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Comparison of methods for evaluating drug-drug interaction

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1. ABSTRACT

The goal of the present report is to compare several published methods of analyzing drug-drug interaction data. The compared methods are the curve-shift analysis, isobologram, combination index, and universal surface response analysis, and the comparison was based on analysis of published cytotoxicity data of combinations of two anti-folate agents. Major findings are as follows. The curve shift analysis enabled the inspection of the experimental data and visual evaluation of the approximate parallelism between the dose response curves. Isobologram analysis provided the range of concentration ratios where maximal synergy was obtained. The combination index analysis readily provided quantitative estimation of the extent of synergy or antagonism. The universal surface response method summarized drug-drug interaction in a single parameter, facilitating comparison of larger arrays of combinations. Only the curve shift analysis and the universal surface response method yielded a statistical estimate of differentiation between synergy, additivity, and antagonism. In summary, curve shift analysis, isobolograms, combination index analysis, and the universal response surface method are useful methods for analyzing drug-drug interaction, and provide complementary information.

2. INTRODUCTION

Evaluation of drug-drug interaction is important in all areas of medicine. The nature and the extent of drug interaction are usually determined in *in vitro* studies. Computational approaches have been used to analyze experimental data for the nature of interaction, i.e., synergistic, additive or antagonistic. *In situ*ations where the mechanisms of drug actions and drug-drug interactions are well understood, mechanism-based pharmacodynamic modeling is a valuable tool (1). However, in the more common situations where there are insufficient mechanistic understandings to allow a well defined method, empirical methods based on Loewe additivity can be applied (2-4). The theoretical basis and methods for analyzing drug-drug interaction have been reviewed previously (5, 6).

Loewe additivity has become the basis for the following contemporary methods used to analyze drug-drug interaction. The isobologram analysis (7) evaluates the nature of interaction of two drugs, i.e., drug A and drug B, at a given effect level. Operationally, the concentrations required to produce the given effect (e.g., IC_{50}) are determined for drug A ($IC_{x,\ A}$) and drug B ($IC_{x,\ B}$) and indicated on the x and y axes of a two-coordinate plot, forming the two points ($IC_{x,\ A}$, 0) and (0, $IC_{x,\ B}$). The line

connecting these two points is the line of additivity. Then, the concentrations of A and B contained in combination that provide the same effect, denoted as ($C_{A,x}$, $C_{B,x}$), are placed in the same plot. Synergy, additivity, or antagonism is indicated when ($C_{A,x}$, $C_{B,x}$) is located below, on, or above the line, respectively.

Combination index (CI) is calculated by Eq. 1.

$$CI = \frac{C_{A,x}}{IC_{x,A}} + \frac{C_{B,x}}{IC_{x,B}} \quad (1)$$

A CI of less than, equal to, and more than 1 indicates synergy, additivity, and antagonism, respectively.

Our laboratory recently described the curve-shift analysis and proposed the simultaneous use of isobologram, combination index, and curve-shift analyses for the evaluation of interaction in anticancer agents (8). Curve-shift analysis is a two-dimensional graphical data representation that directly compares the concentration-effect curves obtained for each of the dilution series associated with the selected concentration ratios in the typical experimental design. Concentrations of single agents and combinations are normalized to the corresponding IC_{50} equivalents of single agents, as previously introduced (5, 9-11), and analyzed by nonlinear regression using the Hill equation. A leftward shift of combination concentration-effect curves relative to the curves for both of the single agents indicates Loewe synergy and a rightward shift indicates Loewe antagonism. Because of the two-dimensional format, visual inspection of goodness of fit of experimental data points, and of differences in slopes of the family of the dose response curves is facilitated.

We showed that non-linear regression analysis in fitting model equations to effect data represented an improvement over linear regression analysis in fitting model equations to transformed effect data, which has been frequently used for the combination index analysis (8).

An additional analysis method, proposed and applied by Greco *et al.* (6,12-13), is the "universal response surface method". This method assumes that the concentration-effect relationship for each drug separately follows the Hill equation and is designed to simultaneously fit all combination data to a single function. The fitting function (Eq. 2) defaults to Loewe additivity when the "synergism-antagonism parameter" alpha has a value of zero. Deviation from additivity results in a positive fitted value of alpha for synergistic interaction, and a negative value of alpha for antagonistic interaction.

$$1 = \frac{C_{A,x}}{IC_{50,A}\left(\frac{E}{E_{max}-E}\right)^{1/m_A}} + \frac{C_{B,x}}{IC_{50,B}\left(\frac{E}{E_{max}-E}\right)^{1/m_B}} + alpha\frac{C_{A,x}C_{B,x}}{IC_{50,A}IC_{50,B}\left(\frac{E}{E_{max}-E}\right)^{1/(2m_A)+1/(2m_B)}} \quad (2)$$

Considerable debate remains with respect to the method-of-choice for analyzing drug-drug interaction data (14). The goal of the present report is to compare several methods of data analysis. The comparison used the literature data on the combination effect on tumor cell growth of two anti-folate agents, i.e., the dihydrofolate reductase inhibitor trimetrexate and the glycinamide ribonucleotide formyltransferase inhibitor AG2034 (12). The anti-proliferation effects of these agents, alone or in combination, were studied in the presence of low and high concentrations of folic acid to determine the effect of folates on the interaction between the two agents acting through inhibition of different members of the de novo purine and thymidylate synthesis pathways; the results were analyzed using the universal response surface method. The current study compared the results of curve shift analysis, isobolograms and combination index analysis to the results of the universal response surface method.

3. COMPARISON OF DRUG-DRUG INTERACTION ANALYSIS METHODOLOGIES – APPLICATION TO PUBLISHED DATA

3.1. Description of dataset

The experimental data was provided by Dr. William Greco (Roswell Park Cancer Institute, Buffalo, NY) and was previously reported by Faessel *et al.* (12). In brief, exponentially growing mycoplasma-free HCT-8 human ileocecal adenocarcinoma cells were treated with AG2034 alone, trimetrexate alone, and their combinations, for 96 h. The trimetrexate -to-AG2034 concentration ratios were 1:0.1, 1:0.2, 1:0.5, 1:1.25, 1:2.5, 1:5, 1:10, 1:20, 1:50, 1:125, and 1:250 in the presence of 2.3 microM folic acid, and 1:1, 1:2, 1:5, 1:12.5, 1:25, 1:50, 1:100, 1:200, 1:500, 1:1250, and 1:2500 in the presence of 78 microM folic acid (5 replicates per data point). To examine the effects of folic acid, the culture medium was supplemented with either low or high concentrations of folic acid (i.e., 2.3 or 78 microM).

Drug activity was measured by the sulforhodamine B (SRB) method; the absorbance readings (OD values) were corrected for the reported, extrapolated background reading of 0.133 (12). We usually correct with the asymptotic minimum OD value for each dilution series (8), but deviated slightly from this practice for a more direct comparison with the data analysis presented in Faessel *et al.* (12), which used a single background value for correction. The deviation was minimal, averaging 0.9 % of the OD reading for control cells. All SRB absorbance readings at zero drug concentration are averaged and the mean is used as OD at control. The drug effect is measured by (OD at control-OD after treatment)/ OD at control *100%.

3.2. Methodologies

Isobologram, combination index, and curve shift analysis are derivatives of Loewe additivity model (5-6), which is based on the assumption that a drug cannot interact with itself.

3.2.1. Isobologram analysis

The isobologram analysis provides a graphical presentation of the nature of interaction of two drugs, i.e., drug A and drug B (7). First, in a two-coordinate plot with one coordinate representing concentration of drug A and the other representing concentration of drug B, the concentrations of drugs A and B required to produce a defined effect x (e.g., $IC_{50,A}$ and $IC_{50,B}$ when x=50%),

when used as single agents, are placed on the x and y-axes, respectively. The line of additivity is constructed by connecting these two points (e.g., ($IC_{50,A}$, 0) and (0, $IC_{50,B}$) for a 50% effect isobologram plot). Second, the concentrations of the two drugs used in combination to provide the same effect x (e.g., x=50%), denoted by point ($C_{A,x}$, $C_{B,x}$), are placed in the same plot. Synergy, additivity, or antagonism is indicated when this point is located below, on, or above the line, respectively.

3.2.2. Combination index analysis

Combination index provides a quantitative measure of the extent of drug interaction at a given effect level (5, 6, 15). That is, the combination concentrations of drug A and drug B to produce a effect x, $C_{A,x}$ and $C_{B,x}$, are normalized by their corresponding concentrations that produces the same effect as a single agent, $IC_{x,A}$ and $IC_{x,B}$, respectively. The sum of $C_{A,x}/IC_{x,A}$ and $C_{B,x}/IC_{x,B}$ is defined as the combination index at effect x as indicated by Eq. 1. If not available from experimental data, predicted concentrations of $C_{A,x}$ and $C_{B,x}$, based on regression-derived Hill parameters of the studied combination ratio, were used to calculate combination index at any effect x (8, 15). Therefore, combination index curves can be generated by plotting combination indices against a series of effect levels. It is worth noting that combination index curves generated by Zhao *et al* (8) did not use the CALCUSYN program made available by Chou and Talalay (15), and instead were obtained by performing data fitting using nonlinear regression without logarithmic transformation.

3.2.3. Curve shift analysis

Curve shift analysis allows simultaneous presentation of the studied concentration-effect curves of singe-agent and combination treatments in a single plot.

Single agent dose-response relationships were analyzed using the Hill equation (Eq. 3)

$$E = E\max \cdot \frac{C^n}{IC_{50}^n + C^n} \quad (3)$$

Where E is the measured effect; C is the drug concentration; Emax is the full range of drug effect, and was set at 100%; IC_{50} is the drug concentration producing the median effect of 50%; and n is the curve shape parameter describing the steepness of the concentration-effect relationship.

The combination concentrations of drugs were normalized to their respective single agent IC_{50}. Eq. 4 states the IC_{50}-equivalent concentration of Drug A or Drug B, used alone or in combination with each other, required to produce x% effect. Note that for single agent, one of the two terms ($C_{A,x}$ or $C_{B,x}$) on the right hand side of the equation becomes zero.

$$IC_{50}\ Equivalent\ Concentration = \frac{C_{A,x}}{IC_{50,A}} + \frac{C_{B,x}}{IC_{50,B}} \quad (4)$$

Where $IC_{50,x}$ is the IC_{50} value of drug X. Substituting Eq. 4 into Eq. 3 yielded Eq. 5, which describes the effects of combination therapy as a function of IC_{50}-equivalent concentrations. $IC_{50,combo}$ and n_{combo} are the values for the combination therapy.

$$Combination\ Therapy\ Effect = \frac{E_{\max}\left(\frac{C_{A,x}}{IC_{50,A}} + \frac{C_{B,x}}{IC_{50,B}}\right)^{n_{combo}}}{\left(\frac{C_{A,x}}{IC_{50,A}} + \frac{C_{B,x}}{IC_{50,B}}\right)^{n_{combo}} + (IC_{50,combo})^{n_{combo}}} \quad (5)$$

Plotting the effects of single agents and combinations against IC_{50}-equivalent drug concentrations enables the simultaneous presentation of these concentration-effect curves in a single plot. Due to the normalization, the curves for the single agents will have an IC_{50} value of one " IC_{50} equivalent", while synergistic combinations will have a lower IC_{50} value resulting in a leftward shift, and antagonistic combinations will show a rightward shift.

3.3. Computer software packages and procedures

All programming codes and calculations used SAS language and procedures (SAS, Cary, NC). Nonlinear regressions were performed using the SAS/STAT Proc NLIN routine with the unweighted Marquardt iteration method. Graphical presentations were generated by S-plus (Insightful, Seattle, WA).

4. COMPARISON OF THE ANALYSIS OUTPUT

4.1. Curve-shift analysis

Figure 1 shows the dose response curves for trimetrexate, AG2034, or their combinations, in the presence of 2.3 or 78 microM folic acid. Table 1 summarizes the nonlinear fitting results.

In general, the plots showed well-spaced concentration points, with several data points near the IC_{50} value. The experimental design used approximately three-fold steps in concentration dilution; this practice provided, in most curves, at least two points in the middle range of approximately 20 to 80% effect. All concentration-effect curves for various trimetrexate and AG2034 combinations were situated close to or to the left of the curves for the two single agents, indicating additivity or synergy. Differences were observed for the curves obtained at low and high folic acid concentrations.

At the high folic acid concentration (78 microM), all concentration-effect curves for the combinations exhibited a strong leftward shift compared to single-agent curves, indicating synergistic interaction between trimetrexate and AG2034. The IC_{50} equivalents for the combinations ranged from 0.1 to 0.72. The corresponding extent of synergy ranged from a 1.5- to 10-fold leftward shift in the concentration-effect curves. The maximal 8- to 10-fold synergy was observed at about 1:50 trimetrexate:AG2034 molar concentration ratio. Note that most of the concentration-effect curves were in parallel,

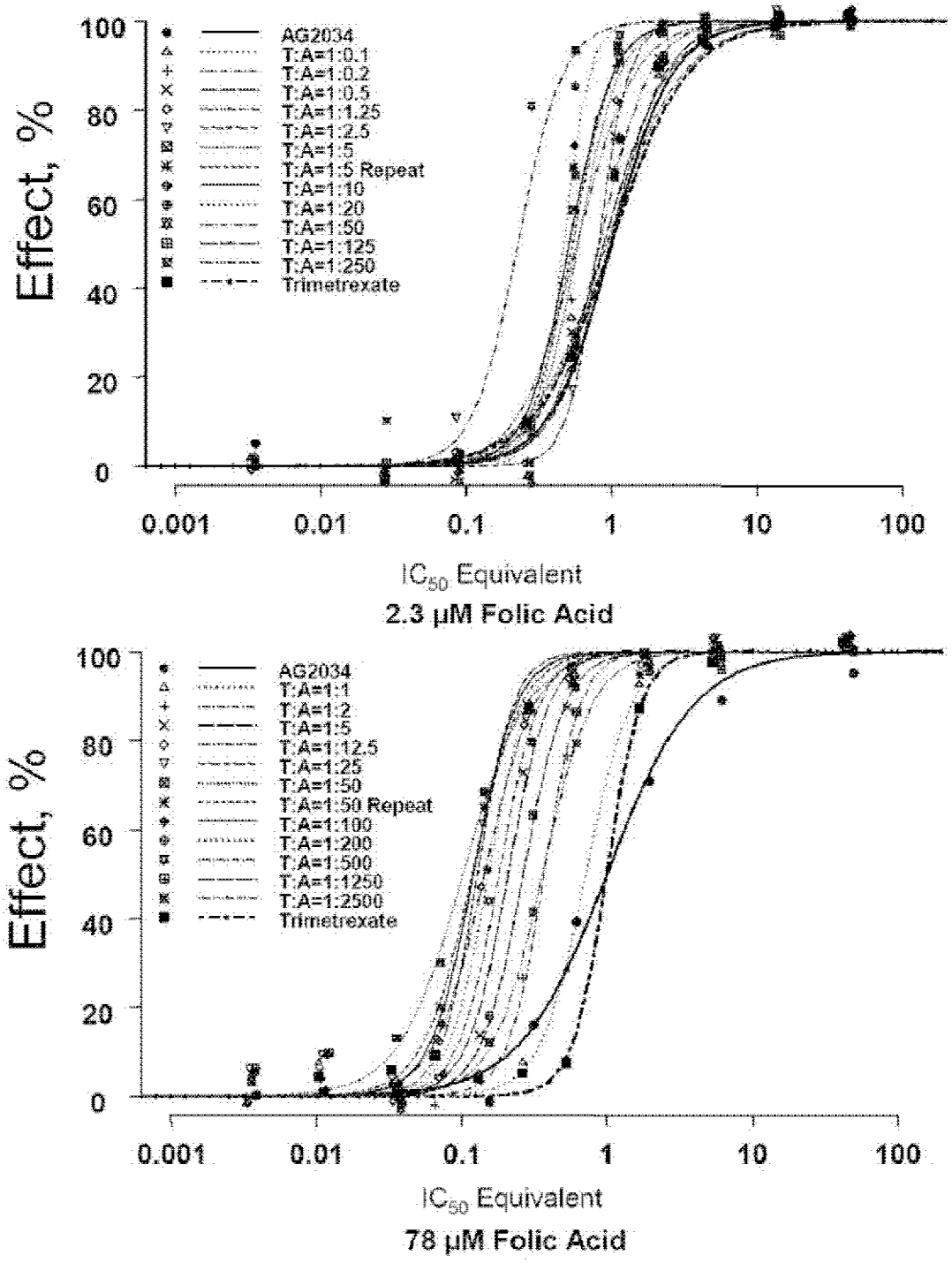

Figure 1. Curve shift analysis. The experimental combination concentrations were normalized to IC_{50}-equivalents of single agents. Data were analyzed using nonlinear regression without weighting. The data points are mean values of five replicates. The lines are best-fitted regressed lines. A leftward shift of concentration-effect curves for combinations when compared to single agent curves indicates synergism, and a rightward shift indicates antagonism. T:A indicates trimetrexate-to-AG2034 ratios in their molar concentration. Experiment with ratio of T:A=1:50 has been performed twice; the second experiment is labeled T:A=1:50 repeat. Note that the legend gives the molar concentration ratios of the trimetrexate:AG2034 mixtures. However, the X axis (logarithmic scale) is the total concentration of trimetrexate plus AG2034 expressed in $IC_{50,\ equivalents}$ as calculated by Eq. 4.

Table 1. Results of curve shift analysis

PteGlu, microM	Combination Ratio	Initial concentration before dilution		$IC_{50,\ combination}$ IC_{50} equivalents	n	Evaluation at 50% Effect Level
	Trimetrexate:AG2034	Trimetrexate, microM	AG2034, microM	Mean+/-SE	Mean+/-SE	
2.3	AG2034 alone	0.00	2.78	1.00+/-0.06	3.12+/-0.53	
	1:0.1	0.55	0.05	0.96+/-0.06	2.29+/-0.28	Additive
	1:0.2	0.54	0.11	0.91+/-0.08	2.26+/-0.42	Additive
	1:0.5	0.51	0.25	0.90+/-0.10	2.82+/-0.77	Additive
	1:1.25	0.45	0.56	0.89+/-0.03	3.81+/-0.31	Synergy
	1:2.5	0.37	0.93	0.89+/-0.06	5.00*	Additive
	1:5	0.28	1.39	0.63+/-0.02	5.00*	Synergy
	1:5 repeat	0.28	1.39	0.58+/-0.02	5.00*	Synergy
	1:10	0.19	1.85	0.56+/-0.01	4.92+/-0.43	Synergy
	1:20	0.11	2.22	0.53+/-0.03	5.00*	Synergy
	1:50	0.05	2.53	0.26+/-0.01	5.00*	Synergy
	1:125	0.02	2.67	0.99+/-0.07	3.19+/-0.59	Additive
	1:250	0.01	2.73	0.60+/-0.02	3.87+/-0.57	Synergy
	Trimetrexate alone	0.56	0.00	1.00+/-0.64	2.32+/-0.33	
78	AG2034 alone	0.00	27.78	1.00+/-0.14	1.45+/-0.23	
	1:1	0.55	0.54	0.72+/-0.05	2.80+/-0.43	Synergy
	1:2	0.54	1.07	0.37+/-0.02	3.53+/-0.49	Synergy
	1:5	0.51	2.53	0.21+/-0.01	3.12+/-0.52	Synergy
	1:12.5	0.45	5.56	0.14+/-0.01	2.54+/-0.35	Synergy
	1:25	0.37	9.26	0.13+/-0.01	3.55+/-0.74	Synergy
	1:50	0.28	13.89	0.10+/-0.01	2.00+/-0.10	Synergy
	1:50 repeat	0.28	13.89	0.12+/-0.01	2.59+/-0.42	Synergy
	1:100	0.19	18.52	0.12+/-0.01	2.96+/-0.34	Synergy
	1:200	0.11	22.22	0.15+/-0.01	3.21+/-0.54	Synergy
	1:500	0.05	25.25	0.18+/-0.02	3.16+/-0.82	Synergy
	1:1250	0.02	26.71	0.27+/-0.02	3.03+/-0.69	Synergy
	1:2500	0.01	27.24	0.36+/-0.04	2.55+/-0.61	Synergy
	Trimetrexate alone	0.56	0.00	1.00+/-0.08	3.73+/-0.62	

Experiments were conducted at two levels of folic acid. Combination ratio indicates the trimetrexate-to-AG2034 ratio in their actual concentrations (microM). Initial concentrations are the starting concentrations, which were subsequently diluted at fixed concentration ratios. $IC_{50,\ combination}$ is the combination concentration in IC_{50} equivalents, as calculated by Eq. 4. SE is the corresponding standard error. The critical value for the inverse cumulative T-distribution (type I error rate = 0.025, two sides, degrees of freedom = 53) is equal to 2.01. $IC_{50,\ combination}$ +2.01 SE less than 1, $IC_{50,\ combination}$ −2.01 SE <1< $IC_{50,\ combination}$ +2.01 SE, and $IC_{50,\ combination}$ −2.01 SE >1 indicate synergy, additivity, and antagonism, respectively. Parameter n is the curve shape parameter describing the steepness of the concentration-effect relationship. IC_{50} values for pure agents are as follows. AG2034 in 2.3 microM folic acid: 0.0063±0.0.004 microM, in 78 microM folic acid: 0.56±0.0.8 μM (universal response surface estimates: 0.0035 and 0.414 microM, respectively). Trimetrexate in 2.3 microM folic acid: 0.0014±0.0009 microM, in 78 microM folic acid: 0.013±0.001 microM (universal response surface estimates: 0.0015 and 0.013 microM, respectively).***:** The fitted value of n is limited to 5.00. At this value, effect declines over the effect range (e.g. from 90% to 3% effect) between adjacent data points at the employed $\sqrt{10}$ or 3.16-fold sequential dilution, and higher values cannot be accurately estimated.

with the exception that AG2034 showed a shallower slope. The analysis of nonparallel curves for drug-drug interaction is considered more challenging compared to parallel curves (4, 5).

At low folic acid concentration (2.3 microM), several differences were observed. First, the IC_{50} values for single agents AG2034 and trimetrexate were about 10 and 100 fold lower compared to their IC_{50} values at high folic acid concentration. Second, not all concentration-time curves for the combinations showed an apparent leftward shift; five of the twelve combinations overlapped with the curves of single agents. This indicates additivity, which is in agreement with the finding that their combination concentrations expressed in IC_{50} equivalents (as calculated by Eq. 4) were not statistically different from 1.0 at 50% effect level (Table 1). A second cluster of six curves showed a shift to the left; the combination concentrations expressed in IC_{50} equivalents were between 0.5 and 0.6 at 50% effect level, indicating a synergy of about two-fold at this level. Finally, one combination (trimetrexate:AG2034 ratio of 1:50) showed the furthest shift to the left, which appeared to be largely the result of a single data point.

4.2. Isobologram analysis

In contrast to curve shift analysis, which provides the entire spectrum of effect levels, isobologram analysis is typically conducted for single effect levels, e.g., 50% effect level. Figure 2 shows the isobolograms at 50% effect level, and Table 2 summarizes the results. At the high folic acid concentration, the isobologram analysis showed extensive synergy, with the maximum extent of about 10-fold synergy occurring at a fairly broad range of concentration ratios (the median ratio was slightly higher than 1.0).

At the low folic acid concentration, all data points for trimetrexate and AG2034 combinations were below the line of additivity, indicating synergy. Maximal synergy of approximately 2-fold was achieved at a trimetrexate-to-AG2034 $IC_{50,\ equivalent}$ concentration ratio close to one.

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