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Application of *in vitro* neurotoxicity testing for regulatory purposes: Symposium III summary and research needs

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ABSTRACT

Prediction of neurotoxic effects is a key feature in the toxicological profile of many compounds and therefore is required by regulatory testing schemes. Nowadays neurotoxicity assessment required by the OECD and EC test guidelines is based solely on *in vivo* testing, evaluating mainly effects on neurobehavior and neuropathology, which is expensive, time consuming and unsuitable for screening large number of chemicals. Additionally, such *in vivo* tests are not always sensitive enough to predict human neurotoxicity and often do not provide information that facilitates regulatory decision-making processes. Incorporation of alternative tests (*in vitro* testing, computational modelling, QSARs, grouping, read-across, etc.) in screening strategies would speed up the rate at which compound knowledge and mechanistic data are available and the information obtained could be used in the refinement of future *in vivo* studies to facilitate predictions of neurotoxicity.

On 1st June 2007, the European Commission legislation concerning registration, evaluation and authorisation of chemicals (REACH) has entered into force. REACH addresses one of the key issues for chemicals in Europe, the lack of publicly available safety data sheets. It outlines a plan to test approximately 30,000 existing substances. These chemicals are currently produced in volumes greater than 1 ton/year and the essential data on the human health and ecotoxicological effects are lacking. It is estimated that approximately 3.9 million test animals (including 2.6 million vertebrates) (Hartung T, Bremer S, Casati S, Coecke S, Corvi R, Fortnaer S, et al. ECVAM's response to the changing political environment for alternatives: consequences of the European Union chemicals and cosmetics policies. ATLA 2003;31:473–81) would be necessary to fulfill the requirements of REACH if the development and establishment of alternative methods is not accepted by regulatory authorities. In an effort to reduce animal use and testing costs within this tonnage band, the European Commission has advocated the use of alternative approaches. Neurotoxicity testing is not directly addressed within REACH, however when alerts are observed based on organ specific toxicity studies then neurotoxicity assessment has to be performed.

This session at the 11th International Neurotoxicology Association Meeting provided a forum to openly discuss and debate the potential of *in vitro* testing strategies that could be relevant for neurotoxicity evaluation in the context of regulatory requirements. The EU FP6 project A-Cute-Tox was presented as an example of a possible *in vitro* testing strategy for prediction of human acute systemic toxicity. Other presentations focused on the characterization of the available *in vitro* models (cell lines and primary culture) and neuronal specific endpoints, with a special emphasis on electrical activity, metabonomics and modulation of vesicular neurotransmitter release as possible neuronal endpoints relevant for *in vitro* neurotoxicity testing. Finally, it was underlined that *in vitro*

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systems (strategies) that have the potential to be applied for neurotoxicity assessment have to be formally validated under standardised conditions that have been recognised by national and international validation bodies.

1. ECVAM strategy for *in vitro* neurotoxicity testing in the context of the new political changes

Dr. Anna Price presented the European Centre for the Validation of Alternative Methods (ECVAM) core activities that presently are driven by REACH and the 7th amendment to the Cosmetic legislation where the application of *in vitro* testing strategy for toxicity testing is included (Hartung et al., 2003). ECVAM's main mission is to assign a statement of validity to relevant, reliable and robust *in vitro* toxicological test systems for regulatory purpose in the field of human health. In the view of current testing requirements for human health, neurotoxicity is evaluated during acute systemic toxicity, repeated-dose toxicity, subchronic, chronic and reproductive/developmental toxicity. These studies are based only on *in vivo* methods where the neurotoxic potency of drugs/chemicals is mainly determined by neurobehavioral and neuropathological effects. Unfortunately, at present, validated *in vitro* methods for neurotoxic hazard assessment are not available. This is due to the anatomical and physiological complexities of the central nervous system (CNS), where various cell types (neuronal and glial) are required to maintain a complicated and integrated structure to be able to function as *in vivo*. Additionally, cell–cell interactions are highly complex, through unique protein interactions where functional coupling via synapses, gap junctions, signalling molecules and growth factors has to be preserved. Presently, *in vitro* studies are proposed as complementary to whole-animal testing by providing cellular/mechanistic understanding of processes underlying normal or pathological nervous system function (Harry and Tiffany-Castiglioni, 2005).

A range of available *in vitro* systems of increasing biological complexity is available for toxicity testing, from single cell types (cell lines) to systems that preserve some aspects of tissue structure and function (primary mixed neuronal and glial cultures, re-aggregated culture or organotypic brain slices). Each model has various advantages and limitations (Costa, 1998; Harry et al., 1998) and the selection of any particular system depends on the question addressed, the intended use of the data and available information on the suspected mechanism of neurotoxicity. Using *in vitro* approaches neurotoxicity is assessed by the determination of cell viability, general but critical cell functions (energy metabolism, oxidative stress or calcium homeostasis) and neuronal specific functions (neurite outgrowth and axonal transport, neurotransmission and vesicular release, signalling between various types of neurons and glia, receptor pharmacology, ion-channel activation, electrical activity, etc.) (Table 1). A number of various approaches can be applied to identify neurotoxic effects of drug/chemical to be able to discriminate between general toxic responses and specific neuroxic effects. In such studies, by comparing the responses of neuronal versus non-neuronal cells to the same set of chemicals a different dose–response curve between neuronal and non-neuronal cells can be established (Gartlon et al., 2006). Another approach to discriminate between cytotoxicity and neurotoxicity is based on the comparison between concentration-dependent curves determined by using general cytotoxicity endpoints and endpoints specific for neuronal/glial cell function (mentioned above). At the same time, it is important to define the concentrations with no effect level (NOEL) and the lowest exposure level required to produce an effect, up to the concentrations that cause cell death to be able to distinguish between a pharmacological and a neurotoxic effect. There is a general consensus that *in vitro* methods provide useful information concerning basic biological processes underlying neurotoxicity and specific information concerning a chemical mechanism of action. *In vitro* systems are not yet capable of fully replacing *in vivo* testing to predict neurotoxicity, especially when the site of action is unclear or unknown. However, at this stage of methods development and validation *in vitro* techniques can provide valuable information that complement established *in vivo* neurotoxicity testing strategies as it was proposed for developmental neurotoxicity (Coecke et al., 2007).

In addition, in some cases it is important to incorporate into testing strategy also *in vitro* blood–brain barrier (BBB) models, especially when a new compound is tested and it is not known whether it penetrates into the CNS. The establishment of *in vitro* BBB models makes a whole testing strategy complex and should be applied only when necessary. There are important qualitative differences between various BBB cell culture systems as different *in vitro* and non-animal models can be used (primary cells, immortalised brain endothelial cells, cell lines of non-cerebral origin or cell free systems (partition coefficients) (Prieto et al., 2004). At present, the *in vitro* model that closely mimics the *in vivo* situation is the co-culture of primary cultured endothelial cells with primary astrocytes (Boveri et al., 2006). This *in vitro* BBB model possesses well-differentiated (morphologically and physiologically) tight junctions as measured by high trans-endothelial electrical resistance and low permeability.

It is important that screening as well as mechanistic assays used in *in vitro* neurotoxicology are validated. Validation of alternative tests is the process by which the reliability and the relevance of the test are established for a particular purpose. In collaboration with external experts, ECVAM has established guidelines on the validation of alternative toxicological methods, which are applicable for testing chemicals, pharmaceuticals, food ingredients and biologicals. Recently, ECVAM proposed to make the validation process more flexible by replacing various steps with an independent module for assessing test validity (Hartung et al., 2005). Furthermore, ECVAM plays a leading role in standardisation of the validation process and actively contributes to the drafting of guidance documents such as Good Cell Culture Practice (GCCP) guidelines (Coecke et al., 2005) to establish the principles for greater international harmonisation, rationalisation and standardisation of cell and tissue culture based laboratory practices.

2. *In vitro* neural models to evaluate human acute neurotoxicity: the European project A-Cute-Tox

Dr. Cristina Suñol pointed out that *in vitro* approaches, based on general cytotoxicity assays, have demonstrated that they may reasonably predict mammalian acute systemic toxicity. Among these studies, the Registry of Cytotoxicity (RC) (Halle, 2003) and the Multicentre Evaluation of *In vitro* Cytotoxicity (MEIC) suggest that cytotoxicity assays reached a level of prediction of rodent and human toxicity up to around 70% (Ekwall, 1999). Other studies also found a high correlation ($r = 0.81$) between *in vitro* cytotoxicity IC_{50} values and *in vivo* rodent oral LD_{50} values (Kitagaki et al., 2006). At present, new regulations like the REACH in the EU and the High