ANALYSIS OF COMBINED EFFECTS OF DISCODERMOLIDE WITH CLINICAL ANTICANCER AGENT, PACLITAXEL

by

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ABSTRACT

Study of multiple drug usage is common in chemotherapy. Many research studies examine the effect multiple usage of these anticancer drugs. The study of these drugs together could help clarify the biological effects of agents that affect microtubules. This could guide future cancer treatment for patients especially these with breast cancer, an important public health issue. The main purpose of this paper is to apply and compare methods for examining the combined effects of anticancer drugs. We focus on Paclitaxel and Discodermolide. While in the process of modeling the single drug effect, we noticed that these anticancer drugs may be unable to kill all the cells even at high concentration, or that, some subpopulation is far less sensitive: there may be a mixed population of two types of cells with very different sensitivities. According to this description, one population of cell is very sensitive to the drug and the other one is nearly resistant to the drug. This model is difficult to fit. One approach is to fit the low-concentration and high-concentration portion of the dose-response curve separately, and then combine them. We constructed a simple method to predict the combined drug effects while adjusting for the assumption of mixed cell populations. We will summarize the commonly used methods for evaluating drug combination. Two models are commonly used as reference models to test the drug additive effect, which are dose-additive models and effect-additive models. For effect-additive models, we focus on the "mutually exclusive" and "mutually non-exclusive" additive effect models, and another additive effect model based on a population log kill mixture model.

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1.0 INTRODUCTION

1.1 PURPOSE OF STUDY

One possible cause for super-additivity could be heterogeneity in drug sensitivity. We wish to develop a general statistical method to study for drug interaction while taking into account the factors of plateau of drugs or a mixed population of cells that other traditional methods may not consider.

The cell heterogeneity assumption is that within each well of the experiment plate, there are four kinds of cell population. These four populations include, the population of cells that are sensitive to both drug A and drug B, the population of cells that are ONLY sensitive to drug A or drug B; and the population of cells that is resistant to both drug A and drug B. The proportions that are according to these four cell population are giving labels as P_{AB}, P_A, P_B, R_{AB}, respectively.

One paper has recognized the importance of issue of cell heterogeneity when one wants to estimate the effect of chemotherapy on the tumor cell lines.[1] This heterogeneity may create a problem when fitting standard model to estimate the affected fraction. The dose response curve may flatten or even reach a plateau at high dose. However, we cannot be sure if this

plateau is an experiment artifact or if there truly exists a plateau for drug effect. The model we present here is based on the working assumption that a plateau truly exists.

Some pairs of drugs may have already been discovered to have a super-additive effect when they are combined in chemotherapy. Paclitaxel and Discodermolide would be an example. [2] This paper will only focus on Discodermolide and Paclitaxel. We performed analysis on the Paclitaxel and Discodermolide while adjusting for the mixed cell population assumption to verify if the apparent non-additivity still holds and construct a model for predicting the combined drug effect.

To develop a simple way to fit this cell heterogeneity assumption, we use two approximations; one includes the wells treated with low concentrations; the other indicates the wells treated with high concentrations. The first may reveal the parameters governing a more sensitive subpopulation. The second may reveal parameters governing a less sensitive or even resistant subpopulation.

To evaluate drug additivity, there are two different concepts of additive models; they are dose-additive and effect-additive models. Dose-additivity is the usual benchmark agent if want to evaluate "synergism", using the combination method. We also examine on effect-additive model, well-known examples are the mutually exclusive and mutually nonexclusive additive models. By fitting Paclitaxel and Discodermolide with mutually exclusive and mutually nonexclusive models, we can check if our models would produce the same result as other papers have stated. In the future, maybe the models we present here can be used to understand more about the mechanism of other microtubule perturbing agents, either known or unknown. We will also base our mixed population assumption on effect-additive model.

The terminology used in discussion of combination of agents is still ambiguous. "Synergism", "synergy", and "super-additivity" are used exchangeably, but with multiple meanings. Super-additivity sometimes is called synergism, which is when two drugs work together to create an effect that is "greater than expected" (dose super-additivity or effect super-additivity). The methodology of how to evaluate the drug effect when they work together is developing and there are competitive ways of doing this. As statisticians, we can evaluate from the data if there is more than an additive effect relative for a particular additive model, but the data can only suggest, not prove, any mechanistic biological interaction between the drugs. Studying combinations of drugs could help to reduce the dose the patients need and possibly reduce the side effect of the drugs. We will be using "dose" and "concentration" interchangeably in this paper.

2.0 RELEVANT LITERATURE

2.1 THE DRUGS

Anticancer drugs have several working mechanisms to stop the cancer growth. One of the ways is to condense microtubules of cancer cells and stabilize them from deploymerization. Paclitaxel and Discodermolide are two examples that work to condense microtubules and interrupt the mutated cell from further splitting. [2][4] Paclitaxel is the generic name of Taxol. Taxol has been used in ovarian, breast, and non-small lung cancer. Discodermolide was isolated from a marine sponge and reported to induce the assembly of micrutubles in vitro more rapidly than Taxol and cause mitotic arrest and microtubule bundling.[2] There are several papers that study the combined effect of Paclitaxel and Discodermolide. Most studies use the median-effect method and combination index from Chou and Talalay [3] to determine if the agents work together would produce a super-additive effect. However, they failed to take into account other statistical approaches, which to be discussed in the paper.

Figure 1 is the graph where you can see how cell division works and *METAPHASE* is the phase where these agents work.

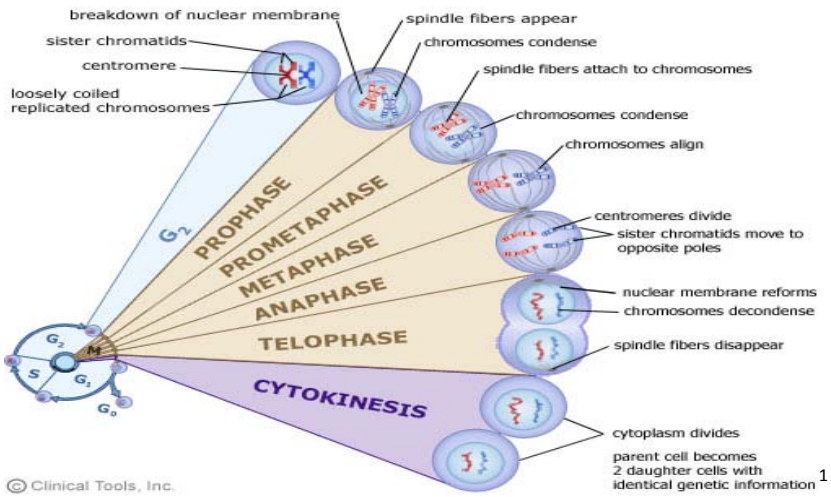

Figure 1 Cell Cycle

A study was reported on the effect of three new agents, epothilones, eleutherobin, and discodermolide, that were identified, which have similar mechanism with Taxol. [2] This paper was focusing to compare and contrast these new agents in Taxol - sensitive and –resistant cell lines. They used Taxol-sensitive cell lines to see if some of these agents can be used as substitute for Paclitaxel. The researchers observed in Taxol- resistant cell line that "the presence of Taxol significantly amplified the cytotoxicity of discodermolide, and this phenomenon was not observed in combinations of Taxol with either the epothilones or eleutherobin." Thus, the paper suggested that Taxol and discodermolide may produce a super-additive effect in chemotherapy. The researchers in this paper have considered the assumption of mixed cell populations, so they isolated the taxol-resistant cell line for Discodermolide to works on. Therefore, heterogeneity of cell population is an important when we wish to evaluate the combined drug effect.

[1] https://eapbiofield.wikispaces.com/CHAPTER+12+THE+CELL+CYCLE+WH

Another research done at the University of California, Santa Barbara, and Albert Einstein College of Medicine, Brownx, New York, demonstrated that Paclitaxel and Discodermolide inhibited the growth of lung cancer cells by 41 percent when used together.[5] Discodermolide and Paclitaxel works alone only inhibited proliferation the cancer cell by 9.6 and 16 percent, respectively. They suggested that Paclitaxel and Discodermolide would synergistically block cell cycle progression. It was known that Paclitaxel and Discodermolide have similar mechanism and they are competitive for the same binding site on microtubules, so it was surprising to see that these two agents would work together to create super-additive effect, quoted by the paper.

One thing needs to be aware is these conclusion were based by using lung cancer cell lines, but the data we have here is using breast cancer cell lines.

2.2 THE MODELS FOR SINGLE DRUG

We will give a summary of the models that are used to evaluate the combined drug effect. We start the summary from the single drug models. We can combine then combined these single drug models to study the combined drug effect. We will also introduce the mixed cell population assumption and present how to modify the model for this assumption.

2.2.1 Median-effect model

Median-effect model is derived from the mass-action law and describes the dose-effect relationship. The median-effect equation is

$$\frac{fa}{fu} = \left(\frac{D}{Dm}\right)^m \quad (1)$$

where fa is the fraction of the cells that is affected by drug, fu is the fraction of the cells that is unaffected, D is the dosage or the concentration of a drug, D_m is the median-effect dose, and m is the slope of median-effect plot that signify the shape of dose-effect relationship, and m<1, =1, and >1 indicate flat sigmoidal, hyberbolic, and sigmoidal dose-effect curves. Median-effect plot is an important step for plotting the combination index plot, because we can drive the value of m and Dm from this particular plot and plug these values into the equation of combination index. [3][6][7][8]

The median effect equation can be transformed to

$$\log_{10}\left(\frac{fa}{fu}\right) = m\log_{10}(D) - m\log_{10}(D_m) \quad (2)$$

which follows the classic straight line equation y= m*x+ b, where m is the slope of the median effect plot, and b is the y-intercept.

2.2.2 Log(kill) model

The log(kill) model assumes

$$f_u = 10^{-kd} \quad (3)$$

, where fu is the fraction of cell unaffected by the drug, d is the dose of the drug, and k is the killing rate of drug based on power of 10. As the dose increases, the fraction of cells unaffected would decrease. We name this equation as log(kill) model.

2.2.3 Heterogeneity model

However, after some analysis, we discover that maybe there are just a mixed population of cancer cells one portion is very sensitive to drug and the other part is nearly resistant to the drug. This is the concept we want to focus on in this paper.

We suggest that heterogeneity of cells is an important concept to consider when we want to evaluate the drugs effect. It needs to be aware that the cell population we label as resistant to the drug does not mean they are 100% resistant to the drugs, but they appears resistant to the dose range that we have. Perhaps, ff the dose increases to infinitely large, all the cells can still be killed.

We alter the log(kill) model to this assumption

$$\frac{Z(d_D)}{\text{avg.\# of control cells}} = (P_S + P_D)\theta_{D(1)}{}^{d_D} + (1 - (P_S + P_D))\,\theta_{D(2)}{}^{d_D}$$

$$= \psi_D \theta_{D(1)}{}^{d_D} + (1 - \psi_D)\theta_{D(2)}{}^{d_D} \qquad (4)$$

where $Z(d_D)$ is the cell count corresponds to different dose of drug and D indicates which drug is used. $\psi_D = P_S + P_D$, P_S is the population proportion of cells that are sensitive to both of drugs and P_D is the population proportion of cells that are sensitive to only one of the drugs. $\theta_{D(1)}{}^{d_D} = \left(e^{-K_{D1}}\right)^{d_D}$ is the proportion of cells that are highly sensitive to the drug D even when the drug increase in small scale, and $\theta_{D(2)}{}^{d_D} = (e^{-K_{D2}})^{d_D}$ represents the second portion of cells that react slowly to the drug, which is at end of high level of concentration.

2.3 THE MODELS FOR COMBINED DRUGS

There are two primary classes of additive models used as references when evaluating the combined effects of drugs: dose additivity and effect additivity.

2.3.1 Dose Additivity

In a dose additive model, the two drugs act as if they are equivalent up to a dose conversion factor, or potency ratio, K. If K is equal to one, then two drugs are equally potent. If K is not equal one, then one drug can be treated as a more concentrated or more diluted form of the other drug. Then the combined effect is given by

$$e(d_1, d_2) = e(d_1 + kd_2, 0) = e(0, k^{-1}d_1 + d_2). \quad (5)$$

In the dose additive model, the transformation of effect is not important. However, thechoice of effect transformation is important for the error model. It is assumed that the dose is untransformed.

2.3.2 Combination Index (CI) Analysis

Combination index is frequently used to describe deviations from dose additivity between two drugs. Combination-Index was developed in 1983 by Chou and Talalay for comparing data to a dose additive model assuming median effect single drug models.[3][6][7]

$$CI = \frac{(D)_1}{(D_X)_1} + \frac{(D)_2}{(D_X)_2} = \frac{(D)_1}{(D_m)_1\left[\frac{fa}{1-fa}\right]^{\frac{1}{m1}}} + \frac{(D)2}{(D_m)_2\left[\frac{fa}{1-fa}\right]^{\frac{1}{m2}}} \quad (6)$$

where $(D)_1$ and $(D)_2$ indicates the dosage of drug 1 and drug 2 respectively. $(D_X)_1$ and $(D_X)_2$ is the dose of drug 1 or drug 2 that inhibit x% of cell growth. f_a is the fraction of cell that is killed by the drugs. ${F_1}^{-1}\left(\frac{f_a}{f_u}\right) = (D_X)_1$ and ${F_2}^{-1}\left(\frac{f_a}{f_u}\right) = (D_X)_2$, where F_1 and F_2 are dose response curves. Dm is the dose that is required to reach the median effect and can be calculated from the median-effect equation (2) as

$$D_m = 10^{-\frac{b}{m}} \quad (7)$$

where m is the slope of the median effect plot, and b is the intercept (constant term).

CI <1, =1, and >1 are sometimes interpreted as synergism, additivity, and antagonism, respectively. [3][6][7][8] Chou and Talalay combines the single drug median-effect model estimation for F1 and F2 with Equation (6).[3][6][7][8]

In order to plot the CI, we can to first fit the median-effect models for each drug to estimate Dm and m value for each drug. Next, plug these values into the CI equation (6), to get the CI value for different dose combinations.

Equation (6) is not limited to use the median effect dose to find the CI value. The CI can be interpreted as follows.

According to the dose-additivity model, we can construct parallel lines for different values of effects, f_a. Suppose x% effect can be reached for drug 1 and drug 2 alone, with $(D_X)_1$ and $(D_X)_2$. However, x% is reached with d_1 and d_2, that is the point $CI(d_1, d_2)$ in Figure 2.,

instead of our expected doses, and assume we know the potency, k, between drug 1 and drug 2. Use d_1, d_2, and k, we can reconstruct the dose-effect line that d_1 and d_2 should have corresponded to, for example y%, which should parallel to the dose-effect line of x%. We can then calculate the single drug doses that are expected to reach y%. For example, $(d_y)_1 = d_2 * k + d_1$. If we divided $(d_y)_1$ by $(D_X)_1$, this will reproduce the equation (6) and this will be the CI value.

$$\mathrm{CI} = \frac{(d_y)_1}{(D_X)_1} = \frac{(d_y)_2}{(D_X)_2} \tag{8}$$

If lines are parallel.

It is important to know that to be able to interpret the CI value this way is valid when m_1, m_2 in Equation (6) are equal. Different values of m change the sigmoid shape of the median effect line and the isoboles will not be parallel with each other. If the isoboles are not parallel with each other, Equation (8) is no longer true.

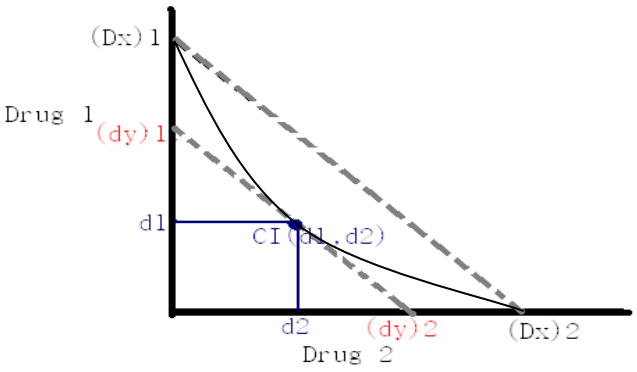

Figure 2 Isobole

2.3.3 Effect additivity

In an effect-additive model, given the effect of each drug, when the two drugs are combined, the predicted effect is the sum of two single drugs' effects relative to the chosen sacle.

$$f(e(d_1, d_2)) = f(e(d_1, 0)) + f(e(0, d_2)) \quad (9)$$

where e is the effect of the drug measured as the fraction of cells killed.

Transformation of effect is crucial because whether effect-additivity holds depends on the scale on which the effect is measured.

If there is an effect greater than the expected, then there exists a super-additivity effect.

$$f(e(d_1, d_2)) > f(e(d_1, 0)) + f(e(0, d_2)). \quad (10)$$

2.3.4 Effect-additivity: mutually exclusive

In statistical definition, "mutually exclusive events are two or more events for which the occurrence of one event precludes the occurrence of the others" [9]. In equation form it will simply be $e=e_1+e_2$, no interaction term involves. In pharmacology, mutually exclusive drugs are two drugs that have the same or similar modes of action. [2] Since two drugs have the same mechanism and they are competitive with each other for the same working site, two drugs cannot work simultaneously on the same working site. Due to the law of mass action, this takes an altered form.

The effect-additive equation for the mutually exclusive model is

$$\frac{e}{1-e} = \left(\frac{e_1}{1-e_1}\right) + \left(\frac{e_2}{1-e_2}\right) \quad (11)$$

where e= effect of the drugs. e_1, e_2 are effect for drug 1 and drug 2 alone. This equation is equivalent to Equation (9) with transformation f(e)= $\frac{e}{1-e}$.

Research has shown that Discodermolide is competitive with Paclitaxel for microtubule binding and Discodermolide has higher affinity, so Discodermolide and Paliclitaxel might fit a mutually exclusive model. [4][5]

2.3.5 Effect-additivity: mutually nonexclusive

Alternatively, mutually non-exclusive drugs mean two drugs are non-competitive. For the *mutually nonexclusive* model

$$\left(1 - \frac{e}{1-e}\right) = \left(1 - \frac{e_1}{1-e_1}\right) * \left(1 - \frac{e_2}{1-e_2}\right) \quad (12)$$

Subtracted one from the left side and the most right side and multiple by -1. Then

$$\frac{e}{1-e} = \frac{e_1}{1-e_1} + \frac{e_2}{1-e_2} - \left(\frac{e_1}{1-e_1}\right) * \left(\frac{e_2}{1-e_2}\right) \quad (13)$$

where e is the effect.

and this equation is equivalent to Equation (9) with different transformation, which is f(e)=log$(1 - \frac{e}{1-e})$.

From the view point of a mutually exclusive model, this looks as though there is an interaction term, which is the product of the previous two terms, times -1, suggesting inhibition. This shows how crucial the choice of effect scale is.

This concept is similar, but not identical to statistical independence of two events.

If the two events, "cell survives drug 1" and "cell survives drug 2", are *independent*

P(a cell would survive if under treatment with drug 1 *AND* drug 2)

=P(a cell would survive under drug 1 only)*P(a cell would survive under drug 2 only).

The model can be written as

$$f(e_{12}) = f(e_1) + f(e_2), \text{ where } f(e_d) = \log(1 - e) \qquad (14)$$

where d=1 or 2, indicates which drug is used.

2.3.6 Effect-additivity: Heterogeneity of cell population

The models that we have discussed so far require that for as the dose goes to infinite large, the cell count approaches zero. The data seems to contract this behavior. This leads us to the assumption of mixed cell populations.

Even within the same cell lines, the cells treated are not likely to be homogeneous with respect to cytotoxicity. There may be some portion of the cell populations that each drug is unable to kill. However, it does not mean this proportion of cell populations cannot be killed because other kinds of drugs may have the ability to kill this portion of cell population due to different biological components or functions. The fraction of cells that is sensitive to drug D but not to the other drug is the probability of cell population that is only sensitive to this drug, so it

is called P_D = P (sensitive to drug D), where D indicates drug A or drug B. Furthermore, the probability of a cell belonging to the cell population sensitive to both drugs A and B would be P_{AB} = P (sensitive to both drug A and B). Vice versa, a cell population that is resistant to both drugs would be labeled as R_{AB} = P(resistant to both drug A and B).

Table 1. Heterogeneity of cell population

P(Sensitive to A, Sensitive to B) = P_S	**P(Sensitive to A, Resistant to B) = P_A**	**P(sensitive to A) = $\Psi_A = P_S + P_B$**
P(Resistant to A, Sensitive to B) = P_B	**P(Resistant to A, Resistant to B) = R_{AB}**	**P(Resistant to A)**
P(Sensitive to B)= $\Psi_B = P_S + P_B$	**P(Resistant to B)**	**=100%**

3.0 METHODOLOGY

The experiment was performed in purpose to test the additive effect within the anti-cancer agents. There are four different anti-cancer agents that were being tested in this data; they are Vincristine(Vinc), , Disorazole C1(Diso) , Paclitaxel, and Discodermolide(Disco). We want to confirm the existing proven case of super-additve effect between Discodermolide and Paclitaxel.

3.1 DATA COLLECTION

The data is from Dr. Vogt, who is the associate director of Fiske Drug Discovery Laboratory of University of Pittsburgh Drug Discovery Institute. The experiments initially were performed for wishing to evaluate the effect between Disorazole C1, the novel anticancer agent, with other anticancer agents, such as Taxel and Vincristine here.

The experiments were performed as follow:

1. On Day 0, plate 1,000 MDA-MB-231 breast cancer cells in plates and separate them into control (non-treated) and case (treated) cells. There are 24 columns and 16 rows, which

adds up to 384 wells, in each plate. Among these 384 wells, there are 96 combinations of different drugs or at different concentration and each combination or concentration has four replications. Also, there are four columns of controls in each plate.

2. Stain the nuclei within cells with fluorescent DNA-binding dye, Hoechst 33342.
3. Treat cells with compounds in 10 two-fold serial dilutions starting at highest concentration.
4. Incubate cells for 72 hours at 37C, 5% CO_2.
5. On Day 4, enumerate the cell number and determine cell density, which is cells per image field, by automated image acquisition and batch image analysis on the ArrayScan II.
6. Calculate growth inhibitory activity as percent cell survival compared to control based on cell expansion over the duration of the study.

The ratio between two drugs is being kept constant by automated a serial dilution.

The measurement of drug effect, which is cells affected fraction in this data, is within the range of 0 to 1.

$$\text{Fraction} = 1 - \frac{\text{Treated Cells}}{\text{Controls (Untreated Cells)}} \tag{15}$$

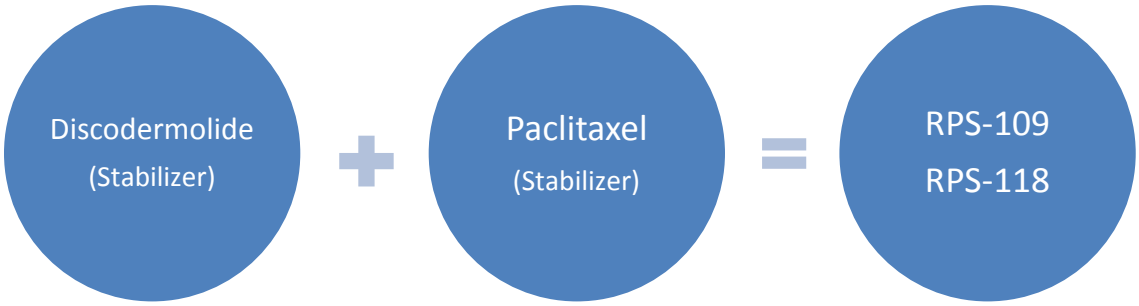

Figure 3 Different experiments have different combinations of drugs. We will only focus on analyzing Paclitaxel and Discodermolide.

The pair we focus on is Discodermolide versus Paclitaxel from RPS-118. The concentrations were as follows

Table 2. RPS-118 Discodermolide vs. Paclitaxel

	Paclitaxel (μM)	Discodermolide(μM) at 1:5	Discodermolide(μM) at 1:2
1	50	250	100
2	25	125	50
3	12.5	62.5	25
4	6.25	31.25	12.5
5	3.125	15.625	6.25
6	1.5625	7.8125	3.125
7	0.78125	3.90625	1.5625
8	0.390625	1.953125	0.78125
9	0.195313	0.976563	0.390625
10	0.097656	0.488281	0.195313

In RPS-118, the drug combinations are Disorazole C1 versus Paclitaxel at 2:1, Disorazole C1 versus Vincristine at 1:1, and Discodermolide versus Paclitaxel at 2:1 and 5:1. There is a plate

First, the data is reorganized in Excel to be suitable for SAS to read and use. The data used here is the cell survival count per field in the wells. There are 384 wells within each experiment plates. Among these 384 wells, 64 of them are control wells and the rest 320 wells

are treated with different concentrations of drugs. There are 10 concentrations of each drug alone and 10 concentration combinations of each drug pair. There are four replications for each concentration combinations and we treat each replication as an independent experiment output. We choose to exam our methods on RPS-118 because the controls in RPS-118 do not have any particular systematic error and it is easier for us to explain the outcome bases on such controls. APPENDIX Table 1A is the plate layout.

3.2 DATA QUALITY ISSUES

One thing to be kept in mind is that we are not sure all these survival cell counts scanned by the machine truly count only living cells. We know the nuclei of cell are stained with fluorescence, but we are not sure if the scanner would definitely only pick up the viable cells. Or it could be that a plate was not clean enough, so there was some unknown chemical left on the plate. Therefore, what we present here is based on the assumption that these cell counts truly count only living cells.

3.2.1 Data Recording

The data we used here must be handling with care because the experiments had several problems which appeared when we examined the data. The data we initially received was in terms of a calculated unaffected fraction (f_u), rather than the original count data. The unaffected fraction that is above one had been deleted from the data, because affected fraction

(f_a) = 1 - f_u will become negative, and calculating CI value cannot include such data. This could cause systematic error in model estimated. Thus, we requested the raw data, the cell counts that were recorded for each well, including controls, 394 wells for each experiment plate.

3.2.2 Outliers

Over-dispersion and outliers are two issues to consider when we want to fit the model appropriately. For the Poisson distribution, the mean is equal to the variance. Outliers could influence the model fit if the variance greatly exceeds the mean. Outliers can be easily detected when we draw the residual plot. When there are outliers, we should be aware that our model fits may be influenced by these outliers and we should be really careful on making conclusion based on such model. One thing we could do is to delete the outliers and fit the model again, this way the model would be less biased. However, deleting outliers is not always the best as this may create other bias problem.

3.2.3 Systematic Errors

We wish to consider other factors that may influence the cell count besides the drugs. We will examine the systematic errors only on the control wells because we can rule out the effect of drugs and it will be easier to detect if there are other factors that would influence the cell counts. The concentrations were not randomly placed on the plate, so row and column may be two factors needed to be considered besides any biological effect of the drugs. We fitted only the counts from control wells with only row and column number and treated row and column number as numeric parameters, not as categorical parameters. Cell counts can be assumed

initially to have the Poisson distribution. The results from SAS show linear row effect is highly statistical significant and they should be considered when we want to fit the model. We can see from Figure 4 and Figure 5 that there is an increasing row effect, but there is no particular trend that suggests column effect. Ideally we should control for the row effect in each model. This has not yet been done.

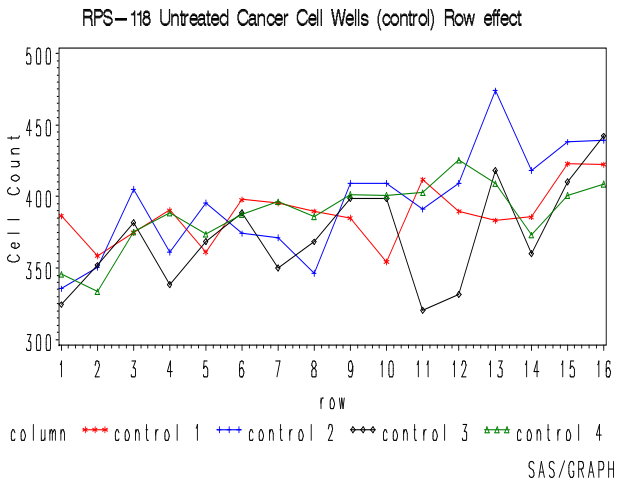

Figure 4. Control wells show increasing row effect

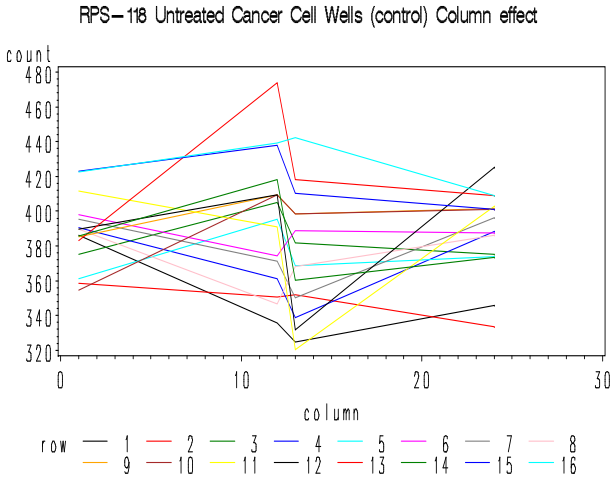

Figure 5. Control wells do not show column effect

3.2.4 Baseline Effect

We do not know how many cells are in each well before we apply our treatments. It would be difficult to keep the number of cells fixed in each well. The survival cell counts in control wells were expected to be random, and drawn from the same distribution as the unknown initial counts in the treated wells. Therefore, this should be added to the model as a

random variable when we estimate the model. To simplify the model fitting process, we fixed the baseline control value by calculating the average of all control wells.

3.3 Fitting Single Drug Effect

Interaction can be defined in several ways when it comes to drugs. By Chou and Talalay, when one drug interferes with another drug's working pathway, it is sometimes called an interaction. Also, when one drug can improve another drug' working pathway, it is sometimes called a synergism effect. We will focus on the drug pair of Paclitaxel and Discodermolide to illustrate our method.

When one wants to evaluate if the model is a good fit to the data, people usually would plot the residual plot of the model to check model fit and see if any outlier exists. If the residual plot has any particular pattern or trend, the model may not be a good fit for the data. If the points are scattered around randomly around 0, the model may be acceptable. If there is point that has a large residual and locates far away from most of other points, then this point could possibly be an outlier. We will plot the residual plots to help us determine if the model is a good fit.

3.3.1 Median-Effect Model

One of the important features of median-effect plot is to flatten the sigmoidal curve in the dose-effect plot by taking log of both side of the median-effect Equation (1). Then we

compare the median-effect model to the data by taking logarithm of concentration and $\frac{fa}{fu}$. The plot would have y-axis as logit (fa) and x-axis as log(dose).

The median effect model is fitted by PROC REG for least squares on the logit scale of f_a. The linear regression fitted will be in the form of equation (2).

3.3.2 Log(kill) Model

The log(kill) model can be plot with log (based 10) for fraction unaffected on the y-axis and the dose of drug but (not logged) on x-axis. The model is fitted by least square on the scale of log(based 10) for fraction unaffected by using PROC REG.

3.3.3 Log(kill) Mixture Model

We plot the effect, the cell count divided the average of control wells, of each drug against the dose. This heterogeneity assumption will lead to different effect-additive model because the proportion populations are varied to each drug.

This outcome is a fraction, the cell count of the treated well divided by the average of four control columns. Then to separate equation (4) into two portions that one corresponds to low concentration of drug and the other portion corresponds to high concentration of drug.

According to our mixed population assumption, the sensitive and resistant cells should have different survival rate. Therefore, we should assign the appropriate survival rate for these two populations instead of fitting all together with only one survival rate, K. We separated Equation (4) into two parts with two set of concentration ranges.

First, let the concentration of drug approach zero and use the fact that $e^{-\alpha} \approx 1 - \alpha$, so Equation (4) will transform to

$$\frac{Z(d_D)}{\text{avg.\# of control wells}} \approx \psi_D\ (1 - K_1 d_D) + (1 - \psi_D)(1 - K_2 d_D)$$

$$= 1 - d_D(\psi_D K_1 + (1 - \psi_D)K_2) \qquad \text{low concentration (16)}$$

As for the high concentration of drug, let the concentration of drug be very large; Equation (4) transform to

$$\frac{Z(d_D)}{\text{avg.\# of control wells}} \approx (1 - \psi_D)\theta_{D(2)}{}^{d_D} \qquad \text{high concentration (17)}$$

After derive the equations for high and low dose, we now would need to determine the range of dose for each equation of each drug, which are Paclitaxel and Discodermolide. We fit two analysis models to Equation (16) and Equation (17) with PROC GENMOD, but with different details. For equation (16), the model is assumed to be Poisson distribution with identity link function and intercept sets to be 1. From this analysis, the parameter estimate of slope would be $(\psi_D K_1 + (1 - \psi_D)K_2)$. As for equation (17), the error model is still assumed to be Poisson distribution, but instead of identity link function, log link function is used here. So equation (17) will be

$$\log\left(\frac{Z(d_D)}{\text{avg.\# of control wells}}\right) \approx \log(1 - \psi_D) - K_2 d_D \qquad (18)$$

The parameter estimated fit by equation (18) will be K_2 and the intercept will estimate $\log(1 - \psi_D)$, from which we can derive ψ_D. Then we can plug K_2 and ψ_D into the slope we derived from equation (16) to estimate K_1.

3.4 FITTING COMBINATION EFFECT

3.4.1 Combination Index Model

We mentioned earlier in the paper that m_1, m_2 in Equation (6) needs to be the same in order for the CI to be clearly interpreted. Therefore, we fit the both single-drug dose responses at the same time with an altered form of Equation (2) and we can force the m to be the same for both drugs.

$$\log_{10}(\frac{fa}{fu}) = m\log_{10}(D) - m\log_{10}(D_m)_A + k * Z = b + m\log_{10}(D) + k * Z \qquad (19)$$

where $b = -m\log_{10}(D_m)_A$, and if it is drug A, Z=0, if it is drug B, Z=1, and k is the coefficient parameter for the indicator I.

The equation for calculating median dose for drug A, $(D_m)_A$, would be the same as Equation (7). $(D_m)_A = 10^{-\frac{b}{m}}$. However, the equation for drug B would be somewhat different than Equation (7), $(D_m)_B = 10^{-\frac{(b+k)}{m}}$. We would fit Equation (19) with least square on the logit scale of fa and the estimate slope would be m. We can use $(D_m)_A$ and $(D_m)_B$ to fit Equation (6) and calculate the combination index values, and we can also plot the isobole similar to Figure 2. Once we have estimated $(D_m)_A$ and $(D_m)_B$, we can also calculate the potency between drug A and drug B, which would be $\frac{(D_m)_A}{(D_m)_B} = 10^{\frac{k}{m}}$

3.4.2 Mutually Exclusive Model

The other purpose of this paper is to fit the data with our models and to hope we can gain a little information about the drugs' working mechanism. We know Paclitaxel and Discodermolide is a mutually exclusive drug pair.[5] We fit the data with Equation (11) and check if the model fits the data.

In RPS-118, the combined effect is measured at a 2:1 ratio of concentration of Discodermolide to Paclitaxel. Since the data we have are counts, we divided all the cell counts by average number of all 64 control wells, which will be fu. To calculate fa, fa=1-fu. In Equation (11), we need the ratio, $\frac{f_a}{f_u}$. We estimated combined drug ratio and single drug ratios for both drugs. To check if the model fits the data well, we plotted the observed ratio against the predicted ratios.

3.4.3 Mutually Nonexclusive Model

In addition to the mutually exclusive model, we proceeded to fit the mutually nonexclusive model, Equation (12) and Equation (13). We were interested to see if one of the two models, mutually exclusive model or mutually nonexclusive model, would fit much better than the other.

3.4.4 Heterogeneity

Previously in the paper, we presented Equation (4), which is for a single drug. In Equation (4), we have three unknown parameters, $K_1, K_2, \text{and } \psi$. By fitting low and high

equation (16) and (18), we estimated $K_1, K_2,$ and ψ. Now, we combined Equation (4) for each drug and used the estimates.

To construct the combined effect model.

$$\frac{Z(d_1,d_2)}{\text{Avg .\# of control cells}} =$$

$$P_S\theta_{A(1)}\theta_{B(1)} + P_A\theta_{A(1)}\theta_{B(2)} + P_B\theta_{A(2)}\theta_{B(1)} + (1 - P_A - P_B - P_s)\theta_{A(2)}\theta_{A(2)} \quad (20)$$

where P_S is the population proportion of cells that are sensitive to both of drugs, and P_A and P_B are the population proportion of cells that are sensitive to either drug A or drug B, respectively. $\theta_{A(1)}\theta_{B(1)} = (e^{-K_{A1}K_{B1}})^{d_A d_B}$ is the proportion of cells that are sensitive to both drugs. $\theta_{A(1)}\theta_{B(2)} = (e^{-K_{A1}K_{B2}})^{d_A d_B}$ is the proportion of cells that are sensitive to drug A but not drug B. $\theta_{A(2)}\theta_{B(1)} = (e^{-K_{A2}K_{B1}})^{d_A d_B}$ is the proportion of cells that are sensitive to drug B but not drug A. And $\theta_{A(2)}\theta_{A(2)} = (e^{-K_{A2}K_{B2}})^{d_A d_B}$ is the proportion of cells that are resistant to both drug A and drug B.

In Equation (20), we have three unknown parameters, $P_A, P_B,$ and P_S. However, once we find the value for P_S, we can easily find the values for P_A, P_B by Table 1.

4.0 RESULTS/ FINDINGS

4.1 SINGLE DRUGS

4.1.1 Median-effect model

The red line is the cell counts and the blue line is the fitted value for median effect model. The y-axis is the logit scale of f_a. The median-effect axis is 0 which represents that $\frac{f_a}{f_u} = 1$ and $f_a + f_u$ need to be equal to 1. [6][7]

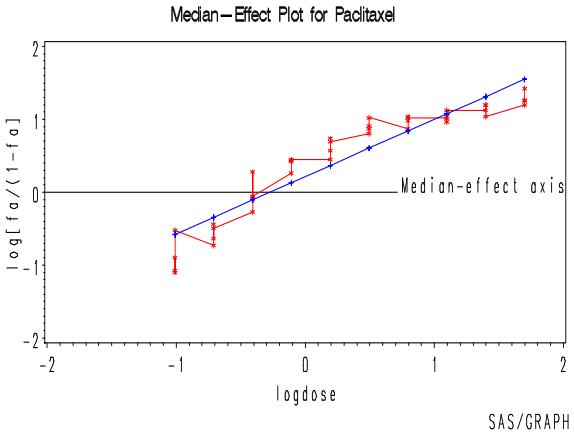

Figure 6 Median effect for Paclitaxel

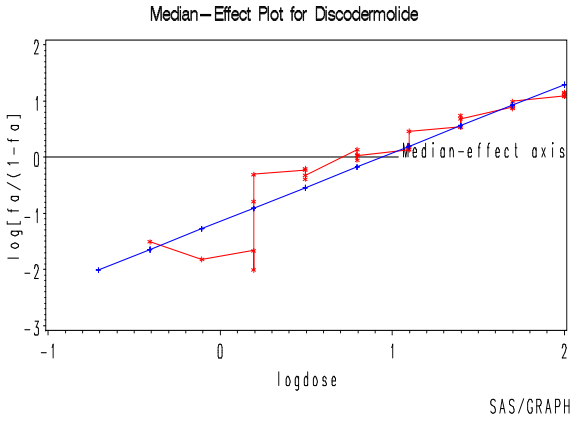

Figure 7 Median effect for Discodermolide

The median effect plot of Paclitaxel is roughly a good fit; however, if we look closely, the cell counts line could be separated into two parts, the first part from -1 to 0.5 $\log_{10}$(dose)is more steep and the second of 0.5 to about 1.8 is more flat. This seems to correspond to our assumption of two kinds of cell populations.

For Discodermolide, there is a dramatic drop around 0.2 $\log_{10}$(dose) level, which could possibly suggest a sensitive cell population exist within and correspond to our assumption of heterogeneity cell population.

We also plotted the residual plot to see if the median-effect model fits the data well enough. In Figure 6 there is an obvious curvature, which indicates the median-effect model is not a good fit for Paclitaxel. As for Discodermolide, the residual points seem to decrease slowly for these that is close to 0 residual and there are possible outliers exist at the bottom of the graph. Therefore, the median effect model is not a good fit for this data either.

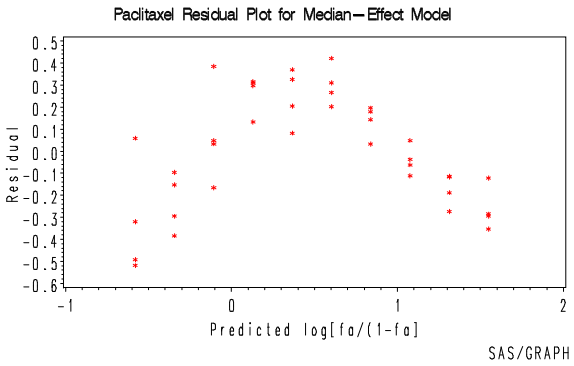

Figure 8 Residuals for Paclitaxel Median Effect Model

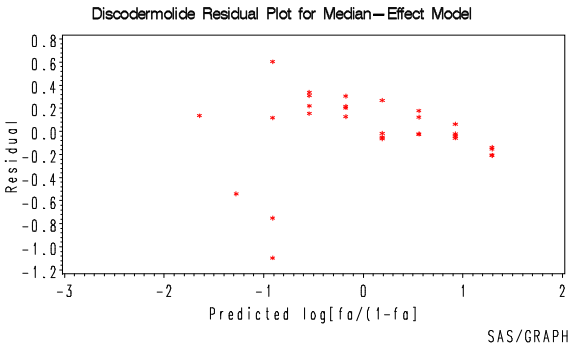

Figure 9 Residuals for Discodermolide Median Effect Model

4.1.2 Log(kill) Model

We plotted the $\log_{10}(f_u)$ on the y-axis and the concentrations of Paclitaxel or Discodermolide on the x-axis. The model we fitted is shown in red, and the cell counts are shown in blue.

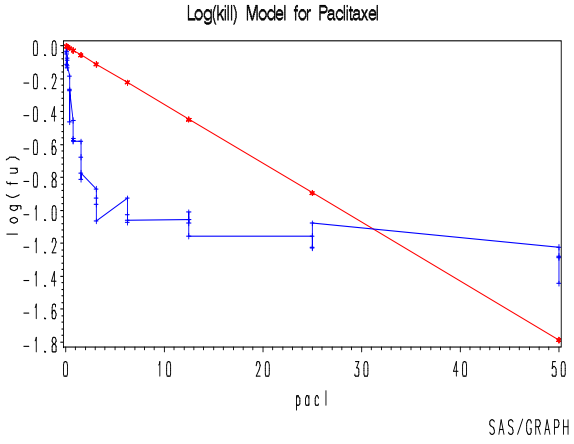

Figure 10 Log(kill) model for Paclitaxel

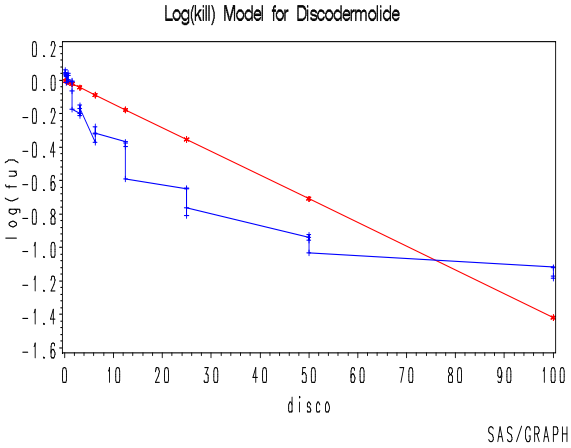

Figure 11 Log(kill) model for Discodermolide

Both log(kill) models suggest that there is a plateau effect of affected effect exist because the lines would approaches nonzero high-dose asymptote, which could be the largest effect the drug alone can achieve. However, it may also represent that the dosage we used weren't large enough to kill all the cancer cells. If somewhat we continuously increase the dosage, we may be able to kill all the cancer cells. The log kill model is very poor fitted, and this could suggest heterogeneity.

4.1.3 log(kill) mixture model

From the single drug graphs, we can see that cell survival decrease dramatically at low concentration, but when the concentration increases in large scale the killing rate would slow

down and appear to reach a plateau effect. One question emerges from the single drug graph is why the cell would respond dramatically to low concentration drug instead of high concentration. We then propose an assumption that instead of one population of cell which has plateau effect, there is a mixed population of two kind of cell that one reacts fast to the drug, which would correspond to the portion of drug that is killed by low concentration, and the other one is insensitive to the drug, which is the portion of high concentration.

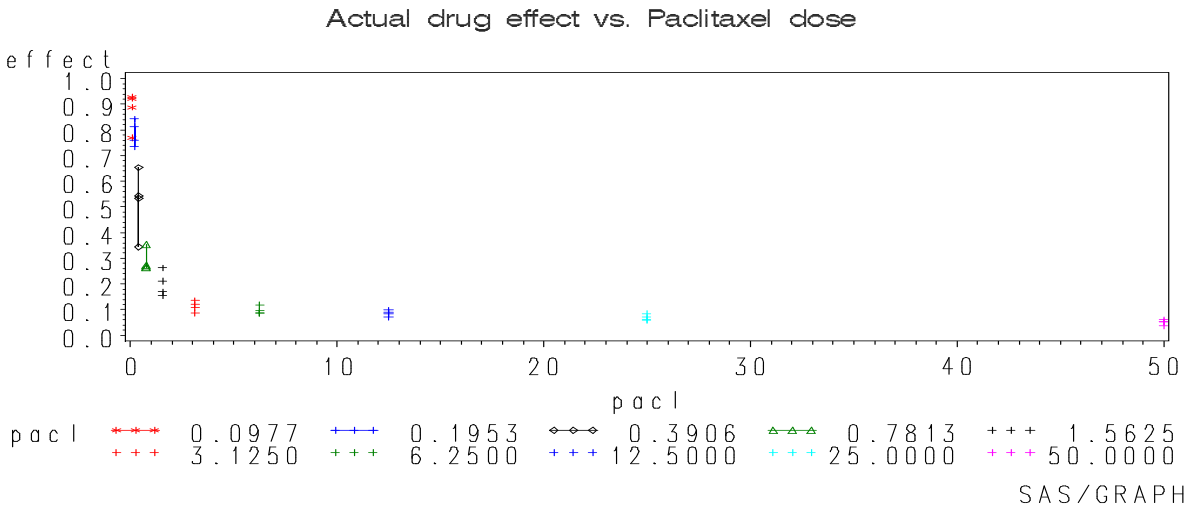

Figure 12 Actual drug effect vs. Paclitaxel dose

Figure 12 is the actual drug effect plot against the dose of Paclitaxel, and using this graph, we can try to determine the dose ranges for fitting Equation (16) and Equation (18).

Table 3 and 4 shows how the dose ranges for the low-dose and high-dose approximations have selected. The total number of points included equals the number of doses times four replications. The estimates of slope change every time we re-fit the models with more points included. The standard error of the slope, and the scale parameter indicate goodness of fit. We can use the scale parameter to determine if the model fits the data well enough. If the scale parameter increases, this suggests that adding the data for this extra

concentration would increase the deviation between predicted values and the actual cell counts too much.

Table 3 Low dose approximation for Paclitaxel

Highest dose included	# of points included	Estimate of Slope	S.E of slope	Scale Parameter
0.1953	8	1.1249	0.1332	0.0652
0.3906	12	1.2155	0.0925	0.1093
0.7813	16	0.9450	0.0386	0.1297
1.5625	20	0.5458	0.0286	0.2749
3.125	24	0.3299	Can't fit	Can't fit

Table 4 High dose approximation for Paclitaxel

Lowest dose included	# of Points included	Estimate of Slope	S.E of slope	Estimate of Intercept	S.E of intercept	Scale Parameter
25	8	0.0124	0.0052	-2.3780	0.1974	0.0445
12.5	12	0.0140	0.0031	-2.3074	0.0932	0.0414
6.25	16	0.0150	0.0025	-2.2728	0.0630	0.0420
3.125	20	0.0169	0.0025	-2.2083	0.0532	0.0475
1.5625	24	0.0260	0.0052	-1.9406	0.0859	0.1026

We conclude from these two tables, the range of low dose for Equation (16) is from 0.0977μM to 0.7813μM. Adding 1.5625μM, the scale parameter increases more than doubles, which means this point should not be added to the model. When tried to include 3.125 μM, the model cannot even fit this value. As for Equation (18), the range of dose should include from 1.5625μM to 50 μM. Fitting Equation (18), we would get the estimate of K_2 from estimate of slope, and $\psi_{pacltaxel}$ from estimate of intercept. K_1, K_2, and $\psi_{\text{pacltaxel}}$, and we got $\psi_{\text{pacltaxel}}$ =0.89011, $K_1 = 1.05958$and K_2=0.016895, which seems to fit the plot because it is around 0.1 of cells are hard to kill and K_1 is supposed to be very large compare to K_2 because K_1 corresponds to the proportion of drugs that is very sensitive to the drug and decay very fast.

Figure 13 and Figure 14 are predicted effect against actual effect plots for low dose equation (16) and high dose equation (18). The red line is predicted effect and the blue line is the actual effect.

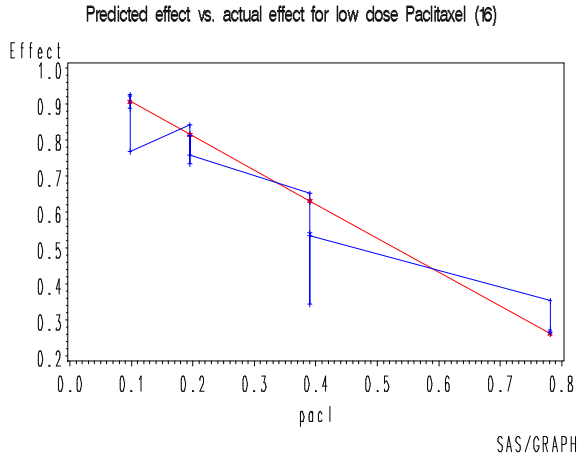

Figure 13 Low dose approximations for Paclitaxel

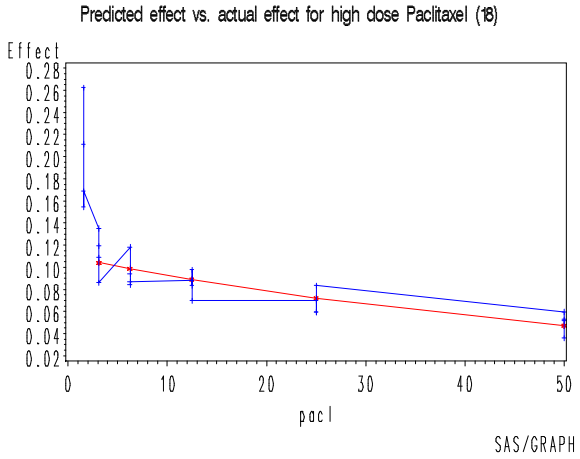

Figure 14 High dose approximations for Paclitaxel

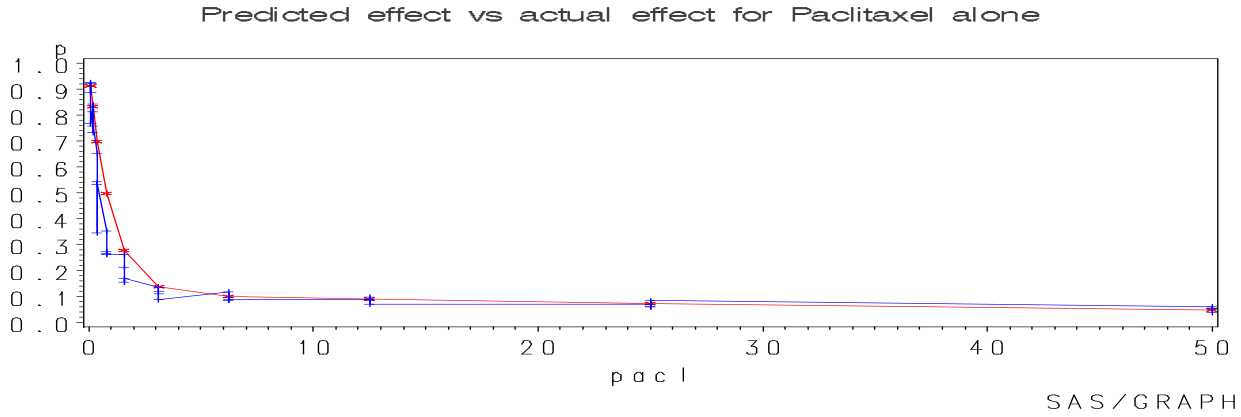

Figure 15 Predicted effect vs. actual effect for Paclitaxel alone

DISCODERMOLIDE:

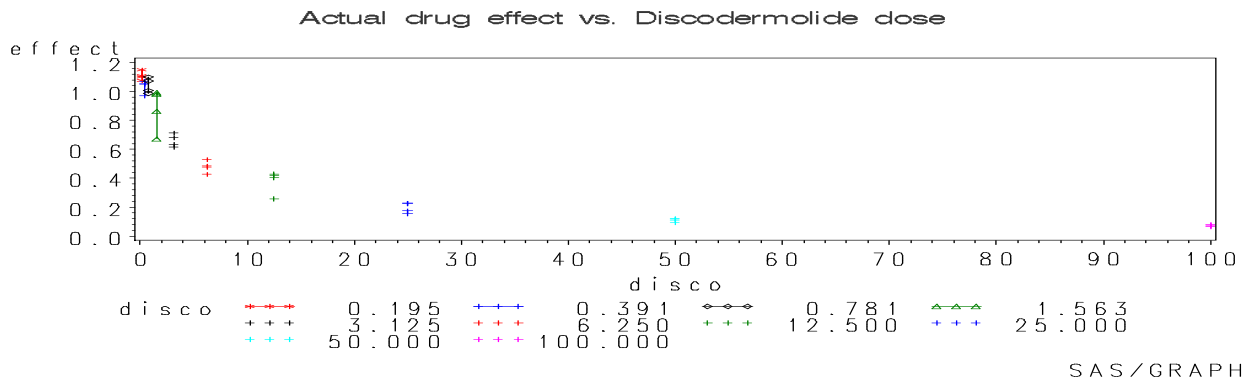

Figure 16 Actual drug effect vs. Discodermolide dose

Applying the same procedures with Discodermolide, first we look at the graph and see which dose point may be the boundary point for Equation (16) and Equation (18). Table 5 is a summary table of fitting Equation (16) and from Table 5, we can roughly decide the range of low dose equation should be from 0.195 μM to 3.125 μM because after 3.125 μM, the scale parameter start to increase. Table 6 is derived from fitting Equation (18), and we can see the dramatic difference in scale parameter when the dose decreases from 25 to 12.5. Therefore, the range of dose to be used in Equation (18) should be from 25 μM to 100 μM. Figure 22 shows the fitted model seems to fit better than before because the predicted effects are close to the observed effects. By fitting Equation (16) and (18), we estimate K_1, K_2, and ψ_{disco} to be 0.12342, 0.0138 and 0.74436, respectively.

Table 5. Low dose approximation for Discodermoolide

Highest Dose included	# of points included	Estimate of Slope	S.E of slope	Scale Parameter
0.3906	8	-0.1074	0.0489	0.0419
0.7813	12	-0.0645	0.0258	0.0451
1.5625	16	0.0457	0.0276	0.1011
3.125	20	0.0963	0.0127	0.1047
6.25	24	0.0856	0.0049	0.0996
12.5	28	0.0547	0.0030	0.1543
25	32	0.0341	0.0014	0.2167
50	36	Cannot fit	Cannot fit	

Table 6. High dose approximation for Discodermolide

Lowest dose included	# of Points included	Estimate of Slope	S.E of slope	Estimate of Intercept	S.E of intercept	Scale Parameter
50	8	0.0086	0.0014	-1.7844	0.1036	0.0291
25	12	0.0138	0.0020	-1.3640	0.1084	0.0664
12.5	16	0.0211	0.0032	-0.8909	0.1203	0.1288
6.25	20	0.0245	0.0029	-0.7178	0.0842	0.1314
3.125	24	0.0288	0.0032	-0.5378	0.0724	0.1520
1.5625	28	0.0338	0.0041	-0.3614	0.0724	0.1942
0.7813	32	0.0384	0.0047	-0.2244	0.0672	0.2201

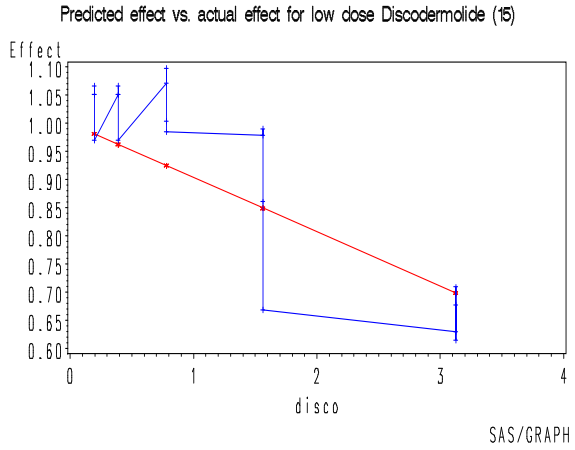

Figure 17 Low dose approximations for Discodermolide

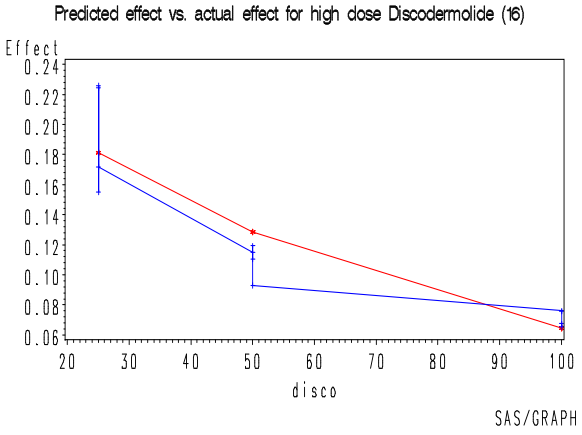

Figure 18 High dose approximations for Discodermolide

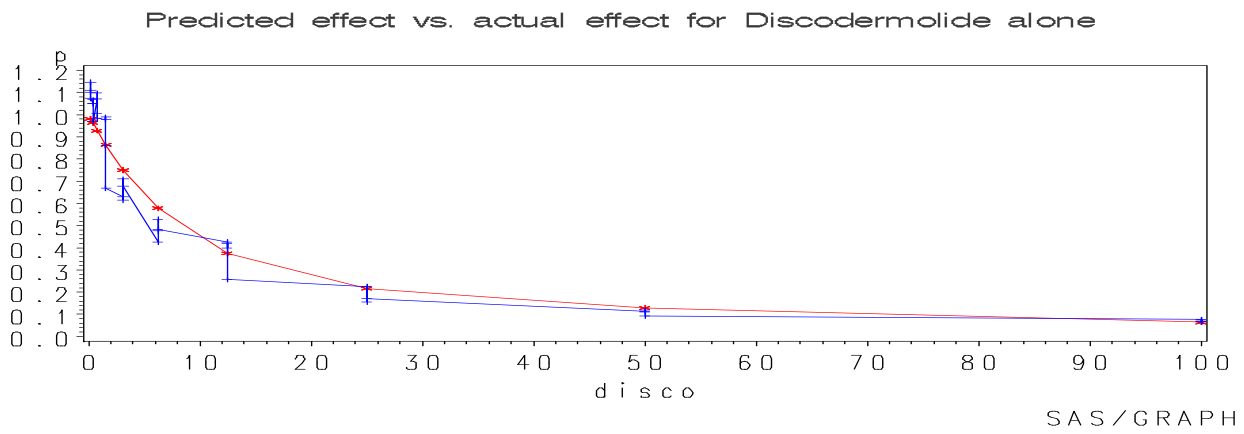

Figure 19 Predicted effect vs. actual effect for Discodermolide alone

After several trying to fit the model, finally the model for Paclitaxel seems to work fine now. However, there is still room for the fit for Discodermolide to improve. Looking at the actual effect graph of Discodermolide again, the residuals do not add to zero, suggesting that the informal fitting approach taken here should be replaced by a true maximum likelihood approach.

4.2 COMBINATION OF DRUGS

4.2.1 Combination Index model

We fitted Equation (19), and the estimated m is 0.92156. The median doses we calculated for Paclitaxel and Discodermolide is 0.65811 and 8.28330, respectively. Using these two median doses, we plotted the isobole which has the slope $10^{-\frac{k}{m}} = 10^{-\frac{-1.01363}{0.92156}} = 12.5865$. We also plotted the residual plot of fitting Equation (19). The curvature in residual plot suggests possible quadratic function. We used these median effect doses to calculate combination index.

However, our calculated combination index values mostly are greater than 1, which is not what we expect for Paclitaxel and Discodermolide. This may suggest that our model here is not an appropriate one to interpret this data, and this may relate to the quadratic function suggests by the residual plot.

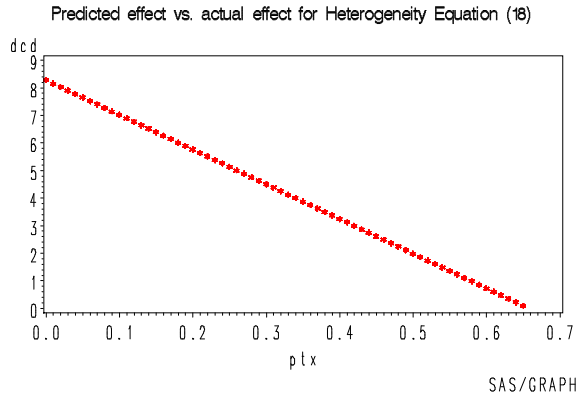

Figure 20. IC_{50} Isobole.

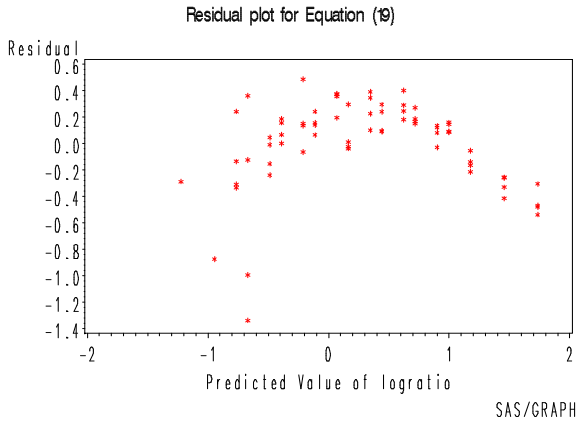

Figure 21. Residual plot of Equation (19)

4.2.2 Mutually Exclusive Model

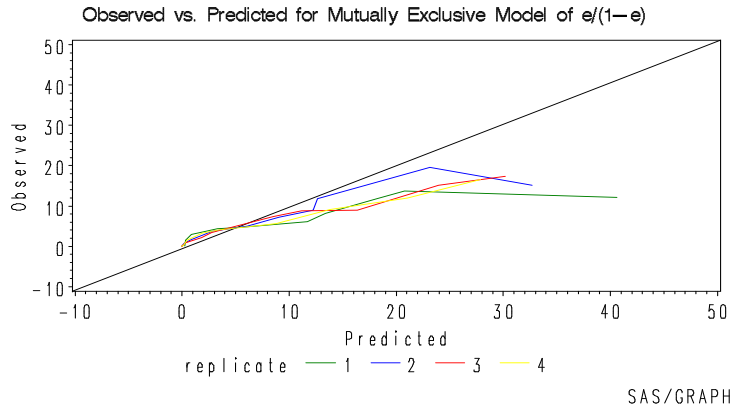

Figure 22. Mutually Exclusive model of $\frac{f_a}{f_u}$

We plotted the observed ratios, $\frac{f_a}{f_u}$, against the predicted ratios we calculated by using Equation (11). We can see that the predicted values are really close to the observed one because

the slope between observed points and predicted points are close to 1. Therefore, we can conclude that our mutually exclusive model can fit the data well here, which can suggest the relation between Paclitaxel and Discodermolide might be mutually exclusive.

4.2.3 Mutually Nonexclusive Model

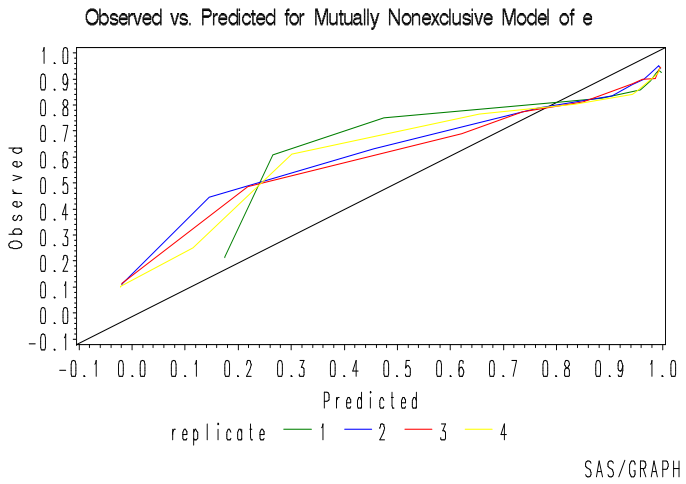

Figure 23 Mutually nonexclusive model of f_a

We fitted the model by using Equation (13). We plotted the observed affected fraction, f_a, against predicted effect. We can see in Figure 23 that the predicted effects are not close to observed effects, the predicted values are lower than observed effect. Therefore, the mutually nonexclusive model is not a good fit for our data, and this could suggest that Paclitaxel and Discodermolide is not a mutually nonexclusive drug pair.

4.3 MIXED POPULATION OF CANCER CELLS

4.3.1 Heterogeneity

After fitting low and high dose Equations (16) and (18) for each drugs, we estimated the $K_1, K_2,$ and ψ for each drugs. Table 7 organized the values.

Table 7. Table of K_1, K_2, Ψ_D

	K_1	K_2	$\Psi_D = P_S + P_D$
Paclitaxel	1.05958	0.016895	0.89011
Discodermolide	0.12342	0.0138	0.74436

At this point, we can get a valid range of P_S by using Table 7. From the column of Ψ_D, we can see that the largest possible value for P_S is 0.74436. We know all the proportions, $P_S + P_A + P_B + R_{AB}$, need to add up to 1, and $P_S + P_A + P_B < 1$. We can use this inequality and derive the lowest possible value for P_S.

$P_S + P_A + P_B = (P_S + P_A) + (P_S + P_B) - P_S = \Psi_A + \Psi_B - P_S = 0.89011 + 0.74436 - P_S < 1$,

and $P_S \geq 0.63$. We got a range for P_S, $0.63 \leq P_S \leq 0.74$. Once we know the value of P_S, we can calculate the rest of proportions using Table 1.

To find the most likely value of P_S, we estimated the likelihood of P_S to find the maximum value of P_S. The maximum likelihood of P_S would be the most likely value of P_S. We calculate the likelihood of P_S assuming the binomial distribution for the error model. The likelihood equation for the binomial distribution is

$$l_i = [r_i \log(\mathrm{P_i}) + (\mathrm{n_i} - \mathrm{r_i}) \log(1 - \mathrm{P_i})] \quad (21)$$

where i is the number of observations, n_i would be the average cell counts of control wells, which is a fixed number, and r_i would be the number of cells are killed, calculated by subtracting each well's count from average cell counts.

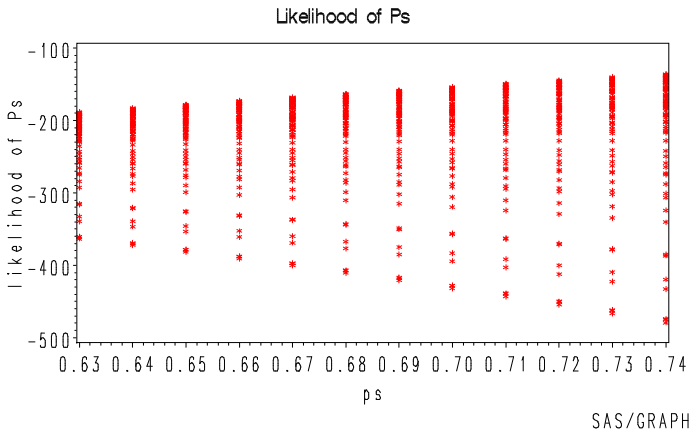

Figure 24 Likelihoods of P_S

Once we calculated all the likelihood value, we noticed the likelihood increase as the value of P_S increases. Therefore, the maximum likelihood value of P_S is at P_S=0.74. Put P_S into Table 1, where A indicates Paclitaxel and B indicates Discodermolide, and we can calculate the rest of proportions.

Table 8. Calculate the proportions

P(Sensitive to A, Sensitive to B) = P_S = 0.74	**P(Sensitive to A, Resistant to B) = P_A=0.15**	**P(sensitive to A) = Ψ_A = 0.89011**
P(Resistant to A, Sensitive to B) = P_B=0	**P(Resistant to A, Resistant to B) =R_{AB}=0.11**	**P(Resistant to A)**
P(Sensitive to B)= Ψ_B=0.74436	**P(Resistant to B)**	**=100%**

We got some interesting results from Table 8, which shows P_B, the proportion that corresponds to the cell population that is only sensitive to Discodermolide, is equal to 0. This result could suggest that probably the cells that are sensitive only to Discodermolide do not

exist in this data. The cells we observed are killed under treatment of Discodermolide may be the cells that are sensitive to both Paclitaxel and Discodermolide.

We plugged all the calculated values from Table 8 back into Equation (20), and calculated the predicted values of effects. We then plotted the predicted values with the observed effect. The red line is the observed effects and the blue line is the predicted effect.

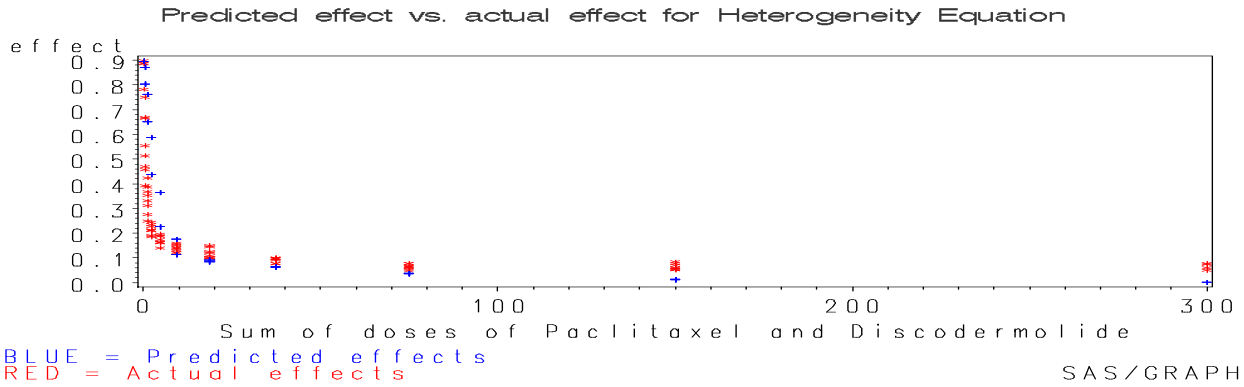

Figure 25 Predicted effect vs. actual effect for heterogeneity assumption

We can see in Figure 25 that after we adjusted for the cell heterogeneity assumption, the predicted values are much closer to the actual effects. Therefore, we can conclude that consider the cell heterogeneity assumption can help us to improve the prediction of combined drug effects.

5.0 DISCUSSION

As we mention earlier, we cannot be sure if these survival cell counts are really from viable cells. It can be machine's error or the plate's problem. What we assume here is only the working assumption, but this assumption may still prove to be true in the future.

We focused our analysis on the drug pair of Paclitaxel and Discodermolide. We first performed the single drug analysis with median-effect model and log(kill) model. Their large deviations from the actual effects lead us to the mixture of cell population assumption. We noticed that around 10% of cells survive individual drugs and their combination at high concentration. We later hypothesized that there may be a mixture of cell populations that consists of a population that is sensitive to both drugs, two populations that are each sensitive to only one of the drugs, and a population that is relatively resistant to both drugs.

We analyzed the combined drug effects focus on using models for effect-additivity and log kill models for mixtures. To avoid difficult optimization problems, we fitted the normalized (f_a) data to low and high dose range equation separately and plotted them. Then we combined the estimates we derived from low and high dose equations to construct the mixed population model. After adjusting for the mixed cell population, the predicted effects are quite close to the

actual effects. Therefore, we would suggest that considering the cell heterogeneity assumption can improve the prediction of drug effects.

We also fitted the mutually exclusive and mutually nonexclusive models to check if our models can tell us the same drug relation other papers have stated. And our results have showing that Paclitaxel and Discodermolide only fitted well in mutually exclusive model, which corresponds to other paper have stated. Thus, our models maybe can be useful for the future investigation in combination of microtubule perturbing agents with known and unknown mechanisms of action.

However, several weaknesses of the experiment need to be kept in mind. The problems with baseline of controls, the outliers, and the way data recorded, and the importance of row and column factors, should be considered when we want to estimate the data more precisely.

6.0 CONCLUSIONS

As other research papers may conclude that it is surprising to see Paclitaxel and Discodermolide works together to produce super-additive effect because theoretically they are competitive with each other for their similar mechanism and working site, it is not what we try to prove here. To be able to explain how two agents which are supposed to compete with each other can produce a super-additive effect requires an extensive and detailed experiment that takes into account many other things, such as the specific working sites of agents, different time gaps between using drugs on the cell lines, etc., that we do not have here in this experiment.

The main purpose for this paper is to provide a summary of methods that can be used to analyze drug combinations while adjusting for the mixed cell population assumption. WE want to emphasize the importance of this assumption when one wants to understand drug effects. Fitting the heterogeneity assumption into our models for predicting the dr drug effects improves our prediction. These methods may be extended for further analysis of other drug pairs, such as Disorazole C1 with Paclitaxel, and Vincristine with Paclitaxel, using data which we have not yet analyzed. Also, our models, such mutually exclusive model, may be useful in the future when one want to investigate the combination of drugs of either known or unknown mechanisms.

What we present here is a statistical analysis that cannot prove any biological conclusion until further cancer cell experiments are done. Also, drug "synergism", whatever that might mean, biologically, might not be desirably clinically. These drugs may combine well against cancer cells, but they may also work too well on normal cells and increase damage on human body.

APPENDIX: TABLE 1A. LAYOUT OF EXPERIMENT PLATE

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	CONTROL																							CONTROL
2		Vinc 100 (µM)	Vinc 50	Vinc 25	Vinc 12.5	Vinc 6.25	Vinc 3.125	Vinc 1.5625	Vinc 0.78125	Vinc 0.390625	Vinc 0.195313	CONTROL	CONTROL	Pacl 50	Pacl 25	Pacl 12.5	Pacl 6.25	Pacl 3.125	Pacl 1.5625	Pacl 0.78125	Pacl 0.390625	Pacl 0.195313	Pacl 0.09765625	Pacl 0.09765625 + Disco 0.195313
3																								
4																								
5		Diso C1 100 + Pacl 50	Diso C1 50 + Pacl 25	Diso C1 125 + Pacl 12.5	Diso C1 12.5 + Pacl 6.25	Diso C1 6.25 + Pacl 3.125	Diso C1 3.125 + Pacl 1.5625	Diso C1 1.5625 + Pacl 0.78125	Diso C1 0.78125 + Pacl 0.390625	Diso C1 0.390625 + Pacl 0.195313	Diso C1 0.195313 + Pacl 0.0976562			Pacl 50 + Disco 250	Pacl 25 + Disco 125	Pacl 12.5 + Disco 62.5	Pacl 6.25 + Disco 31.25	Pacl 3.125 + Disco 15.625	Pacl 1.5625 + Disco 7.8125	Pacl 0.78125 + Disco 3.90625	Pacl 0.390625 + Disco 1.953125	Pacl 0.195313 + Disco 0.976562	Pacl 0.09765625 + Disco 0.48828125	
6																								
7																								
8																								
9		Diso C1 100	Diso C1 50 + Vinc 50	Diso C1 25 + Vinc 25	Diso C1 12.5 + Vinc 12.5	Diso C1 6.25 + Vinc 6.25	Diso C1 3.125+ Vinc 3.125	Diso C1 1.5625+ Vinc 1.5625	Diso C1 0.78125 + 0.78125100	Diso C1 0.390625 0.390625100	Diso C1 0.195313+ 0.195313100			Pacl 50 + Disco 100	Pacl 25 + Disco 50	Pacl 12.5 + Disco 25	Pacl 6.25 + Disco 12.5	Pacl 3.125 + Disco 12.5	Pacl 1.5625 + Disco 3.125	Pacl 0.78125 + Disco 1.5625	Pacl 0.390625 + Disco 0.78125	Pacl 0.195313 + Disco 0.390625	Pacl 0.09765625 + Disco 0.195313	
10																								
11																								
12		Diso C1 100 + Vinc 100	Disco 50	Disco 25	Disco 12.5	Disco 6.25	Disco 3.125	Disco 1.5625	Disco 0.78125	Disco 0.390625	Disco 0.195313			Diso C1 100	Diso C1 50	Diso C1 25	Diso C1 12.5	Diso C1 6.25	Diso C1 3.125	Diso C1 1.5625	Diso C1 0.78125	Diso C1 0.390625	Diso C1 0.195313	
13																								
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