Expanding the H295R steroidogenic assay using LC-MS/MS and an ER-alpha reporter gene assay as read-outs using azole fungicides as test compounds

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published in
Toxicology Letters
2021

DOI (link to publisher)
10.1016/S0378-4274(21)00401-X

document version
Publisher's PDF, also known as Version of record

associated with model inputs is propagated onto the model outputs and what impact this has on the final risk assessment. This approach was implemented in the open-source modelling framework Flame (https://github.com/phi-grib/flame). The method was validated by comparing the predicted and observed arrhythmogenic and torsadogenic properties of a panel of drugs with well-known clinical effects.

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SOC03-03
Next generation risk assessment for skin sensitization combining non-animal data and read-across: A case study with resorcinol

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Since animal testing has been prohibited in the EU, new approach methodologies enabling a Next Generation Risk Assessment (NGRA) have to be used for the risk assessment of cosmetic ingredients. A NGRA framework for evaluation of skin sensitization has been proposed by Gilmour *et al.* [1]. It relies on data integration from NAMs covering various key events of the skin sensitisation AOP in defined approaches (DA) and read-across.

This study illustrates the evaluation of the resorcinol skin sensitisation potential according to the NGRA framework when used at 0.2% in a face cream as well as defining the maximum acceptable concentration to avoid the sensitisation of consumers.

The DA applied in this case study is a stacking meta-model integrating several *in silico*, *in chemico* and *in vitro* methods with a combination of 5 statistical models [2]. This DA is a two-tier strategy with sequential prediction of skin sensitisation hazard and potency.

Resorcinol, predicted as sensitiser in the first tier hazard prediction (Cat.1) with a high probability of 92% was classified as moderate/weak (Cat.1B) in the second tier. To increase the confidence in the potency categorization, read-across was performed in addition to the DA to set the point of departure for the risk assessment. Search for analogues based on structural features followed by selection on the basis of protein reactivity profiling and skin sensitisation data availability identified 39 suitable analogues. Three approaches were then applied to select the final set of suitable analogues and the outcome was compared to gain insight into potential differences in analogue selection and POD determination. All three approaches for analogue selection resulted in the conclusion of a moderate potency for skin sensitisation and determined nearly identical POD values (3.8% in approach 1 and 2, 3.6% in approach 3). The POD of 3.6% was used for the final risk assessment as it was derived from the EC3 values of 3 analogues and was therefore considered to be more robust than the EC3 value from a single analogue determined in approach 1 and 2. Consequently, a higher maximum safe concentration of 0.36% in face cream was identified for resorcinol.The case study confirms that the applied NGRA is sufficiently protective for the consumer and allows an exposure led skin sensitisation risk assessment of cosmetic ingredients without generating new *in vivo* data.

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SOC03-04
Expanding the H295R steroidogenic assay using LC-MS/MS and an ER-alpha reporter gene assay as read-outs using azole fungicides as test compounds

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There is great concern on gynaecologic and female reproductive disorders related to exposure to endocrine disrupting chemicals (EDCs) that interact with steroid hormone synthesis, e.g. via food or personal care products. Our EU-funded FREIA project intends to improve testing strategies to assess female reproductive disorders caused by EDCs for implementation in regulatory frameworks.

We have developed a platform that combines both a reporter gene bioassay of estrogen receptor (ER) activation and quantification of steroid levels using LC-MS/MS. H295R cells were pre-incubated with forskolin (10 µM, steroidogenic inducer) for 24 hours. Then, cells were exposed to various concentrations of selected azole fungicides. Subsequently, exposure media were transferred to an ER-reporter gene assay (VM7Luc4E2) to determine ER transactivation and steroid profiles were assessed using LC-MS/MS. For the LC-MS/MS analysis, medium samples (100 µl) were extracted with solid phase extraction with Agilent Bond Elut Plexa 30 mg in a 96 well format and analysed with a SCIEX 6500+ triple-quadrupole mass spectrometer with electrospray ionization. Lastly, the samples are derivatized with dansylation and re-analyzed to improve the sensitivity for estrogens.

The azoles alone displayed no ER agonism and moderate ER-antagonistic activity in the ER reporter gene assay with IC50 values >10 µM. All azoles caused a concentration-dependent inhibition of estrogen production in forskolin pre-induced H295R cells, as shown by decreased ER activation in the reporter gene assay. The order of potency for the fungicides to inhibit estrogen formation (IC50) was letrozole (0.1 µM) > triflumizole (0.2 µM) > clotrimazole (0.4 µM) > flusilazole (0.6 µM) > ketoconazole (0.8 µM) > tebuconazole (5.3 µM) > propiconazole (25 µM). LC-MS/MS analysis showed that exposure to ketoconazole (1 µM) compared to vehicle control decreased both dehydroepiandrosterone and androstenedione levels by 42%, 17-hydroxyprogesterone by 30%, 17-hydroxypregnenolone by 15%, testosterone by 67%, 17β-estradiol by 24% while 17α-estradiol, estrone, estriol levels were unchanged. Levels of 5α-androsterone and 5α-androstanedione, associated with the backdoor steroidogenic pathway, were reduced more than 50% upon ketoconazole exposure. Moreover, we found that deconjugation with β-*glucuronidase* and arylsulfatase increased the levels of progesterone by 60-fold, pregnenolone by 25-fold, dehydroepiandrosterone by 6-fold, 11-deoxycorticosterone by 2-fold, indicating phase II metabolism in H295R cells.

Together, these data show that a quantitative analysis of steroid hormones using LC-MS/MS provides a more in-depth information on interaction of chemicals with steroidogenesis. The H295R assay followed by a reporter gene assay seems a straightforward approach to explore interaction with steroidogenesis, provided that direct interaction of the test compounds with the nuclear receptor is accounted for.

SOC03-05
A novel prediction model to evaluate genotoxicity based on a gene signature in metabolically competent human HepaRG™ cells

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The research was funded by the Chair M. Aerens at the Vrije Universiteit Brussel and the Research Foundation Flanders

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Genotoxicity testing is essential to ensure the safety of newly developed substances for human health. Traditionally, a stepwise standardized approach is applied, starting with a battery of *in vitro* tests. Despite its wide applicability and high sensitivity, the current *in vitro* genotoxicity battery is facing several limitations, among which the high number of misleading positive results which provoke unnecessary and costly follow-up *in vivo* animal studies [1-3]. To improve the predictive capacity of genotoxicity testing, mechanistic information at the molecular level is needed which can be obtained *via* new approach methodologies including gene expression biomarkers [4-7]. In this context, we previously developed GENOMARK to identify genotoxic substances in metabolically competent human HepaRG™ cells. GENOMARK consists of 84 biomarker genes, derived from whole genome transcriptomics data and selected to cover diverse modes of action, including bulky adduct formation, DNA alkylation, cross-linking, radical generation causing DNA strand breaks, inhibition of tubulin polymerization and base analogues [8, 9]. Briefly, cells are exposed for 72 hours to a concentration of the test chemical inducing a low level of cytotoxicity. Afterwards, gene expression levels are evaluated with RT-qPCR and results are automatically analyzed with a support vector machine (SVM)-based model to classify chemicals as genotoxic or non-genotoxic. In the present study, an improved prediction model was developed based on a different algorithm called random forest (RF). To this extent, the existing reference dataset of 24 chemicals was enlarged to 38 by selecting additional *in vivo* genotoxic and non-genotoxic chemicals for which new test data were generated. Next, two supervised machine learning algorithms in R software, *i.e.* SVM and random RF were applied on the extended dataset and their predictive capacity was compared. Both prediction models showed the same predictive accuracy (*i.e.* 92.3%), although the RF model displayed a higher sensitivity and a lower specificity compared to the SVM model. As the RF model is also less sensitive to outliers, this model was selected and further applied to predict the genotoxicity of 6 misleading positive chemicals. Four of these (2-Methyl-4-isothiazolin-3-one, 4-Amino-3-nitrophenol, Sodium benzoate and Dihydroxyacetone) were correctly classified as non-genotoxic by the GENOMARK biomarker. One chemical was classified as equivocal (hydroxybenzomorpholine) and one as genotoxic (1-napthol). These results demonstrate that GENOMARK could be useful as a follow-up of the *in vitro* genotoxicity test battery to de-risk misleading positives in a Weight of Evidence approach and may contribute to the paradigm shift in genetic toxicology to move towards a more human-relevant genotoxicity testing.

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SOC03-06
Emerging role of epigenetic alterations in the lung inflammation and tissue remodeling induced by air pollution-derived $PM_{2.5}$ in mice

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Purpose: Even though clinical, epidemiological and toxicological studies have progressively provided a better knowledge of the underlying mechanisms by which air pollution-derived particulate matter (PM) exerts its adverse health effects, further studies are still needed. Emerging data suggested the possible alterations of some critical epigenetic marks by air pollution in general, and, in particular, its fine particle fraction (i.e., aerodynamic diameter ≤ 2.5 µm, $PM_{2.5}$). Hence, in this work, we tried to better determine whether epigenetic marks (i.e., DNA methylation, histone acetylation/deacetylation, miRNA) occurred and persisted in the lungs of mice exposed to urban $PM_{2.5}$ (Lille, France), 24 h after their acute exposure, and 24 h and 2 months after their subchronic exposure.

Methods: A/J mice (male, specific and opportunistic pathogen free, 10 weeks, n = 6/group) were exposed either for 24 h by one intranasal instillation, or for 28 days by 3 intranasal instillations/week, to 0, 10, 50 or 100 µg of urban $PM_{2.5}$ suspended in 30 µl of sterile saline. Mice were sacrificed either 24 h after their acute exposure, or 24 h or 2 months after their subchronic exposure. Global DNA methylation, gene promoter methylation of some critical cell cycle regulators, DNA methyltransferase (DNMT) activity, histone H3 post-translational modifications (i.e., H3K9ac, H3K14ac, and H3K27ac), histone acetyltransferase (HAT) and deacetylase (HDAC) activities, and miRNA profiles were studied in lungs by ELISA, EpiTectMethyl II PCR mouse