

# ***New Applications of the Comet Assay in Genetic Toxicology Testing***

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***Genetic Toxicology Association Meeting***  
***Sep 10-11,2008***

## Outline

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- ❑ Definition
- ❑ Methodology
- ❑ *In Vitro Comet Assay*
- ❑ *In Vivo Comet Assay*
- ❑ Summary

## ***Comet Assay***

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- Single Cell Gel Electrophoresis
- Micro-electrophoretic technique which detects DNA damage and repair in individual cells
- *In vitro* and *in vivo*
- Under alkaline conditions it can detect DNA single and double strand breaks, and single strand breaks as a result of alkali-labile sites or nucleotide excision repair
- Levels of DNA damage is correlated to the length and amount of fragmented DNA that migrates outside the cell nucleus (comet tail)

# Comet Assay

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- Ostling and Johanson (1984), microgel electrophoresis technique pH 8

[1984: O. Ostling and K.J. Johanson: Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. *Biochem. Biophys. Res. Commun.* 123: 291-298]

- Singh et al. (1988), microgel technique alkaline conditions (pH >13)

[1988: N.P. Singh, M.T. McCoy, R.R. Tice, and E.L. Schneider: A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.*, 175: 184-191]

- Olive et al. (1990), DNA Damage and repair & Tail Moment

[1990: P.L. Olive, J.P. Banath, and R.E. Durand: Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using a "comet" assay. *Radiat. Res.* 122: 86-94]

## ***Advantages***

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- Almost any eukaryotic cell population can be used (different cell types or animal tissues)
- Data collection at the level of individual cells, giving information on intercellular distribution of DNA damage and repair
- Small number of cells are required (i.e. 10,000-500,000)
  - Broad spectrum of DNA damage detected
  - Easy to integrate into existing assays

## ***Disadvantages***

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- Single Cell Data (rate limiting step)
- Sensitivity (technical variability needs to be controlled)
  - Not all DNA damage is pre-mutagenic
    - Cytotoxicity?
  - Relevance and reliability?

# ***Applications***

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Clinical Trials

Radiosensitivity & Chemotherapeutics

Genetic Diseases

DNA Repair

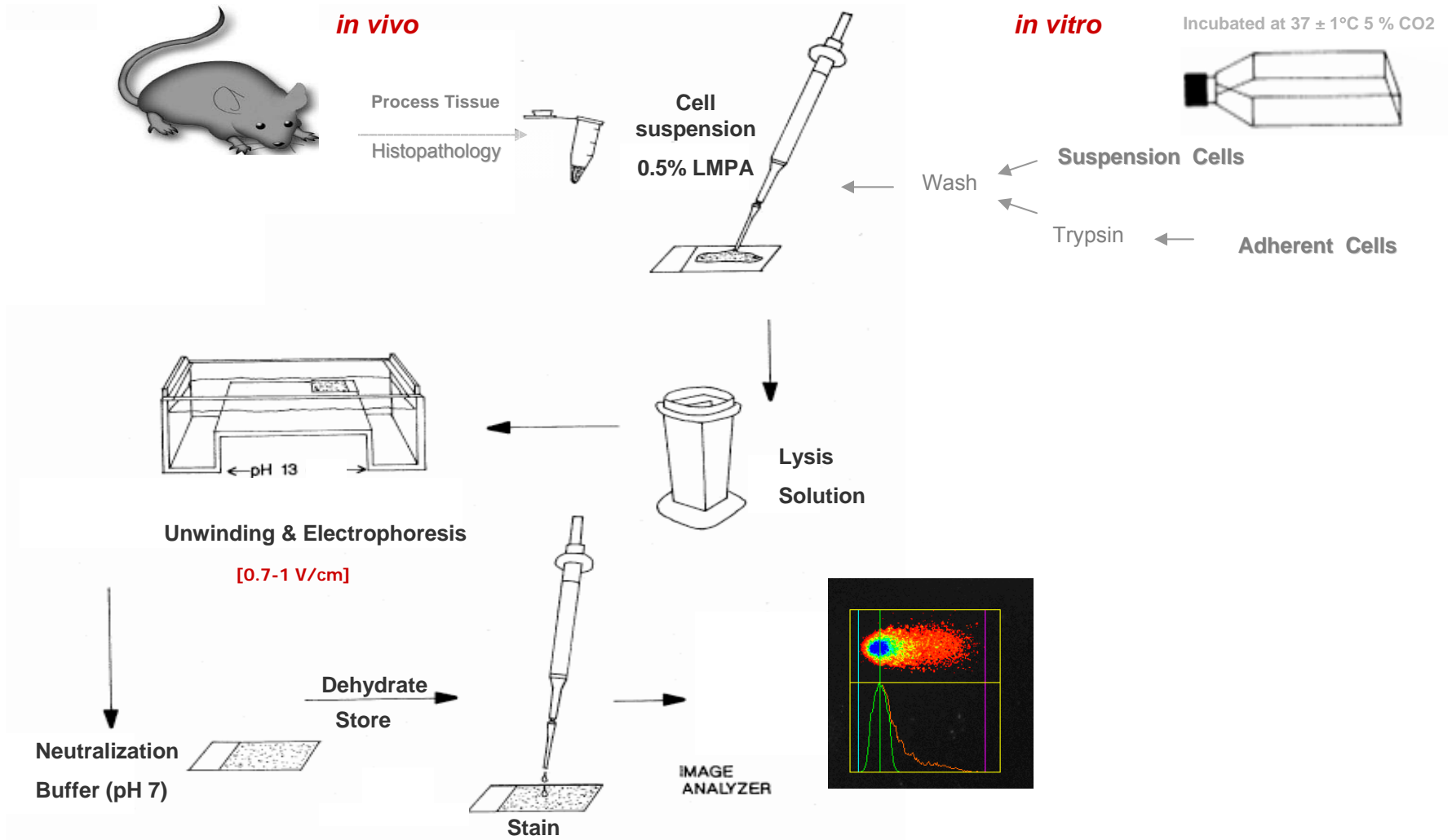
Human Biomonitoring

**Genetic toxicology (*in vitro* & *in vivo*)**

Comet Assay Interest Group Website ([www.cometassay.com](http://www.cometassay.com))

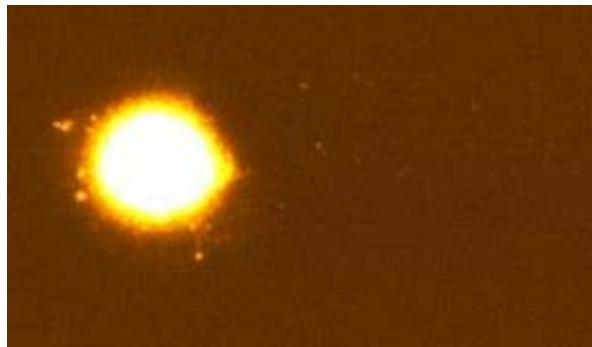
A forum for the free exchange of information on the Comet assay. (Developed by T.S. Kumaravel)

# Methodology



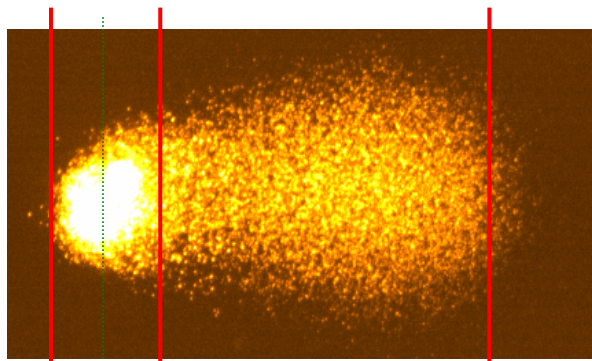
# Data Collection

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Head

Tail



Tail migration

## Tail Length

DNA migration length from center of the head to smallest detectable fragment

## % Tail Intensity

Amount of DNA fragments in the tail

## Olive Tail Moment

[(% Tail intensity) vs. (tail length)]

## Tail Migration

DNA migration length from the edge of the head to Smallest detectable fragment

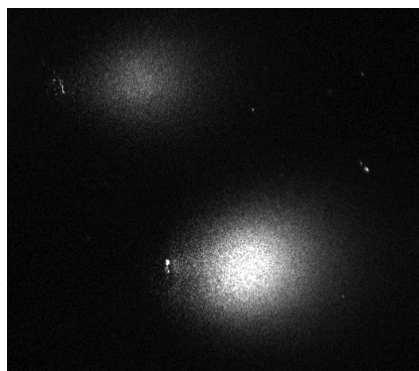
# Cytotoxicity/Apoptosis

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**Viability or cell proliferation assays**  
(i.e. trypan blue, ATP, etc)

**Clouds, hedgehog or ghosts**

Possible indications of cytotoxicity  
but not of apoptosis

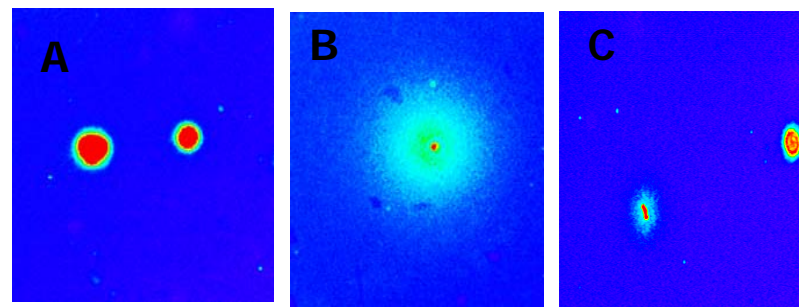


## Neutral diffusion assay

Cells with diffuse DNA indicative of low  
molecular weight DNA (i.e. apoptosis)

A → Control cells with high molecular weight  
DNA

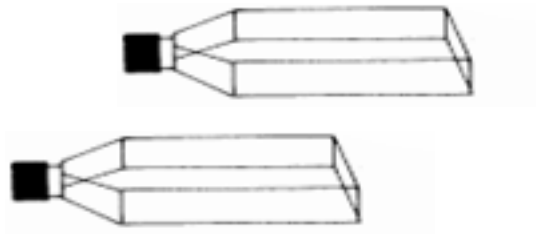
B/C → Show a progression of cells with low  
molecular weight DNA



NDA pictures provided by Dr. Ray Tice

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***In Vitro***  
**Comet Assay**



## ***Why do in vitro Comet Assay?***

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- Genotoxic mechanism
- Mechanism of action
- Predictive of genotoxicity
- As an alternative to in vitro clastogenicity tests for early drug candidate selection

## ***In vitro Comet Assay***

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- **Preliminary toxicity assay**  
Serves as a dose range finding assay for the definitive portion of the study
  
- **Definitive Comet assay**  
Evaluates the potential of a compound to induce DNA damage

## ***Definitive in vitro Comet Assay***

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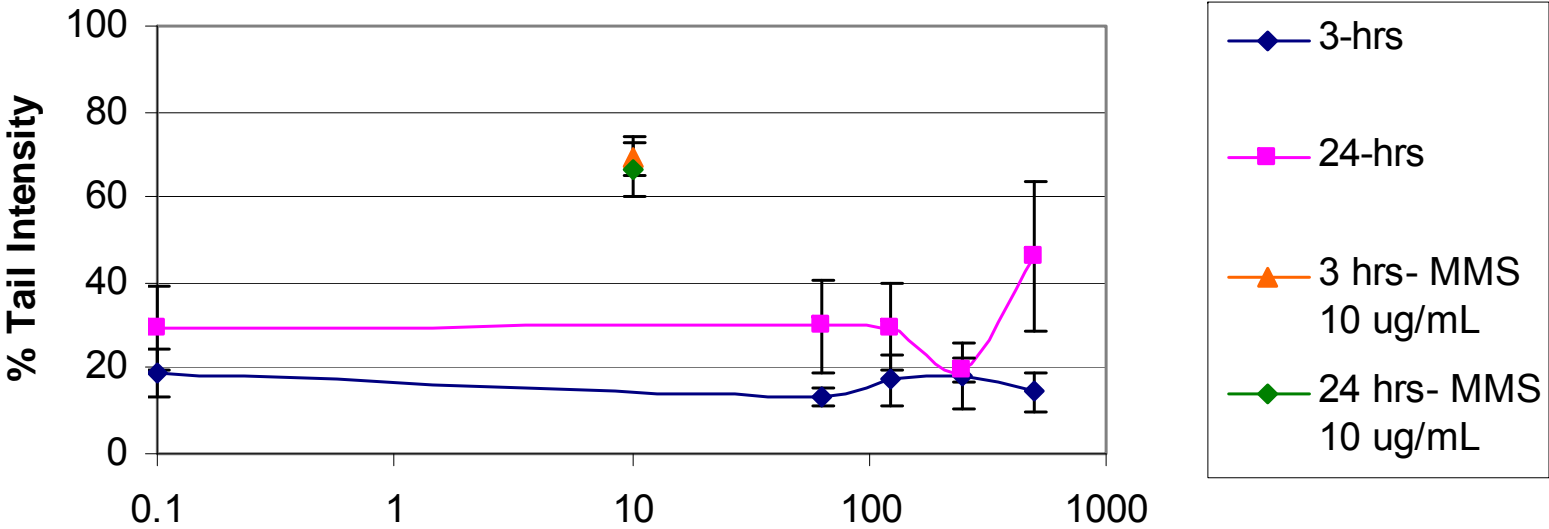
- **Test System (i.e. CHO, V-79, Lymphocytes, etc)**
- **At least 5** Concentrations of Test Article
- Positive and vehicle controls
- Presence and absence of metabolic activation system (-S9/+S9)
- 3- 4 hours Treatment
- ~ 3-4 hours Treatment plus 16-20 hrs recovery
- PH and Osmolarity
- 6 -12 well plates
- **Highest dose: Non-toxic test articles - lowest precipitating dose**
  - ~ 30 % toxicity
  - 5 mg/ml or 10 mM**

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***Data***

# V-79 cells

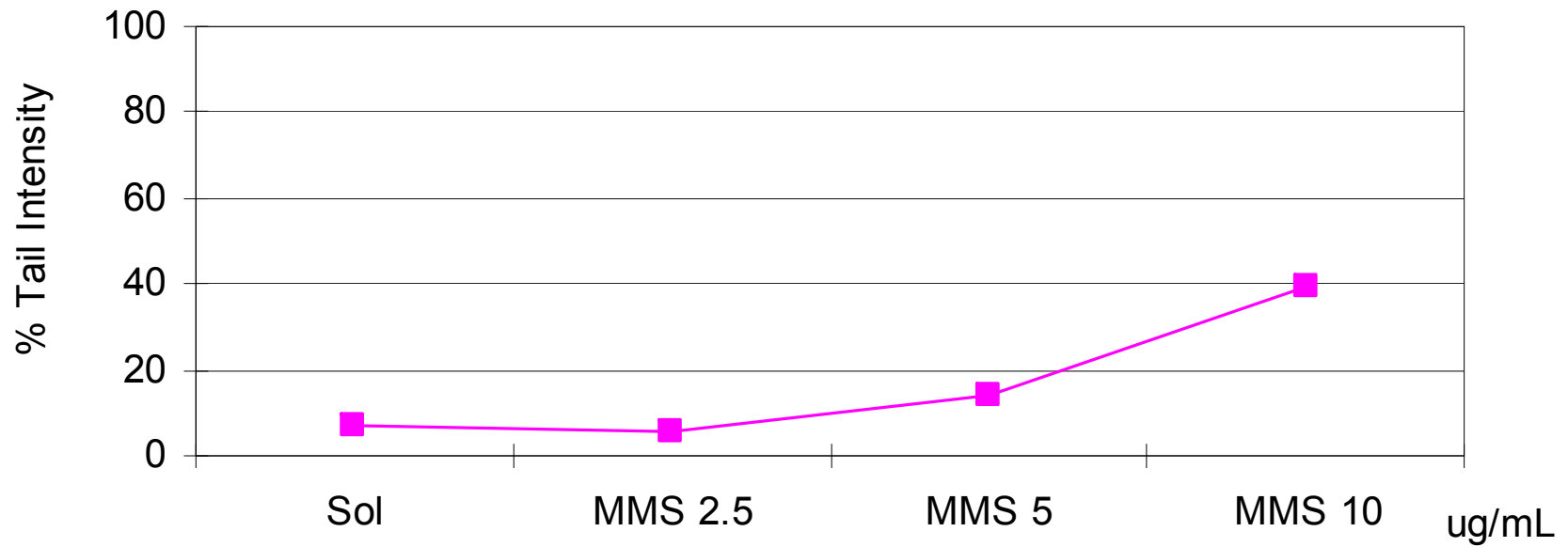
## Detection of DNA Damage in CHO Cells by Comet assay after 3 and 24 hours Exposure to Sodium Salicylate (SS) and Methyl methanesulfonate (MMS)



# SHE cells

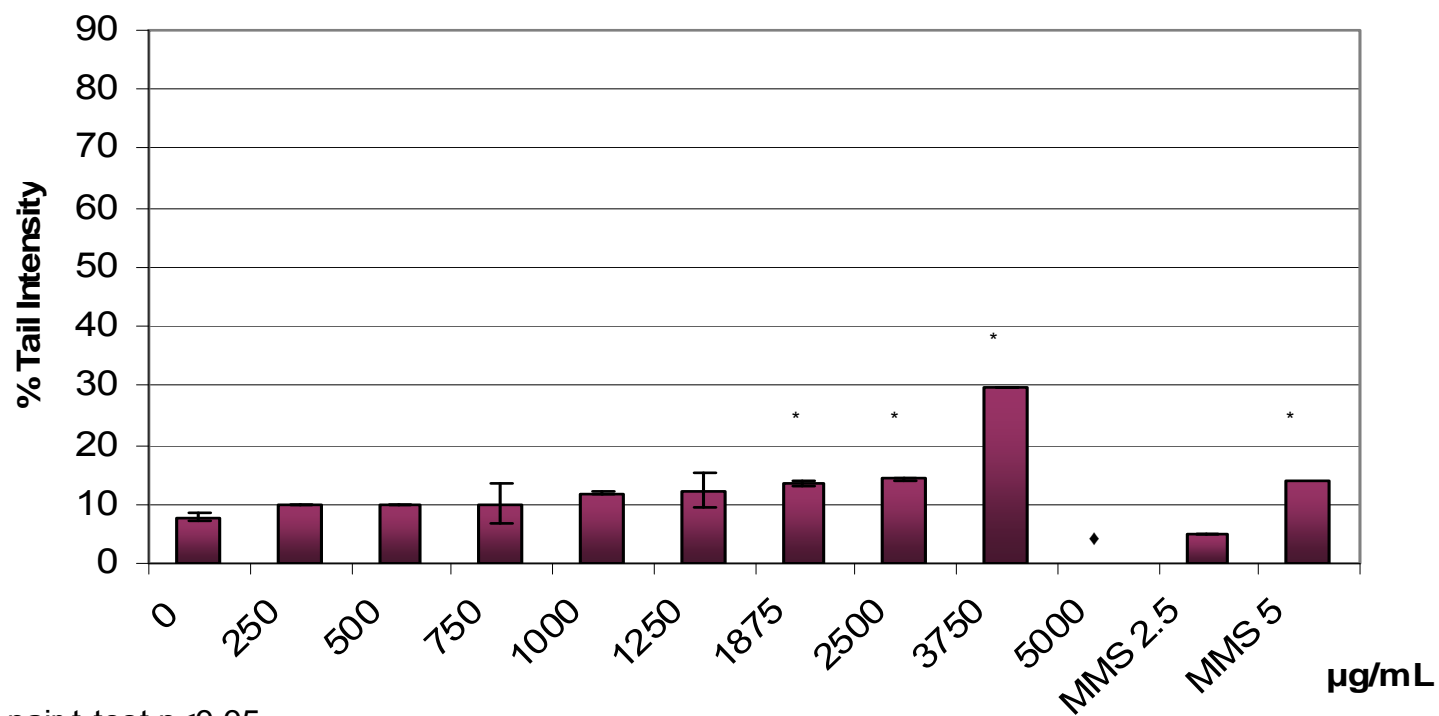
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## DNA Damage in Syrian Hamster Embryo Cells (SHE)



# SHE cells

## DNA Damage in Cells after 24 Hours Exposure to 2,4 diaminotoluene

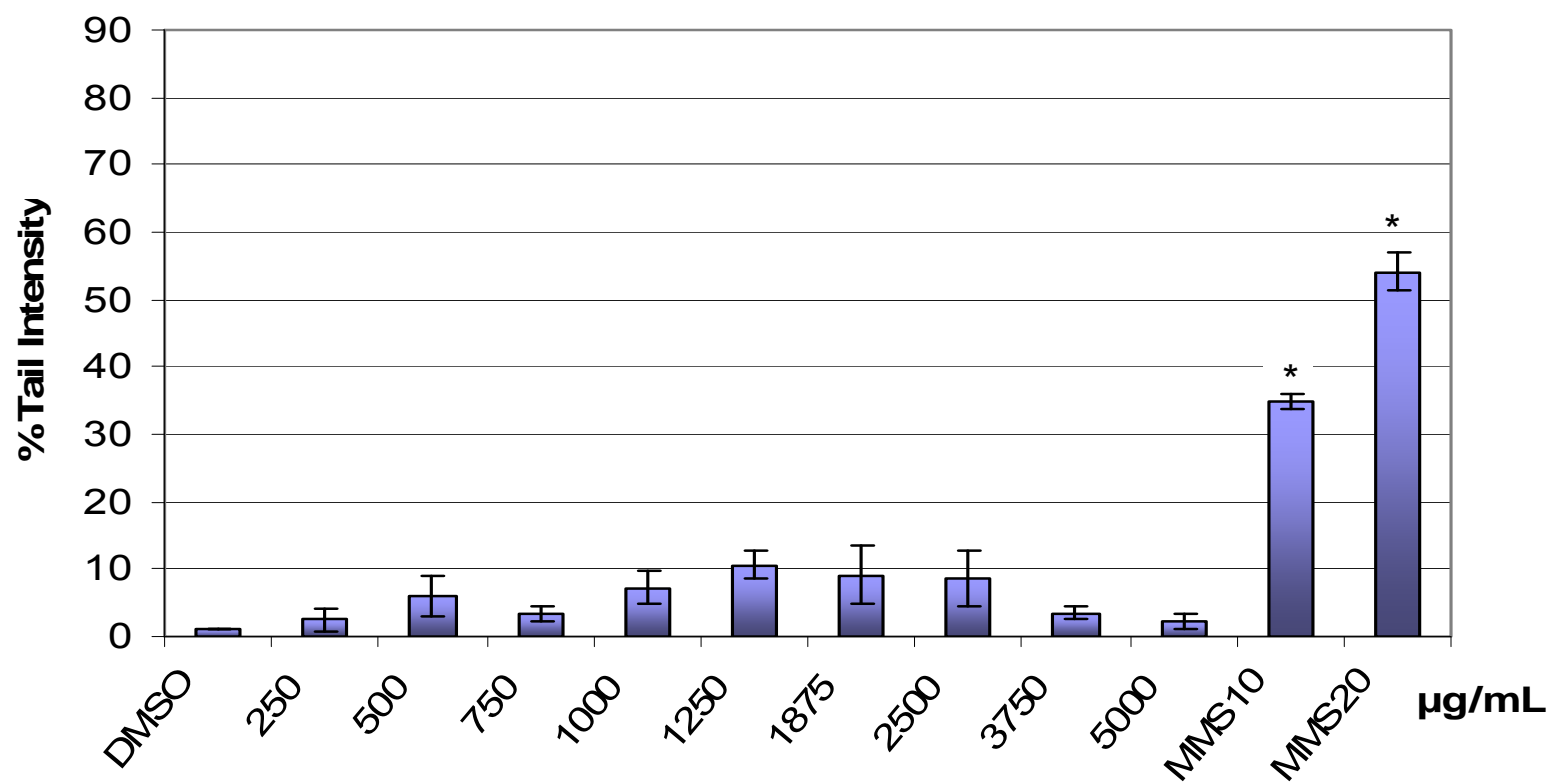


\* pair t-test  $p < 0.05$

◆ Too toxic

# SHE cells

## DNA Damage in SHE Cells 24 hrs after 2,6 diaminotoluene Exposure



\*Pair-t test  $p < 0.05$

2,4-Dichlorophenol (TK6)

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**Data pending to be  
published**

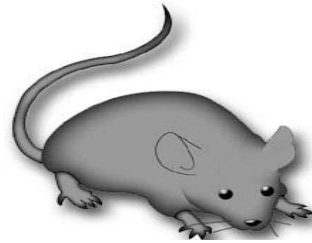
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**What is going on with the *in vitro*  
Comet Assay?**

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- Tice R.R., E. Agurell, D. Anderson, B. Burlinson, A., Hartmann, H. Kobayashi, Y. Miyamae, E. Rojas, J.C. Ryu and Y.F. Sasaki (2000) Single Cell Gel/ Comet Assay: **Guidelines for in vitro and In vivo genetic toxicology testing.** EMM 35:206-221.
  - Witte I, Plappert U, de Wall H, Hartmann A. Genetic Toxicity Assessment: Employing the Best Science for Human Safety Evaluation Part III: **The Comet Assay as an Alternative to In Vitro Clastogenicity Tests for Early Drug Candidate Selection.** Toxicol Sci. 2007 May;97(1):21-6.
  - **In vitro Comet Assay: Validation coordinated by JaCVAM and supported by ICCVAM and ECVAM**

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*In Vivo*  
**Comet Assay**



## ***Why do in vivo Comet Assay?***

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- Genotoxic mechanism
- Analyze possible target organ-specific genotoxicity
- Investigate the *in vivo* relevance to positive *in vitro* genotoxicity
- Elucidate contribution of genotoxicity to tumor formation
- When combined with MN can provide enough evidence of genotoxicity effects *in vivo*

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**Investigate the *in vivo* relevance to  
positive *in vitro* genotoxicity**

## **1<sup>st</sup> Dose-range finding phase**

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- **Evaluate toxicity of the test article**

Animals dosed by a single or multiple oral gavage

Animals observed for 3 days for body weight changes, clinical signs of toxicity and mortality

- **Selection of doses for the definitive phase**

Highest Dose:

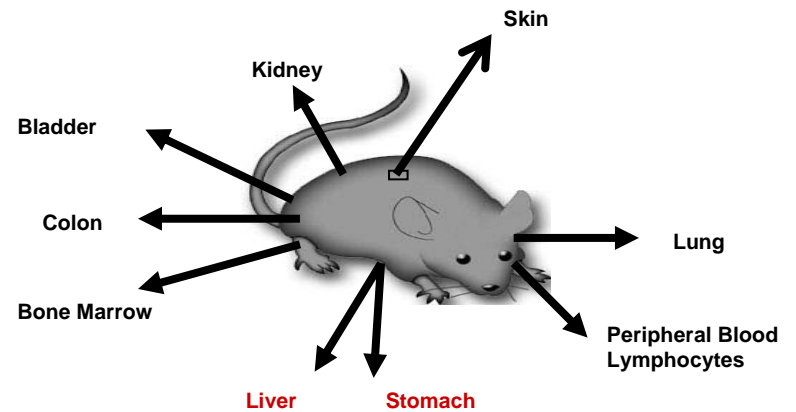
2000 mg/kg bw, in the absence of toxicity

Maximum tolerated dose (MTD)

## 2<sup>nd</sup> Definitive Phase

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- 5 **males**/females per group
- Three concentrations of TA
- Positive and vehicle controls
- Single, **Double** or multiple dosing
- **Oral gavage**
- Tissue collection time 3, 24 hours or T<sub>max</sub>
- Tissues: Comet & Histopathology



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**Data removed for web  
presentation**

# EXAMPLES

## Drug candidate with genotoxicity data sets triggering *in vivo* Comet assay studies

Chemical Class/ Name	Available results of genotoxicity tests	Tissue(s) investigated in the Comet Assay	Results of the Comet Assay	Consequence for Compound	References
Cyanomethyl- carbamoyl- cyclohexyl-propyl- piperazin-benzamide (Drug)	BMT - MNT V79 - CAT V79 + CAT HuLy - MNT BM rat -	Liver, leukocytes (oral administration; 3 and 24- hour sampling)	Negative	OK for health authorities/ EC for multiple dose clinical studies in patients	Genotoxicity/Comet Assay:15
Carbonyl-amino- indanyl-carbamic acid ester (Drug)	BMT - MNT V79 - Comet Huly - CAT V79 + MNT BM rat -	Liver (oral administration; 3 and 24- hour sampling)	Negative	OK for health authorities/ EC for first administration to humans	Genotoxicity/Comet Assay:15
Pyridinyl- suldanylmethyl- octahydro- benzoquinolinol (Drug, topical application)	BMT +/- MNT V79 + CAT V79 + CAT HuLy +/- MLA TK + MNT BM rat -	Liver, Leukocytes (Subcutaneous administration; 3 and 24-hour sampling)	Negative	OK for health authorities/ EC for first (topical) administration to humans	Genotoxicity/Comet Assay:15
Abbreviations: BMT: Bacterial mutation test; CAT: Chromosomal aberration test; MNT: Micronucleus test; HuLy: Human lymphocytes; MLA TK: Mouse lymphoma thymidin-kinase assay; BM: Bone marrow; EC: Ethical committee; UDS: Unscheduled DNA synthesis; CHO: Chinese hamster ovary cell line; V-79: Chinese hamster lung fibroblast cell line; -: negative; +: Positive; +/-: Positive and negative in independent experiments.					

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What is going on with the *in vivo* Comet Assay and  
**regulatory guidelines?**

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### **IWGTP-1999 (Washington DC)**

RR Tice, E Agurell, D Anderson, B Burlinson, A Hartmann, H Kobayashi, Y Miyamae, E Rojas, JC Ryu, YF Sasaki. (2000) Single cell gel/comet assay: Guidelines for *in vitro* and *in vivo* genetic toxicology testing. Environ Molec Mut 35:206

### **4<sup>th</sup> International Comet Assay Workshop-2002 (Ulm, Germany)**

A Hartmann, E Agurell, C Beevers, S Brendler-Schwaab, B Burlinson, P Clay, A Collins, A Smith, G Speit, V Thybaud, RR Tice (2003) Recommendations for conducting the *in vivo* alkaline Comet assay. Mutagenesis 18:45 (2003)

### **IWGT-2005 (San Francisco)**

B Burlinson, RR Tice, G Speit, E Agurell, SY Brendler-Schwaab, AR Collins, P Escobar, M Honma, TS Kumaravel, M Nakajima, YF Sasaki, V Thybaud, Y Uno, M Vasquez, A Hartmann (2006) 4<sup>th</sup> International workgroup on genotoxicity testing: results of the *in vivo* comet assay workgroup. Submitted for publication.

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- ***In vivo* Comet Assay Validation**

**JaCVAM Initiative International Validation on *in vivo* Comet Assay supported by ECVAM and ICCVAM/NICEATM**

**Main objective Generate an OECD Test Guideline**

- **Possible second *in vivo* assay in the newly proposed S2(R1) ICH guidelines**
- **PHRMA/ESPIA collaboration**

# In vivo Comet Assay Validation Study (JaCVAM)\*

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- 1rst Phase** → **Optimize study protocol (2006)**
  
- 2nd Phase** → **Examine within/between lab variability**  
**Determine acceptance criteria**  
**(2007)**
  
- 3rd Phase** → **Reconfirm data acceptance criteria**  
**based on 2nd phase validation**  
**data, and to further optimize**  
**the standard protocol (2008)**
  
- 4th Phase** → **Investigate predictive capacity of**  
**genotoxic carcinogens (now**  
**planning & start 2009)**

\* Information provided by JaCVAM validation Managemet Team

# Summary

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## *In vitro*

- Any Eukarotic cell (cell lines, primary cultures, 3D models; adherent or suspension)
- Proliferating and non-proliferating cells
- Easy to combined with other endpoints (cell proliferation, apoptosis,etc)

But....

- Will it have the same rate of false positives as other genotox assay?
- What added value will bring over existing assay?

## *In vivo*

- Majority of organ tissues can be used
- Combine with in vivo MN assay
- Add on to 14/28 day studies

But.....

- cell isolation induce high background levels of DNA damage
- Sensitive assay, how about specificity; Carcinogenicity predictivity; Reproducibility?

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## **Acknowledgment**

**BioReliance Corporation**