Use for regulatory decisions

Example of a drug candidate with an indirect and threshold mechanism of genotoxicity

Drug	Available results of genotoxicity assays	Question from Health Authority	in vitro Comet Assay	Consequence for compound
X	**Ames -** **V79 MNT +** **CAT HuLy -** **Mouse Lymphoma tk assay +** **MNT BM mouse (+)**	**produce other data particularly to DNA reactivity**	**negative**	**ok from health authorities, obtaining marketing authorization**

Drug candidates with genotoxicity data sets triggering comet assay in vivo

Drug	Available Results	Tissue(s) Comet assay	Comet Assay	Consequence for compound
A	Ames - MNT V79 - CAT V79 + CAT HuLy - MNT BM rat -	**Liver, leukocytes (oral 3 and 24-hour sampling)**	**negative**	**ok from HA / EC for multiple dose clinical studies in patients**
B	Ames - MNT V79 - Comet HuLy - CAT V79 + MNT BM rat -	**Liver (oral; 3 and 24-hour sampling)**	**negative**	**ok from HA/ EC for first administration to humans**
C	Ames +/- MNT V79 + CAT V79 + CAT HuLy +/- MLA TK + HPRT CHO - Comet V79 + MNT BM mouse - UDS liver rat -	**Liver, leukocytes (sub-cut; 3 and 24-hour sampling)**	**negative**	**ok from HA / EC for first (topical) administration to humans**

Examples of drug candidates with tumour findings triggering comet assay in vivo

	Available results	Tumor target organ	Tissue and time Comet assay	Result of Comet Assay	Conclusion
D	Ames - MNT V79 - CAT V79 - HPRT V79 - MNT BM mouse - UDS liver rat -	Small intestine Rat	Jejunum, Liver (oral admin.) 3 and 24 hour sampling	negative	No unacceptable health risk (non-genotoxic mechanism, epigenetic mechanism)
E	Ames - MNT V79 - Comet V79 - CAT CHO - HPRT V79 - HPRT CHO - MNT BM rat -	**Liver** **Rat**	**Liver** **(oral 3 and 24 hour sampling**	Negative	No unacceptable health risk (non-genotoxic mechanism, epigenetic mechanism)

Examples of drug candidates with data of comet assay in vivo using tissue of site of first contact

Drug	Available results	Tissue(s) investigated	Result	Outcome
F	Ames +/- MNT V79 (+) CAT HuLy - MNT BM mouse - DNA binding rat - MutaMouse -	Jejunum, liver (oral;3 and 24-hour sampling)	negative	ok from health authorities to proceed into multiple dose clinical trials
G	Ames - MNT V79 + Comet V79 + CAT HuLy + MNT BM rat -	Stomach mucosa (oral; sampling time 3 hours)	Retarded DNA migration (reason: DNA-protein crosslinks)	Termination of development

Other Comments

- Lack of agreed criteria for collection, presentation and analysis of results
- Uncertainty about toxicity
- Large volume of published data – lots of false "positives"

- But assay accepted in EU

Andrew Smith (UK HSE)

- Large number of procedures (protocols)
- Variance in presenting results
- Little regulatory experience in evaluation.
- Common for "new" assays

Jon Battershill (DoH UK)

- Has important potential but many technical shortcomings
- Considered a tier 2 test for following up ICH battery or
- To exclude genotoxic MOA in positive carcinogenicity studies

David Jacobson-Kram FDA

Conclusions

- Obviously still concern over
  - the many methods being used
  - Type of data produced
  - Quality of data produced
- However, the assay is seen as an important adjunct to human risk assessment process

- The comments from regulators highlight the need for clear, concise, workable guidelines.
- The JaCVAM initiative is timely and with the eventual OECD acceptance should provide all that is required.

Use of the Comet Assay for Human Risk Assessment – a Case Study in the Gastro-Intestinal Tract.

P Clay

Syngenta CTL, Alderley Park, Macclesfield, Cheshire, UK, SK10 4TJ

Site of contact toxicity as a result of exposure to industrial or agro chemicals is a significant issue in human risk assessment. The more commonly used *in vivo* genotoxicity tests, such as the bone marrow micronucleus test and the liver UDS test are designed to assess systemic effects. The *in vivo* Comet assay is different in that the assay can be performed in almost any tissue, including those where site of contact toxicity can be an issue such as skin, nasal tissue and the gastro-intestinal tract.

Folpet is a chloroalkylthiodicarboximide fungicide widely used in commercial agriculture. Previous studies have shown the induction of adenocarcinomas in the duodenum of mice treated with Folpet. Mechanistic studies have shown proliferative changes in the crypt region of the duodenum of treated animals and therefore it is cells specifically from this area that are considered to be of interest when investigating the possible genotoxic origin of the tumours as part of the human health risk assessment. Novel methods have been developed to sample predominantly this cell type for use in an *in vivo* Comet assay to assess the genotoxicity of Folpet in the duodenum.

Preliminary studies established the methodology and confirmed the absence of confounding effects by examining the effects of cysteamine, a toxic but non-genotoxic substance. Folpet was administered to female mice as a single oral dose at dose levels of 2000mg/kg and 1000mg/kg. Crypt cells from the duodenum were sampled 2 and 6 hours post dose. Concurrent histopathology was included in the study design to confirm sampling of the correct cell type. No significant increases in group mean % tail DNA values were observed at either dose level or sampling time. These data support the conclusion that the duodenal tumours seen in the oncogenicity studies on Folpet are non-genotoxic in origin and contribute to a favourable human risk assessment for the chemical.

Data from the evaluation of a further chemical in the gastro-intestinal tract will be presented for consideration and discussion of the significance of small effects in these assays.

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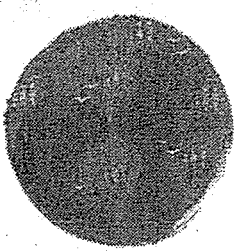
National Toxicology Program Center for the Evaluation Of Alternative Methods

Interagency Coordinating Committee on the Validation of Alternative Methods

Pros and Cons of the Comet Assay for Human Risk Assessment



Raymond Tice, Ph.D.
Deputy Director,
NTP Interagency Center for the Evaluation of Altenative Toxicological Methods
NIEHS
Research Triangle Park, NC

Risk Assessment (1)*

- Hazard Identification – qualitative assessment of the inherent toxicity of an agent. Is there potential for human genotoxicity?
- Dose-Response Assessment – relationship between the dose of an agent and the induction of an adverse (genotoxic) effect.
- Exposure Assessment – determination of the extent of human exposure.
- Risk Characterization – description of the nature and likelihood of genotoxicity risk to humans, including attendant uncertainty.

*Cimino (2006) EMM 47:362

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Risk Assessment (2)*

- Risk associated with both germ cell and somatic cell mutations.
- Mutations carried in germ cells may be inherited by future generations and may contribute to genetic disease, whereas somatic cell mutations may be implicated in the etiology of several disease states, including, but not limited to, cancer.
- Additional endpoints of concern include blood disorders such as sickle-cell anemia, cardiovascular disease, reproductive/developmental effects, neurobehavioral effects, aging.

*Cimino (2006) EMM 47:362

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Risk Assessment (3)*

- Current focus is on a more mechanistic understanding of possible induced toxicity by an agent.
- Mechanistic approach includes a mode of action analysis of the possible and probable genotoxic activity.
- Mode of action analysis is based on physical, chemical, and biological information that helps explain key events in agent's induction of genotoxic damage.
- Newer assays (e.g., Comet assay) and technologies (e.g., DNA microarrays, proteomics) provide opportunity to gather more detailed mechanistic and molecular-based info.
- Mode of action data and exposure data combined allow better consideration of relevancy of genetic risk to humans.

*Cimino (2006) EMM 47:362

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Testing Schemes – USA*

	EPA		FDA			
	Toxics	OPP	CFSAN	CDER	CVM	CDRH
	Industrial	Pesticides	Food additives	Human drugs [ICH S2B]	Veterinary drugs	Devices & radiologics
1st tier	bacterial gene mutation (GM)	bacterial GM	bacterial GM	bacterial GM	bacterial GM	
	in vitro mammalian GM (pref., L51)	*in vitro* L51 or AS52 or CHO GM & CA	*in vitro* structural CA or *in vitro* L51 (pref.)	*in vitro* structural CA or *in vitro* L51 (pref.)	*in vitro* mammalian GM	
					in vitro cytogenetics	3 *in vitro* tests
	in vivo MN or CA	same	*in vivo* MN or CA	*in vivo* MN or CA	*in vivo* MN or CA	
	based upon info for related chemicals, other tests may be required instead of/in addition	same		Tumorigenic chemicals negative in above tests may require other genotox tests		
2nd tier	effect in mammalian gonad *in vivo* (e.g., UDS, AE, SCE, CA in testicular tissues, or RDL.	same				
3rd tier	effect transmitted to offspring of exposed parents (e.g., biochemical or visible SLT, HT, plus quant. risk assessment	same				

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Testing Schemes – EU (ICH S2B)*

	Pharmaceuticals	Biocides	Food additives	Food contact	Hair dyes
1st tier	bacterial GM	same	same	same	same
	in vitro mammalian cell, CA or L51	*in vitro* mammalian cell	same	same	same
		in vitro MN or CA	same	same	*in vitro* CA
					in vitro MN
					in vitro UDS
	in vivo MN or CA	if *in vitro* pos, *in vivo* MN or CA	if *in vitro* pos, *in vivo* test	same	same
		if 1st *in vivo* pos, 2nd *in vivo* in diff tissue			
	in some situations, other tests may be required instead of/in addition	same			if oxidative ingredient, *in vitro* SHE CT
2nd tier		if *in vivo* pos, possible test for germ cell effects			
3rd tier					

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Testing Schemes – Japan*

	ICH (guidance S2B)	Medical devices	
1st tier	bacterial GM	bacterial GM	bacterial GM
	in vitro mammalian cell, CA, or L5178Y	*in vitro* mammalian cell mutation assay	*in vitro* L5178Y
		in vitro CA	
	in vivo MN or CA		
	in some situations, other tests may be required instead of/in addition	in some cases, an *in vivo* MN	in some cases, an *in vivo* MN
2nd tier			
3rd tier			

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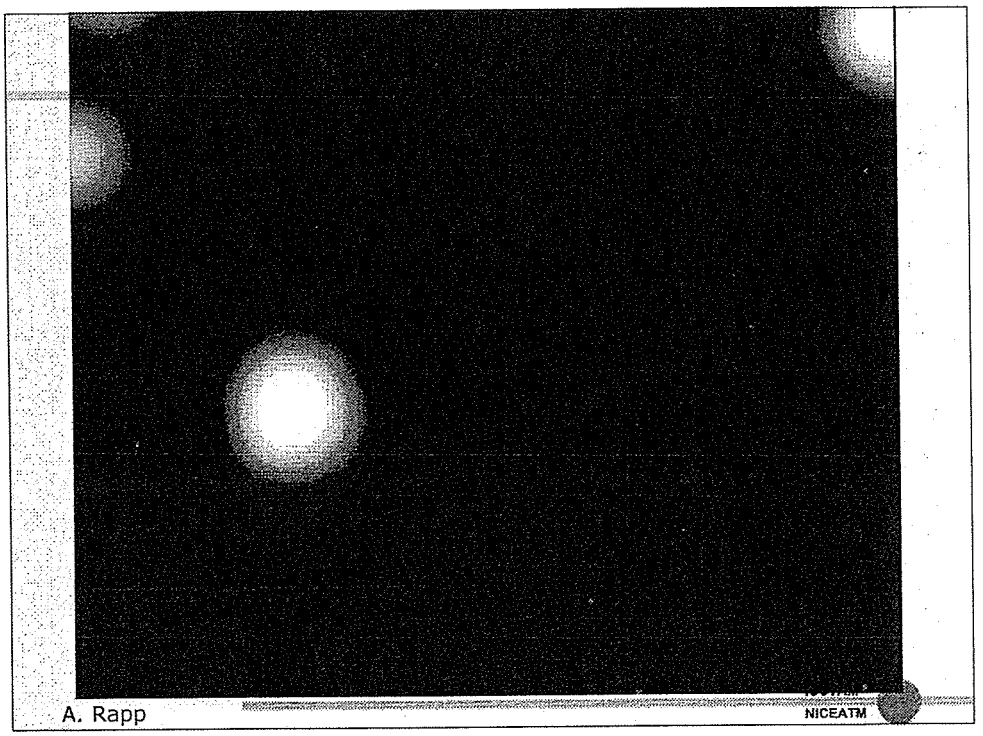

A. Rapp

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The Alkaline (pH>13) Comet Assay

- *In vitro* applications
  - Mechanistic studies
  - Screening for genotoxicity
  - Regulatory test?
- *In vivo* applications
  - Mechanistic studies
  - Regulatory test (alternative or replacement for *in vivo* hepatocyte UDS assay)

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The *In Vivo* Alkaline Comet Assay

PROs

- Detects DNA damage (strand breaks, alkali-labile sites, crosslinking) and incomplete repair events
- Applicable to (virtually) any eukaryote cell and can be applied to any tissues/organs of laboratory animals (site-of-contact of special interest)
- Only a relatively few cells are needed, making it releatively easy to integrate the assay into other studies
- Appears to be more predictive of carcinogenicity than *in vivo* hepatocyte UDS assay

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5

The *In Vivo* Alkaline Comet Assay

CONs

- Considering the number of cells scored per sample, is there a potential for scoring bias response?
- Not all DNA damage is pre-mutagenic (i.e., what is the relevance of DNA damage as an endpoint for human disease?)
  - Sasaki et. al. *in vivo* database on 208 chemicals including 165 rodent carcinogens tested
- Potential confounders exist
  - Cytotoxicity-related DNA degradation (but there are methods for assessing)
  - ?
- Reliability (within and across lab reproducibility) has yet to be adequately demostrated (semi-standardized protocol's exist)
- Applicability domain is not yet adequately established

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What is Test Method Validation? (at least to ICCVAM)

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ICCVAM

- Established in 1997
  - Replaced ad hoc ICCVAM (1994-1997)
  - Implemented NIEHS directives in Public Law (P.L.) 103-43
- ICCVAM Authorization Act of 2000 (P.L. 106-545) - December 19, 2000
  - Established a "permanent" ICCVAM that consists of 15 Federal regulatory and research agencies
  - Established a Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) composed of 15 voting members representing various stakeholders to advise ICCVAM and NICEATM regarding ICCVAM activities and NIEHS and NICEATM regarding NICEATM activities

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ICCVAM's Purpose

- To increase the efficiency and effectiveness of U.S. Federal agency test method review
- To eliminate unnecessary duplicative efforts and share experiences between U.S. Federal regulatory agencies
- To optimize the utilization of scientific expertise outside the U.S. Federal government
- To ensure that new and revised test methods are validated to meet the needs of U.S. Federal agencies
- To reduce, refine, or replace the use of animals in testing, where feasible

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ICCVAM's Responsibilities

- To consider petitions from the public for review and evaluation of validated test methods
- To review and evaluate new, revised, and alternative test methods
- To submit test method recommendations to U.S. Federal agencies and make agency responses (due within 180 days) available to the public
- To facilitate and provide guidance on:
  - test method development
  - validation criteria and processes
- To facilitate:
  - acceptance of scientifically valid test methods
  - interagency and international harmonization

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- Administers ICCVAM
- Provides operational/technical support to ICCVAM
- Supports/organizes workshops, expert panels, and peer reviews
- Disseminates information
- Communicates with stakeholders
- Forms partnerships with stakeholders
- Conducts independent validation studies

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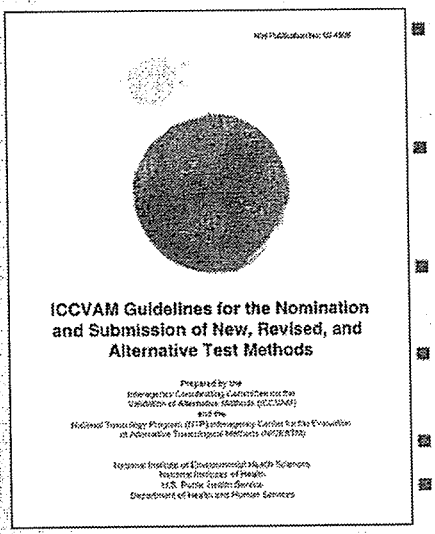
ICCVAM Guidelines



- Provides format for test method background review documents (BRDs)
- Provides basis for decisions on standardized protocols and validation study designs
- Provides criteria for validation and regulatory acceptance
- Describes **nominations** versus **submissions**
- Explains "Performance Standards"
- Explains the process for regulatory acceptance

ICCVAM Guidelines for the Nomination and Submission of New, Revised, and Alternative Test Methods. September 2003. [NIH Publication No. 03-5408; http://iccvam.niehs.nih.gov/subguide.doc

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Performance Standards (PS)

- Provide basis for evaluating the acceptability of proposed test methods that are mechanistically and functionally similar to an adequately validated *and* accepted reference test method
- To communicate the basis on which new proprietary (e.g. copyrighted, trademarked, registered) and nonproprietary test methods have been determined to have sufficient accuracy and reliability for a specific testing purpose
- Regulatory authorities can use or refer to the PS when they communicate acceptance of a new test method

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9

Criteria for Prioritization (1)

1. The extent to which the proposed method is:
   - Applicable to regulatory testing needs
   - Applicable to multiple agencies/program
2. The extent of expected use or application and impact on human, animal, or ecological health
3. The potential for the method, compared to current methods, to:
   - Refine animal use (i.e., decrease or eliminate pain and distress)
   - Reduce animal use
   - Replace animal use

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Criteria for Prioritization (2)

4. The completeness of the submission with regard to ICCVAM test method submission guidelines
5. The potential for the method to provide improved prediction of an adverse health or environmental effect, compared to current methods
6. The extent to which the test method provides other advantages, such as reduced cost and time to perform, compared to current methods

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Test Method Validation

- The process by which the *reliability* and *relevance* of a test method are established for a specific purpose.
  - *Reliability:* A measure of the extent to which a test can be performed reproducibly within and among laboratories over time.
  - *Relevance:* The extent to which a test method will correctly predict or measure the biological effect of interest.
- A determination of the usefulness and limitations of a test method for a specific purpose.

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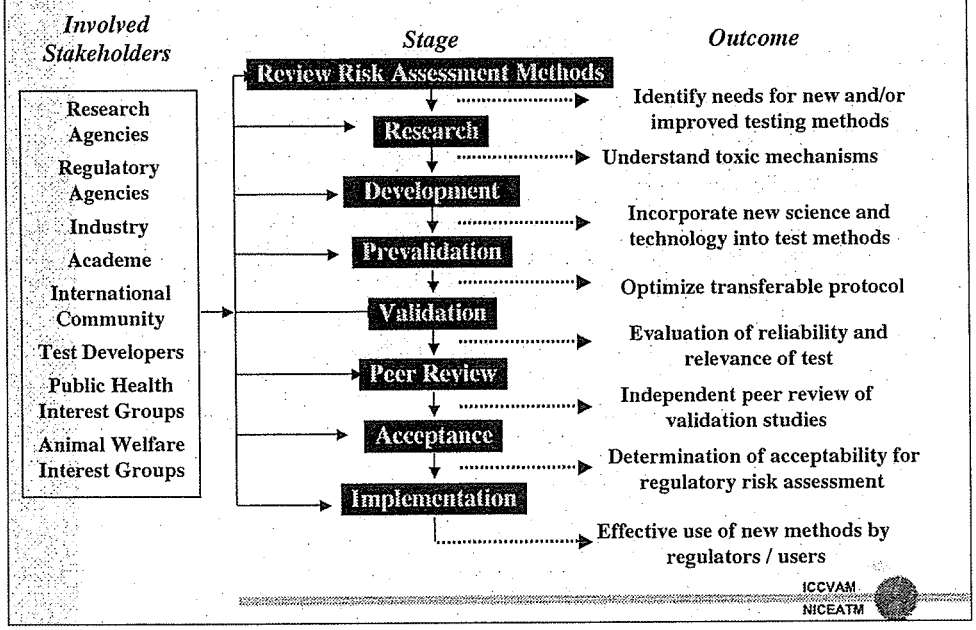
Process for New Test Methods



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11

Test Method Acceptance Criteria

1. Fits into the regulatory testing structure
2. Adequately predicts the toxic endpoint of interest
3. Generates data useful for risk assessment
4. Adequate data available for specified uses
5. Robust and transferable
6. Time and cost-effective
7. Adequate animal welfare consideration (3Rs)

*Adopted from: Validation and Regulatory Acceptance of Toxicological Test Methods: A Report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods; NIH Pub. No. 97-3981, 1997. NIEHS, Research Triangle Park, NC. http://iccvam.niehs.nih.gov/fraiccre.htm

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ICCVAM Peer Review Panels

- National and international experts
- Public meeting with the opportunity for public comment
- Develop scientific consensus on the usefulness of test methods for specific human health or ecological risk assessment purposes
- Product: Peer Review Report

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Peer Review Panels Consider

- The completeness and the accuracy of the test method BRD
- The appropriateness of draft ICCVAM test method recommendations
  - Draft proposed regulatory use
  - Draft standardized test method protocol
  - Proposed (if any) future optimization/validation efforts
  - Draft performance standards (if applicable)
    - Essential test method components
    - Minimum list of reference chemicals
    - Minimum test method accuracy and reliability values

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LIMITATIONS OF THE COMET ASSAY (1)

THE PROS AND CONS OF THE COMET ASSAY IN HUMAN RISK ASSESSMENT

Dr. Brian Burlinson (Huntingdon Life Sciences)

The comet assay is gradually gaining popularity with both industry and regulators as an important adjunct to the current genotoxicity testing strategy. Its speed and ease of use confer upon it a great deal of flexibility allowing it to fit easily into a development program, be it for medicinal, industrial, or agrochemical products. Furthermore, the ability to investigate virtually any cell type makes it an assay perfect for mechanistic studies in cases where chemicals, which were negative in the standard genotoxicity test battery, give rise to neoplasia in longer term toxicity studies.

The question however, is just how useful is the comet assay in human risk assessment? Is the assay capable of providing data of a standard and reliability to convince regulators (and industry) that a potentially carcinogenic material should be allowed into human contact either deliberately as a medicine or accidentally as an agro or industrial chemical? In this presentation I will endeavour to discuss what we are trying to do with risk assessment and whether the comet assay is a tool that will help us do it?