Fundamental concepts in the assessment of interaction of biological response modifiers with other agents

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WR GRECO, WE DEMBINSKI. Fundamental concepts in the assessment of interaction of biological response modifiers with other agents. Can J Infect Dis 1992;3(Suppl B):60B-68B. The universal response surface approach (URSA) was developed to assess the nature and intensity of drug interactions, ie, synergism, antagonism and additivity. URSA consists of fitting a concentration-effect surface to experimental data with maximum likelihood approaches, with the estimation of parameters, including: ID₅₀s, concentration-effect slopes and the synergism-antagonism parameter α . When α is positive, synergism is indicated, when α is negative, antagonism is indicated and when α is zero, additivity is indicated. URSA was applied to data from a set of 45 in vitro growth inhibition experiments with the L929 mouse cell line. The cytokine, tumour necrosis factor (TNF), was combined with one of nine eicosanoid synthesis inhibitors: indomethacin, Ro 20-5720, ibuprofen (cyclooxygenase inhibitors); nordihydroguaiaretic acid, nafazatrom, esculetin (lipoxygenase inhibitors); or BW-755C, phenidone, timegadine (cyclo- and lipoxygenase inhibitors). Five different schedules were used with various orders and durations of drug exposure. Examples of all three types of drug interaction were found for combinations of TNF with all three classes of drugs. The largest synergism found was for TNF plus BW-755C ($\alpha = 4.30 \pm 1.3$ [SE]). In general, for each combination, the degree of synergism was greater for schedules in which cells were exposed to TNF before exposure to the other agent.

Key Words: Antagonism, Cyclooxygenase, Lipoxygenase, Synergism, Tumour necrosis factor, Universal response surface approach

Concepts fondamentaux dans l'évaluation de l'interaction entre les modificateurs de la réponse biologique et d'autres agents

RÉSUMÉ: L'approche URSA (*universal response surface approach*) permet d'évaluer la nature et l'intensité des interactions médicamenteuses – synergisme, antagonisme et additivité. L'URSA consiste à adapter l'effet dose au données expérimentales au moyen des méthodes les plus appropriées pour estimer les paramètres tels: ID₅₀, la pente de l'effet-concentration et le paramètre synergisme-antagonisme alpha. Quand alpha est positif, il y a synergisme; quand alpha est négatif, il y a antagonisme et quand alpha est nul, il y a additivité. Cette approche a été appliquée à un ensemble de 45 tests d'inhibition de la croissance avec une lignée cellulaire de souris L929. La cytokine, le facteur de nécrose tumorale (TNF), a été combinée avec un des neuf inhibiteurs de la synthèse des eicosanoides: indométhacine, Ro 20-5720, ibuprofène (inhibiteurs de la cyclo-oxygénase); NDGA, nafazatrom, esculetin (inhibiteurs de la lipoxidase); ou BW-755C, phénidone, timégadine (inhibiteurs de la cyclo-oxygénase et de la lipoxidase). Cinq différents protocoles incluant des horaires variés et divers temps d'exposition aux médicaments furent utilisés. Des exemples des trois types d'interaction médicamenteuse ont été trouvés pour le TNF combiné aux trois classes de médicaments. Le synergisme le plus important est observé lorsque le TNF est associé au BW-755C; $\alpha = 4,30 \pm 1,3$ (SE). En général, pour chaque combinaison, le synergisme était supérieur dans les expériences où les cellules avaient été exposées au TNF avant d'être exposées à un autre agent.

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THE ASSESSMENT OF THE JOINT EFFECTS OF DRUGS OR other agents is ubiquitous in biology and medicine. The claim of synergism for combinations which include biological response modifiers (BRMs) is especially prevalent. For example, a search of the biomedical literature with MEDLINE from 1983-90 found 14,863 citations in English for synergism, 3560 citations for tumour necrosis factor (TNF), a specific BRM, and 480 citations for the joint occurrence of the two terms. This report discusses several general issues surrounding the highly controversial topic of synergism, and describes an application of a new approach, universal response surface approach (URSA) (1-4) to the quantitative assessment of agent interactions. The specific application of URSA is to a set of 45 individual *in vitro* growth inhibition experiments in which TNF is combined with one of nine other agents using one of five different schedules of administration.

UNIVERSAL RESPONSE SURFACE APPROACH

Figure 1 describes the steps necessary to assess the nature of drug interactions, ie, synergism, antagonism and additivity. With synergism one observes a more intense pharmacological effect for a combination of active agents than one would predict from a good knowledge of the individual actions of the agents, with antagonism one observes a less intense effect and with additivity one observes the predicted effect.

Step 1 is to choose a good concentration-effect (dose-response) structural model for each agent when applied individually. A common choice is the nonlinear form of the median-effect model (5,6) shown in Figure 2 for an inhibitory drug. This equation is fundamentally the same as the Hill-Sigmoid-Emax equation (7-10). In this equation, E is the measured effect (response), such as the number of cells remaining in a culture vessel after drug exposure; D is concentration of drug; E_{max} is the full range of response which can be affected by the drug; D_m is the median effective dose of drug (or ID_{50}); and m is a slope parameter. When m is negative, the curve falls with increasing drug concentration; when m is positive, the curve rises with increasing drug concentration. The specific curve in Figure 2 was simulated with the three parameters, E_{max} , D_m and m , assigned values of 100, 1 and -2, respectively. The concentration-effect curve in Figure 2 can be thought of as an ideal curve formed by data with no discernible variation, or as the true curve known only to God or to Mother Nature, or as the average curve formed by a billion data points at each of a billion evenly spaced concentrations.

Since real experiments always generate data which do not fall on the ideal curve, step 2 in Figure 1 is to choose an appropriate data variation model. Model candidates include the normal distribution for continuous data, such as found in growth assays in which the absorbance of a dye bound to cells is the measured

signal; the binomial distribution (11) for proportions of failures or successes, such as in acute toxicology experiments; and the Poisson distribution for low numbers of counts, such as in clonogenic assays. A composite model is formed from one structural model plus one data variation model and eventually is used for fitting to real experimental data.

In step 3, most approaches can be categorized into one of two main strategies. In step 3a, a structural model is derived for joint action of two or more agents with the assumption of no interaction (additivity). Then after the experiment is designed and conducted, data from the combination of agents are compared with predictions of joint action with no interaction. In contrast, in step 3b, a structural model is derived for joint action which includes interaction terms. After the experiment is designed and conducted, the full interaction model is fit to all of the data at once, and interaction parameters are estimated. Both the left- and right-hand strategies end in a set of guidelines for making conclusions. Examples of approaches which employ the left-hand strategy include the classical isobologram approach (12), the method of Gessner (13), the methods of Berenbaum (14,15) and the method of Chou (5,6). Examples of approaches which employ the right-hand strategy include URSA (1-4) and the response surface approaches of Carter (16).

Although most, and possibly all, approaches for assessing drug combinations may fall under the scheme presented in Figure 1, the approaches differ from each other in many respects. The approaches developed by

Figure 1) Schematic diagram of a general strategy for assessing the nature of interactions of drugs and other agents

Figure 2) Theoretical concentration-effect (dose-response) curve simulated from the equation and parameters shown in the figure. The meaning of the variables in the equation can be seen from the graph. Definitions of the variables are included in the text

pharmacologists usually stress structural models, eg, the approach of Chou (5,6), whereas the approaches developed by statisticians usually stress data variation models, eg, the approaches of Finney based on Probit analysis (17). There are differences in the definitions of key terms, especially that of synergism. Some approaches only yield a qualitative conclusion (synergism, antagonism or additivity), such as the classical isobologram approach, whereas others also provide a quantitative measure of the intensity of the interaction, such as URSA. There are differences in the degree of mathematical and statistical rigor, ie, some approaches are performed entirely by hand (eg, the classical isobologram approach), whereas others require a computer (eg, URSA). Some approaches employ parametric models (1-4), whereas others emphasize nonparametric models (18). The suggested designs for experiments differ widely among the different approaches. It is, therefore, not surprising that it is possible to generate widely differing conclusions on the nature of a specific drug interaction when applying different methods to the same data set. As described previously (1), URSA was developed by adapting and combining elements from many well-established approaches for assessing drug interactions (5,6,12,14,16,17,19-22) and from modern nonlinear statistical methodology (23-25).

BIOMEDICAL RATIONALE

TNF was discovered (and is best known as) a protein which causes hemorrhagic necrosis and complete regression of some murine and human cancer cells transplanted into mice (26,27). Experimental evidence has accumulated which indicates that TNF also plays a key role in the body's response to infection, injury and inflammation (28). It is an important factor for the host defence system (29), graft-versus-host disease (30), differentiation of B cells (31), regulation of expression of human immunodeficiency virus (32) and numerous other processes (33). Effects of TNF have been linked to the arachidonic acid cascade (34). TNF has been reported to cause the activation of phospholipase A₂ in osteoblasts (35), synovial cells (36) and fibroblasts (37). Phospholipase A₂ releases arachidonate which is converted into leukotrienes by the action of 5-lipoxygenase, or into prostaglandins or thromboxane by the action of cyclooxygenase. Prostaglandins and other eicosanoids rapidly alter the activity of the cells in which they are synthesized and the activity of adjoining cells (55). Arachidonate metabolites, thus, are considered second messengers. Glucocorticoids, quinacrine and other inhibitors of phospholipase A₂ have been found to decrease the cytolytic activity of TNF (38). These findings imply involvement of phospholipase A₂ and/or

arachidonic acid and/or its metabolites in TNF-mediated processes. In an attempt to increase TNF efficacy and to gain more insight into the mechanisms of action of TNF, studies were conducted to investigate the effect of a set of eicosanoid synthesis inhibitors (more specifically, a set of cyclooxygenase and/or lipoxygenase inhibitors) given in various schedules on the cytolytic/antiproliferative activity of TNF.

MATERIALS AND METHODS

Chemicals: Homogeneous recombinant human TNF, with a specific activity of 2×10^6 units/mg protein (39), was a gift of the Asahi Chemical Industry Co Ltd, Tokyo, Japan. Indomethacin, ibuprofen, phenidone, nordihydroguaiaretic acid (NDGA) and esculetin were purchased from the Sigma Chemical Company, Missouri. Ro 20-5720 was a gift from Hoffman-La Roche Inc, New Jersey. BW-755C was a gift from the Wellcome Research Laboratories, Beckenham, United Kingdom. Timegadinine was a gift from Lovens Kemiske Fabrik (Leo Pharmaceutical Products), Ballerup, Denmark. Nafazatrom was a gift from Miles Pharmaceuticals, Division of Miles Laboratories, Inc, Connecticut.

Cell culture growth assay system: Murine connective tissue cells, line L929, were cultivated in tissue culture dishes (100x20 mm; Becton Dickinson Labware, New Jersey) in RPMI 1640 medium (Gibco Life Technologies Inc, New York) supplemented with 5% fetal bovine serum (Gibco) and 0.05 mg/mL gentamicin (Gibco). Cells were seeded at 3000 cells/well in the same supplemented medium in each of the 60 inner wells of a 96-well plate (Becton Dickinson Labware). Sterile water was added to the outer wells.

Figure 3 shows the five different schedules (A to E) of administration of combinations of TNF plus one other drug in supplemented medium at 37°C, pH 7.4. Day 0 is the time of TNF addition; cells were seeded two days before. TNF was added at final concentrations of 0, 0.1, 1, 10 or 100 units/mL (0, 0.05, 0.50, 5, 50 ng/mL). The final concentrations of indomethacin, ibuprofen, Ro 20-5720 and nafazatrom were: 0.2, 4, 8, 16, 32, 64, 128 or 256 µg/mL; of BW-755C: 0.1, 2, 4, 8, 16, 32, 64 or 128 µg/mL and of phenidone, timegadinine, NDGA and esculetin: 0.0, 25, 0.5, 1, 2, 4, 8, 16 or 32 µg/mL. Each experiment included four wells with no drugs (controls); eight wells with TNF alone (four concentrations in duplicate), 16 wells with the second drug alone (eight concentrations in duplicate) and 32 wells with all of the different combinations (in singlet). For all schedules (except schedule C) cells were incubated in 100 µL of compound-supplemented medium at the first stage and 200 µL of compound-supplemented medium at the second stage. In schedule A, the second drug was added one day before TNF was added. In schedule B, the second drug was removed, and the cells were washed three times with 100 µL of phosphate buffered saline, pH 7.4, before the addition of TNF. In schedule

Figure 3) Schematic diagram of the five different schedules of drug administration used in this *in vitro* study. TNF Tumour necrosis factor

C, the second drug and TNF were added together (200 µL) on day 0. In schedules D and E, TNF was added on day 0 and the second drug was added one day later. Schedule E includes TNF removal and a wash step before the addition of the second drug. Cells were allowed to grow for six days, medium was removed, cells were fixed with 100 µL of 4% (v/v) formaldehyde for 15 mins. The formaldehyde was aspirated and the cells were stained with 100 µL of 0.02% (w/v) methylene blue for 15 mins. The stain was aspirated, residual stain was removed by immersing the plate in a beaker of fresh tap water several times, and the plate was allowed to dry. After adding 100 µL of 0.33 N hydrochloric acid, released dye was measured spectrophotometrically at 665 nm using a Microplate Reader, Model 2550 (BioRad Laboratories, California).

Data analysis: The following equation was fitted to the complete data set from each experiment (60 measurements) with weighted least squares nonlinear regression, and parameters estimated (1-4).

Equation 1 contains the respective drug concentrations [TNF] and $[D_2]$ as inputs and the measured effect, the absorbance from a well after cell staining, a surrogate of cell mass, E , as the output. The seven estimable parameters include: E_{max} , the maximum effect at zero drug concentration; the respective ID_{50} s or median effective concentrations, Dm_{TNF} , Dm_2 ; the respective concentration effect slope parameters, m_{TNF} , m_2 ; and the synergism-antagonism parameter α . When

Figure 4 Three-dimensional concentration-effect surfaces for three representative experiments. The cell line was murine L929. The x- and y-axes are drug concentrations on a linear scale. The z-axis (% control) is the effect (observed or predicted) divided by the best estimate of E_{max} . The combination of TNF plus BW-755C, schedule E, shows synergism; TNF plus phenidone, schedule A, shows additivity; and TNF plus esculetin, schedule B, shows antagonism. Fishnet surface, predicted concentration-effect surface, estimated from fitting Equation 1 to the data with nonlinear regression as described in the text; Points, measured response from the absorbance from dyed cells in single culture wells. Solid points are above the surface open points fall below the surface. Vertical lines are from the observed points to the fitted surface

α is positive, synergism is indicated; when α is negative, antagonism is indicated; when α is zero, no interaction (or additivity) is indicated. The equation, along with these three assignments, comprises the rigorous quantitative set of interaction definitions promised in the 'Introduction'.

The equation allows the slopes of the concentration-effect curves for the two drugs to be unequal. The equation was derived (1) with the assumption that each individual concentration-effect curve follows the equation presented in Figure 2, using an adaptation (1) of the guidelines of Berenbaum (14). A convention used in the equation is that as drug concentration(s) increases, the measured response (absorbance) decreases; the slope parameter, m , is negative.

The weighting factor for each data point was equal to the reciprocal of the square of the predicted response, with two exceptions. To avoid overweighting data near the limit of detection, if the predicted response was less than 4% of the maximum response, then the weighting factor was assigned a relative value of $1/(0.04E_{max})^2$.

$$1 = \frac{[TNF]}{Dm_{TNF} \left(\frac{E}{E_{max}-E} \right)^{1/m_{TNF}}} + \frac{[D_2]}{Dm_2 \left(\frac{E}{E_{max}-E} \right)^{1/m_2}} + \frac{\alpha [TNF] [D_2]}{Dm_{TNF} Dm_2 \left(\frac{E}{E_{max}-E} \right)^{1/2m_{TNF}} \left(\frac{E}{E_{max}-E} \right)^{1/2m_2}}$$

Also, to avoid overweighting poorly fit data for some experiments when the estimated α was negative (antagonism), if the observed effect was less than the 4% cutpoint and the predicted effect was greater than 20% of the maximum effect, then the weighting factor was assigned a value of zero. All weights were normalized such that the sum of the final weights for one experiment equalled the number of data points.

Equation 1 was fitted to data using custom software called SYNFIT which was written in the computer language, MicroSoft FORTRAN (MicroSoft Corp, Washington). SYNFIT uses a version of the Marquardt algorithm (24) for nonlinear regression as modified by Nash (40). The output of the program includes parameter estimates, asymptotic standard errors, 95% confidence limits for the parameters, residual analyses and two-dimensional graphs. Since the equation is not in closed form, a one-dimensional bisection root finder (eg, 41) was used to calculate predicted values of E . The SAS/PC software package, Version 6 (42), was used to generate the three-dimensional graphs of Figure 4. All software was run on IBM PC-compatible microcomputers. (Inquiries regarding distribution of the custom software package, SYNFIT, should be addressed to WR Greco.)

RESULTS

A total of 45 experiments was conducted: nine different compounds, all with effects on arachidonic acid metabolism, were combined with TNF for the five different schedules of administration. Figures 4 and 5, and Table 1 show the results from three representative

TABLE 1

Parameter estimates ($\pm$ SE) for the fit of Equation 1 to data from three representative growth inhibition experiments in which L929 cells were exposed by various schedules (A-E) to tumour necrosis factor plus a second drug

Second drug	Schedule	Dm_{TNF} (U/mL)	m_{TNF}	Dm_2 (μ g/mL)	m_2	E_{max} (OD)	α	Interaction
BW-755C	E	5.14 ± 1.3	-1.22 ± 0.13	18.3 ± 2.8	-1.71 ± 0.16	0.528 ± 0.039	3.14 ± 1.0	SYN
Phenidone	A	1.51 ± 0.34	-0.724 ± 0.041	4.27 ± 0.18	-7.34 ± 0.60	0.752 ± 0.039	-0.00295 ± 0.035	ADD
Esculetin	B	2.15 ± 0.56	-0.698 ± 0.049	5.82 ± 0.32	-4.85 ± 0.53	0.614 ± 0.033	-0.266 ± 0.052	ANT

ADD Additivity; ANT Antagonism; OD Optical density units; SYN Synergism

experiments. The combination of TNF plus BW-755C, an inhibitor of both cyclooxygenase and lipoxygenase (43) (schedule E) showed synergism. The combination of TNF plus phenidone, an inhibitor of both cyclooxygenase and lipoxygenase (44) (schedule A) showed additivity. The combination of TNF plus esculetin, an inhibitor of lipoxygenase (45) (schedule B) showed antagonism. Figure 4 is a three-dimensional representation of the raw data and the estimated concentration-effect surface. The vertical or z axis is the response divided by the estimated E_{max} value (% control). The x and y axes are drug concentrations on a linear scale. Solid points lie above the fitted surface, and open points lie below. Vertical lines are drawn from the points to the fitted surface. Note the general shape of the curves and the goodness of fit of the raw data to the fitted surface. For the synergism and additivity examples in Figure 4, and for the other experiments which showed either synergism or additivity, the overall fit was good. The main systematic deviations of the surface from the points occur for the example of antagonism, TNF plus esculetin, (schedule B) in the region of joint high concentrations of both compounds. This systematic poor fit at joint high concentrations also occurred for other antagonistic combinations in this set of experiments. Clearly, the antagonism model is only useful in this set of experiments for smaller joint concentrations.

Figure 5 is a two-dimensional isobolographic representation at the 50% effect level of the three-dimensional surfaces in Figure 4. When the isoeffect contour bows downward, synergism is indicated, when it bows upward, antagonism is indicated, and when it is a straight diagonal line, additivity is indicated. The ordinate and abscissa are drug concentrations normalized by their respective ID_{50} s. It should be emphasized that the isobols in Figure 5 are not hand drawn, but rather were simulated from the equation using the parameters estimated from fitting the equation to the data. The degree of bowing, a geometric and visual indication of the intensity of interaction, is related to the magnitude of the interaction parameter α (1). The isobols in Figure 5 can also be described as slices through the three-dimensional surfaces of Figure 4 at the 50% effect level.

The parameter estimates with associated standard errors from the fit of equation 1 to the data are listed in

Table 1. Note that most of the standard errors are from 5 to 20% of the estimated parameter, indicating a good overall fit of the model to the data. The 95% confidence intervals (the range from the parameter estimate minus about twice the standard error to the estimate plus about twice the standard error, 3) for α provide a simple quantitative test to confirm the qualitative type of interaction. For example, the 95% confidence interval for α for the combination of BW-755C plus TNF was 1.08 to 5.19. Since the interval is positive and does not encompass zero, synergism is concluded. For the combination of TNF plus esculetin, schedule B, the 95% confidence interval was from -0.370 to -0.161 . Since the interval is negative and does not encompass zero, antagonism is concluded. For the combination of TNF plus phenidone, schedule A, the 95% confidence interval was from -0.0733 to 0.0674 . Since the interval is small and encompasses zero, neither synergism nor antagonism can

Figure 5) Estimated two-dimensional isobol contours at the median effective dose, ID_{50} , for the three concentration-effect surfaces shown in Figure 4. The curves were estimated as described in Figure 4 and in the text. This two-dimensional representation accents the degree of bowing of the surfaces away from the straight-line prediction of additivity. Downward bowing is an indication of synergism, upward bowing is an indication of antagonism

Figure 6) Alpha estimates for 45 growth inhibition experiments in which a second drug was applied along with tumour necrosis factor to L929 cells. A positive α indicates synergism; a negative α indicates antagonism. The bars around each α estimate indicate the 95% confidence interval. When the 95% confidence interval encompasses 0, then a conclusion of additivity, or no interaction, cannot be firmly discarded. The results for nine drugs, from three classes, combined with TNF (with five different administration schedules) are displayed

be concluded, and thus, a default conclusion of no interaction (or additivity) is made.

Figure 6 is a summary of the results for all 45 experiments. The 95% confidence intervals for α , the interaction parameter, are plotted for all combinations of TNF with nine different drugs (from three classes) administered via five schedules. Overall, the class of compound (cyclooxygenase inhibitor, lipoxigenase inhibitor or cyclo- and lipoxigenase inhibitor) did not seem to influence the pattern of interactions. BW-755C (cyclo- and lipoxigenase inhibitor, 43), nafazatrom (lipoxigenase inhibitor, 46), phenidone (cyclo- and lipoxigenase inhibitor, 44) and NDGA (lipoxigenase inhibitor, 47) seemed to interact the most synergistically with TNF, whereas timegodine (cyclo- and lipoxigenase inhibitor, 48), Ro 20-5720 (cyclooxygenase inhibitor, 49), ibuprofen (cyclooxygenase inhibitor, 50) and indomethacin (cyclooxygenase inhibitor, 51) seemed to interact the most antagonistically with TNF. Four drugs, ibuprofen, Ro 20-5720, indomethacin and esculetin showed both antagonism at some schedules and synergism at other schedules. The main tendency showed by this group of nine drugs, when used in combination with TNF, was for α (the degree of syner-

gistic interaction) to increase from schedule A to E. Thus, overall, exposing cells to TNF 24 h before a second drug (see Figure 3) seemed to enhance synergism, whereas exposing cells to the second drug prior to TNF seemed to enhance antagonism.

Table 2 showcases the ID_{50} s and ms (slope parameters) for the concentration-effect surfaces, estimated from fitting Equation 1 to the raw data from each of the 45 experiments. Note that there is one ID_{50} and one m listed for each second drug at each schedule, but that for TNF, which was present in every experiment, a mean and standard error were calculated for each schedule from the individual estimates from nine experiments. Some trends are evident. The slope parameter, m , was much smaller in magnitude (a shallower slope) for TNF, a BRM, than for the nine other compounds in this system. There is no clear overall pattern for the magnitude of the slope parameters across drug class or across schedule. Overall, the smallest ID_{50} s (highest potencies) for the second drugs were found with schedule C, and for TNF with schedules A, B and D. The largest ID_{50} s (smallest potencies) for the second drugs were found with schedules D and E, and for TNF with schedule E. However, there are numerous exceptions to these generalizations for specific compounds and schedules.

DISCUSSION

A brief description of the URSA (1-4) has been presented. Since BRMs will most likely be applied therapeutically in combination with other agents, rigorous quantitative approaches to the assessment of BRM interactions at the in vitro, in vivo and clinical levels are critical. Feedback among these levels of investigation is facilitated by rigorous quantitative descriptions (mathematical models).

The specific set of experiments presented, that of TNF plus nine different compounds, each which interfere with arachidonic acid metabolism, against murine L929 cells in vitro, may serve as a paradigm for work with TNF combinations in other biological and biomedical systems, as well as for work with other combinations of BRMs and chemotherapeutic agents. However, some comments on definitions, classifications and semantics must be included here. It should be noted that the classification of eicosanoid synthesis inhibitors (three groups: cyclooxygenase, lipoxigenase, and cyclo- and lipoxigenase inhibitors) used in this report is not absolute and may not be useful for all biological systems. Also, although the nine eicosanoid synthesis inhibitors used in this study all inhibited the proliferation of L929 cells, none of them is commonly classified or used clinically as anticancer chemotherapeutic agents.

The general patterns seen in Table 2 for the ID_{50} s and slope parameters across drug classes and schedules may reflect complex interplays among the proliferative state of the cells at the time of drug ex-

TABLE 2

Estimates of Dms (ID₅₀s) and slope parameters (m) for concentration-effect curve of second drug added to tumour necrosis factor (TNF) in combination against L929 cells (and of TNF) for various drug exposure schedules (A-E). Parameters were estimated by fitting Equation 1 to the full data set from one experiment

Drugs		A		B		C		D		E	
		Dm*	m	Dm	m	Dm	m	Dm	m	Dm	m
Cyclooxygenase inhibitors	Ibuprofen	114	-5.75	116	-4.24	59.7	-3.24	193	-7.71	125	-2.98
	Ro 20-5720	33.3	-7.81	46.4	-4.81	26.2	-5.48	51.5	-3.24	57.0	-3.14
	Indomethacin	30.0	-2.45	63.7	-2.86	25.6	-1.82	123	-1.82	58.0	-2.32
Cyclo- and lipoxigenase inhibitors	BW-755C	11.3	-2.44	17.5	-1.95	14.6	-1.90	49.6	-3.00	18.3	-1.71
	Phenidone	4.27	-7.34	3.66	-21.2	3.48	-20.9	13.7	-6.05	6.69	-5.65
	Timegadine	3.97	-7.15	4.49	-11.2	3.22	-5.05	4.42	-9.35	3.74	-5.94
Lipoxygenase inhibitors	NGDA	4.14	-6.23	4.91	-7.78	2.34	-4.89	8.43	-4.91	6.47	-4.89
	Nafazatom	53.1	5.68	92.1	-3.63	34.0	-2.68	97.3	-3.73	66.2	-1.94
	Esculetin	4.37	-7.68	5.82	-4.85	6.72	-5.97	7.86	-1.62	7.27	-0.756
TNF†		1.81	-0.773	1.76	-0.720	2.75	-0.653	1.79	-0.589	3.97	-0.926
		±0.23	±0.030	±0.19	±0.033	±0.28	±0.053	±0.20	±0.021	±0.040	±0.052

* The units for the Dms for the nine eicosanoid synthesis inhibitors are µg/mL; for TNF, U/mL; m is unitless. † The recorded values for TNF are means of nine experiments ± SE

posure, the length of drug exposure, the influence of the cell rinsing procedure, and the differential heterogeneity of the cells to the cytotoxic and cytostatic effects of the 10 inhibitory compounds. Overall, the concentration-effect slopes were much steeper for the nine eicosanoid synthesis inhibitors in this study than has been observed in other studies of anticancer chemotherapeutic drugs by the authors' group (eg, 1,2,52,53).

The overall main conclusion of this study is that the exposure of cells to TNF, before the exposure to these second agents, enhances the potential for synergism. Since it is well known from many other studies that both TNF and the other nine compounds in this report have profound effects on arachidonic acid-associated pathways (26-38,43-51), it is reasonable to hypothesize that the observed empirical interactions between TNF and the other agents were due to biochemical interactions in the arachidonic acid cascade. Characterization of the specific points of biochemical interaction will require an intensive research effort. For therapeutic applications of combinations of TNF (or anti-TNF monoclonal antibodies, eg, 54) with inhibitors of cyclooxygenase and/or lipoxigenase, the order of administration of the agents may be critical. It may be useful in future clinical investigations of such combinations to explore the efficacy of different schedules.

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