Dose-response for predicting skin sensitization potency with the GARD®skin method

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Abstract: Allergic contacts dermatitis is a major health problem in the world and the disease is caused by a type IV hypersensitivity to a small chemical called a hapten. These substances are common in cosmetics, perfumes, pharmaceuticals and house-hold products, and risk factors include both inherent factors and frequent or high exposure. Hazard assessment and potency classification of sensitizers are thereby of great need, to enable withdrawal of problematic substances or decide on a dose with minimal adverse effects. One of the key events in the initial sensitization phase is the activation of dendritic cells. The GARD® assays use a prediction signature to analyze the immunological response of this key event that through a support vector machine give a positive decision value if a chemical is sensitizing. The first method of the GARD® assays, GARD®skin was developed for skin sensitization hazard assessment. Current study analyzed the dose-response relationship of GARD®skin for the potential to predict potency. A four-parametric (min, max, slope, ED_{50}) log-logistic curve was used to form predicted dose-response curves to the experimental data points from seven chemicals of different CLP/GHS regulatory classifications. Comparison of the concentration at the inflection point to sensitizing values and the response variables to regulatory, human potency, and murine values suggest that GARD®skin through utilization of dose-response data has potential for skin sensitization potency classification.

Introduction

The prevalence of allergic contact dermatitis (ACD) to at least one sensitizer is about 20% in Europe (1, 2) and 50% in North America (3). These substances are common in e.g. cosmetics, perfumes, pharmaceuticals and house-hold products. Frequent exposure results in an increased risk to develop ACD. Inherent factors including age, ethnicity, gender, and genetics also affect the risk. Occupational contact dermatitis is a huge problem due to the unawareness of hazards and poor working practices, and minimization or removal of risk factors can decrease the problem (2, 3). Therefore, proper classification and determination of acceptable levels of sensitizers in finalized consumer products is essential.

The Adverse Outcome Pathway (AOP) provides a schematic overview of the key events (KE) of the development of a type IV hypersensitivity reaction resulting in ACD (4). However, as highlighted in recent reviews by Martin (5) and Koppes (6), new aspects of skin sensitization still needs to be investigated. In summary, sensitizers are low molecular weight chemicals, called haptens, which are able to access the viable epidermis and form immunogenic protein-hapten complexes with endogenous proteins, mainly through covalent bonds. This happens either directly, or after chemical activation through air oxidization (pre-hapten) or keratinocyte facilitated metabolization (pro-hapten) (KE1). In the skin, keratinocytes mediate a proinflammatory microenvironment upon production of mediators, such as cytokines, and thus, stimulate the initiation of an immune response through secretion of danger signals (KE2). Cutaneous dendritic cells (DC) internalize and process the allergenic complex, and upon maturation upregulate co-stimulatory molecules and change their expression of adhesion molecules, chemokine/cytokines and their receptors (KE3). During maturation, DCs migrate to the local lymph nodes where the allergen is presented by the major histocompatibility complex (MHC) to naïve T-cells, which subsequently start proliferating and become polarized into helper T-cells (CD4+) or cytotoxic effector T-cells (CD8+) (KE4). The exposed individual is now referred to as sensitized and upon repeated exposure the clinical symptoms of ACD, such as eczema, will appear (4, 6).

Increased knowledge of the development of ACD have led to regulations in order to limit the contact with sensitizers and improve testing methods. To protect the human health and environment the European Classification, Labelling and Packaging (CLP) regulations require labeling of sensitizing chemicals (category 1) into subcategorizes 1A (strong) and 1B (weak) in accordance to United Nations' Globally Harmonized System of Classification and Labelling of Chemicals (GHS) (7). In addition, REACH demands skin sensitization testing and proper labeling of all large-scale productions (>1 ton/year) in Europe (8). Historically, animal-based guinea pig methods (9) or versions of the murine LLNA method (10-13) have been used. The Three Rs (Replace, Reduce, Refine) published in 1959 discussed the ethics of animal methods and the need to work towards less suffering, reduction of animals used and finally replace animals with alternative methods (14). To date, five non-animal methods (*in vitro*, *in silico*, or *in chemico*) have been validated by regulatory agencies and accepted by the Economic Co-operation and Development (OECD) (15-18). In addition, animal-based methods have been banned from the cosmetics industry in Europe (19). A classification system of chemicals' relative human skin sensitization potency (HP) has also been suggested for more human like skin sensitization potency categorization. It is based on available human data of the No Observed Effect Level (NOEL) and frequency of allergy in the general population (20). However, none of the OECD accepted non-animal methods satisfies the regulatory requirements of both hazard assessment (sensitizer vs. non-sensitizer) and potency classification (CLP 1A or 1B). Therefore, integrated approaches to testing and assessment (IATA), combing non-animal methods have been suggested for the decision making by OECD (18, 21). In addition, other promising non-animal methods have been included into the

OECD Test Guidelines programme. One of these, the *in vitro* assay GARD®skin (18), is used in the current study.

The GARD® (Genomic Allergen Rapid Detection) (22-24) assays are performed in a myeloid DC-like cell-line and are based on measurements of the transcriptional levels of genomic biomarkers, referred to as the prediction signatures. GARD®skin is based on a prediction signature comprising 200 biomarkers with high classification accuracy for hazard assessment (25-27) by mimicking KE3 of the AOP of skin sensitization. An initial screening of a test chemical's cytotoxicity determines the concentration of the chemical to be used for stimulation (stimulation concentration) of the DC-like cells. Based on the RNA expression levels, a decision value (DV) generated from a support vector machine (SVM) is used for classification, where a positive DV means that a chemical is a sensitizer (22-24). In addition to hazard assessment, potential for potency predictions has been observed for GARD®skin (22). GARD®potency was developed to provide subcategorization of sensitizers according to their relative sensitizing potency no cat (non-sensitizer), CLP 1A (strong) and CLP 1B (weak) (28). Today, GARD®skin and GARD®potency may be combined in a tiered approach to provide full risk assessment of chemicals (SenzaGen AB, Lund, Sweden).

The stimulation concentration and following DV of GARD®skin have been hypothesized to correlate with the skin sensitizing potency of a chemical. Strong sensitizers (CLP 1A) have been observed to often be cytotoxic and assayed at low stimulation concentrations to induce relatively high DVs according to the GARD®skin prediction model (22, 26). In this study, the dose-response relationship of the GARD®skin assay was examined to observe if it can be utilized in determining skin sensitizing potency of assayed compounds. Substances with various sensitizing potencies, as defined according to CLP categories (7), LLNA data (11) and human NOEL values (20), were evaluated in a range of exposure concentrations. The obtained and herein presented results indicate that the relationship between dosage and the response of GARD®skin may provide a useful tool for risk assessment of chemicals related to their sensitizing potency.

Materials and Methods

Chemicals

Seven well characterized compounds with known classifications were used for cell stimulations (Table I). The selected chemicals were; 2,4-dinitrochlorobenzene (DNCB), formaldehyde (FA), cinnamic aldehyde (CA), resorcinol (RC), eugenol (EG), ethyl vanillin (EV) and 1-butanol (BUT). Dimethyl sulfoxide (DMSO) or deionized water (dH_2O) was used as solvent. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Table I. Compounds included in the dose-response analysis and respective potency classifications according to CLP/GHS, LLNA and HP.

Compound	**Abbreviation**	**CAS No**	**CLP/GHS**	**LLNA (10)**	**HP (20)**
2,3-dinitrochlorobenzene	DNCB	97-00-7	1A (29)	Extreme (29)	1
Formaldehyde (37% solution in dH_20)	FA	50-00-0	1A (29)	Strong (29)	2
Cinnamic aldehyde	CA	104-55-2	1A (30)	Moderate (31)	2
Resorcinol	RC	108-46-3	1B (31)	Moderate (32)	4
Eugenol	EG	97-53-0	1B (30)	Weak (31)	3
Ethyl Vanillin	EV	121-32-4	No cat.	Non-sensitizer (33)	Not found
1-Butanol	BUT	71-36-3	No cat.	Non-sensitizer (34)	6
Dimethyl sulfoxide	DMSO				
Deionized water	dH_20				

Cell maintenance and cell stimulations for dose-response

The SenzaCell cell system (SenzaGen AB, Lund, Sweden) was used as an *in* vitro model of DC. Cell maintenance, phenotypic analysis, cytotoxicity screening, and cell stimulations were performed according to published GARD® protocols (22-24) with deviation in the number of concentrations and replicates used to adapt for dose-response analysis. In short, the cells were maintained at 200 000 cells/ml with media exchange and cell count every 3-4 days. Prior to chemical exposures, phenotypic analysis with flow cytometry was performed to assess cell viability and level of maturation. Cells were either stained with propidium iodine (PI) (BD Biosciences, San Diego, CA), specific mouse mAbs conjugated to FITC (CD86, HLA-DR, CD34 (BD Bioscience), CD1a (DakoCytomation, Glostrup, Denmark)) or PE (CD54, CD80 (BD Biosciences), CD14 (DakoCytomation)), or mouse IgG1 FITC- or PE-conjugated isotype controls (BD Biosciences). Data acquisition of 10,000 events/sample was done on the FACSVerse Instrument with BD FACSSuite v1.06 software (BD Biosciences). Data analysis was done on the FlowJo® v10 software (FlowJo, LLC, Ashland, Oregon) setting gates to exclude cell debris and non-viable cells and quadrants to detect the signals from the specific mAbs. The included chemicals' toxic effect to the cells were examined in the cytotoxicity screening with flow cytometry. 24 hours after incubation stimulated cells and controls were stained with PI, apart from extra wells of unstimulated cells that were left unstained. 10,000 events/sample were acquired with gates set for the cell population (Appendix A) and absolute viability (Appendix B), eliminating cell debris and nonviable cells. The obtained result was used to determine an optimal concentration range for the main chemical stimulations for dose-response analysis.

Cytotoxicity screenings of 16-18 concentrations, in biological duplicates, were run per chemical using flow cytometry (Table II). The range was selected to include concentrations with no toxicity, the optimal dosage as described below and possible toxic concentrations. Controls of unstimulated cells and a negative control with final in-well concentration of 0.1% DMSO were also analyzed. Chemical replicates and necessary controls were done in the same experiment. For biological duplicates, two separate wells of the cells from the same batch were exposed to identical chemical stimulation concentration, or control before 24 hours of

incubation. Two additional wells with unstimulated cells were prepared. For chemical replicates the stimulated samples and control were divided into two different wells after the 24 hours of stimulation. The unstimulated cells were divided into three different wells. In flow cytometry screenings two out of three unstimulated replicates would be PI-stained. The third replicate represent the extra well of unstained, unstimulated cells (Appendix A and B).

The relative viability (Rv) was calculated from the PI-stained flow cytometry results of the cytotoxicity screenings according to the following formula (Eq. 1) (22-24):

$$Relative\ viability = \frac{Fraction\ of\ viable\ stimulated\ cells}{Fraction\ of\ viable\ unstimulated\ cells} * 100$$

10 concentrations per chemical, equally distributed around the concentration inducing a Rv of 90% (Rv90) were selected (Table II). Endpoints were set to comprise the highest concentration without observed cytotoxicity and the highest concentration constrained by cell viability, with the aim to include the concentration when the final GARD®skin decision value (DV) is 0 ($C_{DV(0)}$) regardless of the substance classification. In the same experiment and time-point, cells stimulated with chemicals were prepared in 1-2 biological replicates and unstimulated cells in triplicates by separation in different wells. Incubation, preparation of samples in technical duplicates for flow cytometry and the flow cytometry analysis were performed as described in GARD® protocol (22-24).

Table II. Vehicle and concentrations of importance for test compounds used.

Compound	Vehicle	Expected Rv90 (22) (µM)	Cytotoxicity screenings (µM)	Main stimulations (µM)
DNCB	DMSO	4	0,04-40	0,5-30
FA	DMSO	80	0,8-800	10-145
CA	DMSO	120	1,2-1200	10-145
RC	dH_20	500	5-2000	10-1500
EG	DMSO	300	3-2000	10-1500
EV	DMSO	500	5-2000	10-1500
BUT	DMSO	500	5-2000	10-1500

Extraction, isolation and quality control of RNA

Harvesting and lysing of cells, RNA isolation and RNA quality control (QC) were performed as described in GARD® protocols (22-24). The harvested cells were lysed with the TRizol reagent (Thermo Scientific, Waltham, MA) and stored at -20°C. The RNA was isolated from the cells and purified with the Direct-zol™ RNA MiniPrep column purification kit (Zymo research, Irvine, CA) according manufacturer's protocol and stored at -80°C. Integrity and total concentration of the purified RNA was measured on the Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) as RNA QC. (24, 26)

Gene expression quantification and data analysis

One RNA sample of 100 ng (5 µl at 20 ng/µl) per chemical concentration was prepared and run on the NanoString GEN2 nCounter Analysis System according to instructions by the supplier (NanoString Technologies, Seattle, WA). Hybridized RNA was processed at the NanoString GENE2 nCounter Prep Station 5s with the high sensitivity protocol. The gene expression was individually quantified at the NanoString Digital Analyzer 5s with the customized NanoString CodeSet for the GARD®skin prediction signature (GPS) under maximal resolution (555 Fields of View) (24, 26). The CodeSet and equipment used were obtained from Nanostring Technologies (NanoString Technologies, Seattle, WA).

The received NanoString data was analyzed with the GARD®skin prediction model in the GARD® Data Analysis Application (GDAA), using unstimulated samples as reference samples for normalization purposes. The DV was generated by a support vector machine

(SVM) trained on the GPS of GARD®skin, where a positive DV is indicative of a sensitizer. (24)

Dose-response analysis

The acquired, actual GARD® DV was set as the response variable (y) and plotted against the stimulation concentration as the dosage (x). A Boltzmann fit, four-parametric log-logistic sigmodal curve (Eq. 2) (35, 36) was used to investigate the dose-response relationship for the sensitizing chemicals:

$$f(x,(b,c,d,\bar{e})) = c + \frac{d-c}{1+e^{b(\bar{e}-logx)}}$$

with predicted GARD® DV as the response variable (f(x,(b,c,d,ē))), the stimulation concentration as the dosage (x), b represented the slope of the linear part of the curve, c and d were the lowest respectively highest limit of response and ē was the logarithm of the effective dose found at 50% of the highest limit of response (ED_{50}). (35) The highest response (maximum) establish the efficacy of a substance while the dose or concentration at ED_{50} often determines the therapeutic index in toxicological studies (37).

The best fit of the predicted response to the actual, experimental DVs were determined by the non-linear least squares method (Eq. 3) (36):

$$\sum_{i=1}^{n}(y_i - f(x,(b,c,d,\bar{e})))^2$$

by finding the slope and ED_{50} value that minimized the resulting residual sum of squares (RSS). The total sum of squares (TSS) (Eq. 4) (36):

$$\sum_{i=1}^{n}(y_i - \bar{y})^2$$

with ȳ as the average of the actual response values was together with the RSS result used to evaluate the fit of the predicted results to the actual results. The regression coefficient R^2 (Eq. 5) (36):

$$R^2 = 1 - \frac{RSS}{TSS}$$

was together with RSS, and TSS calculated from the average DV of the experimental unstimulated samples to the experimental maximum DV of each chemical. All dose-response related calculations were performed in Microsoft® Excel® for Office 365 with add-on feature Solver to fit the predicted data points to the actual data points.

Results

In the current study, the dose-response relationship of the GARD®skin assay was explored for the potential to be used as a predictive tool in skin sensitizing potency assessment. A reference panel of seven chemicals with known sensitizing properties were used. The cytotoxic properties of the chemicals were determined after stimulating the human DC-like SenzaCells. RNA from a selected range of relevant concentrations was extracted, isolated and purified. The gene expression was analyzed with the GARD®skin prediction signature on the NanoString technology. A GARD®skin trained SVM generated DVs, giving a positive value for sensitizers.

Dose-response dependent cytotoxicity

The toxicity profile of studied reference chemicals was investigated by quantifying Rv and the frequency SenzaCells over the concentrations tested in the cell stimulations using flow cytometry. The DMSO vehicle control was used as a reference and ensured to not affect the cells. The initial cytotoxicity screenings were visualized to select a range from around the highest concentration without observed cytotoxicity to the highest concentration constrained by cell viability to use in the GARD®skin analysis. Tested concentrations were distributed evenly around the expected Rv90 concentration of a chemical and referred to as the main stimulations (Table II). Prior to initial toxicity screenings and main stimulations, the phenotypic profile had been examined as previously described (22-24).

The cytotoxicity profiles of cells exposed to DNCB, FA, CA, EG, RC, EV, or BUT were assessed using PI-staining and flow cytometry (Fig. 1-7). Rv (%, black data points) was calculated against the unstimulated cells (Appendix B). The cell frequency (%, blue data points) was derived from the FCS/SSC plot (Appendix A). The initial screenings are visualized with the dotted line and cross data points. The main stimulations are viewed on the full line with round data points.

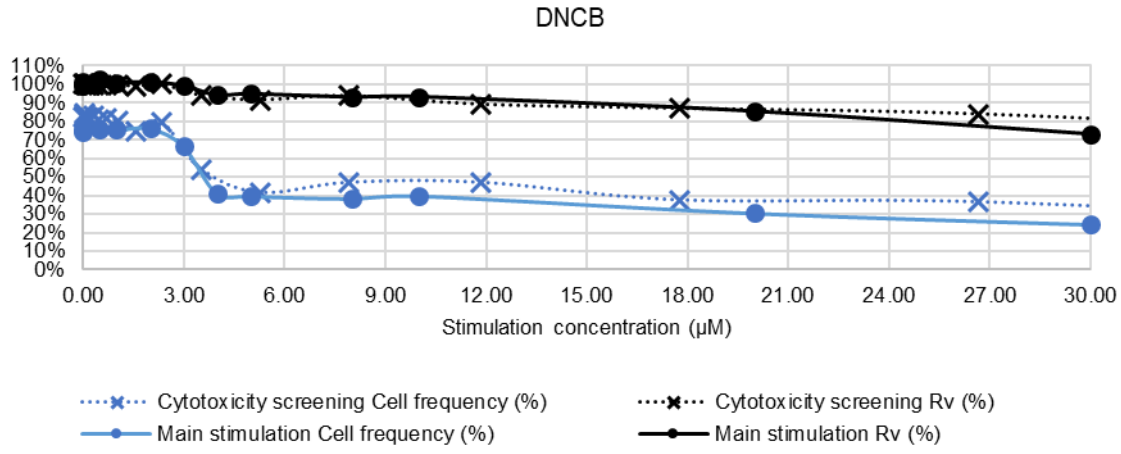

Figure 1. Cells stimulated with DNCB. Main stimulations were performed from 0.5 µM to 30 µM based on cytotoxicity and cell viability in initial cytotoxicity screening.

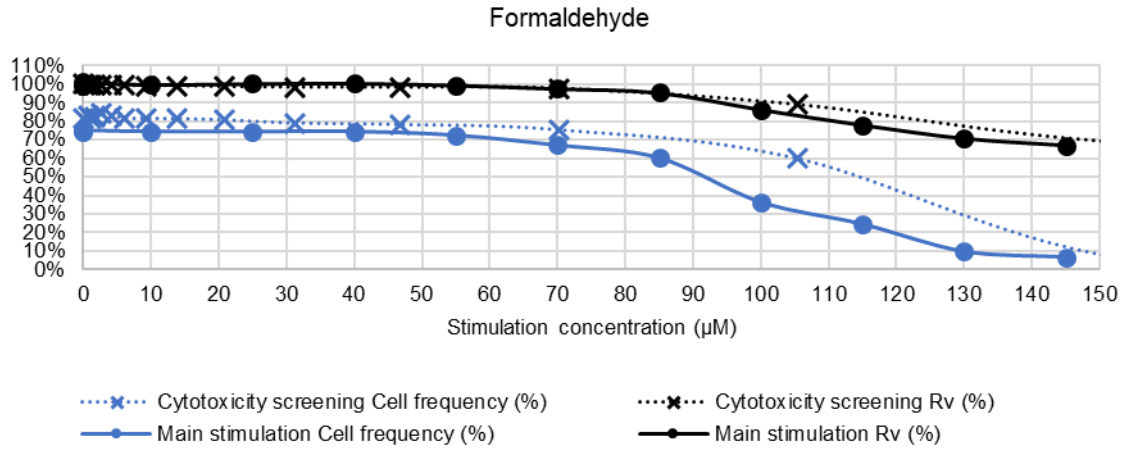

Figure 2. Cells stimulated with Formaldehyde. Main stimulations were performed from 10 µM to 145 µM based on cytotoxicity and cell viability in initial cytotoxicity screening.

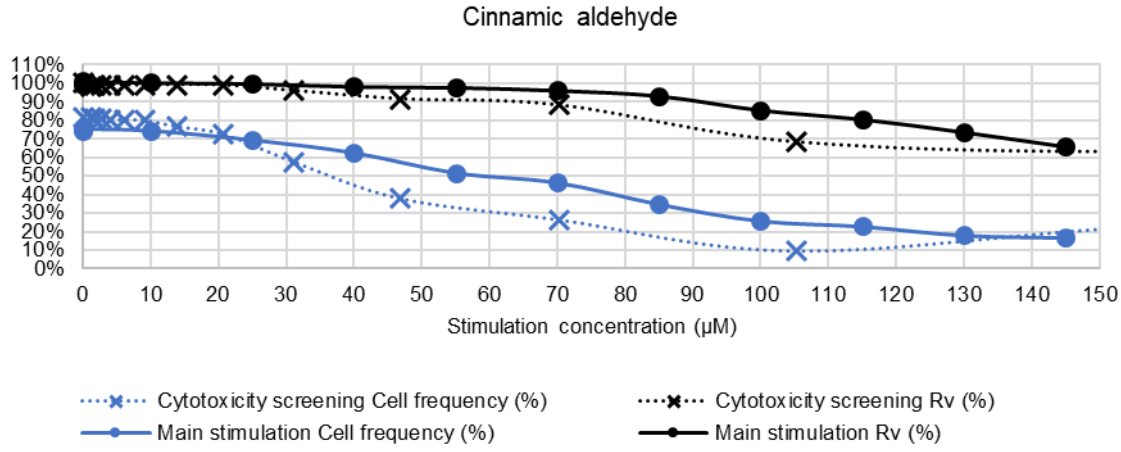

Figure 3. Cells stimulated with Cinnamic aldehyde. Main stimulations were performed from 10 µM to 145 µM based on cytotoxicity and cell viability in initial cytotoxicity screening.

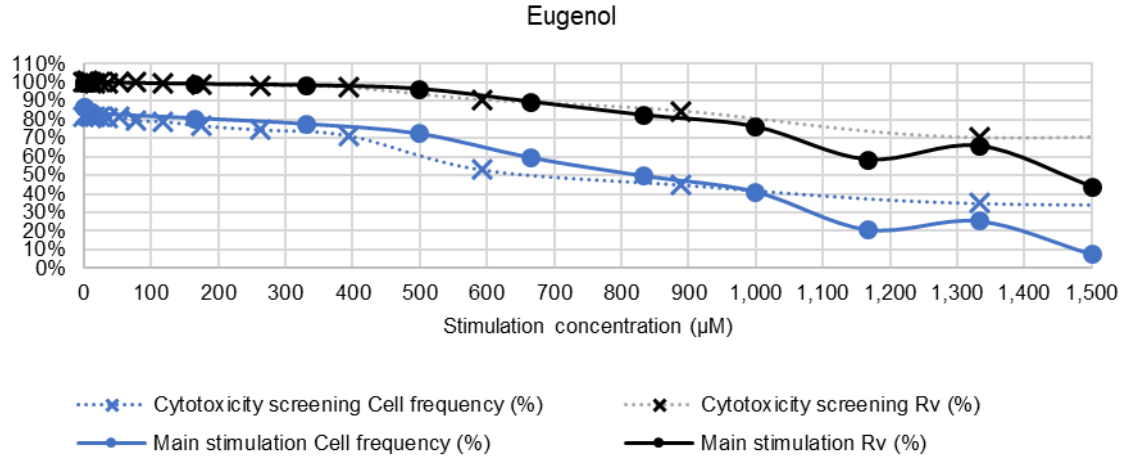

Figure 4. Cells stimulated with Eugenol. Main stimulations were performed from 10 µM to 1500 µM based on cytotoxicity and cell viability in initial cytotoxicity screening.

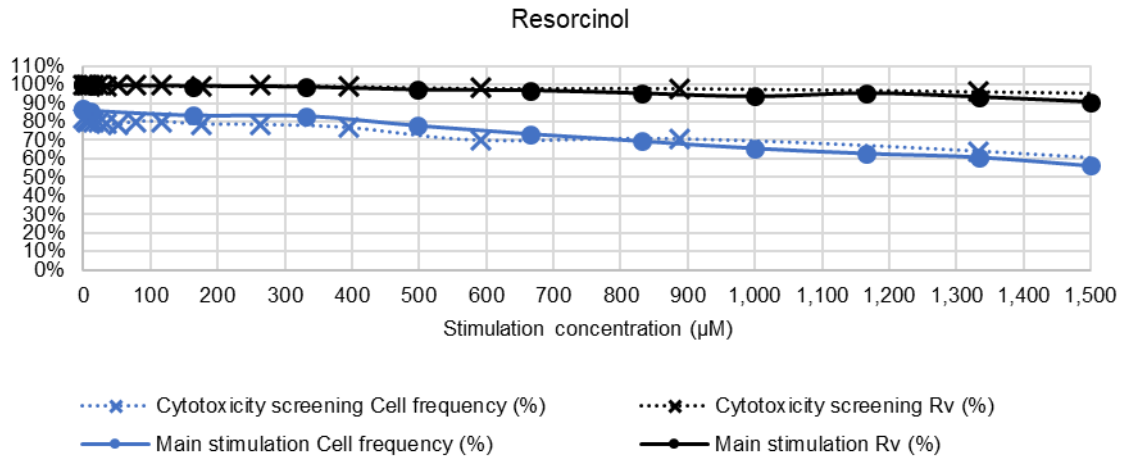

Figure 5. Cells stimulated with Resorcinol. Main stimulations were performed from 10 µM to 1500 µM based on cytotoxicity and cell viability in initial cytotoxicity screening.

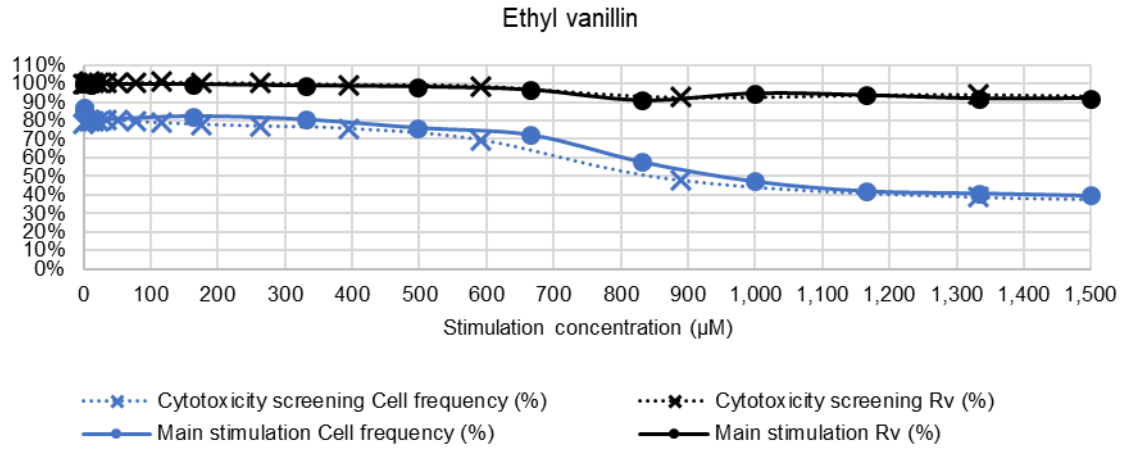

Figure 6. Cells stimulated with Ethyl vanillin. Main stimulations were performed from 10 µM to 1500 µM based on cytotoxicity and cell viability in initial cytotoxicity screening.

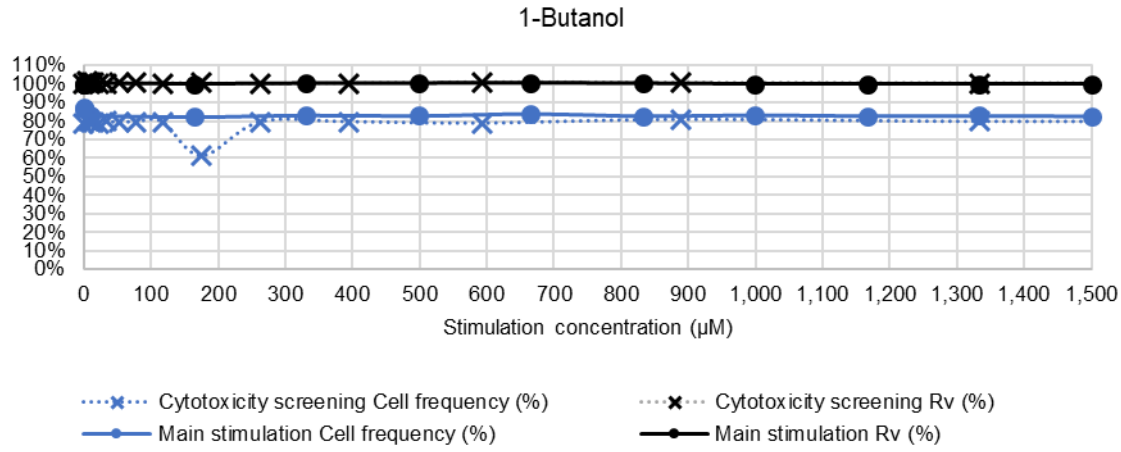

Figure 7. Cells stimulated with 1-Butanol. Main stimulations were performed from 10 µM to 1500 µM based on cytotoxicity and cell viability in initial cytotoxicity screening.

Dose-response dependent GARD®skin assessment

Five of the samples did not pass the RNA QC, due to RNA concentration below required 20 ng/µl for NanoString preparations. These were the two highest stimulation concentrations of DNCB and FA, and the highest stimulation concentration of CA.

The GARD®skin dose-response relationship was assessed using cells stimulated with of DNCB, FA, CA, EG, RC, EV, or BUT (Fig. 8-14). The actual, experimental DVs (blue data points) were obtained from the GARD®skin SVM. Predicted data (grey line and black data points) were calculated with a four-parametric model (max, min, ED_{50}, slope) (Eq. 2) and non-linear least square regression (Eq. 3-5). The minimum DV was set as an average of unstimulated controls (-1.13) and the maximum was the highest experimental DV obtained for each chemical. The RSS was minimized to find the best fitting concentration for ED_{50} and slope for the predicted curve in between minimum and maximum DVs. Empty experimental data points were not used in calculation of the predicted dose-response curve as they were either above the maximum DV or below minimum DV. The R^2 was calculated from RSS and TSS. Overall, the predicted curves correlated well with the experimental data points by R^2 values ≥0.95 for all sensitizing chemicals, but FA. Variances in the response over increasing concentrations resulted in a low R^2 value of 0.60 for FA. The predicted curve and parameters were used to calculate the dose when DV is 0 ($c_{DV(0)}$, the black square data point) and the DV at ED_{50} (the black triangle data point).

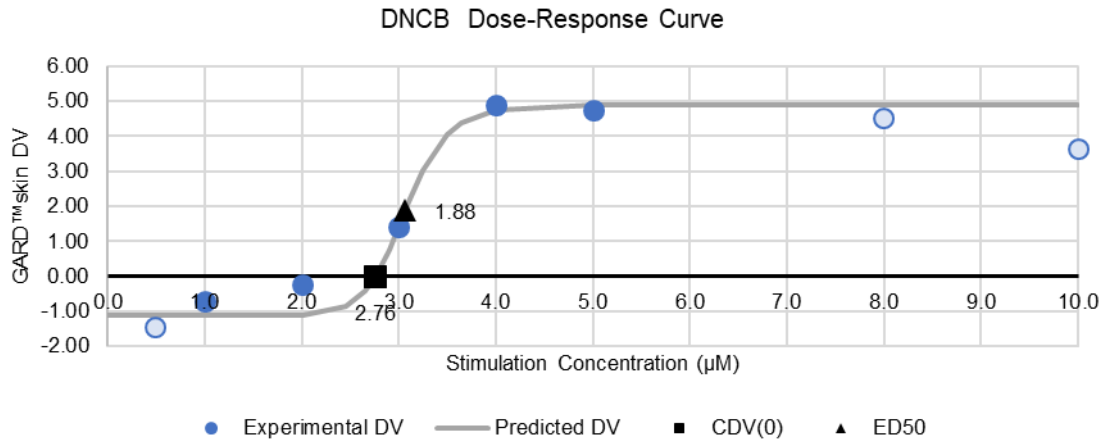

Figure 8. Dose-response relationship for DNCB. Eight stimulation concentrations from 0.5 µM to 10 µM were tested as the two highest main stimulation concentrations did not pass the RNA QC. The concentration for DV = 0 was estimated to about 2.76 µM and ED_{50} was estimated to be at 3.07 µM and a DV of 1.88. Max DV was 4.89 and the slope estimated to 31.90. The non-linear regression gave a RSS of 0.98 and R^2 of 0.97. Empty data points were not used in calculation of the predicted dose-response curve as they are either above the max DV or below the min DV.

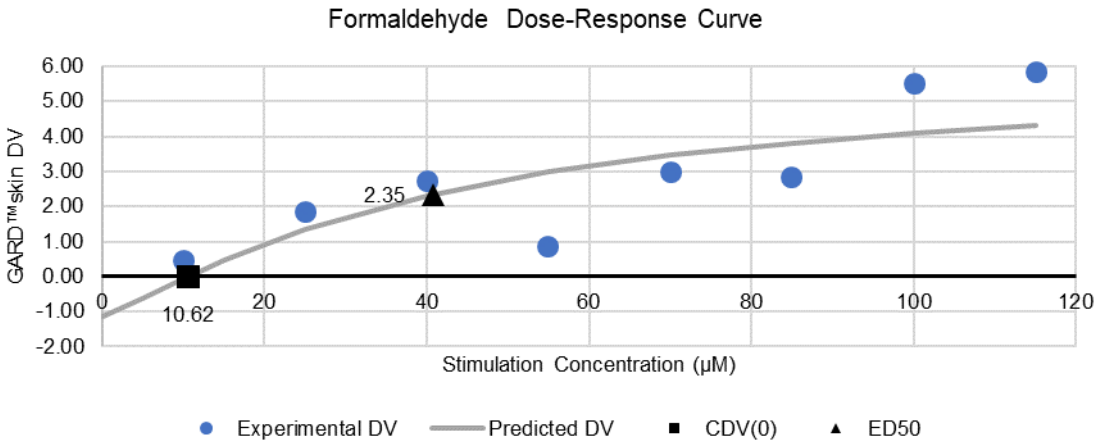

Figure 9. Dose-response relationship for Formaldehyde. Eight stimulation concentrations from 10 µM to 115 µM were tested as the two highest main stimulation concentrations did not pass the RNA QC. The concentration for DV = 0 was estimated to about 10.62 µM and ED_{50} was estimated to be at 40.80 µM and a DV of 2.35. Max DV was 5.83 and the slope estimated to 2.81. The non-linear regression gave an RSS of 10.80 and R^2 of 0.60.

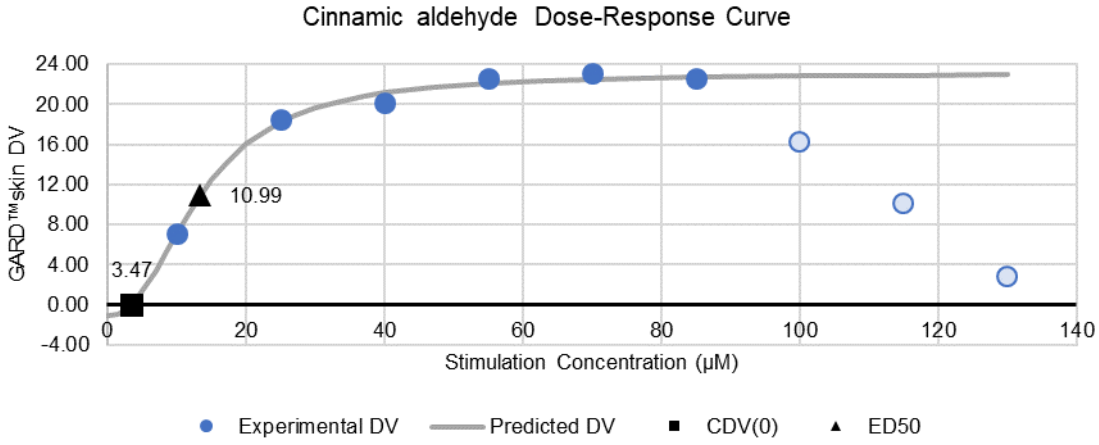

Figure 10. Dose-response relationship for Cinnamic aldehyde. Nine stimulation concentrations from 10 µM to 130 µM were tested as the highest main stimulation concentration did not pass the RNA QC. The concentration for DV = 0 was estimated to about 3.47 µM and ED_{50} was estimated to be at 13.44 µM and a DV of 10.99. Max DV was 23.11 and the slope estimated to 5.14. The non-linear regression gave a RSS of 1.79 and R^2 of 0.99. Empty data points were not used in calculation of the predicted dose-response curve as they are above the max DV.

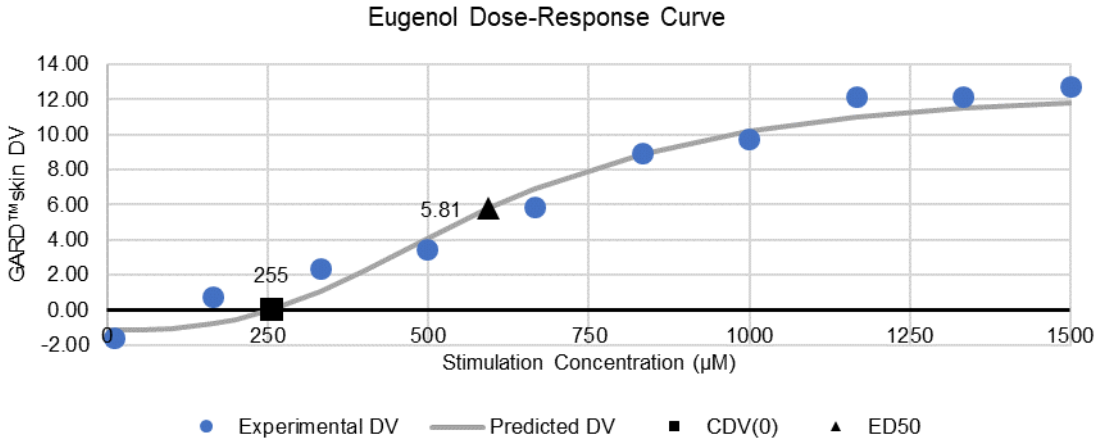

Figure 11. Dose-response relationship for Eugenol. All stimulation concentrations passed the RNA QC and were tested. The concentration for DV = 0 was estimated to about 255 µM and ED_{50} was estimated to be at 594 µM and a DV of 5.81. Max DV was 12.75 and the slope estimated to 6.60. The non-linear regression gave an RSS of 13.42 and R^2 of 0.98.

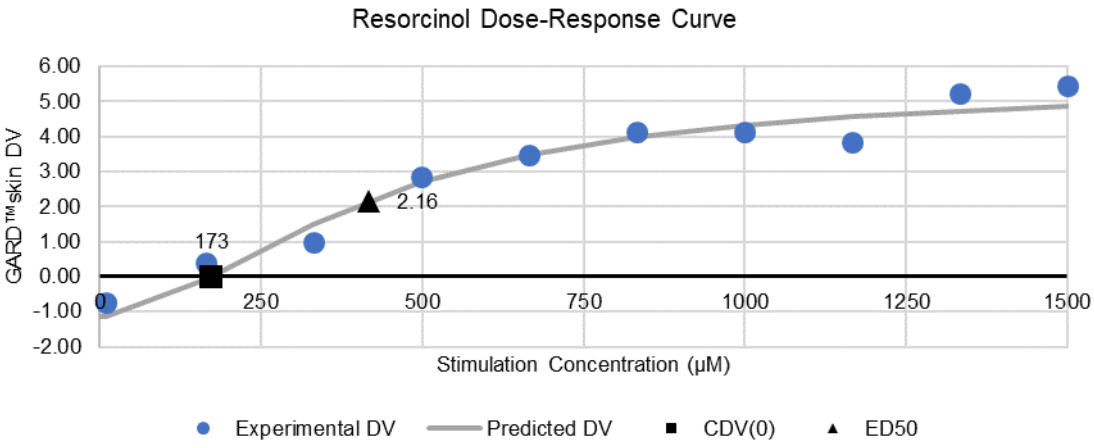

Figure 12. Dose-response relationship for Resorcinol. All stimulation concentrations passed the RNA QC and were tested. The concentration for DV = 0 was estimated to about 173 µM and ED_{50} was estimated to be at 417 µM and a DV of 2.16. Max DV was 5.46 and the slope estimated to 4.13. The non-linear regression gave an RSS of 1.86 and R^2 of 0.95.

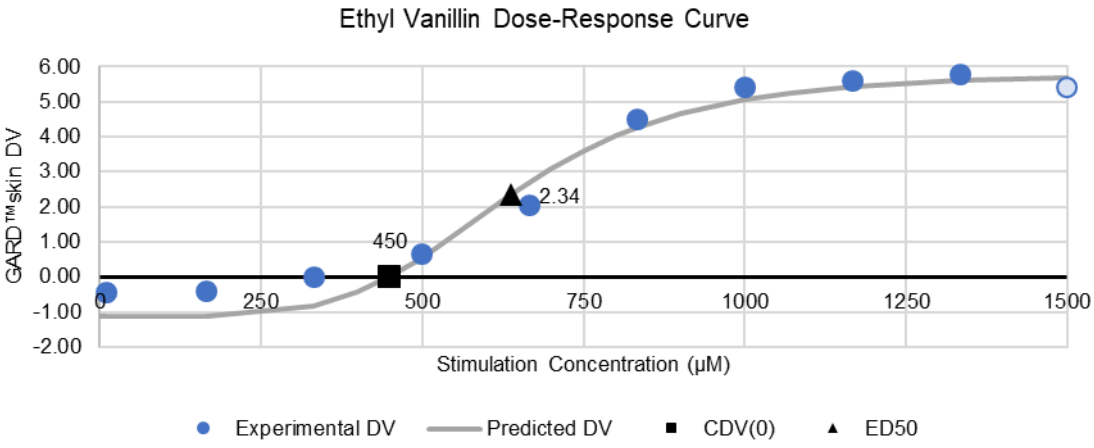

Figure 13. Dose-response relationship for Ethyl vanillin. All stimulation concentrations passed the RNA QC and were tested. The concentration for DV = 0 was estimated to about 450 µM and ED_{50} was estimated to be at 637 µM and a DV of 2.34. Max DV was 5.81 and the slope estimated to 10.82. The non-linear regression gave an RSS of 4.55 and R^2 of 0.96.

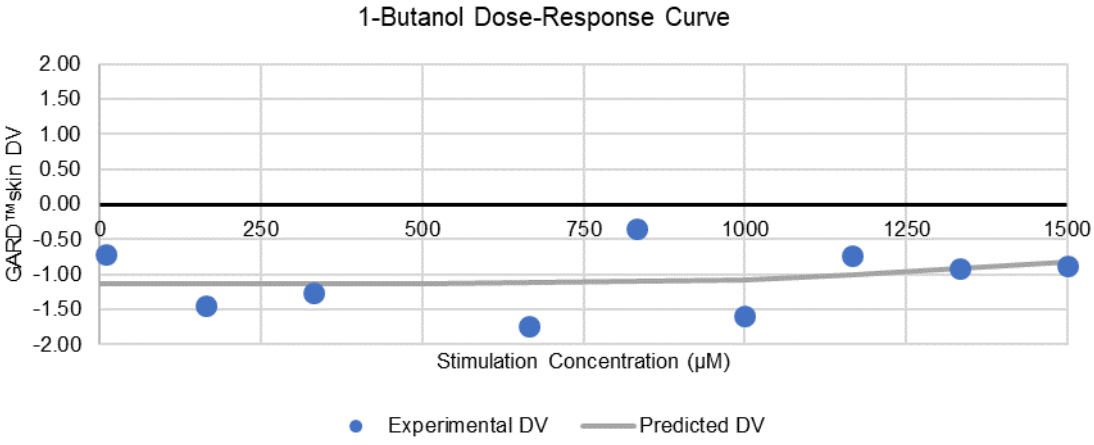

Figure 14. Dose-response relationship for 1-Butanol. All stimulation concentrations passed the RNA QC and were tested. All concentrations were below DV = 0 and 1-Butanol was thereby a true non-sensitizer. ED_{50} was estimated to be placed at 1623 µM. Max DV was -0.35 and the slope estimated to 11.77.

Predictive modeling parameters

Based on the experimental data the four modeling parameters (max, min, slope, ED_{50}) were used to calculate the predicted GARD®skin dose-response curve together with the non-linear least square regression parameters (Table III). The predicted curves between the maximum and minimum DVs were acceptable for all sensitizing chemicals, except for FA. The TSS and R^2 values were only calculated for sensitizing chemicals following the predicted dose-response relationship.

Table III. Modeling parameters, statistical significance of the predicted dose-response curve of GARD®skin. The residual sum of squares (RSS) was minimized to find the most suitable ED_{50} value and slope. The R^2 was calculated from the total sum of squares (TSS) and RSS of sensitizing chemicals. The R^2 is close to 1 for all but FA.

Chemical	**DNCB**	**FA**	**CA**	**EG**	**RC**	**EV**	**BUT**
ED_{50} (µM)	3.07	40.80	13.44	593.94	416.45	636.76	1626.27
Max (DV)	4.89	5.83	23.11	12.75	5.46	5.81	-0.35
Min (DV)	-1.13	-1.13	-1.13	-1.13	-1.13	-1.13	-1.13
Slope	31.90	2.81	5.14	6.60	4.13	10.82	11.77
RSS	0.98	10.80	1.79	8.74	1.86	2.40	2.62
TSS	28.61	26.70	189.31	245.16	39.25	60.48	-
R^2	0.97	0.60	0.99	0.96	0.95	0.96	-

Correlation between obtained results and existing skin sensitization potency classifications

The calculated $c_{DV(0)}$ and DV for ED_{50} was hypothesized to assist in skin sensitization potency prediction with GARD®skin together with the common skin sensitization potency classifications (Table IV). The ED_{50} value is distinctively higher for CA and EG compared to the other chemicals. The $c_{DV(0)}$ increased with less sensitizing classification, apart from the weak sensitizing EG and RC.

Table IV. Calculated $c_{DV(0)}$ (µM) and ED_{50} (DV) values and the correlation to existing skin sensitization potency classifications.

Chemical	**DNCB**	**FA**	**CA**	**EG**	**RC**	**EV**	**BUT**
$c_{DV(0)}$ (µM)	2.76	10.62	3.47	255	173	450	NS
ED_{50} (DV)	1.88	2.35	10.99	5.81	2.16	2.34	NS
CLP/GHS	1A (29)	1A (29)	1A (30)	1B (30)	1B (31)	No cat.	No cat.
HP (20)	1	2	2	3	4	-	6
LLNA(10)	Extreme (29)	Strong (29)	Moderate (31)	Weak (31)	Moderate (32)	Non-sens. (33)	Non-sens. (34)

The predicted dose-response curves were plotted in the same graph in logarithmic value of the concentrations (Fig. 15). The sigmodal curves had different outlooks, but a clear distinction of the $c_{DV(0)}$ intercept was observed between the strong sensitizing chemicals (black and grey), the weak sensitizers (dark blue), but also the non-sensitizers (light blue) according to CLP/GHS classifications.

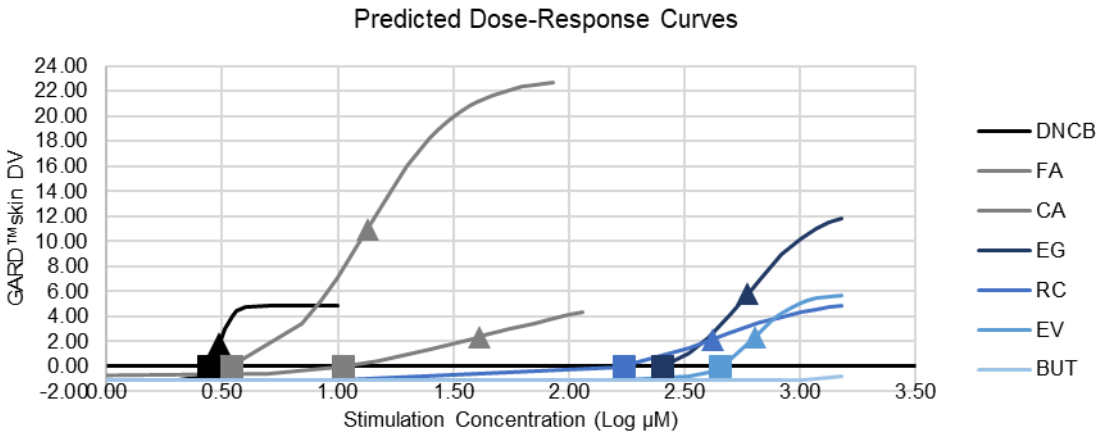

Figure 15. Predicted Dose-response curves for all tested chemicals. The $c_{DV(0)}$ values are marked with a square at the interception of the y-axis and the ED_{50} DVs are labeled with a triangular dot on the curves.

The calculated $c_{DV(0)}$ (light blue column on the left) and ED_{50} values (dark blue column on the right) for the predicted dose-response curves were (secondary vertical axis) plotted against the six HP categories (20) (black dots with connecting black line, primary vertical axis) for each chemical (horizontal axis) (Fig. 16). The outcome of both potency and efficacy consideration was visualized with the middle blue column displaying $c_{DV(0)}$ (µM) divided by ED_{50} (DV). EV has not been estimated with HP and was not labeled. BUT was non-sensitizing and neither have a calculated $c_{DV(0)}$ (µM) nor a ED_{50} (DV) value.

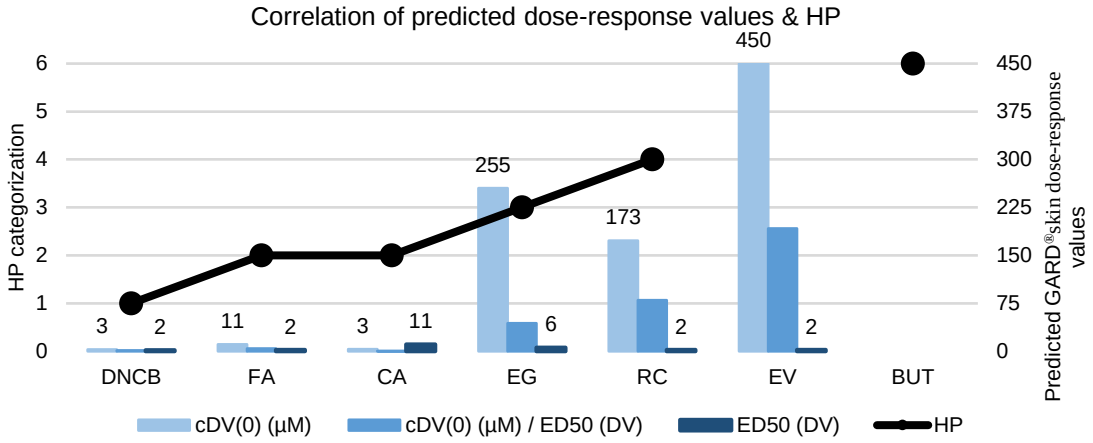

Figure 16. Correlation between the six HP categories (primary vertical axis) and calculated $c_{DV(0)}$ (µM) and ED_{50} (DV) (secondary vertical axis) for the tested chemicals. The effect of both potency and efficacy was visualized with the middle blue column displaying $c_{DV(0)}$ (µM) divided by ED_{50} (DVs).

Discussion

The current study examined the potential to use the dose-response relationship of GARD®skin in prediction of skin sensitization potency. The first method of the *in vitro* GARD® assays, GARD®skin is under OECD validation for hazard determination (sensitizer or non-sensitizer) after high accuracy in multiple blind studies (24-27). In a tiered approach GARD®potency (28) is available for full risk assessment. However, results from previous studies have arisen the hypothesis that the decision value (DV) of GARD®skin correlate with the cytotoxicity, potency classification and stimulation concentration of the chemical (22, 26). To investigate the hypothesis and determine the potential to further develop GARD®skin, the current study analyzed the dose-response relationship of compounds of regulatory potency classification groups (CLP 1A, CLP 1B and non-sensitizer). A Boltzmann fit, log-logistic four parametric (min, max, slope, ED_{50}) curve of the GARD®skin DVs against the simulation concentration was used to predict the dose-response relationship (35) and an estimated concentration for DV(0) ($c_{DV(0)}$). A decreasing value of GARD®skin $c_{DV(0)}$ correlated to a more cytotoxic and potent chemical. The varying magnitude of response in ED_{50} and maximum DV was thought to potentially be connected to the chemical efficacy. In addition, based on overall high R^2 values the predicted curve fit well to the actual, experimental values.

The dose-response relationship is important for determination of chemicals' relative potency and the risk assessment to prevent skin sensitization and the severity of ACD. Susceptibility to be sensitized varies among the population (2), but frequent contact and high initial exposure levels to a sensitizing chemical increase the risk for anyone (38). The relative potency in the murine LLNA method is based on the EC3 value, which is the substance dose responsible for a stimulation index (SI) above three. This means a three times higher T-cell proliferation in the draining lymph nodes of the chemically exposed mouse compared to the vehicle control (11). The human potency (HP) six-category classification system use human data to label chemicals based on the human threshold level (NOEL) and frequency of people sensitized. In regulatory terms, the HP categories 1-2, 3-4 and 5-6 represent CLP/GHS 1A, CLP/GHS 1B and non-sensitizer respectively (20). Since the dose needed to elicit a response often is higher in animal-based methods, both LLNA and HP were interesting categories for comparison of GARD®skin results. The NOEL or NOAEL (no observed (adverse) effects level) is a common reference dose for potency classification in e.g. toxicology (39-41) where a lower concentration of NOEL often means a higher potency. The concentration of GARD®skin when the DV was about 0 ($c_{DV(0)}$) was estimated as NOEL and compared among test chemicals.

The calculated $c_{DV(0)}$ of included chemicals correlated well with the regulatory CLP/GHS sensitization potency classification and the HP categorization. Strong sensitizers DNCB, FA and CA had the lowest $c_{DV(0)}$ of 2-11 µM. Weak sensitizers RC and EG had $c_{DV(0)}$ in a range between 100 µM and 300 µM. Finally, for the non-sensitizers, EV had a $c_{DV(0)}$ of 450 µM and could be estimated as a very weak sensitizer, while BUT proved to be a true non-sensitizer not showing any sensitizing characteristic at all. Thereby, the result suggested that the lower the value of $c_{DV(0)}$, the more potent chemical, similar to the HP NOEL values (20). In addition to the three CLP/GHS categorizations, differences in $c_{DV(0)}$ between the HP 1 and HP 2, HP 3-4 and non-sensitizer EV were observed. However, since HP 3 EG and HP 4 RC were close to non-sensitizer EV further tests would need to strengthen the difference between weak sensitizers and non-sensitizers. The value of $c_{DV(0)}$ was also higher for HP 3 EG than HP 4 RC suggesting that this parameter is not sufficient to determine the skin sensitization potency alone.

All chemicals in current study, except for EV, have been estimated by the HP classifications as seen in Table II. However, vanillin is classified as HP class 5, which is a sensitizer that elicit an immune response to only part of the population if exposed to at a high concentration (20). Both vanillin and EV consist of a benzene ring with a formyl group on the R (1^{st}) position, and a hydroxyl group at the para (4^{th}) position. The difference between vanillin and EV is a methoxy group or ethoxy group respectively at the meta (3^{rd}) position to the formyl group. Vanillin is a pro-hapten that is thought to be activated to a sensitizer through demethylation and oxidation. De-ethylation does not occur as easily as demethylation and therefore EV has been assumed to be a non-sensitizer while vanillin is a weak sensitizer (33). However, based on the $c_{DV(0)}$ results from current study EV is speculated to possibly cause sensitization if exposed to at high concentrations, which correlates to the definition of HP 5. The $c_{DV(0)}$ for EV was higher than the value of the CLP 1B classified compounds, while the HP 6 non-sensitizer BUT did not show any sensitization. Since EV and vanillin are categorized as non-sensitizer both by LLNA and in CLP regulations, EV is likely to be categorized as HP 5. However, more experiments are needed to confirm this suggestion.

A combined evaluation of the dose-response parameters was hypothesized to grow in importance for weaker sensitizers. The difference between HP 3 and HP 4 chemicals was speculated to be explained by the magnitude of the response in ED_{50} or maximum DV connected to the chemical efficacy. The predictive dose-response parameters (max and min, ED_{50} and the slope) are important for efficacy prediction in toxicology (39, 41) and dose-response curves are common also in skin sensitization research (42). The NOEL dose or concentration of a test is often directly translated to the potency of a chemical. However, the efficacy of chemical exposure, such as the usual fraction of the population sensitized at a certain concentration may vary. Therefore, a better understanding of the GARD®skin response values and how it relates to efficacy of chemical exposure could be beneficial for skin sensitization potency classification with GARD®skin. The $c_{DV(0)}$ for HP 3 EG and HP 4 RC in the current study was lower for RC, but the maximum DV and ED_{50} DV was significantly larger for EG. The maximum DV of EG was about 12 while it was about 5 for RC and the same relationship was seen for the response at ED_{50}. EG was also observed to be more cytotoxic than RC in the cytotoxicity screenings. However, since LLNA classify EG as weak sensitizer and RC as moderate sensitizer, alongside the $c_{DV(0)}$ values, the efficacy hypothesis need to be further investigated through more replicates, concentrations and studies of other weak sensitizers.

Weak sensitizers have been considered difficult to classify since they like non-sensitizers often are not cytotoxic. The stimulation concentration of GARD®skin is usually based on cytotoxicity, either at Rv90 or 500 µM. (26, 28) The cytotoxic chemicals, mainly strong sensitizing chemicals tested at the Rv90 concentration had positive GARD®skin DVs already at non-cytotoxic levels. Cytotoxicity was detected at the lowest concentration for HP 1 chemical DNCB, followed by quite similar Rv90 concentrations for FA and CA of HP 2. However, the frequency SenzaCells had to be observed for reliable results since it dropped fast at cytotoxic levels. Weak sensitizer RC, and non-sensitizers EV and BUT were scarcely to not at all cytotoxic. EG labeled as HP 3 became cytotoxic at high concentrations. Therefore, chemical stimulations of all weak and non-sensitizers were conducted in the same range of concentrations. HP 6 non-sensitizer BUT never gave a sensitizing DV. However, non-sensitizer EV and weak sensitizers EG and RC all gave positive DV at 500 µM. This could cause classification issue between HP class 3-5 or CLP 1B and non-sensitizers.

An additional importance of dose-response curves was seen in the slope of the dose-response relationship for the strong sensitizer CA. It had a bell-shaped curve, with a fast inclining slope over the lower dosages reaching a high maximum DV. Then as the stimulation concentrations

continued to increase the DVs started to decrease (Fig. 10), possibly due to e.g. cell death (Fig. 3). The same decrease at the highest sensitization dose was observed in a murine experiment in 1985 (43). This highlights an important application of the dose-response curve and the slope. Skin sensitization potency need to be established at the inclining slope to find the true $c_{DV(0)}$ of GARD®skin. The usual stimulation concentration of CA based on Rv90 is found at the declining slope in this study, but still resulted in a positive DV. In an earlier publication CA was observed as an extreme transcriptional outlier (22). Therefore, it is suggested that chemicals not following the general pattern could benefit extra from dose-response studies in potency classifications based on $c_{DV(0)}$. Without the dose-response curve to see if the response is increasing with higher dosage, the downslope could easily be used instead of the increasing slope, leading to a larger $c_{DV(0)}$ and a possible misclassification of CA as CLP 1B.

In further studies additional replicates, concentration and chemicals should be tested to strengthen the observed dose-response relationship of GARD®skin for determination of potency and potentially efficacy. The predicted dose-response curves correlated well with the experimental DVs confirmed by high R^2 for most chemicals. However, testing various concentrations was prioritized before replicates to establish a representative dose-response curve and different levels of variances in the response is observed for the stimulated cell populations. Additional uncertainty factors included the usage of the average DV of unstimulated cells as the minimum response although the DV of some chemicals were lower. It was done since not all chemicals were measured at a negative response, but caused the inflection point $c_{DV(0)}$ to be predicted a little off the observed inflection point. To statistically strengthen the results and minimize the deviations between the predicted dose-response curve, including the value of $c_{DV(0)}$, and the experimental values more replicates and concentrations should be tried. By testing more chemicals, the value of $c_{DV(0)}$ for potency classification, and maximum and ED_{50} responses for efficacy could also be evaluated more in-depth. It would also be interesting to study whether other chemicals than CA would reach the highest response, stabilize and then display a decreasing DV. In addition, since the vehicle is thought to be important for the ability of a chemical to cause sensitization it would also be interesting to test the effects of other vehicles than DMSO and water.

The current study suggests a dose-response relationship of GARD®skin and a correlation to regulatory and human-based potency classifications of skin sensitizers. Although more replicates, concentrations and chemicals should be tested to show the full potential of dose-response, the test results obtained in the current study suggest a potential to use GARD®skin for potency classifications. Overall high R^2 values proved that the predicted curves fit well to the expected curves and a dose-response can be obtained with GARD®skin. A low value of GARD®skin $c_{DV(0)}$ correlated to a more potent and cytotoxic chemical as previously suggested (22, 26), and the slope of the dose-response curve reassured the correct $c_{DV(0)}$ to be found. The $c_{DV(0)}$ also differentiated strong, weak and non-sensitizers according to CLP/GHS and the majority of subcategories of HP. Thereby, it is suggested that a dose-response relationship and DVs of GARD®skin can be useful in skin sensitization potency classification with GARD®skin.

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Appendices

Appendix A

Cell frequency was identified as the percent SenzaCells of the flow cytometry counted events using the forward scatter (FSC-A) on the x-axis and side scatter (SSC-A) on the y-axis. FSC-A was estimated as proportional to the cell size and SSC-A was estimated as proportional to the cell granularity. The SenzaCells were gated based on the unstained, unstimulated cells (Fig. 17) to exclude cell debris. The gate was transferred to the PI-stained cell populations (Fig. 18). A chemical stimulation (Fig. 18B) that causes cell toxicity (cytotoxicity) result in a decreasing cell frequency.

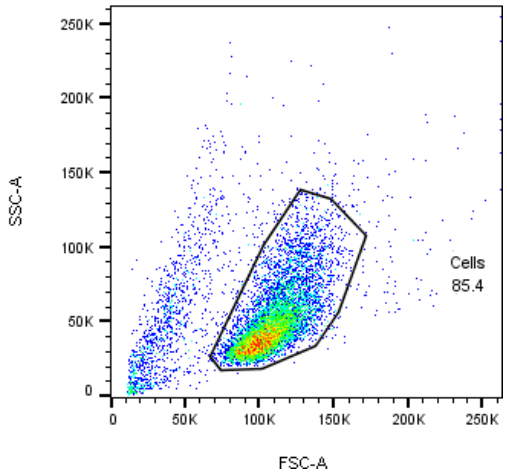

Figure 17. Unstained, unstimulated cells with SenzaCells gated. The for estimated SenzaCells was based on size and granularity and the gate is transferred to PI-stained cells.

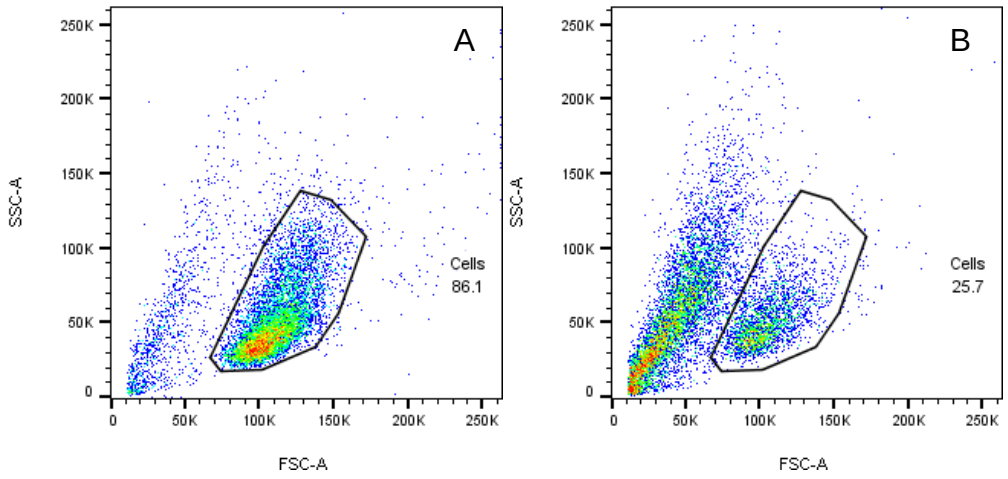

Figure 18. PI-stained cells with SenzaCells gated A) PI-stained unstimulated SenzaCells B) PI-stained stimulated SenzaCells

Appendix B

Absolute viability was identified as the percent viable cells of the full cell count screened with fluorophore FITC (Comp FITC-A) on the x-axis and fluorophore PE (Comp PE-A) on the y-axis. Stimulated cell samples, controls and one unstimulated cell sample were stained with PI, which emits fluorescence on the PE-scale after binding to the DNA of the cells. For the PI to bind the DNA the cell barrier needs to be broken which happens as the cells are dying. An unstained, unstimulated cell sample was used to set the gate for the absolute viability of the cells (Fig. 19). The gate was then transferred to the PI-stained samples (Fig. 20) that were used for calculation of Rv (Eq. 2).

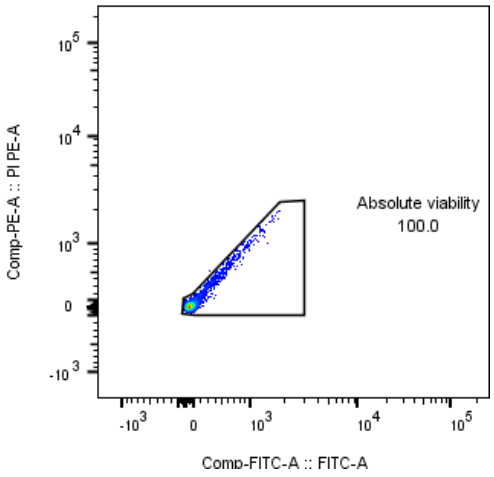

Figure 19. Absolute viability of unstained, unstimulated cells

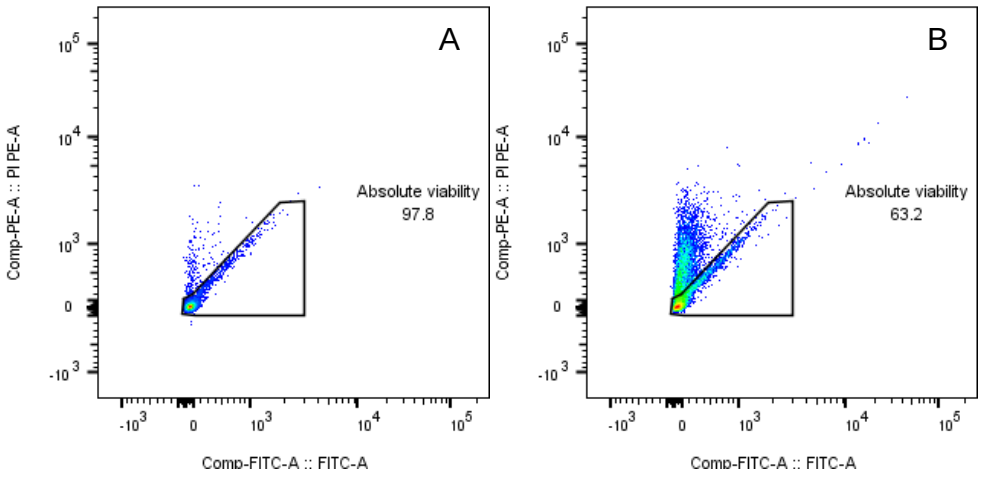

Figure 20. Absolute viability of PI-stained cells A) PI-stained unstimulated cells B) PI-stained stimulated cells