A Mode-of-Action Approach for the Identification of Genotoxic Carcinogens

Lya G. Hernández¹, Jan van Benthem¹, George E. Johnson²

¹ Laboratory for Health Protection Research, National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands, ² Institute of Life Science, College of Medicine, Swansea University, Swansea, Wales, United Kingdom

Abstract

Distinguishing between clastogens and aneugens is vital in cancer risk assessment because the default assumption is that clastogens and aneugens have linear and non-linear dose-response curves, respectively. Any observed non-linearity must be supported by mode of action (MOA) analyses where biological mechanisms are linked with dose-response evaluations. For aneugens, the MOA has been well characterised as disruptors of mitotic machinery where chromosome loss via micronuclei (MN) formation is an accepted endpoint used in risk assessment. In this study we performed the cytokinesis-block micronucleus assay and immunofluorescence mitotic machinery visualisation in human lymphoblastoid (AHH-1) and Chinese Hamster fibroblast (V79) cell lines after treatment with the aneugen 17-β-oestradiol (E2). Results were compared to previously published data on bisphenol-A (BPA) and Rotenone data. Two concentration-response approaches (the threshold-[Td] and benchmark-dose [BMD] approaches) were applied to derive a point of departure (POD) for in vitro MN induction. BMDs were also derived from the most sensitive carcinogenic endpoint. Ranking comparisons of the PODs from the in vitro MN and the carcinogenicity studies demonstrated a link between these two endpoints for BPA, E2 and Rotenone. This analysis was extended to include 5 additional aneugens, 5 clastogens and 3 mutagens and further concentration and dose-response correlations were observed between PODs from the in vitro MN and carcinogenicity. This approach is promising and may be further extended to other genotoxic carcinogens, where MOA and quantitative information from the in vitro MN studies could be used in a quantitative manner to further inform cancer risk assessment.

Citation: Hernández LG, van Benthem J, Johnson GE (2013) A Mode-of-Action Approach for the Identification of Genotoxic Carcinogens. PLOS ONE 8(5): e64532. doi:10.1371/journal.pone.0064532

Editor: Stefan Wölfl, Universität Heidelberg, Germany

Received December 14, 2012; Accepted April 16, 2013; Published May 13, 2013

Copyright: © 2013 Hernández et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: Funding for the E2 work was provided by the Food Standards Agency (contract number T01029) and the European Union "Protection of the European population from aneugenic chemicals" (PEP-FAC) project (contract number QLK4-CT 2000-00058). Dr. George E. Johnson was also supported by a Swansea University Bursary as well as United Kingdom Environmental Mutagen Society (UKEMS) bursaries. More recently, he was supported by a Strategic Award from the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs, contract number NC/K500033/1). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: G.Johnson@swansea.ac.uk

Introduction

Cancer risk assessment is based on low-dose extrapolation of the risk of chemical carcinogens based on their mode of action (MOA). Genotoxic carcinogens, which are clearly DNA reactive and initiating, follow a linear approach for risk assessment, while indirect and non-DNA reactive carcinogens such as aneugens, and topoisomerase poisons follow a non-linear or threshold approach [1,2]. Thus establishing the MOA of substances is important for deciding which approach to use for risk characterization. Aneugens are agents which affect cell division and the mitotic spindle apparatus resulting in the loss or gain of whole chromosomes, in comparison to clastogens which are agents that induce breaks in chromosomes leading to sections of the chromosomes being added, deleted or rearranged, or mutagens which are agents which induce mutations. Aneugens were the first class of genotoxic compounds to have well established non-linear dose-responses [3,4] and the underlying mechanisms responsible for these thresholds are important for hazard and risk assessment. Therefore, distinguishing between aneugens and other genotoxic compounds such as clastogens and mutagens has important implications in cancer risk assessment.

Genotoxicity tests are often used to determine the mutagenic potential of substances because the accumulation of mutations is essential for tumour development, albeit in a qualitative manner. Efforts are presently being made to compare data from in vitro genotoxicity tests and carcinogenicity studies to determine if a quantitative relationship between these two endpoints exists. The main goal here is to investigate whether in vitro or in vivo genotoxicity tests can provide information on genotoxic MOA (linear versus non-linear concentration/dose-response curves observed for clastogens and aneugens, respectively). A recent study showed a correlation between the in vivo MN and carcinogenicity for numerous carcinogens with different MOAs [5], and this approach was of interest for the current work. This quantitative framework has gained international interest, particularly with the International Life Science Institute (ILSI) Health and Environmental Sciences Institutes (HESI) in vitro genotoxicity testing (IVGT, renamed Genetic Toxicology Technical Committee (GTTC) in 2012) quantitative subgroup which has led to the implementation of different concentration- and dose-response modelling approaches for different compounds [6]. The main objective being to put more
emphasis on genetic toxicity data to reduce follow up animal testing and to see if the in vitro data can be used in a quantitative fashion [7]. Most of the work to date has focused primarily on DNA reactive genotoxic compounds. Our work is novel in that we aim to apply the various concentration-response approaches on the well-characterised aneugens 17-β-oestradiol (E2), bisphenol-A (BPA) and Rotenone.

Several methods are currently available for testing the genotoxic potential of chemicals in vitro. The cytokinesis-block micronucleus (CBMN) assay is an accepted test for determining the genotoxic potential of a substance [8–11]. Micronuclei (MN) can be formed in dividing cells that either contain chromosome breaks lacking centromeres or whole chromosomes that are unable to travel to the spindle poles during mitosis [12,13]. The CBMN assay is a convenient and reliable test for the measurement of both chromosome breakage as induced by clastogens and chromosome loss as induced by aneugens. The term aberrant mitotic machinery is defined as the disruption of the microtubules and centrosomes. This can occur by multiple centrosomes being induced by these spindle poisons, resulting in tri, tetra and multi-polar cells, compared to the normal bipolar mitotic cells. Therefore, by visualising the mitotic machinery using immunofluorescence to target γ or β-tubulin (microtubules), γ-tubulin (centrosomes) and DNA by using 4’6-diamidino-2-phenylindole (DAPI), possible MOA for aneugens can be determined. This information can be visualised at concentrations surrounding the no-observed effect level (NOEL) for MN induction to obtain a possible MOA for aneuploidy [14,15]. Here, we put forth an alternative method to the commonly used fluorescence in situ hybridization (FISH) for discriminating between aneugens and clastogens.

In this study we performed the CBMN assay and immunofluorescence mitotic machinery visualisation (IMMV) in the human lymphoblastoid cell line (AHH-1) and the Chinese Hamster fibroblast cell line V79 (V79) exposed to various concentrations of E2. Results from E2 were compared to previously published data for BPA and Rotenone [14]. Different concentration-response analyses were performed including the threshold-dose (Td) and the benchmark-dose (BMD) approach to determine a POD for in vitro MN induction alongside MOA analysis via IMMV. BMD analysis was also performed for carcinogenicity studies to determine an in vivo POD. The lowest in vitro MN POD was compared to the lowest in vivo carcinogenicity POD to investigate whether comparable rankings were observed. This analysis was extended to include 5 additional aneugens (nocodazole, colchicine, mebendazole, carbendazim, and diethylstilbestrol (DES)), 5 clastogens (bleomycin, thioacetazone, chlorambucil, mephalan, and urethane) and 3 mutagens (cytosine arabinoside, 5-fluorouracil and methylmethane sulfonate (MMS)) to see if similar trends were observed between the lowest POD and concentration-response characteristics from the in vitro MN assay and in vivo carcinogenicity studies.

Materials and Methods

Chemicals

All chemicals including cytochalasin-B (CAS number: 14930-962) were purchased from Sigma-Aldrich (Poole, UK) unless otherwise stated. DPX was purchased from Fisher Scientific (Loughborough, UK). Phosphate Buffer Saline (PBS) was prepared using tablets purchased from Sigma, which were dissolved in 1 litre of deionised H2O. PBT was prepared using PBS +0.1% Tween 20 (CAS number: 9005-66-7). 17-β-oestradiol (E2, CAS number: 50-28-2) was dissolved in dimethylsulphoxide (CAS number: 67-68-5, DMSO).

Figure 1. Acridine orange stained AHH-1 cells. (a) Binucleate and (b) binucleate cell with micronucleus, BN-MN. doi:10.1371/journal.pone.0064532.g001

Cell Lines

The human lymphoblastoid cell line AHH-1 was obtained from ATCC (CRL-8146, USA, http://www.1gstandards-atcc.org/
In vitro Cytokinesis Blocked Micronucleus (CBMN) Assay for E2 (Figure 1)

The in vitro CBMN assay in human lymphoblastoid cell line AHH-1 or the Chinese Hamster fibroblast cell line V79 was used to detect both structural and numerical chromosome damage by measuring the formation of MN in interphase cells that have been through a mitotic division [12]. Examples of binucleate cells (BN) with and without MN are shown in Figure 1. In order to determine the effects of E2 upon MN induction and chromosome segregation, actively growing cell cultures were exposed to graded concentrations of E2 dissolved in DMSO. AHH-1 cells were grown in RPMI 1640 medium (Gibco-Invitrogen, Paisley, UK), 10% horse serum (Gibco-Invitrogen, Paisley, UK) and 1% L-glutamine (Gibco-Invitrogen, Paisley, UK). Cultures were exposed for a complete cell cycle (22 to 26 hours dependent upon any cell cycle delay) in the presence of 3 μg/ml of the actin-inhibitor cytochalasin-B. Cells were washed and centrifuged. Suspensions were then deposited on slides using a cytocentrifuge. This treatment resulted in the production of binucleate cells from those cells that have undergone cell division in the presence of the test chemical and cytochalasin-B. Slides were fixed with methanol and stained with either Giemsa (CAS number: 51811-82-6) or acridine orange (CAS number: 10127-02-3) to detect MN. MN for both control and treated cultures were scored according to previously established criteria [12,16].

Aberrant Mitosis Assay: Multiple Centrosomes Induced by E2

Sterile glass microscope slides were placed in Petri dishes on which V79 cells were seeded at approximately 7.5×10⁴ cells/ml and grown overnight in fresh medium consisting of Dulbecco’s Modified Eagles Medium (DMEM) without phenol red (Gibco-Invitrogen, Paisley, UK), and supplemented with 10% foetal bovine serum (FBS) (Gibco-Invitrogen, Paisley, UK). Cells were then incubated for 20 hours in the presence of the E2 dissolved in DMSO. Colchicine (COL) (CAS number: 64-86-8) was used as a positive control. The highest concentration of E2 used did not exceed 50% induced cell toxicity consistent with the OECD guideline (2010) [11].

Conventional Spindle Staining for E2 (Figure 2)

The cells were washed in PBS and then fixed in 3:1 methanol:acetic acid (CAS number: 64-19-7) (3×14 minutes). Slides were air-dried and then placed in 5% perchloric acid (7601-90-3) at 4°C overnight. 0.5% Brilliant blue (CAS number: 6104-59-4, BB) and 0.5% safranin O (CAS number: 477-73-6, SO) in 15% v/v acetic acid (CAS number: 64-19-7) was added to the slides after washing 10× in distilled water. Slides were air-dried and mounted using DPX (Fisher, Loughborough, UK).

Immunofluorescence Mitotic Machinery Visualisation (IMMV) for E2 (Figure 3)

Cells were washed once in ice-cold PBS, and then fixed for 30 minutes in 90% methanol (CAS number: 67-60-1). Slides were then air dried and stored at −20°C. Frozen slides were placed in 90% methanol at −20°C for 20 minutes and then for 20 s in acetone (CAS number: 67-64-1) at −20°C. Following PBT
rinsing, cells were incubated for 2 hours in a humidified chamber at 37°C with a diluted mouse anti γ-tubulin antibody (diluted 1:200 with PBS) (Sigma, Poole, UK). Then slides were rinsed with PBT and incubated for 2 h at 37°C in a humid chamber with TRITC-conjugated secondary anti-mouse antibody (diluted 1:32 with PBS). After extensive washing in PBT, cells were kept for 1 h in the presence of a mouse monoclonal anti-α-tubulin conjugate clone (diluted 1:100 with PBS). DNA was counterstained with DAPI [17,18].

BPA and Rotenone

We have previously characterised the MOA of BPA by employing kinetochore staining [15,18] which shows if the MN contains a chromosome fragment (i.e. compound is clastogenic), or a whole chromosome (i.e. compound is aneugenic) [11,18]. In addition, a summary of NOELs and/or LOELs from genetic toxicity tests after treatment with BPA is illustrated in Table 1.

**In vitro MN Analysis of Other Genotoxic Compounds**

An analysis was performed of the literature in search for *in vitro* MN data from different human lymphocyte cell line studies, in addition to the E2, BPA and Rotenone derived by Johnson and Parry (2008) [14]. *In vitro* MN data on human lymphocytes exposed to aneugens nocodazole, colchicine, mebendazole, and carbendazim, and the alkylating agent MMS were derived from Elhajouji et al. (1997) [3]. *In vitro* MN data on human lymphocytes exposed to aneugens colchicine and DES, the nucleoside analogues cytosine arabinoside and 5-fluorouracil, and the clastogens bleomycin, urethane and thiabendazole were derived from Clare et al. (2006) [19]. *In vitro* MN data on human lymphocytes exposed to clastogens chlorambucil and melphalan were derived from Efthimiou et al. (2007) [20].

**Derivation of in vitro POD from MN Studies**

**Threshold dose approach.** *In vitro* MN concentration-response analysis was performed from the data generated in this study for E2, and for BPA and Rotenone data derived from...
Johnson and Parry (2008) [14]. Two methods were used for concentration-response analysis: Td and BMD. Threshold modelling used a similar approach to Gocke and Wall (2009) [21] and Johnson et al., 2009 [22]. This was performed using a 4 step approach. Briefly, Step 1 involved a one-way ANOVA for a dose-related effect (SPSS version 16.0.1). Step 2 involved a comparison of linear and quadratic models using the coefficient of determination ($R^2$, SPSS version 16.0.1). The $F$ distribution was then used to calculate $P$ values in Microsoft Excel 2007. Step 3 involved the determination of no-observed-genotoxic-effect level (NOGEL) or lowest-observed-genotoxic-effect-level (LOGEL) values using a one-sided Dunnett’s test on either untransformed or log-transformed data (SPSS version 16.0.1). Linear and quadratic models were then compared at the NOGEL and below in the same way as Table 1.

**Table 1.** Summary of NOELs and/or LOELs from genetic toxicity tests after treatment with BPA [28–34].

| End Point | Cell Line | NOEL | LOEL | Reference |
|-----------|-----------|------|------|-----------|
| **In vitro** | | | | |
| Chromosome aberrations | SHE | 200 µM/46 µg/ml | | Tsutsui 1998 |
| DNA adducts | SHE | 50 µM/11.5 µg/ml | | Tsutsui 1998 |
| DNA adducts | Rat liver | 100 µM/23 µg/ml | | Atkinson and Roy 1995 |
| Chromosome aberrations | CHO | 350 µM/80.5 µg/ml | 400 µM/92 µg/ml | Hilliard 1998 |
| Chromosome aberrations | CHO | 220 µM/50 µg/ml | | Ivett 1989 |
| SCE | CHO | 130 µM/30 µg/ml | | Ivett 1989 |
| Aberrant spindles | V79 | 100 µM/23 µg/ml | | Ochi 1999 |
| γ-tubulin | V79 | 100 µM/23 µg/ml | | Ochi 1999 |
| Multipolar division | V79 | 100 µM/23 µg/ml | | Ochi 1999 |
| Microtubule | Bovine-MT | 50 µM/11.5 µg/ml | 100 µM/23 µg/ml | Pfeiffer 1997 |
| Metaphase arrest | V79 | 50 µM/11.5 µg/ml | 100 µM/23 µg/ml | Pfeiffer 1997 |
| Micronuclei | V79 | 100 µM/23 µg/ml | | Pfeiffer 1997 |
| **In vivo** | | | | |
| DNA adducts | Rat | 200 mg/kg | | Atkinson and Roy 1995 |

NOEL, No-observed effect level; LOEL, lowest-observed effect level; SHE = Syrian Hamster Embryo; SCE, sister chromatid exchange; CHO = Chinese Hamster Ovary; V79 = Chinese Hamster fibroblast cell line; CMTC, cytoplasmic microtubule complex.

doi:10.1371/journal.pone.0064532.t001

Table 2. Analysis of carcinogenicity data from the National Toxicology Program (http://ntp.niehs.nih.gov/).

| Compound | Sex | Tissue | BMD$_{10}$ (mg/kg/day) | BMDL$_{10}$ (mg/kg/day) | BMDU$_{10}$ (mg/kg/day) |
|----------|-----|--------|------------------------|------------------------|------------------------|
| DES      | F   | Pituitary gland adenoma | 0.001 | 0.0005 | 0.0024 |
| DES      | F   | Cervix squamous cell carcinoma | 0.07 | 0.05 | 0.12 |
| DES      | M   | Testes interstitial cell tumor | 0.017 | 0.014 | 0.02 |
| DES      | M   | Pituitary gland adenoma | 0.003 | 0.002 | 0.005 |
| DES      | F   | Pituitary gland adenoma | 0.039 | 0.03 | 0.06 |
| DES      | F   | Cervix squamous cell carcinoma | 0.029 | 0.02 | 0.04 |
| DES      | F   | Mammary gland adenocarcinoma, Type B | 0.032 | 0.011 | 0.091 |
| DES      | F   | Cervix adenoacanthoma | 0.086 | 0.06 | 0.13 |
| DES      | M   | Testis interstitial cell tumor | 0.0066 | 0.004 | 0.008 |
| DES      | F   | Mammary gland carcinoma | 0.0003 | 0.00003 | 0.0014 |
| DES      | F   | Pituitary gland adenoma | 0.0009 | 0.0004 | 0.0019 |
| DES      | M   | Testis interstitial cell tumor | 0.0094 | 0.007 | 0.012 |
| DES      | F   | Pituitary gland adenoma | 0.0007 | 0.0004 | 0.0014 |
| E$_2$    | F   | Mammary gland adenocarcinoma | 0.56 | 0.28 | 2.02 |
| BPA      | F+M | Leukemia | 42.8 | 25.99 | 114.9 |
| BPA      | M   | Leukemia | 38.5 | 20.94 | 201.7 |
| BPA      | F   | Leukemia | 56.5 | N/A | N/A |
| Rotenone | M   | Parathyroid glad adenoma | N/A | N/A | N/A |

doi:10.1371/journal.pone.0064532.t002
described in Step 2. Data that had a flat or zero dose-response slope at the NOGEL and below were then suitable for bilinear or hockey stick analysis. Step 4 involved a comparison of linear versus hockey stick models using the R software package (version 12.2) recommended by Lutz and Lutz (2009) [23]. Parameters, $y$-intercept, $T_d$, and slope above $T_d$ were estimated for best fit of a hockey stick model by minimizing the residual sum of squares. Confidence intervals (CI) were estimated for all parameters using an $F$ distribution [23]. If the 95% CI of the derived $T_d$ value does not encompass zero, the model is considered a good fit to the data.

**Benchmark dose approach.** The BMD approach was

| Compound | CP, Most sensitive tissue | CP, BMDL$_{10}$ (mg/kg/day) | POD (in vivo, $\mu$M) | POD (in vitro, $\mu$M) | POD (in vitro, $\mu$M) |
|----------|---------------------------|------------------------------|-----------------------|-----------------------|-----------------------|
| DES      | mammary gland carcinoma   | 3.00 E-05                    | 1.1 E-04              | 0.42                  | 0.40                  |
| E$_2$    | Leukaemia                 | 0.28                         | 0.74                  | 0.42                  | 0.40                  |
| BPA      | Leukaemia                 | 20.94                        | 91.73                 | 2.58                  | 2.58                  |
| Rotenone | parathyroid gland adenoma?  | no DR                        | no CR                 | 1.24 E-04             | 1.24 E-04             |

Table 3. Summary of the BMD$_{10}$ from carcinogenicity studies, and td-L-CI and BMCL$_{10}$ values from AHH-1 and the Chinese Hamster fibroblast cell line V79 cells exposed to E$_2$, BPA, and Rotenone.

Figure 4. Using the CBMN to assess % binucleate cells with MN (%BN-MN) and cell cytotoxicity and/or cytostasis (% Cell Viability) in AHH-1 cells after E$_2$ treatment at super-physiological concentrations. $3 \times 1,000$ binucleate cells were examined for the presence of BN-MN. Cell viability (%) was calculated from the cytokinesis-block proliferation index (CBPI) measure (OECD, 2010) by scoring approximately $8,000$ cells per dose. $0.8 \, \mu M$ E$_2$ and above were significant to $p<0.05$ for BN-MN, by comparison to the control using Dunnett’s.

Figure 5. Using the Aberrant Mitosis Assay to show the activity of 17$\beta$-oestradiol (E$_2$) as a spindle poison, and to give the concentration-response relationship at super-physiological concentrations. Tripolar (Tri) was calculated using number of tripolar cells compared to number of mitotic cells ($3 \times 100$ in total) using IMMIV. Mitotic Index (MI) was calculated using number of mitotic cells compared to number of interphase cells ($3 \times 1,000$ cells in total) using conventional spindle staining. $0.8 \, \mu M$ E$_2$ and above were significant to $p<0.05$ for both MI and Tri, by comparing to the control using Dunnett’s.

**Mode of Action Approach for Carcinogens**
performed using the statistical package PROAST [24] to derive BMC10 (in vitro) and BMD10 (in vivo) values for each data set with a benchmark response of 10% as previously done for in vivo and in vitro genotoxicity data [25]. The BMD approach estimates a dose (i.e., the BMD or BMC) that produces some predetermined, and presumably biologically relevant, increase in the response over control (i.e., the benchmark response). The approach employs mathematical dose–response modeling that takes factors such as sample size and shape of the curve into account [26]. BMC10 and BMD10 values were derived using the dose-response modeling software package PROAST, developed at the National Institute for Public Health and the Environment (RIVM) in the Netherlands (www.proast.nl). The models used were the exponential models recommended by the European Food Safety Authority [27]. Model selection was performed using the log-likelihood ratio test that assesses whether a statistically significant improvement in the fit is achieved by adding additional parameters. The model with additional parameters is only accepted if the difference in log-likelihoods exceeds the critical value at $P=0.05$. This is automatically performed in PROAST by selecting the “automatic selection of optimal model from nested family” option. A log-likelihood value is also provided for the “full” model, which is simply the set of the geometric means of the observations at each dose (together with the residual variance). The log-likelihood ratio test can be used to compare the selected model with the full model using a goodness-of-fit test. The model is accepted when the log-likelihood value of the fitted model is significantly better than that of the full model. The BMC10 and BMD10 with their associated lower (BMDL) confidence limits were then derived from the selected model. Therefore, a BMDL10 refers to the estimate of lower 95% CI of a dose that produces a 10% increase over the fitted background level for continuous endpoints, and 10% extra risk for quantal endpoints.

**Derivation of in vivo POD for Carcinogenicity**

Carcinogenicity data were taken from the National Toxicology Program (NTP) and Carcinogenic Potency Databases (CPD). The BMD approach was used to derive a dose that increases the tumor response by 10% over the modelled control (BMD10), with its respective upper (BMDU10) and lower (BMDL10) confidence limit (Table 2). The lowest confidence limit of the BMD10 (BMDL10) from the most sensitive tumor endpoint was selected as the POD for carcinogenicity data.

**Results**

**17-β-oestradiol (E2)**

Aneugenicity, cytotoxicity and cytostasis testing of E2 was conducted in AHH-1 cells using the CBMN assay and the aberrant mitosis assay. These endpoints were chosen to observe the genotoxic effects of E2 and give a greater understanding of the MOA. E2 was found to induce MN at super-physiological levels of E2 (0.8–1.0 μM) with a significant decrease in cell viability ($P<0.05$) at the same concentrations (Figure 4). The td-L-CI for MN induction was observed at 0.74 μM and for effects on spindle formation (Tripolar) at 0.45 μM (Table 3). The first significant ($P<0.05$) increase (LOEL) for MN induction and for effects on
Spindle formation (Tripolar) was observed at 0.8 μM. The BMD approach showed a BMCL10 of 0.40 and 0.02 μM for MN and spindle formation induction, respectively (Table 3). If there was a decrease of 50% cell viability or less at a genotoxic (i.e. clastogenic) effect, then the MOA was said to potentially be a cytotoxicity related secondary mechanism and not a true genotoxic response [11]. However, there was only a decrease of 10%–20% cytotoxicity and/or cytostasis when a 2–3× fold increase in MN is observed, which indicated that E2 was genotoxic through a non-cytotoxicity related MOA. The NOEL was defined as the lowest value produced between the Td-L-CI and BMCL10 in both the chromosome loss (MN) and spindle formation effects. This was justified because non-disjunction is known to occur at lower concentrations than chromosome loss ([4](Table 3). With this criterion, the NOEL for E2 was 0.02 μM. The most sensitive carcinogenicity endpoint with the lowest BMDL10 was observed in the mammary gland with a BMDL10 of 0.28 mg/kg/day (1.03 μM/day; Tables 2 and 3).

**Discussion**

The goal of this analysis was to investigate whether carcinogenic potency information (i.e. cancer potency ranking) could be derived from *in vitro* MN data. For this, several quantitative dose-response methods were investigated for the selection of a suitable *in vitro* POD for BPA, E2, and Rotenone. In order to investigate which method was more appropriate for POD derivation, the traditional method for analysing *in vitro* genotoxicity data (i.e. derivation of no-observed-effect-level (NOEL) or a lowest-observed-effect-level (LOEL)) was compared to more recent quantitative methods. First, a summary of BPA’s effects in different genetic toxicity tests is represented in Table 1. From Table 1, it was clear that the NOELs vary significantly between the different
Figure 8. BMD analysis of studies by Johnson and Parry (2008, [14]) for (a) E2, (b) BPA and (c) Rotenone; by Elhajouji et al. (1997, [3]) (d) nocodazole, (e) colchicine, (f) mebendazole, (g) carbenazim, and (h) MMS; by Clare et al. (2006 [19]) for (i) colchicine, (j) DES, (k) cytosine arabinoside, (l) 5-fluorouracil, (m) bleomycin (n) urethane, and (o) thiabendazole; and by Efthimiou et al. (2007, [20]) for (p) chlorambucil and (q) melphalan.
doi:10.1371/journal.pone.0064532.g008
concentration-response was observed with Rotenone (Table 3). E2 characterised thresholds for genotoxic activity [3,4], while no spindle effects, respectively. Therefore, the POD for BPA was 2.58 × 10^{-22} M, respectively. This is to be expected as hormones are presumed to have non-linear concentration and dose-responses [1], and this study confirms these observations (Figures 4–7). In addition, comparisons of PODs between \textit{in vitro} MN and carcinogenicity were made. Table 3 demonstrated that the BMCL_{10} for \textit{in vitro} MN were ranked as E2>BPA>>Rotenone. The carcinogenicity ranking of the most sensitive tumour endpoint (Table 2) was also E2>BPA>>Rotenone (Table 3). These results, although with limited number of compounds, were very promising indicating the potential for deriving carcinogenic potency information from \textit{in vitro} MN studies.

Given the promising ranking results observed with E2, BPA and Rotenone, we extended our analysis and performed a literature search for \textit{in vitro} MN data in human lymphocytes [3,14,19,20]. With this analysis we wanted to explore the applicability of using concentration-response analysis to extrapolate information in regards to linear versus non-linear concentration-responses and carcinogenic potency. Based on the concentration-response curves in Figures 8a-q, a clear distinction in the shape of the concentration-response curves from \textit{in vitro} MN for aneugen are E2>BPA>>Rotenone (Figure 8). The carcinogenicity ranking of the most sensitive tumour endpoint (Table 2) was also E2>BPA>>Rotenone (Table 3). These results, although with limited number of compounds, were very promising indicating the potential for deriving carcinogenic potency information from \textit{in vitro} MN studies.

Table 4. Summary of BMCL_{10,5} derived from different human lymphocyte and AHH-1 cell line studies [3,14,19,20].

| Compound | Classification | CP, Most sensitive tissue/endpoint | CP, BMCL_{10} (μM/day) | \textit{In vitro} BMCL_{10} (μM) | \textit{In vitro cell line} | Reference |
|----------|----------------|----------------------------------|------------------------|-------------------------------|--------------------------|-----------|
| E2       | aneugen        | mammary gland adenocarcinoma      | 1.03                   | 0.40                          | AH4H-1 cell line         | Johnson and Parry (2008) |
| BPA      | leukemia       |                                    | 91.73                  | 5.48                          | AH4H-1 cell line         | Johnson and Parry (2008) |
| Rotenone | aneugen        | parathyroid gland adenoma?        | no DR                  | no CR                         | AH4H-1 cell line         | Johnson and Parry (2008) |
| Nocodazole| aneugen       | N/A                               | 0.0026                 | human lymphocytes             | Elhajouji et al. (1997)  |
| Colchicine| aneugen       | promoter in two-stage skin tumor model | N/A                     | 0.004                         | human lymphocytes        | Elhajouji et al. (1997)  |
| Mebendazole| aneugen    | N/A                               | 0.107                  | human lymphocytes             | Elhajouji et al. (1997)  |
| Carbendazim| aneugen     | hepatocellular adenomas and carcinomas | 62.08                  | 0.26                          | human lymphocytes        | Elhajouji et al. (1997)  |
| MMS      | alkylation agent| N/A                               | 7.51                   | human lymphocytes             | Elhajouji et al. (1997)  |
| Colchicine| aneugen       | N/A                               | 0.005                  | human lymphocytes             | Clare et al. (2006)      |
| DES      | aneugen        | mammary gland carcinoma           | 0.0001                 | 6.90                          | human lymphocytes        | Clare et al. (2006)      |
| Cytosine arabinoside | nucleoside analogue | N/A | N/A | 0.53 | human lymphocytes | Clare et al. (2006) |
| S-Fluorouracil | nucleoside analogue | lung and lymphoreticular system | 22.76 | 48.20 | human lymphocytes | Clare et al. (2006) |
| Bleomycin | clastogen     | N/A                               | N/A                    | 0.0002                        | human lymphocytes        | Clare et al. (2006)      |
| Urethane | clastogen (requires metabolic activation) | lung alveolar-bronchiolar adenoma | 0.11 | no CR | human lymphocytes | Clare et al. (2006) |
| Thiabendazole | clastogen | no positive in CPD | no DR | no CR | human lymphocytes | Clare et al. (2006) |
| Chlorambucil | clastogen     | Lymphosarcoma                     | 0.0007                 | 0.006                         | human lymphocytes        | Efthimiou et al. (2007)  |
| Melphalan | clastogen     | tumor-bearing animals mixed        | 0.013                  | 0.002                         | human lymphocytes        | Efthimiou et al. (2007)  |

CP, carcinogenic potency was derived from studies carcinogenic potency database (www.berkley.org) and National Toxicology Program (NTP); POD, point of departure; MI, mitotic index; MN, frequency of micronuclei formation, DR, dose-response; CR, concentration response.
doi:10.1371/journal.pone.0064532.t004

in \textit{vitro} genotoxic endpoint ranging from 25 to 250 μM. The NOEL or LOEL are not the ideal method for performing concentration-response analysis. Comparison of the NOELs from chromosomal aberrations in Chinese Hamster Ovary (CHO) cells between Hilliard et al. [29] and Efthimiou et al. [20] 220 μM clearly demonstrates how deriving the NOEL using the traditional method is highly dependent on experimental conditions. In addition, NOEL only used one concentration and not the entire data set, and no confidence limits can be derived. In contrast, quantitative methods such as the Td and BMD approach use all the data, are not so dependent on experimental conditions and provide confidence limits [26]. For this reason, the Td and BMD approach were selected for the derivation of POD from \textit{in vitro} MN studies. The lowest reported NOEL observed was 50 μM or 11.5 μg/ml for metaphase arrest in the V79 cell line (Table 1) [30]. For the current study in which we carried out extensive statistical modelling on our previously published BPA data [14], the td-L-CI for MN-induction and disruption in spindle formation was observed at 2.58 and 15.42 μM, respectively (Table 3). Similarly, BMCL_{10} were 5.46 and 3.13 for MN and spindle effects, respectively. Therefore, the POD for \textit{in vitro} MN and BPA was 2.58 μM.

E2 and BPA (xenostrogens) are spindle poisons with well-characterised thresholds for genotoxic activity [3,4], while no concentration-response was observed with Rotenone (Table 3). E2 and BPA showed clear thresholds for MN, mitotic index (MI) and tripolarity. This is to be expected as hormones are presumed to have non-linear concentration and dose-responses [1], and this...
For the study by Efthimiou (2007) [20], the genotoxicity ranking was chlorambucil>melphalan while the genotoxicity ranking was melphalan>chlorambucil. No true conclusions can be made until more substances are tested. To the best of our knowledge, this is the first study to attempt to investigate whether carcinogenic potency information can be derived from in vitro MN studies in human lymphocytes using quantitative approaches. Although inconclusive, these results were promising and more in vitro MN studies under the same conditions (treatment schedule and recovery) and carcinogenicity studies are needed.

Conclusions
Here we demonstrated that combining the micronucleus assay along with aberrant mitotic analysis in AHH-1 and V79 cells, has risk assessment applications for the identification of aneugens, and the derivation of PODs using Td and BMD statistical modelling approaches. The traditional NOEL method for deriving POD is less suitable for analyzing in vitro genotoxicity data and quantitative approaches such as the Td and BMD are recommended for future POD derivation. The concentration-response curves from the in vitro MN in AHH-1 and human lymphocytes provide useful information on linear versus non-linear concentration-response which has risk assessment implications. Comparison of POD ranking between the in vitro MN and carcinogenicity were comparable with E2, BPA and Rotenone but comparisons with other clastogens and mutagens were inconclusive.

Further analysis is needed to investigate whether POD derivation from in vitro MN studies may provide carcinogenic potency information.

Author Contributions
Conceived and designed the experiments: GEJ LGH JvB. Performed the experiments: GEJ LGH JvB. Analyzed the data: GEJ LGH. Contributed reagents/materials/analysis tools: GEJ LGH JvB. Wrote the paper: GEJ LGH JvB.

References

1. Bolt HM, Degen GH (2004) Human carcinogenic risk evaluation, part II: contributions of the EUROTOX specialty section for carcinogenesis. Toxicol Sci 81: 3-6.
2. Foth H, Degen GH, Bolt HM (2005) New aspects in the classification of carcinogens. Arch Hig Rada Toksikol 56: 167–175.
3. Elhajouji A, Tibaldi F, Kirsch-Volders M (1997) Indication for thresholds of genotoxicity risk assessment.; 2009; Firenze-Italy. EEMS. 65.
4. Elhajouji A, Van Hummelen P, Kirsch-Volders M (1995) Indications for a threshold of chemically-induced aneuploidy in vitro in human lymphocytes. Environ Mol Mutagen 26: 292–304.
5. Hernández LG, Slab W, van Steeg H, van Bentham J (2011) Can carcinogenic potency be predicted in vivo using genotoxicity data? a meta-analysis of historical data. Environmental and Molecular Mutagenesis 52: 518–528.
6. Gollapudi BB, Dearfield KL, Hernandez LG, Jeffrey AM, Johnson GE, et al. (2012) Toward A Quantitative Approach for Assessing Genotoxicity Environ Mol Mutagen Environ Mol Mutagen In Press.
7. MacGregor JT. The ILSI-HESI project committee on the relevance and follow up of positive results in vitro genetic toxicity testing (IVGT): Quantitative aspects of genotoxicity risk assessment.; 2009; Firenze-Italy. EEMS. 65.
8. El-Zein R, Vral A, Ezel CJ (2013) Cytosine-blocked micronucleus assay and cancer risk assessment. Mutagenesis 26: 101–106.
9. Kirkland D (2010) Evaluation of different cytotoxic and cytostatic measures for the in vitro micronucleus test (MNtest): introduction to the collaborative trial. Mutat Res 702: 135–150.
10. OECD (1997) OECD Guideline for the Testing of Chemicals. In vitro Micronucleus Test. Mammalian Cell Gene Mutation Test: 476.
11. OECD (2010) OECD Guideline for the Testing of Chemicals. In Vitro Mammalian Cell Micronucleus Test. In Vitro Micronucleus Test.
12. Fenech M (2000) In the in vitro micronucleus technique. Mutat Res 453: 81–95.
13. Yang AH, Kaushal D, Rehen SK, Kriech K, Kingsbury MA, et al. (2003) Chromosome segregation defects contribute to aneuploidy in normal neural progenitor cells. J Neurosci 23: 10454–10462.
14. Johnson GE, Parry EM (2008) Mechanistic investigations of low dose exposures to the genotoxic compounds bisphenol-A and rotenone. Mutat Res 651: 56–63.
15. Parry EM, Parry JM, Corso C, Doherty A, Hardell F, et al. (2002) Detection and characterization of mechanisms of action of aneugenic chemicals. Mutagenesis 17: 509–521.
16. Parry JM, Parry EM (2006) The use of the in vitro micronucleus assay to detect and assess the aneugenic activity of chemicals. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 607: 5–8.
17. Izzo M, Antocea A, Degrassi F, Tanzarella C (1998) Immunohistochemical analysis of diazepam-induced mitotic apparatus anomalies and chromosome loss in Chinese hamster cells. Mutagenesis 13: 445–451.
18. Parry EM CC, Quick E, Johnson GE, Parry JM (2002) An Analysis of the in vitro aneugenic activity of Bisphenol A. Mutagenesis: 556.
19. Clare MG, Lorenzoz G, Akhurst EC, Marzin D, van Delft J, et al. (2006) SFTG international collaborative study on in vitro micronucleus test II. Using human lymphocytes. Mutat Res 607: 37–60.
20. Efthimiou M, Andraniopoulos C, Stephanou G, Demopoulos NA, Nikolaropoulos SS (2007) Aneugenic potential of the nitrogen mustard analogues melphalan, chlorambucil and p-N-N-bis[2-chloroethyl]amino)phenylacetic acid in cell cultures in vitro. Mutat Res 617: 125–137.
21. Goeke E, Wall M (2009) In vivo genotoxicity of EMS: statistical assessment of the dose response curves. Toxicol Lett 190: 298–302.
22. Johnson GE, Doak SH, Griffiths SM, Quick EL, Skibinski DO, et al. (2009) Non-linear dose-response of DNA-reactive genotoxins: Recommendations for data analysis. Mutat Res 678: 95–100.
23. Lutz WK LR (2009) Statistical model to estimate a threshold dose and its confidence limits for the analysis of sublinear dose-response relationships, exemplified for mutagenicity data. Mutat Res 678: 118–122.
24. Slab W (2002) PROAST: Software for dose-response modeling and benchmark dose analysis. RIVM.: http://www.rivm.nl/en/Library/Scientific/Models/PROAST. Accessed 2013 April 17.
25. Gollapudi BB, Johnson GE, Hernandez LG, Potterger LH, Dearfield KL, et al. (2013) Quantitative approaches for assessing dose-response relationships in genetic toxicology studies. Environ Mol Mutagen 54: 8–18.
26. Crump KS (1984) A new method for determining allowable daily intakes. Fundam Appl Toxicol 4: 854–871.
27. EFSA (2009) European Food Safety Authority. Guidance of the scientific committee on use of the benchmark dose approach in risk assessment. The EFSA Journal 1150: 1–72.
28. Hillard CA AM, Bradt CI, Hill RB, Greenwood SK (1998) Chromosome aberrations in vitro related to cytotoxicity of nonmutagenic chemicals and metabolic poisons. Environ Mol Mutagen 31: 316–326.
29. Ivet JL, Brown BM, Rodgers C, Anderson RE, Resnick MA, et al. (1989) Chromosomal aberrations and sister chromatid exchange tests in Chinese hamster ovary cells in vitro. IV. Results with 15 chemicals. Environ Mol Mutagen 14: 165–167.
30. Pfeiffer E, Rosenberg B, Deuschel S, Metzler M (1997) Interference with microtubules and induction of micronuclei in vitro by various bisphenols. Mutat Res 390: 21–31.
31. Atkinson A, Roy D (1995) In vitro conversion of environmental estrogenic compounds. Environ Mol Mutagen 26: 424–433.
32. Atkinson A, Roy D (1995) In vivo DNA adduct formation by bisphenol A. Environ Mol Mutagen 26: 60–66.
33. Ochi T (1999) Induction of multiple microtubule-organizing centers, multipolar spindles and multipolar division in cultured V79 cells exposed to diethylstilbestrol, estradiol-17beta and bisphenol A. Mutat Res 431: 103–121.

34. Tsutsui T, Tamura Y, Yagi E, Hasegawa K, Takahashi M, et al. (1998) Bisphenol-A induces cellular transformation, aneuploidy and DNA adduct formation in cultured Syrian hamster embryo cells. Int J Cancer 75: 290–294.