WP6 and WP7.3: Alerts and correctors in toxicity screening (II and V): Role of metabolism (P3, P7, P15, P17, P23, and P31) and hepatotoxicity (P3, P12, P15, P28, P31)

Integrated strategy to alert about hepatotoxic (intrinsic and/or bioactivable) compounds, as a part of a general AcuteTox testing platform (Integrating results from WP6 and WP7.3) (P3, 12, 15, 23, 31)

The scientific purpose was to set up an integrated strategy to alert about intrinsic hepatotoxic compounds, as well as metabolism-dependent toxicity (bioactivation) by assaying cytotoxicity on three cell systems. The idea of generating "alerts" for warning about a possible deviation of a compound in the 3T3 cytotoxicity test, rather than assessing potential hepatotoxicity, has prevailed in the final decision of adopting a simple, yet mechanistically-based test. The issue addressed was whether such a cell-based test would be capable of discriminating bioactivable compounds as well compounds showing a preferential action on hepatocytes.

1. Some compounds may elicit preferential toxicity on hepatic cells (hepatocytes and HepG2) in comparison with the non-hepatic cell line 3T3, indicating that such compound affects hepatic cells without requiring biotransformation.
2. A bioactivable compound is expected to cause more toxicity in hepatocytes than in non-metabolising cells (HepG2 and 3T3).
3. Finally, the compound may show similar toxicities in the three cell types, indicating that it primarily exerts basal cytotoxicity.

The standardised operation protocols (SOPs) for cell cultures, as well as for MTT assay as end-point for basal cytotoxicity, have been written by P3 and distributed to all the partners of WP6 and 7.3. ECOD activity was determined in rat hepatocyte cultures as quality control criteria. An excel template to introduce and store raw data of the experiments has been defined and provided to the partners.

Selection of reference chemicals with hepatotoxic potential

The compounds selected in WP6 were: amiodarone, verapamil, rifampicine, tetracycline and orphenadrine and in WP7.3 amiodarone, 17α-Ethynylestradiol, rifampicine, tetracycline and cyclosporine A. In addition some extra compounds which were reported in the literature as bioactivable (amphetamine sulphate, tamoxifen and cyclophsphamide) or hepatotoxic (chlorpromazine) were also tested by P3 as a proof of concept. This first set of compounds was tested at concentration up to 1 mM and sodium lauryl sulphate was used as a positive control.

The experience gained in the master laboratory (P3) after the transfer of the method to other laboratories and testing the first set of 21 reference chemicals, strongly recommends to routinely include three compounds as internal control in each assay, in order to minimise inter-assay variability. The results showed that absolute IC_{50} values obtained in the different runs differed between runs and labs making comparisons among the cell systems not reliable. These limitations were, in a way, anticipated at earlier stages of the project, and led us to consider the need for development of an appropriate software that could circumvent these limitations (ACUSOFT). By doing so, even in the case that the IC_{50}'s differ among assays, or among laboratories, toxicities still would be possible to be compared and an assessment on the compound could be

made with the aid of ACUSOFT. Three compounds were selected as internal reference compounds out of the approved list of chemicals, because have good solubility, do not attach to plastic, cover a range of toxicity (i.e., amiodarone 1E-05 M "high", SLS 1E-04 M "moderate" and sodium valproate 1E-02 M "low") and quite consistently show ICs in the same order of magnitude, in repeated assays and in different laboratories. It was observed that the relationship of the toxicity of a test compound to any of the three internal reference compound was maintained. Consequently, the second set of 41 reference chemicals was tested at concentrations up to the limit of solubility in culture media in HepG2, rat hepatocytes and 3T3 through MTT assay. Most chemicals from the first set were retested by P31 using the same experimental strategy (acetaminophen, atropine, carbamazepine, sodium valproate, malathion, tetracycline and isopropyl alcohol). A total of 61 chemicals have been tested in WP6 and 7.3.

The need of a software to alert bioactivation/hepatotoxicity

The need of developing an adequate software to calculate ICs and/or compare concentration-toxicity curves of each compound in two or three cellular models to ascertain whether xenobiotic bioactivation is required to produce toxicity, was identified. P3 has assisted Noraybio since 2007 in the development of the ACUSOFT software under the sponsorship of AcuteTox, not only to determine the IC_{50}s, but also to compare dose-response curves in metabolism-dependent toxicity and hepatotoxicity studies and to manage and integrate all the data. Alerts can be derived from the comparison of this relative toxicity values in the three cellular systems. When IC_{50} cannot be estimated by the simple logit transformation (curves incomplete or not sigmoid ones), the ACUSOFT generates a numerical factor MRTE (Mean Relative Toxic Effect) that allows comparison of the curves of the assayed compounds with the three internal reference standards. The MRTE value of each compound provided by the ACUSOFT allows comparison of relative toxicities among different experiments. The software performs the analysis of variance (ANOVA) providing significance of differences between two set of data (cells lines, runs, laboratories, etc.,) enabling to compare the concentration-effect curves for a given compound in the two or three cellular models.

At present, the following are available: 1) Beta-3 version, which can be installed in a PC computer (Windows), 2) User's Manual (pdf), 3) The commitment of P3 and Prof Prof. Hergo Holzhütter (University of Berlin) to continue improving the performance of the software until the end of the guarantee period (2 years).

Sensitivity, reproducibility, robustness and potential transferability of the assay

To examine the robustness of the strategy, the intra-assay, inter-assay as well as the intra-laboratory variability was evaluated for each cell system in each laboratory. A low variability (%CV<10%), both intra-plate and intra-assay, was obtained in all laboratories. However, the analysis of the first 21 compounds showed that the *intra-laboratory* variability needs to be reduced. A similar *intra-laboratory* variability and the lack of reproducibility of the cytotoxicity data was found with the second set of 41 compounds. The variability in HepG2 found in the assay run in a *robotic system* (Bayer, P17) was very similar to that of the other partners, thus, manipulation seems not to be the main cause of the observed intra-laboratory variability. In addition, broad *inter-laboratory* variability (%CV of IC_{50}) has been found in the 5 participant laboratories in WP6 and 7.3, both in the 21-first and in the 41-second set of compounds, despite the

fact that all labs used the same cell models and the same SOPs. Other factors responsible for this intra- and inter-laboratory variability were further examined.

The independent statistical analysis of the data shows that the 3T3/MTT and the HepG2/MTT (with the exception of a few outlying CVs larger 60%) assays show similar variability as 3T3/NRU cytotoxicity assay, while control response variability is apparently larger for the primary rat hepatocytes/MTT assay. Variability of control response was investigated also separately for every partner per cell line combination. Different variability in control response was observed for different partners, and variability differed between the three cell lines. In general, more variability in control responses by P12 was observed compared to other partners. The analysis of the variation (% CV) of EC_{50} estimates of the reference chemicals (SLS, amiodarone and sodium valproate) clearly differs between the partners (Table 3): P3 produced much more reproducible results than all the other partners. Additionally, comparison of EC_{50} estimations for the three reference compounds revealed that the quality of the results obtained in the primary rat hepatocytes/MTT assay was highly variable among partners: P17 (robotic testing) performed the best in arranging the three reference compounds while P31 generated the worst results.

Table 3. Intra- and inter-laboratory (P3, P15, P23 and P31) variability of IC_{50} of reference compounds

		SLS		**AMIODARONE**		**S. VALPROATE**	
		IC_{50} (M)	**CV (%)**	**IC_{50} (M)**	**CV (%)**	**IC_{50} (M)**	**CV (%)**
Primary rat Hepatocytes	P3	3,39E-04	**11,3**	4,38E-05	**10,8**	1,64E-02	**11,5**
	P15						
	P23	7,02E-05	**29,1**	2,25E-05	**12,7**	5,69E-03	**29,0**
	P31	2,48E-04	**19,7**	4,53E-05	**13,0**	1,44E-02	**43,3**
HepG2	P3	3,51E-04	**2,0**	2,38E-05	**6,6**	1,76E-02	**10,9**
	P15						
	P23	2,29E-04	**28,8**	8,09E-05	**14,4**	3,26E-02	**38,3**
	P31	1,65E-04	**37,0**	4,37E-05	**19,3**	6,62E-02	**38,9**
3T3	P3						
	P15	1,81E-04	**19,0**	4,16E-05	**28,5**	3,04E-02	**26,6**
	P23						
	P31	1,61E-04	**26,1**	2,78E-05	**26,1**	2,29E-02	**44,4**

In conclusion, inter-assay variability of cytotoxicity has been too large, even for reference compounds and under these circumstances the software cannot help to analyse and compare the curves.

Possible causes for the intra- laboratory variability

Lack of reproducibility could be due to the physicochemical properties of the compounds assayed, many of them showing hydrophobicity and poor water solubility (digoxin and parathion). In addition, insolubility when diluting in culture medium in order to prepare working concentrations may occur (precipitation, crystal formation) Adsorption to plastic (cycloheximide) could also occur. Volatile compounds also show great variability among experiments.

Predictability/Indication of alert of bioactivation and/or hepatotoxicity

Table 4 show 25 compounds with low cytotoxicity (IC_{50}>1E-03). For 13 compounds IC_{50}s were higher than the highest soluble concentration of the compounds in culture medium (incomplete concentration-effect curves). In these cases, the MRTE value calculated by the software was used in the comparison of all the curves in the analysis of the data. First cytotoxicity in heptocytes and HepG2 (WP6) were compared to alert bioactivation and thereafter hepatocytes, Hep G2 and 3T3 were compared to alert bioactivation/hepatotoxicity.

In view of the high % CV in the data obtained in all cellular models (even for reference compounds; Table 3), the analysis of variance (ANOVA) does not identify statistical significant differences in the comparisons among different cells lines for a huge number of chemicals making it very hard to identify *alerts* of hepatotoxicity and/or bioactivation.

Hepatotoxicity alert

Some hepatotoxic compounds (acetaminophen, acetylsalicylic acid, tetracycline hydrochloride, verapamil hydrochloride, chlorpromazine, rifampicine, orphenadrine hydrochloride and parathion) showed very high %CV in all cellular models and were classified as *undefined* (Table 4). Other hepatotoxic compounds (classified as cytotoxic): ethanol, sodium valproate and amiodarone (reported steatosic), chlorpromazine, cyclosporine A and 17α-ethynylestradiol (reported cholestatic), pentachlorophenol and lindane (reported genotoxic), parathion (reported mainly neurotoxic) were not alerted, likely due to the fact that these toxic effects are chronic and not acute. Cyclophosphamide (low toxicity IC_{50}>1E-03) and carbamazepine was not alerted, while tamoxifen and acrylaldehyde were correctly alerted as hepatotoxic.

Bioactivation alert

Ten compounds reported as bioactivable in the literature were analysed. Acetaminophen and verapamil showed very high %CV in all cellular models and are classified as *undefined.* Several reasons justify that certain compounds were not alerted as bioactivable: orphenadrine hydrochloride (very high %CV in all cellular models), tamoxifen (data reported in human liver/hepatocytes), carbamazepine, cyclophosphamide (low toxicityIC50>1E-03), pentachlorophenol (reported genotoxic), sodium valproate (reported steatosic), and 17α-ethynylestradiol (reported cholestatic). Therefore, these compounds were not alerted, likely due to the fact that these toxic effects are chronic and not acute. However, amphetamine sulphate reported in the literature as bioactivable was correctly alerted in the assay.

Cytotoxic compounds

No difference was obtained in cytotoxicity among the three cellular models for the rest of compounds, any of them were reported as hepatotoxic and/or bioactivable, therefore they were correctly classified as cytotoxic.

In summary

The results suggests that only if intra-laboratory data (indeed inter-laboratory) for a given chemical are reproducible (%CV <15%) in all cellular models (especially for the reference compounds) the software may deliver consistent information for both ICs and MRTE. Consequently the analysis of variance (ANOVA) can identify statistical significant differences in the comparisons among different cell systems allowing identification of reliable *alerts* of hepatotoxicity and/or bioactivation.

The last part of the analysis is based on the comparison of the cells using the cut off configured by the user in order to know which cell type shows higher toxicity or if all of them are similar. Tentatively, cut-off values for IC_{50} ratios >2 have been proposed to alert on bioactivation or hepatotoxicity; lower ratios, are considered not reliable. When compounds elicited very weak toxicities ($IC_{50} \geq$ 1E-02 M) the ratio of IC_{50} values between pairs of cell models should be ≥ 5, to be considered an "alert".

Table 4. Predictability/Indication of alert of bioactivation and or hepatotoxicity

Chemical	**Number**	**Comment**	**IC_{50} > limit solubility**	**IC_{50}>>1E-03**	**Partner**	**Literature classification**	**Alert *in vitro***
25 compounds with IC_{50} > 1E-03							
Acetaminophen	1			>1E-02	P 3, 15,23,31	B/H	Undefined
Acetylsalicylic acid	2	No retested		>1E-03	P3, 15,23,31	(H St) C	Undefined
Caffeine	4			>1E-03	P, 15,23,31	C	NB/C
Colchicine	6			>1E-03	P, 15,23,31	C	NB/C
Isopropyl alcohol	10			>1E-02	P 3,15,23,31	C	NB/C
Phenobarbital	14	Uncomplete curve	1,00E-03	>1E-03	P, 15,23,31	C	NB/C
Nicotine	33			>1E-02	P23/P15	C	NB/C
Ethanol	37			>1E-02	P23/P15	(H St) C	NB/C
Parathion	38	High variability	1,00E-03	>1E-02	P31	(B/H? Neuro) C	undefined
Diethylene glycol	43			>1E-02	P23/P15	C	NB/C
Diquat dibromide	44	High variability, Uncomplete curve	1,00E-03	>1E-02	P31	C	NB/C
Sodium fluoride	48			>1E-03	P15/ P3	C	NB/C
N,N-Dimethylformamide	51			>1E-02	P15/ P3	C	NB/C
Ethylene glycol	54			>1E-02	P15/ P3	C	NB/C
Methanol	55			>1E-02	P15/ P3	C	NB/C

Sodium chloride	57			>1E-02	P31	C	Undefined
Lithium sulfate	60			>1E-02	P15/ P3	C	NB/C
Thallium sulphate	66		1,00E-03	>1E-03	P31	C	Undefined
Warfarin	67		6.5E-03	>1E-03	P31	C	Undefined
Acetonitrile	92			>1E-02	P15/ P3	C	NB/C
(-)Epinephrine (+)bitartrate	96			>1E-03	P15/ P3	C	NB/C
Chloral hydrate	76			>1E-03	P15/ P3	C	NB/C
Chlorpromazine	Extracompound			>1E-03	P3	(H Cho) C	NB/C
Amphetamine Sulphate	Extracompound			>1E-03	P3	B/H/C	B
Cyclophsphamide	Extracompound			>1E-02	P3	B/H	NB
13 compounds with uncomplete concentration-effect curves							
Digoxin	9	Uncomplete curve	1,00E-04		P, 15,23,31	C	NB/C
5-fluorouracil	17	Uncomplete curve	8,00E-03		P23/P15	C	NB/C
Pyrene	23	Uncomplete curve	3.5E-05		P23/P15	C	NB/C
Hexachlorobenzene	26		3.5E-03		P31	C	NB/C
Tetracycline hydrochloride	31		3,00E-05		P, 15,23,31	(H St) C	Undefined
Lindane	34	Uncomplete curve	1,00E-04		P23/P15	(H/Gen) C	NB/C
Glufosinate-ammonium	41		3.5E-05		P31	C	Undefined
Ochratoxin A	45	Uncomplete curve	1.25E-04		P31	(Nephro, Gen) C	NB/C
Propranolol hydrochloride	63		1,00E-05		P15/ P3	C	NB/C
Arsenic trioxide	64	Uncomplete curve	1.5E-05		P15/ P3	C	NB/C

2,4-dichlorophenoxyacetic acid	77		1,00E-03		P31	C	NB/C
Cyclosporine A	46	Uncomplete curve	1,00E-05		P, 15,23,31	(H Cho) C	NB/C
Strychnine	80	Uncomplete curve	5,00E-04		P15/ P3	C	NB/C
23 compounds with complete concentration-effect curves							
Sodium selenate	91				P15/ P3	C	NB/C
Diazepan	8				P, 15,23,31	C	NB/C
Amitryptiline hydrochloride	53				P15/P3	C	NB/C
Tamoxifen	Extra compound				P3	B?/H	NB/H
Atropine sulfate monohydrate	3				P 3,15,23,31	C	C
Carbamazepine	5				P 3,15,23,31	B/H	C
Malathion	11				P 3,15,23,31	B Neuro/C	NB/C
Mercury (II) chloride	12				P 3,15,23,31	C	NB/H
Pentachlorophenol	13				P, 15,23,31	(B/H Gen) C	NB/C
Sodium lauryl sulfate	15				P 3,15,23,31	C	C
Sodium valproate	16				P 3,15,23,31	(B/H St) C	C
Tert-butylhydroperoxide	19				P23/P15	C	NB/C
Acrylaldehyde	20				P23/P15	H	NB/H
Cadmium (II) chloride	21				P23/P15	C Neuro	NB/C
Amiodarone hydrochloride	28				P 3,15,23,31	(H St) C	C
Verapamil hydrochloride	29				P, 15,23,31	B/H	Undefined

Rifampicine	30				P, 15,23,31	(H Cho) C	Undefined
Orphenadrine hydrochloride	32				P, 15,23,31	B/H	NB/C
Dichlorvos	39				P31	(B?/H? Neuro) C	Undefined
Physostigmine	40				P31	C	Undefined
Cis-diammineplatinum (II) dichloride	42				P23/P15	C	NB/H
17a-ethynylestradiol	47				P, 15,23,31	(B/H Cho) C	NB/C
Cycloheximide	7	Binding to plastic			P, 15,23,31	C	C

NB: Non- bioactivable, C: Cytotoxic, B: Bioactivable, H: Hepatotoxic, Cho: Cholestatic, St: Steatosic, Gen: Genotoxic, Neuro: Neurotoxic, Nephro: Nephrotoxic

New strategies to incorporate metabolic capabilities into cell lines (WP6: P3 and P15)

Recombinant-defective adenoviral vectors encoding for major CYP genes (CYP 1A2 2A6, 2E1 and 3A4) involved in foreign compounds metabolism have been generated by Partner 3. These adenoviruses have been used to deliver selected CYP genes into hepatoma cell lines. This procedure requires the use of cells having all the other elements required for the functional expression of drug metabolising enzymes (such as cytochrome reductase, heme synthesis etc.). These adenoviruses have been used to deliver selected CYP genes into hepatoma cell lines. Following aspects have been examined, standardised and defined:

Evaluation of adenoviral vector stability

Amplification, titulation, and testing activity of adenoviral stocks for CYP 1A2 2A6, 2E1 and 3A4 have been performed. Furthermore, evaluation of adenoviral vector stability (freeze/thaw) upon time, cytotoxicity viral particles assessment have been performed: Evaluation of linearity between MOI (number of active viral particles) and cell viability for adenovirus encoding for CYPs on HepG2 and assessment of the correlation of number of viral particles vs. enzymatic activity on HepG2 cells have been performed. No cytotoxicity was observed when cells were infected with the recombinant adenovirus for 24h at a 60 and 100 MOIs. The protocols have been transformed from P3 to P15 to be standardised and adapted to a higher throughput format. Inter-laboratory variability is being investigated with respect to activity measurement through fluorescent substrates (in collaboration with P3) using different plate fluorescence readers. Assay miniaturization was also accomplished, allowing the use of 96 well plates and decrease the virus amounts necessary, thus making the process more time and cost effective and amenable to HTS toxicity platforms. The goal was to demonstrate the applicability of the method developed as an *in vitro* screening tool to study CYP metabolic dependent toxicity.

Proof of concept of CYP3A4 and CYP2E1 bioactivation

To verify the applicability of this model, a test compound which requires CYP3A4 bioactivation to be toxic, was evaluated. CYP3A4, the most abundant P450 in human liver, constitutes a good example for the proof of concept of the proposed approach to study CYP metabolic dependent toxicity. After assessment of metabolic transfected cell capability, transfected and non transfected cells were incubated for 24h with the selected drug. At the end of this period cell viability was measured by MTT test. Cytotoxicity of tamoxifen and cyclosporine A, compounds which are known to be metabolised to toxic metabolites by CYP3A4, and of tetracyclin, with unknown toxicity mechanism, has been investigated in HepG2 transfected with Ad-CYP3A4 cells and with HepG2 cells as control. In order to minimize the effect of GSH detoxification, HepG2 cells were treated with l-buthionine S,R-sulphoximine a potent GSH depletion agent. The results show a clear concentration-dependent cytotoxic effect of Tamoxifen and Cyclosporine A on HepG2 cells expressing CYP3A4 (infected with Ad-CYP3A4), while no effect was observed in HepG2 control cells. The protocols were transferred from P3 to P15 in order to be standardised and adapted to a higher throughput format. Repeats (n = 3 acceptable runs) were compared between laboratories of P15 and P3 to investigate inter-laboratory variability of the assay, and reproducibility of the results was good.

Implementation of new easy-to-use in vitro models with transient CYP 3A4, 2E1 expression in HepG2 cells to evaluate metabolism-dependent toxicity

The Adeno-CYP HepG2 model was adapted to 96-well plates. To verify the applicability of this assay, a short list of compounds within ACuteTox list was screened by the Adv-CYP3A4

and Adv-CYP2E1 models in a easy-to-use model. Tamoxifen, tetracycline, cyclosporine A, amiodarone, atropine sulphate, verapamil-HCl and SLS were tested in the new ready-to-use models (adenoCYP3A4- and 2E1-HepG2) at 8 concentrations in triplicate and in 3 independent experiments . Reproducibility of the assay was evaluated. CYP3A4 and CYP2E1 are very important human CYP450 involved in xenobiotic metabolism, thus constitutes a good way of studying CYP metabolic dependent toxicity. To verify the system applicability, a test compound which requires bioactivation to be toxic was evaluated. Tamoxifen is a well-known compound which is specifically metabolized by CYP3A4 into *N*-desmethyl-Tamoxifen, a very toxic compound. Results demonstrate the applicability of the *in vitro* system as a predictive screening tool for CYP mediated toxicity of chemicals. This model may be useful as a rapid relatively inexpensive *in vitro* assay for the prediction of CYP metabolism mediated toxicity and allow modifying the assay by simply transfecting (transiently) with an adenovirus of a different CYP isoform a good example to study CYP metabolic dependent toxicity. In conclusion, the results demonstrate the applicability of the developed *in vitro* model as a predictive screening tool, as well as an easy-to-use system to elucidate the mechanism, for CYP bioactivation-mediated toxicity of xenobiotics,

Computer-based prediction of metabolism and integration of metabolism data into toxicity screening (WP6: P7)

Measurements of ID, purity, stability and solubility data of 17 of the initial selected ACuteTox reference compounds were performed using established screening methods at P7.

Evaluation of computer-based prediction models for toxicity combining in vitro data on toxicity and PBBK-TD modelling:

The results obtained support the conclusion that PBBK modelling is a promising tool that is likely to improve the possibility to predict toxic doses after oral administration based on *in vitro* data, but also that it is reasonable to question the approach of using only basal cytotoxicity data for performing such predictions. GastroPlus™, a computer program presently used at P7, to simulate absorption and pharmacokinetics for orally dosed drugs based on their physicochemical and ADME properties. The recalculations were made using the permeability converter included in GastroPlus™: The results presented support the conclusion that PBBK modelling is a promising tool that is likely to improve the possibility to predict toxic doses after oral administration based on *in vitro* data.

Metabolic Stability of ACuteTox reference chemicals in hepatocytes and microsomes

In vitro half-life and intrinsic clearance of compounds on the ACuteTox list have been analysed, using rat liver microsomes (15 compounds analysed) human liver microsomes (32 compounds analysed), primary rat hepatocytes (15 compounds analysed) and cryopreserved human hepatocytes (21 compounds analysed). Data is presented as *in vitro* clearance (in µl/min/mg microsomal protein or µl/min/10^6 cells) as well as calculated Clint, hepatic clearance and extraction ratio using established scaling factors. Species differences as well as large differences between the metabolic stability in the presence of liver microsomes and hepatocytes were found for some of the compounds. Data on protein binding in human plasma for 29 compounds using microdialysis and LC-MS-MS was also generated.

***Computer-based prediction of metabolism and integration of metabolism data into toxicity screening*:**

METEOR predictions of 5 piperazines and 2 ACuteTox compounds have been evaluated using experimental as well as literature data. Major metabolites of a number of test

compounds (amiodarone, acetaminophen, acetylsalicylic acid, atropine, caffeine, carbamazepine, colchicine, cycloheximide, diazepam, nicotine, orphenadrine, phenobarbital, valproate and verapamil) identified *in vitro* by LC-MS-MS or found in the literature (*in vivo* or *in vitro* data) were compared to metabolites predicted by the METEOR software at different levels of probability. METEOR was found to predict most of the major metabolites (81%). In 7 out of 14 compounds all major metabolites were predicted correctly.

Evaluation of the METEOR and DEREK software, using in vivo data available in the literature and/or in vitro data generated for selected reference compounds

Evaluation of the DEREK predictive software using selected reference substances was included in the ACuteTox program. The toxicity of 17 substances from the ACuteTox program was predicted. For twelve of these substances, one or several DEREK alerts were obtained. Four of the substances where not flagged at all by the software. For one of these substances, carbamazepine, a possibly toxic metabolite carbamazepine epoxide was predicted by METEOR (confirmed *in vivo*). When a prediction for this metabolite was performed, several DEREK alerts were obtained. It is concluded that the combined use of DEREK and METEOR is likely to improve the possibility to predict the toxicity of an unknown substance and or its major metabolites. Further analysis needs to be performed before any general conclusion regarding the usefulness of DEREK for this type of analysis can be made.

A preliminary investigation on the possibility to use DEREK for ranking of compounds regarding their potential to induce acute toxicity has been performed. The rational for doing this, although acute toxicity is not an established endpoint included in DEREK, is that compounds that have the potential to induce acute toxicity (at least compounds being potent enough) might be expected also to induce one or more different types of subchronic or chronic toxicities at lower doses. It was found that nearly all substances in the dataset used (482 substances) that obtained one or more DEREK "hits" (310 compounds) had an oral LD_{50} dose close to or below 100 mg/kg. Only 2 of the compounds with an oral LD_{50} above 100 mg/kg (N-hexane and butyric acid) obtained any flag (both compounds received 1 flag). However, since only 16 of the compounds in the dataset had an oral LD_{50} above 100 mg/kg further analysis are needed before any general conclusions can be made.

Possible improvement of in vitro predictions of LD_{50}

Different approaches for possible improvement of predictions of LD_{50} doses based on *in vitro* data have been investigated by P7 (approach 1 and 2 n=~50 compounds, approach 3-5 n=~20 compounds [there were only ~20 compounds were fu in rat plasma, metabolic stability in rat microsomes and permeability in the Caco-2 cell model were available.]): 1) use of the software simCYP (Simcyp Ltd, version 8.00) for predictions of fraction absorbed/bioavailability and volume of distribution based on calculated properties. 2) same approach as 1) but in addition using the IC_{50}/fu instead of IC_{50} as estimates for the lethal target concentration. 3) use of experimentally derived *in vitro* kinetic data (Caco-2 data, IC_{50}/fu). 4) same as 3) but only including values on free fraction in plasma for compounds with very high protein binding. 5) same as 4) using IC_{50} values instead of IC_{50}/fu as estimates of lethal concentrations. It was concluded that approach 1 – 5 did not seem to give any improvements of the correlation between *in vitro* predictions of LD_{50} (in mg/kg) and *in vivo* mean LD_{50} in rat (obtained from AcutoxBase) as compared to a "direct" correlation.

New indicators for acute hepatotoxicity (WP7.3: P12)

<u>Biochemical parameters to assess hepatotoxicity</u>

P12 has explored end-point parameters that could be more predictive (specific) of hepatic damage, or better displayed in hepatic cells. The starting hypothesis was that the sensitivity (and perhaps the specificity) of these parameters could be better than the use of a basal cytotoxicity test, such as the MTT test. Therefore, P12 has explored the effect of the first set of 21 reference compounds on other functions as new indicators for hepatic toxicity.

Following biochemical functions were examined: cellular ATP levels, the formation of reactive oxygen species (ROS) as an index of oxidative stress, cellular protein content and the mitochondrial membrane potential. As a function of these results, the next question addressed was whether these early or late markers of hepatotoxicity would be incorporate as a part of a more general "alert" screening procedure. The results were compared with the MTT assays and suggest that neither ATP levels, ROS formation or protein content, when measured at early times (5 hours of incubation) or late times (24 hours of incubation), allowed a better discriminating effect than the one obtained by the MTT test. The performance of the MTT assay seems to be reliable under certain limits, however, too low number of model bioactivable compounds have been analyzed. In general, P12 found similar results with the investigated chemicals when using the above mentioned parameters, as well overlapping values.

As a function of these results, the next question addressed was whether these early or late markers of hepatotoxicity should be incorporated as part of a more general "alert" screening procedure. P12 has tested several end-point parameters that could be predictive of acute toxicity in cellular models. Toxicity of many compounds of the first 21 set of compounds was also tested in hepatocytes, HepG2 and 3T3 by the MTT test.

Development of an in vitro method to assess the effects of chemicals on the impairment of hepatocyte bile acids and bilirubin transport (WP7.3: P3 and P28)

<u>Generation of Cells Expressing Hepatocellular Uptake Transporters for Organic Anions</u>

P28 established a cell bank of 5 CHO cell lines expressing 5 human organic anion transport proteins (NTCP, OATP1A2, OATP1B1, OATP1B3, OATP2B1) and rat organic anion transport proteins (Ntcp, Oatp1a1, Oatp1a4, Oatp1b2). These transporters are expressed in human and rat liver respectively, and are involved in the uptake of endogenous substances such as bile salts and of xenobiotics (drugs and toxins) into hepatocytes. Each of these established cell lines heterologously expresses only one individual hepatocellular transporter. Hence, these cell lines are suitable for testing the substrate specificity of individual human and rat organic anion transporters. Furthermore, they can be used to test, whether the transfected transporters can mediate uptake of given xenobiotics into human or rat liver. These cell lines were predominantly used by partner 3 to characterize the transport properties newly developed test substrates for hepatocellular transporters.

<u>Development of Novel Substrates for Hepatocellular Organic Anion Transporters</u>

P3 has focused on the development of novel assays for the evaluation of impairment of hepatocyte bile acids and bilirubin transport. The strategies made use of chemically modified molecules mimicking bile salts to measure uptake of bile acids, and fluorescent conjugates to measure transport across cell membrane. New fluorescent analogues of bile acids have been

developed to be used to analyse the alterations in bile acid transport by hepatocytes. Freshly isolated suspended rat hepatocytes are an *in vitro* model to examine the bile acid uptake and the interactions between drugs and bile acids in this process. Two different types of fluorescent derivatives have been prepared: First, to synthesise a carboxylic acid derivative of cholic acid, fluoresecein was chosen as the fluorophore. In a second stage, a smaller fluorophore (4-nitrobenzo-2-oxa-1,3-diazole (NBD)) was chosen to prepare fluorescent analogs of cholic acid by derivatisation at positions C-3 and C-7. Later, pilot experiments for the use of the stably transfected cell lines developed by P28 (uptake systems), has been explored for its amenability in robotic systems. To this end, already synthesised compounds, as well new ones bearing new fluorophores were investigated.

Generation of Cellular Systems for Measuring Canalicular Secretion of Organic Anions

P28 established a prototypic LLC-PK1 model cell line stably expressing the basolateral bile salt uptake system Ntcp and the cananlicular bile salt eporter Bsep. This system allows simultaneous testing the interaction of xenobiotics both with uptake and export of bile salts, which can used as marker for liver disease. P28 also generated four recombinant baculoviruses expressing rat and human ABC-transporters expressed in hepatocytes (Mrps, Bsep, Mrp6 and BSEP). These baculoviruses are suitable to express the ABC-transporters of interest in the Sf9 insect cell line. Vesicles from infected Sf9 cells can be isolated and used to study the transport properties of individual ABC-transporters in the absence of other mammalian ABC-transporters using a standard protocol and radioactively labelled substrates. Since vesicle transport assays on the basis of radioactively labelled substrates are not easily applied in a high throughput set up, an alternate protocol assay was developed. This assay is based on the principle that ATP-dependent substrate transport leads to stimulation of ATP-hydrolysis by the respective transport substrate. ATP-hydrolysis can then be followed by detection of absorbance or fluorescence changes. Finally, a protocol outlining a methodological approach for a test system to identify alerts for substances, which are potentially hepatotoxic, was developed by P28. It is based on the clinical observation that inhibition of BSEP by drugs and xenobiotics constitutes an important pathophysiological mechanism of acquired cholestasis. As a result of both combined efforts, a protocol outlining a test system to identify (alert for) substances which are potentially hepatotoxic because of their capability to impair hepatic transport is expected.