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Inclusion of an extended treatment with recovery improves the results for the human peripheral blood lymphocyte micronucleus assay

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Abstract

The *in vitro* micronucleus (IVMN) test was endorsed for regulatory genotoxicity testing with adoption of the Organisation for Economic Co-operation and Development (OECD) test guideline (TG) 487 in 2010. This included two equally acceptable options for extended treatment in the absence of metabolic activation: a treatment for 1.5–2.0 cell cycles with harvest at the end of treatment (Option A) or treatment for 1.5–2.0 cell cycles followed by recovery for 1.5–2.0 cell cycles prior to harvest (Option B). Although no preferences were discussed, TG 487 cautions that Option B may not be appropriate for stimulated lymphocytes where exponential growth may be declining at 96 h after phytohaemagglutinin (PHA) stimulation. Following revision of TG 487 in 2014 and 2016, emphasis has been placed on using Option A. Given the purpose of the IVMN assay is to determine both clastogenic and aneugenic potential, the authors believe the assay is compromised if an extended treatment with recovery is not included for sensitive detection of certain classes of chemical. In this study, average generation time (*via* bromodeoxyuridine incorporation) of human peripheral blood lymphocytes (HPBL) was measured up to 144 h after PHA stimulation. In addition, the HPBL micronucleus (MN) assay was performed using Option A and B treatment schedules. Cytotoxicity (replication index) and MN induction were determined following treatment with 14 chemicals. The data demonstrate that lymphocytes actively divide beyond 96 h after PHA stimulation. Furthermore, MN induction was only observed with some aneugenic chemicals and nucleoside analogues in HPBLs following extended treatment with a recovery period. For the majority of chemicals tested the magnitude of MN induction was generally greater and MN induction was observed across a wider concentration range following the Option B treatment schedule. In addition, steep concentration-related toxicity following treatment without recovery is more common, making selection of suitable concentrations (within regulatory toxicity limits) for MN analysis challenging.

Introduction

The *in vitro* micronucleus (IVMN) test was endorsed for regulatory genotoxicity testing with the adoption of OECD test guideline 487 (OECD 487) in 2010 (1). This guideline included two equally acceptable options for extended treatment in the absence of metabolic activation:

Option A (without recovery): Treat for 1.5–2.0 normal cell cycles in the presence of cytochalasin B (Cyto-B); harvest at the end of treatment
Option B (with recovery): Treat for 1.5–2.0 normal cell cycles; remove test chemical; add fresh medium and Cyto-B; harvest cells 1.5–2.0 normal cell cycles later.

Although no preferences were discussed, OECD 487 (1) indicated that it might be more appropriate to follow Option A 'for stimulated lymphocytes where exponential growth may be declining at 96 hrs following stimulation'. This text remains in the updated OECD 487 (adopted September 2014 (2), updated 2016 (3)) without any reference to the basis of this statement. Following the revisions of OECD 487 in 2014 and 2016, the description of the cell treatment and harvest times were simplified with emphasis placed on performing the extended treatment without recovery (Option A) with sampling or recovery times extended by up to a further 1.5–2.0 normal cell cycles where justified. However, the extended Option B schedule was removed from the main proposed treatment table ('cell treatment and harvest times').

Following a review of past literature and validation studies in support of the IVMN assay (4–6), the preference for Option A was difficult to determine. The IVMN working group (7) in 2003 made specific recommendations over treatment and recovery times for human lymphocytes based on data made available from the French Society of Genetic Toxicology (SFTG), Japanese collaborative studies, European pharmaceutical industry validation studies along with data from Lilly Research Laboratories. Treatment and recovery times recommended included a recovery period in the absence of test article for all treatment conditions (short and extended). A commentary on the SFTG conducted validation trials by Fenech (4) indicated the following:

> The most efficient way to perform the MN assay in human lymphocytes that minimises false negative results is to;
>
> 1) Conduct treatment for 24 hour duration after 48 hour of mitogen stimulation to (cover all phases of the cell cycle)
> 2) Harvest cells 24 and 48 hours post Cytochalasin B (Cyto-B) addition in the recovery phase (to allow for any cell cycle delay in damaged cells).

It is, therefore, difficult to reconcile the current OECD 487 recommended treatment and recovery times, particularly as it is known that upon encountering DNA damage proliferating mammalian cells will initiate cell-cycle arrest to complete repair of DNA damage before continuing with cellular division, or if the damage is too great undergo apoptosis. In mammalian cells the tumour suppressor gene (p53) plays a key role in safeguarding the integrity of the human genome *via* modulating cell-cycle arrest, DNA repair and apoptotic processes (8,9). Consequently for chemicals that may interfere with the mitotic process, target-specific stages of the cell cycle or require multiple rounds of DNA division, it would seem prudent that a recovery phase be included to allow damaged cells to progress through to the next interphase and express micronuclei.

In this study, we measured average generation time (AGT) of human peripheral blood lymphocytes (HPBL) following phytohaemagglutinin (PHA) stimulation at various sample times up to 144 h to determine whether Option B is compromised by declining HPBL proliferation. AGT was determined at each sampling interval using Hoechst plus Giemsa differential chromatid staining following bromodeoxyuridine (BrdU) incorporation.

In addition, the HPBL micronucleus (MN) assay was performed using both Option A and Option B treatment schedules to compare the ability to detect a range of aneugenic and clastogenic chemicals. On the basis of HPBL cell-cycle time in our laboratory (13 ± 2 h), Option A equates to a 24 h-treatment without recovery (24 + 0 h) schedule (including Cyto-B at time of treatment) and Option B to a 24-h treatment with 24-h recovery (24 + 24 h) schedule (including Cyto-B for the 24-h recovery period only). Cytotoxicity [measured by relative replication index (RI)] and MN induction were determined following treatment with a variety of chemicals (encompassing a range of mechanism) including multiple aneugens and nucleoside analogues (detailed in Table 1). The data from both extended treatment options were compared and recommendations made.

Table 1. Referenced chemicals selected for treatment

Chemical	Category	Reference for published *in vitro* data	Molecular weight	CAS No.	Treatment times (h)
AZT	Nucleoside Analogue	(10–12)	267.24	30516-87-1	24 + 0/24 + 24/3 + 21[a]
Cytosine arabinoside		(3,6)	243.22	147-94-4	24 + 0/24 + 24/3 + 21
5-FU		(6)	130.08	51-21-8	24 + 0/24 + 24
Mitomycin C	DNA crosslinker	(3,6,13,14)	334.33	50-07-7	24 + 0/24 + 24/3 + 21[a]
Vinblastine	Aneugenic chemicals	(3,15,16)	810.97	865-21-4	24 + 0/24 + 24/3 + 21
Diethylstilbestrol		(17,18)	268.36	56-53-1	24 + 0/24 + 24/3 + 21[a]
Carbendazim		(19)	191.19	10605-21-7	24 + 0/24 + 24
Taxol		(12,20)	853.91	33069-62-4	24 + 0/24 + 24/3 + 21
Nocodazole		(21)	301.32	31430-18-9	24 + 0/24 + 24/3 + 21[a]
Noscapine		(22)	413.42	128-62-1	24 + 0/24 + 24
Naladixic acid	Topo II poison	(23–25)	232.24	389-08-2	24 + 0/24 + 24
Di(2-ethylhexyl) phthalate	Negative chemicals	(12)	390.56	117-81-7	3 + 21/24 + 0/24 + 24
Pyrene		(26)	202.25	129-00-0	3 + 21/24 + 0/24 + 24
Sodium chloride		(26)	58.44	7647-14-5	3 + 21/24 + 0/24 + 24

Chemicals were selected to cover a range of mechanism with emphasis on those class that are known to target the mitotic process (aneugenic chemicals, nucleoside analogues) which are known to be problematic in a standard 24-h treatment methodology to compare the two extended treatment regimens. Mitomycin C was included as a standard indirect acting clastogenic positive control. Negative chemicals were selected based on recommendations made from past versions of the draft OECD 487 (26) as part of a general testing set for demonstration of assay proficiency.

[a]Experiment 2 only.

Objectives

- Investigate whether PHA-stimulated lymphocytes are still actively dividing beyond 96 h after PHA stimulation to determine their suitability for extended treatment with recovery (Option B).
- Compare Option A and Option B treatment schedules for their ability to detect clastogenic and aneugenic chemicals.

Short 3-h treatments were also included (as per OECD 2016 (3) requirements) for a number of chemicals to provide a context for any differences in response observed.

Materials and Methods

Chemicals and reagents

All chemicals, unless otherwise stated were obtained from Sigma-Aldrich, UK, and were equal to or greater than 98% pure. Test chemicals were formulated in reagent grade dimethyl sulphoxide (DMSO) or water. Stock solutions of test chemicals were prepared at 100× (DMSO) or 10× (water) the required final concentrations ~3–4 h prior to treatment and diluted 100- or 10-fold (respectively) directly in the cultures with mixing. The 14 chemicals selected for testing are given in Table 1.

A concurrent positive control chemical; mitomycin C (clastogen) was included within the 24 + 0-h treatments in the absence of S-9 for each experiment. For all experimental occasions the positive control induced a statistically significant ($P \leq 0.05$) increase in micronucleated binucleate (MNBN) cells over the concurrent vehicle control response with replicate cultures exhibiting MNBN cell frequencies that exceeded the laboratory historical vehicle control ranges and were consistent with the laboratory historical positive control data. All positive control responses demonstrated the acceptability of the tests (these data are not reported).

Cell culture establishment

HPBLs were obtained from two healthy donors. Donors of the same sex were used per experiment, but may have varied between experimental occasions. Blood donor characteristics are summarised in Table 2.

The heparinised blood was pooled and cultures established in sterile centrifuge tubes by placing 0.4 ml blood into a sufficient volume of HEPES-buffered RPMI medium (Life Technologies, UK) supplemented with 10% (v/v) heat inactivated foetal calf serum (HIFCS; Life Technologies) and 0.52% penicillin/streptomycin (Sigma-Aldrich, UK). The mitogen, PHA (reagent grade; Life Technologies) was included in the culture medium at a concentration of ~2% to stimulate the lymphocytes to divide. Blood cultures were incubated at 37 ± 1°C for ~48 h and rocked continuously, prior to treatment.

Average generation time

Blood cultures were treated with 10 µM BrdU (Sigma-Aldrich) for a period of 24 h at various times following PHA stimulation (two independent experiments) as detailed in Table 3.

Four cultures were treated at each time point in two independent experiments. Following Experiment 1, additional harvest times were included in Experiment 2 to more clearly define the point at which lymphocyte exponential growth was declining.

Cells were arrested in metaphase with addition of 0.1 mg/ml colchicine ~2 h prior to harvest followed by slide preparation. BrdU incorporation was assessed using Hoechst plus Giemsa differential chromatid staining (also known as the fluorescence plus Giemsa staining technique) allowing metaphases that have progressed through first, second and third divisions to be easily distinguished by light microscopy.

Standard blood donor AGT times for donors used in this laboratory are 13 ± 2 h.

The proportion of cells in first, second and third division were recorded from 50 cells per culture (200 cells per harvest time) and AGT (27) calculated as follows:

$$\text{AGT (h)} = \frac{\text{Time of BrdU incorporation (h)} \times \text{number of cells scored}}{\text{M1} + (2 \times \text{M2}) + (3 \times \text{M3})}$$

where M1, M2 and M3 are the relative proportion of cells in that division (i.e. first, second and third division, respectively).

MN Experiments

Comparative assessments were conducted between 24 + 0- and 24 + 24-h-treatments. However, for some chemicals additional data were obtained from 3 + 21-h treatments (Figure 1).

48 h after PHA addition, quadruplicate cultures were treated for vehicle controls and duplicate cultures for each test chemical and positive control.

One exception to this was for nocodazole (Noc) where for Experiment 1, single cultures were treated with test article and duplicate cultures for vehicle.

All treatments were performed in the absence of metabolic activation. Each culture received 150 mM KCl [5% (v/v)] followed by treatment with the vehicle, test article or positive control, 1% (v/v) per culture. Cyto-B was added at 1% (v/v) to 24 + 0-h treatment cultures to give a final concentration of 6 µg/ml, as detailed in Figure 1.

The final culture volume was 10 ml at the time of treatment. All cultures were incubated at 37 ± 1°C for the designated exposure time with gentle agitation on rocking platforms.

Post-treatment medium change

For either short (3 + 21 h) or extended (24 + 24 h) treatments, cells were pelleted by centrifugation at ~300 × *g* for 10 min and treatment medium removed. Cells were then washed twice with sterile saline (pre-warmed in an incubator set to 37 ± 1°C) and resuspended

Table 2. Blood donor details

Cell Line	Human peripheral blood lymphocytes
Sex of donors	Either (donors from Covance, Harrogate). Same gender used per experiment
Age range	18–35 years
Selection criteria	Non-smokers, not heavy drinkers of alcohol, not on any form of medication (contraceptive pill excluded), not suspected of any virus infection, or exposed to high levels of radiation or hazardous chemicals
Cell-cycle time (h)	13 ± 2

Table 3. Treatment of HPBLs with BrdU for determination of AGT

	Hours post PHA addition (time 0 h)[a]									
Treatment set	Set 1	Set 2	Set 3	Set 4	Set 5	Set 6	Set 7	Set 8	Set 9	Set 10
Experiment 1										
BrdU addition (h)				48		72		96		120
Cell harvest (h)				72		96		120		144
Experiment 2										
BrdU addition (h)	12	24	36	48	60	72	84	96	108	120
Cell harvest (h)	36	48	60	72	84	96	108	120	132	144

Addition of BrdU at 2 h prior to each harvest to allow for sampling at either 24 or 12-h intervals (Experiments 1 and 2, respectively) over the range of 36–144 h following PHA stimulation.

[a]See culture establishment.

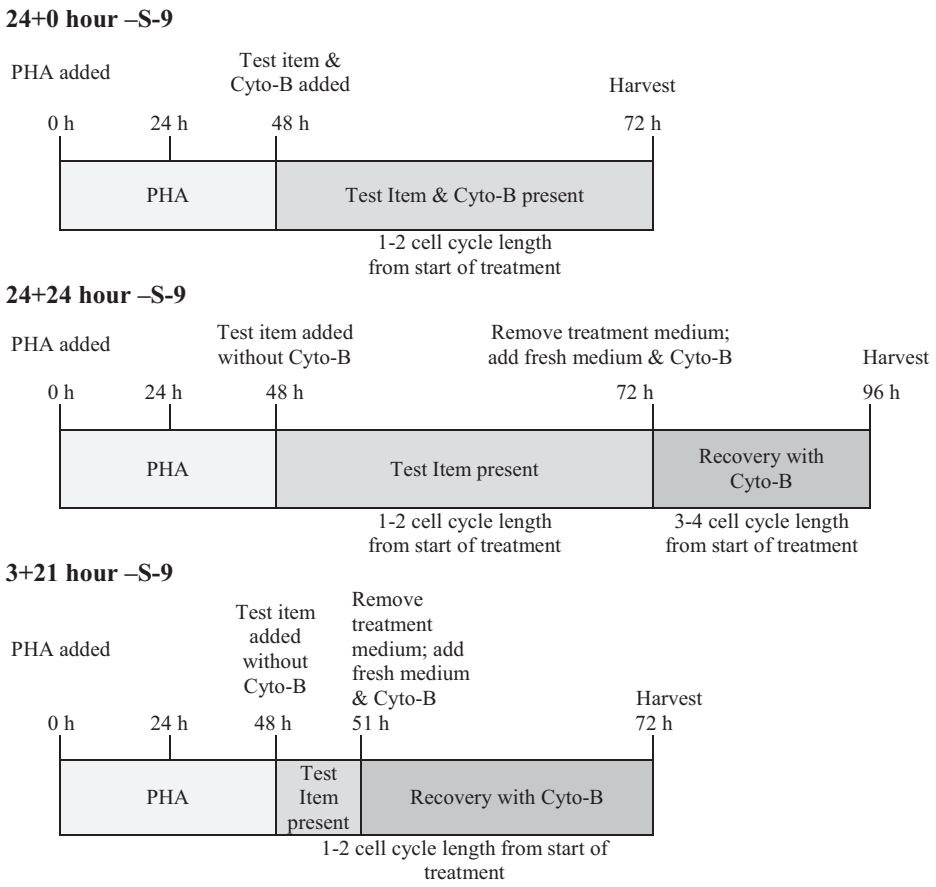

Figure 1. Treatment schedules. Illustration of 24 + 0-, 24 + 24-h and 3 + 21-h treatment schedules from PHA stimulation to cell harvest.

in fresh pre-warmed complete medium containing Cyto-B to give a final concentration of 6 µg/ml per culture. Cultures were incubated at 37 ± 1°C until cell harvest.

Harvest and slide preparation

At the specified sampling time (Figure 1) cells from each treatment culture were sampled for measurement of cytotoxicity and micronuclei as appropriate.

Cells from each culture for MN analysis were collected by centrifugation and treated with a hypotonic solution of 75 mM KCl at 37°C for 4 min to swell the cells to aid microscopic analysis. Cells were fixed with methanol: acetic acid (7:1) fixative using several washing, and centrifugation steps. Slides were prepared and stained with a solution of 125 µg/ml acridine orange (Sigma-Aldrich) and stored in the dark (typically for no longer than 1 week) until slide analysis.

Selection and scoring of concentrations for MN analysis

Slides were examined, uncoded, for cytotoxicity to a minimum of 500 cells per culture.

From these data, the RI which indicates the relative number of cell cycles per cell during the period of exposure of Cyto-B, was determined to calculate cytotoxicity (3), defined as:

$$\frac{[(\text{Number of binucleated cells}) + (2 \times \text{Number of multinucleate cells}) / \text{Total number of cells treated cultures}]}{[(\text{Number of binucleated cells}) + (2 \times \text{Number of multinucleate cells}) / \text{Total number of cells control cultures}]} \times 100$$

Cytotoxicity (%) is expressed as 100 – relative RI

A suitable range of concentrations was selected for MN analysis based on toxicity data in line with current regulatory guidelines for the IVMN assay (3).

The concentrations were selected (where possible) to fulfill the following criteria:

- Highest concentration: inducing ~50–60% cytotoxicity.
- Intermediate concentration(s): Inducing ~20–40% cytotoxicity.
- Lowest concentration: Inducing less than 20% cytotoxicity.

Where no limiting cytotoxicity or post-treatment precipitate was evident, the highest concentration analysed was limited to either 10 mM or 2000 µg/ml (3). The single exception to this was for pyrene, where limiting cytotoxicity was observed following extended 24 + 24-h treatment in Experiment 2 alone. Concentrations were analysed for this chemical to a maximum of 500 µg/ml (Experiment 1) or 750 µg/ml (Experiment 2), equivalent to either ~2.5 or 4 mM (Experiments 1 and 2, respectively).

All slides for MN analysis were coded and scored using fluorescence microscopy under blind-scoring conditions. Prior to analysis, several drops of phosphate-buffered saline were added to the acridine orange-stained slides and the slides coverslipped. At least 1000 binucleate cells per culture (2000 cells per concentration where possible) were analysed for the presence of MN.

Binucleate cells were only accepted for analysis if the cytoplasm remained essentially intact and the daughter nuclei were of approximately equal size. A MN was recorded if it had the same staining characteristics and a similar morphology to the main nuclei, was separate in the cytoplasm or only just touching a main nucleus and was smooth edged and smaller than approximately one third the diameter of the main nuclei. These criteria were in keeping with the principles as described by Fenech *et al.*(28).

Data analysis

After scoring, slides were de-coded and the proportion of MNBN cells at each treatment concentration was compared with the concurrent solvent (negative) control using the Fisher's exact test (one-sided analysis) with probability values of $P \leq 0.05$ accepted as significant (29). MN frequency was also assessed against the historical solvent control (normal) range (95% reference range, based on percentiles of the observed data (30)).

For all tests the following acceptability criteria were fulfilled:

- The mean frequency of cells with micronuclei in concurrent solvent controls fell within the historical solvent control (normal) ranges.
- A minimum of 50% of cells had gone through at least one cell division (as measured by binucleate + multinucleate cell counts).

Analysis of the positive control chemical was included under the 24 + 0 h –S-9 treatment condition for each chemical and fulfilled the positive criteria as stated later.

A test chemical was assessed as positive in this study if all of the criteria given below were met (indicated in Figure 2 by a 'red' colour).

- A statistically significant increase in the frequency of MNBN cells at one or more concentrations was observed.
- The incidence of cells with MN at such a concentration exceeded the 95th percentile of the observed historical vehicle control (normal) range in both replicate cultures.

Category	Chemicals	24+0 hour Treatment	24+24 hour Treatment	3+21 hour Treatment
Nucleoside Analogue	Azidothymidine	Negative	Positive	Negative
	Cytosine arabinoside	Negative	Positive	Negative
	Fluorouracil	Negative	Negative	-
DNA cross linker	Mitomycin C	Positive	Positive	Positive
Aneugenic chemicals	Vinblastine	Positive	Positive	Positive
	Nocodazole	Positive	Positive	Positive
	Noscapine	Positive	Positive	-
	Diethylstilbestrol	Negative	Positive	Negative
	Carbendazim	Positive	Positive	-
	Taxol	Positive	Positive	Positive
Topo II poison	Naladixic acid	Negative	Weak Positive	Negative
Negative chemicals	DEHP	Negative	Negative	Negative
	Pyrene	Negative	Negative	Negative
	Sodium chloride	Negative	Negative	Negative

Topo II: Topoisomerase II

DEHP: Di (2-ethylhexyl) phthalate

	Positive for MN (all criteria fulfilled)
	Negative for MN (no criteria fulfilled)
-	Not tested under this treatment

Figure 2. Summary of outcome of *in vitro* micronucleus tests in the presence of cytochalasin B with human lymphocytes comparing 24 + 0-, 24 + 24- and 3 + 21-h treatments.

A test chemical was considered negative in this study if none of the aforementioned criteria were met (indicated in Figure 2 by a 'green' colour).

Evidence of a concentration-related effect was considered useful but not essential in the evaluation of a positive result (31).

Results

HPBL AGT experimental work

Results for AGT are shown in Figure 3.

Experiment 1 was performed with cell harvests at 72, 96, 120 and 144 h after PHA stimulation. AGTs of 12.4, 12.8 and 14.7 h were recorded at 72, 96 and 120 h, respectively, with the majority of cells in first division (M1) at 144 h.

A second experiment was performed with cell harvest at 12 hourly intervals between 36 and 144 h following PHA stimulation. The majority of cells were in M1 at 36, 48 and 144 h after PHA stimulation. AGTs of 12.25–13.09 h were observed between 60 and 120 h after PHA stimulation, with 17.90 and 21.79 h AGTs observed following 132 and 144 h after PHA stimulation, respectively.

These data demonstrated that between 60 and 120 h following PHA stimulation, the AGT of the proliferating lymphocytes remains generally constant (between ~12 and 14 h). Assay harvest times of 72 and 96 h correspond to 24 + 0 h (Option A) and 24 + 24 h –S-9 (Option B) treatment, respectively, and as such, fall within this range.

This is further supported from comparisons of RI values in vehicle-control-treated cultures across 24 + 0- and 24 + 24-h treatments (Figure 4) which indicate no change in proliferative state between the two sample times (72 and 96 h following PHA stimulation).

Comparison of MN induction

The results for the different chemicals are discussed later. Results tables are illustrated in Figures 5–10 and summarised in Figure 2.

Azidothymidine (CAS Number 30516-87-1).

Results for azidothymidine (AZT) are shown in Figure 5a–d.

AZT is a nucleoside analogue of thymidine (reverse-transcriptase inhibitor used as an antiretroviral medication for human immunodeficiency virus and acquired immune deficiency syndrome). It has been used as a treatment of tumours with its growth inhibitory effects associated with a reduction in telomerase activity, S and G2/M phase cell-cycle arrest and enhanced DNA damage. Positive carcinogenicity has been observed (vaginal squamous cell carcinomas) in mice (10–12).

AZT was tested in the absence of S-9 for 24 + 0- and 24 + 24-h treatment conditions at concentrations up to a maximum of 1500 or 2000 µg/ml, respectively. Limiting toxicity (reduction in RI of greater than 50–60% of control values) was apparent following 24 + 0-h treatments but little to no toxicity was apparent following 24 + 24-h treatments. In Experiment 2, a further short treatment (3 + 21 h) was also included, testing at concentrations up to 2000 µg/ml, with no concentration limiting cytotoxicity apparent.

AZT: 24 + 0-h MN data

With the exception of a small statistically significant ($P \leq 0.05$) increase in MNBN cell frequency at a single low concentration (25.19 µg/ml, inducing 18% cytotoxicity) analysed after Experiment 1, frequencies of MNBN cells were similar to those observed in the concurrent vehicle controls for all concentrations analysed (both experiments). All group mean MNBN cell frequencies (both experiments) fell within normal values. These data, therefore, indicated a negative response.

AZT: 24 + 24-h MN data

Statistically significant ($P \leq 0.01$) increases in MNBN cell frequency were observed for all concentrations analysed in Experiments 1 and 2. With the exception of the highest concentration analysed in Experiment 1, frequencies of MNBN cells clearly exceeded normal range values for both replicate cultures at all concentrations analysed (both experiments). These data indicated a clear positive response.

AZT: 3 + 21-h MN data

No statistically significant ($P \leq 0.05$) increases in MNBN cells were observed. Normal frequencies of MNBN cells were observed for all treatment cultures (all concentrations). These data indicated a negative response.

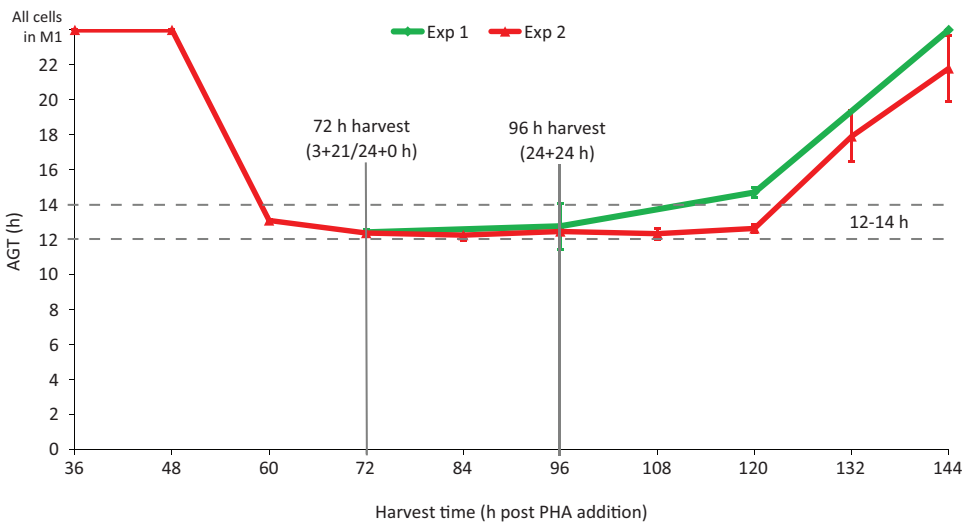

Figure 3. AGT of cultured HPBLs sampled at either 24 or 12 h intervals (Experiments 1 and 2, respectively) over the range of 36–144 h following PHA stimulation.

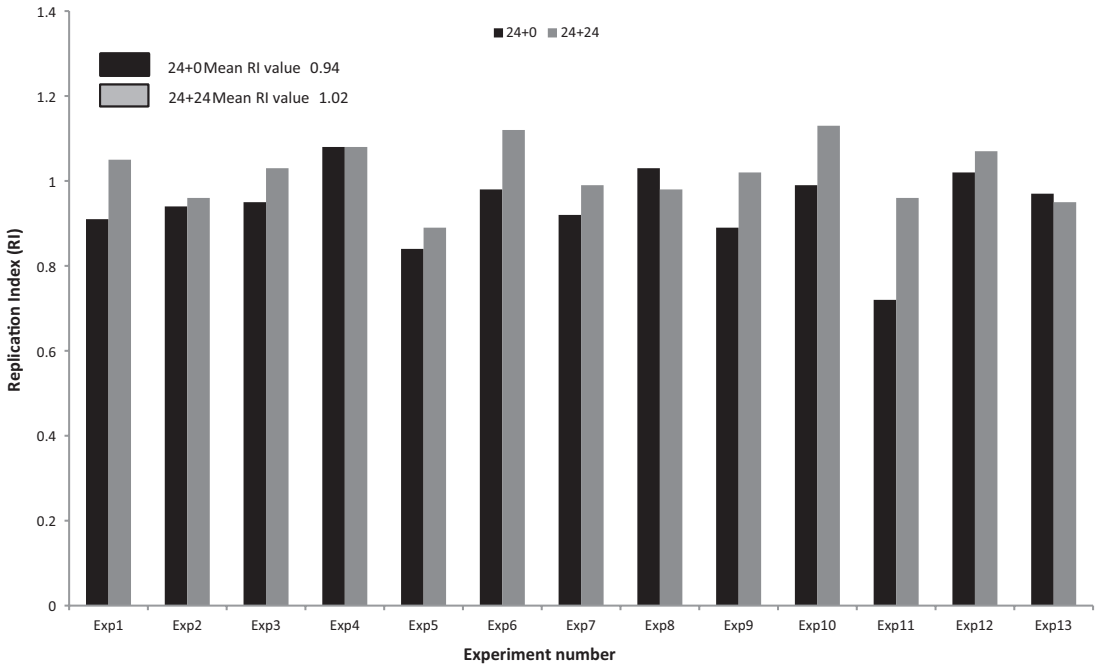

Figure 4. RI comparison. RI values of duplicate vehicle control cultures following either 24 + 0 or 24 + 24-h treatment in the absence of S-9 were taken from 13 separate experiments (conducted as part of this study).

Cytosine arabinoside (147-94-4).

Results for arabinoside (Ara-C) are shown in Figure 5e–h.

Cytosine Ara-C, a nucleoside analogue of cytosine is a chemotherapy medication used to treat acute myeloid leukaemia, acute lymphocytic leukaemia, chronic myelogenous leukaemia and non-Hodgkin's lymphoma. Cytosine arabinoside's mode of action is inhibition of DNA synthesis and Ara-C is recommended as a suitable chemical for either testing a laboratory's profficiency in conducting the IVMN assay or as inclusion as a suitable clastogenic positive control (3,6).

Ara-C was tested in the absence of S-9 for 24 + 0- and 24 + 24-h treatment conditions at concentrations up to a maximum limited by cytotoxicity. A 3 + 21-h treatment was also included. Steep concentration-related toxicity curves were observed across all treatments though this was far more pronounced following the 24 + 0-h protocol where maximum concentrations analysed (limited by cytotoxicity) for micronuclei were 0.06 or 0.1 µg/ml in Experiments 1 and 2, respectively, compared to 8 µg/ml for 24 + 24-h treatments, or 10–40 µg/ml for 3 + 21-h treatments.

Ara-C: 24 + 0- and 3 + 2-h MN data

Frequencies of MNBN cells were similar to and not significantly ($P \leq 0.05$) higher than those observed in concurrent vehicle controls for the majority of all concentrations analysed (Experiments 1 and 2). Exceptions to this were observed for the two highest concentrations analysed following 24 + 0-h treatment in Experiment 2 where statistically significant increases were apparent. However, these statistical increases were small, set against a low concurrent vehicle control response, with the MNBN cell frequencies of both treated cultures at each of these concentrations (and all others, both experiments) falling within normal ranges. There was no evidence of any concentration-related effect. These data indicated a negative response (24 + 0 and 3 + 21 h).

Ara-C: 24 + 24-h MN data

Statistically significant ($P \leq 0.01$) increases in MNBN cell frequency were observed for all concentrations analysed in Experiments 1 and 2. Frequencies of MNBN cells exceeded normal range values for both replicate cultures at all concentrations analysed (both experiments). These data indicated a clear positive response.

5-Fluorouracil (CAS Number 51-21-8).

Results for 5-fluorouracil (5-FU) are shown in Figure 5i and j.

5-FU is an antimetabolite fluoropyrimidine analogue of the nucleoside pyrimidine and is a nucleoside metabolic inhibitor. It interferes with DNA synthesis (nucleic acid synthesis inhibitor) and is used as a chemotherapy drug to treat various forms of cancer [breast, bowel, skin, stomach, oesophageal (gullet) and pancreatic cancer] (6).

5-FU was tested in the absence of S-9 for 24 + 0- and 24 + 24-h treatment conditions in a single experiment at concentrations up to a maximum of 130 µg/ml (equivalent to ~1 mM; molecular weight 130.08). Similar toxicity profiles were obtained between the two treatments with maximum concentrations analysed restricted by cytotoxicity.

5-FU: 24 + 0- and 24 + 24-h MN data

Frequencies of MNBN cells were similar to and not significantly ($P \leq 0.05$) higher than those observed in concurrent vehicle controls for all concentrations analysed, following both treatments. The MNBN cell values for all treated cultures (all concentrations, both treatments) fell within normal ranges. These data indicated a negative response.

Mitomycin C (CAS Number 50-07-7).

Results for mitomycin C (MMC) are shown in Figure 6a and b.

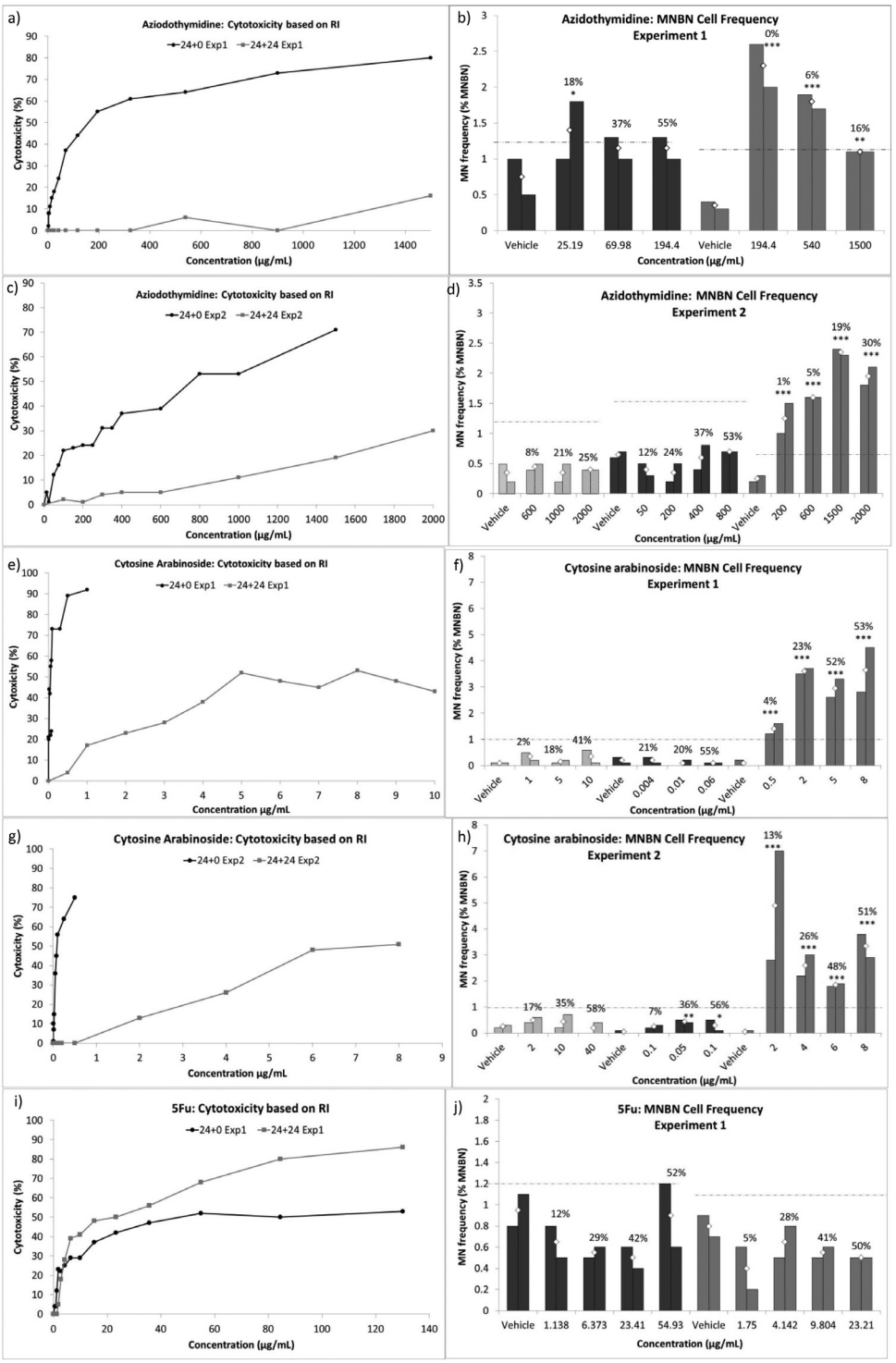

Figure 5. Nucleoside analogues. Comparison of cytotoxicity (based on RI) following 24 + 0- and 24 + 24-h –S-9 treatments. Comparison of MN induction in HPBLs following 3 + 21- (where performed), 24 + 0- and 24 + 24-h treatments in the absence of metabolic activation (S-9). The line charts represent cytotoxicity curve (RI) (**a–i**). The bar charts display MN frequency of individual replicate cultures together with mean MN frequency (**b–j**) and respective cytotoxicity. Mean MN frequency (Diamond); $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ (statistical significance compared to concurrent solvent control using one-tailed Fisher's' exact test); MN historical control (normal) range (Dashed line).

MMC is a chemotherapeutic drug that requires enzymatic bioreduction to exert its biological effects. Upon reduction, MMC is converted into a highly reactive bis-electrophilic intermediate that alkylates nucleophiles (leading to crosslinking of strands of double helical DNA) and inhibition of DNA synthesis (13,14). MMC is recommended as a suitable chemical for either testing a laboratory's efficiency in conducting the IVMN assay or as inclusion as a suitable clastogenic positive control (3).

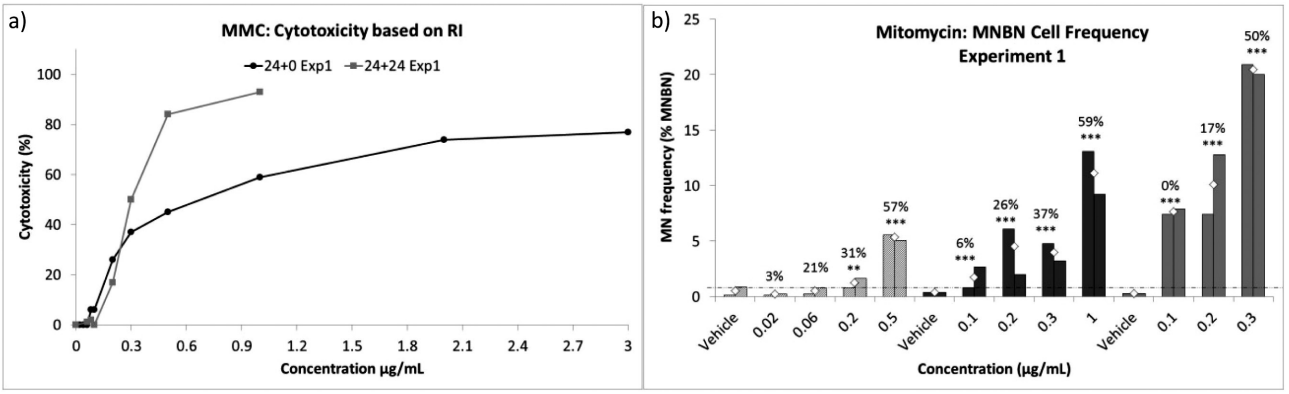

Figure 6. DNA cross linker. Comparison of cytotoxicity (based on RI) following 24 + 0- and 24 + 24-h –S-9 treatments. Comparison of MN induction in HPBLs following 3 + 21- (where performed), 24 + 0- and 24 + 24-h treatments in the absence of metabolic activation (S-9). The line charts represent cytotoxicity curve (RI) (**a**). The bar charts display MN frequency of individual replicate cultures together with mean MN frequency and respective cytotoxicity (**b**). Mean MN frequency (Diamond); $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ (statistical significance compared to concurrent solvent control using one-tailed Fisher's' exact test); MN historical control (normal) range (Dashed line).

MMC was tested in the absence of S-9 for 24 + 0- and 24 + 24-h treatment conditions at concentrations up to a maximum limited by cytotoxicity in a single experiment. A 3 + 21-h treatment was also included. Similar steep toxicity profiles were observed between 3 + 21- and 24 + 0-h treatments, though a steeper profile was observed following 24 + 24-h treatment.

MMC: 24 + 0- and 24 + 24-h MN data

Statistically significant ($P \leq 0.05$) increases in MNBN cell frequency were observed for all concentrations analysed. Frequencies of MNBN cells exceeded normal range values for both replicate cultures for the three highest concentrations analysed under each treatment regimen, though a greater magnitude of MNBN cell induction was observed following 24 + 24-h treatment These data indicated a clear positive response.

MMC: 3 + 21-h MN data

Statistically significant ($P \leq 0.05$) increases in MNBN cell frequency were observed for two of the four concentrations analysed. Frequencies of MNBN cells exceeded normal range values for both replicate cultures at the highest concentration and for a single culture at the intermediate concentration analysed. The general response was weaker than observed for either of the longer treatment regimens.

Vinblastine (CAS Number 865-21-4).

Results for vinblastine (VIN) are shown in Figure 7a–d (Experiments 1 and 2) and Figure 8 (additional investigative experiments).

VIN is an antimitotic drug used as an antitumour agent (e.g. Hodgkin's disease, lymphocytic lymphoma and advanced carcinoma of the testes) and known aneugen. Its mode of action is inhibition of microtubule formation, resulting in disruption of the mitotic spindle assembly and arrest of cells in the M phase of the cell cycle. VIN is recommended for inclusion in the standard testing protocol as a suitable aneugenic positive control (3,15,16).

VIN was tested in the absence of S-9 for 24 + 0- and 24 + 24-h treatment conditions at concentrations up to a maximum limited by cytotoxicity in two MN experiments (a short 3 + 21-h treatment was also included).

Steep toxicity profiles were observed for all treatments though this was far more pronounced following 24 + 0-h treatment where targeted concentration selection (to a limit of 50–60% cytotoxicity) was difficult to achieve (where a concentration interval of 0.005 µg/ml separated concentrations inducing 36% and 79% cytotoxicity). This inconsistent pattern of toxicity was also observed in both experiments following 3 + 21-h treatments. In contrast, a more consistent and less steep toxicity curve was associated with the 24 + 24-h treatments.

The toxicity data of 11 consecutive 24 + 24-h and 7 consecutive 3 + 21-h treatments (which included the experiments detailed earlier) were assessed to further investigate and compare patterns of toxicity and MN response (Figure 8).

For the 24 + 24-h treatments, the same concentration of 0.04 µg/ml was tested and demonstrated a stable and predictable response between experiments (Figure 8a) whereas for the 3 + 21-h treatments, a pattern of inconsistent toxicity was confirmed between treatments (Figure 8c).

VIN: 24 + 0-h MN data

For Experiment 1 (Figure 7a and b) due to steep toxicity the maximum concentration analysed (0.01 µg/ml) induced 31% cytotoxicity (below the regulatory-recommended target of ~50%). At this concentration a weak but statistically significant increase in MNBN cells was observed. However, only one of the two replicate cultures demonstrated a MNBN cell response exceeding the normal range. No other increase (above concurrent vehicle or historical) was observed at lower concentrations analysed.

Following Experiment 2 treatments (Figure 7c and d), steep toxicity was again apparent with concentrations analysed of 0.01, 0.02 and 0.025 µg/ml inducing 19%, 36% and 79% cytotoxicity, respectively. Statistically significant increases in MNBN cells were observed at the highest two concentrations though clear increases in MNBN cells (that clearly exceeded the normal range) were apparent in just one of two treated cultures at 0.02 µg/ml and in both cultures at 0.025 µg/ml where excessive cytotoxicity was observed.

VIN: 24 + 24-h MN data

Statistically significant increases in MNBN cells values were observed for all four concentrations analysed in Experiments 1 and 2 (Figure 7a–d) with the MNBN cell values of the majority of replicate cultures (per concentration analysed) exceeding historical

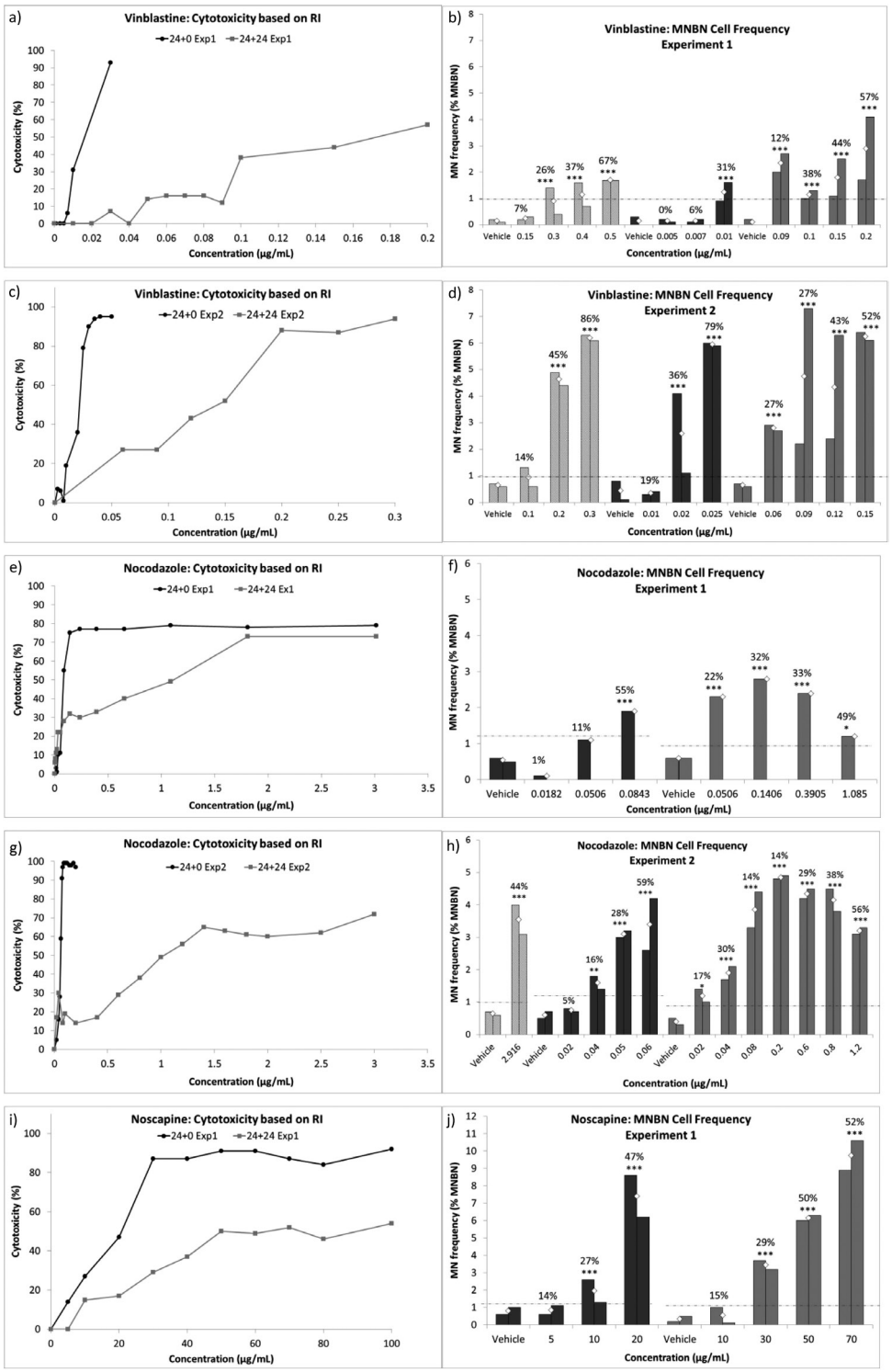

Figure 7. Aneugens. Comparison of cytotoxicity (based on RI) following 24 + 0- and 24 + 24-h –S-9 treatments. Comparison of MN induction in HPBLs following 3 + 21- (where performed), 24 + 0- and 24 + 24-h treatments in the absence of metabolic activation (S-9). The line charts represent cytotoxicity curve (RI) (**a–q**). The bar charts display MN frequency of individual replicate cultures together with mean MN frequency (**b–r**) and respective cytotoxicity. Mean MN frequency (Diamond); $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ (statistical significance compared to concurrent solvent control using one-tailed Fisher's' exact test); MN historical control (Dashed line).

vehicle control range values. A clear positive response was observed across a range of cytotoxicity (12%–57%, Experiment 1 and 27%–52%, Experiment 2).

VIN: 3 + 21-h MN data

For Experiment 1, significantly ($P \leq 0.05$) elevated MNBN cell frequencies were observed for the highest three concentrations analysed

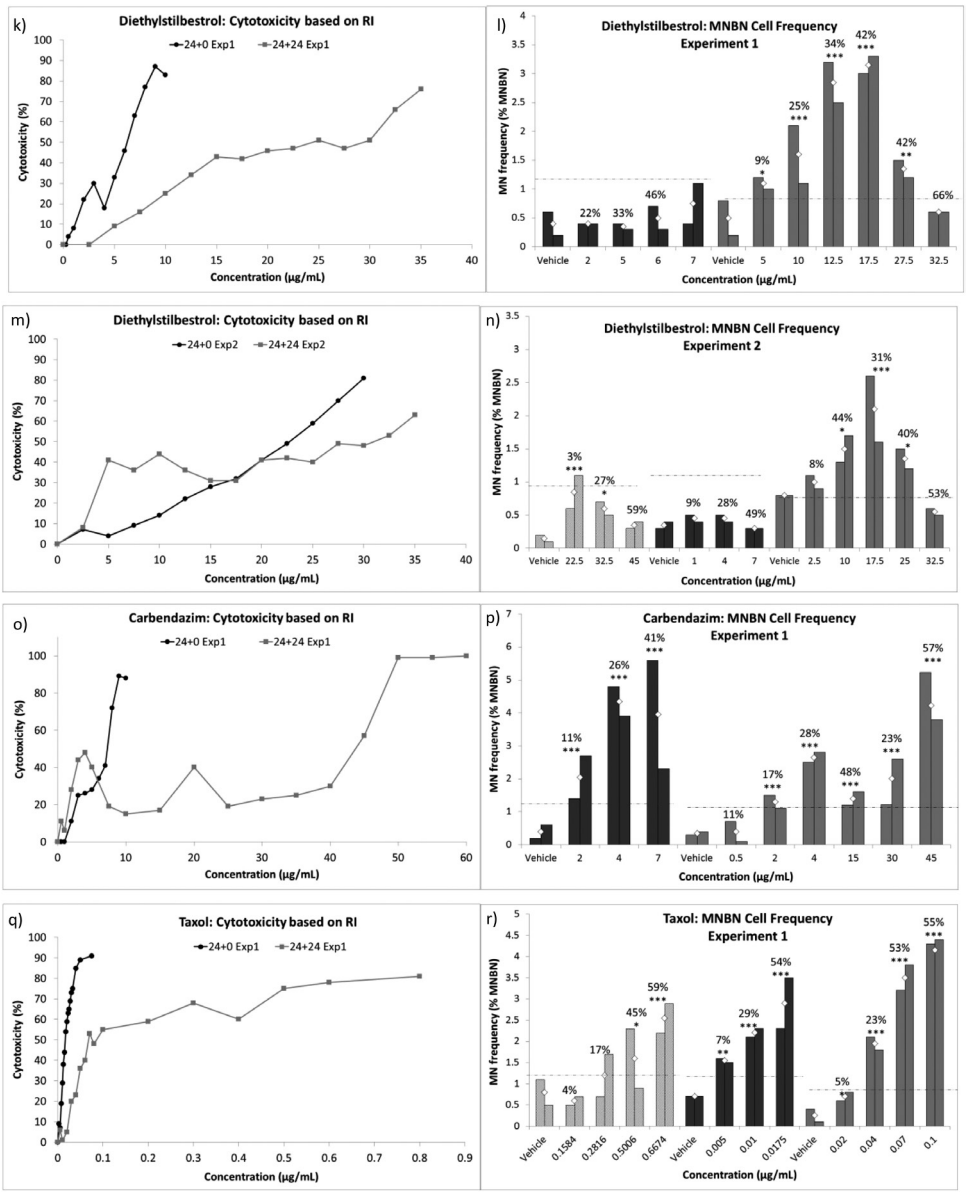

Figure 7. Continued

(Figure 7a and b). Only at the high concentration did both replicate cultures exhibit MNBN cell values exceeding the normal range.

For Experiment 2, significantly ($P \leq 0.05$) elevated MNBN cell frequencies were observed at the highest two concentrations analysed (Figure 7c and d). For both concentrations, both replicate cultures exhibited MNBN cell values that clearly exceeded the normal range.

To further investigate the pattern of MN response following 3 + 21-h treatment, MNBN cell data was compared across 5 additional experiments (Figure 8c and d). Large variations in MNBN cell frequency were observed between experimental occasions (associated with poorly predictable toxicity responses). For Experiments 3 and 4 where the same concentrations of 0.2, 0.3 and 0.4 µg/ml were tested, toxicity of 3%, 54% and 63%, respectively, was observed in Experiment 3 and 95%, 97% and 96%, respectively in Experiment 4. Although both experiments provided a clear positive response in terms of MNBN cell induction, this was not the case for Experiments 5, 6 and 7 where concentrations analysed in the range of 0.01–0.2 µg/ml resulted in low variable cytotoxicity and low MNBN cell responses only marginally exceeding historical vehicle control range values indicating weak and/or questionable positive responses.

In contrast, comparison of the MN response across 11 separate 24 + 24-h experiments (Figure 8a and b) where VIN was included as a positive control at a single concentration of 0.04 µg/ml, stable (and fairly predictable) MN and toxicity responses were observed with mean MNBN cell frequencies between 4.1% and 8.2% and toxicity between 31% and 49%.

Noc (CAS Number 33069-62-4).

Results for Noc are shown in Figure 7e–h.

Noc is a synthetic tubulin-binding agent with antineoplastic activity. Noc binds to β-tubulin and disrupts microtubule assembly/disassembly dynamics. This prevents mitosis and induces apoptosis in tumour cells. Noc is a known aneugen (21).

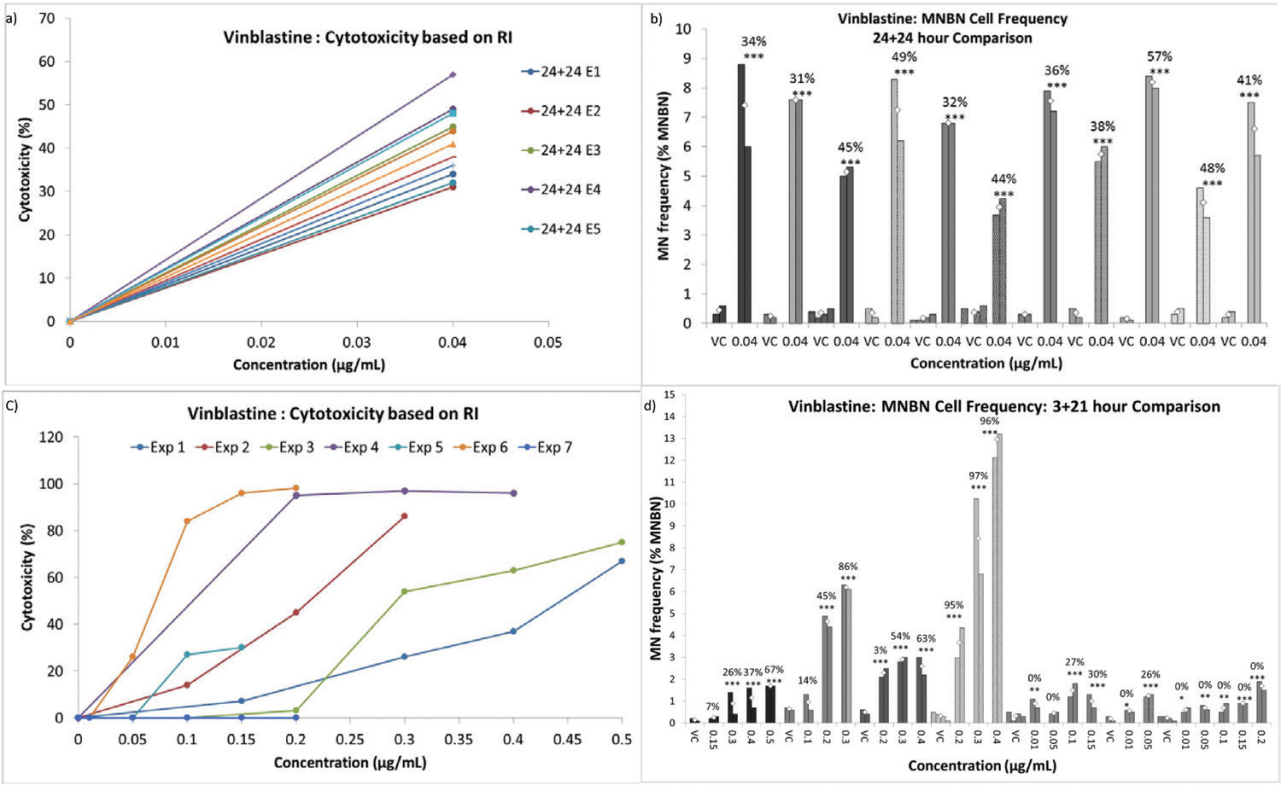

Figure 8. Vinblastine additional experiments. Vinblastine 24 + 24-h cytotoxicity profile (a) and MN response (b) across 11 experiments. Vinblastine 3 + 21-h cytotoxicity profile (c) and micronucleus response (d) across 7 experiments. Line charts represent cytotoxicity curve (RI) and bar charts represent MN frequency of individual replicate cultures together with mean MN frequency and respective cytotoxicity. Mean MN frequency (Diamond); $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ (statistical significance compared to concurrent solvent control using one-tailed Fisher's' exact test).

Noc was tested in the absence of S-9 for 24 + 0- and 24 + 24-h treatment conditions at concentrations up to a maximum limited by cytotoxicity in two experiments. A 3 + 21-h treatment was included in Experiment 2. Steep toxicity profiles were observed following all treatments, though this was more pronounced following the 24 + 0-h treatment where concentrations spanning 0.02–0.06 µg/ml (Experiment 2) induced between 5% and 59% cytotoxicity, compared to the 24 + 24-h treatment where concentrations spanning 0.02–1.2 µg/ml induced between 17% and 56% cytotoxicity.

Noc: 24 + 0-h MN data

Frequencies of MNBN cells were significantly ($P \leq 0.05$) higher than those observed in concurrent vehicle controls for either the highest concentration analysed in Experiment 1 or for three of four concentrations analysed in Experiment 2. Clear concentration-related increases in MNBN cell frequency were apparent. The MNBN cell values for single cultures tested in one-third of concentrations (Experiment 1), or for both treated cultures in three-fourths of concentrations analysed in Experiment 2 exceeded normal ranges. These data indicated a positive response.

Noc: 24 + 24-h MN data

Frequencies of MNBN cells were significantly ($P \leq 0.05$) higher than those observed in concurrent vehicle controls for all four concentrations analysed in Experiment and for all seven concentrations analysed in Experiment 2. The MNBN cell values for all treated cultures (all concentrations analysed, both experiments) exceeded normal values with concentration-related effects apparent. For both experiments a greater magnitude of MNBN cell induction was apparent as compared to the concurrent 24 + 0-h response.

Noc: 3 + 21-h MN data

A 3 + 21-h treatment was included as part of Experiment 2, but due to steep concentration-related toxicity, just a single concentration (2.916 µg/ml, inducing 44% cytotoxicity) was analysed. At this concentration a statistically significant ($P \leq 0.05$) increase in MNBN cells was apparent with both replicate cultures exhibiting MNBN cell values that exceeded the normal range. These data indicated a positive response.

Noscapine (CAS Number 128-62-1).

Results for Noscapine (Nosc) are shown in Figure 7i and j.

Nosc hydrochloride is a non-narcotic (derivative of opium), centrally acting antitussive drug widely used in cough suppressants. It has also been used effectively in reducing death rates from strokes and at higher doses been shown to have anticancer properties. It disrupts the mitotic spindle in mammalian cells and has been shown to induce aneuploidy as well as polyploidy in cultured human lymphocytes (22).

Nosc was tested in the absence of S-9 for 24 + 0- and 24 + 24-h treatment conditions at concentrations up to a maximum limited by cytotoxicity in a single experiment. A steeper toxicity profile was observed following the 24 + 0-h treatment where a concentration range spanning 5–30 µg/ml induced between 14% and 87% cytotoxicity

with a plateau occurring between 30 and 100 µg/ml. Following 24 + 24-h treatment, a concentration range spanning 5–100 µg/ml induced between 0% and 54% cytotoxicity with a plateau occurring between 50 and 100 µg/ml.

Nosc: 24 + 0-h MN data

Frequencies of MNBN cells were significantly ($P \leq 0.05$) higher than those observed in concurrent vehicle controls for two of the three concentrations analysed with a concentration-related effect apparent. However, only at the high concentration did the MNBN cell values of both treated cultures clearly exceed the normal range. These data indicated a positive response.

Nosc: 24 + 24-h MN data

Frequencies of MNBN cells were significantly elevated over the concurrent vehicle control response for three of the four concentrations analysed with a concentration-related effect apparent. The MNBN cell values of both replicate cultures at each of these three concentrations clearly exceeded the normal range. These data demonstrated a greater magnitude of MNBN cell induction compared to the 20 + 0-h treatment.

Diethylstilbestrol (CAS Number 56-53-1).

Results for diethylstilbestrol (DES) are shown in Figure 7k–n.

DES is a synthetic non-steroidal form of the female hormone oestrogen. It was prescribed to pregnant women between 1940 and 1971 to prevent miscarriage, premature labour and related complications of pregnancy. In 1971 prenatal DES exposure was linked to a type of cancer of the cervix and vagina called clear cell adenocarcinoma in a small group of women. DES is now known to be an endocrine-disrupting chemical, one of a number of substances that interfere with the endocrine system to cause cancer, birth defects and other development abnormalities. According to the Fourth Annual Report on Carcinogens (17), DES has been listed as a known carcinogen.

DES inhibits the hypothalamic–pituitary–gonadal axis, thereby blocking the testicular synthesis of testosterone, lowering plasma testosterone. It has also been shown to bind covalently to DNA inducing adducts in a number of tissues and is a known aneugen (18).

DES was tested in the absence of S-9 for 24 + 0 and 24 + 24-h treatment conditions at concentrations up to a maximum limited by cytotoxicity in two experiments. A 3 + 21-h treatment was included in Experiment 2. Steep toxicity curves were observed following the extended treatments, though this was far more pronounced following the 24 + 0-h treatment where maximum concentrations analysed (limited by cytotoxicity) for micronuclei were 7.0 µg/ml (in both Experiments 1 and 2) compared to 32.5 µg/ml for 24 + 24-h treatments, or 45 µg/ml for 3 + 21-h treatments.

DES: 24 + 0-h MN data

Frequencies of MNBN cells were similar to and not significantly ($P \leq 0.05$) higher than those observed in concurrent vehicle controls for all concentrations analysed, both experiments. The MNBN cell values for all treated cultures (all concentrations, both experiments) fell within normal ranges. These data indicated a negative response.

DES: 24 + 24-h MN data

Statistically significant ($P \leq 0.05$) increases in MNBN cell frequency were observed for the majority of all concentrations analysed (both experiments). Clear concentration-related increases in MNBN cells were observed with the MNBN cell frequencies of both cultures for either five-sixths of concentrations Experiment 1 or, three-fifths of concentrations analysed in Experiment 2 exceeding normal range values. These data indicated a clear positive response.

DES: 3 + 21-h MN data

Weak but statistically significant increases in MNBN cells were observed for the lowest two concentrations analysed, however, with the exception of a single culture at the low concentration the MNBN cell frequencies of all DES-treated cultures (all concentrations) fell within normal values and there were no concentration-related increases apparent. These data were considered weak evidence of a test article-related effect and would require further experimentation to provide a clear conclusion.

Carbendazim (CAS Number 10605-21-7).

Results for Carbendazim (CBZ) are shown in Figure 7o and p.

CBZ is the International Standardisation Organization (ISO)-approved common name for methyl 2-benzimidazole carbamate, a systemically active benzimidazole fungicide that inhibits the synthesis of β-tubulin, disrupting microtubule assembly, preventing the formation of spindles at cell division resulting in the malsegregation of chromosomes during cell division. CBZ is a known aneugen (19).

CBZ was tested in the absence of S-9 for 24 + 0- and 24 + 24-h treatment conditions at concentrations up to a maximum limited by cytotoxicity in a single experiment. Steep toxicity profiles were observed following both treatments, though this was far more pronounced following the 24 + 0-h treatment where concentrations of 2, 4, 7 and 8 µg/ml induced 11%, 26%, 41% and 72% cytotoxicity, respectively. Following 24 + 24-h treatment the toxicity curve was less steep with concentrations in the range of 0.5–45 µg/ml inducing 11%–57% cytotoxicity.

CBZ: 24 + 0-h MN data

Statistically significant ($P \leq 0.05$) increases in MNBN cell frequency were observed for all three concentrations analysed with the MNBN cell frequencies of both cultures at each concentration exceeding normal ranges. These data indicated a clear positive response.

CBZ: 24 + 24-h MN data

Statistically significant ($P \leq 0.05$) increases in MNBN cell frequency were observed for five of the six concentrations analysed. Clear concentration-related increases in MNBN cells were observed with the MNBN cell frequencies of both cultures for two of the five concentrations analysed clearly exceeding normal range values. These data indicated a clear positive response.

Taxol (paclitaxel) (CAS Number 33069-62-4).

Results for Taxol are shown in Figure 7q and r.

Taxol is used as a chemotherapy medication for treatment in a number of types of cancer. This includes ovarian cancer, breast cancer, lung cancer, Kaposi sarcoma, cervical cancer and pancreatic cancer. Unlike other tubulin-targeting drugs, such as colchicine that inhibit microtubule assembly, Taxol stabilises the microtubule polymer and protects it from disassembly. It effectively blocks cells in the G2/M phase of the cell cycle and as such cells are unable to form a normal mitotic apparatus impacting chromosome segregation, and cell division. Taxol is a known aneugen (12,20).

Taxol was tested in the absence of S-9 for 3 + 21-, 24 + 0- and 24 + 24-h treatment conditions at concentrations up to a maximum limited by cytotoxicity in a single experiment. Steep toxicity curves

were observed following all treatments, though this was far more pronounced following the 24 + 0-h treatment where concentrations of 0.005, 0.01 and 0.0175 µg/ml induced 7%, 29% and 54% cytotoxicity, respectively. Following 24 + 24-h treatment, the toxicity profile was slightly less steep with concentrations of 0.02, 0.04, 0.07 and 0.1 µg/ml inducing 5%, 23%, 53% and 53% cytotoxicity, respectively.

Taxol: 24 + 0- and 24 + 24-h MN data

Frequencies of MNBN cells were significantly ($P \leq 0.05$) higher than those observed in concurrent vehicle controls for all concentrations analysed with concentration-related effects apparent. The MNBN cell values for both treated cultures for the highest three concentrations analysed (both treatments) exceeded normal ranges. These data indicated a clear positive response. A greater magnitude of MNBN cell induction was observed following 24 + 24-h treatment.

Taxol: 3 + 21-h MN data

Frequencies of MNBN cells were significantly ($P \leq 0.05$) higher than those observed in concurrent vehicle controls for two of the four concentrations analysed with a concentration-related effect apparent. However, only at the highest concentration analysed did the MNBN cell frequency of both replicate cultures exceed the normal range. These data indicated a positive response though the magnitude of MNBN cell induction was lower than observed for either the 24 + 0- or the 24 + 24-h treatments.

Naladixic acid (CAS Number 389-08-2).

Results for naladixic acid (NA) are shown in Figure 9a–d.

NA is a bacterial gyrase inhibitor [weak topoisomerase II (topo II) poison] that prevents the supercoiling of DNA that is necessary for compacting chromosomes in the bacterial cell (inhibiting DNA synthesis). This was the first widely used quinolone antibiotic and used for treatment of urinary tract infections (as it concentrates in the renal tubules and bladder). It has been surpassed by more potent-related products (fluorination of the quinolone structure was subsequently found to provide compounds up to 60 times more active that NA and killed a wider range of organisms). These more active derivatives also possess mammalian cell DNA activity at higher concentrations than those that impact bacterial DNA (23–25).

NA was tested in the absence of S-9 for 24 + 0- and 24 + 24-h treatment conditions at concentrations up to a maximum limited by cytotoxicity in two experiments. A 3 + 21-h treatment was included in both experiments [testing to 500 µg/ml in Experiment 1 and to 2300 µg/ml (equivalent to 10 mM) in Experiment 2, with no concentration limiting cytotoxicity observed]. Similar toxicity profiles were observed following both extended treatments, though a slightly less steep toxicity profile was observed following 24 + 24-h treatment in Experiment 2.

NA: 24 + 0- and 3 + 21-h MN data

Frequencies of MNBN cells were similar to and not significantly ($P \leq 0.05$) higher than those observed in concurrent vehicle controls for all concentrations analysed in both experiments. The MNBN cell

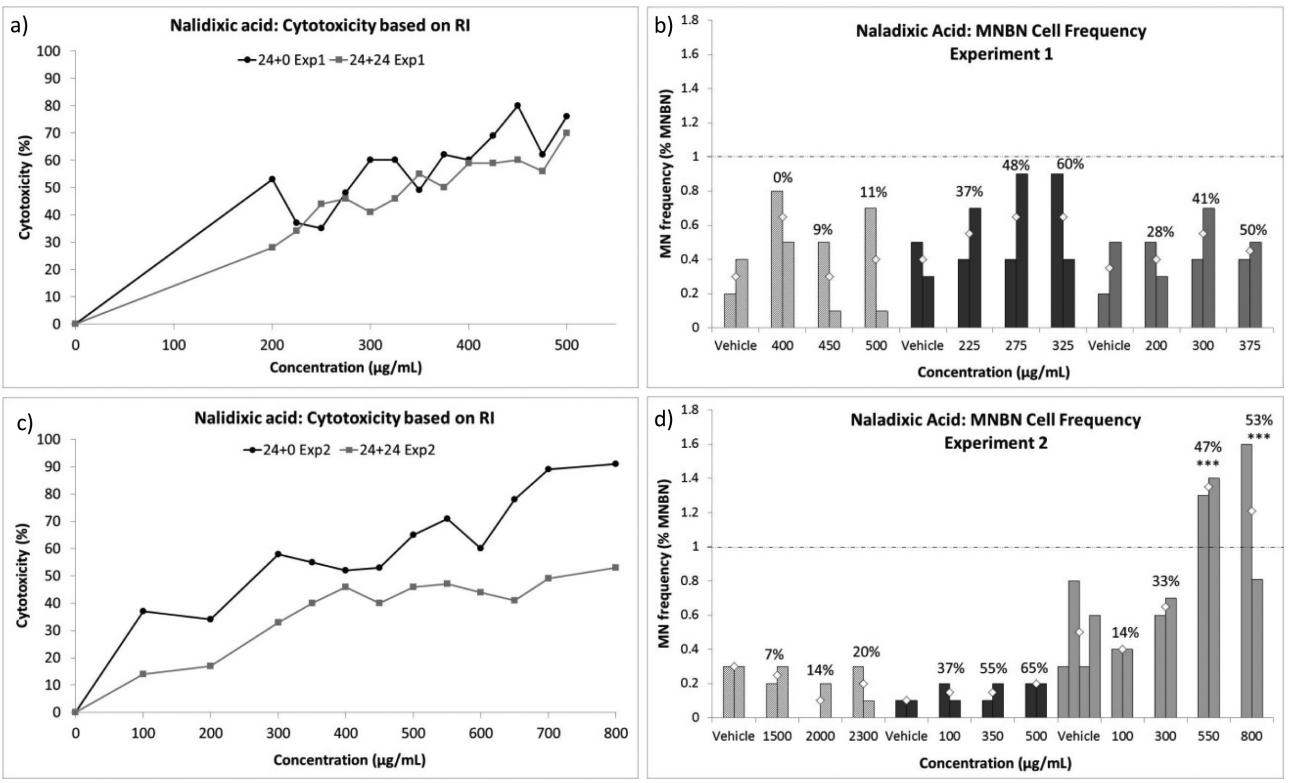

Figure 9. Topo II Poison. Comparison of cytotoxicity (based on RI) following 24 + 0- and 24 + 24-h –S-9 treatments. Comparison of MN induction in HPBLs following 3 + 21- (where performed), 24 + 0- and 24 + 24-h treatments in the absence of metabolic activation (S-9). The line charts represent cytotoxicity curve (RI) (**a, c**). The bar charts display MN frequency of individual replicate cultures together with mean MN frequency and respective cytotoxicity (**b, d**). Mean MN frequency (Diamond); ***$P \leq 0.001$ (statistical significance compared to concurrent solvent control using one-tailed Fisher's' exact test); – · ·, MN historical control (normal) range (Dashed line).

values for all treated cultures (all concentrations, both experiments) fell within normal ranges. These data indicated a negative response. Maximum concentrations analysed following 3 + 21-h treatment were not restricted by cytotoxicity. A maximum concentration of 500 µg/ml (Experiment 1) or 2300 µg/ml, Experiment 2 (equivalent to ~10 mM) were analysed. These data indicated a negative response (3 + 21- and 24 + 0-h treatments).

NA: 24 + 24-h MN data

Frequencies of MNBN cells were similar to and not significantly ($P \leq 0.05$) higher than those observed in concurrent vehicle controls for all concentrations analysed following Experiment 1 treatments, with the MNBN cell frequency values for all treated cultures falling within normal ranges. Concentrations were analysed at 200, 300 and 375 µg/ml, inducing 28%, 41% and 50% cytotoxicity, respectively. Following Experiment 2 treatments, a slight shift in toxicity was observed such that higher maximum concentrations were analysed (concentrations of 100, 300, 550 and 800 µg/ml, inducing 14%, 33%, 47% and 53% cytotoxicity, respectively). In Experiment 2, a clear concentration-related increase in MNBN cell frequency was observed with both replicate cultures at 550 µg/ml and a single culture at 800 µg/ml exhibiting MNBN cell values that exceeded the normal range. These data indicated evidence of a weak induction of MNBN cells.

Di(2-ethylhexyl) phthalate (CAS Number 117-81-7).

Results for di(2-ethylhexyl) phthalate (DEHP) are shown in Figure 10a–d.

DEHP is used in the production of polyvinyl chloride and has been included as a negative (non-DNA reactive) chemical in this test set, though has been observed to be a non-genotoxic liver carcinogen in rats and mice (due to peroxisome proliferation). It has been shown to be negative *in vivo* for chromosome aberrations, micronuclei, unscheduled DNA synthesis and transgenic mutations as well as negative in the *in vitro* mouse lymphoma and chromosome aberration assays (12).

DEHP was tested in the absence of S-9 for 24 + 0- and 24 + 24-h treatment conditions at concentrations up to a maximum limited by cytotoxicity in two experiments. A 3 + 21-h treatment was included in both experiments. Similar toxicity curves were observed following both extended treatments.

DEHP: 3 + 21-, 24 + 0- and 24 + 24-h MN data

Frequencies of MNBN cells were similar to and not significantly ($P \leq 0.05$) higher than those observed in concurrent vehicle controls for all concentrations analysed, (all treatments, both experiments). The MNBN cell values for all treated cultures (all concentrations, all treatments) fell within normal ranges. These data indicated a negative response.

Pyrene (synonym: benzo[def]phenanthrene) (CAS Number 129-00-0).

Results for pyrene are shown in Figure 10e–h.

Pyrene is a polycyclic aromatic hydrocarbon consisting of four fused benzene rings, resulting in a flat aromatic system. Pyrene forms during incomplete combustion of organic compounds. It was included as a negative chemical in this study based on recommendations presented in the draft OECD 487 guideline of November 2, 2009 (Annex 3—reference chemicals recommended for assessing performance) (26).

Pyrene was tested in the absence of S-9 for 24 + 0- and 24 + 24-h treatment conditions at concentrations up to a maximum of 500 µg/ml (Experiment 1), or 750 µg/ml (Experiment 2). A 3 + 21-h treatment was included in both experiments. Similar toxicity profiles were observed following both extended treatments though cytotoxicity was seen to plateau following the 24 + 0-h treatments in Experiment 2 with concentration limiting cytotoxicity only observed following the 24 + 24-h treatment in Experiment 2. The maximum concentration of 750 µg/ml (inducing 41% cytotoxicity) was analysed as part of the 24 + 0-h treatment in Experiment 2.

Pyrene: 3 + 21-, 24 + 0- and 24 + 24-h MN data

Frequencies of MNBN cells were similar to and not significantly ($P \leq 0.05$) higher than those observed in concurrent vehicle controls for all concentrations analysed, (all treatments, both experiments). The MNBN cell frequency for all treated cultures (all concentrations, all treatments) fell within normal ranges. These data indicated a negative response.

Sodium chloride (CAS Number 7647-14-5).

Results for sodium chloride are shown in Figure 10i–l.

Sodium chloride is an ubiquitous sodium salt that is commonly used to season food. It was included as a negative chemical (i.e. not expected to induce micronuclei) in this study based on recommendations presented in the draft OECD 487 guideline ofNovember 2, 2009 (Annex 3—reference chemicals recommended for assessing performance) (26).

Sodium chloride was tested in the absence of S-9 for 24 + 0- and 24 + 24-h treatment conditions at concentrations up to 10 mM in two experiments. A 3 + 21-h treatment was included in both experiments. Little to no cytotoxicity was observed following all treatments.

Sodium chloride: 3 + 21-, 24 + 0- and 24 + 24-h MN data

Frequencies of MNBN cells were similar to and not significantly ($P \leq 0.05$) higher than those observed in concurrent vehicle controls for the majority all concentrations analysed, (all treatments, both experiments). Exceptions to this were observed at the highest two concentrations analysed following 24 + 24-h treatment in Experiment 2. However, these statistical increases were weak (set against a particularly low concurrent vehicle control response), with no concentration-related effect apparent. Furthermore, the MNBN cell frequency of both replicate cultures at each of these concentrations (and all others, all concentrations, all treatments, both experiments) fell within normal ranges. These data indicated a negative response.

Summary of MN experiments.

For the expected positive chemicals, 10 of the 11 tested were positive following the extended treatment with recovery (24 + 24 h) compared to 6 of 11 following the continuous treatment (24 + 0 h) (Figure 2). The one chemical (5-FU) seen to be negative following 24 + 24-h –S-9 treatment was also negative following 24 + 0-h treatment and has been shown to be problematic in previous mammalian lymphocyte cell studies (6). This is discussed in more detail later.

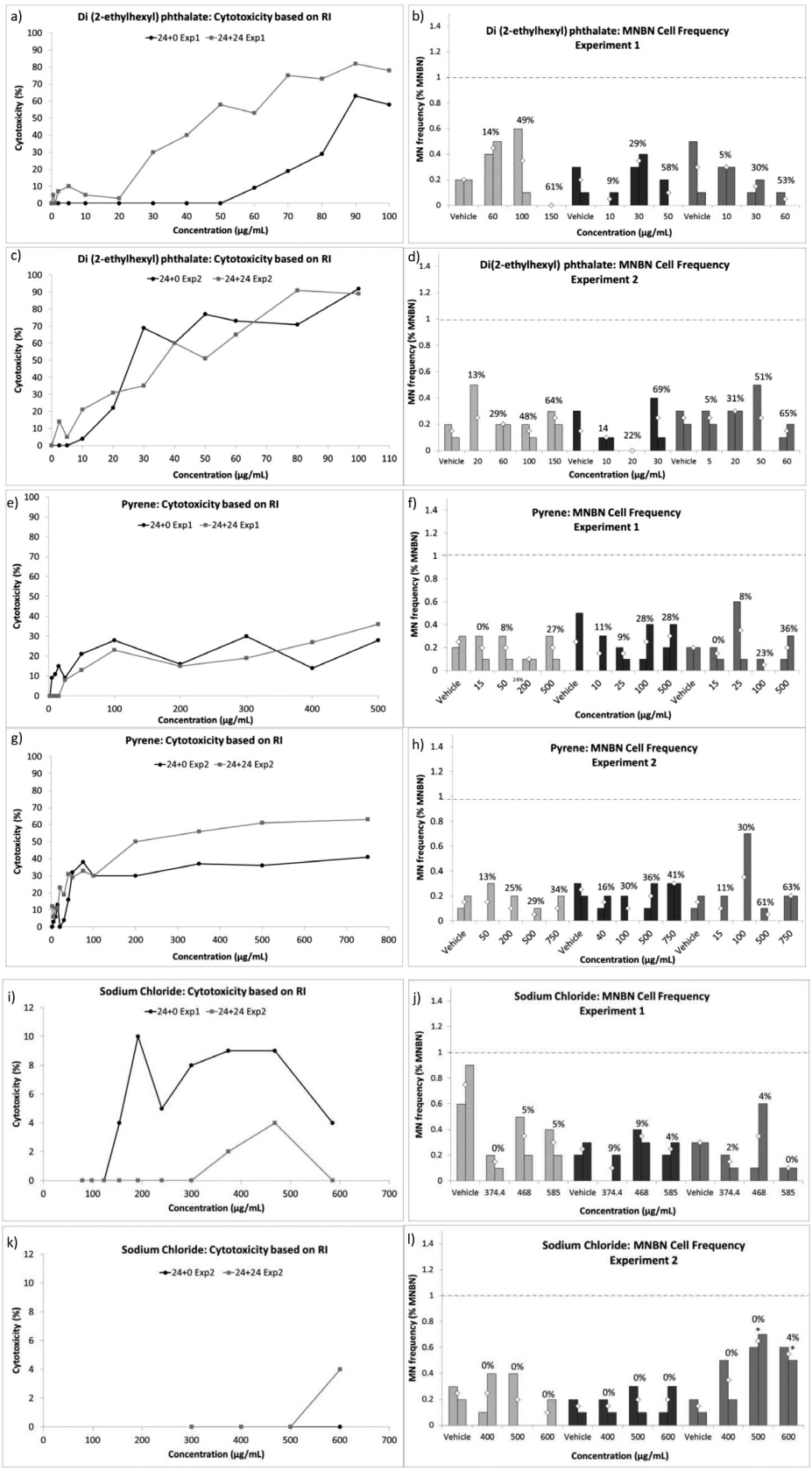

Figure 10. Negative chemicals. Comparison of cytotoxicity (based on RI) following 24 + 0- and 24 + 24-h –S-9 treatments. Comparison of MN induction in HPBLs following 3 + 21- (where performed), 24 + 0- and 24 + 24-h treatments in the absence of metabolic activation (S-9). The line charts represent cytotoxicity curve (RI) (**a–k**). The bar charts display MN frequency of individual replicate cultures together with mean MN frequency and respective cytotoxicity (**b–l**). Mean MN frequency (Diamond); $*P \leq 0.05$ (statistical significance compared to concurrent solvent control using one-tailed Fisher's' exact test); MN historical control (normal) range (Dashed line).

Of the nucleoside analogues tested (AZT, Ara-C and 5-FU), AZT and Ara-C were strongly positive following 24 + 24-h treatment but were negative following both 3 + 21- and 24 + 0-h treatments. AZT and Ara-C demonstrated far steeper toxicity profiles following the 24 + 0-h treatment.

Of the aneugenic chemicals tested (VIN, Noc, CBZ, Nosc, DES and Taxol), DES was negative following both 3 + 21- and 24 + 0-h treatments but was clearly positive following 24 + 24-h treatment. All remaining aneugens were positive following both extended treatment regimens (and the 3-h treatment where tested). However, continual treatment without recovery (24 + 0 h) resulted in steep toxicity profiles where concentration selection [within the OECD target cytotoxicity limits (of 55 ± 5%)] was difficult. The toxicity/MN response was often acute where very small concentration intervals separated high and low cytotoxicity and also MN response. This variability in cytotoxicity/MN response was also apparent with VIN following 3 + 21-h treatment where comparative MN responses across similar concentration ranges analysed for seven separate experiments gave conflicting positive and negative MN responses and large variability in magnitude of MN induction.

In contrast, the opposite was apparent following 24 + 24-h treatment where a higher magnitude of MN induction was generally observed for all aneugenic chemicals over a broader concentration range analysed, with more gradual toxicity profiles. This was illustrated following further investigation with VIN where toxicity and MN responses were compared from 11 experiments using the same single concentration (0.04 µg/ml). These data showed a consistent and predictable MN/toxicity response. For the topo II poison (NA) a positive response was observed following 24 + 24-h treatment but a negative result obtained following both 3 + 21- and 24 + 0-h treatments. NA is a bacterial gyrase inhibitor (weak topo II poison). The functionally more potent derivatives of this parent compound such as ciprofloxacin also exhibit genotoxicity (single and double strand DNA breaks) at higher concentrations in eukaryotic test systems. Although previous publications have concluded a lack of genotoxic potential, they have either failed to include a recovery period following an extended treatment incubation period, or, do suggest small increases in DNA damage above control values (23,24). A more recent investigation of micronuclei induction of NA by Sobol *et al.* (32) using human lymphoblastoid TK6 cells also concluded negative. However, these investigations used a continuous extended treatment without recovery [27 + 0-h treatment in the absence of a rat liver metabolic activation system (S-9)] and short 4-h treatments with extended recovery (4 + 40- h treatments –S-9 and +S-9). They did not look at using an extended treatment that included a recovery period. Furthermore, a randomised patient monitoring study (25) demonstrated that NA-treated patients exhibited significantly elevated frequencies of sister-chromatid exchange (SCE) events. As NA is a weak topo II poison and has been shown to induced SCE events, the weak positive induction of MNBN cells observed in this study is not suprising.

The direct acting clastogen MMC (DNA cross linker) demonstrated a greater magnitude of MN induction following 24 + 24-h treatment compared to both the 3 + 21- and 24 + 0-h treatments. Conventional thinking suggests the use of an extended recovery time with such direct short acting clastogenic chemicals may result in MN cells being lost due to apoptosis/necrosis. Work conducted by Sobol *et al.* (32) using human lymphoblastoid TK6 cells found a similar pattern using a short 4-h treatment with an extended three cell cycles (40 h) of recovery (as compared to the recommended 1.5–2.0 cell-cycle recovery). The author observed the same when looking at benzo(a)pyrene, cyclophosphamide and VIN which all provided a greater magnitude of MN induction when extended recovery times were used.

In the current study, the three expected negative chemicals (DEHP, pyrene and sodium chloride) were all negative following all three treatment regimens (3 + 21, 24 + 0 and 24 + 24 h).

Discussion

HPBL culture AGT

Micronuclei are produced from acentric fragments or whole chromosomes that are unable to attach to the spindle at mitosis (33). To detect the induction of micronuclei it is essential that mitosis has occurred either during or shortly following exposure to a test chemical and it is, therefore, important to ensure that cells are still actively dividing during treatment and any post-treatment recovery phase. The standard cell-cycle time in our laboratory for PHA-stimulated HPBL cell cultures is between 13 ± 2 h. HPBLs are exposed to the test chemical following ~48-h PHA stimulation to ensure cells are actively out of their resting (G_0) phase and dividing asynchronously (34).

For an extended treatment with recovery, HPBLs are exposed to the test chemical for 1.5–2 cell cycles followed by removal of the test chemical and a recovery phase for further 1.5–2 cell cycles (1). In our laboratory, this equates to a 24-h treatment followed by a 24-h recovery period (24 + 24-h, following 48-h PHA stimulation), a total of 96 h in culture from PHA stimulation to time of cell harvest. The current guideline for the *in vitro* mammalian MN cells test [OECD 487 (2016) (3)] states that 'When using extended sampling times (i.e. when total 3.0 to 4.0 cell cycle lengths culture time), care should be taken to ensure that the cells are still actively dividing. For example, for lymphocytes exponential growth may be declining at 96 hours following stimulation'. However, no specific cross-reference to any experimental work is given to support this opinion. This also may lead the investigator towards the default 'Option A' treatment schedule that is detailed in OECD 487 (3) under paragraphs 37, 38 and Table 2 for the continuous treatment (24 + 0 h, without a recovery).

From the AGT experiments conducted as part of this study (Figure 3), we have demonstrated that in our laboratory HPBLs are still actively dividing beyond 96 h, up to ~120 h after PHA stimulation such that extended treatment with recovery (Option B) is both viable and potentially appropriate.

RI is a measure of the proliferative state of the culture calculated using the following formulae to provide a ratio:

$$\mathrm{RI} = \frac{\text{number of binucleate cells} + (2 \times \text{number of multinucleate cells})}{\text{total number of cells counted}}$$

The higher the RI value, the more cells have divided at least once during the time of Cyto-B addition. As a general rule of thumb RI values of 0.7 and higher indicate suitably dividing populations over a 24-h period.

When comparing RI values of vehicle-control-treated cultures (between 24 + 0- and 24 + 24-h treatments) across a series of 13 consecutive experiments (Figure 4) the RI of 24 + 24-h treatments were similar to or exceeded that of the 24 + 0-h values [mean RI across all 13 treatments were 0.94 (24 + 0-h treatments) and 1.02 (24 + 24-h treatments)].

These data confirm actively proliferating cell populations at the 96-h sample times following 24 + 24-h treatments.

Overall the AGT and RI data provide clear evidence that HPBL cell cultures are actively dividing beyond 96 h following PHA stimulation, such that inclusion of an extended treatment with recovery (24 + 24 h) is practical and acceptable.

MN data

Of the 11 chemicals tested that would be expected to provide a positive mammalian cell MN response, with the exception of 5-FU (negative across both 24 + 0- and 24 + 24-h treatments), all were correctly identified as positive following the 24 + 24-h treatment schedule. Four of these (AZT, Ara-C, DES and NA) did not induce MN following the 24 + 0-h treatment.

No chemicals tested were positive following 24 + 0-h treatment, but negative following 24 + 24-h treatment.

The results of these MN experiments have also demonstrated a general greater magnitude of MN induction following extended treatment with recovery (across either equitoxic or lower concentrations analysed).

For the nucleoside analogues AZT and Ara-C, negative responses were observed following both 24 + 0- and 3 + 21-h treatments as opposed to clear positive responses following 24 + 24-h treatment. This pattern was also observed with the aneugenic chemical DES where the only clear (and consistent) positive response across two experiments was observed following the 24 + 24-h treatment schedule.

The exception noted earlier was for 5-FU where negative responses were observed across both extended treatments. However, this chemical has been shown to be challenging to assess in human lymphocytes and was investigated as part of the SFTG trials (SFTG II) (6) with conflicting results across the three treatment regimens tested and also between laboratories, hence it was concluded equivocal.

Although OECD 487 (3) allows the use of extended treatment times where certain chemical classes (such as nucleoside analogues) that impact on the cell cycle are to be tested, it would be preferable and more practical from the point of view of the investigator (no need for additional historical control range generation, for example) to have a protocol design that is able to detect these classes of chemical without modification. The adoption of the 24 + 24-h treatment schedule would therefore be worth considering as the default.

The IVMN test is specifically designed to detect both clastogenic (DNA direct acting breakage) and aneugenic (indirect, whole chromosome loss) events within the same assay system utilising treatment and recovery protocols to target both mechanisms of MN induction. Certainly the inclusion of a short treatment with removal of test article and sampling at 1.5–2.0 cell cycles (e.g. the 3 + 21-h treatment described in this study) has the potential to detect the majority of clastogenic chemicals. The test guideline (OECD 473 (35)) for the well-established *in vitro* chromosome aberration test indicates that short treatments should be performed as follows:

> Cells should be exposed to the test chemical without or with metabolic activation for 3–6 hours, and sampled at a time equivalent to about 1.5 normal cell cycle lengths after the beginning of treatment.

As the chromosome aberration assay is looking at chromosomes arrested in metaphase of mitosis, then allowing a further 2–3 h for cells to progress through to anaphase (or the next interphase) of mitosis, the target cells for analysis for the IVMN test, would seem sensible. Indeed, previous validation studies comparing the responses between the chromosome aberration assay and MN assay (where various treatment protocols were used) demonstrated good concordance (36,37), concluding that the IVMN test was reliably able to detect clastogens.

It would, therefore, be easy to justify the same extension to the extended continuous treatment arm of the assay as the default (OECD 473 (35) to OECD 487 (3)). Indeed, OECD 487 includes reference to the SFTG validation trials (5) and provides the following statement (paragraph 36):

> Published data (SFTG I General conditions and overall conclusions of the study) indicate that most aneugens and clastogens will be detected by a short term treatment period of 3–6 hours in the absence and presence of S-9, followed by removal of the test chemical and sampling at a time equivalent to about 1.5 to 2.0 cell cycle lengths after the beginning of treatment.
>
> However, for thorough evaluation, which would be needed to conclude a negative outcome, all three following experimental conditions should be conducted.

The additional treatment is stated as follows:

> Cells should be continuously exposed without metabolic activation until sampling at a time equivalent to about 1.5 to 2.0 normal cell cycle lengths.

However, the authors believe that this statement lacks clarity, particularly as the supporting reference in question is an overall view presented in the SFTG summary publication (5) combining the data obtained from human lymphocytes and rodent cell line testing. The conclusion statement from this review publication (5) is also different to that presented in OECD 487 in that it states:

> optimal detection was ensured when a short treatment followed by a long recovery was associated with a long continuous treatment without recovery.

However, it should be noted that the human lymphocyte testing arm of the SFTG validation trials using human lymphocytes *did not* include a continual treatment without recovery (6).

The actual treatment times used in these investigations (6) all included a recovery period and equated to the following:

> 3 + 26-h treatment after 43 h of PHA stimulation;
> 3 + 45-h treatment after 24 h of PHA stimulation;
> 20 + 28-h treatment after 24 h of PHA stimulation.

Cyto-B was present for the last 26 (3 + 26-h treatment) or 28 h (3 + 45- and 20 + 28-h treatments) prior to cell harvest.

Multiple chemicals including aneugenic, clastogenic and nucleoside analogues as well as several chemicals known to act at various stages of the cell cycle (Ara-C, DES and 5-FU) were investigated in multiple laboratories. The conclusions of these assessments were that all three treatment regimens were required to pick up all of these chemicals with a repeat treatment often necessary to confirm the results. The base analogue 5-FU proving difficult to detect (only picked up at a single concentration inducing 50% cytotoxicity in a single experiment following the 3 + 45-h treatment schedule). The data from our current investigations with 5-FU were, therefore, not surprising.

Furthermore, the discussion text within SFTG I (5) states (specifically in relation to the human lymphocyte data):

> For an unknown compound, the optimal detection would be expected from the combination of a short treatment followed by both a short and a long recovery, and a long treatment followed by recovery.

This statement accurately depicts the results presented in SFTG II (6).

These discussion points are not without further support. In 2003, the International Workshop on Genotoxicity Testing (IWGT) (7) made specific recommendations over treatment and recovery times for human lymphocytes based on data made available from the SFTG trials (5,6), in addition to data from various Japanese collaborative studies, European pharmaceutical industry validation studies and data from Lilly Research Laboratories (38,39). The recommended treatment schedule for human lymphocytes included a recovery period in the absence of test article for all treatment conditions (short and extended) as follows:

> Experiment 1:
> 3 + 45 h –/+S-9/20 + 28 h –S-9 *following 24-h PHA stimulation*.
> If negative or equivocal proceed to Experiment 2.
> Experiment 2:
> 3 + 45 h –/+S-9/20 + 28 h –S-9 *following 48-h PHA stimulation*.

Furthermore, a commentary on the SFTG validation trials by Fenech (4) recommended an experimental design for that included treatment and recovery periods equivalent to 24 + 24-h and 24 + 48-h in this study. This schedule was further presented in a later publication (33).

The IWGT recommendations fed into the first draft OECD 487 guideline for the IVM test published in 2004 (40) where these treatment schedules were presented.

It is, therefore, difficult to reconcile the treatment schedule for human lymphocytes detailed within the current 487 test guideline (3) against the validation data that were considered. The references stated do not endorse the current recommended treatment protocol in relation to the use of HPBLs. Rather, the validation data and data presented as part of this study would suggest including a 24 + 24-h treatment schedule as standard rather than a continual treatment (24 + 0 h) without recovery.

A continuous treatment with no recovery (24 + 0 h) is also counter-intuitive to our present understanding of the biology and mechanics of the cell-cycle progression for cultures encountering genotoxic insult. It is well known that when proliferating mammalian cells encounter DNA damage they initiate cell-cycle arrest to attempt repair of DNA damage prior to continuing with cellular division. If damage is severe then cells may undergo apoptosis and are lost. In these investigations this was born out with the majority of chemicals tested showing steeper toxicity following 24 + 0-h treatment (where Cyto-B is added for the duration of treatment and where cells have no recovery prior to time of cell harvest) as compared to the 24 + 24-h regimen where Cyto-B is added for the 24-h recovery phase.

RI which is used in this assay to measure cytotoxicity is a measure of cellular proliferation from the point of Cyto-B addition to cell harvest. For the 24 + 0-h treatment where test chemical and Cyto-B is added concurrently for the duration of treatment to time of cell sample, any cell-cycle perturbation from treatment will not allow cells to necessarily divide and be able to express damage as micronuclei. For the 24 + 24-h treatment, test article is washed off after 24 h and Cyto-B added for the recovery phase such that damaged cells in cell-cycle arrest from treatment may escape the block, continue through mitosis and be able to express damage as micronuclei. This is seen in the MN and cytotoxicity data from these experimental trials where, with the exception of the clastogenic chemical MMC and the nucleoside analogue 5-FU, higher concentrations were analysed from the 24 + 24-h treatment regimen for all other chemicals demonstrating the impact of including a recovery phase to allow cells to escape from cell-cycle block and also to suitably express induced damage in terms of micronuclei.

For mammalian cells the tumour suppressor gene (p53) is a major factor in safeguarding the integrity of the human genome *via* modulating cell-cycle arrest, DNA repair and apoptotic processes (8,9)). Previous work looking at the relationship between p53 status, apoptosis and induction of MN in different human and mouse cell lines (41) observed that species origin rather than p53 status accounted for differences in MN response between a number of 'misleading positive' chemicals. This was supported by reference to work showing differences between rodent and human cells in terms of DNA repair and cell-cycle control; e.g. human cells have been shown to have a greater stringency in control of cell-cycle progression than rodent cells (42,43), where treatments with colcemid (disrupting tubulin polymerisation), rodent cells were seen to keep replicating in the absence of mitosis and survive, leading to polyploid cells. In contrast, human cells were seen to arrest in the first G2/M cell-cycle phase and then die.

It, therefore, makes sense that for a comprehensive investigation protocol, treatment schedules for MN assessment should allow for removal of chemical from the test system and include a recovery period prior to cellular harvest (to allow optimal detection of MN cells, and progression/escape from cell-cycle block). In human cells this would be of particular importance when testing chemical classes known to impact the cell-cycle apparatus, or that induce cell-cycle delay.

Conclusions

The data from these investigations along with review of validation literature for the IVMN assay using HPBL cell cultures allow the following concluding points to be made:

PHA-stimulated blood lymphocytes remain actively dividing with a more or less constant AGT (between 12–14 h) from 60 to ~120 h. Therefore (contrary to the statement in OECD 487 (3)), there should be no restriction to the use of an extended treatment with recovery (up to and including 96 h following PHA stimulation).

Certain chemicals (DES, NA, AZT and AraC) were only detected following 24 + 24h treatment. Furthermore, the majority of chemicals tested (including the direct acting clastogen MMC) presented a greater magnitude of MN induction across a broader range of concentrations following the extended treatment with recovery schedule (24 + 24 h). The continuous (24 + 0 h) treatment often resulted in steep cytotoxic and/or MN induction responses that varied from experiment to experiment.

No chemical was positive following the 24 + 0-h treatment that was negative following 24 + 24-h treatment.

The data presented for the chemicals tested in this study are consistent with the views expressed and data considered as part of the *in vitro* MNT validation work performed to investigate the most appropriate treatment and harvest times and general conduct of the assay (4–7). The indication that these references are being used to

support inclusion of an extended treatment without recovery when using human lymphocytes is considered misleading.

Overall it is clear that when using HPBL cultures, short and extended treatments should also include a recovery period for optimal detection of micronuclei. The authors would recommend the inclusion of the extended treatment with recovery [1.5–2.0 cell-cycle treatment with 1.5–2.0 cell-cycle recovery (e.g. 24 + 24-h treatment)] over a continuous treatment without recovery [1.5–2.0 cell-cycle treatment (e.g. 24 + 0-h treatment)] schedule.

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