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Different Types of Combination Effects for the Induction of Micronuclei in Mouse Lymphoma Cells by Binary Mixtures of the Genotoxic Agents MMS, MNU, and Genistein

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Distinction between dose addition and response addition for the analysis of the toxicity of mixtures may allow differentiation of the components regarding similar versus independent mode of action. For nonlinear dose responses for the components, curves of dose addition and response addition differ and embrace an "envelope of additivity." Synergistic or antagonistic interaction may then be postulated only if the mixture effect is outside this surface. This situation was analyzed for the induction of micronuclei in L5178Y mouse lymphoma cells by the two methylating agents methyl methanesulfonate (MMS) and N-methyl-N-nitrosourea (MNU) and the topoisomerase-II inhibitor genistein (GEN). All three chemicals reproducibly generated sublinear (upward convex) dose-response relationships. For the analysis of mixture effects, these genotoxic agents were investigated in the three binary combinations. Statistical testing for dose addition along parallel exponential dose responses was performed by linear regression with interaction based on the logarithm of the number of cells that contain micronuclei. For MMS+MNU, the mixture effect was compatible with dose addition (i.e., significantly larger than calculated for the addition of net responses). For MMS+GEN, the measured effect was larger than for response addition but smaller than for dose addition. For MNU+GEN, the measured effect was below response addition, indicative of true antagonism. In the absence of knowledge on the sublinear dose-response relationships for the individual components, a synergistic effect of MMS on both MNU and GEN would have been postulated erroneously. The observed difference between MMS and MNU when combined with GEN would not have been predicted on the basis of a simplistic interpretation of DNA methylation as the mode of action and may be due to differences in the profile of DNA methylations and/or epigenetic effects. We conclude that knowledge of nonlinearities of the dose-response curves of individual components of a mixture can be crucial to analyze for synergism or antagonism and that an in-depth mechanistic knowledge is useful for a prediction of similarity or independence of action.

Key Words: **alkylating agents; genotoxicity; cell culture; dose response; mixture models.**

A major objective of mixture testing is to establish whether the toxicity of a combination of chemicals will deviate from an effect expected for additivity. Bliss (1939) distinguished between independent, similar, and synergistic action and explained this on the basis of differences in modes of action. For the analysis of results of mixture experiments, the respective terms response addition, dose addition, and interaction came into use (see review in Cassee *et al.*, 1998). Response addition is seen when the net effect of a mixture is equal to the sum of the net effects of the components. For dose (or concentration) addition, all chemicals in a mixture are considered to act by the same mechanism, and the mixture effect is determined by the sum of doses after adjustment for differences in potency of the components. The U.S. EPA has selected dose addition as the no-interaction definition for mixture risk assessment, so that synergism would only stand for effects that exceed those predicted for dose addition (Hertzberg and MacDonell, 2002; Hertzberg and Teuschler, 2002). Using their definition, dose addition means that the dose-response curves of the components are considered identical except for dose scaling. If this requirement is met, the components are often considered to act by a similar mode.

One critical aspect concerning dose addition and deviation from additivity relates to the case of sublinear (upward convex) dose-response relationships for the components, as illustrated by Burkart and Jung (1998). Assume that dose x of chemical A produces a response of 1 effect unit, and dose y of chemical B has the same effect magnitude of 1. If under these conditions a mixture of dose x of substance A plus dose y of substance B generated effect level 4, one would on first sight postulate that A and B acted in a synergistic manner. This interpretation is not correct if both components A and B alone show a quadratic dose response, which would result in effect level 4 with dose 2x of A or dose 2y of B. Figure 1 is a graphical representation of the necessity to distinguish between dose additivity and response additivity under the conditions of sublinear dose-response curves for the mixture components. The left-hand panel shows a quadratic dose-response curve for both A and B. The potency may differ, in that dose x of A is equivalent to dose

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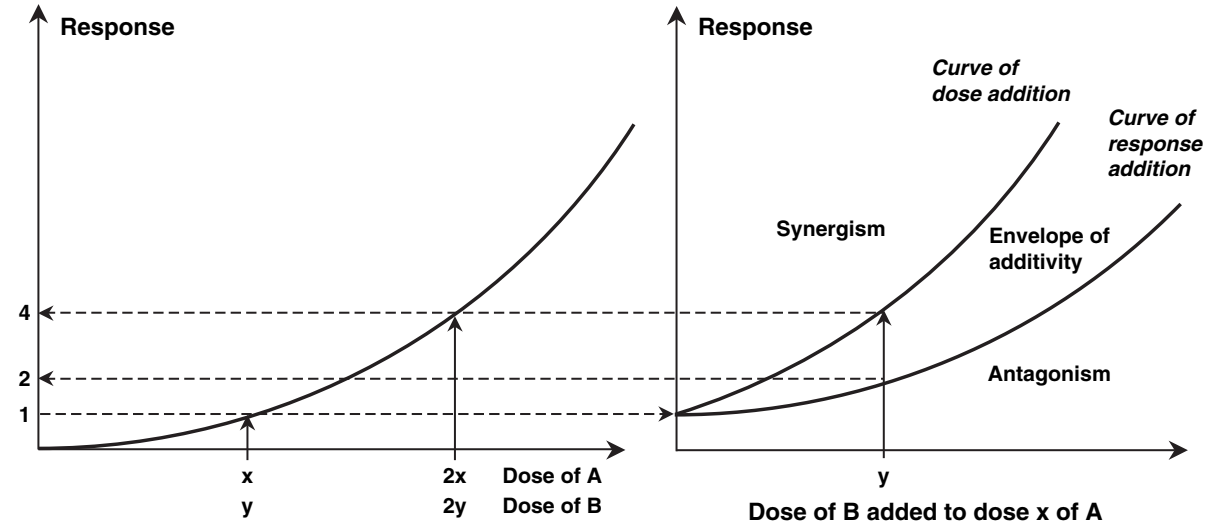

FIG. 1. Schematic representation of a nonlinear (here: quadratic) dose-response relationship (left-hand side; for A or B) and possibilities for the response to a mixture of the two components (right-hand side; dose response for B added to dose x of A).

y of B. The panel on the right-hand side shows two dose-response curves and illustrates the dose response for B added to dose x of A. Two curves are shown. The lower curve describes the situation where B produces the response independent of the process driven by A. The curve has the same shape as the one on the left-hand side, except that it is set off on the y-axis by response level 1. On this basis, y of B added to x of A would result in effect level 2, as shown by the respective dashed line. The upper curve describes the situation where B acts in the same way as A. Addition of B results in moving up along the curve shown in the left-hand panel, but the start is at response level 1 and the slope is steeper, as given by the quadratic function. For y of B added to x of A, this results in effect level 4, but this is not a result of a synergistic interaction of A and B. The two curves mark the boundary of the "envelope of additivity," and only outside the two curves can an interaction be postulated as being synergistic or antagonistic.

While this issue is plausible conceptually, respective observations are scarce (Doty *et al.*, 1992; Mentzer *et al.*, 1999), and have, to our knowledge, not been corroborated by specifically designed experiments. Here, we present and analyze data of experiments that have been planned and performed for this very purpose. They are based on the induction of micronuclei in L5178Y mouse lymphoma cells as an assay for genotoxicity of binary mixtures of components that show sublinear dose-response relationships when tested alone.

MATERIALS AND METHODS

In vitro micronucleus test. Mouse lymphoma L5178Y cells, clone 3.7.2c (Clive *et al.*, 1972) obtained from W.J. Caspary, NIEHS, were cultured in suspension in RPMI 1640 cell culture medium supplemented with antibiotics, 0.25 mg L-glutamine/ml, 107 μg sodium pyruvate/ml, and 10% heat-inactivated horse serum (all from Sigma, Taufkirchen, Germany). Cell cultures were grown in a humidified atmosphere with 5% CO_2 in air at 37°C. The test chemicals methyl methanesulfonate (MMS), N-methyl-N-nitrosourea (MNU), and genistein (GEN) (all from Sigma) were dissolved in dimethylsulfoxide (DMSO) and added to L5187Y mouse lymphoma cells at a density of 2×10^5 cells per ml (final DMSO concentration $\leq$1%). After 4 h of incubation, cells were washed twice, and cytochalasin B was added to a final concentration of 5 μg/ml. Cytochalasin B remained in the culture for the entire expression time of 20 h until the cells were harvested. Mouse lymphoma L5178Y cells tolerate this treatment without showing nuclear extrusions or DNA fragmentation. Cytospin preparations on glass slides were prepared. After 2 h in methanol at −20°C, the slides were incubated with acridine orange (62.5 μg/ml in Sorensen buffer, pH 6.8) for 5 min, washed twice with Sorensen buffer for 5 min, and mounted for microscopy. Two thousand cells (1000 per slide) were evaluated for each treatment. The score (MN) was the number of cells containing one or more micronuclei per 1000 binucleated cells (BN cells). The percentage of binucleated cells was evaluated as a marker of cell proliferation.

Dose selection. The concentration range used for the three chemicals was defined by similar effect magnitude, in order not to exceed the response range of the assay that may be limited by cytotoxicity. Based on pilot experiments, the following concentration steps 0–1–2–3 were selected for the main experiments: MMS: 0, 100, 200, 300 μM; MNU: 0, 700, 1400, 2100 μM; GEN: 0, 15, 30, 45 μM. Mixtures included the four possible combinations of low and medium dose but not top concentrations, in order to keep the mixture effects within the range covered by the dose responses of the components. Two completely independent sets of experiments were run for each pairwise combination. In addition, controls were run in duplicate. If the number of cells with micronuclei observed in a combination exceeded the score of the top dose of either component, the respective combination was not used for the test for dose addition.

Statistical analysis. The hypothesis of response addition (addition of net effects of the components) was tested by the sign test. Under the null hypothesis of response addition and the assumption of symmetrical errors, the observed number of cells with micronuclei is with equal probability greater or less than the number of cells with micronuclei calculated for response addition.

The hypothesis of dose addition was tested with a linear model, using the logarithm of the score MN to linearize the sublinear dose responses for the components in a parallel manner. For the test of deviation from dose addition, we tested for interaction according to

$$\mathrm{Log}_{10}(\mathrm{MN}) = \mathrm{a} + (\mathrm{b} \times \mathrm{A}) + (\mathrm{c} \times \mathrm{B}) + (\mathrm{d} \times \mathrm{A} \times \mathrm{B}) + \varepsilon(\text{error term})$$

Letters A and B stand for the concentrations of the two chemicals. A×B is the interaction term. The coefficients a, b, c, and d were estimated by linear regression. The error was proportional to the score MN. For the statistical handling of the replicates, random effects for the parameters were added if this resulted in a model improvement (mixed effects model).

Note: The absence of an interaction (d = 0) is equivalent to dose addition, as can be shown by replacing B for A by normalization with c/b (last line):

$$\begin{aligned} MN = 10^{(a+b\times A+c\times B+\varepsilon)} &= 10^{a}\times 10^{(b\times A)}\times 10^{(c\times B)}\times 10^{\varepsilon} \\ &= 10^{a}\times 10^{(b\times A)}\times 10^{(b\times c/b\times B)}\times 10^{\varepsilon} \\ &= 10^{a}\times (10^{b})^{A}\times (10^{b})^{(c/b\times B)}\times 10^{\varepsilon} \\ &= 10^{a}\times (10^{b})^{(\mathbf{A}+\mathbf{c}/\mathbf{b}\times \mathbf{B})}\times 10^{\varepsilon}. \end{aligned}$$

RESULTS

In pilot studies with a number of genotoxic chemicals, we found that the two methylating agents methyl methanesulfonate (MMS) and methylnitrosourea (MNU) and the topoisomerase II-inhibitor genistein (GEN) reproducibly produced sublinear (upward convex) dose-response relationships for micronucleus induction in L5178Y mouse lymphoma cells. These genotoxic agents were selected for the investigation of the combination effects of the binary mixtures MMS+MNU, MMS+GEN, and MNU+GEN. The cytochalasin B test modification was used. Cytochalasin B inhibits the formation of two cells after nuclear division, so that the cells are arrested in a binucleated state. This limitation restricts the analysis to those cells that were able to replicate DNA and go through nuclear division so that the result is not biased by dose-related changes in the proportion of cells going through the cell cycle within the period of damage expression.

The panels on the left-hand side of Figure 2 show the reproducibly sublinear dose-response curves for the induction of micronuclei in four independent experiments. The degree of nonlinearity was highest with MMS, lowest with MNU. Logarithmic transformation of the response axis linearized the curves (see right-hand panels in Fig. 2), which was the basis for the use of a linear regression to test for interaction.

Numerical results of all experiments, including the binary combinations MMS+MNU, MMS+GEN, and MNU+GEN, are shown in Tables 1, 2, and 3, respectively. Columns 3 and 5 show the number of cells with micronuclei per 1000 binucleated cells. Columns 4 and 6 give the net treatment-related increase by the components (lines 3–5 and 6–8) and the result calculated for response addition (lines 9–12; mean of controls plus the two corresponding net effects). Comparison of the numbers in lines 9–12, columns 3 versus 4 and 5 versus 6 provides direct information whether the experimental result is consistent with response addition following simple independent action of the two components. Response addition was rejected on the basis of the sign test for all binary combinations. For the combination of MMS+MNU (Table 1) and MMS+GEN (Table 2), the observed score was higher than the sum of net

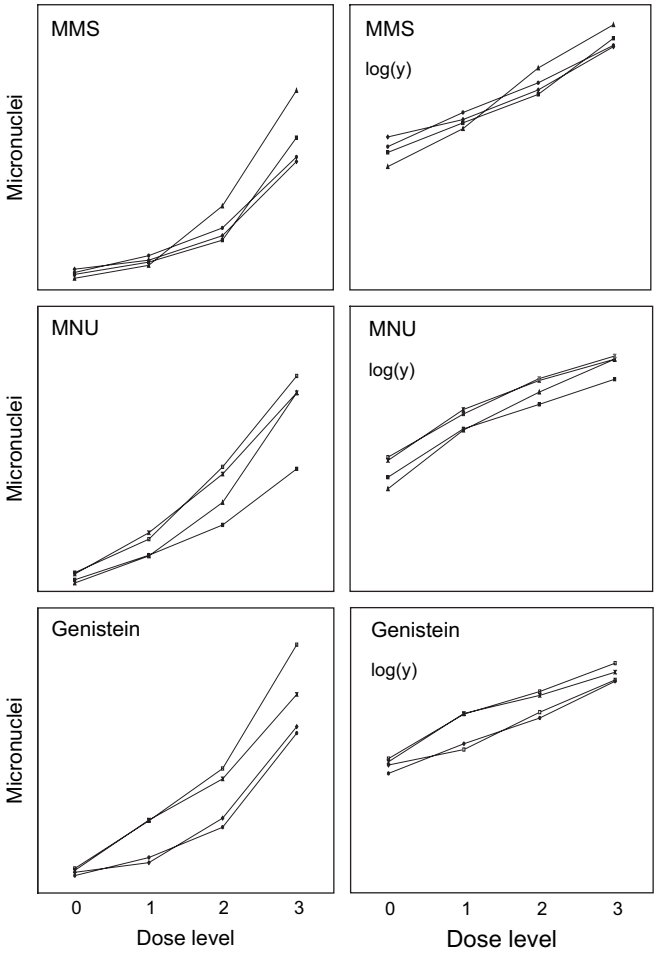

FIG. 2. Shapes of the dose-response relationships observed for methyl methanesulfonate (MMS), N-methyl-N-nitrosourea (MNU), and genistein (GEN) for the induction of micronuclei in L5178Y mouse lymphoma cells. Dose levels 0–3 stand for the following concentration steps: 0, 100, 200, 300 μM for MMS; 0, 700, 1400, 2100 μM for MNU; 0, 15, 30, 45 μM for GEN. The panels on the right-hand side show that logarithmic transformation of the y-axis obliterates the nonlinearity.

effects; for the combination of MNU+GEN the mixture effect was significantly below response addition (two-sided sign test: 8:0; $p = 0.5^8 \times 2 = 0.008$). In the absence of information on the dose-response relationships of the components, one would have postulated a synergistic effect of mixtures of MMS+MNU and MMS+GEN and an antagonistic mixture effect of MNU+GEN.

For the analysis of the hypothesis of dose addition under the assumption of parallel nonlinear dose-response curves, we chose an exponential model based on the logarithms of the response measures and tested for an interaction term of a linear regression. For the combination MMS+MNU, the coefficient d of the interaction term A×B was not significant ($p = 0.96$), so that our dose addition model could not be rejected. This also means that the effect of a mixture of MMS and MNU could lie on the curve of dose addition and may not be synergistic as indicated by the test for response addition. This is indicated in Figure 3, which illustrates graphically the mixture effects of the

TABLE 1
Induction of Micronuclei in L5178Y Mouse Lymphoma Cells as a Function of Concentration of Methyl Methanesulfonate (MMS), N-Methyl-N-Nitrosourea (MNU), and Their Combination

Concentration (μM)		Experiment #1		Experiment #2	
MMS	MNU	No. of cells[a]	Net effect	No. of cells[a]	Net effect
0	0	16	—	11	—
0	0	16	—	13	—
100	0	29	13	26	14
200	0	52	36	88	76
300	0	160	144	210	198
0	700	51	35	50	38
0	1400	93	77	125	113
0	2100	172	156	279	267
			Calc. addn. of net resp.		Calc. addn. of net resp.
100	700	79	64	141	64
100	1400	131	106	256	139
200	700	200	87	230	126
200	1400	235[b]	129	253	201

[a]Number of binucleated cells that show one or more micronuclei per 1000 binucleated cells.

[b]The combination treatment 200 + 1400 was not considered for the analysis of dose addition because this score was higher than the range of scores covered by the individual components.

TABLE 2
Induction of Micronuclei in L5178Y Mouse Lymphoma Cells as a Function of Concentration of Methyl Methanesulfonate (MMS), Genistein (GEN), and Their Combination

Concentration (μM)		Experiment #3		Experiment #4	
MMS	GEN	No. of cells*	Net effect	No. of cells*	Net effect
0	0	21	—	16	—
0	0	23	—	20	—
100	0	31	9	36	18
200	0	57	35	65	47
300	0	135	113	140	122
0	15	32	10	37	19
0	30	79	57	69	51
0	45	175	153	168	150
			Calc. addn. of net resp.		Calc. addn. of net resp.
100	15	47	41	61	55
100	30	89	88	91	87
200	15	85	67	106	84
200	30	152	114	145	116

*Number of binucleated cells that show one or more micronuclei per 1000 binucleated cells.

three binary combinations. For the combination MMS+GEN, the exponential model gave a highly significant negative coefficient d of the interaction term ($p = 4.6 \times 10^{-6}$). Together with the rejection of response addition (see above), this combination effect is postulated to lie in the middle of the envelope of additivity, significantly off both curves (Fig. 3). For the combination MNU+GEN, antagonism had already been shown for response addition. It is therefore not surprising that testing for dose addition also produced a significant negative coefficient d of the interaction term ($p = 5.4 \times 10^{-6}$). In Figure 3, this combination finds its position in the area of antagonism.

The percentage of binucleated cells as a measure for cytostatic effects of the test substance showed a similar dose-dependent decrease with MMS and MNU, from 82% in controls to 34 and 28% at the highest dose of MMS and MNU, respectively. Genistein alone produced only a minor reduction (from 84 to 73%). The decrease seen upon combination of MMS or MNU with genistein was dominated by the effect of the methylating agent. Therefore, the difference between MMS and MNU when combined with genistein cannot be due to different percentages of binucleated cells.

DISCUSSION

We found three significantly different types of combination effects for the induction of micronuclei by binary combinations of the three genotoxic agents MMS, MNU, and genistein. MMS+MNU was compatible with dose addition; MMS+GEN was between dose addition and response addition; MNU+GEN showed antagonism. In the absence of knowledge of the sublinear dose response relationships for the individual

TABLE 3
Induction of Micronuclei in L5178Y Mouse Lymphoma Cells as a Function of the Concentration of N-Methyl-N-Nitrosourea (MNU), Genistein (GEN), and Their Combination

Concentration (μM)		Experiment #5		Experiment #6	
MNU	GEN	No. of cells*	Net effect	No. of cells*	Net effect
0	0	24	—	25	—
0	0	24	—	27	—
700	0	82	58	73	47
1400	0	165	141	175	149
2100	0	279	255	302	276
0	15	77	53	76	50
0	30	120	96	131	105
0	45	209	185	261	235
			Calc. addn. of net resp.		Calc. addn. of net resp.
700	15	74	135	114	123
700	30	138	178	112	178
1400	15	206	218	187	225
1400	30	224	261	258	280

*Number of binucleated cells that shows one or more micronuclei per 1000 binucleated cells.

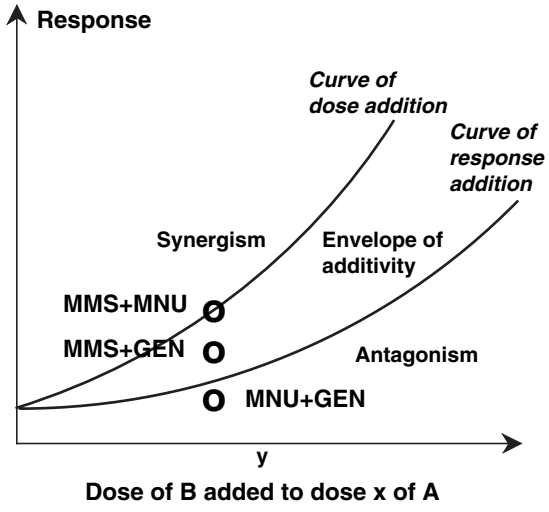

FIG. 3. Conceptual representation of different mixture effects for the induction of micronuclei in L5178Y mouse lymphoma cells by methyl methanesulfonate (MMS), N-methyl-N-nitrosourea (MNU), and genistein (GEN) in binary combinations. The assumption of dose addition was based on parallel nonlinear dose-response curves for all components; response addition was based on the sum of net effects.

genotoxins, MMS+MNU and MMS+GEN would have been misinterpreted to act in a synergistic manner. It illustrates that an interpretation of mixture effects for a putative deviation from additivity (synergism or antagonism) should include the evaluation of the dose-response relationship for the components. This could be particularly important for endpoints related to mutagenicity and carcinogenicity, where a linear-sublinear shape of the dose-response curve can often be explained on mechanistic grounds (Lutz, 1998).

Our analysis was based on an exponential model for the description of the sublinear curves. It requires a minimum number of parameters to be estimated, assumes an error that is proportional to the response, and allows straightforward calculation as a linear model, after logarithmic transformation of the effect measure. However, using log(MN), the same degree of nonlinearity is assumed for all three genotoxins, although Figure 2 indicates some differences. Estimation of the degree of nonlinearity by a nonlinear mixed-effects power model fit by maximum likelihood using $MN \sim a + b \times A^c$ resulted in $c = 2.21$ for MMS, 1.54 for MNU, and 1.72 for GEN. Nevertheless, analysis of the mixture data with a power model produced the same results as the exponential model regarding the postulated difference between the three binary combinations as shown schematically in Figure 3.

Using the exponential model, we assumed parallel dose-response curves and used the definition given in the introduction. The literature also refers to dose addition in a more general way (Berenbaum, 1989), as the method based on isoboles. Isoboles have their particular value for the search for and analysis of dose combinations that produce the same response level. For a given response, isoboles can be used to analyze combinations of agents irrespective of the shape of their dose-response curves (Kortenkamp and Altenburger, 1998). Our experiments included combinations at different dose levels (i.e., $1 + 1$, $1 + 2$, $2 + 1$, $2 + 2$), which resulted in various response levels. For this situation, our model was considered appropriate.

The differentiation between dose addition and response addition has often been explained on mechanistic grounds. In their review article, Cassee *et al.* (1998) define: "With dose addition, each of the chemicals in the mixture contributes to the toxicity of the mixture in proportion to its dose, expressed as the percentage of the dose of that chemical alone that would be required to obtain the given effect of the mixture. All chemicals of concern in a mixture act in the same way, by the same mechanisms, and differ only in their potencies." The question is whether this holds for MMS and MNU, for the combination of which we could not reject dose addition. Both genotoxins methylate DNA, so one could define DNA methylation as their common mechanism. However, while the predominant site of DNA methylation is the nitrogen atom at position 7 of guanine for both genotoxins, there is a difference for the methylation at less nucleophilic sites, such as O^6 of guanine (Lawley, 1984). With true dose addition, one would have to postulate that the common reaction (i.e., guanyl-7-methylation) is primarily responsible for the observed micronucleus induction. This is a reasonable hypothesis, since this type of DNA methylation destabilizes the N-glycosidic bond and facilitates depurination, the repair of which is associated with strand breakage. Following this interpretation, one would have to predict that combination of either MMS or MNU with GEN should produce the same type of mixture effect. This has not been found. While MMS+GEN came to lie between dose addition and response addition, MNU+GEN showed antagonism. Differences between MMS and MNU, for instance for DNA methylation as mentioned above or for DNA synthesis inhibition (Slamenova *et al.*, 1990), did not result in deviation from dose addition, but resulted in different combination effects with GEN. Note, however, that failure to reject the null hypothesis of dose addition is not equivalent with proof of this hypothesis, and that similarity of a mode of action is not equivalent with full mechanistic equality.

The type of a mixture effect may also be specific for a given experimental system. The mouse lymphoma cells L5178Y have a mutated p53 gene. Normal cells could react to DNA damage in a different manner and show another response. Combination effects may also differ between endpoints. While we found an antagonistic mixture effect of MNU+GEN, it was recently reported that genistein stimulated the growth of mammary tumors that had been initiated with MNU (Allred *et al.*, 2004). Consideration of all these limitations is in line with a recently published statement, based on several examples from the pharmacological and toxicological literature, that characteristics of a mixture can hardly be predicted from knowledge on the components (Borgert *et al.*, 2004). On the other hand, our data illustrate that investigation of dose-response relationships for individual components of a mixture

may help avoid misinterpretation of combination effects in terms of synergism or antagonism in the absence of full mechanistic information.

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