Workshop Report

The Role of *In Silico* Tools in Supporting the Application of the Substitution Principle

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1 Introduction

LIFE-EDESIA (LIFE12 ENV/IT/000633; http://www.iss.it/life) is a project funded by the LIFE program, the funding instrument of the European Union (EU), to support actions in favor of the environment and climate. The LIFE-EDESIA objective is to identify safer alternatives to endocrine disrupting chemicals (EDCs) of industrial relevance (bisphenols, phthalates, parabens); the project's scientific strategy is based on the integration of *in silico* and *in vitro* methods. Accordingly, the first LIFE-EDESIA workshop targeted the *in silico* methods and applications used in different contexts to address the identification of EDCs; particular attention has been devoted to the support that *in silico* methods can give to the application of the substitution principle (the replacement of hazardous – or potentially hazardous[1] – chemical substances by less hazardous alternatives) within the REACH framework.

The two-day workshop on "The role of *in silico* tools in supporting the application of the substitution principle" was co-organized by the project coordinator *Istituto Superiore di Sanità* (ISS) and by the project beneficiary *Istituto di Ricerche Farmacologiche "Mario Negri"* (IRFMN) and held on December 10-11, 2014 in Milan at IRFMN; the program included three sessions and a final stakeholders' round-table. The first session introduced LIFE-EDESIA aims and approaches and highlighted the specific aspects of the computational studies in the Project Actions. In the second and third sessions, academic *versus* industrial and regulatory perspectives were considered with particular attention to applications concerning EDCs or or endocrine disrupting (ED)-like activities in the food chain.

The Proceedings of the workshop are available online on the LIFE-EDESIA project website[2]. Here, an overview of the content of each presentation is given.

2 The LIFE-EDESIA project

The opening session, chaired by the LIFE-EDESIA project manager Stefano Lorenzetti (ISS), gave an overview of the scientific and regulatory framework in which the project was presented and how the related concerns are tackled.

Alberto Mantovani gave an *introduction to the LIFE-EDESIA Project* explaining the novelty of the approach proposed. The core idea of the project is to combine *in silico* and *in vitro* approaches to evaluate suitable chemicals for replacing EDCs that are, or may be identified as, of "equivalent concern" to Substances of Very High Concern (SVHC) within REACH. A further and related aim is to demonstrate the feasibility of the selected substitutes in industrial applications. Namely, LIFE-EDESIA thus identified three groups of compounds, phthalates, bisphenols and parabens, used in different industrial applications such as food packaging, medical devices and personal care products. A key element is that these identified (bisphenols, phthalates) or potential (parabens) SVHC are quite extensively investigated in terms of toxicological information; thus, LIFE-EDESIA must provide a robust, consistent and comparative scientific data set supporting that the potential replacers are actually proven to be safer.

Stefano Lorenzetti presented the ideas and approaches adopted in the LIFE-EDESIA project for the *in vitro assessment of endocrine disruptors/EDCs*. He highlighted that most *in vitro* assays currently used to assess ED-like activities use gene transactivation assays, thus highlighting an early part of the mode of action. Hence, they are not suitable to assess whether the observed effect – in terms of modulation of nuclear receptor activities – actually modifies cell function, leading to detrimental effects at organism level. Therefore, the LIFE-EDESIA strategy uses a set of assays capable to catch functional markers relevant

[1] The Substitution Principle, KEMI Report Nr 8/07, http://www.kemi.se/Documents/Publikationer/Trycksaker/Rapporter/Report8_07_The_Substitution_Principle.pdf

[2] http://www.iss.it/life/index.php?lang=2&id=223&tipo=38

to ED-like adverse effects. Such toxicological assays, defined functional assays, take advantage of the exploitation of biomarkers of effects currently used in clinical applications. Hence: i) in a prostate cell line (LNCaP), prostate-specific antigen (PSA) secretion is measured to spot androgen receptor-mediated effects, ii) in a trophoblast cell line (BeWo), β-human chorionic gonadotrophin (β-hCG) secretion is measured to spot estrogen receptor-mediated effects, finally, iii) in a fetal liver cell line (HuH6), alpha-fetoprotein (AFP) secretion and the intracellular lipid accumulation are measured to investigate multiple nuclear receptor (NR)-mediated effects on lipid programming and hepatosteatosis.

Ferdinando Fiorino and **Elisa Perissutti** discussed *the role of chemical synthesis in support of the substitution principle* with a focus on green chemistry. The focus of the presentation was on the substitution principle *per se*, and the possible ways to find alternatives, as well as on the use of laboratory techniques for chemical synthesis that is more environmentally friendly due to reduction of reaction waste. Finally, the support that a functional group approach can give to green chemistry was illustrated, in particular concerning the isosteric replacement of groups that, within given molecules, could be putatively responsible for undesired ED activities.

3 Academic perspective

The second session, chaired by the hosting member Alessandra Roncaglioni (IRFMN), introduced the role of the computational approach within the LIFE-EDESIA project and illustrated the features of different *in silico* tools along with important applications related to the field of EDC toxicology.

Emilio Benfenati introduced *the use of in silico approach in the REACH context in other EC LIFE projects* (CALEIDOS, PROSIL, etc). In particular, results were presented on the use of QSAR models (for instance for bioaccumulation) for chemicals registered at ECHA as part of the CALEIDOS project. PROSIL shares a similar target with LIFE-EDESIA, i.e., to minimize health risks through the identification of safer chemicals in specific industrial sectors (dyes, plant protection products and UVCB substances of organic origin). The importance of integrating QSAR and the read-across approach was discussed; a new tool, developed to assist in the read-across process, called ToxRead (www.toxgate.eu), was also presented.

Alessandra Roncaglioni, speaking about *advantages and disadvantages of in silico tools and their use in addressing EDCs*, illustrated some of the structural features identified in relation to EDCs. Structural features associated to the generic priority list for EDCs or those related to the *in vitro* assessment of the ER pathway within CERAPP (Collaborative Estrogen Receptor Activity Prediction Project) and their statistical performance were presented. Some further models were presented related in general to other detrimental effects, such as reproductive toxicity and developmental toxicity, in order to better fulfill the goal of LIFE-EDESIA. Examples of the application of these approaches were presented by reporting the assessment for some of the identified alternatives for other critical endpoints (carcinogenicity, mutagenicity, reproductive toxicity, persistence and bioaccumulation).

Pietro Cozzini illustrated *a computational approach to NR screening*. His presentation covered all the aspects of the molecular docking approach, dealing with its most critical aspects: the selection of the appropriate receptor structures and conformations, the issue of binding site flexibility and the consensus scoring suggested as an optimal scheme for the scoring of the energetically favorable docking poses. Examples of applications in the food sector in the evaluation of food contaminants against NR targets, such as estrogen and androgen receptors, were given.

4 Industry and regulatory perspectives in risk assessment

The third session, chaired by LIFE-EDESIA coordinator Alberto Mantovani (ISS) and by Action B leader Emilio Benfenati (IRFMN), presented a comparison of different viewpoints and application domains regarding the use of *in silico* tools including read-across, to assess ED-like activities of chemicals present, either intentionally or unintentionally, in food chains and/or environments.

Elena Lo Piparo discussed *industrial approaches towards alternative strategies for EDCs in the food domain*. She presented practical applications of an integrated strategy adopted in the food industry, where *in silico* screening represents the first step. To detect EDCs, virtual screening for the binding to NRs is combined with considerations about metabolic transformation estimated again *in silico*. This approach is integrated with *in vitro* testing and analytical identification of chemicals. The main domain of application is food contact material, either intentionally used substances, or – more important – impurities or unexpected by-products whose toxicological relevance needs to be assessed. This will support the proper management of risks from unwanted releases from food packaging throughout the entire production process.

Raffaella Butera presented *the alternative strategies for EDCs in the domain of man-made chemicals*. She gave an overview of the aspects related to the management of risk for SVHC within the REACH and CLP frameworks, starting from the identified uses of the substances and exposure scenarios through to testing requirements and to the authorization and restriction procedures.

Jürgen Arning presented *the role of regulatory agencies in addressing EDCs in the framework of REACH legislation and*

the support from in silico tools. In particular, he introduced the tool developed at UBA, called ED-scan, to screen and identify SVHC in relation to environmental effects based on *in silico* methods. It consists of 41 2D structural alerts (encoded by SMILES), identifying the potential for binding to ERα/ERβ and androgen receptor. Therefore, the tool currently is designed as a first indicator screen based on *in vitro* data only, without intending to conclude on apical *in vivo* endpoints. The use of these alerts is for a computational rapid screening of substances covered by the C&L inventory and to select candidates for further evaluation based on relevance criteria (e.g., tonnage, use, persistence). Planned developments of the method were presented in relation to the integration of further structural alerts (e.g., for thyroidal pathways or enzymatic regulation of hormone homeostasis), inclusion of alerts for EDC active metabolites, 3D toxicophore, correlation of alerts to apical endpoints and adverse outcome pathways (AOPs).

Maria Teresa Russo introduced the *news from ECHA on alternative methods in compliance with REACH: the read-across* and, in particular, the Read Across Assessment Framework (RAAF). She started by analyzing the results of the second report under Article 117(3) of the REACH Regulation about the use by registrants of alternative testing methods including read-across. She introduced the RAAF scheme, under development at ECHA, to assess the scientific aspects of read-across in a structured and consistent way, providing a convincing scientific explanation to obtain a better indication of scientific robustness and uncertainty of the read-across. When finalized, the scheme will allow to judge on acceptance or rejection of read-across cases submitted to ECHA.

5 Round-table of stakeholders

Finally, Alberto Mantovani (ISS) and Emilio Benfenati (IRFMN) chaired the stakeholders' round-table to which representatives of academy, industry and no-profit organizations were invited.

At the round-table, stakeholders commented on the presentations and the approach proposed by LIFE-EDESIA. **Livia Biardi** (Altroconsuma, a consumers' organization) stressed the importance for scientists to properly communicate with the general public to guide consumers' decisions. **Valeria Croce** (ChemService, a consultant organization) highlighted that different legislations (such as that on plant protection products) have different requirements in relation to the EDC issue and more in general in terms of animal testing requirements; thus, an applicant supporting substances in different fields might have to face different requests from regulators. **Francesca Caloni** (University of Milan) stressed the importance of research to support the 3R principle, by fully exploiting *in vitro* methods and *in silico/in vitro* integration. **Anna Auzzi** (Polynt, a chemical industry) underlined that it is important also from an industrial perspective to search for greener alternatives to compounds having hazard concerns.

6 Conclusions

In silico methods, from QSARs to read-across, have seen an impressive development and now represent a piece of information of growing importance in risk assessment; even the threshold of toxicological concern (TTC) approach, useful to screen the toxicological relevance of substances for which no or insufficient data are available, can be considered an outcome of computational methods. The further development and implementation of *in silico* methods, in terms of application domains and predictive value, will depend on the feedback from experimental data. The effective and comprehensive identification of potential EDCs requires more robust data on EDC-relevant pathways, with priority given to the multiple events that can influence nuclear receptors, resulting in altered cell functions.

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