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Genotoxic thresholds, DNA repair, and susceptibility in human populations

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ABSTRACT

It has been long assumed that DNA damage is induced in a linear manner with respect to the dose of a direct acting genotoxin. Thus, it is implied that direct acting genotoxic agents induce DNA damage at even the lowest of concentrations and that no "safe" dose range exists. The linear (non-threshold) paradigm has led to the one-hit model being developed. This "one hit" scenario can be interpreted such that a single DNA damaging event in a cell has the capability to induce a single point mutation in that cell which could (if positioned in a key growth controlling gene) lead to increased proliferation, leading ultimately to the formation of a tumour.

There are many groups (including our own) who, for a decade or more, have argued, that low dose exposures to direct acting genotoxins may be tolerated by cells through homeostatic mechanisms such as DNA repair. This argument stems from the existence of evolutionary adaptive mechanisms that allow organisms to adapt to low levels of exogenous sources of genotoxins. We have been particularly interested in the genotoxic effects of known mutagens at low dose exposures in human cells and have identified for the first time, in vitro genotoxic thresholds for several mutagenic alkylating agents (Doak et al., 2007). Our working hypothesis is that DNA repair is primarily responsible for these thresholded effects at low doses by removing low levels of DNA damage but becoming saturated at higher doses. We are currently assessing the roles of base excision repair (BER) and methylguanine-DNA methyltransferase (MGMT) for roles in the identified thresholds (Doak et al., 2008). This research area is currently important as it assesses whether "safe" exposure levels to mutagenic chemicals can exist and allows risk assessment using appropriate safety factors to define such exposure levels. Given human variation, the mechanistic basis for genotoxic thresholds (e.g. DNA repair) has to be well defined in order that susceptible individuals are considered.

In terms of industrial exposures to known mutagens, knowing the dose relationships and protective mechanisms involved, offers the possibility of screening workers for susceptibility to mutation through examining DNA repair gene polymorphisms. Hence, thresholds may exist for certain mutagens, but there will undoubtedly be human subpopulations who are more at risk from low dose exposures than others and who should not be exposed, if possible. By studying polymorphisms in DNA repair genes, susceptible individuals may be identified, and additional safety factors appropriately targeted to these populations.

1. Genetic toxicology

Genetic toxicology, which evolved in the latter half of the twentieth century, is involved in the study of DNA damage and mutation and its impact on human health. Genotoxicity describes many different DNA endpoints including DNA adduct formation, point mutation, chromosome breakage and chromosome copy number changes. From its inception, genotoxicity has been used as a surrogate for cancer, as genotoxins are almost always carcinogens and cancer has traditionally been seen as a genetic disease characterised by acquired DNA mutations in growth controlling genes. Consequently, assessment of the genotoxicity of new chemicals is seen as a key regulatory requirement to minimise any deleterious effects that may be produced through genotoxic exposures within human populations. As part of the safety assessment of new chemicals (pharmaceuticals, consumer products, etc.), a tiered approach to genotoxicity testing is currently recommended. This tiered approach is well described by the Committee on Mutagenicity (COM) guidance (Guidance on a Strategy for testing of chemicals for Mutagenicity, 2000) and involves all chemicals entering so-called stage 1 tests where DNA damage induction is assessed in cells cultured in the laboratory. Negative results in these tests reassure the manufacturer that DNA damage is unlikely to be induced

Abbreviations: MMS, methyl methanesulphonate; MNU, methyl nitrosourea; EMS, ethyl methanesulphonate; BER, base excision repair; MGMT, methyl guanine DNA methyl transferase.

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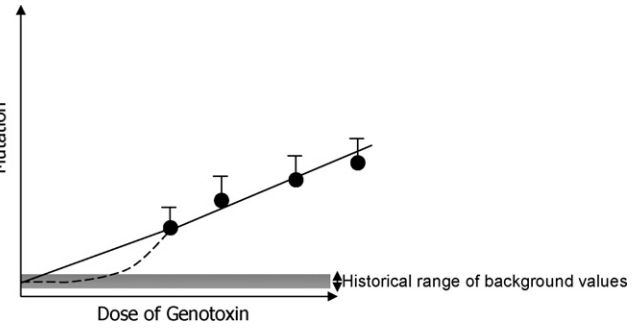


1. Dose–responses induced by genotoxic agents. Theoretical mutagenic e–responses for genotoxic agents are displayed. Linear response (solid line) plies no safe low dose. Note the line does not go through the origin, as background tation levels are detectable in vitro and in vivo. The shaded area represents the kground level (historial background ranges can define this region). Thresholded ponses are depicted by the dotted line in the low dose range. At the low dose ion no increase over background mutation level is seen, followed by a critical e range where mutagenic responses are observed. The boundary between no ect and effect is represented by the no observable effect level (NOEL) and the low- observable effect level (LOEL), and the threshold dose is statistically calculated ere the slope of the graph first increases significantly.

that agent. Stage 2 tests are employed for positives from stage 1 sts and for compounds with medium to high exposure potentials d are carried out in animals. The stage 2 tests are designed to ercome problems with false positive results which can occur in ge 1 tests and also to more rigorously assess the risks to human alth.

Linear dose–response relationships for genotoxins

In genetic toxicology a linear dose–response relationship has ng been assumed to apply for direct acting genotoxic agents enderson et al., 2000). Fig. 1 displays the different theoretical se–response relationships for genotoxins (linear and thresh- ded). In the linear model, DNA damage induction is believed to directly proportional to dose; leading to the implication that ere are no genotoxic doses, however low, devoid of a finite risk genetic damage and hence cancer. This linear model has been plemented partly because of early experimental evidence and rtly due to the precautionary principle. This linear concept has en controversial and has recently been challenged by ourselves d others, as it assumes a binary situation where chemicals are her genotoxic or not, but does not account for the effect of dose. pointed out by Paracelsus in the 16th Century, "only the dose rmits something not to be poisonous". In this context it is inter- ting to note that carcinogenesis has recently been shown to be duced in a non-linear manner with low doses of genotoxic agents ling to drive cancer formation in trout even when large numbers animals were examined (>40,000) (Bailey et al., 2009).

In the case of indirect genotoxins which have non-DNA tar- ts (aneuploidy inducing agents and agents interacting with NA modifying enzymes), thresholds have now been accepted hajouji et al., 1997; Lynch et al., 2003). Hence, this demon- rates the usefulness of solid experimental evidence in altering radigms. However, for direct acting genotoxins, linear models e still assumed to apply. Recently, the role of dose in mutagenicity sting in general has been a major issue in the field and inappro- iately high doses have been suggested to be responsible for many the false positive results in stage 1 tests (Kirkland et al., 2007). gh doses of chemicals have traditionally been used to ensure that NA damaging effects are identified in the available tests (due to st sensitivity constraints) and because it has widely been assumed at the effects are induced in a linear manner, this is then extrapo- ed back to the low dose region. Therefore, if a high dose is positive for genotoxicity, then under this linear paradigm, a low dose will also be positive. Hence, the implications emanating from the linear model for genotoxins can be wide reaching and can impact scientifically and economically on the availability and use of certain chemicals. As the linear model is currently being challenged, this paradigm is subject to change which may affect future regulatory testing and allow some previously unavailable chemicals to be licensed for use in the future.

3. Theoretical arguments against a linear response for genotoxins

The main argument against a linear dose–response for genotoxins is the presence of natural defences which have evolved to cope with our daily exposure to genotoxins. Humans are constantly exposed to genotoxic substances like cytosolic oxidative agents, dietary amines, inhaled hydrocarbons and many others. Low level exposures to these genotoxins have occurred throughout evolutionary time and have led to the development of efficient homeostatic defences to protect organisms against the deleterious mutagenic consequences. DNA repair is one such homeostatic defence mechanism that may impact on the consequences of genotoxin exposure. Indeed, even simple bacteria have intricate defences (like DNA repair) against genotoxins. As multicellular organisms, humans have several tiers of protection against DNA damage including, but not restricted to:

1. Epithelial barriers to genotoxin entry.
2. Detoxification processes leading to excretion of water soluble genotoxins.
3. Compartmentalisation of tissues leading to reduced access for genotoxins.
4. Cellular and nuclear membranes reducing access of genotoxins to the nucleus.
5. DNA repair to remove damaged DNA sequences.
6. DNA redundancy (<1% gives are thought to code for proteins).
7. Apoptosis/autophagy/anoikis to remove damaged cells.

Hence, it is theoretically difficult for genotoxins to cause DNA damage in a manner proportional only to dose. This is due in part to the failure of the genotoxin to readily access the DNA of a target tissue. Even in a simple cell culture system, it is unlikely that true linearity will be seen due to extracellular and intracellular interactions between the genotoxin and non-DNA biomolecules, as well as membrane-based exclusion. Furthermore, once in the nucleus, genotoxins must overcome the homeostatic protection afforded by DNA repair to produce permanent DNA sequence alterations.

DNA repair has over recent decades been shown to function in a complex and, in some cases, in an inducible manner to control the genetic stability of the host cell's genome. Several overlapping DNA repair pathways exist which are responsible for repairing specific DNA damage types (e.g. base excision repair, nucleotide excision repair, homologous recombination, mismatch repair, etc.). DNA repair has been well reviewed elsewhere (Hoeijmakers, 2009; Riches et al., 2008). Hence, it is likely that DNA repair will impact directly on the linearity of genotoxic dose–responses by removing DNA damage, particularly at low doses. At higher doses DNA repair may be saturated and hence not be able to remove newly damaged DNA bases. There is some evidence that DNA adduct formation accrues in a linear fashion (Perera, 1988; Zito, 2001), but it is likely that fixed mutations (point mutations, chromosome damage) will not. One complication with the DNA adduct data is that although the assays for their detection are very sensitive (10^{-8}), they do not define where in the genome the adducts are present. Given that cells have evolved efficient measures to keep gene cod-

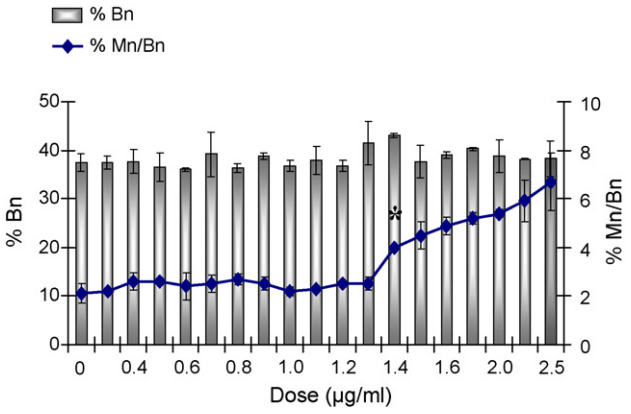


Fig. 2. Example of dose–response threshold for EMS induced micronucleus frequency (chromosome damage: line graph). Columns depict binucleate frequency as a measure of toxicity and clearly show no toxicity in this dose range. Here AHH1 cells were treated with increasing doses of EMS and especially large numbers of cells (up to 10,000 per dose) were assessed for micronuclei to increase the sensitivity of the assay and allow the dose–response to be truly examined. This data is taken from that published previously by ourselves (Doak et al., 2007). EMS clearly does not induce chromosome breakage (line graph) at low dose levels (<1.3 μg/ml), whereas at doses higher than this, chromosome damage is seen to increase. Simple statistical tests (one-way ANOVA with Dunnett's post hoc test) showed that 1.4 μg/ml was the first dose showing significant genotoxicity, but more complex statistical modelling has identified 1.06 μg/ml as the point of inflection in the dose–response and hence the threshold dose (Johnson et al., 2009).

ing sequences damage free at the expense of non-coding regions (Hanawalt, 1994), it is not possible to currently say if DNA adducts accrue in a linear fashion in the coding sequences which form the basis for most genotoxicity tests.

4. Experimental evidence for genotoxic thresholds

Attempts to define genotoxic thresholds have mainly centred on using direct acting DNA damaging genotoxins. This is in contrast to genotoxins requiring metabolic activation, due to the confounding effects of such activation, which may itself impose a non-linear dose–response dependent upon the efficiency of the metabolic enzyme (s) involved. If genotoxic thresholds are accepted for direct acting genotoxic agents, they will certainly exist for agents requiring metabolic activation, and the mechanism involved in the threshold region may additionally involve, for example, P450 enzyme efficiency. Our research group was the first to comprehensively demonstrate that direct acting genotoxins could exhibit thresholds for mutation induction and chromosome breakage in vitro (Doak et al., 2007). These data have led to a paradigm shift in genetic toxicology and has been subject to debate at the highest levels concerning how to modulate safety assessment procedures to account for potential genotoxic thresholds. It is of paramount importance that the data generated to investigate genotoxic thresholds should be appropriately analysed by extensive statistical modelling and that abundant high quality data is solely generated for this purpose. Furthermore, the mechanistic basis for any thresholded effects requires some thought (and some investigation) in order to provide a plausible explanation of the results.

We demonstrated that the well known genotoxic and carcinogenic alkylating agents EMS and MMS displayed genotoxic thresholds, whereby low dose exposures showed no increase in DNA damage above background levels evident in untreated cells (Fig. 2 shows the chromosome damage data for EMS in vitro as an example). A statistical modelling approach has recently confirmed the "hockey stick" shape to the curves for EMS and MMS and highlights the critical threshold doses (Johnson et al., 2009). Our data were accompanied by supporting DNA adduct data showing that DNA adduct formation increased linearly across the same dose range, thus confirming that adequate nuclear exposure was achieved (Swenberg et al., 2008). The identification of DNA adducts at doses where no mutations were found may suggest that DNA adducts were present in the genome but not perhaps in the locus used for mutational analysis (the hprt gene). Crucially, this in vitro threshold data has now been elegantly confirmed in vivo for EMS using the mouse bone-marrow micronucleus test and point mutation at the lacZ locus (Gocke et al., 2009). Furthermore, through comprehensive pharmacokinetic studies, a cross-species analysis has allowed extrapolation from the genotoxic threshold dose observed in the mice to a corresponding threshold in humans. Hence, safe exposure levels for EMS have been identified, providing a precedent for future safety assessment for genotoxic carcinogens with thresholded dose–response curves.

Interestingly, the mouse chromosome damage data showed a potential hormetic effect for micronucleus induction in vivo, suggesting homeostatic mechanisms were induced which also removed some endogenous DNA damage (Gocke et al., 2009). Hormetic effects have never before been observed in genetic toxicology and reinforce the concept that organisms can tolerate low levels of DNA damage. The acceptance of hormetic effects in the low dose region of genotoxin exposure may lead to a significant rethink for regulatory toxicology (Calabrese and Baldwin, 2003). Hormetic effects in genotoxicity further highlight the roles for inducible homeostatic mechanisms, like DNA repair in low dose exposure situations.

It is now possible to say with some confidence that genotoxic thresholds exist both in vitro and in vivo for some chemicals at least. How widespread these genotoxic thresholds will prove to be, can only be answered through further investigation. Therefore, genotoxic carcinogens like EMS and MMS can display dose ranges where there is no elevation of genetic damage above background levels and thus safe exposures can exist for these chemicals. This has important implications for regulating the exposure levels to genotoxic carcinogens. Whilst providing fascinating insight into how genotoxins interact with DNA at low levels, this data does not tell us how cells tolerate low levels of the alkylating agents. These fundamental mechanisms of action underlying genotoxic thresholds have been the focus of our attention for the past 2 years. We showed recently that the DNA repair protein O6-methylguanine-DNA methyltransferase (MGMT), which removes the O^6G alkyl lesion from DNA, was up-regulated by MMS at doses below the threshold, but not above it, suggesting that MGMT might be involved in the threshold by selectively removing the DNA adducts at low doses but becoming saturated (or repressed) at higher doses (Doak et al., 2008). Previous genotoxic studies in vitro have highlighted a potential role for MGMT in altering the shape of dose–responses. In both bacterial and mammalian cells, MGMT knockouts have been shown to alter the shape of mutational dose–responses to a more linear shape in contrast to the sublinear shapes in the wild type cells (Rebeck and Samson, 1991; Kaina et al., 1998; Sofuni et al., 2000). As the alkylating agents EMS and MMS also induce high levels of N7Guanine which is repaired by base excision repair (BER), the BER glycosylase N-methylpurine DNA glycosylase (MPG) is also likely to be involved in the genotoxic thresholds induced by MMS and EMS and is the focus of research in our group at present (see below).

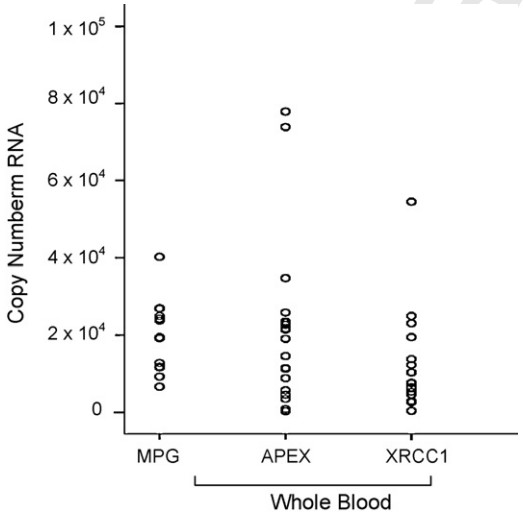
5. Human variation in DNA repair and thresholds

Given that DNA repair appears to be centrally involved in the existence of genotoxic thresholds, there is a potential for population level variation in DNA repair genes which may alter susceptibility to these genotoxins in the low dose region. Indeed, it has previously been suggested that defining a genotoxic thresh-

ble 1
e link between polymorphisms in DNA repair genes involved in correcting ylating agent-induced damage and cancer risk. To demonstrate the functional sig- cance of DNA repair gene polymorphisms, published repair gene polymorphisms ked to cancer susceptibility in molecular epidemiological studies are listed. The is by no means exhaustive, but merely reflects the link between DNA repair gene iation and cancer risk.

Gene	Polymorphism	Cancer type affected	Reference
XRCC1	−71 T/C	Lung cancer link	Vineis et al. (2009)
MGMT	I143V	Oesophageal adenocarci- noma	Doecke et al. (2008)
MGMT	K178R	Lung cancer	Crosbie et al. (2008)
MGMT	56 C/T	Colorectal cancer	Ogino et al. (2007)
MGMT	K178R	Lung cancer	Povey et al. (2007)
APEX	I64V	Lung cancer	Zienolddiny et al. (2006)
MPG	8603 C/T, 12235 G/A	Lung cancer	Rusin et al. (1999)

d for a population might be impossible, due to genetic variation nongst individuals (Lutz, 2000). There is certainly the possibility at individuals will exist in a population who are more sensitive a genotoxin due to possession of a DNA repair variant protein th lower than average efficiency. Table 1 summarises some of e polymorphisms known to be present in some of the DNA repair nes pertinent to alkylating agent-induced DNA damage. As can see from Table 1, possession of some of these alleles is linked increased risk to several cancer types, presumably due to lower NA repair capacity. It is however, fair to say, that some conflicting idence is present in the literature that requires some resolution. ssue specificity of DNA repair gene expression is also a compli- ting factor here. Some of the polymorphisms noted in DNA repair nes have high allele frequencies, e.g. the MGMT I143V (which is disequilibrium with K178R) is detected in up to 20–25% of some nnic groups (reviewed in Pegg et al., 2007). Our own research has entified inter-individual variation in the gene expression levels some genes involved in BER (Fig. 3) highlighting the heterogene- that is possible. Indeed in our studies (Fig. 3), there appeared



. 3. Gene expression levels for DNA repair genes (MPG, APEX, XRCC1) in blood en from healthy individuals. Expression levels (absolute mRNA levels, utilising a ned target for standard curve generation) show considerable variation (8–250- d) in the different individuals. Mpg shows least variation (8-fold), XRCC1 shows 5-fold variation, whilst APEX shows 250-fold variation in mRNA copy number. ne variation may be induced due to environmental exposures (smoking status,) as well as host genetics. Data taken from paper recently submitted (Zaïr et al., omitted for publication).

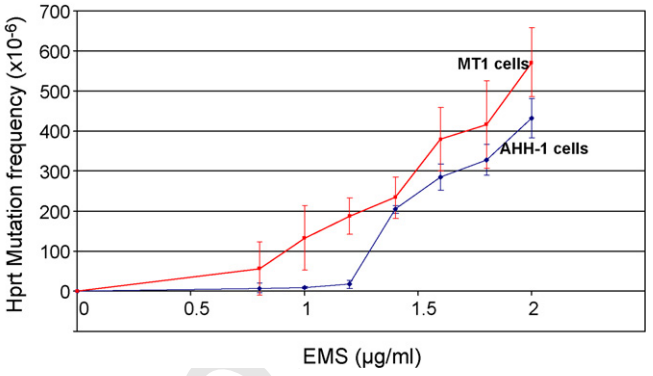


Fig. 4. Hprt dose–responses for AHH1 and MT1 cells exposed to EMS are compared. The dose–response for MT1 (repair deficient cells) is shifted to the left suggesting greater sensitivity to EMS. Indeed, the lowest observable effect level (LOEL) or the first dose giving a positive increase in mutation frequency is 1 μg/ml for MT1 cells compare to 1.4 μg/ml for AHH1 cells.

to be an 8–250-fold variation in repair gene expression in the individuals monitored, although some of this variation could be due to differences in environmental exposure and not due to genetic differences (Zaïr et al., submitted for publication). Similar differences in MGMT expression have also been noted, ranging from ∼8-fold in lymphocytes to over a 100-fold in lung tissue (reviewed in Kaina et al., 2007; Povey et al., 2007, respectively).

In extreme cases, heritable DNA repair deficiencies occur and can be linked to syndromes which include a greater propensity to certain cancer types (e.g. Xeroderma Pigmentosum and skin cancer) as well as neurological and developmental abnormalities (Cockaynes syndrome) and in some cases accelerated aging (progeria). However, alleles leading to more subtle variations in repair efficiency (low penetrance), whilst being much more widespread are likely to have a lesser impact on susceptibility. Indeed, it is often the combination of alleles in a family of genes which will influence overall susceptibility. Unpicking the haplotypes (combinations) of DNA repair gene polymorphisms which influence susceptibility to mutation and cancer is likely to be a complex process. An example of a gene defect in DNA repair of higher penetrance is seen in Hereditary Non-Polyposis Colorectal Cancer (HNPCC). HNPCC patients inherit defects in one of 5 mismatch repair genes (MLH1, MSH2, MSH6, PMS1, PMS2) and have increased risks of colorectal cancer in particular, which are characterised by microsatellite instability, due to lack of MMR (Jascur and Boland, 2006). HNPCC is responsible for ∼5% of colorectal cancers. Theoretically, if MMR were found to be the key mediator of a threshold response to a genotoxin and safe exposure levels in humans were calculated based on a threshold dose in proficient models, then patients with HNPCC would be more susceptible to mutation and perhaps cancer, whilst the general population would be tolerant of exposure. In short, the threshold dose calculated in this example would not necessarily apply for these HNPCC individuals. The importance of DNA repair proficiency in genotoxic thresholds is illustrated in Fig. 4. Fig. 4 displays EMS induced hprt mutations in a repair deficient cell line (MT1) compared to the AHH1 cell line used in our previous studies (both being lymphoblastoid cell lines). MT1 cells are known to be mismatch repair deficient, through loss of MSH6 (Szadkowski et al., 2005) and hence may be more susceptible to alkylating agent-induced mutagenesis. Clearly, the dose–response for the repair deficient cells is different to that of the DNA repair proficient cells and might suggest the impact that DNA repair proficiency has on genotoxic dose–responses in populations.

Of course, the fact that MMR (and indeed other repair processes) can unwittingly facilitate chromosome damage induction (Armstrong and Galloway, 1997) complicates this view as efficient

MMR can drive chromosome damage and may represent a double edged sword in mutagenesis. Moreover, due to the overlapping specificity of the different DNA repair processes, other functional counterpart pathways may well compensate the reduced efficiency of one pathway. Therefore, failure of more than one pathway may be necessary to radically alter any population level threshold dose. Repair haplotypes may better define susceptible individuals, allowing a better understanding of risk assessment in terms of exposure to genotoxic carcinogens. Furthermore, whilst it is accepted that MGMT and BER are centrally involved in the repair of alkylating agent-induced DNA damage, nucleotide excision repair (NER) can also contribute to this repair effort as shown by increased alkylation sensitivity in NER deficient cells (Op het Veld et al., 1997). This is particularly true for larger alkyl groups (ethyl, isopropyl) compared to methyl groups (Kaina et al., 2007). In the context of DNA repair heterogeneity and DNA repair deficiencies, it is interesting to note that inherited deficiencies of BER are rare, with BER deficient embryos often being non-viable (Hasty et al., 2003). Hence, this shows the primary importance of some DNA repair pathways to normal development. Therefore whilst genotoxic thresholds based on BER processes may not have to contend with grossly susceptible subpopulations, as gene deficiency is likely to be rare, subtle variations (polymorphisms) can still modulate BER efficiency in individuals (as shown in Table 1).

Certainly, safety factors used in risk assessment need to take account of genetic variation in DNA repair genes (currently included for inter-individual variation in general), particularly when risk assessing a genotoxin with a thresholded dose–response. Mechanistic studies to better understand the biological basis for genotoxic thresholds are essential as they highlight the key protective factors (like DNA repair) underlying the thresholds. In fact, before suggesting safe exposure limits to known genotoxins (thresholded or not), adequate characterisation of any protective mechanisms should be undertaken in order to inform the risk assessments necessary. The identification of these protective processes and the genes involved, can then lead to the search for susceptible groups before setting safe exposure levels.

6. Conclusion

In conclusion, genotoxic thresholds have been demonstrated for two alkylating agents and are likely to be found for further chemicals in the coming years. DNA repair plays a major role in these threshold responses by removing the DNA damage/mutation induced at low levels. This DNA repair is often inducible at these low doses and may be responsible for hormetic effects observed for EMS in vivo. As DNA repair gene polymorphisms exist and modulate an individual's repair proficiency, it is important to consider these polymorphisms (or combinations of polymorphisms) when calculating safety factors for safe exposure levels.

Conflicts of interest

The authors have no conflicts of interests.

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