



TRADITION

JUST BECAUSE YOU'VE ALWAYS DONE IT THAT WAY
DOESN'T MEAN IT'S NOT INCREDIBLY STUPID.

www.despair.com

The Comet Assay: *Breaking From Tradition*

Marie Z. Vasquez
Helix3 Inc.

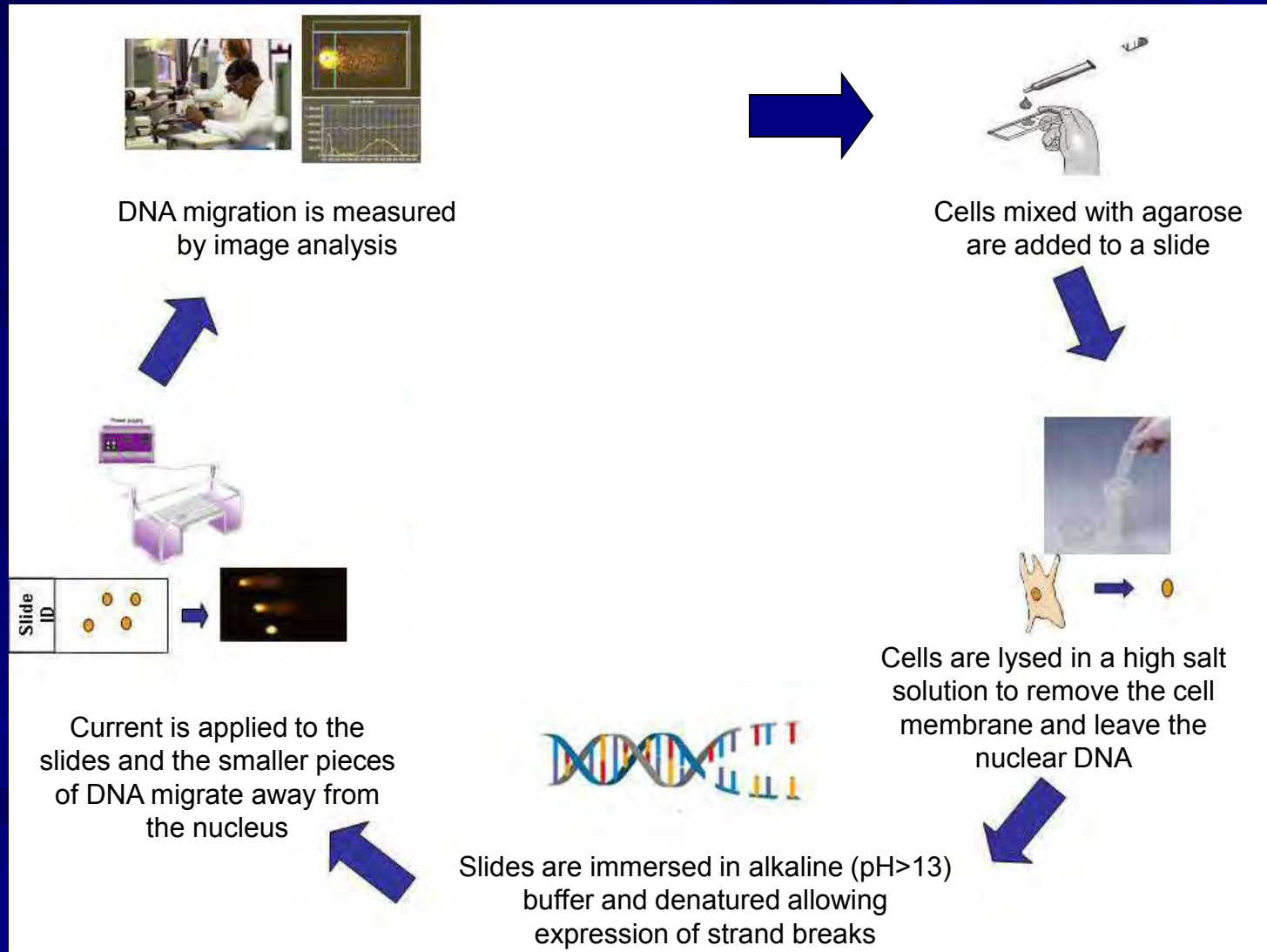


“Relative to other genotoxicity tests, the advantages of the SCG (Comet) assay include its **demonstrated sensitivity** for detecting low levels of DNA damage ...[and] its **ease of application...**”

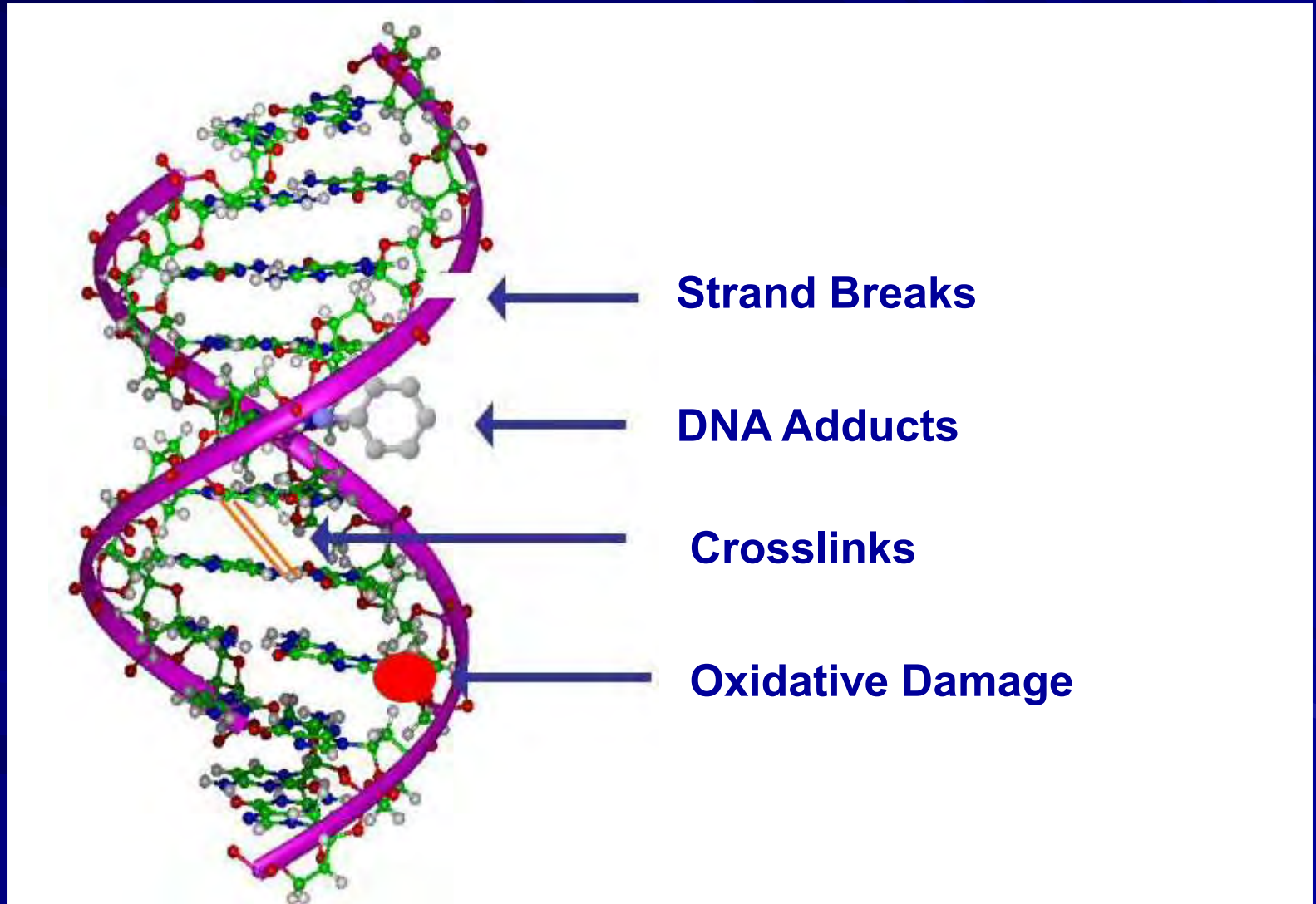
Tice, RR, Agurell, E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ Mol Mutagen. 2000;35(3):206-21.



Comet Methodology



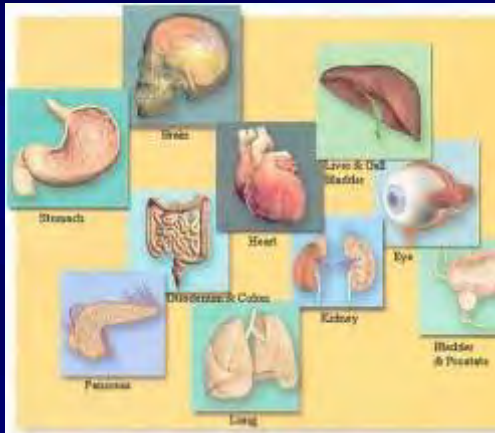
Types of Damage Detected



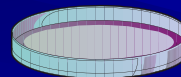
Applications



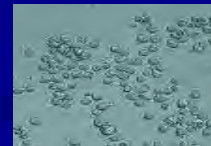
In Vivo



Human Biomonitoring



In Vitro



Advantages

Flexibility

- Detects various forms of DNA damage
- Does not require cell division
- Applicable to any eukaryotic cell type (e.g. *in vitro*, somatic, germ, endocrine)

Advantages

Sensitivity

- Detects low levels of DNA damage
- Measures degree of damage in each cell (continuous data) versus the percentage of cells with damage (discrete data)
- Shorter exposures
- Lower doses
- Minimal / no animal distress

Disadvantages

Flexibility

- Increases DNA migration
 - Strand breaks
 - Excision repair
 - Cell division
 - Cytotoxicity
- Decreases DNA migration
 - Crosslinks
 - Cytotoxicity
- Different tissues = Different responses

Disadvantages

Sensitivity

- Technical variability with inexperience
- Environmental requirements
- Study design considerations
(e.g. Dose, exposure, tissue selection)
- Data interpretation considerations

Genotoxicity Testing with Comet

- Dose Selection
- Cytotoxicity
- Treatments and Sample Time
- Interpretation of Migration Patterns
- Toxicology Study Integration

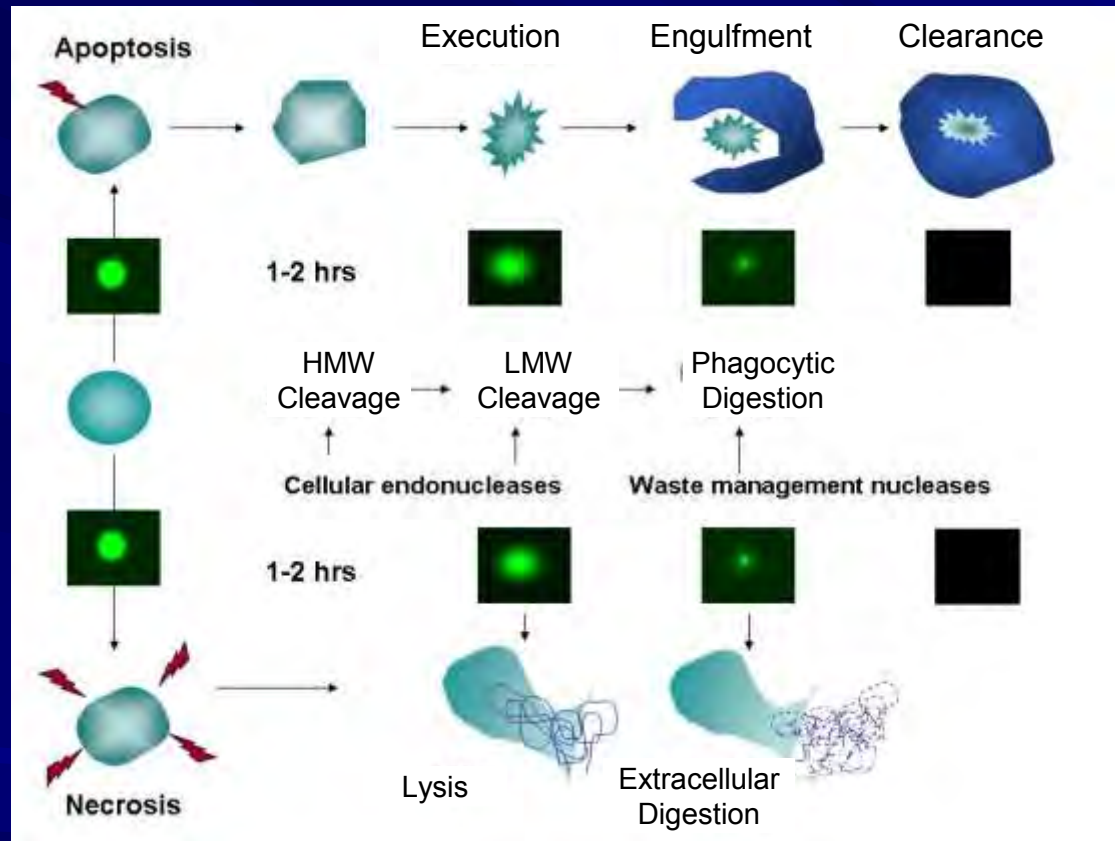


Terms

- Non-toxic compound: Compound that does not induce clinical signs of toxicity and/or mortality in independent toxicity studies
- OTM: Olive Tail Moment; Combination of %Tail and Tail length measurements for DNA migration (multiplied by 4 for combined viewing with %LMW data)
- %LMW: % Cells with Low Molecular Weight DNA Diffusion



Low Molecular Weight (LMW) Diffusion Assay



- Replicate comet slide used

- % Diffused = dead/dying

- Single cell cytotoxicity

- Interpretive tool for comet

- More sensitive than histopath

Dose Selection

“Consistent with current relevant OECD guidelines, the limit dose for nontoxic substances is 2000 mg/kg body weight for single or multiple treatments up to 14 consecutive days.”

Tice, RR, Agurell, E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ Mol Mutagen. 2000;35(3):206-21.



Dose Selection

“For short term (usually 1 to 2 administrations) protocols, the top dose recommended for genotoxicity assays is the limit dose of 2000 mg/kg if this is tolerated, or [the] maximum tolerated dose defined as the dose producing signs such that higher dose levels...would be expected to produce lethality.”

ICH Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals
Intended for Human Use S2(R1) Current version dated 6 March 2008



Case Study 1:

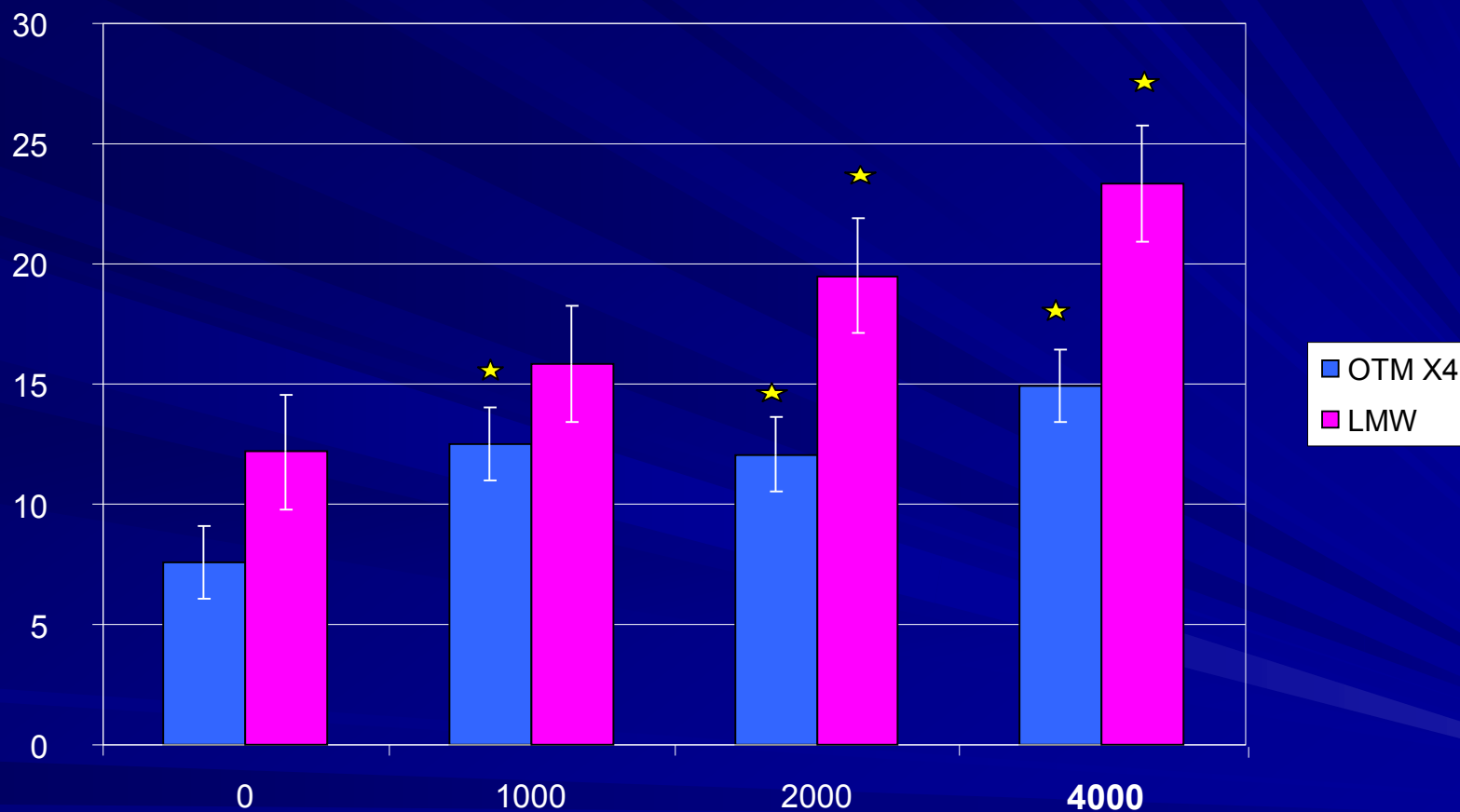
Nontoxic Compound in Liver

- Single oral administrations of non-toxic compound
- Dosed on 2 consecutive days (20 hrs apart)
- High dose: 2000 mg/kg/administration (4000 mg/kg total dose administered)
- Sample Time: 4 hrs after last dose
- Tissue: Liver



Case Study 1:

Nontoxic Compound in Liver



Total Dose Administered (mg/kg bodyweight)

Genotoxic or Cytotoxic?



Dose Selection

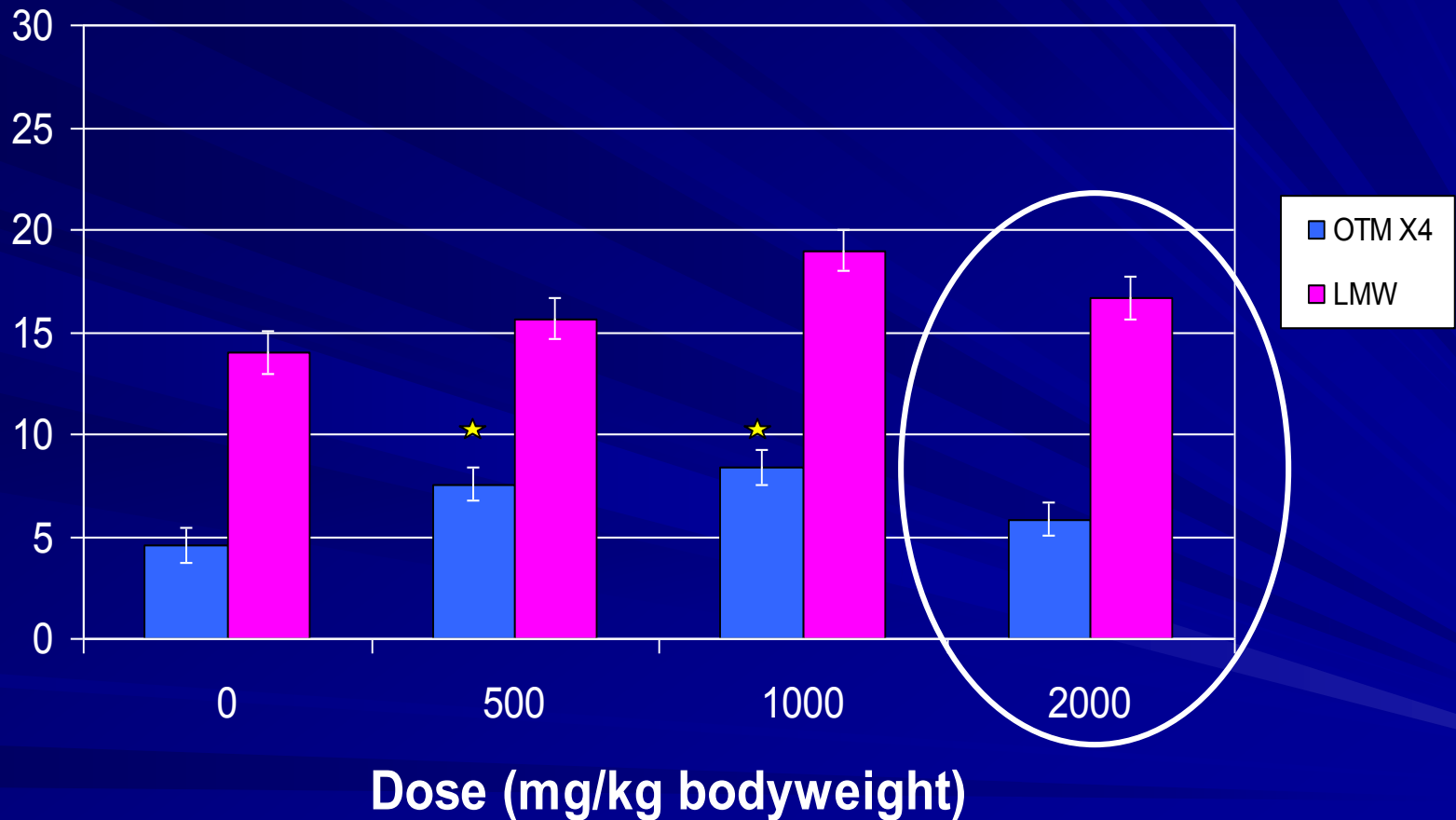
“In the absence of animal or tissue specific toxicity, it may be acceptable to test **only the limit dose**.”

Tice, RR, Agurell, E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ Mol Mutagen. 2000;35(3):206-21.



Case Study 2:

Single Dose Administration Sampled from Liver at 24 hrs



Positive or Negative?



Dose Selection

- Nontoxic \neq Non-cytotoxic
- MTD \neq Non-cytotoxic
- Testing the only the limit dose \neq smart



Cytotoxicity

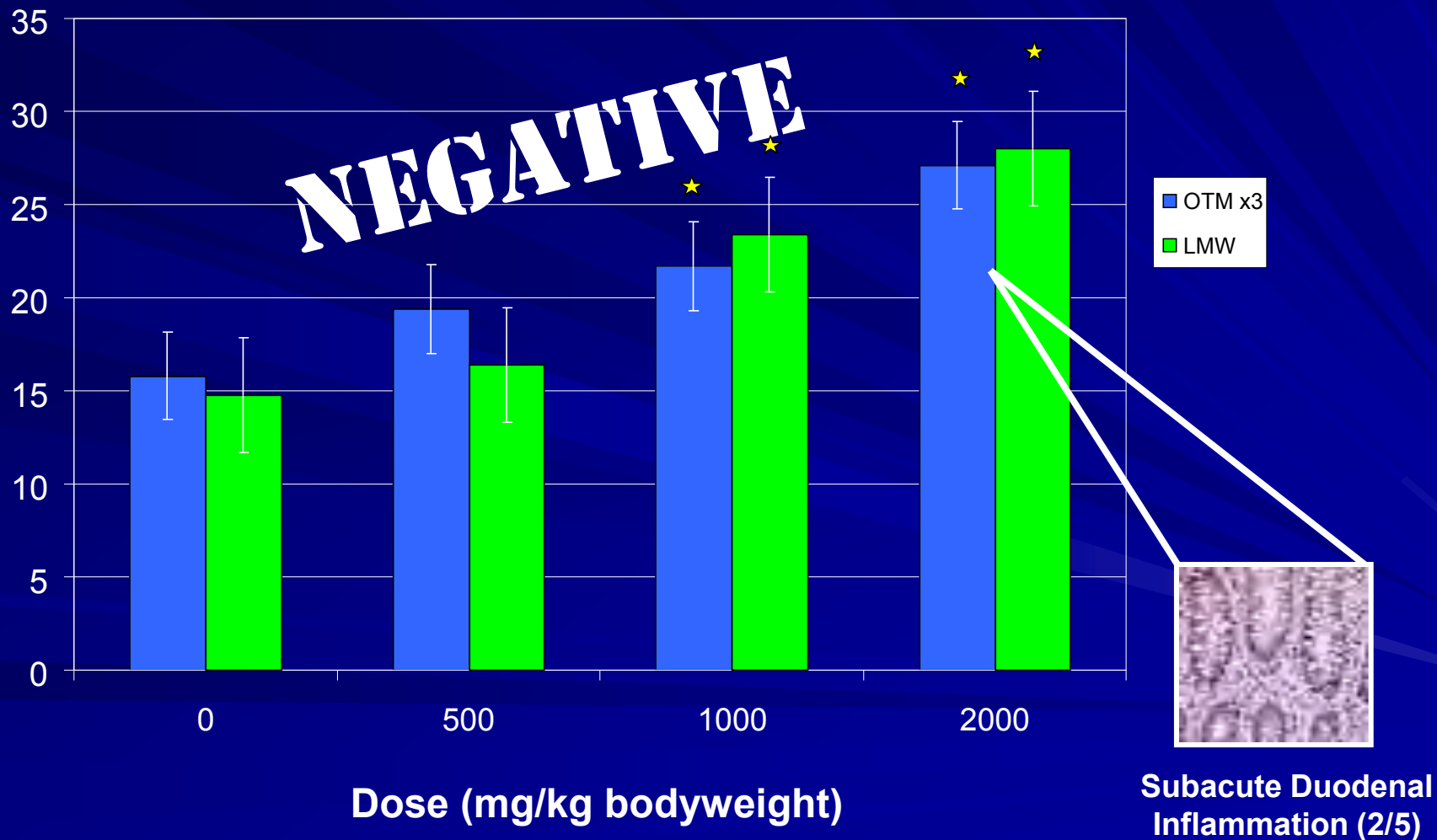
“...cytotoxicity ... did not lead to false positive test results (in the comet assay). “

Hartmann et. al. Influence of cytotoxicity and compound precipitation on test results in the alkaline comet assay. Mutat Res. 2001 Oct 18;497(1-2):199-212.



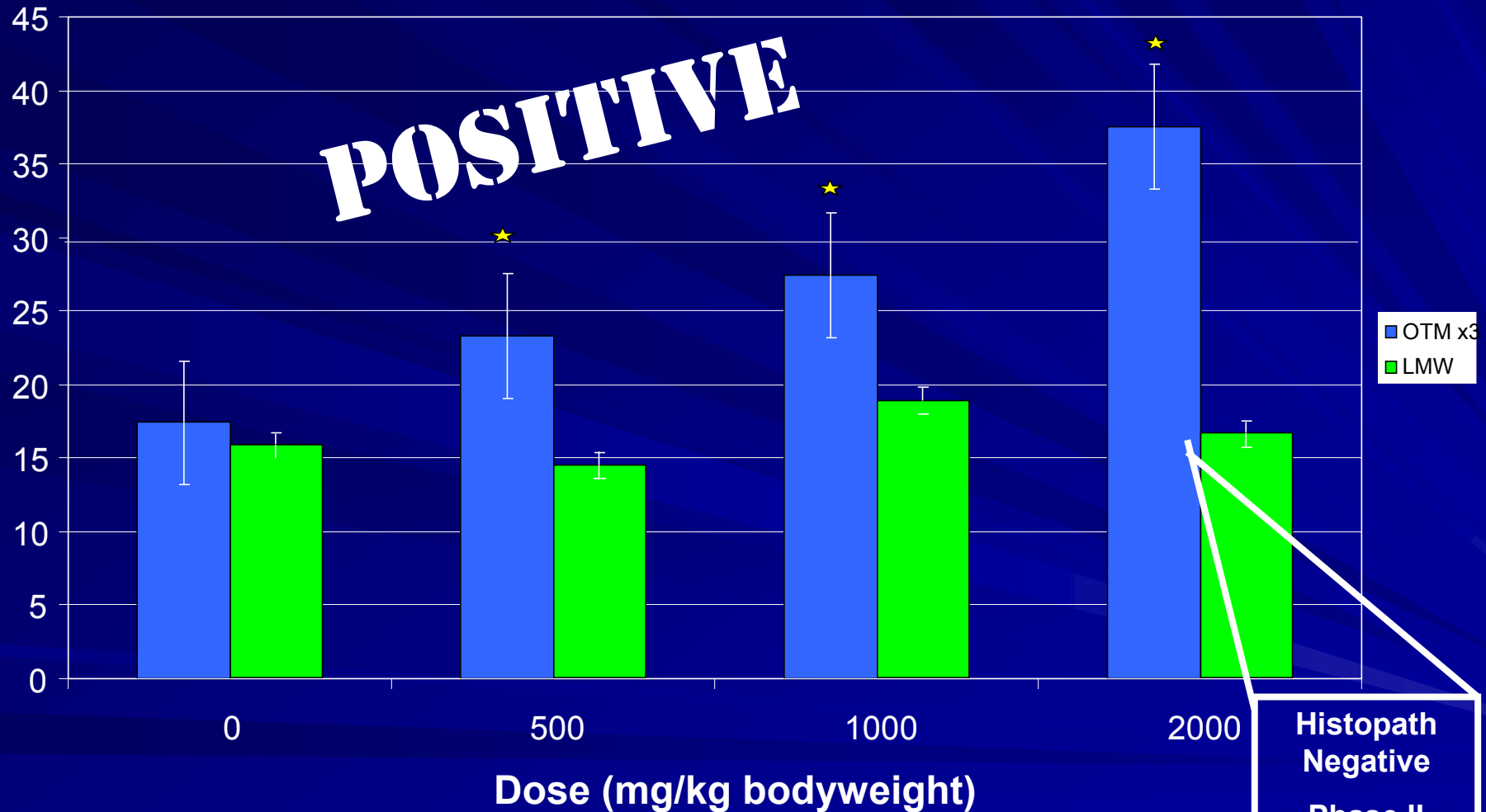
Compound A:

Nontoxic compound in Duodenum



Compound B:

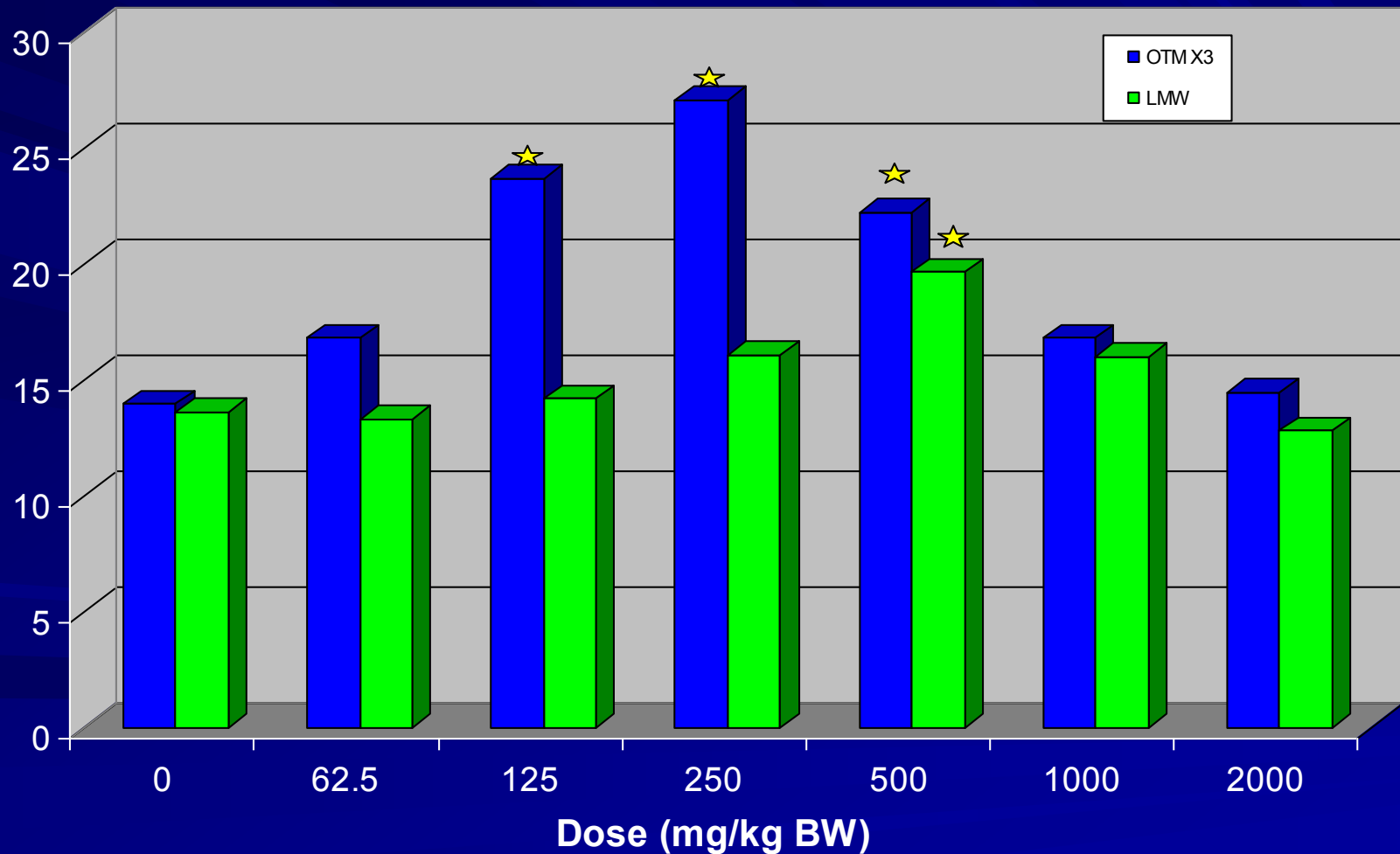
Nontoxic Compound in Duodenum



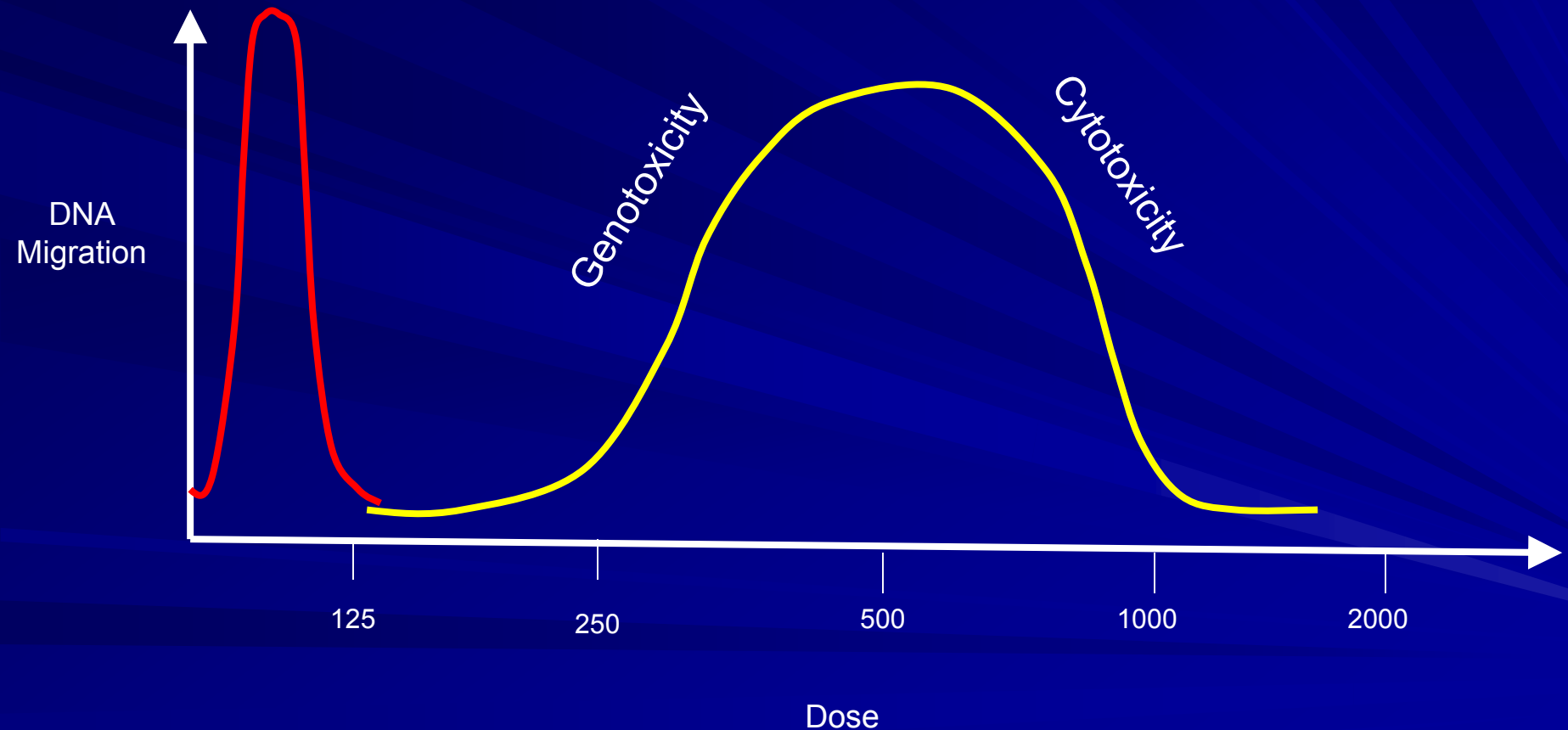
Histopath
Negative
Phase II
Clinical Trial
Stopped

Compound C:

Nontoxic Compound in Colon



Possible Dose Response Curves



Cytotoxicity

- Cytotoxicity can increase DNA migration due to endonuclease activity (false positive)
- Cytotoxicity can appear to decrease DNA migration due to loss of dead/dying cells (false negative)



Treatments and Sample Time

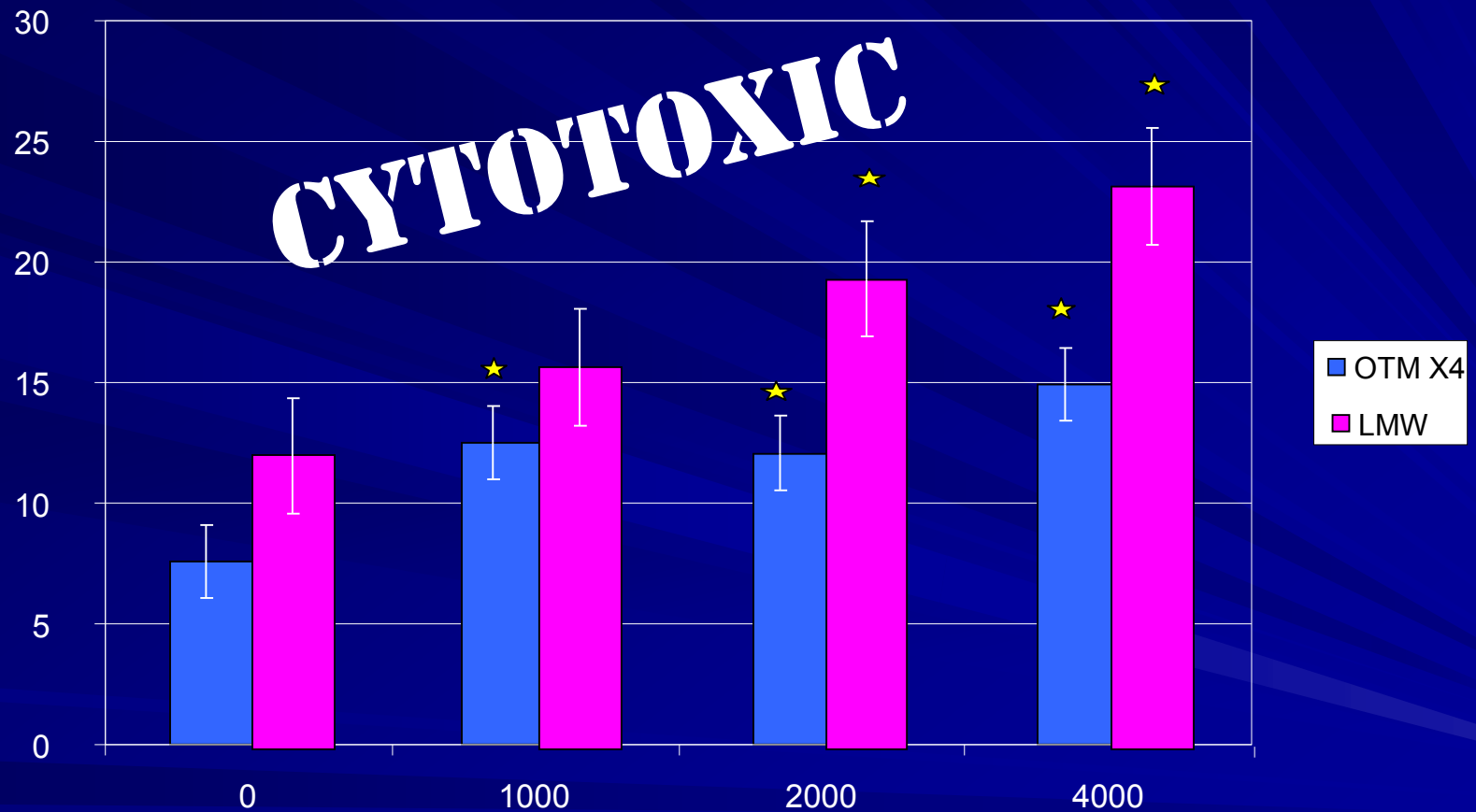
“In relation to the time of test substance administration, tissue/organ **samples are obtained 2-6 and 16-26 hours after dosing.**”

Hartmann A, Agurell E, Beevers C, Brendler-Schwaab S, Burlinson B, Clay P, Collins A, Smith A, Speit G, Thybaud V, Tice RR. Recommendations for conducting the *in vivo* alkaline comet assay. *Mutagenesis* 2003;18(1): 45-51.



Compound D:

Nontoxic Compound in Liver Sampled at 4 and 24 hrs



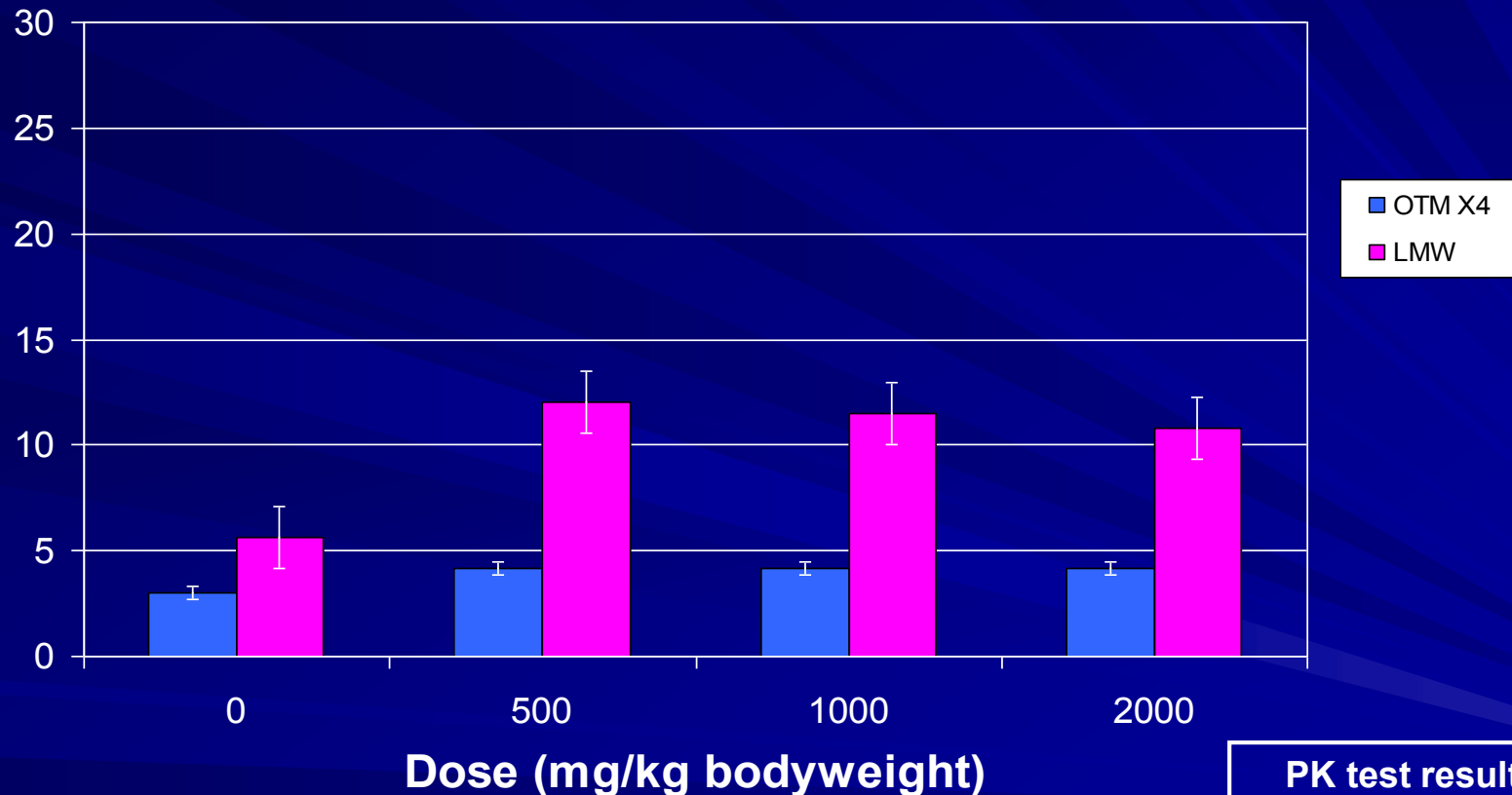
Total Dose Administered (mg/kg bodyweight)

Genotoxic or Cytotoxic?



Compound D:

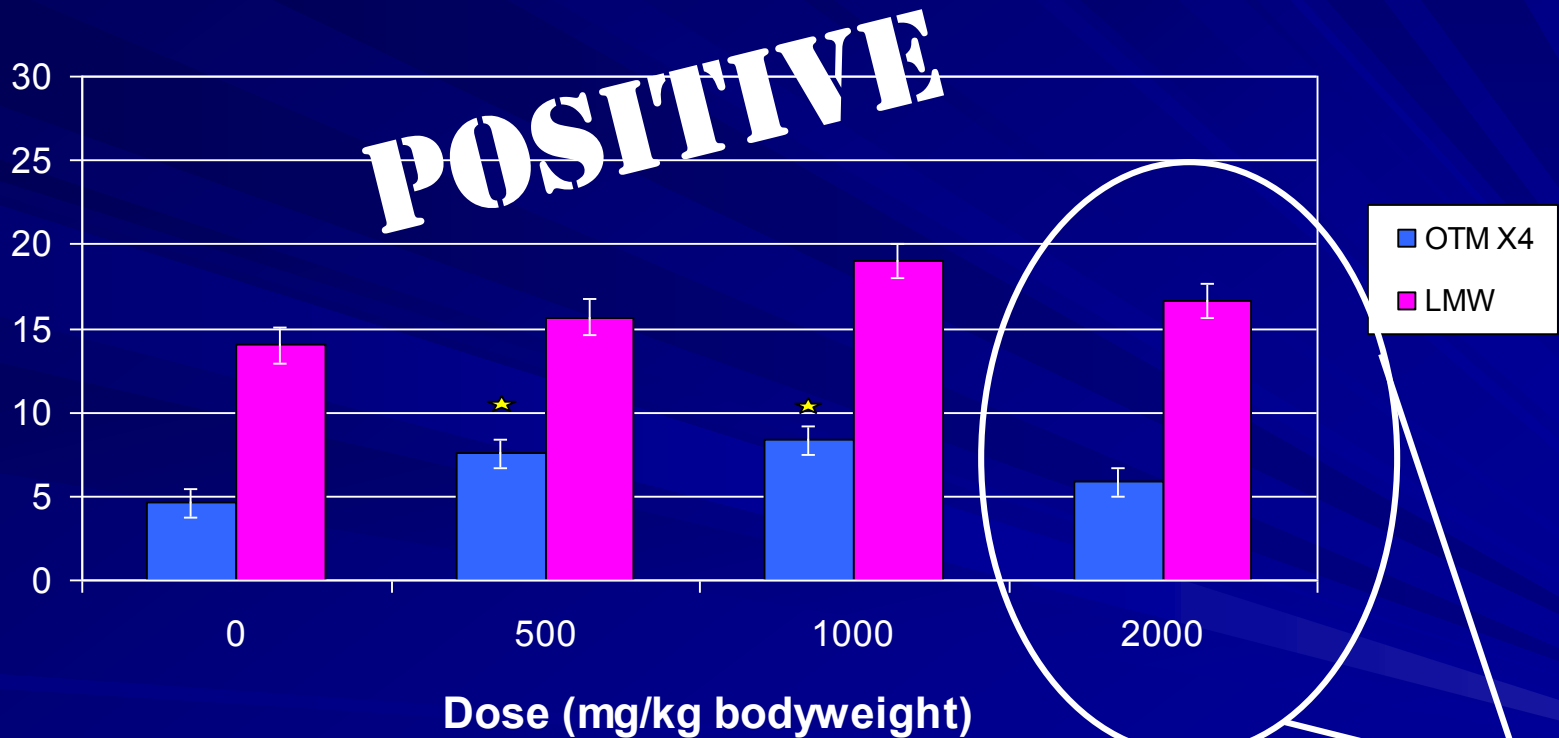
Single Dose 4 hr Sample Time



PK test result:
TA not
metabolized until
after 20 hrs

Compound D:

Single Dose 24 hr sample time

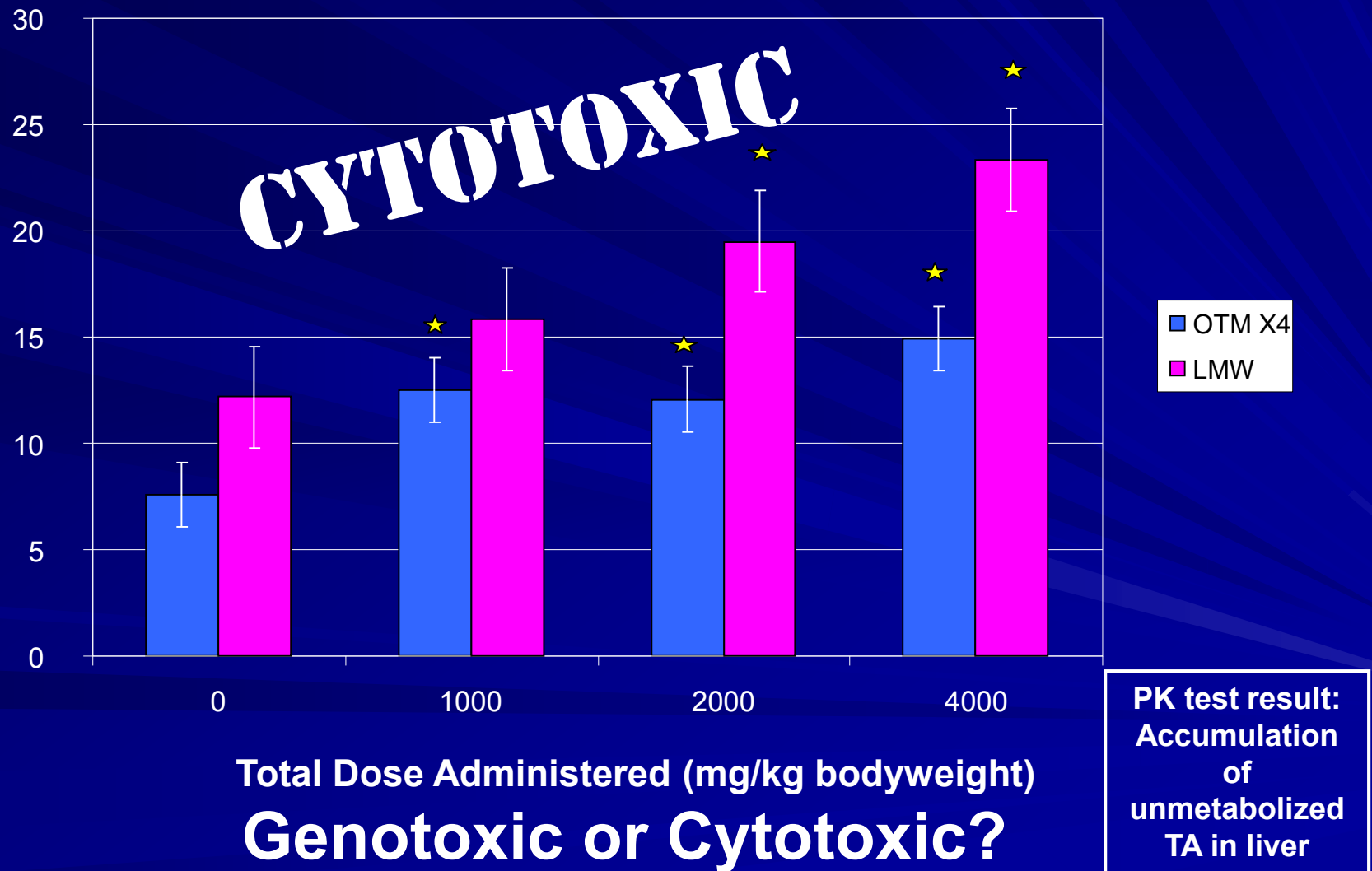


Positive or Negative?

PK test result:
TA not
metabolized at
≥2000 mg/kg

Compound D:

Nontoxic Compound in Liver



Treatments and Sample Time

- The best sample time is not always 2-6 and/or 16-26 hrs after dosing
- Higher dose \neq Higher exposure
- The pharmacokinetics of the compound determines the most appropriate sample time



Interpretation of Migration Patterns:

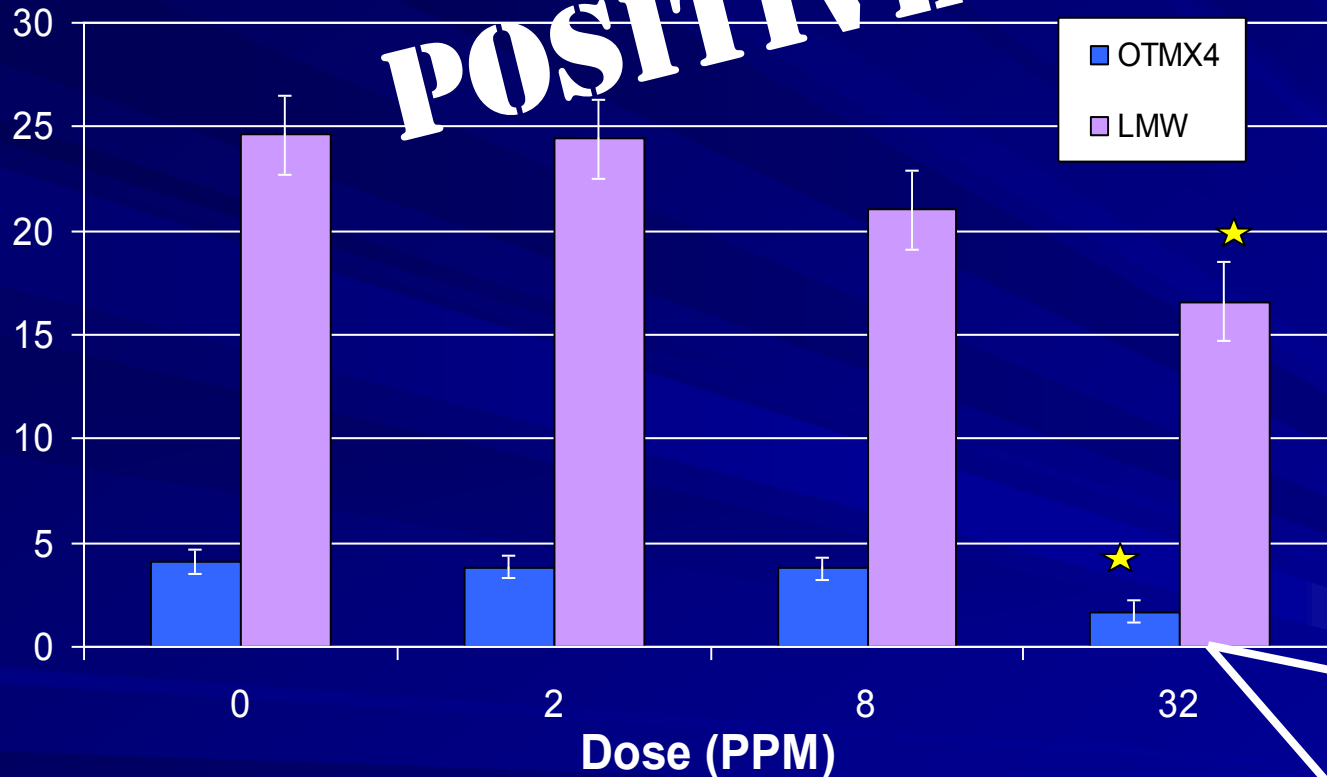
√ Cytotoxicity

▶ Crosslinking

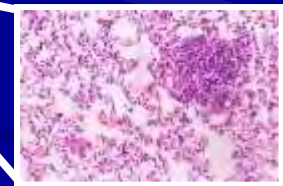


Compound E: 28 day inhalation

POSITIVE



↑ image intensity
↓ DNA diffusion
↑ DNA condensation



**Lung
Hyperplasia
& Metaplasia**

“The electrophoresis duration should result in an average
DNA migration in the negative control group of 1-8%
DNA in the tail”¹
or

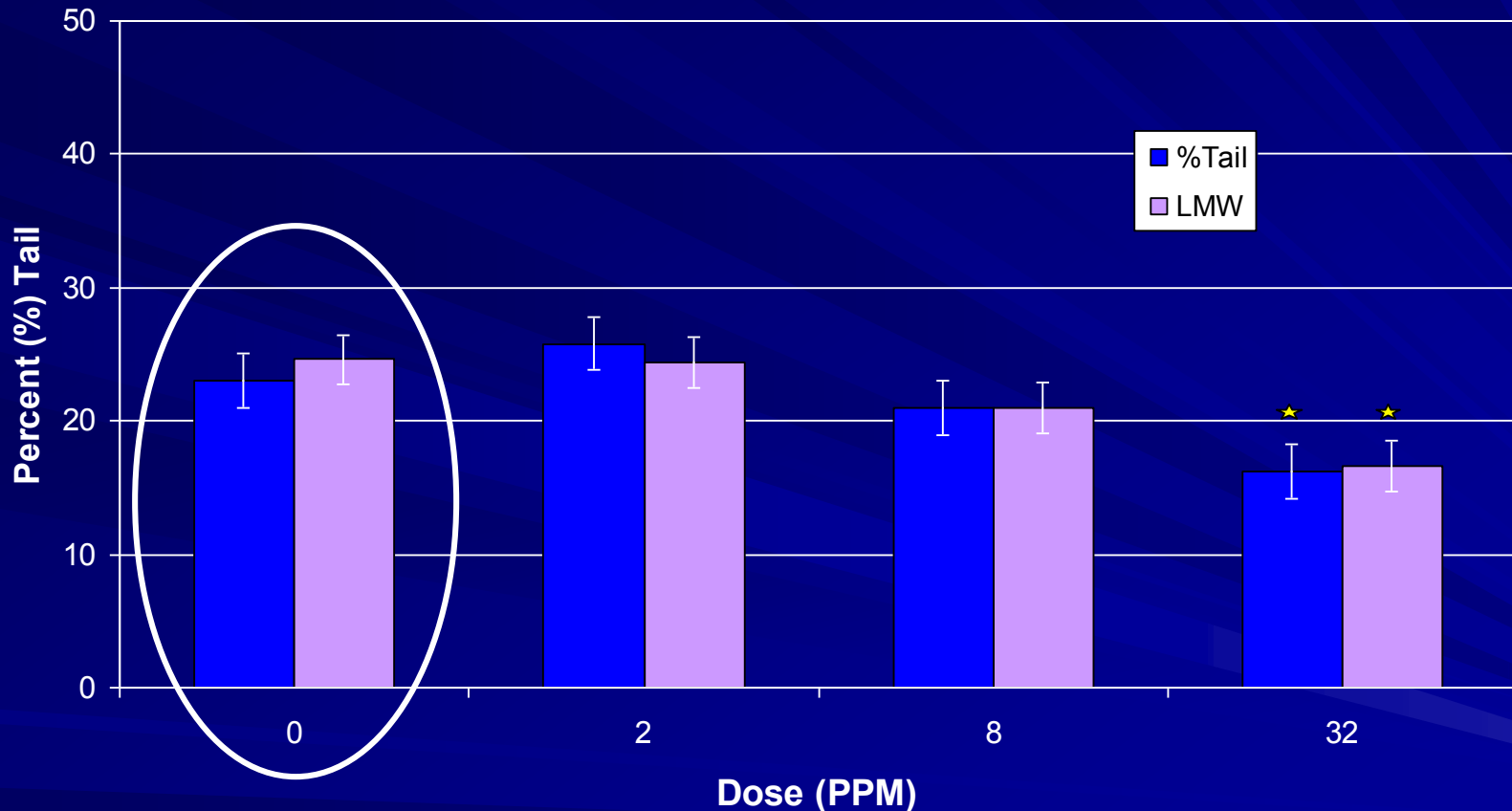
“electrophoresis [should] be conducted for 20 minutes at
<10°C”²

¹ International Validation of the *In Vivo* Rodent Alkaline Comet Assay for the Detection of Genotoxic Carcinogens (Version 13 Revised March 31, 2008)

² EFPIA/PhRMA/JPMA Draft Study Design for Integrating Comet Assay and Micronucleus Test into General Tox Studies (Issued August 27, 2008)



Compound E: 28 day inhalation



Electrophoresis: 40 minutes at room temperature (21-22°C)



Compound E

In Vitro Comet:

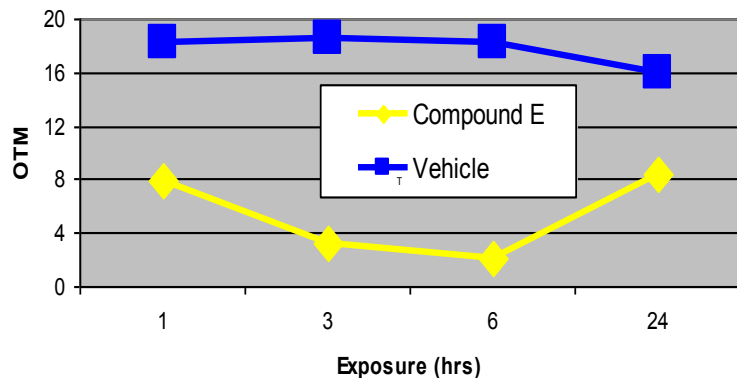
- Replicate conditions/doses of *in vitro* CA Study
- CA Study Results: aberrations induced only at cytotoxic doses (-S9)
- CA Sample Time: 24 hrs



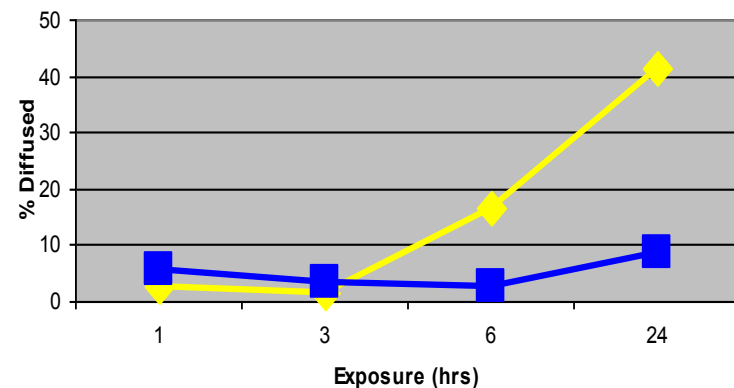
Compound E:

In Vitro Time Course in CHO-K1 Cells

DNA Migration (OTM)



LMW Diffusion (%)



Crosslinks Induced

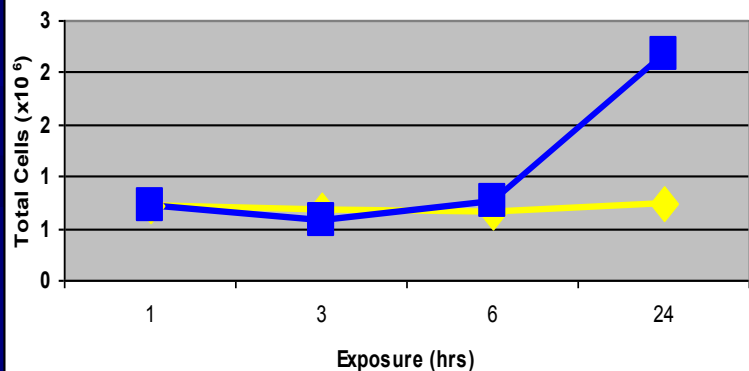
Excision Repair attempt / Cell Division starts

Apoptosis

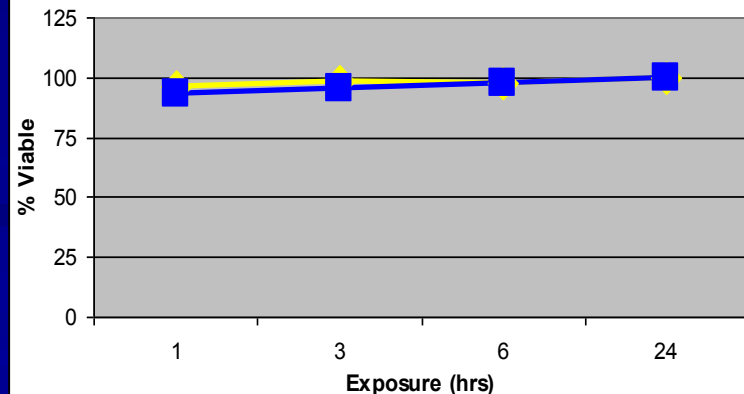


Cell Cycle arrest

Cell Concentration ($\times 10^6$)



Viable Cells (%)



Crosslinking

- Decreases DNA migration
- Can be induced after ≤ 1 hour of exposure
- Can lead to cell cycle arrest and cytotoxicity detectable at later time points (~6-24 hours)
- Can indirectly induce chromosomal aberrations as a result of cytotoxicity



Toxicology Study Integration:

- More Cytotoxicity
- Dosing and Sampling
- Use of Positive Controls



Toxicology Study Integration

More Cytotoxicity

Compound F:

- 7-day Dermal application at MTD
- Sample Time: 24 hours after final administration
- Tissue: Bladder

Animal Observations: Blood in urine at all doses and starting on Day 1

Necropsy Observation: Very thin bladder tissues

Result: No increase in DNA migration



Toxicology Study Integration

More Cytotoxicity

Compound G:

- 28-Day Inhalation at MTD
- Sample Time: 4 hours after last administration
- Tissues: Liver, heart, lung, kidney, blood

Animal Observations: None

Necropsy Observation: dissolving tissues in all dose groups

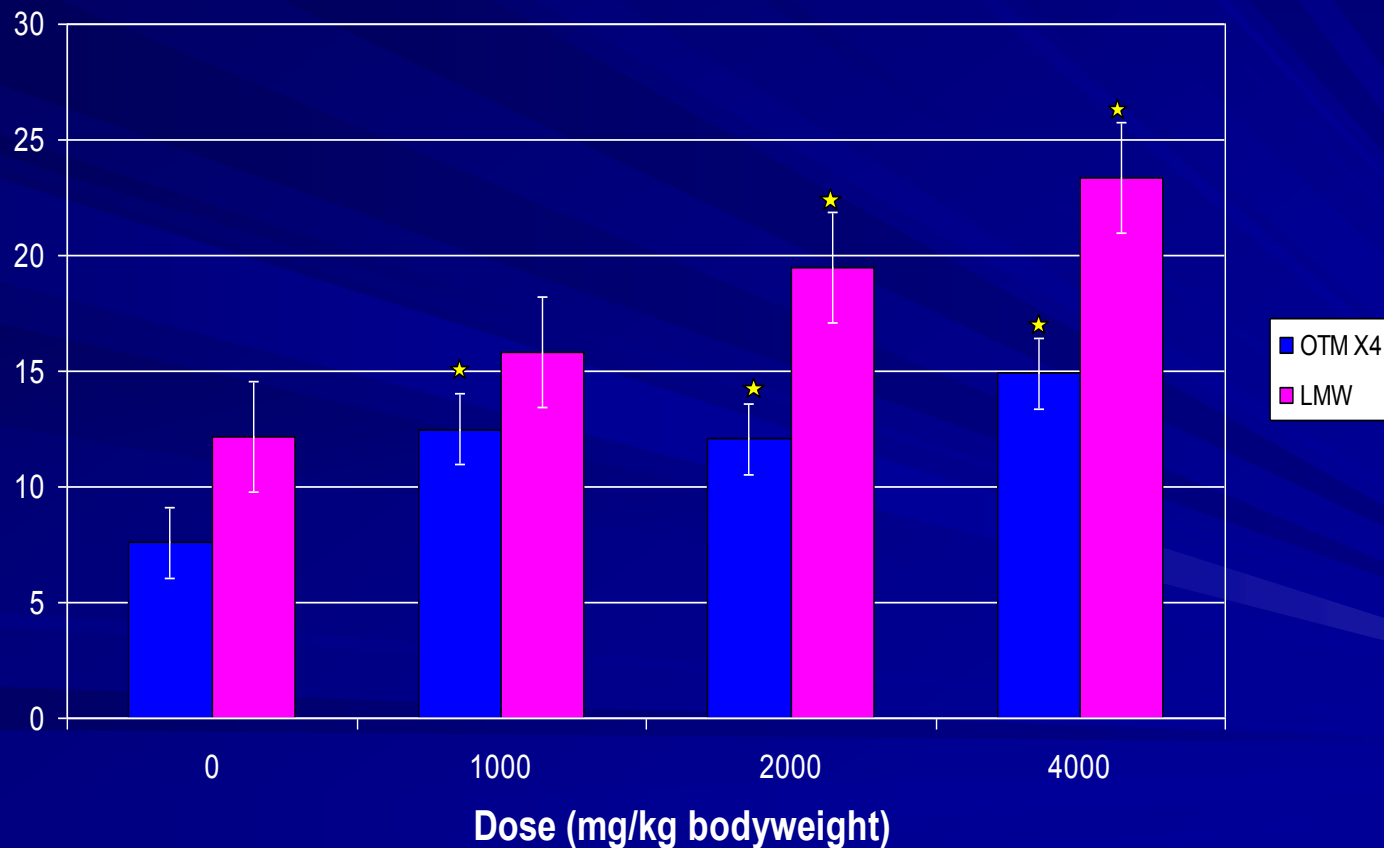
Result: No increases in DNA migration



Dosing and Sampling

Compound D:

Nontoxic Compound in Liver



Toxicology Study Integration

Cytotoxicity, Dosing & Sampling

- Risk of extreme tissue cytotoxicity
- Dose effects can be cumulative . . . or not
- The appropriate dosing / sampling schedule depends on whether the dose effect is cumulative or not



Use of Positive Controls

“...concurrent treatment of animals with a positive control agent may not be necessary and **control of staining and scoring procedures** may be accomplished by including appropriate reference samples obtained previously from animals that are not part of the current experiment.”

Toxicological Principles for the Safety Assessment of Food
Ingredients: Mammalian Erythrocyte Micronucleus Test. U.S. FDA
/ CFSA IV.C.1.d (2000)



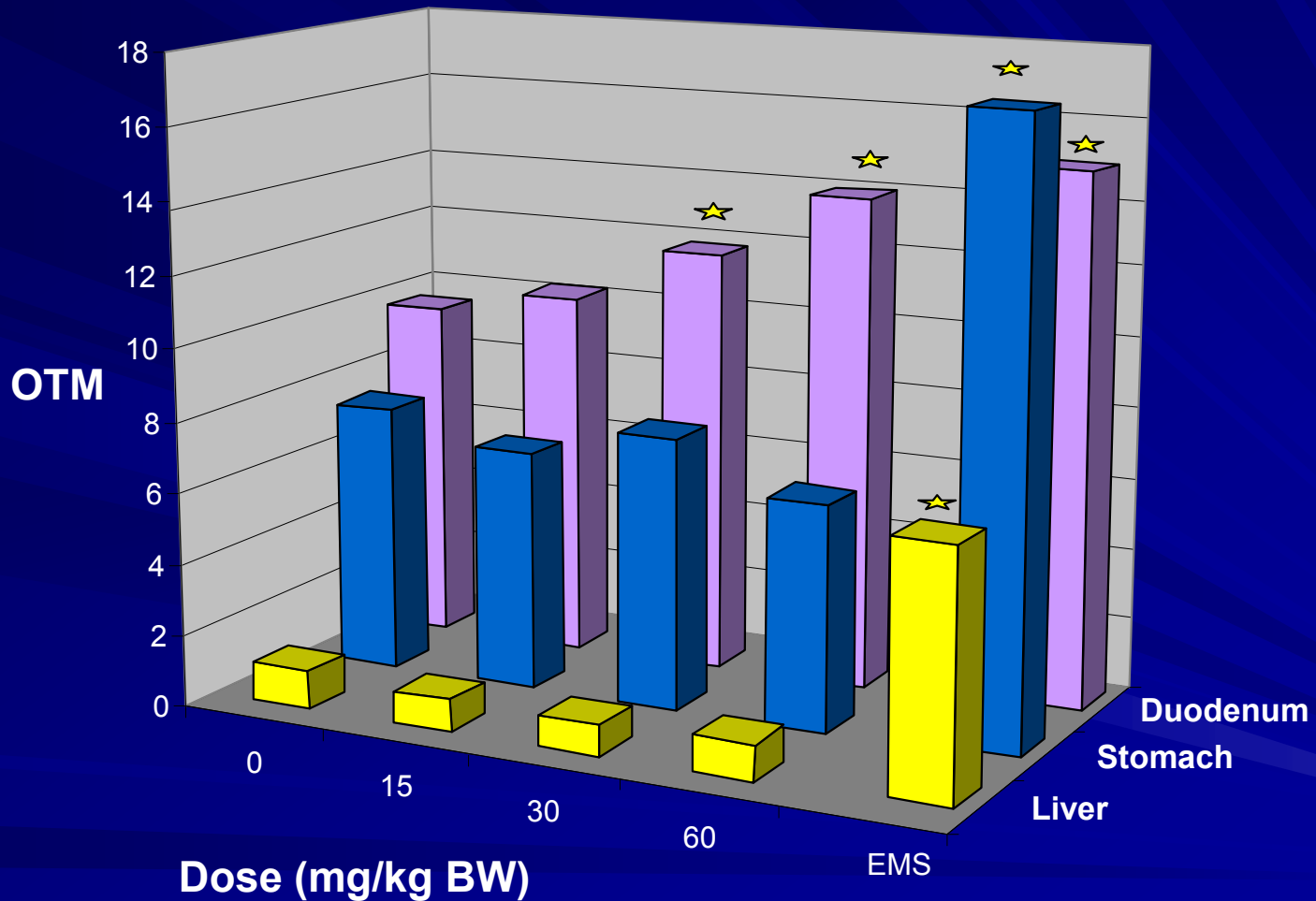
Use of Positive Controls

“For *in vivo* studies, it is not necessary to include concurrent treatments with positive controls in every study, after a laboratory has established competence in the use of the assay.”

ICH Guidance on Genotoxicity Testing and Data Interpretation for
Pharmaceuticals Intended for Human Use S2(R1) Current version
dated 6 March 2008



Multiple Tissues Collected from the Same Animals After 4 hrs



Target Organs Tested at Helix3

Adrenal gland

Blood

Bone marrow

Cecum

Colon

Duodenum

Hypothalamus

Kidney

Liver

Lung

Mammary gland

Ovary

Pineal gland

Pituitary gland

Prostate

Skin

Spleen

Stomach

Testis

Thyroid gland

Urinary bladder

Uterus



Use of Positive Controls

- For comet, merely demonstrating control of the staining and scoring methods is **NOT** the critical factor for a valid test
- A **concurrent** positive control dose group is necessary **in each study** to verify the appropriateness of the experimental conditions
- A **concurrent** positive control is necessary **for each tissue sampled** to verify the sensitivity of the assay in that tissue
- Excluding a positive control group of 5-6 animals can result in the necessity for repeat studies (>6 animals)



If “...repeated dose 14-28-day tox studies are used to characterize the toxicity [and/or] pharmacokinetics of a test compound”¹

and

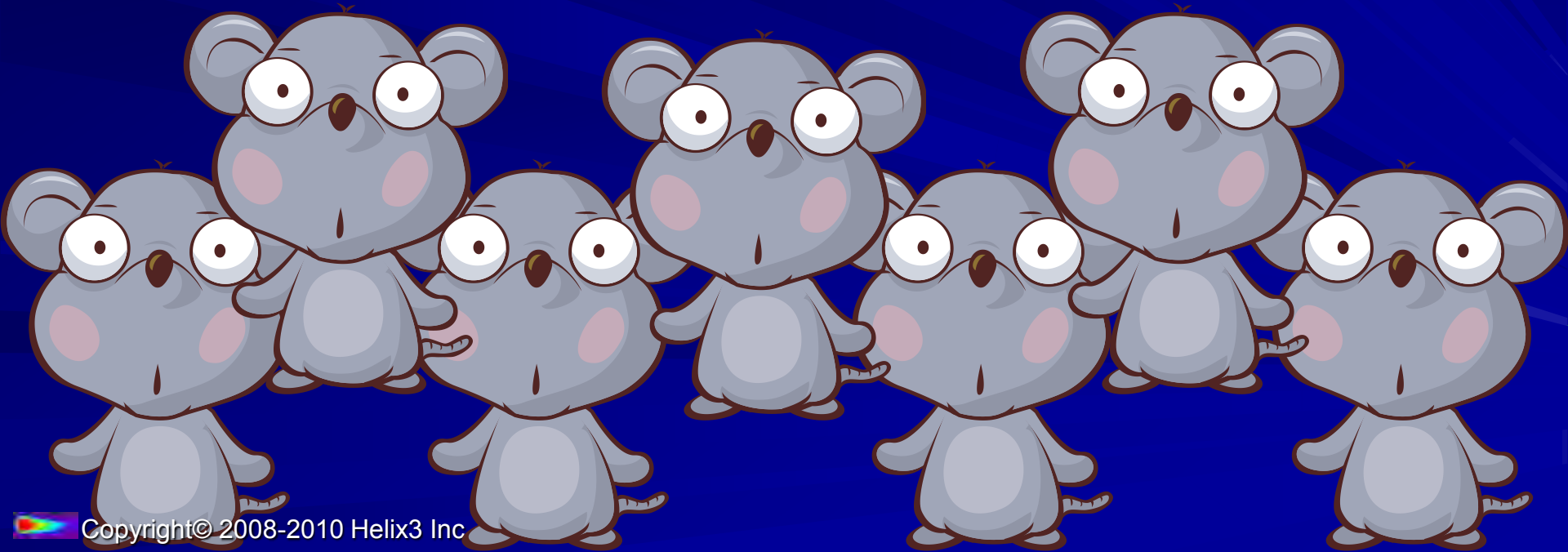
the comet assay is integrated into these ongoing studies ...

¹OECD Guideline 407 for the Testing of Chemicals (Adopted July 1995, Updated May 2007)

- ④ How will you ensure that tissue samples are collected from animals exposed to non-cytotoxic doses?
- ④ How will you ensure tissues are sampled at the appropriate time (T_{\max})?
- ④ How will you ensure the tissues in which the test article is most concentrated are the ones sampled for comet?

Use more animals!

- Additional dose groups
- Larger dose groups
- Wasted time, effort, and animals



Summary

- Guidelines specific to comet must be drafted—not just borrowed from other assays
- Poorly planned comet studies will waste more time and more lives than it will save



Summary

- The comet assay provides valuable organ-specific and multiple organ risk assessment data
- The flexibility and sensitivity of comet can provide critical mechanistic / WOE information
- Well planned comet studies can minimize both the use of resources and the risk to humans