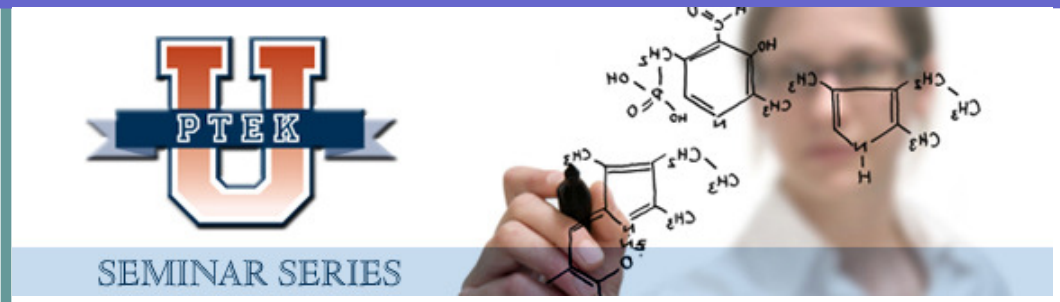
Genotoxicity Testing for Pharmaceuticals Current and Emerging Practices



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17 April 2008

Nonclinical Safety Testing

- The majority of small molecule nonclinical safety programs are comprised of 3 major components
  - Safety pharmacology
  - **Genetic toxicology**
  - Mammalian toxicology

Significance of Genotoxic Effects

- Gene mutations
  - Correlated to carcinogenicity and turmorigenicity
- Chromosomal aberrations
  - Can lead to DNA damage, in turn leading to abnormal and/or carcinogenic growth of cells

Genetic Toxicology
ICH Guidelines

- S2A Guidance on Specific Aspects of Regulatory Tests for Pharmaceuticals (July 1995)
- S2B Genotoxicity: A Standard Battery for Genotoxicity Testing for Pharmaceuticals (July 1997)

ICH Standard Test Battery

1. Test for gene mutation in bacteria
2. In vitro test with cytogenetic evaluation of chromosomal damage with mammalian cells **or** an in vitro mouse lymphoma tk assay
3. In vivo test for chromosomal damage using rodent hematopoietic cells

ICH Standard Test Battery

The test battery approach is designed to reduce the risk of false negative results for compounds with genotoxic potential, whereas a positive result in any assay for genotoxicity does not necessarily mean that the test compound poses a genotoxic/carcinogenic hazard to humans.

Genotoxicity of Marketed Pharmaceuticals

- 1999 PDR & peer-reviewed literature
- 467 marketed drugs
  - Excluding anti-cancer, nucleosides, steroids, biologicals & peptide-based drugs
    - 115 had no published genetox data
      - Acutely administered (antibiotics, antifungals, anthistamines, anesthetics)
    - 352 had at least one standard genetox test result
      - 101 (29%) had at least one positive assay
        - Bacterial mutation 27/323 (8%)
        - *In vitro* cytogenetics 55/222 (25%)
        - Mouse tk lymphoma 24/96 (25%)
        - *In vivo* cytogenetics 29/252 (12%)

ICH Standard Test Battery

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The Bacterial Reverse Mutation (Ames) Assay

- Detects relevant genetic changes & majority of genotoxic rodent carcinogens
- *Salmonella typhimurium*
  - Defect in one of the genes involved in histidine biosynthesis
- *Escherichia coli*
  - Defect in one of the genes involved in tryptophan biosynthesis

The Bacterial Reverse Mutation (Ames) Assay

- Use of several bacterial tester strains enables detection of base substitution mutagens and frame-shift mutagens
- Bacterial tester strains
  1. TA98
  2. TA100
  3. TA1535
  4. TA1537 or TA97 or TA97a
  5. TA102 or E. coli WP2 uvrA or E. coli WP2 uvrA (pkM101)

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In Vitro Cytogenetic Assays

- Mammalian cell test systems
  - CHO cells
  - CHL cells
  - HPBLs
- Division stimulated with phytohemagglutin
- Division arrested in metaphase using a spindle inhibitor
- Evaluation by light microscopy

In Vitro Cytogenetic Assays

Chromatid gap	Translocation	Ring chromosome
Chromasome break	Triradial	Dicentric chromosome
Chromosome gap	Quadraradial	Minute chromosome
Chromatid deletion	Pulverized chromosomes	More than 10 aberrations
Fragment	Pulverized cells	Polyploidy
Acentric fragment	Complex rearrangement	hyperdiploid

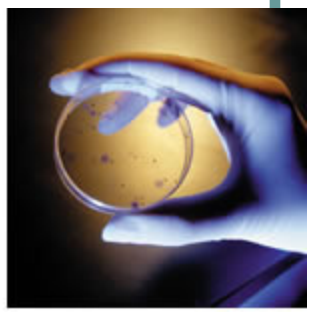
- A biologically significant increase in frequency of cells with structural or numerical aberrations indicates clastogenic or aneugenic effects, respectively.

In Vitro Mouse Lymphoma $TK^{+/-}$ Assay

- Mouse $TK^{+/-}$ lymphoma cells
  - Contain thymidine kinase (TK) enzyme involved in salvage pathway for incorporation of thymidine into cells via phosphorylation
  - Trifluorothymidine (TFT) also phosphorylated by TK
  - Cells containing TK are sensitive to toxic effects of TFT
  - Forward mutations from $TK^{+/-}$ to $TK^{-/-}$ result in loss of TK activity and acquisition of TFT resistance
  - Mutant cells quantitated by cloning in soft agar medium supplemented with TFT

In Vitro Mouse Lymphoma $TK^{+/-}$ Assay

- Assay can detect
  - Gene mutations
    - Point mutations involving base substitutions, deletions, frame shifts and rearrangements
  - Changes in chromosomal integrity
    - Clastogenic lesions involving multiple genes and multi-locus deletions
  - Chromosome loss
- Colony sizing can distinguish mutagens from clastogens



Which *in vitro* test system is best?

- No one test system is superior to the others
- The various in vitro tests are considered interchangeable
- *However, comparative trials have shown conclusively that each in vitro test system generates both false negative and false positive results in relation to predicting rodent carcinogenicity.*

ICH Standard Test Battery

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In Vivo Tests for Chromosomal Damage

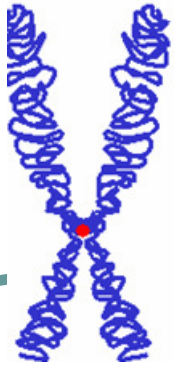
- Assess the potential for DNA damage that may affect chromosome structure or interfere with the mitotic apparatus causing changes in chromosome number
- Include relevant factors which may influence genotoxic activity
  - ADME
  - DNA repair
- May detect some genotoxic agents ‘missed’ in *in vitro* systems

In Vivo Tests for Chromosomal Damage

1. Mammalian Bone Marrow Chromosomal Aberration Assay
2. Rodent Erythrocyte Micronucleus Assay

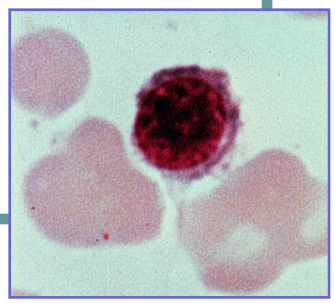
Mammalian Bone Marrow Chromosomal Aberration Assay

- Mice, rats or Chinese hamsters
- Bone marrow target tissue
- Cell division arrested 3-5 h prior to sacrifice
- Bone marrow cells examined by light microscopy
- Data similar to that reported for in vitro cytogenetics assays
  - Clastogenic effect
  - Aneugenic effect



Rodent Erythrocyte Micronucleus Assay

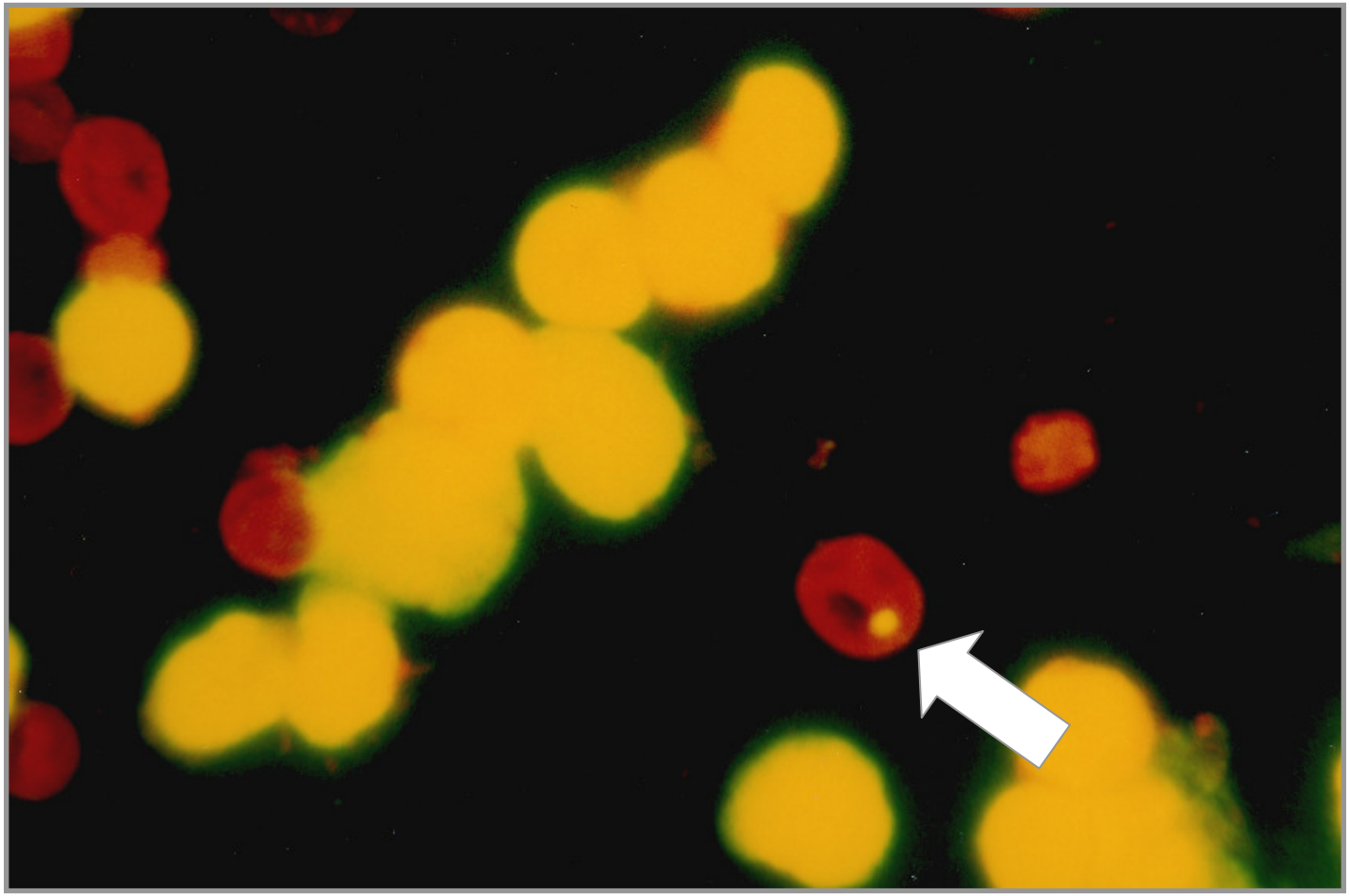
- More common assay (vs. bone marrow chromosomal aberration assay)
- Rats or mice
- Endpoint is frequency of micronucleated PCEs (MPCEs)
  - In bone marrow
  - In peripheral blood
- Methods of analysis
  - Manual enumeration by light microscopy
  - Automated analysis
- An increase in the frequency of MPCEs in treated animals is an indication of induced chromosome damage



What the heck is a micronucleus??

- A small structure containing nuclear DNA that has arisen from chromosome fragments or whole chromosomes that were not incorporated into daughter nuclei at anaphase of mitosis
- Formed by
  1. Mitotic loss of acentric chromosome fragments
  2. Mechanical consequences of chromosomal breakage and exchange
  3. Mitotic loss of whole chromosomes
  4. Apoptosis

What the heck is a micronucleus??



ICH Standard Test Battery (ca. 1996-today)

- Test for gene mutation in bacteria
- In vitro test with cytogenetic evaluation of chromosomal damage with mammalian cells *or* an in vitro mouse lymphoma tk assay.
- In vivo test for chromosomal damage using rodent hematopoietic cells.

In Vitro Micronucleus Assay

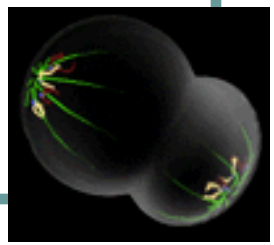
- Not an accepted component of the ICH standard test battery (circa 1996), but…

The continuing evolution of short-term tests and test methodologies will afford new, more sensitive, more practical, more expeditious, and more economical techniques for detection of genotoxic compounds. Some of these may ultimately replace the genotoxicity tests used for regulatory purposes. Among the more promising tests, the *in vitro* micronucleus test appears to offer potential for screening purposes.

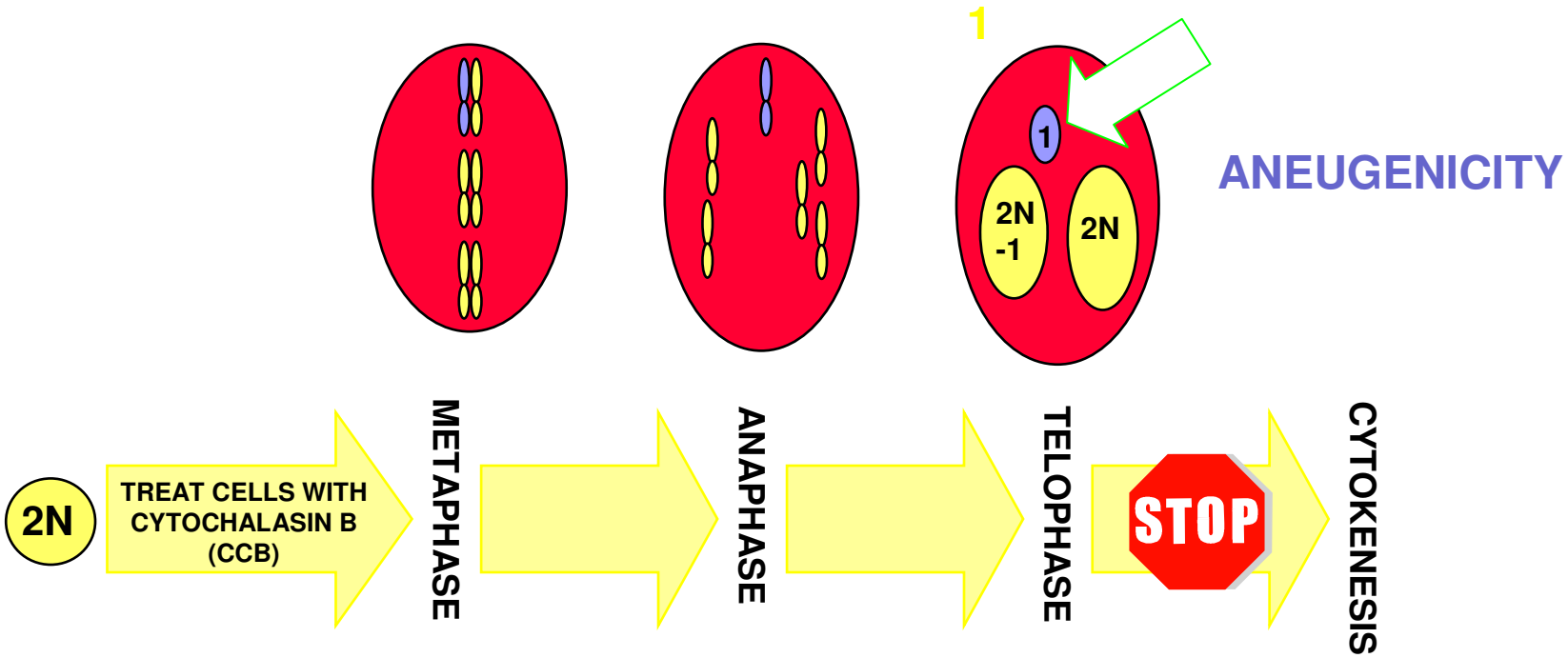
From: ICH Tripartite Guideline Genotoxicity: A Standard Battery for Genotoxicity Testing for Pharmaceuticals (S2B)

In Vitro Micronucleus Assay

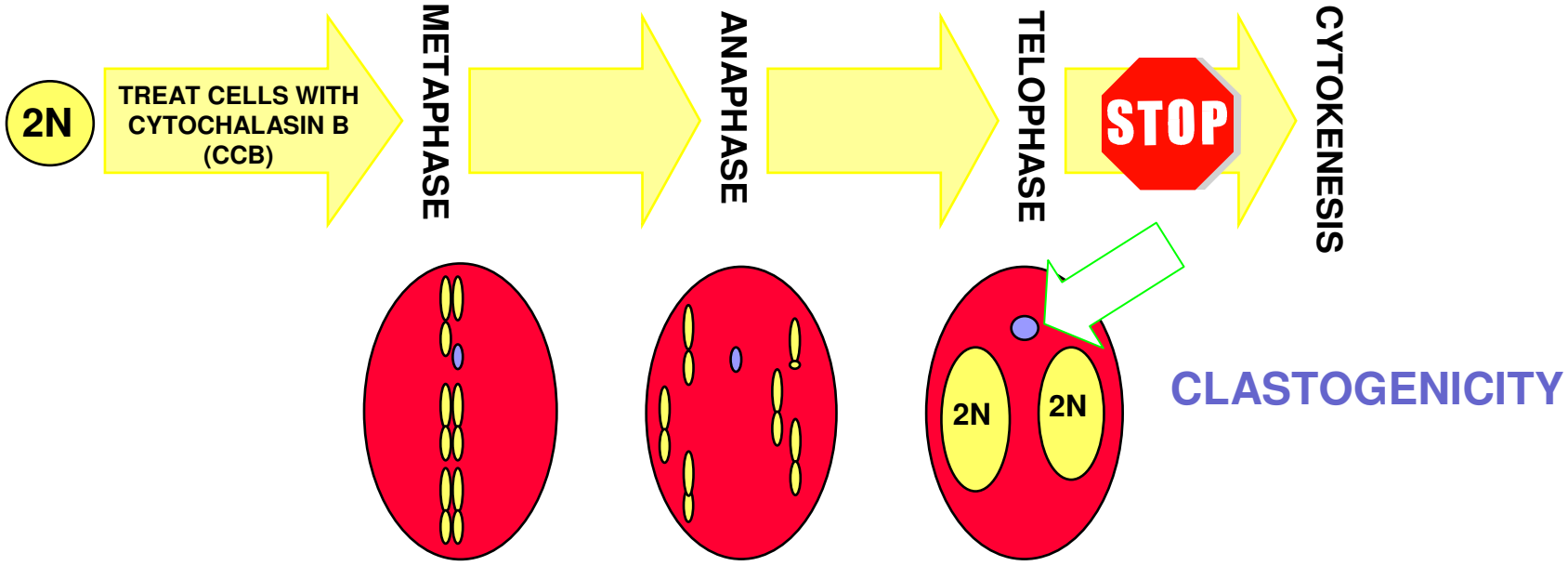
- Cell lines or human lymphocytes
- Micronuclei examined in binucleate cells



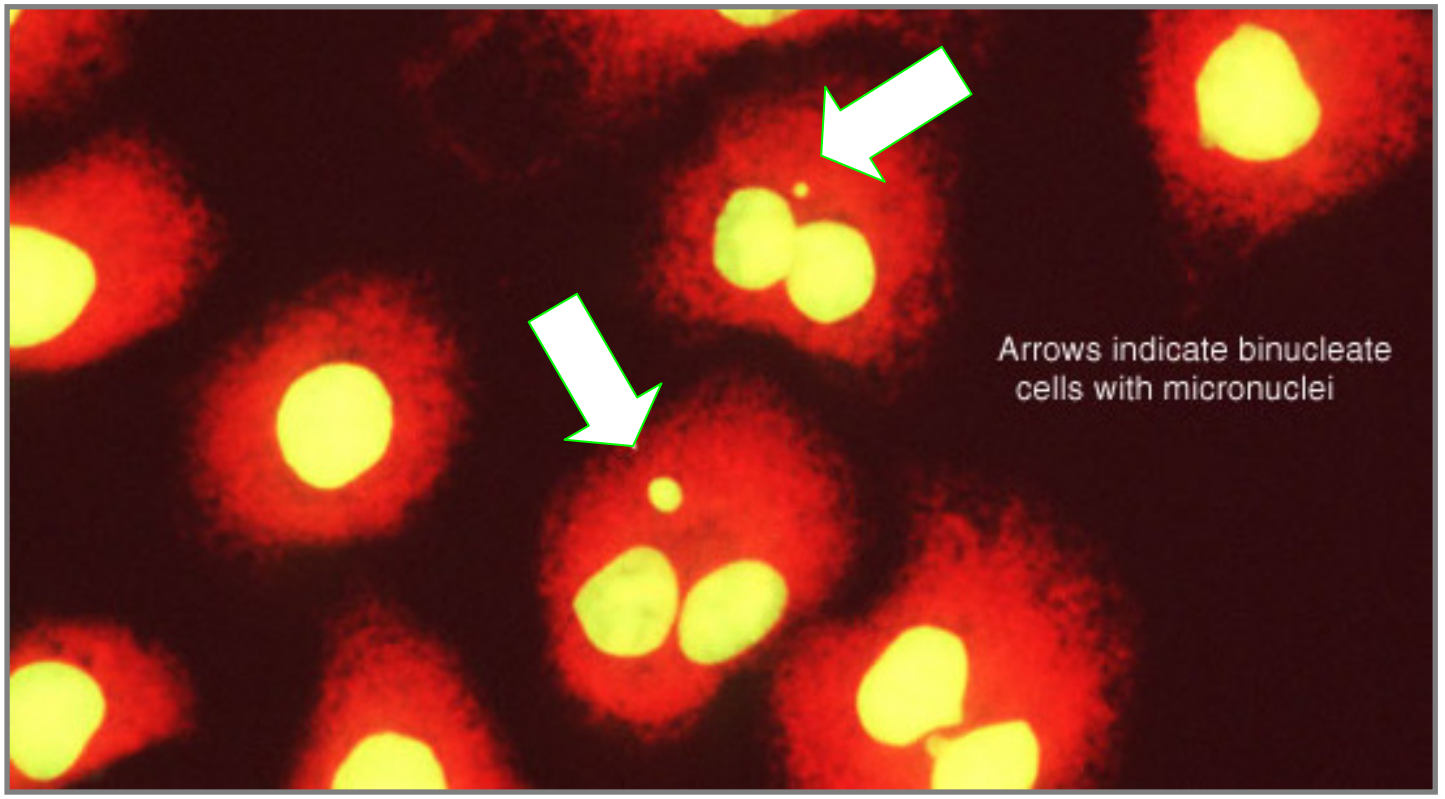
In Vitro Micronucleus Assay



In Vitro Micronucleus Assay



In Vitro Micronucleus Assay



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- **S2(R1): Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use**
  - Released for consultation (Step 3) March 2008

ICH Standard Test Battery

New & Improved!

2 OPTIONS

Option #1

1) Test for gene mutation in bacteria
2) A cytogenetic test for chromosomal damage (*in vitro* metaphase chromosome aberration test or *in vitro* micronucleus test) *or* an *in vitro* mouse lymphoma tk gene mutation assay
3) 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

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New & Improved!

2 OPTIONS

Option #2

1) Test for gene mutation in bacteria
2) An *in vivo* assessment of genotoxicity with two tissues, usually an assay for micronuclei using rodent hematopoietic cells and a second assay.
    - Second assays
        - DNA strand break assays such as the single cell gel electrophoresis (“Comet”) assay and alkaline elution assay
        - i*n vivo* transgenic mouse mutation assays
        - DNA covalent binding assays
        - liver unscheduled DNA synthesis (UDS) assay

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Options #1 and #2

Under both standard battery options, the *in vivo* genotoxicity assays can often be integrated into repeat-dose toxicity studies when the doses are sufficient.

ICH Standard Test Battery

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SUMMARY OF MAJOR POINTS OF REVISIONS

- S2A and S2B guidance documents merged into one
- Options provided for the test battery
  - Battery with in vitro mammalian cell assay
  - Battery without in vitro mammalian cell assay but two in vivo endpoints
- In vitro mammalian cell assay
  - Reduction in top concentration from 10 mM to 1 mM
  - Tightened acceptable cytotoxicity limits
  - No longer requires testing of precipitating concentrations
- Bacterial reverse mutation assay no longer requires duplicate assay

ICH Standard Test Battery

New & Improved!

SUMMARY OF MAJOR POINTS OF REVISIONS

- Integration of genotoxicity endpoints into routine toxicology studies
  - Stringent criteria defined for acceptability of top dose
- Advice on choice of second in vivo genotoxicity endpoint
  - Includes option of the Comet assay and decreases emphasis on liver UDS assay
- Provides advice on weight of evidence and data evaluation to determine relevance of positive findings

ICH Standard Test Battery

New & Improved!

Benefits of test battery revision include:

- Shorter development timelines
- Incorporates accumulated knowledge specific to testing of pharmaceuticals
- Takes advantage of new(er) technologies
- More strategic options for genotoxicity testing
- Reduction in delays caused by dealing with ‘non-relevant’ in vitro positive genotoxicity results
- More efficient use of dollars, API and animals
- Improved interpretation of genotoxicity data while allowing continued safety for patients and volunteers

ICH Standard Test Battery

New & Improved!

S2(R1) published in the Federal Register, March 26, 2008, Volume 73, Number 59, Page 16024-16025. Deadline for comments: May 10, 2008.

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