Genotoxicity Testing Strategies: application of the EFSA SC opinion to different legal frameworks in the food and feed area

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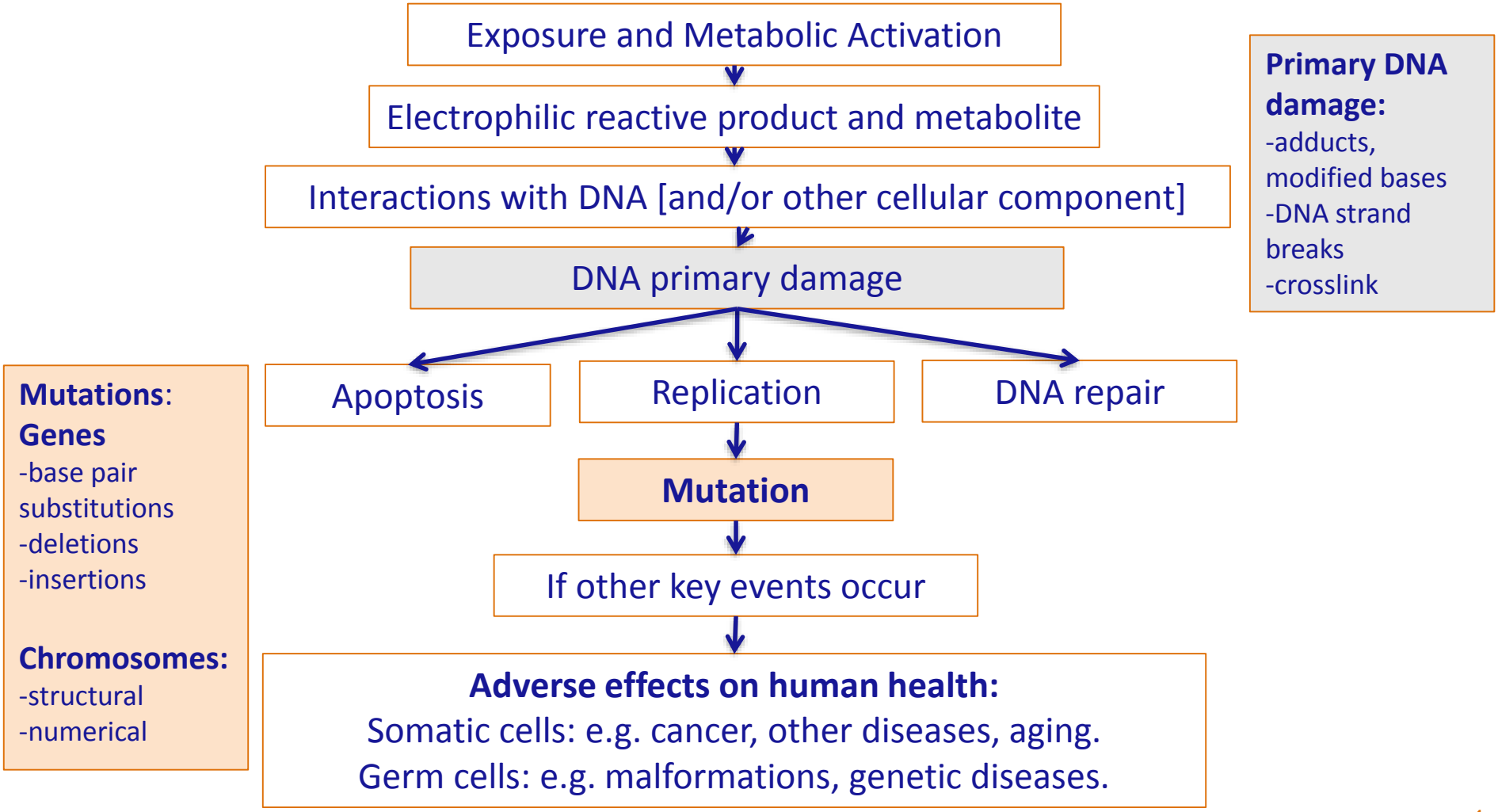
Exposure and Metabolic Activation
Electrophilic reactive product and metabolite
Interactions with DNA [and/or other cellular component]
DNA primary damage
Primary DNA damage:
-adducts, modified bases
-DNA strand breaks
-crosslink
Apoptosis
Replication
DNA repair
Mutations: Genes
-base pair substitutions
-deletions
-insertions
Chromosomes:
-structural
-numerical
Mutation
If other key events occur
Adverse effects on human health:
Somatic cells: e.g. cancer, other diseases, aging.
Germ cells: e.g. malformations, genetic diseases.

GENOTOXICITY

Implications in the risk assessment:

- Under the EU legislation no substance which is considered as mutagenic can intentionally be added to food, at any dose level.
- **Genotoxicity *per se* is an end-point**: Genetic damage in somatic or germ cells is associated with serious detrimental health effects, including cancer, heritable diseases and degenerative conditions.
- **Non-threshold for genotoxicity**: no point of departure for setting health-based reference values (exceptions: e.g. some aneugenic compounds).

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- Genotoxicity: implications in the risk assessment.
- Scientific Committee Opinion: the basic battery, follow-up of positive results and *in vivo* studies.
- EFSA: Regulated substances and products.
- Specific guidance and requirements for different regulated substances and products.
- Recurring issues in genotoxicity testing of pesticides and examples.
- (Q)SAR and read-across for genotoxicity assessment of pesticides metabolites.
- Genotoxic impurities in pesticide active substances.

SCIENTIFIC COMMITTEE OPINION*

Most commonly used *in vitro* methods for:

- Gene mutation:
  - Bacterial reverse mutation test in Salmonella typhimurium and Escherichia coli (OECD TG 471)
  - *In vitro* mammalian cell gene mutation test (OECD TG 476)
- Chromosome aberrations:
  - *In vitro* mammalian chromosomal aberration test (OECD 473)
  - *In vitro* mammalian cell micronucleus test (OECD TG 487)

* EFSA Scientific Committee; Scientific Opinion on genotoxicity testing strategies applicable to food and feed safety assessment. EFSA Journal 2011;9(9):2379. [69 pp.] doi:10.2903/j.efsa.2011.2379. Available online: www.efsa.europa.eu/efsajournal

SCIENTIFIC COMMITTEE OPINION

Most commonly used *in vivo* methods for:

- Gene mutations:
  - Transgenic rodent somatic and germ cell gene mutation assays (OECD TG 488)
- Chromosome aberrations:
  - Mammalian erythrocyte micronucleus test (OECD TG 474)
  - Mammalian bone marrow chromosome aberration test (OECD TG 475)
- Primary DNA damage:
  - *In Vivo* Mammalian Alkaline Comet Assay (OECD TG 489)
  - Unscheduled DNA synthesis (UDS) test with mammalian liver cells *in vivo* (OECD TG 486)

SCIENTIFIC COMMITTEE OPINION

- End-points to be considered:
  - Gene mutation
  - Structural and numerical (aneuploidy) chromosome aberrations.
- Test strategy:
  - No single test system can detect all three end-points, a battery is needed.
- Step-wise (tiered) approach:
  - Tier 1: *In vitro*
  - Tier 2: *In vivo*, driven by test results.

SCIENTIFIC COMMITTEE OPINION

Tier 1: the basic battery:

- Bacterial reverse mutation test in Salmonella typhimurium and Escherichia coli (OECD TG 471): end-point considered- gene mutation.
- *In vitro* mammalian cell micronucleus test (OECD TG 487): end-points considered-structural and numerical chromosome aberrations.
- Outcome:
  - Negative: No further testing*
  - Positive: *In vivo* testing

*unless available information indicate the inadequacy of *in vitro* systems.

SCIENTIFIC COMMITTEE OPINION

Tier 2: Follow-up*of positive results for:

- Gene mutation:
  - Transgenic rodent somatic and germ cell gene mutation assays (OECD TG 488).
  - *In Vivo* Mammalian Alkaline Comet Assay (OECD TG 489) as indicator test in terms of sensitivity.
- Chromosome aberration:
  - Mammalian erythrocyte micronucleus test (OECD TG 474).

*to be selected case-by-case based on in vitro test results, SAR, metabolic and toxicokinetic considerations…

SCIENTIFIC COMMITTEE OPINION

Outcomes of *in vivo* genotoxicity testing:

- Outcome:
  - Negative*: No further testing
  - Positive: Genotoxic hazard

  * With evidence of target cells exposure.

Tier 3: No tier 3 genotoxicity testing:

- Substances positive in tests in somatic cells are assumed to reach germ cells and to be germ cell mutagens too;
- Even in presence of negative carcinogenicity data, genotoxicity in vivo in somatic cells is considered an adverse effects *per se*.

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EFSA: THE REGULATED PRODUCTS

Pesticides: Panel and Peer review.

GMO: Panel.

Feed additives: Panel.

Nutrition: Panel.

Food Ingredients and Packaging: additives, flavourings, enzymes, contact materials.

SPECIFIC GUIDANCE AND REQUIREMENTS

Regulated substances	Data requirement.	Follow the SC Opinion 2011.	Specific Guidance	Legislation
GMO	Only performed if needed	Yes	GD GM Plants and derived food and feed (2006; 2011). GD GM Plants for non-food and feed purposes (2007).	Regulation (EU) No 503/2013
Feed Additives	Yes	Yes	GD Consumer Safety (2012).	Regulation (EC) No 429/2008.
Food Ingredients and contact materials.	Yes	Yes	GD Additives (2012). GD Flavouring (2010). GD Enzymes (2009). GD Contact Materials (2008)	Regulation (EC) No 234/2011.

SPECIFIC GUIDANCE AND REQUIREMENTS

Regulated substances	Data requirement.	Follow the SC Opinion 2011.	Specific Guidance	Legislation
Pesticides active substance.	Yes, specific studies in Regulation (EU) No 283/2013.	Not entirely.	No.	Regulation (EU) No 283/2013.
Pesticides metabolites in groundwater	Genotoxicity studies conducted if occurs in groundwater above 0.1µg/L according to EC (2003)	No.	European Commission (2003) on the relevance of groundwater metabolites.	Regulation (EU) No 283/2013.
Pesticides metabolites in food/feed	Yes but not specific studies mentioned in Regulation (EU) No 283/2013.	Yes, if no exclusion of genotoxic potential based on (Q)SAR and read-across according to EFSA PPR GD (2016)	EFSA PPR GD (2016) on Residue Definition to be adopted.	Regulation (EU) No 283/2013.
Pesticides impurities	Yes but not specific studies mentioned in Regulation (EU) No 283/2013	Yes	European Commission (2012) on assessment of equivalence of technical materials. EFSA SC Statement (2012).	Regulation (EU) No 283/2013.

PESTICIDES: ACTIVE SUBSTANCES

What is different?:

- Additional option for basic *in vitro* test battery to include gene mutation in mammalian cells.
- *In vivo* study always required.
- *In vivo* Comet assay not included.

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RECURRING ISSUES IN GENOTOXICITY TESTING OF PESTICIDES*

In vivo follow-up for *in vitro* gene mutation:

- *"If either of the in vitro gene mutation tests is positive, an in vivo test to investigate the induction of gene mutation shall be conducted, such as the Transgenic Rodent Somatic and Germ Cell Gene Mutation Assay (TGR)."* (Regulation (EU) No 283/2013)
- Historically the *in vivo* UDS test was the common *in vivo* follow-up of positive results in either of the in vitro gene mutation tests but of low recognized sensitivity.

*EFSA (European Food Safety Authority), 2016. Technical report on the outcome of the pesticides peer review meeting on general recurring issues in mammalian toxicology. EFSA supporting publication 2016:EN-1074. 24 pp.

RECURRING ISSUES IN GENOTOXICITY TESTING OF PESTICIDES

Aneugenicity:

- *"If the in vitro micronucleus test for numerical chromosome aberrations on mammalian cells is positive or the in vitro mammalian chromosome test is positive for numerical chromosome changes, an in vivo micronucleus test shall be conducted. In case of positive result in the in vivo micronucleus assay, appropriate staining procedure such as fluorescence in-situ hybridisation (FISH) shall be used to identify an aneugenic and/or clastogenic response"… (*Regulation (EU) No 283/2013)
- Test battery including only the *in vitro* clastogenicity assay: aneugenicity might not be properly assessed.

RECURRING ISSUES IN GENOTOXICITY TESTING OF PESTICIDES

Tissue exposure:

- *"There shall be convincing evidence (i.e. cell toxicity or toxicokinetic data) that the relevant tissue will be reached by the chosen exposure route and application method"*. (Regulation (EU) No 283/2013)

- Discussion on whether the intraperitoneal route might be used (where no evidence of tissue exposure at the limit dose by oral route); no agreement.

EXAMPLE 1

Genotoxicity	
In vitro	• Ames test: -ve (±S9) • Mammalian cell gene mutation: +ve (±S9) • Mammalian chromosome aberration test: -ve (±S9)
In vivo	• Micronucleus test: -ve • Unscheduled DNA synthesis: -ve
Genotoxic potential	?
Reference values	?

EXAMPLE 2

Genotoxicity	
In vitro	• Ames test: -ve (±S9) • Mammalian cell gene mutation: -ve (±S9)
In vivo	• Micronucleus test: -ve
Genotoxic potential	?
Reference values	?

EXAMPLE 3

Exception: Carbendazim as example

- Carbendazim caused numerical chromosome aberrations both *in vitro* and *in vivo* as a result of the interference with mitotic spindle proteins, a threshold concentration for aneugenic activity *in vitro* was estimated to be between 0.2-0.6 µg/mL, and the NOEL for aneuploidy *in vivo* is 50 mg/kg bw. Carbendazim did not cause gene mutations or structural chromosomal aberrations.
- Uncertainties with regard to species differences, influences of the methodology used (i.e. endpoint for aneuploidy measured *in vivo* (micronucleus) less sensitive than assessed *in vitro* (non-disjunction)) and the possible effects of exposure conditions (i.e. single vs. repeated administration).
- Reference values: NOAEL of 10 mg/kg bw per day in the developmental toxicity studies, UF of 500.

European Food Safety Authority; Conclusion on the peer review of the pesticide risk assessment of the active substance carbendazim. EFSA Journal 2010; 8(5):1598. [76 pp.]. doi:10.2903/j.efsa.2010.1598. Available online: www.efsa.europa.eu

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(Q)SAR PREDICTION AND READ ACROSS

Assessment of genotoxicity of pesticides metabolites

- EFSA PPR GD on Residue Definition (2016)*.
- Exclusion of genotoxicity is the 1st module.
- Based on recommendations of JRC Report (2010) on the applicability of (Q)SARs for the assessment of pesticides metabolites
- Read-across to maximise sensitivity and specificity of (Q)SARs prediction.
- Systematic assessment of read-across using computational tools like the OECD QSAR toolbox.

EFSA PPR Panel, 2016. **Draft** Guidance on the establishment of the residue definition for dietary risk assessment. EFSA Journal 2016;14(issue):NNNN, 180 pp. doi:10.2903/j.efsa.2016.NNNN https://www.efsa.europa.eu/sites/default/files/consultation/160307_4.pdf

(Q)SAR PREDICTION AND READ ACROSS

JRC (2010): Analysis

- Analysis of Ames test results from 181 pesticides and SP and SE from individual models (Pesticides DB).
- Analysis of Ames test results from 748 substances and SP and SE from individual models (DSSTox DB).
- Analysis of 113 EU classified mutagens and SE from individual and combination of models

JRC (2010). Applicability of QSAR analysis to the evaluation of the toxicological relevance of metabolites and degradates of pesticide active substances for dietary risk assessment. http://onlinelibrary.wiley.com/doi/10.2903/sp.efsa.2010.EN-50/abstract

(Q)SAR PREDICTION AND READ ACROSS

Table 7.1. Genotoxicity prediction results for the pesticides dataset

Number of compounds: 185
Experimental values available: 181
Exp. active compounds: 11
Exp. inactive compounds: 170

SOFTWARE	STATISTICS*										
	TP	TN	FP	FN	EQ	ND	SP	SE	CONC	1-SE	1-SP
CAESAR	7	129	40	4	1	0	0.76	0.64	0.76	0.36	0.24
Derek	6	148	22	4	1	0	0.87	0.60	0.86	0.40	0.13
HazardExpert	5	95	71	5	5	0	0.57	0.50	0.57	0.50	0.43
Lazar (Kazius/Bursi)	7	127	41	4	0	2	0.76	0.64	0.75	0.36	0.24
Lazar (Toxbenchmark)	5	127	41	6	0	2	0.76	0.45	0.74	0.55	0.24
TOPKAT	7	121	48	4	0	1	0.72	0.64	0.71	0.36	0.28
ToxBoxes	4	112	22	0	43	0	0.84	1.00	0.84	0.00	0.16
Toxtree (Benigni-Bossa)	6	117	53	5	0	0	0.69	0.55	0.68	0.45	0.31

TP – true positives; **TN** – true negatives; **FP** – false positives; **FN** – false negatives; **EQ** – compounds predicted as equivocal; **ND** – the number of compounds that were not handled by the software; **SP** – specificity; **SE** – sensitivity; **CONC** – overall concordance; **1-SE** – false negative rate; **1-SP** – false positive rate

(Q)SAR PREDICTION AND READ ACROSS

Table 7.2. Genotoxicity prediction results for the DSSTox dataset

Number of compounds: 1290
Experimental values available: 748
Exp. active compounds: 368
Exp. inactive compounds: 380

SOFTWARE	STATISTICS*										
	TP	TN	FP	FN	EQ	ND	SP	SE	CONC	1-SE	1-SP
CAESAR	315	298	76	52	7	0	0.80	0.86	0.83	0.14	0.20
Derek	299	298	68	62	20	1	0.81	0.83	0.82	0.17	0.19
HazardExpert	285	199	128	72	64	0	0.61	0.80	0.71	0.20	0.39
Lazar (Kazius/Bursi)	245	305	74	123	0	1	0.80	0.67	0.74	0.33	0.20
Lazar (Toxbenchmark)	243	316	64	124	0	1	0.83	0.66	0.75	0.34	0.17
TOPKAT	286	315	55	59	26	7	0.85	0.83	0.84	0.17	0.15
ToxBoxes	300	301	22	24	101	0	0.93	0.93	0.93	0.07	0.07
Toxtree	311	265	115	57	0	0	0.70	0.85	0.77	0.15	0.30

TP – true positives; **TN** – true negatives; **FP** – false positives; **FN** – false negatives; **EQ** – compounds predicted as equivocal; **ND** – the number of compounds that were not handled by the software; **SP** – specificity; **SE** – sensitivity; **CONC** – overall concordance; **1-SE** – false negative rate; **1-SP** – false positive rate

Table 7.8. Ability of software tools to identify classified mutagens

Software (used alone)	ND	EQ	TP	SE	FN	1-SE	No TS
Toxtree (genotoxic carcinogenicity)	0	0	86	0.76	27	0.24	NA
Toxtree (in vivo micronucleus)	0	0	98	0.87	15	0.13	NA
Toxtree (genotoxic carcinogenicity or in vivo micronucleus)	0	0	98	0.87	15	0.13	NA
TOPKAT	1	0	65	0.58	47	0.42	43
CAESAR	1	0	82	0.73	30	0.27	48
HazardExpert	0	5	82	0.77	25	0.23	Not known
Lazar (Kazius/Bursi)	0	0	65	0.58	48	0.42	58*
Lazar (Toxbenchmark)	0	0	56	0.50	57	0.50	60*
Lazar (Kazius/Bursi or Toxbenchmark)	0	0	69	0.61	44	0.39	74*
Derek (mutagenicity or chromosome damage)	0	2	81	0.73	30	0.27	NA
ToxBoxes	0	27	38	0.44	48	0.56	Not known
Software (used in combination)							
Toxtree or CAESAR	0	0	101	0.89	12	0.11	48
Derek or CAESAR	0	0	96	0.85	17	0.15	48
Derek or Lazar	0	0	92	0.81	21	0.19	74*
Derek or TOPKAT	0	0	89	0.79	24	0.21	43
Toxtree or Lazar	0	0	102	0.90	11	0.10	74*
Toxtree or Derek	0	0	104	0.92	9	0.08	NA
HazardExpert or CAESAR	0	0	94	0.83	19	0.17	≥ 48

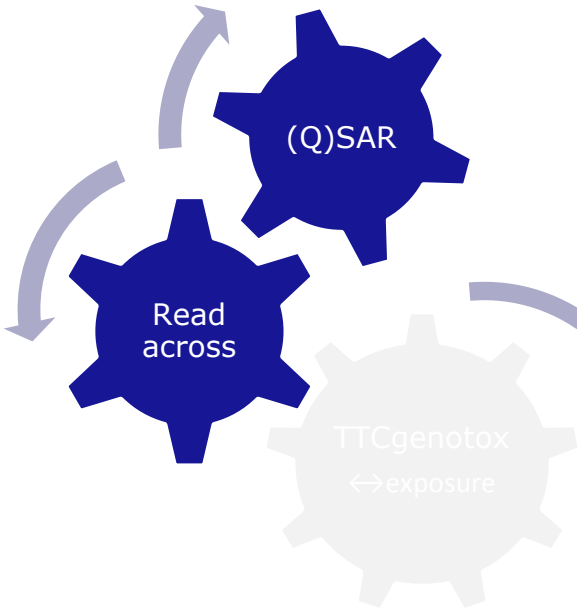
Test set of 113 classified mutagens; ND – not determined; EQ – compounds predicted as equivocal; TP – true positives; SE – sensitivity; FN – false negatives; 1-SE – false negative rate; No TS – number of chemicals already in the training set of the model (where applicable); NA – not applicable

* For Lazar it is not important whether a substance is in the dataset used to build the model, since an instance-based prediction is generated by a local model built from data that exclude the query chemical

JRC (2010):

- recommended the combined use of expert and statistical QSAR
- public available toxtree and Caesar that gave false negative rate of 11%.
- Toxtree and derek (false negative rate of 8%).

(Q)SAR PREDICTION AND READ ACROSS



- genotoxic potential of all identified metabolites is predicted by at least two independent (Q)SAR models for each endpoint
- all metabolites are subjected to read across
- weight of evidence approach for the final conclusion
- in case of different results between (Q)SAR predictions and read across, justification for the decision has to be provided
- Testing if a concern cannot be excluded.

(Q)SAR PREDICTION AND READ ACROSS

Endpoint

- Well defined endpoint
  - Gene mutation and chromosomal aberrations

Similarity

- Well defined and justified similarity
  - molecular initiating events – covalent binding to DNA and/or proteins
  - evaluation of the influence of the rest part of the molecule

Data

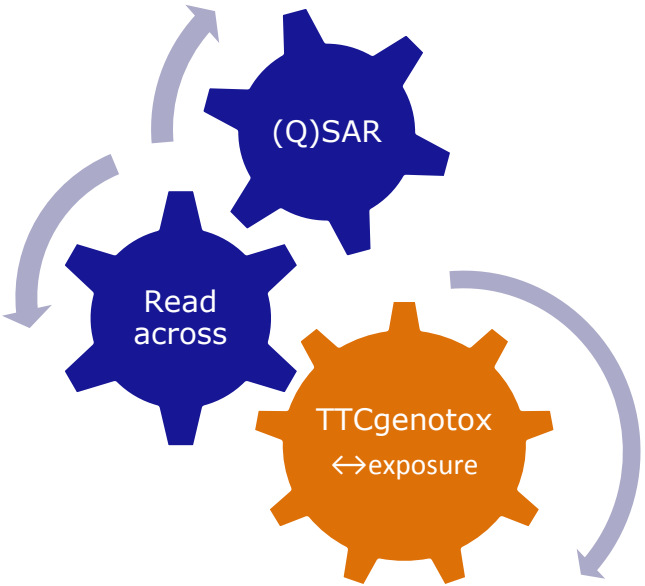
- High quality of the data used
  - Data for the source substance (parent and/or metabolite) - Commission Regulation (EU) No 283/2013

(Q)SAR PREDICTION AND READ ACROSS

- OECD Toolbox:
  - DNA binding by OASIS
  - DNA binding by OECD
  - Protein binding by OASIS
  - Protein binding by OECD
  - DNA alerts for AMES, MN and CA by OASIS
  - In vitro mutagenicity (AMES test) alerts by ISS
  - In vivo mutagenicity (Micronucleus) alerts by ISS
  - Protein binding alerts for Chromosomal aberrations by OASIS

  - Organic functional groups

(Q)SAR PREDICTION AND READ ACROSS



- TTC genotox (optional)
- Exposure assessment for substances **predicted to be of genotoxic concern** (following (Q)SAR prediction and read across)
- Combined exposure (i.e. exposure to all metabolites showing communality in the reaction mechanisms)

  ↔ 0.0025 µg/kg bw/day

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GENOTOXIC IMPURITIES

- Technical specification*:
  - Active substance content: minimum purity
  - Associated impurities: maximum content.
- Relevant impurities:
  - maximum content should be acceptable from the toxicological point of view.
  - Genotoxic impurities: use of the TTC genotox or the Margin of Exposure (carcinogenicity data).

*European Commission, 2012. Guidance document on the assessment of the equivalence of technical materials of substances regulated under Regulation (EC) No 1107/2009. SANCO/10597/2003-rev. 10.1, 13 July 2012.

GENOTOXIC IMPURITIES

Example: ethofumesate

- Purity content: minimum 960 g/kg.
- Relevant impurities: genotoxic impurities ethyl methane sulfonate and/or iso-butyl methane sulfonate could be formed during some manufacturing process.
- A maximum content of 0.1 mg/kg could not be supported from the toxicological point of view (margin of exposure was used; benchmark analysis of TGR data).

EFSA (European Food Safety Authority), 2016. Conclusion on the peer review of the pesticide risk assessment of the active substance ethofumesate. EFSA Journal 2016;14(1):4374, 141 pp. doi:10.2903/j.efsa.2016.4374

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