Application of Mixture Design Response Surface Methodology for Combination Chemotherapy in PC-3 Human Prostate Cancer Cells

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Application of Mixture Design Response Surface Methodology for Combination Chemotherapy in PC-3 Human Prostate Cancer Cells

Richard Vernon Oblad

A thesis submitted to the faculty of Brigham Young University in partial fulfillment of the requirements for the degree of Master of Science

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ABSTRACT

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Master of Science

Combining chemotherapeutics to treat malignant tumors has been shown to be effective in preventing drug resistance, tumor recurrence, and reducing tumor size. We modeled combination drug therapy in PC-3 human prostate cancer cells using mixture design response surface methodology (MDRSM), a statistical technique designed to optimize compositions that we applied in a novel manner to design combinations of chemotherapeutics. Conventional chemotherapeutics (mitoxantrone, cabazitaxel, and docetaxel) and natural bioactive compounds (resveratrol, piperlongumine, and flavopiridol) were used in twelve different combinations containing three drugs at varying concentrations. Cell viability and cell cycle data were collected and used to plot response surfaces in MDRSM that identified the most effective concentrations of each drug in combination. MDRSM allows for extrapolation of data from three or more compounds in variable ratio combinations, unlike the Chou-Talalay method. MDRSM combinations were compared with combination index data from the Chou-Talalay method and were found to coincide. We propose MDRSM as an effective tool in devising combination treatments that can improve treatment effectiveness and increase treatment personalization because MDRSM measures effectiveness rather than synergism, potentiation or antagonism.

Keywords: mixture design response surface methodology, chemotherapy, prostate cancer, combination chemotherapy, naturally occurring compounds, resveratrol, flavopiridol, piperlongumine, cabazitaxel, docetaxel, mitoxantrone
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Table of Contents

Title Page ......................................................................................................................................... i
Abstract........................................................................................................................................... ii
Acknowledgments.......................................................................................................................... iii
Table of Contents ........................................................................................................................... iv
List of Tables ...................................................................................................................................v
List of Figures ................................................................................................................................vi
Manuscript .......................................................................................................................................1
Appendix A ....................................................................................................................................38
Proposed Research .........................................................................................................................39
Literature Review .............................................................................................................................47
Appendix B – Protocols ....................................................................................................................71
Plate Reader Protocol .....................................................................................................................72
Cell Culture Protocols ....................................................................................................................76
AlamarBlue Protocol .....................................................................................................................79
Cell Cycle Analysis Protocol ........................................................................................................80
JMP Protocols .................................................................................................................................81
List of Tables

Table 1 – Ternary Plot Coordinate System Represented as Percentages
Page................................................................................................................................................28

Table 2 – Optimal Concentration and Percentages for Maximizing a Reduction in Cell Viability
Page................................................................................................................................................29

Table 3 – Optimum Combinations for Minimizing Cell Population in the G₁ phase, and
Maximizing Cell Populations in the S, G₂/M, and Sub G₁ Phases, Produced by Cell Cycle
Analysis.
Page................................................................................................................................................30

Supplemental Table 1 – Statistics from the Ic50 Models Shown in Figure 2
Page................................................................................................................................................37
List of Figures

Figure 1 – JMP ternary plot with combination points
Page.................................................................31

Figure 2 – IC50 graphs for chemotherapeutics in PC-3 human prostate cancer cells
Page.................................................................32

Figure 3 – Response surfaces of 12 different mixtures
Page.................................................................33

Figure 4 – Chou-Talalay combination index (CI) chart for all combinations
Page.................................................................35

Figure 5 – Response surfaces produced by cell cycle analysis
Page.................................................................36
Application of Mixture Design Response Surface Methodology for Combination Chemotherapy in PC-3 Human Prostate Cancer Cells

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Abbreviations: Mixture design response surface methodology (MDRSM), mitoxantrone (MIT),
cabazitaxel (CAB), docetaxel (DOC), resveratrol (RES), flavopiridol (FLAV), piperlongumine
(PIP), reactive oxygen species (ROS), signal transducer and activator of transcription 3
(STAT3), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), cyclin
dependent kinase (CDK), cancer stem cell (CSC)
Abstract

Combining chemotherapeutics to treat malignant tumors has been shown to be effective in preventing drug resistance, tumor recurrence, and reducing tumor size. We modeled combination drug therapy in PC-3 human prostate cancer cells using mixture design response surface methodology (MDRSM), a statistical technique designed to optimize compositions that we applied in a novel manner to design combinations of chemotherapeutics. Conventional chemotherapeutics (mitoxantrone, cabazitaxel, and docetaxel) and natural bioactive compounds (resveratrol, piperlongumine, and flavopiridol) were used in twelve different combinations containing three drugs at varying concentrations. Cell viability and cell cycle data were collected and used to plot response surfaces in MDRSM that identified the most effective concentrations of each drug in combination. MDRSM allows for extrapolation of data from three or more compounds in variable ratio combinations, unlike the Chou-Talalay method. MDRSM combinations were compared with combination index data from the Chou-Talalay method and were found to coincide. We propose MDRSM as an effective tool in devising combination treatments that can improve treatment effectiveness and increase treatment personalization because MDRSM measures effectiveness rather than synergism, potentiation or antagonism.
Introduction

Drug resistance is one of the greatest problems facing chemotherapy, and is believed to be the cause of over 90% of failed treatments of metastatic cancer patients in 2005, and continues to be a major problem to this day (Kachalaki et al., 2016; Longley and Johnston, 2005). Personalized and targeted therapies, such as immunotherapy, have shown great potential in combating cancer, but are still affected by chemoresistance (Bozic et al., 2013). Prostate cancer frequently becomes resistant to commonly used treatments (Li et al., 2001; Liang et al., 2010; Theyer et al., 1993; Tilley et al., 1995), and is the second most commonly diagnosed cancer in men in the United States (Siegel et al., 2018). Of the diagnosed prostate cancers 10% are metastatic (Jemal et al., 2011). Metastatic prostate cancer is incurable and highly fatal (Gundem et al., 2015; Hegeman et al., 2004; Kim et al., 2003; Kim et al., 2004; Patel et al., 2004; Pinthus et al., 2004). PC-3 human prostate cancer cells, a cell line derived from metastatic prostate cancer, have been shown to be chemoresistant to some common chemotherapeutics (Li et al., 2001; Theyer et al., 1993; Tilley et al., 1995). One way to combat chemoresistance is combination drug therapy. Combination therapy uses a combination of drugs with different mechanistic actions. It has also been theorized that multi-mechanistic drugs may be helpful in preventing the development of resistance (Zimmermann et al., 2007). Many naturally occurring compounds such as resveratrol (RES), flavopiridol (FLAV), and piperlongumine (PIP) are multi-mechanistic compounds that have been identified as putative chemotherapeutics (Pan et al., 2012). Treatment with RES has been shown to cause increases in cytosolic calcium (Ma et al., 2007; Peterson et al., 2016), bind to various proteins to induce intrinsic apoptosis (Calamini et al., 2010; Hsieh et al., 2014; Locatelli et al., 2005), and cause production of ROS (Miki et al., 2012). FLAV inhibits cyclin dependent kinases (CDK) to disrupt the cell cycle, and causes
production of ROS (Gupta et al., 2010; Iyer et al., 2013; Shao et al., 2016), while PIP has been shown to cause ROS production and inhibit STAT3 and NF-κB (Adams et al., 2012; Ahn, 1994; Bharadwaj et al., 2015; Ginzburg et al., 2014; Gong et al., 2014). Cabazitaxel (CAB), docetaxel (DOC), and mitoxantrone (MIT) are all conventional chemotherapeutics which are used to treat prostate cancer (De Bono et al., 2010; Paller and Antonarakis, 2011; Tannock et al., 2004). CAB and DOC are both tubulin inhibitors (Galsky et al., 2010; Jordan and Wilson, 2004). However, resistance develops to DOC, but not CAB in some forms of prostate cancer likely because CAB has a lower binding affinity to the drug efflux pump, P-glycoprotein (Galsky et al., 2010; OPREA-LAGER et al., 2013; Paller and Antonarakis, 2011). Mitoxantrone is a DNA intercalating agent, and topoisomerase II inhibitor which prevents DNA replication (Ehninger et al., 1990). DOC, CAB, and MIT are all used in treating advanced prostate cancer, and have often been used in combination therapies (De Bono et al., 2010; Petrylak et al., 2004).

The most commonly used statistical model to define and test chemotherapeutic drug combinations is the Chou-Talalay method for measuring drug synergism (two-way complement between drugs) and antagonism (two-way interference between drugs) (Chou, 2010). However, the Chou-Talalay method is limited by: (1) only directly analyzing drug combinations of two drugs, (2) focusing on constant ratio combinations, and (3) measurements of synergism that are not directly correlated with effectiveness. The Chou-Talalay method uses the 1:1 mixtures of the two drugs being tested at concentrations that are multiples of the Ic50 (0.50x, 1.0x, 2.0x, and 4.0x Ic50), and requires collection of data of each drug alone (Chou and Talalay, 1983; Chou and Talalay, 1984). Using Chou-Talalay data you infer how three drug interactions may work, but you never directly measure how synergistic the drugs are together. Chou recognized this limitation when he stated that combinations do not act as two different drugs, but act as a third
drug altogether (Chou, 2010). The advantage of measuring non-constant ratio combinations, is
that non-constant ratios provide a broader range of mixtures that enable the discovery of novel
drug interactions. The Chou-Talalay method can be used to test variable ratio combinations, but
it requires a significant amount of data collection. Lastly, to find the most effective
chemotherapeutic mix one is not necessarily interested in the drugs with the strongest synergism,
but the ultimate goal is to identify the combination that is most effective at reducing the tumor.

Response surface methodologies (RSM) are a compilation of mathematical and statistical
models that create a 3-dimentional map based on interpolating defined data points. A number of
different RSMs have been used in cancer research. For example, the Box-Behnken design was
used to optimize the incorporation of chemotherapeutics into nanoparticles (Gupta et al., 2016).
Additionally, fractional factorial design was used to maximize extraction of putative
chemotherapeutics from plants (Lu et al., 2008) and Plackett-Burman was used to optimize the
growth media for tumor cells to enable the discovery of a potential cancer vaccine (Zhao et al.,
2017). To a lesser extent RSM has also been used to optimize combination chemotherapy. For
example, a multifactorial design was used to identify the most effective chemotherapeutic
combination in prostate cancer (Jia et al., 2017). Given how widely used RSM is in engineering
and other fields it is unclear why it has been underutilized in chemotherapy.

Mixture design response surface methodology (MDRSM) is a type of RSM that can test
combinations with two or more compounds and interpolate effective mixtures from non-constant
ratio combinations. MDRSM can be constrained by desired parameters as designated by the
researcher, such as making the model define a combination that has between 20-80% of one drug
(Dejaegher and Heyden, 2011). MDRSM has been used in many other fields to create the most
effective or desirable product, such as soil composition for a landfill, the most desirable mixture
of ingredients for any given food product, and developing pharmaceutical formulations (Cornell, 2011; Lawson and Erjavec, 2000). MDRSM can be used to develop maximally effective drug combinations for treating cancer, because it is not limited to measuring only synergism, or antagonism, but takes into account any potentiation and indicates the most effective combination. Although a mixture of two or three drugs is most common in drug combination studies, MDRSM can be used to optimize combinations of two or more compounds. The effects of such mixtures on outcomes such as cell viability or cell cycle inhibitions is experimentally determined and normalized to a control. The derived data for percent viability or percent inhibition are then used to construct the response surface. The surface predicts the outcome of any combination of the tested compounds, including the unique blend that would minimize or maximize the desired response. In this study we compare MDRSM to the Chou-Talalay model, and test 12 different combinations of 3 drugs using 6 different chemotherapeutic agents. We demonstrate that MDRSM can be used to determine the efficacy of three drug mixtures in non-constant ratios.

Materials & Methods

Cell lines.

PC-3 human prostate cancer cells were obtained from ATCC (Rockville, MD), and were cultured in F12-K medium containing 10% FBS and 1% antibiotic (penicillin/streptomycin). Cells were grown in an incubator at 37°C and 5% CO₂. Cells from passage numbers 3-17 were used.

Reagents.

Cabazitaxel was obtained from APExBIO (Houston, TX) and dissolved in dimethyl sulfoxide (DMSO) to make a 40 µM stock solution. Docetaxel, flavopiridol, mitoxantrone hydrochloride, and piperlongumine were obtained from Sigma Aldrich (St. Louis, Missouri) and
were dissolved in DMSO to make a 45 µM, 160 µM, 400 µM, and 8 mM stock solution, respectively. Trans-resveratrol was obtained from Cayman Chemical (Ann Arbor, MI) and dissolved in DMSO to make a 75 mM stock solution. Alamarblue was obtained from Remel (Lenexa KS). Propidium iodide was obtained from Sigma Aldrich (St. Louis, Missouri) and dissolved in PBS for a stock concentration of 50 µg/mL.

**Cell Viability.**

Cells were grown near confluency, trypsinized, and 10,000 cells were seeded on 96-well plates. Cells were allowed to adhere to the plate for 24 hours before treatment with the vehicle control (DMSO 0.1%) or 1 of 10 drug combinations and allowed to incubate for 48 hours (37°C and 5% CO₂). DMSO only control was set as 100% viability. Each data point was normalized to the DMSO control and is presented as a percent reduction in cell viability so that the strongest reduction in cell viability would give us a maximum on the surface. Each combination treatment also had its own control where treatment medium, without cells, was analyzed with Alamarblue to eliminate background fluorescence and interactions between the drugs and the Alamarblue reagent. After 48 hours, the cells were treated with 10 µL of Alamarblue (1:10 ratio of Alamarblue to media) and incubated for 6 hours (37°C and 5% CO₂). The fluorescence was then analyzed on a BMG LABTECH FLUOstar OPTIMA plate reader (BMG LABTECH Inc., Cary, NC) using the 544 nm excitation filter and the 612 nm emission filter. All treatments were done with three biological replicates and in triplicate within each biologic replicate.

**IC₅₀’s.**

The cell viability method above was used to collect data for varying concentrations above and below the IC₅₀ as shown in figure 2. GraphPad Prism 7 (Graphpad Software, San Diego, California) was used to fit a variable slope non-linear regression model to the data that had been
normalized to a control (DMSO). Concentrations within the confidence intervals identified by the model were tested in order to find the concentration that consistently produced a half maximal decrease in cell viability. Statistics for the models can be found in supplemental table 1.

Flow cytometry.

Cells were grown near confluency, trypsinized, and seeded on 12-well plates at 150,000 cells per well. Cells were allowed to adhere to the plate for 24 hours before treatment with the specified 10 combinations or the vehicle control (DMSO 0.2%) for 48 hours (incubated at 37° and 5% CO₂). After 48 hours, the treatment medium was removed, and the cells were washed twice with PBS before being trypsinized and harvested. To each aliquot of cells 500 µL of 70% EtOH was added, and samples were stored at 4° C. When analyzed, samples were stained with propidium iodide, and 40,000 counts per sample were collected on a BD Accuri C6 Flow cytometer (BD bioscience). Gates were drawn for the DMSO control using BD Accuri C6 Software, and the same gates were used for data collection from each sample for data collection. Percentages of cells in G₀, G₁, S, and G₂/M phases were calculated and normalized to the DMSO control. All samples were done in triplicate.

Chou-Talalay.

Cell viability assays (Alamarblue) for 0.25x, 0.50x, 1x, 2x, and 4x IC50 for each dual combination of the six drugs was performed and normalized to a 0.8% DMSO control. The data were input into the program CompuSyn (CombioSyn Inc., Paramus, New Jersey), as well as the concentration ratios, to find the combination index for each combination to determine synergism or antagonism. All data was collected and analyzed as directed by the Chou-Talalay method (Chou and Talalay, 1983).
**Mixture design and model.**

The statistical software JMP 12 (SAS Institute, Cary, North Carolina) was used to create and analyze the response surfaces. A simplex lattice (3,2) mixture design, augmented by a center point and 3 axial points, was used (figure 1) (Cornell, 2011). Each mixture combination was replicated three times resulting in a design with 30 total experiments. After conducting each experiment, two responses: 1) cell viability and 2) cell cycle analysis were obtained and recorded. Next, the method of least squares was used with the resulting data to estimate the coefficients in the quadratic mixture model used to define the response surface (Cornell, 2011). The quadratic mixture model is based on a Taylor series expansion (equation 1)

$$f(z) = \sum_{n=0}^{\infty} \frac{f^{(n)}(a)}{n!} (z - a)^n$$  \hspace{1cm} \text{Equation 1}

with a second-degree polynomial (equation 2).

$$\eta = \beta_0 + \sum_{i=1}^{q} \beta_i x_i + \sum_{i \leq j}^{q} \beta_{ij} x_i x_j$$  \hspace{1cm} \text{Equation 2}

The predicted maximum for each combination was identified using the JMP profiler (that allows visualization of cross sections of the predicted response surface as one or two mixture components are varied at a time). The resulting maximum can also be identified on a contour plot of the fitted quadratic model over the simplex design region (for combinations of 3 compounds only). The lack of fit F-test was performed, to check the adequacy of the quadratic model. Statistical significance of the lack of fit (equation 3) was determined by an F-statistic ≤ 0.10.

$$F_{11,20} = \frac{\text{LoF}/11}{3S/20}$$  \hspace{1cm} \text{Equation 3}

*The response surfaces.*
The coordinate system for the simplex is present on each ternary plot. In figure 1 the axes for compound 1 are negative sloping lines, the axes for compound 2 are parallel lines (zero slope), and the axes for compound 3 are positive sloping lines. For example, point 10 on figure 1 represents 16.67% compound 1 (following negative sloping lines to the bottom of the plot), 16.67% compound 2 (following the parallel lines to the left side of the plot), and 66.67% compound 3 (following the positive sloping lines to the right side of the plot). Three combinations are in the corners of the ternary plot (points 1, 2, and 3 of figure 1) and represent the use of only the single compound specified in that corner. Three combinations (points 4, 5, and 6 of figure 1) are in the middle of the axis between two corners and represent a 50-50 combination of the two compounds named at those corners. The other four points (7, 8, 9, and 10 of figure 1) are located in the middle of the ternary plot, and represent mixtures containing various concentrations of all three compounds. The 10 points create an even spread over the plot that produces a strong surface for differentiating mixtures (Dejaegher and Heyden, 2011). Adding more compounds to the mixture design increases the amount of combinations that will need to be empirically tested to define the response surface. For example, optimization of a mixture of 10 compounds would require testing of at least 66 different combinations. Here we focused on combinations of 3 compounds, which require only 10 points. The coordinate system produced on the simplex represents a percentage of a selected maximum concentration for each compound (table 1). The maximum for each compound can be set to any concentration. For example, 100% of compound 1 could be set to the maximum tolerable dose, while the maximum of compound 2 is set to 3X the IC50, and compound 3 to a therapeutic dose. We set the maximum concentration to the IC50 for each compound (figure 2), as determined by Alamarblue cell viability assay in PC-3 human prostate cancer cells. The IC50 was used as the maximum
specifically to have direct comparisons with the 0.5X IC50 combination index from the Chou-
Talalay method (correlates with points 4, 5, and 6 of the MDRSM model). We used Alamarblue
to measure relative cell viability of the 10 points and set our DMSO control as 100% viability.
We subtracted the normalized cell viability from 100 to express the data as a percent reduction in
cell viability so that the strongest reduction in cell viability would give us a maximum on the
surface. The maximum, or ideal combination, can be easily identified by the model. The
predicted points defined by the model are given 95% confidence intervals and allow for
statistical conclusions to be made about that point by comparing the confidence intervals.

Results

Drug combination modeling using MDRSM.

We tested 12 different combinations. We limited each combination to three of the six
total compounds (figure 3). Each data set is represented in a ternary plot. The ternary plots can
be read like topographical maps. The red area indicates the area with the largest decrease in
tumor cell viability. The dark red area is the next most effective chemotherapeutic mixtures with
gray, dark blue, and blue indicating the areas with successively lower decrease in tumor cell
viability. Ten combinations were made with every possible three component arrangements of
five compounds (DOC not included), and two combinations were formulated with DOC and
CAB together. Combinations containing DOC always contained CAB, to determine if MDRSM
could differentiate between possible additive effects produced by drugs with similar
mechanisms, however we found a synergistic interaction between them. CAB was used in the
rest of the combinations because it was effective at a slightly lower concentration than DOC.

We found that CAB is more effective than DOC when combined with PIP, and the
surface favors a combination of CAB and DOC while excluding PIP from the mixture (figure
The model reveals the maximum reduction in cell viability to be 33% DOC, 67% CAB, and 0% PIP (table 2) which causes a 45% decrease in viability. When DOC and CAB are combined with FLAV (figure 3B) the surface favors a combination of FLAV (67.36%) and CAB (32.64%) and causes a 52% decrease in cell viability, indicating that anti-tumor effect of CAB is stronger than DOC when combined with FLAV. This does not match the Chou-Talalay data that will be discussed later. The data support the ability of MDRSM to identify the most effective combinations, even when those drugs have similar mechanisms.

Each compound in the combination has an equal role in defining the surface, even if that compound is not included in the most effective combination. This phenomenon can cause the most effective combination to change between surfaces. For example, the most effective combination for DOC-CAB-FLAV (figure 3B) is 0% DOC, 33% CAB, and 67% FLAV, but the most effective combination for FLAV-PIP-CAB (figure 3D) is 54% FLAV, 0% PIP, and 46% CAB. The change of 13% in concentrations is caused by the presence of PIP in the model, and therefore such results should be confirmed with another combination model, or by reducing the MDRSM model to only look at combinations between the two compounds. The 13% decrease is not a significant difference when the confidence intervals are compared, but with more precise measurements, or a mixture using a higher concentration than the Ic50, the difference may be significant.

Our three drug mixtures also demonstrated that PIP, an effective agent against cancer when used in isolation, decreases the effectiveness of the other compounds when in combination (figure 3A, D, E, F, I, K, L). PIP does not appear to be effective in combination with any of the other compounds that we tested, which the model shows by removing it from any combination that it has been used in. RES has a similar effect to PIP and causes the effective area between
RES (0%)-FLAV (0%)-MIT (100%) (figure 3H) to entirely change as compared to FLAV (56%)-PIP (0%)-MIT (44%) (figure 3E). PIP does not appear to disrupt to the effectiveness of combinations like RES. RES disrupts the effective of combinations, causing the surface to favor the use of only one compound, except between FLAV-CAB (figure 3G). The model reveals that 0.28% RES slightly increases the effectiveness of (potentiates) FLAV and CAB; however, the concentration of resveratrol is not a significant amount, and becomes 0% (Table 2). The presence of RES in the model shifts the concentration higher for FLAV, and lower for CAB as compared to figure 3A (33% CAB and 67% FLAV) to 73% FLAV and 27% CAB (table 2). The handling of RES and PIP in these combinations identify the ability of MDRSM to find the most effective combinations both in the presence of antagonism. The antagonistic actions identified by RES and PIP coincide with antagonism measured by the Chou-Talalay method (figure 4).

Of the 12 combinations performed, only one combination (FLAV-MIT-CAB figure 3C) called for a mixture of all three compounds (table 2). The combinations favor higher percentages of FLAV (55%) and MIT (44%) while only having a minute amount of CAB (1%) figure 3C). However, the predicted maximum viability decreases of 56% and 95% confidence interval of 44% to 69% suggests that there is not a significant difference in reduced viability from that slight addition of CAB (table 2). As stated previously, this may be significant if higher concentrations were used, or if a more precise response was measured.

*The Chou-Talalay method identifies synergism and confirms some MDRSM mixtures.*

MDRSM identifies the optimal concentration, but the Chou-Talalay method differentiates between synergism, additive and antagonistic effects. The combination index curve produced by the Chou-Talalay equation cannot be extrapolated to include combinations with non-constant ratios without collecting significantly more data. As per our design, the equal mixture of 0.5X
IC50 of the Chou-Talalay data is included in the MDRSM data as a 50-50 mixture (points 4, 5, and 6 of Table 1) between two compounds. Interestingly, there is synergism between DOC-CAB at 0.5X IC50 (figure 4) which is close to what is identified as the most effective dose in the MDRSM seen in figure 3A (33% DOC, 67% CAB, 0% PIP). The MDRSM model accounts for that point, and still identifies the ideal combination as 33% DOC and 67% CAB (table 2), although this may be influenced by PIP. In the mixture of DOC-CAB-FLAV (figure 3B) There is stronger synergism between DOC-FLAV (0.63441) than CAB-FLAV (0.79421) or DOC-CAB (0.73087) (figure 4). However, MDRSM places the ideal combination between CAB-FLAV because it is measuring the effectiveness of the combination, and not the synergism. Perhaps this is due to the ability of PC-3 cells to become resistant to DOC more readily than CAB, which makes the CAB-FLAV mixture more effective.

The Chou-Talalay model identified synergism between CAB and FLAV (figure 4) which correlates with most effective dose identified using MDRSM data. MDRSM places the optimal concentration between CAB and FLAV in figure 3B, D, and G. The concentrations vary between mixtures, but this is due to the change in the surface caused by the third compound. The optimal concentration identified by MDRSM appear to correlate strongly with synergistic actions identified by the Chou-Talalay method, and we conclude that the most effective mixture identified by MDRSM is strongly affected by synergism, but not in every circumstance. MDRSM provides and advantage over the Chou-Talalay model because it identifies the most effective combination irrespective of the mechanism.

*Cell cycle data is a valid input for MDRSM.*

To further show the robustness of MDRSM, we performed cell cycle analysis for the 10-point mixture of CAB-FLAV-MIT because all three drugs have a different effect on the cell
cycle. CAB inhibits microtubule formation, FLAV inhibits CDK’s, and MIT inhibits topoisomerase II and prevents DNA unwinding (Ehninger et al., 1990; Galsky et al., 2010; Gupta et al., 2010; Iyer et al., 2013). We treated the samples with the ten combinations of three compounds described above with the maximum concentration being 80 nM CAB, 320 nM FLAV, 800 nM MIT in order to see pronounced effects on the cell cycle. After 48 hours of treatment we harvested the cells, stained them with propidium iodide and used a flow cytometer to analyze the cell cycle phase for cells treated with the 10 mixtures. This data was then normalized to the DMSO control and used to produce the surfaces in figure 5. The combination that gives the largest decrease of cells in the G1 phase is a combination that includes CAB (figure 5A), and the model shows 100% CAB (table 3). However, the lack of fit is significant (p-value = 0.0087), meaning the model does not fit the data properly, or the combination defined by this model is not significant. Maximizing the number of cells in the S phase requires a mixture of MIT and CAB (figure 5B), which is 32% CAB and 68% MIT (table 3). This is perhaps due to the inability of cells to divide, caused by the microtubule inhibitor CAB, and the DNA damage, as well as halting of DNA replication by MIT. The most effective dose at trapping cells in the G2/M phase is 100% CAB because of the inability of the cells to form microtubules and divide (figure 5C). Maximizing cells in the sub G1 phase, or causing the most damage to DNA, requires a combination of MIT and FLAV (figure 5D), and the model shows the ideal combination is 48% MIT and 52% FLAV (table 3). Comparisons between the MDRSM model produced by cell viability (figure 3C), and the models produced here cannot be made because the same concentrations were not used. However, comparisons between cell viability and cell cycle phase, when the same concentrations are used, may help to determine the mechanism by which the drugs are reducing cell viability, whether it be cell death, halting of the cell cycle, or a mixture of
both. It is interesting to note that the ratio between drugs in the cell viability treatment, and that of the sub $G_1$ phase are similar even though the concentrations are different.

Discussion

In this work, we have reviewed the basic uses and application of MDRSM in combination chemotherapy. We show several models of three-drug combinations and the limitations and advantages of MDRSM. We demonstrate that cell viability and cell cycle assays are valid inputs for creating a surface. We have demonstrated beneficial combinations for treating PC-3 human prostate cancer cells between standard chemotherapeutics, and FLAV, a naturally occurring bioactive compound. We have shown that RES and PIP can interfere with standard chemotherapeutics. However, we speculate that RES may potentiate CAB-FLAV. We have also shown that the optimal combination defined by MDRSM is affected by the response that is chosen to define the surface. MDRSM, in companion with the Chou-Talalay method, can be used to identify strong combination treatments that can increase the effectiveness of chemotherapy.

MDRSM has the potential to bring a new perspective to developing drug combinations. MDRSM can be used to measure the effectiveness of drug mixtures targeting specific aspects of cancer such as those defined in the hallmarks of cancer (Hanahan and Weinberg, 2011). Further, by collecting multiple responses for one combinations, such as the effects of the combinations on healthy tissue as well as cancerous tissue, the models for each response can be merged into a new model known as the desirability function. The desirability function can be used to maximize the effect on cancerous tissue, while minimizing the effects on healthy tissue. Developing such a treatment would be a major step forward in improving the quality of life for cancer patients. MDRSM could also be used to develop treatments based on the prominent theory of tumor
heterogeneity (Singh and Settleman, 2010), which is the existence of frequently-chemoresistant tumorigenic cancer stem cells (CSC) that produce a genetically diverse tumor. This theory sites the existence of resistant CSCs to be the cause of chemoresistance and tumor recurrence (Housman et al., 2014; Marusyk and Polyak, 2010). MDRSM could model a combination of common chemotherapeutics along with a targeted treatment of CSCs that could lead to maximum tumor reduction, and minimal risk of tumor recurrence.

Although we collected ten data points for each simplex lattice design, more data points could have been added to the design. Adding more points to a design can allow for fitting a higher order polynomial model (such as cubic or quartic) to the data, however, this is not guaranteed to make the fitted model more reliable. When fitting an empirical model, a higher order terms may just model the random experimental noise. Normally in response surface methods a quadratic model based on the two term Taylor series expansion is sufficient to identify interactions and the combination of compounds predicted to result in the maximum response.

MDRSM is a powerful tool for measuring combination chemotherapy, but the data can easily be overinterpreted. For example, a response for MDRSM could be as simple as a cell viability experiment in a cell model, but the ideal combination given by the surface would only be ideal for reducing cell viability. Response surfaces produced by an apoptotic assay, or cell cycle analysis may have similar results as the cell viability surface, but the differences between ideal combinations will be specific for the response. One can compare multiple responses of the same combination of drugs in MDRSM by merging the models to form a desirability function as mentioned above. Choosing the response for MDRSM should be specific to the cell, or animal model, and should be reflective of a desired effect for combating the specific type of cancer being researched. A potential limitation of MDRSM is the choice of maximum concentrations.
Preliminary data from a Chou-Talalay combination index analysis may be helpful in choosing a maximum concentration.

We propose MDRSM as an effective tool for modeling and defining drug combinations in cell, animal, and clinical models. MDRSM can also be a tool in combating chemoresistance by using a combination of drugs at their maximal effectiveness for decreasing tumor size, while minimizing the ability of the remaining tumor cells to become resistant. MDRSM has the ability to develop combinations using two or more drugs and provides a novel and improved way of measuring the effectiveness of multiple compounds. MDRSM in combination with the Chou-Talalay method can cover a broad area of drug combinations, providing a multiplicity of data while being both cost- and time-effective.
Authorship Contributions

Participated in research design: Richard Oblad, Jason Kenealey, Merrill Christensen

Conducted experiments: Richard Oblad, Hayden Doughty

Contributed analytical tools: Merrill Christensen, John Lawson

Performed data analysis: Richard Oblad, John Lawson

Wrote or contributed to the writing of the manuscript: Richard Oblad, Jason Kenealey, John Lawson, Merrill Christensen, Hayden Doughty
References


Iyer SP, Mejia J, Rosato AE, Grant S and Rosato RR (2013) Flavopiridol-mediated multiple modulatory effects synergistically increase sensitivity to TRAIL-induced cell death in human leukemia cells, AACR.


Oprea-Lager DE, Bijnsdorp IV, Van Moorselaar RJA, Van Den Eertwegh AJM, Hoekstra OS and Geldof AA (2013) ABCC4 Decreases Docetaxel and Not


Footnotes

Grant Support: This work was funded by the Brigham Young University Life Science Startup Grant.

Conflict of Interest: The authors declare no conflict of interest.
Table 1 - Ternary Plot Coordinate System Represented as Percentages - 100% can be artificially set to any desired concentration.

<table>
<thead>
<tr>
<th>Ternary point</th>
<th>Compound 1</th>
<th>Compound 2</th>
<th>Compound 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>2</td>
<td>0%</td>
<td>100%</td>
<td>0%</td>
</tr>
<tr>
<td>3</td>
<td>0%</td>
<td>0%</td>
<td>100%</td>
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<tr>
<td>4</td>
<td>50%</td>
<td>50%</td>
<td>0%</td>
</tr>
<tr>
<td>5</td>
<td>50%</td>
<td>0%</td>
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<td>50%</td>
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<td>33.33%</td>
</tr>
<tr>
<td>8</td>
<td>66.67%</td>
<td>16.67%</td>
<td>16.67%</td>
</tr>
<tr>
<td>9</td>
<td>16.67%</td>
<td>66.67%</td>
<td>16.67%</td>
</tr>
<tr>
<td>10</td>
<td>16.67%</td>
<td>16.67%</td>
<td>66.67%</td>
</tr>
</tbody>
</table>
## Table 2 – Optimal Concentration and Percentages for Maximizing a Reduction in Cell Viability

<table>
<thead>
<tr>
<th>Compound 1</th>
<th>Compound 2</th>
<th>Compound 3</th>
<th>Predicted Decrease in % Cell Viability</th>
<th>95% Confidence Interval</th>
<th>Lack of Fit Prob &gt; F*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percentage of IC50</td>
<td>Concentration</td>
<td>Percentage of IC50</td>
<td>Concentration</td>
<td>Percentage of IC50</td>
</tr>
<tr>
<td>1</td>
<td>DOC, CAB, PIP</td>
<td>33%</td>
<td>15 nM</td>
<td>67%</td>
<td>27 nM</td>
</tr>
<tr>
<td>2</td>
<td>DOC, CAB, FLAV</td>
<td>0%</td>
<td>0 nM</td>
<td>33%</td>
<td>13 nM</td>
</tr>
<tr>
<td>3</td>
<td>FLAV, MIT, CAB</td>
<td>55%</td>
<td>89 nM</td>
<td>44%</td>
<td>176 nM</td>
</tr>
<tr>
<td>4</td>
<td>FLAV, PIP, CAB</td>
<td>54%</td>
<td>86 nM</td>
<td>0%</td>
<td>0 µM</td>
</tr>
<tr>
<td>5</td>
<td>FLAV, PIP, MIT</td>
<td>56%</td>
<td>90 nM</td>
<td>0%</td>
<td>0 µM</td>
</tr>
<tr>
<td>6</td>
<td>PIP, MIT, CAB</td>
<td>0%</td>
<td>0 µM</td>
<td>100%</td>
<td>400 nM</td>
</tr>
<tr>
<td>7</td>
<td>RES, FLAV, CAB</td>
<td>0%</td>
<td>0 µM</td>
<td>73%</td>
<td>117 nM</td>
</tr>
<tr>
<td>8</td>
<td>RES, FLAV, MIT</td>
<td>0%</td>
<td>0 µM</td>
<td>0%</td>
<td>0 nM</td>
</tr>
<tr>
<td>9</td>
<td>RES, FLAV, PIP</td>
<td>0%</td>
<td>0 µM</td>
<td>100%</td>
<td>160 nM</td>
</tr>
<tr>
<td>10</td>
<td>RES, MIT, CAB</td>
<td>0%</td>
<td>0 µM</td>
<td>100%</td>
<td>400 nM</td>
</tr>
<tr>
<td>11</td>
<td>RES, PIP, CAB</td>
<td>0%</td>
<td>0 µM</td>
<td>0%</td>
<td>0 µM</td>
</tr>
<tr>
<td>12</td>
<td>RES, PIP, MIT</td>
<td>0%</td>
<td>0 µM</td>
<td>0%</td>
<td>0 µM</td>
</tr>
</tbody>
</table>

*Values less than or equal to 0.10 indicate that the model does not fit the data
Table 3 – Optimum Combinations for Minimizing Cell Population in the G₁ phase, and Maximizing Cell Populations in the S, G₂/M, and Sub G₁ Phases, Produced by Cell Cycle Analysis

<table>
<thead>
<tr>
<th></th>
<th>Cabazitaxel</th>
<th>Mitoxantrone</th>
<th>Flavopiridol</th>
<th>% Change in Population</th>
<th>95% Confidence Interval</th>
<th>Lack of Fit Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>G₁ phase</td>
<td>100% , 80nM</td>
<td>0% , 0nM</td>
<td>0% , 0nM</td>
<td>(86.37)</td>
<td>(77.84), (94.90)</td>
</tr>
<tr>
<td>2</td>
<td>S phase</td>
<td>32% , 26nM</td>
<td>68% , 543nM</td>
<td>0% , 0nM</td>
<td>237.73</td>
<td>207.65, 267.80</td>
</tr>
<tr>
<td>3</td>
<td>G₂/M phase</td>
<td>100% , 80nM</td>
<td>0% , 0nM</td>
<td>0% , 0nM</td>
<td>537.37</td>
<td>469.21, 605.52</td>
</tr>
<tr>
<td>4</td>
<td>Sub G₁ phase</td>
<td>0% , 0nM</td>
<td>48% , 387nM</td>
<td>52% , 165nM</td>
<td>499.14</td>
<td>311.71, 686.572</td>
</tr>
</tbody>
</table>

* Values less than or equal to 0.10 indicate that the model does not fit the data.
Figure 1 – *JMP ternary plot with combination points*. Ternary plot of a three-compound combination containing the ten points used to define a surface. Concentrations for each point are read by following the left diagonal lines for compound 1, horizontal lines for compound 2, and right diagonal lines for compound 3.
Figure 2 – IC50 graphs for chemotherapeutics in PC-3 human prostate cancer cells. (A) DOC – 45 nM (B) CAB – 40 nM (C) MIT – 400 nM (D) RES – 75 µM (E) FLAV – 160 nM (F) PIP – 7 µM. n=3
Figure 3 – *Response surfaces of 12 different mixtures.* Bright red areas represent the most effective combinations at reducing cell viability. Deep red, grey, and blue are listed in descending order of effectiveness. The numbers within each contour represent the maximum prediction found within that contour, meaning that all predictions within that contour are less
than or equal to the number found within. The mixtures for each ternary plot are: (A) DOC, CAB, PIP (B) DOC, CAB, FLAV (C) FLAV, MIT, CAB (D) FLAV, PIP, CAB (E) FLAV, PIP, MIT (F) PIP, MIT, CAB (G) RES, FLAV, CAB (H) RES, FLAV, MIT (I) RES, FLAV, PIP (J) RES, MIT, CAB (K) RES, PIP, CAB (L) RES, PIP, MIT. All data were obtained by Alamarblue cell viability assay with three biological replicates in triplicate.
Figure 4 – *Chou-Talalay combination index (CI) chart for all combinations.* Red represents synergistic CI’s, grey represents additive CI’s, and blue represents antagonistic CI’s. CI’s were all obtained by Alamarblue cell viability assay with three biological replicates in triplicate.
Figure 5 – *Response surfaces produced by cell cycle analysis.* Bright red areas represent the most effective combinations. Deep red, grey, and blue are listed in descending order of effectiveness. The numbers within each contour represent the maximum prediction found within that contour, meaning that all predictions within that contour are less than or equal to the number found within. (A) Surface for relative decrease in G_1 phase populations. (B) Surface for maximizing S phase populations. (C) Surface for maximizing G_2/M phase populations. (D) Surface for maximizing sub G_1 populations. All data were obtained from flow cytometry of propidium iodide stained cells. All samples were analyzed in triplicate.
Supplemental Table 1 – Statistics from the Ic50 models shown in figure 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ic50</th>
<th>95 % Confidence interval</th>
<th>Hill Slope</th>
<th>R square</th>
<th>Ic50 Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 CAB</td>
<td>35.74 nM</td>
<td>31.83 to 41.89 nM</td>
<td>-0.7528</td>
<td>0.7908</td>
<td>40 nM</td>
</tr>
<tr>
<td>2 DOC</td>
<td>49.1 nM</td>
<td>41.24 to 66.38 nM</td>
<td>-1.848</td>
<td>0.7199</td>
<td>45 nM</td>
</tr>
<tr>
<td>3 MIT</td>
<td>374.3 nM</td>
<td>321.6 to 433.2 nM</td>
<td>-0.9325</td>
<td>0.9200</td>
<td>400 nM</td>
</tr>
<tr>
<td>4 FLAV</td>
<td>155.4 nM</td>
<td>126.3 to 186.9 nM</td>
<td>-0.7571</td>
<td>0.8786</td>
<td>160 nM</td>
</tr>
<tr>
<td>5 PIP</td>
<td>6.706 μM</td>
<td>6.337 to 7.055 μM</td>
<td>-16.26</td>
<td>0.9320</td>
<td>7 μM</td>
</tr>
<tr>
<td>6 RES</td>
<td>85.42 μM</td>
<td>73.78 to 99.51 μM</td>
<td>-1.291</td>
<td>0.9618</td>
<td>75 μM</td>
</tr>
</tbody>
</table>
Proposed Research

Background & Significance

Cancer is the second leading cause of death in the United States, causing the loss of 595,930 lives in 2016 [1]. The main therapies for treating cancer consist of radiation therapy, surgery, and/or chemotherapy.

Problem Statement

Current models for combination chemotherapy are useful for measuring antagonism, synergism, or additive effects, but do not measure actual effectiveness of the combinations. Current models are also limited in their ability to extrapolate to other ratios of combinations or in measuring effects between more than two drugs.

Purpose of the Study

The purpose of this research is to test the applications of mixture design response surface methodology in a combination chemotherapy cancer cell culture model with multiple drugs.

Research Questions

1. What is being studied?
   a. Mixture design response surface methodology in human PC-3 human prostate cancer cells as a model for combination chemotherapy.
2. What will be measured?
   a. Cell response to a combination of drugs such as cell viability and cell cycle phase analysis.
3. How will the model be compared to traditional models?
a. Cell viability measurements will be made with drugs at the Ic50 so that the three 50-50 points in the mixture design response surface model will be compared with the 0.5 X Ic50 measurements from the Chou-Talalay model.

Research Aims and Objectives

The aim of the present study is to test a statistical tool, mixture design response surface methodology, and whether it has application in studying combination drug therapy in cancer.

Objective 1 – Select six drugs, and discover the Ic50 for each drug in PC-3 human prostate cancer via Alamarblue cell viability assay.

Hypothesis 1 (H0) – The drugs have no effect on cell viability at any concentration in PC-3 human prostate cancer cells when measured by Alamarblue P <0.05.

Hypothesis 1 (H1) – The drugs have a concentration dependent effect on cell viability in PC-3 human prostate cancer cell when measured by Alamarblue P <0.05.

Objective 2 – Test cell viability for multiple three drug combinations of the six drugs and use mixture design response surface methodology to identify optimal mixtures for each combination.

Hypothesis 2 (H0) – The drugs have no effect on cell viability in any mixture or combination in PC-3 human prostate cancer cells when measured by Alamarblue and the surface does not fit (measured by lack of fit) P <0.10.
Hypothesis 2 (H₁) – The drugs have an effect in the different mixtures and combinations on cell viability in PC-3 human prostate cancer cells when measured by AlamarBlue, and the surface will fit the model $P > 0.10$. The optimal concentration is identifiable because the model fits.

Objective 3 – Identify strengths and weaknesses of mixture design response surface methodology, and compare the results obtained to current models for drug combinations.

Hypothesis 3 (H₀) – Mixture design response surface methodology will not have similar results to established models, and the weaknesses of the model will prevent it from being used in cancer research.

Hypothesis 3 (H₁) – Mixture design response surface methodology will produce comparable results with established models, and the strengths of the model will be useful in cancer research.

Objective 4 – Test cell cycle analysis as a response that can be used to define a surface as an example of how other assays can be used as a response in mixture design response surface methodology.

Hypothesis 4 (H₀) – Other responses, such as cell cycle analysis, is not a viable responses for mixture design response surface methodology, and the model does not fit $P < 0.10$. 

41
Hypothesis 4 (H4): Cell cycle analysis is a viable response for mixture design response surface methodology, and the optimal concentration is identifiable P > 0.10.

Materials & Methods

Cell lines - PC-3 human prostate cancer cells were obtained from ATCC (Rockville, MD), and were cultured in F12-K medium containing 10% FBS and 1% antibiotic (penicillin/streptomycin). Cells were grown in an incubator at 37°C and 5% CO₂. Cells from passage numbers 3-17 were used.

Reagents - Cabazitaxel was obtained from APExBIO (Houston, TX) and dissolved in dimethyl sulfoxide (DMSO) to make a 40 µM stock solution. Docetaxel, flavopiridol, mitoxantrone hydrochloride, and piperlongumine were obtained from Sigma Aldrich (St. Louis, Missouri) and were dissolved in DMSO to make a 45 µM, 160 µM, 400 µM, and 8 mM stock solution, respectively. Trans-resveratrol was obtained from Cayman Chemical (Ann Arbor, MI) and dissolved in DMSO to make a 75 mM stock solution. Alamarblue was obtained from Remel (Lenexa KS). Propidium iodide was obtained from Sigma Aldrich (St. Louis, Missouri) and dissolved in PBS for a stock concentration of 50 µg/mL.

Cell Viability - Cells were grown near confluency, trypsinized, and seeded on 96-well plates at a concentration of 10,000 cells per well (100,000 cells/mL). Cells were allowed to adhere to the plate for 24 hours before treatment with the vehicle control (DMSO 0.1%) or 1 of 10 drug combinations and allowed to incubate for 48 hours (37°C and 5% CO₂). Each combination treatment also had its own control where treatment medium, without cells, was analyzed with Alamarblue to eliminate background fluorescence and interactions between the drugs and the Alamarblue reagent. After 48 hours, the cells were treated with 10 µL of
Alamarblue, and incubated for 6 hours (37° and 5% CO2). The fluorescence was then analyzed on a BMG LABTECH FLUOstar OPTIMA plate reader (BMG LABTECH Inc., Cary, NC) using the appropriate filters. All treatments were done with three biological replicates and in triplicate within each biologic replicate.

Diagram for Ic50:

<table>
<thead>
<tr>
<th>PBS</th>
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</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>DMSO</td>
<td>Conc 1</td>
<td>Conc 2</td>
<td>Conc 3</td>
<td>Conc 4</td>
<td>Conc 5</td>
<td>Conc 6</td>
<td>Conc 7</td>
<td>Conc 8</td>
</tr>
<tr>
<td>PBS</td>
<td>DMSO</td>
<td>Conc 1</td>
<td>Conc 2</td>
<td>Conc 3</td>
<td>Conc 4</td>
<td>Conc 5</td>
<td>Conc 6</td>
<td>Conc 7</td>
<td>Conc 8</td>
</tr>
<tr>
<td>PBS</td>
<td>DMSO</td>
<td>Conc 1</td>
<td>Conc 2</td>
<td>Conc 3</td>
<td>Conc 4</td>
<td>Conc 5</td>
<td>Conc 6</td>
<td>Conc 7</td>
<td>Conc 8</td>
</tr>
<tr>
<td>PBS</td>
<td>DMSO</td>
<td>Conc 1</td>
<td>Conc 2</td>
<td>Conc 3</td>
<td>Conc 4</td>
<td>Conc 5</td>
<td>Conc 6</td>
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<td>Conc 8</td>
</tr>
</tbody>
</table>

Diagram for Combinations:

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</tr>
</tbody>
</table>
**Flow cytometry** - Cells were grown near confluency, trypsinized, and seeded on 12-well plates at a concentration of 150,000 cells per well (200,000 cells/mL). Cells were allowed to adhere to the plate for 24 hours before treatment with the specified 10 combinations or the vehicle control (DMSO 0.2%) for 48 hours (incubated at 37° and 5% CO₂). After 48 hours, the treatment medium was removed, and the cells were washed twice with PBS before being trypsinized and harvested. To each aliquot of cells 500 µL of 70% EtOH was added, and samples were stored at 4° C. When analyzed, samples were stained with propidium iodide, and 40,000 counts per sample were collected on a BD Accuri C6 Flow cytometer (BD bioscience). Gates were drawn for the DMSO control using BD Accuri C6 Software, and the same gates were used for data collection from each sample for data collection. Percentages of cells in G₀, G₁, S, and G₂ phases were calculated and normalized to the DMSO control. All samples were analyzed in triplicate.

**Response surface and mixture design** - The statistical software JMP 12 (SAS Institute, Cary, North Carolina) was used to create and analyze the response surfaces. A simplex lattice (3,2) mixture design, augmented by a center point and 3 axial points, was used [2]. Each mixture combination was replicated three times resulting in a design with 30 total experiments. After conducting each experiment, two responses: 1) cell viability and 2) cell cycle analysis were obtained and recorded. Next, the method of least squares was used with the resulting data to estimate the coefficients in the quadratic mixture model used to model the response surface [2]. The mixture combination predicted to result in the maximum cell viability was located using the JMP profiler (that allows visualization of cross sections of the predicted response surface as one or two mixture components are varied at a time). The resulting mixture combination predicted to result in maximum cell viability can also be identified on a contour plot of the fitted quadratic
model over the simplex design region. The lack of fit F-test was performed, to check the adequacy of the quadratic model.

Chou-Talalay — Cell viability assays (Alamarblue) for 0.25x, 0.50x, 1x, 2x, and 4x IC50 for each dual combination of the six drugs was performed and normalized to a 0.8% DMSO control. The data were input into the program CompuSyn (CompoSyn Inc., Paramus, New Jersey), as well as the concentration ratios, to find the combination index for each combination to determine synergism or antagonism. All data was collected and analyzed as directed by the Chou-Talalay method [3].

Diagram for Chou-Talalay Method: (0.25 X refers to 0.25 X IC50 etc.)

Plate 1:
Plate 2:

<table>
<thead>
<tr>
<th>PBS</th>
<th>PBS</th>
<th>PBS</th>
<th>PBS</th>
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</thead>
<tbody>
<tr>
<td>PBS</td>
<td>DMSO</td>
<td>0.25X Comb</td>
<td>0.5X Comb</td>
<td>1X Comb</td>
<td>2X Comb</td>
<td>4X Comb</td>
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<tr>
<td>PBS</td>
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<td>0.25X Comb</td>
<td>0.5X Comb</td>
<td>1X Comb</td>
<td>2X Comb</td>
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<tr>
<td>PBS</td>
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<td>0.25X Comb</td>
<td>0.5X Comb</td>
<td>1X Comb</td>
<td>2X Comb</td>
<td>4X Comb</td>
<td>PBS</td>
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<td>0.25X Blank</td>
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References


Literature Review

Cancer and Treatment

Cancer is the abnormal growth of cells that may have the ability to metastasize or move to other tissues. Cancer is the second largest cause of mortality in the United States, and caused the loss of 595,930 lives in 2016 (2017 data is not available at this time) [1]. Prostate Cancer is the second most diagnosed cancer in men, and the third largest killer among cancers in men [2]. Metastatic prostate cancer makes up 10% of said cases, and has a 90% mortality rate [3]. Stage IV prostate cancer is an aggressive metastatic prostate cancer that has metastasized to other tissues and bone[4]. Treatments for stage IV prostate cancer include [4]:

*Hormone therapy* – the addition, blockade, or removal of hormones to slow or stop the growth of prostate cancer.

*Hormone therapy combined with chemotherapy* – the use of anticancer drugs to kill cancerous cells, along with hormones to slow or stop the growth and spread of prostate cancer.

*Radiation therapy* – the use of high energy radiation to kill prostate cancer cells.

*Surgery* – removing the prostate and possibly some surrounding tissue (radical prostatectomy) or removing one, or both, of the testicles (orchiectomy).

Chemotherapy, radiation therapy and surgery are common treatments in many types of cancer. Chemotherapy was first used in humans in the 1940’s when doctors began to use
nitrogen mustards (developed from mustard gas) to treat lymphomas [5]. In the 1960’s combination chemotherapy came about as a new “cure” for more advanced forms of cancer, because chemotherapy could attack metastases more easily than surgery or radiation therapy [5]. Chemotherapy often uses cytotoxic drugs, which will reduce tumor size, but cause life altering side effects such as hair loss, and gastrointestinal distress. Cytotoxic drugs may also cause carcinogenic mutations in previously healthy tissue, causing formation of a new tumor. Cancerous tissue may also become resistant to the cytotoxic drugs, which limits the effectiveness of chemotherapy [6]. Cancer cells can either have primary or acquired resistance. Primary drug resistance occurs when the cancer cell can survive the cytotoxic effects of a drug on the first exposure, meaning that the cell already had a mechanism in place to reduce the effectiveness of the drug. Acquired resistance occurs when the cancer cell evolves a mechanism of resistance sometime after the first exposure to the drug. Whether the cancer exhibits primary or acquired resistance the mechanism is usually an increase in drug transport (efflux), an increase in drug metabolism, or a mutation in the drug target [7, 8].

The tumor microenvironment, specifically cancer stem cells (CSCs) and carcinoma associated fibroblasts (CAFs), plays an important role in drug resistance [9]. Two models of the tumor microenvironment are currently under debate. The first model explains tumor heterogeneity as random spawn and mutation, and that every cell in the tumor is tumorigenic. The genetic diversity within the tumor causes some cells to be resistant to drugs, and after treatment with said drug, the resistant cells can begin to grow a new tumor [10]. The second theory includes CSCs, which generate specialized cells (CAFs) that divide and form a tumor [10]. The CSCs are tumorigenic, but do not replicate often. Most chemotherapeutics target rapidly dividing cells, which makes the CSCs more resistant to standard chemotherapy. The
CAFs are rapidly dividing, and are therefore sensitive to chemotherapy. This causes a reduction in the tumor, but the resistant CSCs do not die, and can then form a new resistant tumor [10]. Prostate cancer has been shown, in current CSCs models, to become resistant after treatment with cytotoxic chemotherapeutics through WNT signaling within the tumor microenvironment [9]. Human prostate cancer PC-3 cells, a cell line derived from metastatic prostate cancer, have been shown to be chemoresistant to some common chemotherapeutics [11-13]. The PC-3 cell model is an aggressive form of prostate cancer that does not express the androgen receptor (AR), or prostate specific androgen (PSA) [14]. Absence of AR and PSA allows for PC-3 cells to grow in the absence of androgen, and therefore is immune to hormone therapy [15]. Such characteristics make PC-3 prostate cancer cells a good model for an aggressive form of prostate cancer (that would be treated with chemotherapy), and a good model for testing cytotoxic chemotherapeutics.

**Combatting chemoresistance**

As previously discussed, common mechanisms of chemoresistance include downregulating target proteins, upregulating metabolizing enzymes, and upregulating transport proteins that can remove the drug from the cell [7]. Treating with multiple compounds simultaneously has been shown to decrease chemoresistance. Cells that become resistant to one drug, are killed off by the second or third compound used in combination, and are therefore unable to replicate and pass on the resistant characteristics [16]. Combination therapies with new drugs, such as immunotherapies, may be the best way to combat chemoresistance, and reduce cancer mortality rates. However, immunotherapy can be very costly, which reduces the ability of patients to receive it, let alone receive combination treatments [16]. Therefore, having a tool to maximize combination effectiveness is very important in improving both the best
treatments available, as well as treatments that are more affordable. The National Cancer
Institute has also identified combination chemotherapy as an area of focus for future research so
that better combinations can be discovered, and streamlined into clinical trials, and therapies
[17].

Several tools for maximizing effectiveness of combinations have been developed over the
past century [18]. Perhaps the most prominent is the method for defining synergy and
antagonism known as the Chou-Talalay method. The Chou-Talalay method is based on the
median-effect equation, derived from the mass-action law principle [19]. The equation takes
from Michaelis-Menten, Hill, Henderson-Hasselbalch, and Scatchard equations to define first
order, and higher order dynamics between single and multiple entities [19]. The resulting
combination index shows antagonism (>1), synergism (<1), or additive (=1) effects. The focus
of combinations is on constant ratio mixtures such as IC50:IC50 or 2X IC50:2X IC50, which
defines the strongest synergism within the constant ratio mixtures. The method can incorporate
non-constant mixtures, but that is not the focus of the median-effect equation [20, 21].
Performing mixtures between more than two compounds also becomes difficult when using the
Chou-Talalay method. The methodology tests the combinations of the three compounds
separately (for compounds A, B, and C they would test AB, AC, and BC), but never tests all
three compounds together (never testing ABC) [19]. Therefore, methods testing non-constant
ratios, as well as multiple compounds need to be improved to create better treatments.

Other methods for testing combinations have involved response surface methodology, but
have fallen short of testing more than two compounds, and suffer from complex functions, which
makes interpretation difficult [22]. In general, the approach for response surfaces in
combinations is creating a grid of mixtures for two compounds, and testing them to require a
response in order to create a surface. From the developed surface, various equations are used to define synergy, or antagonism. The culmination of response surface modeling of combination treatments has come down to nonparametric, and parametric response surface models. Nonparametric models, such as the Sühnel model, have a direct approach of combination mixing that allows the exploration of many different combinations without any preset conditions [23]. However, this model requires extensive data collection, and does not provide a summary of interaction intensity or provide a statistical strength for the conclusions made [22]. Parametric models combine the statistics of non-linear regression, and the student’s t-test to provide confidence in conclusions drawn from the surface [24]. This allows quantitative conclusions to be drawn from a surface, and reduces the amount of data that is needed to form a surface, but also incorporates complexity into the models. The complexity allows for evolution of the model to incorporate new parameters, and more complex models, but becomes more difficult to apply to other fields due to lack of understanding and resources [22]. Response surface methodology has also failed to incorporate more than two drugs in their models, which puts a limit on developing more complex combinations with two or more compounds, and therefore limits the efficacy of combination therapies.

One method that could be used to fill the holes in drug combination development, while remaining uncomplicated, is mixture design response surface methodology (MDRSM). MDRSM is based on response surface methodology, but it is specifically designed to fit a surface with the parameters of a mixture of two or more compounds. MDRSM is used in several industries to define the ideal mixture to bring about a desired response [25, 26]. For example, MDRSM could be used to find the right mixture of soil types that would create the fastest decomposition in a landfill. Applications of response surface methodology in cancer has been
limited, but MDRSM has not been used previously. One application would be to simply measure cell viability while testing a mixture of compounds, and the surface created by the results would be used to find the most effective concentrations of compounds to decrease cell viability. The model is also able to test 2 or more compounds. Testing three compounds would require ten different mixtures of three compounds, while testing ten compounds would require sixty-six combinations of the ten compounds. The basic model for testing three compounds is a simplex lattice (3,2) mixture design, augmented by a center point and 3 axial points. The ten points that would be tested are shown in the table below.

<table>
<thead>
<tr>
<th>Ternary point</th>
<th>Compound 1</th>
<th>Compound 2</th>
<th>Compound 3</th>
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<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>100%</td>
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<tr>
<td>3</td>
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<td>4</td>
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<td>9</td>
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<td>16.67%</td>
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<tr>
<td>10</td>
<td>10</td>
<td>16.67%</td>
<td>16.67%</td>
</tr>
</tbody>
</table>

The maximum concentration of each compound (100%) is set to any desired concentration, but the response being used to define the surface should be about the same for the 100% mark of all three compounds.
Defining mixtures through MDRSM can also be constricted to certain areas of the surface [27]. For example, if a drug has high toxicity above a certain concentration, the surface can be restricted to only include ideal mixtures within the set concentration limits. Or, if a certain drug is required for the mixture above a certain concentration to reduce side effects, the ideal mixture will be found within the constrained concentrations of that drug. Overall, MDRSM is a multifaceted tool that can be used to define mixtures of two or more compounds, that provides the most effective combination by incorporating both non-constant and constant ratio mixtures.

**Standard Chemotherapeutics**

**Cabazitaxel and Docetaxel:**

![Chemical structures of Cabazitaxel, Docetaxel, and Taxol](image)

Cabazitaxel and docetaxel are semisynthetic medicines from the natural compound taxol (paclitaxel) which was isolated from the Pacific yew [28]. The toxoids (semisynthetic compounds based on taxol) are commonly used chemotherapeutics in many types of cancers based on inhibition of microtubule formation, and inability of the cell to continue through the cell cycle [29]. Cabazitaxel and docetaxel have been used in combination with many drugs, and
have been used in hundreds of clinical trials as single drug trials, and combination trials [30-34]. Cabazitaxel, although active by itself, undergoes demethylation in the cell, and continues its activity as docetaxel [35]. However, cabazitaxel is used as a second line of defense against advanced forms of cancer, such as castration resistant, and chemoresistant forms of prostate cancer, even after docetaxel administration [36-40].

The difference in effectiveness of docetaxel and cabazitaxel is due to a difference in the cell’s ability to become resistant to each taxoid. Cancer cells appear to be less likely to develop resistance to cabazitaxel than to docetaxel [34, 38-41]. The ATP-binding cassette (ABC) 4 transporter in prostate cancer has been seen to cause resistance to docetaxel, but not cabazitaxel, and is therefore one mechanism by which the cell responds differently to the similar compounds [41]. The ABC transporter family are cellular transporters that expel toxic compounds from the cell. ABC transporters have low specificity for substrate and therefore are able to expel many different chemotherapeutics from the cell, making them resistant [42].

These compounds will be interesting to study because one compound is more sensitive to resistance than the other, and modeling them together will reveal if the model can differentiate a combination that is more effective due to lower resistance. Cabazitaxel is eventually metabolized to docetaxel, and therefore it will be interesting to view them together in our model, as well as the Chou-Talalay model, to see if the compounds work together synergistically due to resistance or additively due to metabolism.
Mitoxantrone:

Mitoxantrone is a DNA intercalating agent, and an inhibitor of topoisomerase II [43]. Mitoxantrone binds to double stranded DNA to inhibit DNA replication machinery from opening and copying DNA. Stalling of replication machinery makes it impossible for the cell to go through the cell cycle [44-46]. However, mitoxantrone binds to DNA via electrostatic interactions, unlike other intercalating agents such as doxorubicin, and causes double strand breaks. This mechanisms also explains why mitoxantrone resistance is not correlated with doxorubicin resistance [47]. Topoisomerase II is an enzyme that hydrolyzes ATP to cut and reattach both strands of DNA to relieve supercoiling during DNA replication. Inhibition of topoisomerase II leads to inhibition of DNA repair, and replication. Higher concentrations of mitoxantrone are needed in order to inhibit Topoisomerase II in breast cancer and other cell culture cancer models [45, 46]. Apart from typical side effects of chemotherapy, mitoxantrone can be very damaging to cardiac muscle, and is usually administered with cardiac monitoring [48, 49].
Resistance to mitoxantrone is caused by the mitoxantrone resistance-associated protein, part of the ABC transporter family [50-52]. Apart from removing mitoxantrone from the cell, resistant cells reduce topoisomerase II activity, as well as reduce its expression [53].

**Putative Chemotherapeutics**

Resveratrol:

![Resveratrol structure](image)

*Resveratrol*

Resveratrol is a naturally occurring stilbene found in grapes, cocoa and nuts that has been studied in many cancers [54]. Resveratrol activates apoptosis through many pathways while having low toxicity in healthy tissues [55]. However, resveratrol has been limited in its effectiveness because of fast metabolism which leads to low bioavailability [56].

Resveratrol has been seen to induce a calcium signal in both breast cancer [57], and prostate cancer (research from our lab that is not currently published) by inhibiting activity of the plasma membrane calcium ATPase (PMCA). PMCA is a calcium transporter that uses ATP to expel calcium from the cytosol against the electrochemical gradient. Inhibiting PMCA leads to high levels of calcium inside the cell, which can signal apoptosis. The calcium signal of resveratrol has been shown to be in part responsible for the apoptotic effect of resveratrol [58]. However, the mechanism by which the calcium signal activates apoptosis remains undiscovered, but it is related to activation of calcium dependent proteases (calpains) [59].
Resveratrol has also been shown to increase levels of p53 [60], a tumor suppressor that activates DNA repair proteins, and stops the cell cycle in order for repairs to take place. Mutations in the tumor suppressor p53 gene are the most common mutations in cancers, perhaps due to the ability of p53 to halt the cell cycle, and signal for apoptosis when DNA damage is irreparable [61].

Resveratrol also creates reactive oxygen species in the presence of copper, which is stored in higher levels in cancer cells, and used to increase BRAF signaling in aggressive cancers [62, 63]. Mutations in BRAF are common in aggressive forms of cancer, and cause an increase in signaling pathways that drive cell division and differentiation. The presence of copper in the cancer cell allows resveratrol to create hydroxyl radicals, which can cause DNA damage, and lead to cell death [64].

Flavopiridol:

Flavopiridol is a semisynthetic flavonoid derived from rohitukine, and has undergone numerous clinical trials for treating cancer. Rohitukine was originally extracted from the Pithraj (rohituka) tree (*Dysoxylum binectariferum*) from India and the surrounding area. Rohitukine has been used to treat rheumatoid arthritis, but much of the research focused on its anti-tumor
activity [65]. Flavopiridol was identified by the National Cancer Institute during a screening for compounds that inhibit the epithelial growth factor receptor tyrosine phosphokinase, and was seen to halt cell cycle progression in the G1 and G2/M phases by inhibiting cyclin-dependent kinases (CDK) by competing with ATP in the binding site. Cyclin-dependent kinases are phosphorylating proteins that are active when bound to cyclins. Cyclins are a family of proteins that are expressed at different stages of the cell cycle. When the CDKs bid to cyclins, they allow progression in the cycle by activating processes such as microtubule formation, and chromatin formation. Further research found that flavopiridol is a potent inhibitor of CDK1, CDK2, CDK4, and CDK7, which makes it a unique compound for being the only compound yet known that potently and specifically inhibits nearly all CDKs [66]. Flavopiridol may also induce apoptosis in some cell types, and has similar activity to standard cytotoxic drugs, but is superior to them at least in prostate carcinoma [66]. In PC-3 human prostate cancer cells, an aggressive form of prostate cancer, flavopiridol exhibited significant down-regulation of Bcl-2, a key regulator of apoptosis, which further highlights the chemotherapeutic potential of flavopiridol [67].

In early clinical trials, clinical effects of flavopiridol were obtained, with some minor toxicities, but anti-tumor effects were disappointing [68]. The results from single drug treatment trials in androgen-independent prostate cancer were also disappointing, but significant changes in clinical markers indicated that combination treatments might increase the anti-tumor activity of flavopiridol [69]. Approaching the problem from a pharmacokinetic perspective, Byrd et. al. were able to increase the efficacy of flavopiridol by using a carefully calculated administration schedule to maintain plasma concentrations over time. The results were especially promising for those with genetically high-risk chronic lymphocytic leukemia [70]. By modeling combination chemotherapy in lung cancer cells, Bible and Kaufmann, discovered that a scheduled regimen
improved the effectiveness of flavopiridol. Treating cells with a standard chemotherapeutic, and following that treatment with flavopiridol 24 hours later significantly reduced cell viability, and gave a more favorable combination index (more synergistic combination) using the median effect method [71]. Following this model of pretreatment with a standard chemotherapeutic (paclitaxel), followed by flavopiridol exhibited clinical activity in patients with advanced esophagus, lung, and prostate cancer solid tumors [72].

The poor performance of flavopiridol in clinical trials is often blamed on the low availability in the tumor environment due to binding of flavopiridol to blood serum proteins. However, current research compared flavopiridol binding to fetal bovine serum proteins, and found that flavopiridol does not have a high-affinity for blood serum proteins, and therefore new models should be developed that may improve the clinical performance of flavopiridol [73].

Piperlongumine:

![Piperlongumine structure](image)

_Piperlongumine_

Piperlongumine, also known as piplartine, is a naturally occurring compound from the Long pepper, a native plant in southeast Asia and southern India [74]. It has been identified as a putative chemotherapeutic [75], and shows promise in selectively killing cancer cells over normal/non-tumorigenic cells [76]. The ability of piperlongumine to kill cancer cells has been attributed to several mechanisms such as inhibition of STAT3, NF-κB, and production of
reactive oxygen species [77-80]. Signal transducer and activator of transcription (STAT) 3 is a transcription factor that has been identified as a possible target for cancer therapies for its role in controlling cell growth and apoptosis. Nuclear factor kappa-light-chain-enhancer (NF-κB) is a protein complex that controls transcription of genes involved in cell survival. Interestingly, piperlongumine is a known silencer of the GSTP1 gene (encodes the glutathione S-transferase P protein which removes toxins from the cell by binding them to reduced glutathione) which plays an important role in driving cell metabolism to create energy and materials needed for cell division. GSTP1 has been identified as a drug target in triple negative breast cancer [81].

Piperlongumine may be interesting in combinations because of its ability to silence GSTP1 which would reduce the ability of a cancer cell to complex drugs with reduced glutathione and allowing the drugs to be removed from the cell. However, most the research of GSTP1 and piperlongumine has been done in breast cancer, and the gene may be less necessary in advanced forms of prostate cancer, therefore limiting the effectiveness of piperlongumine.

All chemical structures were created using molview.org.

References:
[4] PDQ Prostate Cancer Treatment, in: PDQ Adult Treatment Editorial Board, National Cancer Institute, Bethesda, MD.


resistant prostate cancer is independent of the presence of AR-V7 in circulating tumor cells, European urology, 68 (2015) 939-945.


[71] K.C. Bible, S.H. Kaufmann, Cytotoxic synergy between flavopiridol (NSC 649890, L86-8275) and various antineoplastic agents: the importance of sequence of administration, Cancer research, 57 (1997) 3375-3380.


Appendix B
Plate Reader Protocols

1. Log onto the computer:
   a. Username: Lab User
   b. Password: davidson

2. Open the “FLUOstar OPTIMA” icon located on the desktop

3. Under “User” select User, and then select “Run” on the bottom of the window

4. Select the “Plate out” Button, insert your plate, and then select the “Plate in” button.

5. Select the “Setup” drop down menu, and select “Reader Configuration”

6. Make sure that “Fluorescence Intensity and Time-Resolved Fluorescence” is selected, then select “ok”

7. Make sure that you have the right filters on by opening the top of the machine and rotating the Emission filter to blue, and the Excitation filter to yellow.

8. Select the “Test Setup” drop down menu and select “Test Protocol”
Test Protocol

1. Double click on the desired test protocol
2. Select the “Layout” tab at the top of the window
3. You may clear the previous layout by selecting a new layout over them, or by selecting the wells and then pressing delete
4. If you need to adjust the gain you can do so by selecting the multichromatic tab, and adjust the gains under the “gain” column
5. Press “ok” at the bottom of the window when your protocol is set correctly
6. Press the “Measure” button (the green street light)
7. Select the prepared Protocol
8. Name the experiment, and select “Start test run”
9. Once the protocol is finished you may view the results by pressing the button that looks like an excel spreadsheet
10. This will open up an excel spreadsheet and your experiment should be located at the top of the list. Double-click on the experiment and it will show you the results.

<table>
<thead>
<tr>
<th>Date</th>
<th>Test Name</th>
<th>Id</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>FURA-2</td>
<td>RO Fura</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FURA-2 W/PUMP</td>
<td>RO-10NO091614</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FURA-2</td>
<td>Test091214</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FOLIC ACID</td>
<td>CAP-RR691014P1R2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FOLIC ACID</td>
<td>CAP-RR691014P1R1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FOLIC ACID</td>
<td>CAP-VV896814P2R1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

11. Copy the results onto a new document, and evaluate them as desired.

Setting the Incubator

1. Press the button the main screen that looks like a thermometer
2. Make sure that the incubator is set to 37 degrees Celsius
3. Select the “Incubator On” button and close the window

Using the Pump

1. The solution you want to pump into the solution will need to be in a 15mL falcon tube
2. In order to set the pump, you must click on the button that shows an Erlenmeyer flask with a tube in it
3. The pump will be filled with distilled water, and you will need to backflush it by selecting “backflush” and by pressing the “prime pump 1 button.” When you are sure the distilled water has been removed from the pump you may proceed
4. Place your solution into the top of the machine, and place the input tube (A small tube pushed through a small whole of a falcon tube cap) in your solution and secure the cap tightly.

5. Place the needle cap (a falcon tube cap with a larger hole) on an empty 15mL falcon tube (labeled with the name of your solution as well) and insert needle into the tube.

6. Select “Prime” and adjust the prime volume to the desired volume (1000mL is suggested, 500mL for expensive reagents)

7. Once the pump is primed you need to insert the needle into the pump 1 hole which is the hole closer to the back of the machine.

8. To run the experiment, it must be an experiment that is set up to use the pump

9. Open the desired test protocol and select the “Concentrations/ Volumes/ Shaking” tab
10. On the right-hand side of the window under volume 1 you may input the desired volume of injection
   a. Make sure that the wells you want to inject are numbered properly, and that the desired injection coincides with that well.

11. You may now run the experiment

Cleaning the Pump

1. You will first need to backflush your solution out of the pump by selecting “backflush” and then by pressing “prime pump 1” in the pump control window

2. When your solution is removed you must then prime the pump with distilled water.
   Prime the pump 2 or three times in order to clean it out

3. Leave the distilled water in the pump, and place the needle in the resting area

Cell Culture Protocols

Freezing Down Cells:

1. Prepare at least five cryogenic vials (label with your initials, date, cell type, and passage number)

2. Trypsinize cells (standard protocol).
   a. Remove media
   b. Rinse with PBS
   c. Add Trypsin (2 mL for t-75… other values)
   d. Put into 37°C incubator for 5 min
3. Remove from incubator. Add PBS and transfer to 15 mL falcon tube. Invert once or twice to mix. Take 10 uL from solution for count on hemocytometer, centrifuge falcon tube at 1000 RPM 5 min (count cells during centrifuge)
   a. During centrifuge count with hemocytometer

4. After centrifuge, remove from centrifuge and use vacuum inside hood to decant the supernatant. Be sure not to disrupt the pellet of cells

5. Re-suspend pellet of cells with media
   a. Your goal is to get the cells to about 1 million cells per mL of media. Do calculations to get in that area.

6. Transfer one million cells in solution (preferably around 1 mL of solution) per cryogenic tube

7. Add 5% DMSO to each tube (50 uL/mL)

8. Quickly close cryogenic tube and put into -80° freezer overnight

9. Transfer vials to N₂ tank for indefinite storage

**Thawing Cells:**

1. Place media in water bath to warm up

2. Remove cells from the liquid nitrogen and put IMMEDIATELY on ice (you want to be quick with all of this. The cells are stored in DMSO, which is toxic to the cells. Be as quick as you can to get the DMSO washed out quickly)
   a. The liquid nitrogen container is stored in the northeast corner of Dr. Tessem’s lab. Once the cap is removed from the tank, you will see four handles. Our box is stored in the orange handle.

3. Put 10 mL of warmed up media into a 15 mL falcon tube
4. Warm sample quickly (with hands). As sample is partially melted, pipet some of the media in the falcon tube into the cryogenic vial to speed the thawing process.

5. Quickly transfer the thawed sample into the 10 mL media in the falcon tube and centrifuge for 5 minutes.

6. Discard the supernatant.

7. Add 3 mL media to the pellet and mix well.

8. Plate on a t-25. Check that everything looks good under the microscope.

Feeding Cells:

1. Remove media from the fridge and place it in the water bath.

2. When the media is warm remove it from the water bath, dry it, spray it with ethanol and wipe it down then place it in the hood.

3. Using the pump and an autoclaved pasteur pipette, aspirate off the media in your flask.

4. Using the pipetman and a pipette, remove media from your bottle and pipette it into your flask (T-25 uses 3.33 mL of media, T-75 uses 10 mL of media, T-175 uses 20 mL of media).

5. Cap the flask and place it back in the incubator.

6. Put the media back into the fridge and put the pipette and pasteur pipette into the biohazard disposal.

Trypsinizing Cells:

1. Remove media and the trypsin from the fridge and place them in the water bath.

2. When they are warm remove it from the water bath, dry it, spray it with ethanol and wipe it down then place it in the hood.

3. Aspirate off the media from your cells, wash with PBS, and aspirate it off.
4. Pipette the trypsin onto your cells (T-25 uses 667 uL of Trypsin, T-75 uses 2 mL of Trypsin, T-175 uses 4 mL of Trypsin)

5. Place the trypsinized cells in the incubator for 5 minutes.

6. Remove the cells from the incubator and remove the cells from the flask by pipetting in the appropriate amount of media/PBS (T-25 uses 3.33 mL of media, T-75 uses 10 mL of media, T-175 uses 20 mL of media).

7. Remove 10uL of cell solution to count your cells

8. Place the PBS/trypsin/cell solution into a falcon tube and centrifuge the solution for 5 minutes (Be sure to balance the centrifuge).

9. Aspirate off the supernatant and resuspend the pellet in the necessary amount of media by pipetting the media up and down at least 10 times.

10. Count the cells using the hemocytometer and record your results in the cell count book, and in your lab notebook.

11. Plate out your cells at your desired concentration for your experiments (be sure to mix the solution every 3-6 wells to ensure an even suspension of cells).

12. Place your cells into a new flask, fill it with media, then write the cell type, your passage number and your name on the flask and place it in the incubator (T-25 needs 333,000 cells, T-75 needs 1,000,000 cells, T-175 needs 2,000,000 cells).

AlamarBlue Protocol

1. After cells have been plated and treated the Alamar blue reagent can be used to measure cell viability. When plating cells for this assay, you should leave three wells of cells for each treatment, as well as three wells that will contain the treatment and the media, but will not
contain any cells. These wells will serve as the control to see if the treatment will react with the reagent. Be sure to do this for every treatment, and every concentration of treatment.

2. After the treatment incubation time has finished, add Alamar Blue to every well (3 wells of cells + treatment, and 3 wells of treatments without cells). The amount of alamar blue that should be added to each well should be 1/10 the volume of the well (ex. 10uL of alamar blue for 100uL of treatment).

3. Incubate the alamar blue treated plates at 37°C for 6 hours (incubation may vary based on cell type and cell number).

4. After the six-hour incubation, the plate can be read on the plate reader with the alamar blue program. Be sure to change the machine setup, as well as the hoses in the machine.

5. Analyze the data by averaging the three wells with cells, and subtract the average of the three wells without cells. These can then be compared to a control to determine cell viability.

**Cell Cycle Analysis Protocol (Propidium Iodide Staining for Flow Cytometry)**

This protocol was written by Jordan Hastings and was used with his consent.

1. Plate out your cells in a 6 or 12 well plate
   a. To determine how many to plate out know that you want at least 50k cells for analysis at the end of treatment i.e., if you treat with resveratrol for 24hr at the IC_{50} you should plate out at least 50k cells per well (which will double before being cut in half)

2. After cells adhere (generally 24 hr) remove media and add media + treatment
   a. Total volume should be 500 uL for 12 well, 1mL for 6 well

3. Wait the desired amount of time

4. Trypsinize each well and put cells in their own falcon tube
5. Wash each well with PBS to remove any remaining cells and add them to the well specific tube

6. Add 500µL 70% ethanol in purified water dropwise to each tube while vortexing

7. Let cells sit for 3+ hours at 4°C

8. Pellet cells down and CAREFULLY remove the ethanol

9. CAREFULLY wash with PBS
   a. if you suck up your cells here there will be nothing to analyze

10. Add 200 µL PI
    a. PI is made at 50 µg/ ml in 1x PBS and kept in the fridge for up to a month

11. Add 1.3 mL PBS and vortex gently.

12. Transfer the contents of each falcon tube to a 1.5mL Eppendorf tube
    a. Make sure to label your tubes

13. Run the flow cytometer for 50k counts

**JMP 12 Protocol**

1. From the JMP home screen, select the design of experiments (DOE) tab, and select mixture design from the dropdown list.

2. From the new window, you can name your response, and the compounds you are using.
   To add more than the standard three compounds, enter the number of compounds you would like to add in the text box under the factors subheading on the page, and press “add.”

3. Select “Continue” at the bottom of the page, and then select “ABCD Mixture Design” from the new selectable options.

4. At the bottom of the page enter 2 in the number box in order to have 3 total sets of data.
5. Select “Keep the Same” under “Run Order,” then select “Make Table.”

6. Insert data into the table, label the three compounds appropriately, as well as the response being used to create the data.

7. Select “Fit Model” under the “Analyze” tab, and select the response for the Y value. Then select all three compounds together, and select “Mixture Response Surface” from the “Macro” tab. Select “Run Analysis.”

8. Using the first red arrow on the results page, select “Save Prediction Formula,” under the “Save Columns” option.

9. Using the same red arrow, select “Factor Profiling,” and then select “Profiler” from the drop-down list.

10. Scroll down to the profiler and, using the red arrow over that section, select “Maximize Desirability.” The profiler will then give you the ideal combination.

11. Return to the data table, and select the “Graph” tab. Then select “Ternary Plot.”

12. Input the three compounds under the X, and under “Contour Formula” select the saved prediction formula column and then select “OK.”

13. Using the red arrow on the left, select “Contour Fill,” and then “Fill Below.” The graph can then be used.

JMP 13 Protocol

1. From the JMP home screen, select the design of experiments (DOE) tab, then select classical and then select mixture design from the dropdown list.

2. From the new window, you can name your response, and the compounds you are using. To add more than the standard three compounds, enter the number of compounds you
would like to add in the text box under the factors subheading on the page, and press “add.”

3. Select “Continue” at the bottom of the page, and then select “ABCD Mixture Design” from the new selectable options.

4. At the bottom of the page enter 2 in the number box in order to have 3 total sets of data.

5. Select “Keep the Same” under “Run Order,” then select “Make Table.”

6. Insert data into the table, label the three compounds appropriately, as well as the response being used to create the data (if you have not already done so).

7. Select “Fit Model” under the “Analyze” tab. If the roles have not already been filled in, select the response for the Y value, then select all three compounds together, and select “Mixture Response Surface” from the “Macro” tab. Select “Run.”

8. Using the first red arrow on the results page (top left), select “Save Columns,” and select the “Prediction Formula” option.

9. If the “Prediction Profiler” is not open at the bottom of the screen, using the same red arrow as the previous step, select “Factor Profiling,” and then select “Profiler” from the drop-down list.

10. Using the same red arrow, select “Regression Reports” and select “Summary of Fit,” “Analysis of Variance,” and “Lack of Fit.”

11. Scroll down to the profiler and, using the red arrow over that section, select “Optimization and Desirability” then select “Maximize Desirability.” The profiler will then give you the ideal combination.
12. You can then hold down the “s” key on the keyboard and click on the profiler to highlight and copy it out into a word document. Copy the “Summary of Fit,” and “Analysis of Variance,” and “Lack of Fit” tables as well.

13. Return to the data table, and select the “Graph” tab. Then select “Ternary Plot.”

14. Input the three compounds under the X, and under “Contour Formula” select the saved prediction formula column and then select “OK.”

15. Using the red arrow on the left, select “Contour Fill,” and then “Fill Below.” The graph can then be used. You can adjust the size of the contours by entering new values into the text boxes on the right-hand side of the window.

16. You can then hold down the “s” key on the keyboard and click on the graph to highlight and copy it out into a word document.