[Frontiers in Bioscience E2, 399-410, January 1, 2010]

A synergism model for PPARalpha and PXR agonist effects on HDL-cholesterol and apoA1

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1. ABSTRACT

Synergism between gemfibrozil and CDD 3540, drugs used to elevate mouse serum HDL cholesterol and apoprotein A1, is modeled using nonlinear response surface techniques. This approach employs a common simple pharmacological model to describe the dose-response function. Its parameters are modeled as functions of drug mixture fractions using models borrowed from mixture experiment analysis methods. This study advances previous *in vitro* synergy studies in three key areas. First, it was *in vivo*, with the associated additional variability. Second, the sample size was much smaller than in previous studies. Finally, this was the first specially designed study with this type of statistical analysis in mind. The design consisted of replicated observations along each of five rays at combination amounts chosen employing the principles of D-optimality. Also, the observed *in vivo* synergism of the combined use of these drugs, elevated levels of HDL-C and apoA1, and the experimental results and statistical models may provide important clues regarding the biological mechanisms of action of the two compounds.

2. INTRODUCTION

The study of drug synergism has a long history. Approaches have ranged from isobolographic (1-2) and other graphical approaches (3-4) to the response surface approaches of Hewlett (5), Greco (6-7) and Weinstein *et al* (8). We favor the nonlinear response surface approach due to its completeness in terms of flexibility, predictive capability, its full employment of the available data, and the fact that it can be subjected to statistical verification.

Our recent studies have introduced a method of hierarchical nonlinear response surface modeling for the effects of two or more agents using two large *in vitro* studies on anti-cancer drugs (9-10). These studies have demonstrated our capability of producing models that can describe complex patterns of synergism and antagonism for a) two drugs with a modulating factor and b) three drugs. In each case, the four-parameter sigmoidal Hill model was employed as a base model with mixture experiment polynomial models used to model the Hill parameters as functions of the drug fractions and modulator

concentration. These polynomial models have been seen to exhibit the flexibility required to effectively describe complex patterns of synergy and antagonism. The models represent the first successful attempts to model synergism for both complex patterns and for modeling the effects of three compounds used simultaneously.

One key question left unanswered by these studies is if the nonlinear mixture experiment hierarchical methodology could ever successfully be applied to smaller datasets such as those that would be encountered in an *in vivo* study. In such a study one would also typically encounter a higher degree of variability, breeding even more uncertainty as to the applicability of our models. This paper will describe the successful application of this statistical approach to such a study. In addition, we will describe in detail how the ideas of D-optimal design were employed as an ingredient that was crucial to this successful attempt.

This study involves two drugs whose HDL-cholesterol (HDL-C) elevating capabilities are to be explored. The motivation for this study is that atherosclerosis has been implicated by the Framingham heart study as a major cause of coronary heart disease (11). Whereas low density lipoproteins (LDL) are known to be positively correlated with risk of this disease, there is evidence from epidemiological studies that an increase in high density lipoproteins (HDL) and HDL-C is associated with a *decreased risk* of the disease (12). In addition to HDL-C, the second outcome variable considered in this study is the apolipoprotein, apoA1. The HDL surface is composed of a monolayer of apolipoproteins, phospholipids, and unesterified cholesterol, and there is a strong relationship between the predominant apolipoprotein, apoA1, with HDL concentration. These observations, along with a study on subjects treated with an allelic variant of apoA1 (13), have suggested the possibility that apoA1 is even more important than HDL-C in protecting against atherosclerosis.

This protective mechanism is based on the process of "reverse cholesterol transport" or RCT. This is the mechanism by which cholesterol is delivered back to the liver for the purpose of keeping it in proper balance in the bloodstream. The rate limiting step in RCT is the efflux of cholesterol onto apoA1, which initiates the extra-hepatic formation of HDL particles. It is these particles that carry the cholesterol to the desired destination, the liver, for processing.

Most currently available treatments for atherosclerosis aim to decrease low density cholesterol (LDL-C). Recently, however, more attention has been given to increasing the levels of HDL-C as a primary target (14). The most efficient way to do so is the up-regulation of the major component of HDL, which is apoA1. When considering pharmacologically-based methods for increasing levels of HDL-C and/or apoA1, the role of the recently discovered nuclear receptor (NR), PXR, (15-17) is under study in this investigation. PXR has been shown to be the major determinant for regulation of cytochrome P4503A (CYP3A) levels (18). We have characterized the PXR agonist effects of a number of novel imidazoles (19). We have recently shown in WT mice and in PXR knockout mice that PXR appears to play a role in the regulation of HDL-C and apoA1 in rodents (20). In this study, we employ gemfibrozil as an agonist for one NR (PPARalpha) and CDD3540, an imidazole, as an agonist for PXR, the NR under recent study. The major question to be addressed is whether these compounds, acting on two different NRs, will act synergistically in the elevation of HDL-C and/or apoA1.

3. MATERIALS AND METHODS

3.1. Materials

CDD3540 was obtained from the Department of Medicinal Chemistry at the College of Pharmacy, The University of Toledo. Gemfibrozil, methylcellulose and monobasic 1.0 M potassium phosphate buffer were purchased from Sigma-Aldrich (St. Louis, MO). Liquid Direct HDL-C assay kits were purchased from Amresco Inc. (Solon, OH). Lipi+Plus (direct lipid control set) was purchased from Polymedco, Inc. (Cortland Manor, NY). Affinity purified anti-mouse apoA1 (goat) was purchased from Rockland Inc. (Gilbertsville, PA), and purified mouse apoA1 was purchased from Biodesign International (Saco, ME).

3.2. Animals and treatment

Male wild-type (WT; C57BL/6) mice weighing 20-30 g were purchased from Harlan Sprague/Dawley, Inc., Indianapolis, IN. WT mice were treated with varying doses of either gemfibrozil or CDD3540 administered individually or in combination. In a preliminary study, gemfibrozil was administered singly in doses of: 2, 5, 10, 20, 50, 100 mg/kg; CDD3540 was administered singly in doses of: 10, 20, 50, 75, 100 mg/kg. In a follow-up experiment, fixed dose combinations of both gemfibrozil and CDD3540 along five rays, each having a constant drug ratio, or, equivalently, constant drug fractions, were administered. Doses for the fixed combinations (drug fractions) were established using a scaling and design procedure described below.

In all cases 1% methylcellulose was used as a vehicle control. Each dose was administered in 1% methylcellulose by gavage using a 20 G-1 ½ in. feeding needle. The animals were treated in groups of three. Each treatment was given once daily in the morning for 7 days.

After completion of treatment (i.e. on day 8) blood samples were collected from the tail of the animals for serum HDL-C and apoA1 determination. Blood samples were allowed to clot for 2 hours, centrifuged at 14,000 rpm for 20 minutes, and serum was collected. The serum samples were then stored at -20°C for up to 24 h before quantitative determination of HDL-C and apoA1.

3.3. HDL-C and apoA1 assay

HDL-C was determined quantitatively in the serum using Liquid Direct HDL kits. The assay has been described in detail (20). An immuno-turbidimetric assay

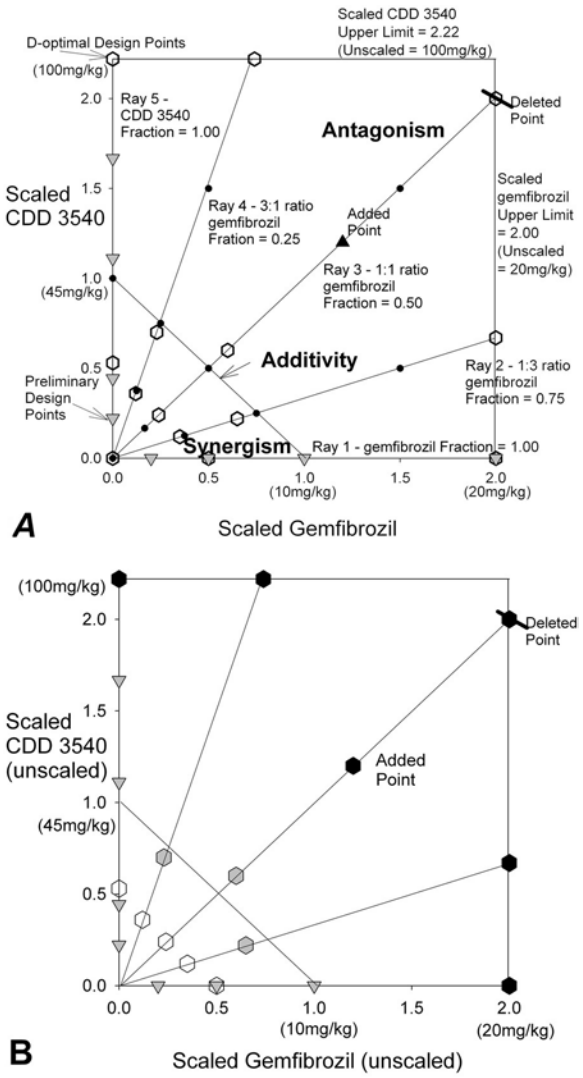

Figure 1. In Figure 1A, black circles show the hypothetical patterns of synergism, additivity, and antagonism for developing the experimental design. Triangles pointing down show the points on the preliminary design that we used for initial scaling of the drug doses. In Figure 1B, hexagons show the design points derived from considerations of D-optimality and used in the final design. Open hexagons correspond to the points combined from assuming synergism and additivity, gray points are from antagonism, and black are from the extreme values displayed in Table 1. On both plots, the upper and right-hand edge lines show the dosage boundaries established to avoid toxicity as indicated by changes in behavior, weight loss, or death. The lower and left-edge lines show the two individual drug rays, while the three internal lines show the idealized combination rays for the 1:3, 1:1, and 3:1 ratios of the mixture. The origin was a design point yielding control observations in both the preliminary and final designs.

was developed for quantitative determination of apoA1 in mouse serum. Details of this assay have also been previously published (20).

3.4. Experimental design

The type of experimental design that optimizes overall information on model parameters for response surfaces is the D-optimal design (21). A fully D-optimal design in this case would provide the drug mixture doses that should be studied in order to yield estimates of the model parameters with the smallest overall uncertainty. However, construction of such a D-optimal design requires prior knowledge of the model to be used to fit the data and, in the case of nonlinear models, prior knowledge of the parameter values is required as well (see, for example, Bezeau and Endrenyi, (22)). In such a circumstance, one is faced with the chicken-and-egg conundrum; if we knew the model and parameter values, we would not require the experiment. Two possibilities mitigate this concern. One is the possibility of performing a pilot study, or using previous similar studies to guess the model and the parameter values. The other is related, and revolves around the notion of robust design. Here we hope that the efficiency of the design is not particularly sensitive to the selection of these parameter values, at least within reasonable ranges.

We employed the notions of D-optimal experimental design in ways that would provide such a robust design. Since here we had little *a priori* knowledge of the model and could not know about the presence or absence of synergism or antagonism, and since we must insist on incorporating certain features beyond D-optimality into our study, the structure of the experiment was not D-optimal overall, but had D-optimal components. The development of the statistical models in our previous studies relied heavily on ray designs where observations were concentrated along fixed drug-ratio rays emanating from the origin (control observations). Hence, the predominant feature of our design was that observations were set along five rays each having constant drug ratios, or, equivalently, constant drug fractions. With these five rays, we attempted to cover the domain of the response surface as fully as possible. We say here "attempted" because drug fractions were determined relative to a data-dependent drug concentration scaling procedure to be described below. We aimed to have fractions of gemfibrozil and CDD 3540 in the set {0.00, 0.25, 0.50, 0.75, 1.00}; stated alternatively, along with drugs administered singly, the three mixtures are in ratios of 1:3, 1:1, and 3:1. Below we describe how we have employed the notions of D-optimal designs along each of these rays individually. Also, in what follows, we refer to gemfibrozil and CDD 3540 by their first letters, G and C.

Next, in view of the limited data to be available, only simple smooth models for synergism or antagonism were tractable. For the purpose of determining an optimal experimental design along each fixed ratio ray, and in order to "hedge our bets", we allowed for three possibilities: a smooth symmetric pattern of synergism, the flat pattern of additivity, or a smooth symmetric pattern of antagonism. The three patterns used are illustrated schematically (Figure 1A), which will be recognized as containing typical isobolograms.

Table 1. Maximum allowable doses of gemfibrozil and CDD 3540

Ratio	f (gem)	Scaled[1] Max (gem)	Scaled[1] Max (CDD)	Scaled[1] MaxAmt	Unscaled[2] Max (gem)	Unscaled[2] Max (CDD)
CDD 3540	0.0	0.00	2.22	2.22	0.00	100
1:3	0.2	0.74	2.22	2.96	7.40	100
1:1	0.5	2.00	2.00	4.00	20.0	90.0
3:1	0.7	2.00	0.67	2.67	20.0	30.0
gemfibrozil	1.0	2.00	0.00	2.00	20.0	0.00

[1]units of individual drug ED_{50}, here and in what follows, [2]units of mg/kg, here and in what follows.
Maximum allowable doses are shown as functions of gemfibrozil fraction. gem, CDD and Amt are used here and in what follows as abbreviations for gemfibrozil, CDD 3540, and total amount of the drug combination, respectively. These maximum doses are included in the optimal experimental designs and are not repeated in Table 2.

Table 2. D-optimal design points for mixtures

Ratio	f (gem)	Synergism Pattern	Hypothetical ED_{50}	Scaled Max Amt	Scaled Mixture Amt	Scaled gem	Scaled CDD	Un-scaled gem	Unscaled CDD
CDD only	0.00	add	1.0	2.22	**.530**	**0.00**	**.530**	**0.0**	**23.7**
1:3	0.25	syn	0.5	2.96	.374	.094	.280	0.9	12.6
1:3	0.25	add	1.0	2.96	.597	.149	.448	1.5	20.2
Collapsed values →					**.486**	**.120**	**.360**	**1.2**	**16.4**
1:3	0.25	ant	2.0	2.96	**.930**	**.232**	**.698**	**2.3**	**31.5**
1:1	0.50	syn	0.33	4.00	.286	.143	.143	1.4	6.4
1:1	0.50	add	1.0	4.00	.667	.333	.334	3.3	15.0
Collapsed value: →					**.476**	**.240**	**.240**	**2.4**	**10.7**
1:1	0.50	ant	3.0	4.00	**1.20**	**.600**	**.600**	**6.0**	**27.0**
3:1	0.75	syn	0.5	2.67	.364	.273	.091	2.7	4.1
3:1	0.75	add	1.0	2.67	.572	.429	.143	4.3	6.4
Collapsed values →					**.476**	**.35**	**.12**	**3.5**	**5.3**
3:1	0.75	ant	2.0	2.67	**.868**	**.65**	**.218**	**6.5**	**9.9**
gem only	1.00	add	1.0	2.00	**.500**	**.50**	**0.00**	**5.0**	**0.0**

Along with the origin for control observations, the eight, **Bold Faced** points are included in the final design in addition to the five maximum points shown in Table 1. For each gemfibrozil fraction optimal design points for scaled mixture amounts were computed for hypothetical patterns of synergism, additivity and antagonism. For the mixtures the proximity of values for synergism and additivity led us to collapse the suggested design points.

To give precise definition to this procedure, and thereby identify the parameters' "best guess estimates" necessary for deriving the optimal designs, we must first describe the procedure for scaling drug concentrations, a procedure that is also foundational to our modeling approach. Simply put, doses for each drug individually are scaled to units equal to that drug's ED_{50}, the dose required for 50% of the maximal effect. The complication is that these ED_{50} values are estimates that change as we proceed through a sequence of analytic procedures. These procedures include setting up the design using preliminary data and intermediate and global analyses using the final data. For the purpose of experimental design, we relied on a preliminary experiment studying the two drugs separately. Using the program Table Curve 2D (Jandel Scientific), estimates of the ED_{50} values for G and C for HDL-C were 7 & 40 mg/kg, respectively, and for apoA1 they were 15 & 47 mg/kg, respectively. Since HDL-C and apoA1 were each to be measured in the same mice, we did not have the luxury of dosing for the two outcome variables separately. Hence, loosely defined average ED_{50} values of 10 mg/kg and 45 mg/kg were selected for G and C for the experimental design, respectively, for studying both endpoints.

In addition to the ED_{50}, the other parameter for the Emax model is the maximum effect, E_{max}. The D-optimal design does not depend upon this parameter. However, the optimal design does depend upon the maximum allowable dose. Based on previous work with these compounds and the preliminary study, these were set at 20 mg/kg for G and 100 mg/kg for C. For G, doses above 20 mg/kg actually elicited an inverse (or hormetic) dose-response relationship. For C, doses above 100 mg/kg began to elicit signs of toxicity. As stated previously, our analytical method depends upon scaling drug doses to units of the ED_{50}. Hence, the necessary parameters for the scaled doses are, for G, ED_{50} = 10/10 = 1 and maximum dose = 20/10 = 2, and for C, ED_{50} = 45/45 = 1 and maximum dose = 100/45 = 2.22. For the purposes of determining the optimal design for each ray/synergism status combination, we followed the rule that the maximum allowable amount of the *mixture* is the largest amount that keeps *each component* within its prescribed limit. Another way of describing this rule is to say that the design in scaled units is confined to the rectangles shown (Figure 1), with right edge (along the scaled G axis) at 2 and upper edge (along the scaled C axis) at 2.22. Shown are the maximum mixture amounts, along with the associated individual drug doses, both scaled and unscaled (Table 1).

The D-optimal design for the Emax model for each ray/synergism status combination calls for two points. One is the maximum allowable dose and the other is derived and given (Table 2). These values were derived using the mathematical software Matlab (The Mathworks,

Inc., 2002) employing the approaches outlined in Atkinson and Donev (21). In terms of the scaled drug doses, the table first lists the hypothetical ED_{50} values used in the derivation of the design. The other point in each D-optimal design, the maximum possible mixture amount, is already given (Table 1). The only exception to this rule is that the maximum dose in the 1:1 ratio turned out to be toxic. This design point was replaced by 60% of these maxima, i.e., a scaled mixture amount of 2.4 as shown (Table 2). The design points can also be seen (Figure 1B).

For all three ratios, to minimize animal use and expense, it was decided to collapse the points determined for synergism and additivity since these design points were relatively close together. The collapsed values are shown (Table 2 and Figure 1B). In addition, new data was obtained along each axis - the individual drug rays. Finally, masked in all of this is the fact that the baseline values for HDL-C and apoA1 do not really equal zero at control observations (no drug) but are a positive value. Our approach was to subtract the mean of the control observations, hence forcing the modeled control values to zero, which is required for the Emax model. Hence, in addition, we obtained 3 control observations for each group of mice studied so that these means could be estimated and subtracted (group-wise) from the data. Also, observations at all design points were replicated three times for the purposes of reducing error and identifying outliers. It should be observed that though the final designs on each ray are several steps removed from the originally calculated D-optimal designs, important features of the D-optimal designs, such as the number of design points and their approximate locations on the rays, remain.

3.5. Statistical modeling

Our ultimate aim for each end-point is a hierarchical global model with three main components. The first component is a formula providing the base model for outcome as a function of drug combination dose. The second is an error structure model for the variation around that base function. Third is models for the parameters of the base model as functions of the drug combination fractions. For each of these three components, we seek both accuracy and simplicity. Simplicity is particularly critical given the small volume of data available.

The Emax model provides the simplest possible set of sigmoidal dose-response curves that can serve as a base model for this data. For the error structure, we will employ normally distributed error terms unless the data indicates otherwise. Finally, for the parameter models, low-order polynomial functions will be used since they comprise the natural class of functions that serve as simple approximations to typical smooth functions. Each of these choices represents the simplest reasonable option.

In the process of developing such a model, we employ several intermediate modeling steps. The purpose of these steps is two-fold. First, they are useful to identify a plausible structure for the global model. This includes confirmation that the Emax model and normal error structure are appropriate, and discovery of the likely order for the polynomials that will be needed for the Emax model parameters. Second, these steps are needed for the nonlinear estimation procedures to find good starting values for the search routines. Without these intermediate steps, it would not be possible to guess the structure of the global model, nor would it be possible to have any confidence in the parameter estimates that were produced.

4. RESULTS

4.1. Two-stage modeling

We begin with an intermediate analysis of the data studying the dose-effect pattern for each ray alone using the two-parameter Emax model,

$$y = \frac{E_{\max} Dose}{(ED_{50} + Dose)} + error \ ,$$

where E_{max} is the maximum observed effect and ED_{50} is the dose of the combination yielding 50% of the maximum effect. Note that this model forces the mean effect through the origin since we subtract control means. Recall also that *Dose* is the total of the scaled drug doses, scaled by the current estimates of the individual drug $ED_{50,*}$. That is,

$$Dose = [G]/ED_{50,\,G} + [C]/ED_{50,\,C} \ ,$$

where [D] denotes the un-scaled dose of drug D, and the single drug $ED_{50,*}$ values are estimates. We assume a constant error variance model, a reasonable choice both from observing the data (Figure 2) and in principle, since the original observations are all reasonably large quantities roughly between 30 and 100 (HDL-C) and 90 and 500 (apoA1). In a second stage the parameter estimates from all five rays for E_{max} and ED_{50} were modeled as simple polynomial functions of the drug fractions. We chose arbitrarily to model the parameters as functions of the gemfibrozil fraction, G.

Next the single drug data was used to obtain ED_{50} estimates for each drug. Shown are the single drug data and models for the four outcome variable/drug combinations (Figure 2) and the results for the two outcome variables for each of the drug combinations studied (Figure 3 and Figure 4). The SAS procedure NLIN (SAS Inst., 1999) was employed for all nonlinear modeling. Due to the fact that maximum drug doses were selected conservatively in order to avoid toxicity (C) or hormetic inversion of the dose-response curve (G), it can be observed (Figure 2, Figure 3 and Figure 4) that the dose-response curves fit to the Emax model did not reach plateaus at the maximum dose. This implies that it is difficult if not impossible to estimate E_{max}. In a one-drug study, we would thus be constrained by these experimental limitations to take E_{max} to be the maximum observed value. However, since for each of the two outcome variables, we had one maximum observed value for each of the five drug combination rays, we took E_{max} for the five rays to be a smoothed version of these observed maxima (Figure 5 and Table 3). We acknowledge here that these approximations of the E_{max} parameter are potentially

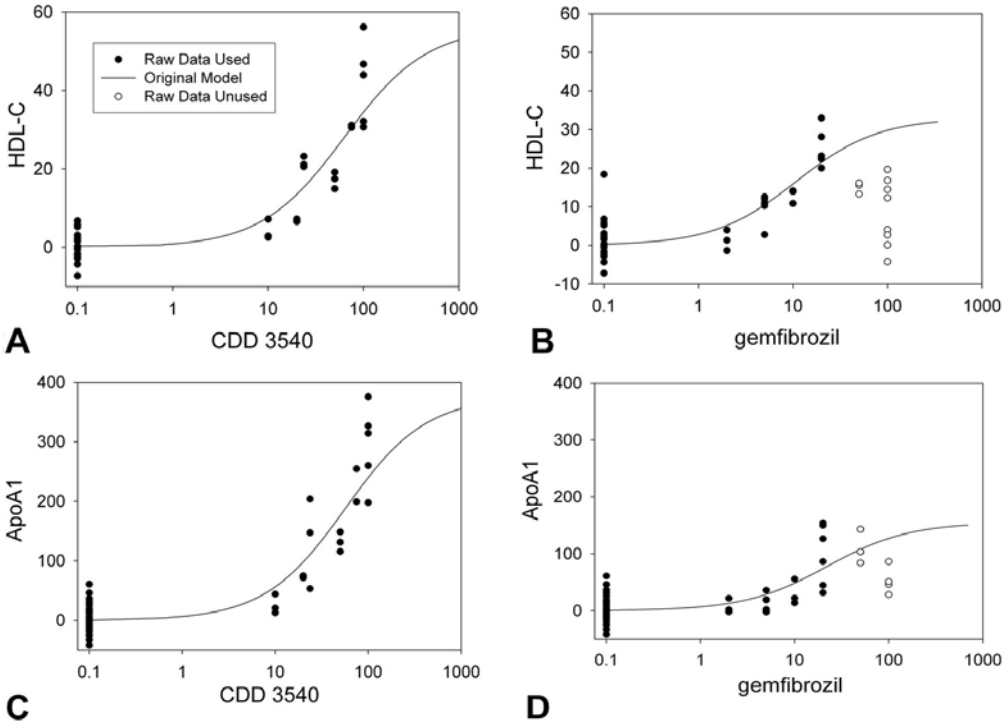

Figure 2. Dose-Response curves and raw data for the initial pair of single drug experiments. Higher doses of CDD3540 than those that appear in 2A and 2C are toxic. Data in the open circles in 2B and 2D was not employed due to the inversion of the dose-response curve for large doses of gemfibrozil. We attribute this inversion to pleiotropic effects that in some way offset the PPARα–mediated effects of gemfibrozil. Design points are those from the D-optimal design points listed in Tables 1 and 2 plus those from the preliminary study used to obtain initial estimates of the two individual drug ED_{50} values.

underestimates due to the limitations on dose imposed by toxicity or inversion of the dose-response curves. By smoothed here we mean a quadratic fit of the observed maxima as a function of G. Arriving at the fit is an iterative process, since the E_{max} values selected affected the individual drug ED_{50} values and these ED_{50} values in turn altered the scaling of the drugs and then the scaled gemfibrozil fraction. For the purpose of arriving at quadratic E_{max} models for the two-stage procedure, two iterations were regarded as sufficient. The formulas are

$$E_{\max}(HDL-C) = 57.57 - 68.58 \times f(G) + 44.16 \times f(G)^2$$

and $E_{\max}(apoA1) = 389.9 - 283.1 \times f(G) + 55.1 \times f(G)^2$.

SAS NLIN was then used to estimate ED_{50} values for all combination rays using the fitted E_{max} values (Table 3). ED_{50} estimates with standard errors and 95% confidence limits are shown (Table 4). For HDL-C and apoA1, displayed are the associated Emax models for each ray (Figure 3 and Figure 4). Next, to complete the intermediate hierarchical modeling steps, following the pattern initiated in our previous papers, these ED_{50} estimates were fit by the model $\log(ED_{50}) = Beta_0 \times f(G) \times (1 - f(G))$. For HDL-C, this intermediate estimate of $Beta_0$ is -2.09 with standard error 0.83 and 95% confidence interval $(-4.40, 0.23)$. For apoA1, the intermediate estimate of $Beta_0$ is -2.80 with standard error 0.28 and 95% confidence interval $(-3.58, -2.02)$. Isobolographic plots containing the ED_{50}'s, their confidence intervals, and the estimated ED_{50} curves are shown (Figure 6).

4.2. The global model

A global model was developed using all data for each outcome variable. The information gleaned from the intermediate hierarchical models was employed to provide a starting point for discovery of the best fit global model. The parameters estimated for this global model were the two individual drug scale parameters and the polynomial coefficients necessary to adequately describe the Emax model parameters as functions of the scaled drug fractions. The SAS NLIN procedure was again employed for this purpose. The model structure and starting guesses for parameter estimation were borrowed from the two-stage analysis. When we checked the model structure by extending the ED_{50} model to a higher order polynomial, the higher order parameter estimates were not statistically significant; hence the synergism model was the same as above, $\log(ED_{50}) = Beta_1 \times f(G) \times (1 - f(G))$. Nonlinear estimation procedures converged. Parameter estimates are shown (Table 5).

We note that this table contains the key result for both outcome variables. For both endpoints, the parameter estimate for $Beta_1$ is negative and statistically significant, with 95% confidence intervals excluding zero. The signs on

Table 3. Quadratic Emax models and resulting ED_{50} estimates

HDL-C				
f (gem)	maxHDL-C	Emax Fit	ED_{50} Preliminary Individual	ED_{50} Scaled Combination
0.00	56.2	57.58	63.15	1.00
0.29	46.1	41.35	NA	0.43
0.55	27.8	33.25	NA	0.92
0.79	33.2	31.04	NA	0.62
1.00	33.0	33.07	10.74	1.00
ApoA1				
f (gem)	maxApoA-1	Emax Fit	ED_{50} Preliminary Individual	ED_{50} Scaled Combination
0.00	376	390.2	56.71	1.00
0.29	397	346.2	NA	0.66
0.55	229	294.4	NA	0.61
0.79	269	233.2	NA	0.46
1.00	155	162.0	21.89	1.00

These are the models developed along each design ray individually

Table 4. Two-Stage ED_{50} estimates with 95% confidence intervals

HDL-C		
f (gem)	ED_{50} Estimate (SEM)	95% Confidence Interval
0.00	1.00 (.12)	(0.77,1.23)
0.30	0.43 (.09)	(0.24,0.63)
0.55	0.92 (.21)	(0.50,1.34)
0.79	0.62 (.15)	(0.31,0.93)
1.00	1.00 (.16)	(0.68,1.32)
ApoA-1		
f (gem)	ED_{50} Estimate (SEM)	95% Confidence Interval
0.00	1.00 (.11)	(0.79,1.21)
0.16	0.66 (.11)	(0.45,0.87)
0.36	0.61 (.10)	(0.40,0.82)
0.63	0.46 (.08)	(0.29,0.62)
1.00	1.00 (.19)	(0.62,1.38)

Table 5. Global model parameter estimates – No Emax proportionality constant

Parameter	**Estimate (SEM)**	**95% Confidence Interval**
HDL-C Gemfibrozil ED_{50}	11.79 (2.11)	(7.58, 16.00)
HDL-C CDD 3540 ED_{50}	59.79 (6.37)	(47.13, 72.46)
HDL-C $Beta_1$	-2.16 (0.81)	(-3.78, -0.54)
ApoA1 Gemfibrozil ED_{50}	24.87 (7.00)	(10.97, 38.77)
ApoA1 CDD 3540 ED_{50}	55.17 (6.40)	(42.43, 67.86)
ApoA1 $Beta_1$	-1.98 (0.55)	(-4.43, -1.03)

$Beta_1$ indicate that the two drugs follow a pattern of synergism throughout the observed dose combination range. More precise details about the pattern are unavailable from such a small study. With more data, it is possible that a more complex pattern of synergism could become apparent.

The most questionable aspect of this analysis might be the use of the observed maxima to generate the E_{max} values employed in the model. If, for the sake of avoiding toxicity, the drug doses used are short of those required for maximal effect, then these maxima could underestimate the true E_{max} parameters and the ED_{50} estimates are substantially dependent upon the choice for E_{max}. This situation is actually a likely scenario for an *in vivo* study such as this. In order to ensure that our main result, that of synergism as reflected by the statistically significant parameter $Beta_1$, is robust to the E_{max} values used, we repeated the global analysis with two alternative approaches to the selection of the E_{max} values. One of these alternatives was to multiply the quadratic E_{max} fitted value by a fixed constant (greater than one to reflect the notion that E_{max} may be larger than the observed maxima). Several such constants were tried, with qualitatively the same result arising in each case. Hence we discuss results only for the representative value of 4/3. The second approach is to attempt to estimate the proportionality constant from the data. It turns out that while the E_{max} parameter cannot be estimated for each ray separately, using the pattern provided by the quadratic fit, we were able to estimate this proportionality constant from the full dataset. Under both of these alterations, the ED_{50} values do indeed change, increasing as expected with increased E_{max}, but in a consistent pattern that leaves the synergism parameter $Beta_1$ relatively unchanged. Hence the result showing synergism is robust to alterations in the assumptions regarding the E_{max} employed. Results for these two approaches are shown (Table 6 and Table 7).

The results of the global model are shown graphically (Figure 3 and Figure 4) in the fitted global curves superimposed on the original data. The ED_{50} curves are also displayed on the isobole plots (Figure 6). Finally, the three-dimensional response surfaces are displayed (Figure 7).

5. DISCUSSION

The result of synergism has potential implications in terms of biology, pharmacology, and statistical methods.

Table 6. Global model parameter estimates – Emax proportionality constant fixed at 4/3

Parameter	Estimate (SEM)	95% Confidence Interval
HDL-C Gemfibrozil ED_{50}	18.61 (2.42)	(13.80, 23.42)
HDL-C CDD 3540 ED_{50}	100.40 (7.85)	(84.80, 116.00)
HDL-C $Beta_1$	-1.79 (0.56)	(-2.92, -0.67)
ApoA1 Gemfibrozil ED_{50}	35.08 (7.84)	(19.51, 50.65)
ApoA1 CDD 3540 ED_{50}	93.02 (8.26)	(76.63, 109.4)
ApoA1 $Beta_1$	-2.29 (0.63)	(-3.53, -1.04)

Table 7. Global model parameter estimates – Emax proportionality constant estimated

Parameter	Estimate (SE)	95% Confidence Interval
E_{max} prop constant (HDL-C)	1.90 (0.33)	(1.23, 2.58)
HDL-C Gemfibrozil ED_{50}	32.2 (8.99)	(14.31, 50.09)
HDL-C CDD 3540 ED_{50}	173.90 (46.08)	(82.24, 265.60)
HDL-C $Beta_1$	-1.35 (0.47)	(-2.28, -0.42)
E_{max} prop constant (ApoA1)	1.69 (0.30)	(1.09, 2.30)
ApoA1 Gemfibrozil ED_{50}	49.67 (16.18)	(17.55, 81.79)
ApoA1 CDD 3540 ED_{50}	136.40 (39.62)	(57.79, 215.1)
ApoA1 $Beta_1$	-1.98 (0.55)	(-3.07, -0.90)

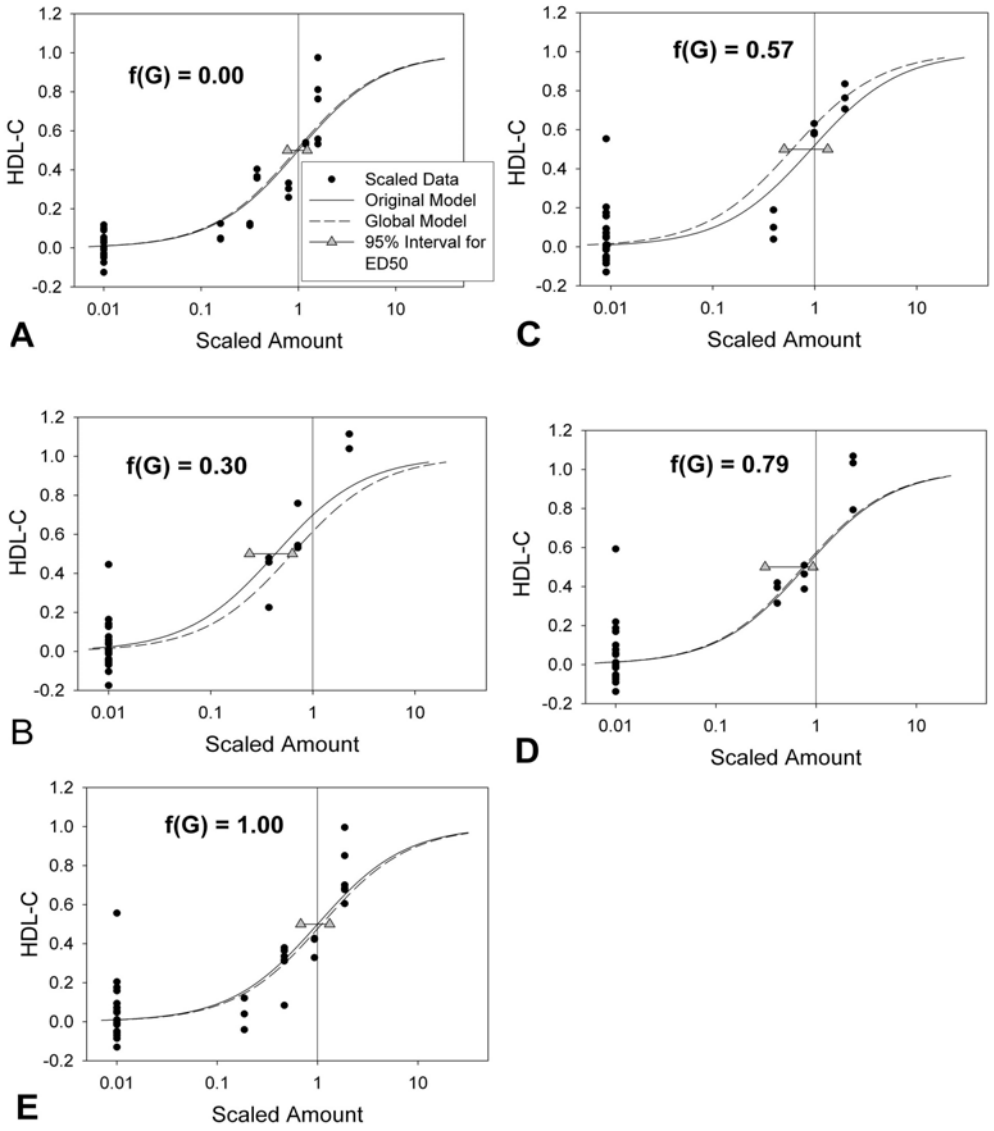

Figure 3. Dose-Response plots for the final set of experiments, for HDL-C. Included in each graph is a 95% confidence interval for the ED_{50} for the combination represented. The lower and upper bounds are shown by the gray triangles. Control observations, also having their own means subtracted, are shown schematically on the log scale at Scaled Amount = 0.01.

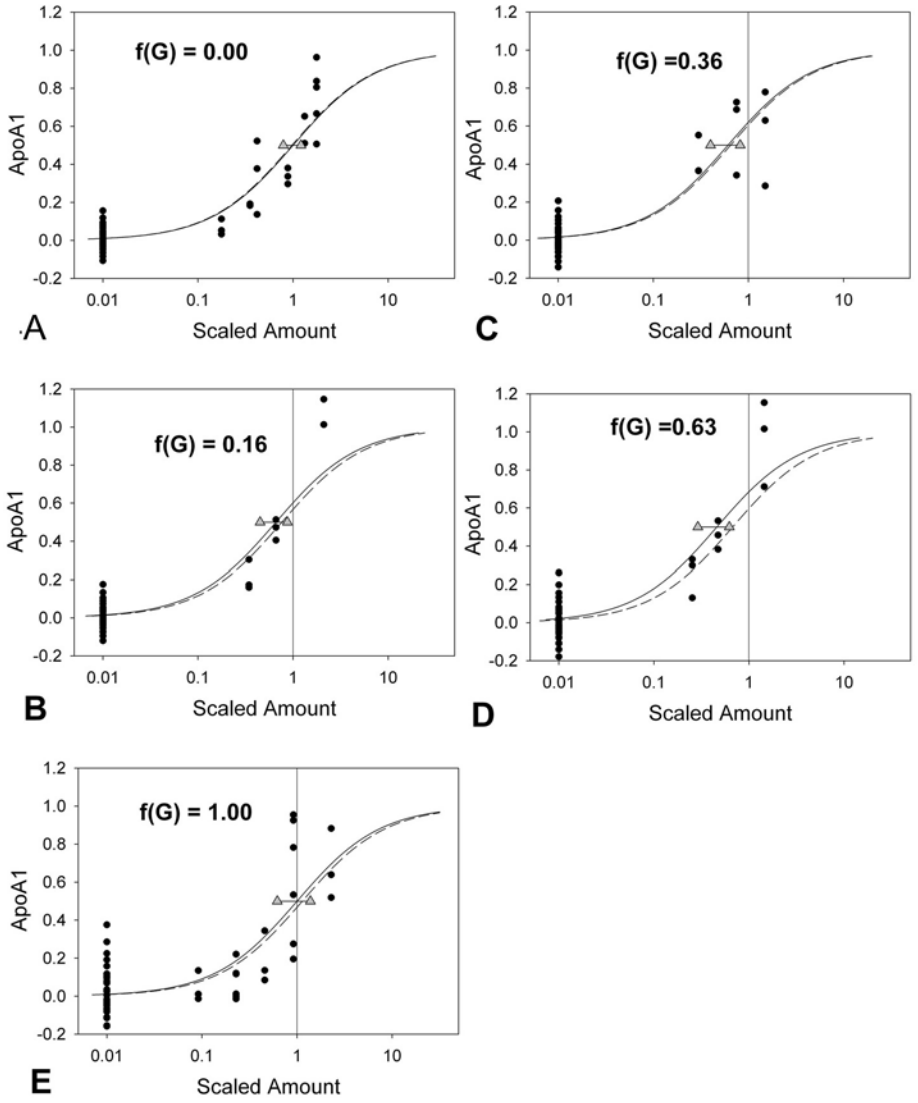

Figure 4. Dose-Response plots for the final set of experiments, for apoA1. Included in each graph is a 95% confidence interval for the ED_{50} for the combination represented. The lower and upper bounds are shown by the gray triangles. Control observations, also having their own means subtracted, are shown schematically on the log scale at Scaled Amount = 0.01.

We first consider the biological message. Since synergistic effects are more likely to be present when two drugs operate by different mechanisms, and particularly with the consistent results showing synergism between gemfibrozil and CDD3540 for apoA1, our results suggest that the two drugs are likely to have different mechanisms of action. This, in turn, supports our hypothesis that CDD3540 acts through activation of PXR (20), however a dispositional interaction between these two drugs cannot be ruled out, either. In terms of pharmaceutical benefit, the results of this study indicate that interactions between CDD3540 and gemfibrozil fulfill the requirements for an advantageous combination. In combination, from the results for the global model and also from the two-stage modeling at all of the ratios employed, the drugs exerted a synergistic (supra-additive) interaction in the up-regulation of HDL-C and apoA1.

For this study, however, the implications for statistical methodology for the study of synergism are also of substantial interest. First we consider the area of experimental design. Out of many possible approaches to acquisition of the most valuable information from the small number of mice available for the study, we have selected five rays to cover the rectangle of allowed doses, and a set of D-optimal designs, two for each of these five rays. The two were originally three – one for hypothetical synergism, one for hypothetical additivity, and one for hypothetical antagonism. It was felt that this was the judicious approach given that D-optimal designs for nonlinear models require knowledge of both the models and parameters to be

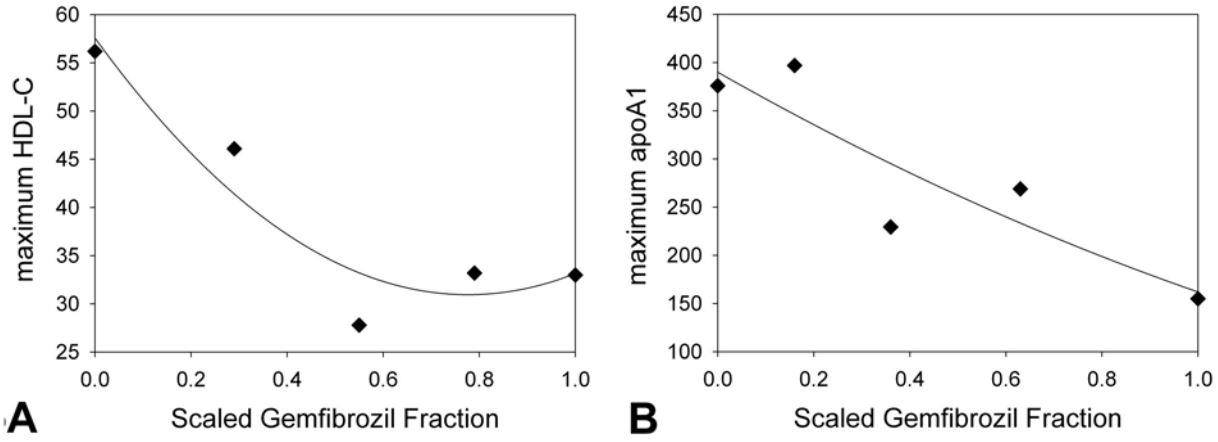

Figure 5. Fitted quadratic models for Emax for HDL-C and apoA1, respectively.

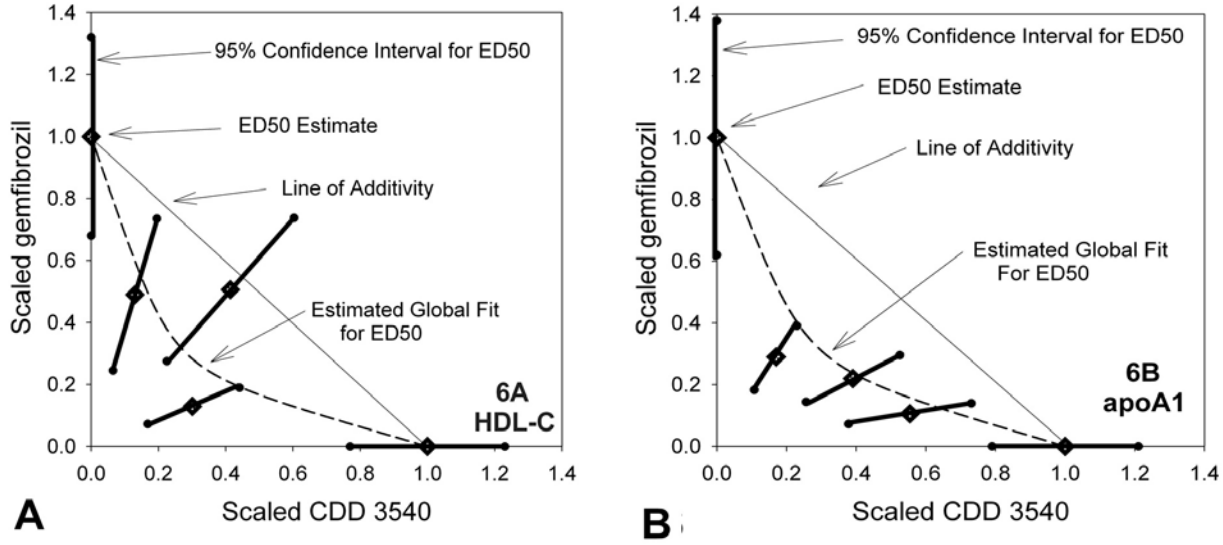

Figure 6. Isobolographs for HDL-C and apoA1, respectively. Each plot includes the estimated ED_{50} values using individual ray data along with 95% confidence intervals and the estimated ED_{50} curve using the global model as estimated from the entire dataset.

employed, and there was substantial *a priori* uncertainty in our knowledge of these models and parameters. Furthermore, for example, an overall D-optimal design for such an experiment would employ very few design points, and hence would provide very little feedback on the structure of the model and would also be substantially non-robust against discrepancies between supposed and actual parameter values.

Second we consider the issue of sample size. For both endpoints, synergism was demonstrated by values for ED_{50} that were shifted two-fold to the left, i.e., the amount of the drug combination required to achieve 50% of the maximal effect was only half that expected under additivity. These results can be seen for the individual combination rays in Figures 3-5 and for the global model (Figure 6). We note that this represents a moderate level of synergism. This, in conjunction with the result that the global model synergism parameter was statistically different from zero for both endpoints, suggests the surprising result that this type of analysis can be performed on *in vivo* experiments where the sample size is of necessity smaller than those typically found in *in vitro* studies. Additionally, it is important to note that the sample size was sufficient for coping with the increased variability inherent to *in vivo* experimentation.

Finally we consider our approaches to the modeling process itself. We acknowledge here that while we were able to obtain valid statistical answers to the key questions addressed by this study, the small sample size did force us to make some compromises along the way. First we note that, in part due to the limited number of drug combination rays employed, only simple models for the ED_{50} parameter as a function of drug fractions were reasonable. Second, limitations of preliminary sample size,

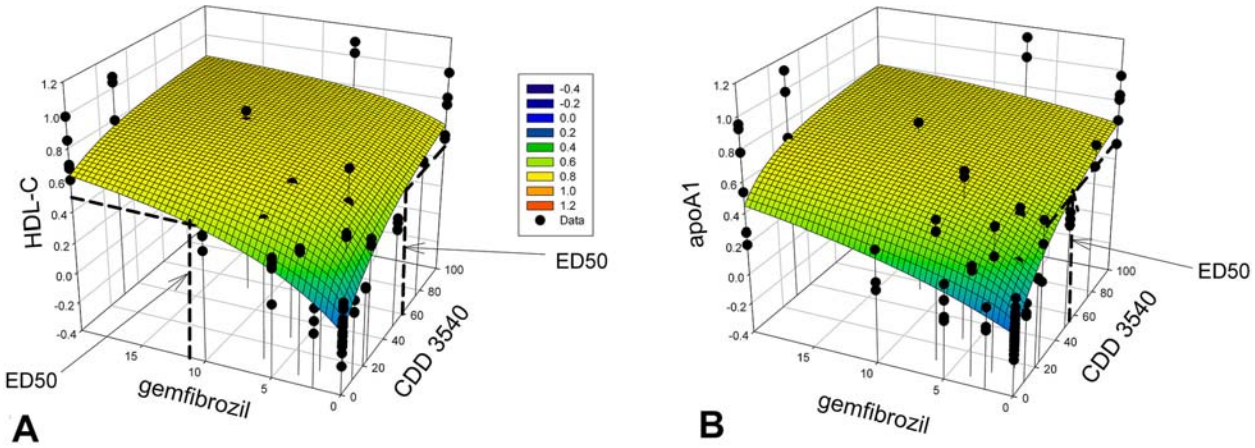

Figure 7. Three-dimensional plots of the raw data and global response surface model for HDL-C and apoA1, respectively.

toxicity-limited range of drug dosage for CDD 3540, and inversion of the dose-response relationship for gemfibrozil at doses exceeding 20 mg/kg, led to substantial uncertainty in our preliminary estimates of the individual drug ED_{50} values. This led to some errors in constructing the experimental design since the design was set in terms of the scaled doses.

For the completed dataset, the same limitations of sample size and dosage range made the simultaneous estimation of both Emax model parameters, E_{max} and ED_{50}, problematic. The primary issue is the limited dosage range available in this *in vivo* study due to toxicity and inversion of the dose-response curve at high doses. In such an experiment where the Emax model is to be employed for effective estimation for both parameters, it is important to obtain data in both the region where the model function is rising (dosage somewhere in the vicinity of the ED_{50}), and in the region where the model function is relatively flat (dosage where the observed effect is close to the E_{max}). High dose toxicity precluded us from obtaining the necessary information from this "flat" region. Since answering the key question regarding synergism requires high quality information about the ED_{50} parameters for the various drug combinations, and since high dose toxicity prevented us from estimating both ED_{50} and E_{max} well, we adopted an approach where information on E_{max} was utilized in a non-standard way. In our modeling scheme, the nonlinear model parameters (in this case E_{max} and ED_{50}) are to be written and estimated as simple canonical mixture polynomial functions of the drug dose fractions. Due to the limitations described above, here we have employed multiples of a smoothed version of the maximum observed value in place of these estimates. In nonlinear estimation with the Emax model, there is a high correlation between the estimates of the two parameters. Hence the ED_{50} values themselves will be affected by any inaccuracies in the E_{max} values used. This is why we were careful to attempt several different modifications with our handling of E_{max}. The result was that, while we maintained the observed pattern of E_{max} across the combination fractions, for several multiples accounting for the likely possibility that the observed maxima underestimate E_{max}, the ED_{50} values were affected but the synergism parameter was not. Hence though we were forced by limitations in available data to modify our modeling approach, we have found that the final conclusion of synergism is robust against a wide range of these modifications.

In conclusion, we have processed drug combination data for a small *in vivo* dataset through a hierarchical modeling approach that has successfully identified statistically significant synergism between gemfibrozil and CDD3540 in increasing HDL-C and apoA1 in mice. This represents a substantial step forward in this type of nonlinear modeling in the presence of a severely limited amount of data. Further, through our statistical modeling procedures, we believe that these two drugs might be found to exhibit clinically valuable synergism and we have learned important facts about their mechanisms of action.

6. ACKNOWLEDGEMENTS

Supported in part by The University of Toledo's DeArce Memorial Endowment Fund, and The University of Toledo's Distinguished University Professor Award (for Dr. Bachmann).

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Key Words: high density lipoproteins; apoprotein A1; synergism; nonlinear response surface; mixture experiment

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