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Evaluation of drug-induced neurotoxicity based on metabolomics, proteomics and electrical activity measurements in complementary CNS *in vitro* models

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ABSTRACT

The present study was performed in an attempt to develop an *in vitro* integrated testing strategy (ITS) to evaluate drug-induced neurotoxicity. A number of endpoints were analyzed using two complementary brain cell culture models and an *in vitro* blood–brain barrier (BBB) model after single and repeated exposure treatments with selected drugs that covered the major biological, pharmacological and neuro-toxicological responses. Furthermore, four drugs (diazepam, cyclosporine A, chlorpromazine and amiodarone) were tested more in depth as representatives of different classes of neurotoxicants, inducing toxicity through different pathways of toxicity.

The developed *in vitro* BBB model allowed detection of toxic effects at the level of BBB and evaluation of drug transport through the barrier for predicting free brain concentrations of the studied drugs. The measurement of neuronal electrical activity was found to be a sensitive tool to predict the neuroactivity and neurotoxicity of drugs after acute exposure. The histotypic 3D re-aggregating brain cell cultures, containing all brain cell types, were found to be well suited for OMICs analyses after both acute and long term treatment.

The obtained data suggest that an *in vitro* ITS based on the information obtained from BBB studies and combined with metabolomics, proteomics and neuronal electrical activity measurements performed in stable *in vitro* neuronal cell culture systems, has high potential to improve current *in vitro* drug-induced neurotoxicity evaluation.

1. Introduction

Neurotoxicity testing of new compounds with the desired pharmacological effects represents one of the major bottlenecks in drug development since it is time consuming and requires large numbers of animal experiments. Indeed, neurotoxicity is one of the causes for withdrawal of pharmaceuticals from the market (Kola and Landis, 2004). Therefore, the large number of hits identified from primary high throughput discovery screens requires early,

Abbreviations: BBB, blood brain barrier; DMSO, dimethylsulfoxide; EC, endothelial cells; DIV, day *in vitro*; IPA, Ingenuity Pathway Analysis; ITS, integrated testing strategy; LY, Lucifer Yellow; MEA, micro-electrode array; RH, Ringer HEPES medium; SPSS, Statistical Package for the Social Sciences.

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rapid and robust preclinical screening testing strategy to assess whether compounds with desirable characteristics are neurotoxic, prior to safety and efficacy testing.

Neurotoxicity is the outcome of complex interactions of xenobiotics at the molecular, cellular and tissue level of the central and/or peripheral nervous system causing an adverse effect. An adverse effect can be caused by changes of neuronal and/or glial cell chemistry, structure and/or function. Therefore any *in vitro* testing strategy for drug-induced neurotoxicity evaluation has to be based on the combination of relevant *in vitro* models that possess the necessary molecular mechanisms and pathways that can be evaluated in a quantitative manner by sensitive, neuronal and glia-specific endpoints (Bal-Price et al., 2010; Crofton et al., 2011).

The EU 7th Framework project, Predict-IV, was established in order to develop mechanistic strategies for predictive toxicology, including neurotoxicology. The major aim of this large-scale integrated project was to provide the drug discovery community with a general test system to predict toxicity prior to pre-clinical testing. The selected compounds covered the major biological, pharmacological and toxicological responses observed in drug-induced toxicity at the level of different organs (liver, kidney and brain). One of the main criteria for the drug selection was that these three subgroups of drugs, linked to three different organs may share basic, well-known pathways, reliable biomarkers and various biochemical processes although they cause organ-specific toxicity. Thus, the selection of chemicals was based on the well documented adverse drug reactions (ADR) due to the pharmacological effect that triggers toxicity in different organs.

Based on the publicly available databases and literature search, 12 central nervous system (CNS) relevant drugs were selected to represent four different categories: (I) neuroactive and neurotoxic: CNS-drugs with strong neurotoxicity (amiodarone, buflomedil, chlorpromazine), (II) neuroactive and non-neurotoxic: CNS-drugs with no or weak neurotoxic effects (carbamazepine, diazepam, propofol); (III) non-neuroactive but neurotoxic: non-CNS drugs with significant neurotoxic effects (cisplatinum, ciprofloxacin, cyclosporine A); (IV) non-neuroactive and non-neurotoxic: non-CNS drugs with no or weak neurotoxic effects (loperamide, nadolol, ondansetron). However, these four drug groups were created only for the purpose of the electrophysiological studies to evaluate whether this end-point is sensitive enough to discriminate between drugs that are neuroactive and/or neurotoxic or non-neuroactive and/or neurotoxic after acute exposure. Among these 12 drugs four such as cyclosporine, amiodarone, diazepam and chlorpromazine were selected for an in-depth proteome and metabolome analysis. Based on the literature search amiodarone, cyclosporine and chlorpromazine should be neurotoxic and diazepam should be neuroactive but non-neurotoxic after acute exposure but could produce some toxicity after long term exposure.

Cyclosporine A and chlorpromazine were selected as they induce toxicity across different organs allowing to study whether different cell types (liver, kidney and the CNS) responded differently to the same treatment through cell specific toxicity pathways or observed toxicity was due to general cytotoxic effects. Amiodarone was selected as it produces side effects in the CNS but were originally designed for treatment of various pathologies (cardiac dysrhythmias) and diazepam was chosen because of its application as CNS drug in order to treat anxiety and seizures. Additionally, kinetics of selected drugs was studied in all three organ cultures (hepatocytes, kidney and neuronal cells) and the results obtained are described separately in this volume (e.g. Bellwon et al., 2014; Pomponio et al., 2014).

In this project we aimed to develop a novel *in vitro* approach for more comprehensive drug-induced neurotoxicity testing by providing insight into mechanisms of neurotoxicity. Since the CNS represents a high level of anatomical and physiological complexity (multiple neuronal and glial cell types) and OMICs-profiling techniques have proven to be powerful new tools (van Vliet et al., 2008; Wilmes et al., 2013) for studying complex biological processes (Csermely et al., 2013; Kleinjans, 2014; Nemes et al., 2013), in this study proteomics and metabolomics analyses were performed after long term exposure to the selected drugs. Comprehensive investigations of responses to a drug-induced perturbation on the transcriptome, proteome, and metabolome levels should lead to a better understanding of the biochemical and biological mechanisms in complex systems such as the CNS.

The study aimed for identification of possible biomarkers of neurotoxicity among the deregulated metabolites and proteins. Furthermore the OMICs analyses were combined with measurements of neuronal electrical activity after acute exposure to evaluate whether such a combination of assays will be a reliable approach for *in vitro* neurotoxicity testing. The applied endpoints were analyzed using two complementary *in vitro* brain cell cultures, cortical networks (2D) and re-aggregating brain cells (3D) after acute, sub-chronic, and repeated-exposure chronic treatments at non-cytotoxic concentrations of the selected drugs.

However, when neurotoxicity of new chemicals with unknown mechanisms of neurotoxicity has to be evaluated, firstly their possible toxicity at the blood–brain barrier (BBB) should also be considered as a fully functional BBB is of key importance for maintaining the homeostasis of the brain (Coecke et al., 2006b). Therefore the effects of 14 days of repeated treatment with the 12 selected drugs on the functionality of the BBB have been evaluated. Furthermore, the BBB is the principal route for the entry of most molecules into the CNS as well as it is the major hurdle that prevents many drugs from eliciting pharmacological or toxicological effects in the brain (Harry and Tiffany-Castiglioni, 2005). Consequently, to evaluate the neurotoxicity of compounds *in vitro* it is crucial to predict whether a drug will reach the CNS in amounts sufficient to cause toxicity. The CNS exposure is a function of several factors such as plasma protein binding, BBB permeability and brain tissue binding (Hallier-Vanuxeem et al., 2009). The ratio between the unbound concentrations in brain and plasma (Cu,br/Cu,pl) is considered as a major pharmacokinetic parameter in the CNS drug discovery (Becker and Liu, 2006; Friden et al., 2007; Kalvass and Maurer, 2002) and recently, the possibility to directly generate Cu,br/Cu,pl ratios in a single *in vitro* model of the BBB has been evaluated (Culot et al., 2013). Therefore, this alternative method has been applied here to obtain *in vitro* Cu,br/Cu,pl ratios which could then be used to estimate the Cu,br based on the plasma concentration of the studied drugs in human plasma. Based on the BBB evaluation, the estimated drug concentrations, relevant to human exposure were taken as an indication for the concentrations selected for *in vitro* neurotoxicity studies using to two mixed neuronal/glial cell culture models, mice neuronal networks (2D) and rat brain aggregates (3D).

The 2D tissue culture model of mice neuronal networks was introduced by the lab of G.W. Gross and developed over the years to a powerful tool to directly study the effects of acute exposure to test compounds effects on the electrical network communication (Gross et al., 1997). Brain region-specific networks consisting of neurons and astroglia can be cultivated for months and provide a phenotypic screening system that is being often applied in testing of both desired and unwanted effects on neuronal communication during early drug development (Johnstone et al., 2010; Lefew et al., 2013; Novellino et al., 2011).

The second mixed neuronal/glial *in vitro* model applied was a 3D rat brain aggregate model that presents a higher level of cell organization, similar to *in vivo* brain tissue cyto-architecture and function as indicated by the final ratio of neurons to glial cells, the formation of an organotypic cyto-architecture, the correct timing and extent of developmental events such as cell proliferation,

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