FORMATION AND DEVELOPMENT OF GREEN TOXICOLOGY

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Abstract. Green Toxicology refers to the application of predictive toxicology in the sustainable development and production of new less harmful materials and chemicals, subsequently reducing waste and exposure. Built upon the foundation of "Green Chemistry" and "Green Engineering", "Green Toxicology" aims to shape future manufacturing processes and safe synthesis of chemicals in terms of environmental and human health impacts. Being an integral part of Green Chemistry, the principles of Green Toxicology amplify the role of health-related aspects for the benefit of consumers and the environment, in addition to being economical for manufacturing companies. Due to the costly development and preparation of new materials and chemicals for market entry, it is no longer practical to ignore the safety and environmental status of new products during product development stages. However, this is only possible if toxicologists and chemists work together early on in the development of materials and chemicals to utilize safe design strategies and innovative in vitro and in silico tools. This paper discusses some of the most relevant aspects, advances and limitations of the emergence of Green Toxicology from the perspective of different industry and research groups. The integration of new testing methods and strategies in product development, testing and regulation stages are presented with examples of the application of in silico, omics and in vitro methods. Other tools for Green Toxicology, including the reduction of animal testing, alternative test methods, and read-across approaches are also discussed.

Keywords: Green Toxicology, Green Chemistry, predictive toxicology, animal testing, *in vitro* research, Green Toxicology in medicines development.

Introduction. Over the past two decades, the movement of Green Chemistry has become a new standard embraced for the development of less harmful materials and chemicals that are safer for both the environment and consumers [1, 2]. Many of these goals, along with the principles of Green Engineering [3], strive for sustainability with chemical synthesis and molecular design [2] and are adopted by major industries (e.g. pharmaceutical and chemical). However, currently and for the future, the inclusion of aspects related to consumer and environmental health has become more and more important. Thus, considerations about the possible toxic activity of a certain molecule or material during its development for the market are crucial not only for the economic

success but also for its consumer acceptance. Taking this aspect into account, Green Toxicology will strengthen the marketing process and avoid serious setbacks.

Green Chemistry practices have been adopted into mainstream research and manufacturing since the early 1990s. Success stories of the application and study of Green Chemistry include the use of microbes as environmentally benign synthetic catalysts [4] as well as the development of fully biodegradable bags with the use of compostable polyester film [5]. The efforts of Green Chemistry have resulted in the reduction of hazardous waste in a cost-effective manner that has maintained the need, efficacy and safety of products for consumers.

A complementary tool for Green Chemistry and Green Engineering that incorporates the toxicological risk and hazard assessment of the design to disposal of products and materials is the concept of Green Toxicology. Green Toxicology [6] describes the application of predictive toxicology in the design, manufacturing, use and disposal of new materials and chemicals. The objective of such an application is to contribute to products, which are safer for humans and the environment by using intelligent and predictive testing strategies of toxicology. Maertens et al. [1] outlines several considerations, which might form the basis of future principles of Green Toxicology, the basis of which are: benign-by-design (also known as safety-by-design); test early – produce safe; avoid exposure and thus testing needs; and make testing sustainable. Green Toxicology aims to expand the respective principles of Green Chemistry to develop and produce products that are less toxic, with safer processes that result in less waste and exposure, utilizing toxicological tools and strategies. While there are many overlapping features and principles among the Green Chemistry, Engineering and Toxicology, the key difference of Green Toxicology is that it promotes the incorporation of toxicological considerations throughout the discovery, development, and production of new materials and chemicals, which are discussed in this paper.

The aim of the study. Providing a framework for developing chemicals that are safer for humans and the environment by using new and innovative toxicology prediction tools and strategies. Conduct an analysis to improve the integration of green toxicology with green chemistry practices to produce safer and less harmful products.

Materials and Methods. The analysis of systematic reviews on Green Toxicology and its development into drug development for human safety assessment is involved.

Results and Discussion. Traditionally, the development of alternative testing methods in Europe was largely driven by ethical rationales such that studies were

targeted for their use of many animals, or for their high potential to result in pain and suffering (e.g. skin and eye irritation testing). Regulatory rationales were also an additional driver of alternative test methods, particularly those that identified compounds with alerts for "cut-off" hazards, such as mutagenicity and endocrine disruption. Therefore, the currently validated in vitro assays particularly apply to the aforementioned endpoints [7, 8].

The traditional *in vivo* Draize irritation test for skin and eyes, in which a restrained, conscious animal is exposed (dermal and ocular, respectively) to a test substance for a set amount of time to determine toxicological effects, has long since been criticized for the limitations in species differences, subjective scoring, and experimental variability. The replacement of the Draize test for skin irritation was historically one of the first steps towards the full replacement of animal testing. BASF, and similar chemical companies, use two methods suitable to provide data for classification as corrosive (Epiderm™ skin corrosion test) or irritant (Epiderm™ skin irritation test) to the skin. These tests are employed within the context of a simple testing strategy described elsewhere [9-12]. In brief, a test substance is applied topically to a reconstructed human epidermis (RhE) that closely mimics the biochemical and physiological properties of the upper parts of the human skin using human derived non-transformed keratinocytes as cell sources. The indication of corrosive and irritant test substances is determined by their ability to decease cell viability.

Skin sensitization is a process more complex than skin or eye irritation, and includes several key events such as dermal penetration, protein reactivity, inducing stress responses in keratinocytes, activation of immune cells (dendritic cells) in the skin, and their translocation to the lymph nodes. Given this complexity, it is difficult to imagine one single test that would be able to incorporate all of these steps [13]. Therefore, the development of an *in vitro* testing approach for skin sensitization resulted in the best solution [14]. Protein reactivity is measured in the Direct Peptide Reactivity assay (DPRA) [15], stress responses are measured in Keratinocytes in either KeratinoSens [16, 17] or LuSens assays [18, 19], and immune cell activation is measured in the Human Cell Line Activation Test (h-CLAT) [20]. Empirical evidence for more than 200 compounds has shown that the best match with known human skin sensitizers is obtained by a "majority rule", such that if two or more assays are positive, the compound is a skin sensitizer, while if two or more are negative, it is not [21]. With this testing strategy, a correlation with human skin sensitizers is obtained, which is slightly better than that obtained in the local lymph node assay (LLNA) [22, 23].

To screen for compounds with endocrine effects, two *in vitro* systems are often used that address the most common causes for endocrine activity: agonist or antagonist effects on the androgen receptor (AR) or estrogen receptor (ER) and interference with steroid synthesis. There are a variety of in vitro, wildlife and mammalian screen tests available to screen for endocrine disruptor activity, with details on each provided elsewhere [24]. In particular, the *in vitro* Yeast Estrogen Screen (YES)/Yeast Androgen Screen (YAS) assays are often used to screen for analyse effects on the AR and ER. The YES and YAS assays consist of yeast cell lines in which the human AR and ER have been introduced and coupled with a reporter gene that produces an enzyme. Activation and deactivation of either receptor are monitored by the change in colour of a dye sensitive to the activity of the enzyme. If deemed necessary, a follow-up is carried out at later stages of testing for endocrine activity with a refined 14- or 28-day study in which a blood metabolome analysis is included. Additional testing strategies for endocrine testing have been reported elsewhere [25, 26].

Another important aspect of systemic toxicity, with respect to avoidance of chemicals, with a problematic hazard profile is neurotoxicity. For screening purposes, the “neurons on a chip” assay is utilized [27, 28]. In this assay, primary neurons are grown on chips connected with a device that measures the spontaneous firing of the neurons. Compounds that stimulate or attenuate neuronal activity can be monitored by the changes in the firing rates of the neurons [29, 30].

In contrast to household and consumer chemicals, where the optimization process of the properties during product development is often independent of the safety assessment, the drug development process can be seen as a series of iterative steps to optimize efficacy and simultaneously lower the safety as early as possible. Therefore, the early assessment of toxicity before the first application to man plays a pivotal role in this process. Compounds for which the preclinical toxicological assessment identifies an adverse effect profile that exceeds the expected benefit for the patient will be excluded from progression in the development pipeline. Preclinical toxicology is hereby facing two challenges: on the one hand, the predictivity of the applied toxicological assays should be improved on a continuous basis to avoid false predictions (both false positives and false negatives), while on the other hand, the predictions should be made as early as possible during the process of drug candidate selection. This early assessment causes a shift from in vivo to in vitro to in silico methods. Maertens et al. [1] stress the parallels between the Green Toxicology movement and the strive for early and reliable safety assessment (“front-loading”) in

the pharmaceutical industry, such that the achievements in meeting the abovementioned challenges will contribute to the objectives of Green Toxicology.

Some toxicological effects can in the meantime be predicted based on in silico methods with reasonable reliability, such as mutagenicity, phospholipidosis, and to a lesser extent skin sensitization [31]. It can be foreseen that integrated testing strategies will evolve with the advent of AOPs and a better understanding of the mechanisms of toxicological effects, which comprise a combination of in silico and in vitro tools to predict toxicological effects. For example, models that predict pharmacokinetic behaviour (absorption, distribution) of compounds based on physicochemical properties could be combined with predictions of liver transport based on QSAR transporter models. The inclusion of subsequent results from in vitro toxicity assays with hepatocytes or mitochondria will help to identify compounds that have a propensity towards drug-induced liver toxicity (DILI). Such complementary tools may limit and remove the most problematic candidates in early phases or allow medicinal chemistry departments to optimize the structure early on.

Over the last two decades, nanomaterials are more and more in the focus of scientists, production companies, but also of regulators. This family of relatively new compounds and materials is different from the normal definition of chemical compounds. Chemical substances are usually described by their chemical composition but in the case of nanomaterials, additional descriptors such as particle size, shape or composition of core and coatings are needed to specify and distinguish them from each other. As a consequence, a virtually unlimited number of different nanomaterials can be identified, which may result in a burdensome request for a large amount of toxicological data for regulatory hazard assessment. It is important to ensure that the development of new nanotechnology occur in the presence of Green Toxicology and Chemistry practices. A framework for chemists and material developers is needed to clearly outline design rules that integrate health, safety, and environmental concerns into nanotechnology development [32]. Thus, for Nanotechnology as a relatively young technology, the opportunity exists to start early on with the implementation of the principles of Green Toxicology.

Cosmetics, especially sunscreens, should protect us from ultraviolet (UV)-light induced sunburn and skin cancer. This protection has been achieved by a multitude of chemicals with different structures, some of which are under suspicion of being endocrine disruptors or of having other effects in environmental organism in receiving aquatic environments. Over the last two decades, nanoparticles consisting of ZnO or TiO_2 have been used as very efficient physical UV-blocking materials. As the natural

background for TiO_2 is relatively high in surface water, such as lakes and rivers [33], the use of TiO_2 as a UV-blocking agent is less hazardous than the "normal" chemical cocktail in sun creams. However, recently an intense discussion was started on the possible carcinogenic effect in the lung after inhalation of sun screens, as the International Agency for Research on Cancer (IARC) stated: "Titanium dioxide is possible carcinogenic to humans based on sufficient evidence in experimental animals and inadequate evidence from epidemiological studies" [34]. This example brings together considerations about a product that has been on the market for decades, despite outcomes of experiments describing relatively severe effects in cells or animals. The idea of Green Toxicology may help to resolve this problem by introducing specific information about the materials used and by establishing relationships between the properties of the TiO_2-particles and the predicted outcomes. Comparisons of the materials used for the critical animal studies with that produced for the sunscreens should allow for the determination of the similarities in the materials and if the benign-by-design principle should be considered more thoroughly for future development of sunscreens.

Conclusions. The cases shown above for chemical and pharmaceutical companies, as well as nanotechnology development, clearly demonstrate that Green Chemistry, together with the principles of Green Toxicology more specifically related to the environmental and health effects of compounds or materials, may achieve a sustainable and safe production scenario of new chemicals. However, in the case of pharmaceutical compounds, there may also be limitations with regard to achieving safe and efficacious drugs that are at the same time environmentally friendly. As the examples from the European Environmental Agency (EEA) demonstrate, it is now the duty of all the stakeholders to implement such rules for a responsible production of new compounds and materials based on common principles. Taking the ideas of Maertens et al. as a basis, the principles of Green Toxicology may be further expanded. It is not only important to test early, but to also try to achieve safety-by-design of the compounds, to use predictive test systems, and to avoid exposure. Overall, testing itself must be sustainable and safe by avoiding solvents that may be hazardous or energy consuming, and testing should help to reduce the need of experimental animals. Moreover, the ideas and fundamental rules of toxicology should be familiar for all chemists but also to physicists and engineers. Thus, a transdisciplinary education in toxicology would be helpful to implement this knowledge in the processes for chemical development.

References

1. Maertens A., Anastas N., Spencer P.J. et al. Food for thought: Green Toxicology. ALTEX Altern Anim Exp. 2014;31:243–249.
2. Anastas P., Eghbali N. Green Chemistry: principles and practice. Chem Soc Rev. 2010; 39:301–312.
3. Tang S.Y., Bourne R.A., Smith R.L., Poliakoff M. The 24 principles of Green Engineering and Green Chemistry: "Improvements Productively" Green Chem. 2008;10:268–269.
4. Kambourakis S., Draths K., Frost J. Synthesis of gallic acid and pyrogallol from glucose: replacing natural product isolation with microbial catalysis. J Am Chem Soc. 2000;122:9042–9043.
5. Siegenthaler K., Künkel A., Skupin G., Yamamoto M. Ecoflex® and Ecovio®: biodegradable, performance-enabling plastics. In: Rieger B, Künkel A, Coates GW, Reichardt R, Dinjus E, Zevaco TA, editors. Synthetic biodegradable polymers. Berlin: Springer; 2011. pp. 91–136.
6. Anastas N.D. Green Toxicology. In: Zhang W, Cue B, editors. Green techniques for organic synthesis and medicinal chemistry. West Sussex: John Wiley & Sons; 2012. p. 1–23.
7. Lilienblum W., Dekant W., Foth H. et al. Alternative methods to safety studies in experimental animals: role in the risk assessment of chemicals under the new European Chemicals Legislation (REACH) Arch Toxicol. 2008;82:211–236.
8. Worth A., Barroso J., Bremer S. et al. Alternative methods for regulatory toxicology – a state-of-the-art review. Joint Res Counc Sci Policy Rep EUR. 2014;26797:1–470.
9. Kolle S.N., Sullivan K.M., Mehling A. et al. Erratum to "Applicability of in vitro tests for skin irritation and corrosion to regulatory classification schemes: substantiating test strategies with data from routine studies" Regul Toxicol Pharmacol. 2013;65:366–378.
10. OECD (2015) OECD guideline for the testing of chemicals: acute dermal irritation/corrosion. Paris, France: Organisation for Economic Co-operation and Development Test No. 404, p. 1–8.
11. OECD (2016) OECD guideline for the testing of chemicals: in vitro skin corrosion: reconstructed human epidermis (RHE) test method. Paris, France: Organisation for Economic Co-operation and Development Test No. 431, p. 1–26.
12. OECD (2015) OECD guideline for the testing of chemicals: in vitro skin irritation: reconstructed human epidermis test method. Paris, France: Organisation for Economic Co-operation and Development Test No. 439, p. 1–21.

13. Rovida C., Alepee N., Api A.M., et al. Integrated testing strategies (ITS) for safety assessment. Altex. 2015;32:25–40.
14. Mehling A., Eriksson T., Eltze T. et al. Non-animal test methods for predicting skin sensitization potentials. Arch Toxicol. 2012;86:1273–1295.
15. OECD (2015) OECD guideline for the testing of chemicals: in chemico skin sensitisation: direct peptide reactivity assay (DPRA). Paris, France: Organisation for Economic Co-operation and Development Test No. 422C, p. 1–19.
16. Andreas N., Caroline B., Leslie F. et al. The intra-and inter-laboratory reproducibility and predictivity of the KeratinoSens assay to predict skin sensitizers in vitro: results of a ring-study in five laboratories. Toxicol In Vitro. 2011;25:733–744.
17. OECD (2015) OECD guideline for the testing of chemicals: in vitro skin sensitisation: ARE-Nrf2 luciferase test method. Paris, France: Organisation for Economic Co-operation and Development Test No. 442D, p. 1–20.
18. Ramirez T., Mehling A., Kolle S.N. et al. LuSens: a keratinocyte based ARE reporter gene assay for use in integrated testing strategies for skin sensitization hazard identification. Toxicol In Vitro. 2014;28:1482–1497.
19. Ramirez T., Stein N., Aumann A. et al. Intra-and inter-laboratory reproducibility and accuracy of the LuSens assay: a reporter gene-cell line to detect keratinocyte activation by skin sensitizers. Toxicol In Vitro. 2016;32:278–286.
20. OECD (2016) OECD guideline for the testing of chemicals: in vitro skin sensitisation Human Cell Line Activation Test (h-CLAT). Paris, France: Organisation for Economic Co-operation and Development Test No. 422E, p. 1–21.
21. Urbisch D., Mehling A., Guth K. Assessing skin sensitization hazard in mice and men using non-animal test methods. Regul Toxicol Pharmacol. 2015;71:337–351.
22. OECD (2010) OECD guideline for the testing of chemicals: skin sensitisation local lymph node assay. Paris, France: Organisation for Economic Co-operation and Development Test No. 429, p. 1–20.
23. Ashikaga T., Sakaguchi H., Sono S. et al. A comparative evaluation of in vitro skin sensitisation tests: the human cell-line activation test (h-CLAT) versus the local lymph node assay (LLNA) ATLA Altern Lab Anim. 2010;38:275–284.
24. OECD (2012) OECD environment, health and safety publications series on testing and assessment: guidance document on standardised test guidelines for evaluating chemicals for endocrine disruption. Paris, France: Organisation for Economic Co-operation and Development Test No. 150, p. 1–524.

25. Kolle S.N., Ramirez T., Kamp H.G. et al. A testing strategy for the identification of mammalian, systemic endocrine disruptors with particular focus on steroids. Regul Toxicol Pharmacol. 2012;63:259–278.
26. OECD (2016) Series on testing and assessment: testing for endocrine disrupters. http://www.oecd.org/chemicalsafety/testing/seriesontestingandassessmenttestingforendocrinedisrupters.htm
27. Hu C., Dillon J., Kearn J. et al. NeuroChip: a microfluidic electrophysiological device for genetic and chemical biology screening of Caenorhabditis elegans adult and larvae. PLoS ONE. 2013;8:e64297.
28. Sung J.H., Srinivasan B., Esch M.B. et al. Using physiologically-based pharmacokinetic-guided "body-on-a-chip" systems to predict mammalian response to drug and chemical exposure. Exp Biol Med. 2014;239:1225–1239.
29. Defranchi E., Novellino A., Whelan M. et al. Feasibility assessment of micro-electrode chip assay as a method of detecting neurotoxicity in vitro. Front Neuroeng. 2011;4:1–12.
30. Vassallo A., Chiappalone M., Lopes R.D.C. et al. (2017) A multi-laboratory evaluation of microelectrode array-based measurements of neural network activity for acute neurotoxicity testing. Neurotoxicology.
31. Steger-Hartmann T. In silico toxicology – current approaches and future perspectives to predict toxic effects with computational tools. In: Pfannkuch F, Suter-Dick L, editors. Predictive toxicology: from vision to reality. Weinheim: Wiley-VCH Verlag GmbH & Co. KGaA; 2014. pp. 13–32.
32. Hansen S.F., Maynard A., Baun A. et al. Nanotechnology – early lessons from early warnings. In: Gee D, Grandjean P, Hansen F, van den Hove S, MacGarvin M, Martin J, Nielsen G, Quist D, Stanners D, editors. Late lessons from early warnings II: science, precaution, innovation. Copenhagen: European Environment Agency; 2013. pp. 530–559.
33. Wagner S., Gondikas A., Neubauer E., Spot the difference: engineered and natural nanoparticles in the environment – release, behavior, and fate. Angew Chem Int Ed. 2014;53:12398–12419.
34. IARC (2010) IARC monographs on the evaluation of carcinogenic risks to humans. World Health Organization, International Agency for Research on Cancer, Lyon, France Vol 93: Carbon Black, Titanium Dioxide, and Talc, p. 1–466.