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REVIEW

Potential thresholds for genotoxic effects by micronucleus scoring

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The concept of thresholds in genotoxicity has been open for debate in the last decades. The micronucleus (MN) test contributed to a large extent in understanding the dose–response relationship for aneugens and clastogens. The threshold concept for aneuploidy is well accepted by the scientific community based on the data and for mechanistic reasons. The concept of threshold for clastogens is still challenging. Acceptance is based on a case-by-case basis together with thorough mechanistic understanding of the different steps from the mutagen–target interactions to MN formation for this class of genotoxicants. This review summarises the significant achievements in the assessment of threshold for genotoxins using the MN test and concludes with an overview of knowledge gaps and recommendations.

Introduction

The micronucleus (MN) test has been extensively used in a variety of exploratory and mechanistic investigations with the aim to understand the basic mechanisms underlying genotoxicity. The simplicity and the readiness to be applied to a variety of cell types either *in vitro* or *in vivo* made it a versatile tool that contributed to a large extent in our understanding of key toxicological issues related to genotoxins and their effects at the cellular and organism levels. Recently, the final acceptance of the *in vitro* MN test Organisation for Economic Co-operation and Development (OECD) guideline 487 (1) together with the standard *in vivo* MN test OECD guideline 474 (2) will position the assay to become a key driver in the determination of the genotoxicity potential in exploratory research as well as in the regulatory environment. In this manuscript, we review the major achievements using the MN test in understanding the dose–response relationship of genotoxicants and specifically the non-linear dose–effect relationship where the threshold mode of action (MOA) is expected or demonstrated. It is widely accepted that indirect-acting genotoxicants may show a threshold MOA and exhibit a level of exposure below which no genotoxic effect may be observed. Among these thresholded MOA one may mention aneuploidy, high level of cytotoxicity, inhibition of protein/DNA synthesis and ionic imbalance (3). For some mechanisms, a threshold is expected, although hard to prove (4). On the contrary, chemicals which directly react with DNA are expected to induce mutation as a result of a single reaction of the compound with the DNA and therefore exhibit a non-threshold response (5–9). However, this hypothesis does not take into account the cellular protective mechanisms of the exposed cell or organism (9,10). Cytoprotective mechanisms such as DNA repair or detoxification should be considered when the threshold is determined.

Significant achievements

Aneugens

Aneugens may act on non-DNA targets such as the inhibition of spindle function with no direct interaction with DNA and it is assumed that a critical number of target sites must be occupied before the biological effect occurs and therefore these mechanisms may have a threshold (11). Therefore, the risk for adverse effects following human exposure to an aneugen could be minimal, if the threshold of activity has been clearly demonstrated *in vivo* and *in vitro* (Table I) and providing the human exposure level is below this threshold. Spindle poisons have been used as model compounds to demonstrate existence of thresholds for aneuploidy induction. Multiple interactions of spindle poisons with the tubulin molecule are necessary to disturb microtubule dynamics and affect chromosome segregation leading to aneuploidy and therefore a threshold dose–response relationship is expected.

In studies by Elhajouji *et al.* (12,13), a threshold effect for chromosome loss and chromosome non-disjunction have been determined *in vitro* in human lymphocytes for spindle poisons such as colchicine, carbendazim, nocodazole and mebendazole. Flow cytometry sorting and further fluorescence in situ hybridization (FISH) analysis with pancentromeric DNA probes was performed to distinguish between centromere-positive (MNCen+) versus centromere-negative (MNCen−) MN. The cytokinesis-block MN assay, in combination with centromeric probes for chromosomes 1 and 17, was used to identify non-disjunction. The non-disjunction threshold values for colchicine, carbendazim, nocodazole and mebendazole were 0.020, 1.046, 0.033 and 0.135 μM, respectively, while those for chromosome loss were 0.037, 2.61, 0.066 and 0.271 μM, respectively. Elhajouji *et al.* (12) investigated *in vitro* MN in human lymphocytes with a discontinuous regression model which reflected a ‘jump’ in the regression line for the possible threshold evaluation. A piecewise linear regression or break-point model was fitted to the centromere positive versus centromere-negative cells. In the second paper, Elhajouji *et al.* (13) defined a threshold in two ways; firstly, as the point where a statistically non-significant increase (last statistically non-significant concentration) changes to a statistically significant increase (first statistically significant concentration) and secondly, by estimating inflection points (the point where there is a change from concavity to convexity of a curve) using

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Table I. Overview of the NOEL values for aneugenic compounds based on literature data and discussed in this review

Compound	CAS number	Mechanism	*In vitro*/*in vivo*	Cell system	NOEL value	References
Colchicine	64-86-8	Spindle poison	*In vitro*	Human lymphocytes	n.d. 0.020 μM, ch.l 0.037 μM	(12,13)
			In vivo	MN in mouse peripheral blood	0.49 mg/kg	(14)
Carbendazim	10605-21-7	Spindle poison	*In vitro*	Human lymphocytes	n.d. 1.046 μM, ch.l 2.61 μM	(12,13)
			In vitro	Human lymphocytes	3.2–4.3 mM	(15)
			In vivo	MN in mouse bone marrow	66 mg/kg	
			In vivo	MN in mouse bone marrow	8 μg/ml	
Nocodazole	31430-18-9	Spindle poison	*In vitro*	Human lymphocytes	n.d. 0.033 μM, ch.l 0.066 μM	(12,13)
		Spindle poison	*In vitro*	Human lymphocytes and mouse splenocytes	50 nM	(16)
Mebendazole	31431-39-7	Spindle poison	*In vitro*	Human lymphocytes	n.d. 0.135 μM, ch.l 0.271 μM	(12,13)
Benomyl	17804-35-2	Spindle poison	*In vitro*	Human lymphocytes	3.8–4.1 mM	(15)
			In vivo	MN in mouse bone marrow	100 mg/kg	
Nitrobenzene and benzonitrile	98-95-3 and 100-47-0	Spindle poison	*In vitro*	MN induction in V79 cells	0.001–0.005 μM	(17)
Paclitaxel	33069-62-4	Spindle poison	*In vitro*	Human lymphocytes	2.5 nM	(16)
			In vitro	Mouse splenocytes	0.5 nM	(16)
Bisphenol-A	80-05-7	Spindle poison	*In vitro*	MN assay in AHH-1, MCL-5 and V79 cell lines; tripolar mitotic	10.8 μg/ml	(18)
				spindle induction in V79 cells	7 μg/ml	
Rotenone	83-79-4	Spindle poison	*In vitro*	MN assay in V79 cell line	0.25 ng/ml	(18)
				Spindle induction in V79 cells	80 pg/ml	
Vincristine	57-22-7	Spindle poison	*In vivo*	MN in mouse bone marrow	0.01 mg/kg	(19)
			In vivo	MN in mouse peripheral blood	0.017 mg/kg	(14)
Vinblastine	865-21-4	Spindle poison	*In vivo*	MN in mouse peripheral blood	0.35 mg/kg	(14)

n.d., non-disjunction; ch.l, chromosome loss.

a polynomial model as an approximate determination of a threshold. These authors have also demonstrated that the threshold for non-disjunction is lower than that for chromosome loss. This finding is to be expected since less damage to microtubules is needed to induce a mono-oriented chromosome (one kinetochore captured by microtubules) than to induce chromosome loss where none of the kinetochores are captured by microtubules. A study by Bentley *et al.* (15) was conducted to determine a threshold mechanism of action for benomyl and carbendazim-induced aneuploidy *in vitro*. Using binucleate human lymphocytes and FISH technique, the authors were able to characterise a threshold mechanism of action for the two spindle inhibitors. Induction of chromosome non-disjunction, chromosome loss and gain and polyploidy were evaluated for six different chromosomes (1 and 8, 11 and 18 and X and 17) in interphase nuclei. The threshold response was nearly identical among the six analyzed chromosomes for both benomyl and carbendazim. The concentrations at which statistically significant increases in non-disjunction first appeared ranged from 3.8 to 4.1 mM for benomyl and from 3.2 to 4.3 mM for carbendazim. Threshold concentrations were also determined for chromosome loss (benomyl and carbendazim) and for MNCen+ for benomyl only. These were found to be equal to or, in most cases, greater than the threshold concentrations for non-disjunction (15). Potential thresholds have also been established for spindle poisons nitrobenzene and benzonitrile. MN induction by these test compounds in V79 cells, as well as effects on the formation and stability of microtubules and on motor protein functions was studied by Bonacker *et al.* (17). The observed data clearly demonstrate a chromosomal end point of genotoxicity, expressed as aneugenic effects, for both nitrobenzene and benzonitrile at remarkably low concentrations, with a lowest-effect concentration at 0.01 μM and no-effect concentrations between 0.001 and 0.005 μM.

In a study by Steiblen *et al.* (16), the threshold levels for nocodazole and paclitaxel were identified using the comparison of the *in vitro* MN assay in human lymphocytes and mouse splenocytes. For nocodazole, a no observed effect level (NOEL) has been identified as 50 nM in both human lymphocytes and mouse splenocytes. The results were comparable with those reported by Elhajouji *et al.* (13), who found that under the same experimental conditions, the NOEL of nocodazole was 33 nM in human lymphocytes. For paclitaxel, the NOEL was 0.5 nM in mouse splenocytes, while it was 2.5 nM in human lymphocytes, which indicates a higher degree of sensitivity of the mouse splenocyte compared with the human lymphocyte in this case (16).

Recent studies by Johnson and Parry (18) have demonstrated that the plastics component bisphenol-A (BPA) and the natural pesticide rotenone, both known as spindle poisons, induce MN and modify the functioning of the microtubule organising centres of the mitotic spindles of cultured mammalian cells in a dose-dependent manner. The cytokinesis-blocked MN assay in human lymphoblastoid cell lines AHH-1 and MCL-5 and in Chinese hamster V79 cell lines was used for these studies. For a mechanistic evaluation of the aneugenic effects of BPA and rotenone, fluorescently labelled antibodies were used to visualise microtubules (alpha-tubulin) and microtubule organising centres (gamma-tubulin). A NOEL at 10.8 μg/ml BPA was observed for MN induction. Rotenone showed a small increase in MN induction with the first significant effect at 0.25 ng/ml in V79 cells but there was no significant effect in the metabolically competent cell line, MCL-5. The NOELs for tripolar mitotic spindle induction in V79 cells were 7 μg/ml for BPA and 80 pg/ml for rotenone (18).

The existence of such thresholds *in vitro* is regarded as evidence for the existence of a safety margin; however, this cannot be used directly for risk evaluation in humans. It is not known how concentrations found *in vitro* in a particular type of cells, e.g. lymphocytes, can be extrapolated to somatic and/or germ cells *in vivo* and further investigations are therefore required (3). Evaluation of the biological threshold for induction of non- maturing, naked mouse oocytes and *in vitro* cultured human lymphocytes exposed to nocodazole suggest that isolated *in vitro* maturing oocytes are not more sensitive to classic aneugens such as nocodazole when compared to mitotically dividing cells, despite the differences in spindle formation and cell cycle regulation (3,20).

Some experimental data with aneugens *in vivo* were published by Seiler *et al.* (21). In this study, micronucleated erythrocytes, induced by carbendazim, were investigated in mouse bone marrow. Carbendazim was found to inhibit tubulin association at the concentration of 10 µg/ml (100 mg/kg) and, thus, it was concluded that the threshold concentration was 8 µg/ml. This data is supported by the study of Bentley *et al.* (15) where NOEL for benomyl was demonstrated at 100 and 66 mg/kg for carbendazim. In experiments by Tinwel and Ashby (19), the minimal detectable level for vincristine was demonstrated in mouse bone marrow at a dose of 0.01 mg/kg. In a study by Asano *et al.* (22), authors showed that a non-linear dose–response relationship exists for the spindle poison colchicine. A recent study by Cammerer *et al.* (14), with the aneugens vinblastine, vincristine and colchicine, demonstrates a non-linear dose–response relationship, clearly indicating the presence of a threshold dose for all tested compounds. Studies were performed in mouse peripheral blood after single administration of the test compound. The last not statistically significant and the first statistically significant concentrations were identified with the Dunnett's test. Further, assuming that in case of a non-linear dose response, the threshold concentration will be situated between these points; the linear regression analysis was performed independently with the set of data lower than the first statistically significant concentration and higher than the last statistically non-significant concentration. In a next step, the determination of the intersection point for both regression lines was performed and the obtained concentration was considered as a threshold. Based on the mathematical modelling performed, the threshold level for aneuploidy induction is 0.35 mg/kg for vinblastine, 0.017 mg/kg for vincristine and 0.49 mg/kg for colchicine (14). As summarised in Table I the available literature data clearly indicate the presence of threshold dose–response relationships for aneugens that target microtubules.

Clastogens

Table II provides an overview of the clastogens discussed in this review and includes the NOEL values as estimated in the respective publications.

Ionising radiation. Ionising radiation induces DNA damage by direct interaction with DNA or via reactive oxygen species (ROS). It has been reported that low-dose radiation

Table II. Overview of the NOEL values for clastogenic compounds based on literature data and discussed in this review

Compound	CAS Number	Mechanism	*In vitro*/ *in vivo*	Cell system	NOEL value	References
X-rays	—	Ionizing radiation	*In vivo*	Bone marrow/CBS/lac male mice/ Chronic 0.07 mGy/h	35–61 cGy	(23)
Genistein	446-72-0	Topoisomerase II inhibition	*In vitro*	Mouse splenocytes	12.5 µM	(24)
				L5178Y mouse lymphoma cell line	0.5 µg/ml	(8)
Ciprofloxacine	85721-33-1	Topoisomerase II inhibition	*In vitro*	L5178Y mouse lymphoma cell line	25 µg/ml	(8)
Etoposide	33419-42-0	Topoisomerase II inhibition	*In vitro*	L5178Y mouse lymphoma cell line	0.005 µg/ml	(8)
Doxorubicin	25316-40-9	Topoisomerase II inhibition	*In vitro*	L5178Y mouse lymphoma cell line	0.002 µg/ml	(8)
Potassium bromate	7758-01-2	ROS inducer	*In vitro*	TK6 human lymphoblastoid cell line/ 1-h treatment	0.9 mM	(4)
				TK6 human lymphoblastoid cell line/ 2-h treatment	0.2 mM	
				TK6 human lymphoblastoid cell line/ 3-h treatment	5.05 mM	
				TK6 human lymphoblastoid cell line/ 24-h continuous treatment	0.025 mM	
Glucose oxidase	9001-37-0	Oxidation of beta-D-glucose to produce gluconic acid and H_2O_2	*In vitro*	TK6 human lymphoblastoid cell line/ 1-h treatment	1.8 10^{-5} U/ml	(4)
Bleomycin	9041-93-4	ROS inducer	*In vitro*	TK6 human lymphoblastoid cell line/ 1 h treatment	0.05 nM	(4)
				TK6 human lymphoblastoid cell line/ 3 h treatment	0.025 nM	
				TK6 human lymphoblastoid cell line/ 6 h treatment	6.25 nM	
				TK6 human lymphoblastoid cell line/ 24 h continuous treatment	6.25 nM	
Methyl methanesulfonate	66-27-3	Alkylatying agent	*In vitro*	AHH-1 human lymphoblastoid cell line	0.8 µg/ml	(10)
Ethyl methanesulfonate	62-50-0	Alkylatying agent	*In vitro*	AHH-1 human lymphoblastoid cell line	1.35 µg/ml	(10)
			In vivo	Male Winstar Rats	50 mg/kg	(25)
				B6C3F1 mice/bone marrow	Two times 50 mg/kg	(26)
				B6C3F1 mice/pheripheral blood	Two times 50 mg/kg	(26)
				CD-1 (IRC) Mice/bone marrow	80 mg/kg/day	(27,28)
Acrylamide	79-06-1	Combination of mechanisms	*In vivo*	B6C3F1 mice/bone marrow RET	4 mg/kg/day	(29)
				B6C3F1 mice/bone marrow NCE	6 mg/kg/day	(29)
				B6C3F1 mice/bone marrow NCE/ haemoglobin adducts as dose metric	1 or 2 mg/kg/day	(29)

may induce defence mechanisms including antioxidant formation and repair of DNA double-strand break. (30). After low-dose radiation, growing human fibroblasts could repair DNA double-strand breaks completely to the level of unirradiated control (30,31). This may suggest a threshold type of response curve. In this context, the literature data presented in 1978 (32) suggest the deviation from linearity in bone marrow MN test after X-rays exposure (CBA male mice). However, the non-linear response in MN formation has been explained by the cell cycle delay induced by radiation (32). It has been proven using mice bone marrow MN test that the chronic exposure to low-dose ionising radiation (0.07 mGy/h) produces the non-linear threshold response with a threshold located between 35 and 61 cGy of accumulated irradiation, whereas the acute treatment resulted in a linear response curve (23).

Topoisomerase II inhibitors. Topoisomerase II is a group of enzymes that play a crucial role in topographical organisation of DNA during replication, transcription and DNA repair processes (8,33,34). Many compounds are known to disrupt either the catalytic cycle of topoisomerase II (catalytic inhibitors) or stabilise the topoisomerase II–DNA transient cleavage complex (topoisomerase II poisons). In contrast to catalytic inhibitors that disrupt the enzyme physiology, topoisomerase II poisons by stabilisation of the cleavage complex may result in fixed DNA double-strand breaks if the DNA damage is not repaired (8).

In 1995, Record *et al.* (24) examined the effects of genistein, genistin and etoposide in mouse splenocytes *in vitro*. The high MN level was observed for all tested compounds, however, the positive effect for genistein started at the third tested concentration (25 μM) suggesting a thresholded mode of action (MOA) with a NOEL value at 12.5 μM. In this perspective, Lynch *et al.* in 2003 (8) examined the threshold concept for selected topoisomerase II poisons with different clastogenic potential and topoisomerase II affinity. Etoposide, doxorubicin, genistein and ciprofloxacine were tested for MN induction in L5178Y mouse lymphoma cell line *in vitro*. Using mathematical modelling of the experimental data, the threshold values for etoposide, doxorubicin, genistein and ciprofloxacin were 0.00236, 0.00151, 1 and 40 μg/ml, respectively. In contrast, the clastogenicity of genistein has not been confirmed *in vivo* in mice and rats (24,35).

Oxidative stress inducers (ROS inducers). Many genotoxicants act through formation of ROS. In physiological state, ROS are formed constantly as a consequence of metabolic and biochemical processes. ROS also play a role in signalling pathways and gene expression (4,36). In the normal conditions, the amount of ROS is regulated by intracellular scavengers like vitamin C, glutathione, uric acid or antioxidant enzymes like glutathione peroxidase, superoxide dismutase or catalase. Additionally, mammalian cells possess very efficient DNA repair systems that limit the ROS-induced DNA damage. Therefore, actual oxidative stress occurs when ROS exceeds the amount neutralised by the natural defence barrier of the organism and causes damage to macromolecules like proteins, lipids and DNA. Some of the DNA damage, when not fixed, may lead to mutations (4). Thus compounds producing ROS are expected to show a NOEL, below which no statistically significant increase in DNA damage/mutation (4,36) is seen, as far as the formation of ROS would not exceed the capacity of the organism to neutralise its harmful effects.

In 2007, Brink *et al.* (37) analysed the MN induction in L5178Y mouse lymphoma cell line after hydrogen peroxide and cumene hydroperoxide treatment. The response curve after hydrogen peroxide was reported as supralinear.

In the context of threshold, Platel *et al.* (4) have investigated dose-related response in the MN test using human lymphoblastoid cell line TK6 after treatment with three DNA oxidising agents: thio-dependent $KBrO_3$, bleomycin (BLM) and hygrogen peroxide (H_2O_2) via glucose oxidase (GOx). For each compound, three statistically significant plateaus were observed and the highest concentration of the first plateau was considered as the NOEL. For $KBrO_3$, NOELs were different dependent on the treatment duration and were 0.9, 0.2 and 5.05 mM for 1-, 2- and 3-h treatment time, respectively. For GOx, NOEL was 1.8×10^{-5} U/ml for 1-h treatment. The BLM showed 0.05, 0.025 and 6.25 nM values for NOEL at 1, 3 and 6 h, respectively. The shape of the response curve obtained for short-term treatment with BLM was confirmed by long-term treatment (with three plateaus), with the NOEL value of 6.25 nM. In contrast, long-term treatment with $KBrO_3$ exhibited a different concentration–response relationship when compared to short-term treatment. A two-plateau curve was noted with a NOEL value of 0.025 mM. For all compounds, the calculated and the obtained values of NOEL showed a positive correlation (R^2 values ranging from 0.50 to 0.93) (4). It has been speculated that the first plateau may correspond to the first level of physiological defence such as scavengers (vitamin C, vitamin E, GSH and others). By conjugation, they may prevent from reaction of ROS with DNA, resulting in the NOEL (4).

Mutagens that induce DNA adducts alkylating agents. An important class of DNA-reactive compounds also used in chemotherapy is the alkylating agents. The MOA and the DNA targets have been well characterised and numerous members of this class of chemicals are either suspected or proven carcinogens. Alkylating agents may be classified as mono- or bifunctional, depending on the number of reactive groups. They are electrophilic, therefore have high affinity to electron-rich nucleophilic centres of DNA or proteins. The standard parameter to characterise the reactivity of alkylating agents is the Swain Scott constant (*s* value). Chemicals with high s value target high nucleophilic centers (e.g. N^7-G, N^3-A). A low s value indicates lower sensitivity to nucleophilicity of the target and therefore the compounds are able to alkylate O atoms (e.g. O^6-G) much more efficiently than compounds with higher s value (10).

In order to scrutinise the type of response curve, Doak *et al.* (10) investigated induction of MN *in vitro* in human lymphoblastoid cell line AHH-1 after treatment with classical alkylating agents methylmethane sulfonate (MMS, s value $>$ 0.83), ethylmethane sulfonate (EMS, s value = 0.67), methylnitrosourea (MNU, s value = 0.42) and ethylnitrosourea (ENU, s value = 0.26). No clear NOEL was detected for MNU and ENU. A NOEL value was defined as 0.8 μg/ml for MMS and 1.35 μg/ml for EMS. Lowest observed effect level (LOEL) was 0.85 μg/ml for MMS and 1.4 μg/ml for EMS (10). The different results obtained for MMS versus MNU and EMS versus ENU may be explained by different mechanisms involved in DNA repair of alkylated N^7-G and O^6-G. N^7-alkyl-G adducts are repaired after involvement of base excision repair (BER) pathway and to lesser extent by mismatch repair, whereas in the removal of O^6-alkyl-G, the methylguanine DNA methyltransferase (MGMT) is primarily involved, though nucleotide excision repair may also be involved when the MGMT is insufficient. Therefore the non-linear response on EMS and MMS may be caused by efficient BER and MGMT. MNU and ENU form far more O^6-alkyl-G adducts, which possibly may not be sufficiently repaired by MGMT. Additionally, they form O^4-alkyl-T and O^2-alkyl-T adducts that are poorly repaired (10,26,27,38,39). Further research conducted by Brink *et al.* (37) proved the upward convex response curve of MN induction when L5178Y cell line was treated with MMS for 4 h; however, the data did not allow a conclusion about the potential threshold for concentrations <50 μM of MMS. A no-threshold response for MNU treatment has been confirmed in the following studies using several variants of mouse lymphoma assay, where possible threshold is suggested for MNU placed in 0.69 μM (40). However, the threshold dose calculated for MMS and presented by Pottenger *et al.* (40) is in a good correlation with the Doak *et al.* data (10).

A sublinear dose–effect response following EMS treatment *in vivo* was published by various groups without defining a NOEL dose level (41–43). A more recent study performed by Cammerer *et al.* (25) estimated a NOEL at 50 mg/kg for peripheral blood MN induction in male Wistar rats following 2-day treatment. Sublinear response to EMS treatment was also presented by Witt *et al.* (44) when B6C3F1 mice and Fisher 344 rats have been treated on three consecutive days with EMS (doses 50, 100, 200 and 300 mg/kg/day). The dose of 50 mg/kg did not show a statistically significant MN induction in bone marrow and in peripheral blood. In a study by Gocke *et al.* (26,27), mice have been treated by oral gavage for 7 days (EMS: 0, 1.25, 2.5, 5, 20, 80, 140, 200 and 260 and ENU: 0, 1.11, 4.45 and 17.8 mg/kg/day). Doses up to 80 mg/kg/day showed no increase in bone marrow micronucleated polychromatic erythrocytes, whereas in higher doses, a clear increase in MN induction was observed. In contrast, the dose–response curve for ENU treatment did not show any indication for a threshold. Using mathematical modelling, the threshold dose for EMS was 89.812 mg/kg/day with a confidence range between 56.664 and 118.245 mg/kg/day (26–28,45). However, the EMS threshold has not been confirmed on the level of protein and DNA adducts formation (26–28). This may suggest that an error-free DNA repair system provides successful DNA damage repair resulting in a lack of fixed mutation up to a threshold dose.

Acrylamide. Many authorities classify acrylamide as a potential human carcinogen based on its carcinogenicity in rats and genetic toxicity (29). The metabolic profile of acrylamide has been well established for humans, rats and mice (29,46). The primary pathway involves conversion to the reactive epoxide and glycidamide by cytochrome P450 2E1 (CYP 2E1). Glycidamide is next metabolised by hydrolases and conjugated with glutathione. In humans, ~15% of acrylamide is converted to glycidamide, which reacts with DNA and forms N^7-G and to a lesser extend N^3-G adducts. In another mechanism, acrylamide induces oxidative stress in rodents. It may also bind to kinesin proteins and therefore modify the chromosome segregation and spindle formation at very low doses though (29).

Hence, Zeiger *et al.* (29) presented data which evaluated the shape of mouse bone marrow MN dose–response curve within the range of low doses. In the study, B6C3F1 mice were treated for 28 days with 0 (deionised water), 0.125, 0.25, 0.5, 1, 2, 4, 6, 8, 12, 16 and 24 mg/kg/day of acrylamide. One million normochromatic erythrocytes (NCEs) and $>200\,000$ reticulocytes (RETs) per mouse were scored. NOEL was noted up to 4 and 2 mg/kg/day for MN–RET and MN–NCE, respectively. MN induction was within these concentrations in the range of historical control values. Statistical analysis based on administrated doses and MN–RET or MN–NCE could not discriminate between linear and non-linear dose–effect relationships. However, when the regression analysis was based on the glycidamide haemoglobin adducts as the dose metric, non-linear regression models fitted the data much better than the linear regression model. The authors concluded that the acrylamide-based MN induction showed a threshold dose response (29).

Knowledge gaps and recommendations

For theoretical considerations together with published data, it is widely accepted that aneugens exhibit a threshold dose–effect relationship. However, regarding clastogens, the situation is still challenging and the demonstration of a threshold requires a clear mechanistic investigation to corroborate experimental data that stipulate a threshold dose–effect relationship. Acceptance of a non-linear extrapolation for clastogens will require exploratory studies to identify the cellular targets and to scrutinise the different steps from the mutagen–target interactions to MN formation. The situation is even more demanding when dealing with complex mixtures since the overall MN frequency may result from different overlapping but still distinct genotoxic insults (3).

The influence of the genotype on the activation/detoxification of genotoxins is well documented and a great variability between individuals has been observed (reviewed in ref. 47), therefore the selection of donors should also be taken into consideration. Experimental design, adequate dose spacing and the duration of exposure (chronic versus acute) are key for threshold identification especially for *in vivo* experiments. Statistical aspects and appropriate mathematical modelling should drive threshold estimation. However, based on the complexity and the variability in the experimental data, a more elaborated mathematical modelling should be developed to address the specificities of genotoxicity data taken into account the sensitivity of the measured end point, i.e. MN and the statistical power of the assay specially with the increasing use in the future of automated MN analysis systems, e.g. image analysis or flow cytometry. The recognition that a significant number of chemicals operate by indirect mechanisms of genotoxicity and showing a possibility of a threshold presents new challenges for the regulation of chemicals. The regulatory schemes should be modified to take into account the MOA and possible threshold effects. When it is demonstrated that a genotoxic substance has a threshold MOA, the threshold level should be identified quantitatively. In addition, it has to be assessed whether the margin of safety between threshold and human exposure is sufficient for health protection. This assessment should also take into account variability in the experimental data, the intra- and inter-species variation, the differences in exposure and the individual susceptibility.

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