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Original article

Development of a prediction method for skin sensitization using novel cysteine and lysine derivatives

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ABSTRACT

Introduction: The Direct Peptide Reactivity Assay (DPRA) was developed as an alternative simple and versatile method for predicting skin sensitization. Here, we describe a novel Amino acid Derivative Reactivity Assay (ADRA) involving 2 amino acid derivatives: *N*-(2-(1-naphthyl)acetyl)-L-cysteine (NAC) and α-*N*-(2-(1-naphthyl)acetyl)-L-lysine (NAL), in which each amino-terminal residue is introduced into a naphthalene ring. ADRA measurements were conducted at 281 nm, which improved baseline stability, and were less influenced by other substances in the reaction solutions than DPRA measurements that are conducted at 220 nm. **Methods:** Chemically synthesized NAC and NAL were dissolved in phosphate buffers of pH 9.5 and 12.0, respectively. Each solution, test chemical solution, and phosphate buffer, were mixed in 96-well microplates and incubated in the dark for 24 h at 25 °C. Following incubation, samples were diluted 10 times with a mixed solution of 25% acetonitrile/0.5% trifluoroacetic acid (TFA) in water, and NAC and NAL levels were quantified in each sample and control using a high-performance liquid chromatography (HPLC) system. The reactivity of NAC/NAL was calculated as the percent depletion based on the decrease in the non-reacted NAC/NAL concentration in the samples relative to the average concentration in the control; the average NAC/NAL reduction score was calculated. A 2-class classification model was developed using ADMEWORKS/ModelBuilder, and an optimal average score that could discriminate between sensitizers and non-sensitizers was determined. **Results:** A total of 82 test chemicals were applied to ADRA for comparison against DPRA. The prediction accuracy of ADRA was 88%, which was similar to that of DPRA. **Discussion:** ADRA was used to quantify NAC/NAL at 281 nm, which showed high accuracy for the prediction of skin sensitization, similar to that of DPRA. Therefore, ADRA could be used to expand the range of chemicals tested in skin sensitization analyses.

1. Introduction

Allergic contact dermatitis is the clinical manifestation of skin sensitization and is caused by a wide range of chemicals after prolonged or repeated contact with the skin. Skin sensitization/allergic contact dermatitis occurs in 6 steps: (1) skin bioavailability, (2) haptenation, (3) epidermal inflammation, (4) dendritic cell (DC) activation, (5) DC migration, and (6) T-cell proliferation, and various methods for predicting or evaluating skin sensitization corresponding to each step have been developed (Adler et al., 2010; Basketter & Kimber, 2010). For example, for steps 2–6, DPRA, the KeratinoSens test method (Aeby, Python, & Goebel, 2007; Basketter & Kimber, 2009; Emter, Ellis, & Natsch, 2010; Natsch, Emter, & Ellis, 2009; Natsch & Gfeller, 2008; Uchino, Taketzava, & Ikarashi, 2009; Vandebriel & van Loveren, 2010), the myeloid U937 skin sensitization test (MUSST) (Python, Goebel, & Aeby, 2007), the human cell line activation test (h-CLAT) (Ashikaga et al., 2006; Sakaguchi et al., 2009), the in vitro DC-based migration assay (Ouwehand et al., 2010), and the CAATC assay (Aliahmadi et al., 2009), have been proposed, respectively.

In the initial process of skin sensitization, the sensitizers are generally electrophilic and reactive toward nucleophilic sites on proteins, and these characteristics have been used to develop non-animal approaches for evaluating the skin sensitization potential of a number of chemicals (Ahlfors, Sterner, & Hansson, 2003; Alvarez-Sanchez, Basketter, Peace, & Leopoittevin, 2003; Meschkat, Barratt, & Leopoittevin, 2001), among which DPRA is currently the most widely used method (Gerberick et al., 2004; Gerberick et al., 2007). Subsequently, a method using the heptapeptide Cor1-C420

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(Natsch & Gfeller, 2008) and similar approaches (Aleksic et al., 2009; Böhme et al., 2009; Roberts, Schultz, Wolf, & Aptula, 2010; Schultz, Yarbrough, & Johnson, 2005) were developed. Among these methods, DPRA has been the most extensively validated, and a prediction accuracy of 89% was demonstrated for a data set that included 82 chemicals (Gerberick et al., 2007). To detect pro-hapten skin sensitizers, a peroxidase peptide reactivity assay using horseradish peroxidase/peroxide was recently proposed (Gerberick et al., 2009; Troutman et al., 2011).

However, the peptides used in DPRA must be detected at 220 nm because of their minimal absorption at longer wavelengths, and various chemical substances also have UV absorption at short wavelengths such as 220 nm. Therefore, these peptides cannot be measured precisely when they co-elute with test chemicals or reaction products in HPLC, as shown by Natsch and Gfeller (2008). Therefore, we chemically synthesized 2 amino acid derivatives with an absorption maximum at 281 nm by introducing a naphthalene ring to the cysteine and lysine residues. We named these derivatives NAC and NAL, respectively, and used them in a novel Amino acid Derivative Reactivity Assay (ADRA). First, to identify the most appropriate conditions for ADRA, we determined the appropriate buffer and buffer pH for the reaction. Furthermore, to allow the experiments to be performed effectively, we facilitated the handling of multiple samples at the same time by using 96-well microplates. Next, to determine the efficacy of ADRA for predicting skin sensitization, we performed a validation test for 82 test chemicals, as reported by Gerberick et al. (2007).

2. Materials and methods

2.1. Test chemicals

The test chemicals for evaluation were those used in the Direct Peptide Reactivity Assay (Gerberick et al., 2007). The chemicals used as test chemicals for ADRA are summarized in Table 1. CD3 (25646-71-3) was supplied by the Synthetic Organic Chemistry Laboratories of FUJIFILM Corporation.

L-cysteine and ε-*N*-BOC-L-lysine for synthesis of NAC and NAL were purchased from Wako Pure Chemical Industries (Osaka, Japan), and 2-(1-naphthyl)acetic acid for synthesis was purchased from Tokyo Chemical Industry (Tokyo, Japan).

2.2. Synthesis of NAC and NAL

2-(1-Naphthyl)acetyl chloride (11.3 g, 55 mmol) was added dropwise into an ice-cooled solution of L-cystine (6.0 g, 25 mmol) and sodium hydroxide (4.2 g, 105 mmol) in water (105 ml), and the mixture was stirred at room temperature for 2 h. Concentrated hydrochloric acid (5 ml) was added to the mixture and the resulting precipitate was collected by filtration. The crude product was recrystallized from ethyl acetate (200 ml) to obtain *N*,*N*′-di[2-(1-naphthyl)acetyl]-L-cystine (8.7 g, 60%). Zinc powder (9.8 g, 150 mmol) was added to a mixture of *N*,*N*′-di[2-(1-naphthyl)acetyl]-L-cystine (14.5 g, 25 mmol), and acetic acid (150 ml) was then added and stirred for 6 h at 60 °C. The reaction mixture was poured into 0.5 M hydrochloric acid (300 ml), extracted with ethyl acetate (450 ml), washed with water (150 ml), washed with brine (150 ml), dried over magnesium sulfate, filtered, and evaporated. The residue was crystallized from isopropanol (40 ml) to give NAC (*N*-2-(1-naphthyl)acetyl-L-cysteine) (7.4 g, 54%) (Fig. 1).

2-(1-Naphthyl)acetyl chloride (4.9 g, 24 mmol) was added dropwise to an ice-cooled solution of ε-*N*-Boc-L-lysine (0.5 g, 20 mmol) and sodium hydroxide (0.8 g, 20 mmol) in water (20 ml), and the pH was maintained at 11 by adding 10% sodium hydroxide aqueous solution. The reaction mixture was stirred at room temperature for 4 h and then acidified with concentrated hydrochloric acid (1.6 ml). The mixture was extracted with hot ethyl acetate (30 ml) and the extract was cooled in an ice bath. The precipitated white solid was filtered to obtain ε-*N*-Boc-α-*N*-2-(1-naphthyl)acetyl-L-lysine (6.1 g, 74%). Trifluoroacetic acid (22 ml, 280 mmol) was added to the solution of ε-*N*-Boc-α-*N*-2-(1-naphthyl)acetyl-L-lysine (5.9 g, 14 mmol) in dichloromethane (35 ml). The mixture was stirred for 1.5 h and then the solvent was removed in vacuo. The residue was dissolved in water (20 ml) and the pH was adjusted to 6 by adding 20% sodium hydroxide aqueous solution. The mixture was extracted with ethyl acetate (50 ml) and evaporated. The residue was purified by column chromatography on a silica gel. The obtained product was crystallized from ethyl acetate/methanol to give pure NAL (α-*N*-2-(1-naphthyl)acetyl-L-lysine) (0.5 g, 11%) (Fig. 1). NAC and NAL were identified using the 3200 QTRAP LC/MS/MS system (AB SCIEX, Ontario, Canada). MS of NAC m/z (%): 290(9) ($[M + H]^+$), 141(100), 122(26), 115(48), and 76(9) (Fig. 2). MS of NAL m/z (%): 315(54) ($[M + H]^+$), 141(100), 129(21), 115(63), and 84(75) (Fig. 2).

2.3. NAC and NAL reactivity assay

Disodium hydrogen phosphate for buffer preparation, acetonitrile, trifluoroacetic acid (TFA) for HPLC, DMSO, and acetone were purchased from Wako Pure Chemical Industries.

Sodium phosphate buffers for NAC and NAL (100 mM; pH 9.5 and pH 12.0) were prepared with disodium hydrogen phosphate and sodium hydride solution, respectively.

Stock solutions of NAC and NAL were prepared to a final concentration of 1.25 mM in 100 mM phosphate buffer at pH 9.5 and pH 12.0, respectively. Test chemical solutions at a concentration of 100 mM were prepared in acetonitrile, water, acetone, or acetonitrile containing 10% or 20% DMSO. Triplicate reactivity samples were prepared in a 96-well microplate. For NAL reactivity, 80 μl of the NAL stock solution and 50 μl of the test chemical stock solution were added to 70 μl of sodium phosphate buffer (pH 12.0). For NAC reactivity, 80 μl of the NAC stock solution, 10 μl of the test chemical stock solution, and 40 μl of acetonitrile were added to 70 μl of sodium phosphate buffer (pH 9.5). As controls, samples containing solvent rather than test chemicals were also prepared in triplicate. The 96-well microplates were sealed by Plate Seal (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) and gently shaken and incubated in the dark for 24 h at 25 °C in an incubator (MIR-253, SANYO Electric Co., Ltd. Osaka, Japan). Following incubation, samples were diluted 10 times with a mixed solution of 25% acetonitrile/0.5% TFA in water, and 96-well microplates were sealed by Sealig Mat (AxyGen Scientific, Inc., Union City, CA). In addition, standards used for defining the calibration curve were prepared without the test chemicals for each amino acid derivative at concentrations ranging from 0.0625 mM to 0.5 mM.

2.4. HPLC analysis of NAC and NAL

NAC and NAL in all samples and standards were quantified using a LC-20A HPLC system (Prominence, Shimadzu Scientific Instruments, Kyoto, Japan) on a CAPCELL CORE C18 column (2.7 μm, 3.0 × 150 mm, Shiseido Co., Ltd., Tokyo, Japan) with UV detection at 281 nm using a photodiode array detector (SPD-M20A, Shimadzu), which scanned wavelengths from 200 nm to 400 nm. The UV spectrum was collected from 200 nm to 400 nm to verify the identity of the NAC and NAL. The temperature of the column oven (CTO-20A, Shimadzu) and autosampler (SIL-20 AC, Shimadzu) was controlled at 40 °C and 4 °C, respectively.

The mobile phase had a flow rate of 0.3 ml/min and consisted of 98/2 water/acetonitrile with 0.1% TFA (A) and 90/10 acetonitrile/water with 0.1% TFA (B). Separation was achieved by holding at the initial conditions (20% B) for 7 min, followed by a linear gradient to 100% B for 3.5 min and then back to the initial conditions for a total analysis