New Pesticide Regulation: Innovative Aspects and Emerging Problems

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Genotoxicity testing strategies: updated recommendations for the assessment of pesticides

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Assessment of genotoxicity of active ingredients (a.i.) of plant protection products (PPP)

Aims:

- Identification of the intrinsic genotoxic properties of the a.i. (*hazard identification*) $\rightarrow$ classification and labelling
- Characterization of the mode of action of a.i. with carcinogenic properties (*hazard characterization*)

Early national legislation concerning the marketing of plant protection products.

- DPR 3 August, 1968 n.1255 "*Regolamento concernente la disciplina della produzione, del commercio e della vendita di fitofarmaci e dei presidi delle derrate alimentari immagazzinate*": Mutagenicity studies are indicated without giving details

- Public Health Ministry, May 1979 "Mutagenicity tests according to the guidelines set by the CMT Committee are required for the registration of new pesticides for agricultural use"

Expert Committee on Carcinogenesis, Mutagenesis e Teratogenesis (CMT)

Guidelines for the evaluation of mutagenic, carcinogenic and teratogenic effects of chemical substances, 2 December 1977

The following battery of tests is recommended:

- Two tests for gene mutations (one in bacteria and one in eukaryotic cells)
- Two tests for chromosomal effects (one in vitro and one in vivo)
- One test for DNA damage/repair

National Advisory Committee on Toxicology (Commissione Consultiva Tossicologica Nazionale, CCTN)

*Guidelines for the evaluation of toxic effects of chemical compounds. Part I: Mutagenic, carcinogeni and teratogenic effects. ISS, 1987**

The following battery of four in vitro assays is recommended:

- One bacterial assay for gene mutations
- One eukaryotic assay for gene mutations (in yeast, Drosophila, mammalian cells)
- One cytogenetic assay for chromosomal effects
- One assy for DNA damage/repair (e.g. UDS or SCE)

** also adopted for a.i. of PPP since 1988*

Council Directive 91/414/EEC concerning the placing of plant protection products on the market

- Annex I – positive list of active substances that are authorised for use in plant protection products within the Community
- Annex II – list of the tests and studies required for an active substance to support its inclusion in Annex I
- Annex III – list of the tests and studies required on the plant protection product active substance of an authorized substance

Council Directive 91/414/EEC, Annex II
5. Toxicological and metabolism studies

5.4 Genotoxicity

These studies are of value in:

- - the prediction of genotoxic potential
- - the early identification of genotoxic carcinogens
- - the elucidation of the mechanism of action of some carcinogens

Council Directive 91/414/EEC, Annex II
5. Toxicological and metabolism studies; 5.4. Genotoxicity

In vitro studies

In vitro mutagenicity tests (bacterial assay for gene mutation, test for clastogenicity in mammalian cells and test for gene mutation in mammalian cells) must always be performed.

Acceptable test guidelines are:

- Directive 92/69/EEC Method B14 - Salmonella Typhimurium reverse mutation assay
- Directive 92/69/EEC Method B10 - in vitro ma mmalian cytogenetic test
- Directive 87/302/EEC, Part B - in vitro mammalian cell gene mutation test

If all the results of the in vitro studies are negative further resting must be done. The test can be an in vivo study or an in vitro study using a different metabolizing system from that/those previously used.

Council Directive 91/414/EEC, Annex II
5. Toxicological and metabolism studies; 5.4. Genotoxicity

In vivo studies in somatic cells

If the in vitro cytogenetic test is positive, an in vivo test using somatic cells (metaphase analysis in rodent bone marrow or micronucleus test in rodents) must be conducted.

If either of the in vitro gene mutation tests are positive, an in vivo test to investigate unscheduled DNA synthesis or a mouse spot test must be conducted.

- Acceptable test guidelines are:
- Directive 92/69/EEC Method B12 - Micronucleus test,
- Directive 87/302/EEC Part B - Mouse spot test,
- Directive 92/69/EEC Method B11 - In vivo Mammalian Bone-Marrow cytogenetic test

Council Directive 91/414/EEC, Annex II
5. Toxicological and metabolism studies; 5.4. Genotoxicity

In vivo studies in germ cells

When any result of an in vivo study in somatic cells is positive, in vivo testing for germ cell effects may be justified. Suitable tests would need to examine interaction with DNA (such as the dominant lethal assay), to look at the potential for inherited effects and possibly make a quantitative assessment of heritable effects.

Draft Commission Regulation amending Annexes II and II to Council Directive 91/4141/EEC (July 2010)

5.4 Genotoxicity testing

5.4.1 In vitro studies (always to be performed)

- Bacterial assay for gene mutation
- Combined tests for structural and numerical chromosome aberrations in mammalian cells
- Test for gene mutation in mammalian cells

Methods B.13/B.14, B.10, B.17, B.18, OECD 471, 473, 476, 482, in vitro comet assay (when justified)

(OECD 487??)

Draft Commission Regulation amending Annexes II and II to Council Directive 91/4141/EEC (July 2010)

5.4.2 In vivo studies in somatic cells

- If all the results of in vitro studies are negative, at least one in vivo study with demonstration of exposure (based on toxicity/toxicokinetic data) shall be done

Recommeded test methods: B.12, B.11, OECD 474, 475, 486 (micronucleus, chromosomal aberrations, unscheduled DNA synthesis)

Draft Commission Regulation amending Annexes II and II to Council Directive 91/4141/EEC (July 2010)

5.4.1 In vitro studies

- Appropriate staining procedures (e.g. FISH) can be applied to highlight alterations in chromosome copy number; if non-disjunction is observed, a reference concentration (NOEC, $BMDL_{10}$) should be reported

- Bacteriostatic substances shall be tested in two mammalian cell gene mutation tests

- Substances bearing structural alerts may necessitate additional testing under conditions optimized for such alerts

Draft Commission Regulation amending Annexes II and II to Council Directive 91/4141/EEC (July 2010)

5.4.2 In vivo studies in somatic cells

- In the follow-up of in vitro positives the same end-point should be tested:

in vitro		*in vivo*
CAs or MN	→	metaphase analysis or MN or comet
aneuploidy	→	micronucleus
gene mutation	→	liver UDS

- In case of positive results in the in vivo micronucleus test, appropriate staining procedures to highlight alterations in chromosome copy numbers should be used

Draft Commission Regulation amending Annexes II and II to Council Directive 91/4141/EEC (July 2010)

5.4.3 In vivo studies in germ cells

- For most somatic cell mutagens no further genotoxicity testing is necessary since they will be considered to be potential genotoxic carcinogens and potential germ cell mutagens

Suitable test to provide information on genotoxicity to germ cells, when needed: B23 and OECD 483 (spermatogonial chromosome aberration test); supplementary tests: comet assay in germ cells, rodent sperm-FISH assay, alkaline elution of testicular DNA, DNA adducts in gonad cells, transgenic animal models

Draft Commission Regulation amending Annexes II and II to Council Directive 91/4141/EEC (July 2010)

5.4 Genotoxicity testing

5.4.1 In vitro studies (three tests, always to be performed)

- Bacterial assay for gene mutation
- Combined tests for structural and numerical chromosome aberrations in mammalian cells (CA and/or in vitro micronucleus test?)
- Test for gene mutation in mammalian cells (no preference for *tk* mouse lymphoma assay)

Ability of in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens (data from Kirkland *et al.*, 2005)*

	Ames	*MLA*	*MN*	*CA*
Sensitivity (% of carcinogens positive)	**58.8**	**73.1**	**78.7**	**65.6**
Specificity (% non-carcinogens negative)	**73.9**	**39.0**	**30.8**	**44.9**

- Data from a database of 553 *carcinogens and 183 non-carcinogens;*
- *Ames test, MLA, mouse lymphoma assay, MN, micronucleus in vitro, CA, chromosomal aberrations in vitro*

Prediction of rodent carcinogenesis by genotoxicity tests in combination

	Ames+MLA	*Ames+CA*	*Ames+CA+MLA*
Sensitivity (% carcinogens positive)	**81.0**	**75.3**	**81.3**
Specificity (% non-carcinogens negative)	**32.4**	**34.6**	**22.9**

from Kirkland *et al.*, 2005

Analysis of carcinogens & in vivo genotoxins database

- A combination of Ames + MNvit (or CA where MNvit data not obtained) clearly detects 316/405 (78.0%) *in vivo* genotoxins with available *in vitro* data

- By adding the MLA to this battery of 2 tests, only an additional 6 *in vivo* genotoxins are detected (322/405 = 79.5%)

Kirkland, EEMS 2010

Recommendation of a Working Group of the Gesellschaft fuer Umwelt-Mutationsforschung (GUM) for a simple and straightforward approach to genotoxicity testing

Stage I (Basic testing)

- Gene mutation test in bacteria + in vitro MN test.

  Such combination covers all the relevant end-points of genotoxicity (gene mutation, structural and numerical chromosomal aberrations) which must be addressed by the initial testing.

(Pfhuler et al., Toxicol. Sci. 97, 237-240, 2007)

ECVAM retrospective validation of the *in vitro* micronucleus test (Corvi *et al.*, Mutagenesis 4, 271-283, 2008)

Concordance between MNvit e Cavit:

- All compounds (113) 83.2%
- Clastogens (71) 87.3%
- Aneugens (27) 77.8

"The in vitro MN test is reliable and relevant and can therefore be used as an alternative method to the in vitro CA test" *ECVAM Scientific Advisory Committee*.

ICH Topic S2 (R1)
Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals
Intended for Human Use

Option 1

i. A test for gene mutation in bacteria.

ii. A cytogenetic test for chromosomal damage (the *in vitro* metaphase chromosome aberration test or *in vitro* micronucleus test), or an *in vitro* mouse lymphoma *tk* gene mutation assay.

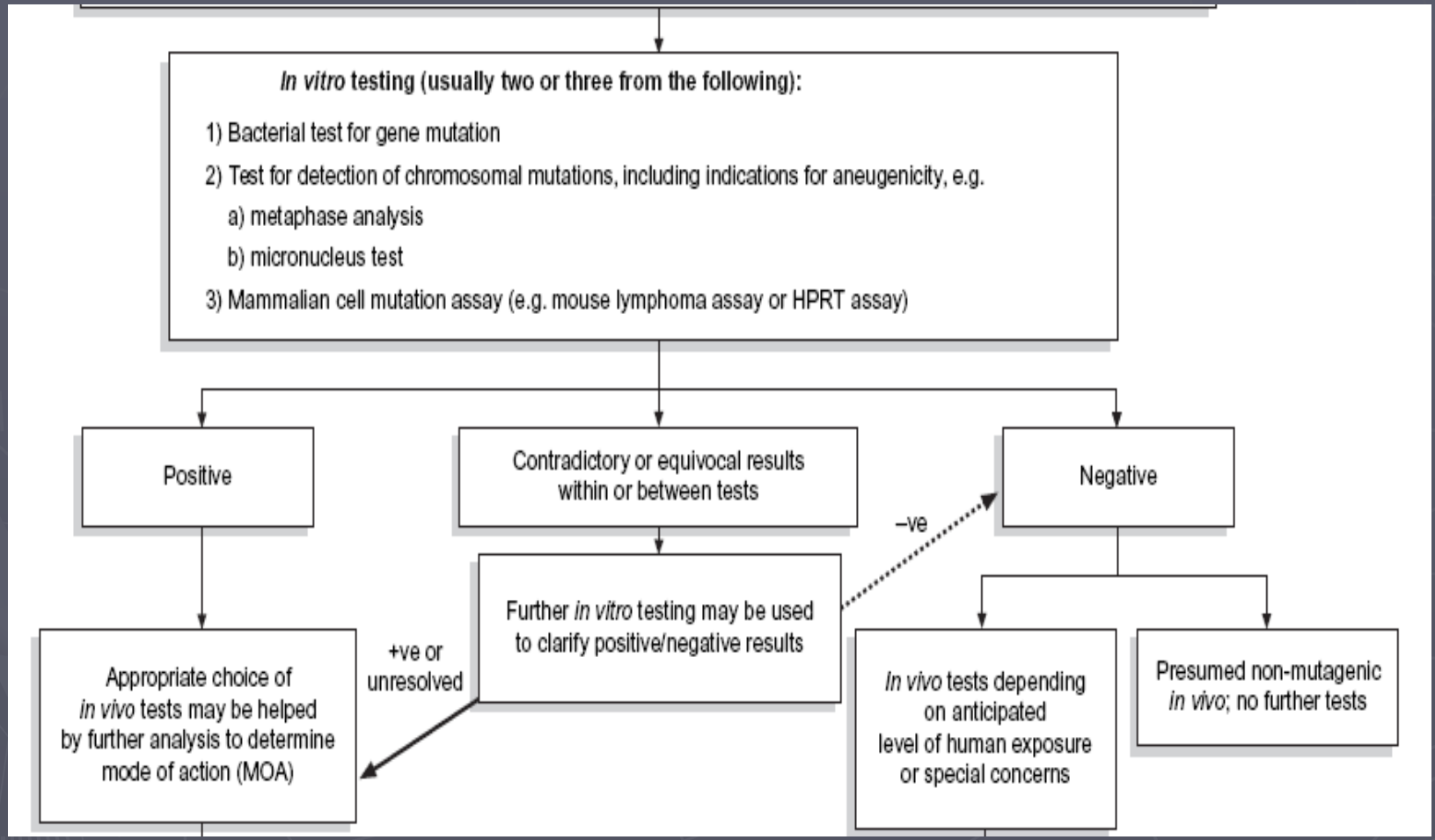
iii. An *in vivo* test for genotoxicity, generally a test for chromosomal damage using rodent hematopoietic cells, either for micronuclei or for chromosomal aberrations in metaphase cells.

Option 2

i. A test for gene mutation in bacteria.

ii. An *in vivo* assessment of genotoxicity with two tissues, usually an assay for micronuclei using rodent hematopoietic cells and a second *in vivo* assay.

WHO/IPCS Harmonized Scheme for Mutagenicity Testing (2009): screening iniziale



WHO/IPCS Harmonized Scheme for Mutagenicity Testing (Eastmond et al., 2009)

- Screening should be based on a limited number of tests that are well validated and informative. Usually two or three in vitro assay in bacteria and mammalian cells to cover the end-points of gene mutation, clastogenicity and aneuploidy.
- A substance negative in all test systems under appropriate conditions in vitro is anticipated not to be mutagenic in vivo.
- In vivo studies are performed to clarify he relevance of in vitro positives (follow-up). For substances negative in vitro, in vivo testing is recomended in case of “high” or “moderate and sustained” human exposure, or for substances otherwise of high concern.

Batteries of short-term tests recommended for the assessment of genotoxicity

- Food contact materials (EFSA): Ames, MCGM, CAvit
- Food additives (EFSA): Ames, MCGM, CAvit
- Cosmetics (SCCNFP): Ames, MCGM, Mnvit
- Botanicals (EFSA): Ames, MCGM, CAvit/MNvit
- Biocides (OECD): Ames, MCGM, CAvit
- Industrial chemicals (ECHA): Ames, CAvit/MNvit (MCGM)

- Medicinal drugs (ICH): Ames, MCGM/CAvit + 1 test in vivo
- Pesticides (UE): Ames, MCGM/CAvit + 1 test in vivo

Opinion of the Scientific Panel on Plant Protection Products and their Residues on a request from the Commission related to the revision of Annexes II and III to Council Directive 91/414/EEC concerning the placing of plant protection products on the market – EFSA Journal (2007) 449, 1-60

5.4.2. In vivo studies in somatic cells

Circumstances in which required

If all of the in vitro studies are negative, at least one in vivo study must be done with demonstration of exposure (e.g. cell toxicity and/or toxicokinetic data)

"The PPR Panel proposes that an in vivo study is not needed in this circumstance.

Reason: The PPR Panel knows of no compounds that are consistently negative in in vitro studies but positive when tested in vivo. The Panel's proposal is in line with the approach applied to biocides."

Draft Commission Regulation amending Annexes II and II to Council Directive 91/4141/EEC (July 2010)

5.4.2 In vivo studies in somatic cells

- In the follow-up of in vitro positives the same end-point should be tested:

in vitro		*in vivo*
CAs or MN	→	metaphase analysis or MN or comet
aneuploidy	→	micronucleus
gene mutation	→	liver UDS

- In case of positive results in the in vivo micronucleus test, appropriate staining procedures to highlight alterations in chromosome copy numbers should be used

Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens. III: Appropriate follow-up testing. Kirkland & Speit, Mutation Research 654, 2008, 114-132

Which further test in the in vivo follow-up when the first cytogenetic test is negative?

67 carcinogens negative in the mouse bone marrow micronuclue test

	% positive results
Comet assay	> 90
TG assay	~ 50
UDS assay	< 20

WHO/IPCS Harmonized Scheme for Mutagenicity Testing (2009): follow-up in vivo

"The choice of an in vivo follow-up test should be guided by the spectrum of genotoxic events observed in vitro as well as knowledge of bioavailability, distribution, metabolism and target organ specificity ofthe substance.

Typically, a bone marrow micronucleus or clastogenicity test is conducted. However, if there are indications that point to a more appropriate assay, then this assay should be conducted instead (e.g. mutagenicity study with transgenic animals and/or comet assay in potential target tissue"

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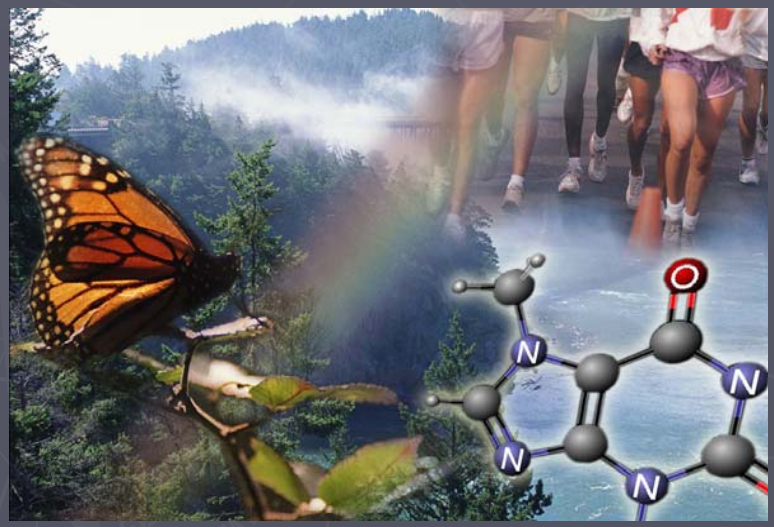
"Liver UDS: Indicator test. Uncertain acceptability and questionable sensitivity."

Guidance on information requirements and chemical safety assessment Chapter R.7a: Endpoint specific guidance



May 2008

Follow-up in vivo

- Sostanze con disponibilità sistemica: test del micronucleo in roditori (sangue o midollo osseo), aberrazioni cromosomiche nel midollo osseo, comet assay, mutazione genica in topi transgenici, UDS nel fegato del ratto.
- Sostanze senza disponibilità sistemica: comet assay, mutazione genica in animali transgenici, addotti al DNA

A few recent and emerging issues in the field of genetic

- Animal welfare (*3R = Replacement, Reductions and Refinement of animal testing*):

  Development of multi end-point protocols (e.g. micronucleus and comet assay in one study)

  Integration of genotoxicity testing in repeat dose toxicity studies

- (Q)SAR, *in silico*, read-across

- Improvement of the specificity of in vitro mammalian tests (e.g. reduction of top dose, use of cell line p-53 proficient or metabolically competent, etc)

Recommendation of a Working Group of the Gesellschaft fuer Umwelt-Mutationsforschung (GUM) for a simple and straightforward approach to genotoxicity testing

Stage II (Follow-up testing)

- A combination of the MN test in bone marrow with the *in vivo* comet assay in relevant tissue is proposed.

  Such combination covers systemic genotoxic effects and local effects (site of contact tissue and target organ for toxicity).

(Pfhuler et al., Toxicol. Sci. 97, 237-240, 2007)

ICH Topic S2 (R1)
Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals
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Impact of the revision of the ICH S2 genotoxicity guideline on animal testing (May 2008)

Animals are still an essential part in the assessment of genotoxicity of pharmaceuticals...however the number of animals used can be significantly reduced taking into account that

1) Use of one sex only in animal studies is sufficient
2) It is recommended to integrate in vivo genotoxicity assessment into existing repeat dose toxicity studies
3) When in vivo assessment of genotoxicity with tw tissues is required, the guideline encourages to incorporate two genotoxicity assays in one study using the same animal

Available online at www.sciencedirect.com
ScienceDirect
Mutation Research 628 (2007) 31–55
Genetic Toxicology and Environmental Mutagenesis
www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

How to reduce false positive results when undertaking *in vitro* genotoxicity testing and thus avoid unnecessary follow-up animal tests: Report of an ECVAM Workshop☆

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Reduction of misleading ("false") positive results in mammalian cell genotoxicity assays. I. Choice of cell type

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Analysis of published data for top concentration considerations in mammalian cell genotoxicity testing

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Introduction

The EU legislation REACH (Regulation, Evaluation, Authorisation and restriction of CHemicals) foresees the safety assessment of thousands of chemicals within the next decade

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Further analysis of Ames-negative rodent carcinogens that are only genotoxic in mammalian cells *in vitro* at concentrations exceeding 1 mM, including retesting of compounds of concern

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and *in vitro* mammalian cell gene mutation and chromosome damage tests was undertaken. The standard CA test yielded the greatest amount of information on clastogenic potential, but some data concerning chromosome mutation were also avail-

Conclusions

- For what concerns genotoxicity assessment, the draft new pesticide regulation mainly reconfirms the recommendations issued in the Council Directive 91/414/EEC.
- In particular, a battery of three in vitro genotoxicity tests, including two mammalian ones, and one in vivo assay to be always performed, is reiterated.
- Such recommendations are considered at variance with current harmonized criteria on genotoxicity testing, which normally only rely on in vitro data for the initial screening.
- Moreover, it is noted that the draft document does not give proper consideration to important issues such as the low specificity of redundant in vitro test batteries, the application of novel methods, animal welfare and related optimized testing strategies.