Supplementary Information

In Vitro Study of the Effects of Five Chemically Modified Tetracycline (CMT) Analogs on Human Epidermal Melanogenesis: Potential as Novel Anti-Melanogenic Agents

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Supplementary Methods

Coculture Assay

Epidermal keratinocytes derived from a neonatal donor, commonly referred to as normal human epidermal keratinocytes (NHEK), were cultured in Medium 154, a commercially available cell culture medium provided by Cascade Biologics. The culture medium was supplemented with 1% human keratinocyte growth supplement (Cascade Biologics), and 1% antibiotics. Cocultures comprising of HEMn-DP and NHEK cells were established in accordance with our previous studies [35,36]. NHEK cells were inoculated onto a 6-well plate and incubated for a duration of 24 h. Subsequently, DP cells were introduced into the culture using Medium 154, with a specific ratio of 1:3 for melanocyte and keratinocyte. The cultures were then sustained for an additional 48 h. After 48 h, the compounds (CMT-1, CMT-4, and CMT-8 all at a concentration of 10 μM) were added, while control group was treated with 0.4% DMSO (solvent-control) and the cocultures were incubated in Medium 154 for a duration of 3 d. Following the incubation period, the cells were subjected to fixation using a 4% paraformaldehyde solution. Subsequently, the application of the Fontana Masson stain was carried out in accordance with the methodology outlined in our earlier work [36]. The cocultures were examined using light microscopy in order to observe the distribution of melanosomes across all groups.

Supplementary Figures

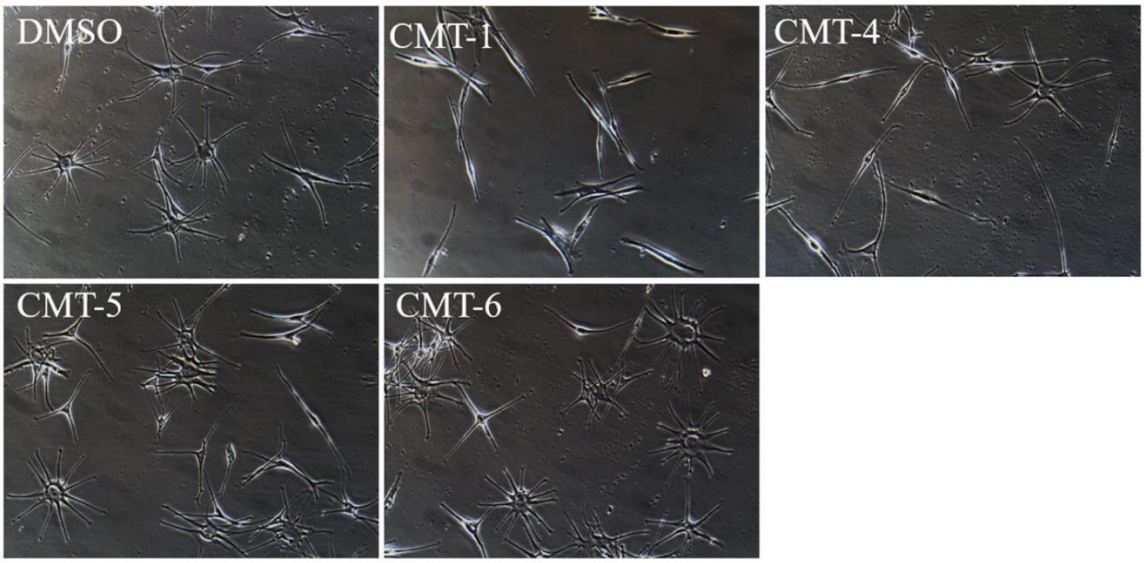

Figure S1: Representative phase-contrast images of HEMn-DP cells treated for 72 h with CMT-1, CMT-4, CMT-5, and CMT-6 at a concentration of 25 μM; CMT-8 was not included due to cytotoxicity at 25 μM ; DMSO group represents solvent-control (cells treated with 0.4% DMSO).

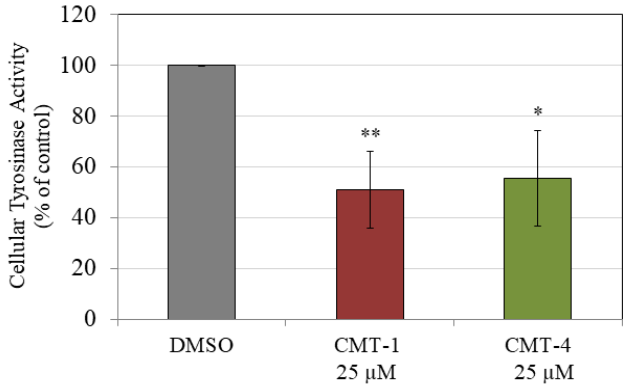

Figure S2: Tyrosinase activity in HEMn-DP cells after a 72-h treatment with CMT-1 and CMT-4 at 25 μM for 72 h. Data is mean ± SD of three independent experiments; * $p < 0.05$ and ** $p < 0.01$ vs. Ctrl by one-way ANOVA with Tukey's test.

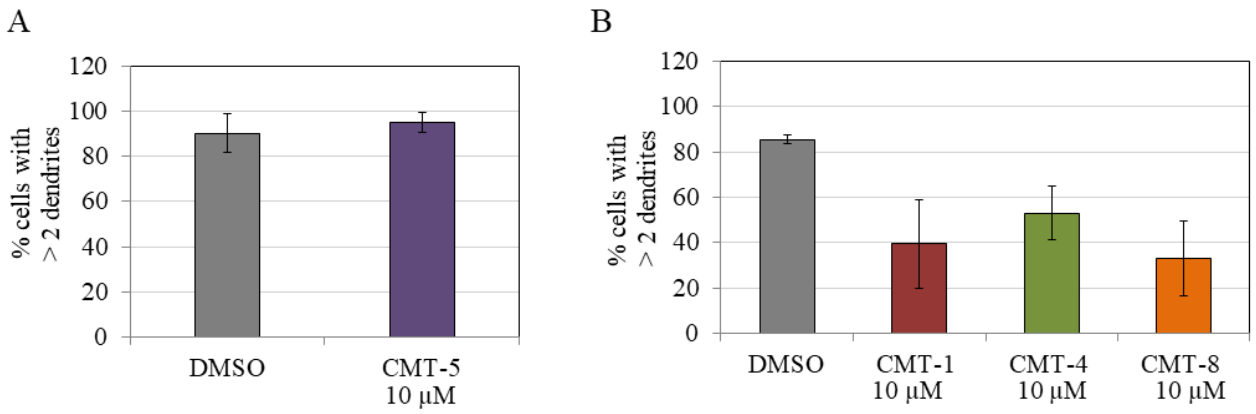

Figure S3: The percentage of cells with > 2 dendrites were calculated for HEMn-DP cells treated with **(A)** CMT-5 (10 µM) and; (**B)** CMT-1, CMT-4, and CMT-8 all at a concentration of 10 µM for a duration of 72 h; a total of up to 50 cells were evaluated for each group from three independent experiments for (A), while a total of 60 cells were evaluated for each group from at least two independent experiments for (B).

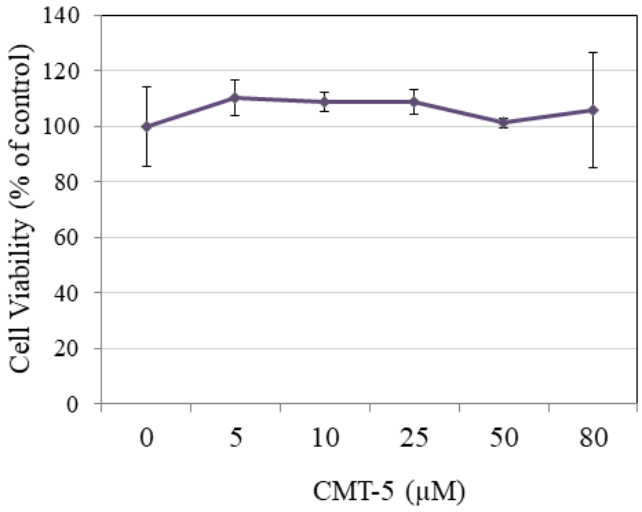

Figure S4: Viability of HaCaT cells after a 72 h treatment with varying concentrations of CMT-5; data are mean ± SD (n = 3 per group).

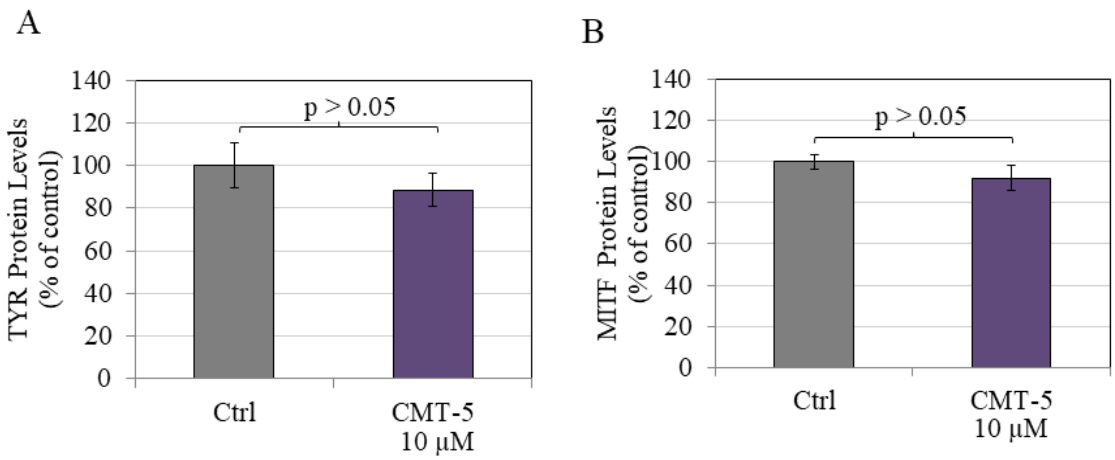

Figure S5: Protein levels of (**A)** TYR and (**B)** MITF in HEMn-DP cells treated with CMT-5 at a concentration of 10 μM for 72 h; $p > 0.05$ vs. Ctrl by Student's t-test; all data are mean ± SD of triplicates.

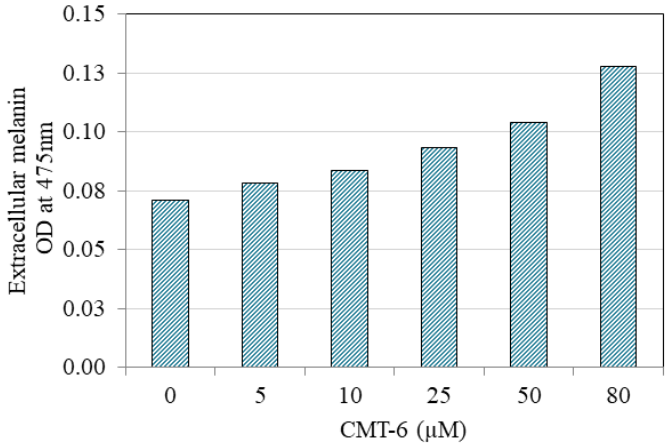

Figure S6: Absorbance of the extracellular culture medium of HEMn-DP cells after a 72-h treatment with CMT-6 at various concentrations (0–80 μM); data are mean of duplicates.

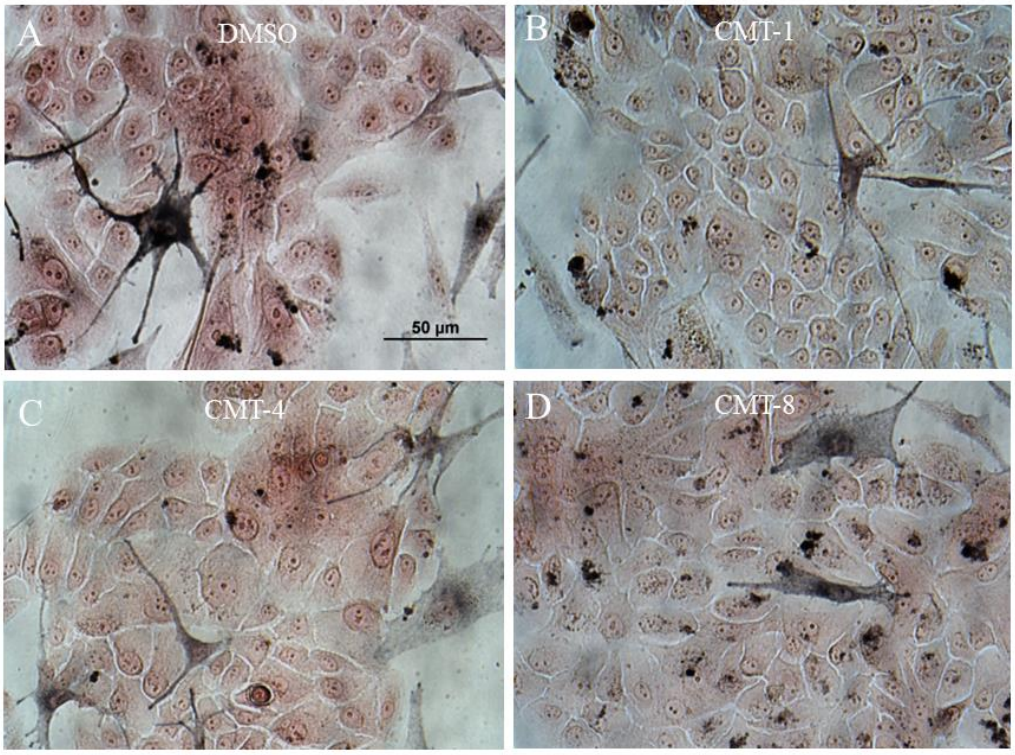

Figure S7: Bright-field micrographs (at 40× objective magnification) of HEMn-DP: NHEK cell cocultures that were stained with Fontana Masson (FM) after a 72-h treatment with various CMT compounds (CMT-1, CMT-4, and CMT-8) all a concentration of 10 µM.

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