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Dose-Response Relationship of Temozolomide, determined by the *Pig-a*, Comet and Micronucleus assay

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Abstract

Temozolomide (TMZ), a monofunctional alkylating agent was selected as a model compound to determine its quantitative genotoxic dose-response relationship in different tissues (blood, liver, jejunum) and endpoints (*Pig-a* -, comet- and micronucleus assay (MNT)) in male rats. TMZ was administered p.o. over 5 consecutive days (day 1-5), followed by a treatment free period of 50 days (day 6-56) and a final administration prior to necropsy (day 57-59).

TMZ showed a dose-dependent increase in DNA damage in all interrogated endpoints. A statistically significant increase in *Pig-a* mutant phenotypes was observed on day 44 starting at 3.75 mg/kg/d for mutant reticulocytes (for RET^{CD59-}) and at 15 mg/kg/d for mutant red blood cells (RBC^{CD59-}), respectively. In addition, a statistically significant increase in cytogenetic damage, as measured by micronucleated reticulocytes, was observed starting at 3.75 mg/kg/d on day 3 and 1.5 mg/kg/d on day 59. DNA strand breaks, as detected by the comet assay, showed a dose dependent and statistically significant increase in liver, blood and jejunum starting at doses of 3.75, 3.75 and 7.5 mg/kg/d, respectively.

The dose-response relationships of the *Pig-a*, MNT and comet data were analyzed for possible points of departure (PoD) using the benchmark-dose (BMD) software PROAST with different critical effect sizes (CES) ($BMD_{0.1}$, $BMD_{0.5}$, BMD_1; BMD_{1SD}). Overall, PoD values show a high concordance between different tissues and endpoints, underlining the suitability of this experimental design to explore quantitative dose response relationships in a variety of different tissues and endpoints, while minimizing animal use.

Keywords: temozolomide, Pig-a, dose-response relationship, multiendpoint

Introduction

Temozolomide (TMZ) is a monofunctional alkylating agent widely used in various cancer therapies. Its cytotoxic mechanism is mainly due to the methylation of O^6 guanine, but also of N7-guanine and N3-adenine (Zhang et al. 2012), leading to various types of DNA lesions, such as mutations (mainly point mutations) as shown in bacteria and in rats (Bodell et al. 2003; Geiger et al. 2006) as well as chromosomal aberrations in vitro (Vernole et al. 2003). Therefore, TMZ was chosen as a model monofunctional alkylating agent to determine its quantitative genotoxic dose-response relationship in different tissues and endpoints in vivo, and to expand on the dose-response data for this compound class that is predominantly represented by ethyl- and methyl methanesulphonate (EMS, MMS) and their corresponding nitrosoureas, summarized by Guérard et al. (Guérard et al. 2015). In recent years, several working groups and consortia acknowledged the need for quantitative data and recommendations on different approaches and the integration of such information to minimize the genotoxic risk have been made (Gollapudi et al. 2013; Guérard et al. 2015; MacGregor et al. 2015b). Recently, the IWGT Working Group on Quantitative Approaches to Genetic Toxicology Risk Assessment (QWG) recommended the use of benchmark dose (BMD) over no observed genotoxic effect level (NOGEL) for quantitative genotoxic risk assessment. In this context, the QWG also highlighted the importance of the selection of endpoints and tissues in vivo, considering metabolism, exposure and genotoxic mode of action (MacGregor et al. 2015a)

Overall, the experimental design of this study was chosen in alignment with previously performed studies (Zeller et al. 2015). In brief, male Wistar rats were treated with TMZ over five consecutive days, followed by a treatment-free period, then a second treatment over three consecutive days, prior to tissue sampling (Figure 1). This experimental scheme was chosen to allow the combination of several endpoints determining DNA damage within one experiment: *Pig-a* assay, Micronucleus test (MNT) and Comet Assay. We chose six closely spaced dose levels to study the dose-response relationship of respective endpoints and to

determine points of departure (PoD). The combination of the different endpoints within one experiment is in line with recommendations of 3-R (replace, reduce and refine animal experiments) and further allows a more precise comparison of different DNA lesions in different tissues not only between individual animals but even within the same animal. Due to the known toxicity of TMZ, the treatment was limited to five days and the top dose of 15 mg/kg/d.

The dose-response relationships of the *Pig-a*, MNT and comet data were analyzed for possible PoDs using the benchmark-dose (BMD) software PROAST. The choice of critical effect size (CES) is crucial for the results obtained through PROAST. Several ways of deriving CES, such as CES 0.1, 0.5 and 1 (corresponding to 10, 50 and 100% increase over control) as well as CES 1SD (i.e. an increase of one standard deviation of the concurrent controls) were used. In addition, the No Observed Genotoxic Effect level (NOGEL), expressed as the highest not statistically significant dose level was evaluated and compared to PROAST results.

Material and methods

Chemicals and dose volumes

Temozolomide (TMZ, CAS 85622-93-1) was purchased from AK Scientific with a purity of 99.4% and HPMC (0.18 % methylparaben, 0.02% propylparaben, 1.25 % hydroxypropylmethylcellulose, 0.10% docusate sodium) was used as a vehicle with fresh formulation preparations on each administration day.

TMZ were administered via oral gavage at a volume of 5 ml/kg body weight, based on most recent recorded body weight. Body weights were determined daily during the treatment period and once weekly during the treatment-free time.

Animal husbandry, treatment and dose-levels

Eight week old Male Wistar rats (stock RccHan:WIST (SPF)) were purchased from Harlan, Netherlands and were acclimatized for approximately one week. The study was performed in conformity with the Swiss Animal Welfare Law (Swiss Animal Welfare Law (Tierschutzgesetz) (Tierschutzgesetz 2005) and in accordance with Roche-internal SOPs and guidelines for care and use of laboratory animals.

Two males per cage had *ad libitum* access to pelleted standard rodent diet and tap water from the domestic supply. Animals were kept in an air-conditioned animal room under periodic bacteriological control, at 22°C ± 2°C with monitored 40% - 80% humidity, a 12-hour light/dark cycle and background radio sound coordinated with light hours. They were assigned randomly and identified by cage card, color code for group and individually by marking the head, back or tail.

As shown in Figure 1, TMZ was administered once per day to 6 animals per dose group at dose levels of 0.375, 0.75, 1.5, 3.75, 7.5 and 15 mg/kg/d over 5 consecutive days, followed by a treatment-free period of 50 days (day 6 to 56). The dose levels were chosen based on a previously performed dose range finding experiment, where a single animal was given a dose

of 30 mg/kg/d over 5 consecutive days, followed by a treatment-free period of 10 days. At this dose, no clinical signs of toxicity were observed. On day 14 after the first administration, blood samples analyzed for the induction of *Pig-a* mutant phenotypes revealed a strong response, both in red blood cells and reticulocytes (data not shown). Thus, it was decided to limit the high dose for the main experiment to 15 mg/kg/d and the administration period to 5 consecutive days.

For the main study, blood for the *Pig-a* assay was sampled before (day -5) and after (day 29, 44) the administration period. Furthermore, comet assay and micronuclei induction was assessed in peripheral blood on day 3 (blood sampling around 1h post-dose). For an additional determination of micronuclei as well as comet assay in liver and jejunum, TMZ was administered 48, 24 and 1h (day 57-59) prior to necropsy on day 59. The 1h time point corresponds approximately to the Cmax observed in rat following oral administration of TMZ (Reyderman et al. 2004).

Tissue collection

During the study, blood was sampled sublingually into EDTA tubes from rats under light isoflurane anesthesia. During necropsy, blood was taken directly from the heart, following asphyxiation with CO_2. For the micronucleus test approximately 50 µl of blood were diluted with anticoagulant (1:7) from which 100 µl were immediately transferred into ultra-cold methanol, mixed and stored at approximately -70°C until further processing for FACS analysis. For the analysis of *Pig-a* mutation, approximately 80 µl of blood were diluted with 100 µl anticoagulant and immediately processed according to instructions of the Prototype Stage III MutaFlow® kit. Blood (approximately 50 µl) for the comet assay was collected in K-EDTA tubes and directly diluted (1:1) with 5 mg/ml Heparin, immediately embedded into agarose and further processed for the assay.

Liver and jejunum for comet assay were sampled during necropsy in Hank's Balanced Salt Solution (1xHBSS), containing 25 mM EDTA and 10 % DMSO, and stored on ice until a single cell suspension was prepared (mincing with a pair of tweezers).

For histopathological evaluation, the left lateral lobe of the liver, as well as samples from jejunum (longitudinal and transversal sections), bone marrow (femur), thymus and testes were collected and fixed in 10 % neutral buffered formalin for approximately 24 h. The samples were then embedded in paraffin, cut at 4 µm and stained with haematoxylin and eosin.

Pig-a assay (blood, flow cytometry)

As described previously (Zeller et al. 2015) and in accordance with Rat MutaFlow kit instructions (Litron 2009), *Pig-a* analyses were performed on blood samples collected on days −5, 29 and 44. 'Precolumn' samples consisted of a small aliquot of each fully labelled and stained sample that was analyzed for approximately 1 min in order to provide % RET measurements as well as RBC to counting bead and RET to counting bead ratios. The majority of each sample was then used for an immunomagnetic separation procedure that utilized Miltenyi MACS LS columns and anti-PE Microbeads. The resulting 'post-column' eluates were analyzed for approximately 3 min to provide mutant phenotype RBC to counting bead and mutant phenotype RET to counting bead ratios. As described previously, pre- and post-column data were used to calculate mutant erythrocyte (RBC^{CD59-}) and mutant reticulocyte (RET^{CD59-}) frequencies. For the current study, this typically provided for the evaluation of $>2 \times 10^6$ RET and approximately 150×10^6 RBC equivalents per rat per time point. An Instrument Calibration Standard was generated on each day of data acquisition. As approximately one half of these erythrocytes were not incubated with anti-CD59-PE, these samples contained a high prevalence of mutant-mimic cells and provided a means to define the location of GPI anchor-deficient erythrocytes. A Becton-Dickinson FACSCanto II flow

cytometer running Diva 6.1.2 software (BD Biosciences, San Jose, CA, USA) was used for Pig-a data acquisition and analysis.

The baseline levels (day -5) of each individual animal were subtracted (on the logarithmic scale) from the respective number of mutant RBCs and RETs at study days 29 and 44. Baseline corrected values (log fold changes) were then modelled as a function of TMZ dose by linear models. The model assumptions were verified and statistical comparisons of interests (all study doses versus vehicle control) were done via one degree of freedom contrasts with Dunnett multiple comparison correction. Analyses were done with PROC MIXED in SAS v9.2.

Micronucleus test (blood)

For the micronucleus assay in peripheral blood, the test methodology was in accordance with requirements of the OECD Guideline 474 (OECD 474 2014) and current literature (Hayashi et al. 1994; MacGregor et al. 1987; Mavournin et al. 1990).

The Rat MicroFlow® Plus kit from Litron Laboratories, Rochester, NY, USA was used to perform the micronucleus test in peripheral blood *via* flow cytometry. After at least one week following the sampling of blood specimens in ultra-cold methanol, cells were washed with PBS buffer, followed by centrifugation at 400 × g for 10 min. After removal of the supernatant, cells were resuspended and labeled with fluorescent antibodies against CD71 and platelets. RNA was degraded enzymatically; DNA was stained with propidium iodide. Flow cytometric measurement was performed on a Becton-Dickinson FACS Canto II flow cytometer using FACS Diva software. A target of 20,000 reticulocytes (RETs) was interrogated for the presence of micronuclei. Results are given as percentage of RETs containing micronuclei (MN-RET). All steps were performed on kit-supplied negative-, positive- and calibration-controls (i.e., calibration *via* malaria-infected rodent blood samples).

The number of micronucleated cells showed substantial over-dispersion both on day 3 and 59. Micronuclei readouts were thus analyzed by generalized linear models, where the

number of MN cells was assumed to follow a negative binomial distribution with an offset equal to the log of the total number of cells evaluated. Risk ratios of MN cells at each dose with respect to vehicle were obtained as a measure of risk of genetic damage. Statistical analyses were implemented in R v3.02.

Comet assay

The standard protocol for sampling of liver tissues, preparation of a single cell suspension and slides, followed by DNA unwinding, electrophoresis and staining of slides being used follows recommendation given by several expert committees (Burlinson et al. 2007; Hartmann et al. 2003; JaCVAM January 14, 2013; Tice et al. 2000). Isolated cells were embedded into agarose on a slide, lysed overnight, followed by alkaline unwinding for 20 minutes. Electrophoresis was performed at approximately 26 V (corresponding to 1 V/cm), 300 mA for 40 minutes at approximately 4°C. Coded slides were analyzed by Metafer/Relosys 4 (Metasystems, Germany). A total of 150 comets per slide were measured. After rejections of artefacts, the median of the first 100 cells per slide was calculated. Two slides per animal and tissue were analyzed. The median value of each replicate was calculated for each animal and from these two slides the mean value of the dose group was calculated. The number of 'clouds' or hedgehogs (a morphology indicative of highly damaged cells often associated with severe cytotoxicity, necrosis or apoptosis (Burlinson et al. 2007)) out of 100 cells was scored manually for each replicate. The statistical analysis of the Comet data was performed by transforming individual tail intensity values using the arcsine of the square root, and based on this, the median was calculated per slide, followed by calculating the mean per animal across replicates (Zeller et al. 2015). Linear mixed-effects models in combination with contrasts were used to test treated versus vehicle control groups. In addition, the treatments were compared with the negative control using the Dunnett's test. The analysis was performed in a PipelinePilot-R (v2.10.1) implementation with the package 'nlme' (non-linear mixed effects model).

Determination of points of departure (PoD)

The terms BMD, BMDL and BMR should – in a strict sense – only be used in conjunction with the US EPA's benchmark-dose software while CED, CEDL and CES should be used in conjunction with RIVM's PROAST software. For the present manuscript we used BMD and BMDL despite calculations being performed in PROAST, but keep "CES" because "BMR" is often used in the context of standard deviation of controls.

Various means of deriving PoD were used, such as BMDL (lower boundary of BMD), which is gaining support as the preferred PoD metric (Johnson et al. 2014; MacGregor et al. 2015b). For the dataset with TMZ, PoD values using critical effect sizes (CES) of 0.1, 0.5 and 1; 1SD) were calculated by using PROAST (v38.9, described in more detail in (Slob and Setzer 2014)). The CES is used as a small effect size, in order to define a lower bound (CEDL) for use as a PoD, and an upper bound (CEDU) where the ratio of CEDL:CEDU is a measure of precision of the CED estimate. If data points with value zero occurred, a constant of 0.01 was added to all values to enable log transformation. Covariates were not taken into account for the PROAST analysis. Further, the No Observed Genotoxic Effect level (NOGEL), expressed as the highest non-statistically significant dose level was determined.

Tolerance intervals as well as the 95 % percentiles of historical negative controls were determined using available data from our laboratory, including: 254 specimens for the *Pig-a* assay; 89 specimens for the micronucleus assay; 151 specimens for the liver -, 63 for the blood- and 123 for the jejunum- comet assay. The functions 'nptol.int' or 'normtol.int', from the R package 'tolerance' were used to estimate one-sided tolerance limits, where 95 % of the historical negative control population lies with a confidence of 95 % (Hahn-Meeker or Howe's method respectively). Although the Shapiro-Wilk test rejected the normality assumption for the micronucleus assay in peripheral blood, a residuals inspection showed that historical negative control residuals were highly symmetric and in strong agreement with

the theoretical normal quantiles (qq-plot) despite the presence of only a few outliers. We thus estimated Normal Tolerance Intervals for micronucleus assay historical control data.

Results

TMZ was administered p.o. at dose levels of 0.375 to15 mg/kg/d over 5 consecutive days, which was generally well tolerated without any clinical signs of toxicity. The highest dose was chosen based on a previously performed dose range finding experiment that revealed a strong response for the induction of mutant phenotype in RBC and RET, following p.o. administration of TMZ at 30 mg/kg/d over the same duration (data not shown) .

Animals treated with 0-3.75 mg/kg/d showed a slight increase in body weight gain, while animals treated with 7.5-15 mg/kg/d showed no increase in body weight gain over the five-days of treatment period.

Histopathological evaluation of the vehicle control and the two highest dose levels (7.5 and 15 mg/kg/d) was done from several highly proliferating tissues (liver, jejunum, bone marrow, thymus and testes) of three animals per group. In none of the tissues and dose levels, a compound related effect was seen (see Suppl. info). Therefore, the observed genotoxic effects described in the following are judged to be not impacted by cytotoxicity.

Pig-a assay

The percentage of reticulocytes (%RET) as well as the frequencies of *Pig-a* mutant phenotype cells (i.e. RBC^{CD59-} and RET $^{CD59-}$) were evaluated before (day -5) and after (day 29 and 44) the administration of TMZ. A decrease in the proportion of %RET was observed over time in animals treated with the vehicle control, and similarly in all animals treated with TMZ (Figure 3). This is in line with previous observations (Zeller et al. 2015), and is related to the ageing process of the animals. Overall, no compound related effect on the %RET was observed.

Frequencies of RBC^{CD59-} and RET $^{CD59-}$ determined before the treatment (day -5) were within the 95 % percentile and tolerance interval of historical solvent controls. The same is true for all control animals sampled on study day 44. Three control animals sampled on day 29

showed RBC^{CD59-} and RET $^{CD59-}$ frequencies that exceeded the 95% tolerance interval of historical solvent controls. Following treatment with TMZ, two sampling timepoints were used. On day 29, no relevant increase of mutant frequencies was observed (no statistical significance, marginally exceeding the reference range of historical solvent controls). On day 44, a statistically significant increase in the frequency of RBC^{CD59-} was observed at ≥ 3.75 mg/kg/d and for RET^{CD59-} at ≥ 7.5 mg/kg/d, respectively (i.e. $NOGEL_{RBC}$: 1.5 mg/kg/d; $NOGEL_{RET}$: 3.75 mg/kg/d, Figure 3). The effect on RBC^{CD59-} was dose-related, with a low variability of mutant phenotype frequencies for the vehicle control groups of both cell types (Figure 3). Most of animals showed RBC^{CD59-} mutant frequencies exceeding the historical control reference range already at 3.75 mg/kg/d. Overall, a considerably higher variability for both RBC^{CD59-} and RET $^{CD59-}$ was observed within the vehicle controls on day 29 as compared to day -5 and day 44.

For RET^{CD59-} a rather large inter-animal heterogeneity was observed on day 44 with increasing dose: for instance at the highest dose level of 15 mg/kg/d three out of six animals showed a strong response, (i.e. RET^{CD59-} > 7.5 10^{-6}), while for the other three animals RET^{CD59-} frequency was well within the reference intervals and comparable to the concurrent negative control.

Micronucleus test and comet assay

The determination of micronuclei and DNA strand breaks (comet assay) in peripheral blood was performed on day 3 specimens and additionally on day 59. At the latter time point, DNA strand breaks were also determined in the liver and jejunum. Therefore, animals were exposed approximately 48, 24 and 1 h prior to necropsy. A comparable dose-dependent increase in the frequency % MN-RETs was observed at both time points. For instance at 0 and 15 mg/kg/d, the median %MN-RET were 0.08 % and 0.61 % on day 3, respectively and 0.05 % and 0.53 % on day 59 respectively (Figure 4). At the highest dose a lower proportion of reticulocytes was observed at both time points. On day 3, an increase of MN-RET

threefold higher as compared to the concurrent vehicle control was found at 3.75 mg/kg/d of TMZ ($p < 0.001$). On day 59 a highly significant risk ratio of MN-RET (2 fold relative to vehicle control) was found already with 1.5 mg/kg/d ($p = 0.0018$)). The frequency of MN-RET was within the 95% percentile historical reference up to and including 1.5 mg/kg/d. Given the short life span of reticulocytes and our historical reference of the MN-RET we consider a biologically relevant increase for both time points at doses of 3.75 mg/kg/d and above and thus, the $NOGEL_{MN, day3, day57}$ is 1.5 mg/kg/d.

In the comet assay in blood, a dose dependent increase of %tail intensity was observed on day 3 of the study with a statistically significant increase at 7.5 mg/kg/d ($p= 0.001$) and higher (i.e. $NOGEL_{blood}$: 3.75 mg/kg/d ; Figure 5). Blood samples for comet assay that were taken during necropsy (day 59) had to be invalidated, since some values of the negative control group were substantially outside the laboratories' reference range. For jejunum and liver a significant and biological relevant increase of tail intensity was observed starting at 3.75 mg/kg/d ($p<0.001$) and 7.5 mg/kg/d ($p<0.001$), respectively (i.e. $NOGEL_{liver}$: 1.5 mg/kg/d; $NOGEL_{jejunum}$: 3.75 mg/kg/d). Generally, the observed levels at doses ≤ 3.75 mg/kg/d in liver, and ≤ 1.5 mg/kg/d for jejunum were around or just outside the reference range of historical controls. We therefore suggest that the small differences in NOGEL values between peripheral blood and jejunum versus liver are rather due to inter-individual differences than tissue specific effects. The number of hedgehog cells was generally low for all investigated tissues (Table 1).

Dose-response assessment

Individual data from the various endpoints and treatment days have been evaluated by the NOGEL approach, defined as the highest non-statistically significant treatment dose, as well as by the BMD approach using PROAST. To this end, four different CES values were chosen: 0.1, 0.5, 1 and 1SD reflecting increases of 10%, 50%, 100% and one SD over mean

concurrent solvent controls as critical effect sizes (Table 2). Individual plots of the modeling are shown as supplementary information (Link/Ref).

BMD and BMDL (the lower 90% confidence limit of BMD) with CES set at 0.1, 0.5 and 1 were always lower than the corresponding NOGELs in the Pig-a endpoint. In the MN and Comet endpoints, BMDs calculated with a CES of 1 were greater than the respective NOGELs. This was also the case for BMDLs calculated with a CES of 1 in the Comet with liver tissue, but not in the other endpoints. Since setting the CES values to 0.1, 0.5 or 1 is a rather arbitrary choice, the CES 1SD was also used to calculate PoDs. The BMD_{CES1SD} were typically much closer to the NOGEL than those obtained from arbitrarily chosen CES (approx. 1 – 3 fold lower than NOGEL), in the Comet endpoint in peripheral blood did the BMD_{CES1SD} even marginally exceed the NOGEL. For the $BMDL_{CES1SD}$ the situation is similar but they never exceeded the corresponding NOGELs (approx. 2 – 15 fold lower). The ratios of BMDU/BMDL are usually below approx. 10 with the exception of RBC^{CD59-} on day 44, RET^{CD59-} on day 29 (n.a. due to infinity/zero division problem) and %MN-RET on day 59 (partly), indicating lower BMD precision for these three endpoints.

Discussion

The low dose region of monofunctional alkylating agents has gained attention in recent years, and some agents like EMS, ENU and their methyl counterparts (MMS, MNU) have been extensively been studied for their genotoxic potential (Doak et al. 2007; Dobo et al. 2011; Gocke and Müller 2009; Guerard et al. 2015; Johnson et al. 2012; Zaïr et al. 2011). Like those four agents, TMZ acts by alkylating DNA, such as the highly mutagenic O6-Guanine. Nevertheless, each of those compounds differ in their specific pattern of alkylation and adduct pattern, depending on the chemical properties, which has been reviewed recently (Guerard et al. 2015).

In this study, TMZ has been evaluated for its genotoxic dose response relationship in different tissues (blood, liver jejunum) and different endpoints (*Pig-a* assay, comet assay and micronucleus test). It was administered over 5 consecutive days and blood was taken at several occasions during the study for the evaluation of mutagenicity in the *Pig-a* assay, as well as chromosome breaks using the micronucleus test. In addition, DNA strand breaks were assessed in several tissues using the comet assay: (1) blood, to compare to the *Pig-a* assay and MNT; (2) jejunum as a first-site of contact organ following p.o. administration of TMZ and (3) liver, as the major organ of metabolization.

A statistically significant and dose-related increase in DNA damage was observed for all interrogated endpoints and tissues. While for MNT and comet assay, rather consistent effects were observed for any given time point and dose, a higher degree of variability was noted in the Pig-a assay for some time points. On day 44, within a given dose group, some animals showed a strong response on RET^{CD59-} , while for others, values were only slightly above the concurrent vehicle control and well within the historical control range of the laboratory (groups treated with 1-15 mg/kg/d). On the same day, the effect in RBC^{CD59-} was much more uniform, and all animals of the 3.75 and 15 mg/kg/d dose group exhibited mutant phenotype frequencies considerably greater than the concurrent vehicle control while also

exceeding the historical control range, which aligns with earlier MMS experiment observations (Zeller et al. 2015). We suggest that three non-mutually exclusive factors likely contributed to the lower variation and higher resolving power of mutant RBCs compared to mutant RET: i) a higher number of RBCs is evaluated for the mutant phenotype relative to RETs (approximately 1.5×10^8 versus 2×10^6); ii) the shorter half-life of reticulocytes means RET^{CD59-} provide information about mutant frequency over a short period of time (hours to days), whereas mutant RBC represents a moving average that integrates mutational events over a longer duration, days to weeks; and iii) at late post-exposure time points induced RET^{CD59-} frequencies can be reduced as mutated erythroid precursor cells lose their self-renewal capacity. On day 29, for both RET^{CD59-} and RBC^{CD59-} populations, no dose group was significantly different from controls. This is possibly related to the relatively high variability in the control group of day 29, since both RET^{CD59-} and RBC^{CD59-} frequencies of the 15 mg/kd/d group are significantly different from pre-dosing samples (all animals) and both arithmetic mean and median of the highest dose groups (15 mg/kg/d) RBC^{CD59-} frequency exceed the 95% tolerance interval of the historical solvent control range. It is assumed that the stronger response on day 44 may be related to the mean life span (60 days) of the erythrocyte population (Derelanko 1987) and thus, the time for mutated hematopoetic cells to expand and cause a measurable effect in the Pig-a assay.

A review on how specific genotoxic mechanism of action, including those at play for monoalkylating agents, may impact the dose response relationship has been published recently (Guerard et al. 2015). The reported non-linear dose-response relationship was demonstrated for different monoalkylating agents, which most likely involves DNA repair processes such as via O-6-methylguanine-DNA methyltransferase and base excision repair (Christmann and Kaina 2013; Kaina et al. 2001; Zaïr et al. 2011). This has also been shown for TMZ, which along with EMS, MMS, ENU and MNU has been studied in *Salmonella typhimurium*, including strains with different complements of O6-alkylguanine-DNA-alkyltransferase genes ogt and ada (Tang et al. 2012). PoD values were either completely

abolished (EMS, MMS, ENU) or considerably lower (MNU, TMZ) in ogt- and ada-deficient bacteria as compared to the respective proficient bacterial strains. In wildtype (i.e. DNA proficient) bacteria, the determined PoD values were comparable between the sulphonates EMS, MMS and TMZ, but considerably lower for the nitrosourea compounds ENU, MNU.

Recently, in a study by Muto and colleagues,(Muto et al. 2016 (in press)) a dose-dependent and statistically significant increase in RBC^{CD59-} and RET^{CD59} following a single oral dose of 25-100 mg/kg of TMZ was demonstrated. The strongest effect in RBC^{CD59-} was observed on day 29 with a maximum response of approximately 300 x 10^{-6} cells vs. 1-2.3 x 10^{-6} in the control and for RET^{CD59-} on day 15 with a maximum response of approximately 600 x 10^{-6} cells vs. 1.5-4.8 x 10^{-6} in the control. The described effects were much higher as in the current study, especially considering that the highest cumulative dose of this study of 75 mg/kg (5x15 mg/kg/d) is roughly in the same range. This suggests, that TMZ-induced mutation, as detected by the *Pig-a* assay, is not additive, which may be due to the involvement of DNA repair processes as demonstrated by the above mentioned experiment in bacteria (Tang et al. 2012). The fact that TMZ-induced DNA lesions are not additive could further support the hypothesis why there is no increase in mutant frequencies on day 29. Only after additional 15 days of a treatment free period (day 44), allowing DNA damaged hemaopoetic cells to expand, the mutant frequencies starts to increase.

Similar observations, such as dose fractionation, resulting in a lower response compared to a single high dose administration, have also been made for EMS with the Muta™Mouse gene mutation endpoint (Gocke et al. 2009). Further, Muto et al. compared the kinetics and potencies of MNU and MMS, with those from TMZ for the induction of RBC^{CD59-} : at 25 and 50 mg/kg similar responses between the three compounds were observed, but a higher number of RBC^{CD59-} and RET^{CD59-} were induced at 100 mg/kg of TMZ, followed by MNU and MMS. This is in line with observations made in wild type (i.e. DNA repair proficient) bacteria (Tang et al. 2012). In the same study, PoD values of MMS were comparable to those of TMZ.

The different PoDs derived from the various tissues and endpoints of TMZ, are in the same range as for MMS, derived from a multi-endpoint study with MMS (Zeller et al. 2015), previously performed using a similar design in our laboratory.

Different PoD values, such as BMD at 0.1, 0.5, 1 and1SD over control, as well as the NOGEL have been determined. It is still a matter of debate, which CES values should be applied to best reflect the dose response relationship for DNA damage in each assay. The CES parameter in PROAST is often set to 5% (i.e. 0.05) or 10% (i.e. 0.1) or 1 SD increase over controls (AMU 2009; Gollapudi et al. 2013; Johnson et al. 2014), which is primarily based on recommendations originally suggested for the assessment of studies outside the area of genotoxicity. The CES has a major impact on the derived BMDL metric used for the PoD, and is therefore currently a matter of debate. The main point of debate is whether one approach is more suitable than others for genotoxicity endpoints, and if the validity of a 'one size fits all' approach for genotoxicity datasets is meaningful or whether endpoint specific CES are required. It is certainly questionable that an increase of 5 or 10 % over control (i.e. a BMD_{10}) is a useful parameter for all of the assays, considering the huge differences in maximum response, background level and variation, and other parameters. Based on the presented data, "arbitrary" low CES values (i.e. 5 or 10 % increases over average solvent controls) seem to be overly conservative for most genotoxicity endpoints. Therefore, endpoint-specific CES values were calculated as well. Continuing the lines of argumentation of Sand et al (Sand et al. 2011) and Edler (Edler 2014), a *critical* effect should be discernible from the background noise of a biological assay and take into account its statistical power. Deriving CES from the standard deviation of the controls (CES 1SD) inversely correlates this parameter to the variability within the study controls. Based on the previous analysis using MMS (Zeller et al. 2015), and characteristics of the TMZ data, CES parameters of 0.5 and 1 could be suitable for BMD calculations for MN, comet or *Pig-a* assay.

The study was designed in a way to allow a most accurate determination of the dose response relationship and respective PoD values of TMZ-induced DNA damage, by using six closely spaced dose levels and several endpoints. At the same time, this combination is in line with recommendations on 3R. The close concordance of the determined PoD for BMD 0.5 and 1 throughout the different tissues and endpoints is striking and allows a precise evaluation of the genotoxic potential of a compound. The comet assay is often referred to as an indicator assay that detects mainly primary DNA lesions that could still undergo DNA repair, in contrast to the micronucleus test or *Pig-a* assay that both determine heritable DNA damage. Nevertheless, we could demonstrate that the comet assay is a suitable model to determine PoD values for TMZ and most likely other monoalkylating agents. Overall, the advanced experimental and mathematical approaches used within this study, can considerably improve the derivation of PoDs, while supporting the goals of the 3R concept.

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Tables

Table 1: Proportion of hedgehog cells (n=6 animals per group; Mean±SD) evaluated in a total of 100 cells.

Dose (mg/kg/d)	Liver (%)	Jejunum (%)	Blood (%)
0	1.75 ±0.87	5.08±4.08	0.17±0.39
0.375	1.83 ±1.34	3.08±1.38	0.08±0.29
0.75	4.33±4.60	4.83±0.94	0.33±0.78
1.5	2.75±1.71	5.17±2.66	0.30±0.48
3.75	3.83± 3.04	3.00±1.41	0.17±0.39
7	4.40± 1.51*	9.70±5.42*	0.25±0.45
15	8.17±4.53	24.25±9.24	3.42±3.42

*n=5

Table 2 Points of departure (benchmark dose, NOGEL) determined for different endpoints and time points (r

liver, jejunum at 7.5 mg/kg/d: n=5).

dose (mg/kg/day)	Pig-a RBC^{CD59-} d29	Pig-a RBC^{CD59-} d44	Pig-a RET^{CD59-} d29	Pig-a RET^{CD59-} d44	% MN-RET d3	% MN-RET d59
CES1SD	0.84	0.84	1.54	1.54	0.38	0.61
1SD BMD	10.3	0.55	14.30	6.14	1.15	0.46
1SD BMDL - BMDU	7.5 - 58.4	0.1 - 3.2	0 - ∞	3.8 - 10.4	0.6 - 2	0.1 - 1.3
BMDU/BMDL ratio at CES 1SD	7.7	32.5	-	2.7	3.5	9.6
0.1 BMD	7.60	0.04	10.9	4.54	0.46	0.06
0.1 BMDL - BMDU	1.9 - 13.0	0 - 0.8	0 - ∞	1.4 - 6.3	0.15 - 1.1	0.01 - 0.4
BMDU/BMDL ratio at CES 0.1	6.9	-	-	4.5	7.2	56.1
0.5 BMD	9.27	0.29	12.8	5.4	1.38	0.4
0.5 BMDL - BMDU	6.2 - 65.4	0.04 - 2.3	1.5 - ∞	1.5 - 7.5	0.7 - 2.3	0.1 - 1.1
BMDU/BMDL ratio at CES 0.5	10.6	57.8	-	5.0	3.0	11.5
1 BMD	10.8	0.68	13.70	5.81	2.26	0.81
1 BMDL - BMDU	7.9 - 60.4	0.13 - 3.6	4.3 - ∞	2.1 - 8.8	1.4 - 3.3	0.3 - 1.8
BMDU/BMDL ratio at CES 1	7.7	26.9	-	4.2	2.3	6.3
NOGEL	15	1.5	15	7.5	1.5	1.5
fold diff 1SD BMD vs NOGEL	1.5	2.7	1.0	1.2	1.3	3.3
fold diff 1SD BMDL vs NOGEL	2.0	15	-	2.0	2.5	16.0

*CES1SD reflects an increase of one standard deviation over the mean study controls

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