Toxicology in Vitro 27 (2013) 1312–1319

Contents lists available at SciVerse ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

The resolving power of *in vitro* genotoxicity assays for cigarette smoke particulate matter

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ARTICLE INFO

Article history:
Received 9 December 2012
Accepted 25 February 2013
Available online 7 March 2013

Keywords:
Genotoxicity
In vitro
Tobacco smoke
Particulate matter
Replication levels
Statistical analysis

ABSTRACT

In vitro genotoxicity assays are often used to compare tobacco smoke particulate matter (PM) from different cigarettes. The quantitative aspect of the comparisons requires appropriate statistical methods and replication levels, to support the interpretation in terms of power and significance. This paper recommends a uniform statistical analysis for the Ames test, mouse lymphoma mammalian cell mutation assay (MLA) and the *in vitro* micronucleus test (IVMNT); involving a hierarchical decision process with respect to slope, fixed effect and single dose comparisons. With these methods, replication levels of 5 (Ames test TA98), 4 (Ames test TA100), 10 (Ames test TA1537), 6 (MLA) and 4 (IVMNT) resolved a 30% difference in PM genotoxicity.

1. Introduction

In vitro tests for genotoxicity are an important part of regulatory toxicology in many sectors, e.g. food and pharmaceuticals, especially in the detection of potential carcinogens (Combes et al., 2007; DOH, 2000; ICH, 1997; Kirkland et al., 2003; Pfuhler et al., 2007). The Ames test, mouse lymphoma mammalian cell mutation assay (MLA) and *in vitro* micronucleus test (IVMNT) are among the most effective methods. The Ames test measures bacterial mutagenicity, the MLA measures mammalian mutagenicity and the IVMNT measures structural and numerical chromosome changes. IVMNT has been validated for the detection of genotoxic carcinogens (Anon, 2006; Corvi et al., 2008; Matsushima et al., 1999). Ames test, MLA and IVMNT methods have been recommended by the Organisation for Economic Cooperation and Development (OECD), the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) or the United Kingdom Environmental Mutagenesis Society (UKEMS). (Gatehouse et al., 1990; ICH, 1995, 1997; OECD, 1997a,b, 2010; UKEMS, 1989). The methods include statistical analysis and replication levels to aid the qualitative interpretation of the results.

Tobacco smoke contains gas and particulate phases. The latter can be trapped on glass fibre filters, and extracted as particulate matter (PM). PM is used for *in vitro* tests, because its preparation is well defined, it gives clear dose responses and there is a large amount of historic control data. PM is genotoxic in the Ames test, MLA and IVMNT (Baker et al., 2004; Clive et al., 1997; Cobb et al., 1989; DeMarini, 2004; Kier et al., 1974; Mitchell et al., 1981; Richter et al., 2010; Rickert et al., 2007, 2011; Roemer et al., 2002, 2004; Sato et al., 1977). The Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA) has recommended descriptive statistics, to characterise PM dose responses in the Ames test and IVMNT (CORESTA, 2004, 2010).

The *in vitro* methods have also been able to quantitatively differentiate PMs from a variety of cigarettes (DeMarini et al., 2008; Guo et al., 2011; Roemer et al., 1998). Novel tobacco materials can reduce PM genotoxicity (Combes et al., 2012; McAdam et al., 2011). Quantitative comparison of PMs' genotoxicity could support the development of Reduced Toxicant Prototype tobacco products,

Abbreviations: ANCOVA, analysis of covariance; CORESTA, Cooperation Centre for Scientific Research Relative to Tobacco; DMSO, dimethyl sulphoxide; ICH, International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; ISO, International Standards Organisation; IVMNT, *in vitro* micronucleus test; MCMC, Markov Chain Monte Carlo; MF, mutation frequency; MLA, mouse lymphoma mammalian cell mutation assay; MnBn, micronucleated binucleate cells; OECD, Organisation for Economic Cooperation and Development; PM, particulate matter; RPMI, Roswell park memorial institute medium; RTP, Reduced Toxicant Prototype; SEM, standard error of the mean; S9, post-mitochondrial supernatant; UKEMS, United Kingdom Environmental Mutagenesis Society.

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http://dx.doi.org/10.1016/j.tiv.2013.02.015

by contributing to an integrated, hypothesis led assessment framework (Proctor and Ward, 2011).

The aim of this paper is to recommend statistical methods and replication levels for the quantitative comparison of test and control PMs in the Ames test, IVMNT and MLA.

2. Materials and methods

2.1. Test materials

3R4F cigarettes were obtained from the University of Kentucky. These are filtered American blend reference cigarettes, with a PM yield of approximately 11 mg/cigarette under International Standards Organisation (ISO) machine smoking conditions (Roemer et al., 2012).

PM preparation was as described by McAdam et al. (2011). Briefly, cigarettes were conditioned according to ISO 3402 (ISO, 1999), then smoked on a RM20CSR smoking machine (Borgwalt-KC, Hamburg, Germany) according to ISO 3308 (ISO, 2000). An appropriate number of cigarettes were smoked to obtain up to 300 mg PM on a 44 mm Cambridge filter pad. PM was eluted in dimethyl sulphoxide (DMSO) to a concentration of 24 mg/ml. Samples were shipped at −80 °C to an independent laboratory for *in vitro* tests, where they were stored at −80 °C in single-use aliquots, and used within 1 month.

To confirm the *in vitro* assays' resolving power, two 3R4F PMs were tested. These were from the same PM stock solution, but one sample was diluted to 70% (v/v), to simulate a 30% difference between PMs.

2.2. In vitro toxicology testing

All *in vitro* tests were performed in an independent Good Laboratory Practice laboratory. Post-mitochondrial supernatant (S9), prepared from male Sprague Dawley rats, induced with Aroclor 1254, was used for metabolic activation.

The Ames test was performed as described by McAdam et al. (2011), with the exceptions that only three *Salmonella typhimurium* strains were used (TA98, TA100 and TA1537), in the presence of S9, and there were 8 replicate plates per dose. Results are presented as mean revertants/μg PM ± standard error of the mean (SEM), within each experiment.

The MLA was performed as described by McAdam et al. (2011), with the exception six replicate cultures per dose were exposed to PM for 24 h without S9. Data are plotted as the means of replicate cultures ± SEM, within each experiment.

The IVMNT was performed as described by McAdam et al. (2011), with the exception that six replicate V79 cell cultures per dose were pulsed with test samples for 3 h followed by a 21 h recovery, without S9. Data are plotted as the means of replicate cultures ± SEM, within each experiment.

The exceptions to McAdam et al. (2011) represent the selection of the most sensitive treatment conditions and an increase in replication levels, to evaluate the statistical methods. The replication levels were selected following a review of historical data, indicating the scope to increase resolving power.

2.3. Statistical analysis

Different outlier, transformation and linearity methods were evaluated using recent PM data, as follows.

Dixon's test (Böhrer, 2008) and boxplot quartiles (Tukey, 1977) were used to identify potential outliers.

The assumed distributions for the Ames test, MLA and IVMNT were Poisson (Roller and Aufderheide, 2008), log-normal (Murphy et al., 1988) and binomial (Hayashi et al., 1994), respectively. A generalised linear model was used, to accommodate response variables that have other than a normal distribution. This required logarithmic transformations for the Ames test and MLA, and a probit transformation for the IVMNT (Armitage and Berry, 1987a).

Two ways to identify the linear part of the dose response (Bernstein et al., 1982) were evaluated. The first was to use a linear regression model and partition the residual error into pure error and lack-of-fit (Draper and Smith, 1998). The linear portion of the response was identified by systematically excluding doses from the model until the lack-of-fit test was non-significant. The second method fitted a generalised linear model with linear and quadratic terms for dose (Roller and Aufderheide, 2008). If the quadratic term was significant ($p < 0.01$), the same model was fitted again with the highest dose excluded, continuing until the quadratic term was not significant or less than three doses remained.

Dose responses were compared and significance tested using analysis of covariance (ANCOVA) for slopes and pooled data, and *t*-tests for individual concentrations (Werley et al., 2008).

Following ANCOVA (Pocock et al., 2002) or *t*-tests, resolving power was calculated using standard formulae (Armitage and Berry, 1987b).

3. Results

3.1. Evaluation of statistical methods

3.1.1. Identification of potential outliers

Dixon's test occasionally identified single values as potential outliers, when the other replicate values were close together. The quartiles method required more than 6 replicates per dose. Furthermore, removing potential outliers did not improve the resolving power of the assays, except for TA1537 data in the Ames test. With sufficient replication (>6 replicates per dose), the quartiles method was used to improve the resolving power of TA1537 data, by identifying potential outliers for removal, before further statistical analysis. Outlier analysis was not applied in the other assays.

3.1.2. Transformations

Examination of the residuals confirmed that the number of revertants in an Ames test were Poisson distributed (Roller and Aufderheide, 2008), the proportion of micronucleated binucleate cells (MnBn) in the IVMNT were binomially distributed and mutation frequency (MF) in the MLA was normally distributed on the log scale, consistent with the assumed distributions of these transformation methods.

3.1.3. Linearity

Identification of linearity through partitioning residual errors from simple linear regression was shown to be unreliable, because the errors were not always normally distributed. Fitting a generalised linear model with linear and quadratic terms for dose, and removing the highest dose until the quadratic term was not significant, also identified the linear part of the dose response, and the residuals were consistent with the method's assumptions.

3.1.4. Significance tests

The linear portion of the curve was used to compare the slopes of dose responses. A test for difference in slopes was investigated using an analysis of covariance model containing terms for dose, PM and a PM-by-dose interaction term. Where PM-by-dose was significant ($p < 0.05$), the difference in slopes was statistically significant. Occasionally, linear dose responses were parallel (PM-by-dose $p \geqslant 0.05$). The PM samples were then compared for differences in overall magnitudes (mean responses). This was done