Genotoxicity, Mutagenicity and Carcinogenicity
and
REACH

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Outline

• Introduction to REACH and OECD
• DNA lesions/modifications
• DNA repair
• DNA mutation – cancer
• Regulatory mutagenicity testing
• Test batteries
ECHA-European chemical agency

Regulations

The new EU chemicals legislation applies to all industry sectors dealing with chemicals and along the entire supply chain. It makes companies responsible for the safety of chemicals they place on the market.

**REACH**

REACH is a regulation of the European Union, adopted to improve the protection of human health and the environment from the risks that can be posed by chemicals, while enhancing the competitiveness of the EU chemicals industry. It also promotes alternative methods for the hazard assessment of substances in order to reduce the number of tests on animals.

› Read more

**CLP**

The CLP Regulation ensures that the hazards presented by chemicals are clearly communicated to workers and consumers in the European Union through classification and labelling of chemicals.

› Read more

**Biocidal Products Regulation**

The Biocidal Product Regulation (BPR, Regulation (EU) 528/2012) concerns the placing on the market and use of biocidal products, which are used to protect humans, animals, materials or articles against harmful organisms, like pests or bacteria, by the action of the active substances contained in the biocidal product.

› Read more

**Prior Informed Consent Regulation**

The Prior Informed Consent Regulation (PIC, Regulation (EU) 649/2012) administers the import and export of certain hazardous chemicals and places obligations on companies who wish to export these chemicals to non-EU countries. It implements, within the European Union, the Rotterdam Convention on prior informed consent procedure for certain hazardous chemicals and pesticides in international trade.

› Read more
• REACH is the result of a comprehensive consultation process in the EU and a decision was made to reform the policy on chemicals.

• REACH will ensure the safety of old and new chemicals produced or imported at 1 tonnes or more per year.

• If REACH succeeds in reducing chemical-related diseases by only 10% the health benefits are estimated at EUR 50 billion over 30 years.
Annex V details the testing requirements for production and import volumes of:

- **1 tonne or more/year (Annex VI and VII)**
  - Toxicological endpoints that should be assessed using existing data and *in vitro* data are:
    - Skin irritation/corrosivity
    - Eye irritation
    - Skin sensitisation
    - Mutagenicity (*in vitro* bacteria tests/Ames)
    - Acute oral toxicity

- **10 - 100 tonnes per annum (ANNEX VIII)**
  The additional data requirements at this level include:
  
  Toxicological data
  - *In vivo* tests for skin irritation and eye irritation (not required for corrosive substances, nor if hazardous effects identified in *in vitro* tests
  - Bacterial and mammalian *in vitro* mutagenicity tests
  - Mammalian *in vitro* cytogenicity test
  - Inhalation and/or dermal toxicity, as appropriate
  - Repeated dose toxicity 28 d (90 d if necessary)
  - Reproductive toxicity
  - Toxicokinetic behaviour
What is genetic toxicology?

- Assesses effects of chemical and physical agents on the hereditary material (DNA) and on the genetic processes of living cells.

- **Genotoxicity and mutagenicity**
  - **Genotoxicity** covers a broader spectrum of endpoints than mutagenicity, includes DNA damage assessments. DNA damage are not themselves necessarily transmissible to the next generation of cells, pre-mutagenic.
  - **Mutagenicity** refers to the production of transmissible genetic alterations.
    - Somatic cell genotoxicity may lead to cancer.
    - Germ cell genotoxicity may lead to infertility or diseased children.
Primary DNA damage

- AP sites
- Cross-links
- Pyrimidine dimers
- Strand breaks (single or double) adducts
- Oxidative damage
Other types of genotoxicity

- Non-genotoxic agents

  *Secondary genotoxicity*: no direct reactivity with DNA

  Lead to genotoxic events through:
  - Forced cell growth
  - ROS formation

  - Epigenetics
Structural DNA modification

- Alkylation
- Oxidation
- Adducts & cross-links
- Hydrolysis: deamination

Electrophilic attack

Oxidation

AP-site
DNA repair mechanisms

- Direct repair
- DNA-photolyase
- Base excision repair (BER)
  - Endonuclease, DNA-Polymerase, Ligase
- Nucleotide excision repair (NER)
Mutagenesis

4 categories:

1. *Primary damage: formation of adducts*

2. *Secondary modifications: strand breaks, DNA repair*

3. *Fixation of damage*

4. *Creation of mutant DNA*
types of DNA mutation

- Gene mutations
  - Point mutation: missense, nonsense, silent or neutral mutations; frame-shift
- Chromosome mutations (clastogenes)
  - Deletion, insertions, inversion, translocation, dicentric chromosomes, sister chromatid exchange (SEC)
- Genome mutations (anugenesis)
  - Polyploidy, aneuploidy
clastogenesis
How do we test for genotoxicity?

**In vitro**

Table R.7.7-2 *In vitro* test methods

<table>
<thead>
<tr>
<th>Test method</th>
<th>GENOTOXIC ENDPOINTS measured/PRINCIPLE OF THE TEST METHOD</th>
<th>EU/OECD guideline^a</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial reverse mutation test (Ames)</strong></td>
<td>Gene mutations / The test uses amino-acid requiring strains of bacteria to detect (reverse) gene mutations (point mutations and frameshifts).</td>
<td>EU: B.13/14 OECD: 471</td>
</tr>
<tr>
<td><strong>In vitro mammalian cell gene mutation test – hprt test</strong></td>
<td>Gene mutations / The test identifies substances that induce gene mutations in the hprt gene of established cell lines.</td>
<td>EU: B.17 OECD: 476(^b)</td>
</tr>
<tr>
<td><strong>In vitro mammalian cell gene mutation test – Mouse lymphoma assay</strong></td>
<td>Gene mutations and structural chromosome aberrations / The test identifies substances that induce gene mutations in the tk gene of the L5178Y mouse lymphoma cell line. If colonies in a tk mutation test are scored using the criteria of normal growth (large) and slow growth (small) colonies, gross structural chromosome aberrations (i.e. clastogenic effect) may be measured, since mutant cells that have suffered damage to both the tk gene and growth genes situated close to the tk gene have prolonged doubling times and are more likely to form small colonies.</td>
<td>EU: B.17 OECD: 476(^b)</td>
</tr>
<tr>
<td><strong>In vitro mammalian chromosome aberration test</strong></td>
<td>Structural and numerical chromosome aberrations / The test identifies substances that induce structural chromosome aberrations in cultured mammalian established cell lines, cell strains or primary cell cultures. An increase in polyplody may indicate that a substance has the potential to induce numerical chromosome aberrations, but this test is not optimal to measure numerical aberrations. According to this test guideline, this test was designed not to measure numerical aberrations.</td>
<td>EU: B.10 OECD: 473(^b)</td>
</tr>
<tr>
<td><strong>In vitro micronucleus test</strong></td>
<td>Structural and numerical chromosome aberrations / The test identifies substances that induce micronuclei in the cytoplasm of interphase cells. These micronuclei may originate from acentric fragments or whole chromosomes, and the test thus has the potential to detect both clastogenic and aneugenic substances.</td>
<td>EU: B.49 OECD: 487(^b)</td>
</tr>
</tbody>
</table>

Guidance on Information Requirements and Chemical Safety Assessment, Chapter R.7a: Endpoint specific guidance, August 2014
Ames Test
In vitro genotoxicity test-mammalian
## In vivo genotoxicity tests

**Table R.7.7.3 In vivo test methods, somatic cells**

<table>
<thead>
<tr>
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<tr>
<td>In vivo mammalian bone marrow chromosome aberration test</td>
<td>Structural and numerical chromosome aberrations / The test identifies substances that induce structural chromosome aberrations in the bone-marrow cells of animals, usually rodents. An increase in polyplody may indicate that a substance has the potential to induce numerical chromosome aberrations, but this test is not optimal to measure numerical aberrations and is not routinely used for that purpose. Accordingly, this test guideline is not designed to measure numerical aberrations.</td>
<td>EU: B.11 OECD: 475b</td>
</tr>
<tr>
<td>In vivo mammalian erythrocyte micronucleus test</td>
<td>Structural and numerical chromosome aberrations / The test identifies substances that cause micronuclei in erythroblasts sampled from bone marrow and/or peripheral blood cells of animals, usually rodents. These micronuclei originate fromacentric fragments or whole chromosomes, and the test thus has the potential to detect both clastogenic and aneugenic substances.</td>
<td>EU: B.12 OECD: 474b</td>
</tr>
<tr>
<td>Unscheduled DNA synthesis (UDS) test with mammalian liver cells in vivo</td>
<td>DNA repair / The test identifies substances that induce DNA damage followed by DNA repair (measured as unscheduled “DNA” synthesis) in liver cells of animals, commonly rats. The test is usually based on the incorporation of tritium labelled thymidine into the DNA by repair synthesis after excision and removal of a stretch of DNA containing a region of damage.</td>
<td>EU: B.39 OECD: 486</td>
</tr>
<tr>
<td>Transgenic rodent (TGR) somatic and germ cell gene-mutation assays</td>
<td>Gene mutations and chromosomal rearrangements (the latter specifically in the plasmid and Spi- assay models) / Since the transgenes are transmitted by the germ cells, they are present in every cell. Therefore, gene mutations and/or chromosomal rearrangements can be detected in virtually all tissues of an animal, including target tissues and specific site of contact tissues.</td>
<td>EU: B.58 OECD: 488</td>
</tr>
<tr>
<td>In vivo alkaline single-cell gel electrophoresis assay for DNA strand breaks (comet assay)</td>
<td>DNA strand breaks / The DNA strand breaks may result from direct interactions with DNA, alkali labile sites or as a consequence of incomplete excision repair. Therefore, the alkaline comet assay recognises primary DNA damage that would lead to gene mutations and/or chromosomal aberrations, but will also detect DNA damage that may be effectively repaired or lead to cell death. The comet assay can be applied to almost every tissue of an animal from which single cell or nuclei suspensions can be made, including specific site of contact tissues.</td>
<td>EU: none OECD: 489</td>
</tr>
</tbody>
</table>

Guidance on Information Requirements and Chemical Safety Assessment, Chapter R.7a: Endpoint specific guidance, August 2014
In vivo test methods

- Micronucleus test (erythrocytes, bone marrow)
- Comet assay-tissue specific (may include germ cells)
  - An international validation study on the in vivo alkaline comet assay (2006 - 2014) was coordinated by the Japanese Centre for the Validation of Alternative Methods (JaCVAM) and this lead to an approved OECD guide line study (OECD 489)
- TGR - transgenic rodent somatic and germ cell mutation assay
- PigA- in vivo somatic mutation assay
**In vivo genotoxicity test-mammalian**

**Rodent bone-marrow micronucleus test**

Rats/mice dosed with compound, three doses, seven animals / group. Animals sacrificed 24 or 48 hours later

Micronuclei may be formed by loss of whole chromosome during division or by chromosome breakage. The erythrocyte’s nucleus is extruded leaving any micronuclei behind

Bone marrow cells spread onto slides. Slides fixed and stained (acridine orange)

Femurs removed and bone marrow aspirated

2000 cells analysed per animal, number of micronucleated immature erythrocytes scored
In vivo comet assay

Tissue

Nuclei in gel with possible DNA damage

Electrophoresis

Comet

% Tail DNA

Target organ may be analysed
High throughput comet assay

Molding cells onto a cold Gelbond™-film

Lysis at 4°C, 1h-ON:

Enzyme treatment: Fpg, EndoIII

Alkaline treatment (unwinding at pH 13.2)

Electrophoresis

• Neutralize
• Fixation in EtOH and drying ON.
• Staining with SybrGold™
• Scoring with computer image analysis, Perceptives, IMSTAR

Test on single cell level
High sensitivity

Gutzkow et al., 2013
PigA- *in vivo* somatisk mutasjons tes

- Phenotypic mutation rates in red blood cells (RBC) and reticulocytes (RET)

- Pig-a gene product – GPI (glycosylphosphatidylinositol) anchors to the cell surface and bind CD24

- Pig-a is localised on X-chromosome – only one allele has to mutate!

- No GPI in the membrane is caused by a Pig-a mutation – *visualized as no CD24 staining*

- Pig-a mutation is *neutral* – no influence on survival
## Germ cell genotoxicity tests

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<tr>
<td>Mammalian spermatogonial chromosome aberration test</td>
<td>Structural and numerical chromosome aberrations / The test identifies substances that induce structural chromosome aberrations in mammalian, usually rodent, spermatogonial cells and is, therefore, expected to be predictive of induction of heritable mutations in germ cells. An increase in polyploidy may indicate that a substance has the potential to induce numerical chromosome aberrations, but this test is not optimal to measure numerical aberrations and is not routinely used for that purpose. Accordingly, this test guideline is not designed to measure numerical aberrations.</td>
<td>EU: B.23 OECD: 483²</td>
</tr>
<tr>
<td>Rodent dominant lethal test</td>
<td>Structural and numerical chromosome aberrations / The test identifies substances that induce dominant lethal effects causing embryonic or foetal death resulting from inherited dominant lethal mutations induced in germ cells of an exposed parent, usually the male. It is generally accepted that dominant lethals are due to structural and numerical chromosome aberrations. Rats or mice are recommended as the test species.</td>
<td>EU: B.22 OECD: 478²</td>
</tr>
<tr>
<td>Transgenic rodent (TGR) somatic and germ cell gene mutation assays</td>
<td>Gene mutations and chromosomal rearrangements (the latter specifically in the plasmid and Spi-assay models) / Since the transgenes are transmitted by the germ cells, they are present in every cell. Therefore, gene mutations and/or chromosomal rearrangements can be detected in virtually all tissues of an animal including specific site of contact tissues and germ cells. Delayed sampling times may need to be considered in order to detect mutations in different stages of spermatogenesis.</td>
<td>EU: none OECD: 488</td>
</tr>
</tbody>
</table>
• Any tissue
• Big Blue – cII gene
• Positive selection of mutants
• Specific information of cells in different stages of spermatogenesis, including stem cells (spermatogonia)
Genotoxic vs non-genotoxic

**Genotoxic**
Primary genotoxicity: Direct interaction with genetic material
Causes indicator effects

*No threshold*
Induce DNA damage linearly related to dose

**Non-genotoxic**
Secondary genotoxicity: no direct reactivity with DNA

Lead to genotoxic events through:
- Forced cell growth
- ROS formation
Testing substances on mutagenicity

• Evaluating negative test results:
  – Concentration of the test substance high enough?
  – Test system sensitive enough?
  – Volatility of the substance?
  – Metabolism activated \textit{in vitro}?
  – Test substance taken up by the test system \textit{in vitro}?
  – Reaching the target organ \textit{in vivo}?
  – Route of exposure
  – Dose response

• Evaluating contradictory results
  – Difference in metabolism, uptake or genetic material between non-mammalian and mammalian systems (mammalian tests are considered to be of higher significance). Additional data may be needed.
  – Results of mutagenicity tests are generally of higher significance than indicator tests such as Comet.
  – \textit{In vivo} tests are of higher degree of reliability than \textit{in vitro} tests.
  – If a test system is known to produce a more accurate response it would be given higher priority.
  – Followed OECD guide line?

• \textit{Weight of evidence}
  – GLP?
Thank you for the attention 😊