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Review

Investigating Mechanisms for Non-linear Genotoxic Responses, and Analysing Their Effects in Binary Combination

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A recent shift by the scientific and regulatory community, towards accepting the existence of non-linear dose responses for certain DNA reactive genotoxic agents, has unveiled a myriad of questions regarding their biological basis. The mechanisms responsible for 'genotoxic tolerance' at low doses are wide ranging but poorly understood, yet this information is essential when analysing non-linear dose responses for hazard and risk assessment. For DNA reactive genotoxins, non-linear dose responses can arise from many different biological mechanisms, including DNA repair. Recent work from our group explored the contributory role of DNA repair to nonlinear genotoxic dose responses, in human cells exposed alkylating agents. Here we discuss the involvement of the repair enzymes methylpurine DNA-glycosylase and methyl-guanine methyl-transferase in modulating the non-linear dose responses observed in human cells exposed to ethyl methanesulfonate (EMS) and *N*-methyl-*N*-nitrosourea, respectively. We also discuss the exposure of binary mixtures, and how combinations of the dissimilar acting agents Benomyl and EMS at their no observed genotoxic effect levels, induce a significant increase in micronuclei.

Key words: alkylating agents, thresholds, mode of action, DNA repair, mixtures

Introduction

The dose-response of a given genotoxic compound is a fundamental principle of toxicology and pertinent to hazard identification, environmental risk assessment and medicinal drug assessment. For direct acting DNA reactive compounds, the dose-response relationship for genotoxic and carcinogenic end points has long been assumed to be linear and have no threshold. Consequently regulatory agencies were quantifying risk at low doses where risk was assumed to increase linearly with dose, and no exposure dose was deemed safe (EPA 2005). This linear model was originally extrapolated from a series of radiation studies in the 1950s (1) based on the underlying assumption that DNA reactive genotoxic compounds followed the 'one hit one target' theory. That is, chemicals which directly react with DNA, induce mutation as a result of a single reaction of the compound with the DNA, thus a linear response is portrayed (2). Several case studies have since contested this model, and have shown that for some genotoxins, such as alkylating agents, a non-linear dose response for gene mutation and chromosome damage exists both *in vitro* (3–5) and *in vivo* (6–9). That is to say that several alkylating agents are considered to have a threshold dose response, whereby certain compounds have no observed effect in the low dose range, and are only considered genotoxic at doses above a point of departure (PoD) (9). PoD is used here, as a more general term that covers threshold dose, no observed genotoxic effect level (NOGEL) and benchmark dose (BMD) (9).

Due to new high content *in vitro* and *in vivo* genetic toxicology test systems, such as the flow cytometric or automated image analysis based micronucleus assay, extensive dose ranges with high statistical power are becoming more common, and this enables a high number of cells (events) to be scored. Such high power dose responses allow for more detailed analysis of the 'true' shape of a compound's dose-response curve. Not only is it possible to discern between a linear and a non-linear dose-response, but further statistical analysis is now possible which enables usable PoDs to be derived for compounds that once were shown to be linear, such as with *N*-methyl-*N*-nitorosourea (MNU) (9). In this mini-review we will discuss recent studies that allude to clear PoD in mammalian cells exposed to low doses of MNU, and point to DNA repair and certain homeostatic pathways as being a mode of action (MOA) for the observed non-linear dose responses. When we investigated MNU

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at lower doses than tested before (10), the initial analysis revealed a potential hormetic response. A predicated mechanism for this, is that cells overcompensate by over-increasing the repair response when exposed to low levels of an alkylating agent, thereby establishing an almost 'beneficial' effect within that low dose range. When carrying out the risk assessment, the high power dose response must be partnered with clear MOA data, such as key mechanisms relevant to the adverse effects of the chemical, to provide biological meaning. Therefore, the dose response can be accounted for experimentally using gene expression analysis, where significant changes at these low exposure levels are often seen. This is supported by knockdown of certain homeostatic pathways which often shift the PoD to a lower dose.

Recent adjustments to risk-assessment methods in EPA now require MOA evaluations in dose-response assessment. EPA's *Guidelines for Carcinogen Risk Assessment* (11) state that if a compound is deemed ''DNA reactive and [to] have direct mutagenic activity'' or to have high human exposures or body burdens ''near doses associated with key precursor events'' (11), a no-threshold approach is applied and a MOA determined. A dose with no associated risk can be calculated (e.g., reference dose, RfD), for carcinogens with sufficient MOA data to conclude nonlinearity at low doses (11). Several possible MOA that may elicit a non-linear dose-response are outlined in Fig. 1. This mini-review discusses recent studies that evidence DNA repair as one MOA required for non-linear dose responses induced by direct acting DNA damaging compounds. Furthermore, it discusses recent preliminary data on the role of methyl guanine methyl transferase (MGMT) in AHH-1 cells exposed to the super-mutagen MNU (12,13), and the effect in altering MGMT function to the proportion of HPRT mutations.

Clear dose responses of genotoxic compounds with

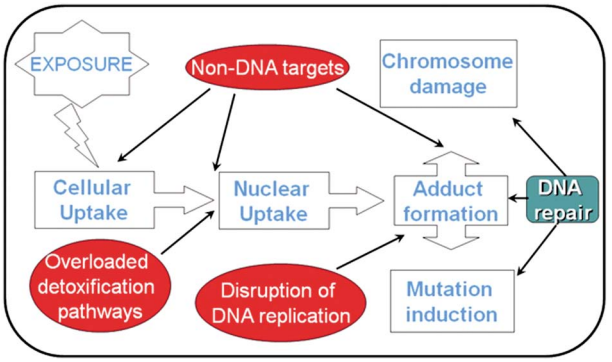

Fig. 1. Route of exposure for a genotoxic compound, shown from cellular uptake to DNA damage. Adapted from Jenkins et al. (14) and COM 2010. Text within the red ovals are existing genotoxic threshold mechanisms representative of the proposals made by the Committee of Mutagenicity (15).

clearly defined MOA, also aid in the investigation of binary mixtures. It has long been assumed that two compounds that have similar genotoxic mechanisms of action behave through dose addition, while dissimilar agents act independently, via response addition (16). This is shown mathematically, if R(x) is the dose-response function of two compounds A and B, the response for a mixture with standardised dose x_A of A and x_B of B, is $R(x_A + x_B)$ for dose addition (expected for similar acting agents), and $R(x_A) + R(x_B)$ for response addition (expected for dissimilar acting agents) (16). Removal of control values, is key to these calculations. These theories have not been tested at low doses, and are not even addressed in much detail in non-pesticide risk assessment. For example, does a combination of two genotoxic impurities found at their NOGELs, cause a positive response? This is a very important area that is currently poorly understood, and much is left to theoretical approaches. Here we will look at different combinations of ethyl- and methyl-methanesulfonate (EMS and MMS) and the fungicide Benomyl, to identify whether such mixtures fit into the current compound mixture models. These experiments investigate how similar the MOA of each compound is, as well as asking the questions of whether mixtures need to be included in hazard and risk assessments, or whether the current theories for dose addition and response addition (17) are suitable when assessing mixtures at their NOGELs.

In summary, this mini-review investigates PoDs for alkylating agents and provides guidance on how to define their underlying MOA. A testing strategy for investigating binary mixtures at low doses is also presented.

DNA Repair as a MOA for the Clastogenic and Mutagenic PoDs Induced by Alkylating Agents

Once the PoD was defined with high statistical power for the alkylating agents tested here, the biological relevance of these responses was then required. To achieve this, the dose response was repeated in the same cells, but with DNA repair pathway knocked down using RNAi or enzyme inhibitors. Considerations for risk assessment include the possibility of the stimulation of DNA repair following low doses of mutagens. Indeed repair processes have a finite capacity, and thus the ability to remove only a fixed number of adducts may give rise to an apparent threshold. DNA repair, as the predicted MOA for DNA reactive genotoxic agents, has been shown experimentally by many global experts (18,19). However, it has never been directly linked to a PoD defined with high statistical power, along with a supporting MOA. Our group has tackled this for chromosome damage induced by EMS *in vitro* (20). We showed the role of *N*-methylpurine DNA glycosylase (MPG), an initiator glycosylase for the base excision repair (BER) pathway, as a key modulator in the clasto-

genic threshold response observed in human lymphoblastoid cells exposed to EMS, through a series of gene expression analysis and knockdown studies (20). Deletion of MAG1, a eukaryotic homolog of MPG was also associated with increased sensitivity and lowered threshold of 0.0001% for MMS and EMS in *Saccharomyces cerevisiae* (21) thus substantiating our findings in humans.

BER was also accountable in the threshold response observed in AHH-1 cells exposed to H_2O_2 where the dose-response curve shifted by modulating the antioxidant glutathione (22). The observed homeostatic mechanisms permitting a pro-oxidant PoD further highlights the relevance of the MOA in aiding risk assessments. The authors would also like to highlight that they are not proposing these enzymes of these DNA repair pathways to be the sole mechanisms of action for the dose response, just a mode of action (MOA) for the response. By this, we predict that by knocking down certain DNA repair enzymes, the PoD moves to the left, at which point the next backup pathway causes a lower PoD, and therefore sequential knockouts of different compensatory pathways would lead to sequential shifting of the PoDs to lower doses.

Within prokaryotes, similar studies elucidated to the role of translesion synthesis (TLS) as a MOA for PoD induced by MMS. TLS is a multi-DNA polymerase process, where specialized DNA polymerases take over blocked DNA replication, to bypass DNA lesions when the chromosomal replication by conventional DNA polymerases is blocked (23). Using fungal model systems Dot-1, an activator of the Rad53 check point kinase to translesion synthesis was shown to modulate tolerance to the genotoxic effects of MMS in a PCNA ubiquitylation-dependent fashion (24). The mechanism by which this is proposed to occur is via alteration in the abundance of the key TLS factor Rev1, which directly binds to chromatin (24). An equivalent study using eukaryotic cells would prove of interest. Thus, it is likely that sequential knockdown of TLS, then BER, followed by a double knockdown TLS + BER in three separate experiments, would sequentially shift the PoDs to lower doses, respectively.

It is well established that DNA repair is elicited in response to a cell exposed to direct acting DNA damaging compounds, and has more recently been attributed to PoDs as an active MOA. However, how is it that for particular alkylating agents, a linear response is often observed? A typical example would include the alkylating agents MMS, which has a PoD, and MNU which has been reported to have a linear dose response (10). It is highly unlikely that the DNA repair pathways elicited for MNU exposure could be overwhelmed at such low doses, and perhaps more realistic to surmise that the linear model itself may not be accurate in this case.

High Power Dose-Response Studies Reveal a Hormetic Response at Low Dose Concentration

Questions about the shape of dose-response curves at low doses for genotoxic effects, such as mutations or clastogenic events, have indeed been debated for decades. Calabrese and Baldwin (25) have long since argued for the existence of a hormetic dose-response for carcinogenic agents, which is an even more controver-

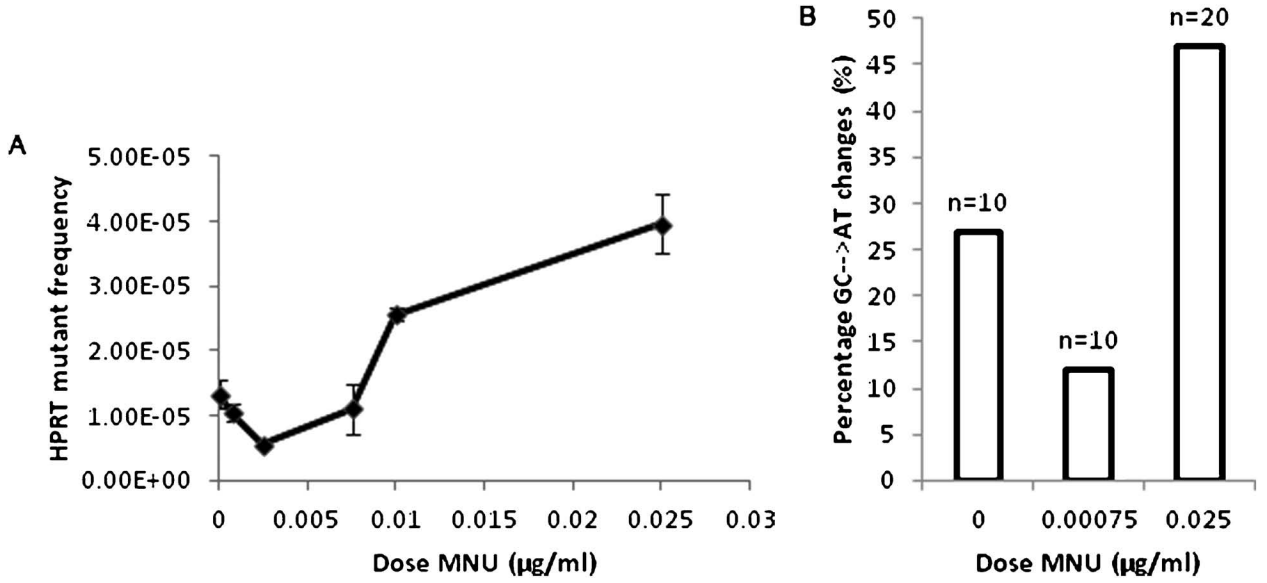

Fig. 2. Preliminary data showing frequency of HPRT mutants at increasing concentrations of MNU (A), and percentage GC > AT mutations at dose 0.00075 μg/ml MNU below the NOGEL, and 0.025 μg/ml which is above the LOGEL (B). Mean and standard deviations plotted for HPRT mutant frequency from duplicate experiments totalling $>66 \times 96$-well mutant plates per dose.

sial one than the threshold dose response. The advent of recent high power dose-response studies has made genotoxic assays more amenable to proving whether a biphasic hormetic response exists. Preliminary data from our group have shown a potential J-shaped hormetic response in AHH-1 cells exposed to low levels of MNU (Fig. 2a). MNU is a super-mutagen with a proportionally high level of O^6-methyl-guanine (O^6MeG) lesions in its adduct spectrum, compared to other alkylating agents (14). This potent mutagenic DNA adduct leads to GC→AT mutations when left unrepaired. In this instance, cells exposed to 0.00075 μg/mL MNU displayed a reduced number of AT mutations when compared to cells that were not exposed to MNU (Fig. 2b). This possibly links a GC→AT mutagenic MOA to the hormetic dose response, with O^6MeG and MGMT being potential candidates for this reduction in observed transition mutations. The authors predict that MGMT may show increased repair efficiency for some 'endogenous' O^6MeG lesions, formed after the mutant cleansing and treatment steps of the HPRT assay. We are not suggesting that fixed mutations are being repaired, and therefore predict that this may be a post-adduct, pre-mutation MOA, with further experimental work required to support this hypothesis.

In order to address regulatory requirements for a MOA for non-linear dose responses (11), we explored DNA repair. MPG was not involved with the threshold response observed for the mutagenic dose response for EMS (10,20,26). We thus focussed on the repair enzyme methyl-guanine methyltransferase (MGMT), which solely removes O^6MeG and prevents O^6MeG induced GC→AT transitions (18,19). Removal of methyl adducts from O^6guanine proceeds stoichiometrically where, the methyl adduct is abstracted to cysteine 145 within the active site of MGMT. This inactivates MGMT and initiates protein degradation via ubiquitin-dependent proteolysis (27). The repair kinetics for MNU are therefore very different to that for EMS, which employs a multi-step repair pathway comprised of several compensatory repair enzymes. Here we propose that MGMT is limited in its capacity, and therefore whilst overcompensation in the repair of O^6MeG may occur at low doses where MGMT production is abundant, a 'beneficial' response is observed. Doses above a given concentration may produce O^6MeG levels disproportionate to the turnover of MGMT protein, and thus a positive genotoxic response ensues. Indeed preliminary studies have shown that in cells where MGMT has been inactivated, an increase in mutations was observed at the NOGEL of 0.0075 seen in wild type AHH-1 cells (Fig. 3).

Similar findings were shown in the response of human keratinocytes to a low dose of the well-known methylating agent, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine

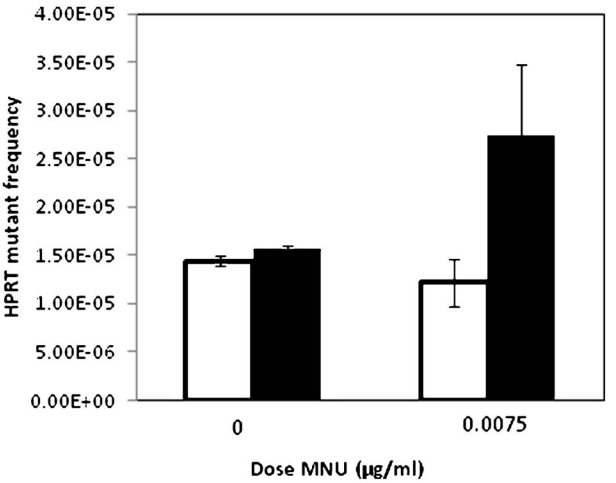

Fig. 3. HPRT mutant frequency following MNU treatment in AHH-1 cells. Wild type active MGMT (white) and O6-benzyl guanine inactivated MGMT cells (black) (28). MGMT is present in the AHH-1 cell line, shown at the protein and mRNA level (5). Data presented from duplicate experiments totalling >66×96-well mutant plates per dose.

(MNNG). It was found that at concentrations ranging from 0.05–50 nM MNNG unwinding and DNA strand breaks were significantly reduced, relative to high doses where they were significantly enhanced compared with the control case (29). Inhibition activity regarding DNA damage at the low doses was attributed to the activation of poly(ADP)-ribose (29). This possible commonality in low dose effects of an alkylating agents relationship to DNA repair could well point to DNA repair as a MOA for all of this class of compounds. However, this saturation of DNA repair at low doses may alter when adding 2 compounds with similar MOA, or more complex effects may be seen when adding compounds that are repaired or coped with through different MOA.

Compound Mixtures and Their Impact on Non-linear Dose-response

When considering mixtures of genotoxic compounds, the dose responses and MOA have previously been considered and used to predict whether the compounds were response additive, dose additive or interactive (30). However, these have been based on high dose data and extrapolated down, whereas binary combinations in and around the low dose region (e.g., NOGEL and lowest observed genotoxic effect level (LOGEL)) have not been suitably assessed. For cells exposed to EMS and MMS, both previously shown to induce NOGELs *in vitro*, a typical dose additive response was observed for micronuclei (Fig. 4a), along with a slight increase in toxicity and cytostasis (Fig. 4b). These findings were consistent with previous studies that looked at MMS and MNU mixtures in murine lymphoma cell lines (17). However, the effect at NOGEL + NOGEL is of particu-

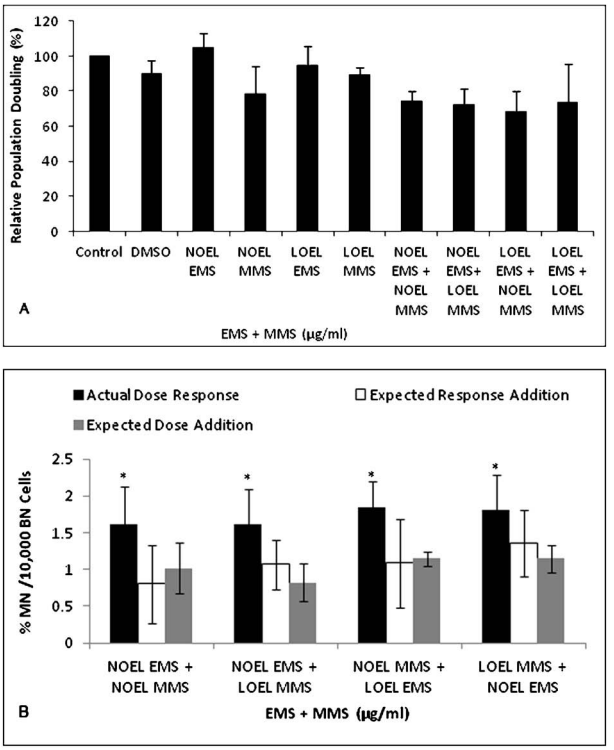

Fig. 4. Cytotoxicity and cytostasis measured by relative population doubling (A) and cytokinesis blocked micronuclei (B) in human lymphoblast cells TK6 cells, following treatment with EMS and MMS in binary mixtures. Mean control value of 0.535 %MN-BN subtracted from each response. Expected response addition and dose addition effects, calculated from single dose responses carried out in parallel, data not presented. Expected dose addition calculated from EMS dose response carried out in triplicate scoring 10,000 BN cells or more per dose. Mean and standard deviations are presented from triplicate data. %MN/BN are binucleated cells with one or more micronuclei scored from 10,000 BN cells, using the automated Metafer system (22). EMS NOEL (1.2 μg/ml), EMS LOEL (1.4 μg/ml) and MMS NOEL (0.4 μg/ml), MMS LOEL (0.52 μg/ml). *Dunnett's test compared to control values ($P < 0.05$).

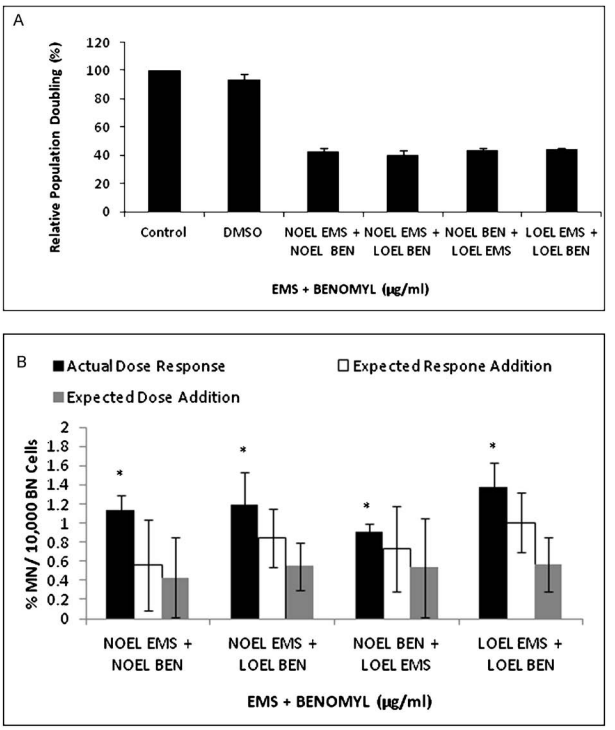

Fig. 5. Cytotoxicity and cytostasis measured by relative population doubling (A) and cytokinesis blocked micronuclei (B) in human lymphoblast cells TK6 cells, following treatment with EMS and Benomyl (BEN) in binary mixtures. Mean control value of 0.816 %MN-BN subtracted from each response. Expected response addition and dose addition effects, calculated from single dose responses carried out in parallel, data not presented. Expected dose addition calculated from EMS dose response carried out in triplicate scoring 10,000 BN cells or more per dose. Mean and standard deviations are presented from triplicate data. %MN/BN are bi-nucleated cells with one or more micronuclei scored from 10,000 BN cells, using theautomated Metafer system (22). EMS NOEL (1.2 μg/ml), EMS LOEL (1.4 μg/ml) and Benomyl NOEL (0.764 μg/ml), Benomyl LOEL (0.86 μg/ml). *Dunnett's test compared to control values ($P < 0.05$).

lar interest, as this explicitly shows that two genotoxic agents with similar MOA have a positive response when added together at their NOGELs. This has long been predicted for similar acting agents, but until now, not proven experimentally using suitable statistical power. Current guidelines state that this phenomena is not predicted (16) for dissimilar acting agents, where two agents combined at their NOGELs are assumed to have a non-significant response.

However, when EMS (clastogen and mutagen) was combined with the dissimilar acting genotoxic agent Benomyl (aneugen, mitotic spindle poison), a deviation in the additive dose response was produced (Fig. 5a) and a significant reduction in the number of surviving cells was observed (Fig. 5b). Benomyl is currently used as a fungicide. Its mechanism of toxicity has not been fully characterised, but generally mitotic spindle poisons act via mitotic arrest induced cell death or cytostasis, which both result in a decrease in 'relative population doubling' (RPD) (31). EMS induced adduct formation also causes cell death or cytostasis through DNA repair induction, abasic site formation, DNA strand breaks and halting of DNA replication. When Benomyl and EMS are added together, there is some kind of interactive effect which results in a synergistic increase in cell death and cytostasis. (Fig. 5a). The authors predict that both chemicals act on the cell cycle checkpoint/s with synergistic MOAs.

For genotoxicity, as measured through the *in vitro* cytokinesis block MN assay, the responses did not clearly deviate from response addition with standard deviations clearly overlapping, and were therefore in the envelope of additivity (17). Nevertheless, the 2× NOGELs unexpectedly produced a positive response, which is more in line with dose addition and not with the

expected result of response addition. This unexpected finding, goes against the most comprehensive guidance on mixtures to date, which was written by the Committee of Toxicity for the assessment of pesticides and residues. It states that dissimilar acting agents act via response addition, and it is the similar acting agents which act via dose addition (16). This difference highlights the importance of low dose mixtures studies. To explore this further, mechanistic experiments must be carried out, and this mini-review highlights our first steps in understanding the effects of binary mixtures in and around the NOGELs for genotoxicity. High statistical power was required to define these effects, and this was possible through use of the Metafer automated micronucleus assay (22). This gives statistical confidence to these findings, and accurately defines these small increases observed above the control values. As discussed previously, it also offers opportunities for MOA to be accurately defined alongside the micronucleus responses. Therefore high content low dose studies are of great importance when investigating genotoxic mixtures. An issue with this is that a high content gene mutation assay in mammalian cells *in vitro*, is not currently available with an OECD guideline. This issue has been recognised *in vivo*, where the MutaMouse studies used to define thresholds for EMS (6) were hugely expensive and certainly not common practise. This is where the *in vivo* phosphatidylinositol N-acetylglucosaminyltransferase subunit A (Pig-a) assay becomes very useful, and it has already shown very high power can be achieved for mutagenicity (8,32–37). Taking this into consideration, the *in vitro* Pig-a assay in human cell lines (38), should be explored further.

Conclusions

Low dose studies on model alkylating agents, provide a platform for using genetic toxicology data in a more quantitative fashion. To provide the biological relevance for these dose responses, MOA analysis can then be carried out using gene expression analysis and knockdown approaches for particular homeostatic pathways. Further care should be taken, to ensure that low dose effects of mixtures are also considered. We show that two × NOGEL of different compounds, even with different modes of action, are positive for MN. This potentially opens up a 'can of worms', where a lot of further experimental testing is required to define or support the current default assumption that dissimilar acting agents act via response addition.

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