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$GABA_A$ receptor and cell membrane potential as functional endpoints in cultured neurons to evaluate chemicals for human acute toxicity

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ABSTRACT

Toxicity risk assessment for chemical-induced human health hazards relies mainly on data obtained from animal experimentation, human studies and epidemiology. *In vitro* testing for acute toxicity based on cytotoxicity assays predicts 70–80% of rodent and human toxicity. The nervous system is particularly vulnerable to chemical exposure which may result in different toxicity features. Acute human toxicity related to adverse neuronal function is usually a result of over-excitation or depression of the nervous system. The major molecular and cellular mechanisms involved in such reactions include GABAergic, glutamatergic and cholinergic neurotransmission, regulation of cell and mitochondrial membrane potential, and those critical for maintaining central nervous system functionality, such as controlling cell energy. In this work, a set of chemicals that are used in pharmacy, industry, biocide treatments or are often abused by drug users are tested for their effects on $GABA_A$ receptor activity, GABA and glutamate transport, cell membrane potential and cell viability in primary neuronal cultures. $GABA_A$ receptor function was inhibited by compounds for which seizures have been observed after severe human poisoning. Commonly abused drugs inhibit GABA uptake but not glutamate uptake. Most neurotoxins altered membrane potential. The $GABA_A$ receptor, GABA uptake and cell membrane potential assays were those that identified the highest number of chemicals as toxic at low concentrations. These results show that *in vitro* cell assays may identify compounds that produce acute neurotoxicity in humans, provided that *in vitro* models expressing neuronal targets relevant for acute neural dysfunctions are used.

1. Introduction

Toxicity risk assessment for chemical-induced human health hazards relies mainly on data obtained from animal experimentation, human studies and epidemiology. The nervous system is particularly vulnerable to chemical exposure; its complexity results in multiple potential target sites with different toxicity features. Acute human toxicity related to adverse neuronal function is mainly a result of over-excitation or depression of the peripheral or central nervous system (CNS). The major molecular and cellular mechanisms involved in such effects include GABAergic, glutamatergic and cholinergic neurotransmission, regulation of cell and mitochondrial membrane potential, and those critical for maintaining CNS functionality, such as controlling cell energy. Severe disturbance of these mechanisms may result in convulsions, fatal central depression and cell death. Compared to other tissues, nerve cells have little ability to replace themselves or regenerate, which limits full recovery when cell damage occurs. Therefore, it is crucial to develop models that predict and can detect neurotoxic chemicals. Recently implemented regulations such as the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) in the EU (EC 1907/2006 directive) (http://ec.europa.eu/environment/chemicals/reach/reach_intro.htm), and the High Production Volume (HPV) Program in the USA (http://www.epa.gov/HPV), are designed to manage the risks from chemicals and provide safety information on them. In this context, non-animal testing allowing high-throughput analysis of a huge number of chemicals is foreseen and promoted in the REACH program.

In vitro testing for acute toxicity based on general cytotoxicity assays, such as the Registry of Cytotoxicity (RC) and the Multicentre Evaluation of *In Vitro* Cytotoxicity (MEIC), can predict 70–80% of rodent and human toxicity [8,19,22]. There are many reasons for the 20–30%

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failure: i) chemical metabolism, ii) barrier passage, iii) biokinetics, and iv) organ-specific toxicity (neurotoxicity, hepatotoxicity, nephrotoxicity, etc.). Alternative testing strategies, including biokinetic models and endpoints for organ specific toxicity, aim to define alerts and correctors to improve this failure rate in prediction when using exclusively cytotoxicity-based assays. Such strategies would either reveal factors that improve the correlation between *in vitro* cytotoxic data and acute human toxicity, or define alerts that identify outliers (compounds for which the cytotoxicity *in vitro* data give a false evaluation of their acute human toxicity).

Neurotoxic events are the consequence of the failure of one or several molecular mechanisms (see [29,39]) such as the functioning of ion channels (voltage- or ligand-operated ionotropic receptors, such as $GABA_A$, NMDA, AMPA/kainate, and nicotinic acetylcholine), the transport of amino acidergic and aminergic neurotransmitters, neurotransmitter synthesis and degradation involving enzymes, cell energy control, the regulation of cell and mitochondrial membrane potential, intracellular calcium homeostasis and control of the production and inactivation of reactive oxygen species. The amino acids γ-aminobutyric acid (GABA) and glutamate are, respectively, the most common inhibitory and excitatory neurotransmitters in the CNS. Once released into the synapse they act on ionotropic receptors of GABA ($GABA_A$) and glutamate (NMDA and AMPA/kainate). Whereas excessive potentiation of the $GABA_A$ receptor activity results in central depression, inhibition of $GABA_A$ receptor activity results in overall excitatory symptoms and convulsions in mammals [26,28]. Conversely, excessive activation of ionotropic glutamate receptors results in excitatory symptoms and in degeneration of neurons through a process known as excitotoxicity [23,31].

In vitro evaluation of these neuronal specific and general endpoints requires well-characterized cell culture systems. Primary neuronal cultures of cortical and cerebellar granule cells are enriched in GABAergic and cholinergic neurons [41,42,48], and in glutamatergic neurons [14,38], respectively. Transport of the neurotransmitters GABA and glutamate can be evaluated by determining the uptake of [^{3}H]GABA in cortical neurons and of [^{3}H]aspartate or [^{3}H]glutamate in cerebellar granule neurons, respectively (aspartate being an analogue of glutamate that is taken up by the cells through the glutamate transport system). Furthermore, both cultured cortical neurons and cerebellar granule cells express functional ionotropic $GABA_A$ and glutamate receptors, which are neural targets for depressant, convulsant and excitotoxic compounds. For a review of the functional characteristics of these *in vitro* systems see [41].

In the present work we select a set of compounds (Table 1 in Supplementary Material) based on the European project ACUTETOX (www.acutetox.org). We then test the effect of these compounds on several neuronal functional endpoints and on cell viability in primary cultured neurons. Most of the reference compounds were chosen from the MEIC project [10] and the NICEATM/ECVAM *In Vitro* Cytotoxicity Validation Study [1] and are pharmaceuticals, pesticides, and industrial chemicals, for which data on their acute human toxicity exist. Both compounds whose acute systemic toxicity was well predicted by general cytotoxicity tests and compounds identified as outliers (i.e., their toxicity was poorly predicted) were included in the list. In addition, some of the reference compounds were selected as positive control compounds for specific target endpoints in the nervous system, kidney and liver. The ACuteTox project aims to improve predictability by combining a handful of simple and robust tests that measure complementary parameters such as absorption, distribution and metabolism, as well as organ specific toxicity. The objective of these research activities is to increase knowledge of the mechanisms by which these compounds cause toxicity and to identify corrector/alert assays in order to improve the *in vivo*/*in vitro* correlation with the aim to use the in vitro test strategy for regulatory classification and risk assessment. The compounds selected have been tested by different research groups using relevant *in vitro* assays and *in silico* approaches including basal cytotoxicity, barrier passage, metabolism, hepatotoxicity, renal toxicity and neurotoxicity. Here we present results concerning GABAergic and glutamatergic neurotransmission (as the main depressant and excitatory neural systems) and cell membrane potential (which determines neural electrical excitability) in primary cultures of cortical neurons and of cerebellar granule cells.

2. Methods

2.1. Materials

Pregnant NMRI mice (16th day of gestation) and 7-day-old NMRI mice were obtained from Charles River, Iffa Credo (St. Germain-sur-l'Arbreste, France). Plastic multi-well culture plates were purchased from Nunc™ (Roskilde, Germany). Foetal calf serum was obtained from Gibco (Invitrogen, Barcelona, Spain) and Dulbecco's modified minimum essential medium (DMEM) from Biochrom (Berlin, Germany). $^{36}Cl^-$ (111–532 Mbq/g), [^{3}H]-flunitrazepam (≈3 TBq/mmol), [^{3}H]-GABA (≈3 TBq/mmol) and [^{3}H]-D-aspartate (1 TBq/mmol) were obtained from Amersham Life Science (Buckinghamshire, UK). Optiphase Hisafe 2 liquid scintillation cocktail was obtained from Wallace Oy (Turku, Finland). The FMP membrane potential assay kit (blue) was from Molecular Devices (Sunnyvale, CA, USA). 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenyl-formazan (MTT) was from Sigma Chemical Co. (St. Louis, MO, USA) and the lactate dehydrogenase (LDH) cytotoxicity kit was from Roche (Manheim, Germany). Fluo-3/AM was obtained from Molecular Devices.

2.2. Chemicals

Of the first set of reference compounds: acetaminophen, acetylsalicylic acid, carbamazepine, cycloheximide, diazepam, digoxin, mercury (II) chloride, phenobarbital, sodium lauryl sulphate (SDS), verapamil hydrochloride, nicotine, (±)methadone hydrochloride, d-amphetamine sulphate, sodium valproate, pentachlorophenol and isopropyl alcohol were obtained from Sigma Chemical Co; atropine sulphate monohydrate, caffeine, colchicine and ethanol were obtained from Fluka (St. Louis, MO, USA); and malathion, lindane and glufosinate ammonium were obtained from Riedel de Haen (St. Louis, MO, USA).

Of the second set of reference compounds: 5-fluorouracil, tert-butylhydroperoxide, rifampicine, tetracycline hydrochloride, cadmium (II) chloride, cyclosporin A, 17α-ethynylestradiol, lithium sulphate, warfarin, 2,4-dichlorophenoxyacetic acid, strychnine, pyrene, hexachlorobenzene, amiodarone hydrochloride, parathion, dichlorvos, physostigmine, cis-diamminiumplatinum (II) dichloride, (−)-epinephrine bitartrate, ochratoxin A, sodium chloride, thallium sulphate, sodium selenate, dimethylformamide, amitriptyline hydrochloride, ethylene glycol, methanol, lithium sulphate, arsenic trioxide, chloral hydrate, acetonitrile and propanolol hydrochloride were obtained from Sigma Chemical Co; acryaldehyde, orphenadrine hydrochloride and diethylene glycol were obtained from Fluka; and diequat dibromide was obtained from Riedel de Haen.

The chemicals were dissolved and diluted in HEPES-buffered saline solution or in DMSO. When dissolved in DMSO, a 200× concentration was prepared, thus the concentration of DMSO in the testing solution was 0.5%. Controls contained the same amount of DMSO, when so required.

2.3. Neuronal cultures

Primary cultures of cortical neurons were obtained from neocortices from 16-day-old mice foetuses, using 10–12 foetuses from one pregnant mouse for each culture preparation. Pregnant animals were anesthetized with isofluorane (FORANE®, Abbott Laboratories SA, Madrid, Spain) and killed by cervical dislocation. Primary cultures of cerebellar granule cells were obtained from 7-day-old pups, using a litter of 10 pups per culture. Pups were killed by decapitation.