

## 4,4'-Dihydroxybiphenyl as a New Potent Tyrosinase Inhibitor

You Jung KIM,<sup>a,1)</sup> Jae Kyung NO,<sup>b,1)</sup> Ji Hyeon LEE,<sup>b</sup> and Hae Young CHUNG<sup>\*,b</sup>

<sup>a</sup>Department of Cosmetology, Pusan Women's College; Busanjin-Gu, Busan, 607–843, Korea; and <sup>b</sup>Department of Pharmacy, Pusan National University; Kumjeong-Gu, Busan, 609–735, Korea.

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The color of mammalian skin is determined by many factors, for which visible ones are the degree and distribution of melanin pigmentation. Because tyrosinase, (polyphenol oxidase) is the key enzyme for melanin biosynthesis, the use of various tyrosinase inhibitors is a common practice for whitening purpose in cosmetics. In the present study, the inhibition of tyrosinase by 4,4'-dihydroxybiphenyl (44'-BP) was investigated. In addition to tyrosinase inhibiting activity, melanin biosynthesis was assessed in B16F10 melanoma cells (B16 cells). The results showed that 44'-BP exhibits a strong anti-tyrosinase activity with  $IC_{50}=1.91\ \mu M$ . The kinetic analysis of tyrosinase inhibition revealed that 44'-BP acts a competitive inhibitor ( $K_i=4.0\times 10^{-4}\ M$  at  $2.5\ \mu M$  and  $K_i=2.1\times 10^{-5}\ M$  at  $5\ \mu M$ ). Furthermore, data on melanin biosynthesis indicated that the amount of melanin was clearly suppressed by 44'-BP.

**Key words** 4,4'-dihydroxybiphenyl; anti-tyrosinase activity; melanin; B16F10 melanoma cell

Tyrosinase (monophenol, dihydroxy-L-phenylalanin: oxygen oxidoreductase, EC 1.14.18.1) is a copper-dependent protein widely distributed in nature.<sup>2)</sup> The enzyme catalyzes two different reactions: the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity), both utilizing molecular oxygen.<sup>3)</sup>

The enzymatic browning of fruits and vegetables is mostly related to the oxidation of endogenous phenolic containing compounds catalyzed by polyphenol oxidase.<sup>4)</sup> Tyrosinase also is responsible for melanization in animals, and is the key enzyme for the regulation of melanogenesis in mammals.<sup>5)</sup>

Melanin is synthesized in epidermal melanocytes, and then is transferred into epidermal keratinocytes via the melanocytes' dendrites.<sup>6)</sup> Melanogenesis is the process by which melanin is produced (and subsequently distributed) by melanocytes within the skin and hair follicles.<sup>7)</sup> This process results in the synthesis of melanin pigments, which plays a protective role against skin photocarcinogenesis.<sup>8)</sup> The main physiological stimulus of melanogenesis is the ultraviolet radiation of solar light, which can act directly on melanocytes or indirectly through the release of keratinocyte-derived factors such as MSH ( $\alpha$ -melanocyte stimulating hormone).<sup>9)</sup>

One of the biggest causative agents of hyperpigmentation is probably UV light.<sup>10)</sup> However, skin darkening can be suppressed, at least partially, by deactivating of tyrosinase.<sup>11)</sup> Therefore, tyrosinase inhibitors have become increasingly important in the cosmetic and medicinal products used in the prevention of hyperpigmentation.<sup>12)</sup> Many compounds, such as hydroquinone,<sup>13)</sup> kojic acid,<sup>14)</sup> and benzaldehyde-*O*-alkyl-oximes<sup>15)</sup> have been reported as tyrosinase inhibitors.

The established murine B16F10 melanoma cell (B16 cells) line offers a model system with readily quantifiable markers that are characteristic of differentiating, including melanogenesis.<sup>16)</sup> In B16 cells,  $\alpha$ -tocopheryl ferulate was shown to have depigmenting effect.<sup>17)</sup> Although 4,4'-dihydroxybiphenyl (44'-BP), a bisphenol derivative was known as a radical scavenger for methacrylate polymerization,<sup>18)</sup> but its efficacy on tyrosinase inhibition and melanin biosynthesis has not been reported.

The aim of this study was to characterize inhibition mode of 44'-BP on mushroom tyrosinase. We also examined the inhibitory effect of 44'-BP on melanin biosynthesis, and cell viability in B16 cells as a biomarker for the potential cytotoxicity.

### MATERIALS AND METHODS

**Materials** Mushroom tyrosinase, L-tyrosine, 44'-BP, MSH and all other chemical reagents were purchased from Sigma Chemical Co. (St. Louis, U.S.A.).

**Cell Culture** B16 cells (from Korean Cell Line Bank) were cultured in DMEM with 10% fetal bovine serum (FBS; Gibco) and penicillin/streptomycin (100 IU/50  $\mu g/ml$ ) in a humidified atmosphere containing 5% CO<sub>2</sub> in air at 37 °C. B16 cells were cultured in 24-well plates for the melanin quantification and enzyme activity assays.

**Determination of 44'-BP for Cell Viability** The cell proliferation assay was carried out by the method of Tada *et al.* using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co., U.S.A.).<sup>19)</sup> Briefly,  $5\times 10^4$  cells/well were plated in a 24-well plate. After cells were exposed to 44'-BP at concentrations of 50, 100, 200, and 400  $\mu M$  for 24 h, MTT solutions were added and the insoluble derivative formed by cellular dehydrogenase was solubilized with EtOH-DMSO (1:1 mixture solution); the absorbance of each well was read at 560 nm using a microplate reader. The optical density (O.D.) of formazan formed by control cells was taken as a 100% enzymatic assay for tyrosinase activity. Results are shown from three determinations.

**Assay on Tyrosinase Activity** A) Mushroom tyrosinase was used in this study as the tyrosinase source. Tyrosinase activity was determined as described previously with a minor modification.<sup>20)</sup> Briefly, 20  $\mu l$  of aqueous solution of mushroom tyrosinase (1000 units) was added to a 96-well microplate (Nunc, Denmark), in a total volume of 200  $\mu l$  assay mixture containing 1 mM L-tyrosine solution, and 50 mM phosphate buffer (pH 6.5). The assay mixture was incubated at 25 °C for 30 min. Following incubation, the amount of

\* To whom correspondence should be addressed. e-mail: hyjung@pusan.ac.kr

dopachrome produced in the reaction mixture was determined spectrophotometrically at 492 nm ( $OD_{492}$ ) in a microplate reader (Hewlett Packard).  $IC_{50}$  refers to the concentration of a substance that inhibits a standard response by 50% of the activity. In the present experiments, the  $IC_{50}$  values were derived from the X-axis of the plot, and to determine the  $IC_{50}$ , dose-dependent inhibition experiments were performed in triplicate. We calculated individual  $IC_{50}$  when Y-axis revealed 50% of the inhibition percentage.

B) Tyrosinase activity in B16 cells was estimated by measuring the rate of oxidation of L-DOPA.<sup>21)</sup> Cells were plated in 24-well dishes at a density of  $5 \times 10^4$  cells/ml. B16 cells were incubated in the presence or absence of 100 nM MSH and then treated for 24 h at various concentrations (10–200  $\mu$ M) of 44'-BP. The cells were lysed in 100  $\mu$ l of 50 mM sodium phosphate buffer (pH 6.8) containing 1% Triton X-100 (Sigma) and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and then frozen at  $-80^\circ\text{C}$  for 30 min. After thawing and mixing, cellular extracts were clarified by centrifugation at 12000 rpm for 30 min at  $4^\circ\text{C}$ . The supernatant (80  $\mu$ l) and 20  $\mu$ l of L-dopa (2 mg/ml) were placed in a 96-well plate, and the absorbance at 492 nm was read every 10 min for 1 h at  $37^\circ\text{C}$  using an ELISA plate reader. The final activity was expressed in  $\Delta OD/\text{min}$  for each condition. The experiments were performed three times.

#### Kinetic Analysis on Tyrosinase Inhibition by 44'-BP

Various concentrations of L-tyrosine (0.25 to 4 mM) as substrate, 20  $\mu$ l of aqueous solution of mushroom tyrosinase (1000 U/ml), and 50 mM potassium phosphate buffer (pH 6.5), with or without test sample (2.5  $\mu$ M and 5  $\mu$ M of 44'-BP), were added to a 96-well plate in a total volume of 200  $\mu$ l for the assay mixture. The initial rate of dopachrome formation from the reaction mixture was determined as the increase of absorbance at wavelength 492 nm per min ( $\Delta OD_{492}/\text{min}$ ) by using a microplate reader. The Michaelis constant ( $K_m$ ) and maximal velocity ( $V_{\text{max}}$ ) of the tyrosinase activity were determined by Lineweaver-Burk's plot using various concentrations of L-tyrosine substrate, as was utilized in our earlier study.<sup>20)</sup> The reaction kinetics required a modification of the Michaelis-Menten equation due to competitive inhibition by 44'-BP together with substrate inhibition by L-tyrosine. Results are shown from three experiments.

**Determination of Melanogenesis in B16 Cells** Determination of melanin content was performed using a modified method of Bilodeau *et al.*<sup>22)</sup> The amount of melanin was used as an index of melanogenesis in the current study. The B16 cells ( $5 \times 10^4$ ) were plated on 24-well multi-dishes and incubated in the presence or absence of 100 nM MSH. The cells were then incubated for 24 h with or without 44'-BP at concentrations ranging from 10 to 200  $\mu$ M. After washing twice with PBS, samples were dissolved in 100  $\mu$ l of 1 N NaOH. The samples were incubated at  $60^\circ\text{C}$  for 1 h and mixed to solubilize the melanin. Absorbance at 405 nm was compared with a standard curve of synthetic melanin. The experiments were performed three times.

**Statistical Analysis** The inhibitory effects of 44'-BP on the tyrosinase activity were expressed as a percent of inhibition based on:  $100 - [(A \cdot 100)/B]$ , where  $A = OD_{492}$  with test sample and  $B = OD_{492}$  without test sample. Data were collected as mean  $\pm$  standard error ( $n=3$ ), and the biological significance  $p < 0.05$  was determined by the Student's *t*-test.

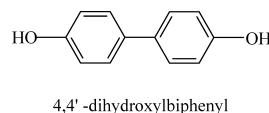


Fig. 1. Chemical Structure of 44'-BP

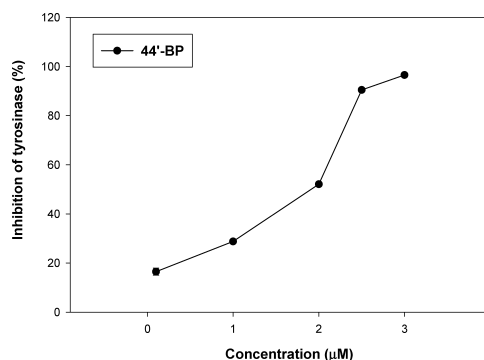


Fig. 2. Dose-Dependent Inhibitory Effects of 44'-BP (●) on Tyrosinase Activity

Tyrosinase activity was measured using L-tyrosine as the substrate. Values are mean  $\pm$  S.E. of  $n=3$  determination. O.D. (optical density) value of control at 492 nm was  $0.486 \pm 0.007$ .

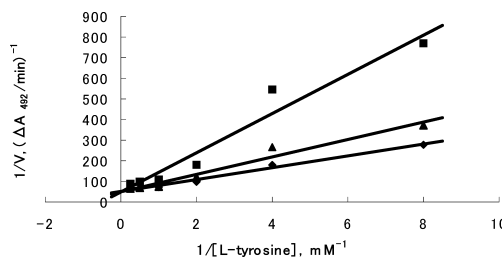


Fig. 3. Lineweaver-Burk Plot of Mushroom Tyrosinase in the Presence of 44'-BP

Data were obtained as mean values of  $1/V$ , inverse of the increase of absorbance at a wave-length 492 nm per min ( $\Delta A_{492}/\text{min}$ ), of three independent tests with different concentrations of L-tyrosine as a substrate. Inhibitors of the enzyme were with 44'-BP 5  $\mu$ M (rectangle), 2.5  $\mu$ M (triangle) and no 44'-BP (circle). The modified Michaelis-Menten equation is:  $1/V_{\text{max}} = 1/K_m (1 + [I]/K_i)$ .  $V$ =velocity of the reaction,  $S$ =L-tyrosine concentration,  $K_i$ =inhibitor constant.

## RESULTS

To know more about 44'-BP for its chemistry, the structure formula of 44'-BP is shown in Fig. 1. And Fig. 2 shows the results of the inhibition of tyrosinase activity by 44'-BP as monitored by a spectrophotometric assay. Data show that the inhibitory potency of 44'-BP on mushroom tyrosinase was  $16.48 \pm 1.46\%$  at 0.1  $\mu$ M,  $28.78 \pm 0.45\%$  at 1  $\mu$ M,  $52.10 \pm 0.65\%$  at 2  $\mu$ M,  $90.48 \pm 0.30\%$  at 2.5  $\mu$ M and  $96.53 \pm 0.26$  at 3  $\mu$ M; indicating a dose-dependent inhibition of tyrosinase activity by 44'-BP. It also showed the inhibitory action on oxidation of L-DOPA by mushroom tyrosinase with an  $IC_{50}$  of 1.91  $\mu$ M. The mode of inhibition of the enzyme was determined by Lineweaver-Burk plot analysis as shown in Fig. 3, in which the inhibitory kinetics exhibited  $K_i = 4.0 \times 10^{-4}$  M at 2.5  $\mu$ M and  $K_i = 2.1 \times 10^{-5}$  M at 5  $\mu$ M. 44'-BP at 2.5 and 5  $\mu$ M showed the same  $V_{\text{max}}$  value of 49.368 and  $K_m$  values of  $8.5 \times 10^{-7}$  M and  $1.96 \times 10^{-6}$ , respectively.

The results from the cell viability assay using MTT for

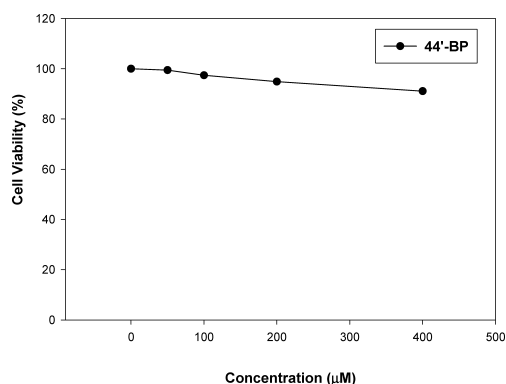


Fig. 4. The Effect of 44'-BP on the Viability of B16 Cells

Cells were treated with various doses of 44'-BP (50–400 μM) and were examined by MTT assay. Data are expressed as % of cell viability.

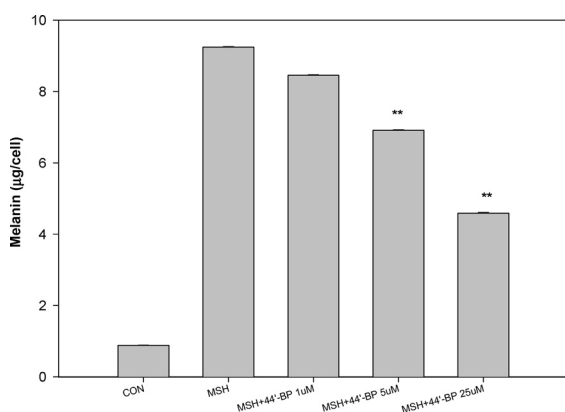


Fig. 5. Inhibitory Effect of 44'-BP in the Presence of Treatment of 100 nM MSH in B16 Cells

Melanin contents were measured at 405 nm. Data are expressed as μg/well and each column represents the mean ± S.E. of three determinations. Asterisks indicate a significant difference between MSH group and MSH plus 44'-BP groups, \*\* $p < 0.01$ .

B16 cells are given in Fig. 4. At growth doses of 50, 100, 200, and 400 μM/ml of 44'-BP, cell viability was 99.4%, 97.4%, 94.8%, and 91.0% in 24 h treatments. These data clearly showed non-cytotoxic nature of 44'-BP in B16 cells. To gain evidence on 44'-BP's involvement in tyrosinase and melanogenesis, the inhibition of melanin production in B16 cells was examined. As shown in Fig. 5, the melanin content of the cells that were treated with 44'-BP decreased. Melanin levels in treated B16 cells decreased in dose-dependent manners, showing 8.5 μg/cell at 1 μM, 6.9 μg/cell at 5 μM, and 4.6 μg/cell at 25 μM compared to the MSH treated group (9.2 μg/cell). In addition, we investigated the inhibitory effect of 44'-BP, on tyrosinase activity of B16 cells treated with the MSH (Fig. 6). After 24 h incubation with 44'-BP tyrosinase activities were suppressed to 144.4% at 10 μM, 137.2% at 25 μM, 92.6% at 50 μM, 55.4% at 100 μM, and 16.9% at 200 μM compared to MSH treated group (164.4%), which correlates well with the inhibitory effect on melanin content.

## DISCUSSION

Melanin pigmentation protects the tissue from absorption and dissipation of UV light,<sup>23,24</sup> and epidermal melanin syn-

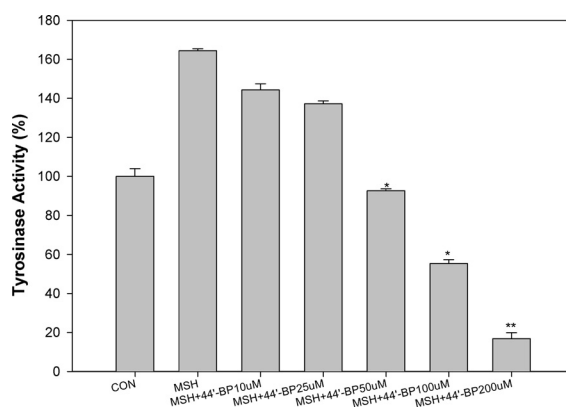


Fig. 6. Inhibitory Effect of 44'-BP in the Presence of Treatment of 100 nM MSH in B16 Cells

B16 cells were treated with various doses of 44'-BP (10–200 μM) for 24 h. The cells were harvested and measured tyrosinase activity. Absorbance at 492 nm was read every 10 min for 1 h at 37 °C using on ELISA plate reader. Data are expressed % of control and each column represents the mean ± S.E. of three determinations. Asterisks indicate a significant difference between MSH group and MSH plus 44'-BP groups, \* $p < 0.05$ ; \*\* $p < 0.01$ .

thesized by melanocytes, resulting from a cascade of enzymatic reactions.<sup>25</sup> is responsible for skin darkening. However, overproduction or dysregulation of melanin pigments can cause skin hyperpigmentation disorders such as freckles, melasma, ephelides, senile lentigo, and age spots.<sup>26</sup>

Tyrosinase has long been known to be essential for melanization. In vertebrates, tyrosinase (EC 1.14.18.1), the enzyme responsible for the initial steps of melanin synthesis, is closely associated with specialized organelles, melanosomes that are found in melanocytes.<sup>27</sup> Although melanin levels vary in the human population, the expression of tyrosinase does not vary significantly among human skin colors, and the levels of tyrosinase mRNA were found to be similar in cultured melanocyte system collected from black and white skin.<sup>28</sup>

The inhibition of tyrosinase has been the subject of numerous studies,<sup>29–31</sup> and the several inhibitors are used as cosmetic additives and medicinal products in the treatment of hyperpigmentation. A recent ever-growing global market expansion demands more for new products for depigmenting, cosmeceutical and skin lighting purposes.<sup>32</sup> The most popular whitening agent has been hydroquinones since the introduction in 1961, but their use were curtailed as cosmetic ingredients because of the adverse cutaneous toxicity.<sup>29</sup> Additionally, several other phenolic compounds have been studied as depigmenting agents as their chemical structure is related to tyrosinase inhibitory activity. It has been suggested that the presence of hydroxylic group and of an electron donor group in position para is a primary requirement for an effective action as alternative substrate of tyrosinase.<sup>30</sup> During our continuous effort on a search for potent tyrosinase inhibitors, we found 3,4-dihydroxyacetophenone that has a highly effective inhibitory activity against and tyrosinase melanin synthesis, which is more potent than a well known kojic acid (manuscript in preparation). In the present study, we compared tropolone and arbutin,<sup>31</sup> which are currently marked as skin lightening compounds with 44'-BP on tyrosinase inhibition, and found that 44'-BP indicated stronger inhibitory effect than tropolone and arbutin (data not shown).

The action of 44'-BP as a radical scavenger has been re-

ported by investigating kinetic property of 2,2'-azobisisobutyronitrile (AIBN)- and benzoyl peroxide (BPO)-induced methyl methacrylate (MMA) polymerization, in which the polymerization was found to suppress as free radicals scavenged by phenolic moiety decreased.<sup>18)</sup> However, at present, other bioactivities of 44'-BP are not well known, and to date, the inhibitory effects of this radical scavenging 44'-BP on tyrosinase activity have not been reported.

In the present study, we investigated measuring the effect of 44'-BP on tyrosinase inhibition in a hope to find a new effective substance for skin whitening purpose and the prevention of hyperpigmentation, and found that 44'-BP clearly reduced tyrosinase activity in a dose dependent manner. A kinetic study of the inhibition of mushroom tyrosinase by 44'-BP showed that this substance behaves as a classical competitive inhibitors, is evaluated by a kinetic analysis of inhibition with  $K_i=4.0 \times 10^{-4}$  M at 2.5  $\mu$ M and  $K_i=2.1 \times 10^{-5}$  M at 5  $\mu$ M, without influencing  $V_{max}$ .

In general, this competitive inhibition may be related in part to the similarity between the dihydroxyphenyl group in L-dopa and the hydroxyphenyl group in 44'-BP.<sup>33)</sup> In our study, 44'-BP showed potent inhibitory activity against mushroom tyrosinase; the concentration causing 50% inhibition ( $IC_{50}$ ) was 1.91  $\mu$ M. Low  $IC_{50}$  indicates that the potency is significantly higher than that of kojic acid (22.16  $\mu$ M), a well known inhibitor of tyrosinase.<sup>34)</sup>

Considering the possible cytotoxic effect, it would be interesting to know exactly how much of 44'-BP actually able to permeate into the cells to elicit the inhibition of tyrosinase. Although we have no quantitative information about the permeability of 44'-BP in the present study, based on what we see on the inhibition of tyrosinase in B16 cells, a sufficient amount is expected to permeate through the membrane to cause the inhibition. If 44'-BP were freely permeable, it might be toxic, but in fact as we have shown, the cytotoxicity is minimal, yet it was able to suppress melanogenesis (see below).

In B16 cells cultured system, we found tyrosinase activity and melanin level were significantly reduced by the addition of 44'-BP to the incubation medium containing cells pretreated with MSH. 44'-BP effectively inhibited tyrosinase of B16 cells. It is worth pointing out that 44'-BP exhibited a stronger effect on mushroom tyrosinase activity as compared to murine tyrosinase activity. One would question on the plausible reason on reduced tyrosinase activity in cell lysates, and several factors may be involved. Included are damaged tyrosinase due to cell lysis, conformational changes of the enzyme structure, or the altered inhibitor-enzyme interaction, just name a few.

Treatment of B16 cells with 44'-BP significantly inhibited melanin formation. Less amount of melanin was formed when treated with 44'-BP at various doses (1–25  $\mu$ M) for 24 h, when induced by 100 nM MSH. The melanin content decreased in a dose-dependent manner by 44'-BP treatments. As shown in Fig. 5, it is known that MSH-induced melanogenesis in mouse melanoma cells occurs by an increase of intracellular cyclic AMP content, an accumulation of tyrosinase mRNA, and a stimulation of tyrosinase activity.<sup>35)</sup> Diarylheptanoids from *Alnus hirsute* Turcz showed melanogenesis inhibitory activities in B16 cells,<sup>36)</sup> and cucurbitacins from *Trichosanthes kirilowii* showed inhibitory effects for tyrosi-

nase activity and melanin synthesis in B16 cells.<sup>37)</sup> These inhibitory effects reported by others in B16 system were similar to what found the current study.

As a potential desirable skin lightening agent, 44'-BP should be safe without adverse side effect. Results from the cell viability test presented in Fig. 4 show that 44'-BP suppresses tyrosinase activity and total melanin content without causing an adverse effect on cell viability. These data suggest that 44'-BP has a strong depigmenting activity without any discernable cytotoxicity. Based with the current data, we conclude that 44'-BP is a potent tyrosinase inhibitor that can be considered for useful skin lightening purpose.

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