EUDIPHARM 2011

Module B
Pre-Clinical development and Drugs toxicity/Safety

Single & Repeated Dose Toxicity

Lyon, October 24th, 2012
Single Dose/Repeated Dose Toxicity

Single Dose Toxicity
Single Dose/Acute Toxicity

• Single dose
  administration of one single dose

• Acute toxicity
  adverse effects after short time (24h) following single or multiple doses
  – Usually done in single dose studies
  – Determine mode of death
  – Determine approximate lethal dose

• Current view
  – Information can be obtained in other studies
  – Animal use can be reduced (3Rs)
Single Dose/Acute Tox

EMA/CHMP/SWP/81714/2010 - 24 June 2010
Withdrawal of 'Note for Guidance in single dose toxicity'

• Guideline on single dose toxicity removed (Eudralex Vol 3; 3BS1a Single Dose Toxicity)
• „extended“ single dose toxicity study (GLP) remains (ICH M3 R2) for specific situations (e.g. oncology products)
• Acute toxicity can be assessed from dose escalation studies or short-duration dose-ranging studies
• Data from 1 species and 1 route of administration are considered sufficient
• Data may be obtained from non-GLP studies
• Valuable source may be safety pharmacology studies according to ICHS7A (general) and S7B (QT)
Single Dose (Acute) Toxicity

ex-EU-Guideline 3BS1a (for medicinal products)

General
Clinical observations recorded for period of 14 days after single administration of high doses (up to sub-lethal or lethal)

Objectives
- Reveal signs of acute toxicity
- Mode of death determined (macroscopic evaluation, no clinical pathology)
- Quantitative evaluation of approximate lethal dose
- Dose-effect relationship

Design
- 2 Mammalian species (rodents), equal number of both sexes
- Doses to achieve spectrum of toxicity

Single Dose (Acute) Toxicity

Classical LD50-Method po

OECD 401 (guideline deleted 2002)

- Dose finding with 1-3 animals (depends on expected profile and results)
- 3 Doses, e.g. 250, 350, 500 mg/kg are usually used
- 10 animals per dose (total 31-33 animals)

→ calculation of median lethal dose (LD50)
Single Dose (Acute) Toxicity

Acute Toxic Class (ATC) Method (p.o.)

OECD 423 *(guideline in use for chemicals)*

- 1st Dose depends on expected profile
- 25, 200 or 2000 mg/kg are usually used
- 3 animals per step (total 6-12 animals)

allocation of toxicity class

Single Dose (Acute) Toxicity

Summary

• Traditional single dose toxicity studies removed from EU guidance
  – Very little value for overdose prediction
  – No need to be performed, acute toxicity information taken from various sources incl. non-GLP toxicity studies and safety pharmacology studies

• „extended“ single dose toxicity studies remain in use for special purposes, e.g. certain indication areas or mechanistic studies (tailor-made no standard)
Single Dose/Repeated Dose Toxicity

Repeated Dose Toxicity
Repeated Dose Toxicity

Guidance for Pharmaceuticals

- **CPMP/SWP/1042/99 Rev 1**
  Guideline on repeated dose toxicity
  (CHMP adopted March 2010)
  This guideline is a revision of previous Note for Guidance on Repeated Dose Toxicity, which was first adopted in October 1983 and second in July 2000
  
  [Link to guideline](http://www.ema.europa.eu/pdfs/human/swp/104209enrev1.pdf)

- **CPMP/ICH/286/95 - ICH M3 (R2)**
  Non-Clinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals
  (Approval by CHMP June 2009)
  This guidance represents consensus that exists regarding type and duration and timing of non-clinical safety studies to support conduct of human clinical trials and marketing authorization

Peter-J. Kramer
Darmstadt, Germany
Repeated Dose Toxicity

Objectives

- Characterise toxicological profile (e.g. target organs/tissue, exposure/response relationships, potential reversibility of toxic effects)
- Determination of no observed adverse effect level (NOAEL)
- Determination of highest dose for subsequent studies
# Repeated Dose Toxicity

Studies in Rodents and Non-Rodents

<table>
<thead>
<tr>
<th>Duration</th>
<th>Route of administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 weeks</td>
<td>p.o. and/or i.v.</td>
</tr>
<tr>
<td>4 weeks</td>
<td>p.o. and/or i.v.</td>
</tr>
<tr>
<td>13 weeks</td>
<td>p.o. or i.v.</td>
</tr>
<tr>
<td>26 weeks</td>
<td>p.o. or i.v.</td>
</tr>
<tr>
<td>39 weeks</td>
<td>p.o. or i.v.</td>
</tr>
<tr>
<td>52 weeks</td>
<td>p.o. or i.v.</td>
</tr>
</tbody>
</table>
Tox Studies in Dev. Process Pharma

Disc Res. | Pre - | Clinical | Development
--- | --- | --- | ---
In silico / in vitro | animals | volunteers | patients

Discovery Research
- Screening
- Early (pilot) studies
- Exploratory studies
- Early Safety Pharmacology

Tox
- Early ADME
- Metab. enzyme
- ‘Polymorphism’ (critical CYPs)
- Exploratory
- Kinetics

DMPK
- Reg. ADME
- Toxicokinetics

P 0 regulatory
- Repeat dose tox
- Mutagenicity
- Pilot
- Teratogenicity
- Explicatory tox
- SP core battery

P I
- Chronic studies 3, 6, 9, 12 months (2 species)
- Reproduction tox (fertility, teratogenicity, peri/postnatal study)
- Carcinogenicity studies (2y, 2 species)
- Explicatory (mechanistic) studies
- SP follow-up & supplemental studies

P IIa/b
- Bioanalytics
- Metabolism
- in Man

P III
- In silico / in vitro
- Support expl. Pharm. Dev.
- Bioanalytics for PK in Patient
Tox-Animal Species

Most ‘human-like‘ relating to pharmacodynamic effect, pharmacokinetic profile and biotransformation

Often Dog and Rat but also: Monkey, Mouse, (Mini-)Pig, Guinea Pig, Rabbit, etc.

Peter-J. Kramer
Darmstadt, Germany
## Repeated Dose Toxicity

### Selection and Number of Species

2 Mammalian species (rodent and non-rodent)

<table>
<thead>
<tr>
<th>Species</th>
<th>Predominant</th>
<th>Practical Reasons</th>
<th>Background Knowledge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rodent</td>
<td>predominant rat</td>
<td>practical reasons</td>
<td>large amount of background knowledge</td>
</tr>
<tr>
<td>Non-Rodent</td>
<td>predominant dog</td>
<td>practical reasons</td>
<td>large amount of background knowledge</td>
</tr>
</tbody>
</table>

Other: Non-human primate and pig
Repeated Dose Toxicity

Number of Groups

General:
1 Control Group
3 Treatment Groups
  - Low Dose
  - Medium Dose
  - High Dose
Repeated Dose Toxicity
Selection of doses

→ One of most critical issues in design of repeated dose toxicity studies

**Dose selection** should be based on data from all available studies (pharmacology, acute and kinetic studies). In general preliminary dose finding studies are conducted.

**High dose** should show clear toxic effects

**Low Dose** should be in the range of therapeutical human dose
Repeated Dose Toxicity

Route of administration

General: should be similar to intended human usage

Special: may be different if similar distribution/kinetic profile
Repeated Dose Toxicity

Frequency of administration

Usual: once daily

but

kinetic variables must be taken into account
Repeated Dose Toxicity

Control groups

Recommended:

Control animals be dosed with vehicle at same rate as test group animals

When vehicle may cause effects or affect action of test substance, a second (sham- or untreated) control group should be considered.
Repeated Dose Toxicity

Number of animals

Number of animals per sex per group should be sufficient to allow meaningful interpretation of the data.

Minimum (e.g. Rats)

4w Study: 10 males/10 females per group

6m Study: 20 males/20 females per group
# Repeated Dose Toxicity

## 4 Week Rat - Typical Study Design

<table>
<thead>
<tr>
<th>4 weeks + 2 weeks</th>
<th>Treatment period + Recovery period</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wistar rat HsdCpb:WU</td>
<td>Species</td>
</tr>
<tr>
<td>4</td>
<td>Groups</td>
</tr>
<tr>
<td>based on the results of a dose finding study</td>
<td>Doses</td>
</tr>
<tr>
<td>15 (10 main kill, 5 end of recovery)</td>
<td>No. of rats/sex/group</td>
</tr>
<tr>
<td>3</td>
<td>Satellite rats/ sex/group for toxicokinetics</td>
</tr>
<tr>
<td>120 (60M + 60F) + 24 (12M + 12F)</td>
<td>Total No of animals</td>
</tr>
<tr>
<td>daily</td>
<td>Mortality, behavior, clinical symptoms</td>
</tr>
<tr>
<td>weekly</td>
<td>BW, FC, WC</td>
</tr>
<tr>
<td>in weeks 4 (10 rats/sex/group) and 7 (5 rats/sex/group)</td>
<td>Hematology, clinical chemistry, urinalysis (standard parameters)</td>
</tr>
<tr>
<td>predose and in week 4 (10 rats/sex/group)</td>
<td>Ophthalmology</td>
</tr>
<tr>
<td>all rats</td>
<td>Necropsy</td>
</tr>
<tr>
<td>standard organs</td>
<td>Organ weights</td>
</tr>
<tr>
<td>all rats, all organs</td>
<td>Histopathology</td>
</tr>
<tr>
<td>on days 1 and 28, one, 3, 6, and 24 hours after administration</td>
<td>Toxicokinetics</td>
</tr>
</tbody>
</table>
# Repeated Dose Toxicity
## 26 Week Rat - Typical Study Design

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment period</td>
<td>26 weeks</td>
</tr>
<tr>
<td>Interim kill (optional)</td>
<td>13 weeks</td>
</tr>
<tr>
<td>Recovery period</td>
<td>8 weeks</td>
</tr>
<tr>
<td>Species</td>
<td>Wistar rat HsdCpb:WU</td>
</tr>
<tr>
<td>Groups</td>
<td>4</td>
</tr>
<tr>
<td>Doses</td>
<td>dependent on the results of the 4 week study</td>
</tr>
<tr>
<td>No. of rats/sex/group (interim kill)</td>
<td>25 (10 each, for interim kill, main kill and 5 each for recovery)</td>
</tr>
<tr>
<td>No. of rats/sex/group (no interim kill)</td>
<td>20 (15 each, for main kill and 5 each for recovery)</td>
</tr>
<tr>
<td>Satellite rats/sex/group for toxicokinetics</td>
<td>3</td>
</tr>
<tr>
<td>Total No of animals</td>
<td>224 (112M + 112F) or 184 (92M + 92F) without interim kill</td>
</tr>
</tbody>
</table>
# Repeated Dose Toxicity

## 26 Week Rat - Typical Study Design

<table>
<thead>
<tr>
<th>Test Parameter</th>
<th>Frequency/Timepoints</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mortality, behavior, clin. symptoms</td>
<td>daily</td>
</tr>
<tr>
<td>BW, FC</td>
<td>weekly</td>
</tr>
<tr>
<td>Hematology, clinical chemistry, urinalysis (standard parameters)</td>
<td>in weeks 4, 13, 26, 34 (10 rats/sex/group)</td>
</tr>
<tr>
<td>special investigations (e.g. hormones)</td>
<td></td>
</tr>
<tr>
<td>Ophthalmology</td>
<td>once predose, and in weeks 4, 13, 26 (10 rats/sex/group)</td>
</tr>
<tr>
<td>Necropsy</td>
<td>all rats</td>
</tr>
<tr>
<td>Organ weights</td>
<td>standard organs</td>
</tr>
<tr>
<td>Histopathology</td>
<td>all rats, all organs</td>
</tr>
</tbody>
</table>

Peter-J. Kramer  
Darmstadt, Germany
**Repeated Dose Toxicity**  
26 Week Dog - Typical Study Design

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment period</td>
<td>26 weeks</td>
</tr>
<tr>
<td>Recovery period</td>
<td>8 weeks</td>
</tr>
<tr>
<td>Species</td>
<td>Beagle Dog</td>
</tr>
<tr>
<td>Groups</td>
<td>4</td>
</tr>
<tr>
<td>Doses</td>
<td>dependent on the results of the 4 week study</td>
</tr>
<tr>
<td>No. of dogs/sex/group</td>
<td>5 (3 each, for main kill, 2 each for recovery)</td>
</tr>
<tr>
<td>Total No of animals</td>
<td>40 (20 M + 20 F)</td>
</tr>
</tbody>
</table>
# Repeated Dose Toxicity

## 26 Week Dog - Typical Study Design

<table>
<thead>
<tr>
<th>Test Parameter</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mortality, behavior, clin. symptoms</td>
<td>daily</td>
</tr>
<tr>
<td>BW, FC</td>
<td>weekly</td>
</tr>
<tr>
<td>ECG</td>
<td>Predose, week 1, 4, 13, 26, 34 all dogs</td>
</tr>
<tr>
<td>Hematology, clinical chemistry, urinalysis (standard parameters)</td>
<td>Predose and in weeks 4, 13, 26, 34 (all dogs)</td>
</tr>
<tr>
<td>special investigations (e.g. hormones)</td>
<td></td>
</tr>
<tr>
<td>Ophthalmology</td>
<td>once predose, and in weeks 4, 13, 26 (10 rats/sex/group)</td>
</tr>
<tr>
<td>Necropsy</td>
<td>all dogs</td>
</tr>
<tr>
<td>Organ weights</td>
<td>standard organs</td>
</tr>
<tr>
<td>Histopathology</td>
<td>all dogs, all organs</td>
</tr>
</tbody>
</table>
Repeated Dose Toxicity

Physiological Investigations

- Blood Pressure (Routine)
- Electrocardiogram (ECG) (Routine) (most important measurement: Q-T)
- Electroencephalogram (EEG)
- Electroretinogram (ERG)
Hematology - Erythropoiesis

Maturation of the red blood cell and ejection of the nucleus

www.graphicpulse.com/medill/wblood.html
Repeated Dose Toxicity

Hematology

- Erythrocyte count (RBC)
- Leukocyte count (total and differential) (WBC)
- Platelet count
- Reticulocyte count
- Hemoglobin (Hgb)
- Hematocrit (volume erythrocytes / plasma)
Hematology - Differential Blood Count

Semiautomated systems perform
• Red blood cell counts (RBC)
• White blood cell counts (WBC)
• Platelet counts
• Hemoglobin (Hgb) measurements
and in some cases, partial differential WBC counts.

Most instruments use impedance technology: cells produce a voltage pulse as they pass between a pair of electrodes. Size of cell is reflected by size of voltage pulse; cells are identified by size and counted.
Hematology - Differential Blood Count (traditional)

- 100 WBC or multiples thereof (preferred) are classified at 1,000× magnification.
- The total number of each cell type is expressed as a percentage value.
- Absolute values of each cell type are obtained by multiplying counted percent value by total WBC count.
- Changes in WBC numbers and morphology are called the leukocyte response or leukogram.
Hematology – Blood cell numbers

• Total blood cell numbers change in disease states, some species variation. Pattern of blood cell alteration may provide information about disease process present.

• Anemia: result of blood loss, hemolysis, or decreased RBC production

• WBC numbers increase: acute bacterial infections, leukemias, tissue necrosis, hemolysis, and chemical or metabolic intoxication
  – Neutrophilia: bacterial infection or stress
  – Increased numbers of monocytes and lymphocytes: associated with immune response or chronic inflammatory process
  – Eosinophilia: allergic responses
  – Numerous immature blood cells with abnormal morphologic features are often the first indicators of a leukemic alteration of blood-forming tissues
Packed Cell Volume (PCV) (Hematocrit)

Method:
- Capillary tube filled with anticoagulated blood, sealed, and centrifuged for 5 min in a special microhematocrit centrifuge. Heparin-coated capillary tubes available for taking samples directly from animals.
- Centrifuged sample is placed on hematocrit tube reader, and percent PCV value is obtained from underlying scale.

PCV provides: accurate, practical evaluation of RBC status, (inaccuracy of manual RBC counts, technical effort required for accurate RBC determinations).

Decreased PCV: not seen for 12-24 hr after acute blood loss (time needed for redistribution of interstitial fluid to replace lost blood volume).
- Low PCV indicates anemia.
- Reticulocyte count or evaluation of a blood smear may help determine if the anemia is regenerative.

Increased PCV: most often caused by dehydration but can also occur with primary or secondary polycythemia.
Repeated Dose Toxicity

Hematology

Calculated Parameters

MCV  mean corpuscular volume
Hematocrit / no of erythrocytes (normal range 80 - 100 fl)

MCH  mean corpuscular hemoglobin
Hemoglobin-Conc. / no of erythrocytes (normal range 28 - 35 pg)

MCHC  mean corpuscular hemoglobin concentration
Hemoglobin-conc. / Hematocrit (normal range 310 - 360 g/l)
# Repeated Dose Toxicity

## Hematology (WBCs)

<table>
<thead>
<tr>
<th>Neutrophils</th>
<th>40 – 75 %</th>
<th>↑ tissue necrosis, bacterial infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
<td>20 – 50 %</td>
<td>↑ acute infection, hepatitis</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>1 - 6 %</td>
<td>↑ allergy, poisoning</td>
</tr>
<tr>
<td>Monocytes</td>
<td>1 – 5 %</td>
<td>↑ poisoning</td>
</tr>
<tr>
<td>Basophils</td>
<td>&lt; 1 %</td>
<td>↓ hyperthyreodism</td>
</tr>
</tbody>
</table>
Peripheral Blood Cells

A. Erythrocytes; B. Large Granular Lymphocyte; C. Neutrophil; D. Eosinophil; E. Neutrophil; F. Monocyte; G. Platelets; H. Lymphocyte; I. Band Neutrophil; J. Basophil
Repeated Dose Toxicity

Hematology

• B Lymphocytes
  – Plasma cells – antibody (immunoglobulins)-secreting B cells

• T Lymphocytes
  - Lymphokine-secreting T cells
    - T helper cells (T<sub>H</sub>) (CD4)
      induce B cells, activate macrophages
    - cytotoxic T cells (T<sub>C</sub>) (CD8)
      kill virus-infected and malignant cells
    - suppressor T cells (T<sub>S</sub>) (CD8)
      inhibit T<sub>H</sub> cells (immune modulation)
Repeated Dose Toxicity

Hematology

Specialized assays

- **Coagulation testing** (e.g. Protime (PT), Activated Partial Thromboplastin Time (APTT), fibrinogen, thrombin time)

- **Hemoglobin fractions** including *methemoglobin* quantitative assessment

- **Platelet functions** including morphology, counting, aggregation and measurement of aggregation markers (e.g. thromboglobulin, serotonin release)
### Repeated Dose Toxicity

#### Clinical Chemistry

<table>
<thead>
<tr>
<th>Sodium</th>
<th>ALT (Alanine Aminotransferase)</th>
<th>Total Bilirubin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium</td>
<td>AST (Aspartate Aminotransferase)</td>
<td>Total Protein</td>
</tr>
<tr>
<td>Chloride</td>
<td>AP (Alkaline Phosphatase)</td>
<td>Albumin</td>
</tr>
<tr>
<td>Blood Urea N</td>
<td></td>
<td>Globulin (calculated)</td>
</tr>
<tr>
<td>Nitrogen</td>
<td></td>
<td>Alb/Glob Ratio</td>
</tr>
<tr>
<td>Glucose</td>
<td></td>
<td>Inorganic Phosphorus</td>
</tr>
<tr>
<td>Creatinine</td>
<td></td>
<td>Calcium</td>
</tr>
<tr>
<td>Cholesterol</td>
<td></td>
<td>Triglycerides</td>
</tr>
</tbody>
</table>
Repeated Dose Toxicity

Clinical Chemistry

Hormones

• Thyroid hormones (total T3, total T4, TSH)

• Reproductive hormones (estradiol, testosterone, DHT, progesterone, FSH, LH)

• Bone regulatory hormones (PTH, calcitonin, vitamin D)

• General endocrinology (insulin, cortisol, corticosterone)
Repeated Dose Toxicity

Clinical Chemistry

Examples of Special Tests:
• Bile Acids, Iron, Magnesium
• Amylase
• N-acetyl glucosaminidase (NAG)
• Lipase
• Sorbitol dehydrogenase (SDH)
• 5'-nucleotidase (5'-NT), Cholinesterase (EPA method)
• Glucose-6 phosphate dehydrogenase (G-6PDH)
• Fatty acids, Hydroxybutyrate, Ammonia
• Inflammatory proteins
• Lipoproteins measured in different fluids using electrophoretic and immunonephelometric techniques.
# Repeated Dose Toxicity

## Urinalysis

<table>
<thead>
<tr>
<th>Component</th>
<th>Measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes</td>
<td>pH</td>
</tr>
<tr>
<td>Blood</td>
<td>Specific Gravity</td>
</tr>
<tr>
<td>Glucose</td>
<td>(Osmolality)</td>
</tr>
<tr>
<td>Ketones</td>
<td>Color</td>
</tr>
<tr>
<td>Nitrite</td>
<td>Clarity</td>
</tr>
<tr>
<td>Urobilinogen</td>
<td>Microscopic Examination</td>
</tr>
<tr>
<td>Protein</td>
<td>(Crystalluria, Sediment)</td>
</tr>
<tr>
<td>Bilirubin</td>
<td></td>
</tr>
</tbody>
</table>
Repeated Dose Toxicity

Urinalysis - Sediment

- Erythrocytes
- Leukocytes
- Spermia
- Tubular Kidney Cells
- Epithelia
- Hyaline Cylinders

- Granulated Cylinders
- Phosphate
- Urea
- Amino acids

15 min, 2000 rpm
### Repeated Dose Toxicity Pathology

**Standard Tissues Gathered at Necropsy**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenals</td>
<td>Harderian Gland</td>
</tr>
<tr>
<td>Aorta</td>
<td>Heart</td>
</tr>
<tr>
<td>Bone Marrow</td>
<td>Ileum</td>
</tr>
<tr>
<td>Brain</td>
<td>Jejunum</td>
</tr>
<tr>
<td>Cecum</td>
<td>Kidneys</td>
</tr>
<tr>
<td>Duodenum</td>
<td>Liver</td>
</tr>
<tr>
<td>Esophagus</td>
<td>Lungs</td>
</tr>
<tr>
<td>Eyes</td>
<td>Mammary Gland</td>
</tr>
<tr>
<td>Femur</td>
<td>Mandibular Lymph Node</td>
</tr>
<tr>
<td>Gallbladder (not in Rat)</td>
<td>Mesenteric Lymph Node</td>
</tr>
<tr>
<td>Muscle</td>
<td></td>
</tr>
</tbody>
</table>
Repeated Dose Toxicity Pathology

Standard Tissues Gathered at Necropsy

- Ovaries
- Pancreas
- Peripheral Nerve (Sciatic)
- Pituitary
- Prostate
- Rectum
- Salivary Glands
- Seminal Vesicles
- Skin
- Spinal Cord
- Spleen
- Stomach
- Testes / Epididymides
- Thyroids / Parathyroids
- Thymus
- Tongue
- Trachea
- Urinary Bladder
- Uterus
- Gross Lesions and Masses
- Macrophotographic Documentation
Repeated Dose Toxicity Pathology

Histology

- Standardized trimming of wet tissues
- Bone sectioning (if necessary)
- Tissue processing (fixation) / embedding
- Microtomy: routine, serial, level or frozen sectioning
- Routine H&E staining
- Wide variety of histochemical stains, incl. immunohistochemistry, e.g. glial fibrillary acidic protein, GFAP
- Routine Slide Preparation
Repeated Dose Toxicity Pathology

Microscopical Evaluation

- Histopathological Evaluation
- Cytological Evaluation
- Histomorphometry
- Bone marrow cell counts
- Sperm Analysis
- Microphotographic Documentation
- Peer Review
Arbeitsablauf in der Pathologie

Organentnahme
Fixation

Histologischer Schnitt
Färbung

Zuschnitt
Paraffineinbettung
Mikrotomschnitte
<table>
<thead>
<tr>
<th>Hals-lymphknoten</th>
<th>Speicheldrüsen</th>
<th>Magen</th>
<th>Uterus (Corpus/Cervix)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aorta thoracalis</td>
<td>Trachea/Oesophagus mit Schilddrüsen</td>
<td>Duodenum</td>
<td></td>
</tr>
<tr>
<td>Mesenterium mit Gefäße u. Mesenterial-lymphknoten</td>
<td>Thymus</td>
<td>Jejunum</td>
<td>Vagina</td>
</tr>
<tr>
<td>Nebennieren</td>
<td>Zwerchfell</td>
<td>Caecum</td>
<td>Ovarien</td>
</tr>
<tr>
<td>Harnblase</td>
<td>Pankreas</td>
<td>Colon</td>
<td>Ovidukt</td>
</tr>
<tr>
<td>Nervus ischiadicus</td>
<td>Milz</td>
<td>Rectum</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nieren</th>
<th>Haut mit Milchrüse (2X)</th>
<th>Kopf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muskel</td>
<td>Femur + Sternum</td>
<td></td>
</tr>
<tr>
<td>Fett</td>
<td>Rückenmark (3x)</td>
<td>Augen</td>
</tr>
<tr>
<td>Lunge</td>
<td>Zunge</td>
<td>Leber</td>
</tr>
</tbody>
</table>

Peter-J. Kramer
Darmstadt, Germany
Histo-Path Liver-Examples

Fatty liver, dog

Liver, mouse, control

Liver, rat, carcinoma

Liver, mouse, hypertrophy
Pathology

Necropsy

Peer review

Peter-J. Kramer
Darmstadt, Germany
Liver - Proliferation

Peter-J. Kramer
Darmstadt, Germany
Liver - Necrosis

Peter-J. Kramer
Darmstadt, Germany
Organtoxicity

Livertoxicity
(Hepatotoxicity)
Why is Hepatotoxicity so important?

• Greatest single cause of worldwide withdrawal of numerous drugs from market was hepatic damage (32 drugs, 26.4%)

• USA: Drug-induced liver disease is the most common cause of acute liver failure (responsible for 33-50% of all cases)

• ~50% of NCEs show some preclinical animal hepatotoxicity
Liver – Important facts

- Central organ in metabolism
  - Degradation/modification of endogenous and exogenous compounds (incl. hormones)
  - Fuel metabolism
  - Synthesis of proteins
  - Gland function (secretion of bile, hormones)
  - Supplementation of vitamins and trace elements

- Prominent organ faced with exogenous compounds like drugs after gastro-intestinal absorption

- Capacity to metabolize compounds (phase I and II drug metabolism) that could lead to de-activation and excretion or toxification
Architecture of the liver

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Hepatic microcirculation

central terminal venule
sinusoidal channels
bile ductule
arteriosinusoidal branch
inlet venule
terminal branch of hepatic portal vein
terminal branch of hepatic artery

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Hepatic architecture - lobule and acinus
Morphology of the liver

[a) zum Herz
V. cava inf.
V. hep.
A. hep.
V. portae
A. lien.
V. lien
V. mes. sup.
vom Darm

b) Z
B
E
K
S
G
A
P

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Liver - Normal Structure
Liver architecture histology

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Liver - Major Drug Target

A broad variety of pathologies/toxic effects can occur:

- auto-immune disease
- inflammatory response
- cirrhosis (hepatocyte necrosis, acute cell death)
- lipid accumulation (steatosis)
- inhibition of bile acid transport (cholestasis)
- neoplasia, tumor formation

The liver has an outstanding regenerative capacity that can promote tumor growth

Peter-J. Kramer
Darmstadt, Germany
# Hepatotoxicity Marker

<table>
<thead>
<tr>
<th>Prototypical Liver toxicant</th>
<th>Symptoms / Mode of action</th>
<th>Marker</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CCl₄</strong>&lt;br&gt;Carbon tetrachloride</td>
<td><strong>cirrhosis</strong>&lt;br&gt;necrosis, apoptosis</td>
<td>caspase 3 activity, ATP content, GSTa, serum ALAT, SDH et al. histo-path</td>
</tr>
<tr>
<td><strong>Valproic acid</strong></td>
<td><strong>steatosis</strong>&lt;br&gt;fatty acid b-ox ↓</td>
<td>GSTa/FABP/MnSOD↑&lt;br&gt;cellular lipid accumulation&lt;br&gt;histopathtochondrial membrane potential ↓&lt;br&gt;ATP content ↓</td>
</tr>
<tr>
<td><strong>Antimycin a, CN⁻</strong></td>
<td><strong>mitochondrial uncoupling</strong></td>
<td></td>
</tr>
</tbody>
</table>
### Hepatotoxicity Marker (contd.)

<table>
<thead>
<tr>
<th>Prototypical Liver toxicant</th>
<th>Symptoms</th>
<th>Mode of action</th>
<th>Marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol, naphthyl isocyanate (ANIT)</td>
<td><strong>cholestasis</strong></td>
<td>inhibition of bile flow/formation</td>
<td>Mg-ATPase↓, p-glycoproteins ↓↑, GSTπ serum AP, gGT</td>
</tr>
<tr>
<td>Phenobarbital Estradiol Aflatoxin</td>
<td><strong>neoplasia / tumor formation</strong></td>
<td>DME induction hyperproliferation mutation/transformation</td>
<td>histo-path, GSTp ↑ CYP, UGT induction/activity c-myc/cyclins ↑</td>
</tr>
<tr>
<td>Halothan</td>
<td><strong>hepatitis</strong></td>
<td>immune response</td>
<td>Antibody production</td>
</tr>
</tbody>
</table>
Hepatotoxicity Marker (contd.)

These are in part general stress response marker:
Regenerative process can be assessed in tissue only (in vivo system),
but: cellular stress markers (like hsp, AP-1, myc) can be measured in vitro!

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Hepatotoxicants
Traditional Classification

Intrinsic Human Hepatotoxicants

- Compounds that are directly hepatotoxic or produce a toxic reactive metabolite
- Predictable (in vitro and/or animal models of toxicity)
- If detected in Preclinical toxicology studies or Phase I, project generally not advanced

Idiosyncratic or Immune-mediated Hypersensitivity

- Low frequency in a population and typically demonstrate “host dependency” or “individual susceptibility”
- Unpredictable (?)
- Often not detected in animal models of toxicity or in human clinical trials
- When detected, generally trial stopped or use limitations

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Darmstadt, Germany
### Characteristics of Hepatotoxicity

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Intrinsic</th>
<th>Idiosyncratic/ Hypersensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose-dependent</td>
<td>Yes: classical D-R, reduce risk by reducing dose</td>
<td>No/Yes: (low to therapeutic doses) Dose reduction doesn’t reduce risk</td>
</tr>
<tr>
<td>Frequence of Response</td>
<td>High: 1/10 to 1/100</td>
<td>Low: 1/5000 to 1/100000</td>
</tr>
<tr>
<td><em>In vitro</em> and/or Animal Models</td>
<td>Yes</td>
<td>No/Maybe: “Biomarker”</td>
</tr>
<tr>
<td>Latency of onset: (Human)</td>
<td>Short: few days to weeks</td>
<td>Long/ Intermediate: up to 1 year/ 1-8 weeks</td>
</tr>
<tr>
<td>Elevations in Serum Enzymes: ALAT/ASAT: (Human)</td>
<td>High: 8-10 X ULN</td>
<td>Low: 2-3 X ULN. Often early, transient and asymptomatic</td>
</tr>
<tr>
<td>Reversibility: (Animal and Human)</td>
<td>Yes: upon cessation or dose reduction</td>
<td>Yes/No: upon cessation/re-challenge and/or sensitization</td>
</tr>
<tr>
<td>Metabolism effects: (Animal and Human)</td>
<td>Yes: Often bioactivation/altered detoxification involved.</td>
<td>Yes: Genetic polymorphisms; species differences; D-D interactions; lifestyle factors Delayed changes in met./repair</td>
</tr>
<tr>
<td>Reactive Metabolite</td>
<td>Maybe: parent compound toxicity vs.reactive met.</td>
<td>Maybe: not all reactive metabolites are toxic</td>
</tr>
</tbody>
</table>

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Organtoxicity

Kidney toxicity
(Nephrotoxicity)
Kidney (Ren) - Anatomy

- Cortex and Medulla
- Cortex contains renal corpuscles (glomeruli) + convoluted tubules
- Medulla about 10 – 20 medullary pyramids (collecting duct + loop of Henle)
- Tip of each pyramide: papilla
Kidney (Ren)

- About 1 Mio. nephrons / kidney
- Nephron has 2 main components:
  - Renal corpuscle (glomerulus)
  - Cortical and medullary tubular systems
- Renal corpuscle (glomerulus) capillary network intruding into hollow sphere of epithelial cells called Bowman’s capsule (urinary space)
- Ultrafiltration occurs in glomerular capillary network, filtrate passes into urinary space, passing down the tubular system
- Kidney generates 180 liters of filtrate a day, 178 liters are reabsorbed allowing about 2 liters of urine

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Nephron - Kidney‘s Functional Unit

- Ultrafiltration of molecules < 60 kd
- Concentration of filtrate in tubules through reabsorption of water, ions, salts, amino acids, sugars, and other carbohydrates plus small proteins (active process)
Renal corpuscle

- Glomerulus

Glomerulus

Capillary network intruding into hollow sphere of epithelial cells called Bowman’s capsule (urinary space)

Glomerular capillary membrane coated by highly specialized cells (podocytes)

Figure 14-3. A. Schematic of the ultrastructure of the glomerular capillaries. B. Cross section of the glomerular capillary membrane with the capillary endothelium, basement membrane, and epithelium podocytes. [From Guyton AC, Hall JE, 1996, p 32, with permission.]

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Kidneys (Ren) - Function

1. Excretion of wastes like urea and ammonium (urine)
2. Prevents leakage of essential elements or compounds from the organism - reabsorption of important substances like water, glucose, and amino acids
3. Homeostatic functions
   • regulation of electrolytes
   • maintenance of acid-base balance
   • regulation of blood pressure
4. Endocrine functions: synthesis of Erythropoietin, Renin and activation of Vitamin D
Nephrotoxic Substances - Overview

• Medical Drugs
  Aminoglycosides
  Acetaminophen (Paracetamol)
  Amphotericine B

• Heavy metals
  Cadmium
  Mercury

• Halogenated Hydrocarbons
  Trichloroethylene
  Tetrachloroethylene
  Hexachlorobutadiene
  Chloroform

• Herbizides
  Paraquat
  Diquat

• Mykotoxines
  Ochratoxin A
  Citrinin
  Aflatoxin B1

• Aliphatic Hydrocarbons
  (α2μ Globulin mediated)
  2,2,4-Trimethylpentan
  Decalin
  D-Limonen

• Cytostatics
  Cisplatin
  Methotrexate
  Mitomycin D
Nephrotoxic Compounds
Aminoglycoside-Antibiotics

- Bacterial toxins (Streptomyces-species)
- Broad efficiency against gram-negative bacteria
- Very polar and difficult transport through membranes
- Limited use because of nephrotoxicity (reduced GFR, Serum-Creatinine↑, Glucosuria, Proteinuria)
- Target cell: cells of proximal tubulus
- Mechanism: next page
Nephrotoxic Compounds
Aminoglycoside-Antibiotics

Figure 14-13. Renal handling of aminoglycosides: (1) glomerular filtration, (2) binding to the brush-border membranes of the proximal tubul, (3) pinocytosis, and (4) storage in the lysosomes. [From De Broe ME: Renal injury due to environmental toxins, drugs, and contrast agents, in Berl T, Bonventre JV (eds): Atlas of Diseases of the Kidney. Philadelphia: Current Medicine, 1999, p 11.4, with permission.]
Kidney-Function Tests

• Urine Tests
  – Standard urinalysis
  – Creatinine and urea clearance
  – Urine osmolarity analysis
  – Urinary protein analysis

• Blood Tests (in Routine Tox Studies)
  – BUN blood urea
  – Creatinine analysis
  – Determ.: Na, K, Cl, Bicarbonate, Ca, Mg, P, Protein,Glc

• New Urine Biomarkers (validated for humans and rats) (urine)
  – Clusterin-α und Kim-1 (Kidney Injury Molecule-1) (early markers!)
Repeated Dose Toxicity

Immunotoxicology

Objectives

Identify adverse effects of drug and/or ist metabolites on the immune system

Adverse effects

• Immunosuppression: decreased immune function (e.g. infectious complications, malignancies)
• Antigenicity: specific immune reactions elicited
• Hypersensitivity: immunological sensitization
• Autoimmunity: immune reactions to self-antigens
• Adverse immunostimulation: non-antigen specific activation
Repeated Dose Toxicity

Immunotoxicology

Methods

Variety of methods to evaluate potential drug-induced alterations in immune functions

28 day Repeated Dose Toxicity Study: lymphoid tissues, immune cell populations (bone marrow cellularity, lymphocyte subsets, NK-cell activity, antibody response etc.)

Functional Assays

- Plaque Assay (SRBC, anti-sheep red blood cell IgM antibody response assay)
- Host Resistance Assays (experimental infections or implanted tumors)
- DTH-Assays (delayed-type hypersensitivity response)

...
Repeated Dose Toxicity

Immunotoxicology

Further endpoints in repeated dose tox studies

- **Lymphoid cell morphology** (bone marrow, thymus, spleen, and lymph nodes) (morphometric analysis) (altered maturation of immunocompetent cells)
- **Lymph node weights** (mandibular, mesenteric)
- **Phenotype distribution analysis of leukocytes** (flow cytometry with mAb (e.g. CD4, CD8))
- **Quantitation** IgM, IgG, IgE etc.
- ...
CLOZAPINE, an atypical antipsychotic drug (Schizophrenia)

- Significant risk of **Agranulocytosis**, a potentially life-threatening adverse event
  - Patients must have baseline WBC count and absolute neutrophil count before initiation of treatment as well as regular counts during and after treatment

- Increased risk of **fatal myocarditis**, especially, not limited to 1st month of therapy
  - Patients in whom myocarditis is suspected treatment must be promptly discontinued
Repeated Dose Toxicity

Data Presentation

- Individual data should be provided
- All data should be summarised (tabular form)
- Numerical data should be evaluated by appropriate and generally acceptable statistical method
- Discussion of results and conclusion must be provided
Repeated Dose Toxicity

Test Report (must include the following information)

Test substance

• Physical nature, purity and physico-chemical properties

• Identification data

Vehicle (if appropriate)

• Justification for choice of vehicle, if other than water
Repeated Dose Toxicity

Test Report (must include the following information)

Test Animals

- Species and strain used
- Number, age and sex of animals
- Source, housing conditions, diet, etc.
- Individual weights of animals at the start of the test
Repeated Dose Toxicity

Test Report (must include the following information)

Test Conditions

• Rationale for dose level selection

• Details of test substance formulation/diet preparation, achieved concentration, stability and homogeneity of preparation

• Details of administration of test substance

• Actual doses (mg/kg body weight/day), and conversion factor from diet/drinking water test substance concentration (ppm) to actual dose, if applicable

• Details of food and water quality

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Repeated Dose Toxicity

Test Report (must include the following information)

Results

• Body weight and body weight changes
• Food consumption and water consumption if applicable
• Toxic response data by sex and dose level, including signs of toxicity
• Nature, severity and duration of clinical observations (whether reversible or not)
• Results of ophthalmological examination
Repeated Dose Toxicity

Test Report (must include the following information)

Results

- Sensory activity, grip strength and motor activity assessments (when available)
- Hematological tests with relevant base-line values
- Clinical biochemistry tests with relevant base-line values
- Terminal body weight, organ weights and organ/body weight ratios
- (Electro)physiological investigations (ECG, BP)
Repeated Dose Toxicity

Test Report (must include the following information)

Results

• Necropsy findings
• A detailed description of all histopathological findings
• Absorption data if available
• Statistical treatment of results, where appropriate
• Indication of No Observed Effect Level (NOEL) or No Observed Adverse Effect Level (NOAEL)

Discussion of Results

Conclusions
Time Schedule of a Toxicity Study

Example:
9 month toxicity study in beagle dogs

1.5 months  |  9 months  |  2 months  |  6 months

Pretreatment | Treatment  | Recovery   | Evaluation | Report

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