Toxicology and Applied Pharmacology 274 (2014) 480–487

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Introduction of a methoxymethyl side chain into *p*-phenylenediamine attenuates its sensitizing potency and reduces the risk of allergy induction

Carsten Goebel [a,*], John Troutman [b], Jenny Hennen [c], Helga Rothe [a], Harald Schlatter [a], G. Frank Gerberick [b], Brunhilde Blömeke [c]

[a] The Procter & Gamble Co., Central Product Safety and Communications, Darmstadt, Germany
[b] The Procter & Gamble Co., Central Product Safety, Cincinnati, OH, USA
[c] Dept. of Environmental Toxicology, Trier University, Trier, Germany

ARTICLE INFO

Article history:
Received 4 September 2013
Revised 4 November 2013
Accepted 26 November 2013
Available online 10 December 2013

Keywords:
Skin sensitization
Hair dyes
Innate immune response
Alternative methods
2-Methoxymethyl-*p*-phenylenediamine
Skin metabolism

ABSTRACT

The strong sensitizing potencies of the most important primary intermediates of oxidative hair dyes, *p*-phenylenediamine (PPD) and *p*-toluylenediamine (PTD, i.e. 2-methyl-PPD) are well established. They are considered as the key sensitizers in hair dye allergic contact dermatitis. While modification of their molecular structure is expected to alter their sensitizing properties, it may also impair their color performance. With introduction of a methoxymethyl side chain we found the primary intermediate 2-methoxymethyl-*p*-phenylenediamine (ME-PPD) with excellent hair coloring performance but significantly reduced sensitizing properties compared to PPD and PTD: In vitro, ME-PPD showed an attenuated innate immune response when analyzed for its protein reactivity and dendritic cell activation potential. In vivo, the effective concentration of ME-PPD necessary to induce an immune response 3-fold above vehicle control (EC3 value) in the local lymph node assay (LLNA) was 4.3%, indicating a moderate skin sensitizing potency compared to values of 0.1 and 0.17% for PPD and PTD, respectively. Finally, assessing the skin sensitizing potency of ME-PPD under consumer hair dye usage conditions through a quantitative risk assessment (QRA) indicated an allergy induction risk negligible compared to PPD or PTD.

Introduction

Oxidation hair dyeing with the *p*-amino aromatic compound *p*-phenylenediamine (PPD) has a long tradition (Corbett, 1999). This key primary intermediate is still used in many permanent hair dye products based on its excellent color performance. Following oxidation of the primary intermediate by hydrogen peroxide, color performance is dependent on the high degree of reactivity of its oxidized form with couplers to form dimeric and trimeric reaction products within a short period of time (Scientific Committee on Consumer Safety (SCCS, 2010b)).

Apart from the excellent hair coloring property PPD is well known as the most important allergen associated with hair dye-related allergic contact dermatitis reviewed in (Goebel et al., 2012; Krasteva et al., 2009). Historically, introduction of a side chain into the PPD molecule in the 2 position was studied to assess if the skin sensitizing properties can be modulated while keeping the coloring performance. Successful attempts regarding performance were the 2-methylderivative *p*-toluylenediamine (PTD) and to a lesser degree the 2-hydroxyethyl derivative hydroxyethyl-PPD (HE-PPD). However, no major reduction of the sensitizing potency was found: based on maximized potency data derived from skin sensitization tests such as the local lymph node assay (LLNA), PTD and PPD are both categorized as extreme sensitizers, while HE-PPD is categorized as strong sensitizer (SCCS, 2013a).

Advancements in understanding the mechanistic steps underlying skin sensitization revealed in the case of pre-haptens, such as PPD, that oxidation, epidermal bioavailability, protein reactivity, and dendritic cell (DC) activation are critical parameters (Aeby et al., 2009; Jenkinson et al., 2009; McFadden et al., 2011). In addition, aromatic amine hair dyes are known to undergo enzymatic *N*-acetylation in the skin (Goebel et al., 2009; Kawakubo et al., 2000; Nohynek et al., 2005) and *N*-acetylation of PPD was shown to prevent skin sensitization in the LLNA (Aeby et al., 2009).

Abbreviations: PPD, *p*-phenylenediamine; PTD, *p*-toluylenediamine; ME-PPD, 2-methoxymethyl-*p*-phenylenediamine; LLNA, local lymph node assay; QRA, quantitative risk assessment; SCCS, Scientific Committee on Consumer Safety; HE-PPD, hydroxyethyl-PPD; WoE, weight-of-evidence; NESIL, no expected sensitization induction level; MEL, measured exposure level; DC, dendritic cell; HRP/P, horseradish peroxidase and hydrogen peroxide; DMSO, dimethylsulfoxide; DNCB, 2,4-dinitrochlorobenzene; SI, stimulation index.

* Corresponding author at: Wella ZN, The Procter & Gamble Company, Berliner Allee 65, 64274 Darmstadt, Germany. Fax: +49 6196 8921 963.
E-mail address: goebel.c.1@pg.com (C. Goebel).

0041-008X/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.taap.2013.11.016

Therefore, in the present paper we asked how introduction of a methoxymethyl side chain into PPD, resulting in 2-methoxymethyl-*p*-phenylenediamine (ME-PPD), impacts the skin sensitizing properties including skin metabolism.

For this purpose, we investigated a) the bioavailability and the metabolic fate using human skin *ex vivo*, b) the reactivity towards protein using the peptide reactivity assay in the presence and absence of an oxidizing system (Gerberick et al., 2009) and c) the ability to activate THP-1 cells, used as surrogate DCs, for the expression of the costimulatory molecule CD86 (Migdal et al., 2013; Yoshida et al., 2003).

In the LLNA we further assessed, how the interplay of the individual mechanistic steps analyzed is related to specific T lymphocyte recognition and proliferation, because the magnitude and vigor of the proliferative response induced determine the extent to which skin sensitization will be acquired (Kimber et al., 2011). Thus, we determined the skin sensitizing potency of ME-PPD by calculating the EC3 value (concentration necessary to induce a 3-fold increase above the vehicle control) representing the threshold for the induction of skin sensitization, referred to as the non expected skin sensitization induction level (NESIL).

Finally we applied a quantitative risk assessment (QRA) approach for hair dyes (Goebel et al., 2012) to determine if usage of ME-PPD in hair dye products may be associated with the risk to induce skin sensitization by comparing the skin sensitizing potency (NESIL) with the measured exposure level (MEL) of the skin following a typical hair coloring application.

Material and methods

Analytical standards of all mono and di-acetylated derivatives, parent compounds as well as the hair color formulations used for the *ex-vivo* skin studies were from the Procter and Gamble Service GmbH, Darmstadt, Germany. All other chemicals were of the highest grade from Sigma Aldrich. Radioactive ME-PPD [ring-U-14C]ME-PPD-sulfate with a specific activity of 20 mCi·mmol-1 was used (GE-Healthcare, Cardiff, UK).

Skin exposure, bioavailability and metabolism

Firstly, an experimental design was used to specifically assess the skin exposure to ME-PPD following standardized average use conditions of oxidative hair dyeing with an exposure time of 0.5 h relevant for risk assessment applications. For this purpose, split thickness pig skin samples from back and flank from three different donors (total of 11 skin samples) were used. Due to regulatory requirements for hair dye ingredients in the European Union the data has been assessed by the Scientific Committee of Consumer Safety, and their assessment is publicly available (SCCS, 2013b) and can be referred to for the experimental details. However, it is important to notice that the data was generated by the authors and has not been published in a peer reviewed format yet.

Secondly, a comparable experimental design with living human skin *ex vivo* was used to focus on the metabolite assessment following exposure to ME-PPD in an oxidative hair dye product for 1 h. For this purpose, fresh split-thickness skin samples from four different donors (total of 12 skin samples, 3 per donor, source: cosmetic surgery), trimmed of fat and cut to a thickness of approximately 500 μm, were mounted onto a two-compartment static model (van de Sandt et al., 2004). The skin membranes were transferred into 6-well plates on a Netwell™ insert (Costar, 500 μm mesh), which keeps contact between the receptor fluid (1.2 ml DMEM/Ham F12 media supplemented with epidermal growth factor (10 ng/ml), hydrocortisone (400 ng/ml), gentamycin (50 μg/ml) and foetal calf serum (10% v/v), pH 7.5) and the basal membrane of the skin, while the stratum corneum remains exposed to the air. After mixing an equal amount of the hair dye formulation (containing the coupler 4-amino-2-hydroxytoluene as reaction partner) with developer containing 6% hydrogen peroxide, the mixture was spread evenly on the surface of the skin samples to obtain a final dose of 100 mg/cm^2. The final formulation contained 3% ME-PPD sulfate, spiked with radiolabeled [ring-U-14C]-ME-PPD sulfate and was removed from the skin surface by washing with water and shampoo after an exposure period of 1 h. For the entire experimental period of 24 h samples were kept in a humidified incubator (32 ± 2 °C, 5% CO_2, 40% O_2). Receptor fluid was collected at 3 h and 24 h and frozen until analysis. At 24 h, the stratum corneum was removed from the skin by tape-stripping 15 times. The remaining skin discs were dissolved in 1.5 M KOH in 20% ethanol and were stored at −70 °C until analysis. All samples (surface access, stratum corneum, skin, and receptor fluid) were subjected to determination of radioactivity by scintillation counting. Mass balance was calculated relative to the actual administered dose of [14C]-ME-PPD and only individual diffusion cells with a recovery of 100 ± 10% were considered valid. Viability of the skin was shown to be acceptable by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) conversion of 78% to 103% of the initial activity after 24 h (data not shown). [14C]-ME-PPD metabolites were detected by HPLC-UV-RAD from pooled receptor fluid samples (freeze dried pooled samples per group and time point) and pooled skin compartments (pulverized pooled tissue dissolved in 80% methanol). Analysis by HPLC-UV-RAD was done after organic phase evaporation under a dry stream of nitrogen gas and reconstitution in appropriate mobile phase, and by comparison to reference standards for the parent compound, potential mono- and di-acetylated and sulfated metabolites of ME-PPD.

Both experiments were conducted following OECD guideline 428 (OECD, 2004a, 2004b). For comparison purposes, all data provided are converted to the free base by applying a conversion factor of 0.61 based on the molar ratios (for molecular weight differences see table 2).

Peptide reactivity

Reactivity of test chemicals towards a cysteine-based synthetic peptide was determined as previously described (Gerberick et al., 2009; Lalko et al., 2013). Briefly, reactivity was determined in triplicate reactions containing cysteine peptide (20 μM) at target test chemical concentrations of 0.32, 1.6, 8.0, 40 and 200 μM. Enzyme-mediated reactivity was determined in samples containing horseradish peroxidase and hydrogen peroxide (HRP/P) at a final concentration of 3 U/mL and 100 μM, respectively. Reactivity in the absence of enzyme (direct reactivity) was assessed in test chemical incubations devoid of HRP/P. Following the 24 h incubation period under ambient lab conditions, samples were diluted and processed for analysis by HPLC/MS/MS. Peptide depletion in samples with test chemical was calculated by comparing the peak area ratio in samples containing test chemical to the average of the peak area ratio that was calculated from the corresponding pair of bracketing zero-depletion control samples. Peptide depletion values for individual replicates were determined and then used to calculate mean and standard deviation for both the direct (no HRP/P) and enzyme-mediated (with HRP/P) reactivity determinations.

CD86 expression of THP-1 cells

THP-1 cells were cultivated and CD86 expression of test chemicals was determined as previously described (Hennen et al., 2011). In brief, cells (0.5 × 10^6/ml) were exposed to test chemicals for 24 h, then expression of CD86 as well as cell viability (exclusion of propidium iodide stained cells) was analyzed via flow cytometry, using allophycocyanin-labeled monoclonal anti-CD86 antibody (clone 2331 [FUN-1]) and related isotype control. Data were analyzed with CellQuest Pro software, and RFI of CD86 was calculated as ratio of mean fluorescence