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Alternative testing of inhalable aerosols: A complementary in vitro/ex vivo model for acute inhalation toxicity

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An in vitro model consisting of a human alveolar lung cell line (A549) in an air-lifted interface (ALI) culture situation and exposure using the P.R.I.T. ExpoCube device was combined in an experimental strategy with an ex vivo model consisting of an isolated perfused lung model (IPL) from the rat to achieve a testing strategy using only low amounts of testing materials (< 1 g), short testing periods and a high relevance of results as documented by correlation to literature data from acute inhalation toxicity in vivo animal testing. Test items for model validation included 5 commercial crop substances (Chlorothalonil, Captan, Mancozeb, Fosetyl-AL, Ethiprol) and two additional control items (sodium dodecyl sulfate (SDS) and n-dodecane). Test items were applied in a short-term / high concentration exposure design in both models. Acute inhalation toxicity was estimated from effects on cellular viability (in vitro) and on tidal volume and lung weight (ex vivo). Moreover, effects on the breathing mechanics could be explored using the ex vivo IPL approach. Dosimetry considerations were carried out on the basis of the specific particle deposition characteristics of the ExpoCube expose device (in vitro) respectively the multiple-path particle dosimetry model (MPPD, ex vivo) to estimate surface loads in vitro (µg/cm²) or lung loads (µg/lung) ex vivo. Comparison to in vivo literature data indicated highly correlated estimations of the acute toxicity in vitro in a nearly quantitative way without interference by test item solubilities also including the range of surface-load values in vitro and in vivo in the range of 1 to 100 µg/cm². Ex vivo results clearly enabled detection of harmful substances and, moreover, also enabled detection of test items exhibiting adverse effects on the mechanical breathing behavior, possibly by affecting the surfactant system of the lung (Fosetly-AL, SDS, dodecane). In conclusion, it is proposed to apply the test system in a tiered approach (in vitro/ex vivo/in vivo) to achieve a relevant acute inhalation toxicity testingusing only small amount of test substances and short testing periods in the sense of the "3R-principle" for replacing, refinement and reduction of animal experimentation.

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The SILIFE Project: production, toxicity screening, and industrial application of quartz species with reduced lung toxicity

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In 1997 the IARC classified respirable crystalline silicas (RCS) as human carcinogens (category 1), strongly affecting work places in silica-dependent industries. IARC acknowledged differences among RCS species, based on source, chemical, thermal and mechanical history. As abundance, density and heterogeneity of surface silanol groups/radicals seem to be involved in RCS-mediated lung toxicity, adverse RCS lung effects might be reduced by coatings, covalently blocking these groups. Persistent problems with silicosis and the diversity of quartz applications stimulated issuance of the EU-project SILIFE, aiming at developing a dry surface-coating technology to abate RCS toxicity on industrial scale. The development process included choice of feasible, cost-effective coating additives (i.e. organosilanes and catalysts), definition of treatment parameters (technology, reaction time, dosage, application), proof of coating effectiveness and toxicity-reducing functionality by physico-chemical (e.g. ζ potential) and predictive in vitro and in vivo toxicity tests, with final implementation of coated RCS into industrial processes. Primary rat alveolar macrophages (4 h of incubation, 75 µg/cm2 of pristine/coated quartzes) served as sensitive in vitro toxicity screening model with membrane (LDH-release) and DNA damage (Comet assay) as relevant endpoints. The very promising in vitro results were validated in a 28-/90-days intratracheal instillation study in male Wistar rats (pristine v. coated industrial quartz species; 2 mg/lung; positive control: quartz DQ12, 1 mg/lung). In bronchoalveolar lavage fluid both latency and variable, quartz species-dependent adverse reactivity of three industrial quartz samples was noted, using classical inflammatory parameters (differential cell count, LDH, ß-glucuronidase, total protein) and pro-inflammatory mediators (CINC-1, TXB2) as meaningful readouts. But, more importantly, the study clearly demonstrated that some covalent RCScoatings were indeed able to effectively block RCS lung toxicity in the rat for up to 90 days, without markedly compromising the technical process quality. Thus, covalent surface-coating of biologically reactive RCS species represents a promising strategy to render RCS handling safer. Funding: LIFE 2014: project no. LIFE14 ENV/ES/00238.

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Comparison of NOAEL, ETD10, and BMDL10 Values for Nongenotoxic Carcinogens: Which point of departure is most sensitive?

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Human carcinogens may act by different modes of action, but generally can be classified as genotoxic and non-genotoxic carcinogens. The aim of this study was to better understand which endpoints and/ or point of departures are the most sensitive after chronic oral exposure for non-genotoxic carcinogens. 232 compounds which were classified as non-genotoxic carcinogens, using a simple decision tree, were identified. Following a detailed review of all available peer-reviewed literature, experimental and predicted data, this dataset was reduced to 223 organic compounds. NOAEL values were derived for these 223 compounds from chronic and (if required) subchronic studies following oral application using well established high quality databases e.g. RepDose, ToxRefDB, COSMOS or peer-reviewed publications. As far as possible, NOAEL values were derived from the same study as used in the CPDB database for the calculation of carcinogenic potency (TD50 values). NOAELs were based on either the most sensitive i) adverse apical effect in the entire study; ii) non-neoplastic lesion; or iii) neoplastic lesion. Study quality was considered as one potential confounder. These NOAELs are compared to the effective tumour dose (ETD10) and the benchmark dose level (BMDL10) calculated by model averaging, where a tumor-related effect is expected to be observed in 10% of the animals tested. The comparative analysis of the correlations between NOAEL/EDT/BMDL values revealed that compounds with a concern for bioaccumulation were found among the 5% most toxic compounds. After exclusion of these compounds, the 5th percentile of the chronic NOAELs is in the same range as of BMDL10 values, whereas the 5th percentile of the EDT10 is about 3 times higher than the NOAEL. These results were evaluated with regard to the current threshold of toxicological concern (TTC) threshold. This work received funding from the CEFIC LRI B18_2 project.

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Systematic application of new approach methods to uncover the mode-of-action of herbicides and fungicides with unanticipated toxicological properties: a joint EU-ToxRisk and Syngenta case study

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Fast and reliable identification of toxic effects is crucial in the development of new active substances. New approach methods (NAMs) enable high throughput screening without the costs and ethical concerns of animal tests. Also, NAMs provide insights into underlying mechanisms of toxicity, which can be useful in screening new active substances. Syngenta provided 10 compounds that are either commercially available or research compounds that were discontinued due to developmental and reproductive toxicity (DART) effects. A selection of the EU-ToxRisk in vitro test battery was applied to determine if they can be used in hazard assessment or screening of new active substances for weed and disease control in cereals. 20 CALUX assays and 14 HepG2 BACGFP reporter gene assays were chosen to study activation of (nuclear) receptors and cellular signaling pathways. Cell type specific assays included high content imaging in primary human hepatocytes (PHH) and cell migration (UKN2) or neurite outgrowth assays (UKN4) in human dopaminergic neuronal cell lines. Hierarchical clustering was applied to the points of departure (PoDs) that were determined in each test system. This analysis revealed three distinct clusters of activity patterns that correspond to the known modes of action of the 10 case study compounds (1) Brequinar; (2) ACCase inhibitors; (3) fungicides of unknown mode of action. The results also gave insights into potential mechanisms underlying the toxicities observed in vivo. Group 1 showed a high activity pattern across the in vitro tests with activation of endocrine receptors, steroidogenesis and oxidative stress pathways and inhibition of neuronal function. Group 2 displayed a low activity pattern with activation of cellular stress response pathways and some effects on neuronal cells. Group 3 showed a moderate activity pattern across test systems with activation of several cellular stress response pathways and effects on neuronal function and viability. Combined but not individual analysis of the results gave the best match of clusters to known activity mode. Mechanisms of toxicity will also be analysed through transcriptomic changes in liver (PHH) and kidney (RPTEC/TERT1) cells. Computer models will be applied to determine the oral equivalent doses of the PODs for extrapolation to the human situation in hazard assessment.

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Transcriptomic data of liver and kidney cells treated with valproic acid and analogues to support read-across assessment

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A read-across assessment usually starts with a list of initial source compounds sharing structural and physico-chemical properties. The finally selected source compounds also should exhibit similar toxicodynamic and kinetic properties. The conclusion on similar dynamic properties is often challenging, in particular because mechanistic data are usually not available. Legacy animal repeat dose oral exposure data show longer-chain analogues, like Valproic acid induce microvesicular liver steatosis as primary toxic effect with renal weight loss at higher dosing. Short-chain analogues, like 2-Ethylbutyric acid and Pivalic did not show adverse liver or kidney effects. We treated the human liver cancer cell line HepG2 and the human renal proximal tubule cell with 18 carboxylic acids (six concentrations) and conducted transcriptomic analysis using TempO-Seq. Unsupervised clustering of differentially expressed genes (DEGs) revealed clustering of steatotic versus non-steatotic carboxylic acids using the HepG2 model. Activity increased with increasing side chain length. A similar response was found with RPTEC/TERT1 cells. Further comparative analyses like pathway analyses among all analogues in both HepG2 and RPTEC/TERT1 cells are discussed. In summary, by studying the mode of action on a transcriptional level, we anticipate to support risk assessment by providing qualitative similarity data on a mechanistic level. By testing different cellular models, we will also learn more about the minimal scope needed to conclude on shared modes of action. This work received funding of the EUToxRisk project (Grant agreement No 681002).

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Integration of transcriptome data into the hazard assessment of volatile compounds: a read-across approach

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Read-across is one of the most often used alternative methods in chemical risk assessment. It is often a challenge to conclude on shared toxicodynamic properties within the grouped substances based on observed adverse effects from in vivo studies. New models providing mechanistic information might be useful to support this assessment. ExITox II (Explain Inhalation Toxicity) addresses this question with compounds to which humans are exposed via the inhalation route. Human lung alveolar epithelial (A549) cells were exposed to five different read-across groups of airborne compounds, including gases, liquid aerosols and nanoparticles. The read-across groups comprise structurally related compounds. After repeated inhalation exposure aliphatic diamines, aldehydes and alkyl acrylates induced mainly pulmonary inflammation in rodents, whereas vinylester showed in addition hyperplasia and the nanoparticles led to fibrosis. Cytotoxicity was measured using mainly air-liquid exposure with the P.R.I.T.® ExpoCube® setup and repeated exposure. Whole transcriptome analysis (TempO-Seq) revealed DEGs (differentially expressed genes) per read-across groups in a dose dependent manner, with some nanoparticles having only very few DEGs. With the geneXplain platform, enriched TFBS (transcription factor binding sites) and the corresponding transcription factors (TFs) were identify. These TFs were the input for a master regulator (MR) analysis, the postulated MRs may function as marker/key molecules for the investigated substance groups. Finally group specific functionalities of DEGS, TFs and signaling pathways were described. The experimental validation of the proposed MRs is ongoing by quantitative RT-PCR. Unsupervised clustering was used to group the compounds according to their DEG/MR pattern. Further comparative analysis among the read-across groups providing more evidence on the association of DEGs/MR and signaling pathways with the observed adverse in vivo outcome will be discussed. We intend to support hazard assessment within a read-across approach by providing more evidence on shared toxicodynamic properties using transcriptome data. This work has been funded under grant agreement FKZ 031 L0120A (BMBF program).

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Hazard assessment of diacetyl and structurally related diketones—A read-across approach

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Diacetyl (2,3-butanedione) is a volatile organic compound with a strong buttery flavor that is commonly used as food flavoring ingredient and in electronic cigarettes. Concerns were raised regarding respiratory disorders

(Popcorn Lung) such as bronchiolitis obliterans linked to occupational pulmonary exposure to diacetyl vapors and use of electronic cigarettes. In this read-across study, we investigated the use of new approach methodologies to characterize and differentiate the hazard of diacetyl and other structurally related diketones. For this purpose, three structurally similar groups (α, β and γ diketones) which most likely have different modes of action were included, together with 2 negative compounds aliphatic aldehydes, which do not show any inflammatory response in the available rodent in vivo studies. Primary human bronchial epithelial cells (PBECs) were isolated from tumor-free lung tissues from four donors and differentiated into mucociliary epithelial cells at air-liquid interface (ALI) conditions. The cells were exposed to the case study chemicals under (ALI) conditions using the P.R.I.T.® ExpoCube® device for 1h once or repeatedly on three consecutive days. Cellular viability was measured by LDH-leakage and barrier function by measuring the transepithelial electrical resistance (TEER) 24h after the final exposure. Exposure concentrations ranged from 100 to 1840 ppm (diacetyl) and from 50 to 5000 ppm (other diketone analogues). Chemical-induced transcriptomic responses were investigated utilizing targeted RNAseq with the Templated Oligo Detection Assay (TempO-Seq) based on a 3347 gene panel. TempO-Seq analysis revealed up or down regulated differentially expressed genes (DEGs) in a dose and exposure time dependent manner. Analysis of the gene expression patterns indicated that some of these diketones may share a similar mode of action. Translational analyses were carried out to link these in vitro data to relevant adverse human outcomes like pulmonary fibrosis and inflammation. Acknowledgement: This project received funding from the European Union's Horizon 2020 research and innovation program (grant agreement No 681002).

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ICONS: Integrated testing strategy for mechanistically assessing the respiratory toxicity of functionalized MWCNT

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Toxicological concerns are opposed to the promising technical properties of multiwalled carbon nanotubes (MWCNT). Meeting WHO fiber criteria MWCNT can be carcinogenic (rigid type) or non-carcinogenic (tangled type) depending on fiber length and diameter. The project ICONS focused on the comparison of core MWCNT vs. surface-modified MWCNT (both tangled type) regarding their fibrotic and genotoxic potential. Purification of core MWCNT (chemically or thermally) and surface functionalization (-COOH or -NH2) of the industrially relevant Nanocyl NC7000 were varied. At Fraunhofer ITEM, the eight resulting MWCNT (pristine, milled, purified, and functionalized) were tested for sterility and endotoxin contamination. For in vitro use, they were dispersed using an ultrasound-based protocol, and characterized by light and scanning electron microscopy. Subsequent in vitro (geno)toxicity testing with MRC-5 primary human lung fibroblasts revealed differential inhibition of proliferation (RICC, mitotic index) and induction of membrane damage, DNA-strand breaks and micronuclei. Using primary human mesothelial LP9 cells, a variable number of differentially expressed genes was noted. Based on these in vitro data and the in vivo data, generated by LTAP and NCSU, the COOH-functionalized chemically purified (NC3151) and thermally purified (NC-PlacylCOOH) samples were selected for a 4-wk inhalation study in rats (design based on OECD TG 412; 0.2, 1 and 5 mg/m3), including a 4-wk recovery (validation test). As a reference group, the thermally purified NX7100 sample (non-functionalised core) was included with 5 mg/m3 only. Pre-trials demonstrated feasibility of generating respirable MWCNT aerosols by dry dispersion with pressurized air, supported by a jet mill. The differential cell count analysis, the levels of lactic dehydrogenase, beta-glucuronidase and total protein in bronchoalveolar lavage fluid and the histopathological examination resulted in the following ranking: NC3151 < NX-7100 (core) = NC-PlacylCOOH, indicating that the purification method seems to be important. This ERA-NET SIINN project was funded by the German BMBF (FKZ: 03XP0063).

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Systematic assessment of cellular gene network modulation by valproic acid analogues for biological read-across

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Chemical read-across is commonly evaluated without particular knowledge about the biological mechanisms leading to the observed adverse outcomes in in vivo studies. The integration of data showing a shared mode of action in humans will strengthen the read-across assessment. Here we used a large panel of valproic acid (VPA) analogues to include detailed mode-of-action (MoA) data as a proof-of-concept for read across. In rodents, VPA and some of its analogues cause hepatic steatosis, whereas other analogues do not. Previous studies showed a predictive value for stress pathway response activation for the hepatotoxic potential of VPA analogues. Here, we look into the transcriptomic response of HepG2 cells stimulated for 24 h by the analogues in a dose response range to be able to predict steatotic potential in vivo. We used TempO-Seq targeted high-throughput screening assay to assess the differential expression of more than 3000 genes, referred as the S1500+ geneset, that reflects all biological pathways. Dose response analysis revealed clustering of steatotic-positive versus steatotic-negative VPA analogues. To quantitatively define biological read-across of VPA analogues we used both pathway analysis and our in-house primary human hepatocyte (PHH) TXGMAPr tool, a novel gene expression visualization tool based on weighted gene co-expression network analysis of the Open TG-GATEs database. VPA responses in HepG2 cells displayed high similarity in gene network modulation compared to responses in PHH. Steatotic VPA analogues demonstrated similar pathway activation. Quantitative gene network analysis allowed ranking of biological similarity of VPA analogues. Importantly, free fatty acid synthesis modulation was highly affected by all steatotic VPA analogues, but not by in vivo negative analogues. We defined the most representative genes that reflect steatosis responses in both HepG2 and PHH that could be generally used as markers for steatosis onset. In summary, by studying the MoA on a transcriptional level, we anticipate to support risk assessment by providing quantitative and mechanistic biological information to corroborate a robust read-across approach. This work was part of the EU-ToxRisk project and received funding of the European Union's Horizon 2020 research and innovation program under grant agreement No 681002.

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A combination of a physiologically-based pharmacokinetic (PBPK) model for the inhalational route and an ex vivo model for prediction of lung absorption kinetics

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Information on systemic available concentrations is important for the safety assessment of chemicals and the pre-clinical development of new drugs. In recent years, the inhalational route has gained particular interest. A Physiologically-Based Pharmaco-Kinetic (PBPK) model for airborne subSOT 59th Annual Meeting and ToxExpo 446 stances with focus on the uptake via the lung has been developed. The resulting IVIVE-Lung-PBPK model (in vitro to in vivo extrapolation) shall be applicable to gases, liquid aerosols and (slowly) soluble particles. The lung is divided into three sub-compartments to account for the different clearance and uptake processes in the individual lung sections. The systemic part of the

model is based on a state-of-the-art design and includes the different relevant organs/tissues. For uptake through the lung epithelium, permeation values are derived using in vitro and ex vivo models, like the Isolated Perfused Rat Lung. Further relevant data and processes, such as mucociliary clearance or blood-tissue distribution coefficients are included based on literature data. To investigate the applicability of the model, pulmonary permeability coefficients were determined for ciprofloxacin and a second small molecule substance, for which many further ADME parameter are available from literature. Comparison of the data derived from the PBPK model with the permeability coefficients from the IPL as input parameters to the corresponding human data shows good agreement for the plasma concentration profile and the quantitative concentration levels. According to these investigations, the inhalation PBPK model in combination with the pulmonary absorption parameters determined in the ex vivo model of the Isolated Perfused Rat Lung can successfully predict the systemic uptake in humans for small molecule substances with diffusion-controlled transport mechanisms. Therefore, this model can well contribute to the safety assessment of chemicals with usually very limited availability of in vivo PK data, as well as for pre-clinical studies with inhalable drugs. Currently, the lung PBPK model is further improved in the project Cefic-LRI B21. This includes a more detailed description of different clearance processes, such as macrophage-mediated clearance and dissolution as well as the investigation of the applicability of other biological parameters determined in vitro.

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Apparent permeability coefficients to parameterize PBPK models for inhalation: Impact of the in vitro setup

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This study aimed at the determination of apparent permeability coefficients (Papp) of the inhalable antibiotic ciprofloxacin hydrochloride monohydrate (CHM) in human lung barrier models and to utilize these coefficients as input parameters for a lung PBPK model (physiologically based pharmacokinetic model). Two different cell models were used resembling different lung regions: The Calu-3 cell line expressing features of differentiated small airway epithelial cells as well as functionally immortalized human alveolar epithelial (CI-hAELVi) cells. For both cell models, Papp values were measured in a submerged setting and under air-liquid interface (ALI) exposure conditions. For submerged exposures, the antibiotic CHM was dissolved in culture medium and added to the apical compartment. ALI exposures were done with CHM aerosol generated using the PreciseInhaleTM device. For efficient and precise aerosol exposures, the P.R.I.T.®ExpoCube® was used. CHM concentrations in the different compartments were analysed by LC-MS/MS. In the case of ALI exposures, the CHM mass deposited on the apical cell surface had to be converted to concentration values. This was done by assuming a thickness of 2 µm for the lining fluid layer for Calu-3 and AT-1 cells and dissolving of CHM in the resulting volume. The resulting Papp coefficients were similar for both cell models, the levels being 1.99*10-8 cm/sec for AT-1 and 1.09*10-8 cm/sec for Calu-3, respectively. The cell models under submerged conditions showed higher Papp values of 6.34*10-7 cm/sec for AT 1 cells and 7.24*10-7 cm/ sec for Calu-3 cells. Papp values obtained from both in vitro settings were used as input parameters for the physiologically based pharmacokinetic (PBPK) model to simulate the absorption and distribution processes in the human body. Resulting peak blood concentrations for ciprofloxacin were 5 fold higher when Papp coefficients obtained from submerged experiments were used. However, comparison with human literature data revealed that simulations based on Papp coefficients calculated for ALI exposures produced blood levels that were very close to the human in vivo situation. These findings suggest that the experimental setup is highly important when in vitro data are to be used as input parameters for PBPK models to predict the bioavailable dose after inhalation exposure in humans.

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ToxSIBAR: Predicting toxicity with similarity based descriptors

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The concept of similarity is broadly implemented in predictive toxicology, assuming that chemical compounds with similar chemical structure, similar pharmacological target profiles, similar metabolic, toxicodynamic and or toxicokinetic profiles will exert similar toxicological effects. Being widely known as read-across approaches, such concepts can aid as alternative tools to animal testing for hazard assessment. In addition to a concept aiming to group (or position) compounds based on some sort of similarity measure, compounds with known toxicity can also be used to build a predictive computational model in order to forecast the toxicity of a compound with unknown adverse effect. A prerequisite however, is the availability of enough chemical data as well as the employment of relevant features (describing/encrypting the chemical compounds) being able to sufficiently capture the essentials of that toxic event. The latter, is one of the biggest challenges in computational toxicology nowadays since mechanistic information of the toxic event under study can seldomly sufficiently be encrypted in the chemical structures and derived descriptors. We are proposing a new methodology, utilizing the concept of read-across in the framework of a predictive modeling approach. The ToxSIBAR concept is based on previous work where (euclidean) distances of the training set compounds to a set of reference compounds were utilized as input features for building QSAR models for predicting e.g., transport inhibition. We applied this concept to an in vivo dataset of >1000 diverse compounds measured as positive or negative for hepatic steatosis in rodent studies with repeated oral exposure by using different reference compound sets (e.g., randomly chosen; based on maximum diversity) and by applying different basic descriptors and classifiers. Interestingly, the models utilizing the ToxSIBAR descriptors as input outperformed the baseline models highlighting the potential of the method to be developed and optimized further. This work has received funding from the EU-TOXRISK, a project running under the European Union's Horizon 2020 research and innovation programme, grant agreement No 681002.

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EU-ToxRisk guidance for new approach methods (NAM)-supported read-across EU-ToxRisk guidance for new approach methods (NAM)-supported read-across

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Read-across (RAX) is a valuable approach to fill data gaps for complex toxicological endpoints without the use of de novo in vivo studies. Toxicological data from compounds with certain structural/physicochemical properties can be extrapolated to similar compounds that lack these data. For RAX to be reliable, however, biological similarity has to be accounted for as well. NAMs that describe toxicokinetic and toxicodynamic properties can be deployed to verify this biological similarity, and to establish a scientifically reliable and robust RAX. The use of NAMs goes hand in hand with new challenges like scope of in vitro testing, use of AOPs, interpretation of conflicting results, description of uncertainty, and reliability of test methods. More guidance is needed to assure transparent and accurate use of NAMs and by this promote acceptance of these new data within RAX assessments. In May 2019, the Horizon 2020 European collaborative project EU-ToxRisk - An

Integrated European 'Flagship' Programme Driving Mechanism-based Toxicity Testing and Risk Assessment for the 21st century - together with European agencies (ECHA, EFSA, SCCS), US agencies (NTP, EPA), and global organizations (OECD), hosted the workshop "New Approach Method (NAM)-supported read-across: from case studies to regulatory guidance in safety assessment". Based on case studies that targeted different regulatory applications, the use of NAMs was discussed with a special focus on the regulatory context and the associated regulatory requirements that these approaches have to fulfil. Furthermore, some of the cases have also been reviewed by the OECD IATA working group. This poster highlights the main learnings from the feedback on the NAMbased read-across reports, such as the requirements for data and method descriptions, the application of AOPs in terms of IATAs, the use of positive and negative controls to proof fitness-for-purpose of testing strategies, and uncertainty assessment.