1. Introduction

Established *in vitro* mammalian cell genotoxicity tests, which are used for regulatory testing, have the benefit of high sensitivity for the detection of genotoxic human carcinogens [1]. They also have the disadvantage of less than optimum specificity in terms of their ability to correctly identify non-genotoxic carcinogens and non-carcinogens as negatives. This has been brought into sharp focus by the recent paper of Kirkland et al. [2]. Thus, established *in vivo* genotoxicity assays, such as the rodent bone marrow micronucleus test, are often used to put positive results from *in vitro* genotoxicity assays into perspective, i.e., to determine if the potential of a test compound to induce genotoxic damage *in vitro* is realised in the whole animal where adsorption, distribution, metabolism and excretion are fully functional. Metabolism, including detoxification, is often a key feature in determining the genotoxicity of test agents and metabolic activation may be different *in vivo* compared to *in vitro*. In some apparently rare circumstances the exogenous metabolism provided by rat liver S9, especially from animals pre-treated by cytochrome P450 inducers may be inadequate or inappropriate, so that inherent genotoxicity may be missed *in vitro* (see accompanying paper).

The *in vivo* micronucleus assay, using either bone marrow or peripheral blood, is the focus of this paper. The assay has proved useful for the detection of agents that induce chromosomal damage as: (i) the endpoint is easy to score, (ii) it provides good statistical power as many cells can be scored for the presence of micronuclei, and (iii) there is evidence that chromosomal breakage always leads to micronucleus formation, thus, if the micronucleus test is negative (and the compound and/or its metabolites can be shown to reach the bone marrow), chromosomal aberrations in this tissue can be ruled out. The converse is not true, thus, if the micronucleus assay is positive, one cannot conclude that the mechanism is due to chromosomal breakage without further experiments as will be shown below.

The rodent bone marrow micronucleus test is perceived as having moderate sensitivity for the detection of carcinogens, in that not all genotoxic agents or genotoxic metabolites necessarily reach the bone marrow in sufficient quantities to produce a detectable genotoxic response; however, specificity is perceived to be relatively high, i.e., false positives are apparently rare. Thus, with the regulatory genotoxicity test batteries currently in use, the results of *in vivo* genotoxicity tests, in particular bone marrow cytogenetic tests, play a vital role in risk and hazard assessments. Increases in micronucleated bone marrow cells are given great regulatory weight indicating that the test compound is likely to be a genotoxic carcinogen. However, there is an increasing body of evidence demonstrating that some disturbances in the physiology of treated animals can give rise to apparent increases in cytogenetic damage in the bone marrow that are not due to compound-induced genotoxic damage *per se*. An IWGT Working Group was formed to assess the existing data from the literature on this subject as well as to evaluate previously unpublished data obtained from a questionnaire sanctioned by the IWGT Steering Committee. The questionnaire (Appendix A) was sent to companies and institutes performing or contracting for genotoxicity studies.

The target cell in the bone marrow micronucleus test is the developing erythroblast. During the formation of enucleated erythrocytes, at the final cell division of the erythroblast, the nuclear contents fragment in a process similar to that in apoptotic cells (karyorrhexis) [3] and these fragments are collectively expelled from the cell envelope, contained in a plasma membrane. This is an efficient process, but occasionally some material is left behind in the immature erythrocyte. In haematology, these fragments are termed Howell-Jolly bodies and constitute at least a part of the spontaneous micronuclei seen in cells from untreated animals. Conditions that can reduce oxygen tension in the blood can stimulate the secretion of additional erythropoietin (EPO), which stimulates an increase in cell division of erythroblasts, and hence, increases the numbers of circulating erythrocytes. This improves the overall oxygen carrying capacity of the blood and restores levels of oxygen tension [4]. The increase in cell division will cause more cells to undergo enucleation and this may result in an increase in micronuclei formed 'spontaneously'.

2. Consequences of significant changes in core body temperature

2.1. Cytogenetic effects of hyperthermia and hypothermia in vitro and in vivo

Non-physiological temperatures have been known for some time to induce cytogenetic damage [5]. Transient hyperthermia (40 °C for 24 h or 42 °C for 6 h) induces chromosomal aberrations and micronuclei in CHL cells *in vitro* [6]. This damage is related to the disruption of the mitotic spindle as treatment of dividing cells at 45.5 °C for 5–15 min results in disassembly of the spindle apparatus and disruption of the contractile ring and midbody–cytoplasmic bridge complex [7]. Mild heat shock causes loss of dynamitin/p50 antibody staining from centrosomes and kinetochores. Hsp70 is rapidly

recruited to centromeres following heat shock as a protective measure [8].

Similar effects of hyperthermia occur *in vivo*. Up to four-fold increases in micronucleated erythrocytes have been reported in the bone marrow of mice subjected to whole body hyperthermia of 35–36 °C for 20–32 h [9,10] or 40 °C for 1–2 h [11]. About 25% of the micronuclei were relatively large suggesting the possible presence of whole chromosomes. Thus, it appears that body temperatures of 39.5 °C or higher for more than 30 min induce micronuclei in bone marrow cells due to disturbance of the mitotic apparatus.

Transient hypothermia also induces micronuclei (but not chromosomal damage) in CHL cells (31 or 33 °C for 24 h) [6]. More support for the ability of hypothermia to induce micronuclei comes from the studies of chemically induced hypothermia that are outlined below.

2.2. *Cytogenetic effects of compound-induced hypothermia in the bone marrow micronucleus test*

2.2.1. *Chlorpromazine*

Hypothermia is a known side effect of the binding of neuroleptic drugs to several classes of pharmologically active sites, including dopamine, serotonin, alpha 2 adrenergic receptor and sigma binding sites [12]. Chlorpromazine is one such drug. This phenothiazine compound is negative in assays for gene mutation in bacteria, for chromosome aberrations in mammalian cells and in Drosophila (SLRLT and SMART assays). Analysis of these results suggests that this compound generally is not considered to be a genotoxic agent [13].

Asanami and co-workers carried out a series of mouse bone marrow micronucleus tests on this drug. Doses of 12.5–100 mg/kg chlorpromazine decrease rectal temperature in mice from 37.7 °C to a mean of about 29 °C at 8–11 h after dosing; with the lower doses, body temperature returns to normal after 24 h [6]. Doses of 25–100 mg/kg chlorpromazine produce a significant increase in micronucleated polychromatic erythrocytes (PCEs) at 48 h after dosing (occasional increases were seen for individual doses at 24–48 h). Typical increases were 2.6 micronucleated PCEs/1000 PCEs, in the 100 mg/kg group, compared to 0.8 micronucleated PCEs/1000 PCEs in the controls.

No increases in micronuclei were seen when mice were given 25, 50 or 100 mg/kg chlorpromazine in an environment where the temperature was maintained at 30 °C for 46 h to help keep body temperature in the normal range. This observation provides evidence that the micronuclei seen in chlorpromazine-treated mice were a result of chlorpromazine-induced hypothermia and were not due to another type of inherent genotoxicity of this compound.

In addition, 53–58% of the micronuclei detected in the chlorpromazine-treated animals were relatively large (diameter equal or greater than 0.25 the diameter of the cytoplasm), providing some evidence that the micronuclei were a result of dysfunction of chromosome linkage to the spindle apparatus. It is also possible that hypothermia reduces the oxygen tension of the blood, inducing a stimulation of erythropoietin release and increased cell division of erythroblasts. This could cause an increase in micronucleus formation due to increased rates of cells undergoing enucleation (see Section 1).

Rats are known to be more refractory to the hypothermic effects of phenothiazine drugs such as chlorpromazine. Male SD rats were dosed i.p. with 31.3–250 mg/kg chlorpromazine. This treatment resulted in a decrease in rectal temperature, but only at 250 mg/kg chlorpromazine did the temperature fall below 33 °C for 20 h. This dose also produced a significant increase in micronucleated PCEs (control 0.55/1000 PCEs, test 1.5/1000 PCEs) [14].

2.2.2. *Reserpine*

Reserpine is another phenothiazine, neuroleptic drug. This compound is negative for chromosome aberration induction *in vitro*. Although reserpine has been reported to transform SHE cells, no cytogenetic abnormalities (or other genotoxic effects) were observed [15].

Mouse bone marrow micronucleus tests were conducted with reserpine doses of 1, 4, 10, 100 and 1000 mg/kg and significant increases in micronucleated PCEs were detected 48, 72 and 96 h after treatment with 100 and 1000 mg/kg (controls, 1.5/1000 PCEs, rising to 12/1000 PCEs in the treated animals). These doses produced a decrease in rectal temperature to below 33 °C for the entire 96 h period after dosing, with comparable suppression at 100 and 1000 mg/kg. Temperature suppression was less at 10 mg/kg.

No increases in micronuclei were seen in a study carried out at doses in the range 50–200 mg/kg where the environmental temperature was maintained at 30 °C for 40 h [6].

In rats, which as mentioned for chlorpromazine are more refractory to phenothiazine induced hypothermia, i.p. doses of 500–2000 mg/kg produced neither significant hypothermia nor increases in micronucleated bone marrow erythrocytes [14].

2.2.3. *E-5824*

E-5824 is a triazole pyridine compound that binds to the sigma-1 receptor [12]. It is an atypical antipsychotic

Table 1
Mouse bone marrow micronucleus test of E5824 with and without heating (48 h data only)[a]

Environmental conditions	Dose E5842 (mg/kg)	PCE/NCE	MNPCEs/ 2000 PCEs
RT	0	0.99	1.7
RT	50	1.08	2.1
RT	100	1.28	1.9
RT	200	1.28	3.4*
Heater[b]	0	1.08	1.8
Heater	50	1.30	1.4
Heater	100	0.98	1.4
Heater	200	1.07	1.1

*Statistically significant at $p < 0.05$; ANOVA, followed by Dunnett's test. Groups of five CD-1 mice were dosed by gavage and the bone marrow sampled at 24 h (data not shown) and 48 h following exposure. RT = room temperature; PCE; polychromatic erythrocyte; NCE = normochromatic erythrocyte; MNPCE = micronucleated PCE.

[a] Data taken from Guzman et al. [12].

[b] Animals were housed in Makrolon cages, internal temperature maintained at 30 °C using a Selecta hot air heater (Model 381).

drug. Ames and mouse lymphoma *tk* tests of E-5824 were negative. An increased incidence of micronucleated PCEs was observed in mouse bone marrow 48 h after exposure to 200 mg/kg E-5824 (but not other doses or sampling times) (Table 1). Rectal temperature measurements showed that the test compound decreased core body temperature by more than 7 °C at 50 mg/kg and by almost 13 °C at 200 mg/kg.

As shown in the table, in the repeat test where the environmental temperature was maintained at 30 °C, the micronucleus response disappeared. Measurements of the size distribution of micronuclei showed that the micronuclei induced at 200 mg/kg were skewed towards larger micronuclei, again suggestive that these represented whole chromosome loss from disruption of chromosomal attachment to the spindle due to hypothermia (and/or due to increased rates of enucleation). There are large numbers of sigma 1 binding sites in the hypothalamus [16], a tissue known to be involved in temperature regulation in mammals.

2.2.4. Phenol

Although phenol has promoter activity in the 2-stage skin carcinogenesis model [17], it is regarded as negative in NTP carcinogenicity studies in rats and mice [18]. A more recent IARC assessment stated that there is inadequate experimental evidence for the carcinogenicity of phenol and as such it is not classifiable [19].

CD mice were given single i.p. doses of phenol up to 500 mg/kg. Lethality was seen above 300 mg/kg. At 300 mg/kg, a significant increase in micronucleated PCEs was observed in both male and female animals at 24 and 48 h after dosing (male data are shown in Table 2). In addition, 300 mg/kg phenol (but not the other doses used) induced a significant and prolonged hypothermia where core body temperature was lowered by 7 °C. The authors surmised this might have been responsible for the increases in micronuclei observed. Comparison of these results for phenol with results evaluated by the Working Group indicates that the response is somewhat higher than with other compounds that induce hypothermia. It would be informative to ascertain if this response can be reversed by maintaining the core body temperature of the treated animals.

2.2.5. Covance compound number 4

Compound number 4 is a CNS drug tested under contract. The genotoxicity data were collected using the questionnaire (see Appendix A). *In vitro* genotoxicity assays of the compound were regarded as negative, although a borderline positive was observed in the Ames test using strain TA100 and the pre-incubation protocol. Uniformly negative results were seen in plate

Table 2
Mouse bone marrow micronucleus test of phenol[a]

Test agent	Dose (mg/kg)	Temperature change[b] (°C)	PCE/NCE at 24 h	MN PCEs/1000 PCEs at 24 h	PCE/NCE at 48 h	MN PCEs/1000 PCEs at 48 h
Vehicle control	0	−1.9	1.8	2.1	1.75	1.4
Phenol	30	−0.1	1.08	4.3	1.93	1.3
Phenol	100	−0.3	1.01	3.1	1.87	1.5
Phenol	200	−7.1	1.01	10.8*	0.61	18.3*
Positive control (cyclophosphamide)	120	−0.3	0.59	79.9*	NT	NT

Data shown is from groups of five male CD1 mice (similar data were obtained from female mice) dosed i.p. and sampled 24 and 48 h after dosing. PCE = polychromatic erythrocyte; NCE = normochromatic erythrocyte; MNPCE = micronucleated PCE.

[a] Data taken from Spencer et al. [20].

[b] Average change over a 48 h observation period, temperature measured by a subcutaneous transponder.

* Significantly different from the negative control by Factorial-Rep ANOVA.

incorporation assays and human metabolites were isolated and tested separately in TA100 and were also negative.

The mouse lymphoma L5178Y *tk* test was negative even though test concentrations were used that caused high levels of toxicity. Three *in vitro* chromosome aberration assays were carried out using CHL cells. Negative results were obtained at concentrations that induced up to 50% inhibition of the mitotic index.

Up to 500 mg/kg compound number 4 was negative in the *in vivo-ex vivo* rat liver UDS test. This dose resulted in exposures that were 15 times the human plasma Cmax.

The mouse bone marrow micronucleus test was positive when assays were conducted 48 h after oral dosing (control, 1.6/1000 PCEs; 200 mg/kg compound 4, 4.5/1000 PCEs). There was no difference in micronucleated PCEs in treated groups compared to the control level when sampling was conducted 24 h after dosing. The 200 mg/kg dose of the test compound produced an exposure that was 10-times the plasma Cmax and AUC of humans receiving therapeutic doses.

The 200 mg/kg dose also decreased the rectal temperature of mice from 37 to 24 °C in 6 h; the rectal temperature was still 29 °C, 48 h after dosing. Repeat mouse bone marrow micronucleus studies were conducted using heating pads to prevent hypothermia. Under these conditions, the 200 mg/kg dose of compound number 4 was negative at 48 h (unheated mice confirmed the original positive results) (Table 3).

Table 3
Mouse bone marrow micronucleus test of Covance compound number 4 (with and without heating)

Test agent	Dose (mg/kg)	Heating	PCE/ NCE	MNPCEs/ 1000 PCEs
Vehicle control	0	–	2.4	2.1
Compound 4	100	–	2.2	4.3*
Compound 4	200	–	1.8	5.8*
Reserpine	200	–	1.8*	5.1*
Positive control (MMC)	2	–	0.6*	72.3*
Vehicle control	0	+	2.8	3.3
Compound 4	100	+	1.6*	3.2
Compound 4	200	+	1.7*	2.7
Reserpine	200	+	1.5*	2.7

Male and female ICR mice were used in groups of six and sampled 48 h after oral dosing. Some treated groups were kept without heating pads and some groups were exposed to external heating (via heating pads located under the cages) that raised the body temperature to near-normal temperatures (hypothermia rescue). Data from male animals; female data were similar. PCE = Polychromatic erythrocyte; NCE = normochromatic erythrocyte; MNPCE = micronucleated PCE; MMC = mitomycin C.

* $p < 0.05$.

2.3. Conclusions regarding the effects of hyperthermia and hypothermia on cytogenetic damage

Experimental work shows that hyperthermia and heat shock disrupt the binding of chromosomes to the mitotic spindle inducing chromosome loss. Thus, the results of *in vivo* cytogenetic assays on compounds that induce hyperthermia and hypothermia in animals should be interpreted with caution.

This analysis has highlighted a number of compounds that induce increases in micronuclei in bone marrow cells only when there has been a significant and sustained hypothermia in the treated animals. The work of Asanami and colleagues with chlorpromazine and reserpine provides evidence that a body temperature of less than 33 °C for 11 h induces micronuclei in mouse bone marrow cells. For some of the examples, the increase in micronucleated cells was abolished by artificially maintaining normal core body temperature.

Thus, core body temperature should be measured routinely in bone marrow cytogenetic assays and if increases in chromosome damage occur, these data should be considered when interpreting the results. A flow chart is provided (Fig. 1) to assist in the interpretation process.

3. Consequences of increases in the division of bone marrow cells on the frequency of micronucleated polychromatic erythrocytes

3.1. Effects of compound-induced increases in cell division of bone marrow erythroblasts

Whenever a cell divides there is a low but finite chance that genetic changes will occur. Indeed these spontaneous errors of cell division are often cited as a cause of the background levels of mutation or chromosomal damage that are observed in untreated cells in various genetic toxicology test systems. For erythroblasts, errors can also occur during enucleation resulting in formation of micronuclei in PCEs (see Section 1). Steinheider et al. [21] demonstrated that bleeding can result in elevated frequencies of micronucleated PCEs and normochromatic erythrocytes (NCEs) in the peripheral blood of mice. Peak levels occurred 24–48 h after removal of 0.5 ml of blood.

The following describes the results of *in vivo* micronucleus tests where the test compound appears to increase the rate of cell division within the bone marrow and, as a result, produces increased frequencies of micronucleated PCEs and NCEs.

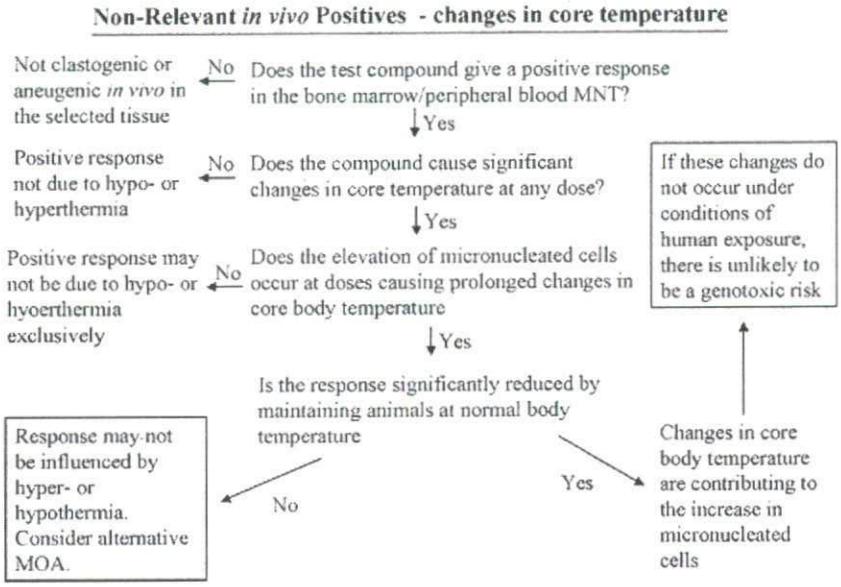

Fig. 1. Flow chart showing the approaches to be taken when a hypothermic or hyperthermic response occurs in rodent bone marrow micronucleus tests. MOA = mode of action.

Some of the examples cited below are from the literature and others from the IWGT questionnaire.

3.1.1. *Erythropoietins*

Comparative studies were conducted on the induction of micronucleated erythrocytes *in vivo* by three recombinant human EPOs, epoetin alpha, epoetin beta, and SNB-5001, and by urinary human EPO (uhEPO) [22]. Small but significant increases in the frequency of micronucleated PCEs were induced in bone marrow of mice 48 h following single i.p. injections of the test compounds. Similar results were seen in peripheral blood reticulocytes at sampling times of 48, 72 and 96 h.

Further research by the same group showed that the increases in micronucleated bone marrow cells induced by high doses of EPO are a consequence of the acceleration of erythroblastic maturation and proliferation [23]. The authors concluded that errors in enucleation or differentiation of erythrocytes and errors in genetic repair processes should be considered equally as possible mechanisms for the increased frequencies of micronucleated erythropoietic cells.

3.1.2. *2-Hydroxy-1,4-naphthoquinone*

2-Hydroxy-1,4-naphthoquinone (HNQ) is the main component of the natural hair dye henna. Data on HNQ were submitted by Covance using the questionnaire, but some of the data were published subsequently [24].

With regard to the results of *in vitro* genotoxicity tests on HNQ, bacterial mutation assays results were mixed in that initial Ames tests gave positive findings for TA98 and TA2637 +S9, but more recent studies were negative at test concentrations up to 600 μg/plate. Chinese hamster V79 *hprt* tests were negative up to accepted levels of osmolality, 1740 μg/ml, while HNQ was reproducibly positive in the mouse lymphoma *tk* assay at test concentrations up to 400 μg/ml (8-fold increases +S9, 3–4-fold −S9). Slight increases in chromosome damage were observed in CHO cells. Finally, a SHE cell transformation assay was negative with a top test concentration of 100 μg/ml.

An *in vivo-ex vivo* UDS test in rat hepatocytes was negative using oral dosing of up to 1500 mg/kg. Similarly, a sex linked recessive lethal test in Drosophila was also negative.

Initial mouse bone marrow micronucleus tests of HNQ, however, gave a positive result 72 h after oral administration using DMSO as the vehicle; the test compound was negative at 24 and 48 h sampling times. A series of four new mouse micronucleus tests were reported (three with oral dosing of HNQ and one with i.p. injection with henna); these assays gave some positive responses, but only at 72 h and when using 110 and 250 mg/kg HNQ (e.g. at 72 h: control, 1.2 micronucleated PCEs/1000 PCEs; 250 mg/kg HNQ delivered in DMSO, 3.6 micronucleated PCEs/1000 PCEs). HNQ was negative in the assay when the solvent was changed

to 0.5% aqueous methyl cellulose (MC), even at the 72 h sampling point.

These results are typical of compounds that induce erythropoiesis or of bleeding where a delayed (i.e. sampling times >24 h) increase in micronucleated cells is seen. HNQ is known to be haematotoxic as is the solvent used in positive tests (DMSO) (Marzin and Kirkland, *ibid*). It is possible that, following bone marrow toxicity due to the combined insult of HNQ plus DMSO, there is a rebound erythropoiesis resulting in increases in micronucleated cells 72 h after dosing.

Support indicating that HNQ is not a true clastogen is provided by two negative chromosome aberration assays, although it is possible that these assays may not have sufficient power to detect very small increases in chromosome damage. The first was conducted in the mouse and the second in Chinese hamsters (maximum doses of 250 and 200 mg/kg, respectively with both 24 and 72 h sampling times). A negative result was also observed in a rat peripheral lymphocyte chromosome aberration assay after 28 days dosing with 100 mg/kg HNQ. Marzin and Kirkland [24] concluded that HNQ does not appear to be a genotoxic agent *in vivo*.

3.1.3. Phenylhydrazine

Phenylhydrazine is used worldwide mainly as a chemical intermediate in the pharmaceutical, agrochemical, and chemical industries. The following data were taken from the review of the safety of phenylhydrazine compiled under the IPCS programme [25].

The mutagenicity of phenylhydrazine has been evaluated in Ames tests on multiple occasions and in multiple laboratories. There is some variability in the findings, although positive results have been obtained in *Salmonella typhimurium* strains TA97, TA100, TA102, TA1537, and TA1538 in the absence of exogenous metabolic activation [24]. There also has been one report (abstract only) of a positive result in the Chinese hamster V79 mutation test (Ouabain resistance) [26]. Concentrations of 0.0144-144 mg/l phenylhydrazine have been assayed in UDS assays using both rat and mouse primary hepatocytes [27]. Although toxicity was measured, no details were given, and quantitative data were not reported. A positive result was obtained in both cell types, although the effect was small.

Phenylhydrazine was tested in an *in vitro* micronucleus assay using primary mouse bone marrow cells [28]. Bone marrow cells were exposed to 1–50 μg phenylhydrazine/ml for 30 min, in the presence and absence of metabolic activation. The percentage of micronucleated PCEs was significantly increased, only in the presence of S9 and only at phenylhydrazine concentrations of 5 μg/ml and greater.

Treatment of mice with phenylhydrazine significantly increases the alkaline elution rates for liver and lung DNA and thus, is considered positive in this assay for DNA damage [29]. In addition, gavage of rats with 65 mg phenylhydrazine/kg body weight produced DNA adducts (N^7-methylguanine and a trace of O^6-methylguanine) in the liver [30]. Other tissues were not examined.

Groups of 11–12 female BALB/c mice were given single i.p. injections of 50 mg phenylhydrazine/kg body weight in saline [21]. Smears of tail vein blood were prepared at 24-h intervals for 7 or 11 days, and reticulocytes and micronuclei were evaluated in NCEs and PCEs. Phenylhydrazine caused a significant increase in the reticulocyte count on days 2–4 post-injection and in the PCE count on day 3. There was a significant increase in the frequency of micronucleated PCEs at 24 h post-injection (from 1 to 4.7/1000 PCEs) and in the frequency of micronucleated NCEs at 48 h post-injection (from 0.7 to 2.3/1000 NCEs). The authors ascribed the increases in micronuclei in part to the haemolysis induced by this compound and the subsequent rebound in erythropoiesis. However, due to the consistently positive results in a range of *in vitro* and *in vivo* genotoxicity assays, clastogenicity cannot be excluded.

Phenylhydrazine induces lung tumours (adenomas and adenocarcinomas) and blood vessel tumours in the liver (angiomas and angiosarcomas) [31,32].

3.1.4. Aniline

Unless otherwise indicated data on aniline were abstracted from the EU risk assessment report on this compound [33].

Some activity has been observed in *in vitro* genotoxicity assays. However, Ames tests were negative (to 5 mg/plate) unless plant S9 was used or norharman was added to the Ames test medium (positive in TA98). Urine from aniline-exposed rats was positive in the Ames test conducted with rat liver S9 [34].

Aniline was positive in four of five mouse lymphoma *tk* assays, with greater activity observed in the presence of rat liver S9. Very high doses of aniline (4650 μg/ml) also were positive in the V79 cell *hprt* gene mutation assays in the presence of S9. Doses of 1 mg/ml and above were positive in chromosome aberration assays with the Chinese hamster cell lines CHO, CHL, and V79 (more potent +S9).

Aniline doses up to 93 μg/ml were negative for UDS in human and rat hepatocytes, whereas studies of the induction of DNA strand breaks in mouse lymphoma cells were equivocal at very high concentrations (21.5 mM).

Aniline produced positive responses in the *in vivo* rat liver alkaline elution assay. An i.p. dose of 210 mg/kg produced a three-fold increase in elution rate. A second study using doses of up to 840 mg/kg confirmed that aniline damaged rat liver DNA; DNA damage also was seen in the kidney (but not in the spleen or bone marrow). Alkaline elution studies in mouse liver, kidney and bone marrow were all negative at doses up to 420 mg/kg.

Aniline is carcinogenic in the rat (producing tumours in the spleen and other abdominal organs) but not in the mouse [35]. It is not clear why there is this species difference, but there are clear differences in aniline metabolism between the rat and the mouse, qualitatively and quantitatively in the proportion of metabolites formed and in the rates of metabolism [36].

A series of bone marrow micronucleus tests of aniline have been carried out in the mouse and rat, using both oral and i.p. dosing. Three studies using i.p. or oral doing of mice reported positive responses at high and toxic doses (up to 1000 mg/kg). One of these studies [37] reported that many of the micronuclei had abnormal shapes, which may suggest an unusual method of induction. A recent mouse bone marrow chromosome aberration study was negative [38].

A rat bone marrow micronucleus study gave a weak positive response at 400 mg/kg that was accompanied by a dose related reduction in PCEs at 24 h and an increase at 48 h. This change in %PCEs is consistent with an initial toxic response followed by a rebound in erythropoiesis [39]. This study was repeated using the high dose only (one sampling time), with negative results, but no cytotoxicity was seen in this study [40].

Aniline induces methaemoglobinaemia mainly due to the formation of its phenylhydroxylamine metabolite. It has been questioned whether the mouse gives false positive results in the bone marrow or peripheral blood micronucleus test due to erythrocyte degradation/iron depletion or due the formation of Heinz bodies (iron-containing inclusions) as a result of the methaemoglobinaemia [38,41]. However, there are examples of other compounds that induce methaemoglobinaemia and that are clearly negative in the bone marrow micronucleus assay, e.g., 2,4,6-trinitrotoluene [42]. Thus, the question of whether increases in erythropoiesis are involved in the responses seen in the micronucleus test *in vivo* after aniline treatment is unresolved.

3.1.5. *Novartis compound GPI562*

Details of the genotoxicity package for GPI562 were obtained via the IWGT questionnaire. This compound is an anti-thrombotic agent.

Ames tests were negative (test concentrations not supplied). Mouse lymphomas *tk* tests were also negative (tested to a maximum of 5 mg/ml). Small increases in the percentage of cells with chromosome aberrations were observed in V79 Chinese hamster cells treated with high-test doses (above 1 mg/ml) for +S9 3 h and harvested after 17 h of recovery. At doses resulting in 50% inhibition of the mitotic index, the maximum percentage of cells with aberrations in the first study was 3.0% at 2.19 mg/ml, compared to 0% in the negative control; the maximum induction in the second study was 3.0% at 1.73 mg/ml compared to 1.5% in the negative control. In a third study, the maximum response was detected at a dose of 0.6 mg/ml GPI562, where 3.0% of cells contained aberrations compared to 0.5% in the negative control. A similar weak effect was seen in *in vitro* micronucleus tests using V79 cells where a 3 h exposure to 0.54 mg/ml produced a micronucleus frequency of 1.3% compared to 0.6% in the negative control. Comprehensive (short and longer exposures) chromosome aberration assays using human peripheral lymphocytes were negative up to test doses of 5 mg/ml where little toxicity was seen.

Three *in vivo* micronucleus tests were carried out. In an intitial mouse bone marrow assay the control value for micronucleated PCEs was 1.2/1000 PCEs, whilst i.v. dosing with 320 mg/kg GPI562 produced a micronucleated PCE frequency of 2.3/1000 PCEs (4000 PCEs scored, statistically significant)(Table 4). There was also a significant reduction in PCEs at this dose. A follow-up study was carried out in the mouse using oral dosing to a maximum of 2.0 g/kg. This dosing regimen produced no bone marrow toxicity, haematological findings or clin-

Table 4
Mouse bone marrow micronucleus test of Novartis compound GPI562[a]

Test agent	Dose (mg/kg)	PCE/ NCE	MNPCEs/ 1000 PCEs
Vehicle control	0	0.52	1.2
GPI562	32.00	0.41	1.3
GPI562	100.00	0.45	1.5
GPI562	320.00	0.25	2.3*
Positive control (TEM)	0.15	0.54	27.9*

PCE = polychromatic erythrocyte; NCE = normochromatic erythrocyte; MNPCE = micronucleated PCE; TEM = triethylenemelamine.

* Statistically different from vehicle control ($p < 0.05$).

[a] Groups of 10 mice (5 males and 5 females) were dosed i.v. and the bone marrow sampled 24 h later. A total of 4000 PCEs per mouse were analysed for micronuclei.

ical signs. This study was negative. Finally, a rat liver micronucleus test was conducted with i.v. doses of up to 5000 mg/kg. Clinical signs were observed at doses of 325 mg/kg and above, but the mitotic index in the hepatocytes of the dosed animals was unchanged. This study was also negative.

A possible explanation for the increases in micronuclei seen in the mouse bone marrow micronucleus study using i.v. dosing is as follows. GPI562 is an antithrombotic which causes an inhibition of blood platelet aggregation. This in turn can lead to erythropoietic stimulation, with a subsequent increase in errors during cell division leading to micronucleus formation. Presumably these effects are more marked after i.v. dosing compared with oral dosing.

3.1.6. GlaxoSmithKline (GSK) compound Y

Details of genotoxicity tests conducted on GSK Compound Y also were obtained via the IWGT questionnaire and are otherwise unpublished in the open literature. This compound is an adenosine A2 receptor agonist.

No *in vitro* genotoxicity data were supplied. In a rat bone marrow micronucleus test, two groups of seven male animals were dosed i.v. with 8 or 16 mg/kg of the test compound. One group was killed 24 h after dosing, the second 48 h after dosing. Small but significant increases in micronucleated PCEs were observed 48 h after i.v. administration of 16 mg/kg of the test compound (Table 5). No significant increases were seen at the lower dose at 24 or 48 h or at 24 h for the higher dose group. There were no effects on the PCE/NCE ratio for any dose group.

Measurements were made on peripheral blood (full blood count, differential leucocyte count and reticulocyte count); bone marrow (nucleated cell count and myeloid:erythroid ratio) and spleen (weight, nucleated cell count and myeloid:erythroid ratio) plus histological examination were conducted at 24 and 48 h after dosing. Both doses produced a reduction in erythrocyte and platelet count after 24 h, although there was no evidence of haemolysis. After 48 h, the platelet count was normal and erythrocyte counts had partially recovered. There was moderate dose-related reticulocytosis in the blood, bone marrow and spleen. These changes were significant in the blood at both doses and in the marrow and spleen at 16 mg/kg. Histological examination of the spleen also showed increased haematopoiesis at both doses. These changes are consistent with an enhancement of EPO production, a property reported for this class of compounds [43], and this is likely to be the cause of the small increase in micronuclei seen in this study.

3.2. Conclusions regarding the effects of increased cell division of red blood cell precursors

The interpretation of bone marrow micronucleus tests can be complicated by compounds that induce toxicity followed by a recovery that results in a rebound in erythropoiesis, or because of their pharmacology, induce cell division in red blood cell precursor cells such as erythroblasts. Steinheider and colleagues [20] demonstrated that bleeding or splenectomy can cause increases in micronucleus frequencies in peripheral blood cells of mice. Recombinant EPOs also cause small increases in micronucleated PCEs [21]. When a compound shows little or no genotoxic potential *in silico* or *in vitro* yet induces small increases in micronucleated PCE frequencies at >24 h or the effect is more pronounced at 48 h compared to 24 h, then an effect on erythropoiesis may be suspected. There often may be pre-existing data from rodent toxicity tests indicating that the compound is toxic to bone marrow cells. In such cases, it is prudent to confirm any effects on erythropoiesis by haematological and histological analysis as was done for GSK compound Y. A flow chart is provided (Fig. 3) to assist in the interpretation process.

Table 5
Rat bone marrow micronucleus test of GSK compound Y

Test agent	Dose (mg/kg)	PCE/NCE/1000 cells		MNPCEs/1000 PCEs[a]	
		24 h	48 h	24 h	48 h
Vehicle control[b]	0	0.41	0.41	0.8	0.8
Compound Y	8	0.45	0.48	1.7	1.5
Compound Y	16	0.41	0.45	1.3	1.8*
Positive control (cyclophosphamide)	20	0.26	Not measured	26.2*	Not measured

Male Han Wistar rats were given single i.v. doses and sampled 24 and 48 h after dosing. PCE = polychromatic erythrocyte; NCE = normochromatic erythrocyte; MNPCE = micronucleated PCE.

[a] 2000 PCEs scored.

[b] 0.9% (w/v) sodium chloride i.v. infusion British Pharmacopea at 10 ml/kg.

* Statistically significant increase compared to the solvent control.

4. Other mechanisms that can lead to increases in micronuclei not related to inherent genotoxicity of the test compound in the rodent bone marrow micronucleus test

4.1. AstraZeneca compound Z, a probable eukaryotic protein synthesis inhibitor

Details of the genotoxicity package on AstraZeneca compound Z (AZ-Z) were obtained via the IWGT questionnaire and have not been published in the open literature. The compound is known to inhibit bacterial protein synthesis.

Negative results were obtained in a standard Ames test using strains TA1535, TA1537, TA98, TA100 and TA102 in plate-incorporation and pre-incubation assays and treatment concentrations up to 500 (−S9) or 1000 (+S9) μg/plate. No evidence of clastogenicity was seen in an *in vitro* cytogenetics assay using human lymphocytes in whole-blood cultures at concentrations up to 0.3 mmol/l. The maximum concentration used for the human lymphocyte assay was limited by changes in chromosome morphology, not toxicity as assessed by reduced mitotic index. AZ-Z was positive in the mouse lymphoma *tk* assay using a 4 h exposure ±S9 or a 24 h exposure −S9; the clearest increases were seen using 4 h exposure −S9 at concentrations of 0.6–0.8 mol/l (Table 6). Under all exposure conditions, the maximum concentrations were determined by the cytotoxicity of the test compound. No evidence of binding to calf thymus DNA was seen in either the presence or absence of S9.

AZ-Z is positive in replicate rat bone-marrow micronucleus tests using oral doses of 1000–2000 mg/kg (Table 7), and i.v. doses of 70–100 mg/kg, the maximum tolerated dose (Table 8).

Since AZ-Z is known to inhibit bacterial protein synthesis, inhibition of protein synthesis in eukaryotic cells was considered to be a possible mode of action. Cycloheximide, an inhibitor of protein synthesis in mammalian cells is negative in TA98 and TA100, but injections (route not specified) of 100–200 mg/kg

Table 6
Mouse lymphoma L5178Y $tk^{\pm}$ test of AstraZeneca compound Z (AZ-Z)

Test agent	Concentration (mmol/l)	Test 1		Test 2	
		RTG (%)	MF ($\times 10^{-6}$)	RTG (%)	MF ($\times 10^{-6}$)
Vehicle control	0	100	85	100	121
AZ-Z	0.6	95	150	23	507
AZ-Z	0.65	84	178	7	498
AZ-Z	0.7	76	181	4	585
AZ-Z	0.75	48	229	4	401
AZ-Z	0.8	27	263		
Positive control (4-NQO)	0.001	44	1174	20	1497

Mouse lymphoma L5178Y $tk^{\pm}$ cells were exposed for 4 h to the test compound in the absence of S9. RTG = Relative total growth; MF = Mutant frequency; 4-NQO = 4-nitroquinoline-*N*-oxide.

Table 7
Rat bone marrow micronucleus test of AZ-Z (oral dosing)

Test agent	Dose (mg/kg)	Micronucleated PCEs/1000 PCEs[a]			
		Test 1		Test 2	
		24 h	48 h	24 h	48 h
Vehicle control	0	0.34	0.29	0.50	0.36
AZ-Z	1000	NT	NT	2.36**	NT
AZ-Z	1500	NT	NT	2.00**	NT
AZ-Z	2000	1.65***	0.43	2.43**	1.65***
Positive control (cyclophosphamide)	20	28.5	NT	19.65	NT

Male rats were given a single oral dose and sampled 24 and 48 h after dosing. PCE = polychromatic erythrocyte; NT = Not tested.
[a] 2000 PCEs scored.
** Positive dose–response relationship at all dose levels ($p < 0.01$).
*** Pairwise comparison $p < 0.001$.

Table 8
Rat bone marrow micronucleus test of AZ-Z (i.v. dosing)

Test agent	Dose (mg/kg)	Micronucleated PCEs/1000 PCEs[a]			
		Test 1		Test 2	
		24 h	48 h	24 h	48 h
Vehicle control	0	0.69		0.54	0.79
AZ-Z	10	0.65	NT	NT	NT
AZ-Z	35	0.57	NT	0.68	NT
AZ-Z	70	NT	NT	1.07*	NT
AZ-Z	100	1.45**	0.57	1.72**	0.72
Positive control (cyclophosphamide)	20	35.67***	NT	25.67***	NT

Male rats were given a single i.v. dose and sampled 24 and 48 h after dosing. PCE = polychromatic erythrocyte; NT = not tested.
[a] 2000 PCEs scored.
* Statistically significant positive trend with dosing, $p < 0.05$.
** Statistically significant positive trend with dosing, $p < 0.01$.
*** Pairwise comparison, $p < 0.001$.

cyclohexamide increase the frequency of micronuclei in mouse bone-marrow cells at a 30 h sampling time [44]. Cycloheximide is also positive in the mouse lymphoma *tk* assay with 4 h treatment in the absence of S9, giving maximum increases of ~2.5× the control at concentrations resulting in ~25% survival [45].

The ability of AZ-Z to inhibit protein synthesis in mouse lymphoma cells was investigated by measuring ^{14}C-leucine incorporation in parallel with estimates of cytotoxicity as either relative suspension growth (RSG) or relative total growth (RTG). Almost complete inhibition of protein synthesis was seen at concentrations showing no significant cytotoxicity (Fig. 2); therefore, the observed mutagenicity in mouse lymphoma cells could be due to its inhibition of protein synthesis.

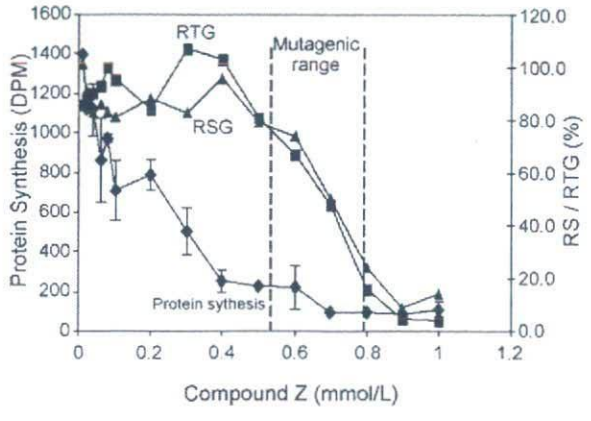

Fig. 2. Effects of AZ compound Z on protein synthesis and cytotoxicity of mouse lymphoma L5178Y $tk^{\pm}$ cells.

Work was initiated to investigate the ability of AZ-Z to inhibit protein synthesis in the bone-marrow of rats, but the studies were discontinued before completion.

5. Discussion

The rodent bone marrow micronucleus assay is used in regulatory test batteries to predict the carcinogenicity of chemical agents through their ability to produce genotoxicity *in vivo*. Because, there is a perception that there are very few (if any) compounds that induce increases in micronuclei in this test without being genotoxic carcinogens, a 'positive' result in this test is often regarded as definitive evidence of *in vivo* genotoxicity and thus, it is highly likely that the test compound is a genotoxic carcinogen. For pharmaceuticals intended to treat diseases that are not life threatening, such results can mean the termination of development. Similarly for any new chemical where there is likely to be widespread human exposure, development could be terminated. The Working Group analysis indicates, however, that compounds that cause either significant increases or decreases in core body temperature for a sustained period, compounds that cause an increase in erythropoiesis in the bone marrow, and compounds that inhibit protein synthesis may induce increases in micronucleated PCEs. These increases may not be a result of any intrinsic direct genotoxic properties of the agent. However, if compound exposure is high enough to induce the same changes in physiology for human exposure, then there still may be a cause for concern. An alternative testing strategy, when such compound-induced changes in physiology have been detected, is to carry out metaphase analysis of bone marrow cells. This test may not be as susceptible to

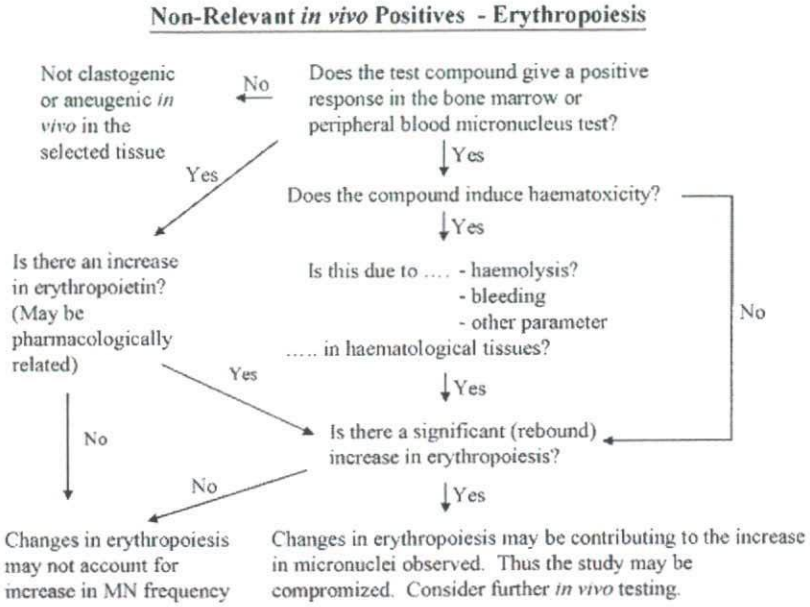

Fig. 3. Flow chart showing the approaches to be taken when an effect on erythopoiesis occurs in rodent bone marrow micronucleus tests.

the same problems, with the possible exception of sustained hyperthermia. The potential for a test agent to operate through any one of these modes of action should be considered when interpreting the results of *in vivo* micronucleus studies. Flow charts are provided to aid in this process (Figs. 1 and 3).

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The Working Group is grateful to all the companies and individuals that supplied data to the IWGT questionnaire on 'irrelevant' positives in *in vivo* tests or supplied data and analyses from their Institutes. Apart from some of the authors of this paper and their Institutes, these include; Robin Fielder (UK COM); David Kikland (Covance); Hans-Joerg Martus (Novartis Pharma AG); Robert Rees (GlaxoSmithKline) and Andrew Smith (UK HSE). The authors are also grateful to James MacGregor (Toxicology Consulting Services) and David Kirkland for constructive comments on the manuscript.

Appendix A. Questionnaire

A.1. Examples of 'non-relevant' in vivo positive compounds

1. Do you have examples in your database of compounds that have given a 'positive' result *in vivo*, but following mechanistic studies or knowledge, the result has been deemed non-relevant to human risk? Examples of such compounds may include, but are not restricted to, those that:
   (i) Induce hypothermia or hyperthermia *in vivo*.
   (ii) Induce an increase in proliferation of the target tissue, e.g., erythropoiesis in the bone marrow.
   (iii) Disturb nucleotide pools following high doses.
   (iv) Act through a different, threshold related mechanism, e.g., aneuploidy.
2. If so, can you give details of:
   (i) Chemical structure (if not confidential).
   (ii) Relevant mechanism as in 1 above.
3. Please list *in vitro* tests used in screening.
4. Please submit summary test data for *in vitro* tests, i.e., test concentrations; mean plate counts for each strain (Ames test); mean *tk+* mutant frequency and RTG scores for each concentration and time point (mouse lymphoma assay); mean aberration counts and mitotic index scores for each concentration and time point (chromosome aberration assays), etc.
5. Please submit summary test data for positive *in vivo* tests, e.g., PCE/NCE ratios and mean micronucleated PCE counts for each time point and dose (*in vivo* micronucleus test), etc.
6. Please give details, justification and rationale for an additional testing.
7. Please give summary details of the results of additional tests and final justification for declaring the 'positive' *in vivo* non-relevant.

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Report of the IWGT working group on strategy/interpretation for regulatory *in vivo* tests II. Identification of *in vivo*-only positive compounds in the bone marrow micronucleus test

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Abstract

A survey conducted as part of an International Workshop on Genotoxicity Testing (IWGT) has identified a number of compounds that appear to be more readily detected *in vivo* than *in vitro*. The reasons for this property varies from compound to compound and includes metabolic differences; the influence of gut flora; higher exposures *in vivo* compared to *in vitro*; effects on pharmacology, in particular folate depletion or receptor kinase inhibition. It is possible that at least some of these compounds are detectable *in vitro* if a specific *in vitro* test is chosen as part of the test battery, but the 'correct' choice of test may not always be obvious when testing a compound of unknown genotoxicity. It is noted that many of the compounds identified in this study interfere with cell cycle kinetics and this can result in either aneugenicity or chromosome breakage. A decision tree is outlined as a guide for the evaluation of compounds that appear to be genotoxic agents *in vivo* but not *in vitro*. The regulatory implications of these findings are discussed.

Keywords: IWGT; Genotoxicity tests; *In vivo*; Rodent bone marrow; Micronucleus test; *In vivo*-only positive compounds; ADME; *In vitro* versus *in vivo* metabolism; Sex-specific metabolism; Influence of gut flora; Pharmacological mechanisms; Kinase inhibitors; Regulatory implication; Pharmaceuticals; Cosmetics; Food additives; *In vitro*-only genotoxicity test batteries

1. Introduction

In vivo genotoxicity tests are included in most regulatory batteries for two purposes. The first is to put any

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positive results obtained *in vitro* into perspective, i.e., to determine if the genotoxic potential observed *in vitro* is realised *in vivo*. The second is to ensure that genotoxic carcinogens that are not detected or are difficult to detect *in vitro*, but do cause detectable genotoxic damage in the tissues of an intact animal, are recognised. This later issue is addressed in the ICH guideline for pharmaceuticals (S2B—Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals) which states that "An *in vivo* test for genetic damage should usually be a part of the test battery to provide a test model in which additional relevant factors (absorption, distribution, metabolism, excretion) that may influence the genotoxic activity of a compound are included". As a result, *in vivo* tests permit the detection of some additional genotoxic agents. Note 5 of the guideline states that, "There are a small but significant number of genotoxic carcinogens that are reliably detected by bone marrow tests for chromosomal damage that have yielded negative/weak/conflicting results in the pairs of *in vitro* tests outlined in the standard battery options . . . Carcinogens such as procarbazine, hydroquinone, urethane and benzene fall into this category".

An IWGT working group was formed to examine the published data supporting the existence of genotoxic agents only detectable *in vivo* and through the use of a questionnaire, determine if there are further unpublished data on additional compounds that may fall into this category [1].

This paper does not review all possible 'unique' *in vivo*-positive compounds from the literature but is focussed on previously unpublished data obtained from company archives via the IWGT questionnaire (Appendix I). The exceptions include urethane, salicylazosulfapyridine, sulfapyridine and morphine. Urethane is regarded by the working group as a well-studied example of an *in vivo*-only positive. Data on the other compounds were brought to our attention in response to the IWGT questionnaire.

2. Analysis of possible *in vivo*-only positives from the literature

2.1. Urethane (ethyl carbamate) and benzene

Urethane has been recognised as a carcinogen since the 1940s and induces a variety of tumours in rodents, including tumours of the lung (alveolar/bronchiolar adenoma or carcinoma) and liver (hemangioma or angiosarcoma) [2]. In addition, lymphomas, melanomas, and vascular tumours have been reported [3]. Urethane has been extensively tested in a variety of *in vitro* tests for genotoxicity. There are sporadic reports of positive results for urethane in *in vitro* tests (usually in the presence of rat liver S9) [4–6], but only when tested at concentrations above internationally agreed-upon limits for relatively non-toxic compounds, i.e., 5 mg/plate in the Ames test and 10 mM in cultured mammalian cell tests. At or below these limits, the compound is uniformly negative. Negative results have been reported for the Ames assay, the human lymphoblastoid TK6 *TK* mutation test, chromosome aberration tests in a variety of cell lines, and the *in vitro* UDS test in primary rat hepatocytes [7].

The situation is very different *in vivo*. Urethane gives strongly positive results in mouse bone marrow micronucleus tests, as has been shown by many laboratories. In a typical study using CBA mice given 900 mg/kg (i.p. or oral), up to 30-fold increases in micronucleated polychromatic erythrocytes (PCEs) were observed relative to concurrent control values [8] (Table 1). In addition, weak, but statistically significant, positive results have been detected in the Muta^{TM}Mouse following single i.p. dosing with 900 mg/kg, where increases in mutant frequency in lung and liver (equivocal results in bone marrow and spleen) were seen [9]. Similar results have also been obtained in an evaluation of mutant frequencies of chemically induced tumours and normal tissues in lambda/*cII* transgenic mice treated with urethane [10]. Urethane-associated adducts are formed in the DNA of lung and liver cells from exposed mice (the principal sites for urethane-induced carcinogenesis) [11].

The most widely accepted hypothesis for the discrepancy between the *in vitro* and *in vivo* genotoxicity profiles of urethane is that the S9 used for metabolic activation in many *in vitro* assays is deficient in the specific cytochromes P450 (CYPs) and possibly other enzymes, necessary to metabolize urethane to its ultimate genotoxic metabolites; by contrast, these metabolites are readily formed *in vivo*. Urethane metabolism is known to require CYP2E1 and carboxylesterase isozyme hydrolase A [12]. The metabolic activation route is thought to involve C-hydroxylation to form vinyl carbamate, which is then converted to an epoxide that can interact with nucleic acids [13]. This scheme is supported by the observation that urethane-induced bone marrow micronucleus frequencies are reduced in CYP2E1-null mice [14]. Attempts have been made to detect urethane *in vitro* using rat liver S9 from animals pre-treated with CYP2E1 inducers such as ethanol, but these were unsuccessful [15].

Another important example is benzene, a known human carcinogen that gives a strong *in vivo* response whilst being weak or negative in *in vitro* assays, benzene undergoes complex metabolism *in vivo*, which may be

Table 1
Activity of urethane in the mouse bone marrow micronucleus test

Agent	Dose (mg/kg)	Route	PCE/NCE ratio (±S.D.)	MNPCE/1000 PCE (±S.D.)
Vehicle control	–	Oral	1.1 (0.05)	2.6 (1.6)
	–	i.p.	1.05 (0.08)	2.9 (0.9)
Urethane	900	Oral	0.66 (0.09)	62.2 (10.8)
	900	i.p.	0.55 (0.05)	67.9 (11.0)

Data taken from Ashby et al. [8]. Assay was carried out in male CBA mice; bone marrow was sampled 24 h after dosing. i.p.: intraperitoneal; PCE: polychromatic erythrocyte; NCE: normochromatic erythrocyte; MNPCE: micronucleated PCE.

difficult to reproduce *in vitro*. The precise metabolites involved in carcinogenicity or indeed the mechanism of carcinogenicity induced by this molecule or its metabolites are not known, but may involve inhibition of DNA topoisomerase II [16].

2.2. *Salicylazosulfapyridine and sulfapyridine*

The mutagenicity profile of salicylazosulfapyridine (SASP), an anti-inflammatory drug used for over 50 years, is detailed by Bishop et al. [17]. Ames tests were negative at concentrations up to 5 mg/plate. Similarly CHO chromosomal aberration (and sister-chromatid exchange [SCE]) studies were negative at concentrations up to 1 mg/ml. However, 20–160 μg/ml produced positive responses in human lymphocyte chromosomal aberration (and SCE) assays.

Bone marrow micronucleus tests in male B6C3F1 mice using single oral doses of SASP of up to 1000 mg/kg were negative; but weak, statistically significant and dose-related increases were seen when animals received 500, 1000 or 2700 mg/kg for 3 days. Micronucleus tests in peripheral blood erythrocytes from male and female B6C3F1 mice were clearly positive at doses of 675, 1350 or 2700 mg/kg given orally for 90 days (Table 2). Sulfapyridine (SP), a major metabolite of SASP, was subsequently shown to have the same profile, i.e., negative for chromosomal aberrations (although SCEs were induced) in CHO cells, but positive in the mouse bone marrow micronucleus test [18]. Further work showed that both SASP and SP are strong inducers of kinetochore-positive micronuclei *in vivo*. Although small increases in kinetochore-negative micronuclei also were observed in SP treated mice, as well as in mice receiving the highest test dose of SASP (Table 3), the results suggest that both chemicals are predominantly aneugens [19]. Increases in micronucleus and SCE frequencies have been reported in patients treated with SASP for inflammatory bowel diseases (IBD) for one or more months, although there were confounding factors in these studies [20].

Bishop et al. [17] suggest that the results they observed *in vivo* could have been due to the induction or exacerbation of folate deficiency. Folate deficiency is known to cause chromosomal aberrations and fragile-site expression. Sulfa drugs, as a class, are known to inhibit *p*-aminobenzoic acid uptake and to inhibit the formation of folic acid by gut flora. SASP therapy is associated with impaired folic acid absorption, although serum folate levels in IBD patients were low, they were still in the normal range when tested [21]. It has now been established that SASP does depress folate levels significantly in treated patients [22,23]. Humans are particularly sensitive to micronucleus induction due to limiting folate or B_{12} levels [24,25].

Reticulocytosis was observed in a 90-day study of SASP in mice [26], which also suggests that SASP may have a rebound erythropoietic effect following a haemo-

Table 2
Mouse peripheral blood micronucleus test of salicylazosulfapyridine (SASP)

Agent	Dose (mg/kg)	MNPCE/1000 PCE (±S.D.)	MNNCE/1000 NCE (±S.D.)
Vehicle control	–	1.71 (0.32)	1.07 (0.08)
SASP	675	2.27 (0.42)	2.46[a] (0.17)
	1350	3.42[a] (0.45)	2.94[a] (0.22)
	2700	3.66[a] (0.41)	2.78[a] (0.20)

Data from male B6C3F1 mice (five per group); peripheral blood was obtained at the termination of a 90-day oral gavage NTP toxicity study. At least 2000 PCEs and 10,000 NCEs from each animal were scored for micronuclei [17]. PCE: polychromatic erythrocyte; NCE: normochromatic erythrocyte; MNPCE: micronucleated PCE; MNNCE: micronucleated NCE.

[a] $p < 0.01$, Cochran–Armitage test.

Table 3
Mouse bone marrow micronucleus tests of SASP and SP with kinetochore (KC) staining

Agent	Treatment (mg/kg)	MNPCE/1000 PCE (±S.D.)		
		Total MN	KC^-	KC^+
Vehicle control	–	1.6 (0.19)	1.6 (0.19)	0.0
SASP	1875	4.5 (0.52)	2.2[a] (0.20)	2.3 (0.44)
	2721	5.3 (0.34)	2.8[a] (0.34)	2.5 (0.27)
	3649	6.3 (0.68)	2.1[a] (0.29)	4.2 (0.58)
SP	2083	7.8 (0.88)	4.2 (0.58)	3.6 (0.37)
	2721	9.5 (0.42)	3.8 (0.25)	5.7 (0.37)
	3472	14.2 (1.56)	5.7 (1.33)	8.5 (0.65)
TEM (positive control)	1.0	100.2 (4.94)	96.3 (4.68)	3.9 (0.58)
VCR (positive control)	0.125	83.0 (2.91)	6.7 (0.94)	76.3 (3.00)

Data from Witt et al. [19]. Five animals per dose group; 2000 PCEs scored per animal; vehicle control was corn oil. PCE: polychromatic erythrocyte; MNPCE: micronucleated PCE; MN: micronuclei; SASP: salicylazosulfapyridine; SP: sulfapyridine; TEM: triethylenemelamine; VCR: vincristine sulfate; KC^-: kineticore negative; KC^+: kineticore positive.

[a] Not statistically significant, otherwise all pairwise comparisons of dosed groups to control were statistically significant at $p < 0.01$.

toxic insult, which may also contribute to induction of micronuclei in bone marrow cells (see accompanying paper, this issue).

SASP treatment increases the number of urinary bladder tumours in F344 rats and liver tumours in B6C3F1mice, when the animals are maintained under *ad libitum* (AL) feeding conditions; under a feed restriction (FR) regimen, these tumours were not increased [23]. With regard to the etiology of the bladder tumours, SASP caused intraluminal bladder changes in the rat (especially males) consisting of chronic urothelial stimulation, concretions, and hyperplasia, which resulted in neoplasia. With regard to the mouse liver tumours, chronic hepatocellular toxicity was observed, resulting in preneoplasia and neoplasia within 2 years. Thus, it is probable that these rodent tumours are not induced as a consequence of the direct genotoxicity of the test agent.

To follow up this work, SASP and its two major metabolites, 5-aminosalicylic acid (ASA) and SP, were tested for the induction of micronuclei in mouse bone marrow, with or without pre-treatment with folate, and for the formation of DNA adducts in rat and mouse liver and urinary bladder [27]. None of the compounds exhibited genotoxicity or DNA reactivity under the protocols used. However, the authors of this paper stated that, without folate supplementation, SASP is an aneugen, and thus the folate deficiency associated with SASP administration is probably responsible for its *in vivo* genotoxicity in lymphocytes and erythrocytes.

From the genotoxicity profile above, it is clear that SASP is detected as a genotoxic agent *in vitro* if chromosome damage is measured in human peripheral lymphocytes, but not if Chinese hamster cells are used. Thus, if SASP were a new drug candidate and the testing laboratory concerned routinely used Chinese hamster cells, this activity would have been missed. Why is there a difference between these two cell types for the genotoxicity of SASP? SASP undergoes acetylation *in vivo*, and CHO cells are known to be poor acetylators [28], whilst acetylated metabolites of SASP have been shown to induce chromosome damage [29]. It is possible that human lymphocytes are better or more rapid acetylators than Chinese hamster cells and that this is responsible for the differences in ability to detect SASP as a genotoxic agent. In terms of the differences in the *in vitro* and *in vivo* genotoxicity profiles, Bishop et al. [17] conclude that the differences in distribution and metabolism of SASP, its cleavage into SP and 5-ASA metabolites by gut flora, the absorption of these metabolites, their acetylation and/or hydroxylation in the liver, their reaction with macromolecules, and their subsequent elimination from the body are all factors for understanding differences between species and cell types in the amounts and types of chromosome damage induced by SASP.

2.3. Morphine

According to the Physicians Desk Reference (PDR) [30], no formal studies to assess the mutagenic potential of morphine have been conducted for the FDA. However, literature studies are cited and include results showing that morphine was non-mutagenic in the *Drosophila melanogaster* sex-linked recessive lethal mutation assay and produced no evidence of chromosomal aberrations when incubated with murine splenocytes. However, as described in the PDR, morphine increased DNA fragmentation when incubated *in vitro* with a human lym-

phoma cell line, and *in vivo*, morphine produced an increase in the frequency of micronuclei in bone marrow cells and in immature red blood cells in the mouse micronucleus test, and induced chromosomal aberrations in murine lymphocytes and spermatids. The product labelling states that some of the *in vivo* clastogenic effects reported with morphine in mice may be directly related to increases in glucocorticoid levels produced by morphine in this species.

Morphine has been reported to produce apoptosis in human peripheral lymphocytes [31,32], and thus the DNA fragmentation in the human lymphoma cell line reported in the product labelling may be attributed to apoptosis. The induction of micronuclei *in vivo* has also been discussed in the literature [33], and it was concluded that the genotoxic response is opioid receptor-mediated because it was abolished in adrenalectomized animals. Further, plasma from morphine-treated animals also induced micronuclei in naive lymphocytes *in vitro*; this response was blocked by inclusion of the steroid antagonist RU 486 in the incubation mixture. Despite this hypothesis for the responses in the *in vivo* micronucleus test, others [34] have concluded that, although basal levels of glucocorticosteroids are required for induction of micronuclei by morphine in murine splenocytes, activation of the hypothalamo-pituitary-adrenal (HPA) axis by morphine does not contribute to the observed response. This is based on studies with *N*-methylmorphine, which did not stimulate the release of corticosterone from adrenal glands, yet induced micronuclei in splenocytes. Also metyrapone, an inhibitor of corticosterone biosynthesis, blocked the morphine-induced increase in corticosterone secretion, but had no effect on the frequency of micronuclei.

An alternative explanation for the *in vivo* micronucleus effect is that it is a consequence of hypothermia, which is caused in rodents by morphine [30,35] (see accompanying paper). Since the data from the chromosomal aberration effects in mouse lymphocytes has not been evaluated independently, it is not clear whether morphine is a unique *in vivo* positive, but this is worthy of further study. Increases in DNA fragmentation (Comet assay) and HPRT mutations have been reported in the human HUT-78 cell line exposed to morphine *in vitro* [36].

3. Candidate *in vivo*-only positives identified by the questionnaire

3.1. Alimta® (pemetrexed)

Alimta® is indicated for the treatment of malignant pleural mesothelioma in combination with cisplatin. It also is indicated for the treatment of locally advanced or metastatic non-small cell lung cancer after prior chemotherapy. Alimta® is an antifolate agent that exerts its action by disrupting folate-dependent metabolic processes essential for cell replication. No carcinogenicity tests have been carried out. However, Alimta® administered at i.v. doses of 0.1 mg/(kg day) or greater to male mice (about 1/1666 the recommended human dose on a mg/m^2 basis) reduced fertility, and induced hypospermia and testicular atrophy.

Ames tests were negative up to 250 μg/plate, which was the lowest precipitating concentration. Alimta® also was negative in chromosome aberration and Hprt mutation assays using CHO cells at concentrations up to 2099 μg/ml (limited by solubility). Two bone marrow micronucleus studies were carried out on Alimta® in ICR mice. Both sexes were dosed intravenously with 393, 787, and 1574 mg/kg. Two doses were given 24 h apart, with the animals killed 24 h after the last dose. Bone marrow cells were stained with Acridine Orange. The ratio of PCEs to normochromatic erythrocytes (NCEs) was not affected at any dose, but Alimta® was positive in both sexes in both tests, with significant differences in two-tailed trend tests and also in pairwise comparisons between individual doses and the controls (Table 4).

Table 4
Mouse bone marrow micronucleus test of Alimta®

Agent	Dose (mg/kg)	PCE/NCE ratio (±S.D.)	MNPCE/1000 PCE (±S.D.)
Vehicle control	–	1.2 (0.3)	1.2 (1.1)
Alimta®	393.5	1.1 (0.4)	5.0 (2.4)
	787.1	0.7 (0.2)	4.8 (2.2)
	1574.1	0.8 (0.1)	4.3[a] (2.2)
Cyclophosphamide (positive control)	25.0	1.0 (0.2)	16.4 (1.5)

Two equal i.v. doses were given 24 h apart to ICR mice, with harvest 24 h after the second treatment. Male mouse data shown (five per group); similar results obtained in female mice. PCE: polychromatic erythrocyte; NCE: normochromatic erythrocyte; MNPCE: micronucleated PCE.

[a] $p = 0.02$, treated groups were significantly increased relative to the vehicle control as determined by a two-tailed trend test.

Table 5a
Rat bone marrow micronucleus test of Pfizer MEK kinase inhibitor 1 (MEK1) in male animals

Agent	Dose (mg/kg)	PCE/NCE ratio	MNPCE/1000 PCE (±S.D.)
Vehicle control	–[a]	7.3	2.0 (0.82)
MEK1	0.3	11.5	1.8 (0.84)
	1.0	8.1	1.8 (0.45)
	3.0	10.0	3.2 (1.3)
Cyclophosphamide (positive control)	20.0	5.3	5.8 (1.1)

Doses were administered to groups of five Sprague–Dawley rats once daily for 2 days by oral gavage and the bone marrow harvested 24 h after the final dose. PCE: polychromatic erythrocyte; NCE: normochromatic erythrocyte; MNPCE: micronucleated PCE.
[a] 0.5% methylcellulose/0.2% Tween 80 in water.

The pharmacology of the test compound indicates that Alimta® causes a decrease in thymidine levels along with a concomitant increase in uridine levels, which may have disrupted normal DNA replication. Why this should affect micronucleus induction in cells *in vivo*, and not *in vitro*, is unclear.

3.2. *Pfizer and AstraZeneca MEK kinase inhibitors*

The genotoxicities of two MEK kinase (Mitogen Extracellular Kinase kinase that activates mitogen-activated protein kinase) inhibitors of the same structural class developed by Pfizer were studied *in vitro* and *in vivo*. In mini-Ames tests, both were negative at concentrations up to 5 mg/plate. *In vitro* micronucleus tests using CHO WBL cells also were negative using a concentration range of 32–125 μg/ml (+/− S9); the highest concentration was limited by cytotoxicity. Similarly chromosome aberration tests using human peripheral lymphocytes (3 h exposure +/− S9 and 24 h −S9) were negative (no increases in chromosome aberrations or polyploidy) when tested at concentrations inducing up to 50% inhibition of the mitotic index (300–400 μg/ml).

The first compound, MEK1, was tested in a rat bone marrow micronucleus test in males and females. The compound was administered once daily for 2 days by oral gavage at doses of 0.3, 1.0 and 3.0 mg/kg and the bone marrow harvested 24 h after the second dose. Increases were seen at the highest dose indicative of clastogenicity, although the effects seen were within the historical control range for this laboratory (Tables 5a and 5b). An *in vivo* metaphase analysis test was carried out in bone marrow cells of Sprague–Dawley rats (Tables 6a and 6b). The compound was administered at the same doses as the micronucleus test, using the same dosing regimen. Although increases were seen in the highest dose group in both sexes, possibly indicating a weak clastogenic effect, the increases were not significant. No aberrations were observed in male control animals whereas at 3 mg/kg MEK1, 2.6% aberrant cells were observed; in females 0.2% aberrant cells were observed in the cells of the negative controls and 1.0% aberrant cells were seen at 3.0 mg/kg MEK1.

MEK2 was tested in the rat bone marrow micronucleus test in males and females. Animals were given two consecutive doses of 50, 100, and 300 mg/kg and the bone marrows were sampled 24 h after the last dose. Significant increases in micronucleated PCE were observed in males at 100 and 300 mg/kg, 2.7- and 4.0-fold relative to the negative control (Table 7), whilst significant

Table 5b
Rat bone marrow micronucleus test of Pfizer MEK kinase inhibitor 1 (MEK1) in female animals

Agent	Dose (mg/kg)	PCE/NCE ratio	MNPCE/1000 PCE (±S.D.)
Vehicle control	–[a]	5.7	2.4 (1.1)
MEK1	0.3	6.1	2.2 (0.8)
	1.0	5.3	2.8 (0.45)
	3.0	5.3	5.4[b] (1.1)
Cyclophosphamide (positive control)	20.0	2.1	10.8[b] (2.4)

Doses were administered to groups of five Sprague–Dawley rats once daily for 2 days by oral gavage and the bone marrow harvested 24 h after the final dose. PCE: polychromatic erythrocyte; NCE: normochromatic erythrocyte; MNPCE: micronucleated PCE.
[a] 0.5% methylcellulose/0.2% Tween 80 in water.
[b] Statistically significant, $p < 0.05$.

Table 6a
In vivo metaphase analysis of rat bone marrow cells from male animals dosed with Pfizer MEK1 inhibitor

Agent	Dose (mg/kg)	% Mitotic index	% Cells with chromosomal aberrations (±S.D.)
Vehicle control	–[a]	5.9	0.0
MEK1	0.3	4.5	0.6 (0.4)
	1.0	6.7	0.6 (0.4)
	3.0	4.2	2.6 (7.0)
Cyclophosphamide (positive control)	60.0	0.9	62.4[b] (5.15)

Doses were administered to groups of five Sprague–Dawley rats once daily for 2 days (except for the positive control which was administered only once, 24 h before bone marrow harvest) by oral gavage and the bone marrow harvested 24 h after the final dose.
[a] 0.5% methylcellulose/0.2% Tween 80 in water.
[b] Statistically significant, $p < 0.05$.

Table 6b
In vivo metaphase analysis of rat bone marrow cells from female animals dosed with Pfizer MEK1 inhibitor

Agent	Dose (mg/kg)	% Mitotic index	% Cells with chromosomal aberrations (±S.D.)
Vehicle control	–[a]	7.2	0.2 (0.2)
MEK1	0.3	6.7	0.2 (0.2)
	1.0	7.6	0.4 (0.24)
	3.0	3.6	1.0 (0.77)
Cyclophosphamide (positive control)	60.0	1.0	89.8[b] (4.8)

Doses were administered to groups of five Sprague–Dawley rats once daily for 2 days (except for the positive control which was administered only once, 24 h before bone marrow harvest) by oral gavage and the bone marrow harvested 24 h after the final dose.
[a] 0.5% methylcellulose/0.2% Tween 80 in water.
[b] Statistically significant, $p < 0.05$.

increases in micronucleated PCEs were seen in females at all doses (3.4- to 6.4-fold relative to the control). A second test was carried out in males with 4 weeks of dosing with lower doses (10, 25 and 50 mg/kg). This test was negative (data not shown) establishing 'no-detectable effect' levels.

AstraZeneca also provided genotoxicity data for a selective inhibitor of human MEK kinase with an *in vitro* IC50 of ~10 nmol/l. The compound was negative in a standard regulatory Ames test and a mouse lymphoma *Tk* assay, but the compound was clearly positive in two independent mouse bone marrow micronucleus tests (Table 8). In the second of these, up to 82% of the micronuclei contained centromere-staining material, indicating a primarily aneugenic mode of action. *In vitro* micronucleus tests in mouse lymphoma cells were inconclusive. MEK is necessary for normal mitotic spindle function [37,38], so it is not surprising that aneugenicity was observed in micronucleus tests. It is possible that the regulatory functions controlled by MEK and

Table 7
Rat bone marrow micronucleus test of Pfizer MEK kinase inhibitor 2 (MEK2)

Agent	Dose (mg/kg)	PCE/NCE ratio	MNPCE/1000 PCE (±S.D.)[a]
Vehicle	–[b]	2.7	4.4 (0.6)
MEK2	50	4.0	4.2 (1.2)
	100	2.3	11.8 (2.8)
	300	1.0	17.5 (2.4)
Cyclophosphamide (positive control)	20	1.9	13.0 (1.1)

Doses were administered to groups of five Sprague–Dawley rats once daily for 2 days by oral gavage and the bone marrow harvested 24 h after the final dose. Data from male animals shown; female animals gave comparable results. PCE: polychromatic erythrocyte; NCE: normochromatic erythrocyte; MNPCE: micronucleated PCE.
[a] Statistical analysis not provided.
[b] 0.5% methylcellulose/0.2% Tween 80 in water.