The establishment of the *in vitro* Comet assay using six genotoxic compounds as positive controls

Patricia Ordoñez[1], David Thorne[2], Annette Dalrymple[2], Ana Belén Sierra[1], Antonio Vila-Coro[1], Pilar Prieto[1], Debbie Dillon[2], Clive Meredith[2]

Corresponding author: ordonez@vivotecnia.com

[1] VIVOTECNIA Research S.L., Parque Científico de Madrid, C/Santiago Grisolía, 2 (PTM), 28760 Tres Cantos, Madrid, Spain

[2] British American Tobacco, Group R&D, Southampton, Hampshire, SO15 8TL, UK

INTRODUCTION

- **The Comet assay is a sensitive & rapid method to measure DNA damage[1-2]**
- **We have used the *in vitro* Comet assay & 6 positive control genotoxic compounds to determine DNA damage by:**
  1. **The Alkaline Comet assay which detects DNA strand breaks, alkali-labile sites (ALS) & DNA-DNA/DNA-protein cross linking**
  2. **The Modified Alkaline Comet assay which identifies oxidised purine & pyrimidine lesions by using formamidopyrimidine DNA glycosylase (FPG) & endonuclease III (ENDO III) repair enzymes, respectively**
- **A published Statistical method[3] was utilised to evaluate the data**

METHODS

❖ Comet assay method

Cell culture

- ✓ NCI-H292 cells were supplied by ATCC (http://www.lgcstandards-atcc.org)
- ✓ The day before Comet analysis, cells were seeded in 6-well plates at $1.2x10^6$cells/well
- ✓ **Alkaline Comet assay:** Confluent H292 cells were incubated for 1 hour at 37ºC with etoposide (250 µM), methyl methanesulfonate (MMS, 750 µM), ethyl methanesulfonate (EMS, 60 mM), hydrogen peroxide (H_2O_2, 400 µM), acetoxymethyl nitrosamino-1-(3-pyridyl)-1-butanone (NNKOAc, 150 µM) or vehicle (0.5% DMSO or 1 x PBS)
- ✓ **Modified Comet assay:** Confluent H292 cells were incubated for 1 hour at 37ºC with two concentrations of potassium bromate ($KBrO_3$, 2.0 mM or 10.0 mM) or vehicle (1 x PBS)

Alkaline & Modified Comet assay procedure

- ✓ **Cell sample preparation:** ~20,000 cells were resuspended in 0.6% low melting-point agarose at 37ºC & placed on Superfrost slides pre-coated with 1.0% collagen & 1.5% normal melting-point agarose
- ✓ **Lysis:** Slides were placed in *Lysis buffer* (2.5 M NaCl, 100 mM EDTA,10 mM Trizma, 0.2 M NaOH, 10% DMSO & 1% Triton X-100, pH 10) for 24h at 5ºC ± 3
- ✓ **Wash:** Slides were rinsed (3x 5 min) with *Enzyme reaction buffer* (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL BSA, pH 8) at room temperature (RT)
- ✓ **Enzyme incubation (Modified Alkaline Comet assay only):** Slides were incubated with FPG (New England BioLabs®) or ENDO III (New England BioLabs®) diluted 1:5,000 or 1:10,000, respectively, in *Enzyme reaction buffer* for 45 min at 37ºC
- ✓ **Alkaline unwinding:** Slides incubated with *Electrophoresis buffer* (1 mM EDTA, 300 mM NaOH, pH >13) within the electrophoresis tank (Thistle Scientific) for 5 min at 5ºC ± 3
- ✓ **Electrophoresis:** 20 min at 25 constant volts in 1.0L *Electrophoresis buffer at* 5ºC ± 3
- ✓ **Neutralisation:** ~3.5 mL of *Neutralisation buffer* (0.4 M Trizma, pH 7.5) was placed on each slide
- ✓ **Fixation:** Slides were air dried for approximately 24h at RT
- ✓ **Comet visualisation:** 20 µL of VECTASHIELD Mounting Medium containing DAPI (Vector Laboratories Inc.) was applied to slides & nuclei visualised using 20x magnification

❖ Determination of cell viability

- ✓ Cell viability was determined by trypan blue dye exclusion using an automated Vi-CELL® cell viability analyser (Beckman-Coulter)

❖ Statistical analysis

- ✓ ~100 cells per slide were assessed. DNA damage was determined using Comet Assay IV image analysis software & percentage of tail intensity (TI) recorded
- ✓ Mean & standard deviation (SD) of TI were calculated
- ✓ Data were analysed by using a published parametric statistical analysis approach[3] **(Fig.1)**

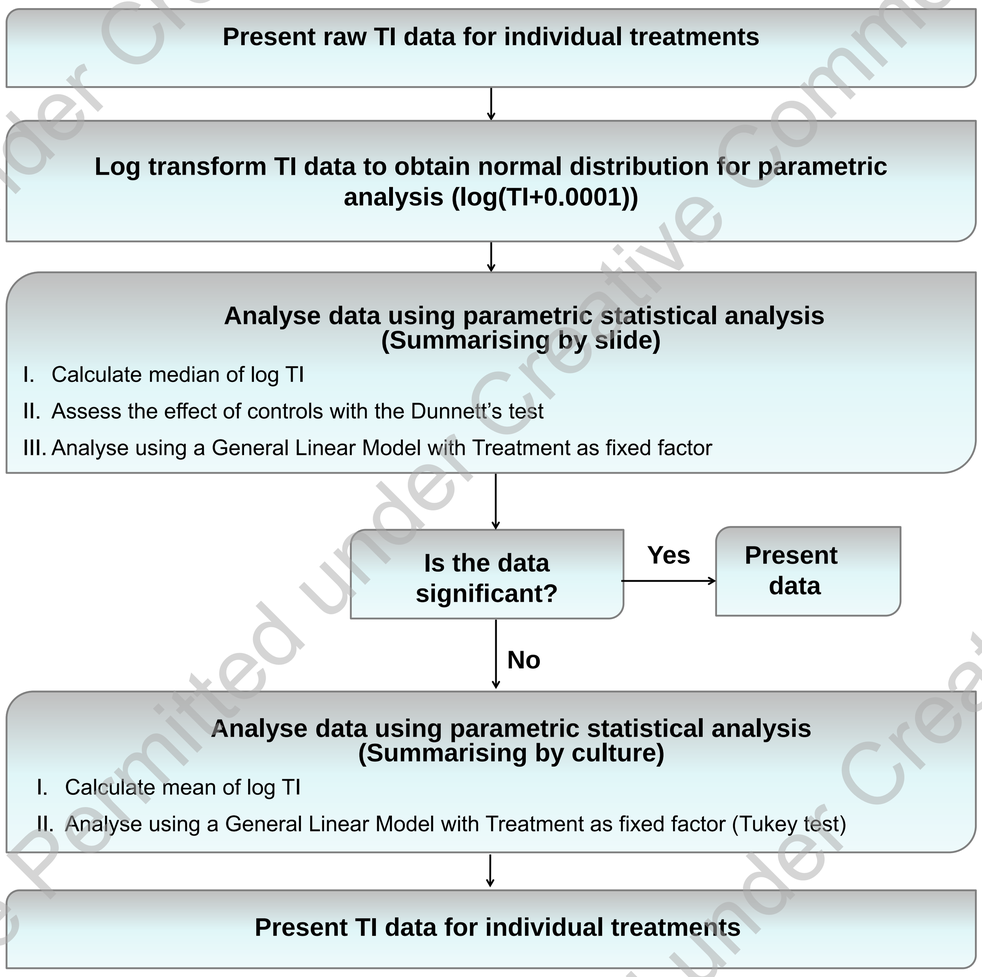

Figure 1.- Parametric statistical analysis flow diagram, based on Bright *et al.* 2011[3]

REFERENCES

1. Brendler-Schwaab S, Hartmann A, Pfuhler S, Speit G. Mutagenesis 2005; 20(4):245-54.
2. Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF. Environ Mol Mutagen. 2000; 35(3):206-21.
3. Bright J, Aylott M, Bate S, Geys H, Jarvis P, Saul J, Vonk R. Pharm Stat. 2011, 10(6):485-93

www.bat-science.com

RESULTS

❖ Alkaline Comet assay

Etoposide, MMS, EMS, H2O2 & NNKOAc treatment

- ✓ Cell viability was >95% after all treatments
- ✓ 250 µM etoposide, 750 µM MMS, 60 mM EMS, 400 µM H_2O_2 & 150 µM NNKOAc induced 54.54 ± 29.40%, 41.87 ± 16.74%, 70.03 ± 11.40%, 71.92 ± 21.87% or 72.19 ± 12.32% DNA damage, respectively **(Fig. 2)**
- ✓ Responses were significant ($p<0.05$) when compared to respect to vehicle control

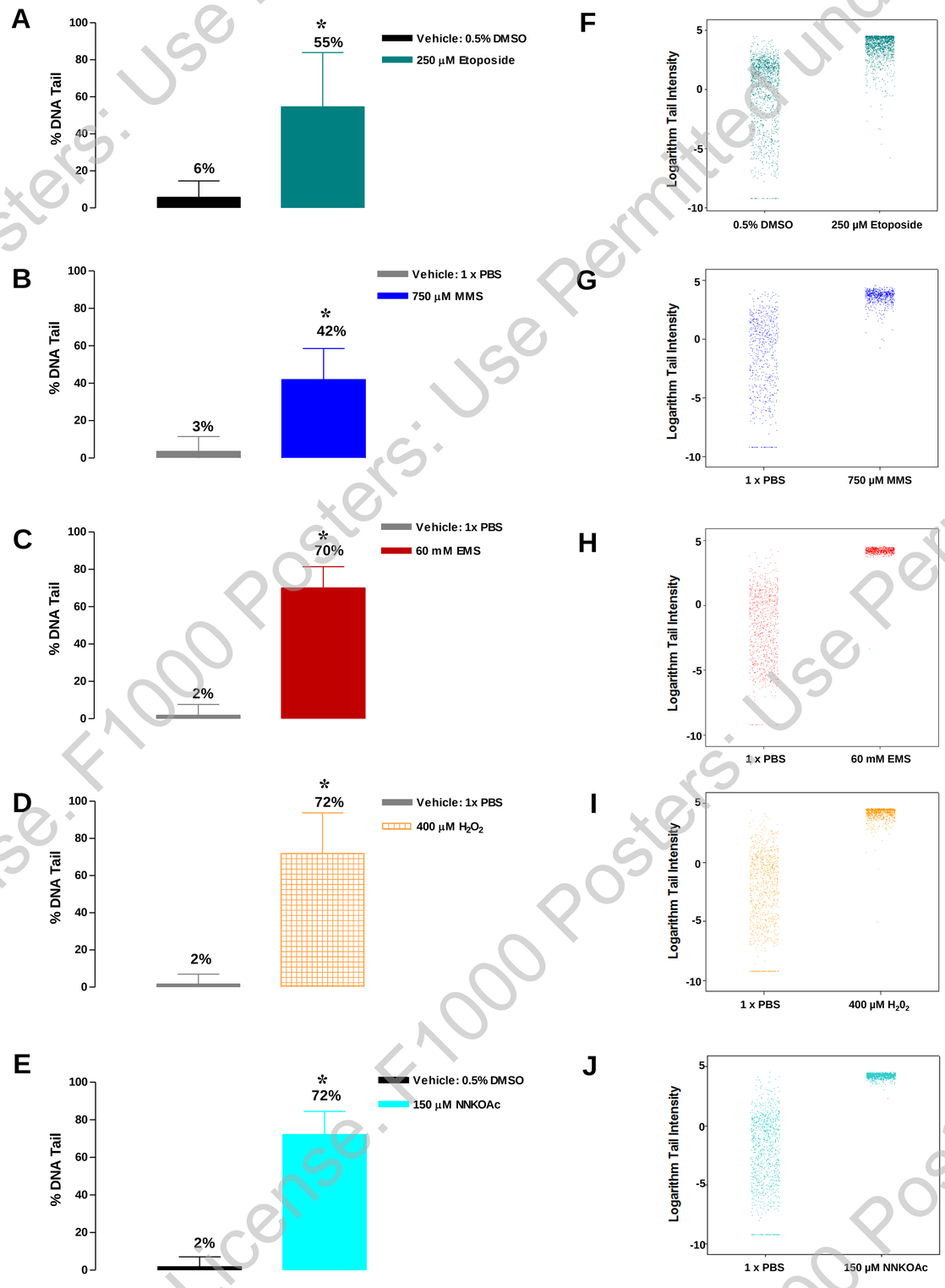

Figure 2.- DNA damage in H292 cells after 250 µM etoposide, 750 µM MMS, 60 mM EMS, 400 µM H_2O_2 or 150 µM NNKOAc treatment. A-E: Bar charts (Mean ± SD of TI) & F-J: Scatter plots (Individual log–transformed TI). * $p<0.05$ when compared to vehicle.

❖ Modified Alkaline Comet assay

Potassium Bromate (KBrO3)

- ✓ Cell viability after 2.0 mM & 10.0 mM $KBrO_3$ treatment was >98%
- ✓ Minimal DNA damage was induced by 2.0 mM or 10.0 mM $KBrO_3$ <9%
- ✓ 2.0 mM $KBrO_3$ + FPG & 10.0 mM $KBrO_3$ + ENDO III induced 70.73 ± 16.72% & 63.63 ± 25.68% oxidative DNA damage, respectively. **(Fig. 3)**

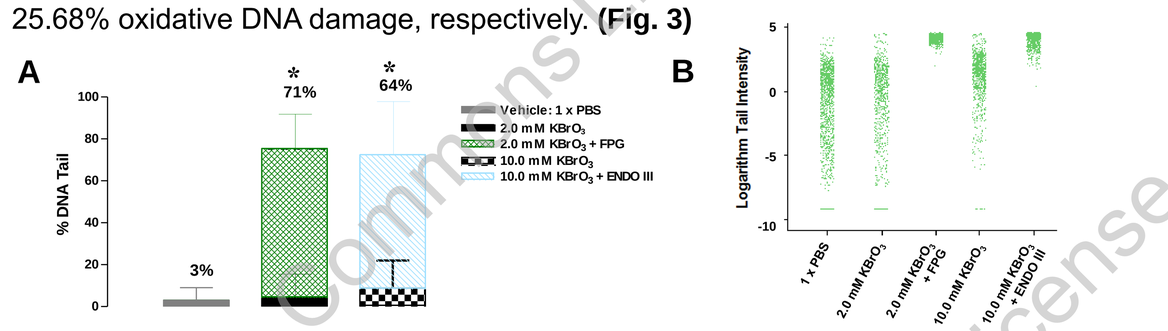

Figure 3.- Strand breaks & oxidative DNA damage in H292 cells following incubation with 2.0 or 10.0 mM $KBrO_3$ & subsequent incubation with FPG or ENDO III. A: Bar charts (Mean ± SD of TI). B: Scatter plots (Individual log–transformed TI). * $p<0.05$ when compared to vehicle.

CONCLUSIONS

1. **We have developed optimal exposure conditions & positive response to all 6 genotoxic compounds in NCI-H292 cells**
2. **A published parametric statistical analysis approach[3] has enabled appropriate, meaningful interrogation & evaluation of the data**

www.vivotecnia.com