



The long and winding road of progress in the use of in vitro data for risk assessment purposes: From “carnation test” to integrated testing strategies



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ABSTRACT

This paper introduces the special issue on quantitative in vitro-in vivo extrapolations (QIVIVE). It highlights important issues in the development of in vitro toxicology towards its implementation in toxicological risk assessment.

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Over the last three decades the area of in vitro toxicology has developed into an important sub-discipline of toxicology. This was made possible by the vast improvements in the areas of cell biology and cell and tissue culture techniques, allowing the culturing of cells and tissues under controlled conditions over a prolonged period of time. The application of these techniques in toxicology opened the possibility to study the effects of chemicals at the cellular or tissue level.

My personal involvement in the area of in vitro toxicology started in the 70s of the previous century, during the preparation of my PhD thesis (Blaauboer, 1978). In this study the emphasis was on the use of cellular systems (i.e. erythrocytes) to understand the processes involved in the formation and reduction of ferrihaemoglobin. In later studies we used mainly freshly isolated cells or primary cultures of hepatocytes and keratinocytes. As in most academic studies the main interest was in the understanding of the mechanisms of toxic action.

A main milestone in our interest in a wider application of in vitro results was sparked by a workshop held in Soesterberg, the Netherlands, entitled “the application of tissue culture in toxicology”. During this workshop a limited number of presentations was given by renowned experts in the field. The emphasis was on what was then described as tissue cultures, but in reality was better describes as isolated cellular systems. The proceedings were

published (Zucco, 1980; Heilbronn, 1980) and from one of the papers (Nardone, 1980) I take two remarkable quotes.

“We, too, have to face up to the problems of 2 cultures in toxicology. For the most part, the origins, tradition, and literature of animal toxicology and tissue culture have been separate, or, at best, only marginally overlapping. . . . This barrier was reinforced further by the inadequate toxicology training of tissue culture workers who jumped into the field without an understanding of the principles of toxicokinetics and toxicodynamics, and how these impinge on experimental design and interpretation. The blame for this barrier must also be shared by the animal toxicologists. They have done relatively little to obtain special insights regarding what skilled practitioners of tissue culture provide for them, including the fundamentals of quality control”

“Some argue that the in vitro test method must be capable of generating the toxic endpoint exactly the way it occurs in vivo. Others, recalling that the primary role of screening studies is prediction with a relatively high level of confidence, care not how the prediction can be made as long as it is reliable. For example, let us assume that a validation study shows that many diverse neurotoxic substances added to a vase kills carnations in 90% of the tests. The death of carnations would then be considered an acceptable end-point for screening neurotoxic substances. While this is disquieting from an intellectual and scientific point of view, it has validity if the test has predictability and other advantages such as speed and low cost”.

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These two quotes from Ronald Nardone (Nardone, 1980), illustrate more or less the state of affairs at that time. I still remember the origin of the “carnation test” quote, which was at the general discussion during the Workshop. Ron was standing in the room and the first thing he could grab was a vase of carnations, which he used to illustrate his point.

Nardone’s statements also reflect the awareness of the fact that the introduction of in vitro data in the area of toxicology – and certainly also in the field of risk assessment – were not taken for granted at that time. There are many “cultural” and technical barriers that prevented the rapid implementation of in vitro toxicity results as the basis for a risk or safety evaluation. This was the case at that time, and presently still is a matter of debate (Blaauboer and Andersen, 2007; Schiffelers et al., 2012).

The Soesterberg meeting was in many respects a memorable one. It brought together a group of about 70 people all working in the field of cell or tissue culture. In fact it was the start of a much broader European cooperation for in vitro toxicology, resulting in the organization of a biennial workshop, later called the INVITOX workshops and since the start of the European Society of Toxicology in Vitro (ESTIV) in 1998 now evolved in the biennial ESTIV Congresses. The meeting also established cooperation across the Atlantic, with input from the Baltimore Centre for Alternatives to Animal Testing (CAAT) and many others. We now also witness the start of the American Society for Cellular and Computational Toxicology (ASCCT) and similar organizations in Asia. The development of the area of in vitro toxicology is also evident in the scientific literature, e.g. by the start of specialized journals such as *Toxicology in Vitro* as early as 1987.

In this special issue, the major trends in the development of in vitro toxicology, with emphasis on the implementation of the results derived from in vitro toxicity testing in the area of a toxicological risk assessment will be highlighted. This is of particular relevance since on the one hand the need for toxicity data is still increasing, e.g. given the requirements laid down in the REACH directives (ECHA, 2008). On the other hand, the implementation of e.g. the EU cosmetics regulation shows the urgency of developing risk or safety assessment procedures that are not relying on studies with animals (Adler et al., 2011; Basketter et al., 2012).

In the early times of in vitro toxicology most emphasis was given to the measurement of cell death as the major parameter for cytotoxicity. The work of Bjorn Ekwall is ground-breaking in this respect. He initiated studies on what then was called “basal cytotoxicity” (Ekwall, 1980). The hypothesis was that especially acute toxicity could be predicted from in vitro cytotoxicity measurements in each cell type if measured with parameters that are linked to the basal household functions of all cell types: outer membrane integrity (e.g. by measuring LDH leakage), maintenance of energy supplies (e.g. with parameters linked to mitochondrial function) or the integrity of cell compartmentalization. This has led to the development of frequently used methods such as the Neutral Red Uptake assay (Babich and Borenfreund, 1987) or the MTT assay. The studies initiated by Ekwall led to his invitation to all working in the field to provide data on basal cytotoxicity with many different in vitro systems, the MEIC studies (Clemmedson and Ekwall, 1999).

Another area in which the use of in vitro systems in toxicology was apparent from the beginning was in genotoxicity testing. One early hallmark was the introduction of the Ames mutagenicity test (Ames et al., 1973b), in which the potential of compounds – and their metabolites (Ames et al., 1973a) – to produce mutations in bacterial genome was measured. Later developments in this field are the in vitro micronucleus assay (Schmid, 1975) and the Comet assay (Ostling and Johanson, 1984). These tests are useful tools in determining the intrinsic ability to produce genomic damage and are therefore frequently used in hazard identification. However,

their usefulness in the process of risk evaluation is limited (Fowler et al., 2012), certainly when used in isolation. For instance, it is often mentioned that the in vitro genotoxicity tests do grossly overpredict genotoxicity – and carcinogenicity – in vivo and this may be explained at least partly by the lack of correlation between the target exposure in the in vitro system and the actual target exposure in vivo. The missing link obviously is the kinetic behaviour of the compound and its metabolites (Kiwamoto et al., 2013), in vitro as well as in vivo.

The same holds true for the measurement of phototoxicity. The test for this property was developed by Spielmann et al. (1994). This probably is the first toxic endpoint that, studied in an in vitro assay, was accepted as a regulatory tool and incorporated in the OECD guidelines (OECD, 2004). The test is based on a clear hypothesis: compounds that on illumination with light of determined wavelengths will absorb light and will be able to transfer the absorbed light energy to molecular oxygen, will produce reactive oxygen species that will cause oxidative stress in any biological system. Thus, when basal cytotoxicity, e.g. in the Neutral Red Assay, is measured with these compounds in the presence of light that will activate these compounds, there will be an increase in their toxicity when compared to exposure in the dark. Here too, however, we are dealing with a test on hazard identification and the results will need to be evaluated in relation to its in vivo relevance, e.g. if a compound when absorbed orally will not reach the skin in a high enough concentration, the compound will not be phototoxic in vivo. A measured phototoxic potential in vitro can thus be considered a false positive. Here too, for a proper risk evaluation the biokinetic behaviour should be considered (Bernauer et al., 2005; Coecke et al., 2006).

As mentioned above, the interest in in vitro toxicology in academia was mainly in the better understanding of the mechanisms of toxic action. On the one hand there was the realization that these mechanisms are very difficult to interpret from in vivo toxicity studies in animals in which the emphasis is strongly on the evaluation of apical endpoints of toxicity processes. On the other hand, also in the 70s it was obvious that there was more to toxic mechanisms than only studying basal cytotoxicity. The more cell- and tissue-specific toxicity of compounds often is related to mechanisms of action that can be described as influencing specific physiological functions in cells and tissues. The vast development in this field has led to an impressive increase in our insights into toxicological processes. One area in which this was evaluated was in the study of the usefulness of in vitro data for predicting acute toxicity. In the EU-sponsored project A-cute-tox not only basal cytotoxicity was studied, but also the disturbance of physiological functionality in in vitro systems derived from e.g. the liver, the nervous system and the haematological system (Clemmedson, 2008). The increase in insights in toxicological processes was also instrumental in the definition of toxicity pathways (Eisenbrand et al., 2002), in vitro biomarkers of toxicity (Blaauboer et al., 2012), stress response pathways or adverse outcome pathways (Jennings, 2013).

One further important milestone is the realization that in vitro data do not stand alone. If one would want to replace a toxicity study in vivo the complexity of the in vivo situation should be taken into account. The physiological networking and feedback loops might very well not exist in the isolation of an in vitro system. In vitro results should therefore be carefully interpreted in that light (Murk et al., 2013). Moreover, the earlier mentioned apparent lack of conformity of the biokinetics in vivo and in vitro should be covered (Gülden and Seibert, 2003; Kramer et al., 2012). This is evident for e.g. the prediction of repeated dose toxicity (Prieto et al., 2006). Many in vitro systems are static, i.e. a test compound is added to the medium of a cell culture and – apart from processes like evaporation, binding to plastic cell culture material or a possible metabolism in the cell culture – remains there until the next

medium change (Broeders et al., 2013). This is quite different from the situation in vivo where the blood flow, excretion processes and metabolism result in a more dynamic situation. The development of perfusion systems and body-on-a-chip techniques are therefore interesting (van Midwoud et al., 2010; van der Meer and van den Berg, 2012).

Many of the issues mentioned above have resulted in a number of reports that highlight the steps taken towards a new paradigm in toxicology and in human and environmental health risk assessment. One of these reports is the National Academy of Sciences report *Toxicity testing in the 21st century* (NRC, 2007). It consists of a shift from the reliance on observing apical toxicity endpoints in animal models towards testing systems that evaluate perturbations of toxicity pathways. It foresees the use of batteries (or suites) of in vitro methods together with the application of computational models describing the dynamics of pathway perturbations, as well as biokinetic models. Thus, the approach relies on the availability of methods studying these toxicity pathways, making use of modern biology methods including systems biology and the “omics”, in combination (integration) with modelling approaches.

The relevance of this paradigm shift for the science of toxicology will be obvious (see e.g. the report by the Health Council of the Netherlands entitled: “*Toxicity testing: a more efficient approach*” (HCN, 2001), and also Combes et al. (2003). Moreover, the development of this area of research is eminent, also in the light of the changes it will encompass for regulatory risk assessment procedures, viz. a number of recent European policy changes (cosmetics; the REACH chemicals policy), explicitly calling for a more extensive use of in vitro and other non-animal methods. The debate on these issues (e.g. Greim et al., 2006; Blaauboer and Andersen, 2007), exemplifies that the implementation of the new approaches will need a considerable investment in time and money, and needs to be supported by the international toxicology community worldwide (Adler et al., 2011; Basketter et al., 2012).

One issue in this approach is the use of biokinetic data. These are of utmost importance in the evaluation of in vitro-derived data on dose-(or concentration)–response relationships for the situation in intact organisms (Bouvier d’Yvoire et al., 2007). A number of examples exist in which the integrated approach of using in vitro toxicity data in combination with kinetic modelling is applied for estimating exposure scenarios in vivo that could be related to the effect levels found in in vitro systems (Dejongh et al., 1999; Gubbels-van Hal et al., 2005; Verwei et al., 2006; Forsby and Blaauboer, 2007). These examples have given the proof of principle for the integrated approach.

In this special issue, devoted to the proper use of in vitro-derived data in the risk assessment process, the most important issues regarding the quantitative in vitro–in vivo risk assessment will be highlighted. We expect that this special issue will contribute to the further incorporation of the integrated testing strategies described herein in the field of regulatory toxicology.

Conflict of interest

The author declares that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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