Differential Effects of Methoxylated *p*-Coumaric Acids on Melanoma in B16/F10 Cells

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ABSTRACT: As an approach to search for chemopreventive agents, we tested *p*-coumaric acid, 3-methoxy-*p*-coumaric acid (ferulic acid), and 3,5-dimethoxy-*p*-coumaric acid (sinapic acid) in B16/F10 melanoma cells. Intracellular melanin contents were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay and cytotoxicity of the compounds were examined by lactate dehydrogenase (LDH) release. *p*-Coumaric acid showed inhibitory effect on melanogenesis, but ferulic acid increased melanin content, and sinapic acid had almost no effect on melanogenesis. Treatment with ferulic acid resulted in a 2 to 3 fold elevation in the production of melanin. Correlatively, cell viability decreased in a dose-dependent manner when treated with ferulic acid. However, ferulic acid did not affect the LDH release from the cells. Treatment with sinapic acid resulted in a 50~60% elevation in the release of LDH when treated with a 200 μg/mL concentration and showed neither cytostasis nor increase of melanin synthesis in a dose-dependent manner. Taken together, *p*-coumaric acid inhibits melanogenesis, ferulic acid induces melanogenesis, and sinapic acid exerts cytotoxic effects in B16/F10 murine melanoma cells. The results indicate that the addition of methoxy groups to *p*-coumaric acid shows the melanogenic or cytotoxic effects in melanoma cells compared to the original compound. Therefore, this study suggests the possibility that methoxylated *p*-coumaric acid, ferulic acid can be used as a chemopreventive agent.

Keywords: methoxy groups, ferulic acid, sinapic acid, cytostasis, cytotoxicity

INTRODUCTION

Melanoma, a type of cancer which forms from melanocytes, is the most lethal skin cancer in humans and its incidence and mortality rate has increased over the last three decades (1). A possibly less toxic approach for treating cancer involves reprogramming tumor cells to encounter irreversible growth arrest and terminal differentiation, referred to as differentiation therapy. In this scheme, neoplastic cells displaying atypical patterns of differentiation upon treatment with an appropriate agent lose proliferative capacity and terminally differentiate. The limitations of current cancer therapies underscore the need to develop less toxic and probably more specific and effective forms of treatment (2). Previous studies on melanoma-cell differentiation have reported that interferon (IFN) and melanocyte stimulating hormone (MSH) induce differentiation in melanocytes and melanoma cells (3,4).

Melanin plays a protective role in the linkage and symbiosis between melanocyte and keratinocyte as an anti-oxidant. Melanogenesis indicates a marker of differentiation in human and murine melanoma cells. The process of melanoma cell differentiation includes melanin synthesis, the transport of melanin and anti-proliferation (5).

Methoxylated flavones with hydroxyl groups capped by methylation are shown to exert biologically and pharmaceutically activities in the regulation of melanogenesis, differentiation, and other physiological conditions. Previous studies have been reported that methoxyflavones enhance metabolic stability and membrane transport in the intestine/liver, which results in the improvement of oral bioavailability (6). Nobiletin, a 5,6,7,8,3',4'-hexamethoxyflavone, triggers melanogenesis and differentiation in B16/F10 melanoma cells and induces neuritogenesis in PC12D cells (2,7,8). Sinensetin, a 5,6,7,3',4'-pentamethoxyflavone, has antiangiogenic effect in zebrafish and inhibited carrageenan-induced paw inflammation in mice (9,10). Tangeretin, a 5,6,7,8,4'-pentamethoxyflavone, inhibits a number of cellular activities such as growth of mammary cancer cells *in vitro* and cytolysis by

natural killer (NK) cells *in vivo* (11).

Recently, studies have shown that melanogenesis is induced by methoxylated compounds, such as nobiletin, tangeretin, sinensetin, 4'-O-methylfisetin, scopletin (7-hydroxy-6-methoxycoumarin), 7-methoxycoumarin, scoparone (6,7-dimethoxycoumarin), and citropten (5,7-dimethoxycoumarin) (7,12-14), and stimulated by hydroxylated compounds, such as quercetin, naringenin, and isoliquiritigenin (15-17), while inhibited by diosgenin, α-tocopheryl ferulate, and 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (18-20).

In this study, we report the chemopreventive effects of ferulic acid and sinapic acid in murine B16/F10 melanoma cells. By determining the intracellular melanin contents and cytostasis of the compounds *in vitro*, the stimulatory effects of the addition of methoxy groups to *p*-coumaric acid on melanogenesis and cytostasis were shown. This study presents the fundamental information on methoxy *p*-coumaric acids as a chemopreventive agent for melanoma.

MATERIALS AND METHODS

Materials
All solvents used were of analytical grade without any further purification. α-Melanocyte stimulating hormone (α-MSH), *p*-coumaric acid, ferulic acid, sinapic acid, and doxorubicin were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Cell culture
B16/F10 murine melanoma cells were maintained in DMEM (Gibco BRL, Rockville, MD, USA) supplemented with 10% FBS (Gibco BRL), and 1% penicillin-streptomycin (10,000 U/mL and 10,000 μg/mL, Gibco BRL). The cells were maintained in a humid atmosphere of 5% CO_2 at 37°C.

Intracellular melanin contents
B16/F10 murine melanoma cells were seeded into 6-well plates at a density of 1×10^5 cells per well. The cells were then treated with or without the test compounds at 37°C for 2 days. Next, the cells were washed with $1 \times PBS$ and then collected in $1 \times trypsin$-EDTA, after which they were lysed with 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and 1% Triton X-100 in 67 mM sodium phosphate buffer (pH 6.8). The samples were then sonicated and centrifuged at 15,000 rpm for 15 min at 4°C. To extract the melanin from the pellets, 1 N sodium hydroxide (NaOH) was added to the pellets and subsequently incubated at 70°C for 4 h. The absorbance was then measured at 405 nm and the corresponding total protein was used to normalize the absorbance.

Cell viability
The cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, cells were seeded into 96-well plates at a density of 1×10^4 cells per well in the presence or absence of *p*-coumaric acid, ferulic acid, sinapic acid, doxorubicin, or α-MSH. After two days of incubation, the mitochondrial enzyme activity, which is an indirect measure of the number of viable respiring cells, was determined using the MTT reagent. Finally, the absorbance was measured at 540 nm and the effect of *p*-coumaric acid, ferulic acid, sinapic acid, doxorubicin, or α-MSH on cell viability was evaluated relative to a control without the test compounds.

Cytotoxicity
The cytotoxicity was determined by the lactate dehydrogenase (LDH) release assay. The cytotoxic effect of *p*-coumaric acid, ferulic acid, sinapic acid, doxorubicin or α-MSH was estimated by the measurement of LDH as the leakage of LDH is a well-known marker of the damage of cellular membrane. The cytotoxicity was expressed as the percentage of LDH released from the culture supernatant (LDH release in medium of α-MSH, *p*-coumaric acid, ferulic acid, sinapic acid, or doxorubicin treatment/maximal LDH release×100). Maximal LDH release was measured after lysis of the cells with 0.5% Triton X-100.

Statistical analysis
All experiments were performed in triplicate. Treatment effects were analyzed using the Student's *t*-test; $P < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Previous studies show that melanogenesis, a marker of melanoma cell differentiation, is stimulated by methoxylated flavones, methoxylated coumarins, and hydroxylated flavones (7,12-17) which suggests that these compounds can be used as the improvement agents for acquired hypopigmentation disorders (21). Moreover, *p*-coumaric acid inhibits cellular melanogenesis induced by α-MSH in B16/F10 melanoma cells (22) and α-tocopheryl ferulate exerts depigmenting effects on human melanoma cells. α-Tocopheryl ferulate is a compound of α-tocopherol and ferulic acid connected by an ester bond (19). The effect of ferulic acid or sinapic acid on melanogenesis has not been previously studied (Fig. 1A). To investigate the effects of *p*-coumaric acid (CA), 3-methoxy-*p*-coumaric acid (FA), or 3,5-dimethoxy-*p*-coumaric acid (SA) on melanogenesis, B16/F10 melanoma cells were incubated with 500 μg/mL of each compound

A

p-Coumaric acid   Ferulic acid   Sinapic acid

B
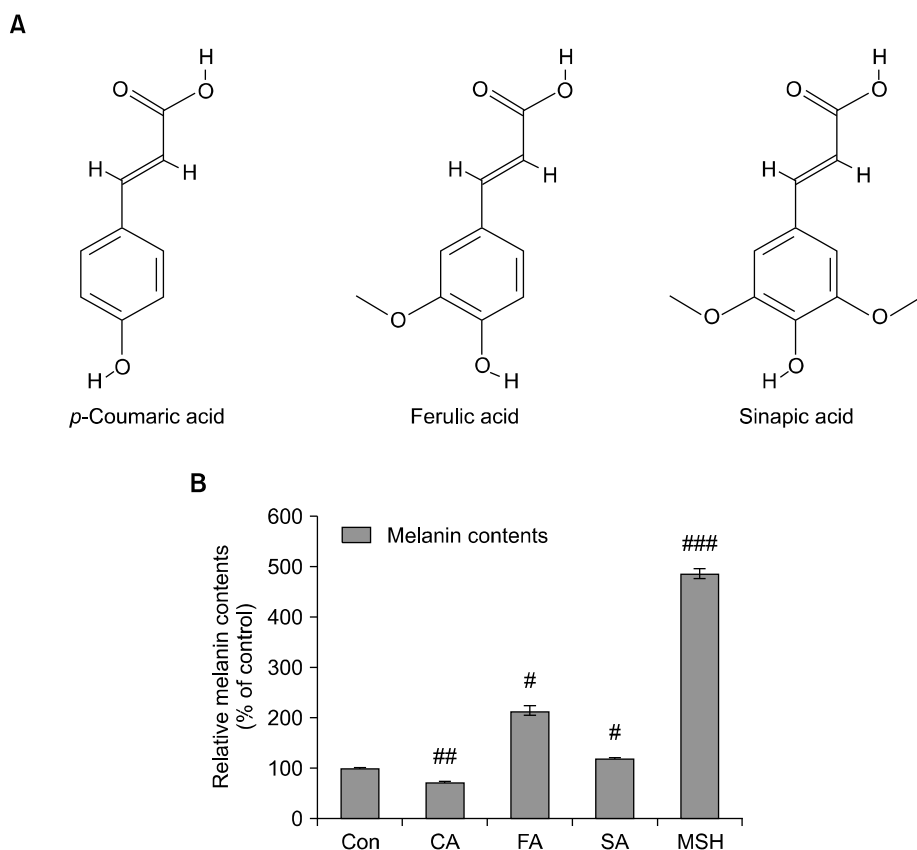
Fig. 1. Effects of *p*-coumaric acid derivatives on melanin production. (A) Chemical structure of *p*-coumaric acid, 3-methoxy-*p*-coumaric acid (ferulic acid), and 3,5-dimethoxy-*p*-coumaric acid (sinapic acid). (B) Relative intracellular melanin contents. [#]$P<0.005$, [##]$P<0.0005$, [###]$P<0.00005$ compared to control without *p*-coumaric acid, ferulic acid, sinapic acid, and α-MSH by Student's *t*-test. Con, negative control without *p*-coumaric acid, ferulic acid, sinapic acid, and α-MSH; CA, 500 μg/mL of *p*-coumaric acid; FA, 500 μg/mL of ferulic acid; SA, 500 μg/mL of sinapic acid; MSH, 50 nM of α-MSH as a positive control. Bars represent means±SD of three independent experiments.

for 48 h. *p*-Coumaric acid showed an inhibitory effect on melanogenesis, but 3-methoxy-*p*-coumaric acid and α-MSH increased melanin content 2- and 5-fold, respectively, and 3,5-dimethoxy-*p*-coumaric acid showed little effect on melanogenesis (Fig. 1B). These findings suggest that the 3-methoxy group added to *p*-coumaric acid (i.e. ferulic acid) is necessary for stimulating melanogenesis.

To confirm the safety and efficacy of ferulic acid for pharmaceutical use, intracellular melanin contents were determined by LDH release and the MTT assay with the doses previously indicated. After exposure of the cultured B16/F10 melanoma cells to ferulic acid or α-MSH, cell viability decreased in a dose-dependent manner when treated with ferulic acid with maximum reduction of cell viability by 40% at 1,000 μg/mL, compared to the control. However, ferulic acid did not affect the LDH release from the cells (Fig. 2A). Correlatively, treatment with ferulic acid resulted in a 2 to 3 fold elevation in the production of melanin. The most marked melanin synthesis occurred in the cells treated with 1,000 μg/mL ferulic acid (Fig. 2B). These data show that ferulic acid exerts a cytostatic effect in B16/F10 melanoma cells rather than a cytotoxic effect. Interestingly, previous studies showed that nobiletin, a citrus hexamethoxy-flavonid, induces melanogenesis and cytostasis except for cytotoxicity in B16/F10 melanoma cells (2,7).

To demonstrate the cytotoxic effect of sinapic acid in B16/F10 melanoma cells, LDH release was determined by intracellular melanin content and the MTT assay. Previous research has revealed that doxorubicin induces DNA fragmentation as a marker of apoptosis (2). Treatment with sinapic acid resulted in a 50~60% elevation in the release of lactate dehydrogenase (LDH) when treated with a concentration of 200 μg/mL which is the highest LDH level detected. However, sinapic acid showed neither cytostasis nor increase of melanin synthesis in a dose-dependent manner (Fig. 3) which suggests that sinapic acid exerts cytotoxic effects, but not cytostatic effects. Furthermore, a subsequent study of sinapic acid will have to be performed to experiment on DNA fragmentation as a marker of apoptosis. With regards to methoxylated compounds, previous studies showed that sinapic acid has a neuroprotective potential in the 6-hydroxydopamine-induced hemi-parkinsonian rat (23) and tangeretin, a citrus pentamethoxyflavonoid, induces apoptosis in human gastric cancer cells (24).

In conclusion, these results indicate that *p*-coumaric acid inhibits melanogenesis, ferulic acid induces melanogenesis, and sinapic acid exerts cytotoxic effects in B16/F10 murine melanoma cells. This study suggests that the addition of methoxy groups to *p*-coumaric acid show the differential effects compared to original compounds. Contrary to *p*-coumaric acid as a hypopigmentation agent, ferulic acid, can be used as a chemopreventive agent.
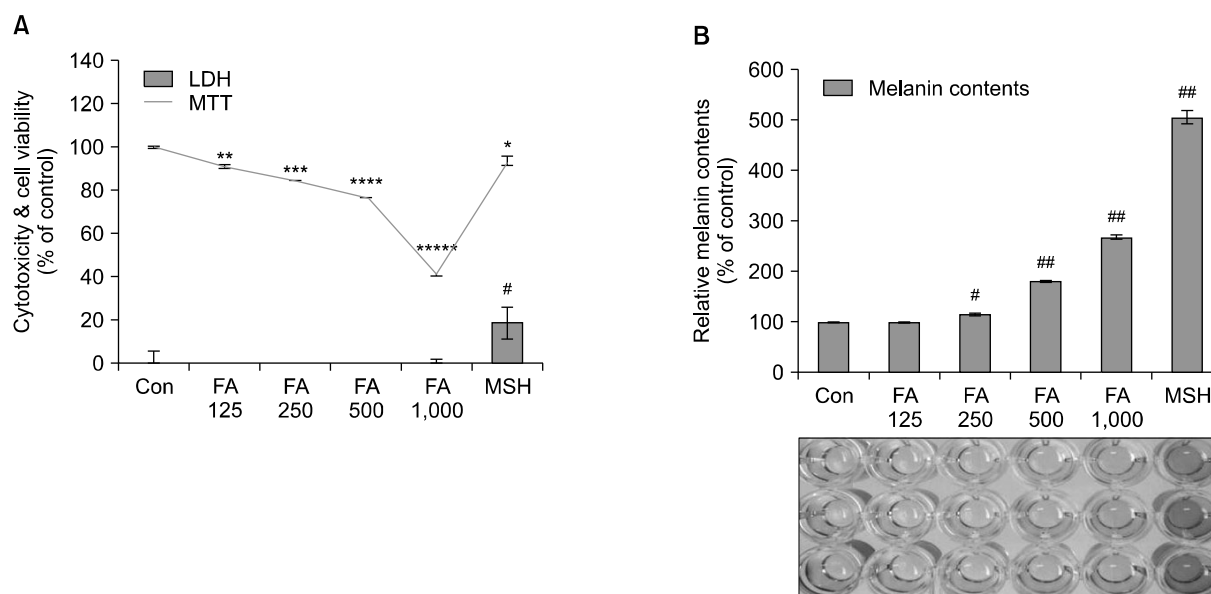
Fig. 2. Cytostatic effects of a 3-methoxy-*p*-coumaric acid (ferulic acid). (A) Cytotoxicity using LDH assay and cell viability using MTT assay of ferulic acid. $^{#}P<0.001$, $^{*}P<0.05$, $^{**}P<0.005$, $^{***}P<0.00005$, $^{****}P<0.000005$, $^{*****}P<0.0000005$ compared to control without ferulic acid and α-MSH by Student's *t*-test. (B) Relative intracellular melanin contents $^{#}P<0.05$, $^{##}P<0.005$ compared to control without ferulic acid and α-MSH by Student's *t*-test. Insert represents the raw data of intracellular melanin contents. Con, control without ferulic acid and α-MSH; FA125, 125 μg/mL of ferulic acid; FA250, 250 μg/mL of ferulic acid; FA500, 500 μg/mL of ferulic acid; FA1000, 1,000 μg/mL of ferulic acid; MSH, 50 nM of α-MSH as a positive control. Bars represent means±SD of three independent experiments.
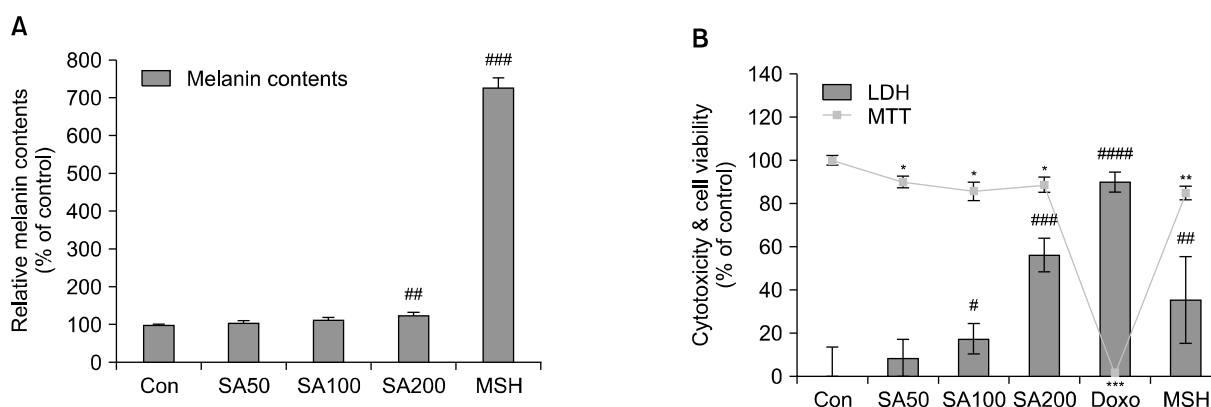
Fig. 3. Cytotoxic effects of a 3,5-dimethoxy-*p*-coumaric acid (sinapic acid). (A) Relative intracellular melanin contents. $^{#}P<0.05$, $^{##}P<0.005$, $^{###}P<0.0005$ compared to control without sinapic acid and α-MSH by Student's *t*-test. B. Cytotoxicity using LDH assay and cell viability using MTT assay of sinapic acid. $^{#}P<0.01$, $^{##}P<0.005$, $^{###}P<0.0001$, $^{*}P<0.05$, $^{**}P<0.005$, $^{***}P<0.0005$ compared to control without sinapic acid and α-MSH by Student's *t*-test. Con, control without sinapic acid and α-MSH; SA50, 50 μg/mL of sinapic acid; SA100, 100 μg/mL of sinapic acid; SA200, 200 μg/mL of sinapic acid; Doxo, 1 μM of doxorubicin as an apoptosis inducer; MSH, 100 nM of α-MSH as a positive control. Bars represent means±SD of three independent experiments.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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