の検討を行う
⑤プレバリデーション終了後には半年程度のデータ解析期間をおき、会議を開いて次の Phase への進行に関して議論する
⑥厚生科学研究の延長を考えても後 5 年で完結したい
⑦OECD ガイドラインへの提案は来年度を目処にしたい（プレバリデーション終了後？）
⑧被験物質の選択が重要（実行委員会で陽性強度、陰性数のバランスを考慮して対応する：林実行委員長）
などの方向性が固まった。

E.結論

画像解析ソフトによるばらつきの検討および国際共同研究の予備試験結果から、統一した試験手順書を確立でき、プレバリデーションを実施する準備が整った。主なプロトコール作成上の決定事項を以下に示す。
①Crl:CD(SD)ラット雄　7-9 週令を 5 匹／群使用、
②投与回数 2 回（投与 21 時間後に 2 回目投与し、その 3 時間後にサンプリング、
③適用臓器は胃および肝臓、
④単一細胞を使用、
⑤電気泳動は冷蔵で実施、
⑥指標はテールに含まれる%DNA 量の平均値

F.健康危険情報

なし

G.研究発表

G-1）論文発表

1）小島肇夫、動物実験代替に関する最近の動向、化粧品技術者会誌、40(4)263-268（2006）
2）小島肇夫、JaCVAM の設立と使命、日皮協ジャーナル、57、129（2007）

G-2)学会発表

1) H.Kojima, Current activities on alternative research in Japan, KSOT/KEMS Spring Annual Meeting, Alternative Toxicology and Marine Ecotoxicology, Korea(2006)
2) 小島　肇、JaCVAM (Japanese Center for the Validation of Alternative Methds) 新規試験法評価室の紹介、日本環境変異原学会　MMS 研究会第 49 回定例会、熱川（2006）
3) H. Kojima, JaCVAM Update, Scientific Advisory Committee on Alternative Toxicological Methods, North Carolina (2006)
4) 小島　肇、JaCVAM の設立と使命、日本産業皮膚衛生協会第 39 回研修会、京都（2006）
5) 小島　肇、特別企画 1 ：動物実験代替法に関する最近の国内外の動向、国内において現在進行中の評価試験プロジェクト紹介、日本動物実験代替法学会第 20 回大会、東京（2006）
6) 小島　肇、動物実験代替法開発の推進とその評価：JaCVAM の設立と役割、日本薬学会第 127 年会、富山（2007）

H.知的財産権の出願、登録状況

なし

I.参考文献

1) 日本トキシコロジー学会教育委員会編集、トキシコロジー、p 142、朝倉書店(2002)
2) FDA　Guidance、
http://www.fda.gov/cder/guidance/index.htm
3) Burlinson B, Tice RR, Speit G, Agurell E, Brendler-Schwaab SY, Collins AR, Escobar P, Honma M, Kumaravel TS, Nakajima M, Sasaki YF, Thybaud V, Uno Y, Vasquez M, Hartmann A; In Vivo Comet Assay Workgroup, part of the Fourth International Workgroup on Genotoxicity Testing Fourth International Workgroup on Genotoxicity testing: results of the in vivo Comet assay workgroup. Mutat Res. 627(1):31-5(2007)
4) Hartmann A, Agurell E, Beevers C, Brendler-Schwaab S, Burlinson B, Clay P, Collins A, Smith A, Speit G, Thybaud V, Tice RR; 4th

International Comet Assay Workshop. Recommendations for conducting the in vivo alkaline Comet assay. 4th International Comet Assay Workshop, Mutagenesis. 18(1), 45-51(2003)

J.添付資料

添付資料 1：MMS seminor -The Pros Con for Comet Assay-

添付資料 2：JaCVAM/MMS seminor 参加者リスト

添付資料 3：Draft International Validation Study Management Chart

添付資料 4：Comet Assay Meeting in August 2006

添付資料 5：Comet Assay Meeting in December 2006

添付資料 6：コメットアッセイ用画像解析ソフトによる結果のばらつきの検討 by Koji Yamakage, FDSR

添付資料 7：JaCVAM initiative International validation on in vivo and in vitro comet assay

添付資料 8：International validation study of in vivo & in vitro come t assay by Makoto Hayashi, NIHS

添付資料 9：OECD Activities on Chemical safety by Nobumasa Nakashima, OECD

添付資料 10：The in vitro Comet assay -Study Plan- by Masaatsu Honma

添付資料 11：Memo about statistical test by Takashi Ohmori, Kyoto Univ.

添付資料 12：About data sheet ver.1.0 for our validation study by Takashi Ohmori, Kyoto Univ.

添付資料 13：Comet Assay Validation: modular approach by Thomas Hartung, ECVAM

添付資料 14：Protocol International Validation of the in vivo Rodent Alkaline Comet Assay for the Detection of Genotoxic Carcinogens (Version 10)

添付資料 15：Draft Minutes International Validation Kick off Meeting on Comet Assay

添付資料 16：Comet -Pre-Validation Study- Check Sheet: Protocol/ SOP

添付資料 17：Data Management Issue

添付資料 18：Before examinations ~Endpoint, Estimate and Effect~

添付資料 19：Data analysis of the pre-validation study with EMS in five leading laboratories

添付資料 20：Data on the protocol ver.10

添付資料 21：One side Dunnett's test

添付資料 22：Slide-to-slide variation by Takashi Ohmori, Kyoto Univ.

添付資料 23：Protocol International Validation of the in vivo Rodent Alkaline Comet Assay for the Detection of Genotoxic Carcinogens (Version 11)

添付資料 24：Draft Minutes The 2nd International Validation Meeting on Comet Assay

添付資料 25：In vivo Comet Assay -International Validation- Plan in 2007-2010 (Draft)

syngenta

Use of the Comet Assay for Human Risk Assessment.

A case study in the gastro-intestinal tract.

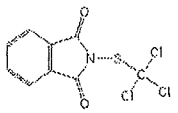
Philip Clay
Syngenta CTL

Introduction

- **Accidental exposure or operator exposure to chemicals can lead to site of contact toxicity.**
- **Standard *in vivo* Genetic Toxicity tests detect systemic effects**
  - UDS – liver
  - Micronucleus – bone marrow, blood
  - Cytogenetics – blood
- ***In vivo* comet assay can measure genotoxicity in site of contact tissues**
  - Skin
  - Respiratory tract (nasal tissue, lung, etc)
  - Gastro-intestinal tract (stomach, intestine, etc)
- **Measurement of genotoxic effects in a relevant tissue**

Gastro-Intestinal Tract

- **Folpet:**
  - phthalimide fungicide

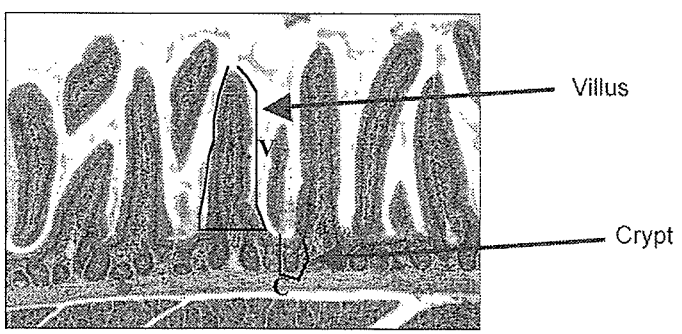


  - Widely used in commercial agriculture on food and oil crops

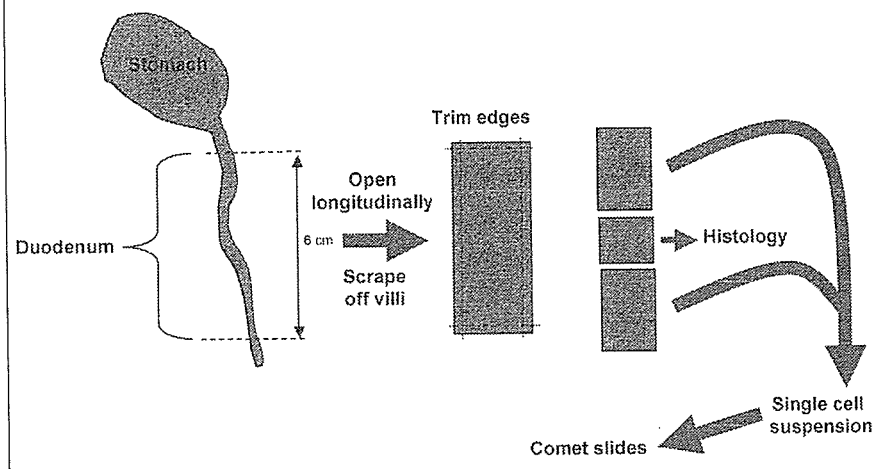
- **Potential for operator exposure during application and ingestion from residues on food crops. Possible exposure of GI tract.**
- **Lifetime studies in mice show adenocarcinomas in the duodenum**
- **Mechanistic studies show proliferative changes in the crypt region of the duodenum**
  - Stem cells
  - Cell division
- **Cells specifically from the crypt region of interest when investigating the possible genotoxic origin of the tumours as part of the human health risk assessment**

Duodenal Crypt Comet

- **Investigate genotoxic origin of duodenal tumours**
- **Develop methods to sample predominantly crypt cells**



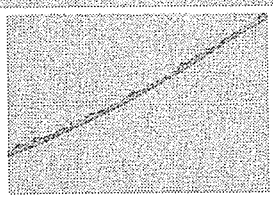
Duodenal Crypt Comet



5

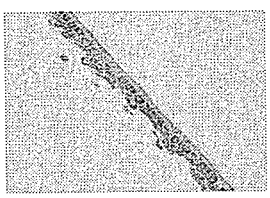
syngenta

Histology



Crypt cells missing - unacceptable

The tissue has been over scraped and the crypts removed as well as the villi



Villi mainly absent – ideal

The majority of the villi have been removed and the crypts remain intact

Villi partially absent – acceptable

Some villi remain intact; there will be some contamination of the Sample with villus cells. Animals from this category were only used if sufficient animals showing villi mainly absent were not available

6

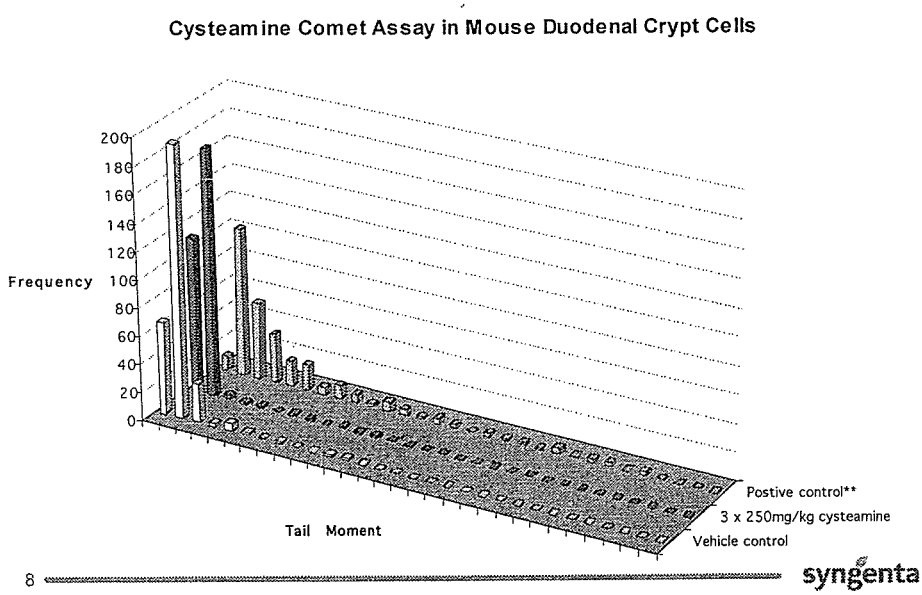
syngenta

Cytotoxicity vs Genotoxicity

- Folpet induced adenocarcinomas associated with continued proliferative pressure due to irritation and loss of villi
- Need to demonstrate that any effects in Comet assay not caused by similar tissue toxicity
- Cysteamine (3 x 250 mg/kg) caused similar pathological changes in duodenum – non genotoxic
- Effects of cysteamine investigated in Comet assay

syngenta

Cysteamine Results



syngenta

syngenta

Study Design - Folpet

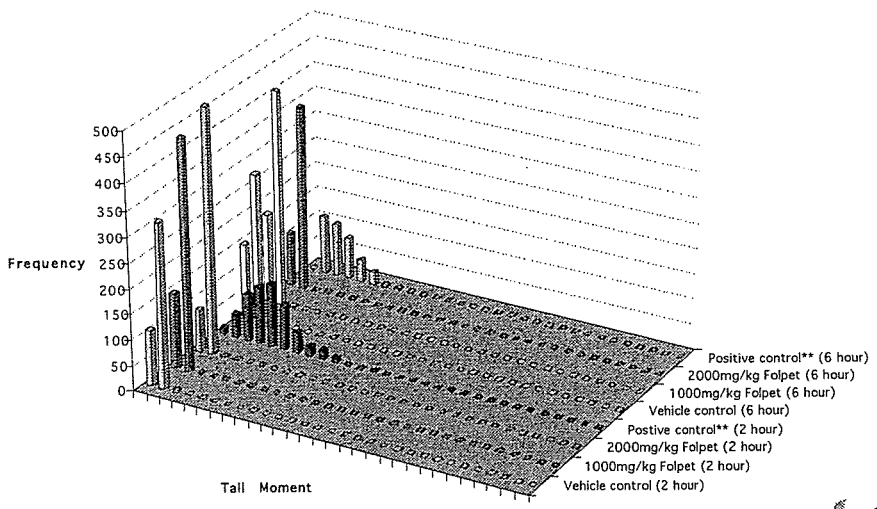
- CD-1 mice - consistency with oncogenicity study
- Females – slightly more sensitive in oncogenicity study
- Dose levels (2000 mg/kg and 1000 mg/kg) based on absence of adverse clinical and pathological effects in dose ranging
- Concurrent vehicle and positive control groups
- 2 sampling times (2 and 6 hours post dose)
- 8 animals per group dosed, 4 per group showing optimal villus removal selected for analysis
- 50 cells/slide, 3 slides per animal analysed

Results

Treatment	Dose (mg/kg)	No. of Animals	No. of cells scored	Mean % tail DNA
2 hours sampling time				
1% CMC	10ml/kg	4	435	0.93 ± 0.47
Folpet	1000	4	600	0.81 ± 0.11
Folpet	2000	4	568	1.53 ± 0.82
MNU	100	4	538	54.75** ± 8.02
6 hours sampling time				
1% CMC	10ml/kg	4	439	0.85 ± 0.44
Folpet	1000	4	600	0.72 ± 0.19
Folpet	2000	4	471	1.11 ± 0.62
MNU	100	4	373	36.84** ± 7.20

Results

Folpet Comet Assay in Mouse Duodenal Crypt Cells



syngenta

Conclusions

- The testing of negative (cysteamine) and positive (MNNG, MNU) controls in these experiments demonstrated that the techniques used are not compromised by the testing of chemicals which can cause pathological damage to the tissue examined and also the ability of the techniques to detect genotoxic chemicals
- Folpet does not induce DNA damage (as measured by Comet formation) in the mouse duodenum in vivo
- These data are consistent with the conclusion that the duodenal tumours seen in the oncogenicity studies on Folpet are non-genotoxic in origin and support the favourable human risk assessment based on these, and other, information

syngenta

syngenta

Further studies in the GI Tract

- Effects of a chemical with an aldehyde type structure investigated in the mouse fore-stomach

- Aldehyde type structures associated with tumour formation in mouse fore-stomach in lifetime oncogenicity studies
- Positive response in the *in vitro* cytogenetics assay
- Male $B_6C_3F_1$ mice, single oral dose, 800 mg/kg (MTD) and 400 mg/kg. Sampled 2 and 16 hours post dose

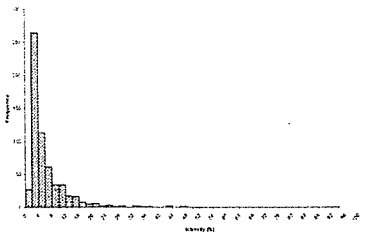
Results - Aldehyde

Treatment	Dose	Group Mean Tail Intensity± SD (%)	
		2 hours	16 hours
Vehicle Control	10 ml/kg	4.3±0.9	4.5±1.2
Test substance	400 mg/kg	7.7*±2.5	5.7±1.3
Test substance	800 mg/kg	9.5±7.8	6.1±2.7
Positive Control	100 mg/kg	65.0**±10.4	38.8**±5.3

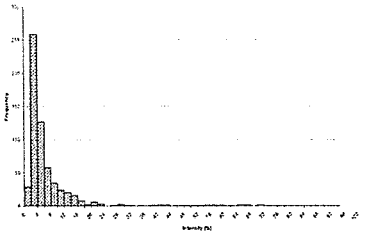
All values based on 4 animals/group
SD = standard deviation
* = statistically significant by Student's T-Test, $P < 0.05$
** = statistically significant by Student's T-Test, $P < 0.01$

Results - Aldehyde

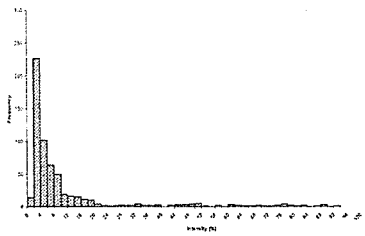
Control – 2 hour



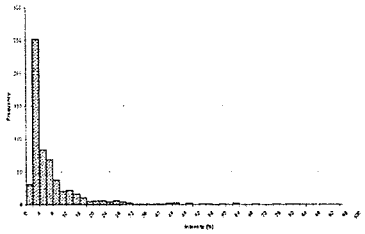
Control – 16 hour



Treated – 2 hour



Treated – 16 hour



15 syngenta

Conclusions - Aldehyde

- Dose related increases in tail intensity at the 2 hour sampling time.
  - Statistical significance at the lower dose level
- Increases in tail intensity at the 16 hour sampling time.
  - Smaller than those seen at the 2 hour sampling time and not statistically significant. Dose related and consistent with the effects seen at the 2 hour sampling time
- Distribution of tail intensities for individual cells within treatment groups shows clear differences between vehicle and test substance treated groups at both sampling times. Much more marked at the 2 hour sampling time.
- Also considered – other parameters, published data for fore-stomach, robust MTD
- Concluded to be a weak positive effect

16 syngenta

syngenta

Comet Assay

- Can be performed in (almost) any tissue
- Tissue examined can be:
  - Relevant to human exposure
  - Relevant to effects seen in experimental animals
- Valuable in human risk assessment

Acknowledgements

- Sponsors
  - Makhteshim Chemical Works Ltd
- Co-workers
  - Emma Goodhead
  - Ranbir Arif
  - Katie Wood

The End

syngenta

Animal Selection

Animal Number	Crypt cells missing	Villi Mainly Absent	Villi Partially absent	Villi Mainly present
1			•	
2		•		
3		•		
4	•	•		
5		•		
6		•		
7		•		
8			•	
9		•		
10		•		
11		•		
12			•	
13			•	
14			•	
15		•		
16		•		
17			•	
18			•	
19	•	•		
20		•		
21			•	
22		•		
23		•		
24		•		
25		•		
26	•	•		
27		•		
28		•		
29	•	•		
30		•		
31		•		
32		•		

• selected for analysis

syngenta

syngenta

CASE STUDY 2 –
Sensitivity of the alkaline Comet Assay to buffer temperature during unwinding and electrophoresis

Escobar Patricia, Do Chuong and Joseph Michael
Genetic Toxicology Department
BioReliance, Invitrogen BioServices

- **Definition**
- **Methodology**
- **Study Designs**
- **Results – Case Study**

Comet Assay

Single Cell Gel Electrophoresis

Micro-electrophoretic technique which detects DNA damage and repair in individual cells

Assay based upon the ability of denatured, cleaved DNA fragments to migrate out of the cell under the influence of an electric field

Comet Assay

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

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

Comet Assay

Under alkaline condition can detect DNA double-strand breaks, single-strand breaks and/or strand breaks induced by alkali-labile sites

Levels of DNA damage is correlated to the length and amount of fragmented DNA that migrates outside the cell nucleus (comet tail)

Advantages

Almost any eukaryotic cell population can be used (different 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)

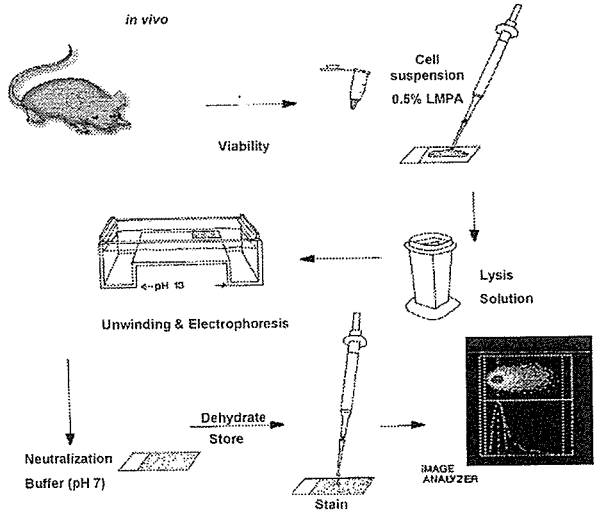
Why do Comet Assay?

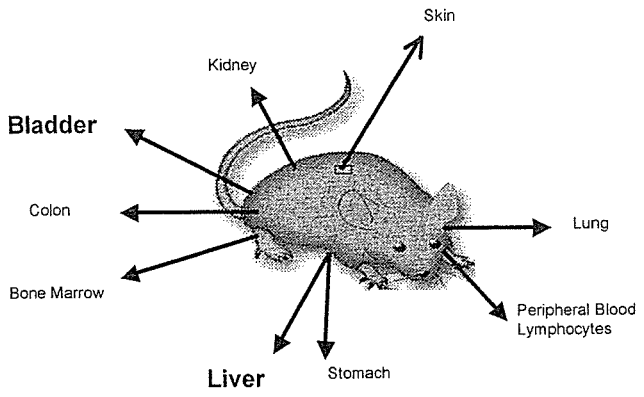
- Genotoxic mechanism
- Analyze possible target organ genotoxicity
- Support carcinogenicity risk evaluation

Why do Comet Assay?

- Investigate the *in vivo* relevance to positive *in vitro* genotoxicity
- Elucidate contribution of genotoxicity to tumor development and target organ-specific gentotoxicity.

METHODOLOGY





Cell Suspension

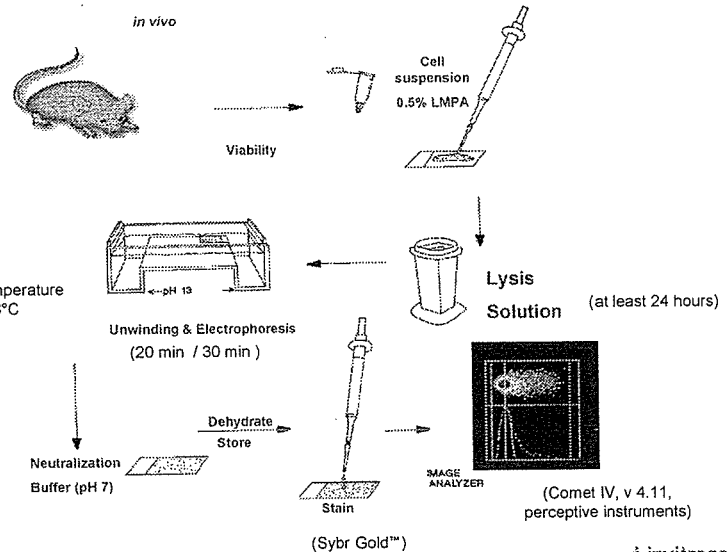
Mincing → **Liver**, Lung, Kidney

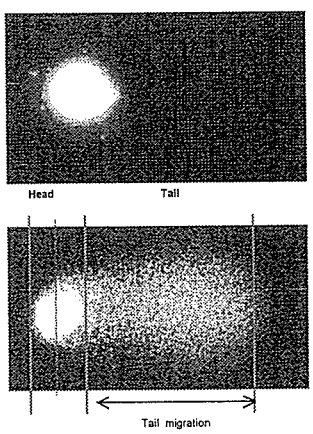
Scraping → Stomach, Colon, **Bladder**

Aspiration → Bone Marrow, Peripheral blood

Enzymatic Digestion → Skin

Comet Assay





Tail Length

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

% Tail Migration

Amount of DNA fragments in the tail

Olive Tail Moment

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

Tail Migration

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

EXPERIMENTAL DESIGN

- 6 males mice per group
- Three Test Compounds
- Positive and vehicle controls
- Oral gavage administration
- Collection time 3 hours
- Tissues→ **Liver & Bladder**