

The Use of NeoHepatocytes for Assessment of Metabolism-Dependent Human Acute Toxicity

DISSERTATION

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Isabelle Pochic

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1.Referent: Prof. Dr. Albrecht Wendel, Uni Konstanz

2.Referent: Prof. Dr. Andreas Nüssler, TU München

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1. Introduction

1.1 The Liver

The liver has to perform a broadly based distribution of responsibilities in the body. These physiological functions include the synthesis of plasma proteins (e.g. albumin), regulation of lipid metabolism (e.g. biosynthesis of cholesterol), the excretion of bile and urea, the storage of glycogen, the release of carbohydrate, and the regulation of blood composition. As major immune organ, the liver has a lymphocyte population that is enriched in macrophages, natural killer and natural killer T cells, which are part of the innate immune system [1]. Additionally, the liver is the most important organ in metabolism of endogenous molecules as well as xenobiotics and their detoxification processes.

The liver cell population is composed of hepatocytes, bile duct-, Kupffer-, Ito-, and endothelial cells. Sinusoidal endothelial cells make up most of the nonparenchymal liver cells; the sinusoid and the bile duct are the transport vessels of the liver [Figure 1]. Thus, products of hepatic metabolism reach the intestine through the bile, and products of intestinal/bacterial metabolism reach the liver through the portal venous circulation. Ito cells store vitamin A and lipids and are found in the plasma-filled space of Disse as are the Kupffer cells, liver specific macrophages. The space of Disse is formed by the sinusoidal lining of the endothelial cells and the hepatocytes. The hepatocytes make up 80% of the volume, they have a polygonal shape with a diameter of 20-30 μm , typically with two or more nuclei.

The xenobiotic metabolism capacity is based on the hepatocytes, therefore they play a central role in the processing of endogenous and exogenous substances. By biotransformation, they detoxify and inactivate compounds such as steroids, bilirubin, and fatty acids, drugs and chemicals. They initiate the formation and secretion of bile salts, phospholipids and cholesterol into bile, one way to excrete waste product from the body. On the other hand, hepatocytes convert ammonia into urea for renal excretion.

There is an increasing demand for liver material and hepatocytes and hepatocyte-like cells in pharmacological and toxicological research and development, and in the more far future also for clinical applications. If metabolically competent, isolated and cultured human hepatocytes represent an important cellular system for the validation of chemicals with toxicological and pharmacological relevance [2].

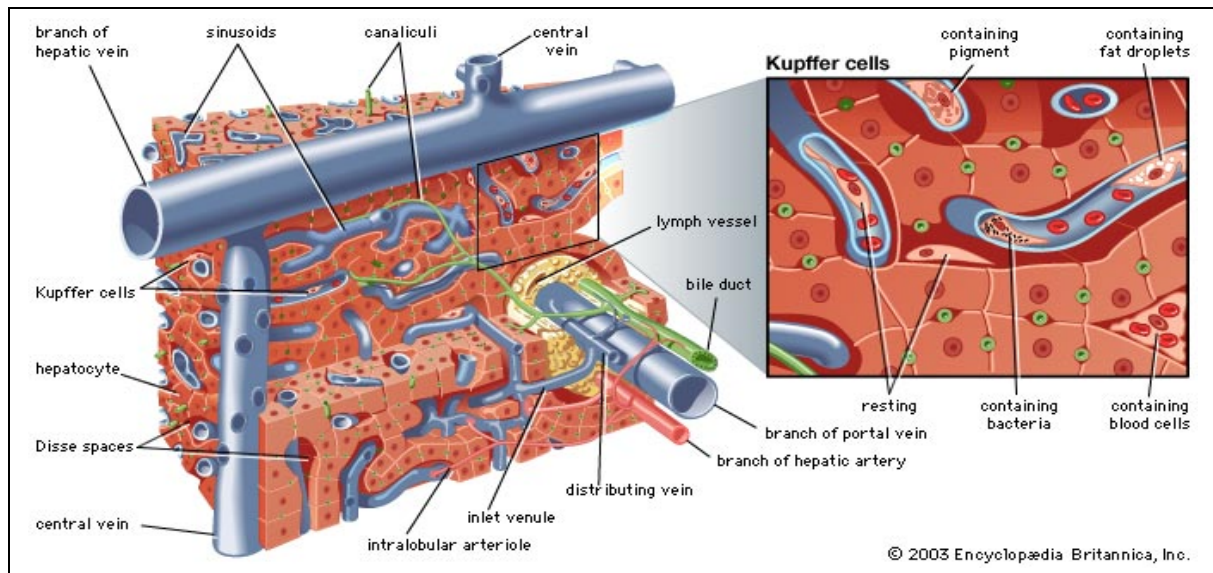


Figure 1
Structure of human liver. Encyclopædia Britannica Online[3]

1.1.1 Definition of a Hepatocyte

The definition of a hepatocyte found in the Merriam-Webster dictionary is as follows: any of the polygonal epithelial parenchymatous cells of the liver that secrete bile [4].

Unfortunately, this characterization is not sufficient to answer the question if any, maybe stem cell derived cell resembles a primary hepatocyte. There is still no consensus reached in the search for an alternative cell to replace primary hepatocytes. In *in vivo* studies, it was found that liver regeneration was due to fusion of transplanted cell with hepatocytes not to transdifferentiation. In addition, the liver-specific microenvironment influences the performance of transplanted cells. Hengstler and colleagues [5] suggest to perform not only qualitative assays (RT-PCR, IHC), but also a quantitative analysis of enzymatic activities as well as measurement of synthesis products (AST, urea, and fibrinogen synthesis) to allow a direct comparison with primary hepatocyte characteristics. A hepatocyte-like cell should have similar enzyme activities and functionalities as primary human hepatocytes. For the analysis of enzyme activities, they suggested substrates and inducers for *phase I* enzymes i.e. CYP1A1, CYP2A6, CYP2B6, CYP2C8/9/19, CYP2D6, CYP2E1, and CYP3A4/5, and *phase II* enzymes i.e. UDP-Glucuronosyltransferases, Glutathione-S-Transferases, and Sulfotransferases. The paper closes with the conclusion that cells lacking some of the mentioned activities/inducible capacities are a valuable tool for metabolism studies but may not be called hepatocyte.

Various liver models address different aspects of liver research, so it is important to ensure the essential features are still present in the used *in vitro* systems e.g. active enzymes are present in cells used for metabolism experiments, receptor are expressed in ligand-cell interaction studies, etc.

1.2 Xenobiotic Metabolism and Detoxification

Xenobiotics are compounds that are not synthesised by the organism and are therefore alien to the body. Everyday people ingest by breathing, through the skin or with the nourishments apart from the necessary nutrients also various chemical substances, i.e. xenobiotics. These compounds originate from natural or man-made sources in our environment like microbial impurity, chemicals, auto mobile exhausts, agriculture (pesticides), everyday life products (cosmetics, food), etc. At best, they are eliminated unmodified or comply with the required effect (medication), in the worst they become toxic and are hazardous or even lethal to the whole organism.

Once incorporated e.g. with food, absorbed in the gastro-intestinal tract, the xenobiotic reaches the blood, binds reversible to plasma proteins, and is transported by the circulation to the various organs. Even though the kidney and the colon play a role in xenobiotic metabolism, the liver is the major metabolic active organ with its hepatocytes. The enzymes, located at the endoplasmic reticulum or soluble in the cytoplasm, modify the substances, mostly lipophilic substances by biotransformation into metabolites for better excretion. Paradoxically, it also occurs that an initially harmless substance is converted in to an electrophilic metabolite, highly reactive, and toxic to the organ and the organism.

Metabolic reactions of biotransformation and detoxification are classified in the two phases functionalisation (*phase I*) and conjugation (*phase II*). *Phase I* includes among others the CYP450 system (see below), the Flavin-containing monooxygenase (FMO) system, the Epoxide hydrolase, the Alcohol dehydrogenase (ADH). *Phase II* are conjugation reactions catalysed by transferases such as Glutathione-S-transferases, Glucuronosyl transferases, Sulfotransferases, N-acetyl transferases, etc.

The term *phase III* is often used to describe reactions affecting the products of *phase II* metabolism, but several publications specify it as the positive removal of xenobiotics from the body i.e. the primary active excretion into bile and this elimination process [6, 7].

1.2.1 The Cytochrome P450 System

One group of the enzymatic system of bioactivation and detoxification in vertebrates is the super family of Cytochrome P450 monooxygenases (CYP). Their expression level is regulated by nuclear receptors [8, 9]. They are heme containing enzymes [10] with very wide substrate specificities due to the large number of isoforms or isoenzymes [11-13].

The monooxygenases have already been located in many different tissues [14]: in liver tissue [15] and in kidney tissue [16], in brain [17] and blood cells [18-20] and in macrophages [21, 22, 20], in the respiratory [23] and in the gastro-intestinal tract [24, 25], in the oesophageal mucosa [26] and in the mammary, ovary and uterus [27-29].

Drug metabolism and consequently drug toxicity or therapeutic ineffectiveness is dependent on genetic polymorphisms, age, sex, nutrition, hepatic disease and endogenous chemicals, and the substance administered [30-34]. Polymorphisms are described for a variety of different proteins involved in the metabolic system, e.g. the multidrug resistance transporter p-glycoprotein MDR1 [35], CYP 2C9, CYP 2D6, and CYP 3A [36]. In literature, human individuals are classified as "poor metabolisers", "intermediate", "extensive" and "ultrarapid metabolisers" [37], depending on the alleles coding for the drug-metabolizing enzymes and transporters and the repetition of the gene.

The Cytochrome P450 monooxygenases of metabolism *phase I* normally convert numerous endogenous substances and ingested toxins into harmless and easily excreted compounds. This includes the enzymatic addition of oxygen or removal of hydrogen. By oxidation, hydroxylation, reduction, or hydrolysis nonpolar molecules become more hydrophilic. Substances already possessing an appropriate group, for example a hydroxy group, may completely bypass *phase I* and be directly conjugated in phase II.

The transformed, reactive metabolites may be the activated therapeutics (codeine → morphine, CYP2D6) or may become toxicants (Paracetamol → NAPQI, CYP2E1) that may also be mutagenic (see next chapter).

In *phase II*, the intermediates are transformed by conjugation reactions (with glucuronic acid, sulfonates, glutathione, amino acids or others small molecules). The majority is then inactive and may be excreted by the renal system.

In conclusion, the role of liver metabolism is to modify substances to be eliminated more easily, but it also forms molecules toxic for the human body. That may lead to liver disease or even to death.

1.3 Hepatotoxicity

Toxicity is the lethal effect on a whole organism, an organ (organotoxicity), or a cell (cytotoxicity) or by chemicals (drugs, gas), biological toxic entities (virus), or physically toxic entities (radiation).

Hepatotoxicity is the noxious effect of substances such as pharmaceuticals and other chemical compounds on the liver. Because it is the major organ of bioactivation (metabolic activation of foreign compounds into reactive, toxic metabolites) and detoxification of xenobiotics, the liver plays the central role in risk assessment of such substances. Other than molecules that are directly toxic, there are substances that develop their toxicity by passage of the liver due to metabolism. The fatal effect may be limited to the liver (oxidative stress, necrosis, hepatitis), but may result additionally transmit to other organs (cerebral oedema, hepatic encephalopathy); or may finally lead to a breakdown of the whole organism (acute liver failure, death).

Gender, age, genetic polymorphisms, and environmental influences [38, 31, 39] play a potential role in the sensitivity of individuals to metabolism dependent hepatotoxic effects.

1.3.1 Paracetamol Toxicity

Paracetamol (APAP, N-acetyl-p-aminophenol; Benuron®, Tylenol®) is widely used in the treatment of slight to moderate pain and/or fever, but has only weak anti-inflammatory properties compared to Non-steroidal anti-inflammatory drugs (NSAIDs). Platelet aggregation is not affected. There is an ongoing discussion in scientific literature about a consequential target of APAP activity [40, 41] [Figure 3]: APAP inhibits Cyclooxygenase (COX) in the brain but not in the peripheral sites, which is held responsible for the analgesic effect. APAP induces the nuclear receptor CAR (Constitutive Androstane Receptor), this increases CYP activity, and CYP2E1 metabolises the prodrug to the short-lived metabolite N-Acetyl-p-Benzoquinonimine (NAPQI). This quinone imine is eliminated from the liver by reaction with the thiol-group of glutathione (GSH), to be excreted by the renal system. If the availability of GSH is insufficient, the toxic quinone will accumulate and react with cellular proteins and nucleic acids in the liver [34]. The accumulation of reactive oxygen species (ROS) is followed by lipid peroxidation and DNA damage. Eventually this yields to fatal hepatic failure. GSH is also one of the important antioxidants in the body; consequently, the antidote of choice is N-Acetylcystein (NAC) [35-37], an antioxidant, to replace the missing thiol.

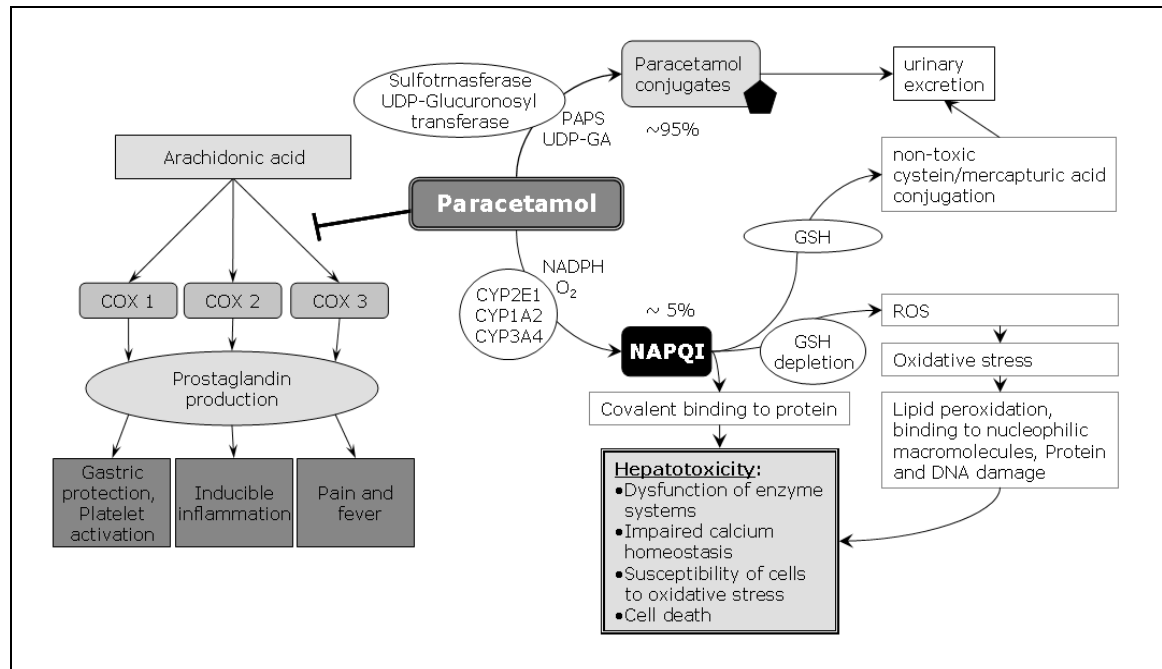


Figure 2
Paracetamol effect and metabolism, adapted from [42],[43], and [44].

Paracetamol hepatotoxicity is the leading cause of drug induced liver failure. Essential work in the past demonstrated that Paracetamol-induced liver cell injury is unlikely to be due to a single mechanism rather than to the coincidence of various deleterious processes.

1.4 Models of Hepatotoxicity

Various approaches [45] exist to predict human hepatotoxicity, ranging from animal models *in vivo* (mainly with rodents) [46], humanised mice [44], isolated perfused liver *in situ* [47, 48] to primary animal cell cultures to human cell cultures and to the gold standard right now, i.e. primary human hepatocytes *in vitro*. However, *in vitro* primary hepatocytes do normally not proliferate and lose their cellular functions (enzyme activities, etc.) after only a short time. Therefore different groups have made the attempt to immortalise primary hepatocytes [49-52].

It is long known that the expression pattern of metabolic active enzymes varies in diverse species [53-55]. Hence, hepatotoxicity is not always predictable based on results from animal models or animal cells. Several drugs had to be withdrawn from the market because of unpredictable specific liver failure. *In vitro* studies of drug-drug interactions or multiple substance toxification based on human tissue material [56] are also of importance.

Different studies of liver metabolism using hepatoma cell lines like HepG2 or HUH-7 are published. But this type of cancer cell lost parts of its ability to metabolise and must therefore be transfected [57] with the desired P450 enzyme for metabolic tests. Many attempts to reach this goal were only partially successful, and the whole spectrum of metabolic enzymes compared to a primary hepatocyte was never reached.

There is an increasing demand of hepatocytes on the market. Since freshly isolated human hepatocytes are only occasionally available, cryopreserved hepatocytes [58] may offer new possibilities. A new approach to the shortage of liver cell material might be offered by stem cell technology.

Stem cells offer the advantage that they can be continuously cultured in an undifferentiated state and may be induced to develop into more differentiated cells of the human body such as heart and liver tissue, bone marrow and blood vessel cells, pancreatic islet cells, nerve cells, etc.

With this great ability, stem cells are predestined for the development of exclusive, *in vitro* model systems to test drugs and chemicals, to predict or anticipate hepatotoxicity in humans. But on reasons other than scientific ones, embryonic stem (ES) cell research evokes controversy all over the world. Alternatively, adult stem cells [59, 60] are analysed for their potential to become hepatocyte-like.

As an alternative to ES, the study in hand started out on the base of dedifferentiation of cells of monocytic origin. Blood cells are induced by growth factors to regress into stem cell-like cells and then hepatocyte-like cells may be generated [61, 62].

The generation of hepatocytes or hepatocyte-like cells from stem cells or progenitor cells offer a long-term alternative as the need arises for donor organs and human hepatocytes.

1.5 REACH - A New Chemical Regulation in the EU

In February 2001, the EU Commission started a regulatory initiative (white paper [63]) setting out the strategy for a future chemicals policy. The regulation known as REACH (Registration, Evaluation and Authorization of Chemicals) [64-68] states the evaluation of risk of chemical substances produced, used or imported in quantities of 1–100 tons per year in the EU.

“The main objective of the new Chemical Strategy is to ensure a high level of protection for human health and the environment, while ensuring the efficient functioning of the internal market and stimulating innovation and competitiveness in the chemical industry.”[69]

REACH Timeline

1st of June 2007	REACH enters into force Pre-registration phase starts
2007 - 2008	PRE-REGISTRATION Pre-registration of all phase-in substances supplied at more than 1 tonne per year includes substances that are: <ul style="list-style-type: none"> • EINECS listed (=existing substances) • EU manufactured • have been existent for 15 years but not yet placed into market • No-longer polymers
2008 - 2011	REGISTRATION First phase of registrations Valid for substances supplied at 1,000 tonnes or more and some other priority high-risk substances
2011 - 2013	REGISTRATION Second phase of registrations Must be completed 6 years after REACH comes into force Will apply to substances supplied at 100 tonnes or more
2013 - 2018	REGISTRATION Final phase of registrations for substances supplied at 1 tonne or more

REACH does not differentiate between new and already existing chemicals; so 30,000 chemical substances that are produced in volumes of more than one tonne per year have to be brought under a single regulatory system.

A new organisation, the European Chemicals Agency (ECA) was launched to handle the first phase: Registration involves submission of a technical dossier of information about the substance; the required data depend on the volume of production or import. The ECA internet database of substances will contain the substance information collected in the framework of the registration procedure, and it will be publicly accessible except for information that is confidential. The following evaluation process will be crucial for the decision if further test will be necessary.

Chemicals that are carcinogenic, mutagenic, toxic for reproduction, very persistent or very bioaccumulating will have to be authorized. If specific safety instructions are required and if measures should be installed to protect human health and environment, will be decided in detail.

The German Federal Institute for Risk Assessment (Bundesinstitut für Risikobewertung) [70] predicts as a consequence of REACH, the need for 45 million animals during the next 15 years. This number may be reduced to 7.5 million animals (mainly rodents) over the same period, if new, including many methods and concepts without animal experiments were to be used without reducing the level of health protection. Thereof 80% of animals would be needed for chemical exposition during gravidity and infantile development.

Hence, there is the urgent need for the development of animal-free methods for risk assessment [71]. In practice, this means that approximately 30,000 chemical compounds have to be re-evaluated regarding their hazardous potential by using alternative *in vitro* test systems [72, 73].

"All in all, REACH will contribute to reduced pollution of air, water and soil as well as to reduced pressure on biodiversity. Improved control of persistent bio-accumulative and toxic substances is needed to ensure these substances are prevented from polluting the environment as once there they are very difficult to remove. In addition, REACH will help to reduce the effects from endocrine disrupting chemicals." DG Environment, European Commission [74].

1.6 The EU Research Project For Alternative Testing - An Integrated Project within the 6th Framework Programme

The main aim of the integrated acute systemic toxicity project (IP ACuteTox) [75] is the optimisation and prevalidation of an *in vitro* test strategy designed to predict human acute toxicity. ACuteTox has to explore innovative tools and cell systems and identify new end points and strategies to better anticipate human toxicity [76]. The risk assessment of chemicals based on *in vitro* studies in combination with computer simulation of their biokinetic behaviour will have to be rendered possible.

The central hypothesis of this EU project is that acute toxicity tests are feasible with cell cultures instead of animal testing.

Since 2005 ACuteTox is assigned to developing an *in vitro* test strategy with the participation of 35 partners in 13 European countries, coordinated by Dr. Cecilia Clemedson, Stockholm, Sweden and Dr. Leila Risteli, Oulu, Finland.

The scientific objectives of ACuteTox are:

1. record, critically evaluate and generate *in vitro* and *in vivo* data for comparative analyses,
2. identify factors (kinetic, metabolism and organ specificity) that influence the *in vitro*–*in vivo* correlation, and define an algorithm accounting for this,
3. explore innovative tools and cell systems to identify new end points and strategies to better anticipate human toxicity,
4. design a simple, robust and reliable *in vitro* test strategy amenable for robotic testing.

1.7 Approach to an *in vitro* Model for Risk Assessment based on NeoHepatocytes

Hepatocyte-like cells were lately made available from terminally differentiated human peripheral blood monocytes, prepared from buffy coats [77, 78, 62]. By culturing those under suitable conditions, monocytes dedifferentiate into PCMO (programmable cells from monocytic origin) and differentiate into hepatocyte-like cells.

1.7.1 Generation of NeoHepatocytes

Prof. F. Fändrich's group [62] cultured peripheral blood monocytes for six days in medium with M-CSF and IL-3 to generate PCMO. In the following differentiation phase the cells were kept in medium with FGF-4 to generate hepatocyte-like cells i.e. NeoHepatocytes [Figure 4]. The PCMO and the NeoHepatocytes were compared to primary human hepatocytes:

PCMO grew confluent by increasing cell size and cell number, *de novo* proliferation and DNA synthesis were measurable. The authors concluded, also based on the FACS analysis of PCMOs (up-regulation of stem cell markers) that the cells are comparable to stem cells.

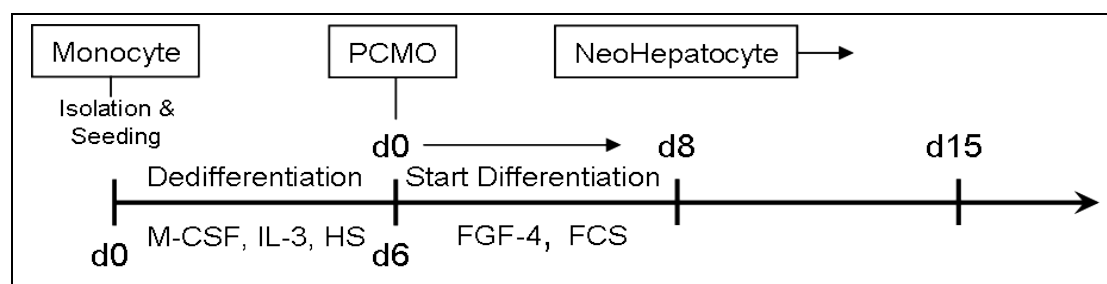


Figure 3

Timeline of NeoHepatocyte generation, cytotoxicity tests were implemented after day 8 of differentiation

After the differentiation phase, NeoHepatocytes showed similarities with primary hepatocytes in a variety of aspects: hexagonal morphology, confluent cell layers with intercellular contacts by gap junctions and expression of connexin 32, expression of epithelial marker proteins cytokeratin 18 and pan-actin. The new cells also expressed functions comparable to primary cells such as expression of fetoprotein, coagulation factor II, albumin and urea synthesis and secretion. In addition transcripts for carbamyl phosphate synthetase I were found. Metabolism phase I activity, i.e. cytochrome P450 enzymes, (incl. CYP1A2, CYP2A6, CYP2B6, CYP2C8–9, CYP2E1, and CYP3A3–5) was successfully induced by treatment with methylcholanthrene and assayed by the generation of 7-OH-coumarin via 7-Ethoxycoumarin O-deethylase. Metabolism phase II activity was assessed as glucuronidation of 4-methylumbelliferone by uridine diphosphate. The authors suggest that NeoHepatocytes resemble primary human hepatocytes with respect to the phenotype and specialised metabolic activities and report that the phenotype was stable *in vitro* for 15 days after the initial dedifferentiation phase.

1.7.2 The use of NeoHepatocytes in Toxicity Assessment

At first glance, NeoHepatocytes seem to be an ideal cellular system suitable for large-scale risk assessment studies. The PCMO can be continuously generated in large amounts. The NeoHepatocytes resemble primary human hepatocytes in morphology, expression of hepatocyte markers, various secretory and metabolic functions, and drug detoxification activities. The maintenance of the metabolic balance is kept well for up to weeks[61].

With particular respect to the new EU regulation concerning REACH, there is an ultimate need for metabolically competent human cellular test systems suitable to perform risk assessment studies on a large scale [79]. High throughput screening (HTS) applied by the pharmaceutical and biotech industries to select hits in extensive compound collections represent an opportunity to increase the capacity of cytotoxicity testing.

For that reason, the aim of this project was to check the suitability and robustness of NeoHepatocytes regarding their potential use in risk assessment for chemicals with a HTS robot, especially focusing on chemical compounds that undergo metabolic activation/toxification in the liver.

Metabolically toxified chemicals with a proven toxicological and pathological background in humans were selected for toxicological studies in cooperation with ECVAM and the IP ACuteTox consortium.

NeoHepatocytes from multiple donors were treated with these substances and cytotoxicity was assessed. With these results, a Standard Operating Procedure (SOP) was set up. Based on that protocol, a comparative study with three different laboratories was carried out. In a final step, the SOP was transferred onto an automated platform at the ECVAM/Joint Research Centre of the European Commission located in Ispra, Italy.

The use of NeoHepatocytes raises the hope for a new *in vitro* metabolising system. It is now a promising candidate in the ACuteTox program with the objective to become part of a validated assay [76].

1.8 Cell Death

There are multiple modes of cell death identified. Apoptosis also referred to as the suicide of cell, plays the counter part to necrosis that occurs as an uncontrolled progressive degradation of the cells. These two cell death processes contrast cytotoxicity that does not define a specific cellular death mechanism. Cytotoxicity is actually just the cell-killing property of a chemical compound (medicinal drug, venom, and toxin) or a mediator or mediator cell (TNF, T-cell), independent from the mechanisms of death.

1.8.1 Apoptosis

Apoptosis plays an important role in proper development (embryogenesis, metamorphosis), tissue homeostasis and malignancy defence i.e. destruction of cells that represent a threat to the integrity of the organism (infected immune cells, tumour cells). With cells that are introduced into the human body, there is always a risk for inflammation or even cancer. If the pathway of receptor induced cell death fails, damaged cells will no longer be eliminated. With regard to clinical application of hepatocyte-like cells, it is important to characterise this important suicidal ability for controlled cell-death.

The apoptotic process is commonly categorised in two parts: the intrinsic or mitochondrial pathway, which is triggered by external signals (UV, chemotherapy) or intracellular processes (DNA damage), and the extrinsic or death receptor mediated pathway (e.g. CD95/CD95L system).

Apoptosis is controlled by the balance between the withdrawal of positive signals, i.e. signals needed for survival, and the reception of negative signals. If the pathway of receptor induced cell death fails, damaged cells will no longer be eliminated. Most cancer cells protect themselves by a reduction of the necessary receptors on the outer membrane. The treatment of cells with ligands such as TNF [80], CD95-L or TRAIL yield information as to their ability of executing apoptosis.

In the context of the potential use of Neohepatocytes for re-population of partially damaged liver, it was therefore of interest in this study, to collect also some orientating data about the preservation of the sensitivity to endogenous apoptosis signals in these artificially created cells.

2. Aims of the study

The aim of this study was to investigate the usefulness and / or limitations of NeoHepatocytes for assessing acute toxicity in a human cellular system capable of metabolism.

The following working packages had to be passed:

- A suitable cytotoxicity assay with NeoHepatocytes for a selected set of chemicals had to be established.
- A Standard Operating Procedure had to be elaborated and harmonized. The delivery, quality control, and use of the cells according to this Standard Operating Procedure had to be adapted to a measurement on a robotic high throughput system
- As an extension, the applicability of this technology as an inexpensive, ethical and more scientifically based testing strategy for acute toxicity had to be critically explored.
- With regard to a future clinical application of NeoHepatocytes, it had to be evaluated whether NeoHepatocytes possess cell death properties comparable to hepatocytes.

3. Materials and Methods

3.1 Materials

3.1.1 Cell culture material

Cell culture plates and other plastic materials were purchased from Greiner (Frickenhausen, Germany). Cell culture medium RPMI 1640, FCS, DPBS, Accutase[®] were purchased from PAA laboratories (Pasching, Austria) and collagen was obtained from Serva (Heidelberg, Germany). FCS was inactivated by water bath incubation at 55°C for 30 min prior to addition to the medium.

The NeoHep II-Medium (containing FGF-4) was delivered with the cells from EUFETS AG (Idar-Oberstein, Germany), also for the experiments in other laboratories basal RPMI-Medium with glutamine was delivered with the NeoHepatocytes from EUFETS AG to each lab.

3.1.2 Animals

Specific pathogen-free C57Bl/6 wild type mice (24-29 g), from the in-house animal breeding station of the University of Konstanz, were maintained under controlled conditions (22°C and 55% humidity, constant day/night cycle of 12 h) and fed a standard laboratory chow.

All steps of animal handling were carried out according to the Guidelines of the National Institute of Health (NIH), the European Council (directive 86/609/EEC), and the national German authorities and followed the directives of Ethical Committee, University of Konstanz.

3.1.3 Cells

HepG2 cells

HepG2 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA).

Primary murine hepatocytes

For the preparation of primary murine hepatocytes from C56Bl/6 male mice the following material was used:

- Set of instruments (2 small rounded forceps, curved pair of scissors, spatula, iris scissors, clamp), black thread for ligatures, 26-gauge intravenous canula, teflon coated, 2 small hooks (bended canula on a rubber tube), lab tape
- Autoclaved beaker with gauze (100 µm), (nylon gauze, mesh size 0,1 mm, Qual. PA - 100/31 Nybolt, Cat. No. 10136, Eckert Franz GMBH (Waldkirch, Germany))

- Collagen coated dishes/plates (Collagen R (4°C) 1:10 in PBS+0,1 % acetic acid, 24-well-plate 200-250 µl/well, 6-well-plate 1 ml/well, overnight incubation at 37°C, 2x washing with PBS or Millipore, storage at -20°C)
- PPM-Addition for preperfusion medium: EGTA 4.75 g; Hepes 298 g; adjust to pH 7.4; Pen/Strep 250 ml; H₂O ad 1 L, use sterile filter 0.2 µm (FastCap™ with PES), 20 ml per 500 ml HBSS w/o Ca/Mg
- PM-Addition for perfusion medium: MgCl₂×6H₂O 4.75 g; Hepes 58.75 g; D-Glucose 25 g; BSA 50 g; adjust to pH 7.4; Pen/Strep 250 ml; H₂O ad 1L, use sterile filter 0.2 µm (FastCap™ with PES), 20 ml per 500 ml HBSS with Ca/Mg
- Pentobarbital (Narcoren®) was purchased from Sanofi Withrop (München, Germany), Stock 16 g/100 ml (1.5 ml), 1:16 dissolved in NaCl 0.9 % (2.5 ml), Heparin (1 ml), ca.100 µl per animal iv. by tail vein.
- Collagenase Typ IV for perfusion, 50 mg/10 ml in Hanks Puffer w/o Ca/Mg, 20x: 1 mg in 200 ml, centrifugation at 4°C/ 4000 rpm for 10 min, storage of aliquots à 10 ml at -80°C
- Percoll, Amersham Biosciences (Freiburg, Germany)

Primary human hepatocytes

Primary human hepatocytes were generously provided by Dr. N. Nüssler (Charité, Berlin). The hepatocytes were isolated from pathological inconspicuous specimens obtained from patients undergoing hepatic resections. Cells were seeded on 24-well plates and after 1 day shipped to Konstanz overnight.

NeoHepatocytes

NeoHepatocytes were obtained from EUFETS AG, Idar-Oberstein, Germany on a routine basis. The seeding, dedifferentiation and differentiation procedure was performed under defined conditions [78] by EUFETS AG, Idar-Oberstein, Germany.

Plate type	numbers of initially seeded cells
6-well	25x10 ⁶ cells per well
24-well	4x10 ⁶ cells per well
96-well	7.58 x10 ⁵ cells per well

The NeoHepatocytes had to be shipped to the test-performing laboratory at Konstanz. The company used to send their cells by parcel service. Therefore, it had to be investigated whether or not the shipment had an impact on the quality of the cells. The first trail deliveries revealed that

the assigned courier service was unable to deliver the cells in time to the right place. In the consequence the parcels contents lost their temperature control and the cells were considered to be stressed. The involved employees at EUFETS AG, at the courier service, and at the delivery department of the University of Konstanz were informed about the modalities of the shipments and were given all required phone numbers and email addresses to ensure a trouble free arrival of the cells in the Konstanz lab. EUFETS AG announced the shipment one day in advance per email. They marked the parcel with adequate signs (recipient's Phone number, "Store at 37°C", etc.) and added an inside/outside digital thermometer. The delivery department was instructed to call the lab right away by the time the parcel arrived.

For the inter-lab experiments in Berlin, Mannheim, Konstanz, and Ispra, the NeoHepatocytes -at day 15 after differentiation-, were sent by a carrier car or transported by a carrier company. Cells were constantly maintained in plates with medium, at a temperature 20-37°C inside the parcel (controlled by In/Out thermometer).

3.1.4 Automated Liquid Handling Workstation

The core of the automated test facility of the IHCP is based on the automatic liquid handling system "MicroLab Star" supplied by the Hamilton Company.

3.1.5 Substances

All testing chemicals used in this work were chosen in accordance to the recommendations of expert commissions ("A-Cute Tox") of ECVAM and the EU. (LSHB-CT-2004-512051)

For the interlaboratory comparison, substances were purchased from one partner and the lot evenly shared between the participating laboratories.

Table 1

Chemical	Distributor	CAS-No.	Solvent
(±)-Verapamil hydrochloride	Sigma, Germany	152-11-4	DMSO
17 α -Ethinylestradiol	Fluka, Germany	57-63-6	DMSO
5-Fluorouracil	Sigma, Germany	51-21-8	Medium
Acetaminophen (Paracetamol)	Sigma, Germany	103-90-2	DMSO
Acetylsalicylic acid	Sigma, Germany	19774-82-4	DMSO
Allyl alcohol	Fluka, Germany	107-18-6	Medium
Amiodarone hydrochlorid	Sigma, Germany	50-78-2	DMSO
Atropine sulfate monohydrate	Sigma, Germany	5908-99-6	Medium
Bromobenzene	Fluka, Germany	108-86-1	Medium
Caffeine	ICN Biochemicals Inc., USA	58-08-2	Medium

Chemical	Distributor	CAS-No.	Solvent
Carbamazepine	Sigma, Germany	298-46-4	DMSO
Colchicine	Sigma, Germany	64-86-8	Medium
Cycloheximide	Sigma, Germany	66-81-9	Medium
Cyclosporine A (Robot)	Sigma, Germany	59865-13-3	DMSO
Cyclosporine A (manually)	Sandoz/Novartis, Switzerland	(59865-13-3)	NaCl 0.9%
Digoxin	Sigma, Germany	20830-75-5	DMSO
DMSO	Sigma, Germany	67-68-5	Medium
Eserine	Sigma, Germany	57-47-6	DMSO
Ethanol	Fluka, Germany	64-17-5	Medium
Isoniazid	Sigma, Germany	54-85-3	Medium
Isopropyl alcohol	Fisher Scientific Chem., Germany	67-63-0	H ₂ O
Mercury chloride (II) (HgCl ₂)	Sigma, Germany	7487-94-7	Medium
Nicotine	Sigma, Germany	54-11-5	Medium
Orphenadrine hydrochloride	Sigma, Germany	341-69-5	Medium
Parathion	Sigma, Germany	56-38-2	DMSO
Rifampicine	Sigma, Germany	13292-46-1	DMSO
Sodium dodecyl sulfate	Sigma, Germany	151-21-3	Medium
Sodium Fluoride	Sigma, Germany	7681-49-4	Medium
Sodium valproate	Sigma, Germany	1069-66-5	Medium
Tetracycline	Sigma, Germany	64-75-5	DMSO

3.1.6 FACS Analysis

FACS-Calibur (Becton Dickinson, Heidelberg) with software CellQuest™ for Mac, Becton Dickinson, Germany

FACS buffer: CellWASH, Cat. No. 349524, BD Biosciences, Germany

Antibodies

CD95-FITC, Cat. No. 340479, Becton Dickinson Immunocytometry Systems, USA

CD14-APC, Cat. No. 555399, TRAIL-PE, Cat. No. 550516, and TNF-R II, Cat. No. 552418, BD Pharmingen™, BD Biosciences, Germany

TNF-R I, Cat. No. AB25471, Biozol Diagnostica Vertrieb GmbH, Germany

3.2 Methods

3.2.1 Cell Culture

Cell number and vitality for primary murine hepatocytes and HepG2 cells were assessed with Trypan Blue solution in a Neubauer-improved hemocytometer.

HepG2 cells

Cells were cultured in RPMI 1640 containing 10 % FCS in a humidified incubator at 5 % CO₂. Cells were split 1:5 three times a week, using Accutase® to detach adherent cells. Cells were allowed to adhere overnight before medium was changed to RPMI 1640 without FCS. Incubation of cells with potential hepatotoxic substances started 15 min after medium exchange. Incubations were carried out at 37°C in an atmosphere of 40% O₂, 5% CO₂, 55% N₂ and 100% humidity.

Primary murine hepatocytes

Isolation of hepatocytes from 8-12 weeks old mice was performed by the two-step collagenase perfusion method of Seglen [81] as modified by Klaunig [82, 83] and Leist [84]. Cells were additionally purified by centrifugation using a Percoll gradient modified from Osypiw [85]. To separate hepatocytes from remaining non-parenchymal cells, the pellet of the second centrifugation step (50×g, 2.5 min) was resuspended in 20 ml HBSS w/o Ca/Mg and mixed with 20 ml of an isotonic Percoll solution, HBSS w/o Ca/Mg ad. 50 ml, mixed by gently turning the tube, followed by centrifugation at 50×g for 11 min at room temperature. To remove remaining Percoll, the pellet was washed with HBSS w/o Ca/Mg by an additional centrifugation step (50×g, 2.5 min). Hepatocytes were plated in 500 µl RPMI 1640 medium with 10% FCS in collagen-coated 24-well plates at a density of 9×10⁴ cells/well. Cells were allowed to adhere for at least 4 h before medium was changed to RPMI 1640 without FCS. Incubation of cells with potential hepatotoxic substances started 15 min after medium exchange. Incubations were carried out at 37°C in an atmosphere of 40% O₂, 5% CO₂, 55% N₂ and 100% humidity.

Primary human hepatocytes

The cells were cultured in RPMI 1640 with L-Glutamin, supplemented with 10% FCS and maintained at 5% CO₂, 37°C, and 100 % humidity. Before the test, medium was changed to RPMI 1640 without FCS. Incubation of cells with potential hepatotoxic substances started 15 min after medium exchange. Incubations were carried out at 37°C in an atmosphere of 40% O₂, 5% CO₂, 55% N₂ and 100% humidity.

NeoHepatocytes

After arrival, medium (RPMI 1640 with 10% (v/v) FCS, 100 Units of penicillin/ml, 100 µg streptomycin/ml, 2 mM L-glutamine, 3 ng FGF-4/ml,

and without phenol red) was changed and plates were equilibrated over night in a humidified incubator at 5% CO₂ / 95% air. Starting with the Interlab tests the medium was changed to RPMI 1640 without FCS the night before the test. The next day medium was changed (RPMI 1640 without FCS).. Incubation of cells with potential hepatotoxic substances started 15 min after medium exchange. Incubations were carried out at 37°C in an atmosphere of 40% O₂, 5% CO₂, 55% N₂ and 100% humidity.

3.2.2 Treatment of cells

For the cytotoxicity study, the cells were incubated only with the chemical. If substances were dissolved in DMSO or Ethanol, the solvent alone was used in control incubations.

Cells were treated approx. 15 min after medium exchange (RPMI 1640 with L-Glutamin) with serially diluted substances. Cells were incubated for 20-22 h at 37°C. After incubation, supernatant (24-w: 100 µl, 96w: 75 µl) was transferred to 96-well plates and stored at 4°C for further LDH determination. Remaining medium was removed and replaced by lysis buffer (Dulbecco PBS with 0.1 % (v/v) Triton X-100). After 15 min, 25 µl of the lysate was transferred to 96-well plates, filled up with medium to 100 µl respectively 75 µl, and stored at 4°C for further LDH determination.

For the apoptosis study, cells were preincubated with ActD, CHX 30min before treatment with TNF α , or CD95L.

3.2.3 LDH-Assay

There are miscellaneous LDH assay kits on the market. The Cytotoxicity Detection Kit (Cat.No. 11 644 793 001, Roche Diagnostics GmbH, Mannheim, Germany). is a colorimetric assay for microplate format that is based on the cleavage of a tetrazolium salt. The measurement of LDH in serum-free supernatant and lysate represents a standardized parameter that has already been used in combination with the particular experimental setup in our lab, therefore cytotoxicity was determined by measuring Lactate dehydrogenase (LDH).

The enzyme Lactate Dehydrogenase (LDH) is a stable cytoplasmic component ubiquitously present in all cells. Upon substantial damage of the plasma membrane, it is released into the supernatant of the cell culture. LDH activity is determined by an enzymatic test: In the first step NAD⁺ is reduced to NADH/H⁺ by the LDH-catalyzed conversion of lactate to pyruvate. In the second step the catalyst (diaphorase) transfers H/H⁺ from NADH/H⁺ to the yellow tetrazolium salt INT, which is reduced to formazan (red). Therefore, the amount of colour formed in the assay is proportional to the LDH-release of the lysed cells. The formazan salt is water-soluble and shows a broad absorption maximum at 492 nm, whereas the tetrazolium salt INT shows no significant absorption at these wavelengths. The use of a spectrophotometric micro-plate reader (ELISA reader) allows the simultaneous measurement of multiple samples and thereby guarantees the easy processing of a large number of samples.

LDH was measured in cell culture supernatants (S) and in the remaining cell monolayers (C) after lysis with 0.1 % Triton X-100. Kit component were mixed 1:46 and added to sample wells, after a change in colour the plates were measured at 492 nm in a spectrophotometer. The percentage of lactate dehydrogenase release was calculated from the ratio of the OD of S/(S+C).

3.2.4 Standard Operating Procedure

A standard operating procedure is a set of instructions, covering features of operations to provide an exact protocol without loss of efficiency. The SOP was written based on the preliminary protocol and results. It was sent to the participating laboratories for the comparative study in advance. The SOPV2.1 is attached to this thesis as appendix.

3.2.5 Adaptation of the SOP for the automated workstation

The established SOP was sent to the laboratory at ECVAM/JRC. The engineer adapted the SOP for the requirements of the robot based on the operating experience (BALB/c 3T3 Neutral Red Uptake (NRU) in vitro cytotoxicity test [86]) offline (*in silico*) and on the HTS system. First test runs with BALB/c 3T3 cells using SLS as control compound were performed to implement the LDH-Assay on the automated platform.

The optimisation of the automated LDH-Toxicity assay for the NeoHep cells followed. The platform was adapted to cell culture plates from Greiner on which the cells were delivered.

Fife runs were performed on the PTP platform. In each run, seven compounds were tested. Paracetamol was tested as a positive control in all runs. Each seeded plate was treated with one test compound.

In one run the cells were serially treated with the compounds, followed by incubation for 22 h at 37°C, collection of the supernatant and lysate and immediate end-point measurement. The collected supernatant and lysate were not stored before measurement.

The following main experimental parameters are summarized in one excel file (OWL): Run ID, date, number of tested compounds, compound application time, chemical name, highest stock concentration, dilution factor, concentrations series, read-out time.

For each test compound, four optical density readings were performed and are reported in the OWL: supernatant at 492 nm and at 600 nm, also lysate at 492 nm and at 600 nm.

3.2.6 FACS-Analysis

Different approaches to get NeoHepatocytes off the plate failed (Trypsin-EDTA, Acutase, Lidoquaine), so finally the cells were scrapped off and the viability was controlled with Trypan Blue dye. The cell contents of three wells were pooled. 2 ml of FACS buffer was added per sample, gently mixed and centrifuged for 6 min at 250xg. The supernatant was discarded so that approx. 120 µl remained in the tube. The volume was split and

redistributed in four small FACS tubes (size 2ml). After vortexing shortly, the antibody was added and slightly mixed again. The samples remained at 4°C for 60 min. Undyed cells were used as control for background fluorescence. After the incubation, the cells were washed with 700 µl FACS buffer, centrifuged for 5 min at 650xg. The supernatant was discarded, 500 µl FACS buffer was added and vortexed. The samples were stored at 4°C until measurement.

The measurement was conducted with the software CellQuest™ (Becton Dickinson, Heidelberg). Forward scatter, sideward scatter were examined in a dot plot and the antibody corresponding channels were assessed. 10,000 events were measured per sample. The frequency of events was depicted in a histogram with the software WinMDI Version 2.9.

3.2.7 Data Analysis and Statistics

A p value <0.05 was considered as being significant. Interpretation of the data was performed using MS Excel (Microsoft), GraphPad Prism® version 4.01 for Windows, GraphPad Software, San Diego California USA. EC₅₀ values were calculated by non-linear regression curves, extrapolated EC₅₀ was not interpreted, but instead the non-sigmoid curve shapes were compared. Spearman Rank Order Correlation Coefficient was calculated by correlation analysis.

FACS-Analysis was performed using CellQuest™ for Mac, Becton Dickinson, Germany and data was interpreted with WinMDI Version 2.9 for Windows, Joseph Trotter, <http://facs.scripps.edu/software.html>.

4. Results

In the first part of this work a suitable cytotoxicity assay was chosen and adapted to the used cell culture systems. NeoHepatocytes were compared to primary human and murine hepatocytes and the human hepatoma cell line HepG2. A Standard Operating Procedure (SOP) for cytotoxicity testing with NeoHepatocytes was developed based on these experiments. The SOP was tested in an interlaboratory comparison study. Finally, the procedure was transferred onto a robot platform (High Throughput System, HTS) for further automatic handling.

4.1 Definition of a Standard Operation Procedure (SOP) for a NeoHepatocytes Based Toxicity Test System

4.1.1 Supply of NeoHepatocytes: Acceptance Criteria

To ensure standardized shipping and storage conditions, "Test Acceptance Criteria" were specified in the SOP. The NeoHepatocytes were regularly inspected by microscopy upon receipt. Cell deliveries that did not comply with the SOP were excluded, i.e. cells were only used if delivered before 11 am (approx. 18hrs after dispatch), temperature never dropped below 20°C inside the parcel, cells were still covered with medium, and no obvious contamination was observed. Additionally cell culture plates with anomalous appearance e.g. different morphology, <50% confluence were excluded and reclaimed at EUFETS AG. In Figure 4 four different samples are shown: acceptable, confluent grown NeoHepatocytes from day 9 (A) and day 16 (B) with hexagonal morphology and cell-cell contact, on the other hand non-confluent cells with small, round shapes (C) or fibroblast-like, small, long cells (D), neither one showing cell-cell contacts.

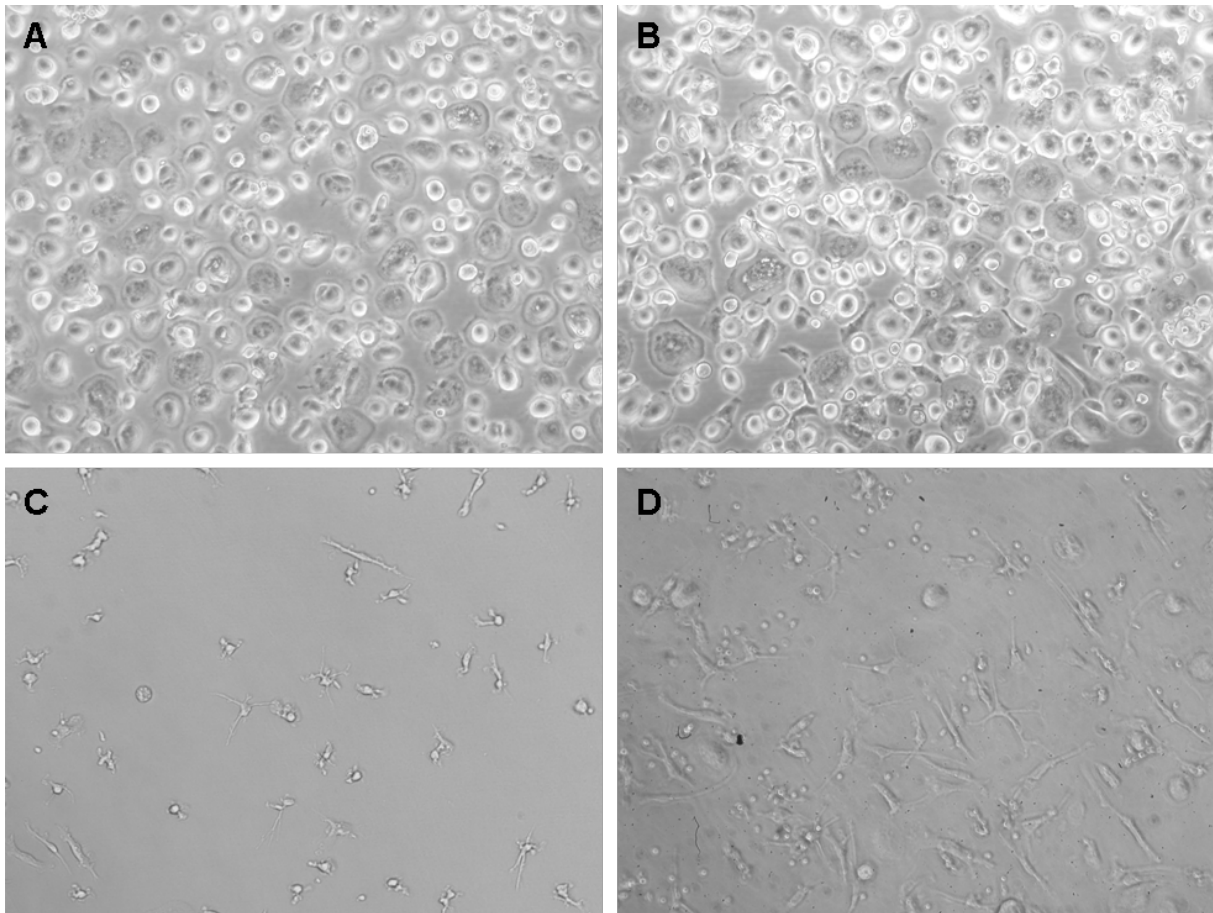


Figure 4: Microscopic pictures of NeoHepatocytes, Samples from different donors received from EUFETS AG: A: day 9; B-D: day 16, preparations C and D were excluded for testing.

4.1.2 Cytotoxicity Assay: Selecting the suitable End Point

The first approach in the assessment of cytotoxicity was to find a suitable assay for the proposed cell culture system based on NeoHepatocytes. Such a test system should be easy to handle manually as well as by High Throughput Screening (HTS), also with different cell types and it should be cost-effective.

Cell viability assays based on MTT or AlamarBlue depend on the numbers of cells in the well. Therefore, these assays detect variable growth rates, e.g. an inhibition of cell proliferation might be misinterpreted as cell death. However, the way of producing the NeoHepatocytes posed a challenge to the measurement of parameters of cell death: the cells are cultured on the plate for weeks and so the cell number per well can differ noticeably. Therefore, a kind of intra-well control was required to cope with disadvantages of the above-mentioned cytotoxicity/cell viability assays.

The lactate dehydrogenase (LDH) is an enzyme found in cells of many tissues, including the heart, liver, kidneys, skeletal muscle, brain, red blood cells, and lungs. It is responsible for converting lactate into pyruvate, an essential step in producing cellular energy. Cellular damage

causes, due to membrane leakage, an elevation in the release of lactate dehydrogenase from cells. Clinically, the LDH serum level is measured in the diagnosis of heart attack, anaemia, and liver disease. Increasing LDH release clearly correlates with the increase of dead cells, therefore the measurement of LDH-release and remaining cytosolic LDH in cells is a useful parameter for cytotoxicity assessment *in vitro*.

By means of the Cytotoxicity Detection Kit (Roche), LDH was measured in cell-free supernatant (S) and in the remaining lysate of cell monolayers (C) after lysis with 0.1 % Triton X-100. The percentage of lactate dehydrogenase release was calculated from the ratio of $S/(S+C)*100\%$. A basal level of LDH release (in the range of 20%) was found with all culture conditions, 100% LDH-release refers to absolute cell death. The background i.e. lysing buffer and medium on the plate was measured. Since background subtraction had no influence on the test results, it was and was therefore neglected in the tests.

When using a cell line or primary cells from the same animal strand to check for cytotoxicity, appropriate positive and negative controls are required. For direct cytotoxicity, sodium lauryl sulfate (a protein denaturant and ionic surfactant) or Triton X 100 treatment is commonly used for that purpose in viability or cell death experiments. The detergents disrupt the cell membrane irreversibly and thereby kill the cells. The negative control consists of untreated cells or cells treated with solvent (e.g. DMSO 1%, etc.).

The suitability of the Cytotoxicity Detection Kit was first tested with the commonly used liver cell line HepG2 on 24-well plates with serum-free medium treated for 20hrs with SDS (sodium dodecyl sulfate), a detergent that chemically disrupts the cell membrane.

The LDH-assay based on that Cytotoxicity Detection Kit showed to be a highly reliable method for general cytotoxicity read-out (Figure 1): The resulting graph for HepG2 cells shows a concentration dependent cytotoxicity with a basal LDH-release of 9% in the control cells and 73% at the highest concentration (0.7 mM SDS).

Then the LDH-assay was tested for reproducibility with other cells, i.e. primary murine and primary human hepatocytes and was compared to NeoHepatocytes (Figures 1.B-D).

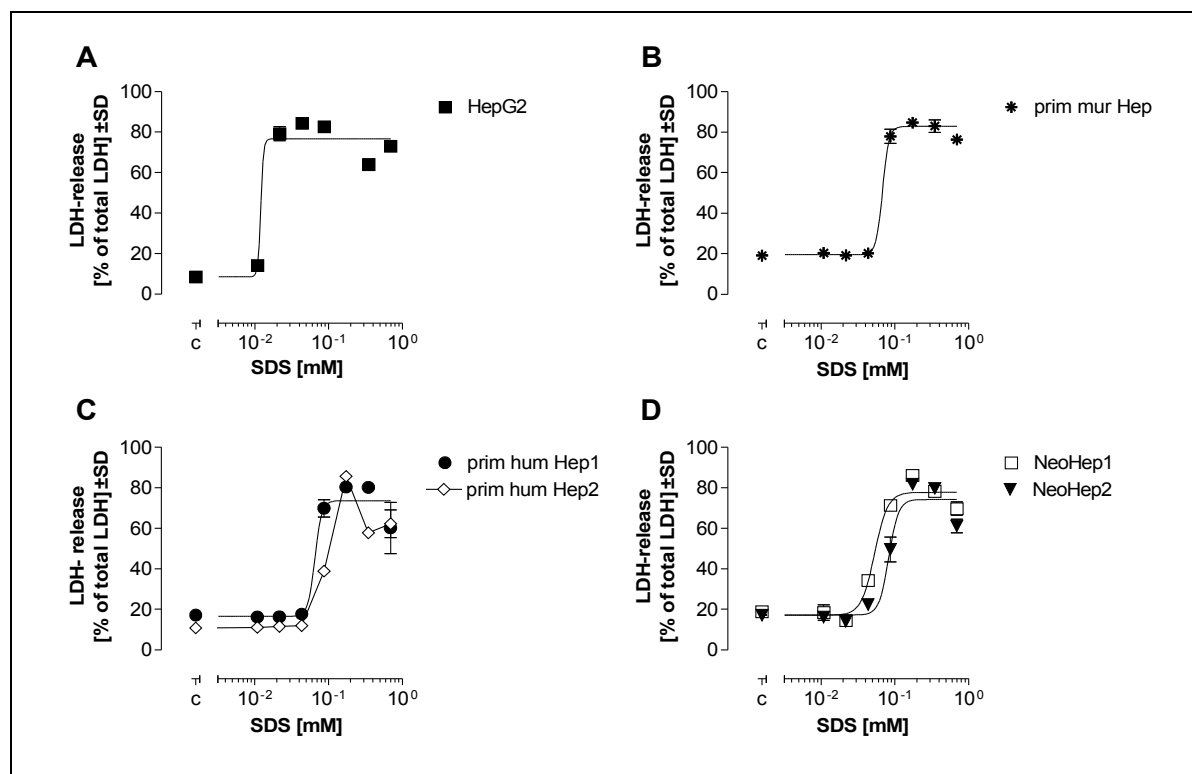
Compounds like SDS are toxic by chemically destroying the cell membrane. This treatment led to direct toxicity in all cell types (Figure 5, A-D). HepG2 cells showed an increasing LDH-release with the very first concentration of SDS, whereas in NeoHepatocytes and primary cells the third concentration of SDS showed an increase of LDH-release. The absolute toxicity was similar, ranging from 44% to 64%. The results from treatment with SDS are summarised in table 2.

Table 2:

Cytotoxicity of SDS on various liver cells as represented by LDH-release after 20hrs of incubation in 24-well plates.
The EC₅₀ was calculated using GraphPad Prism Software. Difference: absolute toxicity calculated from basal minus highest LDH-release [%]. na: No EC50 value could be calculated (*estimated value).

LDH release	NeoHep1	NeoHep2	HepG2	prim hum Hep1	prim hum Hep2	prim mur Hep
basal	19%	17%	9%	17%	11%	19%
SDS Top	70%	61%	73%	60%	62%	76%
Difference	51%	44%	64%	43%	51%	57%
EC ₅₀	0.05 mM	0.08 mM	0.01 mM	0.07 mM	na (0.09*)	0.07 mM

In the set-up with different cell types on 24-well plates and direct toxins the LDH-assay performed well in the application of cytotoxicity assessment.

**Figure 5:**

Concentration dependence of the cytotoxicity of SDS on various liver cells as represented by LDH-release after 20hrs of incubation in 24-well plates. Data represent mean \pm SD.

A: HepG2; B: primary murine hepatocytes; C: primary human hepatocytes; D: NeoHepatocytes.

4.1.3 Liver-specific Toxicity: Paracetamol as Control Compound

Paracetamol was chosen to be the control substance for liver cell specific, indirect cytotoxicity. It is well documented that this drug has only toxic effects if metabolised by phase I/II reactions, i.e. formation of N-Acetyl-p-benzoquinone imine (NAPQI). Therefore only cells with a liver specific metabolism i.e. P450 system, should be fatally affected by the drug.

HepG2 cells, primary murine and primary human hepatocytes, as well as NeoHepatocytes were treated with increasing concentrations of Paracetamol. After 20hrs of incubation, the LDH content were assessed in the supernatant and the remaining cell lysate as described before.

A large variability between HepG2, primary hepatocytes, and different preparations of NeoHepatocytes was found in the concentration dependent LDH-release (Figure 6). The absolute toxicity (basal-top) found in NeoHepatocytes (A) donor 1 and 3 was 39% and 17%, in primary murine hepatocytes (B) 59% and primary human hepatocytes (C) donor 1 44%, donor 2 33%, and donor 3 34%, whereas in HepG2 cells (D, 7%) and NeoHepatocytes from donor 2 (A, 8%) a less sensitive response was observed. (Note: NeoHepatocytes and primary human hepatocytes do not originate from the same donor.) The results from treatment with Paracetamol are summarised in Table 3.

Table 3:

Cytotoxicity of the indirect hepatotoxin paracetamol on various liver cells as represented by LDH-release after 20hrs of incubation in 24-well plates. The EC_{50} was calculated using GraphPad Prism Software. The program extrapolates the data to calculate an EC_{50} , therefore higher values than the concentrations actually used (≤ 50 mM) are excluded (*Data*). Difference: Absolute toxicity calculated from basal minus highest LDH-release [%]. na: No increase in cell toxicity in this concentration range, hence no EC_{50} value is calculated

LDH release [%]	NeoHep 1	NeoHep 2	NeoHep 3	HepG2	prim mur Hep	prim hum Hep 1	prim hum Hep 2	prim hum Hep 3
basal	24%	9%	27%	15%	19%	20%	24%	34%
Paracet. Top	63%	26%	35%	22%	78%	64%	57%	68%
Difference	39%	17%	8%	7%	59%	44%	33%	34%
EC_{50}	4.2 mM	8.4 mM	na	na	5.2 mM	73.7 mM	64.3 mM	na

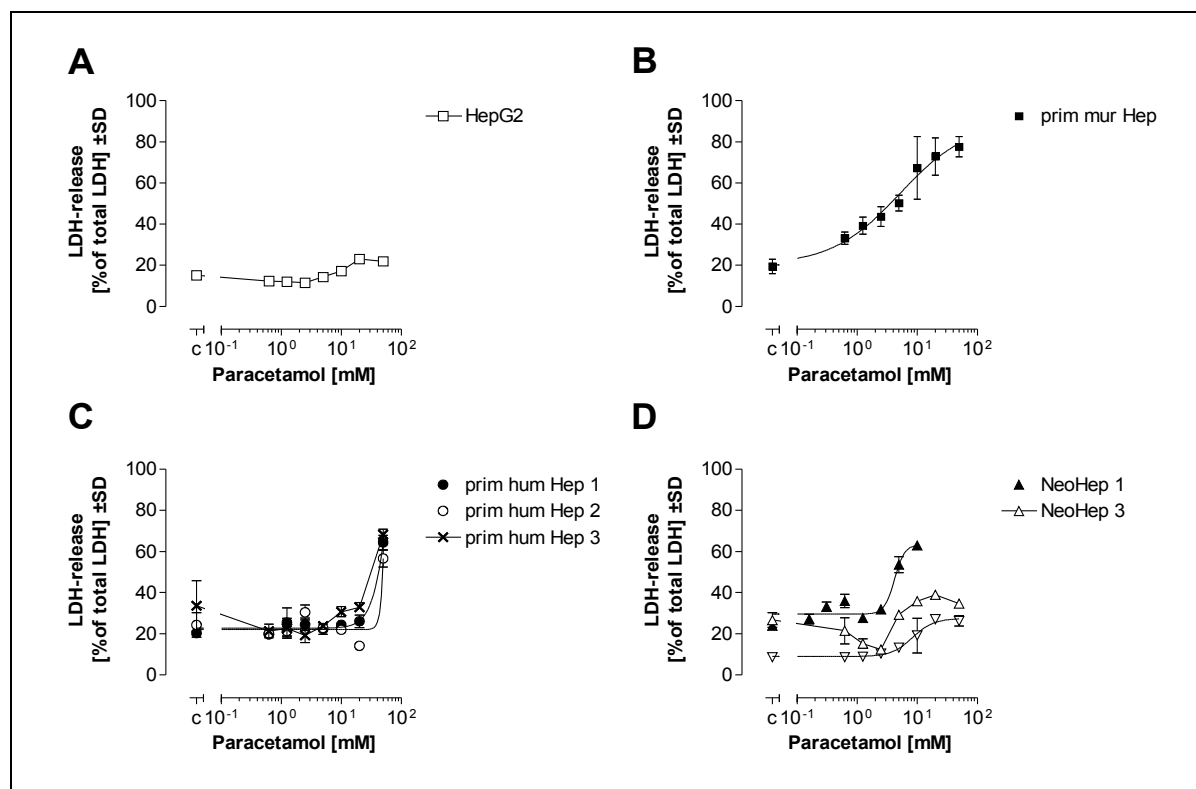


Figure 6:

Concentration dependence of the cytotoxicity of the indirect hepatotoxin paracetamol on various liver cells as represented by LDH-release after 20hrs of incubation in 24-well plates. Data represent mean \pm SD.

A: HepG2; B: primary murine hepatocytes; C: primary human hepatocytes; D: NeoHepatocytes.

It was not possible to induce cytotoxicity in HepG2 in the same extent as in the primary cells and in some NeoHepatocytes. The experiments indicate that the carcinoma cell line can be used to assess direct toxicity but is not a useful *in vitro* model for metabolism dependent toxicity in our study. The Paracetamol toxicity data from the NeoHepatocytes range between the one from the two primary cells. They were less sensitive than the murine cells, but were sensitive in the same concentration range as the primary human cells. The NeoHepatocytes reflect the human situation much better than the HepG2 cells. We therefore conclude that NeoHepatocytes are a better choice than the hepatoma cell line and the best approximation to primary human hepatocytes in metabolism dependent cytotoxicity studies.

4.1.4 Glutathione protects the cell from Paracetamol Toxicity

The concentration dependent toxic effect of Paracetamol is particularly due to decreasing glutathione concentration in the cell. Glutathione, as an antioxidant protects the cell from oxidation reactions, i.e. lipidperoxidation processes. Catalysed by GSH transferases, the reactive metabolite NAPQI rapidly reacts with glutathione (GSH), thereby forming a Paracetamol-GSH conjugate and stoichiometric amounts of Paracetamol and glutathione disulfide (GSSG).

Though *in vitro*, GSH is inefficiently transported into cells, the glutathione monoethyl ester in contrast, in which the glycine carboxyl group is esterified, is transported effectively into the cytosol. In the cell it is hydrolysed to GSH which leads to an increasing cellular GSH level and therefore to a prevention of oxidative stress, certain toxicities, and glutathione deficiency [87-89]. The treatment of Paracetamol in combination with glutathione ethyl ester should therefore prevent cell death in a concentration dependent manner.

NeoHepatocytes were treated with glutathione ethyl ester (3.33 mM), Paracetamol (45 mM) or both. The LDH-assay was performed after 20 hrs. of incubation.

A significant decrease (63% reduction) of the toxic effect was seen in the treatment with GSH ester plus Paracetamol (LDH-release 34%) compared to Paracetamol (LDH-release 54%) alone (Figure 7).

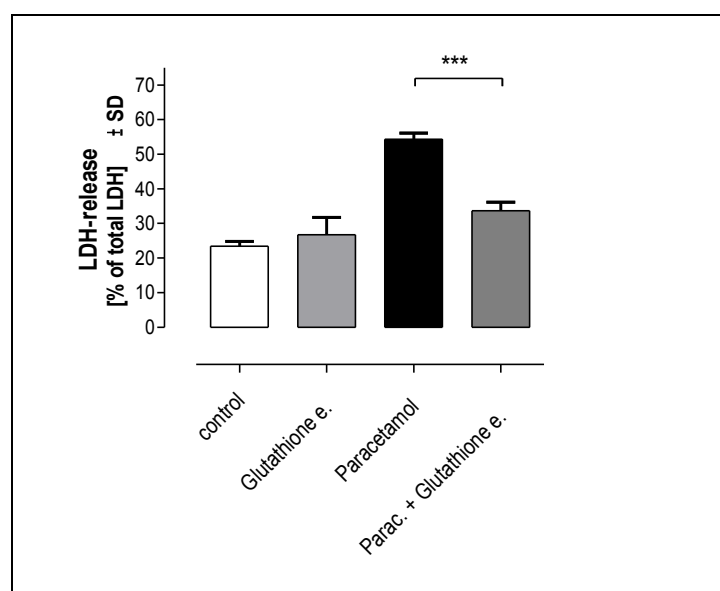


Figure 7:

NeoHepatocytes were treated with GSH ester [3.33 mM], Paracetamol [45 mM] or both. Cytotoxicity was determined by LDH-release after 20hrs of incubation. Data represent mean ± SD. Bonferroni's Multiple Comparison Test: *** P<0.001 for Paracetamol vs. Paracetamol + GSH ester.

4.1.5 Further substances on 24-well plates

It was further analysed if the cytotoxicity of other compounds i.e. direct and indirect toxins was to be measured in experiments with NeoHepatocytes, primary cells and HepG2 likewise (Figure 5). The different cells were treated with increasing concentrations of the specified substances and cytotoxicity was determined by LDH-assay after 20hrs of incubation.

Concentration dependent cytotoxicity (Table 4, Figures 8+9) was induced in all cell types with the following substances (required CYP activity in parenthesis), given is the absolute toxicity: Cyclosporine A (CYP 3A) 53-72% and Sodium valproate (CYP 2C19, 3A) in NeoHep and HepG2 ~29% and in primary hepatocytes 63-69%, Cycloheximide in HepG2 (11%), in NeoHep (30-35%), in primary murine (43%) and primary human hepatocytes (63%). Also Isopropyl alcohol, Mercury chloride (HgCl₂), and Tetracycline showed toxic effects. Because of the volatile character of the alcohol, this data has to be seen with reservation. The results with HgCl₂ showed that there are problems with the assay, because the concentrations dependency is seen in the first four concentrations, higher concentrations cannot be assessed correctly. Despite the observed water solubility of Mercury chloride, it possibly precipitated in the wells occasionally so that the higher concentration did not give a correct result. Tetracycline highly induced toxicity in the primary cells (murine 60%, human 36%) and HepG2 (66%), but to a much lesser extent in NeoHepatocytes (7%). Only one NeoHepatocyte donor reacted upon Colchicine, a tubulin network-disrupting agent (absolute Tox. 15%). Isoniacid, a tuberculostatic agent induced 20% cytotoxicity in primary murine hepatocytes, but less the 10% in the other cell types. No cytotoxicity in the used concentration range was seen with 17 α -Ethinyl estradiol (CYP1A2, 3A) and Caffeine (CYP1A2). Further screening of these substances is required to exclude false negative results.

Table 4:

Cytotoxicity of specified substances on various liver cells as represented by LDH-release after 20hrs of incubation in 24-well plates. Absolute toxicity calculated from basal minus highest LDH-release [%].

absolute Toxicity [%]	NeoHep 1	NeoHep 2	HepG2	prim mur Hep	prim hum Hep 1	prim hum Hep 2	prim hum Hep 3
17 α -Ethinyl estradiol							
Caffeine							
Colchicine	15%				6%	3%	1%
Cycloheximide	35%	30%		43%	63%		
Cyclosporine A	53%		61%	61%	72%		
Isoniacid	9%	3%		8%	21%		
Isopropyl alcohol	12%	61%	43%	65%			
Mercury chloride							
Sodium valproate	30%	63%	29%	69%			
Tetracycline	7%	60%	66%	36%			

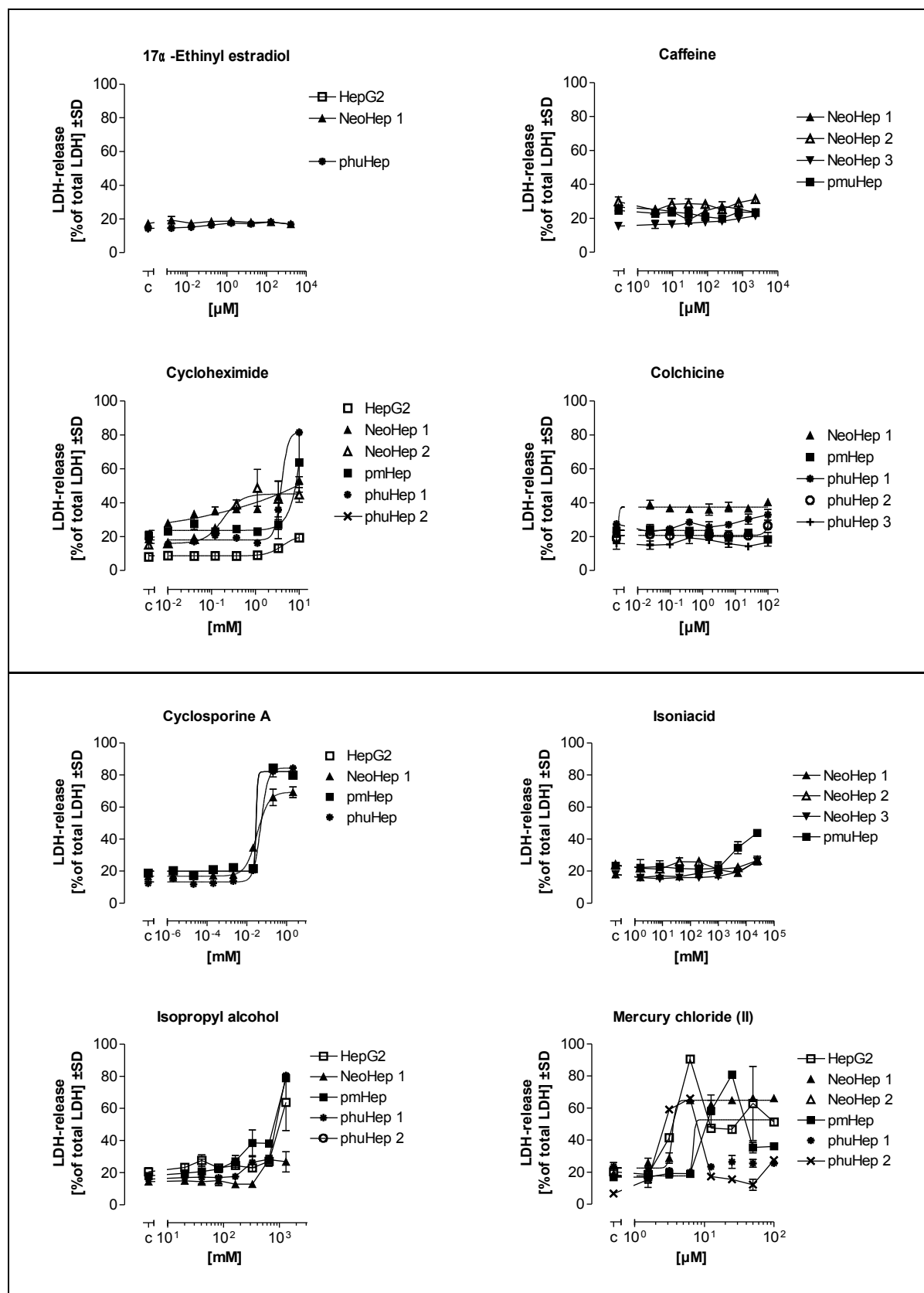


Figure 8:

Concentration dependence of the cytotoxicity of specified substances on various liver cells as represented by LDH-release after 20hrs of incubation in 24-well plates. Data represent mean \pm SD.

HepG2, primary murine (pmHep) and human hepatocytes (phuHep) compared to NeoHepatocytes (NeoHep).

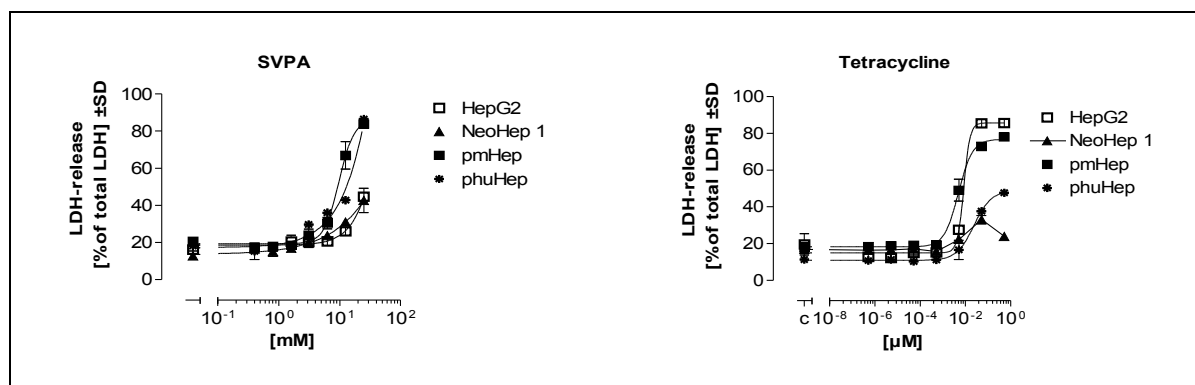


Figure 9:

Concentration dependence of the cytotoxicity of specified substances on various liver cells as represented by LDH-release after 20hrs of incubation in 24-well plates. Data represent mean \pm SD. HepG2, primary murine (pmHep) and human hepatocytes (phuHep) compared to NeoHepatocytes (NeoHep).

The LDH-assay proved to be highly reliable in this setup, giving logical curves (except for HgCl_2) with low standard deviations. It was easy to handle manually, with the various hepatic cells, and showing very low standard deviations. With the knowledge of the mentioned limitations, the LDH-release was chosen as end point measured via the Cytotoxicity Detection Kit for the assessment of cytotoxicity testing with NeoHepatocytes.

4.1.6 Influence of Culture Conditions

Regarding the high throughput screening of substances in the framework of REACH, it is more feasible to use 96-well plates and 386-well plates than the so far used 24-well plates. Therefore, NeoHepatocytes had to be tested in comparison seeded on 24-well plates (4×10^6 cells/well) and 96-well (7.58×10^5 cells/well). The details on the various volumes in the protocol were adapted to the smaller size of the wells. Apart from that, the assay was carried out exactly according to the 24-well experiments. Cells were treated with increasing concentrations of Paracetamol and the LDH-release was assessed 20hrs later.

The data from identical donors was statistically analysed to check if the different plate sizes have an influence on the results. The 1-way ANOVA revealed the correlation: The pairing was significantly effective ($P < 0.0001$), there was no influence on the results due to the plate size (Figure 10).

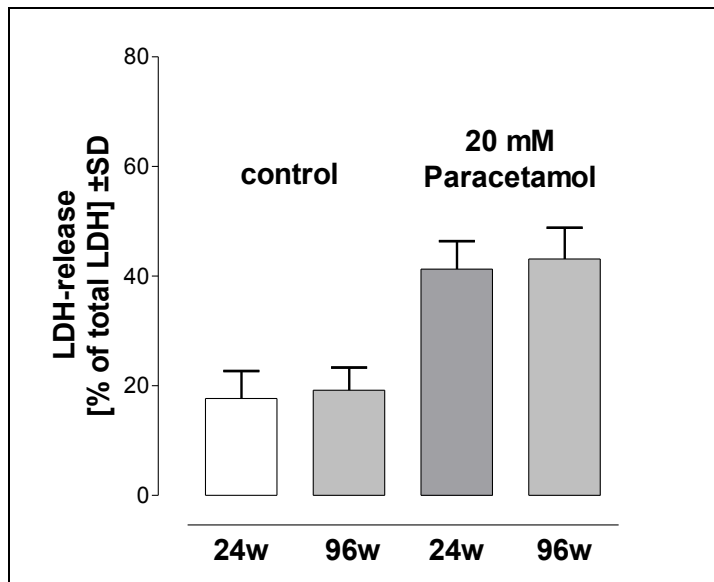


Figure 10:

Cytotoxicity determined by LDH-release after 20hrs of incubation. NeoHepatocytes (donor n=9) cultivated in 24-well and 96-well plates were treated with Paracetamol. Data represent mean \pm SD.

Since the received cell plates were not always matching regarding the donors (one and the same donor on 24-well and 96-well plates) the EC_{50} from all cytotoxicity assays with Paracetamol were analysed by unpaired t-test. The calculated median of the 14 donors were 8.1mM /24w and 10.5mM /96w, the mean were 10.1mM/24w and 10.3mM/96w with minima at 4.2mM/24w and 8.3mM/96w, maxima at 23.4mM/24w and 14.0mM/96w. No significant differences were quantified ($P \geq 0.5$) (Figure 11).

We therefore conclude that the smaller plates can be used equivalently for cytotoxicity testing with NeoHepatocytes.

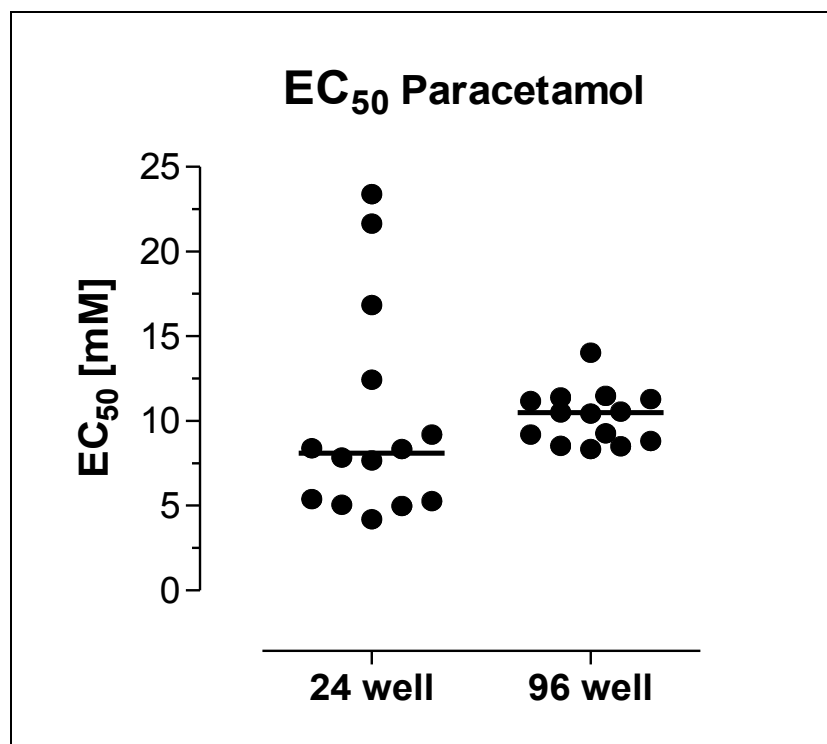


Figure 11:

Mean cytotoxicity (EC_{50}) of Paracetamol on NeoHepatocytes seeded on 24-well and 96-well plates. The EC_{50} were calculated from separate incubations with increasing concentrations of Paracetamol; cytotoxicity was determined by LDH-release after 20hrs. The resulting EC_{50} values from the two groups were analysed by unpaired t test, medians are indicated. No significant differences were found.

4.1.7 Further substances on 96-well Plates

Additionally to Paracetamol, a set of substances was tested on NeoHepatocytes seeded on 96-well plates to check the feasibility of the assay on 96well plates. Cells were treated with increasing concentrations of the specified substances and LDH-release was measured 20 hrs. later. The following graphs (Figures12+13) illustrate the results from different donors in 96-well plates.

Concentration dependent cytotoxicity (Figures 10, 11) was induced with the following substances (required CYP activity in parenthesis), given is the medial absolute toxicity: Acetylsalicylic acid (CYP2C, 2E1) 15%, Digoxine (CYP3A) 28%, Sodium valproate (CYP 2C19, 3A) 45%, and Verapamil (CYP3A) 70%. Direct toxicity was also measured with Cycloheximide 47%, DMSO 27%, Eserine 67%, SDS 39%, Mercury chloride 50%, Sodium fluoride 48%, and Tetracycline 30%. Isopropyl alcohol (39%, 5%, and 20%) showed a variable result indicating problems during treatment. There was a poor induction of cell death (probably due to missing CYP2E1 activation) after the treatment with alcohol, i.e. isopropyl alcohol, suggesting that the assay might be unreliable for the testing of volatile substances. No cytotoxicity in the used concentration range was seen with 17 α -Ethinylestradiol (CYP1A2, 3A), 5-Fluoruracil,

Amiodarone hydrochloride (CYP3A), Caffeine (CYP1A2), Carbamazepine (CYP3A), Cholchicine (CYP3A), Rifampicine (CYP inductor), and Nicotine. Further screening of these substances is required to exclude false negative compounds.

Apart from the above-mentioned limitations, i.e. volatile substances, donor variability, the LDH-assay works properly with the new set-up and the protocol is summarised in the SOP V1.0.

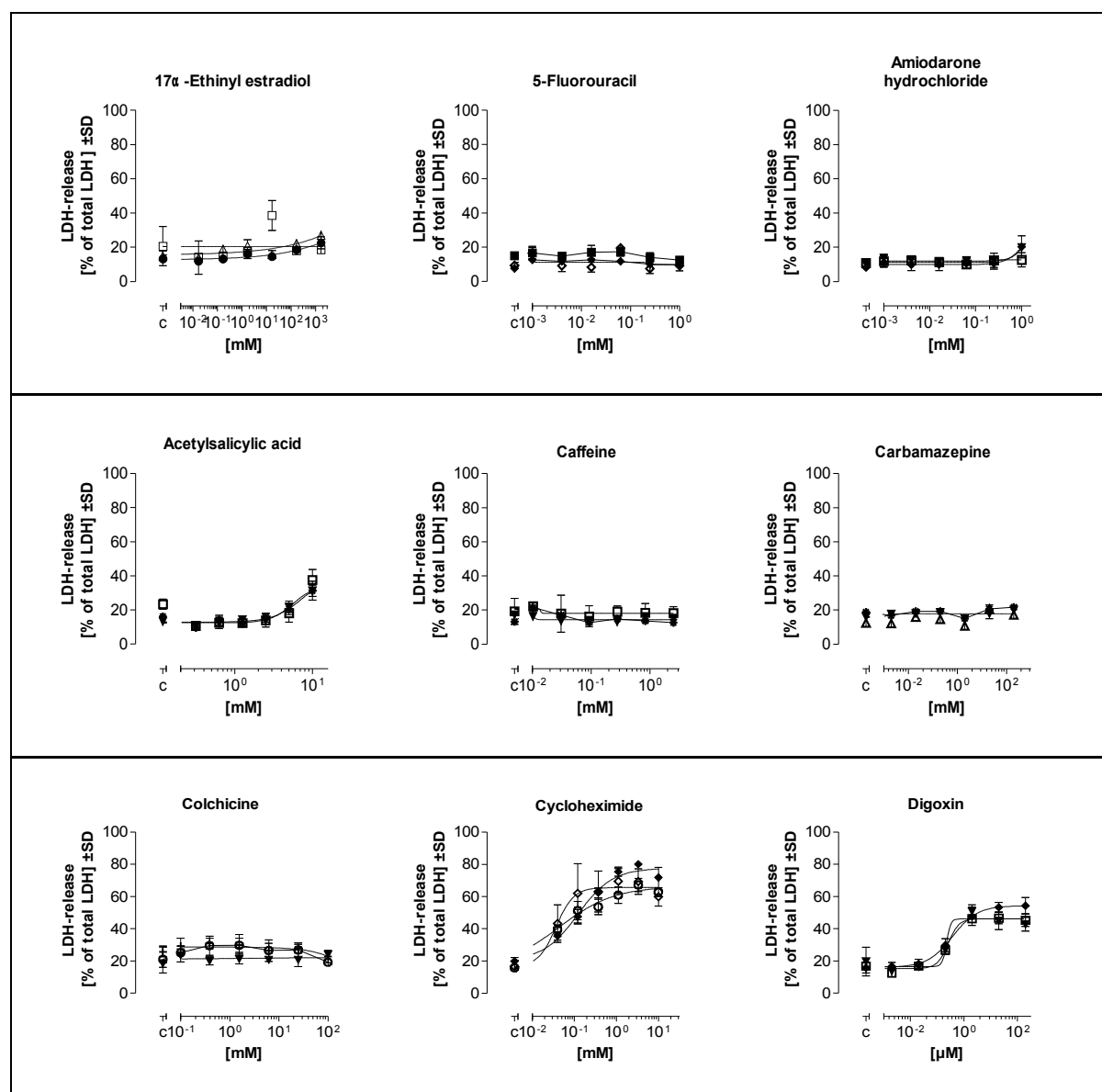


Figure 12:

Concentration dependence of the cytotoxicity of specified substances on NeoHepatocytes as represented by LDH-release after 20hrs of incubation in 96-well plates. Data represent mean ± SD.

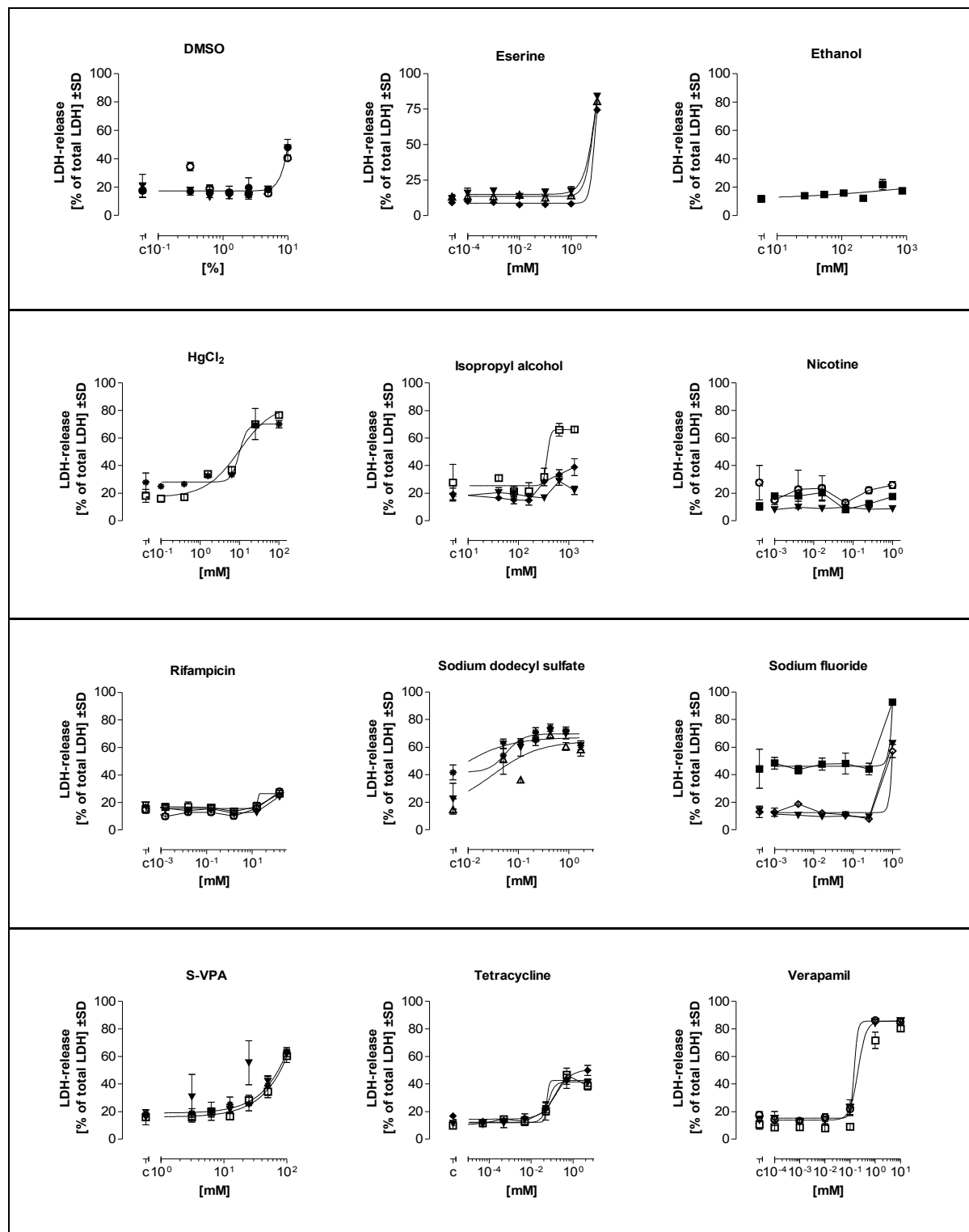


Figure 13:

Concentration dependence of the cytotoxicity of specified substances on NeoHepatocytes as represented by LDH-release after 20hrs of incubation in 96-well plates. Data represent mean \pm SD.

4.2 NeoHepatocytes and Metabolism: Donor Variability

NeoHepatocytes are generated of peripheral blood monocytes from blood donors of various age, sex and blood group. Interindividual variability in expression and catalysis of the various CYP450 enzymes has an influence on the toxicity of metabolites. Considering this, NeoHepatocytes from numerous donors (19 in 24well, 18 in 96well) were assessed in the cytotoxicity experiments. The cells were treated with Paracetamol and the LDH-release was measured as described. The previous results reveal already, that NeoHepatocytes from different donors do react differently to treatments. In the further experiments on 24-well plates this observation was confirmed (Figures 14+15): The absolute toxicity ranged from 10-30% and the EC_{50} values obtained in tests with different cell batches treated with Paracetamol showed a range from 4 mM to 23 mM., Thereby probably reflecting the human population with distribution of polymorphism [90], age, gender etc.

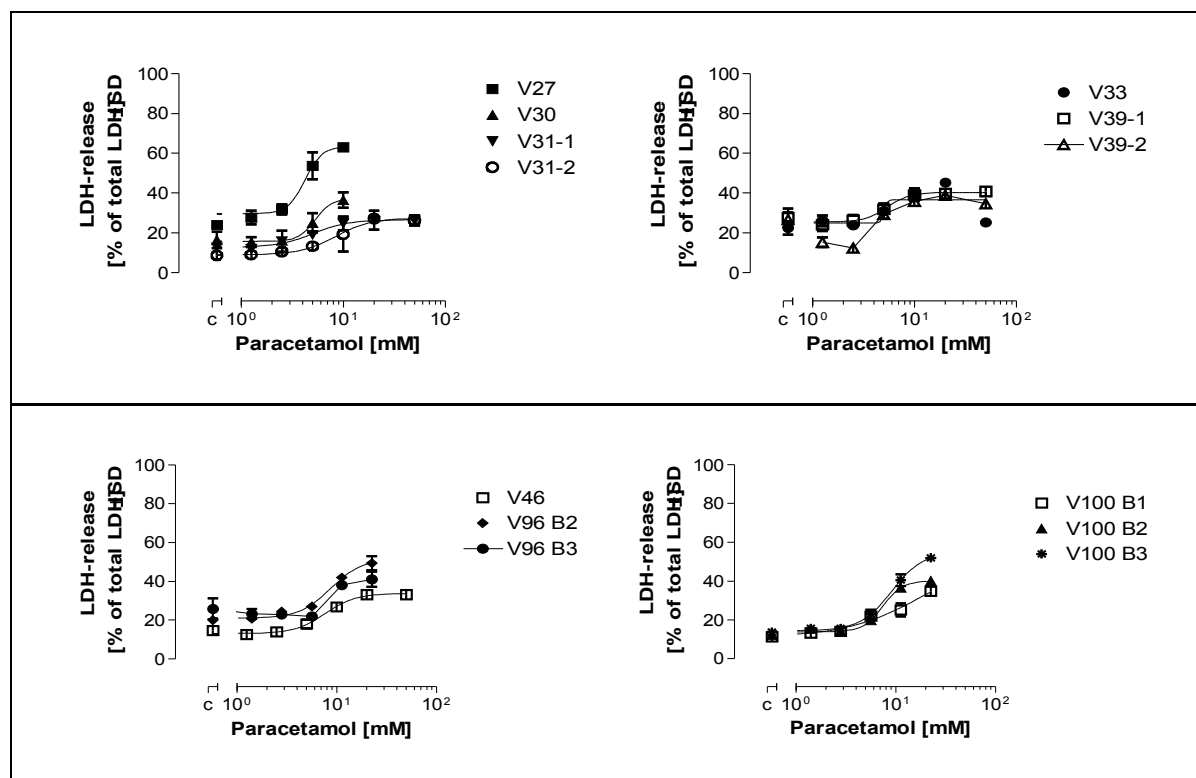


Figure 14:

Concentration dependence of the cytotoxicity of the indirect hepatotoxin paracetamol on NeoHepatocytes as represented by LDH-release after 20hrs of incubation in 24-well plates. Shown are results from 19 different donors. Data represent mean \pm SD.

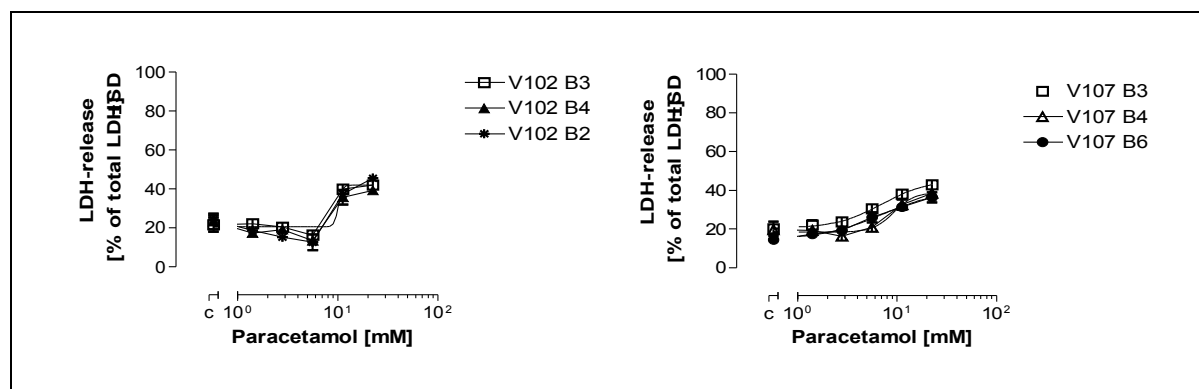


Figure 15:

Concentration dependence of the cytotoxicity of the indirect hepatotoxin paracetamol on NeoHepatocytes as represented by LDH-release after 20hrs of incubation in 24-well plates. Shown are results from 19 different donors. Data represent mean \pm SD.

Consistent with the previous experiments, a distinct donor variability was found in the effect of Paracetamol in 96-well plates. In contrast to the results from the 24-well plates, the EC_{50} values from the 96-well plates (Figure 16) range from 8 mM to 14 mM, with an comparable absolute toxicity of 13-33%. This seems due to the donor variability and additional experiments with NeoHepatocytes are required to ensure powerful statistic analysis.

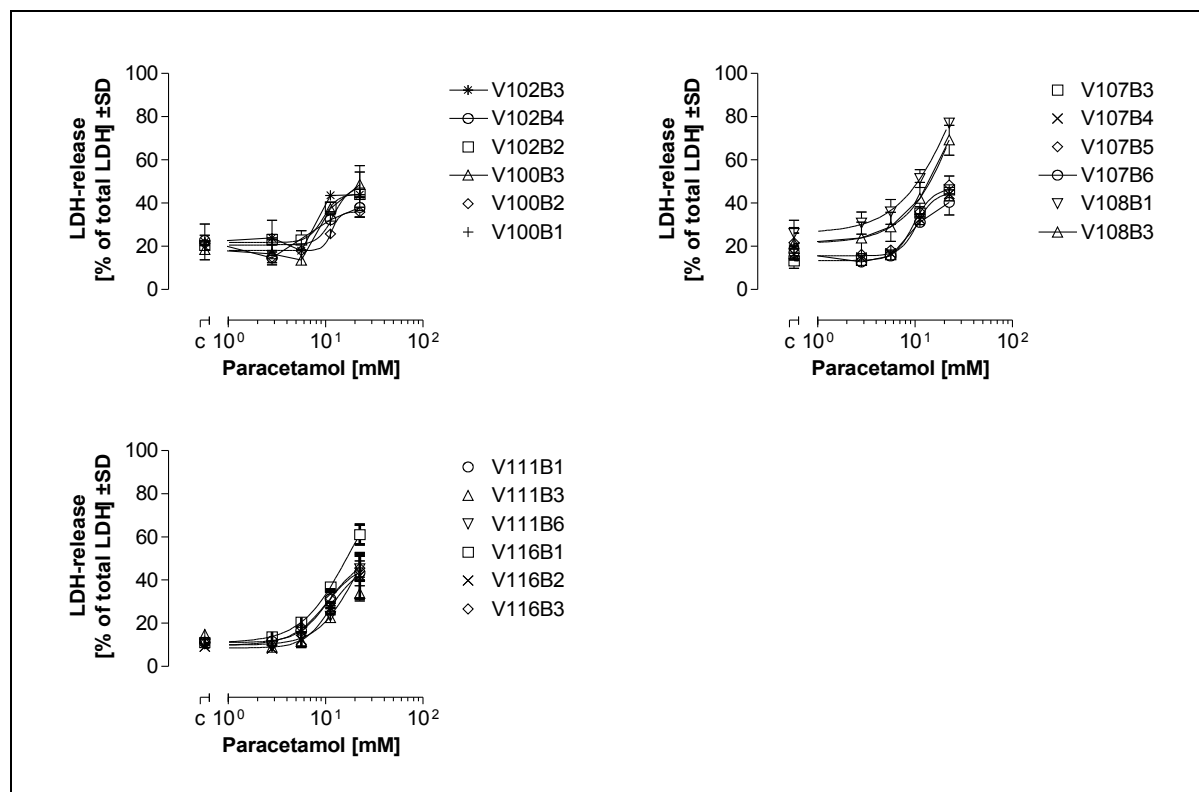


Figure 16:

Concentration dependence of the cytotoxicity of the indirect hepatotoxin paracetamol on NeoHepatocytes as represented by LDH-release after 20hrs of incubation in 96-well plates. Shown are results from 18 different donors. Data represent mean \pm SD.

4.2.1 Pooling of NeoHepatocytes from Multiple Donors

It was considered that a potential approach to overcome donor variability might be the pooling of monocytes from multiple donors in the generation process of NeoHepatocytes. Therefore EUFETS AG was asked to provide plates seeded with cells pooled from different donors and cells of these donors on separate plates.

These cells were then treated with Paracetamol to reveal a potential differences between pooled and individually incubated NeoHepatocytes (3 donors at a time). On a first glance at the graphs, the pooled cells show a concentration dependent increase of LDH-release, slightly earlier than the separate cells (Figure 17).

But the usual visual check-up under the microscope revealed, that in the wells with pooled cells confluence was under 20%, no cell-cell contacts were possible and the cells (Figure 18) were even smaller than monocytes.

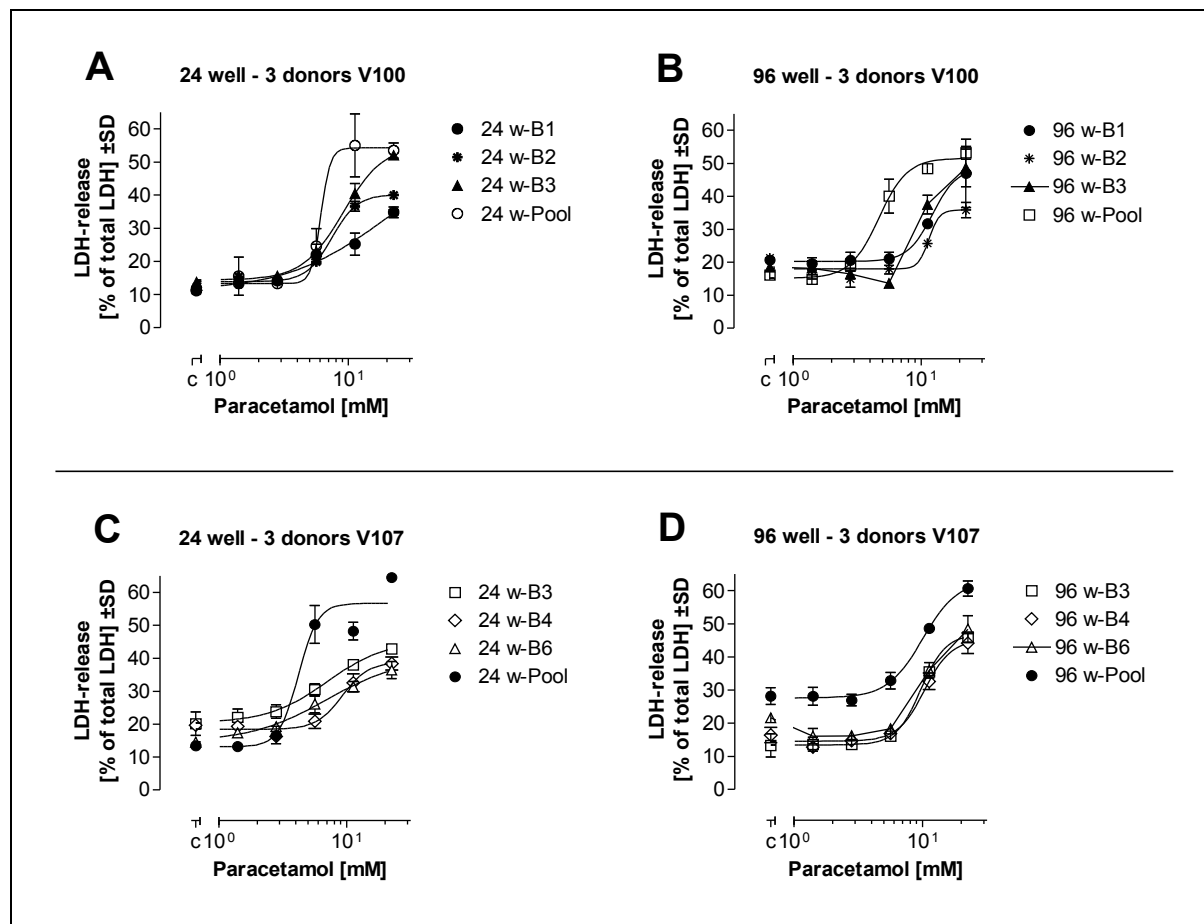


Figure 17:

Concentration dependence of the cytotoxicity of the indirect hepatotoxin paracetamol on NeoHepatocytes as represented by LDH-release after 20hrs of incubation. Shown are results from different donors separate and pooled culture on 24-well (A+C) and 96-well plates (B+D). A+B: Exp.No. V100/ donors B1, B2, B3; C+D: Exp.No. V107/donors B3, B4, B6. Data represent mean \pm SD

Therefore the pooling procedure of cells from different donors was not further pursued. The further experiments were therefore carried out with cells from separate donors.



Figure 18:

Microscopic picture of cells generated over three weeks from a pool of monocytes from three different donors.

4.3 The Standard Operating Procedure (SOP)

V1.0 Initial Protocol

V1.1 improved in Intralab testing

V2.0 Ring Trial (adapted to 96well plates)

V2.1 Ring Trial, improved after first run

V3.0 Adjustment to HTS platform based on V2.1 and NRU/3T3-assay [86]

The SOP V2.1. is attached as [Appendix](#) to this thesis.

4.4 Applicability of the SOP

4.4.1 Intralaboratory Reproducibility

As the LDH-assay proved to be highly reliable, the protocol was brought into the specific form of a Standard Operating Procedure (SOP). To test intralaboratory reproducibility of the test system, two different staff members were chosen to apply the SOP. Each person performed the assay in an independent fashion. Cells from the same donors were treated with increasing concentrations of Paracetamol and the LDH-release was measured after 20 hrs. NeoHepatocytes showed a concentration dependent LDH-release with EC_{50} between 10-14mM (Figure 19).

The resulting data was statistically analysed to check if different experimentators have an influence on the results when using this SOP. The paired t-test revealed a very tight correlation between the data sets obtained by different experimentators ($P < 0.0001$). The SOP was therefore considered as comprehensible and feasible for applications at different laboratories.

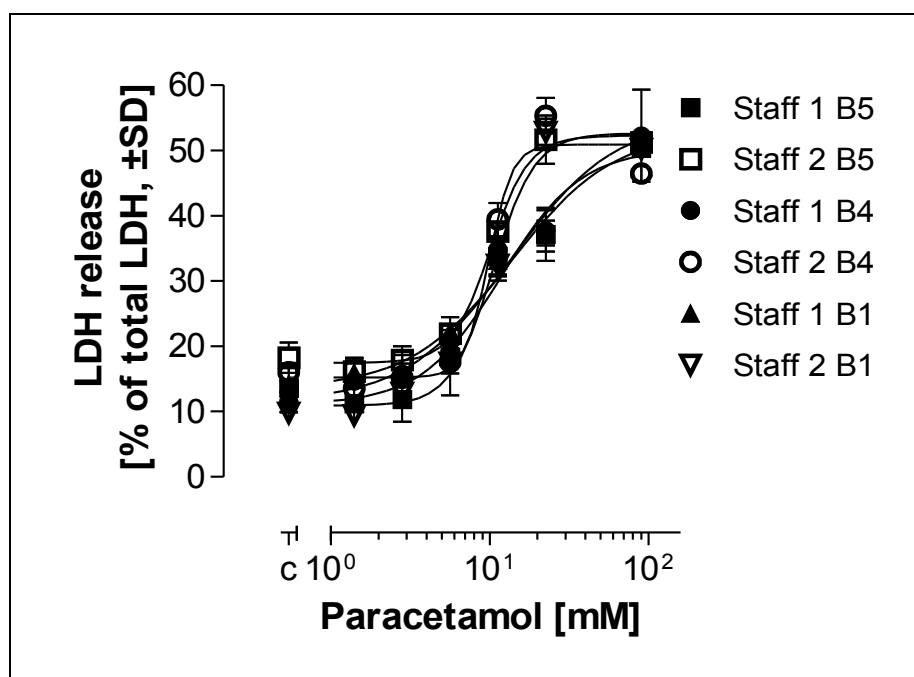


Figure 19:

Concentration dependence of the cytotoxicity of the indirect hepatotoxin paracetamol on NeoHepatocytes as represented by LDH-release after 20hrs of incubation. Cells from three donors (B1, B4, B5) were treated by two staff members. Data represent mean \pm SD.

4.4.2 Interlaboratory Comparison: Participating Laboratories and Data Reporting

The SOP was then tested in an interlaboratory comparison study to check its practicability also in different laboratory locations. The study was conducted in three academic research institutions located in Konstanz, Mannheim and Berlin, Germany. All participants received the provided SOP for the LDH-release assay. Seeded cells (every time from an identical donor), empty plates, assay kits, and chemical compounds were sent to each laboratory to ensure the same conditions for each test run.

The interlaboratory comparison allowed estimating the variability of the measurement process under the given donor variability. By evaluating the ability of each laboratory to produce consistent data, the transferability of the SOP was controlled (Figure 20, Tables 5+6).

Precision varied greatly among laboratories in the first run. After troubleshooting in the concerning lab, the data from the first run (3 donors) was excluded due to technical reasons in the assay procedure. The SOP had to be adjusted and better defined. After a site visit to the mentioned lab and clarification of the protocol, the resulting data showed a matching of EC₅₀ values between the participants.

Where available, the EC₅₀ were compared. When no EC₅₀ calculations by the software were available, the non-sigmoid curve shapes were compared.

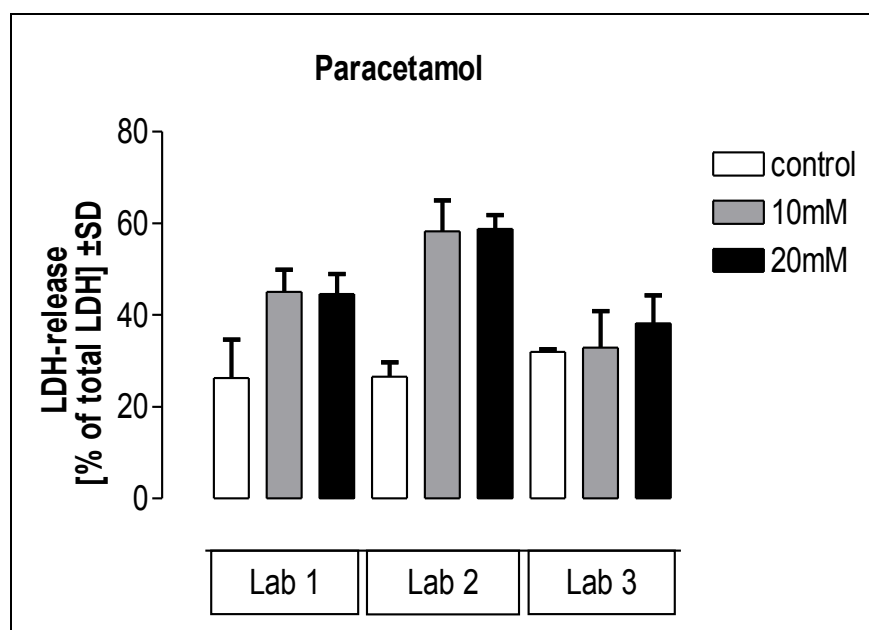


Figure 20:
Cytotoxicity of paracetamol on NeoHepatocytes as determined by LDH-release after 20hrs of incubation on 96-well plates. Treatment of cells from one and the same donor in three different laboratories by three different staff members. EC₅₀: Lab 1–4.0mM; Lab 2–4.4mM; Lab 3–not available. Data represent mean ± SD.

The calculated EC₅₀ (only non-extrapolated values), analysed by paired t-test, were significantly correlated, (not enough paired data points for ANOVA). But there were still significant differences in basal toxicity and response to compounds in some donor cells as well as between the laboratories in the treatment of individual donors (data analysed by 2-way ANOVA). The differences in basal toxicity are astonishing, since it seems

to correlate with the location of the participating laboratories. The mode of shipment may have had an influence as addressed in the discussion chapter of this thesis.

The interlaboratory results showed, that the NeoHepatocytes have a potential in evaluating cytotoxicity based on a SOP in independent laboratories. But there is the statistical need to repeat such a study with a GLP and GCCP-trained (Good cell culture Practice [91]) staff and with a higher number of NeoHepatocyte batches to ensure consistent data.

Table 5:

**Statistic results of the interlaboratory comparison:
Paired t-test of available EC₅₀ pairs from the treatment of NeoHepatocytes with various substances from participating labs, 2-way ANOVA was not possible due to too little data points.**

Parameter	Lab 1- Lab 2	Lab 3 – Lab 1	Lab 2 – Lab 3
Number of data points (EC ₅₀)	16	40	21
Spearman r	0.79	0.77	0.71
P value (two-tailed)	0.0003	0.0001	0.0003
Exact or approx. P value?	Gaussian Approx.	Gaussian Approx.	Gaussian Approx.
Is the correlation significant? alpha=0.05)	Yes	Yes	Yes

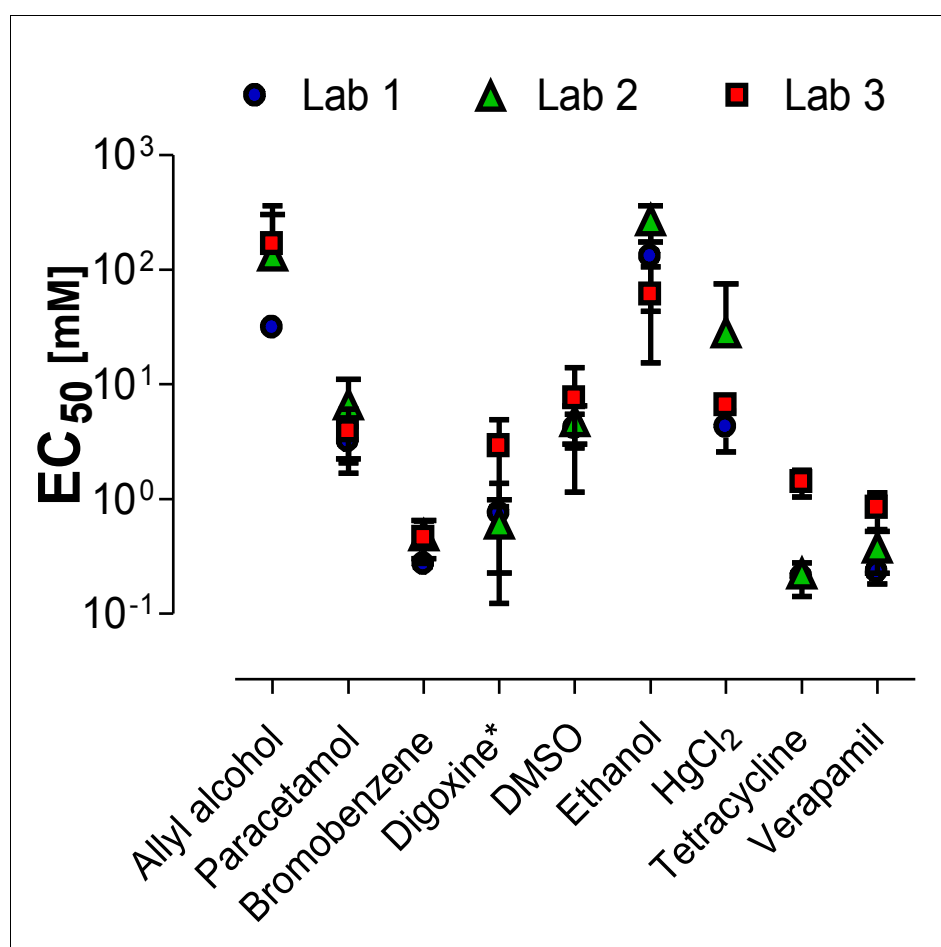


Figure 21:
Summary of Interlab results Cytotoxicity of nine compounds on NeoHepatocytes as determined by LDH-release after 20hrs of incubation on 96-well plates. Treatment of cells from 11 donors in three different laboratories by three different staff members. Data represent mean ± SD.

Table 6:

EC₅₀ values from cytotoxicity experiments in the interlab comparison with NeoHepatocytes (SOP V2.0/2.1)).

The EC₅₀ was calculated using GraphPad Prism Software. The program extrapolates the data to calculate an EC₅₀, therefore higher values than the concentrations actually used were excluded (*Data*). na: No increase in cell toxicity in this concentration range, hence no EC₅₀ value was calculated.

Cell batch	Partner	Allyl Alcohol [mM]	Paracetamol [mM]	Bromobenz en [mM]	Digoxin [µM]	DMSO [%]	Ethanol [mM]	HgCl2 [mM]	Tetracycline [mM]	Verapamil [mM]
1	Lab 1	na	2.39	na	na	64.42	na	3.07	0.23	0.24
	Lab 2	na	10.28	0.01	1.20	8.86	272.70	na	0.26	0.50
	Lab 3	na	na	na	0.37	6.03	na	332.30	0.23	0.25
2	Lab 1	na	10.59	0.24	0.84	4.75	na	0.39	0.23	0.41
	Lab 2	na	na	na	0.34	4.21	na	5.41	0.24	0.24
	Lab 3	na	14.28	0.00	0.94	5.13	na	5.41	0.26	0.40
3	Lab 1	na	3.71E+11	na	2.24	3.12	na	232.20	0.28	0.28
	Lab 2	40.66	7.91	na	0.97	2.53	119.80	8.21	0.22	0.17
	Lab 3	na	na	na	na	na	na	na	1.67	na
4	Lab 1	0.00	3.97	49.18	1.07	2.34	56.79	na	0.04	0.30
	Lab 2	332.50	4.39	na	0.38	2.50	208.50	na	0.20	0.38
	Lab 3	305.90	na	na	3.93	3.85	na	na	na	0.63
5	Lab 1	43.67	4.26	6.60E+15	3.30E+05	4.68	107.10	433.90	0.20	0.17
	Lab 2	na	400.50	na	0.55	379.60	8.45E+04	na	0.18	0.30
	Lab 3	4025.00	23.70	na	2.92	18.19	6.10E+04	6.61	na	0.92
6	Lab 1	4.00E+17	4.59E+04	na	0.76	2.81	4.42E+13	0.03	0.21	0.29
	Lab 2	na	3.01	0.65	0.04	5.51	389.00	na	na	0.25
	Lab 3	29.19	26.29	0.59	0.05	4.82	92.71	na	91.20	0.33
7	Lab 1	na	na	4.67E+18	0.65	5.83	na	1.97E+06	0.21	0.23
	Lab 2	25.13	3.15	0.54	0.31	4.90	3.67E+03	99.00	0.22	0.34
	Lab 3	na	4.76E+03	na	na	12.77	28.64	na	na	0.96
8	Lab 1	na	49.30	5.07E+16	0.19	4.90	na	na	0.25	0.19
	Lab 2	na	2.50	na	0.33	4.29	310.90	na	na	0.30
	Lab 3	na	5.44	0.33	na	4.49	na	4.45E+07	na	1.06
9	Lab 1	31.18	na	0.27	0.34	3.17E+03	225.00	na	na	0.16
	Lab 2	na	1.05	0.48	0.21	4.56	na	na	na	0.74
	Lab 3	603.60	na	na	na	1.47	2.61E+14	na	1.15	0.74
10	Lab 1	na	2.08	na	0.77	3.25	2.58E+25	na	na	0.19
	Lab 2	na	9.44	na	0.92	4.66	313.30	na	0.22	0.33
	Lab 3	na	2.32	na	4.70	20.33	2.86E+04	na	na	1.24

4.4.3 Automation: Adaptation of the SOP to the robot HTS

A future goal for industrial application of such a toxicity test system represents the transfer to automated liquid handling and culturing systems, guaranteeing both high throughput and standardized performance.

The automated platform used is located at the ECVAM Institute, Ispra, Italy. To have the transfer of the LDH-assay as easy as possible, the NRU/3T3-cell SOP, already established on the robot, was compared to the NeoHepatocyte/LDH-assay SOP. Possible problems were communicated and put to the test in Konstanz.

Because the robot unit does not have a cooling device, the components of the assay kit cannot be stored at 4°C during assay runs. Therefore the altered conditions had to be tested. HepG2 cell on 96-well plates were treated with increasing concentrations of SDS. A concentration-dependent LDH-release was assessed after 20 hrs (Figure 21). For the measurement, the Cytotoxicity kit was used as freshly prepared mixture compared to components stored at room temperature (RT) for 5 hrs. No differences between standard conditions and storage at RT were found (Correlation coefficient (r) 0.9995). Thus, the handling of the kit components by the robot was expected to be uncomplicated.

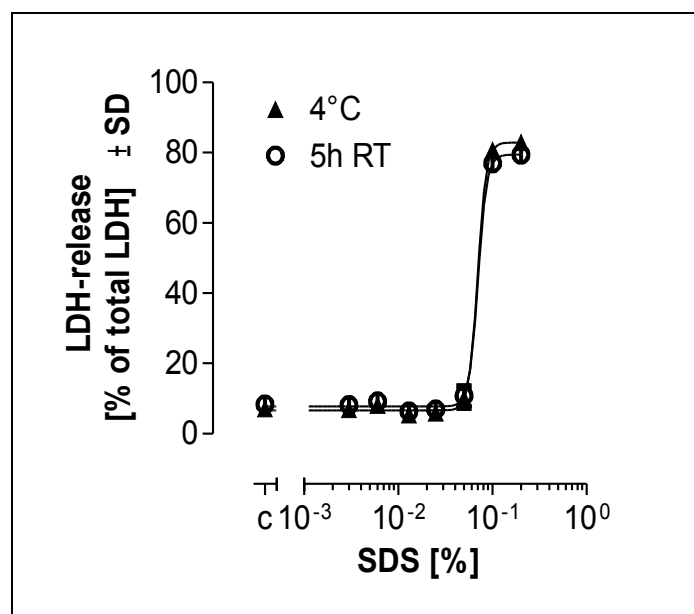


Figure 22:

Concentration dependence of the cytotoxicity of SDS on HepG2 cells as represented by LDH-release after 20hrs of incubation on 96-well plates. LDH-assay kit components were stored at 4° or for 5 hrs at room temperature (RT).

Data represent mean ± SD.

For the type of robot in use at ECVAM, it is not possible to pipette small quantities as 5 μ l for the treatment, or to measure the LDH in supernatant and lysate on the same plate, as documented in the manually-used SOP from Konstanz. Hence, it was decided to use 50 μ l in a trial run, but the final concentration of the test compounds remained the same. The two SOPs were compared in a manually performed assay (Figure 21).

It turned out that the robot-fitted SOP performed much better with HgCl_2 , and the results from Verapamil (donors B2, B6, B7) and Paracetamol (donors B6, B7) were significantly positive correlated. According to these results, the SOP was adapted and the new version was sent to the research assistants of the HTS unit at ECVAM.

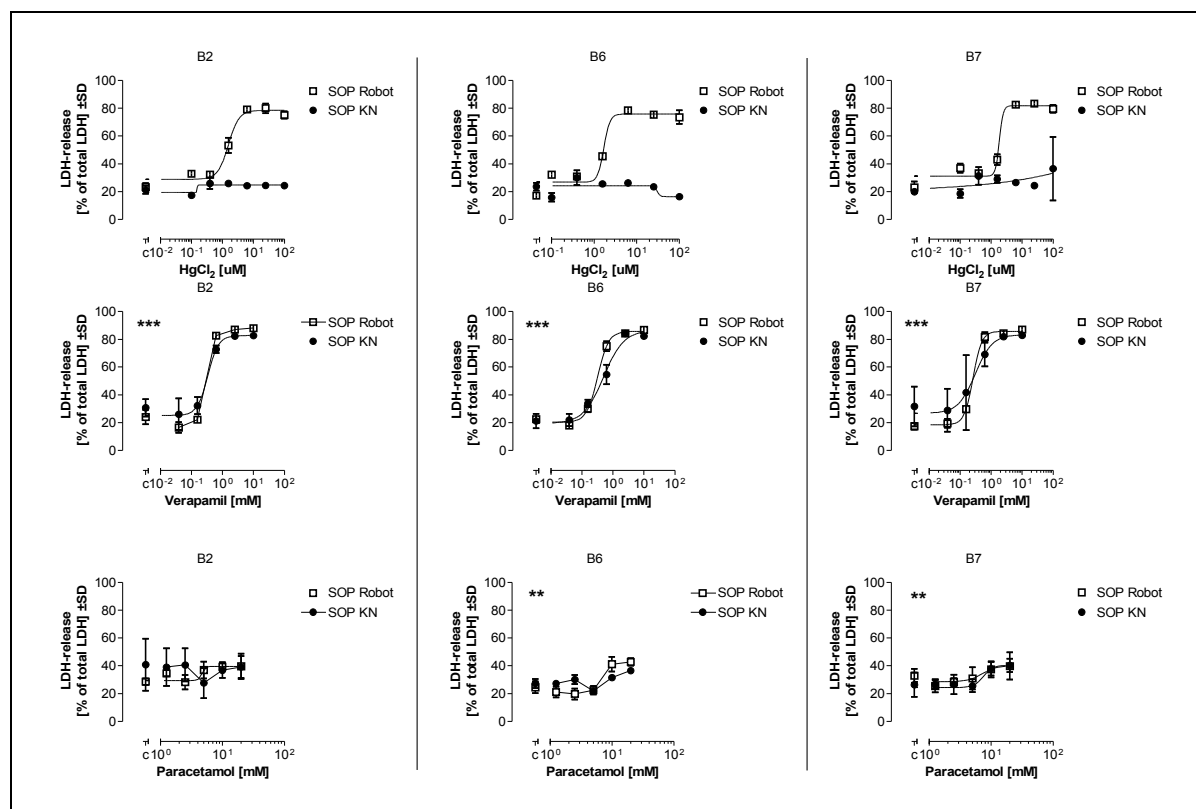


Figure 23:

Comparison SOP KN vs. SOP Robot: Concentration dependence of the cytotoxicity of HgCl_2 , Verapamil or Paracetamol on NeoHepatocytes determined by LDH-release after 20hrs of incubation on 96-well plates. Application of the substances in different volumes (5 μl vs. 50 μl) but same end concentration. Data represent mean \pm SD. Correlation significance: **P<0.01, * P<0.001**

For the first trial runs on the robot platform, it was planned to use 3T3 cells on hand as substitution for NeoHepatocytes. Therefore the adaptability of these cells was checked by staff members from ECVAM and Konstanz in the HTS lab, where the cells are normally prepared for robot runs. The robotic version of the LDH-assay (SOP V3.0) was performed manually with 3T3-cells at different seeding densities (initially 3500 and 700 cells/96-well) and after 24hrs and 48hrs of treatment (Figure 23). The results of all conditions showed a good feasibility of the cells and therefore were to be used for the optimisation of the SOP on the platform.

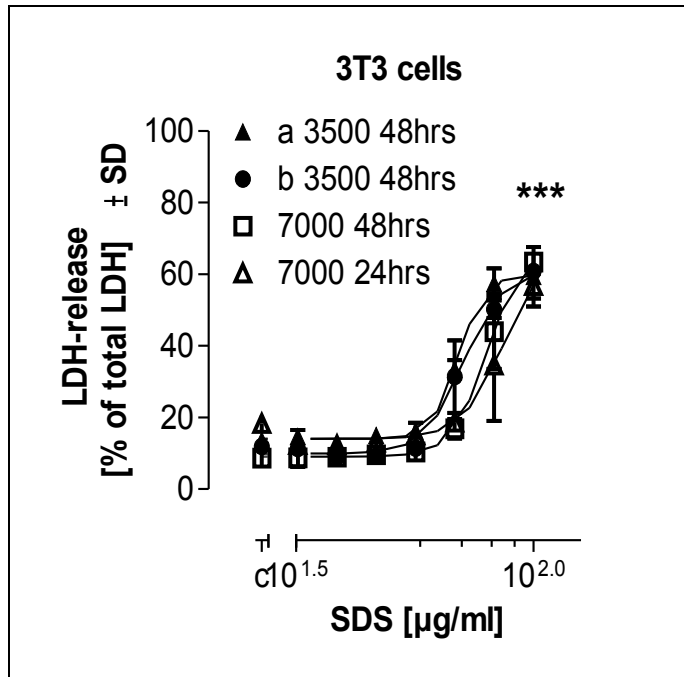


Figure 24:
Concentration dependence of the cytotoxicity of SDS on 3T3 cells determined by LDH-release after 20hrs of incubation on 96-well plates (SOP V3.0). Cells were seeded 3500cells/well or 7000cells/well, treated manually after 24hrs or 48hrs of proliferation. Data represent mean \pm SD. 1-way ANOVA: pairing of data: *** $p > 0.001$

The shipment of NeoHepatocytes has already been an issue at the onset of the study. This time special delivery was chosen: a staff member of EUFETS brought the cells directly to ECVAM. But the same cells were sent simultaneously to Konstanz as usual. The test was repeatedly conducted in parallel in our group and at ECVAM, each time with cells from identical donors. The measured basal toxicity was around 10% less in all samples at ECVAM than at the lab in Konstanz (Figure 24). However, the absolute toxicity was comparable, the obtained data matched well (correlation coefficient > 0.85). Therefore the NeoHepatocytes based LDH-assay was considered suitable for the automated platform application.

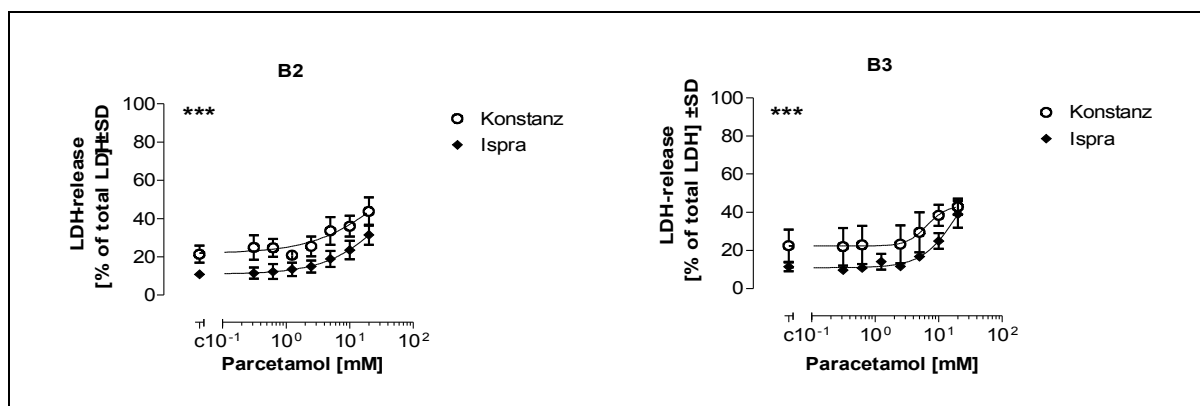


Figure 25:

Concentration dependence of the cytotoxicity on NeoHepatocytes (donors B2 and B3) determined by LDH-release after 20hrs of incubation on 96-well. Cells were treated manually (SOP V3.0) in Konstanz and at ECVAM institute. Data represent mean \pm SD. T-test: Pairing of data: *** $p > 0.001$

Following the successful transfer of the SOP to Ispra, the ECVAM HTS-engineer adapted the manual protocol to the HTS software and hardware (offline + online implementation). Then the SOP was applied to 3T3-cells on the robot and the engineer reported a very good performance.

After the above-mentioned optimisation phase, 30 plates of NeoHepatocytes from one donor per week were ordered at EUFETS AG. Because it is not possible to obtain the required quantity of monocytes from buffy coats, donor cells were gained by aphaeresis. The generated NeoHepatocytes were sent again by direct delivery to ECVAM for the hepatotoxicity testing on the HTS platform.

After the receipt, the cells were placed in the HTS-associated incubator and medium changes were handled manually. Following the SOP V3.0, the NeoHepatocytes were treated in increasing concentrations with various substances by the robot system. Right at the first run (referred to as ID 1) using NeoHepatocytes, the robot encountered difficulties: remains of the additional covering of the plates, a kind of shrink-wrap material stuck to the plates, so that the robot arm was not able to handle the plates correctly. The remaining plates were then manually uncovered and the test went through without further problems. EUFETS AG was contacted about the problem with the adhesive foil. They managed to change the wrapping according to the requirements. The next experiments were performed with covers on the plates to be removed from the robot arm when needed.

Where possible from the resulting data, the EC_{50} were calculated using GraphPad Prism Software. The program extrapolates the data to calculate an EC_{50} , therefore higher values than the actual concentrations used were excluded (Table 7). The standard deviation was very low indicating excellent performance (Figures 25-28). One exception was SDS (Figure 28), higher SD were found in the three highest concentrations: the OD was not measured correctly due to foam formation in the wells. The EC_{50} obtained in the robot tests were in the same range as the results from prior, manually performed experiments. Again, the donor variability was seen in the experiments.

Due to the time required for collection and measurement (approx. 70 min/plate), it is possible to test up to eight compounds per run. Two runs are possible per week on the working station at ECVAM.

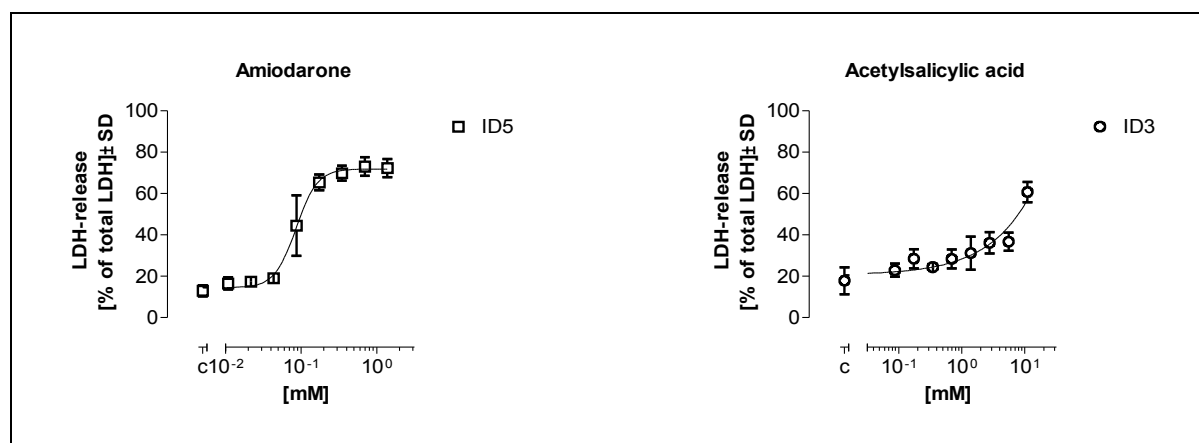
The assay results received from the automated liquid handling system of ECVAM show that controlled quality and standardized testing of compounds are achieved within a stage of manual SOP requirements. The LDH-release cytotoxicity assay, which is now well established and optimised, proves to be easily automated with an acceptable throughput. Since the cells show metabolic activity, but with a donor-dependency, we recommend further experiments with and better characterisation of NeoHepatocytes.

Table 7:

EC₅₀ values from cytotoxicity experiments with NeoHepatocytes on the HTS at ECVAM (SOP V3.0).

The EC₅₀ was calculated using GraphPad Prism Software. The program extrapolates the data to calculate an EC₅₀, therefore higher values than the concentrations actually used were excluded. na: No increase in cell toxicity in this concentration range, hence no EC₅₀ value was calculated.

EC ₅₀ [mM] except*	ID1	ID2	ID3	ID4	ID5	max. Treatm.
Cycloheximid	0.08	0.04		0.04		1.78
SLS (SDS)	0.17	0.02		0.02		0.69
Verapamil	0.21	0.19		0.27		1.02
Digoxin [uM]*	0.57	0.36		0.87		6.40
Tetracycline	1.04	1.11		0.36		4.16
DMSO [%]	683.60	4.01		68.49		10.00
Paracetamol	na	7.46	11.19	5.26	7.70	16.54
Mercury Chloride II (HgCl ₂)			0.00			2.21
Colchicine			0.01			0.13
Sodium valproate			0.45			60.17
Atropine sulfate monohydrate			3.80			14.39
ASS			2235.00			11.10
Carbamazepine			na			4.23
Amiodarone hydrochloride					0.09	1.39
Cyclosporine A [uM]*					26.22	36.83
Isoniazid					67.37	120.34
Orphenadrine hydrochloride					1.03	8.46
Parathion					36.89	1.25
Rifampicin					7.15	0.72

**Figure 26:**

Concentration dependence of the cytotoxicity of specified substances on NeoHepatocytes as represented by LDH-release after 20hrs of incubation in 96-well plates. Treatment and LDH-assay was performed by robot. Data represent mean ± SD.

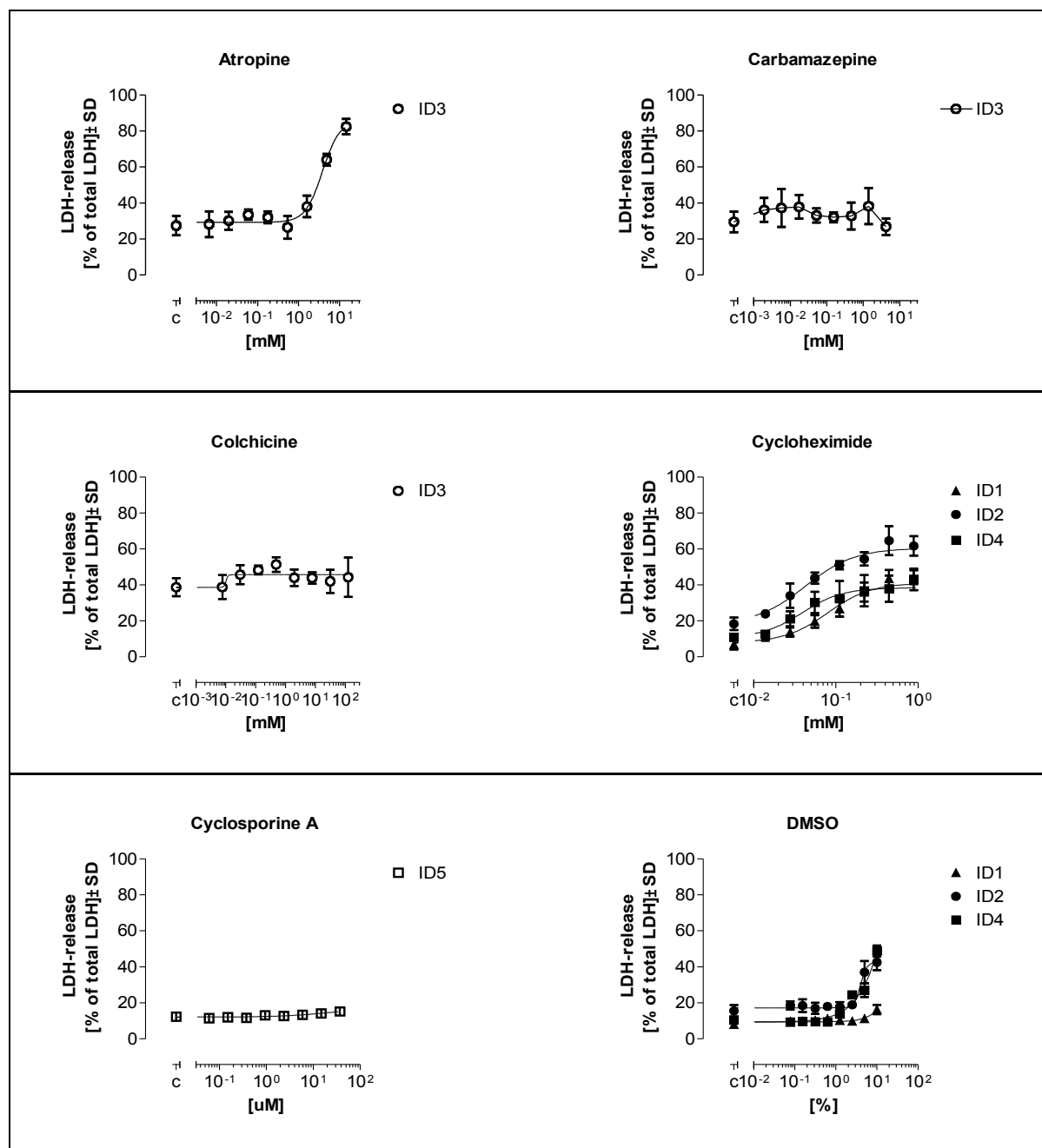


Figure 27:

Concentration dependence of the cytotoxicity of specified substances on NeoHepatocytes as represented by LDH-release after 20hrs of incubation in 96-well plates. Treatment and LDH-assay was performed by robot. Data represent mean \pm SD.

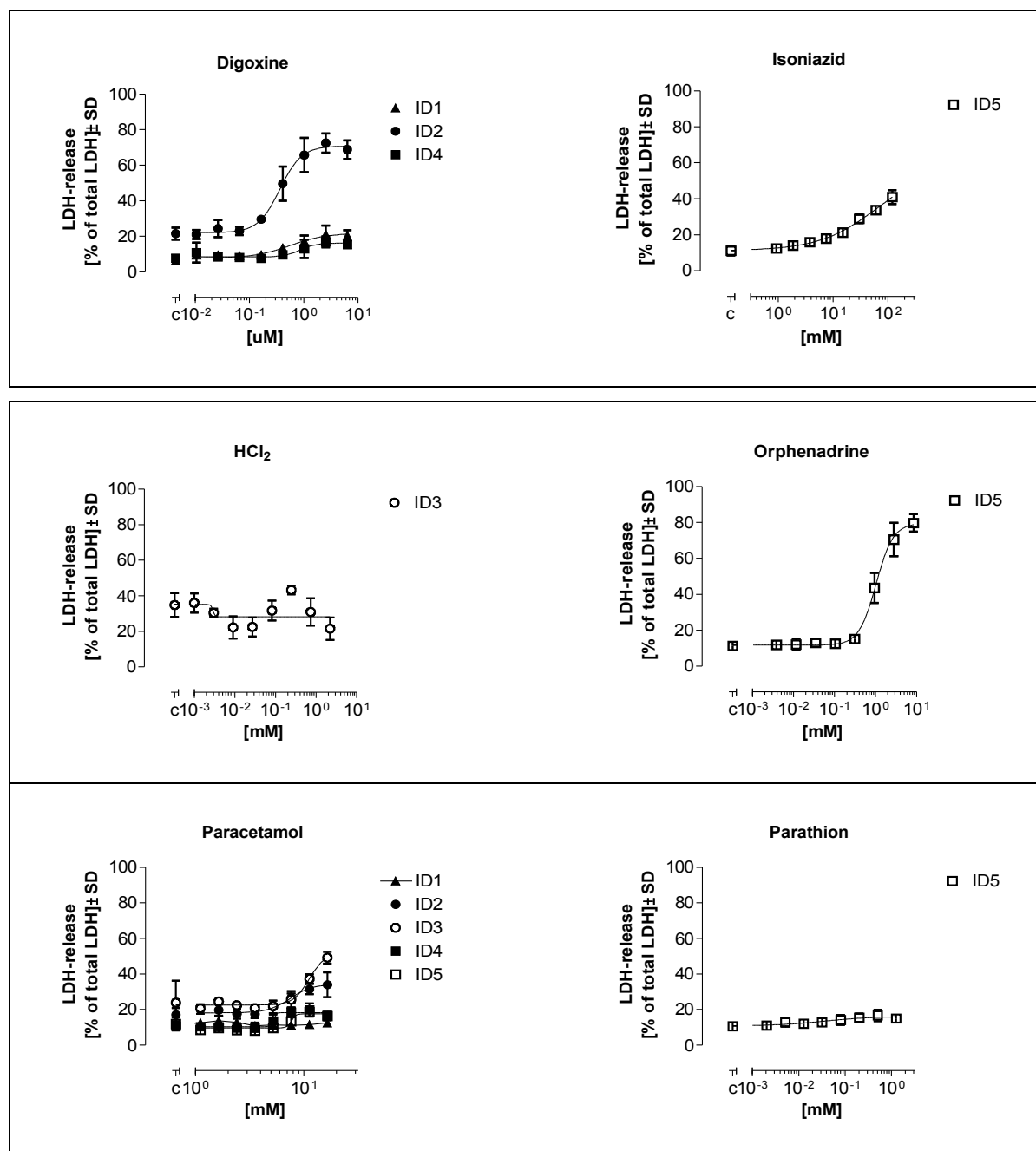


Figure 28:

Concentration dependence of the cytotoxicity of specified substances on NeoHepatocytes as represented by LDH-release after 20hrs of incubation in 96-well plates. Treatment and LDH-assay was performed by robot. Data represent mean \pm SD.

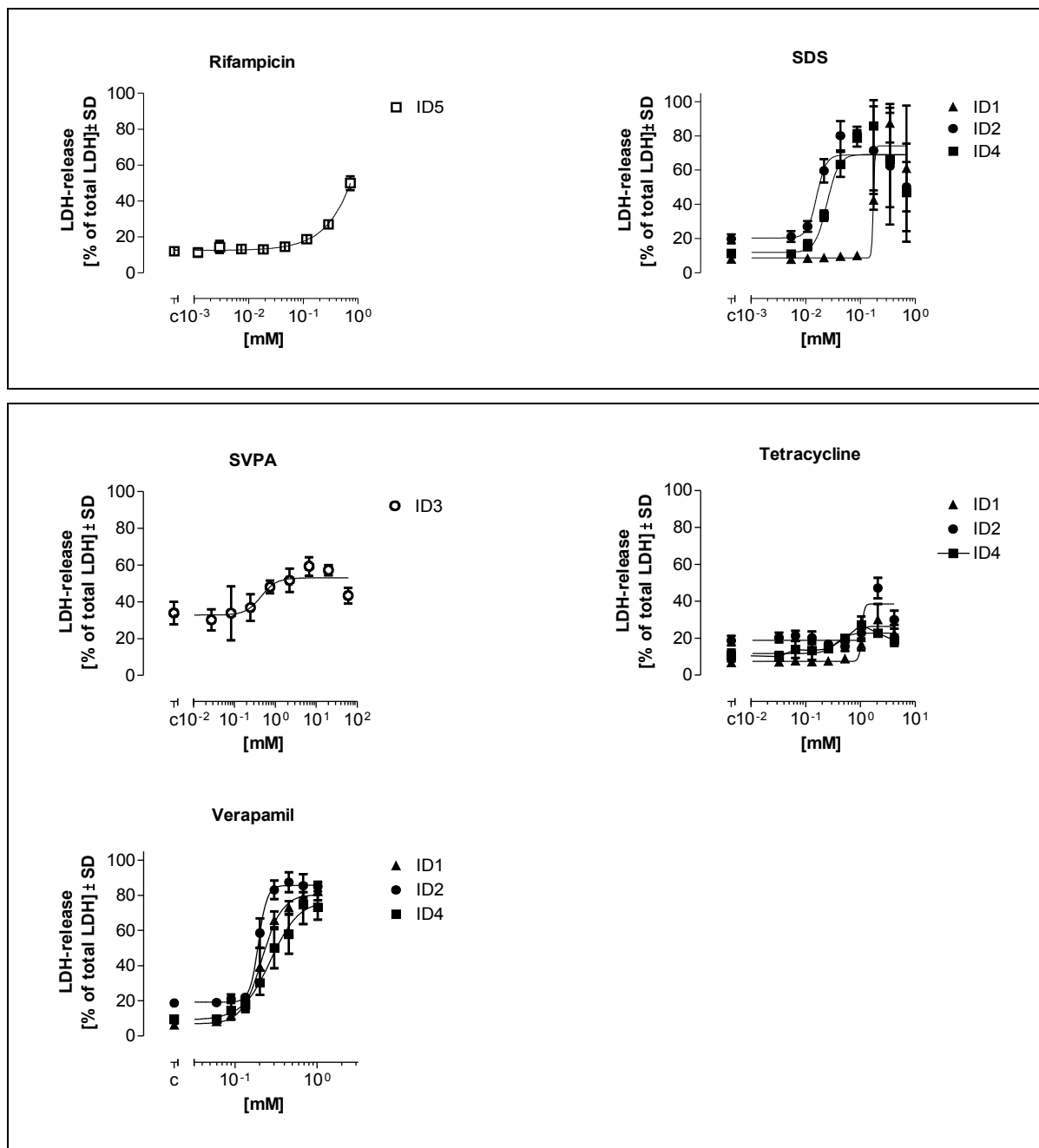


Figure 29:

Concentration dependence of the cytotoxicity of specified substances on NeoHepatocytes as represented by LDH-release after 20hrs of incubation in 96-well plates. Treatment and LDH-assay was performed by robot. Data represent mean ± SD.

4.5 Physiological Cell Death

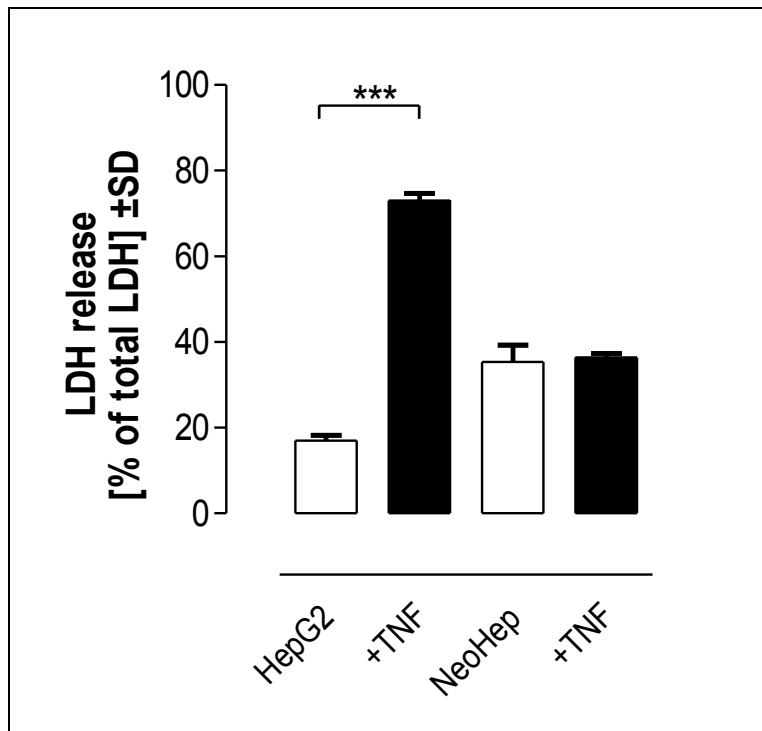
4.5.1 Death-Receptor Induced Cell Death

Apoptosis is the balance between the withdrawal of positive signals, i.e. signals needed for cell survival, and the reception of negative signals. If the pathway of receptor induced cell death fails, damaged cells will no longer be eliminated. With cells that are introduced into the human body, there is always the possibility of benign or even malign development. For that reason, it is very important that cells, which are intended for cell therapy i.e. NeoHepatocytes [61], are still sensitive to apoptotic signals.

It is known that CD95L induces apoptosis in hepatocytes without sensitising treatment. This was first investigated parallel to TNF α treatment, but neither TNF α or CD95L had a cytotoxic effect on NeoHepatocytes. Therefore the cells were sensitized with either Actinomycine D (ActD), a transcription inhibitor, or Cycloheximide (CHX), a peptide synthesis inhibitor, and stimulated with death receptor ligands (TNF [80], CD95L).

Activation of the death receptor pathway by ActD [1 μ g/ml] \pm TNF α [100ng/ml] or CHX [100 μ M] \pm CD95-L [5%] treatment triggers cytotoxicity in HepG2 (Figure 29). In contrast, NeoHepatocytes do not react to any sensitising with ActD or CHX (not shown) and treatment with the death receptor ligands TNF α or CD95L (not shown). The curves with and without treatment proceed in parallel, indicating only the basal toxicity of the sensitising substances (ActD, CHX), increasing concentration dependently.

With these results, the question arises, whether or not the NeoHepatocytes do express the death receptors. Therefore, the cells were analysed in FACS analysis.

**Figure 30:**

HepG2 compared to NeoHepatocytes: Cytotoxicity of ActD/ ±TNF determined by LDH-release after 20hrs of incubation in 24-well plates. Data represent mean ± SD.

4.5.2 Death-Receptor Expression

NeoHepatocytes were prepared for FACS analysis to answer the question of death receptor expression. Since the cells originate from monocytic cells of peripheral blood and are expected to be differentiated into hepatic cells, they were also analysed for CD14 expression (Figure 30). The FACS analysis showed that NeoHepatocytes still express the macrophage marker CD14, but the expression decreases over time. In addition, the cells express the death receptors CD95, TNF R-I (not shown) and TNF R-II on the cell surface, increasing over time.

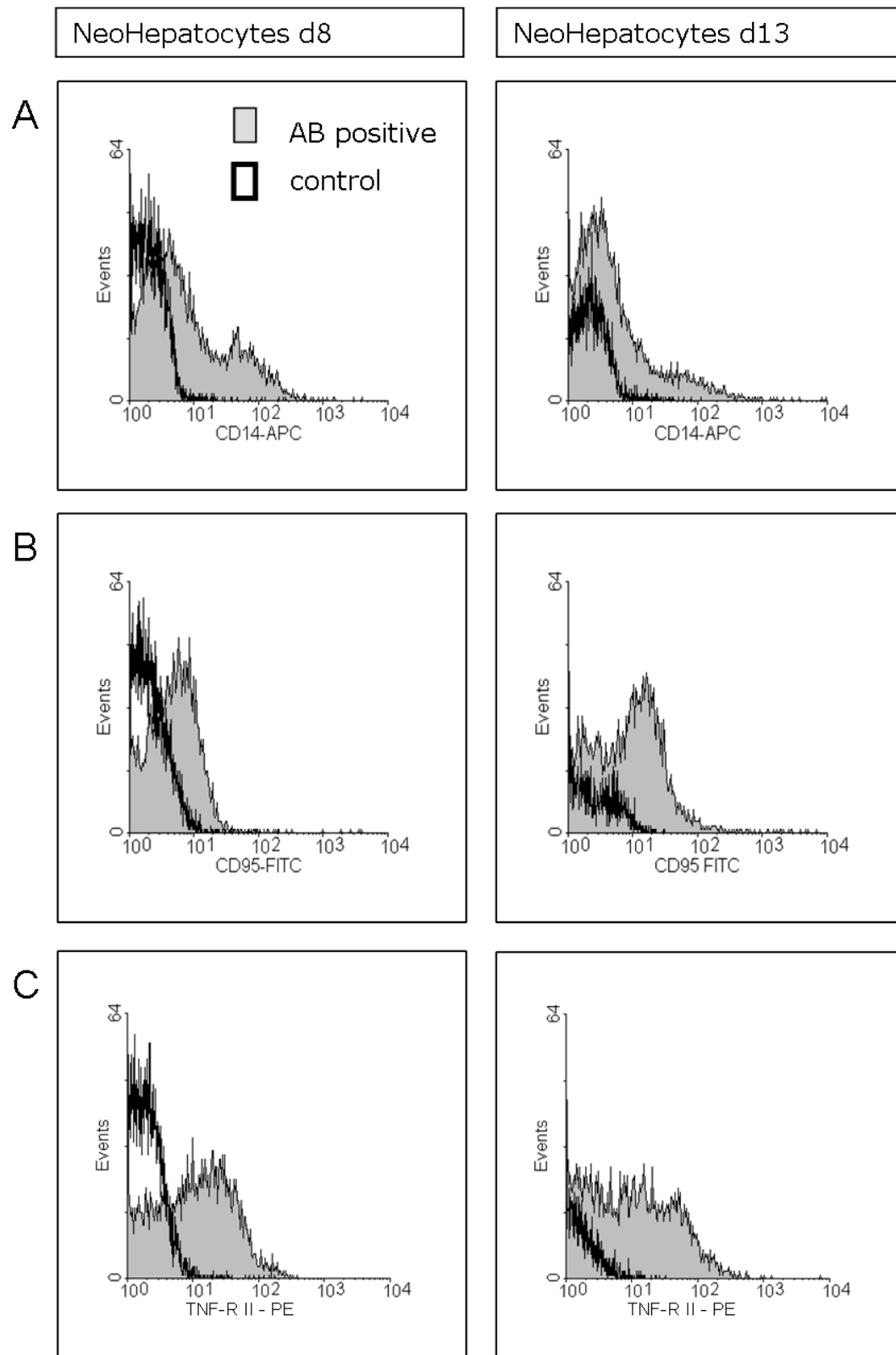


Figure 31:

FACS-Analysis of NeoHepatocytes at day 8 and day 13 after differentiation (n=1). AB: Antibody positive, control: unstained sample

A. CD14 expression, B. CD95 expression, C. TNF-R II expression

5. Discussion

This work was initiated to establish a cytotoxicity assay based on NeoHepatocytes. With the straightforward procedure of their generation, this new cell type raises hope for a liver-like metabolising system. Based on these cells a large-scale *in vitro* test system designed to predict human toxicity is the objective.

5.1 Methodological aspects of a NeoHepatocyte-Based Toxicity Test System

In order to define a SOP (Standard Operation Procedure) for the use of NeoHepatocytes in a cytotoxicity assay, various experiments were performed to define general conditions suitable for high performance testing of chemicals for their toxicological properties.

5.1.1 Selecting the suitable Measurement End Point

An ideal test system should be easy to handle manually as well as by a High Throughput System (HTS) and low priced. Cell viability assays based on MTT or AlamarBlue depend on the numbers of cells in the well. Therefore, these assays detect variable growth rates and an inhibition of cell proliferation, which could be misinterpreted as cell death. However, the way of producing the NeoHepatocytes posed a challenge to the measurement of parameters of cell death: the cells are cultured on the plate for weeks and so the cell number per well can differ noticeably. Hence, a kind of intra-well control was required to cope with disadvantages of conventional cytotoxicity/cell viability assays.

An assay that measures plasma membrane leakage would consequently be a practicable test for membrane integrity and thereby for cytotoxicity. Therefore, the measurement of enzyme release into the supernatant and remaining intracellular enzyme as result of toxicological effect was chosen for the setup of the acute toxicity test with NeoHepatocytes. The resulting data are expressed as the ratio of released to cumulative enzyme in percent.

The measurement of Lactate dehydrogenase (total LDH=cytosolic LDH plus released LDH) represents a standardized parameter that has already been used in combination with the particular experimental setup in our lab. The assay met the requirements defined above. It proved to be highly reliable and reproducible with different cell types and different treatments. Therefore, LDH release was chosen as an end-point measurement of chemical induced acute toxicity in NeoHepatocytes.

5.1.2 Definition of Control Substances for Liver-specific Toxicity

When using cells, i.e. a cell line or primary cells to check for cytotoxicity, appropriate positive and negative controls are required. For direct cell death, sodium lauryl sulfate (a protein denaturant and ionic surfactant) is

commonly used for that purpose in viability and cell death experiments. As the positive control, the detergent SLS disrupts the cell membrane irreversibly and thereby kills the cells, additionally untreated or with the solvent treated cells are used as negative control.

At the beginning of the study, it was decided that the negative control, i.e. untreated cells were to be used in the setup of the LDH-assay, revealing the basal enzyme release. In our tests, we found an average basal LDH-release of 20%. Paracetamol was chosen to be the control substance for hepato-specific cytotoxicity. It is well documented that the toxic effects of this drug after high concentrated applications are due to bioactivation in the liver by phase I/II reactions.

In the first experiments, cytotoxicity was induced by Paracetamol in a concentration dependent manner in primary cells, but not in HepG2 cells. The results with NeoHepatocytes reveal variability between the different donor cells' reaction to Paracetamol. The question stays unanswered if the variability is due to differences in enzymatic activities, differences in the number of gene copies (polymorphism) or because the quality during cell generation is not yet manageable. It is important to keep in mind that the primary human hepatocytes originate from patients. Hence, it is likely that the cells metabolizing enzymes are induced by drugs the patient got prior to or during surgery. The cells may have already enhanced their cellular survival program and therefore react less sensitive to the Paracetamol treatment than the primary murine cells do. Nevertheless, the results with NeoHepatocytes seem to be the best approximation to the ones with human hepatocytes.

Since the NeoHepatocytes are derived from many different individuals, a preliminarily test for the metabolising system may be required for each donor's metabolic enzyme pattern. If the variability is due to the variable metabolising systems of NeoHepatocyte batches, instead of Paracetamol, one control substance per p450 enzyme should be used. (see 5.2).

5.1.3 Influence of Culture Conditions

EUFETS AG offered NeoHepatocytes on various culture plates and flasks. At the launch of this study, 6-well and 24-well plates were at our disposal. The observed morphology of NeoHepatocytes appeared comparable to the published pictures [62]. The cytotoxicity induced by Paracetamol was checked in cells on 6-well plates (data not shown) and 24-well plates, with corresponding results. Indeed the latter were far more suitable for concentration dependent treatment: more data points for the calculation of the regression curve and the EC_{50} with the same cell amount. Therefore, the initial experiments were designed using 24-well plates as a basic set-up for the toxicity test system.

The transfer to a robot pipetting and culturing system guaranteeing both high throughput and a standardized performance is a future goal for the mass application of this toxicity test system. Since these systems work based on 96- and 384-well plates, EUFETS AG adapted its protocol to 96-well plates and was able to deliver this standard plate size. First, the cells

in the smaller wells were examined under the microscope. Some looked very different than their counterparts in the larger wells, by appearing more fibroblast-like. It is still not known if this observation is of relevance for the experiments intended. In the cytotoxicity assay, results showed no significant differences between cells from the same donors on 24 well- and 96 well plates.

Based on these findings, we conclude that 96-well plates represent a promising format for the future toxicity test system. NeoHepatocytes can be cultured and incubated on 96-well plates ensuring that a high number of chemicals are to be tested at the same time in a high throughput fashion.

Based on the protocols of the aforementioned experiments and the experiences during the test, the Standard Operating Procedure (SOP V1.0) was created.

5.2 Generation and Supply of NeoHepatocytes

The unsteady quality of the cells, starting with the seeding and the amount of cells per well was a difficulty during test runs. NeoHepatocytes had to be reordered several times and the proposed timeline was retarded.

The LDH measurement allows for the unequal number of cells per well, but the allocation of cells in the well is also very variable, probably dependent on the seeding process/operator.

On the plates provided by EUFETS AG, we repeatedly found cells of heterogeneous morphology. Frequently cells were produced that looked more like fibroblasts, i.e. long in shape and with filopodia-like characteristics. It is still unknown, which cell type on the plate, is the one that has the ability to metabolise the different substances, and therefore may be called NeoHepatocyte.

Information about irregularities during the production of NeoHepatocytes is only available from EUFETS AG. To our knowledge, no quality control checks were performed to ensure the metabolising ability of the cells after cell culture for several weeks.

The production of NeoHepatocytes must become more standardized for a future use in different fields of application. The composition and definition of medium, serum, cytokines, etc. must be included in a SOP in detail. The individual variability of the cell source might be handled by predefining potential donors (e.g. healthy males between 20-50 years of age).

5.2.1 NeoHepatocytes and Metabolism: Donor Variability

Since monocytes derive from the immune system, pre-treatment of donors and thereby induction of the CYP450 system could have immense influence on the development of NeoHepatocytes and their subsequent ability to metabolise. The donors should probably be tested on their drug treatment and their inflammation status [92] before blood sampling.

Other groups working with NeoHepatocytes showed that these cells have a measurable CYP 450 activity [61, 62, 93, 94]. A new approach could lead to a better quality assessment of the ability of a cell type to metabolise. Pelkonen and colleagues recently published the protocol for an *in vitro* interaction assay [95, 96], with this being able to measure multiple p450 substrates in one single run. In cooperation with ECVAM NeoHepatocytes will be sent to Finland and tested with this protocol.

This would be a practicable test to ensure that donor cells do have a defined set of liver enzymes when using them to test for acute toxicity of substances. Since up to now, no solution is found to solve the problem of donor variability, normalisation for each enzyme is inevitable.

However, one should not forget that this variability represents the human population with slow and fast metabolisers and may bear possibilities in drug development of custom-made or individual-specific prescription and therapy.

5.2.2 Pooling NeoHepatocytes from Multiple Donors

To approach the task of overcoming donor variability, NeoHepatocytes from different donors were pooled for the cytotoxicity assay. However, the use of pooled NeoHepatocyte preparations seemed to be affected by incompatibility reactions, thereby clearly reducing the number of cells and their appearance. In pooled cultures, only a fractional amount (approx. 20%) of cells survived the initial culture phase necessary to differentiate cells from monocytic origin to NeoHepatocytes. This suggests that certain differences in the donor haplotype results in incompatibility reactions finally leading to depletion/cell death of a major number of developing NeoHepatocytes. In consequence, pooled cell cultures were not used for further experiments.

5.3 Applicability of the SOP

5.3.1 Preliminary Experiments

During the cytotoxicity experiments, it became obvious, that it is not suitable for the assessment of volatile fluids e.g. diverse alcohols. Additionally, the cytotoxicity of Mercury chloride II could not be assessed properly in some runs. An explanation is that in these cases, the chemical precipitated in the highest concentrations and thereby prevented a correct measurement.

5.3.2 Intra-and Interlaboratory Experiments

The SOP was first tested in intralaboratory experiments and then in an interlaboratory study. In both cases, the resulting data showed a good correlation between the participants indicating that the SOP can be handled independently of the experimenter and/or the laboratory. Like in the initial experiments the variability between the batches of NeoHepatocytes were found.

It was frequently observed that the basal toxicity was lower in lab 2. The fact that the closest lab had the lowest values raised the question as to whether the distance between the supplier and the test laboratories was significant for the outcome. Another reason for different basal levels might have been the different handling of the cell delivery packages during the shipping. Later observations reinforced the latter thesis.

5.3.3 Automatisations: Transfer to the Robot System

The SOP was successfully transferred to the laboratory at ECVAM. The manual performed LDH-assay showed correlating results when compared with the ones from Konstanz laboratory. Again, a difference in the basal toxicity was observed: it was much lower at ECVAM. Because of the long distance from the cell supplier in Idar-Oberstein, Germany, the cells were handled by one person who drove directly to Ispra, Italy. The NeoHepatocytes for Konstanz were shipped as usual by shipping company, including overnight storage. This may explain why NeoHepatocytes arriving in Italy had a lower basal LDH-release i.e. were less stressed.

Preliminary tests on the robot were effectively performed with the common used 3T3 cell line, treated with SDS. After the optimisation phase NeoHepatocytes were send from EUFETS AG to ECVAM for the hepatotoxicity testing of substances. Due to the time required for collection and measurement (approx. 70 min/plate), it is possible to test up to eight compounds per run and two runs are possible per week on the working station at ECVAM.

The assay results received from the automated liquid handling system of ECVAM show that controlled quality and standardised testing of substances was achieved within a stage of manual SOP requirements. The on LDH-release based cytotoxicity assay was well established and

optimised, and proved to be easily automated with an acceptable throughput.

5.4 Physiological Cell Death

The hepatic character of NeoHepatocytes was controversially discussed during a symposium held in Konstanz in 2007. J. Hutchinson [97] introduced the view that NeoHepatocytes are less analogue to hepatocytes but have in fact a macrophage-like structure [97].

A FACS analysis of NeoHepatocytes (n=2) showed that death receptors CD95, TNF-R I and TNF-R II were present on the cell surface (increasing over time), maybe not in a fully functional form, since NeoHepatocytes differ from the HepG2 and the primary hepatocytes i.e. they do not react upon fatal stimulation with the death-inducing cytokines TNF α and CD95L. In addition, CD14 (a membrane-associated protein, especially expressed at the surface of macrophages) was found. However, the expression of this macrophage marker decreased over time.

Death receptor-induced signalling pathways are differentially regulated in macrophages than in hepatocytes, and growth factor treatment prevents active cell death in macrophages [98]. Therefore, the findings imply that NeoHepatocytes rather resemble these immune cells than liver cells regarding their apoptosis mechanisms. Based on the literature we note that variances during dedifferentiation and/or differentiation phase in medium composition or handling may have an enormous effect on the outcome of the generation of NeoHepatocytes.

In the consequence of these results on apoptosis susceptibility, NeoHepatocytes rise reservations as to the potential use of these cells for repopulation therapy of partially impaired livers in humans. Such a treatment regimen has been proposed by various groups [99, 61, 100, 101]. It cannot be excluded that without a functioning apoptosis signalling transduction in the cells, such neohepatic treatment bears the high risk of off the balance between proliferation of cell and cell death. In other words: the loss of death control might result in uncontrolled growth, i.e. benign or even maligne tumour formation. It seems therefore mandatory to explore the apoptotic signalling pathways in these cells in detail before the cells may be considered for clinical applications. The introduced preliminary results clearly warrant further investigations of the issue.

6. General Conclusions about the technology

The use of primary human cells and human stem cells for safety assessment of chemicals struggles with the availability of cells and ethical considerations. The need for human cells will increase with the ongoing research and development in science as well as in industry. The most important benefit in the use of human cell systems in toxicology is that they avoid extrapolation between species [59].

NeoHepatocytes offer excellent features regarding (automated) test systems for toxic substances, especially hepatotoxic compounds: they are derived from human monocytes, are continuously available, little expensive, free of ethical concerns, and to be kept in culture for up to weeks with ongoing hepato-specific enzyme activity [61, 62, 94].

The evaluation of results from different donors and different laboratories shows the suitability, robustness, and capacity of NeoHepatocytes as a cellular system to be used in risk assessments of chemical compounds. Certainly, the reproducibility of the results depends on a comprehensive protocol for the cytotoxicity assay, the generation, and the handling of the cells themselves. It became evident that the test system is not suitable for volatile substances such as alcohols due to evaporation effects. Only water-soluble or DMSO-soluble substances were tested. The highest possible/necessary dilution should be used to avoid precipitation.

In order to apply the LDH-release assay to a large variety of substances, NeoHepatocytes should be cultured and incubated on 96-well plates for the use on a robot system. The transfer to such a system needs a revision of the SOP for the different handling methods of automatic operating systems. Such a system should be assembled at least with a cell culture incubator, a liquid handling module, and an optical density reader.

As discussed, donor variability is still considered a difficulty. Nevertheless, the source of NeoHepatocytes i.e. the multiple donors in the generation process of the cells must not be considered a problem only: they mirror the human population and this may also offer advantages in *in vitro* cell culture or *in vivo* medication. It will be interesting to investigate whether the enzymatic activity of NeoHepatocytes is identical equal to the one in hepatocytes of a test person/patient.

In conclusion, this study showed that the NeoHepatocyte/LDH-assay has the potential to take a pole position in the testing of metabolised toxins in the REACH program. Before NeoHepatocytes may be used as an alternative for animal testing, they will have to undergo a validation process [6] by ECVAM. Provided, that a standardized cell source is available, a validated toxicity test with NeoHepatocytes is, due to the concerns mentioned above, a better choice than primary animal cells and a pragmatic compromise compared to primary human liver cell cultures.

7. Outlook

7.1 Quality control

As mentioned before, general quality assessment of the cells and thereby reducing donor variability must have priority in the generation of NeoHepatocytes. A variety of possible methods was discussed and should be considered for the future production of this cell type.

7.2 Cost Reduction Strategies

The financial aspect of the test system must be considered for further industrial usage. A commercially available assay kit was used to measure the LDH contents in this study. This kit has already been used routinely in our group with other cell types. Since it is normally used in small quantities, the toxicity testing of a huge amount of compounds would get too expensive in large scale testing. Our group just started to evaluate an assay based on the same principle as the commercial kit by using separate products in a modular assembly concept. It seems to be as reliable as the commercial kit and the compounds are less expensive in procurement. This concept may be an alternative and most likely can easily be adapted to a liquid handling system.

7.3 Further Research Perspectives for Neohepatocytes

In addition to the toxicity testing, the NeoHepatocytes might be used to minimize the use of animals in pharmacovigilance testing:

- Metabolism studies
- Acute toxicity tests in pharmaceutical research and development
- Repeated dose toxicity/ subchronic toxicity
- Reproduction toxicology

These cells might also be a substitute for human hepatocytes in "Artificial liver" systems [11] and the treatment of liver patients (metabolic disease, liver dissection):

- Co-culture system e.g. with Kuppfer cells or replacement
- 3D culture (Reproducible Three-Dimensional Tissue Culture)
- Bioreactor system /liver dialysis
- Cell therapy

8. Summary

REACH (Registration, Evaluation and Authorization of Chemicals) is a regulatory initiative of the EU Commission for the safety of chemicals which came (in force since June, 1st, 2007). At present, a majority of chemical testing is animal-based or on the exposure of hepatocytes from mainly animal species. The European Centre for the Validation of Alternative Methods (ECVAM) validates new, optimally human *in vitro* systems to replace animal experiments.

Here a test system was established, based on human NeoHepatocytes for the assessment of metabolism-dependent, chemically induced acute toxicity. It is inexpensive and suitable for High Throughput Screening (HTS). A Standard Operating Procedure (SOP) was elaborated, adjusted and tested on a robotic platform. As to potential clinical use of NeoHepatocytes, orientating experiments were performed as to their sensitivity towards receptor-dependent apoptotic cell death.

In detail the following results were obtained:

1. A cytotoxicity assay using the release of lactate dehydrogenase (LDH) was adapted for the special culture conditions of NeoHepatocytes.
2. Concentration-dependent cytotoxicity was induced by the indirect hepatotoxin Paracetamol in primary murine and primary human hepatocytes as well as in NeoHepatocytes. Even though varying EC₅₀ values (5mM-20mM) were obtained with NeoHepatocytes from different donors, the data indicate that these cells (mean absolute toxicity 30%) gave the best match to primary human hepatocytes (37%) while the human liver cell line HepG2 (7%) failed to do so.
3. An SOP was established, refined in intralaboratory and interlaboratory studies with nine chemical compounds (thereof direct and indirect toxic substances) and led to reproducible results.
4. The assay was successfully transferred to a computer-controlled robotic platform at the European Centre for the Validation of Alternative Methods (ECVAM), Ispra, Italy.
5. Applicability and performance with NeoHepatocytes was tested on the High Throughput System. Controlled quality and standardised testing of compounds were achieved.
6. FACS-analysis revealed the presence and time-dependent increase of death receptors on the cell surface. However, NeoHepatocytes were not sensitive to ActD or CHX –sensitized cell death induced by TNF α or CD95L.

In summary, the results of this thesis encourage the further development of NeoHepatocytes for risk assessment of direct and particularly indirect hepatotoxins.

9. Zusammenfassung

REACH (Registrierung, Evaluation und Zulassung von Chemikalien) ist eine neue EU-Verordnung zur Sicherheit chemischer Stoffe (seit 1. Juni 2007 in Kraft). Bis heute wird der Großteil der Chemikaliertestung an Tieren durchgeführt oder basiert auf Leberzellen der verschiedensten Spezies. Das Ziel des Europäischen Zentrums zur Validierung von Alternativen Methoden zu Tierversuchen (ECVAM) ist es, neue, möglichst humane *in vitro* Systeme als Ersatz zum Tierversuch zu validieren.

In dieser Arbeit wurde ein, auf menschlichen NeoHepatozyten basiertes Testsystem entwickelt, das zur Bewertung von Metabolismus abhängiger, Chemikalien induzierter, akuter Toxizität dient. Dieses Testsystem ist preisgünstig und geeignet für High Throughput Screening (HTS). Eine Standardarbeitsanweisung (SOP) wurde ausgearbeitet, angepasst und auf einer automatisierten Plattform getestet.

Im Hinblick auf eine mögliche klinische Nutzung von NeoHepatozyten, wurden erste Vorversuche zur Fragestellung gemacht, ob diese Zellen ihre Sensitivität gegenüber der rezeptorabhängigen Apoptose behalten.

Im Einzelnen wurden folgende Ergebnisse erzielt:

1. Ein Zytotoxizitätsassay basierend auf der Freisetzung von Laktatdehydrogenase (LDH) wurde an die speziellen Zellkulturbedingungen der NeoHepatozyten angepasst.
2. Durch das indirekte Hepatotoxin Paracetamol konnte eine konzentrationsabhängige Zytotoxizität in primären murinen und humanen Hepatozyten, so wie NeoHepatozyten induziert werden. Obwohl die mit NeoHepatozyten erzielten EC_{50} variierten (5mM-20mM), zeigen die Daten, dass diese Zellen (mittlere absolute Toxizität bei 30%) im Gegensatz zu HepG2 Zellen (7%) den primären humanen Hepatozyten (37%) gleichkommen.
3. Standardarbeitsanweisung (SOP) wurde erstellt, während Intralabor- und Interlaborversuchen anhand von neun verschiedenen Chemikalien (direkte und indirekt toxische Substanzen) präzisiert und führte zu reproduzierbaren Ergebnissen.
4. Der Assay wurde auf eine computergesteuerte, automatisierte Plattform am Europäischen Zentrum zur Validierung von Alternativen Methoden zu Tierversuchen (ECVAM), Ispra, Italien übertragen.
5. Die FACS-Analyse zeigte das Vorhandensein und die zeitabhängige Zunahme von Todesrezeptoren auf der Zelloberfläche. Allerdings konnten NeoHepatozyten nicht mit ActD oder CHX für den TNF α oder CD95L-induzierten Zelltod sensitiviert werden.

Im Rahmen dieser Arbeit konnte gezeigt werden, dass die Weiterentwicklung von NeoHepatozyten für die Risikobewertung direkter und im Besonderen indirekter Hepatotoxine ein großes Potential birgt.

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11. Abbreviations

ActD	Actinomycin D
CD95L	anti-CD178, FAS ligand
[c]end	final concentration
CHX	Cycloheximide
CO ₂	Carbon dioxide
ctrl	Control
CYP	Cytochrome P450 monooxygenase
DMSO	Dimethylsulfoxide
EC ₅₀	Effective Concentration 50%
ECVAM	European Centre for the Validation of Alternative Methods
EU	European Union
FCS	Fetal calf serum
FGF	Fibroblast growth factor
hrs	Hours
H ₂ O	sterile Water
HGF	Hepatocyte growth factor
IHC	Immunohistochemistry
IHCP	The Institute for Health and Consumer Protection
IP	Integrated Project
JRC	The Joint Research Center, Ispra, Italy
L	Lysate of the remaining cell monolayer
LDH	Lactate dehydrogenase
M-CSF	Macrophage Colony stimulating factor
MgCl ₂	Magnesium chloride
MTT	3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl-tetrazolium bromide
na	not available
nd	not done
NaCl	Sodium chloride
NeoHep	NeoHepatocytes
NSAID	Non-steroidal anti-inflammatory drug
REACH	Registration, Evaluation and Authorization of Chemicals
RT-PCR	reverse transcriptase polymerase chain reaction
S	Supernatant
SDS (=SLS)	Sodium dodecyl sulphate (=Sodium lauryl sulphate)
SOP	Standard Operating Procedure
SVPA	Sodium valproat
TNF α	Tumor necrosis factor α
TRAIL	TNF-related apoptosis-inducing ligand, Apo-2L
v/v	Volume per volume
w	Well plates
w/o	without

12. Appendix

LDH MEASURING CYTOTOXICITY ASSAY

Standard Operating Procedure

Drafted by:	Name	<i>Isabelle Pochic</i>
	Date	<i>24.08.2006</i>
	Signature	

Reviewed by:	Name	<i>Prof. Albrecht Wendel</i>
(expert)	Date	
	Signature	

Approved by:	Name	<i>Dr. Corinna Herrmann</i>
	Date	
	Signature	

Issued by:	Name	
	Date	
	Signature	

*Owner/Trainer: Signature: Date:

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1. INTRODUCTION

Isolated and cultured human hepatocytes represent an indispensable cellular system for the validation of metabolisable chemicals with toxicological and pharmacological relevance. Unfortunately, their use in actual practice is limited by the following facts (1) primary human hepatocytes are only available in limited quantities (2) all cellular test systems - including primary human hepatocytes - are compromised by a rapid and spontaneous down-regulation of metabolizing enzymes, i.e. cytochrome P450 enzymes. Especially with respect to the new strategy for a future chemicals policy, there is an ultimate need for metabolically competent cellular test systems suitable to perform risk assessment studies on a large scale.

Recently, hepatocyte-like cells (NeoHepatocytes) from terminal differentiated peripheral blood monocytes were made available by culturing them under conditions that promote hepatocyte-like differentiation (1). These NeoHepatocytes resemble primary human hepatocytes with respect to morphology, expression of hepatocyte markers, various secretory and metabolic functions and drug detoxification activities (2). The maintenance of the metabolic balance is kept well for up to weeks, and they are suitable for co-cultures with other cells.

2. PURPOSE

The aim of this project is to investigate the use of human monocytes-derived NeoHepatocytes for risk assessment studies of chemicals. Therefore, chemical compounds are tested for their cytotoxic effects by quantification of LDH activity.

3. HEALTH SAFETY AND ENVIRONMENT

All toxic substances should be handled with caution, using gloves and protective clothing and if necessary a protective mask. Disposal of toxic substances should be done in accordance with the legal safety and health instructions.

4. TEST PRINCIPLE

The enzyme Lactate Dehydrogenase (LDH) is a stable cytoplasmic component ubiquitously present in all cells. Upon substantial damage of the plasma membrane, it is released into the supernatant of the cell culture. LDH activity is determined by an enzymatic test: In the first

step NAD^+ is reduced to NADH/H^+ by the LDH-catalyzed conversion of lactate to pyruvate. In the second step the catalyst (diaphorase) transfers H/H^+ from NADH/H^+ to the tetrazolium salt INT, which is reduced to formazan. Therefore, the amount of color formed in the assay is proportional to the LDH-release of the lysed cells. The formazan salt is water-soluble and shows a broad absorption maximum at about 500 nm, whereas the tetrazolium salt INT shows no significant absorption at these wavelengths. The use of a spectrophotometric micro-plate reader (ELISA reader) allows the simultaneous measurement of multiple samples and thereby guarantees the easy processing of a large number of samples.

This assay can be conveniently performed using commercial LDH detection Kits.

5. LIMITATIONS

Some test substances may interact with components of the LDH-assay.

6. OVERVIEW AND GENERAL REMARKS

The assay is performed over a period of 2 days (under sterile conditions).

The procedure is divided in two parts:

1. Incubation of the cultured cells with the chemical compound.
2. Determination of LDH-release by a colorimetric reaction.

7. DEFINITIONS/ABBREVIATIONS

DMSO:	Dimethylsulfoxide
DPBS:	DULBECCO'S Phosphate buffered Saline
ELISA:	Enzyme-linked immunosorbent assay
EUFETS AG:	Europäisches Institut für Forschung und Entwicklung von Transplantationsstrategien AG (an affiliated company of Fresenius Biotech GmbH)
FCS:	Fetal Calf Serum (heat inactivated for 30 min at 56°C)
FGF:	Fibroblast Growth Factor
INT:	Iodotetrazolium chloride
LDH:	Lactate dehydrogenase
NAD^+ :	Nicotinamide adenine dinucleotide
RPMI 1640:	Roswell Park Memorial Medium 1640
RT:	Room temperature

8. MATERIALS AND EQUIPMENT

All materials in contact with cells must be sterile, pyrogen-free, stored and used according to the manufacture's directions unless specified otherwise. Alternative suppliers for materials and equipment may be used if the quality of the material is equivalent, but all changes must be recorded in the appropriate worksheet.

8.a. Cell Type

Human monocytes-derived NeoHepatocytes, supplied by EUFETS AG, Idar-Oberstein, Germany

8.b. Test Substances

All chemicals are chosen in accordance with the recommendations of the expert commissions ("A-Cute Tox") of ECVAM and the EU.

8.b.a. Cytotoxic substances

No.	Substance	Catalog No.	Distributor	Solvent (DMSO [c] end: ≤1%)	concentration range (6 steps)
1	Acetaminophen (Paracetamol)	A7085	Sigma, Germany	DMSO	2-10 mM
2	Digoxin	D-6003	Sigma, Germany	DMSO	0.06 – 20 µM
3	DMSO	D8418	Sigma, Germany	Medium	0.3 – 10 %
4	Ethanol	34852	Riedel de Häen, Seelze, Germany	Medium	6.2 - 200 mM
5	Mercury chloride II	215465	Sigma-Aldrich, Germany	Medium	5-50 mM
6	Bromobenzene	16350	Fluka, Germany	DMSO	0.03 – 1 mM
7	Allyl alcohol	05788	Fluka, Germany	Medium	0.01 – 100 µM
8	Tetracycline	T7660	Sigma, Steinheim, Germany	DMSO	0.01 - 5 mM
9	Verapamil	V4629	Sigma, Steinheim, Germany	DMSO	0.01 - 10 mM

(DMSO [c] end: ≤1%)

8.c. Materials

- RPMI 1640 with L-Glutamine, (supplied by EUFETS)
- DMSO, D8418, Sigma, Germany
- Triton X-100, X100, Sigma, Germany
- DPBS, H15-001, PAA, Austria
- Cytotoxicity detection kit, No. 1644793, Roche, Germany

8.d. Technical Equipment

- Microplate ELISA-Reader, 492 nm and 600 nm filters
- Incubator: 37°C ± 1°C, 90% ± 5% humidity, 5.0% ± 1% CO₂/air
- Laminar flow clean bench/cabinet (standard: "biological hazard")
- Water bath: 37°C ± 1°C
- Inverse phase contrast microscope
- sterile cups (e.g. 1.5 ml)
- sterile 96 well-plates
- Laboratory balance
- Spatula
- Pipettes (multi-channel and single channel)
- sterile pipette tips
- Multichannel reagent reservoir
- Vortex mixer
- Filters/filtration devices
- Refrigerator at 4°C
- Freezer at -20°C
- Computer and software package for data capture and analysis

9. PROCEDURE

All preparations are performed under sterile conditions in a laminar flow box. Please assure that during all steps performed outside the tubes are closed.

9.a. Preparation of Media and Solutions

9.a.a. Media

The test medium (RPMI) will be shipped from EUFETS.

All media shall be stored at 4°C

9.a.b. LDH-Assay Kit

Cytotoxicity Kit (Roche #1644793):

Both reagents (R1: Dye, R2: Catalyst) can be kept at 2-8°C for a maximum of 4 weeks, and then both should be discarded. The reagent mixture is for immediate use only and can not be stored!

R1 (catalyst: diaphorase/NAD⁺ mixture) (blue cup bottle) is stored at -20°C. Reconstitute it by adding 1 ml at RT of distilled water to the lyophilized powder, wait 10 minutes at RT and mix shortly with the vortex.

R2 (dye solution: INT, sodium lactate) (red cup bottle) is stored at -20°C and is ready to use after defrosting.

Prepare the reagent mixture at RT and with dimmed room light (for one 96-well plate with 56 filled wells): 4.5 ml of defrosted dye solution with 100.9 µl of reconstituted Catalyst Solution (blue cup bottle) (Note: check package insert for changes in ratio.), use instantly. Keep in mind that the mix is sensitive to light (e.g. cover with aluminium foil).

9.b. Cells

At EUFETS AG, Idar-Oberstein, Germany, two weeks before the experiment, the monocytic cells are seeded into 96-well culture plates at a density of 7.58×10^5 cells per well. The de-differentiation and differentiation procedure is performed under defined conditions (see SOP EUFETS AG, Idar-Oberstein, Germany).

9.b.a. Shipping

At day 14 after differentiation the cells are sent by courier service over night to the test performing laboratory. Four days in advance the performer is informed by the supplier via e-mail regarding the shipment of the cells.

The cell plates (wrapped in aluminum foil) are sent in a styrofoam parcel that is equipped with a thermometer (In+Out) and heat reservoirs (heated “CoolPacks”).

9.b.b. Quality Check

The cells are inspected immediately after the arrival from the supplier.

Test Acceptance Criteria:

The received cells shall only be used if:

- Arrived before 11 am (ca. 18h after dispatching)
- Temperature is 20-37°C inside the parcel
- Wells are still filled with medium
- No obvious infection/ contamination is observed
- Documentation of the conditions!

9.b.c. Storage

If the quality of the cells is acceptable, cells should be stored at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$, $90\% \pm 5\%$ humidity, $5.0\% \pm 1\%$ CO_2 /air in an incubator. After six hours the old medium is removed and 100 μl /well of new medium (37°C) is added. The cells are then stored again in the incubator overnight.

9.c. Application flow**9.c.a. Dissolving of the test substances**

Solutions of the test substances have to be prepared immediately prior to use and should not be stored. The substances have to be completely dissolved and must not have precipitates. The stock solution for each test chemical should be prepared as concentrated as possible.

There are two different preparation protocols depending on the polarity of the compound.

Non-polar chemicals: Stock solution in DMSO

Polar chemicals: Stock solution in medium

Allow test chemicals to equilibrate to room temperature before weighting.

Weigh the appropriate amount of compound.

Dissolve and dilute in the adequate solvent (medium, DMSO + medium).

9.c.b. Dilution of the test substances

The dilution steps could be prepared in a 96-well plate. (For final concentration on cells see 11. Appendix.)

If the chemical is dissolved in DMSO, prepare a stock solution, then dilute successively using DMSO for all six following dilution steps. The concentration of DMSO shall be the same for each well. For each dilution, one final dilution step is made with medium (to avoid that the DMSO concentration exceeds 1% in the well. (See Fig. 1 yellow wells).

If the chemical is dissolved in medium, prepare a stock solution. Then dilute successively using medium for all five following dilution steps. (See Fig.1 red wells.)

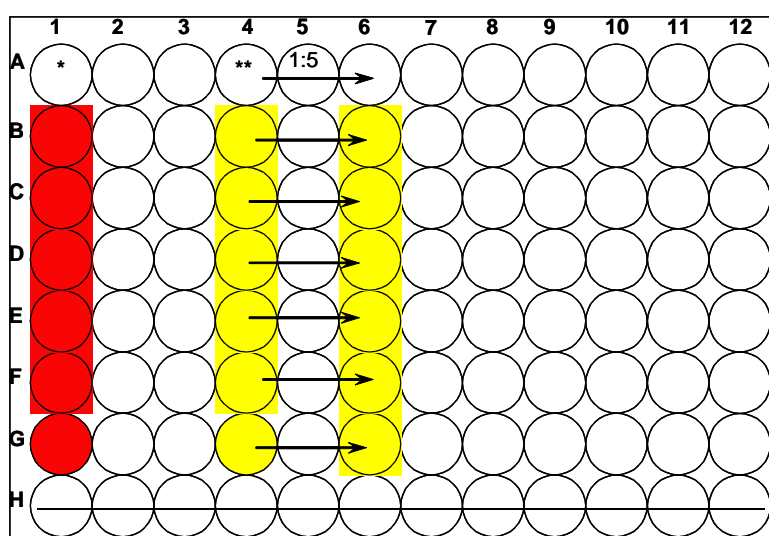


Figure 1: Dilution of test substances in DMSO and medium (e.g. prepare in a 96-well plate, transfer with multichannel pipette to cell plate). Control without compound: * medium, **DMSO

9.c.c. Application of substances

Prior to incubation of test substances, the medium is exchanged (100 μ l/ well, 37°C). Three substances per plate are tested in parallel (5 μ l of one concentration per well), a quadruple set of each concentration. Incubate for 20h at 37°C, 5% CO₂, humidified incubator.

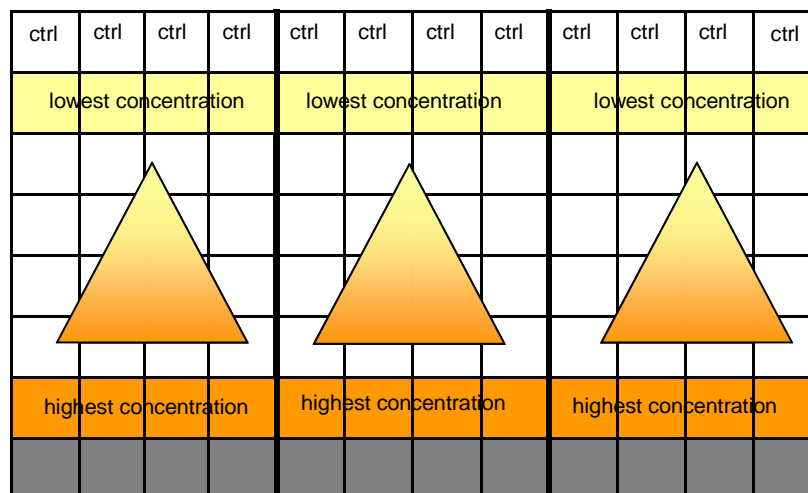


Figure 2: Plate configuration for substance application

9.c.d. Measurement of endpoint

After 20h, 75 μ l of the supernatant medium is transferred to a 96-well plate and stored at 4°C until the LDH-assays is performed (maximum storage time: 1 week).

The remaining medium is removed and replaced by 100 μ L of lysis buffer (DPBS, 0.1% Triton X-100) in order to lyse the cells attached to the bottom of the well.

After incubation for 15 min at 37°C, 25 μ l are transferred to the same 96-well-plate as the supernatant and 50 μ l of medium is added per well. Storage at 4°C until the LDH-assays is performed.

For LDH quantification, the sample plate is incubated with 75 μ L of the reaction mixture (see LDH-Assay Kit) protected from light at 20-25°C for 5-30 min. Add the reaction mixture with a multichannel pipette in horizontal order, so that corresponding wells do have the same incubation time. In horizontally order the LDH activity is measured by its absorbance at 492 nm with reference wavelength 600 nm, values should not exceed OD=1.

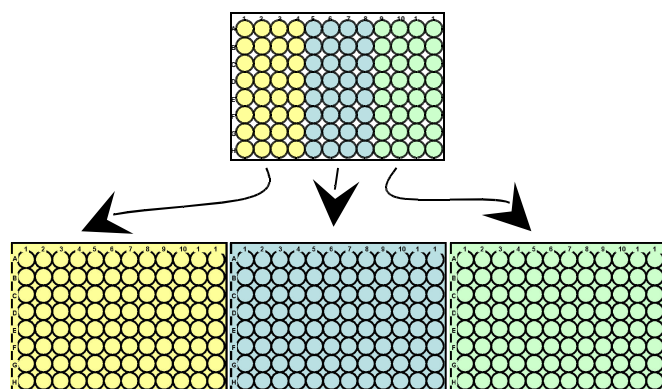


Figure 3: Plate configuration: one third of the initial plate (with cells+compound) is measured on a new 96 well plate.

Corresponding to one well of the test plate there are two wells, one with the supernatant, one with the corresponding lysat:

S ctrl	S ctrl	S ctrl	S ctrl	L ctrl	L ctrl	L ctrl	L ctrl				
S	S	S	S	L	L	L	L				
S	S	S	S	L	L	L	L				
S	S	S	S	L	L	L	L				
S	S	S	S	L	L	L	L				
S	S	S	S	L	L	L	L				
S	S	S	S	L	L	L	L				
S	S	S	S	L	L	L	L				

lowest concentration

highest concentration

Figure 4: Plate configuration for LDH-measurement (S: supernatant, L: lysat)

9.d. Data Analysis and Statistics

Cytotoxic effect is shown as percentage of LDH release of total LDH per well.

Calculation (S: supernatant, L: lysat): $(S/(S+3*L))*100$

EC50 values are calculated by non-linear regression curves.

Interpretation of the data with MS Excel and GraphPad Prism Software.

9.e. Calibration of pipettes

All used pipettes should be calibrated competently prior to the experiment serie.

10. REFERENCES

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- (2) Ruhnke M, Nussler AK, Ungefroren H, Hengstler JG, Kremer B, Hoeckh W, Gottwald T, Heeckt P, Fandrich F. *Human monocyte-derived Neohepatocytes: a promising alternative to primary human hepatocytes for autologous cell therapy*. *Transplantation* 2005; 79:1097-103.

11. Appendix: Table of Concentrations

Absolute concentrations on cells:

	DMSO [%]	Para cetamol [mM]	HgCl ₂ [uM]	Allyl alcohol [uM]	Ethanol [mM]	[mM]Bromo benzene	Vera pamil [mM]	Digoxin [uM]	Tetra cycline [mM]
1st solvent	medium	DMSO	medium	medium	medium	DMSO	DMSO	DMSO	DMSO
2nd solvent (1:5)	-	medium	-	-	-	medium	medium	medium	medium
highest conc.	10,00	20,000	100,00	500,000	857,50 (=5%)	1,000	10,000	20,000 0	5,0000
	5,00	10,000	25,00	250,000	428,75	0,500	2,500	4,0000	1,0000
	2,50	5,000	6,25	125,000	214,38	0,250	0,625	0,8000	0,2000
	1,25	2,500	1,56	62,500	107,19	0,125	0,156	0,1600	0,0400
	0,63	1,250	0,39	31,250	53,59	0,063	0,039	0,0320	0,0080
lowest conc.	0,31	0,625	0,10	15,625	26,80	0,031	0,010	0,0064	0,0016
control	medium	1% DMSO	medium	medium	medium	1% DMSO	1% DMSO	1% DMSO	1% DMSO

Erklärung

Ich erkläre hiermit, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe der Quelle gekennzeichnet. Weitere Personen, insbesondere Promotionsberater, waren an der inhaltlich materiellen Erstellung dieser Arbeit nicht beteiligt. Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Januar 2008, Isabelle Pochic

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