Case Study

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Use of Fractional Factorial Designs in Antiviral Drug Studies

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Experimental design and analysis is an effective and commonly used tool in scientific investigations and industrial applications. Many successful applications have been reported in engineering domains, such as chemical engineering, electrical engineering, and mechanical engineering. However, few cases have been reported in biological research, particularly in virology study. Antiviral drug combinations are increasingly used to reduce possible drug-resistant viral mutant and reduce cytotoxicity. Drug combinations have often been reported to have higher efficacy and lower individual drug dosage. However, the combined antiviral drug effect is generally hard to assess. One important reason is due to the complex interactions between biological systems and drug molecules. We report a study using fractional factorial designs to investigate a biological system with Herpes simplex virus type 1 and five antiviral drugs. The experiment uses a novel composite design that consists of a 16-run fractional factorial design and an 18-run orthogonal array. The results indicate that two chemical drugs, Ribavirin and Acyclovir, are more effective than three Interferon drugs. Furthermore, significant interactions exist within the Interferon drug group and within the Ribavirin-Acyclovir chemical drug group, but the interactions between the Interferon group and the chemical group are not significant. These observations have major implications in the understanding of antiviral drug mechanism towards better design of combinatorial antiviral drug therapy. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: composite design; drug combination; experimental design; fractional factorial design; Herpes simplex virus

1. Introduction

Experimental design and analysis is an effective and commonly used tool in scientific investigations and industrial applications. Factorial and fractional factorial designs, especially two-level and three-level designs, are the most commonly used experimental plans in practice. They have been successfully applied in a wide variety of fields of application, including engineering, physical and chemical sciences, medical and life sciences. Many textbooks on experimental design, such as,[1–6] provide various real applications.

Despite of the aforementioned popularity, few applications have been reported in biological studies using factorial designs with four or more factors, especially in virology. Here, we report a study using fractional factorial designs to investigate a biological system with Herpes simplex virus type 1 (HSV-1) and five antiviral drugs. The goals were to learn what effects the five drugs had on the virus, with a special interest on the interactions among the drugs. Antiviral drug experiments require the preparation of cell culture and viral infection and are very time consuming and costly. A big issue was reproducibility due to the large batch to batch variation and the inherent complexity of the underlying biological system. To overcome this and other issues, we used a novel composite design that consists of a 16-run factorial design with two levels and an 18-run orthogonal array with three levels. The resulting composite design has three levels and 34 runs so that it was possible to conduct the experiment using a single batch of cell samples to reduce cost and variation. The data analysis successfully identified important drugs and drug interactions.

The paper is organized as follows. In Section 2, we describe some background of the antiviral drug experiment and experimental design. In Section 3, we analyze the data from the experiment and discuss the results. Section 4 gives a summary.

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2. Antiviral drug experiment and experimental design

HSV is known to cause diseases of various severities, including mucocutaneous diseases, neonatal herpes, and herpes encephalitis.[7] Recent reports also suggest HSV infection could strongly increase risk for HIV infection.[8] HSV has become one of the most common sexually transmitted diseases in U.S.A., U.K., French, and other western societies.[9–11] Furthermore, HSV encephalitis is the most common form of fatal encephalitis in the U.S., occurring about 2 per 100,000 persons yearly in the U.S..[12] Many therapeutic agents both pharmaceutical and chemical have been developed and used for HSV infections.[13,14] While these agents are generally effective, drug resistance and toxicity concerns have been increasingly reported.[15,16] In order to reduce the possible selecting drug-resistant viral mutants and the cytotoxicity, combinations of different antiviral drugs have been widely used.[17] In general, it is uncertain how the combined antiviral drug effect should be assessed. The main concern is the fact that possible drug interactions hamper prediction of synthetic drug effects. Because of the multitude of potential interactions, each possible drug interaction can hardly be tested individually.

2.1. *Experimental design*

We investigated a biological system with HSV-1 and five antiviral drugs: Interferon-alpha (*A*), Interferon-beta (*B*), Interferon-gamma (*C*), Ribavirin (*D*), and Acyclovir (*E*). The first three are protein drugs, and the latter two are chemical drugs. Before this study, we performed single drug pilot studies and found the minimum effective dosage for each drug at which the drug's antiviral effect reached plateau. This minimum effective dosage was set as the high dosage level in this study. Two more dosage levels were included in the study by setting the middle level to be 32 times diluted from the high level and the low level to be no drug. The dosage of the middle level was decided based on pilot studies. Table I shows the actual dosage levels for five drugs. For analysis purpose, the low dosage level was encoded as -1, the middle dosage was encoded as 0, and the high dosage was encoded as $+1$.

With careful consideration of experimental cost, time, and statistical efficiency, we constructed a novel composite design for this experiment. This composite design consists of two popular fractional factorial designs: (i) a 16-run design with two levels, coded as -1 and 1, and (ii) a 18-run design with three levels, coded as -1, 0, and 1. The 16-run design is a half fractional factorial design with resolution V and is defined by $E = ABCD$. This 16-run design itself enables the estimation of all the linear and interaction effects among the five drugs. The 18-run design is a subdesign of the commonly used three-level orthogonal array $OA(18, 3^7)$; see, e.g., Table 8 C.2 of Wu and Hamada.[5] For any two columns of the 18-run design, there are $3^2 = 9$ possible level combinations, and each combination appears twice. The 18-run orthogonal array allows the estimation of linear and quadratic effects of the drugs, as well as the estimation of some interactions among the five drugs; see, e.g.[18,19] Putting the two designs together, we got a 34-run composite design that could be used to estimate the linear, quadratic, and interaction effects among the five drugs. Furthermore, the 34-run composite design was small enough so that we could conduct the experiment with a single batch of cell samples, saving cost and time. Two researchers carried out the experiment independently with different random orders, yielding two replicates for each run. Table II gives the design and data of the experiment. Each run represented a combinatorial drug treatment, and the outcome, called readout, was the percentage of virus infected cells after that treatment.

A possible alternative to the 34-run composite design would be a 27-run central composite design that consists of the 16-run fractional factorial design of resolution V, 10 axis points, and one center point. Central composite designs are popular designs for response surface modeling.[1–5] A less appealing alternative design would be a Box-Behnken design because it requires more runs (at least 41 runs) and is less efficient in estimating the parameters in a second-order model than the central composite design.[20]

We constructed the 34-run composite design by replacing the ten axis points and the center point with an 18-run orthogonal array. The idea of using an orthogonal array rather than the axis points was inspired by the recent developments in the study of nonregular fractional factorial designs.[21] We opted for the 34-run composite design because it gave more precise estimation on the parameters than both the central composite design and the Box-Behnken design. A paper studying a class of such composite designs will appear elsewhere. Interested readers can request a copy from the corresponding author.

2.2. *Cell culture condition and viral infection*

We provide some technical details of the antiviral drug experiment. NIH 3 T3 cells were chosen as host cells. Cells were initially cultured on 15-mm plates covered with 25-mL culture medium. The culture medium was made from DMEM in the presence of 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin (Pen-Strep). The 15-mm plates were maintained in 37-C incubator filled with 5% CO_2. Cultures were propagated at 10^7 cells/plate every 24 h for two times before use in experiment.

Table I. Factors and levels of the antiviral drug experiment

	Levels		
Factor	Low (-1)	Mid (0)	High ($+1$)
A = Interferon-alpha	no drug	1.56 ng/mL	50 ng/mL
B = Interferon-beta	no drug	1.56 ng/mL	50 ng/mL
C = Interferon-gamma	no drug	1.56 ng/mL	50 ng/mL
D = Ribavirin	no drug	781 ng/mL	25,000 ng/mL
E = Acyclovir	no drug	156 ng/mL	5000 ng/mL

Table II. Design and data of the antiviral drug experiment

	Factor					Readout	
Run	*A*	*B*	*C*	*D*	*E*	Replicate 1	Replicate 2
1	1	−1	−1	−1	−1	69.8	72.0
2	−1	1	−1	−1	−1	66.4	67.4
3	−1	−1	1	−1	−1	83.0	68.6
4	−1	−1	−1	1	−1	16.2	23.4
5	−1	−1	−1	−1	1	46.1	33.6
6	1	1	1	−1	−1	68.6	65.5
7	1	1	−1	1	−1	6.8	7.2
8	1	1	−1	−1	1	15.6	19.1
9	1	−1	1	1	−1	11.1	7.0
10	1	−1	1	−1	1	19.8	20.3
11	1	−1	−1	1	1	3.7	4.7
12	−1	1	1	1	−1	5.8	3.9
13	−1	1	−1	1	1	2.6	4.0
14	−1	1	1	−1	1	42.2	23.2
15	−1	−1	1	1	1	1.8	5.2
16	1	1	1	1	1	3.1	3.4
17	−1	−1	−1	−1	−1	78.6	81.9
18	0	0	0	0	0	13.3	16.7
19	1	1	1	1	1	3.4	3.8
20	−1	−1	0	0	1	21.4	25.2
21	0	0	1	1	−1	8.6	4.4
22	1	1	−1	−1	0	18.0	27.3
23	−1	0	−1	1	0	7.3	2.4
24	0	1	0	−1	1	17.9	23.7
25	1	−1	1	0	−1	52.9	54.3
26	−1	1	1	0	0	13.2	8.8
27	0	−1	−1	1	1	2.1	4.5
28	1	0	0	−1	−1	73.4	73.9
29	−1	0	1	−1	1	19.6	14.6
30	0	1	−1	0	−1	59.1	41.7
31	1	−1	0	1	0	1.4	2.6
32	−1	1	0	1	−1	7.3	4.8
33	0	−1	1	−1	0	22.3	24.0
34	1	0	−1	0	1	14.1	18.3

Cell infection was carried out in 24-well plates. Each well contained 2×10^5 cells in 1-mL culture medium. Cells were allowed to grow for 4 h before viral infection and drug treatments occurred. Drug combinations were added simultaneously with HSV-1 to the host cells in 24-well plates by two researchers. The plates were incubated at 37-C incubator with 5% CO_2 for 16 h.

The virus was engineered to carry the green fluorescent protein (GFP) gene. Thus, cells infected with virus would be GFP positive. GFP served as a biomarker to assess the percentage of infected cells. The readout was defined as the percentage of GFP positive cells after combinatorial drug treatment. The readout was measured through a flow cytometer (BD FACSCanto II, BD Biosciences).

Two researchers carried out the experiments using the same cell stock, drug stock, virus stock, and the measuring instrument. Each researcher had two 24-well plates in their experiments. One well of each plate was used as positive control with viral infection but no drug treatment. The 34 runs were randomly assigned to the remaining 23 wells on the first plate and 11 wells on the second plate (12 wells on the second plate were not used). The two researchers used two different random orders. They independently added virus and drug combinations to the wells and mixed the wells afterwards. Possible experimental errors included the pipetting error (wrong drug dosage) and the mixing error (lack of mixing after each pipetting). One researcher had more experimental experience than the other.

3. Data analysis and results

3.1. *Model fitting*

The main purpose of the experiment was to evaluate the antiviral effect of drug combinations and to detect any interactions among drug components. For this purpose, we fit a second-order model

$$y = \beta_0 + \sum_{i=1}^{5} \beta_i x_i + \sum_{i=1}^{5} \beta_{ii} x_i^2 + \sum_{1=i<j}^{5} \beta_{ij} x_i x_j + replicate + \varepsilon, \tag{1}$$

where y is the response, $x_1, \ldots, x_5$ are the five antiviral drugs (coded as $-1, 0, 1$), $\beta_0, \beta_i, \beta_{ii}$, and β_{ij} are the intercept, linear, quadratic, and interaction (or bilinear) terms, respectively, and $\varepsilon \sim N(0, \sigma^2)$ is the error term. We include the variable *replicate* in the model to capture possible effect from the two researchers. It is coded as -1 for replicate 1 and 1 for replicate 2.

We start with fitting the second-order model (1) using the percentage of viral infection as the response, that is, $y = readout$. The estimates of the parameters are given in column (a) of Table III. The model fits the data quite well with an R^2 value of 96.1%. Table III(a) shows that the linear effects D and E, the quadratic effect E^2, and the interaction DE are significant at the 0.1% level; the linear effect B and quadratic effect D^2 are significant at the 1% level; the linear effect C and interaction AC are significant at the 5% level. The variable *replicate* is not significant, indicating no statistical difference between the replicates from the two researchers. However, residual analysis identifies some problems on the model assumptions. The variance of the residuals increases with the fitted values, and the residuals are positively skewed. It appears that a transformation on the response is needed. We investigate this with the Box-Cox method which suggests a square root transformation on the response.

We refit the second-order model (1) with $y = \sqrt{readout}$, and the estimates are given in column (b) of Table III. The new model has a slightly higher R^2 than the old model. Both models have the same significant effects with the addition that D^2 and AC become more significant in the new model. Residual analysis indicates that the usual assumptions on the error are reasonable. However, run 14 of replicate 1 turns out to be an outlier. This is indeed obvious by inspecting Table II. The two replicates of run 14 have large discrepancy and replicate 1 appears to be too large and suspicious. We remove this outlier and refit the model. Column (c) of Table III gives the results. We observe that significant effects identified earlier remain significant, and three effects, B, C, and AC, become more significant. In addition, the interaction AB becomes significant at the 5% level after the removal of the outlier. We further perform variable selection via stepwise regression and confirm no other significant effects. The final model is

$$\begin{aligned}\sqrt{readout} = {} & 4.21 - 0.09A - 0.30B - 0.21C - 2.03D - 1.23E \\ & -1.13D^2 + 1.41E^2 + 0.17AB + 0.30AC + 0.60DE,\end{aligned} \tag{2}$$

which has an R^2 of 96.9%. We keep the linear effect A in the model because AB and AC are significant.

Table III. Estimates of parameters for the antiviral drug experiment

	(a)	(b)	(c)
Intercept	17.39***	3.99***	4.08***
A	−1.60	−0.13	−0.09
B	−2.36**	−0.23**	−0.27***
C	−2.01*	−0.20*	−0.22**
D	−19.59***	−2.07***	−2.03***
E	−13.95***	−1.22***	−1.24***
A^2	3.65	0.26	0.24
B^2	0.16	0.09	0.06
C^2	−1.70	−0.01	−0.05
D^2	−7.21**	−1.17***	−1.18***
E^2	15.39***	1.41***	1.37***
AB	0.71	0.12	0.17*
AC	2.34*	0.26**	0.29***
AD	1.46	0.08	0.04
AE	−1.32	−0.13	−0.08
BC	1.64	0.14	0.09
BD	−0.28	−0.09	−0.03
BE	1.31	0.13	0.08
CD	−0.66	−0.11	−0.06
CE	0.11	0.05	0.01
DE	9.56***	0.54***	0.57***
Replicate	−0.52	−0.03	0.00
$\hat{\sigma}$	6.095	0.5515	0.4833
R^2	0.961	0.965	0.973

NOTE: The response in the analysis is (a) $y = readout$; (b) $y = \sqrt{readout}$; (c) $y = \sqrt{readout}$. In (c), replicate 1 of run 14 is removed. Significance levels are coded as 0 (***) 0.001 (**) 0.01 (*) 0.05.

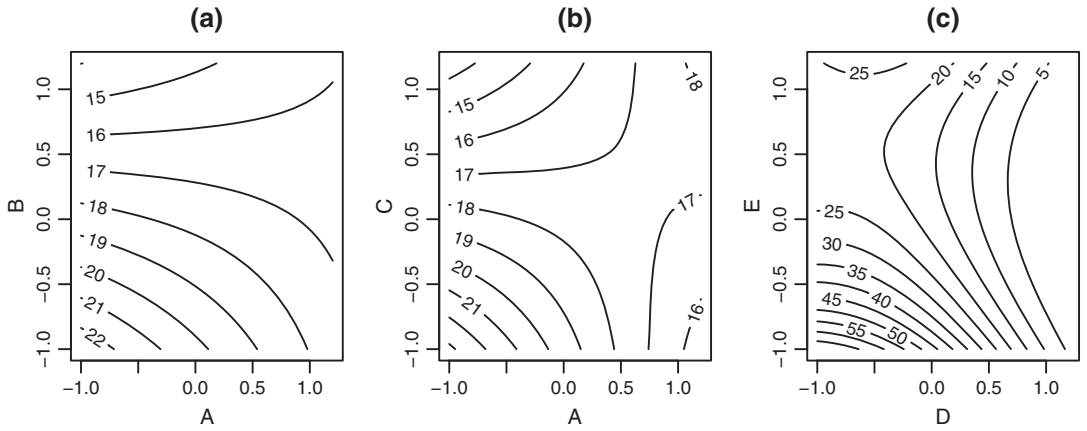

Figure 1. Contour plots of predicted readout

3.2. *Results*

The data analysis identifies that Ribavirin (*D*) and Acyclovir (*E*) are very effective drugs, and both drugs have nonlinear (quadratic) effects on HSV-1. The other three drugs (*A*, *B*, and *C*) have some but much smaller effects compared with Ribavirin and Acyclovir. We further observe strong interaction between *D* and *E*, and possibly significant interactions among *A*, *B*, and *C*. Note that *A*, *B*, and C are Interferon drugs that are cytokines derived from immune system,[22] while *D* and *E* are chemical drugs designed more specific to work at DNA and RNA level.[23,24] The data suggest that the interactions within both Interferon group and chemical drug group are significant, which agrees with published reports from clinical trials.[25,26] However, no significant interactions between Interferon group and Ribavirin/Acyclovir are observed, indicating distinct antiviral pathways between these two drug categories.

To further understand the system, we examine the contour plots of the predicted percentage of viral infection from the final fitted model (2). Figure 1(a)(b)(c) shows the contour plots of the predicted readout in terms of *A* and *B*, *A* and *C*, and *D* and *E*, respectively, when the other drugs are held at the middle level 0. In Figure 1(a)(b), when A, B, and C are all at high level, the viral infection percentage readout is not optimal. The minimum viral infection is achieved when *A* is set at low while *B* and *C* are set at high level. Therefore, overdose Interferon-alpha would indeed interfere Interferon-beta and Interferon-gamma efficacy. In Figure 1(c), although optimal antiviral readout is attained when *D* and *E* are both at high level, we can indeed achieve the same antiviral readout by reducing both *D* and *E*. Lowering drug dosage usually leads to reduced toxicity. Therefore, inhibiting the same amount of viral infection using lower dosage of both Ribavirin and Acyclovir is practically meaningful.

4. Summary

We present the design and analysis of an experiment that studies the system of HSV-1 and five antiviral drugs. The results of this study show that HSV-1 infection can be suppressed by using a combination of Interferon drugs and chemical drugs. The statistical analysis suggests that interactions within Interferon drug group and chemical drug group are significant, but interactions between the two groups are not significant. The interactions as well as the quadratic effects of Ribavirin and Acyclovir suggest a nonadditive relationship between drug dosages and antiviral outcomes. Moreover, our study indicates the same optimal antiviral efficacy can be achieved at lower dosages for both Ribavirin and Acyclovir when used as combination.

The application of experimental design to investigate complicated drug–drug interactions represents an innovative approach in virology. In the present study, we apply a 34-run composite design to study five drugs at three dosage levels. As the number of drugs and level of dosages increase, larger and more complicated designs would be required. As this study demonstrates, experimental design and analysis can play a vital role in the study of more complicated biological systems.

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