In silico, in vitro, and in vivo methods as alternatives to the carcinogenicity bioassay for epigenetic chemicals

Romualdo Benigni

Istituto Superiore di Sanita’
Rome Italy

rbenigni@iss.it
Testing strategy for carcinogenicity pre-screening in REACH

• Pivotal role of genotoxicity short-term tests

• Bioassay may be required in the case of high exposure plus genotoxicity evidence
Testing strategy for carcinogenicity pre-screening in REACH

• Pivotal role of genotoxicity short-term tests

• Bioassay may be required in the case of high exposure plus genotoxicity evidence

• Nongenotoxic carcinogens are negative in genotoxicity tests, and go undetected
Nongenotoxic carcinogens: an overlooked issue

- Theory on nongenotoxic carcinogenicity is much less developed than that on the genotoxic mechanisms.

- Nongenotoxic carcinogens are often considered to represent a lesser risk for human health.

  However

- Nongenotoxic carcinogens are a remarkable proportion of recognized human carcinogens (17 - 27% of IARC Group 1).

**Human Carcinogens** *(IARC Group 1)* negative in genotox assays

<table>
<thead>
<tr>
<th>Chemicals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimethylarsinic acid</td>
</tr>
<tr>
<td>Monosodium methane arsenate</td>
</tr>
<tr>
<td>Beryllium</td>
</tr>
<tr>
<td>Beryllium sulfate tetrahydrate</td>
</tr>
<tr>
<td>Chromium carbonyl</td>
</tr>
<tr>
<td>Cyclosporin</td>
</tr>
<tr>
<td>Estrogens, non-steroidal</td>
</tr>
<tr>
<td>Chlorotrianise</td>
</tr>
<tr>
<td>Estrogen/progesterone therapy</td>
</tr>
<tr>
<td>Estradiol</td>
</tr>
<tr>
<td>Estrogens, steroidal</td>
</tr>
<tr>
<td>Ethynyl estradiol</td>
</tr>
<tr>
<td>Ethanol</td>
</tr>
<tr>
<td>Gallium arsenide</td>
</tr>
<tr>
<td>Nickel sulfate hexahydrate</td>
</tr>
<tr>
<td>Nickel (II) oxide</td>
</tr>
<tr>
<td>Nickelocene</td>
</tr>
<tr>
<td>2,3,7,8-Tetrachlorodibenzo-para-dioxin</td>
</tr>
</tbody>
</table>

...Alternative approaches to the identification of nongenotoxic carcinogens?
Backing up the STTs with Structure-Activity concepts:

more SAs for nongenotoxic carcinogens
more SAs for nongenotoxic carcinogens

• Analysis of mechanistic knowledge

e.g.,


Combined with

• Data Mining of unflagged carcinogens –with mechanistic explanation-
more SAs for nongenotoxic carcinogens: results

- 18 new SAs not contained in the previous rulebase

*mechanistic classes of:*

a) Peroxisome Proliferators (including DNA methylating agents, and chemicals affecting Gap Junction);

b) inducers of Hormonal Imbalance;

c) inducers of Oxidative Stress;

d) Aryl Hydrocarbon Receptor agonist / antagonist chemicals

- 2 new SAs for genotoxic carcinogenicity coded as well

- implemented into Toolbox 2.3, to be implemented into Toxtree
more SAs for nongenotoxic carcinogens: examples

- substituted phenoxyacid
- substituted n-alkylcarboxylic acids
- phthalate (or butyl) diesters and monoesters
- perfluorooctanoic acid (PFOA)
- pentachlorophenol
- quercetin-type flavonoids
- imidazole, benzimidazole
- dicarboximide
- tri (or tetra) chloroethylene
Carcinogenicity prediction:
Old (mainly genotoxic) versus expanded SAs rulebase

-Carcinogenicity databases:
  - ISSCAN
  - Toxcast phase 1
  - ISSPEST

pesticides (PAN)
Non-mutagenicity *in vitro* assays for non-DNA-reactive carcinogens?
Cell transformation assays

in cultured cells, phenotypic alterations characteristic of tumorigenic cells

Data: OECD vol.31 2007
no inorganics
Benigni et al., Mutagenesis, 2011, 26: 455-460
ISSCTA: a database of Cell Transformation Assays results
to be included into the OECD (Q)SAR Toolbox and the ISSTOX database

\[ n = 370 \quad \text{(including inorganics and organics)} \]

**Systems**

*primary normal diploid cells:* Syrian Hamster Embryo cells assay (pH 7)
Syrian Hamster Embryo cells assay (pH 6.7)

*immortalized aneuploid mouse cells:* BALB/c 3T3
C3H10T1/2
Bhas 42

Data: OECD vol.31, 2007
Sakai et al., 2010 Mutat.Res. 702:100-122
Cell Transformation Assays: carcinogenicity prediction

A new analysis on ISSCTA, including inorganics and Bhas 42
CTAs prediction of genotoxic and nongenotoxic carcinogens
CTAs and Rodent Bioassay:
global similarity between responses to the chemicals

Procedure:
Tests similarity matrix, then
Principal Component Analysis

PC1 + PC2: 0.80 Exp. Var.
SHE pH ≥ 7 Cell transformation *versus* rodent carcinogenicity

**Carcinogenicity**

<table>
<thead>
<tr>
<th></th>
<th>neg</th>
<th>pos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negatives</td>
<td>36</td>
<td>18</td>
</tr>
<tr>
<td>Non-DNA-reactive</td>
<td>6</td>
<td>70</td>
</tr>
<tr>
<td>DNA-reactive</td>
<td>5</td>
<td>59</td>
</tr>
</tbody>
</table>

Sensitive to DNA-reactive and non-DNA-reactive carcinogens

*Chemicals n = 194, from OECD vol. 31*
Efficient, alternative testing strategy with tools available today?

*in vitro assays* {Salmonella and Cell transformation} sensitive to both DNA-reactive and non DNA-reactive carcinogens

**Structural Alerts** to predict / rationalize experimental results
A tiered approach to carcinogens identification

<table>
<thead>
<tr>
<th>Tiered Approach A</th>
<th>Tiered Approach B</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Noncarc</strong></td>
<td><strong>Carc</strong></td>
</tr>
<tr>
<td>Initial Sample</td>
<td>36</td>
</tr>
<tr>
<td>After Tier 1</td>
<td></td>
</tr>
<tr>
<td>STY Negative</td>
<td>27</td>
</tr>
<tr>
<td>After Tier 1b</td>
<td></td>
</tr>
<tr>
<td>SA nongeno Negative</td>
<td></td>
</tr>
<tr>
<td>After Tier 2</td>
<td></td>
</tr>
<tr>
<td>SHE Negative</td>
<td>17</td>
</tr>
<tr>
<td>% initial sample</td>
<td>47 %</td>
</tr>
</tbody>
</table>

only ~ 5-10% undetected carcinogens
Meta-"omics" of toxicological endpoints

Panels of chemicals (databases) as mechanistic probes

(\(PC1 + PC2 + PC3:\) 0.74 Exp. Var.)

Panels of chemicals (databases) as mechanistic probes
Carcinogenicity and Transformation

In vivo Micr.

In vitro Clastogenicity

Ames test

<table>
<thead>
<tr>
<th>Name of Observation or Cluster</th>
<th>OB1</th>
<th>OB7</th>
<th>OB2</th>
<th>OB11</th>
<th>OB14</th>
<th>OB13</th>
<th>OB18</th>
<th>OB19</th>
<th>OB16</th>
<th>OB4</th>
<th>OB6</th>
<th>OB9</th>
<th>OB10</th>
<th>OB5</th>
<th>OB15</th>
<th>OB17</th>
<th>OB3</th>
<th>OB8</th>
<th>OB12</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vivo Micronucleus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carcinogenicity and Transformation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ames test</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Do “nongenotoxic carcinogens” lack genotoxic activity (and viceversa)?

Cyproterone acetate and tamoxifen:
Liver carcinogens that had been thought to act by nongenotoxic mechanisms. Later studies indicated that are genotoxic and have tumor-initiating activity.

Steroidal estrogens: nongenotoxic, but also DNA-adducts

Inducers of Oxidative stress: also DNA damage

DNA-reactive chemicals can interact with proteins as well

Animal studies demonstrate that tumor promoters can cause cancer in the absence of an initiating agent
Do “nongenotoxic carcinogens” lack genotoxic activity (and vice versa)? An issue for risk assessment

...classification of carcinogens into genotoxic and non-genotoxic or into initiating or promoting agents may not only be unhelpful but even an impediment to risk assessment.

Once we can accumulate enough relevant mechanistic information about individual chemicals, it will be more reasonable to use this information directly for risk estimation, without expending efforts in classifying them... Yamasaki 1995

Classification systems based on labeling chemicals as genotoxic or nongenotoxic and on presumed mechanisms of action for each class lead to ambiguous reconstructions of the carcinogenic process...

...the existence or absence of threshold dose-responses cannot be determined from current knowledge of carcinogenic mechanisms.

The prudent policy for protecting public health is the one that considers the dose-response of all potential contributing effects of each specific chemical (in each specific stage of carcinogenesis)... Melnick et al. 1996
...some (final) considerations

- **Most reductionist approaches** are weak predictors of reality
  (e.g., chromosomal aberrations and other STTs, DNA-hypomethylation in tissues, omics in pharmacology)

- **Two success stories**
  - **Ames test** maps a large family of DNA- (and protein-) reactive carcinogens
  - **Cell Transformation** as model for cell-cell and cell-stroma interplay in cancer tissues (not a single-cell condition)

- Balance of details and generality as **challenge** in describing complex systems
In a fluctuating (stochastic) environment is governed by the three fundamental laws of conservation:

i) **mass**,  
ii) **energy &**  
iii) **momentum**

Resulting in Navier–Stokes equations

\[
\rho \left( \frac{\partial \mathbf{v}}{\partial t} + \mathbf{v} \cdot \nabla \mathbf{v} \right) = -\nabla p + \nabla \cdot \mathbf{T} + \mathbf{f}
\]

\(v\): flow velocity, \(\rho\): fluid density, \(p\): pressure, \(\mathbf{T}\): stress tensor, \(f\): body forces/volume
...some (final) considerations

• The present strategies for carcinogenicity pre-screening do not defend adequately human health, and need to be updated

• Simpler and more efficient alternative strategies available

• Reliable ground for refinements:
  - Expand SAs for nongenotoxic carcinogens to decrease reliance on experiments (mechanistic knowledge plus data mining)
  - Improve specificity
  - .........
Freely-available tools generated at the ISS

**Structural Alerts** for - canc / *in vitro* mut
- *in vivo* micronucleus

**Toxtree**
http://ihcp.jrc.ec.europa.eu/our_labs/computational_toxicology/qsar_tools/toxtree

**OECD (Q)SAR Toolbox**
http://www.oecd.org/document/54/0,3746,en_2649_37465_42923638_1_1_1_37465,00.html

**Curated toxicological databases**

**ISSTOX**
http://www.iss.it/ampp/dati/cont.php?id=233&lang=1&tipo=7
Acknowledgements

Alessandro Giuliani
Cecilia Bossa
Olga Tcheremenskaia
Chiara Battistelli
Mauro Colafranceschi