

Area: systemic toxicity

Test purpose: ITS for repeated doses on chemicals, screening and pre-clinical studies for pharmaceuticals, to determine the MTD for myelosuppressive drugs

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Impact on animal use

Acute tox

AcuteTox testing strategy that aims to totally replace the animal acute toxicity tests used today for regulatory purposes, should deliver a pre-validated testing strategy by 2009. The implementation of this testing strategy will result in full replacement by 2016 (worst scenario) and 2009 (best scenario).

BEST SCENARIO: Validated and or pre-validated test methods and testing strategies are used

WORST SCENARIO: Only regulatory accepted method are used

Chronic toxicity

The tests listed below could be integrated in a testing strategy. Once validated this strategy can reduce the number of animals used for repeated dose toxicity testing (see Prieto et al. ATLA 2006, in press). The in vitro methods available are all models aimed at the study of individual types of target organ toxicity. However, it is clear that, to adequately replace repeat-dose studies in animals, target organs in addition to those must be considered. At the moment, strategies for integrating such models into a testing programme have not been defined — and the eventual replacement of in vivo repeat-dose toxicity tests will probably involve the integration of in vitro data on target organ toxicity with in vitro/in silico data on ADME parameters.

Test method		Status of validation	Information needed	Timelines
Cell function assay	Rat CFU-GM *	Starting end 2006	in vivo animal data	
Cell function assay	In vitro tests for immune toxicity *	Starting beginning 2007	in vivo animal and human data	

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<p style="writing-mode: vertical-rl; transform: rotate(180deg);">Part of testing strategy</p>	<p>Prevalidation of in vitro models for the prediction of gastrointestinal absorption” **</p> <p>Management Team: Isabella De Angelis (Istituto Superiore di Sanita’, Roma, Italy); Flavia Zucco (CNR, Istituto di Neurobiologia e Medicina Molecolare, Roma, Italy); Pilar Prieto (ECVAM); Sebastian Hoffmann (ECVAM)</p> <p>Coordinator Project: Isabella De Angelis (isabella.deangelis@iss.it)</p>	<p>Started 29.09 2005</p>	<p>Human and animal data on intestinal absorption and bioavailability</p> <p>List of compounds: Ethylene Glycol Sodium Valproate Paraquat Paracetamol Cupric sulfate Colchicine Atenolol Cimetidine Propranolol Nicotine Acrylamide Coumarin Parathion Pentachlorophenol</p>	<p>29.03.07</p>
<p style="writing-mode: vertical-rl; transform: rotate(180deg);">Part of testing strategy</p>	<p>Evaluation of the performance of five selected in vitro blood-brain barrier models for predicting absorption/uptake into the brain” **</p> <p>Management Team: Prof. Romeo CECHELLI (University of Artois, Lens, France); A.G. de BOER (LACDR, University of Leiden, The Netherlands); Bas Blaauboer (IRAS, NL); Pilar Prieto (ECVAM); Sebastian Hoffmann (ECVAM).</p> <p>Coordinator Project: Prof. Romeo CECHELLI (romeo.cecchelli@univ-artois.fr)</p>	<p>Started 31 March 2006</p>	<p>Animal data on Blood Brain Barrier permeability clearance (= BBB transport-rate/plasma concentration)</p> <p>List of compounds: Acrylamide Caffeine Lindane Diazepam MeHg TET Phenytoin Parathion Paraoxon Verapamil Dichlorvos Physostigmine Glufosinate Doxorubicine Glutamate CdCl₂ Pb-acetate Ethanol isopropanol</p>	<p>31.03.2007</p>

METHODS PROFILES
(see list of criteria attached)

CRITERIA	CFU-GM	Immunotox assays	Caco-2	Caco-2/TC7
Ethics				
overall impact on animals under conditions of use	medium	medium	medium	medium
3 R benefit	reduction	reduction	reduction	reduction
increased level of safety	yes	yes	not	not
Regulatory requirement				
covers a specific regulatory purpose	not	yes	not	not
timelines				
Industrial incentive				
transferability	good	good	under evaluation	under evaluation
Applicability across sectors	pharma/chem/food	pharma/chem/food	pharma-chem	pharma/chem
Range of substances concerned	drugs/chemicals/food additives direct toxicants		drugs/chemicals/food additives direct toxicants	
relevance for screening	high	high	high	high
overall costs vs current assays	↓↓↓	↓↓↓	↓↓↓	↓↓↓
high probability to be accepted by authorities	yes	yes	yes	yes
Development status				
Overall performance (predictivity, reliability)	ongoing	ongoing	ongoing	ongoing
Stand alone or part of a future strategy	strategy	strategy	strategy	strategy
availability of in vivo/in vitro data and substances	in vivo data on humans and animals (MTD)		in vivo/in vitro data on animals for pharma absorption can be found	

CRITERIA	HCMEC/D3	MDCKmdr-1	Primary bovine	Porcine primary
Ethics				
overall impact on animals under conditions of use	medium	medium	medium	medium
3 R benefit	reduction	reduction	reduction	reduction
increased level of safety	not	not	not	not
Regulatory requirement				
covers a specific regulatory purpose	not	not	not	not
timelines				
Industrial incentive				
transferability	under evaluation	under evaluation	under evaluation	under evaluation
Applicability across sectors	pharma/chem	pharma/chem	pharma/chem	pharma/chem
Range of substances concerned	drugs/chemicals/food additives direct toxicants	drugs/chemicals/food additives direct toxicants		drugs/chemicals/food additives direct toxicants
relevance for screening	high	high	high	high
overall costs vs current assays	↓↓↓	↓↓↓	↓↓↓	↓↓↓
high probability to be accepted by authorities	yes	yes	yes	yes
Development status				
Overall performance (predictivity, reliability)	ongoing	ongoing	ongoing	ongoing
Stand alone or part of a future strategy	strategy	strategy	strategy	strategy
availability of in vivo/in vitro data and substances	idem	idem	idem	idem

ADDITIONAL INFORMATION

Rat CFU-GM

The test is intended to be used in a testing strategy approach for repeated doses as well as for acute toxicity

The aim of the study is to refine and validate a SOP-based GM-CFU assay that makes use of rat bone marrow as source of myeloid progenitors, cultured in methylcellulose medium type A containing recombinant rat GM-CSF.

To make the assay relevant to toxicology applications governed by regulations in OECD and the US for food contaminants, additives, and non-toxic drugs.

Statement On The Application Of The Cfu-Gm Assay For Predicting Acute Neutropenia In Humans

At its 24th Meeting, held on 20-21 March 2006 at the European Centre for the Validation of Alternative Methods (ECVAM), Ispra, Italy, the Non-Commission Members of the ECVAM Scientific Advisory Committee (ESAC)¹ unanimously endorsed the CFU-GM assay^{2,3} for predicting acute neutropenia in humans as a substitute to using a second species, such as the dog, for this purpose. It should be noted that the test relies on the availability of mouse MTD data and is, therefore, not a full replacement method, but is intended to reduce the overall numbers of animals needed in toxicity testing. Performance standards for the assay should be developed to enable reasonable flexibility in the protocol used.

- Mouse was used because of the large amount of experimental data on MTD and LD10 that were used in the "gold standard" database.
- However, it is not the emphasized toxicology species in the EU or US.
- Building on this success and the verification of the prediction model, now time to extend the CFU-GM concept into the relevant pre-clinical toxicology species of rat and ultimately dog, which are the basis for regulating almost all registered chemicals.
- Establishment of the rat CFU-GM assay is the first step toward major reductions and refinements in the number of animals required for regulatory testing.

What it is needed:

- Data on prevalence of myelotoxic compounds in drug development (preclinical studies) and risk assessment
- Set of compounds to be tested
- Human and animal (rat) in vivo data (bioavailability, kinetics, myelotoxicity)
- dose-effect as well as PK
- human MTD data from the Phase I clinical trial in oncology

IN VITRO TESTS FOR IMMUNOTOXICITY

- To evaluate reproducibility and predictivity of a set of in vitro assays to detect immunotoxicity, to be used in a testing strategy for repeated doses as well as to replace animals in case of ICH recommended in vivo follow-up studies.
- Different endpoints will be assessed and an appropriate number of chemical substances, representatives of the full range of in vivo responses and for which good human and/or animal bioavailability data are available either from databases or literature, will be chosen.

Immunosuppression

An initial evaluation of myelotoxicity should be performed.

An in vitro test to determine lymphotoxicity should be carried out (cell death by necrosis or apoptosis). Such assays would require pre-validation to evaluate their reliability/reproducibility.

Potential effects on cytokine expression should be determined.

Major Limitations

In vitro exposure is most straightforward for direct immunotoxicants. However, materials that require biotransformation would require special culture systems (e.g., culture in the presence of S9).

Compounds Tested

NEGATIVE

URETHANE : is a chemical intermediate in preparation of amino resins, manufacture of pesticides, cosmetics, active ingredient in drugs

FUROSEMIDE:is a loop diuretic drug

POSITIVE

VERAPAMIL: is a calcium channel blocker

BENZO(A)PYRENE: is a product resulting from the incomplete combustion of organic materials

CYCLOSPORIN A: is a potent immunosuppressive agent

TBTC: is an organotin compound used as polyvinyl chloride plastics stabilizer, catalytic agent, agricultural pesticide and rodenticide

The set of methods used agree in classifying the compounds tested as follows:

Compounds	Mouse	Rat	Human
Urethane	No effect	No effect	No effect
Furosemide	No effect	No effect	No effect
Verapamil	13<IC50<30	17<IC50<30	20<IC50<22
Benzo(a)pyrene	10<IC50<18	9<IC50<13	11<IC50<12
Cyclosporin A	0.08<IC50<0.3	0.16<IC50<0.18	1<IC50<8
TBTC	0.002<IC50<0.003	0.007	0.07

Cytotoxicity (murine, rat and human cells)
-LDH

Cytokine release (murine, rat and human cells)
-interferon γ
-supernatant collection for a possible further TNF α dosage

Proliferation and mitogen responsiveness (murine rat and human cells)

- Anti-CD3 and CD28 (human)
- ConA (only on rodents)
- LPS
- Anti-IgM+ IL4

Metabolic processes should be addressed on selected compounds

Classes Of Compounds To Be Tested

- Industrial and environmental chemicals
- Food additives
- Pharmaceuticals

What it is needed:

- Frequency of ICH recommended additional immunotoxicity studies
- Data on prevalence of immunotoxic compounds in drug development (preclinical studies) and risk assessment
- Species used
- Set of compounds to be tested
- Human and/or animal in vivo data (bioavailability, kinetics, immunotoxicity)

IN VITRO MODELS FOR THE PREDICTION OF GASTROINTESTINAL ABSORPTION

To evaluate the reproducibility and the predictive capacity (e.g., sensitivity, specificity, concordance) of two in vitro systems, Caco-2 and Caco-2/TC7, to estimate oral fraction absorbed by using an appropriate number of chemical substances, representatives of the full range of in vivo responses and for which good human and/or animal data are available.

List Of Testing Compounds

Acrylamide	Nicotine
Atenolol	Paracetamol
Cimetidine	Paraquat
Colchicine	Parathion
Coumarin	Pentachlorophenol
Cupric sulfate	Propranolol
Ethylen Glycol	Sodium Valproate

Needed:

In vivo data on absorption and eventually bioavailability -kinetics

EVALUATION OF THE PERFORMANCE OF FIVE SELECTED IN VITRO BLOOD-BRAIN BARRIER MODELS FOR PREDICTING ABSORPTION/UPTAKE INTO THE BRAIN

To evaluate the performance of the most promising BBB in vitro models discussed during the ECVAM workshop by testing an appropriate number of compounds representing low, medium and high passage and measuring the barrier integrity and the rates of transport for

each compound at the no-observed effect level (NOEL) or the lowest-observed-effect level (LOEL) concentrations.

Selected In Vitro Models Under Evaluation

Primary bovine endothelial cells co-cultured with rat primary astrocytes;
HCMEC/D3 human cell line from brain capillary endothelial cells;
Porcine primary endothelial cells cultured in hydrocortisone-conditioned medium;
MDCKmdr-1 cells
ARPE-19 human retinal cell line co-cultured with a human glioma cell line (although this is a leaky model it is included in the study as 3D model for cytotoxicity studies).

List Of Testing Compounds

Acrylamide	Dichlorvos
Caffeine	Physostigmine
Lindane	Glufosinate
Diazepam	Doxorubicine
MeHg	Glutamate
TET	CdCl ₂
Phenytoin	Pb-acetate
Parathion	Ethanol
Paraoxon	isopropanol
Verapami	

Needed:

Animal data on BBB permeability/clearance (transport rate/plasma concentration)

Criteria for prioritization

The following criteria were agreed at the WG5 Workshop as important for the prioritization of validation studies:

A – Ethics

- the number of animals used by the animal and the alternative test (impact data) in the overall context of the actual use of the respective methods,
- the level of diminution of the test severity or 3Rs benefit offered
- the increased level of safety introduced

B – Regulatory demand

- Would the test address regulatory needs ?
 - Does the method cover a specific regulatory testing purpose ?
 - Is it strictly applicable ? ie hazard/risk
 - Are there clear agreed rules/ criteria for how test results would be used for decision making : ie Regulatory and/or Safety
 - Would the test offer full replacement for safety assessment or be used in a tiered strategy ?
- Are there legislative timelines ?

C - Industrial incentive

- Does it have a high probability to be accepted by regulators (liability aspects) ?
- the applicability of the test in different sectors,
- the applicability for all chemicals / or for materials of limited range,
- the frequency of use of the method
- method transferability,
- need for special equipment, trained staff, resources (overall cost)
- reduced time
- Could the test be used for screening purposes?(HTS)
- are there patents involved, would the test be commercially available?

D – Development status

- relevant and consistent responses with positive and negative reference materials
- level of advancement of the test, (optimised protocol , standard operating procedures)and of the method (prediction model)
- part of an overall strategy (basic research still missing),
- availability of data (optimization studies , prevalidation , publications),
- Is this test making use of the best scientific knowledge available?
- probability for success