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Gamma- and delta-tocotrienols inhibit skin melanin synthesis by suppressing constitutive and UV-induced tyrosinase activation

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Dear Sir,

Cutaneous pigmentation provides an important protective mechanism against harmful ultraviolet radiation. In the body, the formation of pigment melanin occurs within the melanosomes of skin melanocytes (Fitzpatrick et al., 1950). This process is regulated by melanogenic enzymes such as tyrosinase and tyrosinase-related protein 1/2 (TRP1/2) (Chen and Chavin, 1966). Specifically, these proteins catalyze the rate-limiting, two-part reaction in melanin biosynthesis: the hydroxylation of L-tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and its subsequent oxidation to dopaquinone (Korner and Pawelek, 1982). The modulation of tyrosinase activity therefore represents a key process for the regulation of cutaneous pigmentation. In addition, considering that cutaneous pigmentation is a hallmark of melanin-generating melanoma disease, the control of tyrosinase activity may provide a basis for treating patients with this type of cancer. A number of biochemical agents are known to either stimulate (melanocyte-stimulating hormones and UVB rays) or inhibit (kojic acid and sodium lactate) melanogenesis in cultured melanoma cell lines.

Tocotrienols (T3) are important plant vitamin E constituents which provide antioxidant activity for all living cells. The common volatile hydroxyl group in T3 and tocopherols (TP) acts to scavenge the chain-propagating peroxyl free radicals. Depending on the level of methylation on the chromanol ring, T3 can be distinguished into four isomeric forms: alpha (α), beta (β), gamma (γ) and delta (δ). Recently, δT3 has been found to decrease melanin levels in murine B16 melanoma cells by inhibiting the oxidative reactions of tyrosinase (Michihara et al., 2009). In another study (Makpol et al., 2009), the palm αT3-rich fraction was also shown to suppress tyrosinase activity in primary human skin melanocytes. However, it should be noted that the experimental data might have been misinterpreted, because the palm tocotrienol-rich fraction (TRF) is known to consist of 75% T3, rather than αT3 as claimed by the authors.

To extend our current understanding of T3 and tyrosinase, we aimed to investigate whether other T3 isomers also inhibit tyrosinase activity in murine and human melanoma cell lines. In addition, our study explores the synergistic interaction of T3 with tyrosinase inhibitors and UVB-induced tyrosinase activation.

Our results show that, with the exception of αT3, the other T3 isomers inhibited the proliferation rate of B16 melanoma cells in a dose-dependent fashion (Supporting Information Figure S1). Among the tyrosinase inhibitors investigated, kojic acid was found to produce an anti-proliferation effect at a concentration of ≥35 mM. Because the anti-melanogenesis effect investigated herein was independent of the anti-proliferation effect, we chose to use the treatment dosage that did not affect the cell proliferation rate in subsequent experiments.

To study the dose response of tyrosinase suppression, B16 melanoma cells were treated with an increasing dosage of palm TRF isomers. Figure 1A shows that a low dose of γ- and δT3 induced significant suppression of tyrosinase in a dose-dependent manner. A similar inhibitory effect was observed for γT3 using human melanoma cell lines (A375 and WM793B). In addition, the treatment of B16 melanoma cells with 20 μM of a palm TRF mixture and its acetate also resulted in consistent suppression of tyrosinase protein expression (Figure 1A), suggesting that palm TRF acetate had been absorbed and hydrolyzed by B16 melanoma cells to the form of native palm TRF (Brisson et al., 2008), thus exerting the inhibitory effect on the tyrosinase gene. Alternatively, the results also suggest that the anti-melanogenesis effect may be unrelated to the antioxidant properties of γ- and δT3, but could be associated with the unsaturated isoprenoid side chain. In contrast, 20 μM treatments with kojic acid and sodium lactate did not result in observable down-regulation of the tyrosinase protein levels. Using a higher dose of kojic acid and sodium lactate (≥3.5 mM), however, led to significant inhibition of tyrosinase protein expression. Similar inhibition of tyrosinase protein expression was also observed for retinoic acid at a treatment concentration of 1.6–16.6 nM (Figure S2).

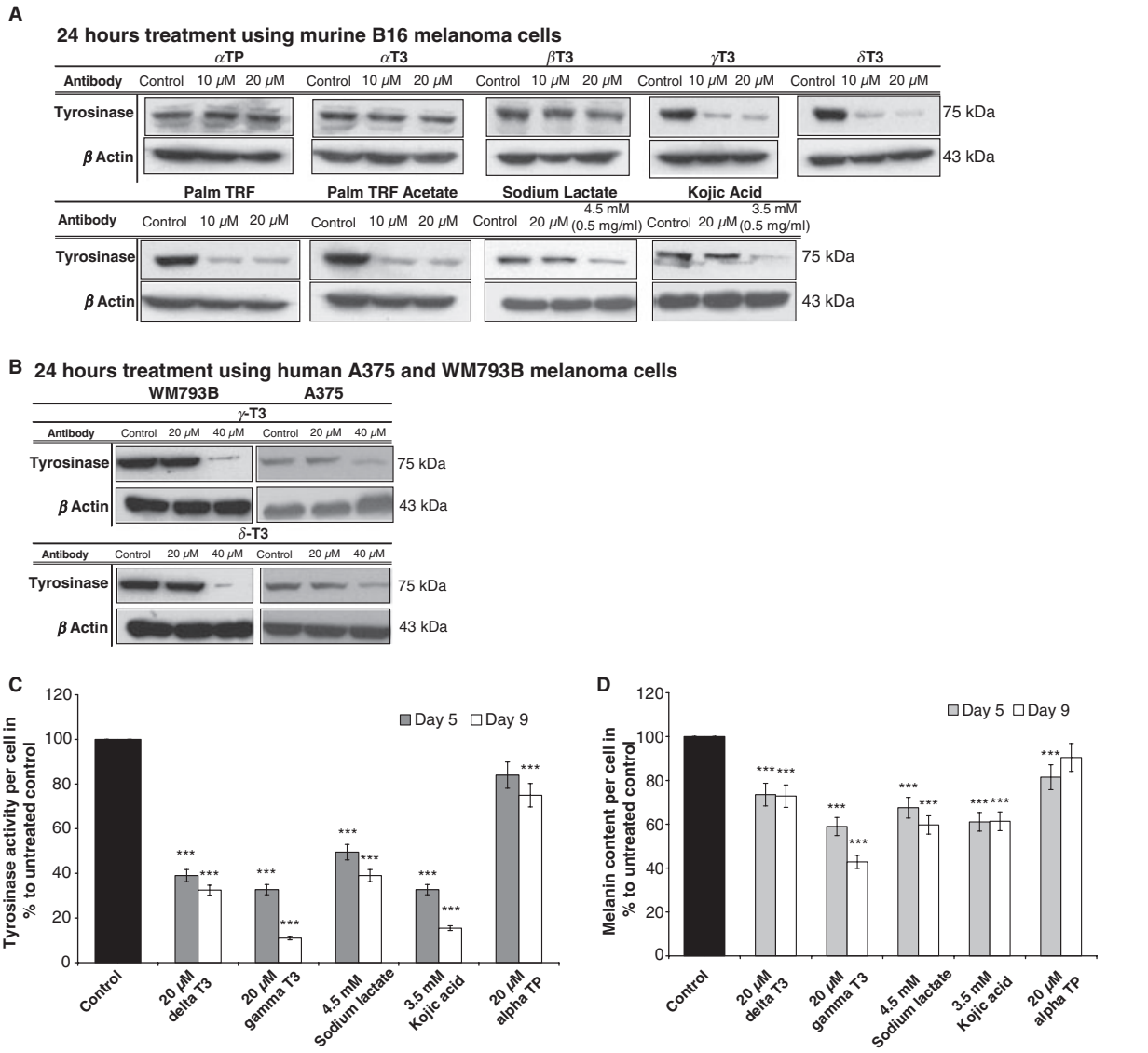

Figure 1. (A) Treatment of B16 cells with γ- and δT3, palm tocotrienol-rich fraction (TRF), TRF acetate, sodium lactate and kojic acid for 24 h inhibited tyrosinase protein expression. It is noteworthy that 20 μM of palm TRF had a lower anti-tyrosinase activity compared to γ- and δT3, whereas αTP had no impact on the suppression of tyrosinase. (B) Treatment of human melanoma cells (A375 and WM793B) with γ- and δT3 suppressed tyrosinase protein expression in a dose-dependent manner. (C) The time-dependent tyrosinase activity of B16 cells was measured on days 5 and 9 after treatment with αTP, γ- and δT3, sodium lactate and kojic acid. (D) The time-dependent suppression of melanin synthesis was measured on days 5 and 9 after treatment with αTP, γ- and δT3, kojic acid, and sodium lactate. Bar chart: average for three assay measurements. Bars: standard deviation. Note that γT3 significantly suppressed the tyrosinase activity and melanin content to day 9. ***Significant one-way analysis of variance test (P value ≤ 0.01) when compared to untreated control group.

The time response of tyrosinase suppression by γ- and δT3, αTP, and tyrosinase inhibitors was also investigated in B16 melanoma cells. The suppression of tyrosinase protein expression by γ- and δT3 isomers was enhanced by increasing the treatment period from 24 to 48 h. However, the opposite effect was observed when the treatment period for sodium lactate and kojic acid was increased from 24 to 48 h, suggesting that the inhibition by the two agents may be short-lived (Supporting Information Figure S2).

The melanin synthesis rates and total melanin content per cell were determined in both control medium and treated medium. After tyrosinase activity was normalized for differences in cell growth by dividing the total activity by the cell number, it was found that B16 melanoma cells treated with γ- and δT3 and palm TRF had <40% of the tyrosinase activity present in the controls. The inhibition of tyrosinase activity continued for up to 9 days after treatment. On day 9, it was found that B16 melanoma cells treated with γT3 had <15% of the

tyrosinase activity that was present in the controls. Figure 1C shows that the tyrosinase activity on day 9 following γT3 treatment was comparable to treatment with 3.5 mM kojic acid. Taking into account the low γT3 treatment concentration, the inhibition of tyrosinase activity by γ- and δT3 was at least 150-fold more potent than treatment with kojic acid and sodium lactate. On day 9, the melanin content of B16 melanoma cell cultures treated with γ- and δT3 was 55 and 30% lower than the controls, respectively (Figure 1D). The melanin content of B16 melanoma cells following γT3 treatment was marginally lower than that in the treatment samples using 4.5 mM sodium lactate and 3.5 mM kojic acid.

In Figure 2A, the photographs show the amount of pigment present in cell pellets that have undergone 5 days of αTP, γT3, δT3, kojic acid and sodium lactate treatments. Lighter pigmentation was observed in samples that were treated with γT3, δT3 and kojic acid compared to controls. In Figure 2B, not only was the pigmentation of in vivo solid tumors lighter in color compared to the controls after 14 days of oral γT3 supplementation (100 mg/kg/day), the tumor size was also significantly smaller for the γT3 group (Chang et al., 2009; Yap et al., 2010) (Figure 2C). Similar tumor shrinkage was observed when human melanoma cells (A375 and WM793B) were xenografted on nude mice (Figure 2C). Immunoblot of tyrosinase in solid tumors indicate lower tyrosinase protein expression of the γT3-treated B16 solid tumors (Figure 2D).

Previous studies have shown that many natural products inhibit tyrosinase activity in a synergistic manner via different mechanisms (Schved and Kahn, 1992). To test whether palm TRF acts synergistically with tyrosinase inhibitors, we compared the effects of palm TRF alone or in combination with kojic acid and sodium lactate. As shown in Figure 3A, the tyrosinase activities per cell following co-treatment with palm TRF, kojic acid and sodium lactate are significantly lower than those following treatment with palm TRF, kojic acid or sodium lactate alone. Using Western blotting (Figures 3B and

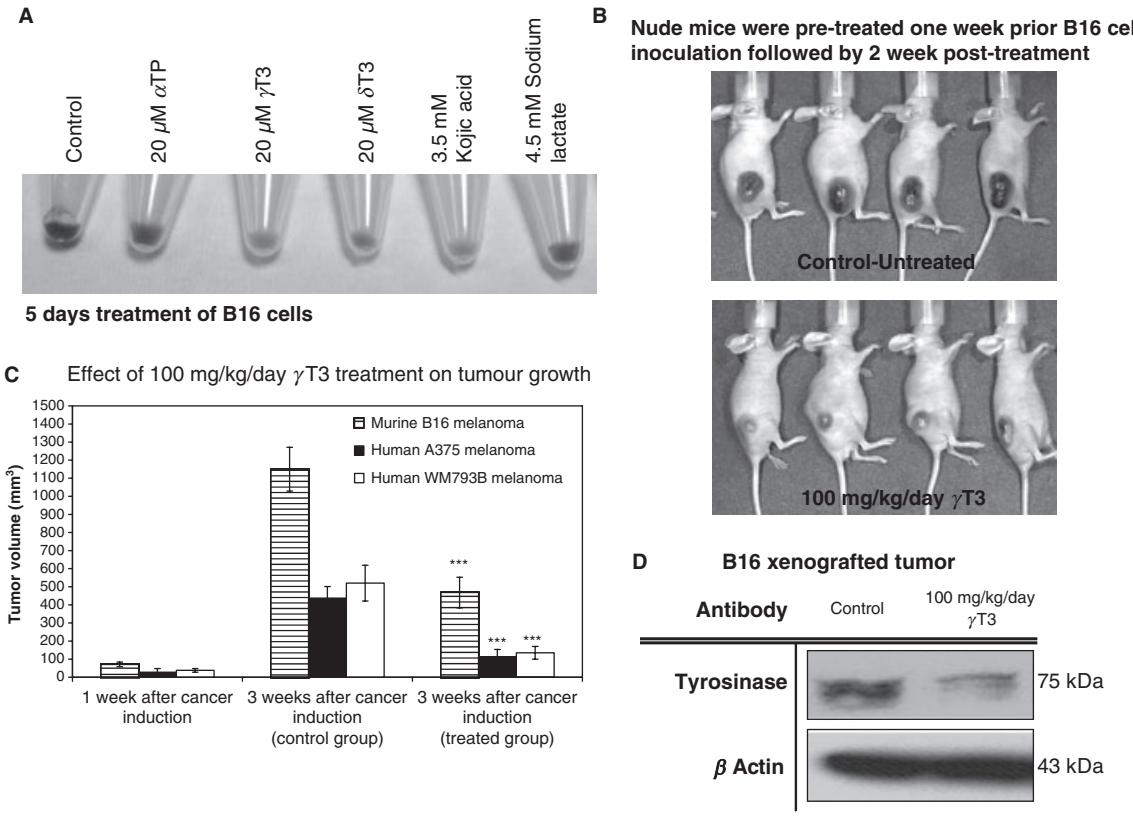

Figure 2. (A) B16 cells were sub-cultured, treated for αTP, γT3, δT3, kojic acid and sodium lactate for 5 days, and then harvested. Photographs of equal density cell pellets were taken. Note that treatments of B16 cells with γT3, δT3 and kojic acid led to lighter cell pigmentation compared to the control group. (B) 5×10^5 B16 cells pre-treated with 20 μM of γT3 for 1 week were xenografted onto the flanks of 10 nude mice. This was followed by a 2-week oral supplementation of γT3 at 100 mg/kg/day after formation of the palpable tumors (1 week). Photographs of the solid tumors were taken at the end of the experiment. (C) Tumor size was measured 1 week after cancer induction (start) and 2 weeks thereafter (end). Bar chart: average for 10 mice. Bars: standard deviation for 10 mice. ***Significant one-way analysis of variance test (P value $\leq$ 0.01) when compared to the untreated control group at the end of the experiment. (D) Immunoblot of tyrosinase of solid tumors from B16 cells injected subcutaneously into nude mice.

S3), we were only able to demonstrate that co-treatment of γ- and δT3, and kojic acid enhanced the suppression of tyrosinase protein expression when compared to treatments of γ- and δT3, or kojic acid alone. However, the same cannot be said for sodium lactate. The reason for this is that sodium lactate inhibits melanin formation by directly targeting tyrosinase catalytic activity (Usuki et al., 2003). Hence, the synergistic interaction of sodium lactate with γ- and δT3 cannot be easily determined using Western blotting. Instead, the effect could be observed through its tyrosinase activity and melanin content (Figure 3A).

Another area of our study concerns tyrosinase activation that is induced by ultraviolent light (UVB). Given that UVB has been reported to stimulate skin melanin synthesis via a different mechanism from the constitutive tyrosinase action in melanin-generating cells (Fitzpatrick et al., 1949), we evaluated the ability of T3 to block UVB-induced melanogenesis in B16 melanoma cells. Figure 3C shows that γT3, δT3 and palm TRF possess a higher sun protection factor (SPF) compared to αTP and palm TRF acetate. Subsequent results show the time-dependent suppression of UVB-induced tyrosinase protein over-expression (Figure 3D) by these molecules. Although δT3 has been found to be more potent than γT3 in suppressing short-term (<10 min) UVB-induced tyrosinase activation, their long-term inhibitory effects were comparable. Consistent with the SPF result, palm TRF acetate and αTP were not able to block UVB-induced activation of tyrosinase (Figures 3D and S4). This observation was not uncommon given that αTP acetate was reported to be unable to prevent UVB photocarcinogenesis in C3H mice (Kramer-Stickland and Liebler, 1998).

Conflict of interest

W. N. Yap, N. Zaiden, C. H. Xu, S. Ong, A. Chen, and Y. L. Yap are employees of Davos Life Science Pte. Ltd., a manufacturer of tocotrienols based in Singapore.

Acknowledgements

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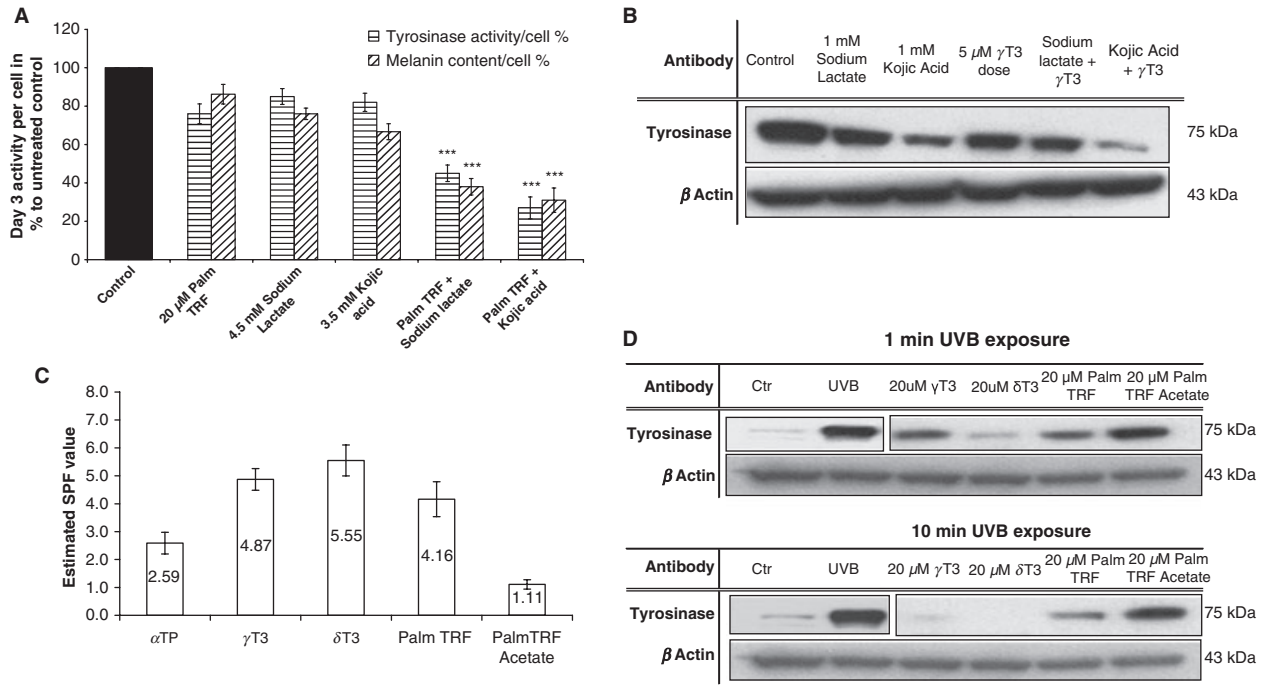

Figure 3. (A) B16 cells were treated with 20 μM of palm tocotrienol-rich fraction (TRF) and either 4.5 mM sodium lactate or 3.5 mM kojic acid for 72 h. The suppression of tyrosinase activity and melanin content following co-treatment was significantly greater than that for the cells treated with either agent alone. (B) Using Western blotting, 5 μM of γT3 co-treatment with 1 mM of either sodium lactate or kojic acid for 24 h resulted in enhanced suppression of tyrosinase protein. (C) Vitamin E isomers were irradiated individually with a series of UV doses using calibrated lamps and the mean applied and transmitted UV doses were computed to obtain the power curve equation [transmitted dose=α(applied dose$^{\beta}$)]. Sun protection factor (SPF) was determined as $(1/\alpha)^{(1/\beta)}$. γT3, δT3 and TRF possess a higher SPF compared to αTP and TRF acetate. (D) B16 cells were exposed to 3.3 mW/cm^2 UVR from an overhead UVB lamp at a distance of 15 cm. The UVB spectrum used was 302 nm, of which the UVB portion is >99%. Using Western blotting, the activation of tyrosinase by UVB was completely blocked by γ- and δT3 after 10 min of incubation time. The same experiment showed that palm TRF only partially blocked UVB-induced tyrosinase activation. In contrast, palm TRF acetate had no effect on UVB-induced tyrosinase activation.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. (A–C) The anti-proliferation effect of T3 isomers and tyrosinase inhibitors was determined by the MTT cell viability assay following 24 h of treatment. Note that β-, γ- and δT3, and kojic acid inhibited B16 cell viability. (D,E) Treatment of B16 cells with β-, γ- and δT3, and kojic acid induced critical apoptotic molecules in a dose-dependent manner (cleaved caspase 3 and PARP). (F,G) DNA fragmentation induced by γ- and δT3 was detected by terminal deoxynucleotidyl transferase (TUNEL assay).

Figure S2. (A) Treatment of B16 cells with retinoic acid for 24 h inhibited tyrosinase protein expression. (B) Suppression of tyrosinase by γ- and δT3 in B16 cells is enhanced after a 48 h incubation period. Conversely, the anti-tyrosinase activities of sodium lactate (NaLac) and kojic acid diminished after 24 h. Of note, 20 μM of palm TRF has a lower anti-tyrosinase activity compared to γ- and δT3, whereas αTP has no impact on the suppression of tyrosinase.

Figure S3. (A) Using Western blotting, 5 μM of δT3 co-treatment with 1 mM of either sodium lactate or kojic acid resulted in enhanced suppression of tyrosinase protein.

Figure S4. (A) Treating B16 cells with 20 μM of αTP does not reverse UVB-induced tyrosinase activation.

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