Identification of Compounds with Weak Skin Irritation Potential Using *in vitro* Methods Based on 3D Reconstructed Human Epidermis Model

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Abstract

Background: The replacement of animal testing with *in vitro* methods has reduced the incidence of false positive results, reduced the number of animal tests, and increased the efficiency in terms of time and cost. Such replacement for alternative methods is supported by the concept of three Rs, which includes replacement, reduction and improvement of individual original methods carried out on animals. In the chemical, pharmaceutical and cosmetic industries, *in vitro* models have replaced many methods used in safety and efficiency testing, including tests for skin sensitization, skin corrosion, skin absorption and skin irritation.

Methods: This work was focused on utilization of an in vitro reconstituted human skin model to identify substances with a weak irritation potential. The *in vitro* method for determining skin irritation according to OECD test guideline 439 and the method for determining skin irritation for extracts from medical devices according to ISO standard - ISO 10993-23:2021, were used to detect the irritation of 15 test articles (TAs) in 5 concentrations (0.1%, 0.5%, 1%, 5%, 10%) in non-polar solvent (sesame oil) and in polar solvent (saline). If no effects were observed at these concentrations, TAs were also tested neat. The concentration that reduced tissue viability to 50% (EC-50) was calculated. ELISA assays were also used to measure the release of pro-inflammatory cytokines, specifically interleukins IL-1α, IL-6 and IL-8.

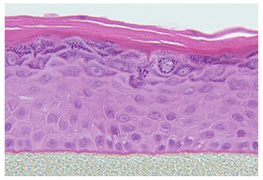
Results: 5 of the TAs (allyl heptanoate, heptyl salicylate, linalyl acetate, methyl laurate, hexyl salicylate) had viability comparable with negative control tissues in all concentrations in both solvents as well as in undiluted form. On the other hand, 7 TAs (10-undecenoic acid, lactic acid, 2-ethoxyehtyl methacrylate, 1-decanol, methyl methacrylate, 2-bromobutane, 50% sodium carbonate) did not cause the irritation effect in all tested concentrations in both solvents, but irritation effect was observed using the TAs. The remaining 3 TAs (heptanoic acid, SDS and 10% sodium hypochlorite) decreased tissue viability below 50%, so the EC-50s could be calculated. When using the skin irritation test for medical device extracts according to ISO 10993-23:2021 we found that 3 compounds (heptyl butyrate, hexyl salicylate, methyl laurate) did not decrease viability at any concentration in both solvents and the viability of the undiluted form was 100%. On the other hand, 2 compounds (methyl methacrylate, allyl heptanoate) caused decreased tissue viability only in the undiluted form. All other compounds caused decreases in viability in at least one solvent as well as in the undiluted form (**Table 2, Figure 2**).

These results were confirmed by release of IL-1α (**Figure 3**), IL-6, and IL-8 (data not shown). Although the relationship between these *in vitro* results and known *in vivo* results remains unclear, we anticipate that the materials that caused *in vitro* cytotoxicity or cytokine release will have some level of *in vivo* response.

Conclusion: This approach represents a promising *in vitro* method that may be utilized to distinguish non-irritants from compounds with weak but existing skin irritation potential.

Methods

EpiDerm Tissue Model: EpiDerm tissues were cultured at the air-liquid interface which attains in vivo-like differentiation and allows for topical application of test articles, see **Figure 1**. EpiDerm tissues are produced from normal human epidermal keratinocytes obtained from neonatl male donors, following Good Manufacturing Practices (GMP) and ISO 9001:2015.



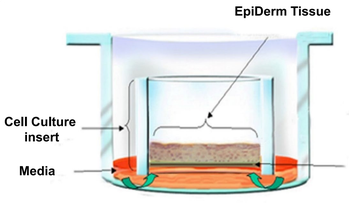


Figure 1: EpiDerm (EPI-200) Model Representative formalin-fixed, paraffin embedded, H&E stained tissue cross-section. Tissue structure closely parallels human epidermis.

Chemicals: 15 chemicals (**Table 1**) were tested within this project. All test chemicals were tested neat and in 5 concentrations (0.1%, 0.5%, 1%, 5%, 10%) in non-polar solvent (sesame oil) and in polar solvent (saline). Within a single experiment, a Negative Control (NC, DPBS), 2 vehicle controls (VC, saline (sal.) and sesame oil (s.o.)) and 2 respective Positive Controls (PC, 5% SDS, 1% SDS in saline and 1% SDS in sesame oil) were concurrently tested on N=3 tissues replicates.

Table 1: Chemicals used in this study.

Compounds	CAS #	Form	In vivo classification	
			GHS	EU CLP
heptanoic acid	111-14-8	liquid	1B	1B
SDS	151-21-3	solid	Cat 2	Cat 2
lactic acid	50-21-5	liquid	Cat 2	Cat 2
10-undecenoic acid	112-38-9	solid	Cat 3	No Cat
heptyl butyrate	5870-93-9	liquid	Cat 3	No Cat
hexyl salicylate	6259-76-3	liquid	Cat 3	No Cat
methyl laurate	111-82-0	liquid	Cat 3	No Cat
allyl heptanoate	142-19-8	solid	Cat 3	No Cat
sodium carbonate (50%)	497-19-8	liquid	Cat 3	No Cat
linalyl acetate	115-95-7	liquid	Cat 3	No Cat
2-bromobutane	78-76-2	liquid	Cat 3	No Cat
2-ethoxyethyl methacrylate	2370-63-0	liquid	Cat 3	No Cat
1-decanol	112-30-1	liquid	Cat 3	Cat 2
methyl methacrylate	80-62-6	liquid	Cat 2	Cat 2
sodium hypochlorite (10%)	7681-52-9	liquid	1B	1B

Table 2: Summary of EC-50 results for 15 chemicals treated in 2 skin irritation tests: EpiDerm skin irritation test (SIT) according to OECD TG 439 and EpiDerm skin irritation test of medical device extracts (SIT-MD) according to ISO 10993-23:2021 in non-polar solvent (sesame oil, s.o.) and in polar solvent (saline, sal.). Chemicals were tested neat and in 5 concentrations (0.1%, 0.5%, 1%, 5%, 10%) in both solvents.

Compounds	In vivo GHS class	EPI SIT (OECD TG 439)			EPI SIT MD (ISO 10993-23)		
		Viability at 100 %	EC-50 s.o. (%)	EC-50 sal. (%)	Viability at 100 %	EC-50 s.o. (%)	EC-50 sal. (%)
heptanoic acid	Cat 1B	3.9	2.93	7.22	2.0	2.27	3.35
SDS	Cat 2	11.6	3.04	1.5	1.9	0.55	0.29
lactic acid	Cat 2	2.5	-	-	5.2	0.55	2.82
10-undecenoic acid	Cat 3	3.7	-	-	2.6	0.75	7.95
heptyl butyrate	Cat 3	100.9	-	-	123.2	-	-
hexyl salicylate	Cat 3	91.5	-	-	103.2	-	-
methyl laurate	Cat 3	115.5	-	-	110.6	-	-
allyl heptanoate	Cat 3	105.2	-	-	5.2	-	-
sodium carbonate (50%)	Cat 3	42.1	-	-	5.9	2.54	-
linalyl acetate	Cat 3	106.1	-	-	3.8	6.69	7.87
2-bromobutane	Cat 3	3.7	-	-	3.8	-	7.42
2-ethoxyethyl methacrylate	Cat 3	11.2	-	-	4.8	1.67	1.64
1-decanol	Cat 3	4.8	-	-	12.7	7.55	-
methyl methacrylate	Cat 3	6.9	-	-	4.2	-	-
sodium hypochlorite (10%)	Cat 1B	3.5	3.66	7.27	1.8	0.68	0.32

EpiDerm Skin Irritation test according to OECD TG 439: The apical surfaces of EpiDerm tissues were exposed to 30 µL compounds at 37 ± 1 °C in humidified incubators at 37°C with 5% CO_2 for 1 hour. After exposure, the tissues were washed, and 42 hours post-incubation followed. Then the viability of the tissues was determined by the MTT assay, and the medium was stored for cytokine analysis.

EpiDerm Skin Irritation test for medical device extracts according to ISO standard ISO 10993-23:2021: The apical surfaces of EpiDerm tissues were exposed to 100 µL compounds at 37 ± 1°C in humidified incubators at 37 °C with 5% CO_2 for 18 hours. After exposure, the tissues were washed, the viability of the tissues was determined by the MTT assay, and the medium was stored for cytokine analysis.

Cytokine analysis: ELISA assays were used to measure the release of interleukins: IL-1α (R&D Systems, cat. #: DLA50), IL-6 (R&D Systems, cat. #: S6050), and IL-8 (R&D Systems, cat. #: S8000C).

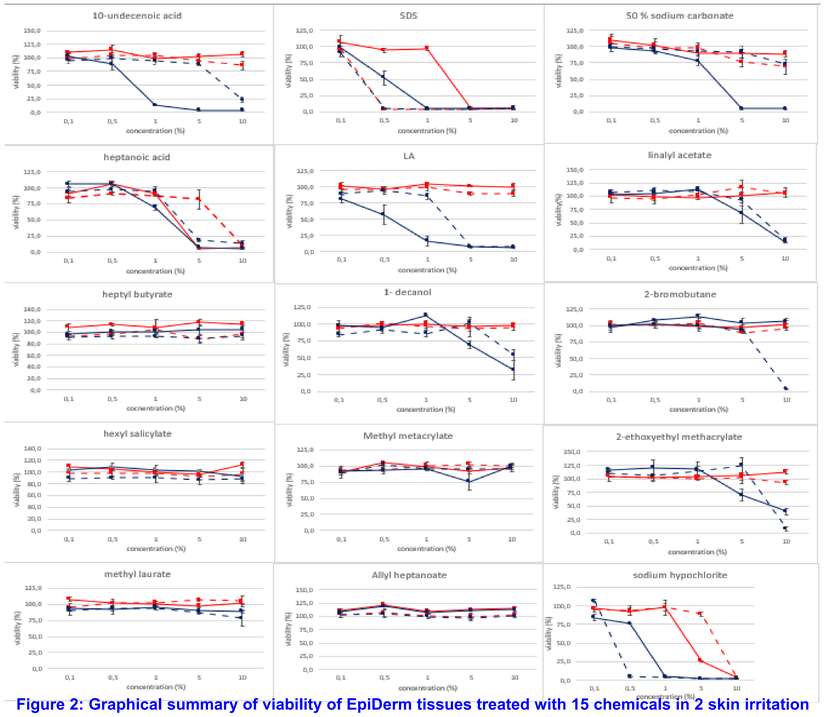
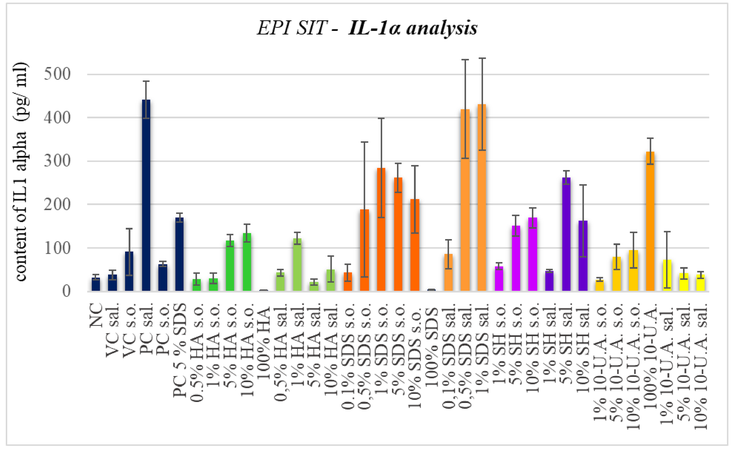


Figure 2: Graphical summary of viability of EpiDerm tissues treated with 15 chemicals in 2 skin irritation tests: EpiDerm skin irritation test (SIT) according to OECD TG 439 and SIT of medical device (SIT-MD) according to ISO 10993-23:2021. Compounds were tested in 5 concentrations (0.1%, 0.5%, 1%, 5%, 10%) in non-polar solvent (sesame oil, s.o.) and in polar solvent (saline, sal.). Results are presented as follows: EPI SIT in s.o. – red solid line, EPI SIT in sal. – red broken line, EPI SIT-MD in s.o. – blue solid line, SIT-MD in sal. – blue broken line.



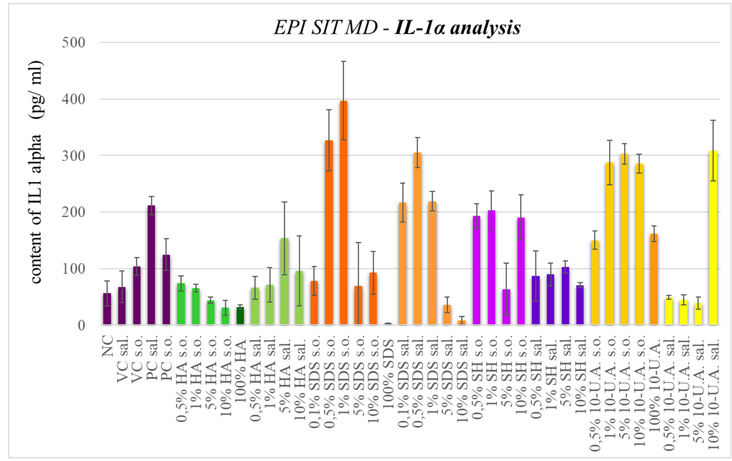


Figure 3: Analysis of IL1α content in the medium from EpiDerm tissues treated with selected compounds and selected concentrations from EpiDerm SIT and SIT-MD test. NC, negative control; VC, vehicle control; PC, positive control; HA, heptanoic acid; SH, sodium hypochlorite; 10-U.A., 10-undecenoic acid; s.o., sesame oil; sal., saline.

Conclusions

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- In vitro results for 15 chemicals with weak skin irritation potential were assessed using two different methods and two different solvents.
- Combination of methods identified the ability of these chemicals to affect viability and/or induce proinflammatory cytokines.
- This approach represents a promising in vitro method that may be utilized to distinguish non-irritants from compounds with weak but existing skin irritation potential.