WP7.1: Alerts and correctors in toxicity screening (III): Neurotoxicity (P4, P5, P13, P16, P20, P37)

Selection of reference chemicals with neurospecific potential and the optimized neurotoxicity test battery

The first 16 general reference chemicals plus the reference chemicals with neurotoxic potential; D-amphetamine sulphate, ethanol, glufosinate ammonium, lindane, methadone hydrochloride, nicotine and verapamil hydrochloride (see table of all reference chemicals in Appendix II) were tested up to 1 mM (ethanol 50 mM) or up to the solubility limit, in all assays listed in Table 5. In addition, physostigmine, dichlorvos and parathion were tested in the acetylcholine esterase assays. Lowest observed effective concentrations (LOEC), EC_{20} and EC_{50} values (i.e. neurotoxic concentrations, NTC) were determined and compared with IC_{50} generated by the general cytotoxicity 3T3/NRU assay. Neurotoxic concentrations which were 0.7 log units lower than the cytotoxic IC_{50} indicated potential neurotoxicity, i.e. alerting acute systemic toxicity by a neurotoxic mechanism. The possibility to correct under-estimated toxicity by substituting cytotoxic IC_{50} values with the neurotoxic concentrations was also used as an indication of the predictive capacity of the neurotoxicity endpoints. Both criteria indicated a subset of assays that identified the most neurotoxic alerts and also had the best correction capacity(Table 6).

Table 5. The original neurotoxicity test methods used for the selection of the "best performing" neurotoxicity test battery to be used for the full screening.

Partner	Endpoint	Cell model	Method
4	Viability/general cytotoxicity	rat CGC	Resazourin ("Alamar-blue")
4	Viable neurons	rat CGC	NF-H, immunohistochemistry
4	Glia cell viability and activation	rat CGC	GFAP, immunohistochemistry
4	Mitocholndrial membrane potential	rat CGC	JC-1, fluoroscence
5	Viability	SH-SY5Y cells, mouse cortical neurons, mouse brain slices	Total LDH activity, photometrically
5	Acetylcholine esterase activity	purified bovine AChE enzyme	Photometrically
5	Voltage-operated ion channel function	SH-SY5Y cells	K^+-induced excitotoxicity
5	mRNA expression: bcl-2, p53, myc, bax, ngf-R, bcl-xL	mouse cortical neurons	Quantiatiative RT-PCR, TaqMan
13	GABAA-receptor activity	mouse cortical neurons	$^{36}Cl^-$ uptake, 5 microM GABA
13	GABAA-receptor inhibition	mouse cortical neurons	$^{36}Cl^-$ uptake, 100 microM GABA
13	GABA transporters	mouse cortical neurons	^{3}H-GABA uptake
13	Membrane leakage	mouse cortical neurons	LDH leakage, photometrically

13	Glutamate/aspartate uptake	mouse CGC	^{3}H-aspartate uptake
13	Transmitter release	mouse CGC	Extracellular glutamate, HPLC
13	Cell membrane potential	mouse cortical neurons	Fluorescence, outliers only
16	Membrane leakage	SH-SY5Y cells	LDH leakage, photometrically
16	Cell membrane potential	differentiated SH-SY5Y cells	Fluorescence
16	Acetylcholine esterase activity	SH-SY5Y cells	Photometrically
16	Monoamine uptake	SH-SY5Y cells	^{3}H-noradrenaline uptake
16	Acetylcholine receptor function	differentiated SH-SY5Y cells	Carbachol-induced Ca^{2+} influx, fluorescence
16	Voltage-operated ion channel function	differentiated SH-SY5Y cells	K^{+}-evoked Ca^{2+} influx, fluorescence
20	Cytotoxicity	Mature rat brain cell aggregates,	Total LDH activity, photometrically
20	Choline acetyltransferase activity	Mature rat brain cell aggregates,	Radiometrically
20	Glutamic acid decarboxylase activity	Mature rat brain cell aggregates,	Radiometrically
20	Glutamine synthethase activity	Mature rat brain cell aggregates,	Radiometrically
20	Acetylcholine esterase activity	Mature rat brain cell aggregates,	Radiometrically
20	2',3'-cyclic nucleotide 3'-phosphohydrolase (CNP) activity	Mature rat brain cell aggregates,	Photometrically
20	Global glycolytic activity	Mature rat brain cell aggregates,	^{3}H-2-deoxyglucose uptake
20	protein synthesis	Mature rat brain cell aggregates,	^{35}S-methionine incorporation
20	mRNA synthesis	Mature rat brain cell aggregates,	^{14}C-uridine incorporation
20	M,H-Neurofilament mRNA expression (neurons)	Mature rat brain cell aggregates,	Quantiatiative RT-PCR, SYBR Green
20	Glial fibrillary acidic protein(GFAP) mRNA expression (astrocytes)	Mature rat brain cell aggregates,	Quantiatiative RT-PCR, SYBR Green
20	Myelin basic protein mRNA expression (oligodendrocytes)	Mature rat brain cell aggregates,	Quantiatiative RT-PCR, SYBR Green
20	PPAR-gamma mRNA expression (cellular stress)	Mature rat brain cell aggregates,	Quantiatiative RT-PCR, SYBR Green
20	iNOS mRNA expression	Mature rat brain cell	Quantiatiative RT-PCR, SYBR Green

	(cellular stress)	aggregates,	
20	HSP32 mRNA expression (cellular stress)	Mature rat brain cell aggregates,	Quantiatiative RT-PCR, SYBR Green
37	Viability	rat CGC	MTT
37	Ca^{2+} homeostasis	rat CGC	Ca^{2+} influx, fluorescence
13	Ionotropic glutamate receptors	rat CGC	Ca^{2+} influx, fluorescence
37	Reactive oxygen species	rat CGC	DCF, flow cytometry
37	Membrane leakage	rat CGC	LDH leakage, photometrically
37	Identification of target genes out of 31	rat CGC	Microarray. Quantiatiative RT-PCR, TaqMan
37	Caspase-3 mRNA expression	rat CGC	Quantiatiative RT-PCR, TaqMan

CGC; Cerebellar granule cells
SH-SY5Y: Human neuroblastoma cell line
PPAR; Peroxisome proliferators-activated receptor
iNOS; inducible nitric oxide synthase
RT-PCR; Real time polymerase chain reaction
HSP; Heat shock protein

Results of the first screening

The assays which indicated most alerts were the transcriptional, metabolic and enzyme activity endpoints analysed in the aggregated rat brain cells, GABAA receptor function in primary cortical neurons, caspase-3 expression in cerebellar granule cells and cell membrane potential and noradrenalin uptake in human neuroblastoma cells. Furthermore, dichlorvos and physostigmine were correctly identified as AChE inhibitors by the 60-minutes test in the neuroblastoma cells, whereas malathion and parathion could be correctly identified as alerts in the same system only after 24 hour of exposure. None of the general cytotoxicity tests analysed in the neuronal cell models (the MTT test, total LDH activity assay and the Alamar Blue test) indicated significant alerts, after 24-72 hours of exposure to the reference chemicals, i.e. general cytotoxicity determined in neuronal models gave similar results as the 3T3/NRU assay. Furthermore, short term exposure did not affect the mithochondrial membrane potential or produced reactive oxygen species. Only sporadic genomic markers were affected, Caspase-3 being the most sensitive, in the cerebellar granule cells. Hence, the assays to be included in the optimised neurotoxicity test battery for further evaluation were (i) the transcriptional markers analysed in aggregates of rat brain cell cultures; NF-H (neurons), GFAP (astrocytes), MBP (oligodendrocytes), HSP32 (cellular stress), (ii) the metabolic markers glucose uptake and total RNA synthesis in aggregates of rat brain cell cultures, (iii) Caspase-3 mRNA expression in rat cerebellar granule cells, (iv) GABAA receptor function in mouse cortical neurons, (v) cell membrane potential and (vi) AChE activity in human neuroblastoma SH-SY5Y cells (Table 6).

Evaluation of the predictive capacity of the neurospecific endpoints for estimation of acute oral systemic toxicity

Thirty three additional reference chemicals were tested up to the solubility limit in the optimised test battery (Table 6). All together, the effects of 59 chemicals on 10 endpoints were analysed. Neurotoxic concentrations (NTC, i.e. LOEC, EC_{20} and EC_{50}) were determined for all the active chemicals on every endpoint and delivered to the AcutoxBase. The correction capacity of every endpoint was analysed together with the general cytotoxicity IC_{50} values generated by 3T3/NRU assay. Neurotoxic alerts were identified if the negative logarithm of the NTC (pNTC) was 0.7 unit higher than the corresponding negative logarithm of the IC_{50} (pIC_{50}) determined in the 3T3/NRU assay.

The selected endpoints were evaluated separately according to the following rationale: If the pNTC showed to be higher than the pIC_{50} (3T3/NRU), i.e. the chemical was regarded as a neurotoxic alert, the NTC was considered as the apparent target tissue concentration. If the pIC_{50} (3T3/NRU) was higher than the pNTC, the IC_{50} value was considered as the estimated target tissue concentration. Hence, the apparent target tissue concentration for every chemical was either the endpoint-specific NTC or the IC_{50} (3T3/NRU), depending on which endpoint was the most sensitive. The pNTC and the pIC_{50} were compared by linear regression models with the negative logarithm of the human lethal blood concentrations (pLC_{50}), which were analysed for 45 of the chemicals from clinical cases (see WP1 and Sjöström *et al*., 2008) (Forsby *et al*., 2009). The LC_{50} values were used as surrogate for the target tissue concentrations, which holds true for chemicals affecting the PNS and for chemicals with free passage over the blood brain barrier.

Table 6. The optimized neurotoxicity test battery.

Endpoints	***In vitro* model**	**Partner**
GABAA receptor function	Primary cortical mouse neurons	13
Cell membrane potential	Neuroblastoma cell line	16
Acetylcholine esterase activity	Neuroblastoma cell line	16, 5
Transcriptional markers (NF-H, GFAP, MBP, HSP32)	Aggregated rat brain cells	20
Metabolic markers (glucose uptake, mRNA synthesis)	Aggregated rat brain cells	20
Caspase-3 mRNA expression	Primary rat cerebellar granule cells	37

Short description of the methods in the optimised neurotoxicity test battery

Transcriptional markers (NF-H, GFAP, MBP, HSP32) and metabolic markers (glucose uptake, mRNA synthesis) in aggregating brain cell cultures

Aggregating brain cell cultures were prepared from mechanically dissociated 16-day embryonic rat brain comprising the telencephalon, mesencephalon and rhombencephalon, and maintained in serum-free chemically defined medium under constant gyratory agitation

(Honegger *et al*., 1979). The initially immature brain cells reorganize spontaneously into thousands of even-sized spheres, and undergo progressive maturation, giving rise to highly differentiated 3D cultures. For the testing of acute neurotoxicity, randomized replicates of the aggregating brain cell cultures were used at an advanced state of maturation (DIV 17 – 25). Each replicate culture contained sufficient material for high content analysis of multiple endpoints. Adverse effects were assessed at 44 h of drug exposure.

The selected set of transcriptional biomarkers included 4 genes, representative for neurons (NF-H), astrocytes (GFAP), oligodendrocytes and myelin (MBP), and cellular stress (HSP32). The total RNA was extracted of each lysed replicate culture, using a kit from Qiagen. The total RNA content per replicate was determined. The reverse transcription (RT) was performed using the TaqMan reverse-transcription reagents from Applied Biosystems. The RT was run with 200 ng of total RNA in a reaction volume of 50 µl. The cDNA obtained was then used for subsequent PCR reactions. The cDNA (3.2 ng) was added to the PCR mixture composed of primers (150-300 nM), 1 x SYBR Green PCR master mix, and H_2O, in a final volume of 10 µl.

The two selected metabolic markers were [^{3}H]deoxyglucose uptake, representative for the rate of glycolytic activity, combined with [^{14}C]uridine uptake, representative for the overall rate of RNA synthesis. The two tracers were added to the replicates 4 h before the harvest. Aliquots of culture homogenates were taken for the determination of the protein content, and for the measurements by liquid scintillation counting to determine [^{3}H]deoxyglucose uptake and the specific incorporation of [^{14}C]uridine into the total RNA.

Cell membrane potential

Neuronal cell function is dependent on a membrane potential and every alteration changes the properties of the electrical signalling. The cell membrane potential (CMP) can be analysed by using fluorescent dyes which track movements of ion fluxes over the cell membrane. Here, the FLIPR membrane potential dye and FlexStationII fluorometer (Molecular Devices) were used to monitor the changes in fluorescence intensity in real time before and during exposure of the differentiated human neuroblastoma SH-SY5Y cells to the reference chemicals (Gustafsson *et al*., 2010). The assay has also been implemented in primary mice cortical neurons (Suñol *et al*., 2008; Galofré *et al*., 2010).

GABAA receptor function

The neuronal GABA-A receptor (GABAR) is a membrane protein which conforms a pore permeable to Cl^- ions. The protein receptor has recognition sites for the neurotransmitter γ-aminobutyric acid (GABA), for therapeutic drugs as benzodiazepines and barbiturates and for toxic chemicals as the convulsant picrotoxinin, and polychlorocycloalkane pesticides. A reasonable correlation exists between these pesticides inhibiting the GABAR and their oral toxicity in rodents. The activity of the GABAA receptor can be determined by analysing the Chloride flux induced by GABA by means of a radiometric assay in primary mice cortical neurons (Suñol *et al*., 2008; Galofré *et al*., 2010).

Acetylcholine esterase activity

Functional acetylcholine signalling is essential for survival and the inactivating enzyme acetylcholine esterase has been a popular target for the development of pesticides and chemical warfare substances. Here, human neuroblastoma SH-SY5Y cells were cultured in 96-well plates and the effect of the reference chemicals on acetylcholine esterase activity during 60 minutes of exposure was analysed by using the Ellman reaction (Ellman *et al*

1961). ACh analogue acetylthiocholine (ASChI) is hydrolysed by cellular AChE into thiocholine and acetate. The formed thiocholine reacts with 5,5´-dithio-bis-(2-nitrobenzoic acid) (DTNB or Ellman's reagent), which results in the formation of the yellow 5-thio-2-nitrobenzoate anion (TNB^{2-}). This yellow product can be measured spectrophotometrically at 420 nm.

Caspase 3 mRNA expression

Primary cultures of cerebellar granule neurons (CGNs) were prepared from 7-day-old Sprague Dawley rat pups. Isolation of total RNA was performed after 16 h of incubation with the chemical to be tested. Total RNA isolation was carried out according with the guidelines of the NucleoSpin® RNA II kit (Macherey–Nagel GmbH & Co. KG, Düren, Germany). Briefly, cell culture medium was removed and cells rinsed with PBS and lysed directly in the culture dish by adding lysis buffer containing β-mercaptoethanol. For each preparation, one Nucleo-Spin® RNA II column was placed in a 2 mL centrifuge tube and the lysate loaded. After centrifugation, 30 s at 8.000 g, the column was desalted, loaded with 95 µL of DNAse I reaction mixture. After 15 min of digestion at room temperature, the RNA was eluted from the column and recovered in 50 µL H_2O (RNAse-free). RNA content in the samples was measured at 260 nm and purity of the samples was determined by the A260/280 ratio. RNA samples were finally stored at -80 ºC until reverse transcription was performed (less than 48 h later). First strand cDNA was reversely transcribed from 25 µL of total RNA, at 0.2 µg/µL, by using a High-Capacity cDNA Archive Kit. Reaction mix was prepared according with the manufacturer's guidelines and the reaction was performed in a Perkin Elmer 2400 Thermal Cycler, according with the following program: Step 1 at 65 ºC for 5 min; Step 2 at 50 ºC for 120 min. The cDNA samples were used for Real-time PCR analysis.

PCR was developed by using Taq-Man® Universal PCR Master Mix, No AmpErase® UNG, and with TaqMan ® Gene Expression Assays of Caspase-3 and GADPH (Applied Biosystems, Foster City, CA, USA). PCR program was set according to the supplier guidelines: initial setup at 95 ºC for 10 min; 40 cycles of step one at 95 ºC for 15 and step two at 60 ºC for 1 min. The method also uses the formula 2 -ΔΔCt to calculate the expression of caspase 3 normalized to GADPH gene expression (N-fold). We used cDNA from colchicine-treated cerebellar cells (1 mM) as positive control of caspase 3 up-regulation. Non-cDNA template sample and total RNA samples (non-reverse transcribed) were used as negative controls. As acceptance criteria, differences in Ct values between negative controls and samples should be greater than 10 Ct units (Folch *et al*., 2009).

Results from testing of 59 reference chemicals in the optimized neurotoxicity test battery

A: Transcriptional markers in aggregating brain cell cultures ("GENE")

Results

The LOEC values of gene expression determined by quantitative RT-PCR for NF-H, GFAP, MBP and HSP32 produced 25 neurotoxic alerts (negative log > 0.7 unit higher than in the 3T3/NRU assay) out of the 59 test compounds (42 %). For the rest of the test compounds, the sensitivity was similar to that of 3T3/NRU. The compounds for which a higher sensitivity was found in aggregating brain cell cultures included mercury(II) chloride, arsenic trioxide, glufosinate-ammonium, lindane, diquat dibromide, malathion, parathion, physostigmine, dichlorvos, epinephrine, diazepam, phenobarbital, amiodarone, ochratoxin A, cyclosporine A, digoxin, pentachlorophenol, 5-fluorouracil, acrylaldehyde, tetracycline hydrochloride,

sodium valporate, cadmium (II) chloride, sodium selenate, methadone hydrochloride and lithium sulphate. Figure 4a shows that the GENE assay in combination with the two metabolic markers corrected most of the outlier chemicals identified by the 3T3/NRU assay in comparison with the human lethal blood concentration (LC_{50}). These findings show that by using 4 representative genes as transcriptional markers together with metabolic markers (see below) in aggregating brain cell cultures most of the CNS-specific toxicants were detected.

Conclusions on variability
Each compound was tested at least thrice, using aggregate cultures from different batches (preparations), and the results were in general highly reproducible.

Limitations
For the time being, culture maintenance is not automated, requiring considerable work for this part of the method. On the other hand, the RNA extraction and qPCR have been already automated.

Possible use of alerts
The alerts thus detected can be taken as indicators for specific organ toxicity. It will require, in addition, the knowledge of ADME, and particularly of the BBB permeability of the compounds, to decide on the effective neurotoxicity of the compounds producing alerts.

B: Metabolic markers in aggregating brain cell cultures ("Metabolism")
Results
Taking into consideration, in addition to the transcriptional markers, also the two metabolic markers, [^{3}H]2-deoxyglucose uptake and [^{14}C]uridine incorporation, 7 additional alerts were detected according to the >0.7 log pIC_{50} limit, namely for cycloheximide, carbamazepine, pyrene, acetylsalicylic acid, nicotine, methadone and propranolol hydrochloride.

Conclusions on variability
Measurements of glycolytic activity and RNA synthesis, when used in a high content approach in combination with transcriptional markers showed considerable variability, because the test conditions were sub-optimal at the end of the period of drug exposure (40^{th}-44^{th} h of exposure). It seems likely that by enlarging the set of representative genes for qRT-PCR (there is no limitation to that, since large amounts of mRNA are obtained from the culture replicates) the compounds affecting specifically metabolic pathways could be detected as well.

Limitations
The method use radioactive solutions.

Variability in high content analyses (see above, "GENES").

C: Cell membrane potential in human neuroblastoma SH-SY5Y cells ("CMP")Results
The LOEC values of cell membrane potential as analysed in differentiated human neuroblastoma cells produced 11 neurotoxic alerts (negative log >0.7 unit higher than in the 3T3/NRU assay) out of the 59 test compounds. 13 additional chemicals induced depolarisation or hyperpolarisation the CMP, whereas 33 chemicals did not acutely affect the

CMP. The compounds for which a higher sensitivity was found in neuroblastoma cell/CMP assay as compared to the 3T3/NRU cytotoxicity included mercury(II) chloride, pentachlorophenol, sodium lauryl sulphate, nicotine, lindane, propranolol hydrochloride, D-amphetamine sulphate, orphenadrine hydrochloride, amitryptyline hydrochloride, sodium chloride and 2,4-dichlorophenoxy-acetic acid. Out of these compounds, an alert for digoxine was recognised in primary mouse cortical neurons by using the CMP assay. The correlation with human lethal blood concentration was improved for these chemicals (Figure 4b) and these findings show that several of the specific neurotoxic chemicals can be correctly detected as alerts by the neuroblastoma/CMP assay.

Conclusions on variability

The response in CMP is highly dependent on the cell physiology, e.g. resting cell membrane potential, receptor or ion channel expression and ion homeostasis. Hence, the status of the cell model used in the assay is crucial for the variability.

Limitations

Some chemicals may interfere with the ion-sensitive fluorescent dye and give false positive or negative fluorescence. Cell-free controls with chemical and dye must be included.
The results are highly dependent on the cell model used (see Variability).

Possible use of alerts

Depending on the cell model used, very specific and sensitive endpoints may be analyzed and identified as alerts, as exemplified by nicotine in this study.

D: $GABA_A$ receptor function in primary cortical neurons from mouse ("GABAA")

Results

The IC_{50} values of $GABA_A$ receptor function as analysed in primary cortical neurons from mouse produced 8 neurotoxic alerts (negative log > 0.7 unit higher than in the 3T3/NRU assay) out of the 59 test compounds. These alerts correspond to lindane, malathion, parathion, strychnine, rifampicine, pentachlorophenol, 17α-ethynylestradiol and sodium lauryl sulfate. Additionally 15 chemicals acutely impaired the $GABA_A$ receptor function, but did not generate neurotoxic alerts according to the 0.7 log unit limit rule (Galofré *et al*., 2010). Diazepam, isopropyl alcohol, mercury (II) chloride, chloral hydrate and phenobarbital enhanced the GABA receptor function, i.e. increased 36chloride influx in the presence of GABA. This information can also be used for alert identification since the effect *in vivo* may result in unconsciousness and even death. Thirty one of the 59 test chemicals did not acutely affect the $GABA_A$ receptor function. These findings show that specific neurotoxic chemicals can be correctly detected as alerts by the $GABA_A$ receptor function assay (Figure 4c).

Conclusions on variability

The variability for each chemical between independent experiments was around 10% for log EC values: coefficients of variability (CV = sd/mean * 100) for the log EC values were 8.2 % for log EC_{20} (n = 26) and 8.6 % for log EC_{50} (n = 21). However, when EC values are expressed in molar concentrations, the variability accounts for around 60%: coefficients of variability (CV = sd/mean * 100) for the EC values were 67 % for EC_{20} (n = 26) and 59 % for EC_{50} (n = 21).

Limitations
The method use a radioactive $^{36}Cl^-$ solution, which must be used in laboratory areas prepared for radioactivity work. The method is not still optimised for high throughput assay.

Possible users of alerts
An inhibition of $GABA_A$ receptor activity may be detrimental to the neuronal signal transmission. Most of the chemicals that inhibit the $GABA_A$ receptor produce toxic excitatory symptoms in humans that can develop into seizures. On the contrary, the chemicals that over-activated the $GABA_A$ receptor possess depressant properties that may result in toxic effects in humans (e.g., barbiturates). It should be noted that some of these latest compounds have therapeutic properties, when used at adequate doses. Hence, alteration in $GABA_A$ receptor activity may give an alert for neurospecific toxicity.

E. Caspase 3 mRNA expression in cerebellar granule cells from rat ("CASP3")

Results
The LOEC values of Caspase 3 mRNA expression as analysed in primary rat cerebellar granule cells produced 15 neurotoxic alerts (negative log > 0.7 unit higher than in the 3T3/NRU assay) out of 57 compounds tested. Nine of the chemicals affected Caspase 3 expression but generated pLOECs that were less than 0.7 log units higher than pIC_{50}. Thirty three of the chemicals did not affect the Caspase 3 mRNA expression. The compounds for which a higher sensitivity was found in Caspase 3 mRNA expression assay included acetylsalicylic acid, carbamazepine, diazepam, digoxin, isopropyl alcohol, malathion, lindane, sodium valproate, ethanol, acrylaldehyde, dichlorvos, verapamil hydrochloride, ochratoxin A, propranolol hydrochloride and warfarine (Figure 4d). These findings show that some of the neurotoxic chemicals can be correctly detected as alerts by Caspase 3 mRNA expression assay.

Conclusions on variability
Each compound was tested at least three times, using primary cultures from different batches (preparations), and the results were in general highly reproducible.

Limitations
Culture maintenance is not automated, requiring considerable work for this part of the method. On the other hand, the RNA extraction and qPCR have been already automated.

Possible use of alerts
The alert thus detected can be taken as indicator for specific organ toxicity. Increases in transcriptional activity of Caspase 3 gene can be considered as a common event in neuronal cells that undergo apoptotic cell death. Thus, results from the testing of a panel of substances agree with the potential value of this gene as a marker of apoptotic death in neuronal cells.

F. Acetylcholine esterase activity in human neuroblastoma SH-SY5Y cells ("AChE")

Results
The LOEC values of acetylcholine esterase activity as analysed in human neuroblastoma SH-SH5Y cells produced 9 neurotoxic alerts (negative log >0.7 unit higher than in the 3T3/NRU

assay) out of the 59 test compounds. Additionally, eight of the chemicals inhibited the AChE activity, but the pNTCs (IC_{50}) were less than 0.7 log unit higher than pIC_{50} determined in the 3T3/NRU assay, whereas 42 chemicals did not acutely affect the AChE activity. The compounds for which a higher sensitivity was found in the AChE activity assay included acrylaldehyde, physostigmine, sodium fluoride, dimethylformamide, ethylene glycol, lithium sulphate, nicotine, dichlorvos and strychnin (Figure 4e). These findings show that some of the neurotoxic chemicals can be correctly detected as alerts by the AChE activity assay. Primary cortical neurons gave similar results after acute exposure whereas 44 hrs exposure in aggregated rat brain cell cultures correctly identified all expected alerts.

Conclusions on variability

The AChE assay displays very low variability but the exposure time is crucial for chemicals that need to be bioactivated by oxidation to become full inhibitors of AChE. The assay was successfully transferred from partner 16 to partner 5 after the first phase of the project when 26 of the chemicals were tested.

Limitations

Herein, 60 minutes exposure was performed in the selected assay, which resulted the loss of alert indication for malathion and parathion. The sensitivity for these AChE inhibitors increased significantly if the exposure time increased to 24 hrs and when primary cultures with metabolising capacity was used.

Possible use of alerts

Chemicals that acutely inhibit AChE activity and chemicals that possess acetylcholine alike activity (e.g. nicotine) can be identified as alerts.

Summary of the results of the extended testing using the optimized neurotoxicity test battery

All outliers identified in the correlation between *in vivo* toxicity data, i.e. human lethal blood concentration (LC_{50}) and LD_{50} in rats, and *in vitro* cytotoxicity, i.e. IC_{50} determined using the 3T3/NRU assay could be identified as potentially neurotoxic by one or more assay in the optimised neurotoxicity test battery, except for acetaminophen, atropine sulphate and thallium sulphate (Table 7). However, atropine sulphate affected the cell membrane potential, $GABA_A$ receptor function and Caspase 3 expression but the difference between the pNTCs and the pIC_{50} was less than 0.7 log units and hence, were not alerting neurotoxicity. Acetaminophen is well known to induce liver toxicity and is not expected to be identified as a neurotoxic compound. Thallium sulphate accumulates in body tissues after *in vivo* exposure, which may result in increasing concentration in the target tissue with time. Hence, the identification of thallium sulphate as an outlier in the in IC_{50} vs. LD_{50} correlation as well as the failure to alert for neurotoxicity may be due to toxicokinetic factors.

The limit for alert identification was set to 0.7 log unit higher pNTC than pIC_{50}. This limit was chosen because the definition of an outlier from the correlation between the log IC_{50} determined in the 3T3/NRU assay and the log LD_{50} or log LC_{50} was set to 0.75 log units. The GENE assay alerted as many as 45 % of the test chemicals as neurotoxic and severe outliers such as e.g. digoxin (chemical 9), malathion (chemical 11) and lindane (chemical 34) were totally corrected to the LC_{50} (Figure 4a). However, it must be remembered that the blood-brain barrier may hinder chemicals to enter the brain parenchyma from blood, which means

that the neurotoxic concentrations determined by the assays presented herein may be significantly higher as compared to the lethal blood concentrations. Furthermore, to be able to perform a correct estimation of the toxic dose (LD_{50}), biokinetic factors such as absorption, distribution, metabolism and excretion shall be taken into consideration.

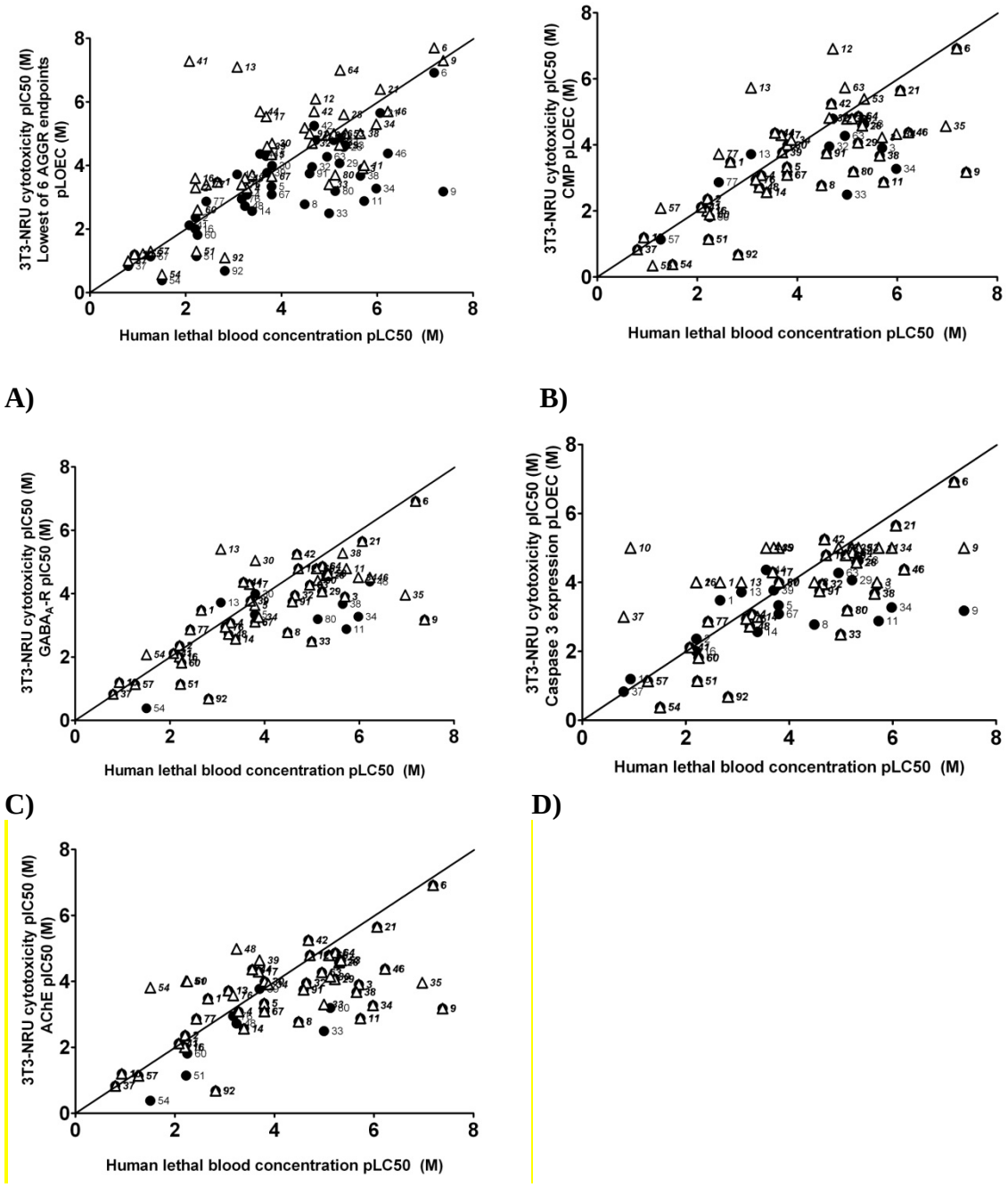

Figure 4. Correlation between the general cytotoxicity in the 3T3/NRU assay in combination with neurospecific toxicity and the human lethal blood concentration for the ACuteTox reference chemicals. A) Lowest LOEC of the multi-parameter endpoint (NF-H, GFAP, MBP, HSP32 mRNA expression, glucose uptake, mRNA synthesis) in AGGR, B) LOEC of altered CMP in differentiated SH-SY5Y cells C) IC_{50} of $GABA_A$ receptor function in PCN D) LOEC of altered Caspase-3 expression in CGC, and E) IC_{50} of AChE activity in SH-SY5Y cells. The toxicity is indicated as the negative logarithm of the neurotoxic concentrations, IC_{50} (3T3/NRU) and LC_{50} (M). Filled circles: 3T3/NRU, opened triangles: neurospecific endpoints or 3T3/NRU when pIC_{50}>pNTC or pNTC is missing. The numbers refer to the chemical identity within the ACuteTox project (Table in Appendix II). Lines: identity 1:1. (Adopted from Forsby *et al*. 2009).

Table 7. Outliers identified as potentially neurotoxic by one or more assay in the optimized neurotoxicity test battery (from Table 2, WP3).

Chemical name	**Outlier in linear regression between IC_{50} and rat LD_{50}**	**Outlier in linear regression between IC_{50} and human LC_{50}**	**Assays indicating alerts when pNTC is > 0.7 log pIC_{50}**
(-) epinephrine	X	No human data	GENES
2,4-dichlorophenoxyacetic acid		X	CMP
5-fluorouracil		X	GENES, Metabolism
Acetaminophen		X	
Atropine sulfate monohydrate		X	
Cis-diammineplatinum (II) dichloride		X	GENES, Metabolism
Codeine		X	Not tested
Cyclosporine A		X	GENES
D-amphetamine sulfate	X	No human data	CMP
Digoxin	X	X	GENES, CASP3, Metabolism
Diqaut dibromide		X	GENES
Formaldehyde	X	X	Not tested
Lindane		X	GENES, CMP, GABAA, CASP3 Metabolism
Malathion		X	GENES, GABAA
Methadone hydrochloride		X	GENES, Metabolism
Nicotine	X	X	CMP, AChE
Ochratoxin A	X	No human data	GENES, CASP3, Metabolism
Parathion	X	No human data	GENES, GABAA, Metabolism
Pentachlorophenol		X	GENES, CMP, GABAA, Metabolism
Phenobarbital	X		GENES
Physostigmine	X	No human data	AChE, GENES, GABAA
Potassium cyanide	X		Not tested
Sodium chloride		X	CMP
Sodium selenate	X		GENES, Metabolism
Strychnine	X	X	GABAA, AChE
Thallium sulfate	X		
Warfarin	X		CASP3

Conclusive remarks of the evaluation of the neurotoxicity assays

In conclusion, the aggregated rat brain cell cultures was the most sensitive cell model and the multi-endpoint GENE assay detecting alterations in transcript markers for neurons, astrocytes, oligodendrocytes and cellular stress, together with a marker for total RNA synthesis seems to be the most complete assay for identification of neurotoxic alerts. However, the assays analysing CMP, GABAA, AChE and CASP3 identified additional alerts, which could not be detected by the GENE-Metabolism analysis. However, taking other issues into account such as variability and limitations, the GENE assay seems to be the most promising candidate to be integrated into the test strategy for identification of acutely toxic chemicals.