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Pyridoxylamine reactivity kinetics as an amine based nucleophile for screening electrophilic dermal sensitizers

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ABSTRACT

Chemical allergens bind directly, or after metabolic or abiotic activation, to endogenous proteins to become allergenic. Assessment of this initial binding has been suggested as a target for development of assays to screen chemicals for their allergenic potential. Recently we reported a nitrobenzenethiol (NBT) based method for screening thiol reactive skin sensitizers, however, amine selective sensitizers are not detected by this assay. In the present study we describe an amine (pyridoxylamine (PDA)) based kinetic assay to complement the NBT assay for identification of amine-selective and non-selective skin sensitizers. UV-Vis spectrophotometry and fluorescence were used to measure PDA reactivity for 57 chemicals including anhydrides, aldehydes, and quinones where reaction rates ranged from 116 to 6.2×10^{-6} M^{-1} s^{-1} for extreme to weak sensitizers, respectively. No reactivity towards PDA was observed with the thiol-selective sensitizers, non-sensitizers and prohaptens. The PDA rate constants correlated significantly with their respective murine local lymph node assay (LLNA) threshold EC3 values ($R^2 = 0.76$). The use of PDA serves as a simple, inexpensive amine based method that shows promise as a preliminary screening tool for electrophilic, amine-selective skin sensitizers.

1. Introduction

Allergic contact dermatitis (ACD) is caused by a wide range of chemicals after prolonged or repeated contact with the skin. In developed countries, 15–20% of the population has contact allergy to one or more chemicals in their environment (Nielsen et al., 2001). Contact allergies constitute 20–50% of occupational contact dermatitis cases and it is estimated that ACD accounts for 7% of all occupations related diseases (Andersen, 2003; Jost, 2003). The main causes of ACD in the USA are the members of the Rhus genus (poison ivy, poison oak, and poison sumac), paraphenylenediamine, nickel, rubber compounds and ethylenediamine hydrochloride (Jost, 2003). Chemical-induced allergy thus remains an on-going challenge and an important occupational and general public health issue. People continue to be exposed to new chemicals making the identification of allergenic chemicals a priority.

Developed in the 1990s, the murine local lymph node assay (LLNA) (Gerberick et al., 2007a) is the preferred *in vivo* assay used for skin sensitization hazard identification and characterization. Even though the LLNA is now accepted as a standalone *in vivo* assay for evaluating potential skin sensitizers, recent changes in the European Union will require non-animal based toxicity testing before the marketing of consumer products such as cosmetics (EU Directive, 2012). There is, therefore, a strong push to develop non-animal based assays to screen products for their skin sensitization potential. The basis of these reactivity-based methods is that a compound must be able, either as such or after metabolic or abiotic activation, to react covalently with skin proteins (haptenation) to form a neoantigen. Despite considerable investment in exploring different approaches to develop alternative methods for skin sensitizer identification and characterization, no validated alternative methods are available to date. Nevertheless, a number of emerging *in chemico*, *in vitro* and *in silico* assays (Gerberick et al., 2004, 2007b) are showing promise for use in the identification and characterization of dermal sensitizers. Further exploration of these assays is warranted in view of the potential for their ability to detect and possibly measure the potency of skin sensitizers. Notably, several peptide reactivity based assays have been reported (Gerberick et al.,

Abbreviations: PDA, pyridoxylamine; NBT, nitrobenzenethiol; ACD, allergic contact dermatitis; LLNA, local lymph node assay; SBF, Schiff Base Formers; MA, Michael acceptor; S_N1/S_N2, Nucleophilic Substitution (1 or 2); S_NAr, Nucleophilic Substitution (aromatic); AA, acylating agents.

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2004, 2007b) where the target moieties on the various peptides have usually been either cysteines or lysines. Model peptides have been used as surrogates for protein binding. Aptula et al. (2006) reported the use of glutathione as a model nucleophile to study the reactivity of several skin sensitizers. The direct peptide reactivity assay (DPRA) which measures loss of parent, unbound peptide after addition of an electrophilic chemical, has been nominated to the European Centre for the Validation of Alternative Methods (ECVAM) for validation after demonstrating good sensitivity and specificity (Aeby et al., 2010; Bauch et al., 2011).

A number of limitations associated with peptide reactivity based assays have been identified as discussed by Natsch et al. (2007). These include solubility incompatibilities between peptides and test chemicals, inability to directly monitor the chemical reaction kinetics in solution resulting in estimated rate constants and the non-specific modifications of the peptides due to oxidative reactions. Occurrence of false positives has been noted with peptide reactivity assays due to oxidative chemistry which may not be relevant to skin sensitization. Utility of HPLC-MS techniques (Aleksic et al., 2009; Natsch and Gfeller, 2008) can add specificity and eliminate false positives due to oxidation, but these add complexity to the assays while making them more costly. A recent review also discusses some of the limitations of these assays (Roberts et al., 2008).

The use of low molecular weight model nucleophiles in place of peptides addresses some of the above limitations associated with use of peptide reactivity assays. Relative binding of a chemical skin sensitizer is not dependent on the protein/peptide nature of the nucleophile, but rather follows the HSAB (hard and soft (Lewis) acids and bases) concept which allows for the use of model low molecular weight chemical nucleophiles as protein surrogates to quantify reactivity of electrophilic agents. The HSAB theory and its relevance to several toxicity endpoints have recently been reviewed by Lopachin et al. (2012). The use of relative reactivity does not depend on identification of the target proteins that are covalently modified in the skin allowing for the use of either model peptides or other nucleophiles in the development of *in chemico* assays. Enoch et al. (2008) discusses the importance of using model nucleophiles in a recent review. A high throughput kinetic profiling assay reported by Roberts and Natsch (2009) utilized a model peptide to determine second order rate constants as a quantitative end point. Solubility problems, which are common in these reactivity assays, were addressed in this method. Schwobel et al. (2011) published an extensive review which highlights the importance of incorporating reactivity based assays in the prediction of a chemical's toxicity such as skin sensitization. The review discusses the importance of using model nucleophiles and the influence of experimental factors on the determination of quantitative end points such as rate constants. Extensive reviews on skin sensitization and the development of non-animal based assays based on chemical reactivity, which results in covalent protein binding, have recently been published by Organization for Economic Co-operation and Development (2012a,b). The importance of proper chemical categorization is highly encouraged in these reviews.

The use of 4-nitrobenzenethiol (NBT), which is a "soft" thiol based nucleophile, to quantify reactivity of more than 20 electrophilic skin sensitizers from different mechanistic domains was previously reported from our laboratory (Chipinda et al., 2010) where correlation of reactivity to LLNA potency was demonstrated across all domains. NBT reactivity to Schiff Base Formers and diones was predictably absent as these chemicals are harder electrophiles with preferential reactivity to amine based nucleophiles. This study reports the utility of pyridoxylamine (PDA), a hard nucleophile, to complement NBT for identification of electrophilic skin sensitizers. Reactivity of electrophilic chemicals spanning the S_N1/S_N2, Schiff Base Formers (SBF) and acylating agents (AA) mechanistic domains, among others, is discussed in terms of its correlation to LLNA data compiled by Kern et al. (2010) and Gerberick et al. (2005).

2. Materials and methods

2.1. Chemicals

Phosphate buffer, acetonitrile (ACN), pyridoxylamine dihydrochloride (PDA; CAS # 524-36-7) and all test chemicals which were reagent grade were purchased from Sigma Chemical Co. (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA) and used without further purification. With a few exceptions, chemicals with available LLNA data were chosen as the test chemicals for reactivity with PDA.

2.2. UV/vis spectroscopy

Absorbance measurements were carried out on a Beckman DU 800 Spectrophotometer (Beckman Coulter Inc., Somerset, NJ) using quartz cells with calibrated 1 cm path lengths. Experiments were carried out at 25 °C with temperature being controlled by a Fisher Scientific Model 9000 circulating water bath (Thermo Fisher Scientific Inc., Waltham, MA). Reaction progress was followed by monitoring the loss of the amine PDA at 324 nm, where it has its highest molar absorptivity coefficient (ε). Test chemicals were dissolved in acetonitrile at concentrations ranging from 1 to 10 mM. These solutions (250 μL) were combined with 50 μL of 0.1 mM PDA in phosphate buffer (PB) (pH 7.4) and a further 200 μL of PB in a cuvette. The aqueous content of all the experiments was fixed at 50%. PDA was thus the limiting reagent in the reactions. Control experiments contained test chemical, acetonitrile and phosphate buffer to determine background absorbance before each experiment was initiated. Five replicates were performed for each chemical at each concentration. Fifty seven test chemicals consisting of known skin sensitizers, non-sensitizers and pre/prohaptens were used to evaluate the potential of this kinetic assay for identification of skin sensitizers.

2.3. Fluorescence spectroscopy

Experiments were performed on a Perkin Elmer Luminescence Spectrometer LS50B (Perkin Elmer Inc., Waltham, MA) with a Czerny quartz lamp. Quartz cuvettes with calibrated 1 cm path lengths were used and a circulating water bath temperature control was set at 25 °C. Excitation of PDA was set at 324 nm with emission at 398 nm. Excitation and emission slit widths were set at 10. Test chemicals were dissolved in acetonitrile at concentrations ranging from 1 to 10 mM. These solutions (250 μL) were combined with 50 μL of 0.1 mM PDA in phosphate buffer (PB) (pH 7.4) and a further 200 μL PB + 1500 μL ACN:PB (50:50) in a 1 cm path length cuvette. Control experiments contained test chemical, acetonitrile and phosphate buffer to determine background fluorescence before each experiment was initiated. Five replicates were performed for each chemical at each concentration. The fluorescence measurements used PDA and test chemical concentrations that were 10-fold less than in the absorbance measurements while maintaining the test chemical:PDA concentration ratios in the reaction mixtures.

2.4. Rate constant determination

The amount of amine remaining at time t, $[PDA]_t$, was calculated using the following equation (N1);

$$[PDA]_t = \frac{A_{PDA_t}}{\varepsilon} \text{ or } [PDA_t] = \frac{F_{PDA_t}}{k'} \quad \text{(N1)}$$