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Prediction of skin sensitization potency sub-categories using peptide reactivity data

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ABSTRACT

While the skin sensitization hazard of substances can already be identified using non-animal methods, the classification of potency sub-categories GHS-1A and 1B is still challenging. Potency can be measured by the dose at which an effect is observed; since the protein-adduct formation is determining the dose of the allergen in the skin, peptide reactivity was used to assess the potency.

The Direct Peptide Reactivity Assay (DPRA; one concentration and reaction-time) did not sufficiently discriminate between sub-categories 1A and 1B (56% accuracy compared to LLNA data, n = 124). An extended protocol termed 'quantitative DPRA' (three concentrations and one reaction-time), discriminated sub-categories GHS 1A and 1B with an accuracy of 81% or 57% compared to LLNA (n = 36) or human (n = 14) data, respectively. The analysis of the Cys-adducts was already sufficient; additional analysis of Lys-adducts did not improve the predictivity. An additional modification, the 'kinetic DPRA' (several concentrations and reaction-times) was used to approximate the rate constant of Cys-peptide-adduct formation. 35 of 38 substances were correctly assigned to the potency sub-categories (LLNA data), and the predictivity for 14 human data was equally high.

These results warrant the kinetic DPRA for further validation in order to fully replace in vivo testing for assessing skin sensitization including potency sub-classification.

1. Introduction

It has been estimated that 15–20% of the general population suffers from allergic contact dermatitis (Bruckner et al., 2000). Accordingly, the identification of skin sensitizing properties of substances forms an important pillar of substance hazard identification and risk assessment. To identify hazards and to determine the need for risk management measures, the outcomes of toxicity tests are translated into hazard categories. Traditionally, hazard and potency are determined by animal studies (Organization for Economic Co-operation and Development (OECD), 2010; OECD test guideline (TG) 406, 1992).

In the EU, the provisions for hazard classification are laid down in *Regulation (EC) No 1272/2008 on the classification, labelling and packaging of substances and mixtures* (CLP; European Parliament and Council, 2008). The CLP Regulation has implemented most of the *United Nation's Globally Harmonized System of Classification and Labelling of Chemicals* (GHS; United Nations, 2015). Specifically, for skin sensitization, the CLP/GHS system prescribes discriminating between category 1 (sensitizers) and no category (non-sensitizers, not classified, NC). Sub-classification of category 1 sensitizers by potency (sub-category (in the following: Cat) 1A/1B) only has to be performed if sufficient data are available. Also under the REACH Regulation (European Parliament and Council, 2006), information on skin sensitization potency is not required if the data are not sufficient for sub-categorisation (in which case sensitizers are assigned to category 1). In general, this is based on data generated by the murine local lymph node assay (LLNA; OECD TG

Abbreviations: A, alanine; AOP, adverse outcome pathway; C, cysteine; Cys, cysteine; DNCB, 2,4-Dinotrochlorobenzene; DPRA, direct peptide reactivity assay; EGDMA, Ethylene glycol dimethacrylate; F, phenylalanine; FN, false negative; FP, false positive; GHS, Global harmonized system of classification and labelling of chemicals; h-CLAT, human cell-line activation test; HPLC, high performance liquid chromatography; HRIPT, human repeated insult patch test; IATA, integrated testing approaches and assessments; K, lysine; LLNA, local lymph node assay; logP, octanol-water partition coefficient (logarithmic form); Lys, lysine; MA, Michael acceptor; MIE, molecular initiating event; n, number of chemicals; OECD, Organization for Economic Co-operation and Development; OECD TG, OECD test guideline; qDPRA, quantitative DPRA; QP, quinone precursor; R, arginine; RC, reactive carbonyl; SB, Schiff 'base former'; S_NAr, Aromatics reacting by nucleophilic substitutions; TN, true negative; TP, true positive

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429; OECD, 2010), although guidance is also provided on how data from other sources, e.g. human or guinea pig data, can be used. The LLNA is preferably used as it includes dose-response assessments, which can be used to determine estimated test substance concentrations that lead to a three-fold increase in the stimulation index (i.e. EC3 values). According to the European Chemicals Agency (ECHA) guidance on classification and labelling (ECHA, 2015), which also describes how to use data derived from human or guinea pigs, substances that yield EC3 value at concentrations ≤ 2% should be considered strong sensitizers, i.e. Cat 1A. Sensitizers with EC3 values above 2% are then classified into Cat 1B (United Nations, 2015). Between weak and strong sensitizers, the relative skin sensitizing potency may vary by up to five orders of magnitude (Basketter et al., 2007).

In the EU, a number of legal acts limit the use of animal testing for regulatory purposes. The REACH Regulation (European Parliament and Council, 2006) prescribes that animal testing for the generation of new data on a toxicological endpoint may only be undertaken as a last resort. The recast *Regulation (EC) No 1223/2009 on Cosmetic Products* (European Parliament and Council, 2009) implemented an animal testing ban with a concomitant marketing ban that came into full force in March 2013 for cosmetic ingredients and products tested on animals after this date. Finally, *Directive 2010/63/EU on the protection of animal used for scientific purposes* (European Parliament and Council, 2010) has implemented the 3Rs principle to replace, reduce and refine animal testing (Russell and Burch, 1959). These and other legal and ethical requirements have fostered the development of non-animal methods for regulatory skin sensitization testing and much progress has been made over the past few years (Mehling et al., 2012). Three non-animal methods have now been successfully validated and adopted as OECD TG (OECD, 2015a; OECD, 2015b; OECD, 2016a, 2016b). Test methods adopted as OECD TG can generally be used for regulatory purposes, i.e. for the hazard identification and risk assessment of substances in an occupational setting or that are intended to be marketed. The revision of Annex VII of the REACH regulation now mandates that non-animal testing has to be conducted and that justification must be given for in vivo testing in order to generate new data to assess skin sensitization potentials (European Commission, 2017).

Currently, a single non-animal method cannot assess the skin sensitization potential of a substance (Basketter et al., 2015; Sauer et al., 2016). Instead, different non-animal methods need to be integrated into testing strategies, which preferably address key events (KE) of the adverse outcome pathway (AOP) for skin sensitization (OECD, 2016a; European Commission, 2017). AOPs describe the sequence of (substance-induced) pathophysiological events, beginning with a specific molecular initiating event (MIE) that initiates a sequence of early cellular events that ultimately result in an observable (toxic) effect (Ankley et al., 2010). Thereby, AOPs provide a framework to describe the mechanisms of toxicity that are relevant for a given toxicological endpoint. The four KE of the AOP for skin sensitization are: KE 1: the covalent binding of electrophilic substances to nucleophilic centers in skin proteins (also considered to be the MIE); KE 2: events in keratinocytes (e.g. inflammatory responses); KE 3: events in dendritic cells (DC; e.g. DC maturation); and KE 4: events in lymph nodes (e.g. T cell priming and proliferation) (Basketter et al., 2013; Basketter et al., 2015; OECD, 2012a; OECD, 2012b). Together, these four KE reflect the induction or sensitization phase of substance-induced allergic contact dermatitis that may become clinically manifest upon secondary exposure to the sensitizing (allergenic) substance (i.e. upon completion of the elicitation or challenge phase) (Mehling et al., 2012). The MIE, KE 1, in the AOP for skin sensitization (i.e. the covalent interaction with skin proteins) can be assessed in the *in chemico* Direct Peptide Reactivity Assay (DPRA) that was originally described by Gerberick and coworkers (Gerberick et al., 2004, 2007) and that has been adopted as OECD TG 442C (OECD, 2015a). In the DPRA, the covalent interaction with proteins is determined by quantifying the peptide reactivity of a substance towards model synthetic heptapeptides that contain either lysine or cysteine (in the following: Lys-peptide and Cys-peptide). Peptide reactivity may depend on a variety of factors including the test substance's electrophilicity, nucleophilicity, the reaction rate and concurring reactions or the stability of resulting conjugates/adducts (Gerberick et al., 2008) as well the presence or absence of abiotic or metabolic activation (Urbisch et al., 2016a). The *in chemico* DPRA is used to assess skin sensitization potential for hazard identification, and a threshold of 6.38% mean Cys- and Lys-peptide depletion is used to discriminate between skin sensitizers and non-sensitizers (in the following: $DPRA_{Cys\ \&\ Lys}$; OECD, 2015a). OECD TG 442C also describes a prediction model (PM) that is based upon Cys-peptide depletion alone. In this PM, a threshold of 13.89% Cys-peptide depletion has been laid down to discriminate sensitizers from non-sensitizers (in the following: $DPRA_{Cys\text{-}only}$; OECD, 2015a). Moreover, the OECD TG 442C PMs include thresholds to quantify peptide reactivity by assigning sensitizing substances to one of three 'reactivity classes', i.e. low, moderate and high reactivity. Noteworthy, these reactivity classes quantify the reactivity (the yield of peptide-adducts) and not per se the sensitization potency. The use of the DPRA and other in vitro methods to determine skin sensitization for REACH has been critically discussed (Sauer et al., 2016) and recently, a new ECHA guidance on REACH Information Requirements and Chemical Safety Assessment came into force which only allows the use of non-animal methods to assess skin sensitization without requiring further animal data (ECHA, 2016).

In the context of skin sensitization, potency can be defined as the amount of an allergen that is needed to sensitize a naïve individual (Kimber et al., 2003). Although other factors, e.g. genetic predisposition or whether specific T-cells are present in the lymph node, will also play a role (not all protein conjugates are sensitizers), the more protein-adducts a potentially sensitizing low-molecular weight substance forms in the skin, the more potent it may be as sensitizer (Friedmann, 2007). This appears plausible, since low-molecular weight substances are generally not allergenic as such but their peptide reactivity determines the amount of the antigen that will be formed in the skin. Hence, skin sensitization potency is related to the peptide reactivity of a substance. Since the DPRA quantifies peptide reactivity, obtained data can be linked to the determination of skin sensitization potency (Gerberick et al., 2007; OECD, 2015a).

While in vivo methods to assess skin sensitization potency are available, non-animal methods to predict the potency of skin sensitizers are still under development (e.g., Hirota et al., 2013;Jaworska et al., 2015;Natsch et al., 2015; Takenouchi et al., 2015). The present study assesses the utility of peptide reactivity data to discriminate skin sensitization potency Cat 1A and 1B according to GHS/CLP. Specifically, the 'standard DPRA' as laid down in OECD TG 442C was compared to a quantitative DPRA (qDPRA) that allows establishing concentration-response relationships (i.e. the concentration dependence of peptide reactivity) and a kinetic DPRA, a modification of the assay published by Roberts and Natsch (2009) that allows investigating the reaction kinetics of peptide reactivity (i.e. whether the concentration dependence of peptide reactivity changes with increasing or decreasing reaction times).

2. Materials and methods

2.1. Test substances and evaluation of available data

For the comparisons between DPRA and LLNA data, previously published in vivo reference data as well as in vitro standard DPRA data were retrieved from Urbisch et al. (2015). The data derived from this paper include standard DPRA data (OECD TG 442) for 199 substances and the corresponding LLNA data (OECD TG 429). For two further substances, standard DPRA data were unavailable in Urbisch et al. (2015), and these data were newly generated in the present study, yielding a total of 201 test substances that were evaluated in the course of the present study.