Toxicology in Vitro 27 (2013) 1416–1424

Contents lists available at SciVerse ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Evaluation of aggregating brain cell cultures for the detection of acute organ-specific toxicity

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ARTICLE INFO

Article history:
Available online 29 August 2012

Keywords:
Acute oral toxicity
Neurotoxicity
3D brain cell cultures
In vitro testing
Pre-validation
Concentration–response analysis

ABSTRACT

As part of the ACuteTox project aimed at the development of non-animal testing strategies for predicting human acute oral toxicity, aggregating brain cell cultures (AGGR) were examined for their capability to detect organ-specific toxicity. Previous multicenter evaluations of *in vitro* cytotoxicity showed that some 20% of the tested chemicals exhibited significantly lower *in vitro* toxicity as expected from *in vivo* toxicity data. This was supposed to be due to toxicity at supracellular (organ or system) levels. To examine the capability of AGGR to alert for potential organ-specific toxicants, concentration–response studies were carried out in AGGR for 86 chemicals, taking as endpoints the mRNA expression levels of four selected genes. The lowest observed effect concentration (LOEC) determined for each chemical was compared with the IC_{20} reported for the 3T3/NRU cytotoxicity assay. A LOEC lower than IC_{20} by at least a factor of 5 was taken to alert for organ-specific toxicity. The results showed that the frequency of alerts increased with the level of toxicity observed in AGGR. Among the chemicals identified as alert were many compounds known for their organ-specific toxicity. These findings suggest that AGGR are suitable for the detection of organ-specific toxicity and that they could, in conjunction with the 3T3/NRU cytotoxicity assay, improve the predictive capacity of *in vitro* toxicity testing.

1. Introduction

As part of the five-year ACuteTox project, aggregating brain cell cultures (AGGR) were evaluated for their capability to detect organ-specific toxicity supposedly missed by using the 3T3/NRU cytotoxicity assay (Clothier et al., 2008) as the most simple, economic and efficient *in vitro* testing approach. To this end, the AGGR and 3T3/NRU test systems were compared by their toxicity data obtained from concentration–response analyses of a total of 86 reference chemicals, 56 representing a training set, and 30 belonging to a subsequent blinded pre-validation exercise.

The principal goal of the EU-funded project ACuteTox (Clemedson et al., 2006) was to develop and pre-validate simple, robust and reliable *in vitro* testing strategies for the prediction of human acute oral toxicity of commercial chemicals. It was based on previous multicenter efforts such as MEIC (Ekwall et al., 1998) comparing various *in vitro* systems with respect to their relevance to predict acute oral systemic toxicity, by testing a set of reference chemicals. These preceding studies showed a fair correlation between *in vitro* basal cytotoxicity data and *in vivo* data (i.e., rat oral LD_{50} values and human blood concentrations estimated to cause 50% lethality); but there was a certain amount of chemicals exhibiting significantly higher or lower *in vitro* toxicity than could be expected from the existing *in vivo* data. Significantly higher *in vitro* toxicity (i.e., overestimated *in vivo* toxicity) was generally attributed to lower accessibility of the biological target(s) *in vivo*, due to a combination of low absorption, slow distribution, rapid degradation, or rapid excretion. Significantly lower *in vitro* toxicity (i.e., underestimated *in vivo* toxicity) was thought to reflect the lack of metabolic activation and/or biological targets in the *in vitro* test

Abbreviations: ADME, absorption, distribution, metabolism and excretion; 3D, three-dimensional; 3T3, "3-day transfer" mouse fibroblast cell line; AGGR, aggregating brain cell cultures; DIV, day *in vitro*; EU CLP, European regulation on classification, labelling and packaging of chemical substances and mixtures; GFAP, glial fibrillary acidic protein; HSP32, heat-shock protein 32; IC_{20}, concentration causing 20% decrease of cell viability; IVTC, *in vitro* toxicity class; LD_{50}, 50% lethal dose value; LOEC, lowest observed effect concentration; MBP, myelin basic protein; MEIC, multicenter evaluation of *in vitro* cytotoxicity; NF-H, high molecular weight neurofilament; NRU, neutral red uptake; OECD TGs, organisation for economic co-operation and development test guidelines.

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http://dx.doi.org/10.1016/j.tiv.2012.06.018

system. This view was supported by the fact that many of the chemicals for which *in vivo* toxicity was either over- or underestimated (termed outliers by Ekwall et al., 1998), were known for their organ-specific actions. One of the goals of the ACuteTox project was, therefore, to identify correcting factors to improve the correlation between *in vitro* and *in vivo* toxicity. The correcting factors examined included biokinetics, metabolism, and organ-specific toxicity. With respect to organ-specific toxicity, the MEIC study showed that a considerable proportion of compounds of which the toxicity was underestimated *in vitro* were known for their action on the nervous system, and in particular on the central nervous system (CNS). Therefore, serum-free AGGR (Honegger et al., 1979, 2011) were included in the ACuteTox program as a representative model system for the detection of organ- and CNS-specific toxicants. These three-dimensional (3D) cell cultures have been shown to reach a highly differentiated phenotype that is maintained for at least two months (Zurich et al., 1998). Previous studies showed their usefulness for neurotoxicological investigations (Braissant et al., 2002; Honegger and Schilter, 1992; Honegger and Werffeli, 1988; Kucera et al., 1993; Monnet-Tschudi et al., 1996, 2000; Zurich et al., 2002, 2000, 1998, 2005).

For the testing of chemicals in the ACuteTox project, AGGR were taken at an advanced stage of maturation, i.e., at days *in vitro* (DIV) 17–25. Randomized replicate cultures were prepared. In each experiment, five chemicals were tested simultaneously, each at three different concentrations, requiring 32 replicate cultures. The exposure to the test compound was initiated by the addition of an aliquot of a concentrated stock solution. After 44 h of exposure at the usual culture conditions under gyratory agitation, the cultures were harvested and assayed for chemically induced adverse effects using a set of relevant biochemical and/or gene expression endpoints. All data generated were stored in an internet-based data base (AcutoxBase) as part of the project (Kinsner-Ovaskainen et al., 2009). During the first phase of the project, the endpoints measured in AGGR included enzymatic activities (i.e., lactate dehydrogenase, glutamic acid decarboxylase, choline acetyltransferase, acetylcholinesterase, glutamine synthetase, 2′,3′-cyclic nucleotide 3′-phosphohydrolase) and overall metabolic activities (i.e., the rate of 2-deoxyglucose uptake and the rate of incorporation of uridine and methionine). Thereafter, gene expression endpoints were included, measuring the mRNA expression of selected genes by quantitative reverse transcription-PCR (qRT-PCR) (Forsby et al., 2009). Of the various genes examined initially, four genes were finally chosen for routine testing, i.e., three genes representing CNS-specific traits: (i) the high molecular weight neurofilament protein (NF-H), (ii) the glial fibrillary acid protein (GFAP), and (iii) the myelin basic protein (MBP); and one known to be up-regulated by cellular stress: the heat-shock protein-32 (HSP-32). The gene expression endpoints were measured in parallel for each replicate culture. Based on the concentration–response data generated with the 56 chemicals of the training set AGGR were selected for a final pre-validation exercise with 30 blind-coded chemicals (Kinsner-Ovaskainen et al., 2013; Prieto et al., 2013). Among the eight selected *in vitro* test methods participating in the pre-validation exercise AGGR was the only nervous system-specific model.

The data generated with the 56 training set compounds and the 32 compounds evaluated in the ACuteTox pre-validation exercise were analyzed for alerts indicating organ-specific toxicity, by the comparison of AGGR with the 3T3/NRU system used for basal cytotoxicity testing (Clothier et al., 2008; Kinsner-Ovaskainen et al., 2013; Prieto et al., 2013). Alerts were determined for test compounds showing consistently higher toxicity in AGGR as compared to the 3T3/NRU cytotoxicity assay.

2. Materials and methods

2.1. Chemicals

The selection of the reference chemicals for the training set (57 chemicals) and the pre-validation exercise (32 blind-coded chemicals) is described elsewhere (Kinsner-Ovaskainen et al., 2013; Prieto et al., 2013). The first 57 chemicals were purchased separately by each group according to the instructions obtained in the ACuteTox project; the blind-coded 32 chemicals were provided from a common pool by the ECVAM (Prieto et al., 2013). Because of problems with solubility, three out of the total of 89 compounds could not be analyzed, i.e., one of the first set of 57 chemicals, and two of the second set of 32 chemicals. All three compounds showed relatively weak *in vitro* toxicity, thus requiring highly concentrated stock solutions.

2.2. Preparation and use of stock solutions

The test chemicals were stored in desiccators at 4 °C. For the preparation of stock solutions, each of these compounds was dissolved in the appropriate solvent, either in aqueous solutions or in Dimethylsulfoxide (DMSO), according to recommendations of the ACuteTox project. For each individual test, fresh stock solutions were prepared. Chemicals dissolved in DMSO were prepared as 2000-fold concentrated stock solutions. Thus, the final DMSO concentration in the medium never exceeded 0.05%. At this concentration, none of the biochemical parameters examined was significantly affected by the solvent. Compounds dissolved in aqueous solutions (either Puck's saline solution D1 or medium) were prepared as 100- to 1000-fold concentrated stock solutions, depending on their solubility. They were sterilized by membrane filtration (0.2 μm) prior to their use. For the treatments, aliquots of the stock solutions were pipetted directly into the culture supernatants. Some of the lipophilic compounds (e.g., Phenobarbital, Lindane, Diazepam) precipitated in the medium when given at the highest concentration. However, due to the gyratory agitation of the medium, these precipitates dissolved subsequently, as indicated by their disappearance and the coherence of the concentration-dependent effects.

2.3. Preparation and maintenance of aggregating brain cell cultures

Serum-free, rotation-mediated AGGR were prepared from 16-day embryonic rat brains as described previously in detail (Honegger et al., 1979, 2011). The dissected brain tissue, comprising the telencephalon, mesencephalon and rhombencephalon, was dissociated mechanically into a single cell fraction by the sequential passage through nylon sieves of 200-μm and 100-μm pore sizes. Through all steps of the preparation, the cells were kept in ice-cold, Ca^{2+}-Mg^{2+}-free saline (Puck's saline solution D_1). The dissociated cells were washed by centrifugation (15 min, $300g_{max}$), and finally resuspended in cold serum-free culture medium (modified DMEM). Aliquots of the cell suspension (4 ml, containing the amount of cells obtained on average from one embryonic brain) were transferred to culture flasks (25-ml modified Erlenmeyer flasks with air-permeable stoppers) and incubated under continuous gyratory agitation (68 rpm) in a CO_2 incubator (10% CO_2, 90% humidified air, 37 °C). After two days, the cultures (4 ml) were transferred to larger flasks (50-ml modified Erlenmeyer flasks with air-permeable stoppers) and supplemented with 4 ml of fresh medium (total volume: 8 ml). The frequency of gyratory agitation was increased progressively from 68 rpm at culture initiation (day 0) to 70 rpm (evening of day 0), then to 73 rpm (day 1), 77 rpm (day 2, after the culture transfer), and then stepwise