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Application of the acquired knowledge and implementation of the Sens-it-iv toolbox for identification and classification of skin and respiratory sensitizers

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ABSTRACT

The contribution of the Sens-it-iv project to the reduction and replacement of animal experimentation is 3-fold. The funding of basic research has expanded the existing scientific knowledge thereby strengthening the understanding of the cellular and molecular mechanisms driving skin and respiratory sensitization. Examples are given on how a better understanding was used to improve existing test concepts. This knowledge was also applied to develop novel test systems. While some of test systems did not reach sufficient maturity for being considered for pre-validation others did and entered into the Sens-it-iv toolbox.

In the process, developments outside the Sens-it-iv orbit were carefully followed and assessed in order to avoid duplication and to assure synergy between the ongoing activities (e.g. Cosmetics Europe Task Force for Sensitization).

Tests from the Sens-it-iv toolbox were submitted to the European Reference Laboratory for Alternative Methods (EuRL-ECVAM) to initiate the rigid procedures for regulatory acceptance by national and international authorities. In spite of not being validated yet, selected tests were already applied in a weight-of-evidence approach in the context of REACH. Furthermore, several chemical, pharmaceutical, cosmetic and consumer product companies are currently assessing selected tests and testing strategies for their value as tools for screening and hazard identification using *in house* compounds and mixtures.

The main points of concern related to transfer to and implementation by industry were cost, throughput and applicability domain, rather than regulatory acceptance. These issues are currently addressed in applied research projects which are financially supported by individual companies, or consortia of companies, representing the various industry sectors.

1. Introduction

The overall objective of the Sens-it-iv project was to develop novel tools that could be used for testing chemical compounds and proteins for their potency to initiate in susceptible individuals an immune response that, upon second exposure, could induce an allergic reaction.

Sensitization is a complex multi-step process (Adler et al., 2011). The overall challenge facing *in vitro* test developers is the creation of test formats that represent key events of the *in vivo* mechanisms of action triggered in humans upon exposure to a sensitizing compound. Defining whether or not a cell-based test reveals adequate *in vivo* functionality is a difficult task requiring solid understanding of the physiological mechanisms occurring in humans *in vivo*. Several of these mechanisms (e.g. CYP activity, receptor expression, (pro-)inflammatory processes) are maintain, suppressed or driven by the microenvironment surrounding the target cell, as well as by cell–cell interactions. Consequently, development of physiologically relevant *in vitro* tests requires an in depth understanding of how microenvironment and neighbouring cells control cell differentiation, dedifferentiation, and responsiveness to, e.g. xenobiotics.

During the first phase of the Sens-it-iv project, progress was made primarily in fundamental research leading to an improved appreciation of the biological processes that occur in humans, and the molecular interactions that are affected, when human tissue is exposed to sensitizing materials (Thierse et al., 2011).

Subsequently, the Sens-it-iv consortium initiated the 'applied research' phase (2008–2010) aiming at the application of the available knowledge and understanding for the development of new assay systems that model sensitization in humans, rather than irritation and toxicity of chemicals and proteins. While focus was on test development, evaluation and refinement, specific fundamental research activities were allowed to continue: (i) specific activities expanding our knowledge about the role of innate immune responses in sensitization, (ii) the interaction between pro/pre-haptens and cells, and (iii) the cellular pathways involved in sensitization. These research

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topics were deemed necessary for assuring optimal exploitation of new opportunities for test development.

Throughout the project research and development activities that were ongoing outside the Sens-it-iv orbit were identified and followed-up. Thus, novel test systems representing human alveolar (University of Mainz, Germany) and bronchial epithelium (Epithelix Sárl, Switzerland) with good potential for respiratory sensitization assessment were successfully evaluated by members of the Sens-it-iv consortium. Tests, such as the dendritic cell (DC) maturation test, for which no added value to existing test (KAO Cooperation, Shiseido Co., L'Oréal) could be demonstrated, in spite of extensive efforts, were discontinued.

The final deliverable of the Sens-it-iv project was a toolbox which contains novel assays selected on the basis of their maturity (i.e. ready for prevalidation) and added value with respect to the tests developed outside the Sens-it-iv orbit. The previous manuscripts in this Special Edition of Toxicology *in vitro* addressed the most advanced Sens-it-iv tests.

None of the potential *in vitro* replacements for the LLNA went yet through the rigid procedures for scientific validation and (hopefully) regulatory acceptance by national and international authorities. Nevertheless, a number of these tests could be ready for non-regulatory use earlier, specifically when used for research purposes or *in-house* in the compound discovery and development process, resulting in a significant reduction of experimental animals.

The major 'post-Sens-it-iv' challenge is now (i) to drive the application of the acquired knowledge, as well as the implementation of these tests by industry, (ii) to improve the industrial applicability in close collaboration with industry, and (iii) to drive regulatory acceptance.

This manuscript wants to report on experiences from scouting of, technology transfer to and implementation by industry of acquired knowledge and selected tests from the Sens-it-iv toolbox.

2. Improved scientific knowledge and understanding guiding tests development and refinement

This section reports on three 'fundamental research' activities of high importance for the understanding of skin sensitization and with high impact on *in vitro* test development and refinement.

2.1. Recognition of the pivotal role of innate immune mechanisms

In vitro and *in vivo* gene knockout mouse systems uncovered a pivotal role of innate immune mechanisms in the initiation of contact sensitivity (Martin and Esser, 2010). These mechanisms were shown to involve Toll-like (TLR) or NOD-like receptors, reactive oxygen species (ROS) and the NLRP3 inflammasome, reflecting closely the signalling cascades induced by microbial infections.

Chemical sensitizers were shown to activate indirectly TLR2 and TLR4 on dendritic cells via TLR ligands produced by enzymatic degradation of high-molecular weight (HMW) hyaluronic acid (HA). The production of HMW HA-degradation was triggered in keratinocytes after exposure to sensitizers but not irritants (Martin et al., 2008).

These results were used to improve existing human keratinocyte-based *in vitro* tests. The targeted tests assess the impact of compounds on the Nrf2-Keap1 pathway which is involved in oxidative stress responses (Natsch, 2010). While this pathway was identified as very prominent in the cellular response to exposure to skin sensitizer, it was observed that about 1/3 of skin sensitizers from the Sens-it-iv chemical list did not affect this pathway (Johansson et al., 2011). Thus, a read-out with a broader chemical applicability domain was to be identified.

The recognition that regular inflammatory processes such as stimulation of IL-18 levels drive the early events in skin sensitization, and the observation that inhibition of the NrF2-Keap1 pathway still allows for the increase of IL-18 levels, supported the decision by the Sens-it-iv project board to focus on a test detecting changes in intracellular IL-18 levels before and after exposure to chemicals of human primary keratinocytes and human keratinocyte cell lines (e.g. NCTC2544, HaCat) (Corsini et al., 2009). Evaluation of the performance of the test indicated that intracellular IL-18 levels (97% accuracy) performs better than Nrf2-Keap1 induction (85%) on the same chemicals (N = 33).

The study of the role of the innate immune response produced a mechanistic description of the interactions between keratinocytes and dendritic cells during exposure to sensitizing compounds. This re-enforced the activities aiming at the development of an immune-competent reconstituted skin model resulting in the establishment of a prototype test system that may prove to be a helpful tool in mechanistic studies (Ouwehand et al., 2011). In addition, the recognition that keratinocyte and DC responses together establish a micro-environment that supports sensitization is currently part of the discussions about the establishment of integrated strategies for safety assessment.

Finally, the acquired knowledge has also potential applications outside the field of testing. Indeed, the better mechanistic understanding has provided new opportunities for treatment of contact dermatitis as well as other skin diseases involving inflammation (Stefan Martin, personal communication).

2.2. Pro-haptens fail to induce IL-8 secretion in DC cell line but induce increase in mRNA levels

Another spin-off example originates from the finding that unlike typical haptens most pre- and pro-haptens (i.e. chemicals requiring oxidative or enzymatic metabolism to become chemically reactive haptens) fail to induce secretion of IL-8 in the human DC line THP-1 within 24 h. This failure to produce IL-8 protein appears to result from chemical-induced destabilization of IL-8 mRNA. Consequently, when measured at earlier time points (i.e. 3 h), elevated levels of mRNA for IL-8 as well as for p38 MAP kinase can be detected after treatment with pre- or pro-haptens (Mitjans et al., 2010).

This new understanding was implemented by replacing IL-8 detection using ELISA by detection of mRNA levels (Emanuela Corsini, personal communication). The test was already applied in a weight-of-evidence approach for the purpose of registration under REACH.

2.3. Identification of signalling pathways

Proteomic and genomic studies designed to identify new biomarkers which differentiate sensitizers from irritants and non-sensitizers became highly successful during the last year of the project (Johansson et al., 2011; Thierse et al., 2011). Proteome analyses of primary human keratinocytes as well as of MUTZ-3 dendritic cells, and gene-chip based analyses of MUTZ-3 cells revealed large numbers of clearly sensitizer-specific gene-changes. Selections of these genes were used to develop the MUTZ-3-based Genomic Allergen Rapid Detection (GARD) test described below and to define a sensitizer-specific proteome signature for keratinocytes (Petra Budde, personal communication).

Identified genes and proteins were analyzed according to their assignment to defined cellular signaling pathways. The most prominent of these pathways for keratinocytes and MUTZ-3 cells revealed several interesting overlaps. Particularly markers relating to the Nrf-2-mediated oxidative response and oxidative stress in general were identified repeatedly, very much in line with the