Deviation from Additivity in Mixture Toxicity: Relevance of Nonlinear Dose–Response Relationships and Cell Line Differences in Genotoxicity Assays with Combinations of Chemical Mutagens and γ-Radiation

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Sublinear dose–response relationships are often seen in toxicity testing, particularly with bioassays for carcinogenicity. This is the result of a superimposition of various effects that modulate and contribute to the process of cancer formation. Examples are saturation of detoxification pathways or DNA repair with increasing dose, or regenerative hyperplasia and indirect DNA damage as a consequence of high-dose cytotoxicity and cell death. The response to a combination treatment can appear to be supra-additive, although it is in fact dose-additive along a sublinear dose–response curve for the single agents. Because environmental exposure of humans is usually in a low-dose range and deviation from linearity is less likely at the low-dose end, combination effects should be tested at the lowest observable effect levels (LOEL) of the components. This principle has been applied to combinations of genotoxic agents in various cellular models. For statistical analysis, all experiments were analyzed for deviation from additivity with an *n*-factor analysis of variance with an interaction term, *n* being the number of components tested in combination. Benzo[*a*]pyrene, benz[*a*]anthracene, and dibenz[*a,c*]anthracene were tested at the LOEL, separately and in combination, for the induction of revertants in the Ames test, using *Salmonella typhimurium* TA100 and rat liver S9 fraction. Combined treatment produced no deviation from additivity. The induction of micronuclei *in vitro* was investigated with ionizing radiation from a ^{137}Cs source and ethyl methanesulfonate. Mouse lymphoma L5178Y cells revealed a significant 40% supra-additive combination effect in an experiment based on three independent replicates for controls and single and combination treatments. On the other hand, two human lymphoblastoid cell lines (TK6 and WTK1) as well as a pilot study with human primary fibroblasts from fetal lung did not show deviation from additivity. Data derived from one cell line should therefore not be generalized. Regarding the testing of mixtures for deviation from additive toxicity, the suggested experimental protocol is easily followed by toxicologists. *Key words:* ^{137}Cs, Ames test, cell line, chemically induced, dose–response relationship, drug effects, drug interactions, ethyl methanesulfonate, gamma rays, genotoxicity, L5178Y, micronuclei, models, mutagens, polynuclear aromatic hydrocarbons, radiation effects, research design, risk assessment, statistics, TK6, WTK1. *Environ Health Perspect* 110(suppl 6):915–918 (2002).
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The question whether mixture effects are additive cannot be answered without information on the dose–response relationship for the single agents. Figure 1 (left) illustrates the problem. Assume that dose level *x* of substance A produces response level *y*. If substance B is added at a dose level that also produces response level *y*, and the combined exposure results in a response level >>2*y*, one is tempted to call this a supra-additive combination effect. However, this result could simply be the consequence of a sublinear dose–response relationship for the single substances; that is, dose level 2*x* of A would on its own result in response level >>2*y*. Therefore, the response obtained with the combination treatment is not the result of an interaction. It is still additive.

Figure 1 (right) illustrates the situation in more general terms. It shows different responses when increasing doses of chemical B are added to a fixed dose *x* of A. If B acts by "simple joint action" as originally termed by Bliss (*1*), the combined response follows the "curve of joint action." This is also called "dose addition" and indicates the same mode of action of the two chemicals. On the other hand, if B produces the same type of response but by a mechanism unrelated to A, the "curve of independent action" is followed. Based on this concept, all data points between the two curves lie on the "surface of additivity." This issue was taken up long ago in radiobiology under the concept of "isoaddition," heteroaddition, and the "envelope of additivity" (*2–5*). In chemical mixture toxicology, it has not gained much attention.

For numerous end points of toxicity (e.g., carcinogenicity), sublinear dose–response relationships are not uncommon (*6*). This can be explained by superimposition of various effects that modulate or contribute to the process of cancer formation (*7*). For instance, DNA repair processes can become saturated with increasing doses of a genotoxic carcinogen, or cytotoxicity at high doses can result in regenerative processes that accelerate the conversion of primary DNA lesions to mutations. Furthermore, cell death elicits an immune reaction that can be associated with oxidative stress, which in turn can result in an increased level of indirect, oxygen-related DNA damage in surviving neighbor cells (*7*). For genotoxic carcinogens, therefore, dose–response linearity could only be postulated for situations in which the effect is dominated by one single mode of action such as DNA adduct formation. At higher doses, saturation phenomena and additional mechanisms result in deviation from linearity.

Environmental exposure of humans is usually in a low-dose range, in most situations below the lowest observable effect level (LOEL). The best approach, therefore, to avoid confounding by nonlinear shapes of the dose response is to work at the limit of detection of a toxic response. This has the additional advantage that the number of experiments can markedly be reduced. If high dose levels are to be included in the evaluation of mixture effects, for instance, if there is interest in accidentally high exposure levels or in pharmacological combination treatments, it will be necessary to investigate the full dose–response relationship for the single agents.

The suggested procedure to investigate deviation from additivity at the LOEL is as follows:

- Determine (or take from literature data) an approximate LOEL for the agents to be tested in combination.
- Divide each LOEL by the number of agents to be combined (*n*).
- Measure the effect of the combination of *n* agents each at LOEL/*n*.
- Analyze the result for the significance of an interaction term by *n*-factor analysis of variance.

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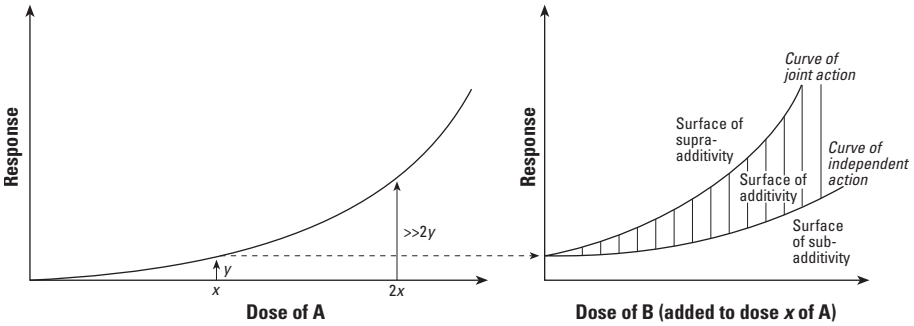

Figure 1. Schematic representation of a sublinear dose–response relationship for agent A (left) and two dose–response curves for combination effects of dose *x* of agent A with increasing dose levels of agent B (right).

In the case of additivity, the response to the combination treatment should just produce the lowest observable effect in the assay.

We used this type of approach first to investigate the combined mutagenicity of three mutagens with similar mode of action, the polynuclear aromatic hydrocarbons (PAHs) benzo[*a*]pyrene (B[*a*]P), benz[*a*]anthracene (B[*a*]A), and dibenz[*a,c*]anthracene (DB[*ac*]A), in the Ames test using *Salmonella typhimurium* tester strain TA100. For the second part, the DNA alkylating agent ethyl methanesulfonate (EMS) and γ-radiation from a ^{137}Cs source were applied and investigated for the induction of micronuclei in different cellular eukaryotic model systems of mouse and human origin. Although the initial modes of action of these two genotoxic agents are different, both lead to the formation of DNA strand breaks and chromosomal breaks.

Materials and Methods

All compounds, media, and stains were from Sigma (Taufkirchen, Germany). L5178 mouse lymphoma cells were supplied by W.J. Caspary (National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, USA). Human cell lines TK6 and WTK1 were purchased from ATCC (Manassas, Virginia, USA). Human fibroblasts were provided by the Department of Human Genetics, University of Würzburg, Germany.

Ames Test with Combinations of Three PAHs

As an approximate LOEL, we used the dose resulting in a doubling of the background numbers of revertants. The corresponding doses for TA100 were taken from the literature (*8*) as 0.3, 3, and 0.3 μg/plate, for B[*a*]P, B[*a*]A, and DB[*ac*]A, respectively.

Treatment solutions were prepared separately for each dose level by weighing the appropriate amount of chemical and dissolving it in dimethylsulfoxide (DMSO). Dilutions were prepared to obtain the required dose in 20 μL DMSO per plate. For the combination experiments, appropriate amounts of the three chemicals were weighed, and the combined portions were dissolved and diluted in DMSO to a final volume of 20 μL per plate. *Salmonella* cultures were grown overnight for approximately 10 hr and had cell titers of 3–4 × 10^9/mL. Liver 9,000×*g* supernatant (S9; protein concentration, 50 mg/mL) from Arochlor 1254–induced male Wistar rats and S9 mix containing 5% S9 and an NADPH-regenerating system were prepared. A modification of the plate incorporation test was used (*9*). We added 100 μL bacterial suspension, 20 μL DMSO containing the test compound(s), and 2 mL top agar containing histidine and biotin to vials prefilled with 500 μL S9 mix. Components were mixed and plated on Vogel-Bonner medium E with 1.5% Bacto-Difco agar and 2% glucose. After 2 days of incubation, revertant colonies were counted with an automated colony counter. Counts were corrected for overlapping colonies with a computer program. The number of replicates was $n = 6$ for the controls, $n = 3$ for the single agents, and $n = 2$ and 4 for the combination experiment at one-third and one "doubling dose," respectively.

In Vitro Micronucleus Test

Cells used were L5178Y mouse lymphoma cells, the lymphoblastoid human cell line TK6, the lymphoblastoid human cell line WTK1, and fibroblasts from lung tissue of 16-week-old human fetus. The method used for the *in vitro* micronucleus test using the L5178Y mouse lymphoma cells and the respective results have been described previously (*10*). In short, treatment included irradiation of the cells from a ^{137}Cs source (662 keV γ-radiation; dose rate, 0.6 or 1 Gy/min), immediately followed by incubation with EMS. After 4 hr, fresh medium was added, and the cells were incubated for 15 hr (30 hr for the human cell lines). Cells were put on glass slides and fixed with methanol, and DNA was stained with Hoechst 33258.

As a modification used for the main experiment with the mouse cells and for all experiments with the human cells, the inhibitor of cytokinesis cytochalasin B was added with the medium change, and acridine orange was used for staining. Cytochalasin B allows the cell to replicate the DNA and form two nuclei but not to form two cells. Scoring of micronuclei only in binucleated cells allowed us to restrict the analysis to cells that have undergone one cycle of DNA replication. This controls for effects of the treatments on the cell cycle.

Pilot studies were performed with all cell lines to investigate the low-dose linear response range. Doses that resulted in a doubling of the control values were chosen for the main experiments. No deviation from linearity was seen at this dose level in any cell line. It has to be noted, however, that background response and susceptibility of the cells to a doubling dose of the mutagens changed from cell batch to cell batch, such that responses usually ranged within a factor of 1.5–3 of the controls. For the main experiments, the number of independent replicates was $n = 3$, except for the mouse lymphoma cells treated with 0.5 Gy alone (one sample lost), for which $n = 2$.

Statistical Evaluation of Deviation from Additivity

For the testing of a putative supra-additive or subadditive effect of the combination treatment, the data were evaluated with an *n* factor analysis of variance with interaction, *n* being the number of agents tested in combination. For the Ames test data with $n = 3$ chemicals, for instance, the underlying model is described by the equation $y = ctr + a + b + c + d + e$, where *y* are the observed numbers of revertants; ctr is the expected value for the background revertants; *a*, *b*, and *c* are the expected effects of the single chemicals; *d* is the interaction term for the simultaneous administration of the three chemicals, describing the additional positive or negative effect obtained by simultaneous administration. Hence, the expected number of revertants for the simultaneous administration of the chemicals is $ctr + a + b + c + d$; *e* is the error term, accounting for the variation within groups. The error is assumed to have a normal distribution with mean 0 and identical standard deviation for all treatment groups. The *p*-value is reported for the test of the hypothesis that $d = 0$. It describes the probability that the observed difference between the effect of the mixture and the sum of the single net effects (=additivity) is different from zero by chance alone. For $n = 2$, this analysis is available in most basic statistics software. More elaborate software also allows for $n > 2$.

Note that for situations with significant nonlinearity in the dose response for the single agents, the above analysis is not appropriate.

Higher-order terms must be introduced and combination effects analyzed by testing for interaction of the higher-order terms.

Results

Ames Test with Combinations of Three PAHs

Results are shown in Figure 2. The solvent background derived from six replicates was 170 ± 26 revertants per plate. The doubling dose considered to represent an LOEL for the Ames test resulted in slightly more than a doubling of the background number of revertants for B[*a*]P (382) and DB[*ac*]A (407) but was only about 1.5-fold for B[*a*]A (262). At one-third of the doubling dose, the net increase was 28–32% of the effect at the doubling dose, indicating a linear dose response in this dose range. For B[*a*]P and DB[*ac*]A, the increase was still statistically significant ($p < 0.05$). Treatment with the combination of the three mutagens produced the result shown by the dark gray bars; the calculated additivity is represented with the dark blue bars. No deviation from additivity was observed.

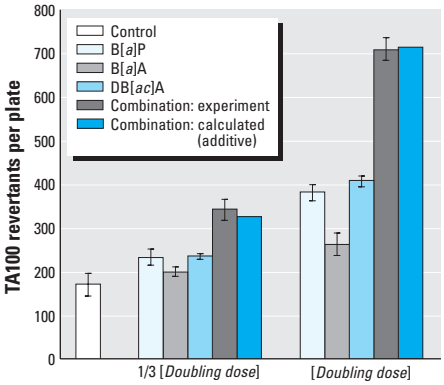

Figure 2. Mutagenicity in the Ames test (*Salmonella typhimurium* TA100/rat liver S9) of B[*a*]P, B[*a*]A, and DB[*ac*]A, tested individually and in combination. Means and standard deviations for the experimental results are given.

Induction of Micronuclei in Eukaryotic Cells by γ-Radiation and EMS

Mouse lymphoma cells L5178Y. A dose-finding study with up to 400 μg/mL EMS or 2 Gy showed no deviation from a linear dose response (*10*). Various combinations of EMS and γ-radiation within that dose range (100–400 μg EMS/mL plus 0.25–1 Gy) reproducibly showed supra-additivity (*10*). An additional experiment performed with the cytochalasin B modification is shown in Figure 3 (left). Supra-additivity by 40% was statistically significant ($p = 0.02$), using the two-factor analysis of variance with an interaction term as described.

Human cell lines TK6 and WTK1. In view of the results with the mouse lymphoma cells, the question was whether the observed supra-additivity for the induction of micronuclei by ionizing radiation and an ethylating agent was a general phenomenon or whether it was specific for a mouse cell line that harbors a mutation in the *p53* tumor suppressor gene (*11*). The lymphoblastoid human cell line TK6 (*12*), which does not have a *p53* mutation (*13*), was tested. Pilot experiments showed a linear dose response up to 1 Gy and 200 μg/mL EMS (data not shown). Combination treatment with 0.125 Gy and 25 μg/mL EMS did not result in a deviation from additivity (Figure 3, center). If at all, a putative deviation would be subadditive.

For the investigation of whether the difference between the mouse lymphoma cells and the human TK6 cell line was because of the difference in the p53 status, the related human cell line WTK1 (*14*), which does have a mutation in the *p53* gene (*13*), was used. After checking for dose–effect linearity (data not shown), the main experiment with 0.15 Gy and/or 50 μg/mL EMS was performed. The results are shown in Figure 2 (right). No deviation from additivity was seen. Again, if there was a deviation at all, it would be subadditive.

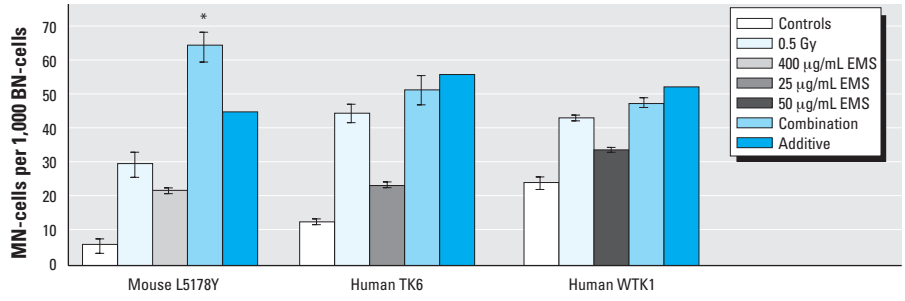

Figure 3. Induction of micronuclei in one mouse cell line and two human cell lines, by treatment with γ-radiation, EMS, and their combination. The horizontally hatched bar represents additivity (calculated sum of the control level plus the two net effects). BN-cells, binucleate cells; MN-cells, micronucleus-containing binucleate cells. Means and standard deviations for the experimental results are given. Asterisk (*) indicates significant deviation from additivity by two-factor analysis of variance with interaction ($p = 0.02$).

Human primary fibroblasts. One problem when using cell lines is that they have lost mortality. The genomic changes associated with this feature might have been responsible for deviation from additivity in the mouse lymphoma cells. Therefore, "normal" human cells should be investigated. The results of a pilot study with fetal human fibroblasts treated with 1 Gy ^{137}Cs irradiation and/or 200 μg/mL EMS are shown in Table 1. There was no indication of a deviation from additivity. Subsequent experiments confirmed this finding (*15*).

Discussion

The basic understanding of the toxicology of chemical mixtures was described more than 60 years ago (*1*). A recent review gives a comprehensive overview on the various concepts, experimental strategies, data analyses, and risk assessment procedures that have been suggested (*16*). Still, there appears to be a lack of simple experimental guidelines. The present contribution is an attempt in this direction. The first point addressed, the problem of nonlinear dose–response relationships, has been a point of concern, and the idea to focus on a dose range near the limit of detection has been put forward before (*17*). Also, statistical procedures that include information on the dose–response relationship of the individual components have been suggested (*18,19*). Our approach combines the two issues with an experimental protocol that is easily followed by toxicologists.

LOEL for the Ames Test at the Doubling Dose?

In view of the relative ease in performing an Ames test, numerous mixture studies have been performed before, but none so far have included dose levels below the doubling dose. The present results did not indicate any deviation from additivity. This was not surprising, however, in view of the same mode of mutagenic action of B[*a*]P, B[*a*]A, and DB[*ac*]A,

Table 1. Induction of micronuclei in human primary fibroblasts by treatment with ionizing radiation from a ^{137}Cs source and EMS, individually and in combination (pilot study with duplicates).

Treatment	MN-cells per 1000 BN-cells (duplicates)		Mean	Net increase
Controls	19	20	19.5	
γ-Irradiation (1 Gy)	118	119	118.5	99
EMS (200 μg/mL)	25	29	27	7.5
Sum of net increases of individual treatments				106.5
Combination treatment	131	116	123.5	104

Abbreviations: BN-cells, binucleated cells; MN-cells, micronuclei-containing binucleated cells.

based on the formation of DNA adducts of similar type. Additivity had been observed before, in connection with spiking diesel exhaust particle extracts with PAHs (*20*).

As a byproduct, the present study shed some light on the rule of thumb that the lowest observable effect in the Ames test is a doubling of the control number of revertants. This simple and convenient rule is still regarded by many as the lowest acceptable sign of mutagenicity (*21*). It is often applied together with the requirement of a dose-dependent response to keep the number of false-positive results low. Although this criterion might be appropriate in terms of biological relevance, it is hardly acceptable from a statistical point of view. Based on the data presented here, it appears that the limit of detection could be lower by a factor of almost 3 for the strain TA100 and when based on three independent replicates.

Combined Exposure to Radiation and Chemicals

Annex H of the UNSCEAR 2000 Report (*5*) is an in-depth discussion of "combined effects of radiation and other agents" and includes an extensive reference list. A review that focuses on human health risks is also available (*22*). Examples of "synergism" outnumber observations of additivity or antagonism. This might in part be the result of nonlinear dose–response relationships when including the high dose levels of exposures at the workplace, in accidents, and from therapeutic procedures. The UNSCEAR Report states:

> In general, for short exposures to high concentrations and for low chronic concentrations, deviations from additivity are small, if at all existent. In most epidemiological and experimental studies, effects exceeding a level predicted from isoaddition have not specifically been demonstrated. (*5*)

Indeed, recent experimental studies rarely show deviation from additivity. This includes the effects of the tobacco-related nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in combination with α-particle radiation in a human–hamster hybrid cell assay for toxicity and mutagenicity (*23*) or the combined effects of γ-radiation and ethylene oxide in human diploid fibroblasts (*24*). The induction of micronuclei in polychromatic erythrocytes of bone marrow of mice also was additive for the combination of X rays with cyclophosphamide or mitomycin C (*25*). An example for synergistic effects induced by the combination of an activation-independent alkylating agent with X rays is the induction of mutation in the *Tradescantia* stamen-hair system (*26*).

Cell Line Differences

The difference between the mouse lymphoma cells and the human cell lines could be due to species differences, the mutation in the *p53* gene, or additional mutations in the mouse cell line (*15*). Species differences were indicated by the background number of micronucleus-containing cells and the susceptibility to the mutagens. Although 0.5 Gy and/or 400 μg/mL EMS were required with the mouse cells, dose levels lower by a factor of 3–16 were sufficient for a comparable effect in the human cells.

Although no difference between the human lymphoblastoid cell lines TK6 (normal *p53*) and WTK1 (mutant *p53*) in the response to the combination treatment was observed in the present study, folate deficiency interacted significantly with EMS for the induction of hypoxanthine–guanine phosphoribosyltransferase mutations in the same two cell lines (*27*). This illustrates again that any result of a mixture effect in a cellular model may be not only species specific and cell-type specific but also agent specific.

Conclusions

- Combination experiments should be performed at the limit of detection of a toxic effect. First, "low dose" is as close as possible to most environmental human exposure levels; second, the danger of a nonlinear dose response for the single agents (which could result in a misinterpretation of the data as showing deviation from additivity) is minimized; third, the number of dose levels to be tested is reduced if high dose combinations are not included.
- Analysis of variance with an interaction term is a readily available statistical procedure that lends itself to the analysis of the mixture data obtained within a linear dose response.
- Results of cellular systems must be interpreted with extreme caution. They may be specific to species, cell type, and agent and may not be extrapolated to other situations.

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