The Growing Network of Genotoxicity AOPs

- Lhasa Limited Virtual AOP Symposium, 2020

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Overview

1. Background: Why are we working on AOPs?
2. AOPs 101 (very brief review)
3. The first genetic toxicology AOP
4. The Health and Environmental Sciences Institute’s GTTC MoA working group
5. The growing network of genetic toxicology AOPs
6. The future and beyond
1. Background – why AOPs?
Genomics in Regulatory and Applied Toxicology (GReAT) Laboratory

1. Improve human health risk assessment of environmental chemicals through enhanced use of toxicogenomic information.

2. Develop and apply novel/improved tools to quantify genotoxicity.

Overarching goal is to modernize regulatory toxicology testing and improve understanding of the impacts of environmental chemicals on health.
Paradigm for use of transcriptomics in risk assessment

1. Extract predictive biomarkers

2. Align to AOPs

3. Dose-response modeling

4. Case studies

Large data sets

At what dose do effects occur?

Risk assessment

Human exposure levels?

Hazard identification
Mode of action analysis
TGx-DDI Biomarker to Predict DNA Damage-Inducing (DDI) Chemicals

An *in vitro* transcriptomic biomarker to predict probability that an agent is DDI or non-DDI.

- Developed using human cells in culture (TK6 cells) using DNA microarrays
- From exposure to 28 prototype DNA damage-inducing (DDI) and non-DDI chemicals
- 64 genes identified as being predictive of DDI potential

**TGx-DDI Publications for Methods Development, Validation, Application:**

**Biomarker development and validation**

**Development of method for use of biomarker with metabolic activation system**
GeneTox21

Test substances via HT in vitro assays assessing genetic damage, mutagenicity, chromosomal abnormalities, molecular alterations.

Quantitative Benchmark Dose Modeling

Use IVIVE to obtain bioactivity-to-exposure (BER) ratios

Analogous to ToxPi approach, provide a single score indicating GeneTox hazard for substance prioritization

Adverse Outcome Pathways to determine mechanisms and evaluate potential adverse effects

Risk assessment

Lead, Paul White, Health Canada
And a large crew of collaborators!
Primary Purposes of AOPs

1. Link **measurable changes** at various levels of biological organization to **endpoints of regulatory significance**

By

2. Efficiently **organizing** the most relevant experimental **evidence** using an easily accessible framework
AOPs integrate complex information

- Add biological (and regulatory) context to measured endpoints
- Rigorous documentation and evaluation of supporting evidence
- Easily accessed: machine readable, searchable, open access, linked
- Easily interpreted by non-experts
2. AOPs 101 (brief refresher)
AOPs

A framework that describes knowledge about how toxicants interact with cellular molecules to lead to an adverse outcome relevant to human or ecological health

- A way to organize information
- Based on biological plausibility and/or statistical inference
- A hypothesis and a set of measurements to test it
Two Primary Building Blocks

**Key Events (KEs)**

*Functional unit of observation/verification*
- Observable Δ biological state (measurable)
- Essential (but not necessarily sufficient)
- Methods for observing/measuring
- Taxonomic/sex/life stage applicability

**Key Event Relationship (KERs)**

*Functional unit of inference/extrapolation*
- Define a directed relationship
- State of KE$_{up}$ provides some ability to predict or infer state of KE$_{down}$
- Supported by plausibility and evidence
- Quantitative understanding
Molecular initiating event (MIE): The initial point of chemical interaction with molecules in the organism that starts the AOP.

Adverse Outcome (AO): An endpoint of regulatory significance, corresponding to:
- an established protection goal,
- an apical endpoint in an accepted regulatory guideline toxicity test.
# Bulk of the work in building an AOP: Assembling the Weight of Evidence for the KERs and overall AOP

<table>
<thead>
<tr>
<th>Modified BH Considerations</th>
<th>Conclusions</th>
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<tbody>
<tr>
<td><strong>Biological Plausibility</strong></td>
<td>KE(R)s are consistent with current biological understanding – plausible.</td>
</tr>
<tr>
<td><strong>Essentiality of KEs</strong></td>
<td>Effects are reversible if the KEs are blocked or the stressor is removed.</td>
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</tbody>
</table>
| **Concordance of Empirical Observations**  | **Dose response** – The KEs are observed at doses below or similar to those associated with the apical effect?  
**Temporality** – The KEs are observed in hypothesized order?  
**Incidence** – The frequency of occurrence of the AO less than that for the KE? |
| **Uncertainties and inconsistencies**       | Clear documentation of inconsistencies between studies/stressors and indication of any uncertainties |

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**Strongest independent evidence**

**Weakest independent evidence**
Important AOP Concepts

1. Individual AOPs are a *pragmatic simplification*

2. AOP networks are the functional unit of prediction (in most cases)

3. AOPs are modular

4. AOPs are “living” documents and published in AOP-wiki
   • Facilitates collaboration and crowd-sourcing
   • Avoids duplicative effort
   • Integration and analysis
   • Accessible and searchable
AOPs are modular: borrow and share AOP components

- KEs and KERs are not unique to a single AOP
- Do not need to be regenerated independently for every new AOP
- AOP Networks result from shared KE
Important AOP Concepts

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   - Created, stored and shared in the AOP-Knowledge Base (AOP-Wiki)
   - Facilitates collaboration and crowd-sourcing
   - Avoids duplicative effort
   - Integration and analysis
   - Accessible and searchable
   - Can be updated at anytime
3. Case study: The first genetic toxicology AOP
Alkylation of DNA leading to heritable mutations

Motivation:
• One of the best characterized modes of action in genetic toxicology.
• Provide context of use for new methods/technologies to detect somatic and germ cell mutations.
• Emphasize testing and data gaps in this field.

• *De novo* mutations are increasingly recognized as contributing to a large array of human genetic diseases.
• Regulatory assessments require consideration of potential heritable effects.
Prototype alkylating agent

- Alkylating agents: chemicals that add alkyl groups (e.g., methyl, ethyl, propyl) to cellular molecules.

- Database on alkylation of DNA is heavily biased towards a few prototype agents.

  - E.g., N-ethyl-N-nitrosourea (ENU)
Alkyl adducts can be repaired

- Primary repair protein is AGT: alkyl guanine transferase.
- AGT irreversibly binds the alkyl group and is inactivated.
- Very efficient repair at LOW doses.
- AGT overwhelmed at HIGH doses = alkyl adducts retained.
- Not very good at repairing all adduct types.
Replication of alkylated DNA causes mutations

• Replication over an alkyl adduct can cause insertion of an incorrect base in the DNA duplex.
• Mutation becomes ‘fixed’ and can propagate to daughter cells.

$O^4$ thymine alkylation = AT-GC transitions
$O^6$ guanine alkylation = GC-AT transitions

Note: some adducts not mutagenic. N-alkyl adducts tend to be bypassed error-free
Selection of measurable endpoints

- Adduct
- Not repaired
- Repaired
- Offspring with mutations
- Sperm with mutation
- Fertilizing egg
- Mutations (somatic)
- Mutations (sperm)
AOP
Alkylation of DNA Leading to Heritable Mutations

Molecular Initiating Event
Alkylation of DNA

Cellular Response
Inadequate DNA repair

Organism Response
Mutations increase
Inherited mutations increase

Offspring: Increased numbers of mutations in all tissues

(Male germ cells)
Example Evidence

Dose Concordance  Temporal Concordance  Incidence Concordance

Swenberget al. (2008)
Example Evidence

Dose Concordance

Adducts > Mutations in dose

Temporal Concordance

Adducts in rodent germ cells: Peak early, some persist to 6 d


Incidence Concordance

Mutations in rodent sperm: Exposed 28 d, mutations occur ≥ 42 days post-exposure

Example Evidence

Dose Concordance

Adducts > Mutations in dose

Temporal Concordance

Adducts > Mutations in time

Incidence Concordance

Adducts per nucleotide

Mutations per nucleotide (SPERM)

Mutations per nucleotide (OFFSPRING)
Example Evidence

<table>
<thead>
<tr>
<th>Dose Concordance</th>
<th>Temporal Concordance</th>
<th>Incidence Concordance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adducts &gt; Mutations in dose</td>
<td>Adducts &gt; Mutations in TIME</td>
<td>Adducts &gt;&gt; Mutations (Sperm) ≈ Mutations (offspring) in frequency</td>
</tr>
</tbody>
</table>
**Example Evidence: Essentiality**

**Essentiality:** Are downstream KEs and/or the AO prevented if an upstream KE is blocked?

- Evidence supports that overcoming DNA repair is required for mutation to occur.
- Knock-down DNA repair, mutations increase; over-express DNA repair, mutations decrease.

Figure derived from data in: Allay et al. (1999) Oncogene: 18(25):3783-3787.
**Quantitative understanding of the KERs (in development)**

<table>
<thead>
<tr>
<th>Extent of Quantitative Understanding</th>
<th>Characteristics</th>
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</table>
| Strong                              | - Change in $\text{KE}_{\text{down}}$ can be precisely predicted based on a relevant measure of $\text{KE}_{\text{up}}$.  
- Uncertainty in the quantitative prediction can be precisely estimated from the variability in the relevant measure of $\text{KE}_{\text{up}}$.  
- Known modulating factors are accounted for in the quantitative description.  
- There is evidence that the quantitative relationship between the KEs generalizes across the relevant applicability domain of the AOP. |
| Moderate                            | - Change in $\text{KE}_{\text{down}}$ can be precisely predicted based on a relevant measure of $\text{KE}_{\text{up}}$.  
- Uncertainty in the quantitative prediction is influenced by factors other than the variability in the relevant measure of $\text{KE}_{\text{up}}$.  
- Quantitative description does not account for all known modulating factors.  
- The quantitative relationship has only been demonstrated for a subset of the overall applicability domain of the AOP (e.g., based on a single species). |
| Weak                                | - Only a qualitative or semi-quantitative prediction of the change in $\text{KE}_{\text{down}}$ can be determined from a measure of $\text{KE}_{\text{up}}$.  
- Known modulating factors are not accounted for.  
- The quantitative relationship has only been demonstrated for a narrow subset of the overall applicability domain of the AOP (e.g., based on a single species). |
Status of alkylation of DNA AOP

- Endorsed by the OECD
- https://aopkb.org/aopwiki/index.php/Aop:15
- See also:
  - Yauk et al. Development of the adverse outcome pathway "alkylation of DNA in male premeiotic germ cells leading to heritable mutations" using the OECD's users' handbook supplement. Environ Mol Mutagen. 56(9):724–50. 2015
  - Yauk et al., Adverse Outcome Pathway on Alkylation of DNA in Male Pre-Meiotic Germ Cells Leading to Heritable Mutations. OECD Series on Adverse Outcome Pathways ISSN: 2415-170X. http://dx.doi.org/10.1787/2415170X
How does this look in the AOP-wiki?

AOP Title Search Results

<table>
<thead>
<tr>
<th>Id</th>
<th>Title</th>
<th>Point of Contact</th>
<th>Author Status</th>
<th>SAAOP Status</th>
<th>MIE</th>
<th>AO</th>
<th>OECD Status</th>
<th>OECD Project</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>Alkylation of DNA in male pre-meiotic germ cells leading to heritable mutations</td>
<td>Carole Yauk</td>
<td>Open for citation &amp; comment</td>
<td>Included in OECD Work Plan</td>
<td>DNA alkylation</td>
<td>heritable mutations</td>
<td>TFHA/WNT Endorsed</td>
<td>1.11</td>
</tr>
</tbody>
</table>
Borrowing KE(R)s to make new AOPs

Alkylation of DNA network

1. Alkylation of DNA
2. Inadequate DNA repair
3. DNA strand breaks Increase
4. Apoptosis Increase
5. Mutations Increase
6. Leukemia
7. Sperm count Decrease
8. Infertility
9. Inherited mutations

(Male germ cells)

(Bone marrow)
Alkylation of DNA (Male germ cells) 

Mutations Increase 

Inadequate DNA repair 

Inherited mutations 

Upstream expansion of network 

(Bone marrow) 

Oxidative DNA damage 

DNA strand breaks, Increase 

Leukemia 

(Male germ cells) 

Sperm count, Decrease 

Infertility 

Bulky DNA adducts 

Apoptosis Increase
4. Health and Environmental Sciences (HESI) and Genetic Toxicology Technical Committee (GTTC)

Mode of Action Working Group
Evaluate and recommend mode of action approaches that identify genotoxicity mechanisms in mammalian cell systems

- molecular targets
- sequence of cellular events
- Dose-response relationships
- defense mechanisms
- define risk based on predicted in vivo exposure

Causality supported by mechanistic understanding

Drafted by Maik Schuler
AOPs can facilitate characterization of MOA and identification of the molecular pathways leading to genotoxic outcome.

Well-described AOPs can inform testing paradigms.

Develop AOPs ending in permanent genomic damage.
Oxidative DNA Damage leading to Mutations and Chromosomal Aberrations

- Oxidative DNA Damage, Increase
- Inadequate Repair
- DNA Strand Breaks, Increase
- Mutations, Increase
- Chromosomal Aberrations
- Inadequate Repair
Challenge: lack of empirical data to support WoE for AOP
Opportunity: use new test methods to support AOP

<table>
<thead>
<tr>
<th>KE</th>
<th>Methods</th>
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<tbody>
<tr>
<td>Oxidative DNA damage</td>
<td>High-throughput fpg comet assay</td>
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<tr>
<td></td>
<td>Transcriptomics</td>
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<tr>
<td>Inadequate repair</td>
<td>Ogg1 inhibitors (test essentiality)</td>
</tr>
<tr>
<td>DNA strand breaks</td>
<td>High-throughput comet assay</td>
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<tr>
<td></td>
<td>Transcriptomics</td>
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<tr>
<td></td>
<td>Multi-flow assay</td>
</tr>
<tr>
<td>Chromosomal aberrations</td>
<td>Flow cytometry micronucleus assay</td>
</tr>
<tr>
<td>Mutations</td>
<td>Error-corrected single-molecule sequencing</td>
</tr>
<tr>
<td></td>
<td>(Duplex)</td>
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Cho et al. (submitted to OECD and *EMM*)
OECD AOP 296
Topoisomerase II inhibition AOP

![Diagram showing the AOP process](image)

**Fig. 4.** Preliminary AOP for binding to DNA–topo II cleavage complex (topo II inhibition) leading to chromosome breaks and rearrangements (AO2) and/or gene mutations (AO1). Abbreviations: AO, adverse outcome; AOP, adverse outcome pathway; KE, key event; MIE, molecular initiating event; topo II, topoisomerase II.
5. The growing network of genetic toxicology AOPs
The growing genomic damage AOP network

DNA damage and Mutation Network

- Binding to DNA-topo II cleavage complex
- Stabilization of cleavage complexes (cleaved DNA)
- Disrupted replication forks
- Apoptosis
- Decreased sperm count
- Infertility
- Chromosome aberrations
- Mutations, Increase
- Heritable mutations, Increase
- Cancer (lung, leukemia)

- Multiple MIEs (ex: chemical binding to DNA polymerase; disruption of dNTP pools)
- Oxidative DNA damage, Increase
- MIEs leading to increases in ROS

- Direct deposition of energy
- Alkylation of DNA

Aneuploidy Network

- Binding to tubulin
- Abnormal depolymerization/stabilization of microtubules
- Mitotic abnormalities
- Cytokinesis inhibition and increase in binucleated cells
- Increase of multipolar spindles in 2nd mitotic division
- Aneuploidy (chromosome loss/non-disjunction)

- Chemical binding to the catalytic domain of aurora kinases
- Catalytic inhibition of aurora kinases
- Activation of the Spindle Assembly Checkpoint (SAC)
- Incorrect chromosome alignment & segregation

- Mitotic abnormalities
Two test articles produce micronuclei in mammalian cells

Centromere staining shows that the micronuclei contain whole chromosomes

Compounds classified as aneugenic

Follow-up: test both compounds in the flow cytometric MultiFlow assay.

Compound A: *increases* phospho-H3 positive cells at the 4-h time point, while inducing an increase of polyploidy at the 24-h time point at concentrations that are significantly higher than the concentration at which micronucleus induction is seen.

Compound B: *decreases* the frequency of phospho-H3-positive cells and causes a large increase of polyploid cells at the 24-h time point at concentrations that are similar to the concentration where micronucleus induction is seen.

Based on the additional assay, it is likely that Compound A induces micronuclei by tubulin binding, whereas Compound B induces micronuclei by interacting with the catalytic domain of AURKB.

Outcome: Use the information to improve on the AURKB selectivity or make changes to the molecule to avoid tubulin interaction.
6. The future and beyond
Uses of genotoxicity AOPs in regulatory context

- To help delineate a MOA
  - predictive toxicology
  - support selection of best fitting assay
- Justification for chemical grouping/read-across
- Weight of evidence to support a MOA – direct/indirect
- Prioritization or regulatory action
- Supports use of New Approach Methodologies (NAMs)
- Extrapolation across species
- Help resolve complex questions and define uncertainties and inconsistencies
- Genotoxic/non-genotoxic carcinogens
- Supports assessment of mixtures
Challenges

- Lack empirical data to support the KER and AOPs
  - Major gaps in quantitative understanding

- Concurrent work by other groups
  - Redundant development of overlapping KEs & KERs
  - Reaching consensus on KE and KER

- Re-use of existing KEs and KERs
  - Adding and modifying content and titles to improve applicability to our AOP
  - Working with the wiki
  - We’re not good at sharing

- The AOP is a very big document to work with
Challenges

- **Labour intensive to develop AOPs**
  - Relatively few OECD-endorsed AOPs
  - Difficult and time consuming to mine the literature to identify the best KEs for the AOPs, the appropriate names, and the measurement methodologies
    - Subjectivity
  - Finding the right studies (of empirical data) to support the KERs and extracting the relevant data
    - Automation and systematic approaches
  - Challenging to identify prototype stressors for the pathways
  - Increasing requirements to define confidence in elements of the AOPs
Opportunities

• Concurrent work by other groups
  • The events and relationships can be re-used by other groups developing genetox AOPs
  • ROS DNA damage and Oxidative Stress will be a central KE in many AOPs
    – Opportunities for collaboration
      • Sharing workload - crowdsourcing
      • Building new AOPs and AOP networks
      • Making connections to additional MIE and KE

• **Case studies to demonstrate use** – advancing regulatory assessment of genetic effects and associated adverse outcomes

• Harness the information in the AOPs to build predictive models (quantitative work needed!)

• Systematic review and systematic mapping tools to build and review AOPs (leadership by the Evidence-based Toxicology Collaboration (John Hopkins University))
Case studies of AOPs in a regulatory context

<table>
<thead>
<tr>
<th>Confidence vs. uncertainty</th>
<th>AOP Continuum</th>
<th>USE</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Correlative/qualitative</td>
<td></td>
<td></td>
<td>“Low” confidence: Membrane disruption (Narcosis) leading to respiratory failure</td>
</tr>
<tr>
<td>Qualitative</td>
<td></td>
<td></td>
<td>“Moderate” confidence: Hepatocellular proliferation leading to cancer</td>
</tr>
<tr>
<td>Semi-quantitative</td>
<td></td>
<td></td>
<td>“High” confidence: Skin sensitization initiated by covalent binding to proteins</td>
</tr>
<tr>
<td>Quantitative</td>
<td></td>
<td></td>
<td>“High” confidence: Aromatase inhibition leading to reproductive dysfunction in fish</td>
</tr>
<tr>
<td>Predictive system</td>
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</tbody>
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Note that confidence in an AOP can be independent of the quantitative understanding of an AOP

One step closer to use of transcriptomics in risk assessment

Large data sets

1. Extract predictive biomarkers

2. Align to AOPs

3. Dose-response modeling

4. Case studies

At what dose do effects occur?

Risk assessment

Human exposure levels?

Hazard identification

Mode of action analysis
Acknowledgements

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