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GENOTOXICITY AND CARCINOGENICITY

A benchmark concentration-based strategy for evaluating the combined effects of genotoxic compounds in TK6 cells

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Abstract

Chemical risk assessment has historically focused on single compounds, neglecting the implications of combined exposures. To bridge this gap, several methodologies, such as concentration addition (CA) and independent action (IA), have been developed. However, a systematic, consistent, and integrated approach across various legislative frameworks is still lacking. The assessment of combined effects of genotoxicants is even more challenging, as genotoxicity data are typically evaluated qualitatively, without considering the effect size. This study aimed to develop a quantitative approach for evaluating the combined effects of genotoxic compounds with both similar and dissimilar modes of action (MoA), based on the benchmark concentration (BMC) principle. A proof-of-concept study was conducted using the in vitro micronucleus (MNvit) test to examine two types of binary mixtures: ethyl methanesulfonate (EMS) and methyl methanesulfonate (MMS), which share similar MoA, and MMS and etoposide (ETP), which have dissimilar MoA. The methodology involved collecting data for individual compounds, calculating BMC values, composing mixtures with different ratios and inducing various effect levels, testing these mixtures, and comparing the experimental results with the modelled data to verify additivity. The findings indicated that for both mixtures, the experimental responses aligned with the predicted additive effects, supporting the validity of the additivity principle. This study highlights the potential of an optimized BMC-based approach as a robust framework for testing chemical mixtures. It should be adopted in future studies to evaluate a wider range of genotoxic compounds, offering a more comprehensive and quantitative strategy for assessing combined chemical exposures.

Keywords In vitro micronucleus test · Mixtures · Genotoxicity · Benchmark dose approach · Principle of additivity

Introduction

Until recently, chemical risk assessment primarily focused on individual compounds, largely neglecting the combined exposure to multiple chemicals. However, in recent years, there has been growing recognition of the need to address the issue of chemical mixtures. This shift highlights the importance to evaluate the combined effects of co-occurring chemicals on human health, with particular attention to genotoxicity due to its critical role in the chemical risk assessment process (European Commission 2012). Different types of combined effects have been described, with synergistic, antagonistic and additive effects being the three principal categories (Lasch et al. 2020; Tralau et al. 2021). Past evaluations of mixture interactions, however, often relied on incorrect or incomplete assumptions, e.g. as (i) assuming that the summation of effects predicts additive expectations or (ii) comparing mixture effects solely with those of individual compounds, without considering null hypotheses about expected additive effects (Berenbaum 1985; Ermler et al. 2014).

Over time, various methodologies for assessing the combined effects of chemicals have been developed and applied by scientists and regulators to address these challenges. Central to these methodologies is the evaluation of whether the principle of additivity applies. According to this principle,

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the combined effect of multiple chemicals can be predicted based on the sum of their individual effects. Two recognized and widely employed models that exemplify this principle are concentration addition (CA) and independent action (IA) (Ermler et al. 2014). CA and IA utilize algorithms to translate effect concentrations or the effects of individual mixture components, respectively, into expected combined effects, thereby providing robust frameworks for evaluating chemical mixtures. Alongside CA and IA, the Chou-Talalay (CT) model has also gained increasing interest over the years, utilizing the median-effect equation to establish a common link between single and multiple entities (Chou 2010). It is important to note that, while the CT method is widely used for assessing mixture effects, it may overestimate these effects due to its focus on the steeper segments of dose–response curves, as discussed by Lasch et al. (Lasch et al. 2020).

However, a systematic, consistent, comprehensive and integrated approach across different legislative frameworks for evaluating the effects of chemical mixtures remains absent (Kienzler et al. 2016). In response to this gap, the European Food Safety Authority (EFSA) published in 2019 its Guidance Document on Harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals (EFSA 2019). This document outlines tiered and stepwise methodologies for both whole mixture approaches and component-based approaches, with EFSA expressing a preference for the latter, as testing every mixture individually is practically unfeasible. Additionally, the whole mixture approach would necessitate excessive animal testing and lacks scientific justification (Tralau et al. 2021). Consequently, they concluded that a key aspiration of mixture toxicology is to anticipate quantitatively the effects of mixtures of chemicals from knowledge about the toxicity of their individual components based on the CA assumption (EFSA 2019).

In the context of genotoxicity, data have traditionally been used in a qualitative way, resulting in a binary (yes/no) answer, complicating the evaluation of combined effects of genotoxicants. At present, a paradigm shift is ongoing, exploring whether genotoxicity data can be used more quantitatively (Menz et al. 2023; White et al. 2020). Significant advancements in this field have been achieved through the introduction of the benchmark dose/concentration (BMC/BMC) concept, hereafter referred to as the BMC approach, as in the present study, it was applied on data collected using an in vitro model. The BMC concept employs computational algorithms to fit mathematical functions to concentration–response data, subsequently interpolating the concentration corresponding to a predefined change above the background (Davis et al. 2011; EFSA 2017). So far, in the field of genotoxicity, the BMC concept has mainly been used for potency ranking (Haas et al. 2023; Wills et al. 2016). However, for non-genotoxic endpoints, BMC-based approaches have proven to be valuable for evaluating combined effects, consistently demonstrating that the principle of additivity generally applies (Alarcan et al. 2021; Karaca et al. 2021; Kienhuis et al. 2015; Staal et al. 2018; van Oostrom et al. 2020; Zoupa et al. 2020). In contrast, for genotoxicity, insufficient research has been done to determine whether the responses of mixtures containing genotoxic compounds can be accurately predicted based on data from individual compounds using the principle of additivity.

The aim of this research was to develop a quantitative approach for assessing the combined effects of genotoxic compounds with both similar and dissimilar modes of action (MoA) by extending the BMC-based strategy previously applied to non-genotoxic endpoints, as outlined by Kienhuis et al. (Kienhuis et al. 2015). To illustrate the practical application of this strategy, a proof of concept study was conducted using the in vitro micronucleus (MNvit) test, which is currently the genotoxicity assay with the most established expertise in quantitative data analysis. Two types of binary mixtures were tested: the first comprised ethyl methanesulfonate (EMS) and methyl methanesulfonate (MMS), both DNA-alkylating agents with similar MoAs; the second mixture contained MMS and etoposide (ETP), a topoisomerase II inhibitor, representing dissimilar MoAs. The strengths and limitations of this strategy are also discussed.

Materials and methods

Chemicals and cell culturing

EMS (CAS 62–50-0), MMS (CAS 66–27-3) and ETP (CAS 33419–42-0) were purchased from Merck Life Science (Belgium). Human lymphoblastoid cryopreserved TK6 cells (Cat. No. 95111735, Merck Life Science, Belgium) were cultivated (37 °C, 5% CO_2, 100% humidity) in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 1% gentamycin, 1% GlutaMAX, 1% sodium pyruvate, 1% non-essential amino acids (NEAA), and 0.1% amphotericin B (Life Technologies, Belgium).

In vitro micronucleus test

The MNvit test was conducted according to the Organisation for Economic Co-operation and Development (OECD) Test No. 487, with some minor modifications (OECD 2023). Two milliliters of TK6 cell suspension with a density of 75 000 cells/mL was transferred into 6-well plates (surface area 9.6 cm^2). Twenty-four hours after seeding, cells were exposed to different concentrations of the individual genotoxic compounds or mixtures for 24 h. Next, 3 µg/mL cytochalasin B (cyt B) was added to the cells for 21 h to obtain binucleated

cells. Subsequently, cells were treated with a hypotonic 0.075 M KCl solution, fixed twice and spread on slides. The solvent was used as a negative control and 2 µg/mL MMS as a positive control. A detailed description on how the MNvit test was performed can be found in Sanders et al. 2022. Results were scored with the automated Metafer system and expressed as % binucleated cells with MN.

The cytokinesis-block proliferation index (CBPI) method was used as an indication for the level of cytotoxicity at the different concentrations. One slide of each condition was stained with DAPI/propidium iodide (PI). First, 100 µL of a 1.5 µg/mL PI solution was pipetted on the slides, and after ten minutes, the slides were rinsed two times with reverse osmosis water. The slides were air-dried for 1 h and afterwards counterstained with DAPI-containing Vectashield antifade mounting medium (Roemer et al. 2015). Using the Axiolmager.Z2 fluorescence microscope (Metasystems, Germany) for visual scoring, the number of mono-, bi- and polynucleated cells was determined for 500 random cells and the CBPI was calculated (Sanders et al. 2022).

Mixture strategy

The strategy applied in this research to test mixtures involved five sequential steps (Fig. 1), which are explained in more detail below.

Collection of individual data

The first step involved collecting MNvit data for each compound in the mixture. To enable quantitative analysis, a minimum of five concentrations per compound were tested, inducing low, moderate, and high response levels. For each concentration, 10.000 cells were scored, and at least three independent experiments were performed. The mean percentage of binucleated cells with MN and the corresponding standard deviation were then calculated based on the results from the three (or more) experiments.

Calculation of benchmark concentrations (BMCs)

In the second step, BMCs for the compounds in the mixture were calculated at different response levels using the data from step 1. BMC covariate analyses were performed using the PROAST web tool (version 70.1–https://proastweb.rivm.nl/) at benchmark response (BMR) values of 0.5, 1, 1.5 and 2, corresponding to 50%, 100%, 150% and 200% changes over the background, respectively (Sanders et al. 2022). The BMCs calculated at ach BMR represent equivalent effect levels for the compounds.

Composition of mixtures

The BMC values calculated at various BMRs (i.e. 50, 100, 150 and 200%) in step 2 were used to compose relevant mixtures. For each set of BMCs (i.e. concentrations calculated at a single BMR), three mixtures were prepared to achieve the same response level, based on the assumption of additivity. The ratios of the two compounds in these mixtures were set at 1:1, 3:1 and 1:3. In the equipotent mixture (1:1), concentrations were chosen to produce an equivalent response level, where the sum corresponds to the BMR under the assumption of additivity. Additionally, two non-equipotent combinations (1:3 and 3:1) were prepared to assess whether mixture effects vary when one compound contributes more significantly to the effect than the other. Since there were four BMRs, a total of 12 different mixtures were composed.

Collection of mixture data

Next, all 12 mixtures were tested using the MNvit assay, following the same protocol as for the individual compounds outlined in step 1. To account for inter-experimental variability when comparing the experimental and modelled mixture data in the final analysis, the tests included the concentrations of each individual compound that corresponded to the respective BMR, referred to as repeat individual concentrations (i.e. the BMC values, 1:0 and 0;1).

Modelling of the mixture data

As a final step, the data from the mixtures, along with the repeats of the individual concentrations, were analysed in R using the PROAST dose-addition model 15. This model uses the experimental responses of the individual compounds obtained within the mixture test to predict the concentration–response curve of the combined responses assuming additivity. The experimental mixture results were then

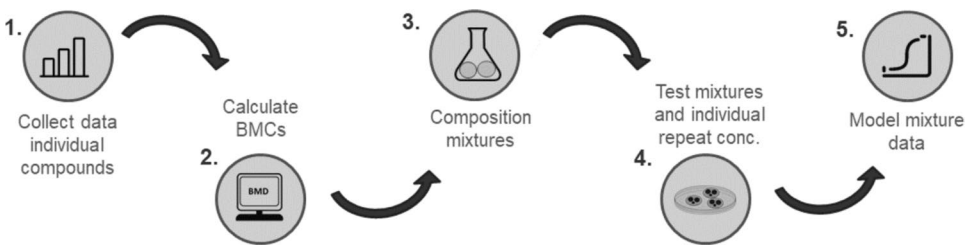

Fig. 1 The five different steps of the mixture strategy. *BMC* Benchmark Concentration, *Conc.* concentration

plotted against the predicted curve to assess whether the principle of additivity applies. If concentration addition is applicable, the mixture data should align with the curve. Deviations from the curve, with rightward shifts indicating antagonism and leftward shifts indicating synergism, would suggest that the principle of additivity does not apply.

Results and discussion

Collection of individual data

Figure 2 displays the MNvit data for the individual compounds (Fig. 2). These graphs present the average results from at least three independent experiments. For each compound, a statistically significant concentration-dependent increase was observed, as determined by the Chi-square test. Additionally, low, medium, and high responses were detected for all three compounds (MMS, EMS, and ETP), indicating that the selected concentration ranges were appropriate. Cytotoxicity remained below the threshold value of 55 ± 5% as specified by OECD Test No. 487 for all three compounds (OECD 2023). The presence of a concentration-dependent effect and the availability of sufficient replicates at each concentration level are crucial for conducting a robust and comprehensive data analysis using the BMC covariate approach (EFSA 2017).

Calculation of benchmark concentration (BMC)

Using the individual compound data obtained in the previous step, BMC covariate analyses were conducted separately for MMS–EMS data and MMS-ETP data at various BMRs (i.e. 50, 100, 150 and 200%) to calculate the BMCs of the compounds. The results for both datasets are displayed in Table 1. For example, the covariate analysis for the MMS–EMS dataset with a BMR of 50% shows that MMS induces a 50% change over the background at a concentration of 0.758 µg/mL, whereas EMS requires a higher concentration of 5.432 µg/mL to achieve the same response. Graphs

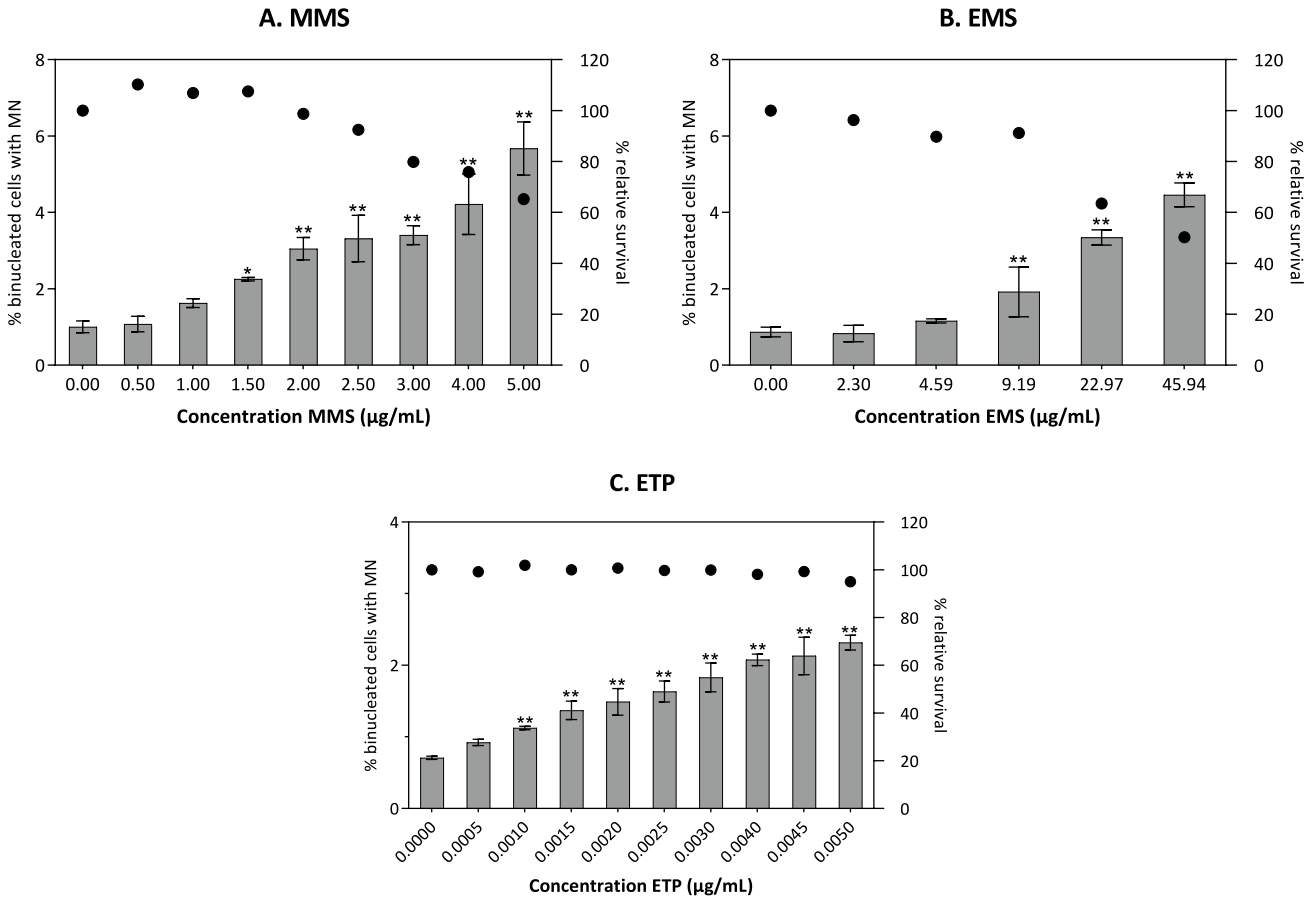

Fig. 2 Results from the MNvit assay for the individual compounds MMS (2A), EMS (2B) and ETP (2C). The bars represent the mean % of binucleated cells with MN, with associated standard deviation (n ≥ 3). The dots indicate the mean % of relative survival, calculated using the CBPI method ($n \geq 3$). *statistically significant difference compared to the negative control based on one-way ANOVA followed by Dunnett's test (*: $p < 0.05$; **: $p < 0.01$ (including $p < 0.001$ and $p < 0.0001$))

Table 1 The benchmark concentrations (BMC) calculated using the BMC covariate approach at various benchmark responses (BMR) for the two data sets (MMS–EMS and MMS–ETP), along with their associated confidence intervals (BMCL-BMCU) derived using the Exponential model in PROAST

Data set	Concentration at specific BMR (µg/mL)			
MMS–EMS	**BMC_{50}** (BMCL-BMCU)	**BMC_{100}** (BMCL-BMCU)	**BMC_{150}** (BMCL-BMCU)	**BMC_{200}** (BMCL-BMCU)
MMS	**0.758** (0.559–0.974)	**1.249** (1.00–1.52)	**1.681** (1.39–2.00)	**2.097** (1.76–2.49)
EMS	**5.423** (3.37–8.66)	**8.940** (5.71–14)	**12.030** (7.73–18.90)	**15.010** (9.63–23.60)
MMS–ETP	**BMC_{50}** (BMCL-BMCU)	**BMC_{100}** (BMCL-BMCU)	**BMC_{150}** (BMCL-BMCU)	**BMC_{200}** (BMCL-BMCU)
MMS	**0.3630** (0.251–0.506)	**0.9032** (0.684–1.160)	**1.4540** (1.15–1.81)	**1.9830** (1.60–2.42)
ETP	**0.0008** (0.0006–0.0010)	**0.0019** (0.0016–0.0023)	**0.0031** (0.0027–0.0036)	**0.0042** (0.0038–0.0048)

MMS methyl methanesulfonate, *EMS* methyl methanesulfonate, *ETP* etoposide, *BMCL* Benchmark Concentration Lower Limit, *BMCU* Benchmark Concentration Upper Limit

of the BMC covariate analyses at the different BMR values are provided in Supplementary Information 1 (for MMS and EMS) and Supplementary Information 2 (for MMS and ETP). BMRs of 50%, 100%, 150% and 200% were selected based on previous studies with MNvit data, which have demonstrated that these values correspond to biologically relevant genotoxic effects (Allemang et al. 2018; Sanders et al. 2022; Wheeldon et al. 2020). BMR values below 50% could be comparable to statistical noise, limiting the usefulness of the derived BMC values for evaluating the principle of concentration-addition for genotoxic compounds. However, minor adjustments in the selected BMR values may be warranted depending on the genotoxic compounds assessed in the MNvit, such as the steepness of the dose–response curve indicated by the parameter "d" in the benchmark dose models employed (EFSA 2017). Additionally, the choice of BMRs may vary based on the specific genotoxicity assay employed (Beal et al. 2023). Consequently, if the proposed approach would be applied to data collected with other in vitro assays, the BMRs may need to be adjusted.

Composition of mixtures

The next step involved composing the mixtures based on the BMC values of the individual compounds (Table 1). Two types of mixtures were tested: MMS–EMS and MMS–ETP (Table 2). Specifically, in Table 2A, the 1:0 and 0:1 ratios represent the BMC values for the individual compounds MMS and EMS, respectively, calculated at various BMRs using the covariate method, corresponding to the BMC values in Table 1. The same applies to Table 2B, where 1:0 and 0:1 ratios correspond to the BMC values for MMS and ETP, respectively. The remaining three ratios (i.e., 1:3, 1:1 and 3:1) in both tables reflect the composition of the binary mixtures as described in the material and methods section. For example, at BMR 50%, the BMCs of MMS and EMS are 0.758 and 5.423 µg/mL, respectively (see Table 2A). The corresponding 1:1 mixture ratio consists of 0.379 µg/mL MMS and 2.711 µg/mL EMS, which are half of the BMC values for each compound (e.g. for MMS $0.758\mu g/mLx\frac{1}{2} = 0.379\mu g/mL$). For the 1:3 ratio of MMS to EMS at BMR 50%, the BMC of MMS is reduced by a factor 4 to 0.190 µg/mL ($0.758\mu g/mLx\frac{1}{4} = 0.190\mu g/mL$), while the BMC of EMS is divided by 4 and then multiplied by 3, resulting in 4.067 µg/mL ($5.423\mu g/mLx\frac{3}{4} = 4.067\mu g/mL$). As mentioned in the material and methods section, the three mixtures selected for each BMR and the individual compound concentrations (1:0 and 0:1), should all induce the same response level assuming additivity. Including the individual compounds ensures that experimental variability is accounted for, as this data will be used in the final mixture analysis.

Collection of mixture data

All mixtures were tested using the same experimental setup as for the individual compounds. If the principle of additivity is valid, the MNvit tests for both individual repeat concentrations and the three distinct mixtures should yield equivalent responses for each BMR. A previous study investigating the combined effects of genotoxic pyrrolizidine alkaloids only used equipotent concentrations (i.e., 1:1 ratio) of the individual compounds in the mixtures (Allemang et al. 2022). However, as highlighted by Kienhuis et al., it is important to include non-equipotent combinations (1:3 and 3:1) to assess

Table 2 A: The selected concentrations of methyl methanesulfonate (MMS) and ethyl methanesulfonate (EMS) for each of the three mixtures (1:3, 1:1 and 3:1) together with individual repeat concentrations indicated by 1:0 and 0:1 at different benchmark responses (BMR). B: The selected concentrations of methyl methanesulfonate (MMS) and etoposide (ETP) for each of the three mixtures (1:3, 1:1 and 3:1) together with individual repeat concentrations indicated by 1:0 and 0:1 at different benchmark responses (BMR)

A	Concentration of compounds within mixtures (µg/mL)							
	BMR 50%		BMR 100%		BMR 150%		BMR 200%	
	MMS	*EMS*	*MMS*	*EMS*	*MMS*	*EMS*	*MMS*	*EMS*
1:0 (BMC)	0.758	0.000	1.249	0.000	1.681	0.000	2.097	0.000
1:3	0.190	4.067	0.312	6.705	0.420	9.022	0.524	11.258
1:1	0.379	2.711	0.624	4.470	0.840	6.015	1.048	7.505
3:1	0.569	1.356	0.937	2.235	1.261	3.008	1.573	3.752
0:1 (BMC)	0.000	5.423	0.000	8.940	0.000	12.03	0.000	15.010
B	Concentration of compounds within mixtures (µg/mL)							
	BMR 50%		BMR 100%		BMR 150%		BMR 200%	
	MMS	*ETP*	*MMS*	*ETP*	*MMS*	*ETP*	*MMS*	*ETP*
1:0 (BMC)	0.36300	0.00000	0.90320	0.00000	1.45400	0.00000	1.98300	0.00000
1:3	0.09075	0.00058	0.22580	0.00145	0.36350	0.00233	0.49575	0.00318
1:1	0.18150	0.00039	0.45160	0.00097	0.72700	0.00156	0.99150	0.00212
3:1	0.27225	0.00019	0.67740	0.00048	1.09050	0.00078	1.48725	0.00106
0:1 (BMC)	0.00000	0.00078	0.00000	0.00193	0.00000	0.00311	0.00000	0.00425

BMC Benchmark concentration

potential variations in mixture effects when one compound has a greater contribution to the effect than the other (Kienhuis et al. 2015).

The graphs based on the experimental results are provided in Supplementary Information 3 for MMS and EMS and in Supplementary Information 4 for MMS and ETP, showing that at each BMR, the three tested mixtures yielded similar responses. Although no conclusions can be drawn solely from these graphs regarding possible deviations from the principle of additivity, the results of the repeat individual concentrations (i.e. 1:0 and 0:1 ratios) can indicate the quality of the tests and the preceding steps. Ideally, these concentrations should produce approximately the same response, as they are selected to induce an equivalent percentage change over the background (i.e., the BMR). Discrepancies suggest that earlier steps may need to be revised to ensure the selection of appropriate concentration ranges and the conduct of accurate BMC analyses, without excessive distortion of the dose–response curves (EFSA 2017; Kienhuis et al. 2015).

Modelling of the mixture data

For each binary mixture, all data were compiled into a single file and analysed in R using the PROAST dose addition model 15 to assess the applicability of the principle of additivity.

In Fig. 3, the black curve represents the predicted concentration response curve of the mixture, derived from the

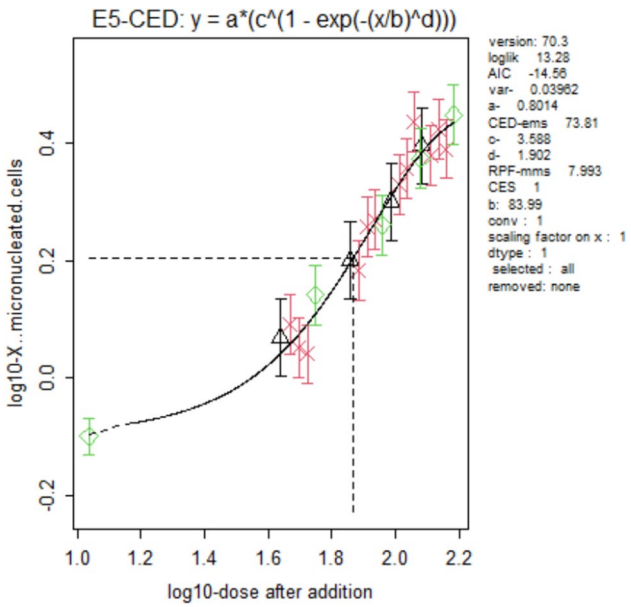

Fig. 3 The black curve represents the predicted concentration response curve of the mixture assuming additivity based on the experimental responses of the individual compounds methyl methanesulfonate (MMS) and ethyl methanesulfonate (EMS) within the mixture experiment using the in vitro micronucleus assay. The marks (green diamonds: individual compound MMS; black triangles: individual compound EMS; red crosses: mixtures) represent the experimental values obtained with the MMS–EMS mixture tests at different BMRs (50%, 100%, 150%, 200% from left to right) with their corresponding 95% confidence intervals

experimental responses of the individual compounds of the EMS–MMS mixture tests, under the assumption of additivity (Fig. 3). The individual experimental responses for MMS and EMS are denoted by green diamonds and black triangles, respectively, while the experimental mixture responses are indicated by red crosses. The intersection of all experimental mixture responses with the fitted curve suggests that the principle of additivity is valid across the entire concentration range for the MMS–EMS mixtures.

The same analysis was conducted for the MMS–ETP mixtures, as illustrated in Fig. 4 (Fig. 4). As before, the experimental mixture responses, represented by red crosses, intersect with the black curve, suggesting additive effects. However, during the first analysis of the mixture data for MMS and ETP, we observed a poor fit of the model to the data. Upon closer examination, we found that parameter "a", which represents the estimated background response, significantly deviated from the experimental control response. This discrepancy likely arose because the model estimate the background frequency based on the entire concentration–response dataset (EFSA 2017). By manually adjusting parameter "a" to match the experimental value of the negative control within the mixture tests, the model's fit improved. Additionally, this adjustment resulted in a BMC

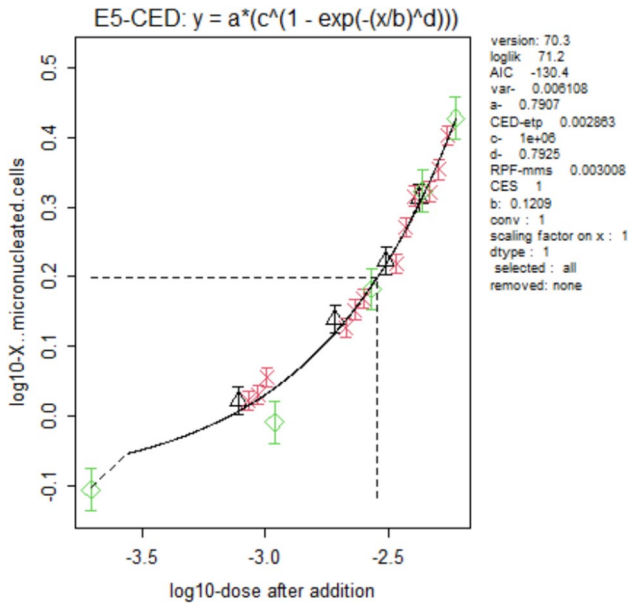

Fig. 4 The black curve represents the predicted concentration response curve of the mixture assuming additivity based on the experimental responses of the individual compounds methyl methanesulfonate (MMS) and etoposide (ETP) within the mixture experiment using the in vitro micronucleus assay. The marks (green diamonds: individual compound MMS; black triangles: individual compound ETP; red crosses: mixtures) represent the experimental values obtained with the MMS–ETP mixture tests at different BMRs (50%, 100%, 150%, 200% from left to right) with their corresponding 95% confidence intervals

of 0.002883 µg/mL for ETP (indicated as CED in the legend of Fig. 4) which closely aligns with the BMC obtained from the individual compound analysis using the covariate approach in step 2, i.e. 0.001931 µg/mL (Supplementary Information 2).

As Kienhuis et al. have emphasized, traditional statistical methods that rely on significance testing for null hypotheses are not suitable for evaluating the combined effects of individual compounds. The objective of this study was not to achieve perfect adherence of genotoxicant mixtures to the principle of concentration addition, but rather to predict mixture responses approximately. Consequently, the results were presented visually, with confidence intervals included to account for statistical variability (Kienhuis et al. 2015). It is important to note that if a single mixture data point deviated from the fitted curve, this would not be sufficient to draw definitive conclusions about synergistic or antagonistic effects. To identify such effects, mixture responses must systematically appear either on the left (indicating synergism) or the right (indicating antagonism) of the curve. In cases where the visual interpretation of mixture results is ambiguous or difficult to assess, an additional quantitative evaluation can be performed. As described by Zoupa et al., this method involves comparing the relative potency factor (RPF) estimation of the compounds, both with and without incorporating the mixture data in PROAST, through a metric known as the "ratio of overlap" (van Oostrom et al. 2020; Zoupa et al. 2020). However, in the present study, the results were clear, and no further data analysis was deemed necessary.

Overall, in this study, we combined and refined existing BMC-based approaches for analyzing complex mixtures in the context of non-genotoxic endpoints and applied the optimized strategy to study mixture effects of genotoxicants. Our method focused on the composition and testing of mixtures based on multiple BMC analyses at various BMR levels, rather than relying on a single BMC analysis with only one BMR value (Allemang et al. 2022; Kienhuis et al. 2015). This approach helps to reduce data variability, thereby enhancing the robustness and reliability of our findings. Furthermore, we utilized the dose addition model 15 in R, which offers a more precise and adaptable framework for our analysis.

The quantitative BMC approach offers several advantages. A key benefit is the highly informative content it provides, as analysing mixtures at different BMRs allows for a comprehensive understanding of concentration–response relationships and potential compound interactions. Moreover, the model's flexibility in adjusting specific parameters, when necessary and justified, is a key strength enhancing the accuracy and adaptability of the analyses. However, as also highlighted by Lasch et al., who used a fictional dataset to compare different methods,

BMC-based approaches also have certain drawbacks. The most notable disadvantage is the time-consuming nature of data generation and processing. The complexity involved in performing multiple BMC analyses and synthesizing the results can be labour-intensive (Lasch et al. 2020). However, creating well-designed templates can significantly reduce the time required for data handling.

To further refine and validate this method, particularly in the context of genotoxic compounds, additional studies are needed. So far, antagonistic and synergistic effects have not been empirically demonstrated using BMC approaches on real-world data. Although Lasch et al. have shown with a fictional dataset that the BMC approach can detect synergism and antagonism, empirical validation with real-world data remains crucial (Lasch et al. 2020). Future research should focus on applying this method to a broader range of genotoxic compounds and experimental scenarios, thereby confirming its effectiveness and expanding its applicability in toxicological risk assessment. Continued refinement and validation of this approach will deepen our understanding of the complexities inherent in chemical mixtures, thereby improving our capacity to protect human health and the environment from potential hazards.

Conclusion

In conclusion, this study introduced an optimized BMC-based approach for evaluating the combined effects of genotoxic compounds, adapted from methodologies applied to non-genotoxic endpoints. By employing multiple BMC analyses and the dose addition model in R, this approach offers high informative content and adaptability. Our proof-of-concept study using binary mixtures of genotoxicants demonstrated the method's robustness and practical applicability, confirming the principle of additivity for the genotoxicant mixtures tested.

Given the critical nature of genotoxicity as an endpoint in risk assessment, which currently lacks extensive data on chemical mixtures, this study provides a comprehensive guide for practical testing of such mixtures. Future research should focus on validating this method across a broader spectrum of compounds to determine whether synergistic or antagonistic effects may also occur, thereby enhancing its utility in toxicological risk assessment and improving the protection of human health and the environment from combined chemical exposures.

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Data availability The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors have no competing interests to declare that are relevant to the content of this article.

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