Impact of genotoxicity in risk assessment of pesticides, their metabolites and degradates

Claudia Bolognesi
Environmental Carcinogenesis Unit, National Institute for Cancer research, Genova

New Pesticide Regulation: Innovative Aspects and Emerging Problems
Joint Meeting SIMA-SITOX Roma 5 ottobre 2010

REGULATION (EC) No 1107/2009 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 21 October 2009
concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/E

The new pesticide regulation the objective of:

- **protecting human and animal health and the environment over the objective of improving plant production**
- **promoting non-animal test methods and other risk assessment strategies**
- **minimizing animal testing**
- **considering tests on vertebrates as a last resort**
- **data sharing related to studies on vertebrates**

(24) **The provisions governing authorisation must ensure a high standard of protection.** In particular, when granting authorisations of plant protection products, the objective **of protecting human and animal health and the environment should take priority over the objective of improving plant production**. Therefore, it should be demonstrated, before plant protection products are placed on the market, that they present a clear benefit for plant production and **do not have any harmful effect on human or animal health, including that of vulnerable groups, or any unacceptable effects on the environment.**

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(21) In addition to active substances, plant protection products may contain **safeners or synergists for which similar rules should be provided.** The technical rules necessary for the evaluation of such substances should be established. Substances currently on the market should only be evaluated after those rules have been established.

(22) Plant protection products may also contain **co-formulants**. It is appropriate to provide a **list of co-formulants** which should not be included in plant protection products.

CHAPTER II
ACTIVE SUBSTANCES, SAFENERS, SYNERGISTS AND CO-FORMULANTS

2. The residues of the plant protection products, consequent on application consistent with good plant protection practice and having regard to realistic conditions of use, shall meet the following requirements:

(a) they **shall not have any harmful effects on human health**, including that of vulnerable groups, or animal health, taking into account known cumulative and synergistic effects where the scientific methods accepted by the Authority to assess such effects are available, or on groundwater;

(b) they shall not have any unacceptable effect on the environment.

For residues which are of **toxicological, ecotoxicological, environmental or drinking water relevance**, there shall be methods in general use for measuring them. Analytical standards shall be commonly available.

SECTION 3
Unacceptable co-formulants

Article 27
Co-formulants

1. **A co-formulant shall not be accepted** for inclusion in a plant protection product where it has been established that:

(a) its residues, consequent on application consistent with good plant protection practice, and having regard to realistic conditions of use, have a harmful effect on human or animal health or on groundwater or an unacceptable effect on the environment; or

(b) its use, consequent on application consistent with good plant protection practice and having regard to realistic conditions of use, has a harmful effect on human or animal health or an unacceptable effect on plants, plant products or the environment.

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Article 3
Definitions

1. '**residues' means one or more substances present in or on plants or plant products**, edible animal products, drinking water or elsewhere in the environment and resulting from the use of a plant protection product, including their **metabolites, breakdown or reaction products**;

Article 3
Definitions

32. ‘metabolite’ means any metabolite or a degradation product of an active substance, safener or synergist, formed either in organisms or in the environment.
A metabolite **is deemed relevant if there is a reason to assume that it has intrinsic properties comparable to the parent substance in terms of its biological target activity, or that it poses a higher or comparable risk to organisms than the parent substance or that it has certain toxicological properties that are considered unacceptable.** Such a metabolite is relevant for the overall approval decision or for the definition of risk mitigation measures;

ANNEX II

Procedure and criteria for the approval of active substances, safeners and synergists pursuant to Chapter II

3.3. Relevance of metabolites

Where applicable the **documentation submitted shall be sufficient to permit the establishment of the toxicological,** ecotoxicological or environmental relevance of metabolites.

ANNEX II

Procedure and criteria for the approval of active substances, safeners and synergists pursuant to Chapter II

3.6.2. An active substance, safener or synergist **shall only be approved if, on the basis of assessment of higher tier genotoxicity testing carried out in accordance with the data requirements for the active substances, safeners or synergists and other available data and information**, including a review of the scientific literature, reviewed by the Authority, **it is not or has not to be classified**, in accordance with the provisions of Regulation (EC) No 1272/2008, **as mutagen category 1A or 1B**. EN 24.11.2009 Official Journal of the European Union L 309/41

Unclassified

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Organisation de Coopération et de Développement Économiques
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English - Or. English

ENVIRONMENT DIRECTORATE
JOINT MEETING OF THE CHEMICALS COMMITTEE AND
THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY

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SERIES ON TESTING AND ASSESSMENT
No. 63 and
SERIES ON PESTICIDES
No. 31

GUIDANCE DOCUMENT ON THE DEFINITION OF RESIDUE (AS REVISED IN 2009)

Guidance Documents:

- *Definition of Residue (series on Testing and Assessment, No.63)*
- *Overview of Residue Chemistry Studies (series on Testing and Assessment, No. 64)*
- *Guidance Document on Pesticide Residue Analytical Methods (series on Testing and Assessment, No. 72)*
- *Guidance Document on Magnitude of Pesticide Residues in Processed Commodities (series on Testing and Assessment, No. 96)*

Test Guidelines:

- *TG 501:Metabolism in Crops,*
- *TG 502: Metabolism in Rotational Crops,*
- *TG 503: Metabolism in Livestock,*
- *TG 504: Residues in Rotational Crops (Limited Field Studies),*
- *TG 505: Residues in Livestock*
- *TG 506: Stability of Pesticide Residues in Stored Commodities*
- *TG 507: Nature of Pesticide Residues in Processed Commodities – High Temperature Hydrolysis*
- *TG 508: Magnitude of Pesticide Residues in Processed Commoditie*
- *TG 509: Crop Field Trial (To be publiched late 2009)*

METABOLITES , DEGRADATES, TRANSFORMATION PRODUCTS

The number of metabolites, degradates and other transformation products varies from pesticide to pesticide and in some cases dozens of compounds can be found.

The continuous improvement in analytical methods and sensitivity results in the detection of an increasing number of compounds at low levels and also in the identification of new compounds.

Relevant metabolites should be included in residue definition for dietary risk assessment

Toxicologically significant metabolites

Metabolites and degradates are identified in:

- metabolism experiments in rat,
- in crops and
- in livestock animals

ESTABLISHMENT OF THE RESIDUE DEFINITION FOR DIETARY RISK ASSESSMENT REQUIRES:

- assessment of the toxicological end points of interest and related reference values

- a decision on which metabolites or degradates, due to their level, significantly contribute to toxicological effects
  - parent compound
  - major metabolites 10% or more of the total radioactive residue
  - minor metabolites less that 10 % of the total radioactive residue

TOXICOLOGICALLY SIGNIFICANT METABOLITES

ADME studies currently available data are very heterogeneous and often inconclusive

- appropriate labelling
- more knowledge on metabolic profile
- detailed investigation upon distribution of metabolites in rat tissues
- consideration of different kinetics of parent substances and their metabolites
- examination of the mode of action of parents and their metabolites

Toxicity studies

- only acute toxicity studies are available for the large majority of the metabolites
- the data available related to the parent compounds and metabolites are obtained in different species (rat or mice) or strains.
- dose spacing. Benchmark dose model may be an alternative to current estimation of NOAELs

TOXICOLOGICALLY SIGNIFICANT METABOLITES

The process of metabolism or degradation of active compounds may give:

- breakdown products retaining the active moiety responsible for the biological activity and for the toxic effects,

or

- the toxic moiety may be modified or totally removed.

In some instances a new toxic moiety may be created with different mechanisms of action.

this knowledge should be taken into account to gain more knowledge on metabolites

THRESHOLD OF TOXICOLOGICAL CONCERN - TTC

The TTC concept is based on establishment of human exposure threshold values for chemicals below which the risk to human health is not appreciable.

The TTC approach allows to identify the threshold values for chemicals without or with very limited toxicity data, **based on their chemical structures and the known toxicity of chemicals which share similar structural characteristics.**

Cramer et al, 1978 separates chemicals into 3 structural classes
via a series of questions I = low, II = medium, III = high toxicity

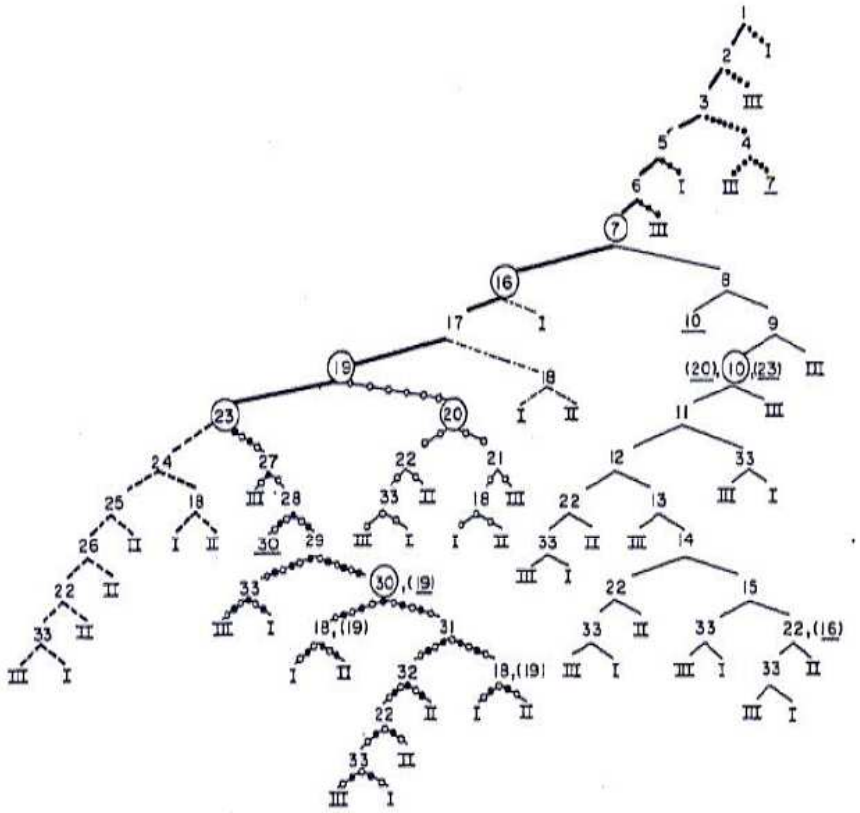


Fig. 1. A schematic diagram of a decision tree for the estimation of probable toxicity. Assessors should (a) start with question 1, (b) proceed by 'no' ↙ or ↘ 'yes', (c) move from any underscored number encountered to same circled number and (d) proceed to final classes I, II or III. Working downwards through the tree, the symbols designate the following groupings: biological normality (●●●), high and low toxicity (●–●–●); heterocyclics (——); terpenoids (–·–·–); aliphatics (–O–O–O); aromatics (–O–●–O); alicyclics (– – –).

Class I
Simple chemical structures and efficient modes of metabolism which would suggest a low order of oral toxicity

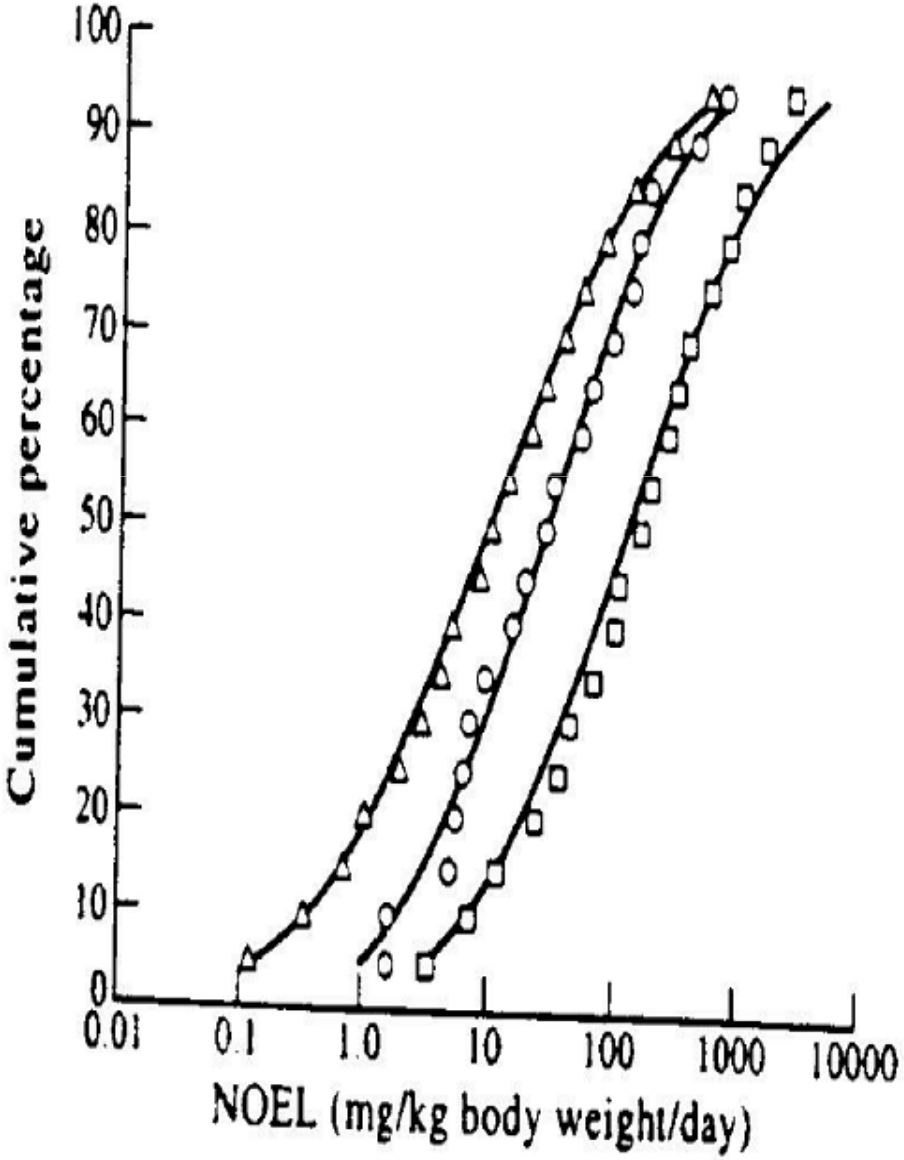
Class II
Structural features which are less innocuous and may contain reactive functional groups

Class III
Structural features that permit no strong initial presumption of safety, or may even suggest significant toxicity

Certain compounds should be excluded from consideration

- **Heavy metals, such as arsenic, cadmium, lead and mercury**
- **Compounds with extremely long half-lives that show very large species differences in bioaccumulation, such as TCDD and structural analogues**
- **Proteins**

Refinement by Munro *et al.al.(1996)(1996)*



Class	5%ile NOEL (mg/kg/day)	Human threshold (µg per day) *
I	3.0	1800
II	0.91	540
III	0.15	90

*calculated as NOEL/100 times 60kg body weight.

Excluding organo-phosphates
- the 5th percentile NOEL for class III is 0.30mg/kg/day giving a TTC value of 180µg/person/day

THRESHOLD OF TOXICOLOGICAL CONCERN - TTC

For carcinogens/mutagens

Analysis of dose-response data for carcinogens identified in cancer bioassays.
Determination of daily intake that would give risk of < 1 in a million
Simple linear extrapolation from the TD50 to a 1 in 10 6 incidence.

0.5 ug/kg of diet (1.5 ug/person/day or 0.025 ug/kg bw/day)
Gold Cancer Potency Database (1995)

Threshold of regulation" (TOR) (1.5 ug/person/day) adopted by USFDA for indirect food additives to assess the acceptable exposure of chemicals to which humans are exposed at low levels

The approach assumes that all biological processes involved in the generation of tumours at high dosages are linear over a 500,000-fold range of extrapolation

THRESHOLD OF TOXICOLOGICAL CONCERN - TTC

For carcinogens/mutagens

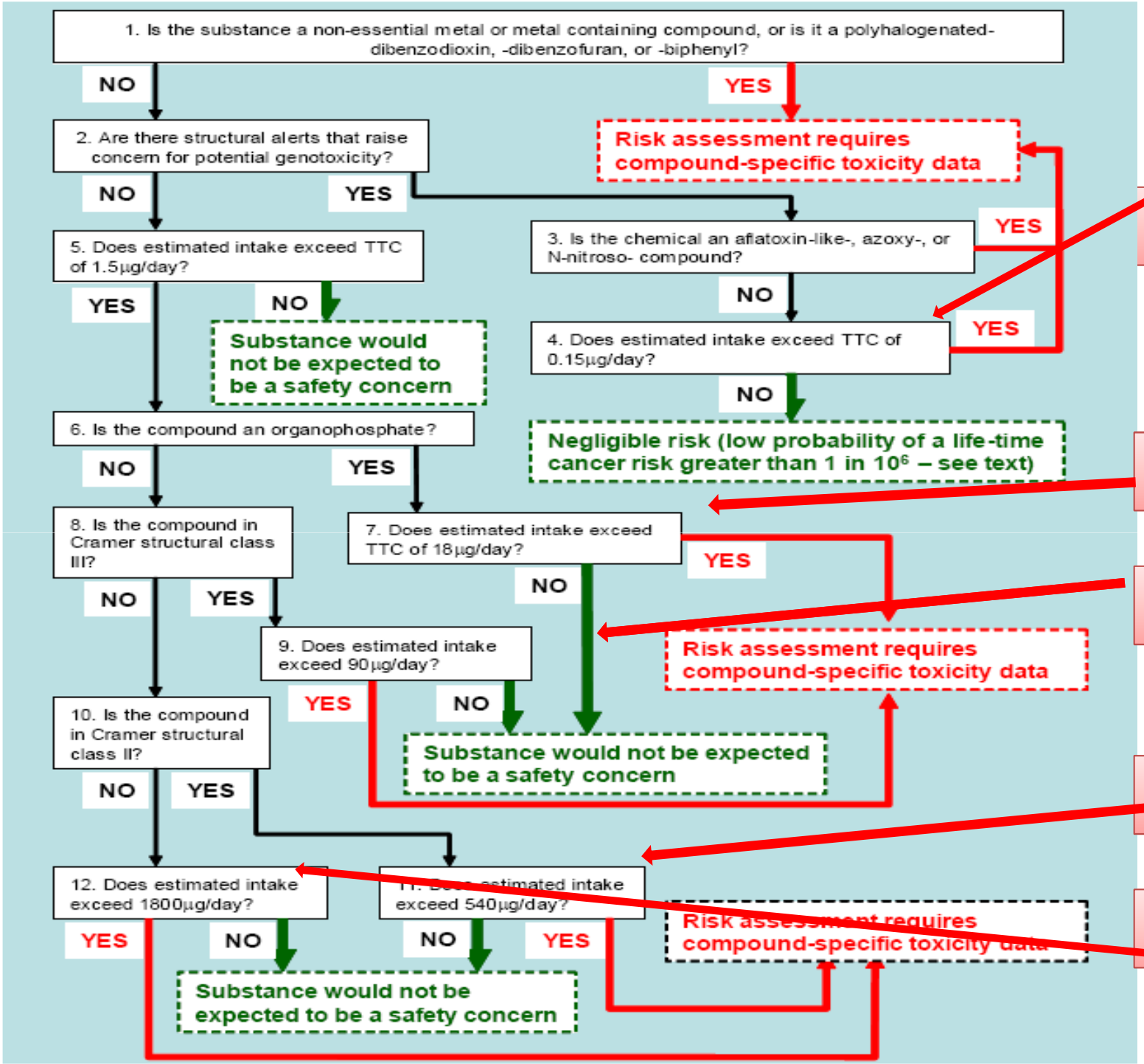
Establishment of the dose giving a 50% tumour incidence (TD50) using data for the most sensitive species and most sensitive site (Cheeseman *et al., 1999).*

- Based on a selected subset of the database containing 730 carcinogenic substances which had adequate estimates of the TD50 following oral dosage.
- Simple linear extrapolation from the TD50 to a 1 in 10 6 incidence.

The approach assumes that all biological processes involved in the generation of tumours at high dosages are linear over a 500,000-fold range of extrapolation

0.05 ug/kg of diet (0,15 ug/person/day or 0.0025 ug/kg bw/day)

“Cohort of Concern”: aflatoxin-like compounds, N-nitroso-compounds, azoxy-compounds, and polyhalogenated dibenzo-p-dioxins and-dibenzofurans.



DECISION TREE

Kroes et al 2004

European Medicines Agency EMA

Impurities identified in pharmaceuticals are usually not directly tested for genotoxicity.
A unidentified impurity was considered non genotoxic if:
if the testing batch of drug substance with the impurity at the level of 0.05% is negative in a genotoxicity battery.

The guidelines recommend a scientific expert review of the synthetic route and the chemical reactions and conditions involved to identify compounds of special concern. This review should include an evaluation of structure-activity relationship (SAR) for genotoxicity.

European Medicines Agency EMA

Genotoxicity testing is not obligatory when a potential genotoxic impurity is controlled at the TTC level 1.5 (10-5 risk justified due to pharmaceutical derived benefit).
Some highly potent structural groups excluded (e.g., aflotoxin-like, N-nitroso-,, unless it belongs to a class of very potent genotoxic carcinogens (N-nitroso and azoxy compounds, or a aflatoxin-like compound), where a case by case decision has to be made.

The absence of a structural alert based on a well-performed assessment (e.g. through application of commonly used QSAR assessment software such as DEREK or MCASE) will be sufficient to conclude that the impurity is of no concern with respect to genotoxicity and no further ‘qualification’ studies or justification will be required.

A negative Ames test (conducted to regulatory acceptable standards) will overrule a structural alert and no further studies would be required providing the TTC level

EFSA PPR Panel
Permanent working group
2008-2012

Scientific opinion on approaches to evaluate the toxicological relevance of metabolites and degradates of pesticide active substances in dietary risk assessment

Guidance document on the establishment of the residue definition for risk assessment in food commodities

TTC APPROACH VALIDATION EXERCISE
(Chemicals Regulation Directorate CRD, UK)

- 100 actives substances randomly selected from a list of 500 compounds that were evaluated under the Directive 91/414/EEC

- Range of toxicity
  - ADIs >1 mg/kg bw to 0.00008 mg/kg bw

- Range of types
  - New, old, accepted, rejected

- Critical toxicity end-points were considered
  - Genotox, carc, repro, development, immuno, neuro
  - QSAR approach DEREK for prediction of: genotoxicity; carcinogenicity; reproductive toxicity; developmental effects; immunotoxicity / sensitisation; neurotoxicity and general toxicity

DECISION TREE (Kroes, 2004)

- Exclude metals, dioxins, potent genotoxins
  - Genotox alerts / data = 0.15ug/person/day
  - Neurotox alert (not just OPs) – 18ug/person/day
  - Cramer class 3 – 90 ug/person/day
  - Cramer class 2 – 540 ug/person/day
  - Cramer class 1 – 1800 ug/person/day

VALIDATION EXERCISE: RESULTS

Genotoxicity alert :
Derek software (version 11) 12 compounds: no SAR alert but positive data
Toxtree 17/30 with SAR alert matching the data

TTC Threshold (µg/person/d)	TTC Threshold (mg/kg bw/d)	No. of substances with an ADI below applicable TTC threshold	Compounds (ADI/TTC)
		Total substances = 100	
0.15	0.0000025	None	
18	0.0003	0	
90	0.0015	3	Aviglycin (0.67) Halaxyfp-R (0.43) Amitrole (0.67)
540	0.009	1	1-MCP is a gas and deriving the ADI involved many assumptions and uncertainties (0.1)
1800	0.03	0	

Summary of DEREK predictions versus results from studies

Endpoint[#]	**SAR Alert**	**SAR alert matches data [#]**	**No SAR alert**	**No SAR alert but Data positive**
Genotoxicity	28	10	72	12
Carcinogenicity	40	19	60	17 (mainly liver)
Reproduction	0	0	100	15
Developmental	4	2	96	15
Immuno / sensitisation	39	14	61	17
Neurotoxicity	11	11	89	10
General toxicity	51	20 (mainly cholinesterase)	49	NA

VALIDATION OF THE DEVELOPED TTC CONCEPT: CASE STUDIES

15 case studies: 79 metabolites

The list cover pesticides with a range of transformation product profiles:

- Few transformation products - predominant residue is parent;
- Few transformation products - predominant residue is not parent;
- Many transformation products;
- Profile of transformation products changes with Pre-Harvest Interval (PHI)
- Profile of transformation products changes with crop;
- Novel transformation products seen in animal transfer studies.
- Active substances of low, medium and high toxicity

DECISION TREE (Kroes, 2004)

- Exclude those with exposure <0.15ug/person/day
- Assume DEREK genotoxicity predictions reliable
- Neurotox alert (Ops and cabamates) – 18ug/person/day

Estimation of metabolite levels

The supervised trials median residue (STMR) for each metabolite was then determined using the median level of parent compound found in the trials data (according to GAP) and the expected ratio of parent to metabolite from the relevant metabolism studies.

Consumer intake assessment

long-term or chronic intakes

$$\text{NEDI} = \frac{\text{STMR} \times \text{food consumption value (kg)}}{\text{Mean body weight for consumer group (kg)}}$$

VALIDATION OF THE DEVELOPED TTC CONCEPT: CASE STUDIES RESULTS

	Total	Exposure <TTC	Expo >TTC
Bitertanol	4	2	2
Boscalid	1	1	0
Carbaryl	4	3	1
Dimethoate	6	2	4
Fenamidone	6	5	1
Fludioxonil	7	7	0
Formetanate	3	1	2
λ-Cyhalothrin	6	5	1
Metconazole	4	4	0
Metiram	7	5	2
Metribuzin	3	3	0
Pirimicarb	13	11	2
Proquinazid	6	6	0
Spirotetramat	7	7	0
Thiodicarb	2	1	1
Total	**79**	**63**	**16 (9 with genotoxic alert)**

HSE

SCIENTIFIC / TECHNICAL REPORT submitted to EFSA

Applicability of thresholds of toxicological concern in the dietary risk assessment of metabolites, degradation and reaction products of pesticides[1]

Prepared by:

Richard Brown (CRD); Julian Carter (CRD); Ian Dewhurst (CRD);

Claire Stephenson (CRD); Sonia Tessier (CRD).

EFSA Project Code

Grant Agreement EFSA/PPR/2008/01

Sponsor

Scientific Panel on Plant Protection Products and their Residues

Working Group on the toxicological relevance of pesticide metabolites

(Q)SAR

SARs and QSARs are theoretical models that can be used to predict in a qualitative or quantitative manner the physico-chemical, biological, toxicological properties and environmental fate of compounds from a knowledge of their chemical structure.

The basic assumption for the application of QSAR analyses in risk assessment is that the biological activities of the chemicals depend on its intrinsic nature and can be directly predicted from its molecular structure and inferred from the properties of similar compounds whose activities are known.

(Q)SAR project

- Objective: explore the predictive performances of a range of software tools for mutagenicity predictions in order to investigate the potential applicability of QSAR analysis in the context of a TTC assessment

- Software tools:
  - based on expert rules (DEREK)
  - based on statistical methodologies (CAESAR, LAZAR, TOPKAT, HazardExpert and ToxBoxes)
  - a hybrid tool implementing both expert rules and statistical methodologies (Toxtree)

MUTAGENICITY PREDICTIONS FOR A SERIES OF PESTICIDES AND THEIR METABOLITES

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

Sensitivity=TP/TP + FN
Specificity =TN/FP +TN
Positive predictivity= %carcinogens/total positive compounds
Negative predictivity= % non carcinogens/total negative compounds

Genotoxicity prediction for the classified mutagen dataset

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

(Q)SAR project conclusions

The results of the QSAR project on the prediction of genotoxicity for PPP metabolites show a wide range of sensitivity from 45 and 100% and specificity from 57-87%.

The accuracy of the prediction is related to the training set data applied, as it demonstrated by the high performance of ToxBoxes and Toxtre in detecting chemicals positive at the Ames test or with the in vivo micronucleus test respectively.

The range of sensitivity and specificity values are in the range of those described in the scientific literature.

To improve the sensitivity of the applied models various two software combinations were tested. The followed rule for this exercise was that if either tool in the combinations gives a positive result then the overall prediction is considered positive.

A reduction of the false positive rate was obtained with the lowest value of 8% for the combined use of Toxtree and Derek.

Two important challenges faced by QSAR models for genotoxicity prediction of pesticide metabolites are the diversity of compound structural space and the multiplicity of structures that can produce the same effect.

JRC
EUROPEAN COMMISSION

ihcp
Institute for Health and Consumer Protection

SCIENTIFIC REPORT submitted to EFSA

Applicability of QSAR analysis to the evaluation of the toxicological relevance of metabolites and degradates of pesticide active substances for dietary risk assessment[1]

Prepared by

Computational Toxicology Group, Institute for Health & Consumer Protection, European Commission - Joint Research Centre, Ispra, Italy

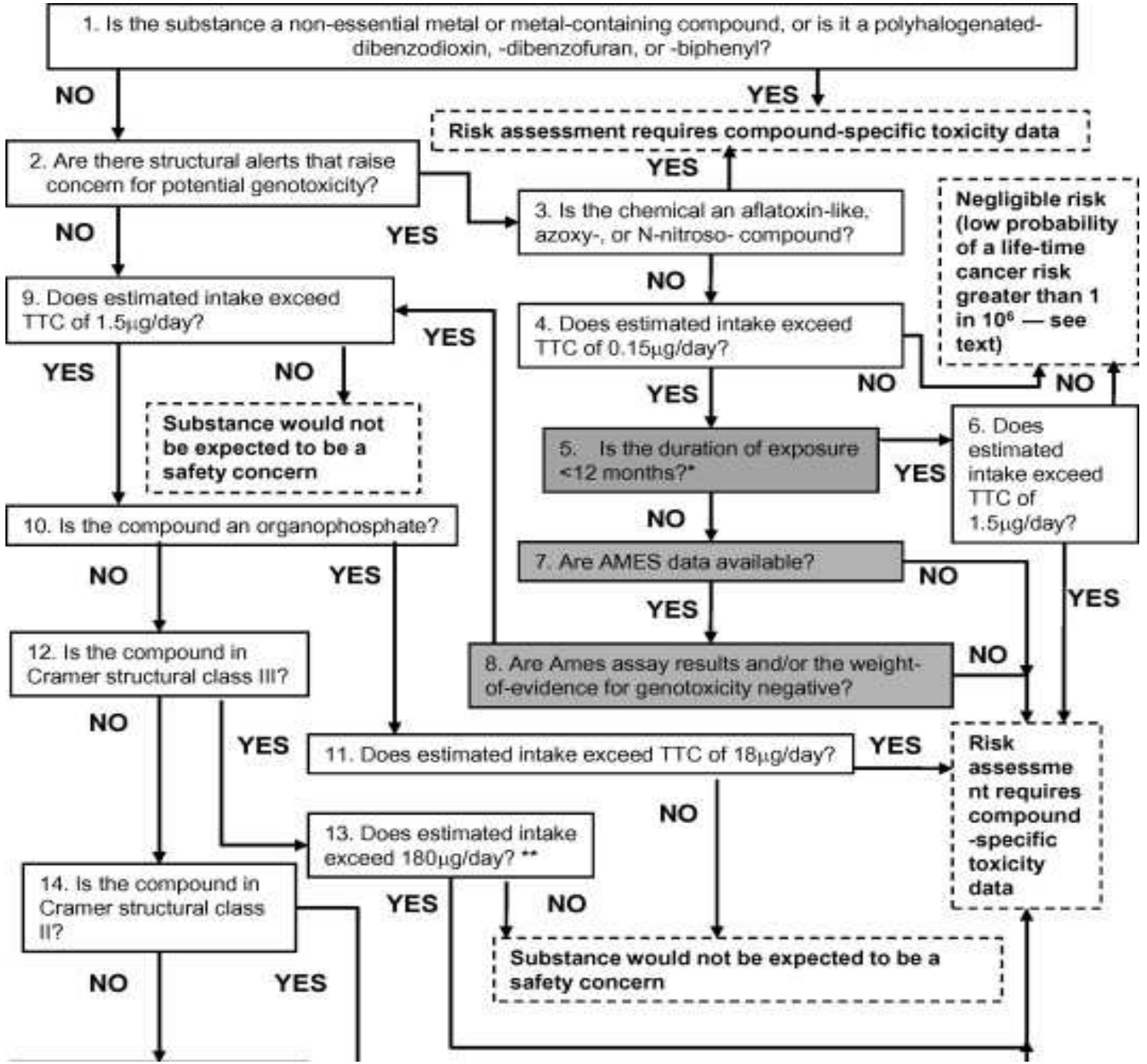
TTC Concept

In almost all cases genotoxicity (alerts) to be considered the first step for assessment followed by structural similar approaches and then necessity of further test studies

Identifies the combination of potential toxicity and exposure that is of concern and requires specific toxicity data

Provides a scheme whereby conclusions are reached early on compounds with very low exposure

Provides a useful tool for formulating advice to risk managers in the absence of toxicity data on the chemical,



Felter et al 2009

Tiered TTC
Carcinogens/Mutagens

Combining exposure and toxicity considerations

Exposure
Major metabolites higher exposure
lower exposure
Minor metabolites

ADME Studies Toxic moiety

Toxicity studies
Acute effects
Chronic effects
Structural alerts
QSAR
Read across
Ames tests

Other mutagenicity tests

Thank you for your attention