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Skin sensitization risk assessment model using artificial neural network analysis of data from multiple *in vitro* assays

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ABSTRACT

The sensitizing potential of chemicals is usually identified and characterized using *in vivo* methods such as the murine local lymph node assay (LLNA). Due to regulatory constraints and ethical concerns, alternatives to animal testing are needed to predict skin sensitization potential of chemicals. For this purpose, combined evaluation using multiple *in vitro* and *in silico* parameters that reflect different aspects of the sensitization process seems promising.

We previously reported that LLNA thresholds could be well predicted by using an artificial neural network (ANN) model, designated iSENS ver.1 (integrating *in vitro* sensitization tests version 1), to analyze data obtained from two *in vitro* tests: the human Cell Line Activation Test (h-CLAT) and the SH test. Here, we present a more advanced ANN model, iSENS ver.2, which additionally utilizes the results of antioxidant response element (ARE) assay and the octanol–water partition coefficient (Log*P*, reflecting lipid solubility and skin absorption). We found a good correlation between predicted LLNA thresholds calculated by iSENS ver.2 and reported values. The predictive performance of iSENS ver.2 was superior to that of iSENS ver.1. We conclude that ANN analysis of data from multiple *in vitro* assays is a useful approach for risk assessment of chemicals for skin sensitization.

1. Introduction

Tests for skin sensitization are required prior to the introduction of novel cosmetic ingredients. Animal models such as the guinea pig maximization test (Magnusson and Kligman, 1969) were historically used to identify whether a chemical has the potential to induce skin sensitization in humans. More recently, the murine local lymph node assay (LLNA), in which cellular proliferation in the draining lymph node is measured after repeated topical application of the test compound onto the ears, has been employed (Basketter et al., 2002). The relative potency of a chemical sensitizer is measured by deriving what is known as the EC3 value, which is the test chemical concentration necessary to produce threshold stimulation of proliferation in draining lymph nodes as compared with concurrent vehicle controls (Basketter et al., 2002). Based on EC3 values, chemicals can be classified into five potency categories (non-sensitizing, and weak, moderate, strong and extreme sensitizers), and it is also possible to predict a safe level of human exposure using a quantitative risk assessment approach (Api et al., 2008).

Due to regulatory constraints and ethical concerns, great efforts have been made to develop alternatives to animal testing to predict the skin sensitization potential of chemicals. In the induction phase of skin sensitization, haptens penetrate through stratum corneum and activate antigen-presenting cells (APCs), including dendritic cells (DCs). Furthermore, the activation is accompanied with augmentation of CD86 and CD54 expression. The activated cells migrate into lymph nodes and present antigens to T cells. At the same time, the Keap1-Nrf2-antioxidant response element (ARE) regulatory pathway is activated as an innate toxic response (Emter et al., 2010). Each of these activation steps can be probed by means of recently developed *in vitro* methods. The human Cell Line Activation Test (h-CLAT) is used to model the activation of dendritic cells by measuring changes in expression of CD86 and CD54 in THP-1 cells upon exposure to chemicals (Ashikaga et al., 2006; Sakaguchi et al., 2006). Similar *in vitro* sensitization methods using U-937 (Python et al., 2007) and MUTZ-3 cell lines (Azam et al., 2006) have also been reported. Hapten–protein complex formation is also an essential step in the process of skin sensitization. Direct peptide reactivity assay (DPRA) (Gerberick et al., 2007) is a method to detect hapten–protein complex formation using two synthetic peptides, which contain either a single cysteine or lysine as a reaction target.

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Further, we have reported the SH test, which measures changes of cell-surface thiols induced by haptens as a model of activation of intracellular signal transduction (Suzuki et al., 2009). The SH test might also reflect hapten–protein complex formation, because changes of cell-surface thiols can be caused by reaction of hapten with cell-surface proteins, and were shown to be correlated with the reactivity of cysteine-containing peptide with hapten in DPRA (Hirota et al., 2013). The ARE assay (Natsch et al., 2008) and KeratinoSens (Emter et al., 2010) are used to evaluate activation of the antioxidant signaling pathway by measuring the enhancement of ARE-dependent gene expression following exposure to chemicals. These two assays reflect emerging innate toxic responses, mainly in keratinocytes.

It is widely recognized that a single *in vitro* test is insufficient to replace animal testing and that integration of results from different *in vitro* tests, as well as *in silico* methods, is needed for prediction of the skin sensitization potential of chemicals (Jowsey et al., 2006; Natsch et al., 2009; Jaworska et al., 2011). Artificial neural network (ANN) analysis is a nonlinear statistical data-modeling tool, which is useful to model complex relationships between inputs and outputs. In our previous report (Hirota et al., 2013), we utilized ANN analysis to integrate data from the SH test and h-CLAT and found that the resulting model, named iSENS ver.1 (integrated *in vitro* sensitization tests by artificial neural network version 1), could predict *in vivo* LLNA thresholds at least in part. In iSENS ver.1, we used the maximum amount of change of cell-surface thiols (MAC value) in the SH test and the minimum value of CV75 (concentration giving 75% cell viability in cytotoxicity assay), EC150(CD86) and EC200(CD54) (threshold concentrations for inducing CD86 and CD54 expression, respectively, in h-CLAT) as descriptors for the input layer in ANN analysis (Hirota et al., 2013). The CV75 is determined as part of the dose-setting procedure for hCLAT. In the present study, we aimed to improve the predictive performance of iSENS ver.1 by adding the water-octanol partition coefficient (Log*P*) as a parameter reflecting the ability of chemicals to penetrate into the epidermis and the result of ARE assay as a parameter reflecting activation of antioxidant signaling, in addition to the above two parameters. The resulting extended ANN model, designated iSENS ver.2, showed improved predictive ability compared to iSENS ver.1.

2. Materials and methods

2.1. Databases

In total, 62 test chemicals were used in this study (53, used as the training set, are listed in Table 1 and the other 9 are listed in Table 3, with their Chemical Abstract Service (CAS) numbers). All the chemicals had been evaluated and classified with the LLNA (Basketter, 2010; Basketter et al., 2007; Basketter and Kimber, 2011; Basketter and Scholes, 1992; Ryan et al., 2002; Gerberick et al., 2004a,b, 2005; Kimber et al., 2003; OECD Test Guideline 429, 2010). They had also been evaluated with h-CLAT and the SH test (Ashikaga et al., 2010; Nukada et al., 2012; Hirota et al., 2013; Hoya et al., 2009).

2.2. Chemicals

The majority of the test chemicals were purchased from Sigma–Aldrich Corporation (St. Louis, MI). As for the others, propyl gallate, linalool, hydroxycitronellal, pyridine, cobalt chloride, propylene glycol were from Wako Pure Chemicals (Osaka, Japan), 2-mercaptobenzothiazole and hexylcinnamic aldehyde were from Tokyo Chemical Industry (Tokyo, Japan), ethylenediamine was from Junsei Chemical (Tokyo, Japan), isopropanol was from (Dojindo Laboratories, Kumamoto, Japan), and dimethyl sulfoxide (DMSO) was from Kanto Chemical (Tokyo, Japan).

Log*P* values of these chemicals were calculated using KOWWIN V.1.67 obtained from the United States Environmental Protection Agency web site.

2.3. Cells and culture

AREc32 cells were obtained from CXR Biosciences, Dundee, Scotland. The cells were maintained in Dulbecco's modified Eagle's medium containing Glutamax supplemented with 10% (v/v) FBS, 100 units/mL penicillin, 100 μg/mL streptomycin (all from Gibco) and 500 μg/mL G418 (Calbiochem) at 37 °C in an incubator under an atmosphere of 5% CO_2 in air.

2.4. Pre-study cytotoxicity assay

AREc32 cells were seeded in 96-well plates at a density of 50,000 cells per well in 180 μL of growth medium and pre-cultured for 48 h. Test chemicals were dissolved in culture medium or DMSO and 6 binary dilutions were made. If DMSO was used as a solvent, the solutions were further diluted 10-fold with culture medium so that the final concentration of DMSO was less than 1%. After the pre-culture period, cells were treated with 20 μL of various concentrations of chemicals for 24 h and then washed twice with phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS(-)). The cell viability was determined using CellTiter-Glo Luminescent Cell Viability Assay Reagent (Promega KK, Tokyo, Japan). The concentration providing a cell viability of 75% (CV75) was calculated for each chemical by interpolation on the log-linear dose–response curve.

2.5. ARE assay

The ARE assay was based on that of Natsch and Emter (2008), but we revised the dose selection protocol as follows in order to match the h-CLAT protocol (Ashikaga et al., 2010). The test doses were set at 1/8*CV75, 1/4*CV75, 1/2*CV75, CV75, 2*CV75, 4*CV75. If the CV75 could not be determined, the test doses were set at 6 binary dilutions from the maximum concentration of 10,000 μg/mL (for water-soluble chemicals) or from 5000 μg/mL (for water-insoluble chemicals) or from the concentration corresponding to the maximum solubility in each solvent.

AREc32 cells were seeded and treated with chemicals in the same way as described for the cytotoxicity assay. At 24 h after addition of the test compound, cells were washed twice with PBS(-) and luciferase activity was measured using the Steady-Glo Luciferase Assay System with a GloMax Luminometer (Promega).

Cytotoxicity of compounds towards AREc32 cells was tested in parallel assays run under the same conditions and with the same test concentrations. At 24 h after addition of the compound, cell viability was measured as described above.

2.6. Data analysis for ARE assay

The assay was repeated three times, with duplicate analysis for each chemical at each concentration in each repetition. A chemical was rated positive if it induced an increase of luciferase activity of more than 1.5 times compared to the vehicle control at any of the tested concentrations, either in all repetitions or in two out of three repetitions. When the cell viability was less than 50%, the relative luciferase activity was not calculated. Based on these experiments, the average maximal induction of luciferase activity (Imax) and the concentration giving maximal induction (CImax) were determined for each test chemical, as described previously (Natsch et al., 2008). The threshold concentration for positivity of a chemical (EC1.5) was calculated for each chemical by interpolation on the log-linear dose–response curve.