[Frontiers in Bioscience E2, 266-278, January 1, 2010]

Application and review of the separate ray model to investigate interaction effects

Roel Straetemans[1], Luc Bijnens[1]

[1]*Johnson and Johnson Pharmaceutical Research and Development, a division of Janssen Pharmaceutica N.V., Beerse Belgium*

TABLE OF CONTENTS

1. ABSTRACT

In this paper we review the application of the Separate Ray Model to analyze drug combination experiments coming from a fixed ratio design. The idea is the joint fit of separate concentration response curves to each ray under investigation leading to an interaction index for each together with a 95 percent Confidence Interval. The approach is a simple and easy to implement parametric modeling approach and allows estimation and testing of drug interactions based on regularly sampling in the entire space of all combinations going from pure compound A to pure compound B. The analysis is implemented using the SAS/STAT procedure NLMIXED. Two datasets were provided for the modeling exercise. One included different qualitative effects with some rays showing synergy, others antagonism and again others additivity. The second dataset involved the presence of very large synergy together with different observed background effects for the individual rays. The Separate Ray Model is able to handle these practical issues making it a flexible tool to investigate drug interaction experiments using a fixed ratio design.

2. INTRODUCTION

In recent years the study of drug interactions in pre-clinical drug development has gained importance among pharmacologists, biologists and statisticians. Two important questions when performing a drug interaction experiment concern the design and the statistical methodology to assess the presence of interaction. Before these questions can be answered however a clear definition of drug interaction, and how to calculate it needs to be available. When two (or more) drugs are applied as a combination in a mixture to a biological system the resulting effect can be equal or different as compared to what is expected from the biological activity of the individual compounds. In the first case no interaction is said to be present while in the second interaction is said to exist. This concept of drug interaction is generally accepted but the reference 'what is expected from the biological activity of the individual compounds' plays a crucial role and to this day is a topic of debate. Two excellent review papers (1, 2) describe existing reference models used to calculate the expected treatment effect under assumption of

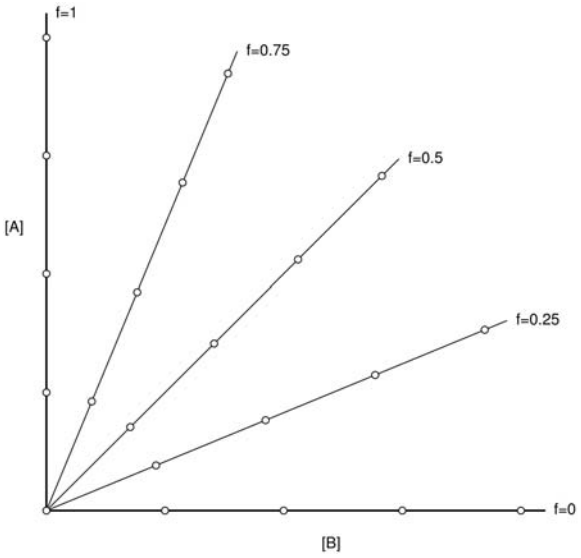

Figure 1. Illustration of the Ray design. The X-axis corresponds to the concentration of compound B and the Y-axis to the concentration of compound A. Each line corresponds to a different ray with a specific *f* value and the dots represent the *m* concentrations within a ray.

no interaction. In accordance to the authors, we consider the so called Loewe additivity model as the most suitable one and is therefore the reference model used in this paper. Next to the choice of reference model there is also debate on how to define drug interaction terms if an interaction is observed. In this paper the so called Sarriselkä agreement (3) on terminology will be used, as well as the assumption that both individual compounds are active.

The additivity term in the Loewe additivity model follows from the dose equivalent concept (4) which leads (5, 6) to the linear isobole of additivity given by equation (1)

$$1 = \frac{C_{X,a,r}}{IC_{X,A}} + \frac{C_{X,b,r}}{IC_{X,B}} \quad (1)$$

where:

$IC_{X,A}$ is the concentration of pure compound A reaching X% inhibition,

$IC_{X,B}$ is the concentration of pure compound B reaching X% inhibition,

$C_{X,a,r}$ is the concentration of compound A present in the concentration of combination (or ray) r that reaches X% inhibition and

$C_{X,b,r}$ is the concentration of compound B present in the concentration of combination (or ray) r that reaches X% inhibition. The definition of ray will be explained in more detail later on.

If this equation on the right, also known as Berenbaum's interaction index I (7), sums to one the mixture is said to be additive. When $I < 1$ Loewe synergy is concluded and when $I > 1$ the mixture is said to be Loewe antagonistic. In the remainder Loewe synergy and Loewe antagonism are abbreviated to respectively synergy and antagonism.

The remainder of the paper is organized as follows. In section 3 the details of the statistical methodology of the Separate Ray Model is explained. Section 4 contains the analysis results for both datasets and an overall discussion and conclusion will be given in Section 5.

3. STATISTICAL METHODOLOGIES

The applied study design of the two experiments is the so called fixed ratio or ray design (8). In this design the two individual compounds are mixed according to some fraction *f* to form a mixture Z, as

$$Z = f\ IC_{X,A} + (1\text{-}f)\ IC_{X,B} \quad (2)$$

with $IC_{X,A}$ and $IC_{X,B}$ being preliminary estimates of the concentrations of the individual compounds required to reach a certain effect level, typically the 50% effect level, and *f*, called the mixture factor, ranging from 0 to 1. Depending on the choice of *f*, different mixtures Z can be prepared. For each mixture Z, *m* dilutions are prepared to study the concentration-response profile of the combination. The *m* dilutions of a specific mixture Z are called a ray. Within a ray the ratio of the two individual compounds is constant or fixed and hence the name fixed ratio design. By choosing appropriate rays the whole spectrum of mixtures going from pure A (*f*=1) to pure B (*f*=0) can be studied. This is illustrated in Figure 1. Because of the constant proportion between the two individual compounds within a ray, the amount of one can be written as a linear expression of the other as

$$C_{X,b,r} = p_r\ C_{X,a,r} \quad (3)$$

where:

$C_{X,b,r}$ is the concentration of compound B present in the concentration of ray r reaching X% inhibition,

$C_{X,a,r}$ is the concentration of compound A present in the concentration of ray r reaching X% inhibition and

p_r is the constant proportion of the two compounds within the ray r.

In the current datasets, 12 different mixtures were prepared, each studied in 11 dilutions. In the provided datasets each ray was identified with the variable curvelo and curvehi in respectively dataset 1 and 2. In Table 1 the link between these original provided mixture identifications in the datasets and identification via ray and slope coefficient p_r in the remainder of the paper, is shown

Table 1. Identification of ray based on used curvelo identification in dataset 1 (2.3µM folic acid) and curvehi identification in dataset 2 (78µM folic acid). The table also contains the linear relation coefficient p_r for each ray

Dataset 1			**Dataset 2**		
curvelo	**ray (r)**	**p_r**	**curvehi**	**ray (r)**	**p_r**
1	1	Pure A	1	1	Pure A
2	2	Pure B	2	2	Pure B
3	3	4.964285714	3	3	49.60714
4	4	2.482453791	4	4	24.80357
5	5	9.934133847	5	5	99.21429
6	6	1.241071429	6	6	12.40179
7	7	19.85714286	7	7	198.4286
8	8	Control data	8	8	Control data
9	9	4.964342857	9	9	49.60714
10	10	0.496429694	10	10	4.960714
11	11	49.64359796	11	11	496.0714
12	12	0.198807	12	12	1.984286
13	13	124.1513778	13	13	1240.179
14	14	0.099294931	14	14	0.992143
15	15	248.4517304	15	15	2480.357
16	16	Control data	16	16	Control data

Notice how ray 1 is the concentration range of pure compound A and ray 2 of pure compound B.

The basic idea of the Separate Ray model is to simultaneously fit a concentration-response model to each ray under investigation (9, 10). Based on these individual nonlinear curves an interaction index *I* for each ray, noted as I_r, will be estimated together with a 100 (1-α)% CI. This calculated 100 (1-α)% CI is then used to conclude interaction or additivity for each specific combination (or ray). When the 100 (1-α)% CI contains 1 the null hypothesis of additivity can not be rejected. Otherwise the null hypothesis of additivity is rejected in favor of synergy when the upper limit is smaller than 1 and antagonism when the lower limit is larger than 1.

3.1. Concentration-response model

The concentration-response curve of our choice to fit to each mixture is the four parameter log-logistic curve where the mean response E(Y) is related to the concentration C b

$$E(Y) = \theta_1 - \frac{\theta_1 - \theta_2}{1 + \exp\left[(\log(C) - \log(IC_{50})) * \theta_3\right]} \quad (4)$$

where:

θ_1 is the extrapolated background at infinite drug concentration,

θ_2 is the expected response at zero drug concentration,

θ_3 is a slope parameter, where a positive value of θ_3 corresponds to a decreasing profile as is seen in the two datasets under study and

IC_{50} is the concentration at which half the effect (difference between θ_2 and θ_1) is reached.

This four parameter log-logistic model is well known to describe a sigmoidally shaped curve for response versus the logarithm of concentration. It's application, although in a simpler parameterization with only 3 parameters, assuming θ_1 to be zero, dates back to Emmens (11) and is a commonly used nonlinear model to describe concentration-response data.

Equation (4) can more generally be written as

$$Y_{r,j} = g(\boldsymbol{\theta}_r, C_{r,j}) + \varepsilon_{r,j} \quad (5)$$

where:

$Y_{r,j}$ is the response of ray r at concentration j,

g is the nonlinear function (4),

$\boldsymbol{\theta}_r = (\theta_{1,r}; \theta_{2,r}; \theta_{3,r}; IC_{50,r})$ is a ray specific parameter vector to be estimated,

$C_{r,j}$ is the concentration j of ray r and

$\varepsilon_{r,j}$ is the random error, assumed to be normally distributed with mean zero and variance covariance matrix $\boldsymbol{\Sigma}$.

3.2. Separate ray model

In the Separate Ray Model the ray specific estimated parameters of each mixture together with those from the pure compounds A and B, respectively ray 1 and 2, will be used to estimate the mixture specific interaction index I_r. The link between I_r and those ray specific parameters is based on the constant proportion of the two individual compounds within the ray as defined in equation (3). For each ray, $C_{X,r,j}$, the concentration j of ray r corresponding to X% inhibition, consists of an amount of A ($C_{X,a,r}$) and B ($C_{X,b,r}$). Specific for 50% inhibition, this corresponds to

$$C_{50,r,j} = IC_{50,r} = C_{50,a,r} + C_{50,b,r}, \quad (6)$$

which after combination with expression (3) becomes

$IC_{50,r} = (1+p_r)\, C_{50,a,r.}$ (7)

Combination of the Loewe additivity model (1), equation (3), equation (7) and replacing 1 by I_r leads then to the following expression,

$$I_r = \frac{IC_{50,r}\left(IC_{50,B} + p_r IC_{50,A}\right)}{IC_{50,A} IC_{50,B}(1+p_r)} \quad (8)$$

which equivalently can be written as

$$IC_{50,r} = \frac{IC_{50,A} IC_{50,B}(1+p_r) I_r}{IC_{50,B} + p_r IC_{50,A}} \quad (9)$$

Expressions (8) and (9) now allow us to calculate for each ray the specific interaction index I_r, based on p_r, a known constant from the design, the parameters $IC_{50,A}$ and $IC_{50,B}$ from pure A and B and the ray specific parameter $IC_{50,r}$. Equation (8) and (9) provide the I_r values at the 50% inhibition level. The above described approach can however be extended to other effect levels using the same principles. For a given effect level X expression (8) can be written as,

$$I_{X,r} = \frac{C_{X,r,j}\left(C_{X,B} + p_r C_{X,A}\right)}{C_{X,A} C_{X,B}(1+p_r)} \quad (10)$$

where:

$I_{X,r}$ is the interaction index for ray r at effect level X% inhibition,

$C_{X,r,j}$ is the estimated concentration j of ray r for which X% inhibition effect is obtained,

$C_{X,A}$ and $C_{X,B}$ are the estimated concentrations of pure compounds, A and B respectively, for which X% inhibition effect is obtained.

The estimation of the interaction index at the 50% effect level requires IC_{50} values, obtained directly as parameters from the model. Any other effect level requires C_x values that are not parameters in the model. Hence they need to be estimated via inverse estimation from the fitted concentration-response model.

The approach is implemented using the SAS/STAT® procedure NLMIXED (12). This procedure is a flexible tool which allows performing the joint fit of the different ray specific concentration-response models and estimation of I_r, the ray specific interaction indices at 50% inhibition, as parameters. In addition the NLMIXED procedure has an ESTIMATE statement which enables the user to estimate any expression from the model parameters and thus estimation of the general expression $I_{X,r}$ via equation (10). The statement uses the delta method to estimate the corresponding standard error and produce a 100(1-α)%CI. Other advantages of the NLMIXED procedure are the possibility to specify non-normal distributions for the response variable and specification of a non-constant variance function. In the two datasets studied, the final model assumed the residual variance in equation (5) to follow a normal distribution with a variance being proportional to a power of the mean, as follows

$\varepsilon_{r,j} \sim N(0, \sigma^2(\, g(\boldsymbol{\theta}_r, C_{r,j}))^{\rho}\,)$. (11)

3.3. Changes to original published work

Application of the Separate Ray Model in drug combination experiments has been published previously (10). Although the methodology has stayed the same, four practical changes compared to the original published work are incorporated. First, the originally used three parameter log-logistic concentration-response model is extended to a four parameter log-logistic model. Second, combination indices for effect levels different from 50% are calculated, using the ESTIMATE statement in the NLMIXED procedure. Third, the model fitted is slightly reparameterized. Compared to the original SAS code provided, the separate Ray model is fitted using the log-logistic parameterization in equation (4), instead of the Hill parameterization in equation (12),

$$E(Y) = \theta_1 - \frac{\theta_1 - \theta_2}{1 + \left(\frac{C}{IC_{50}}\right)^{\theta_3}} \quad (12)$$

and the ray specific interaction indices I_r are fitted or estimated (when different from 50%) as lognormal parameters. This has the advantage that all estimated values and confidence interval of the concentration parameters as well as the ray specific interaction indices are always strict positive. Finally, compared to the model fitted in the original published work, no model adjustment for possible row and column effects is incorporated.

3.4. Hard- and Software

All analyses were performed using SAS v9.1.3 under Microsoft Windows 2000 as operation system. The computer used contained an Intel(R) Pentium (R)M processor of 1.60 GHz and had 1 Gb RAM memory. Graphs were produced using Splus v7.0 with the exception of Figure 1 which was made using PowerPoint.

4. OVERVIEW OF APPLICATIONS

For both datasets, compound trimetrexate (TMQ) and AG2034 are labeled as compound A and B respectively in the analysis.

4.1. First data set: Combination of trimetrexate and AG2034 with 2.3µM folic acid

A plot of the mean observed concentration response profiles by curvelo identification is given in

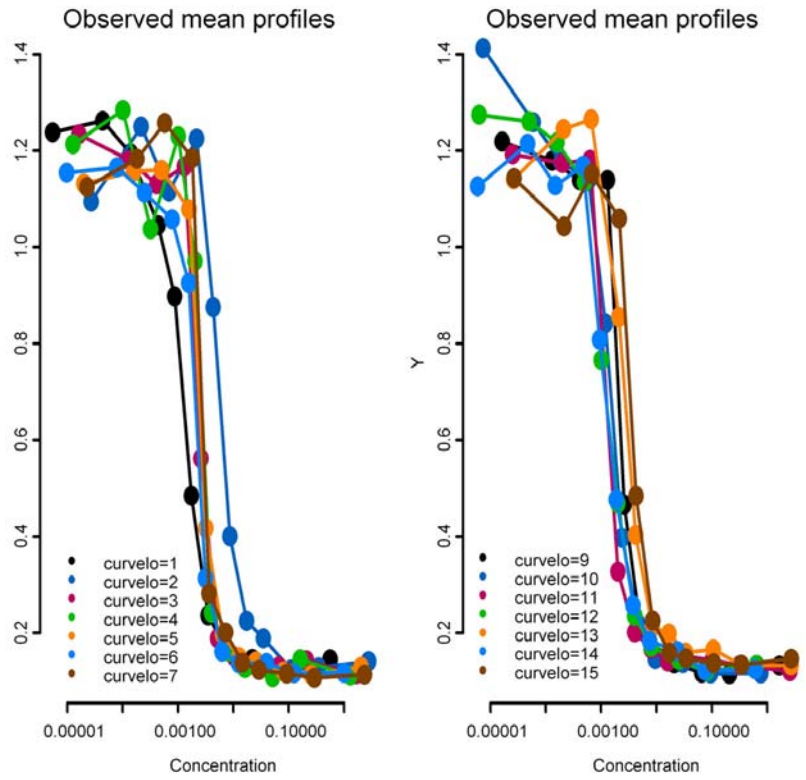

Figure 2. Observed mean concentration response profiles by curvelo identification for dataset 1. Curvelo 8 contained control data and hence is not depicted.

Figure 2. The estimates of the ray specific interaction indices together with their 95%CI are given in Table 2 for 10, 25, 50, 75 and 90% inhibition. The corresponding isobolograms can be found in Figure 3. Figure 4 is a graphical representation of Table 2.

From Table 2 and Figure 4 we notice two important results. First for a given % inhibition the estimated effect changes over the different mixtures, both in a quantitative, since different mixtures have different sizes of I_r as a qualitative meaning, as for some mixtures the null hypothesis of additivity can not be rejected, for others it can be reject in favor of synergy while for some it is rejected in favor of antagonism. Second it is seen how for a given mixture a similar change in quantitative and qualitative result can be observed in function of the inhibition level. An example is curvelo 4 which goes from synergy at the 90 and 75% inhibition level over additivity at 50% inhibition to an antagonistic effect at 25 and 10% inhibition. These results highlight the importance of being able to assess drug interaction for different mixtures of two compounds. A graphical assessment of the model fit, given in Figure 5, shows an acceptable fit for all rays.

4.2. Second data set: Combination of trimetrexate and AG2034 with 78µM folic acid

A plot of the mean observed concentration response profiles by curvehi identification is given in Figure 6. From this graph we should notice how for pure compound B (curvehi = 2) it seems that at the highest drug concentration the lower asymptote is not completely reached. Second for pure compound A (curvehi = 1) no concentrations are present between the two asymptotes. The observations are more clear in Figure 10 where only pure A and B are plotted. The estimates of the ray specific interaction indices together with their 95%CI are given in Table 3 for 10, 25, 50, 75 and 90% inhibition. The corresponding isobolograms can be found in Figure 7. Figure 8 is a graphical representation of Table 3.

Table 3, as well as Figure 8, show a large synergistic effect. Based on the point estimate of I_r all mixtures are synergistic at all levels of % inhibition. For a minority of combinations, curvehi 14 at 90% inhibition and curvehi 13, 14 and 15 at 10% inhibition, the null hypothesis of additivity can not be rejected based on the 95%CI. Notice however how no adjustment for multiplicity has been performed. As a result the overall type I error will be higher than 5%. Although multiple adjustment could be performed, these experiments typically are situated in a screening setting where the main purpose is to pick up certain effects. Those observed will then be confirmed in a second experiment. Moreover for some settings these *in vitro* combination experiments are performed to pick up strong antagonistic effects for safety reasons, meaning that non adjustment is a conservative approach. Hence the multiple comparison adjustment is not considered to be essential here.

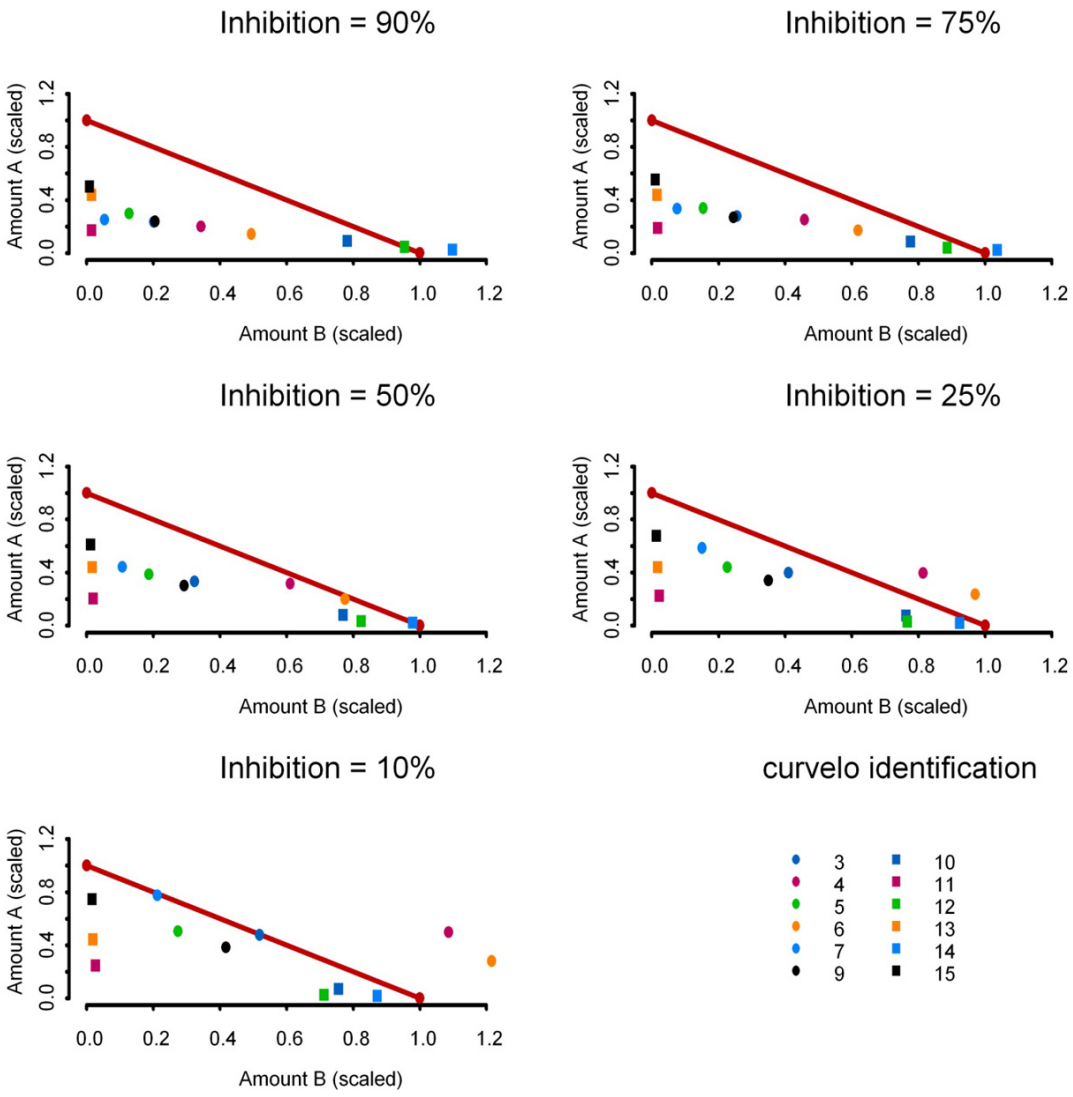

Figure 3. Isobologram for dataset 1 with the red line being the additivity line and the symbols being the different curvelo's. Compound A corresponds to trimetrexate (TMQ) and compound B to AG2034. Curvelo 8 contained control data and hence is not depicted.

A valuable observation in this dataset is the profile for pure compound A. Notice from Figure 10 how almost no data points are available in the decreasing part of the concentration-response curve. This has a clear effect on the curve estimation as shown in table 4 where the estimate of θ_3 for pure compound A is estimated with a large uncertainty. A graphical assessment of the model fit, given in Figure 9, shows an acceptable fit for all rays.

5. SUMMARY AND PERSPECTIVES

In pre-clinical research the study of combination experiments is recognized as being very valuable. A specific application is the field of oncology where efforts are made for finding successful treatments of cancer by combining different treatments. The quantification of combination therapies in clinical trials is expensive and time-consuming. As a result there is a need for the *in vitro* quantification of interaction effects. The success of the *in vitro* quantification depends on both an adequate design as well as a statistical analysis of the results. Preferably both need to be fast, easy and robust. The fixed ratio design is used in industry for these reasons. Several analysis techniques are developed to process the results coming from drug combination experiments and one of them is the Separate Ray Model. In this modeling exercise the Separate Ray Model is applied to two previously analyzed datasets coming from the field of oncology (17).

For the first dataset, the analysis result show how the interaction between TMQ and AG2034 depends both in a quantitative as qualitative manner from the mixture chosen. At each effect level it is seen how the point estimates of the interaction indices range from synergy over no interaction to antagonism. When looking at the 95%CI it can be seen that these differences are statistically

Table 2. Estimated interaction indices by ray (curvelo) for dataset 1 together with the 95%CI at 10-25-50-75 and 90% inhibition

Ray (r)	Curvelo	I_r (95%CI)				
		% inhibition				
		10%	25%	50%	75%	90%
3	3	1.00(0.78,1.27)	0.81(0.68,0.96)	0.66(0.59,0.73)	0.54(0.48,0.59)	0.44(0.37,0.51)
4	4	1.58(1.23,2.03)	1.21(1.02,1.44)	0.93(0.82,1.04)	0.71(0.64,0.78)	0.54(0.47,0.62)
5	5	0.78(0.60,1.00)	0.67(0.56,0.80)	0.57(0.51,0.64)	0.49(0.44,0.55)	0.42(0.36,0.50)
6	6	1.49(1.11,2.01)	1.21(0.98,1.48)	0.98(0.86,1.11)	0.79(0.71,0.88)	0.64(0.54,0.75)
7	7	0.99(0.72,1.37)	0.74(0.59,0.92)	0.55(0.48,0.63)	0.41(0.37,0.45)	0.31(0.26,0.36)
9	9	0.80(0.62,1.03)	0.69(0.58,0.82)	0.60(0.53,0.67)	0.51(0.46,0.57)	0.44(0.38,0.52)
10	10	0.83(0.58,1.18)	0.84(0.65,1.07)	0.85(0.72,1.00)	0.86(0.75,0.98)	0.87(0.72,1.05)
11	11	0.27(0.19,0.39)	0.25(0.19,0.32)	0.23(0.19,0.27)	0.20(0.18,0.23)	0.19(0.15,0.23)
12	12	0.17(0.12,0.25)	0.19(0.15,0.25)	0.22(0.19,0.26)	0.25(0.22,0.29)	0.29(0.23,0.35)
13	13	0.46(0.33,0.65)	0.46(0.36,0.58)	0.46(0.39,0.53)	0.45(0.39,0.52)	0.45(0.36,0.56)
14	14	0.89(0.60,1.32)	0.94(0.71,1.25)	1.00(0.83,1.20)	1.06(0.91,1.24)	1.12(0.90,1.41)
15	15	0.76(0.57,1.02)	0.69(0.56,0.85)	0.62(0.54,0.72)	0.56(0.49,0.65)	0.51(0.42,0.62)

Table 3. Estimated interaction indices by ray (curvehi) for dataset 2 together with the 95%CI at 10-25-50-75 and 90% inhibition

Ray (r)	Curvelo	I_r (95%CI)				
		% inhibition				
		10%	25%	50%	75%	90%
3	3	0.15(0.09,0.24)	0.13(0.09,0.18)	0.12(0.10,0.15)	0.12(0.10,0.14)	0.13(0.10,0.16)
4	4	0.18(0.12,0.27)	0.15(0.11,0.21)	0.14(0.12,0.18)	0.15(0.12,0.17)	0.16(0.13,0.19)
5	5	0.21(0.13,0.35)	0.17(0.12,0.23)	0.14(0.11,0.17)	0.12(0.10,0.15)	0.12(0.09,0.15)
6	6	0.15(0.09,0.23)	0.14(0.10,0.19)	0.15(0.12,0.18)	0.17(0.14,0.19)	0.19(0.16,0.24)
7	7	0.39(0.24,0.64)	0.27(0.20,0.38)	0.19(0.15,0.24)	0.14(0.11,0.17)	0.11(0.08,0.14)
9	9	0.13(0.08,0.21)	0.12(0.09,0.17)	0.12(0.09,0.15)	0.12(0.10,0.15)	0.14(0.11,0.18)
10	10	0.17(0.11,0.27)	0.18(0.13,0.26)	0.20(0.16,0.26)	0.24(0.20,0.29)	0.29(0.23,0.37)
11	11	0.45(0.27,0.73)	0.31(0.22,0.43)	0.21(0.17,0.27)	0.15(0.12,0.19)	0.11(0.08,0.15)
12	12	0.30(0.18,0.50)	0.31(0.21,0.46)	0.34(0.26,0.43)	0.37(0.31,0.44)	0.41(0.32,0.52)
13	13	0.77(0.47,1.27)	0.51(0.36,0.71)	0.33(0.26,0.42)	0.22(0.17,0.28)	0.15(0.11,0.21)
14	14	0.59(0.35,1.00)	0.63(0.42,0.94)	0.69(0.53,0.90)	0.75(0.64,0.90)	0.83(0.69,1.01)
15	15	0.73(0.42,1.26)	0.54(0.37,0.78)	0.40(0.31,0.51)	0.30(0.23,0.39)	0.23(0.15,0.33)

significant as for some the hypothesis of no interaction can not be rejected, for others it can be rejected in favor of synergy while for others in favor of antagonism. Also notice how for some mixtures the same phenomenon of changing interaction, both qualitative as quantitative, is observed when looking at different effect levels. For the second dataset the main message is the presence of large synergistic effect for most mixtures at each effect level. A second main observation is the apparent difference in background response for pure compound B. By applying ray specific concentration-response parameters this effect can easily be corrected for.

The performed analysis highlights some of the most appealing features of the Separate Ray Model. First it allows the individual quantification of interaction for different mixtures, covering the whole spectrum between the two individual compounds, by using ray specific interaction indices. Second the methodology allows the quantification of uncertainty by calculating a 95% CI for the interaction indices. These two features provide information and allow easy visualization on how the interaction, both qualitative as quantitative, can change over the different mixtures. Finally the methodology can be easily implemented using SAS, a commercially available software program. The NLMIXED procedure allows correct modeling of heteroscedasticity, a phenomenon typically observed in these concentration response profiles.

We realize that the discussed approach has its strenghts and drawbacks, compared to a response surface approach. To our knowledge, before 2005, most response surface approaches focused on calculating one single parameter for the assessment of interaction over the whole surface. Noteworthy exceptions were Minto (18) and White (19). More recently new methods such as Kong (20) and Fang (21) seem to be very promising in terms of expanding the response surface methodology to local estimation of interactions. However when the biological focus is on specific fixed fractions the ray approach has the advantage of having a richer and more informative sampling scheme. A clear advantage of the afore mentioned methods is the possibility to investigate local interactions anywhere in the entire surface, which is not possible using our current analysis model.

Finally the authors want to point out that the methodology presented makes use of the linear isobole of

Table 4. Parameter estimates, together with the lower (LL) and upper (UL) limit of the 95% CI, from the concentration response model for pure A and B for data 1 and data 2

Parameter	estimate	LL	UL
Data 1			
$\theta_{2,A}$	3.3816	3.17578	3.6008
$\theta_{2,B}$	3.2982	3.10655	3.5017
$\theta_{1,A}$	1.1479	1.13359	1.1623
$\theta_{1,B}$	1.1512	1.13577	1.1669
$IC_{50,A}$	0.0012	0.00109	0.0014
$IC_{50,B}$	0.0059	0.00533	0.0066
$\theta_{3,A}$	8.3434	5.75948	12.0864
$\theta_{3,B}$	10.9755	7.06277	17.0560
Data 2			
$\theta_{2,A}$	3.0351	2.90336	3.173
$\theta_{2,B}$	3.3639	3.17107	3.568
$\theta_{1,A}$	1.1429	1.12405	1.162
$\theta_{1,B}$	1.1952	1.15986	1.232
$IC_{50,A}$	0.0141	0.01110	0.018
$IC_{50,B}$	0.4351	0.35237	0.537
$\theta_{3,A}$	63.2820	6.73658	594.457
$\theta_{3,B}$	3.6995	2.86364	4.779

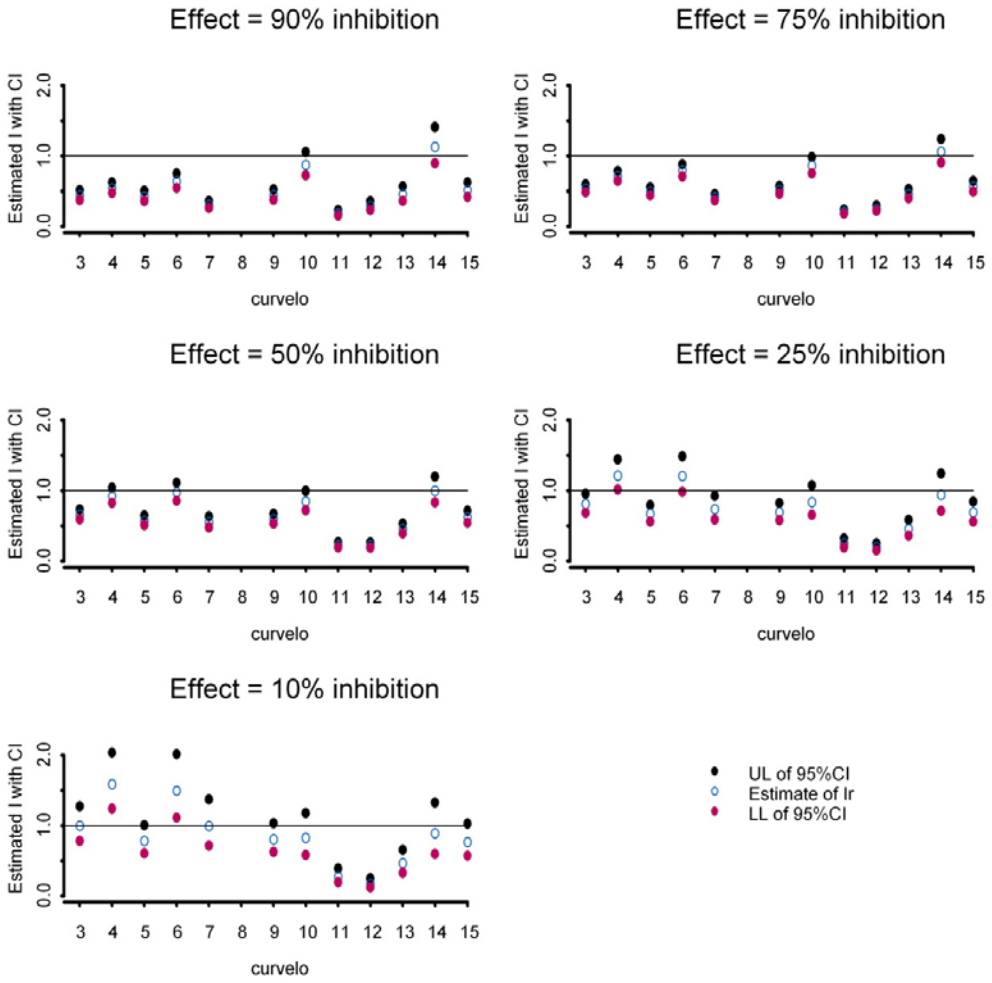

Figure 4. Point estimates and 95%CI for I_r for the different rays, identified as curvelo in dataset 1. Curvelo 8 contained control data and hence is not depicted.

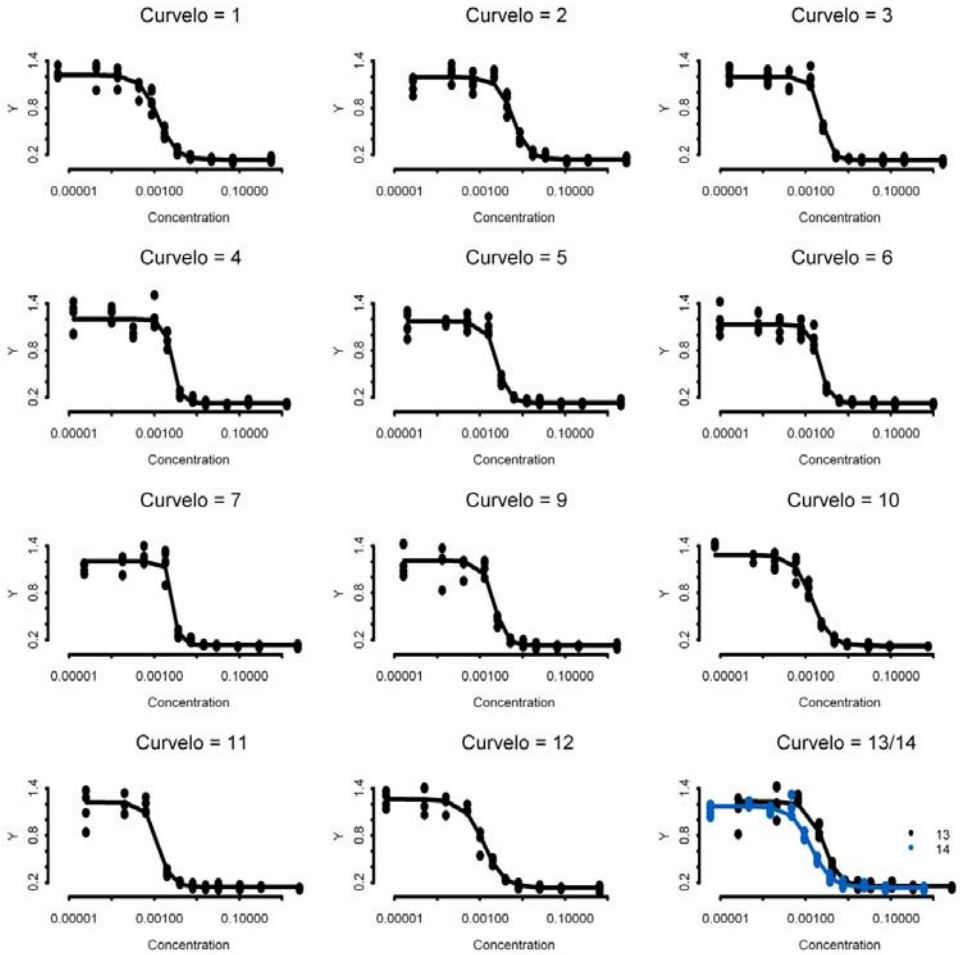

Figure 5. Model fit for dataset 1. The dots are the individual data points the solid line is the model prediction. Curvelo 8 contained control data and hence is not depicted.

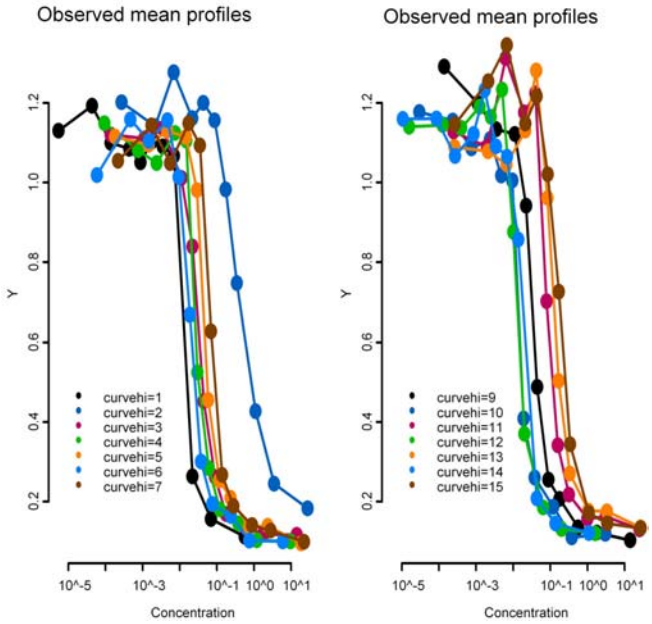

Figure 6. Observed mean concentration response profiles by curvehi identification for dataset 2. Curvehi 8 contained control data and hence is not depicted.

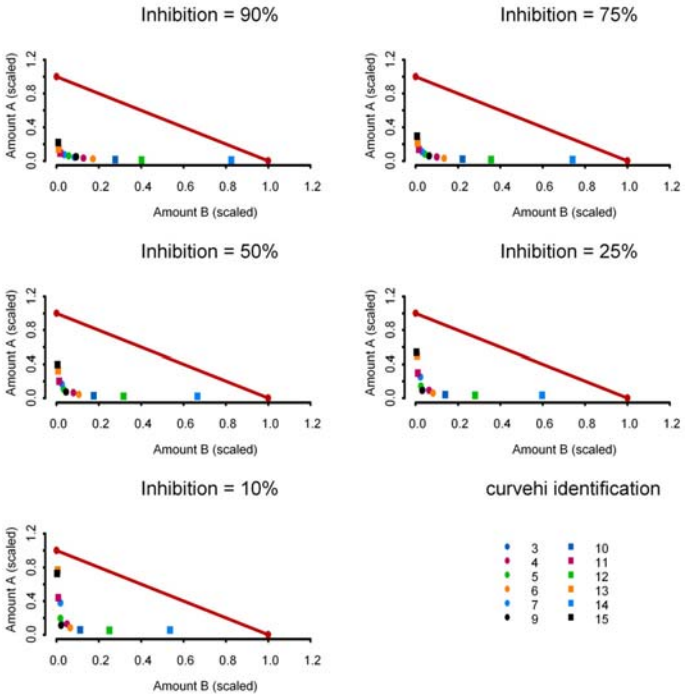

Figure 7. Isobologram for dataset 2 with the red line being the additivity line and the symbols being the different curvehi's. Compound A corresponds to trimetrexate (TMQ) and compound B to AG2034. Curvehi 8 contained control data and hence is not depicted.

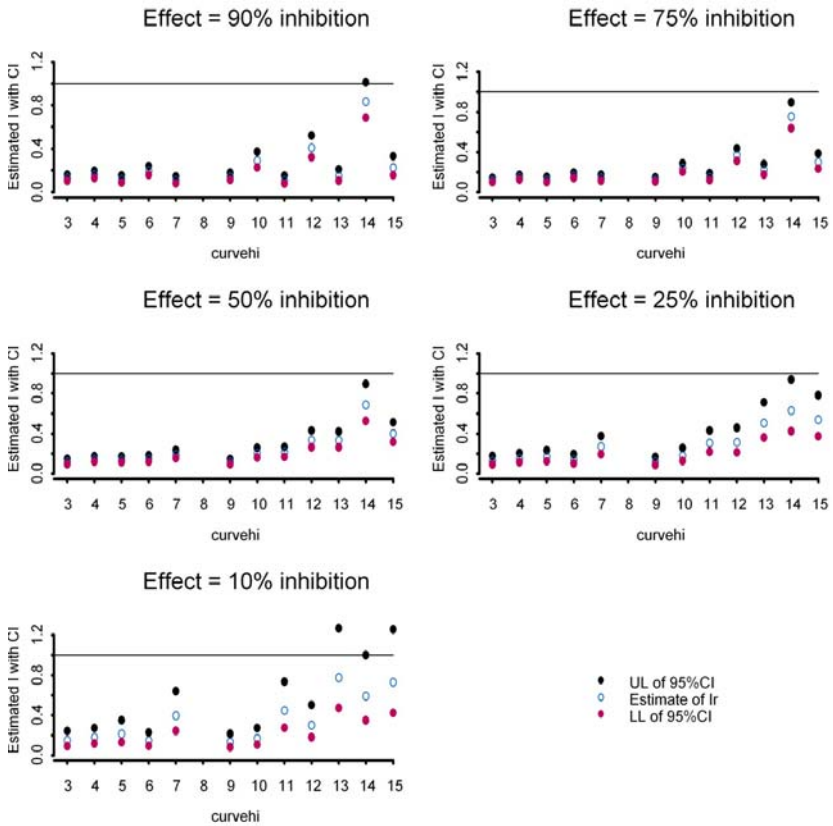

Figure 8. Point estimates and 95%CI for I_r for the different rays, identified as curvehi in dataset 2. Curvehi 8 contained control data and hence is not depicted.

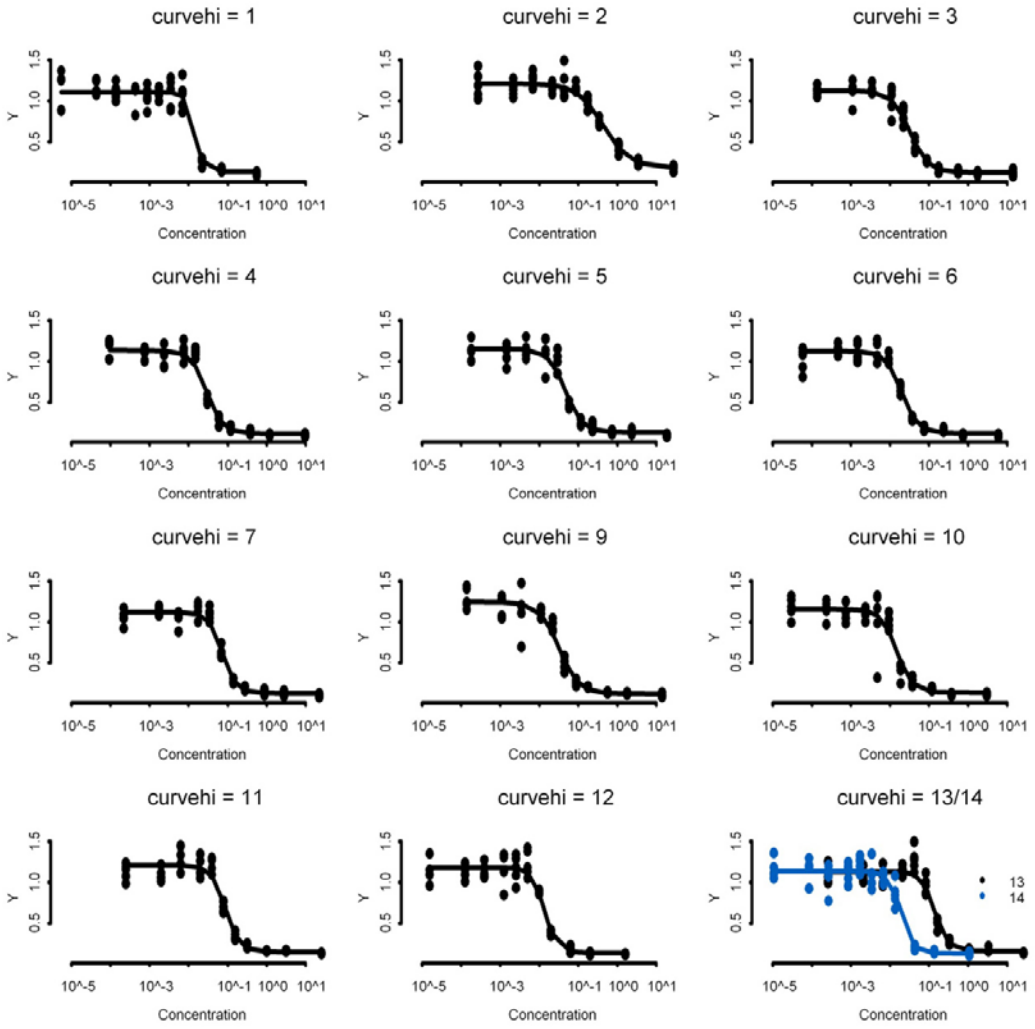

Figure 9. Model fit for dataset 2. The dots are the individual data points the solid line is the model prediction. Curvehi 8 contained control data and hence is not depicted.

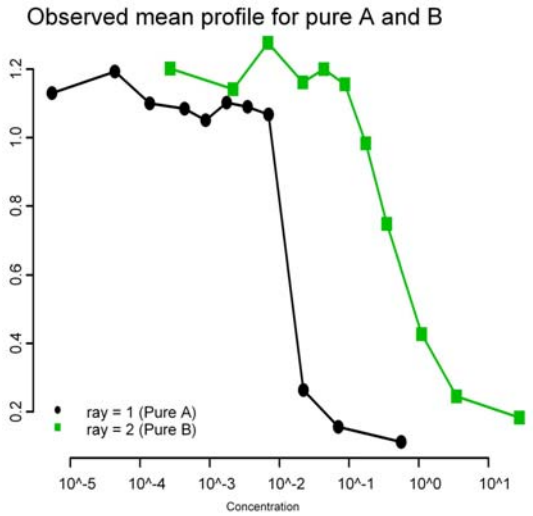

Figure 10. Observed mean profiles for pure compound A and B in dataset 2.

additivity. There is some discussion about the general validity of this linear isobole of additivity and opposing viewpoints can be found. Loewe questioned the validity and allowed the possibility of curved isoboles for which the linear one is a special case in his original paper (4). Other authors do not agree with his view and claim the lineair isobole is generally valid, as exemplified on p.100 in the review by Berenbaum (2). In some recent published work by Tallarida (5) and Grabovsky and Tallarida (6) these authors describe how the concept of dose equivalence leads to the general nonlinear isobole of additivity and claim to have proven the validity of the curved isobole where under the assumption of constant relative potency it reduces to the linear isobole of additivity. It is clear that there is discussion and controversy, a well found basis for science in general, and we would applaud any similar initiative as this current modeling exercise with respect to going in depth into the theoretical arguments of both sides.

6. ACKNOWLEDGEMENTS

The authors would like to thank Bill Greco for organizing this initiative and giving us the opportunity to participate. Also the referees are thanked for providing valuable comments which improved the paper considerably.

7. REFERENCES

1.Greco W.R., G. Bravo and J.C. Parsons: The Search for Synergy: A Critical Review from a Response Surface Perspective. *Pharm Rev* 47, 331-385 (1995)

2. Berenbaum M.C.: What is Synergy? *Pharm Rev* 41, 93-141 (1989)

3. Greco W.R., H.D. Unkelbach, G. Pöch, J. Sühnel, M. Kundi and W. Bödeler: Consensus on concepts and terminology for interaction assessment: the Sarriselkä Agreement. *Arch Complex Environ Studies* 4, 65-69 (1992)

4. Loewe S.: The problem of synergism and antagonism of combined drug. *Arzneim-Forsch* 3, 385-390 (1953)

5. Tallarida R.J.: An Overview of Drug Combination Analysis with Isobolograms. *J Pharmacol. Exp Ther* 319, 1-7 (2006)

6. Grabovsky Y. and R.J. Tallarida: Isobolographic Analysis for Combinations of a Full and Partial Agonist: Curved Isoboles. *J Pharmacol. Exp Ther* 310, 981-986 (2004)

7. Berenbaum M.C.: Synergy, additivism and antagonism in immunosuppression. *Clin Exp Immunol* 28, 1-18 (1977)

8. Tallarida R.J.: Drug Synergism and Dose-Effect Data Analysis. Chapman and Hall/CRC, Boca Raton, Fl. (2000)

9. O'Brien T.E.: Modeling and Design to Detect Interaction of Insecticides, Herbicides and Other Similar Compounds. *Johnson, D. and Milliken G., eds., Proceedings of the 15th Conference on Applied Statistics in Agriculture* 303-321 (2004)

10. Straetemans R., T.E. O'Brien, L. Wouters, J. Van Dun, M. Janicot, L. Bijnens, T. Burzykowski and M. Aerts: Design and Analysis of Drug Combination Experiments. *Biometrical Journal* 47, 299-308 (2005)

11. Emmens C.W.: The dose/response relation for certain principles of the pituitary gland, and of the serum and urine of pregnancy. *J Endocrinol* 2, 194-225 (1940)

12. Online documentation for the NLMIXED procedure can be found at http://support.sas.com/documentation/onlinedoc/91pdf/index.html

13. Feng P. and C. Kelly: An Extension of the Model Free Test to Test Synergy in Multiple Drug Combinations. *Biometrical Journal* 46, 293-304 (2004)

14. White D.B., H.M. Faessel, H.K. Slocum, L. Khinkis and W.R. Greco: Nonlinear Response Surface and Mixture Experiment Methodologies Applied to the Study of Synergism. *Biometrical Journal* 46, 56-71 (2004)

15. Minto C.F., T.W. Schnider, T.G. Short, K.M. Gregg and A. Gentilini: Response Surface Model for Anesthetic Drug Interactions. *Anesthesiology* 92, 1603-1616 (2000)

16. Machado G.W. and G.A. Robinson: A direct, general approach based on isobolograms for assessing the joint action of drugs in pre-clinical experiments. *Stat in Med* 13, 2289-2309 (1994)

17. Faessel H.M., H.K. Slocum, C. Jackson, T.J. Boritzki, Y.M. Rustum, M.G. Nair and W.R. Greco : Super *in vitro* Synergy between Inhibitors of Dihydrofolate Reductase and Inhibitors of Other Folate-requiring Enzymes: The Critical Role of Polyglutamylation. *Cancer Res* 58, 3036-3050 (1998)

18. Kong M. and J.J. Lee: A Generalized Response Surface Model with Varying Relative Potency for Assessing Drug Interactions. *Biometrics* 62, 986-995 (2006)

19. Fang H.B., D. Ross, E. Sausville and M.Tan: Experimental Design and Interaction Analysis of Combination Studies on Drugs with Log-linear Dose Responses. *Stat in Med (epub)*(2008)

20 Minto C.F., T.W. Schnider, T.G. Short, K.M. Gregg, A. Gentilini & S.L. Shafer: Response surface model for anesthetic drug interactions. *Anesthesiology* 92, 1603-1616 (2000)

21 White D.B., H.K. Slocum, Y.F. Brun, C. Wrzosek & W.R. Greco: A new nonlinear mixture response surface paradigm for the study of synergism: a three drug example. *Curr Drug Metab* 2, 399-409 (2003)

Abbreviations: CI: Confidence Interval, TMQ: trimetrexate

Key Words Synergy, Antagonism, Ray Design, Fixed Ratio Design, Loewe, Review

Send correspondence to: Roel Straetemans Turnhoutseweg 30 2340 Beerse, Belgium, Tel: 0032-0-14 60 2744, Fax: 0032-0-14 60 5802, E-mail: rstraete@its.jnj.com

http://www.bioscience.org/current/vol2E.htm