the tumor volumes at the doses of 50 mg/day.

done (**2**) exerts its anti-tumor activity in colon tumor cells through apoptosis.

2.4. In vivo anti-tumor activity

It is well known that high incidence of Ras-mutation and activation is found in colon cancers (50%) and the compound **2** strongly inhibited the growth human colon tumor cells. Therefore, the anti-tumor effect of compound **2**, that is the major component of the fruit of *L. erythrocarpa* was evaluated with HCT116 human colon cancer cells in a human tumor xenograft model in nude mice. The compound in 0.5% Tween 80 was orally administrated at a dose of 50 mg/kg for 20 days. As shown in Figure 6, the growth of tumors was moderately inhibited by the compounds. Compound **2** reduced the tumor volumes 26.6% at final day. Fortunately, the loss of body weight was not observed in nude mice administered with compound **2** at the doses of 50 mg/day.

3. Conclusion

It can be concluded that the cyclopentenediones will be a new class of anti-tumor agents with a couple of biological activities including inducing apoptosis and inhibition of FPTase. Compound **2**, the major component of the extract of fruits of *L. erythrocarpa*, was endowed with a capacity to specifically inhibit the growth of H-*ras*-transformed rat-2 cells versus normal rat-2 cells. In addition, compound **2** strongly inhibits human colon tumor cells and exerts its anti-tumor activity by inducing apoptosis through caspase-3 pathway. Above all, an important point is that it has oral anti-tumor activity, confirmed by a human tumor xenograft model in nude mice. Although further studies are needed to clarify the mechanism of the anti-tumor activities of compound **2**, these results suggest that cyclopentenediones could be a good lead molecule for the development of anti-tumor agents.

4. Experimental

4.1. General

Chemicals and solvents were of reagent grade and were used without further purification. The column fractions were monitored by thin-layer chromatography on precoated Merck Silica gel $60F_{254}$ plates; the spots were visualized by exposure to UV radiation. Melting point of the compounds was determined between two microcoverglasses using Fisher–Johns Melting Point Apparatus. ^{1}H NMR was recorded on a Varian 400 MHz spectrometer in $CDCl_3$, and EIMS was recorded on a JMS-HX 110A/HX 110A spectrometer. UV spectrum was recorded on an Agilent 8453 UV–vis spectroscopy system (Hewlett Packard). Column chromatography separations were carried out by using silica gel 60 (0.04–0.063 mm) and LiChroprep RP-18 (40–63 μm) supplied by E. Merck.

4.2. Plant material

The fruits of *L. erythrocarpa* were collected in July 2004 from the Sockri mountain in Chungbuk Korea. The authenticity of the plant was confirmed by Plant Diversity Research Center and a voucher specimen (018-053 for *L. erythrocarpa*) is deposited in Korea Research Institute of Bioscience and Biotechnology, Korea. The samples were shade dried and milled to powder form, which were then kept in air-tight brown bottle until use.

4.3. FPTase activity assay

FPTase assay was done using a scintillation proximity assay method following the protocol described by the manufacturer except that a biotinylated substrate peptide containing the Ki-Ras carboxyl-terminal sequence was used. The C-terminal peptide of Ki-Ras was used a Biotin-KKKSKTKCVIM synthesized by solid-phase peptide syntheses. FPTase activity was determined by measuring transfer of [^{3}H] farnesyl from [^{3}H] farnesyl pyrophosphate to Biotin-KKKSKTKCVIM. Typical reaction mixtures (100 μL total volume) contained 50 mM HEPES, pH 7.5, 30 mM $MgCl_2$, 20 mM KCl, 5 mM DTT, 0.01% Triton X-100, 150–250 nM [^{3}H] farnesyl pyrophosphate (60 μM, 1 Ci/μL), 25–50 ng (~2.5 to 5 nM) of recombinant rat FPTase or 10 μL of partially purified Q-Sepharose-derived FPTase, the indicated concentration of cyclopentendione or DMSO vehicle control (10%, v/v, final), and 10–200 nM Biotin-KKKSKTKCVIM. After 60 min incubation in water bath at 37 °C, reactions were stopped by adding 150 μL of STOP/bead reagent into each tube. Radioactivities were mixed with vortex and allowed to stand for 30 min at room temperature. Samples were measured in a Wallac 1450 microbeta TRILUX liquid scintillation counter. Percent inhibition was calculated relative to the DMSO vehicle control.

4.4. Extraction and isolation

The dried fruits (175.18 g) of *L. erythrocarpa* were extracted with MeOH (2× 1 L) for 48 h at room tem-

perature. After filtration, the combined extract was concentrated and the residue weighed 11.31 g. The MeOH extract was partitioned between H_2O and EtOAc (1:1, v/v) to give a EtOAc-soluble fraction (9.91 g). The EtOAc fraction showed FPTase inhibition activity and was partitioned again with MeOH to obtain MeOH-soluble fraction (8.5 g) and -insoluble fraction (1.26 g). The MeOH-soluble fraction was concentrated, and then the residue was chromatographed on a silica gel (350 g) column, eluted with a gradient of *n*-hexane/EtOAc (19:1, 18:2, 17:3, 16:4, 14:6, 1:1, each 1.5 L) to provide 45 fractions. Active fractions were collected and concentrated to yield 7.3 g. The active fraction was resubjected to a C-18 column, and it was eluted with a gradient of MeOH/H_2O (6:4, 7:3, 8:2, MeOH, each about 3 L) to provide methyllinderone (432 mg), methyllucidone (1450 mg), lucidone (64 mg), and linderone (43 mg). [13–15]

4.4.1. Methyllinederone (1). Yellow solid, mp 75 °C; EIMS *m/z* (rel. int.): 300 $[M]^+$ (68), 241 (100), 225 (26), 103 (59). HREIMS: $[M]^+$ *m/z* 300.0991 ($C_{17}H_{16}O_5$ calcd *m/z* 300.0997). ^{1}H NMR ($CDCl_3$): δ 4.09 (s, 3H), 4.18 (s, 6H), 7.38 (m, 3H), 7.51 (d, 1H, J = 15.8 Hz), 7.60 (m, 2H), 7.93 (d, 1H, J = 15.8 Hz).

4.4.2. Methyllucidone (2). Yellow solid, mp 126–128 °C. HREIMS: $[M]^+$ *m/z* 270.0763 ($C_{16}H_{14}O_4$ calcd *m/z* 270.0892). ^{1}H NMR ($CDCl_3$): δ 3.92 (s, OCH_3), 3.93 (s, OCH_3), 4.19 (s, OCH_3), 4.20 (s, OCH_3), 7.40 (m, 3H), 7.61 (m, 3H), 7.93 (d, J = 15.8 Hz). 7.98 (d, J = 15.8 Hz).

4.4.3. Lucidone (3). Yellow solid, mp 166.5–168.5 °C. HREIMS: $[M]^+$*m/z* 256.0752 ($C_{15}H_{12}O_4$ calcd *m/z* 256.0735). ^{1}H NMR ($CDCl_3$): δ 3.96 (s, 3H), 7.40 (m, 3H), 7.63 (m, 3H), 7.71 (d, 1H, J = 18 Hz).

4.4.4. Linderone (4). Yellow solid, mp 92–94 °C. HREIMS: $[M]^+$ *m/z* 286.0796 ($C_{16}H_{14}O_5$ calcd *m/z* 286.0841). ^{1}H NMR ($CDCl_3$): δ 4.17 (s, 3H), 4.21 (s, 3H), 7.63 (m, 3H), 7.38 (m, 4H), 7.61 (m, 3H).

4.5. Cell growth inhibition assay

Cells were seeded at a density of 1500 (H-*ras*-transformed rat-2), 4000 (rat-2 vector, A549, and MDAMB-231), 5000 (HEK293), and 6000 (DU145, HCA-7, HCT116, and SW620) cells per well in a 96-well microtiter plate in medium containing 10% FBS. Cells were counted with a hematocytometer. One day after seeding, cells were replenished with fresh complete medium containing compounds or 0.1% DMSO. After incubation for 48 h, cell proliferation reagent WST-1 (Roche, Germany) was added to each well. The amount of WST-1-formazan produced was measured at 450 nm by ELISA Reader (Bio-Rad).

4.6. Western blotting

H-*ras*-transformed rat-2 and HCT 116 were treated with 10 mM of methyllucidone 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). The membrane was blocked with 5% nonfat dried milk in TBS-T (50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.1% Tween 20). H-Ras antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). SW620 cells treated with methyllucidone were harvested and washed with PBS. Cell pellets were lysed in 200 μL of lysis buffer (protease inhibitor cocktail, 30 mM Na_2HPO_4, 50 mM NaF, 1 mM Na_3VO_4, RIPA buffer) for 1 h on ice. Lysates were centrifuged at 1360 rpm for 5 min. Then, protein contents in the supernatant were measured using the ELISA Reader (Bio-Rad). The lysates containing 50 μg of protein were mixed with protein 2X sample buffer (ELPiS) and boiled for 10 min. The proteins were separated on 4–15% SDS–PAGE gels with running buffer and then electrotransferred onto to polyvinylidene difluoride membrane (Roche Applied Science) with transfer buffer at 100 mV for 2 h. The membranes were blocked with 5% nonfat dried milk in TBS-T (50 mM Tris–HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20) for 1 h at room temperature. 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 and washed five times with TBS-T. And the proteins were developed using a chemiluminescence peroxidase reagents (Roche Applied Science) and exposed to X-ray films.

4.7. In vivo activities of methyllucidone

For the evaluation of in vivo anti-tumor activity, HCT116 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 orally administered at a concentration of 50 mg/kg per day for 20 days. Doxifluridine was used as a reference compound and its dosage was 100 mg/kg. Test substances were administrated in a volume of 0.2 ml per 20 g body weight of animals. On day 20, the mice were sacrificed and tumor volumes were estimated [length (mm) × width (mm) × height (mm)/2]. To determine the toxicity of the compounds, the body weight of tumor-bearing animals was measured. Animal experiments were performed under the permission according to 'Institutional Guideline of Animal Experiments' of Korea Research Institute of Bioscience and Biotechnology.

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