Anisaldehyde, a Melanogenesis Potentiator

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Anisaldehyde (4-methoxybenzaldehyde), previously reported as a tyrosinase inhibitor, did not inhibit melanogenesis in cultured B16-F10 melanoma cells but rather enhanced it. This adverse effect of anisaldehyde was accompanied by melanocytotoxicity in a dose-dependent manner up to 2 mM. The melanin content per cell at 1 mM was increased 5-fold compared to control and morphological observations showed the deposition of melanin pigments. Anisaldehyde was also examined against cultured human A375 melanoma cells.

Key words: Anisaldehyde, Tyrosinase, B16-F10 Melanoma Cells

Introduction

Melanins have many functions in living systems, and alterations in melanin synthesis occur in many disease states. Melanins play a crucial role in the absorption of free radicals generated within the cytoplasm and in shielding the host from various types of ionizing radiation. Tyrosinase (EC 1.14.18.1) is a copper-containing enzyme and catalyzes two distinct reactions of melanin synthesis (Lerch, 1987; Sánchez-Ferrer *et al*., 1995). Recently, the effects of anisaldehyde (4-methoxybenzaldehyde) (**1**) on the enzymatic oxidation of L-3,4-dihydroxyphenylalanine (L-DOPA) were reexamined. Although anisaldehyde was previously reported to inhibit the enzymatic oxidation of L-DOPA noncompetitively (Kubo and Kinst-Hori, 1998a; Lee, 2002), it did not inhibit this oxidation if a longer reaction time was observed (Ha *et al*., 2005). In these cell-free spectrophotometric experiments, the enzyme activity was monitored by measuring the dopachrome formation at 475 nm. Dopachrome is a relatively stable intermediate but it is gradually oxidized further. The spectrophotometric method measures only the very initial rate of dopachrome formation to avoid the involvement of secondary reactions (Mayer *et al*., 1966). Hence, the inhibition kinetics reported is based on the data obtained within 1 min, which may not be long enough to evaluate biological application. The enzyme activity was monitored by measuring the oxygen consumption to examine it from a different aspect. L-DOPA was oxidized in the presence of even 1 mM of anisaldehyde, and the amount of dopachrome formed after 60 min was not significantly different from the control (Ha *et al*., 2005). Therefore, although the spectrophotometric method is a sensitive and convenient assay to search for tyrosinase inhibitors from natural sources, the anti-melanogenic action is not simply to inhibit tyrosinase but involves more complex biochemical reactions. Hence, the effects of anisaldehyde and related compounds on melanin production in cultured murine B16-F10 melanoma cells were examined.

Materials and Methods

General procedures

Images of murine melanoma cells were obtained using a Zeiss Axiovert 35 microscope (Carl Zeiss, Thornwood, NY, USA) with a 25 X lens, a Nikon 8008S camera (Nikon, Tokyo, Japan), and Image DV software. Cells were viewed in 96-well plates approx. 72 h after treatment with anisaldehyde.

Materials

Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), and the MTT cell proliferation assay kit were purchased from ATCC (Manassas, VA, USA). Anisaldehyde, anisic acid, benzaldehyde, 2,4-dimethoxybenzaldehyde, cuminaldehyde, arbutin, and L-DOPA were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Quercetin was available from our previous

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work (Kubo *et al.*, 2000). Dimethyl sulfoxide (DMSO) and L-tyrosine were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Cell culture

B16-F10 mouse melanoma cells (CRL-6475) and A375 human melanoma cells (CRL-1619) were obtained from ATCC and cultured in continuous log phase growth in DMEM containing 10% FBS. Cells were seeded in 96-well plates (2000 cells/well for B16 cells, 4000 cells/well for A375 cells) and incubated at 37 °C in 5% CO_2 atmosphere for about 24 h before chemical treatment. Each chemical was applied in duplicate with a final content of 0.1% DMSO, and treated cells were cultured for 72 h before assays.

Cell viability assays

Cell viability was determined by trypan blue exclusion using a hemocytometer. After overnight incubation of cells, viability was also determined by the MTT cell proliferation assay. Both bioassays basically provided the same results but the concentration leading to 50% viable cells lost (IC_{50}) was established by trypan blue assay for steady comparison purpose. The appropriate concentrations of the test chemicals were selected by microscopic observation of the preliminary cell viability assay using a Nikon Diaphoto TMD.

Trypan blue method

Cells were washed with PBS and dispersed with trypsinization, and an aliquot of the cells was mixed with a half volume of DMEM containing 10% FBS, and then mixed with trypan blue solution (final content 0.1%) at room temperature. Unstained cells (viable cells) were counted using a hemocytometer within 10 min after mixing with trypan blue solution.

MTT method

Cell viability was examined also by the MTT cell proliferation kit following the instructions of the manufacturer. MTT is reduced by mitochondrial dehydrogenases in living cells to a blue-magenta colored formazan precipitate. Briefly, cells were washed with PBS, and dispersed with trypsinization, and an aliquot of the cells was seeded in 96-well plates and incubated with DMEM containing 10% FBS at 37 °C in 5% CO_2 atmosphere for 16 to 24 h. At the end of the period, 10 μl of MTT reagent were added to each well, which was then incubated at 37 °C in 5% CO_2 atmosphere for 4 h. Then, 100 μl of detergent reagent were added to each well. The plate was kept at room temperature in the dark for 2 h, and relative amount of MTT reduction was determined based on the absorbance at 570 nm using a SpectraMax Plus spectrophotometer and SoftMax Pro software (Molecular Devices, Union City, CA, USA).

Melanin assay

The melanin content was determined as previously described (Kageyama *et al.*, 2004; Venkatasamy *et al.*, 2004) with minor modification. Cells were washed with PBS, harvested by trypsinization, and centrifuged for 10 min at $1500 \times g$. The cell pellets were then dissolved in 1.0 M NaOH containing 10% DMSO during 2 h incubation at 80 °C. Melanin content was measured at 475 nm using a SpectraMax Plus spectrophotometer and SoftMax Pro software (Molecular Devices).

Statistical analysis

The data were evaluated by either Student's or Welch's *t*-test after examining the variances using F test. $p < 0.01$ was considered to be statistically significant.

Results

The effect of anisaldehyde (**1**) (see Fig. 1 for structures) on the oxidation of L-DOPA catalyzed by mushroom tyrosinase was studied. Anisaldehyde showed a dose-dependent inhibitory effect on this oxidation. As the concentration of anisaldehyde increased, the enzyme activity was rapidly decreased but not completely suppressed. This indicates that anisaldehyde's inhibition of the enzyme is a reversible reaction with residual enzyme activity. The inhibitory concentration leading to 50% activity lost (IC_{50}) was estimated to be 0.40 mM. As long as the enzyme activity was monitored by measuring the dopachrome formation at 475 nm, anisaldehyde inhibited the oxidation of L-DOPA catalyzed by mushroom tyrosinase as a partial mixed type inhibitor without being oxidized. However, anisaldehyde did not inhibit this enzymatic oxidation at a longer reaction time (Ha *et al.*, 2005).

Tyrosinase is one of the key enzymes in melanin synthesis, but the inhibition of mushroom tyrosin-

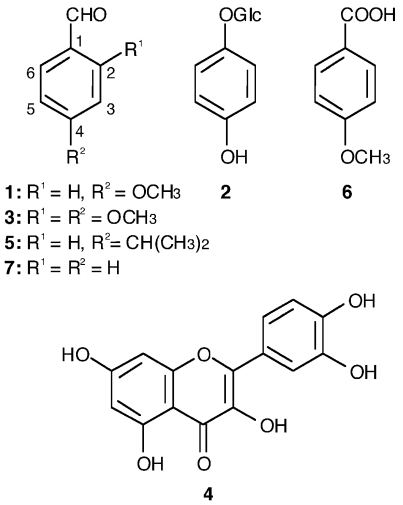

Fig. 1. Chemical structures of anisaldehyde (**1**) and its related compounds arbutin (**2**), 2,4-dimethoxybenzaldehyde (**3**), quercetin (**4**), cuminaldehyde (**5**), anisic acid (**6**), and benzaldehyde (**7**).

ase activity was reported not to correlate with that of cellular tyrosinase or with melanin production in cultured melanocytes (Maeda and Fukuda, 1996; Briganti *et al.*, 2003). On the other hand, arbutin (**2**), a well-known depigmenting agent, did not inhibit the tyrosinase-catalyzed oxidation of L-DOPA (Nihei and Kubo, 2003) but significantly suppressed the melanin formation in cultured murine melanoma cells. Hence, the experiment of anisaldehyde and related compounds was extended to examine their effects on the melanin production in cultured murine B16-F10 melanocytes. Arbutin was also tested as a reference for comparison. Cell viability was determined on the third day for melanocytes using both trypan blue and MTT assays, and basically the same results were observed. However, cell viability in the presence of anisic acid was estimated by microscopic observation. The specificity of melanogenesis inhibition was assessed by dividing the melanin content by the number of cells determined by trypan blue exclusion. The appropriate concentrations of the test chemicals were selected by microscopic observation of the preliminary cell viability assay. In the case of anisaldehyde, the highest concentration tested was 2 mM.

Anisaldehyde exhibited cytotoxicity in a concentration-dependent manner when murine B16 melanoma cells were cultured with this benzaldehyde derivative (Fig. 2A). The number of viable cells was significantly decreased at concentrations greater than 0.25 mM compared to control ($p < 0.01$). The concentration leading to 50% viable cells lost (IC_{50}) was established as 0.80 mM by trypan blue assay. Total melanin production was not suppressed but rather enhanced (Fig. 2B). Compared to control cells, the total melanin production was significantly increased at concentrations greater than 0.5 mM in a dose-dependent manner. At the microscopic level there was a significant increase in melanin pigmentation in anisaldehyde-treated cells. The pigmentation was observed as black spots. The total melanin production was slightly suppressed at concentrations in the range of 0.13–0.25 mM. However, since anisaldehyde exhibited cytotoxicity at this range, the observed action may be due, at least in part, to its melanocytotoxicity. The number of viable cells at 1 mM was less than 50% of the control but the total melanin production was more than 200%. In other words, cellular melanin production was enhanced more than 400% when the cells were cultured with 1 mM of anisaldehyde. Cellular melanin content showed a concentration-dependent increase up to 2 mM (Fig. 2C). Compared to control cells, the melanin content per cell increased 6.6-fold at 2 mM indicating a 560% increase.

On the other hand, 2,4-dimethoxybenzaldehyde (**3**) did not inhibit the oxidation of L-DOPA catalyzed by tyrosinase up to 0.5 mM. However, it exhibited potent cytotoxicity against B16 melanoma cells with IC_{50} of 0.070 mM, indicating that the aldehyde moiety seems requisite to interact with intracellular targets and that the adjacent methoxy group does not hinder this interaction. The precise explanation of melanin enhancing action observed with anisaldehyde still remains unknown, although many possibilities are conceivable (Maeda and Fukuda, 1996). For example, anisaldehyde might cause an increase in melanin production of cultured melanocytes mediated by increasing tyrosinase activity and decreasing other factors such as melanogenic inhibitors (Nagata *et al.*, 2004). In the case of arbutin, melanin production per cell was suppressed when the cells were cultured with arbutin up to 0.4 mM.

Since quercetin (**4**) was previously reported to enhance melanogenesis by increasing tyrosinase activity in human melanoma cells (Nagata *et al.*, 2004), this common flavonol was also examined using the same murine B16 melanoma cells for comparison. Similar to anisaldehyde, quercetin did

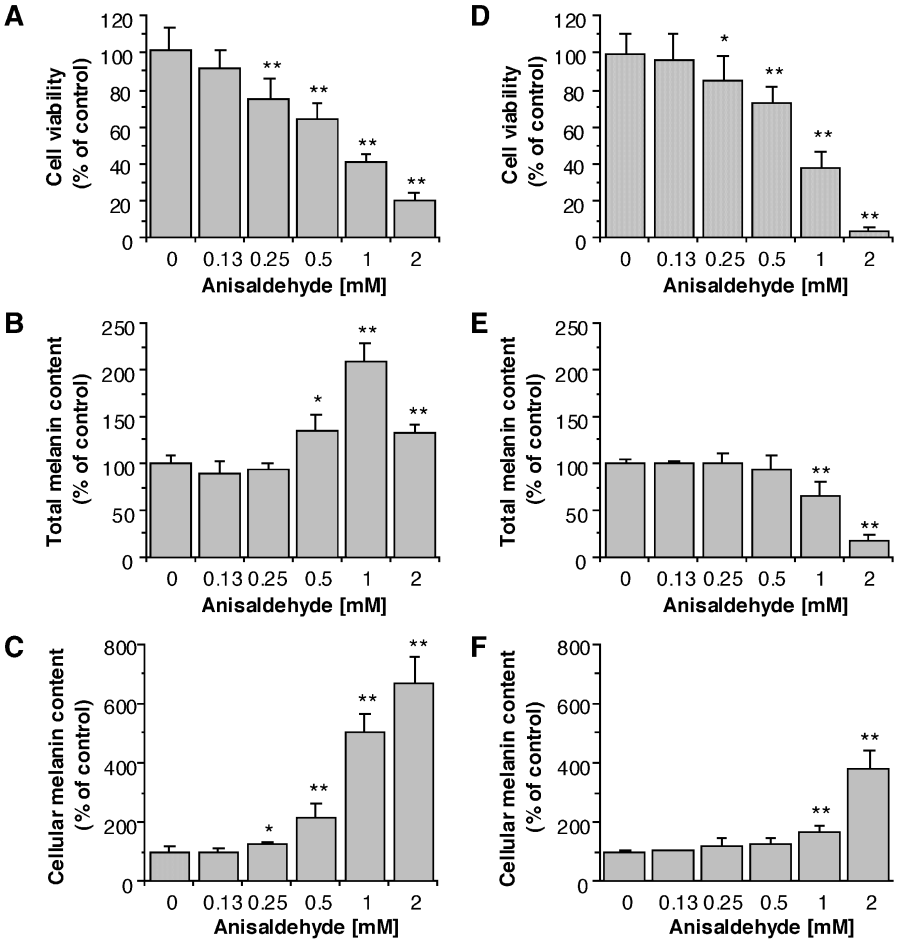

Fig. 2. (A, D) Viabilities of B16 (A) and A375 (D) melanoma cells following treatment with anisaldehyde for 72 h measured as described in 'Materials and Methods'; data are expressed as percentage of the number of viable cells observed with the control, and each column represents the mean ± S. D. of at least 6 determinations. (B, E) Total melanin content in B16 (B) and A375 (E) melanoma cells following treatment with anisaldehyde for 72 h measured as described in 'Materials and Methods'; data are expressed as percentage of melanin content per well observed with the control, and each column represents the mean ± S. D. of 4 determinations. (C, F) Cellular melanin content in B16 (C) and A375 (F) melanoma cells following treatment with anisaldehyde for 72 h measured as percentage of melanin content per cell observed with the control, and each column represents the mean ± S. D. of 4 determinations. The statistical significance of differences was evaluated using Student's or Welch's *t*-test. Significantly different from the control value: * $p < 0.05$; ** $p < 0.01$.

not suppress the cellular melanin production but rather increased it. The total melanin content at 20 μM was not different from that of the control, although this concentration showed a high melanocytotoxic effect. Melanin contents per cell were increased in a dose-dependent manner up to 40 μM when cells were cultured with quercetin (data not illustrated). As long as the melanin content per cell is compared, the activity of anisaldehyde is stronger than that observed for quercetin. Notably, the amount of total melanin production enhanced by anisaldehyde was always more than that of the control cells while that of quercetin was less.

The effects of cuminaldehyde (4-isopropylbenzaldehyde) (**5**) on melanin synthesis in the same cultured murine B16-F10 melanoma cells were also examined for comparison. Total melanin production was suppressed when the cells were cultured with cuminaldehyde in a dose-dependent decrease up to 0.25 mM without affecting the cell

growth. However, cuminaldehyde became cytotoxic at 0.5 mM, indicating that the observed action at 0.5 mM may be due, at least in part, to its melanocytotoxicity. In other words, cuminaldehyde was not cytotoxic up to 0.25 mM in B16 melanocytes, but almost complete lethality was observed at 1 mM (data not illustrated). The IC_{50} was established as 0.70 mM. The results of quantitative measurement of the melanin content per cell showed a concentration-dependent decrease up to

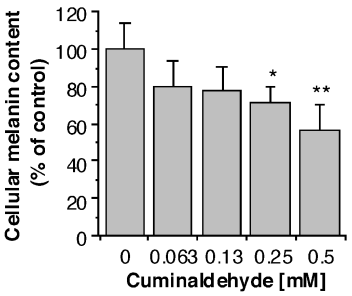

Fig. 3. Cellular melanin content in B16 melanoma cells following treatment with cuminaldehyde for 72 h measured as percentage of melanin content per cell observed with the control, and each column represents the mean ± S. D. of 4 determinations. The statistical significance of differences was evaluated using Student's *t*-test. Significantly different from the control value: * $p < 0.05$; ** $p < 0.01$.

0.50 mM (Fig. 3). The data obtained suggests that the melanin synthesis inhibitory activity of cuminaldehyde on cultured melanocytes is a result of the inhibition of melanin synthesis but not a melanocytotoxic effect up to 0.25 mM. The cellular melanin formation inhibitory activity of cuminaldehyde observed was almost comparative with that of arbutin. As long as the cell-free spectrophotometric assay data are compared, the results obtained with cuminaldehyde (Kubo and Kinst-Hori, 1998b) are similar in pattern to those of anisaldehyde (Kubo and Kinst-Hori, 1998a), but their effects on the melanin synthesis in cultured melanoma cells are markedly different, thus indicating the different effects of methoxy and isopropyl groups at the *para*-position. It appears that a small change in the chemical structure affects the biological activity to a large extent.

The effects of anisaldehyde on human A375 melanoma cells were also examined for comparison. In the case of human melanoma cells, anisaldehyde exhibited a dose-dependent cytotoxicity up to 2 mM (Fig. 2D). The IC_{50} was established as 0.82 mM, and almost complete lethality was observed at 2 mM. Total melanin production was suppressed above 0.5 mM (Fig. 2E). At 1 mM of anisaldehyde, there was approx. 40% suppression of total melanin production. However, the amounts of melanin suppression were always less than that of cytotoxicity by anisaldehyde, suggesting that, along with a melanocytotoxic effect, anisaldehyde can also enhance melanogenesis in the remaining viable cells. This effect of anisaldehyde was visible in the range of 0.25–2 mM, for which the melanin contents per cell were noted to be significantly enhanced compared to control ($p < 0.01$) (Fig. 2F). The enhancement of melanin synthesis by anisaldehyde at higher concentrations was less, however, in human melanoma than in mouse melanoma cells.

Anisic acid (**6**) did not show noticeable cytotoxicity up to 8 mM against the same cultured B16 melanoma cells judged by microscopic examination, but almost complete lethality was observed at 16 mM. This can be explained by the fact that deprotonated acid does not enter into cells through the membrane. The process by which drugs penetrate into cells is usually neglected in cell-free experiments. It may not be illogical to assume that anisic acid did not affect melanin formation because it could not enter into cells. Because of its inferior cytotoxicity, further experiments were not carried out.

On the other hand, the aldehyde moiety alone is not enough to elicit melanogenesis enhancing activity since benzaldehyde (**7**) did not increase the melanin formation when the cells were cultured with benzaldehyde up to 2 mM, indicating that the methoxy group at the *para*-position is requisite to elicit the activity. Benzaldehyde showed no noticeable cytotoxicity up to 2 mM in the cultured melanoma cells. Anisaldehyde may enter the cell by passive diffusion across the plasma membrane. Once inside the cells, in general terms aldehydes react with amino groups forming Schiff bases or imino compounds. In particular, cross-linkages may form in proteins. Reversible reactions with proteins and deactivation of enzymes, which primarily cause inhibition of the protein biosynthesis, are of concern. Hydrophobic interaction with the binding cavity of enzymes significantly affects the binding affinity.

Discussion

The skin sensitization potential of various aldehydes in relation to the development of structure and activity relationships based on consideration of their chemistry was previously reported. A range of 17 aldehydes was evaluated for which differing reaction mechanisms were suspected, and a QSAR defined for a subset of these where the mode of action was considered to be the same. According to the conclusion described, the aryl aldehydes, benzaldehyde and anisaldehyde, are nonsensitizing in the murine LLNA (Patlewicz *et al.*, 2001). Despite this advantage, biological impact of anisaldehyde in melanogenesis is still largely unknown, but it is a potential candidate for the treatment of a dermatological disease such as vitiligo (Gauthier *et al.*, 2003). On the other hand, if the adverse effect of anisaldehyde is also producible in human melanocytes, it would be useful in a different sense since melanins play a crucial role in the absorption of free radicals generated within the cytoplasm and in shielding the host from various types of ionizing radicals, including ultraviolet (UV) irradiation. This role unfortunately seems destined to become even more critical due to the alarming predicted increases in incident UV radiation at the Earth's surface by recent stratospheric ozone depletion. Thus, anisaldehyde is capable of protecting from human skin damage (Shoji *et al.*, 1997), for example by UV irradiation (Eller *et al.*, 1994). The same concept may be applicable to develop an anti-white hair agent, although hair pigmentation involves complex biochemical reactions (Fitzpatrick *et al.*, 1967; Commo *et al.*, 2004). The fact that anisaldehyde was characterized from many edible plants as a flavor substance should cause it to be superior compared to nonnatural melanogenesis enhancing agents.

Although the precise explanation for how anisaldehyde interacts with enzymes on a molecular basis is still largely unknown, formation of a Schiff base with a primary amino group in the enzymes is plausible since the aromatic nucleus is known to stabilize it by conjugation. Thus, anisaldehyde may form a Schiff base with a primary amino group in the enzymes rather than binding to the active site. The Schiff base is largely governed by those factors affecting the stability of the carbon-nitrogen double bond. The introduction of an electron-donating methoxy group at the *para*-position should stabilize the Schiff base and increase the inhibitory activity. It may not be illogical to assume that the primary amino group plays an important role in the tertiary structure of cellular enzymes such as tyrosinase, being involved with hydrogen bonding that is essential to maintain the tertiary structure of the enzymes. The low conformational stabilities of native proteins make them easily susceptible to denaturation by altering the balance of the weak nonbonding forces that maintain the native conformation. Native proteins form a sort of intramolecular micelle in which the nonpolar Schiff base portion is largely out of contact with the water-based test solution. Anisaldehyde should disrupt this interaction and form a Schiff base with the freed amino group, giving rise perhaps to its nonspecific cytotoxicity.

As long as tyrosinase inhibition data obtained by cell-free spectrophotometric experiments are compared, anisaldehyde (Kubo and Kinst-Hori, 1998a) and cuminaldehyde (Kubo and Kinst-Hori, 1998b) are nearly identical. The spectrophotometric method is a sensitive and convenient assay to search for tyrosinase inhibitors from natural sources. However, it is evident that inhibition of mushroom tyrosinase activity is not well correlated to melanin production in cultured B16-F10 melanoma cells. The melanin formation inhibitory action is not simply to inhibit tyrosinase but involves more complex biochemical reactions. The relevance of *in vitro* experiments in simplified systems should be carefully considered. The results so far obtained indicate that their further evaluation is needed from not only one aspect, but from a whole and dynamic perspective.

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