



Development of novel alternative hair dyes to hazardous *para*-phenylenediamine

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ABSTRACT

Most of the permanent hair dye products contain p-phenylenediamine (PPD), a well-known skin sensitizer. PPD may cause cutaneous reactions and leads to allergic contact dermatitis (ACD), a condition with major medical and financial repercussions. Hair dye-induced ACD represents a growing concern both for consumers and the cosmetics industry. In this study we introduced novel side chains on the PPD molecule with the goal of overcoming the hazard potential of PPD. Our strategy relies on the replacement of the colorless PPD with new, larger and intrinsically colored PPD derivatives to reduce dermal penetration and thus the skin sensitization potential. We synthesized two oligomers with bulky side-chains, which displayed 7–8 times lower cytotoxicity than PPD, a significantly weaker sensitization potential (22.0 % and 23.8 % *versus* 55.5 % for PPD) in the Direct Peptide Reactivity Assay, minimal cumulative penetration through excised skin and an intrinsic ability to colour and preserve the nuance when applied on bleached hair. The lower skin permeation and sensitizing potential are absolutely crucial and give a clear advantage of our products over other standards. These novel PPD hair dyes show significantly less hazard potential than PPD and may, upon further risk assessment studies, replace PPD in consumer care products.

1. Introduction

“Permanent” hair dyes are among the most frequently used cosmetic products. Most formulations contain *p*-phenylenediamine (PPD) and *p*-toluenediamine (PTD) (Fig. 1). Besides skin irritation, PPD causes acute, subacute or chronic allergic contact dermatitis (Mukkanna et al., 2017), with a prevalence of positive patch tests reported to range from 4 to over 6% of sensitive patients in Asia, Europe and North America (Krasteva et al., 2009; Thyssen and White, 2008). People at risk span a large age spectrum, with the young and elderly alike vulnerable to contact sensitization following dermal PPD exposure. More worryingly, early-age exposure and sensitization can lead to further reactions to PPD at later life stages (Schwensen and Johansen, 2014; Waard-van der Spek et al.,

2013). Allergic reactions are unpredictable and thus, all consumers of PPD-containing products run a risk of subsequently developing allergy. Consumers sensitive to PPD could face not only obvious physical, medical, and financial burden but also intangible social disruptions with impact on quality of life and incapacity to work due to e.g. occupational allergies in hairdressers. The effluents of PPD hair dyes from salons leak out into the environment, representing not only a potential threat to the wider public but also to aquatic life (Bessegato et al., 2018).

Allergic contact dermatitis (ACD) is an inflammatory skin disease characterised by a cellular delayed-type hypersensitivity response caused by a small molecule (less than 500 Da in molecular weight) - such as PPD- either by direct effects through Toll-like receptors or hapten binding. ACD occurs in two phases. In the initial, clinically indiscernible

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sensitization phase, epidermal Langerhans cells or dermal dendritic cells (DC) are triggered to migrate to skin-draining lymph nodes and present the contact allergen covalently bound to skin proteins to naïve T lymphocytes. This process can either lead to tolerance or sensitization by production of skin-homing CD4⁺ and CD8⁺ effector T cells, which then enter the blood circulation. Subsequent exposure to the same contact allergen leads to the elicitation phase, characterised by the recruitment of various immune cells, which leads to downstream effects such as DCs' production of interleukin IL-8 (An et al., 2009; Yoshida et al., 2003) and finally to the typical acute eczematous skin lesion with spongiotic inflammation (Gibson et al., 2015; Martin et al., 2011). Induction of IL-8 production has been studied with THP-1 human monocytic cell lines as surrogates for DC (Kimura et al., 2018).

The most reliable way for testing epithelial absorption and sensitization potential of new compounds and prospective allergens are on human volunteers with or without specific sensitizations, which is difficult for ethical and logistical reasons. Therefore, animal models such as the local lymph node assay (LLNA) and the guinea pig maximization test (GPMT) have been widely used as predictive tests for the identification of skin sensitizers. However, the European Union and other countries have regulated usage of animal test on cosmetics products and their ingredients. Hence, OECD approved alternative *in vitro*, *in chemico* or *in silico* testing methods to identify the toxicity and sensitization potential of new chemicals (OECD, 2015).

In order to trigger sensitization, chemicals must absorb into and permeate through the skin's *stratum corneum* (SC) into the viable tissue layers. PPD undergoes *N*-acetylation in the skin, yielding mono- and diacetyl-PPD metabolites. These products are considered non-sensitizers, with *N*-acetylation even described as a PPD detoxification pathway (White et al., 2006; Aeby et al., 2009). However, the enzymes responsible for such detoxification may quickly saturate, enabling PPD to pass through the skin unobstructed (Manwaring et al., 2015). Indeed, PPD was found in plasma, urine and faeces of people exposed topically to PPD-containing hair dyes for 5 min (White et al., 2006; Aeby et al., 2009).

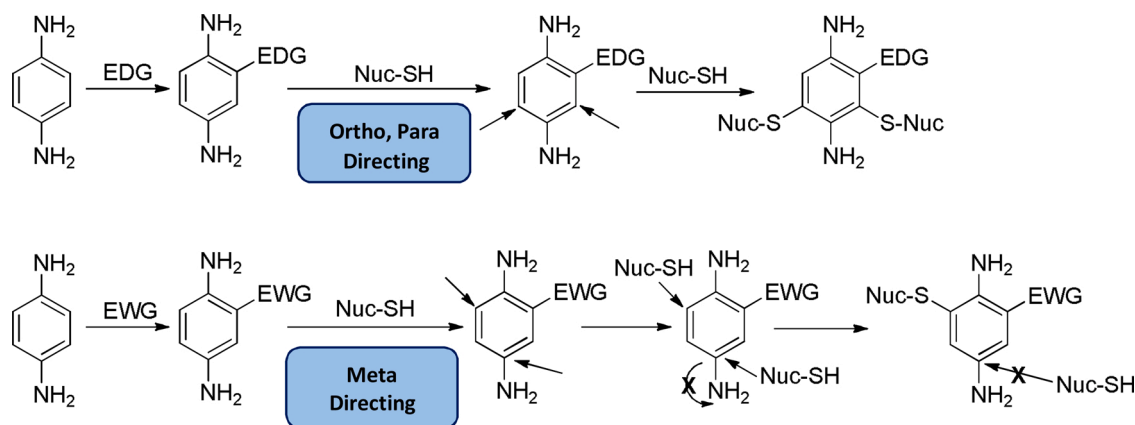
For these reasons, alternatives to PPD in hair dyes are highly warranted. A recent first attempt was the development of the PPD-derivative 2-methoxyethyl *p*-phenylenediamine (ME-PPD) (Goebel et al., 2014) (Fig. 1). ME-PPD displayed reduced sensitization potential in the cysteine Direct Peptide Reactivity Assay (DPRA) compared to PPD and PTD in the absence of enzymatic oxidation. Under oxidizing conditions in the presence of a horseradish peroxidase/hydrogen peroxide system, ME-PPD reactivity was similar to that of PPD and PTD. DC activation, indicated as CD86 surface expression, was significantly weaker for ME-PPD than PPD and PTD. A lower sensitization potential was predicted under LLNA conditions. Clinical studies by the same group

pointed to lower percentages of cross-elicitation by ME-PPD versus PPD in PPD-allergic cohorts (Blömeke et al., 2015; Kock et al., 2016; Zahir et al., 2016). Although these results suggest ME-PPD as a promising alternative to PPD, these small cohort studies in sensitive patients still yielded a sensitizing response in about 30 % of the population.

PPD undergoes *in situ* oxidation, which leads to the synthesis of monomeric, dimeric and trimeric products, some of which have never been isolated since the polymerization process is not interrupted (Zanoni et al., 2015; Hudari et al., 2014). Of note, substances that are unable to penetrate through the *stratum corneum* cannot sensitize the skin or induce allergic reactions (Fitzpatrick et al., 2017) and this is only relevant for healthy and intact skin conditions. Hence, determination of the skin penetration profile of hair dyes is pivotal. The main determinants of skin permeability as identified by quantitative structure-permeability relationships (QSPRs) are the molecular size (expressed as a volume, MV, or weight, MW) and lipophilicity (expressed as the octanol-water partition coefficient $P_{O/W}$) (Tsakovska et al., 2017).

Commercial products such as *p*-toluenediamine (PTD)-containing formulations have an alkyl sidechain at the *ortho* position of the PPD ring. When considering PTD and the library of oligomeric structures of PPD (Table S1) previously developed by our group (Venkatesan et al., 2019) we noticed that the presence of electron donating groups at the *ortho* position was detrimental to HaCaT skin cells. We thus postulated that the presence of moderate electron withdrawing groups at the *ortho* position of PPD would reduce the electron density and thereby decrease the oxidation potential of PPD's benzene ring. Hence, the electron withdrawing resonance effects of the substituents would deactivate the benzene ring system and direct eventual nucleophile substitutions towards the *meta* position of the ring (Scheme 1). Since the *meta* position is unavailable for the nucleophilic substitution due to presence of the amino group, there would be less or no binding of nucleophilic peptides on the benzene ring. To the best of our knowledge, there have been no reports on the substitution of electron withdrawing groups at the *ortho* position of PPD. We expected that, by carefully substituting the *ortho* position of PPD with moderate electron withdrawing groups, we could obtain more biocompatible compounds, further decrease the skin sensitization potential, and retain the same hair-dyeing efficacy as PPD, all while decreasing their hazardous potential to consumers.

To assess the sensitization potential of our new dyes (PPD 6 & 7) with electron-withdrawing groups at the *ortho* position of one of PPD's amine groups (Table 1), the DPRA was conducted using model peptides as putative skin enzymes. In addition, IL-8 cytokine marker profile analysis over THP-1 cells was performed to validate the skin sensitization or ACD potential of the novel hair dyes.



Scheme 1. Proposed mechanism depicting the effect of Electron Donating Group (EDG) and Electron Withdrawing Group (EWG) substitutions at the *ortho* position of *para*-phenylenediamine on the binding affinity of nucleophilic peptides.

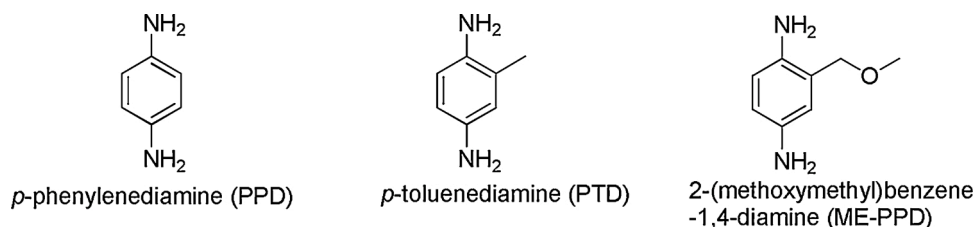
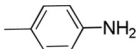


Fig. 1. Commonly used commercial PPD-based hair dyes.

Table 1

Lists of the compounds investigated in this study. Derivatives PPD 1-5 (Venkatesan et al., 2019) are reported in Tables S1 and S2.

Derivative	R ₁	R ₂
PPD 6	H	-COOC ₆ H ₁₃
PPD 7		-COOC ₆ H ₁₃

2. Materials and method

2.1. General information

Unless otherwise mentioned, all commercial materials were used without further purification. NMR spectra were recorded on a Bruker Ultrashield 300 MHz system, Bruker Ultrashield Plus 400 MHz system and Bruker Ultrashield 500 MHz system, at room temperature with DMSO-*d*₆ as the solvent and TMS as the internal standard. ¹H and ¹³C chemical shifts are reported in ppm downfield of tetramethylsilane with reference to the residue solvent peak. Coupling constant (*J*) were reported in Hert (Hz) and signal couplings are reported using the following abbreviations: s, singlet; d, doublet; t, triplet; m, multiplet; p, pentet; q, quartet; br, broad resonance. High resolution mass spectra (HRMS) were obtained on a Finnigan/MAT 95XL-T spectrometer system equipped with electrospray ionization (ESI) and, or Atmospheric Pressure Chemical Ionization (APCI) mode.

Para-phenylenediamine (98.0 %) and resorcinol (≥99.0 %) were purchased from Sigma-Aldrich (Singapore), 2-(methoxymethyl)benzene-1,4-diamine (ME-PPD) (97.0 %) was purchased from ArkPharm Illinois, United States. 2,5-diamino toluene dihydrochloride (>98.0 %) was purchased from Tokyo Chemical Industry Co Ltd (TCI, Japan), Schwarzkopf BlondMe Premium Lift 9+ bleaching powder and Schwarzkopf BlondMe 12 %/40 vol developer solution, Silkpro VitAir

series daily balance shampoo, and Silkpro VitAir series daily treatment Masque (hair conditioner) were purchased from Amazon and used without further purification. 30 % hydrogen peroxide solution was purchased from Merck (Singapore).

HPLC grade acetonitrile (99.0 %), methanol (99.0 %) from Fisher Chemical (Fischer Scientific, Belgium), leucine enkephalin acetate salt hydrate (95.0 %), deferoxamine Mesylate (92.5 %), lysine-containing (Ac-RFAAKAA-COOH, 95.5 %) and cysteine-containing (Ac-RFAA-CAA-COOH, 96.05 %) heptapeptides were purchased from Peptide 2.0 Inc (Chantilly, VA) were purchased from Sigma-Aldrich (Singapore).

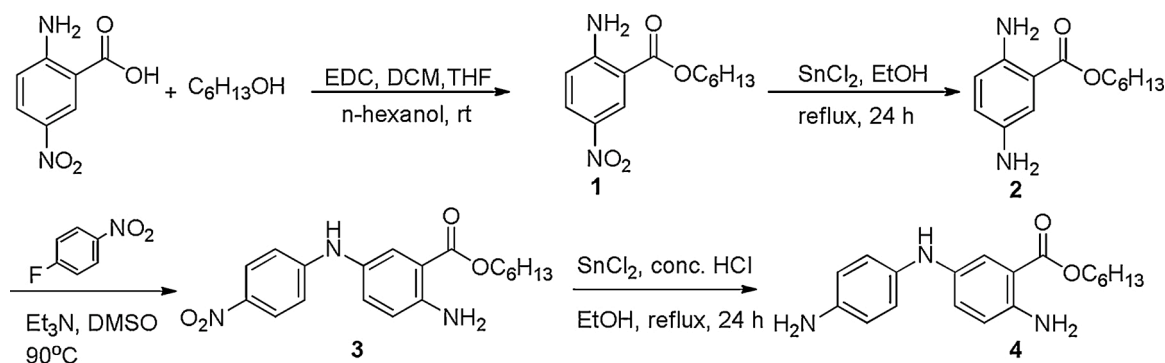
2.2. Synthesis of C-substituted mono PPD derivatives

The synthesis of PPD derivatives PPD 6 and PPD 7 was performed according to Scheme 2.

2.2.1. Hexyl-2-amino-5-nitrobenzoate (1)

5-nitroanthranilic acid (1.05 g, 5.7 mmol) was dissolved in 1:1 mixture of DCM and THF kept under inert atmosphere

and EDC (2.2 g, 11.5 mmol), DMAP (0.35 g, 2.9 mmol) and n-hexanol (0.7 mL, 5.6 mmol) were successively added to the yellow solution. Mixture was stirred vigorously at room temperature for 3 h. During the course of reaction, a yellow precipitate was formed first, which then slowly dissolved. Volatiles were removed under reduced pressure and the residue was dissolved in DCM and the solution was washed thrice with saturated NaHCO₃ solution, thrice with water and finally with brine solution. Evaporation of solvent afforded the crude product as red oil. Pure compound was isolated as bright yellow solid after purification by column chromatography using 60 % dcm/hexane mixture. Yield: 1.4 g (90 %). ¹H NMR (500 MHz, DMSO) δ 8.59 (d, *J* = 2.7, 1 H), 8.09 (dd, *J* = 9.3, 2.8, 1 H), 7.85 (s, 2 H), 6.90 (d, *J* = 9.4, 1 H), 4.27 (t, *J* = 6.6, 2 H), 1.73 (t, *J* = 7 Hz, 2 H), 1.44 – 1.36 (m, 2 H), 1.35 – 1.29 (m, 4 H), 0.88 (t, *J* = 6.9, 3 H). ¹³C NMR (125 MHz, DMSO) δ 165.9, 155.8, 135.1, 128.8, 128.1, 116.7, 107.6, 64.7, 30.8, 28.0, 25.1, 21.9, 13.8.



Scheme 2. Synthesis of C-substituted PPD derivatives PPD 6 (2), PPD 7(4).

2.2.2. PPD derivative 6: Hexyl-2,5-diaminobenzoate (2)

Excess tin(II) chloride (2.3 g, 12 mmol) was added to an ethanolic solution of 0.8 g (3 mmol) of hexyl 2-amino-5-nitrobenzoate. Resulting mixture was refluxed for 24 h. After cooling solvent was evaporated and the residue was taken into water and the pH of the mixture was adjusted at 8 with addition of aqueous K_2CO_3 solution. Mixture was extracted with ethyl acetate and the organic layer was quickly washed with water and finally with brine solution. After solvent evaporation residue was dried under vacuum to get the title compound. Yield: 0.6 g (84 %). ^1H NMR (500 MHz, DMSO) δ 7.00 (d, J = 3 Hz, 1 H), 6.69 (dd, J = 8.7 Hz, 2.7 Hz, 1 H), 6.57 (d, J = 8.6 Hz, 1 H), 5.87 (s, 2 H), 4.43 (s, 2 H), 4.17 (t, J = 6.6 Hz, 2 H), 1.67 (quintet, J = 7 Hz, 2 H), 1.43–1.35 (m, 2 H), 1.35–1.28 (m, 5 H), 0.88 (t, J = 7 Hz, 3 H). ^{13}C NMR (125 MHz, DMSO) δ 168.1, 143.7, 138.3, 123.7, 118.1, 114.3, 110.1, 64.0, 31.4, 28.8, 25.7, 22.5, 14.4. ESI MS (m/z): 237.4 ($M+H$)⁺.

2.2.3. Hexyl [2-amino-5-(4-nitrophenylamino)]benzoate (3)

0.22 g (0.93 mmol) of hexyl 2,5-diaminobenzoate was dissolved in 7 mL of DMSO, and 1.5 equivalent of triethylamine was added. To this mixture 0.12 mL (1.13 mmol) 1-Fluoro-4-nitrobenzene was added and stirred at 90 °C for 24 h. The reaction mixture was cooled and poured into water to induce precipitation. The sticky precipitate was extracted with ethyl acetate and the organic layer was washed thoroughly with water and brine solution. Evaporation of solvent afforded the crude product as red liquid which was purified by silica gel column chromatography using dcm/hexane mixture and the title compound eluted with 30 % dcm/hexane. Yield: 0.2 g (60 %). ^1H NMR (400 MHz, DMSO) δ 8.97 (s, 1 H), 8.02 (d, J = 9.2, 2 H), 7.55 (d, J = 2.3, 1 H), 7.20 (dd, J = 8.7, 2.4, 1 H), 6.85 (d, J = 8.8, 1 H), 6.78 (d, J = 9.2, 2 H), 6.66 (s, 2 H), 4.20 (t, J = 6.5, 2 H), 1.71–1.62 (m, 2 H), 1.36 (mm 2 H), 1.31–1.24 (m, 4 H), 0.84 (t, J = 6.7, 3 H). ^{13}C NMR (75 MHz, DMSO) δ 167.3, 153.1, 149.3, 137.2, 131.0, 127.3, 126.6, 125.3, 118.1, 112.3, 109.4, 64.4, 31.2, 28.5, 25.6, 22.3, 14.1.

2.2.4. PPD derivative 7: Hexyl [2-amino-5-(4-nitrophenylamino)]benzoate (4)

Excess tin(II) chloride was added to an ethanolic solution of nitro derivatives under inert atmosphere. Concentrated HCl (2.5 mL) was added and the mixture was refluxed for 24 h. Resulting mixture was poured into ice-water mixture and the pH of the mixture was set at 12 with 20 % aqueous NaOH solution. Mixture was extracted with ethyl acetate and the organic layer was quickly washed with water and finally with brine solution. After solvent evaporation residue was dried under vacuum to get the title compound.

Yield: 0.60 g (64.7 %). ^1H NMR (400 MHz, DMSO) δ 7.32 (d, J = 2.7, 1 H), 7.00–6.91 (m, 2 H), 6.72–6.62 (m, 3 H), 6.52–6.43 (m, 2 H), 6.13 (s, 2 H), 4.57 (s, 2 H), 4.18 (t, J = 6.5, 2 H), 1.69–1.60 (m, 2 H), 1.40–1.34 (m, 2 H), 1.32–1.27 (m, 4 H), 0.87 (t, J = 6.9, 3 H). ^{13}C NMR (75 MHz, DMSO) δ 167.8, 145.2, 142.2, 135.1, 125.9, 119.4, 118.0, 117.1, 115.4, 109.7, 64.0, 31.2, 28.5, 25.5, 22.3, 14.2. ESI MS (m/z): 328.3 ($M+H$)⁺.

2.3. Hair dyeing tests

2.3.1. Dye preparation

Hair dye formulations were prepared from PPD derivatives PPD 6 and PPD 7 according to a method reported in the literature (Shah et al.,

1972; Venkatesan et al., 2019; Goettel et al., 2003, P&G, 2007). Three formulations were prepared (Types A–C): Type A formulation (non-oxidative type) is an aqueous solution prepared without any oxidizing agent such as hydrogen peroxide, while Type B formulation (oxidative type) is composed of 3% hydrogen peroxide (H_2O_2) solution. In case of Type C formulation (coupler and oxidative type), the dye precursor is mixed with an equimolar amount of coupler resorcinol and 3% H_2O_2 . In both Type A and Type B formulations, pH of the formulation was adjusted to 10–10.5. Dye formulations in solution forms are prepared for the dye precursors PPD, ME-PPD, PTD and PPD 6, 7, as shown in Table S3. Bleached hair of approximately 35 cm in length and 2–5 g in weight were used for dyeing. Then the dye formulation was subsequently applied to blend bleached hair swatches with treatment period of 20 min. to react. Each hair swatch was then removed from the dye preparation and air dried for 20 min.. The same was then washed with deionized water to remove any excess hair dye. The hair samples were then washed twice with Silkpro VitAir Series daily balance shampoo, and were further treated with Silkpro VitAir Series daily treatment Masque (hair conditioner) for 5 min.. The dyed hair samples were then left to be air dried.

The SkinColorCatch (Delfin Technologies Ltd, Kuopio, Finland) was used to measure the CIELAB tristimulus L^* , a^* and b^* values of the dyed hair samples. The difference in the color tone is given by ΔH and the total color difference by ΔE (Venkatesan et al., 2019).

2.4. Skin permeation study

Porcine ears were generously provided by the Department of Surgery, National University of Singapore. The ears were frozen at -20 °C for up to 3 months and thawed at 4 °C on the day of the permeation experiment. Prior to the experiment, the skin was separated from the cartilage and 1 mm thick skin pieces were harvested by using an Acculan™ 3Ti dermatome (Aesculap, Inc., B. Braun, USA). Individual thicknesses were checked using a digital micrometer. PPD and PPD derivatives were dissolved in deionized water at 1% w/v. Skin permeation studies were carried out using vertical Franz diffusion cells (PermeGear, USA) immersed in a 37 °C water bath. The donor and receptor volumes were 900 μ L and 5 mL, respectively; the mean surface area of penetration was 0.64 cm². Upon insertion of the skin pieces into the Franz cells, the skin was equilibrated for 45 min with PBS in the receptor compartment. After equilibration, the skin's trans-epidermal water loss (TEWL) was measured using an Aquaflux AF200 (Biox, UK) to assess the integrity of the skin barrier.

A dedicated coupling (Biox, UK) attached to the Aquaflux probe was used to measure the TEWL of the skin pieces assembled into the Franz cells and in contact with the receptor solution (Elmahjoubi et al., 2009). The donor solution, consisting of PPD or a PPD derivative (n = 3–6), was then added to the donor compartment and the permeation experiments were conducted for 8 h. The receptor solutions were continuously stirred with magnetic stirrers. At fixed time points following application, aliquots of 200 μ L were taken from the receptor solution of each cell, to be assayed by LC–MS/MS for calculation of cumulative amounts that permeated through the skin as a function of time. After each 200 μ L sampling, the receptor solution topped up with 200 μ L of fresh PBS. At the end of the experiment, the donor and receptor solutions were removed and the cells dismantled. Following recommendations by the Scientific Committee on Consumer Safety (SCCS) for the *in vitro* assessment of skin absorption of cosmetic ingredients (SCCS, 2010) mass balances on the PPD and derivatives were performed (Table S4). Following each experiment, the skin pieces and swabs of their outer surfaces were collected and stored in acetonitrile for extraction of the permeant. The donor solution as well as acetonitrile used to thoroughly wash each part of the Franz cells were also collected. PPD and PPD derivatives concentrations in all permeation and mass balance samples were measured by LC–MS/MS (Table S5–S7).

2.5. Cytotoxicity assay on HaCaT cells

Cell viability was determined using the MTT assay as described in the literature (Mosmann, 1983). HaCaT cells were grown in DMEM with 10 % FBS and 1% penicillin/streptomycin. The cells in T-75 cm² culture flasks were incubated with 5% CO₂ at 37 °C in a humidified incubator. The HaCaT cells were grown to expected confluency, then harvested by trypsinization with 0.25 % trypsin/EDTA and incubated at 37 °C for 5 min to obtain complete cell detachment. After incubation, cell pellets were resuspended and washed twice with 1x PBS. The cell suspension in 1x PBS was counted and adjusted to 1 × 10⁵/mL. A HaCaT keratinocyte suspension (100 µL in 1x PBS) with a cell concentration of 1 × 10⁵ cells/mL and a test solution (100 µL in milli-Q water) were combined in each well of two 96-well plates. Test solutions with a desired concentration were prepared by serial dilutions of the stock solutions with 1x PBS.

Cells were seeded at a density of 5 × 10³ in 200 µL of medium into each well of 96-well plates. Cells were seeded as three sets of four replicates for each concentration. After 24 h of incubation, the cells were washed with PBS and cultured for 72 h with 200 µL of 10 % FBS-containing DMEM medium, together with the different concentrations of the test compounds ranging from 500 µM to 1.9 µM for PPD, PPD derivatives, MEPPD, PTD, and positive control standard in a serial dilution. Then the medium was removed and 100 µL of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] (1 mg/mL) in PBS was added. At the end of 4 h incubation, the plates were removed from the incubator and shaken for 5 min. The absorbance at 570 nm was determined using a Bio-Tek plate reader. The cell viability rates were calculated from absorbance readings and represented as percentages of control value (untreated cells).

2.6. Skin sensitization assays

2.6.1. Direct peptide reactivity assay

Incubation mixtures were formulated in glass auto sampler vials to test compound ratios of 1:10 for cysteine peptide and 1:50 for lysine peptide (Venkatesan et al., 2019; OECD, 2015; Wong et al., 2016; Gerberick et al., 2004, 2007). Stock solutions of cysteine (Ac-RFAA-CAA-COOH) and lysine (Ac-RFAAKAA-COOH), containing peptides was prepared fresh immediately before use. Reactivity of PPD derivatives towards the peptides was determined using 20 µM of cysteine and lysine in 0.1 M phosphate buffer (pH 7.5) and 0.1 M ammonium acetate buffer (pH 10.2) respectively, containing 10 µM deferoxamine mesylate.

2.6.2. Cytokine IL-8 analysis on dendritic cells through MTS assay

MTS assay shows specific cell viability profiles for THP-1 models. The MTS assays were performed to quantitate the CV75 dose (concentration showing 75 % of THP-1 cell survival, i.e., 25 % cytotoxicity) required for sensitization assays (Kleinstreuer et al., 2018). The CV75 dose was calculated by performing an assay on different dosages of standard PPD dyes and PPD derivatives and by plotting dose curves. Specific controls (i.e. untreated and DMSO) were used to represent solvent and baseline for cytotoxic profiles. Standard PPD dye, commercial dyes such as PTD, ME-PPD, positive control compound 2,4-dinitrochlorobenzene DNCB and canonical by product of PPD (Bandrowski's base) were included in the assay. The THP-1 cell viability was assessed using the MTS assay by directly adding MTS reagent in suspension culture (1 × 10⁶) and cell viability is measured after 90 min. The THP-1 cell supernatant was collected from the wells after 24 h exposure to the compounds of interest. A Chemokine immunosorbent assay Bio-legend ELISA Max kit was used to quantify the levels of available IL-8 protein. THP-1 cells were treated with CV75 % inhibitory doses of respective compounds obtained from MTS assays with control, DMSO, PPD, ME-PPD, DNCB and PPD derivatives PPD 1-7.

3. Results and discussion

3.1. Synthesis of C-substituted PPD derivatives

Table 1 lists the structure of the newly synthesized derivatives PPD 6 and PPD 7 obtained through the strategic introduction of electron withdrawing esters at the *ortho* position of the PPD monomer and PPD dimer. The detailed characterizations of PPD 6 and 7 are reported in Fig. S1-S6.

The synthetic method is presented in Scheme 2. In the new PPD derivatives, we introduced a carboxylic ester at the *ortho* position of one amine group in PPD to develop PPD analogues with electron-withdrawing properties. The hexyl ester derivative of 2-amino-5-nitrobenzoic acid was synthesized by the Steglich type esterification reaction as reported in Scheme 2 (Tsakos et al., 2015). 5-nitroanthranilic acid was reacted with n-hexanol at room temperature using EDC and catalytic amount of DMAP to obtain the compound 1 in high yields. The nitro group of compound 1 was further reduced with SnCl₂ to obtain hexyl ester-substituted monomeric compound 2 (PPD 6). This newly synthesized PPD derivative (PPD 6) was further functionalized to get a higher oligomer. PPD 6 was treated with 1-fluoro-4-nitrobenzene in the presence of triethylamine to get the nitro-substituted compound 3 which was then reduced by SnCl₂.2H₂O to get the corresponding dimeric compound 4 (PPD 7) with ester long chain.

3.2. Hair dyeing study

The hair colouring properties of the standards PPD, ME-PPD and PTD were compared with the novel derivatives. Fig. 2 and Table 2 show the hair dyeing results obtained for PPD, ME-PPD, PTD, PPD 6 and PPD 7 applied as aqueous basic solutions according to procedures reported in the literature procedure (Shah et al., 1972; Venkatesan et al., 2019; Goettel et al., 2003; P&G, 2007). Type A formulation was prepared to investigate the effect of dyes on the hair shaft without any oxidizing agent. Type B formulation is the one generally used in commercial hair dye formulations with oxidizing agents, whereas type C formulation was prepared to study the effect of a coupler (e.g. resorcinol) in combination with PPD precursors.

Hair coloring results of the commercial dyes and derivatives in formulations A–C are shown in Fig. 2, along with the measured color tone and color difference values (Table 2). Type A formulations of PPD, ME-PPD and PTD dyes showed no colour formation, while type B of ME-PPD and PTD displayed similar brown colour and similar ΔH and ΔE value as type C of PPD (Fig. 2 and Table 2). This suggests that the compounds needed to undergo oxidation in order to impart any color to the hair shaft. Moreover, type B formulation of the ME-PPD, PTD did not produce black colour as PPD, suggesting weaker dyeing properties of both these standards as compared to PPD. Conversely, the dark black color in formulation B of PPD is most likely due to its oxidized metabolite, namely the Bandrowski's base. Interestingly, type C formulation of ME-PPD and PTD showed black colour, which is the representation of the dyes complexed with resorcinol in the presence of an oxidizing agent. ΔH and ΔE values of type C of ME-PPD and PTD were comparable with our new dyes.

According to the chromometric scale, a higher ΔE value is a representation of the lighter colour dye while a low value is associated with darker nuances. Type A formulations of PPD 6, PPD 7 without any oxidizing agent produced a vibrant brown and green colour, respectively, while PPD, ME-PPD, and PTD failed to show any intrinsic colouring properties for type A formulation. This might suggest that, in case of extremely damaged hair, the consumer might be given the choice to opt for H₂O₂-free dyes. Moreover, type B formulation of PPD 6 showed nil ΔE value, which is associated with a darker color in comparison to PPD 7. Finally, the similarity of ΔE values between our new dyes and the commercial standards (e.g., PPD or ME-PPD) indicate that the former maintain suitable hair nuancing properties.

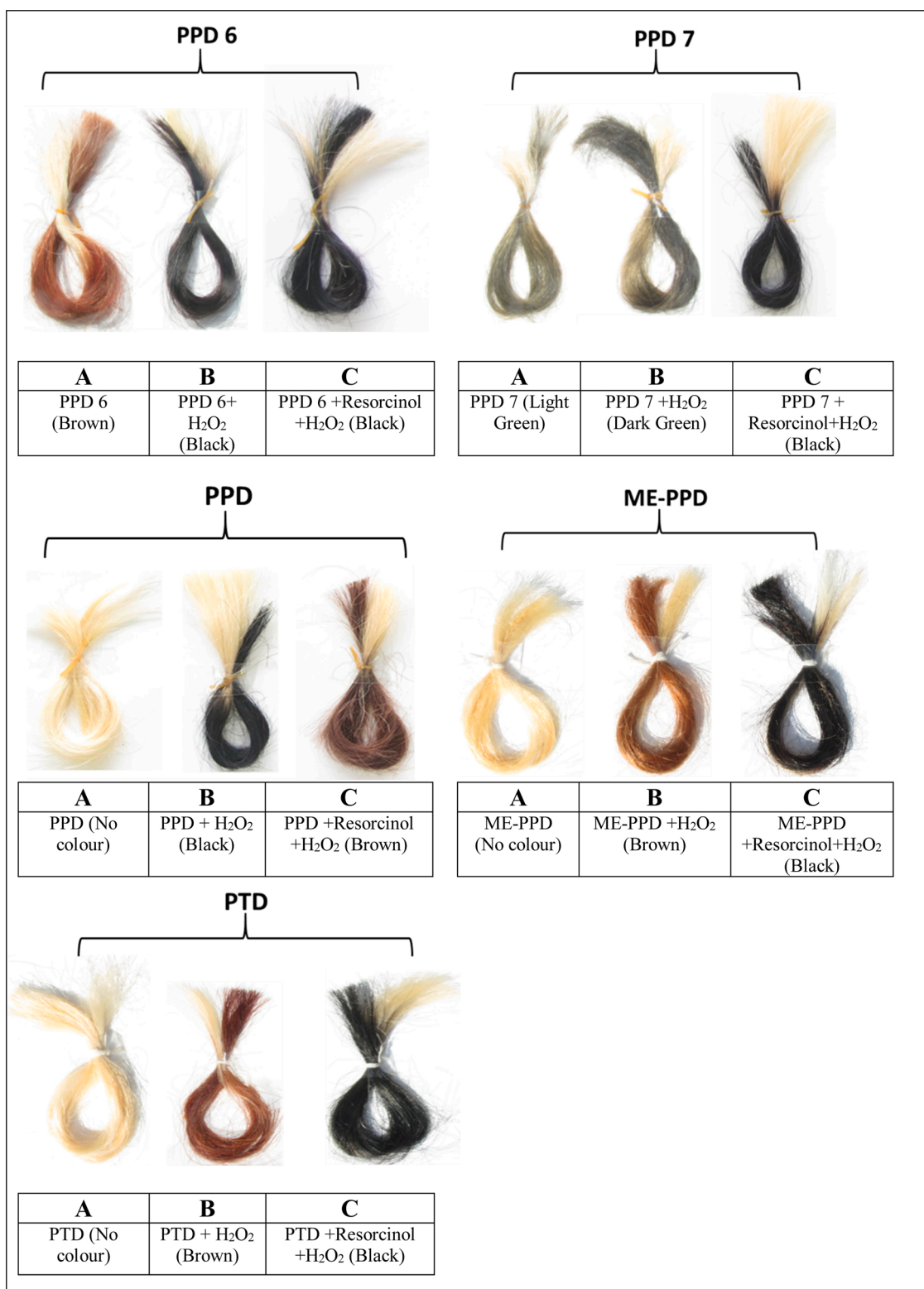


Fig. 2. Dye compositions and colour resulting from the synthesized PPD derivatives applied on hair shafts.

3.3. Skin permeation study

Skin permeation was investigated only for the compounds that dyed the hair fibre. We added our previously reported PPD 1-5 to PPD 6 and PPD 7, whose syntheses are reported herein, to investigate the effects of different chemical properties of PPD derivatives on permeation through porcine skin. The mean excised porcine skin thickness values were

consistent across all experiments, with mean values of (1.2 ± 0.13) mm. TEWL values were consistent at (18 ± 2.8) g/(m² h, in agreement with previously published results for undamaged dorsal porcine ear skin (Klang et al., 2013).

Fig. 3 summarizes the permeation of our synthesized PPD derivatives compared to those of PPD, ME-PPD and PTD. The data are shown as percentages of the dose of compound applied to the skin. Fig. S7 and

Table 2Quantitative hair dyeing performance of PPD 6 and PPD 7, as obtained through chromometric assay. [#] Hair dye data published in Venkatesan et al., 2019.

		L*	L ₀	a*	a ₀	b*	b ₀	ΔE	ΔH
Natural hair			9		0		1		
Derivative	Formulation								
PPD [#]	A	57		0		30		56.1	29
	B	9		1		1		1	0
	C	14		1		3		5.5	2.2
ME-PPD [#]	A	58		0		30		56.9	29.0
	B	15		1		3		6.3	2.2
	C	9		1		1		1	0
PTD [#]	A	58		0		30		56.9	29.0
	B	14		1		3		5.4	2.2
	C	9		1		1		1	0
PPD 6	A	19		1		9		10.8	4.1
	B	9		0		1		0	0
	C	9		1		1		1	1
PPD 7	A	34		1		14		28.1	13.0
	B	31		5		9		23.9	9.4
	C	9		1		1		1	1

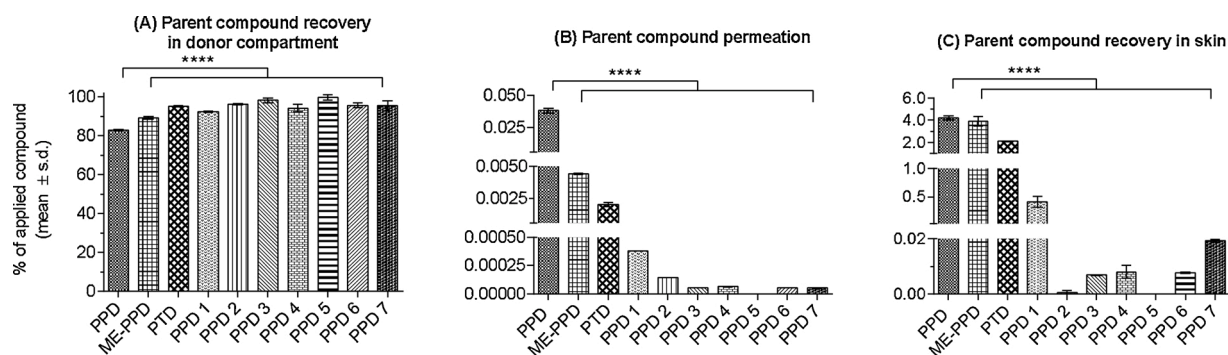
**Fig. 3.** Skin permeation of PPD derivatives compared to PPD, ME-PPD and PTD, expressed as percentage of applied compound dose. Data are means $\pm$ s.d. of 3 to 6 replicates, **** indicates $p < 0.0001$.

Table S4 show the cumulative amount profile of PPD, PPD derivatives, ME-PPD and PTD. Fig. S8 summarizes the extracted Ion chromatogram (XIC) of PPD derivatives as derived from porcine skin permeation study. With regards to skin permeation, PTD and ME-PPD showed 18.8 times ($0.287 \mu\text{g}/\text{cm}^2$) and 10.4 times ($0.519 \mu\text{g}/\text{cm}^2$) lower mean cumulative amounts passing through the skin than PPD ($5.4 \mu\text{g}/\text{cm}^2$), respectively. The ester substituted monomer and dimer derivatives PPD 6 and PPD 7 showed even lower cumulative amount of permeation through the skin, equal to $0.00734 \mu\text{g}/\text{cm}^2$, $0.00712 \mu\text{g}/\text{cm}^2$, respectively. In other words, the monomer ester (PPD 6) showed 735 times lower permeation as compared to PPD, while the dimer ester (PPD 7) displayed 757 times lower permeation than PPD.

The skin absorption of PPD 6 and PPD 7 derivatives was significantly lower than that of PPD. The amounts of applied compound recovered in the donor compartment of the Franz cells at the end of the experiment were $83 \pm 0.5\%$, $89 \pm 0.8\%$ and $95 \pm 0.3\%$ for PPD, ME-PPD and PTD, respectively. For PPD 6 and 7 they ranged from 92 % to 98 % (Fig. 3). The percentages of PPD 6 and 7 derivatives permeated are three orders of magnitude lower than the percentage of PPD permeated, and 40–90 times lower than that of ME-PPD and PTD.

Permeation of the PPD derivatives decreased with increasing molecular weight with the exception to PPD 2. The permeation of the trimeric PPD 2 (363.28 Da) was about two times less than that of PPD 4 (281.22 Da), despite the former having a higher molecular weight (363.28 Da). We speculate the higher permeation of PPD 2 to be due to its lower ionization potential (pKa, Table S2). Indeed, with the stratum corneum being slightly acidic (pH ranging typically from 4.70 to 5.75), solutes penetrate the skin in their unionized form to a higher extent than their ionized counterparts.

3.4. Cytotoxicity assay on HaCaT skin cells

Fig. 4 shows the viability of human skin HaCaT keratinocytes exposed to several concentrations of PPD and its derivatives in PBS along with the negative control without test compounds. Fig. S9 displays the cytotoxicity profile comparison of PPD versus hair dye standards MEPPD, PTD along with its oxidized product, Bandrowski's base, and the positive control Simvastatin. Among the standards, PTD displayed an IC_{50} of $73.13 \mu\text{M}$, and MEPPD displayed the IC_{50} of $334 \mu\text{M}$, which are 3.5 and 14 times, respectively, less toxic than PPD. Simvastatin was chosen as positive control for this study as the effect of statins on HaCaT cells is well documented (Qi et al., 2009). In contrast to PPD 1–5, substitution of an electron withdrawing ester group at the *ortho* position of

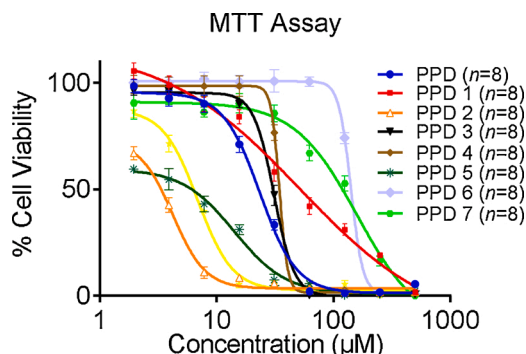
**Fig. 4.** Cell viability profile of PPD derivatives in HaCaT cells.

Table 3

IC₅₀ values of hair dye compounds and positive control simvastatin.

Hair Dye compounds	IC ₅₀ μ M
p-Phenylenediamine	23.45
MEPPD	334.5
PTD	73.13
PPD 1	51.53
PPD 2	4.23
PPD 3	31.04
PPD 4	34.50
PPD 5	14.17
PPD 6	160.1
PPD 7	140.8
Bandrowski Base	6.10
Simvastatin (positive control)	9.27

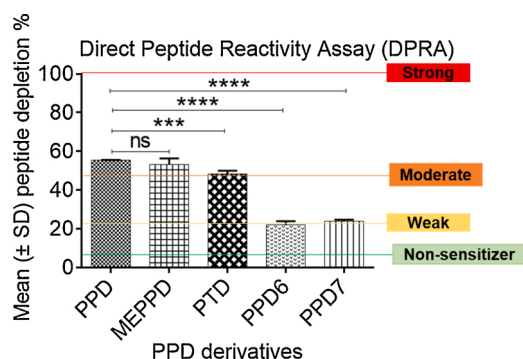


Fig. 5. Mean Peptide depletion % (Cysteine 1:10 /Lysine 1:50) of PPD derivatives at 24 h of incubation. Recommended thresholds (6.38 %, 22.62 %, 42.47 %, and 100 %) of the mean peptide depletion. Data represents mean $\pm$ sd of triplicate replicates, ns; non-significant, ***, p value of < 0.001, ****; p value of < 0.0001.

PPD improved the HaCaT cells viability percentage substantially.

Cytotoxicity results of the PPD 6 and PPD 7 were compared to commercial hair dyes PPD, ME-PPD and PTD and other PPD derivatives. Based on the cell viability data shown in Fig. 4 and the cell viability values listed in Table 3, PPD 6 and PPD 7 showed IC₅₀ values of 160 μ M and 140 μ M, respectively, which are 6–7 times less cytotoxic to HaCaT cells than PPD. Other commercial standards such as PTD, with an ortho methyl group substitution in PPD, displayed two times higher cytotoxicity than PPD 6 and PPD 7. Interestingly, ortho methoxy methyl substituted ME-PPD showed two times lower cytotoxic profile than PPD

6 and PPD 7, proving that additional methoxy side chain substitution to methyl group of PTD molecule is beneficial to HaCaT cells. On the other hand, PPD 6 and PPD7 produced a much better cytotoxicity profiles than other PPD derivatives PPD 1–5, proving that electron withdrawing groups are better than long chain donating groups.

3.5. Skin sensitization assays

3.5.1. Direct peptide reactivity assay

Fig. 5 represents a comparison of mean peptide depletion % of PPD 6 and PPD 7 versus commercial hair dyes PPD, ME-PPD and PTD. DPRA results of the PPD derivatives PPD 1–5 were reported in Venkatesan et al., 2019 (Table S8). In an attempt to decrease the sensitization potential, we substituted a hexyl ester at the ortho position of the PPD monomer (PPD 6) and PPD dimer (PPD 7). PPD 6 displayed a weak skin sensitizing potential, while PPD 7 showed weak/weak-to-moderate sensitizing potential.

The ester form of PPD in the form of PPD 6 and PPD 7 resulted in low mean peptide depletion values (22.0 % and 23.8 %, respectively). These molecules are substituted by a long chain of moderately deactivating electron withdrawing groups. These groups direct the nucleophilic peptide binding to meta position of the ring by blocking the ortho and para position of the CH group of benzene system. As the meta position of both PPD 6 and PPD 7 is already substituted with an amino group, positions 3, 4, and 5 of the benzene ring are unavailable for covalent interaction with peptides. On the contrary, electrophilic CH at positions 2,3 and 4,5 in PPD are available for the covalent binding with nucleophilic peptides, and this leads to its high percentage of peptide depletion (Scheme 1 and Table S1). On the whole, this strategic electron withdrawing group substitution enabled the weak and weak-to-moderate sensitization potential effect for PPD 6 and 7, respectively. Hence, we demonstrated a further improvement in the skin sensitization profile by introducing novel electron withdrawing groups at the ortho position of PPD.

3.5.2. Cytokine IL-8 analysis on THP-1 leukemic monocyte cell models

Immune sensitization assay for modified PPD derivatives was performed on THP-1 cells as a surrogate for dendritic cells. The CV 75 % value, i.e. the concentration at which 75 % of THP-1 cells survive (25 % cell death) was calculated using a dose-response curve (Table S9). The CV75 % value was used to determine the concentration of hair dye chemicals in all following IL-8 expression measurements. These data are required before testing supernatants in THP-1 models for expression of cytokine IL-8 upon treatment with standard PPD and modified PPD hair dye compounds. Most allergens show a specific dose-dependent alteration of secretion of cytokine profiles. However, depending on the nature of the chemical, the alteration pattern may vary.

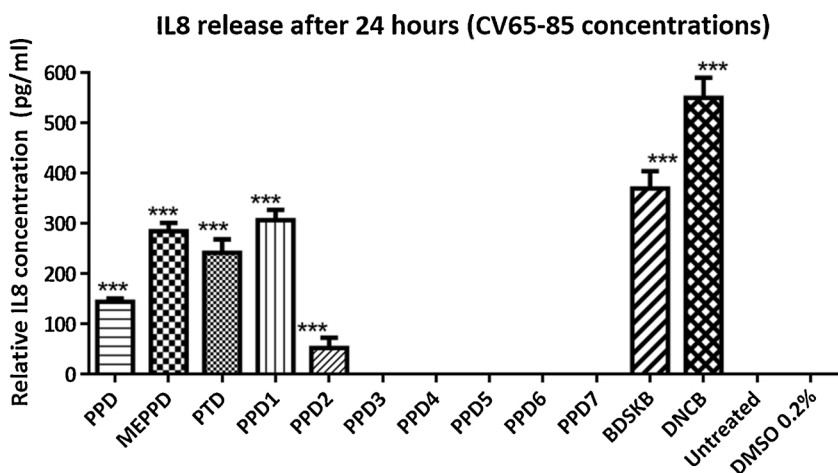


Fig. 6. IL-8 is elicited by specific PPD derivatives in comparison to known PPD and commercial PPD derivatives. Skin sensitization of PPD compared to ME-PPD and PTD, PPD derivatives (PPD1-8, BDKSB, DNCSB, untreated, DMSO 0.2 %) expressed as IL-8 release profile at respective concentrations (pg/mL). Data are means $\pm$ s.d. of four replicates, *** indicates p < 0.0001; * indicates p < 0.1, ns indicates not significant. The PPD derivatives PPD 3-7, untreated and DMSO 0.2 % conditions in comparison to PPD alone did not release significant amounts of IL-8 cytokine and are statistically significant (***) indicating p < 0.0001).

CV75 values were computed based on cell survival dose ranges for each controls, PPD parent compound and all PPD derivatives. The CV75 dose allows us to use specific dose with 75 % viability to assess sensitization in THP-1 dendritic cell models after 24 h duration of treatment. The treatment of THP-1 cells with PPD and its derivatives through ELISA studies showed that IL-8 is differentially secreted, implying some extent of sensitization potential as shown in Fig. 6. Increased expression of IL-8 was observed with standard hair dyes PPD, PTD, ME-PPD, DNCB control, BB and PPD 1 while other modified derivatives PPD 3, 4, 6 and 7 did not stimulate IL-8 expression, confirming the lack of sensitization potential measured through the DPRA assay.

4. Conclusion

In this project, we investigated novel hair dyes with electron withdrawing properties at the *ortho* position of PPD and assessed their hair dyeing efficacy, percutaneous penetration, and toxicity profiles. Our results indicate that PPD 6 and PPD 7 are excellent candidates, with preserved dyeing properties on the hair shaft under oxidative conditions, as confirmed by low ΔE values under the chromometer assay. Further, skin permeation study on excised porcine skin confirmed the lower penetration of our derivatives in comparison to PPD, ME-PPD and PTD. We believe the increase in molecular size of our PPD derivatives mainly contributed to this decrease in skin permeation.

Moreover, low mean peptide depletion values (22.0 and 23.8) of PPD 6 and PPD 7, as obtained from *in chemico* DPRA assay, further substantiate the effect of electron withdrawing properties on decreasing the skin sensitization potential of the hair dyes.

Cytotoxicity assays on HaCaT cells assessed our derivatives' toxicity towards human keratinocytes. Both PPD 6 and 7 showed the highest IC₅₀ (i.e. the lowest cytotoxicity) as compared to previously synthesized PPD derivatives. Even more remarkable, the minimal potential for sensitization was confirmed by assessing the expression of specific marker cytokines (IL-8 secretion) in immune-related THP-1 cells: neither derivative cause any secretion in the inflammatory IL-8, unlike the commercial counterparts (PPD, PTD and even ME-PPD).

In summary, in this study we achieved the proposed objective of reducing skin sensitization potential, decreasing cytotoxicity towards skin cells, and minimizing skin permeation while maintaining permanent hair dyeing properties in the form of our new candidates PPD 6 and 7. Taken together, the results presented here pave the way for clinical studies on patients with proven sensitization to hair dye PPD, which are planned in the near future. Hence, upon further risk assessment studies, our new compounds might have the potential to become less hazardous cosmetic products to end users.

CRediT authorship contribution statement

Gopalakrishnan Venkatesan: Conceptualization, Methodology, Investigation, Writing - original draft, Validation, Visualization, Writing - review & editing. **Yuri Dancik:** Investigation, Validation, Visualization, Writing - original draft, Writing - review & editing. **Arup Sinha:** Writing - review & editing. **Hpone Myint Kyaw:** Investigation. **Ram-asamy Srinivas:** Validation, Visualization. **Thomas L. Dawson:** Writing - review & editing. **Mei Bigliardi:** Writing - review & editing. **Paul Bigliardi:** Conceptualization. **Giorgia Pastorin:** Conceptualization, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jhazmat.2020.123712>.

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