Received: 20 July 2020 | Revised: 7 December 2020 | Accepted: 10 December 2020

DOI: 10.1111/cod.13762

ORIGINAL ARTICLE

CONTACT DERMATITIS WILEY

Assessment of the different skin sensitization potentials of irritants and allergens as single substances and in combination using the KeratinoSens assay

Anna M. A. De Rentiis[1] | Mario Pink[1,2] | Nisha Verma[1] | Simone Schmitz-Spanke[1]

[1]Institute and Outpatient Clinic of Occupational, Social and Environmental Medicine, Friedrich-Alexander-University Erlangen-Nürnberg (FAU), Erlangen, Germany

[2]Department of Chemical and Product Safety, German Federal Institute for Risk Assessment (BfR), Berlin, Germany

Correspondence
Dr Simone Schmitz-Spanke, Institute and Outpatient Clinic of Occupational, Social and Environmental Medicine, Friedrich-Alexander-University Erlangen-Nürnberg (FAU), Henkestrasse 9-11, 91054 Erlangen, Germany.
Email: simone.schmitz-spanke@fau.de

Abstract

Background: People are exposed to mixtures containing allergens and irritants often causing contact dermatitis. Therefore, regulatory authorities require systematic information on the effects of mixtures on the sensitization threshold. In this study a moderate (cinnamal) and a weak (ethylene glycol dimethacrylate) allergen were combined with irritants covering different mechanisms of action (sodium dodecyl sulfate, salicylic acid, and α-pinene). For a systematic approach, the single substances were initially tested using the KeratinoSens assay. Thereafter, each allergen was combined with noncytotoxic concentrations of the irritants.

Method: The KeratinoSens assay was applied for the single substances according to OECD (Organisation for Economic Co-operation and Development) Test Guideline 442D. Based on these results, three noncytotoxic concentrations of the irritants were selected and applied simultaneously with 12 concentrations of the allergens to the KeratinoSens cells. Sensitization threshold and cytotoxicity were measured and compared with the individual testing.

Results: The combinations of allergens and irritants differed from the effects of the single substances and lowered the sensitization threshold. The quantitative approach allowed a clear description of the changes which varied by factors between 1.1 and 10.3.

Conclusions: Overall, the allergen was the prominent compound in the mixture and its nature appeared to determine the degree of the response.

KEYWORDS
allergens, combined exposures, irritants, KeratinoSens assay, mixtures, skin sensitization

1 | INTRODUCTION

Contact dermatitis is a public health problem and affects all age groups with a high prevalence and incidence.[1] Compared to the general population, incidence rates in the occupational environment are significantly higher. Thus, in Germany work-related "severe or recurrent skin diseases," which partly encompass contact dermatitis, are among the most frequently notified occupational diseases.[2] Causative factor of contact dermatitis is the exposure to irritants, which is usually the case in the workplace, and allergens, whereby most

commercial products contain both substance classes in various combinations and concentrations. Regarding the effects on the skin, irritants and allergens share principal inflammatory pathways. Both activate the early immune response via mediators (such as danger-associated molecular patterns), which bind to pattern recognition receptors initiating an inflammation cascade along with the production of proinflammatory cytokines/chemokines and T-cell activation.[3,4] Allergens directly also induce T-cell activation, whereas in the case of irritants the mode of action involves the damaging of keratinocytes with a subsequent increased release of mediators.[5] Therefore, it is very likely that a combination of irritants and allergens in a mixture can boost the acute inflammatory response by considerably enhancing the release of mediators. In addition, irritants facilitate the penetration of allergens by disturbing the skin barrier, which can also intensify the development of contact dermatitis.[3,6]

Simultaneous application of irritants and allergens has been assessed in few studies (eg, reviewed in[3,7,8]). The authors evaluated studies in humans and mice using the local lymph node assay (LLNA) and concluded that the combination of irritants and allergens influences the immune state and the penetration, which results in reduced activation threshold of allergens. In most of the evaluated studies, the effects were additive or synergistic.

Whether the sum effects of the single substances have an additive or synergistic impact on the threshold is of decisive importance for the classification of mixtures. To date, additivity has been the most common approach of regulatory authorities for estimating a mixture response.[9-11] The nonfood Scientific Committees of the European Commission (Scientific Committee on Health and Environmental Risks, Scientific Committee on Emerging and Newly Identified Health Risks, Scientific Committee on Consumer Safety) differentiate between chemicals with common and different mode of actions. If the mode of action is unknown, they recommend an addition of the effects.[11] However, the European Chemicals Agency (ECHA) excludes this approach for skin sensitization, and, as described above, research results indicate different combinatory effects.[9] Therefore, there is a considerable need for systematic studies, in which the impact of initially well-characterized combinations of irritants and allergens on the sensitization threshold will be evaluated in a quantitative approach.

To investigate this, a toxicological endpoint with a good predictivity must be selected, which can be tested in a fast and reproducibly way in a non-animal assay. In this context the adverse outcome pathway for skin sensitization is the ideal framework to identify appropriate endpoints, as it summarizes the key events leading to the development of sensitization.[12] The first key event or molecular initiating event is the covalent binding of electrophilic substances to skin proteins. In the second key event, these protein complexes are incorporated by keratinocytes, leading to a cascade of cellular responses. One key factor in this cascade is the binding of the repressor protein Kelch-like ECH-associated protein 1 (Keap1) and the transcription factor nuclear factor-erythroid 2–related factor 2 (Nrf2) to the antioxidant response element (ARE) leading to activation of ARE-dependent genes.[13] Simultaneously, in the third key event, dendritic cells are activated moving to lymph nodes, where T cells become activated and proliferate in the fourth and final key event. For the first three key events, non-animal tests are available which are fully accepted at a regulatory level: (a) the Direct Peptide Reactivity Assay focuses on the molecular initiating event[14]; (b) the ARE-Nrf2 luciferase tests (KeratinoSens or LuSens) address the second key event,[15] (c) and three tests monitor the activation of monocytes and dendritic cells (human Cell Line Activation Test, U937 Cell Line Activation Test, and Interleukin-8 Reporter Gene Assay).[16] The fourth event is indirectly assessed in the in vivo murine LLNAs.[17] For this study the second key event was selected, which is assessed by the KeratinoSens assay as specified in the OECD (Organisation for Economic Co-operation and Development) 442D Test Guideline.[15] This assay measures in stably transfected HaCaT human keratinocyte cells the induction of the luciferase gene, an ARE-dependent gene product, which reflects the activation of Nrf2-dependent genes. The assay was reported to have a high predictivity and to be promising for evaluating the sensitization potential of mixtures, if it is adapted to the conditions in mixtures with regard to dose selection.[18,19] The latter has been considered in our study design. Because of its highly reproducible and technically simple performance it is a suitable high-throughput instrument for obtaining dose–response information, which enables a quantitative comparison of the sensitization threshold of the single substances with those of the pairwise combination.

This study utilized a moderate (cinnamal) and a weak (ethylene glycol dimethacrylate [EGDMA]) allergen to avoid strong cytotoxic reactions. The allergens were combined with irritants covering different mechanisms of action (sodium dodecyl sulfate [SDS; syn.: sodium lauryl sulfate], salicylic acid, and α-pinene). For a systematic approach, the single substances were initially tested. Thereafter, each allergen was combined with three noncytotoxic concentrations of the irritants. By applying this study design, reliable information on the impact on the sensitization threshold over a wide dose range could be obtained.

2 | MATERIAL AND METHODS

The KeratinoSens assay has been validated by the EURL ECVAM (European Union Reference Laboratory for Alternatives to Animal Testing) and was performed in accordance with the OECD guidance 442D;[15,20] further details can be found in the guideline.

2.1 | Chemicals

Chemicals were chosen and prepared following OECD test guideline 442D. Cinnamal (syn.: cinnamic aldehyde; CAS no. 1437 1-10-9) and salicylic acid (99%; CAS no. 69-72-7) were purchased from Alfa Aesar (Thermo Fisher, Kandel, Germany), EGDMA (>97.5%; CAS no. 97-90-5) was purchased from EMD Millipore Corporation (Merck, Darmstadt, Germany), SDS (99%; CAS no. 151-21-3) from Sigma Aldrich (St. Louis, Missouri, USA), and α-pinene from (CAS no. 80-56-8) Acros Organics (New Jersey, USA). Test chemicals are dissolved in

dimethyl sulfoxide (CAS no. 67-68-5) from PanReac AppliChem (ITW Reagents, Darmstadt, Germany).

2.2 | KeratinoSens cell line

Immortalized human keratinocytes were transfected with a selectable plasmid (KeratinoSens cell line, obtained from Givaudan, Switzerland). Cells were cultured until confluence in a selection medium composed of Dulbecco's Modified Eagle Medium (c.c.pro, Oberdorla, Germany) supplemented with glucose (1 g/L), 2% glutamine, 10% foetal bovine serum, and 500 μg/mL G418 (KeratinoSens normal medium). To perform tests, cells were seeded into 96-well plates (clear plates for cytotoxicity testing, while white plates were used for activation assay) at a cell density of 10 000 cells/well, and adopted to G418-free medium for 24 hours prior tests.

2.3 | KeratinoSens assay (OECD test guideline 442D)

KeratinoSens cells were exposed for 48 hours to 12 concentrations of single substances or combinations. The concentrations ranged from 1 to 2000 μM for single substances and from 0.5 to 1000 μM for the combinations (see the "Study Design" section). After incubation and cell lysis (passive 5× lysis buffer; Promega, Walldorf, Germany), the luminescence was measured using a modified luciferase substrate (20 mM tricine, 2.67 mM magnesium sulfate, 100 μM ethylenediaminetetraacetic acid, 20 mM dithiothreitol, 125 μM adenosine triphosphate, and 100 μM luciferin).

Cell viability was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay as previously described.[21] Briefly, medium was replaced after exposure time with fresh medium containing 5 mg/mL MTT and cells were incubated for 4 hours at 37°C and 5% CO_2. After incubation, supernatant was discarded and MTT solvent (99.4 mL dimethyl sulfoxide, 0.6 mL 100% acetic acid, and 10 g SDS) was added. After a further incubation, the absorbance was measured at 492 nm to the reference wavelength of 620 nm.

The following endpoints were evaluated: I_{max} (maximal average fold induction of luciferase activity observed at any concentration of the tested single substances and mixtures), $EC_{1.5}$ (concentration for which the luciferase expression increased >1.5-fold), and IC_{30} and IC_{50} (concentration at which 30% and 50% reduction of cell viability occurs, respectively). A response was considered positive if for two out of three repetitions the $EC_{1.5}$ value was less than 1000 μM at a cellular viability higher than 70%.

2.4 | Study design

The study was divided into two steps to evaluate the impact of pairs of allergens (cinnamal, EGDMA) and irritants (SDS, salicylic acid, and α-pinene) on the threshold of luciferase induction.

1. The sensitization potential and cytotoxicity of the single substances were evaluated using the KeratinoSens assay.
2. The sensitization potential and cytotoxicity of the pairs were evaluated. Based on the cell viability tests included in the KeratinoSens assay, three noncytotoxic concentrations of the irritants were selected (1, 4, and 16 μM, hereinafter referred to with the prefix L [low], M [medium], H [high]). The concentrations of the allergens ranged from 0.5 to 1000 μM to avoid strong cytotoxicity at higher concentrations caused by the combination with the irritants. Each concentration of an allergen was combined with the three concentrations of the irritants, resulting in 216 pairs. For example, the lowest concentration of cinnamic aldehyde (0.5 μM) was combined with 1, 4, and 16 μM SDS. The pairs were tested according to the first step.

2.5 | Data evaluation and statistics

All experiments were performed at least three times in independent runs unless otherwise stated. Microplate reader readouts of fluorescence or absorbance measurements were normalized to control wells. Control values were set to 100%.

3 | RESULTS

Luciferase induction and cytotoxicity were analysed according to the protocols of ECVAM or OECD 442D.[15,20]

3.1 | Single substances

Salicylic acid, EGDMA, and cinnamal are recommended substances for demonstrating technical proficiency with the KeratinoSens assay.[15] Comparative data are also available for SDS.[13,22] To our knowledge, no data concerning the effect of α-pinene on the luciferase induction are available.

According to the validated protocols, the significant induction of luciferase activity above the threshold ($EC_{1.5}$) and the cytotoxicity were calculated for each substance and compared with the literature for demonstrating the technical proficiency of the KeratinoSens assay (Table 1). The study data were consistent with the literature data and sensitizers and nonsensitizers were correctly predicted in the assay. For cinnamal, different data on cytotoxicity have been published in the literature. While the OECD test guidelines reported an IC_{50} value of over 1000 μM,[15] Natsch et al reported an IC_{50} value of 194.4 μM,[22] which is within the range of our study data.

The quantitative dose–response analysis of the luciferase activity exhibited a dose-dependent increase after exposure to both sensitizers (Figure 1A). The remarkable increased cytotoxicity at higher concentrations of cinnamal stopped further increase of the activity (Figure 1C). The dose–response relationship is in line with the classification of cinnamal as a moderate sensitizer and EGDMA as a weak one, as cinnamal induced a 1.5-fold gene induction at lower concentrations. Of

TABLE 1 Comparison between literature data and study data for demonstration of technical proficiency of the KeratinoSens assay

	Literature data[a]			Study data		
Substance	**$EC_{1.5}$ (μM)**	**IC_{50} (μM)**	**Sensitization class[b]**	**$EC_{1.5}$ (μM)**	**IC_{50} (μM)**	**Prediction**
Cinnamal	25–175	>1000[c] 194.4	Sensitizer (moderate)	57.1	123	Sensitizer
EGDMA	5–125	>500	Sensitizer (weak)	134.4	670	Sensitizer
SDS	>2000	44.7	Nonsensitizer	43.7	40.2	Nonsensitizer
Salicylic acid	>1000	>1000	Nonsensitizer	>2000	>2000	Nonsensitizer
α-Pinene			GPMT: Grad I LNNA: neg.	1838	87.5	Nonsensitizer

Abbreviations: $EC_{1.5}$, concentration for which the luciferase expression increased >1.5-fold; EGDMA, ethylene glycol dimethacrylate; GPMT, guinea pig maximization test; IC_{50}, concentration at which 50% reduction of cell viability occurs; LLNA, local lymph node assay; SDS, sodium dodecyl sulfate.
[a]References[13,15,22].
[b]Sensitization class according to LLNA; for SDS classification human data were taken[13]; for α-pinene different results were reported[23,24].
[c]Different ranges or values were reported[15,22].

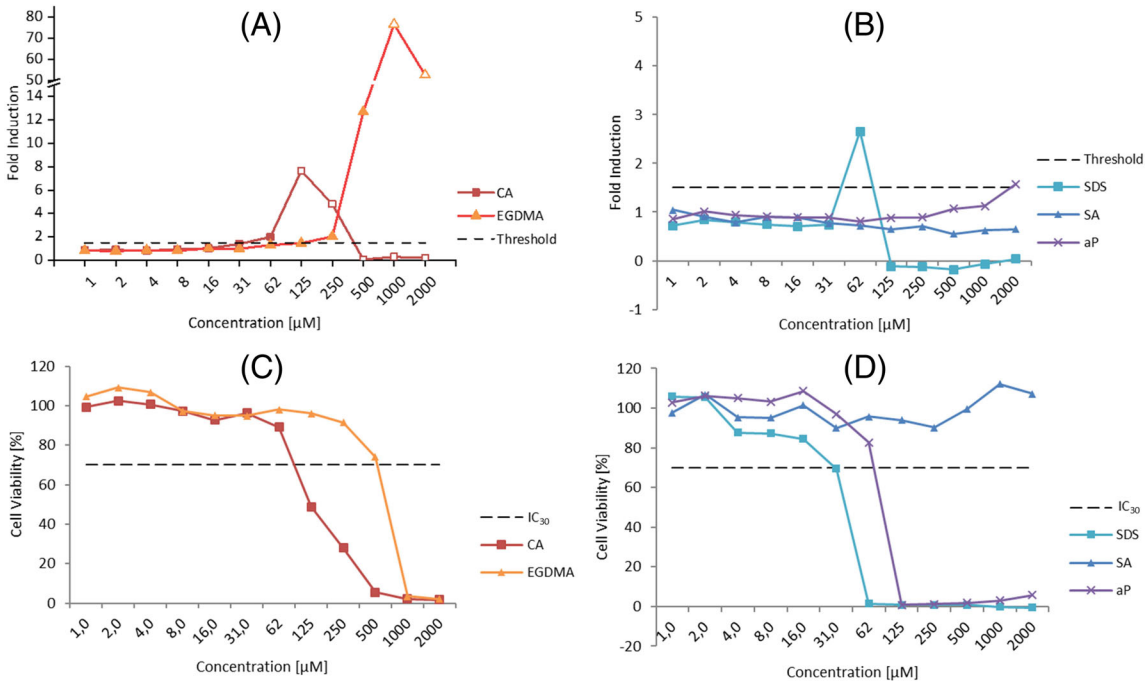

FIGURE 1 The maximal induction factors (I_{max}) of the luciferase activities and the cell viability curves for (**A,C**) sensitizers and (**B,D**) nonsensitizers. The graphs contain threshold lines, which are based on the criteria for a positive prediction. Open squares indicate luciferase activity for cinnamal (CA) at cell viability less than 70%. Please note the break of the *y* axis in (**A**) to show both small and high values of fold induction simultaneously. aP, α-pinene; EGDMA, ethylene glycol dimethacrylate; IC_{30}, concentration at which 30% reduction of cell viability occurs; SA, salicylic acid; SDS, sodium dodecyl sulfate

the irritants tested, SDS reduced cell viability at the lowest concentrations, followed by α-pinene. Salicylic acid had no effect on cell viability in this study (Figure 1D). No response in luciferase activity was observed after exposure to irritants (Figure 1B). Natsch et al reported for salicylic acid and SDS comparable $EC_{1.5}$ and IC_{50} values.[22]

3.2 | Combination of allergens and irritants

The KeratinoSens assay analyses the dose-dependent response to cytotoxicity and luciferase activity of 12 concentrations of the chemical to be tested. Therefore, 12 concentrations of both sensitizers in the range of 0.5 to 1000 μM were used, each combined with 1, 4, and 16 μM of an irritant resulting in 216 pairs. The selected concentrations of the irritants were not cytotoxic in the single experiments.

The allergen cinnamal induced in the individual testing 30% reduction of cellular viability at a concentration of 92 μM (Figure 2A). The combination with the irritants had only a minor effect on viability with a range for the IC_{30} values between 49 and 134 μM. The strongest effect was observed in combination with 16 μM SDS (H-SDS). In this combination, 49 μM cinnamal exceeded the threshold (IC_{30}).

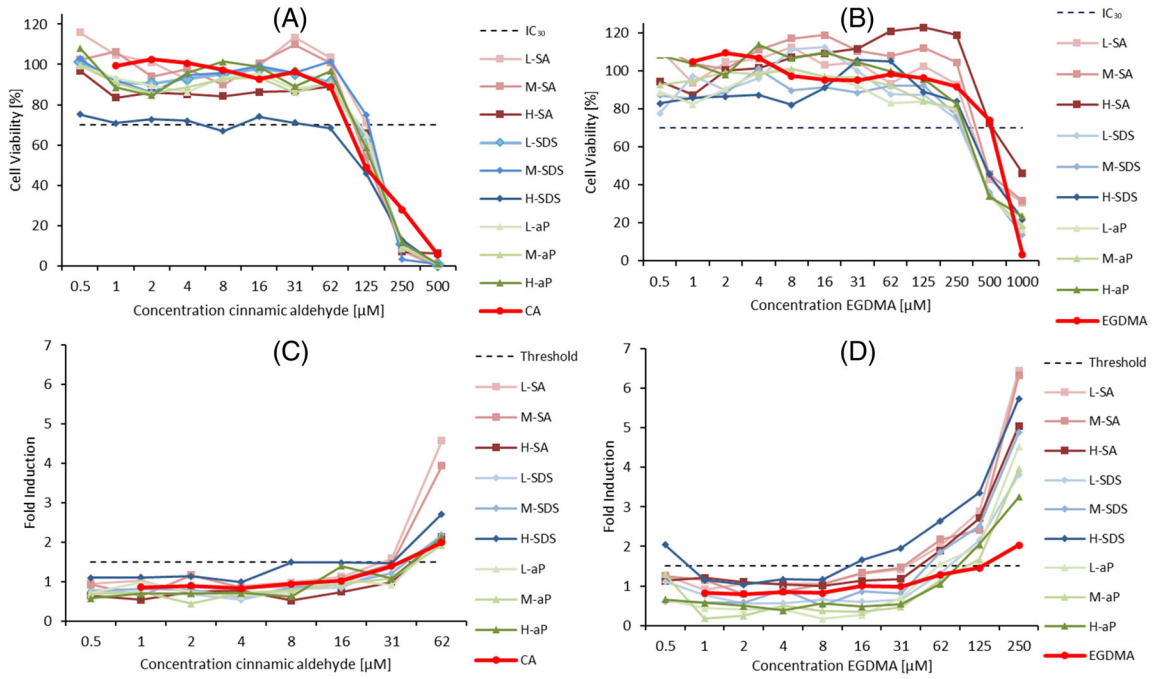

FIGURE 2 The cell viability curves and the maximal induction factors (I_{max}) of the luciferase activities for cinnamal (**A,C**) and EGDMA (**B,D**) plus irritants. For comparison, the individual values of the allergens were also given. The graphs contain threshold lines, which are based on the criteria for a positive prediction. For a better presentation only ranges with relevant changes are shown. aP, α-pinene; CA, cinnamal; EGDMA, ethylene glycol dimethacrylate; IC_{30}, concentration at which 30% reduction of cell viability occurs; SA, salicylic acid; SDS, sodium dodecyl sulfate; prefix L (low), M (medium), H (high) refers to the concentration of added irritants

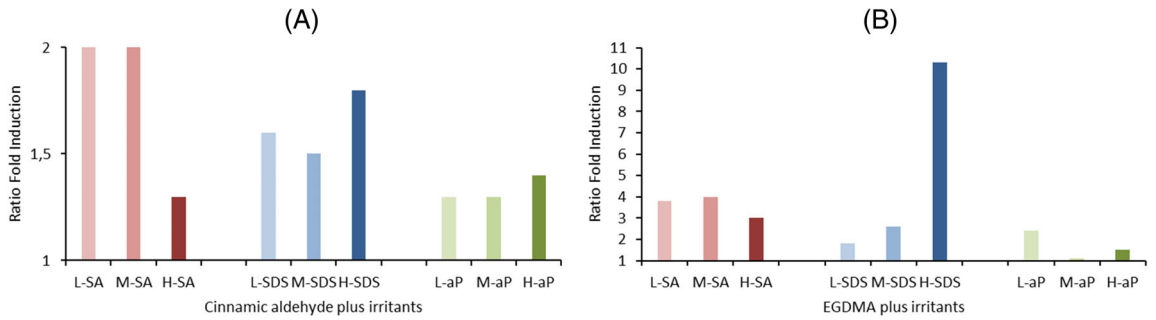

FIGURE 3 The ratio of the $EC_{1.5}$ values of single allergen/pairs. The ratio of the (**A**) combination of cinnamal and irritants, and (**B**) the combination of EGDMA and irritants. Please note the different scaling of the *y* axis. aP, α-pinene; EGDMA, ethylene glycol dimethacrylate; SA, salicylic acid; SDS, sodium dodecyl sulfate; prefix L (low), M (medium), H (high) refers to the concentration of added irritants. $EC_{1.5}$, concentration for which the luciferase expression increased >1.5-fold

EGDMA exerted in the individual testing a considerably lower cytotoxic effect as cinnamal (IC_{30}: 528 µM). The addition of the irritants shifted the IC_{30} values only slightly with a range between 280 and 547 µM (Figure 2B).

The $EC_{1.5}$ represents the concentration for which the induction is above the 1.5-fold threshold. The $EC_{1.5}$ value for cinnamal was 57.0 µM. In combination with irritants, this value shifted slightly to lower concentrations with a range between 28.0 and 45.2 µM (Figure 2C). The addition of a lower and a medium concentration of salicylic acid had an additive effect; all other combinations had a single allergen-to-pair ratio less than 2 (Figure 3A).

The $EC_{1.5}$ value for EGDMA was 134.4 µM, which is in line with its classification as a weak sensitizer. In combination with irritants, this value decreased in part remarkably with a range between 32.5 and 117.4 µM (Figure 2D). The ratio of the $EC_{1.5}$ values ranged from 1.1 to 10.3 (Figure 3B). The response varied depending on the irritants: the combination with α-pinene resulted in ratios between 1.1 and 2.4;

the combination with salicylic acid in ratios between 3.0 and 4.0; and the combination with SDS in ratios between 1.8 and 10.3.

4 | DISCUSSION

In this study two allergens were combined with three different irritants covering different mechanisms of action to investigate their effects on the sensitization threshold of the allergens in a broader approach than in most available studies. The KeratinoSens assay, which assesses the inflammatory response in keratinocytes, was used to compare the effects of the combination with the single test. Based on the results of the single tests, the allergens were combined with noncytotoxic concentrations of the irritants to reflect more closely real-life conditions. The main finding in this experimental setting was that the addition of the irritants did not lead to similar lowering of the sensitization threshold of cinnamal and EGDMA, suggesting that in the combination of allergen/irritant the type of the allergen has a decisive influence on the nature of the response. A comparative conclusion was also drawn after exposure to allergens in combination with irritants using the SENS-IS assay.[25] In the present study, for the weaker allergen EGDMA, both cytotoxicity and $EC_{1.5}$ were considerably more altered by the addition of the irritants than for the moderate allergen cinnamal.

4.1 | Effects of the single substances

Besides other factors such as chemistry, enzyme induction, or kinetics, possible interactions in mixtures are influenced by the mechanisms of action of the single substances.[26] Both allergens are recommended as positive controls to assess keratinocyte activation according to OECD guideline.[14] Both allergens also achieve positive results in the tests addressing key events 1 and 3-4.[22] However, the threshold values for EGDMA in all tests are lower than for cinnamal, whereas the cytotoxicity is less pronounced. Although the KeratinoSens assay has not been adopted to distinguish between weak and strong sensitizers, the results of this study are in agreement with existing literature and correspond to the classification of cinnamal as a moderate and EGDMA as a weak sensitizer. Both substances differ regarding their inflammatory effect during the sensitization phase of the allergic response. In murine keratinocytes, exposure to cinnamic alcohol, a precursor of cinnamic aldehyde, led to an increased release of interleukin-1α (IL-1α),[27] which functions as a damage-associated molecular pattern (DAMP) and plays a role in the early phase of a "sterile inflammation," namely, the induction of the NLRP3 inflammasome.[28] EGDMA failed to achieve such an effect.[27]

SDS is a common test substance for induction of skin barrier damage and the most established substance for testing the combined effect of allergens and irritants.[8,29-33] It affects various aspects of the epidermal barrier,[34] which together can render the skin barrier highly permeable and allow a greater penetration. The KeratinoSens assay does not map the barrier function, enabling the influence of other mechanisms on sensitization to be investigated. At the molecular level it was shown that the interaction of SDS with the cell membranes triggers elevated intracellular levels of calcium and reactive oxygen species as well as the secretion of IL-1α.[35,36] In addition, the chemical reactivity of SDS has generally the potential to cause cellular damage that is required for the release of DAMPs. However, these mechanisms are not sufficient to induce sensitization, which was also observed in this study. Notably, SDS is well known to produce false-positive results in LLNA.[37]

To our knowledge, the other two irritants have not yet been utilized in combination studies, but are both common ingredients in cosmetic formulations. Both substances are classified as irritants in the chemistry database PubChem, whereby α-pinene is regarded as a weakly acting substance. Salicylic acid exfoliates the stratum corneum by desmolysis and exerts anti-inflammatory properties such as counteracting the induction of the high-mobility box group 1 (HMGB1) protein, which is also a prototypic DAMP.[38] α-Pinene is a component of many aromatic dietary plants and induces dermal and sensory irritation.[24,39] Conflicting data are presented in the literature regarding the sensitizing properties of α-pinene. Patch tests revealed allergic reactions and α-pinene was confirmed as a weak allergen in the guinea pig maximization test. However, it failed to induce a positive response in the LLNA.[23,24,40] Besides, pinenes do not have the structural properties to bind covalently to skin proteins.[39] To our knowledge, a KeratinoSens assay has not yet been performed with α-pinene and in this study both irritants failed to induce luciferase activity.

4.2 | Effects of allergens and irritants on inflammation and Nrf2–Keap1–ARE pathway

Both allergens and irritants directly induce keratinocytes to release mediators promoting inflammation. The Nrf2–Keap1–ARE pathway, which is monitored by the KeratinoSens assay, plays a decisive role in the maintenance of cellular homeostasis under inflammatory conditions or cellular oxidative and electrophilic stress. Allergens activate this pathway besides the release of cytokines/chemokines by several mechanisms, such as elevated cellular levels of radical oxygen species, direct or receptor-mediated (via the Toll-like receptor) chemical modification of Keap1.[41] The activation leads to the transcription of antioxidant genes and inhibition of the transcription of several genes encoding proinflammatory cytokines.

Irritants damage the skin barrier followed by an activation of the innate immunity. However, even without disruption of the skin barrier, irritant exposure activates an inflammatory cascade involving the release of the two major proinflammatory mediators, IL-1 and tumour necrosis factor-α, leading to the release of further cytokines/chemokines and expression of cell adhesion molecules such as ICAM-1.[5,42-44] However, in contrast to allergens, irritants, such as SDS, failed to activate Nrf2.[41] In general terms, the inflammation induced by irritants potentiates the allergic response as described decades ago by Kligman.[45] The extent of potentiation by different combinations of

allergen/irritant has so far hardly been considered and is in the focus of the present study.

4.3 | Effects of the combination of allergen and irritant

A simple scheme of possible toxicity interactions of chemical compounds is based on the sum of the effects of the single substances, which can be additive, more than additive (synergistic), or antagonistic.[26] While the type of response can mostly be described, identifying the underlying mechanistic interactions is much more challenging.

The combination of cinnamal with the irritants hardly changed the effect on cell viability of the allergen compared to the single tests, but interestingly, the combination with H-SDS exerted stronger cytotoxicity compared to the single test. The combination of irritants with EGDMA resulted in a more pronounced cytotoxicity than in the combination with cinnamal. This means that in this study, both allergens decisively influenced the cytotoxic effect of the mixtures and enhanced the cytotoxic effect of the irritants. The exception is salicylic acid, which in this study—as in the literature—shows no cytotoxicity in the MTT test when it is used on its own. Even though to our knowledge no studies have yet addressed the combined effects of allergens and irritants on cytotoxicity, it should be mentioned that individuals with a positive colophonium patch test have a lower irritant threshold than normal individuals.[46]

The focus of the study was on the impact of the combination of an allergen with an irritant on the sensitization threshold. In combination, all irritants reduce the sensitization threshold of the allergens, but to varying degrees and are mostly not in a dose-dependent manner. The impact on the threshold was less pronounced in the combinations with cinnamal than in the combinations with EGDMA.

The addition of the low and medium concentration of salicylic acid to cinnamal reduced the threshold concentration by a maximum of half in an additive manner. All other combinations decreased the threshold concentration by a factor of less than 2.

The addition of irritants to EGDMA had, in general, a more pronounced effect. The combination with salicylic acid showed a synergistic trend with factors ranging between 3.0 and 4.0, whereas no dose-dependent effect was observed. Similarly, no dose-dependent effect was observed in combination with α-pinene. The response was less pronounced and ranged between a factor of 1.1 and 2.4. The most striking result showed the combination of EGDMA with SDS. This combination lowered dose dependently the sensitization threshold starting with a factor of 1.8 up to 10.3 at the highest concentration of SDS.

Thus, for both endpoints, cytotoxicity and $EC_{1.5}$ luciferase activity, the allergen in the combination determined the type of the combined response. The combinatory effect of allergens and irritants has been investigated in few studies and altered responses compared to application of single substances were reported.[3,7,8,33] Barrier-injuring detergents were mainly used as irritants in all these studies. However, the strength of this study is that the KeratinoSens assay can be used to explore mechanisms that are not based on alteration of the barrier function.

In a recent study, different allergens were combined with two irritants, SDS and lactic acid, and—as a marker for sensitization—the impact on expression of biomarkers in 3D reconstructed epidermis (SENS-IS assay) was investigated.[25] Nonsensitizing doses of the allergens were combined with nonirritating doses of the irritants. However, in contrast to this study, no quantitative approach and no test according to OECD guidelines were applied. The authors also noted that the SENS-IS assay allows only a very limited differentiation between additive and synergistic effects. Their results differed from those of this study. They reported the most pronounced increase in potency for the combination of both irritants with cinnamal. Equal increases were observed in the combinations with moderate and weak sensitizers (isoeugenol, hydroxycitronella). Even the combinations with other moderate sensitizers did not show a consistent pattern of response (hexyl cinnamal, dihydrocoumarin).

In our quantitative approach, the combination with the weaker allergen showed the stronger response. As described above, EGDMA is significantly less potent in the induction of the NLRP3 inflammasome by IL-1α release.[27] SDS alters intracellular conditions toward increased levels of reactive oxygen species and IL-1α secretion initiating an inflammation, which can potentiate the allergic response.[35] However, in our study the extent of the potentiation is dependent on the nature of the allergen. The inflammatory response to a weaker allergen might be more than additive by the supporting effect of SDS. In combination with a stronger allergen, the comparatively low-inflammatory SDS effect might be less amplifying. It would have strengthened this hypothesis if other allergens had been tested in the study. However, the extreme allergen 2,4-dinitrochlorobenzene was also tested but was not combined with irritants due to its high cytotoxicity (data not shown). Besides the point just addressed, our study has further limitations. It is clear that a single assay is not sufficient to assess the entire adverse outcome pathway. For this reason, assays were developed for the different key events and numerous approaches involving the use of two or more assays were evaluated for their performance.[47] To confirm the described observations, further experiments should be performed focusing on specific mixtures. Ideally, they should cover both key events, the effect on keratinocytes, and the immunological aspect. However, these tests were not feasible with the broad approach of this study.

In conclusion, the quantitative approach in the KeratinoSens assay allows distinguishing between additive and synergistic effects and demonstrates that, in contrast to regulatory recommendations, synergistic effects occur in mixtures. In addition, the allergen appears to be the prominent compound in the mixture determining the response. However, further combinations of allergens and irritants and an expansion of the test battery are a prerequisite for a detailed characterization of the underlying mechanisms. This basic evidence would substantially improve risk assessment of mixtures used in the workplace and in everyday life.

ACKNOWLEDGEMENTS

This work was performed in (partial) fulfilment of the requirements for obtaining the degree "Dr. med."

Open access funding enabled and organized by Projekt DEAL.

AUTHOR CONTRIBUTIONS

Anna De Rentiis: Conceptualization; data curation; formal analysis; investigation; methodology; project administration; validation; visualization; writing-original draft; writing-review and editing. **Mario Pink:** Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; resources; supervision; validation; visualization; writing-original draft; writing-review and editing. **Nisha Verma:** Conceptualization; data curation; funding acquisition; investigation; methodology; resources; supervision; writing-review and editing. **Simone Schmitz-Spanke:** Conceptualization; funding acquisition; resources; supervision; visualization; writing-original draft; writing-review and editing.

CONFLICTS OF INTEREST

The authors have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Anna M. A. De Rentiis https://orcid.org/0000-0001-9526-8592

REFERENCES

1. Brasch J, Becker D, Aberer W, et al. Guideline contact dermatitis: S1-guidelines of the German Contact Allergy Group (DKG) of the German Dermatology Society (DDG), the Information Network of Dermatological Clinics (IVDK), the German Society for Allergology and Clinical Immunology (DGAKI), the working group for occupational and environmental dermatology (ABD) of the DDG, the Medical Association of German Allergologists (AeDA), the Professional Association of German Dermatologists (BVDD) and the DDG. *Allergo J Int*. 2014;23(4):126-138.
2. Diepgen TL. Occupational skin diseases. *J Dtsch Dermatol Ges*. 2012;10(5):297-313; quiz 314-5.
3. Bonefeld CM, Geisler C, Gimenez-Arnau E, Lepoittevin JP, Uter W, Johansen JD. Immunological, chemical and clinical aspects of exposure to mixtures of contact allergens. *Contact Dermatitis*. 2017;77(3):133-142.
4. Rustemeyer T, van Hoogstraten IMW, von Blomberg BME, Gibbs S, Scheper RJ. Mechanisms of irritant and allergic contact dermatitis. In: Johansen JD, Frosch PJ, Lepoittevin J-P, eds. *Contact Dermatitis*. Vol 3. Berlin, Heidelberg: Springer Berlin Heidelberg; 2011:43-90.
5. Lee HY, Stieger M, Yawalkar N, Kakeda M. Cytokines and chemokines in irritant contact dermatitis. *Mediators Inflamm*. 2013;2013:916497.
6. Proksch E, Brasch J. Abnormal epidermal barrier in the pathogenesis of contact dermatitis. *Clin Dermatol*. 2012;30(3):335-344.
7. Pedersen LK, Johansen JD, Held E, Agner T. Augmentation of skin response by exposure to a combination of allergens and irritants - a review. *Contact Dermatitis*. 2004;50(5):265-273.
8. Agner T, Johansen JD, Overgaard L, Volund A, Basketter D, Menné T. Combined effects of irritants and allergens. Synergistic effects of nickel and sodium lauryl sulfate in nickel- sensitized individuals. *Contact Dermatitis*. 2002;47(1):21-26.
9. Corporate authors: European Chemicals Agency, Published: 2017-07-05, Title: Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixture, ISBN 978-92-9020-050-5, DOI 10.2823/124801, Catalogue number ED-02-17-754-EN-N, Link: file:///home/oteru/Zotero/storage/3IVMT5JZ/source-183086287.html
10. U.S.EPA. *Supplementary Guidance for Conducting Health Risk Assessment of Chemical Mixtures*. Washington, DC: Risk Assessment Forum; 2000.
11. Corporate authors: Directorate-General for Health and Consumers (European Commission) Now known as Directorate-General for Health and Food Safety (European Commission), Published: 2013-09-18, Titel: Toxicity and assessment of chemical mixtures, ISBN 978-92-79-30700-3 DOI 10.2772/21444, Catalogue number ND-03-13-259-EN-N, Link: https://op.europa.eu/en/publication-detail/-/publication/ffab4074-6ce5-4f87-89b7-fbd438943b54/language-en/format-PDF/source-183090997#
12. OECD. The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins, OECD Series on Testing and Assessment, No. 168, OECD Publishing, Paris; 2014. https://doi.org/10.1787/9789264221444-en.
13. Natsch A, Emter R. Skin sensitizers induce antioxidant response element dependent genes: application to the in vitro testing of the sensitization potential of chemicals. *Toxicol Sci*. 2008;102(1):110-119.
14. OECD. Test No. 442C: In Chemico Skin Sensitisation: Assays addressing the Adverse Outcome Pathway key event on covalent binding to proteins, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris; 2020. https://doi.org/10.1787/9789264229709-en.
15. OCDE. Test No. 442D: In Vitro Skin Sensitisation : ARE-Nrf2 Luciferase Test Method, OECD Guidelines for the Testing of Chemicals, Section 4, Éditions OCDE, Paris; 2018. https://doi.org/10.1787/9789264229822-en.
16. OCDE. Test No. 442E: In Vitro Skin Sensitisation : In Vitro Skin Sensitisation assays addressing the Key Event on activation of dendritic cells on the Adverse Outcome Pathway for Skin Sensitisation, OECD Guidelines for the Testing of Chemicals, Section 4, Éditions OCDE, Paris; 2018. https://doi.org/10.1787/9789264264359-en.
17. OCDE. Test No. 442B: Skin Sensitization : Local Lymph Node Assay: BrdU-ELISA or -FCM, OECD Guidelines for the Testing of Chemicals, Section 4, Éditions OCDE, Paris; 2018. https://doi.org/10.1787/9789264090996-en.
18. Andreas N, Caroline B, Leslie F, et al. The intra- and inter-laboratory reproducibility and predictivity of the KeratinoSens assay to predict skin sensitizers in vitro: results of a ring-study in five laboratories. *Toxicol In Vitro*. 2011;25(3):733-744.
19. Settivari RS, Gehen SC, Amado RA, Visconti NR, Boverhof DR, Carney EW. Application of the KeratinoSens assay for assessing the skin sensitization potential of agrochemical active ingredients and formulations. *Regul Toxicol Pharmacol*. 2015;72(2):350-360.
20. Corporate authors: Database Service on Alternative Methods to animal experimentation, Title: DB-ALM Protocol n° 155 : KeratinoSens™, Link: https://jeodpp.jrc.ec.europa.eu/ftp/jrc-opendata/EURL-ECVAM/datasets/DBALM/LATEST/online/DBALM_docs/155_P_%20KeratinoSens.pdf (last accessed: January 4th, 2021)
21. Pink M, Verma N, Zerries A, Schmitz-Spanke S. Dose-dependent response to 3-nitrobenzanthrone exposure in human urothelial cancer cells. *Chem Res Toxicol*. 2017;30(10):1855-1864.
22. Natsch A, Ryan CA, Foertsch L, et al. A dataset on 145 chemicals tested in alternative assays for skin sensitization undergoing prevalidation. *J Appl Toxicol*. 2013;33(11):1337-1352.
23. Wei QJ, Wei CN, Harada K, et al. Evaluation of allergenicity of constituents of myoga using the murine local lymph node assay. *Int J Immunopathol Pharmacol*. 2010;23(2):463-470.

24. Wei Q, Harada K, Ohmori S, Minamoto K, Wei C, Ueda A. Toxicity study of the volatile constituents of Myoga utilizing acute dermal irritation assays and the Guinea-pig maximization test. *J Occup Health*. 2006;48(6):480-486.
25. Cottrez F, Boitel E, Berrada-Gomez MP, et al. In vitro measurement of skin sensitization hazard of mixtures and finished products: results obtained with the SENS-IS assays. *Toxicol In Vitro*. 2020;62:104644.
26. McCarty LS, Borgert CJ. Review of the toxicity of chemical mixtures: theory, policy, and regulatory practice. *Regul Toxicol Pharmacol*. 2006; 45(2):119-143.
27. Son D, Na Y, Cho WS, et al. Differentiation of skin sensitizers from irritant chemicals by interleukin-1alpha and macrophage inflammatory protein-2 in murine keratinocytes. *Toxicol Lett*. 2013;216(1):65-71.
28. Borthwick LA. The IL-1 cytokine family and its role in inflammation and fibrosis in the lung. *Semin Immunopathol*. 2016;38(4):517-534.
29. Kartono F, Maibach HI. Irritants in combination with a synergistic or additive effect on the skin response: an overview of tandem irritation studies. *Contact Dermatitis*. 2006;54(6):303-312.
30. McLelland J, Shuster S, Matthews JN. Irritants' increase the response to an allergen in allergic contact dermatitis. *Arch Dermatol*. 1991;127(7):1016-1019.
31. De Jong WH, Tentij M, Spiekstra SW, Vandebriel RJ, van Loveren H. Determination of the sensitising activity of the rubber contact sensitisers TMTD, ZDMC, MBT and DEA in a modified local lymph node assay and the effect of sodium dodecyl sulfate pretreatment on local lymph node responses. *Toxicology*. 2002;176(1–2):123-134.
32. Cumberbatch M, Scott RC, Basketter DA, et al. Influence of sodium lauryl sulphate on 2,4-dinitrochlorobenzene-induced lymph node activation. *Toxicology*. 1993;77(1–2):181-191.
33. Pedersen LK, Haslund P, Johansen JD, Held E, Volund A, Agner T. Influence of a detergent on skin response to methyldibromo glutaronitrile in sensitized individuals. *Contact Dermatitis*. 2004;50(1):1-5.
34. Koppes SA, Ljubojevic Hadzavdic S, Jakasa I, et al. Effect of allergens and irritants on levels of natural moisturizing factor and corneocyte morphology. *Contact Dermatitis*. 2017;76(5):287-295.
35. Mizutani T, Mori R, Hirayama M, et al. Sodium lauryl sulfate stimulates the generation of reactive oxygen species through interactions with cell membranes. *J Oleo Sci*. 2016;65(12):993-1001.
36. Rabolli V, Lison D, Huaux F. The complex cascade of cellular events governing inflammasome activation and IL-1beta processing in response to inhaled particles. *Part Fibre Toxicol*. 2016;13(1):40.
37. Basketter DA, Kimber I. Skin irritation, false positives and the local lymph node assay: a guideline issue? *Regul Toxicol Pharmacol*. 2011; 61(1):137-140.
38. Arif T. Salicylic acid as a peeling agent: a comprehensive review. *Clin Cosmet Investig Dermatol*. 2015;8:455-461.
39. Kasanen JP, Pasanen AL, Pasanen P, Liesivuori J, Kosma VM, Alarie Y. Stereospecificity of the sensory irritation receptor for nonreactive chemicals illustrated by pinene enantiomers. *Arch Toxicol*. 1998;72(8): 514-523.
40. Cachão P, Menezes Brandão F, Carmo M, Frazao S, Silva M. Allergy to oil of turpentine in Portugal. *Contact Dermatitis*. 1986;14(4): 205-208.
41. Helou DG, Martin SF, Pallardy M, Chollet-Martin S, Kerdine-Romer S. Nrf2 involvement in chemical-induced skin innate immunity. *Front Immunol*. 2019;10:1004.
42. Corsini E, Bruccoleri A, Marinovich M, Galli CL. Endogenous interleukin-1 alpha associated with skin irritation induced by tributyltin. *Toxicol Appl Pharmacol*. 1996;138(2):268-274.
43. Willis CM, Stephens CJ, Wilkinson JD. Selective expression of immune-associated surface antigens by keratinocytes in irritant contact dermatitis. *J Invest Dermatol*. 1991;96(4):505-511.
44. Lisby S, Müller KM, Jongeneel CV, Saurat JH, Hauser C. Nickel and skin irritants up-regulate tumor necrosis factor-alpha mRNA in keratinocytes by different but potentially synergistic mechanisms. *Int Immunol*. 1995;7(3):343-352.
45. Kligman AM. The identification of contact allergens by human assay. II. Factors influencing the induction and measurement of allergic contact dermatitis. *J Invest Dermatol*. 1966;47(5):375-392.
46. Smith HR, Holloway D, Armstrong DK, Basketter DA, McFadden JP. Irritant thresholds in subjects with colophony allergy. *Contact Dermatitis*. 2000;42(2):95-97.
47. Kolle SN, Natsch A, Gerberick GF, Landsiedel R. A review of substances found positive in 1 of 3 in vitro tests for skin sensitization. *Regul Toxicol Pharmacol*. 2019;106:352-368.

How to cite this article: De Rentiis AMA, Pink M, Verma N, Schmitz-Spanke S. Assessment of the different skin sensitization potentials of irritants and allergens as single substances and in combination using the KeratinoSens assay. *Contact Dermatitis*. 2021;84:317–325. https://doi.org/10.1111/cod.13762