Title
Feedback System Control: optimizing drug combinations for tuberculosis treatment

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Feedback System Control: optimizing drug combinations for tuberculosis treatment

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in Mechanical Engineering

by

Aleidy Marlene Silva Vite

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ABSTRACT OF THE DISSERTATION

Over the past years, numerous reports have surfaced demonstrating the outstanding superiority of combinatorial therapies over single drug treatments, one such example was the successful treatment of the human immunodeficiency virus with a combination therapy. The main problem faced when designing a multi-drug therapy is that combining a set of drugs at different possible concentrations yields a large testing parametric space, and thus the search of an optimal combination becomes a major challenge. To solve this issue, the Feedback System Control (FSC) optimization scheme has emerged as a better alternative for achieving a therapeutic goal when compared to the typical trial and error methods; FSC's primary advantage is its ability to circumvent the need for detailed information of the cellular functions of the system of interest. It has been demonstrated that only tens of iterations out of a large number of possible combinations are needed to achieve a desired response, as opposed to testing the entire search space. This effort-saving approach actively manipulates the complex biological systems as a whole, rather than controlling the system's individual intrinsic pathways.

To further exploit the capabilities of this platform, FSC has now taken advantage of the benefits offered by multivariable experimental designs such as orthogonal array composite designs; these designs are intended to draw valid correlation conclusions from an experimental data set while further minimizing the number of tests performed. In the context of FSC, they provide the initial conditions to be tested, which facilitate the development of quadratic models describing the relationship between the drug combinations and their efficacies with a reliable statistic correlation. This method is known as FSC.II.

In this project, the FSC.II methodology was used to find a drug combination for tuberculosis treatment. In six iterations, several three and four drug combinations were found to be superior to the standard regimen, which represented a drastic decrease in the number of experiments needed to find the optimal combinations for inhibiting tuberculosis infection on cell

based assays. The results obtained were then verified through a colony forming unit cell based assay to verify tuberculosis killing.

These results will provide a basis of drug combinations to be tested on an animal model, where only a small number of subjects will be needed to find the optimal drug combination. Furthermore, future efforts will focus on using the FSC scheme to model the drug combination efficacy as a temporal function of a drug combination, which would allow the optimization of a drug combination efficacy over time on a single individual subject; this method would be suitable for both animal and human clinical tests and will an outstanding step towards personalized medicine.

The dissertation of Aleidy Marlene Silva Vite is approved.

Yong Chen

Laurent Pilon

Michael Teitell

Chih-Ming Ho, Committee Chair

University of California, Los Angeles

2014

To my parents,

For their endless love and unconditional support.

Feedback System Control: optimizing drug combinations

for tuberculosis treatment

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Acknowledgments

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VITA

Aleidy Silva obtained her bachelor's degree in Biomedical Engineering (2007) at the Instituto Tecnológico y de Estudios Superiores de Monterrey (ITESM), Mexico. While pursuing her degree, she demonstrated an interest in research and development and promptly joined the Electrical Engineering Department, where she worked on the design of micro cantilevers for glucose detection.

During her undergraduate studies, she was selected to participate in the summer research program for undergraduate research at UCLA, where she first got involved in the research carried out at Prof. Chih-Ming Ho's laboratories. She was sponsored by the Institute for Cell Mimetic Space Exploration (CMISE) in 2006 and by the Center for Scalable and Integrated Nano Manufacturing (SINAM) in 2007; during both years she assisted in the characterization of electrokinetic micro-focusers used to manipulate nano particles.

After her bachelor's completion, she worked as a research assistant at the Centro de Investigación y Estudios Avanzados (CINVESTAV), Mexico. She later obtained the UC Mexus – CONACYT Doctoral Fellowship to continue her graduate studies and finally joined Prof. Chih-Ming Ho's research group in 2009, where two years later obtained her Master's Degree in Mechanical Engineering.

Her current research interests lie in driving biological systems into desired states by optimizing given inputs. Her interdisciplinary research combining systems biology, targeted therapeutics, mathematics and engineering will provide new insights into disease and drug resistance development and treatment.

Feedback System Control: Optimizing drug combinations for tuberculosis treatment

I. Introduction

I.I. Motivation

I.I.I. Tuberculosis facts

Tuberculosis (TB) is an ancient disease that has plagued humankind and claimed victims since prehistory, and it reached epidemic proportions in Europe and North America during the 18th and 19th centuries.

The study of TB began in the 19th century with the work of Théophile Laennec, and it culminated with the identification of the tubercle bacillus as the infectious etiologic agent by Robert Koch in 1882 [1]. The tuberculin skin test was not developed until 1907, and its utility was demonstrated three years later as a detection tool for latent tuberculous infection in asymptomatic children [2]. In the late 19th and early 20th centuries, there was a surge of sanatoria that were solely dedicated to the treatment of patients infected with TB. Vaccination against the disease was widely used following World War I, and the modern era of TB treatment started with the discovery of streptomycin in 1944 [3].

Currently, TB remains a major global public health concern. It is the worldwide second leading cause of death from an infectious disease after the human immunodeficiency virus

(HIV). In 2012 only, an estimated of 8.6 million people developed TB, and 1.3 million died from the disease. [4].

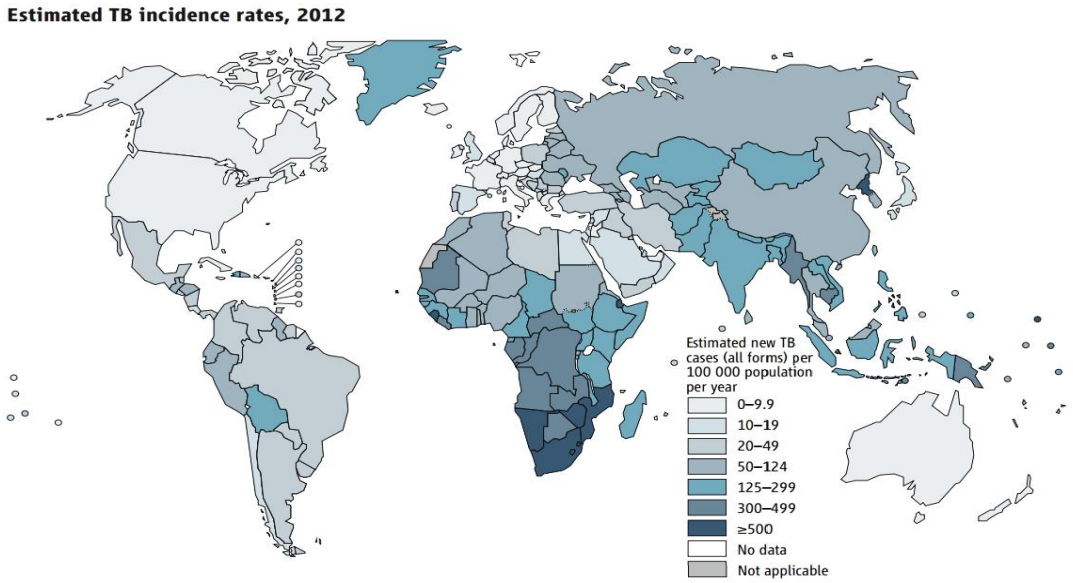


Fig. 1. Estimated TB incidence rates by country, 2012. Reproduced with permission, from *Global Tuberculosis Report 2013,* World Health Organization [4].

TB is highly contagious, since it spreads from person to person through the air. When people with lung TB cough, sneeze or spit, they propel the TB germs into the air; a person needs to inhale only a few of these germs to become infected. Once it becomes active, TB attacks the respiratory system and other organs and destroys body tissue [5].

The control of TB is complicated by the fact that about a third of the world's population has latent TB, that is, they are infected with *Mycobacterium tuberculosis* (MTB), the causative pathogen of TB, but are asymptomatic. MTB's unique cell wall, which has a waxy coating primarily composed of mycolic acids, allows the bacillus to lie dormant for many years [6]. The body's immune system may restrain the disease, but it does not fully clear it. While some people

with this latent infection will never develop active TB, about 10% of those latently infected eventually develop the active disease during their lifetimes [5].

Without treatment, TB mortality rate is high. Effective drug treatments were first developed in the 1940s, and the most effective first line drug against TB, rifampicin, became available in the 1960s [3].

Currently, drug-susceptible TB is treated with a six month regimen of four first line drugs, isonazid, rifampicin, ethambutol and pyrazinamide; whereas multidrug resistant TB (MDR-TB), which is defined as resistance to isonazid and rifampicin, the two most powerful first-line drugs, requires more expensive and more toxic drugs. Second-line treatment options are limited, not always available, and quite lengthy: The World Heath Organization (WHO) recommends twenty months of treatment, and extensive chemotherapy is often required (up to two years).

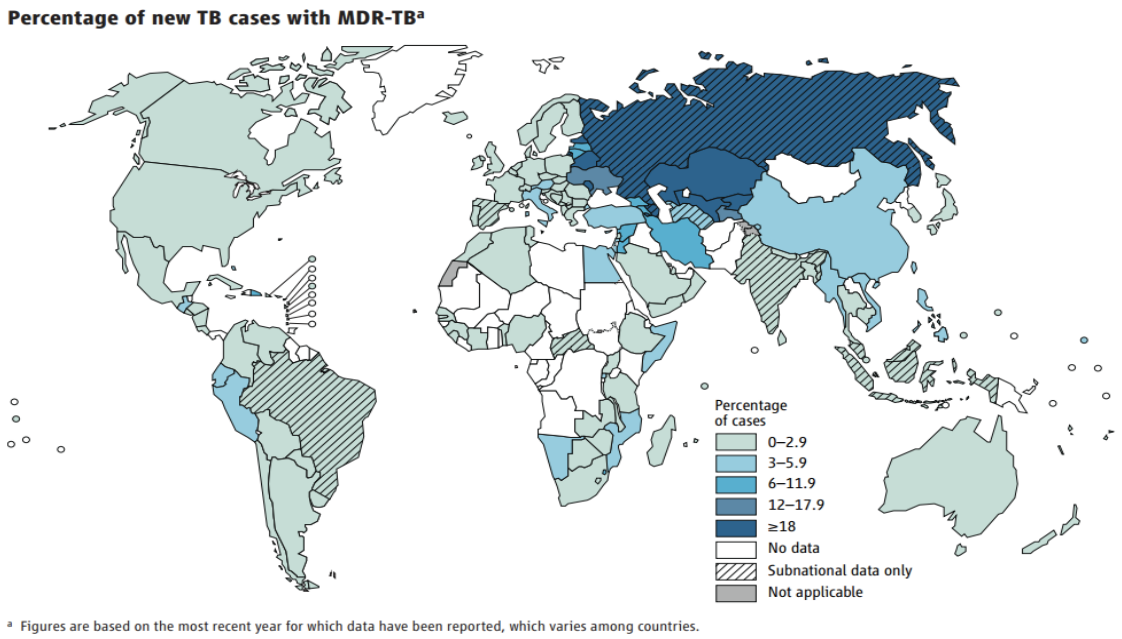


Fig. 2. Percentage of new TB cases with MDR-TB by country, 2012. Reproduced with permission, *Global Tuberculosis Report 2013* , World Health Organization [4].

The treatment of MDR-TB is that much more complex, expensive, and toxic that about a third of all the patients infected with MDR-TB die [8].

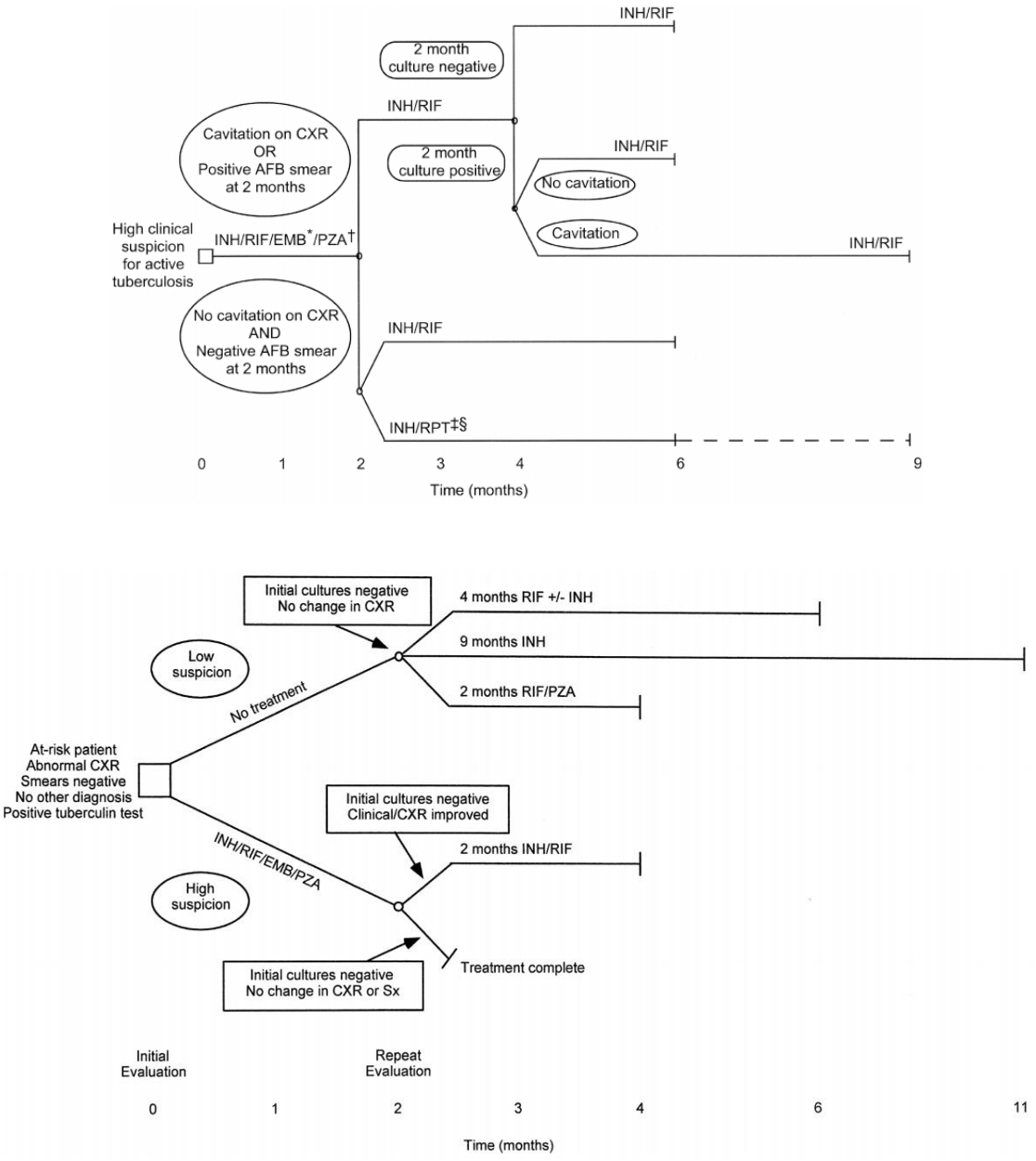


Fig. 3. TB treatment is complex and depends on several variables. Reproduced, *Treatment of tuberculosis, MMWR Recommendations and reports,* Centers for Disease Control and Prevention [7].

The most common causes of MDR-TB are inappropriate treatment, incorrect use of anti-TB drugs, or use of poor quality medicines. In 2012, an estimated 450,000 people developed MDR-TB, and there were 170,000 deaths associated with it [4]. The high complexity and prohibitive cost of the MDR-TB treatment means that less than a fifth of all the MDR-TB patients receive the proper treatment. Without a significantly simpler, faster, cheaper oral treatment for MDR-TB, it is no possible to scale up treatment for the increasing demand. The WHO has issued a target to treat 80% of the MDR-TB cases by 2015, but with the current state of the art this target is not feasible [8].

Recently, a new extensively drug-resistant TB (XDR-TB) strain, also known as extremely drug-resistant TB has emerged. It has been reported by ninety-two countries; on average, 9.6% of MDR-TB cases are XDR-TB. These new strains respond to even fewer available medicines: XTDR-TB is resistant to fluoroquinolone, isoniazid and rifampin, and to at least one of the three injectable second line drugs (capreomycin, kanamycin, amikacin) [8,9].

Fig. 4. Countries that had notified at least one case of XDR-TB, 2012. Reproduced with permission, *Global Tuberculosis Report 2013* , World Health Organization [4].

I.I.II. Goals and challenges for tuberculosis treatment development

Over the past decade TB drug, research and development has experienced a surge powered by an urgent need to control the disease and come up with new, more effective treatments for both drug sensitive and drug resistant TB strains. As a result, there are several products in clinical development, and a large pipeline of early stage compounds aimed to improve efficacy, safety, and shortening if the duration of the treatment for both drug sensitive and drug resistant strains, as well as for latent TB infections [10].

Pharmaceutical companies continue to search for effective treatments for TB, as many strains of the bacteria have developed a resistance to first-line drugs currently used to treat the disease. Moreover, identifying drugs that will shorten treatment and thereby improve adherence is one of the most important goals in improving TB treatment. Ideally, finding such drugs would be based on the understanding of the mechanisms of mycobacterial action; however, there are several challenges that have yet to be addressed to fully achieve these goals.

Currently, both a clear understanding of persistence mechanisms and fully validated animal models the reliably predict human treatment duration are lacking. The mouse model appears to reflect human treatment results but not in all circumstances, and it lacks adequate prospective data to be considered truly validated at this time. Without a fundamental understanding of the TB mechanisms for latency, shortening the therapy to days rather than months is not a feasible goal in the near future; it is realistic, however, to pursue a reduction of the treatment to three to four months even with combinations of the already available and currently in development drugs [10].

A second challenge for TB drug development is the very long timeline of clinical trials. Phase 2 studies for TB drugs usually take at least two years, and pivotal studies require a

minimum of three years. On top of this, the requirement for multidrug therapy adds an additional layer of complexity; since a few weeks of single drug use may lead to the development of drug resistance, it is not allowed to test single drugs beyond Early Bacterial Activity (EBA) studies. Additionally, current therapy should not be withheld for any longer than necessary, thus experimental research on patients is quite limited [10].

Moreover, since people must be treated with a combination of four drugs according to the standard treatment, new drugs should be tested as a single drug replacing one of the components of a current regimen, and this replacement has to be done one at a time. This method would require not a minimum of 6 years, but a minimum of 6 years times four, that is, over two decades just to finalize clinical trials [10].

Added to all these limitations, the spread of MDR-TB and the appearance of XDR-TB pose new challenges for the prevention, treatment, and control of this deadly disease.

I.II. Theoretical Background

I.II.I. The importance of drug combinations

I.II.I.I. A case of success: the human immunodeficiency virus therapy

On June 5th of 1981, the Centers for Disease Control and Prevention (CDC) released a report of five cases of *Pneumocystis carinii* pneumonia in previously healthy young men located in Los Angeles, California; by then, two of them had already died [11]. This report would later be acknowledged as the first published scientific account of what in 1984 would be known as HIV, the causative agent of the Acquired Immune Deficiency Syndrome (AIDS) pandemic [12]. As the death rate kept increasing, several single drugs were used as treatment, but none of them

provided significant results. It wasn't till 1995 that a new breakthrough was achieved: on December 6th, the Food and Drug Administration (FDA) approved the first protease inhibitor Invirase. Protease inhibitors could be used in combination with one or two of the reverse transcriptase inhibitors (NRTIs), this was known as Highly Active Antiretroviral Therapy (HAART) [13]. This new multidrug therapy became widely available in 1996 [14], and it was responsible of a rapid decline in the death rate. During that year, the CDC documented the first overall decline in the annual incidence of AIDS in the United States; the death rate declined 23% within a year, and this trend continued for the following years [15].

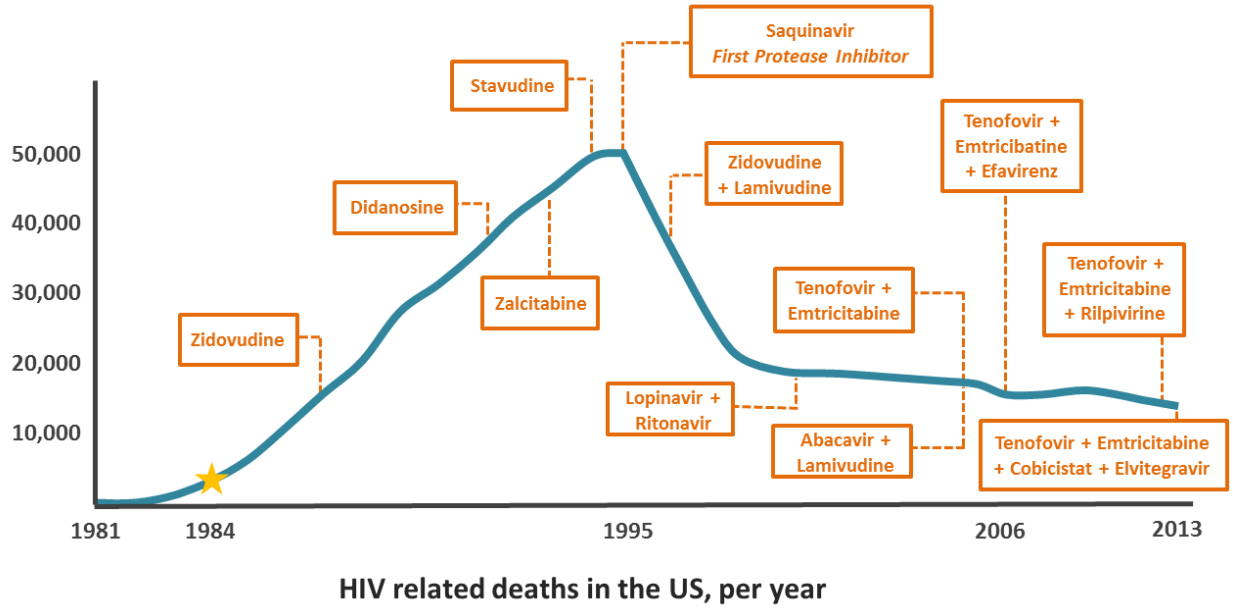


Fig. 5. HIV related deaths in the US, per year. The timeline shows single drugs and drug combinations as they were released [11, 12, 14, 15].

The HIV case is a prime example of how drug combinations can change the course of a pandemic disease. The use of multiple drugs presents numerous advantages over single drug

therapies: drug combinations may be helpful to attack multiple and different targets within a bionetwork, facilitate treatment for heterogeneous populations, or provide a deal with multiple diseases simultaneously [16].

Moreover, the use of drug combinations possesses another important advantage over single that of drug therapy. Monotherapies often lead to disease recurrence and subsequent ineffectiveness of standard treatment due to drug resistance development. Drug resistance can develop by several mechanisms: decreased drug uptake, increased drug efflux, activation of detoxifying systems, activation of DNA repair mechanisms, and evasion of drug-induced apoptosis, among others [17]. Thus, in order to overcome drug resistance, the drug treatment should consist of several drugs simultaneously attacking these mechanisms necessary for the disease progression.

Typically, multicomponent therapies have one or more of the following goals [18]:

- Reduce the frequency at which developed drug resistance arises by combining drugs with minimal cross-resistance, such that emergent resistance requires acquisition of multiple mutations in a rapid fashion.

- Diminish drug doses with non-overlapping toxicity and similar therapeutic profile so as to achieve efficacy with fewer side effects.

- Cell sensitizing to the action of a drug through the use of another drug (chemosenstization) or radiation (radiosenstization), usually by altering the cell cycle.

- Achieve enhanced potency by drug additive effects

Multicomponent drugs are now the standard treatment for multiple diseases, but their development has required arduous empirical testing. The design of such therapies is quite

challenging since the interactions between drugs are not well understood, as the crossover between the affected cellular pathways is quite difficult to comprehend. Besides the inherent complexity of all bio systems, their inner networks dynamically connect and disconnect on a frequent basis. In addition to all these challenges, the response to stimuli of a diseased cell is quite different from a healthy cell; thus, predicting the therapeutic effect of a drug combination is dauntingly complicated.

I.II.I.II. Drug combination characterization techniques

Currently, there are two main techniques used to characterize drug combination performance as compared to monotherapy: the Loewe additivity model and the Bliss independence model [18];

The Loewe additivity model assumes that two drugs act through a similar mechanism by calculating a combination index. According to the theory, the combined effects of two drugs, D_1 and D_2 can be calculated as [19]:

$$\frac{(f_a)_{1,2}}{(f_u)_{1,2}} = \frac{(f_a)_1}{(f_u)_1} + \frac{(f_a)_2}{(f_u)_2} = \frac{(D)_1}{(D_m)_1} + \frac{(D)_2}{(D_m)_2} \quad (1)$$

Where f_a is the fraction affected by D, f_u is the fraction not being affected by D, and D_m is the half maximal effective concentration (EC50) value of D. Since $\log(f_a/f_u) = \log(D/D_m) = m\log D - m\log D_m$, a plot of $y = \log(f_a/f_u)$ with respect to $x = \log(D)$ will be linear with a slope m [19]. If the slope of the plot generated by these equations is not 1, then:

$$\left[\frac{(f_a)_{1,2}}{(f_u)_{1,2}}\right]^{\frac{1}{m}} = \left[\frac{(f_a)_1}{(f_u)_1}\right]^{\frac{1}{m}} + \left[\frac{(f_a)_2}{(f_u)_2}\right]^{\frac{1}{m}} = \frac{(D)_1}{(D_m)_1} + \frac{(D)_2}{(D_m)_2} \quad (2)$$

This combination index CI describes the interactions in between two drugs: if $CI = 1$ the effects are additive, if $CI > 1$ they are synergistic, and if $CI < 1$ the effects are antagonistic. There are several approaches based on the Loewe additivity model such as the interaction index of Berenbaum [20], median-effect method of Chou and Talalay [19], mutually exclusive model method of Berenbaum [21], bivariate spline fitting of Sühnel [22], the parametric response surface approach of Greco [23] and Weinstein [24], the approach of Gessner [25], the parametric response surface approach of Greco and Lawrence [26], and the use of multivariate linear logistic model of Carter [27, 28, 29] and Brunden [30]. Moreover, there are several concepts that are consistent with the Loewe additivity model such as the similar joint action [31], simple similar action [32], and concentration addition [33].

The second main technique to characterize drug combinations is the Bliss independence model [34]. It assumes that two inhibitors act through independent mechanisms, such that

$$f_{u_{12}} = f_{u_1} f_{u_2} \tag{3}$$

That is, each drug has a certain probability of having an effect on the system, and the cumulative effect is the result of combining those probabilities. The effects of each individual drug are calculated based on the Hill model as:

$$E = \frac{Econ\left(\frac{D}{D_m}\right)^m}{1+\left(\frac{D}{D_m}\right)^m} \tag{4}$$

where E is the measured effect after drug exposure, and $Econ$ is the control effect no drug is applied.

The Bliss independence model has several advocate models such as the fractional product method of Webb [35], the method of Valeriote and Lin [36], the method or Drewinko [37],

the method of Steel and Peckham [38], and the method of Prichard and Shipman [39]. Some synonyms for the Bliss independence model include: the independent effects, independent joint action [31], independent action [32], response addition [33], effect summation [40], and effect multiplication [41].

These two techniques yield different outcomes, which generates a heated debate as to which method handles noisy clinical data and uncertainty in a better way. None of these studies offer any information about the mechanism of action of the drug combination; as a result, they have a limited predictive ability. Furthermore, they require extensive experimental work to determine dose-response curves for inhibitors, both individually and in combination. Another limitation is the difficulty to adapt these models to combinations of three or more drugs, since they require large parametric search spaces to be tested in their full extension. And most importantly, none of these models are intended to optimize drug combinations from a big pool of drugs.

I.II.I.III. Current drug combination optimization methods

In order to solve the enormous challenge that is to find an optimal drug combination, a top-down, model free approach combined with sparse search strategies that maximize statistical power while minimizing the number of trials should be considered. Several research studies developed in many different engineering fields have led to powerful control tools to drive systems toward required performances; hence, they can provide an ideal candidate for developing new therapies.

A new approach to solve this issue is to use either deterministic or stochastic search algorithms; deterministic algorithms can be used for linear approximations, while stochastic

models are favored towards searches over highly non-linear spaces. In stochastic optimization, neither the biological model nor an analytical expression of the objective function is needed, making these methods very useful for drug cocktail optimization problems. Among some of the most popular stochastic optimization schemes are the gur game, simulated annealing, and differential evolution. In this regard the Feedback System Control (FSC) scheme, a top-down modeless approach that originally started by taking advantage of these types of search algorithms, serves as a formidable candidate for optimizing drug combinations.

I.II.II. Feedback System Control as an optimization scheme

I.II.II.I. Feedback System Control I

As previously stated, combining several drugs at different possible concentrations yields to a large testing parametric space, which makes the search of an optimal combination a major challenge. Traditional experimental design techniques for drug cocktail optimization start by studying a biological system response to a coarse grid of drug combinations, and once the best combinations are recognized the search is narrowed to the neighboring combinations around them. Although this method can achieve better results than the use of individual drugs, it seldom ends in a local maximum performance [42, 43] Therefore, there is a blatant need to use a different approach to tackle this problem.

It has been previously reported that a closed-loop optimization scheme serves as a better alternative for achieving a therapeutic goal when compared to the typical trial and error method. This method is known as the Feedback System Control (FSC) scheme. The FSC is analog to a traditional closed loop control system (Fig 6 a). According to the classic control theory, any system which is intended to be controlled consists of an input, a plant and an output.

In order to control the system, the output is feed backed and compared to the initial input, then a controller makes the necessary adjustments to achieve the desired output. In this scheme, the plant dynamics have to be known in full detail in order to implement a useful controller.

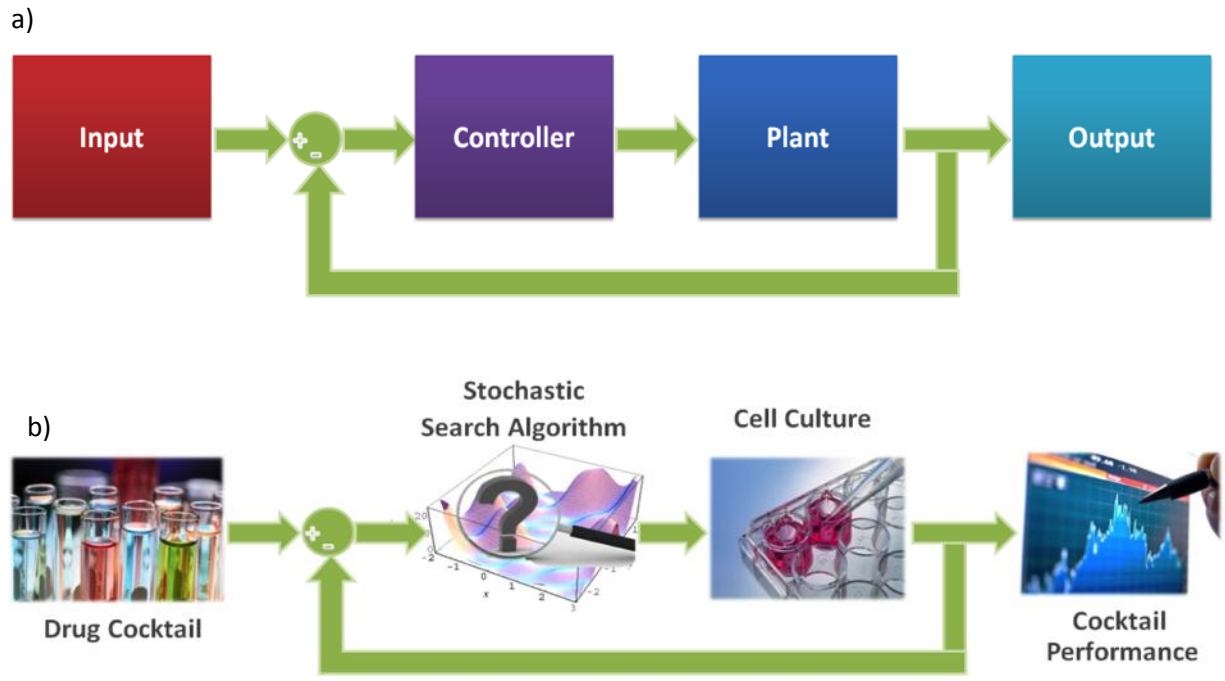


Fig. 6. a) Traditional Closed loop control system. b) Schematic of the FSC.

The FSC, on other hand, aims to circumvent the need for detailed information of the cellular functions of the system of interest. When this method is used to drive a biological system to a desired state, drug cocktails are used as the input to stimulate the system, and the biomarkers indicating the biological responses of interest, such as cell viability, are evaluated. This cocktail performance is then fed back and analyzed by a stochastic search algorithm, which chooses a new drug cocktail to be tried out. This process is repeated until the system is driven into achieving the desired biological response [44].

The stochastic search algorithm is an essential component of the FSC.I scheme. For this method to work, it is required that the chosen algorithm does not require any previous knowledge or training of data to form a metamodel. Different kinds of algorithms can be used, each with different properties, but they all have in common a search departing from initial random conditions. One of the most widely used algorithms for this approach is the differential evolution algorithm; this approach will be explained below to exemplify the operation of FSC.I.

I.II.II.I.I. Example of application of the Differential evolution algorithm for FSC.I

The differential evolution (DE) algorithm is a stochastic search algorithm that optimizes a problem by iteratively improving candidate solutions with regard to a given measure of quality; this algorithm mimics the biological evolution process coined by Charles Darwin as natural selection. In general, the DE algorithm is useful when performing a cost function minimization task, particularly when the cost function is nonlinear, non-differentiable and/or multimodal [45].

In order to illustrate how the algorithm works, suppose there is a pool of 6 different drugs in 10 different concentrations each, and the goal is to find the drug combination which yields the best therapeutic window (Fig. 7).

We start by randomly generating 5 different combinations or searchers, denoted C_i^G, where $i \in \{1,2,3,4,5\}$, and G refers to the G^{th} generation or iteration; these combinations will be modified as the three basic steps of the algorithm are carried out: mutation, crossover and selection.

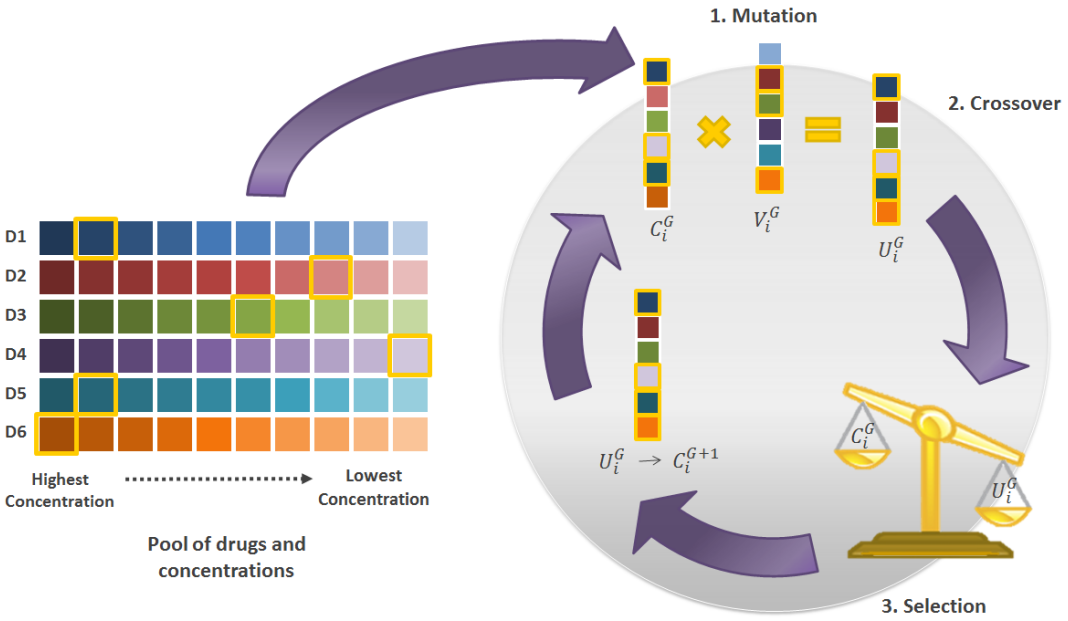


Fig. 7. Differential Evolution Scheme.

During the mutation process, 5 different mutated combinations denoted V_i^G, will be generated based on the original combinations as Eq. 5:

$$V_i^G = C_{r_1}^G + F \cdot \left(C_{r_2}^G - C_{r_3}^G\right) \tag{5}$$

where $C_{r_1}^G$, $C_{r_2}^G$ and $C_{r_3}^G$ refer to three combinations other than the i^{th} combination in the G generation, and F is a mutation factor between 0 and 2 giving more differential variance as it increases its value.

The next step is the crossover, in which a new set of combinations is generated, each denoted as U_i^G and where each element forming a particular combination is generated as Eq. 6:

$$U_i^G = \begin{cases} V_i^G, & rand \leq CR \\ C_i^G, & rand > CR \end{cases} \tag{6}$$

Where $rand$ is a random number between 0 and 1, and CR is a crossover constant. During the crossover, is it ensured that the new combination U_i^G takes at least one element from the mutation vector V_i^G.

The last step of the DE algorithm is the selection, where the new combinations U_i^G are tested, and their performance is compared to the original combinations C_i^G. If they perform better then they become C_i^{G+1}; otherwise the combination C_i^G is retained and becomes C_i^{G+1}. Then the entire process is performed again. For our purposes, the DE generates the next C_i^{G+1} to be used as an input in the FSC scheme.

It has been demonstrated that only tens of iterations out of a large number of possible combinations are needed as opposed to testing the entire search space. This effort-saving approach actively manipulates the complex biological systems as a whole, rather than analyzing the processes through individual signaling pathways in a network [46]. This method has been successfully used to optimize conditions for several and different biological systems where the goal to be achieved is multi-dimensional.

I.II.II.II. Lessons learned from FSC.I and transition to FSC.II

There are two key findings from the multiple FSC.I experiments conducted in the past. First, likely because of synergies among their mechanisms of action, the concentration of each drug in a combination is significantly lower than when used alone. Second, the system response plotted against drug doses is a quadratic surface [47], and these findings have been repeated over and over across different biological systems. This simple shape is surprising, but it can be interpreted from an evolutionary perspective: over millions of years of selection, biological

systems that were too sensitive to environmental perturbation, responded dramatically and failed to survive. Existing biological systems therefore must have developed a robustness and adaptiveness to perturbation, which is reflected in the smooth drug response surface.

The consequence of this is that a relatively small number of drug-dose combinations is sufficient to describe the a system response as quadratic function expressed as:

$$y = \beta_0 + \beta_1 x_1 + \cdots + \beta_n x_n + \beta_{12} x_1 x_2 + \cdots + \beta_{mn} x_m x_n + \beta_{11} x_1^2 + \cdots + \beta_{nn} x_n^2 \quad (7)$$

where y is the efficacy of combinatorial drugs, x_n is the n^{th} drug dosage, β_n is the single drug coefficient of the n^{th} drug, β_{mn} is the synergetic coefficient between the m^{th} and m^{th} drugs, and β_{nn} is the quadratic coefficient for the n^{th} drug.

In the simplest case where only one drug concentration has to be optimized, only three concentrations are needed to determine the parabolic curve; for a two-drug combination, six concentrations are required to determine the shape of the paraboloid. By extension, data from only ~100 combinations of a set of 14 drugs are needed to fit the quadratic surface in 14 dimensional coordinates.

I.II.II.III. Surface Response Methodology for FSC.II

Based on the previous findings, it was suggested that the FSC methodology could benefit from a rational selection of conditions to be tested instead of a random selection of testing points. It was found that there is an existing field intended to achieve suck task: the Response Surface Methodology (RSM) is a collection of statistical and mathematical methods

intended to develop, improve, and optimize processes in which the objective is influenced by multiple variables [48].

RSM has been used for optimization of biochemical processes such as hydrolysis of pectic substrates, enzymatic synthesis of fatty esters, cholesterol oxidase production, alkaline protease production in bioreactors, to name a few [49, 50, 51, 52, 53, 54, 55, 56, 57, 58].

RSM is widely used to analyze the effects of independent variables by generating a mathematical model which describe the process [59, 60], where the data is collected by statistical methods, resulting in valid and objective conclusions.

RSM is carried out in stages. The first stage is intended to screen the most important factors from a large number of factors. The second stage has the purpose of determining the optimum region. The final stage is meant to fit a second order model to the optimum region found on the second stage. FSC.II uses the first and second stages as the initial iteration, and takes the second stage result as the input for a subsequent iteration to complete the third stage. Depending on the number of factors, the FSC.II methodology may require one or more iterations to predict the optimal combination.

Before applying the RSM methodology, it is first necessary to choose an experimental design that will define which experiments should be carried out in order to generate the proper model; there are some experimental matrices for this purpose. Experimental designs for first-order models (e.g., factorial designs) can be used when the data set does not present curvature [61]. One of the most popular experimental designs is orthogonal arrays. They significantly reduce the number of experiments to be performed, while the conclusions drawn from small scale experiments are still valid over the entire region spanned by the experiment factors [62]. An orthogonal design is where the matrix X^TX is diagonal. If the goal is to approximate a response function to experimental data that cannot be described by linear functions, then

experimental designs for quadratic response surfaces should be used, such as three- level factorial, Box-Behnken, central composite, and Doehlert designs [61].

Xu et. Al. introduced a new class of composite designs that are based on a two-level factorial design, and a three-level orthogonal array, these are called Orthogonal Array Composite Designs (OACD) [63]. An orthogonal array consisting of N runs, k factors, s levels and strength t is denoted as $OA(N, s^k, t)$, and is an $N \times k$ matrix in which all s^t level combinations appear equally often in every $N \times t$ submatrix. There are plenty of successful applications using either 2-level factorial designs or 3-level orthogonal arrays [64, 65, 66, 67, 68], but there are only a very few examples of applications using OACDs.

An OACD for k factors, denoted by $x_1, \dots, x_k$,will consist of 3 parts:

a) n_c cube points $(x_1, \dots, x_k)$ where all $x_i = -1$ or 1. This facilitates analysis according to the theorem that states the following: For the first-order model (linear polynomial) and a fixed sample size. If all variables lie between -1 and 1, then the variance of the coefficients is minimized if the design I s orthogonal, and all the variables are at their outer positive or negative limits (i.e., -1 or +1) [61].

b) n_a additional points with all$x_i = \alpha, 0, -\alpha$.

c) n_0 center points will all x_i = 0.

Composite designs have a total of $n_c + n_a + n_0$ points, and 3 or 5 levels depending on whether $\alpha = 1$ or not. The two-level part can be a 2^k full factorial or a regular 2^{k-p} fractional factorial design; these can also be either central composite designs (CCD) or small composite designs (SCD). In any case, $n_a = 2k$ axial points are chosen to be the additional points. For the three-level portion, an orthogonal array that accommodates at least k three-level factors can be

designed, and then the minimum aberration or generalized minimum aberration subset is chosen.

Composite designs are used to fit a second-order model as the one described in equation 7. In order to estimate the quadratic terms β_{nn}, all factors must have at least 3 levels. Thus, using a OACD will allow to estimate the linear effects, β_i and two factor interactions β_{ij} with the 2-level portion, and the linear and quadratic effects β_{nn} with the 3-level portion; each of the linear terms is estimated three times, and each of the bilinear and quadratic effects is estimated twice [63].

In the context of FSC, the OACD can provide the initial conditions to be tested and the drug combinations to be examined during the subsequent iterations. It would facilitate the development of quadratic models describing the relationship between the drug combinations and their efficacy with a reliable statistic correlation.

II. Project description

This project is a collaborative effort between Prof. Marcus Horwitz' research group, affiliated to the Division of Infectious Diseases, School of Medicine, and the Mechanical and Aerospace Engineering Department, School of Engineering. While Prof. Horwitz group contribution consisted on carrying out the cell culture, delivering the drug combinations to the cultured cells, executing the readout assays and collecting all the data, the author arranged the experimental designs, prepared the drug combinations and performed the data analysis.

II.I. Objective

The purpose of this work is to use a novel approach to optimize a drug cocktail intended to treat TB by using the Feedback System Control scheme. Concretely, by using this approach, we expect to:

- Develop a combination of drugs for TB treatment to serve as an alternative therapy.
- Reduce the labor, time and costs associated to drug cocktail design experiments.
- Reduce the potential side effects of our drug cocktail by penalizing the use of aggressive drugs.
- Direct our findings into animal tests and subsequent clinical trials implementation.

II.II. Cell culture & readout essays

For this project, two main readout essays were used: a Green Fluorescent Protein (GFP) Expression for TB inhibition detection, and a Colony Forming Unit (CFU) count for TB killing assessment. The timelines for each essay are described below:

Day 1: Plate MTB-iGFP

Day 2: Expand THP-1 monocytic cells

Day 8: Differentiate THP-1 cells to macrophage-like cells

Day 11: Prepare Drug Combinations

Prepare MTB-iGFP and infect THP-macrophages

Deliver drug combinations and induce IPTG

Day 15: Fix cells and stain nuclei with DAPI

Day 16: Imaging

Day 17-21: Data Readout & Analysis

Readouts are nuclei count and integrated granule intensity. These two readouts lead to the calculation of the integrated bacterial green fluorescence per nucleus, which is normalized to obtain a 0-100% Inhibition output.

A second in vitro assay for assessment of intramacrophage MTB killing by Colony Forming Unit (CFU) determination is planned as follows:

Day 1: Plate MTB

Day 2: Expand THP-1 monocytic cells

Day 8: Differentiate THP-1 cells to macrophage-like cells

Day 11: Prepare drug combinations

Prepare MTB and infect THP-1 macrophages

Deliver drug combinations

Day 12: Lyse infected macrophages 1-day post infection

Serial dilute and plate the lysate on 7H11 agar plates

Incubate the plates at 37°C, 95% air-5% CO_2 for two weeks.

Day 14: Lyse infected macrophages 3-day post infection

Serial dilute and plate the lysate on 7H11 agar plates

Incubate the plates at 37°C, 95% air-5% CO_2 for two weeks.

Day 26: Enumerate CFU on plates from 1-day post infection

Day 28: Enumerate CFU on plates from 3-day post infection

Day 29-30: Data readout & analysis

Readout for this essay is calculated as

$$Fraction\ of\ inhibition = 1 - \left(\frac{Log\ CFU\ of\ Treated\ Group}{Log\ CFU\ of\ Untreated\ Group}\right) \quad (8)$$

II.III. Drug library

For this project, 14 drugs were selected to be part of the drug pool for the combinatorial experiments. These drugs, along with their assigned code are listed below.

D1 Amoxicilin/Clavulanate (A/C): Amoxicilin is a penicilin that inhibits cell wall synthesis, while clavulinic acid is a beta-lacamase inhibitor [69].

D2 Clofazimine (CLZ): The mechanism of action is unknown, but it may be related to DNA binding. It has anti-mycobacterial, anti-inflammatory and immunosuppressive properties [69, 70].

D3 Cycloserine (CYC): It is a structural analogue of D-Alanine, and it inhibits alanine racemase, which forms D-alanine from L-alanine. It also inhibits the incorporation of D-alanine into peptidoglycan pentapeptide, necessary for bacterial wall synthesis [69, 71].

D4 Ethambutol (ETH): An oral chemotherapeutic agent that inhibits mycobacterial arabinosyltransferases, which are involved in the polymerization of D-arabinofuranose to arabinoglycan, an important cell wall component. It may also inhibit the synthesis of spermidine in mycobacteria [69, 72].

D5 Isonazid (INH): It is the most active drug for the treatment of TB caused by susceptible bacteria. It is a nicotinamide analog, which plays a role in the inhibition of synthesis of my colic acids, an important component of mycobacterial cell walls [69].

D6 Linezolid (LNZ): A class of oxazolidinone antibiotic, inhibits protein synthesis at the early phase of protein translation, preventing the binding of formy-methionine tRNA. This drug has been used off-label in combination regimens to treat MDR-TB patients, but its contribution to these combinations is still unclear [69, 73].

D7 Moxifloxacin (MXF): A fluoroquinolone that inhibits bacterial DNA replication by binding itself to and inhibiting topoisomerases II and IV; moxifloxacin has 100 times higher affinity for bacterial DNA gyrase than for mammalian [69, 74].

D8 Nitroimidazopyran (PA824): It is a pro-drug requiring reductive activation, its anaerobic mechanism of action is believed to be inhibition of cytochrome c oxidase by nitric oxide, affecting cell respiration. It also has an aerobic killing effect by inhibiting mycolic acid and protein synthesis. It acts via generation of radicals having non-specific toxic effects [69].

D9 Para-aminosalicylic acid (PAS): Anti-metabolite that interferes with the synthesis of folic acid by binding to pteridinesynthetase. Since bacteria are unable to use external sources of folic acid, their cell growth and multiplication is affected. It may also inhibit the synthesis of mycobactin, an important cell wall component, reducing iron uptake by the bacteria [70, 75].

D10 Prothionamide (PRO): A N-propyl derivative of ethionamide. It is a pro-drug that requires activation by monooxygenase EthA. It inhibits the *inhA* gene product enoyl-ACP reductase, which is an essential enzyme in mycolic acid synthesis [69].

D11 Pyrazinamide (PZA): It is a nicotinamide analog that is converted to the active pyrazanoic acid (encoded by pncA) by pyrazinamidase in susceptible organisms. Pyrazanoic acid lowers pH in the immediate bacteria surroundings, making it unable to grow. It may also function as an antimetabolite of nicotinamide and interfere with the synthesis of NAD, inhibiting the synthesis of short-chain, fatty acid precursors [69].

D12. Rifampicin (RIF): Inhibits bacterial RNA synthesis by binding to the β subunit of bacterial DNA-dependent RNA-polymerase (DDRP). Inhibition of DDRP leads to blocking of the initiation chain formation in RNA synthesis. It is one of the most effective antituberculosis agents available and is is effective for both intra- and extra-cellular bacteria [69].

D13 SQ109: A novel 1,2-ethylenediamine-based ethambutol (ETH) analog; it inhibits cell wall synthesis and acts on multiple cellular pathways, but no specific studies on mode of action are available. ETH is perceived as the weakest component of directly observed therapy [69, 76, 77].

D14 Bedaquiline (TMC-207): A first-in-class diarylquinoline that targets the c subunit of ATP synthase, inhibiting its proton pump action, causing a decrease in cellular ATP levels [69, 78].

II.IV. Controls

There are several controls to be tested: the 60's regimen control (INH, RIF, ETH, Streptomycin), and the 80's regimen control (INH, RIF, ETH, PZA), the uninfected cells with IPTG and no treatment, infected cells without IPTG and no treatment, and the infected cells with IPTG and no treatment.

II.V. Experimental setup

The combinatorial experiments were performed using a Hamilton Starline Robot for liquid handling. A set of two different sequences were performed: drug dilutions preparation and drug combinations preparation.

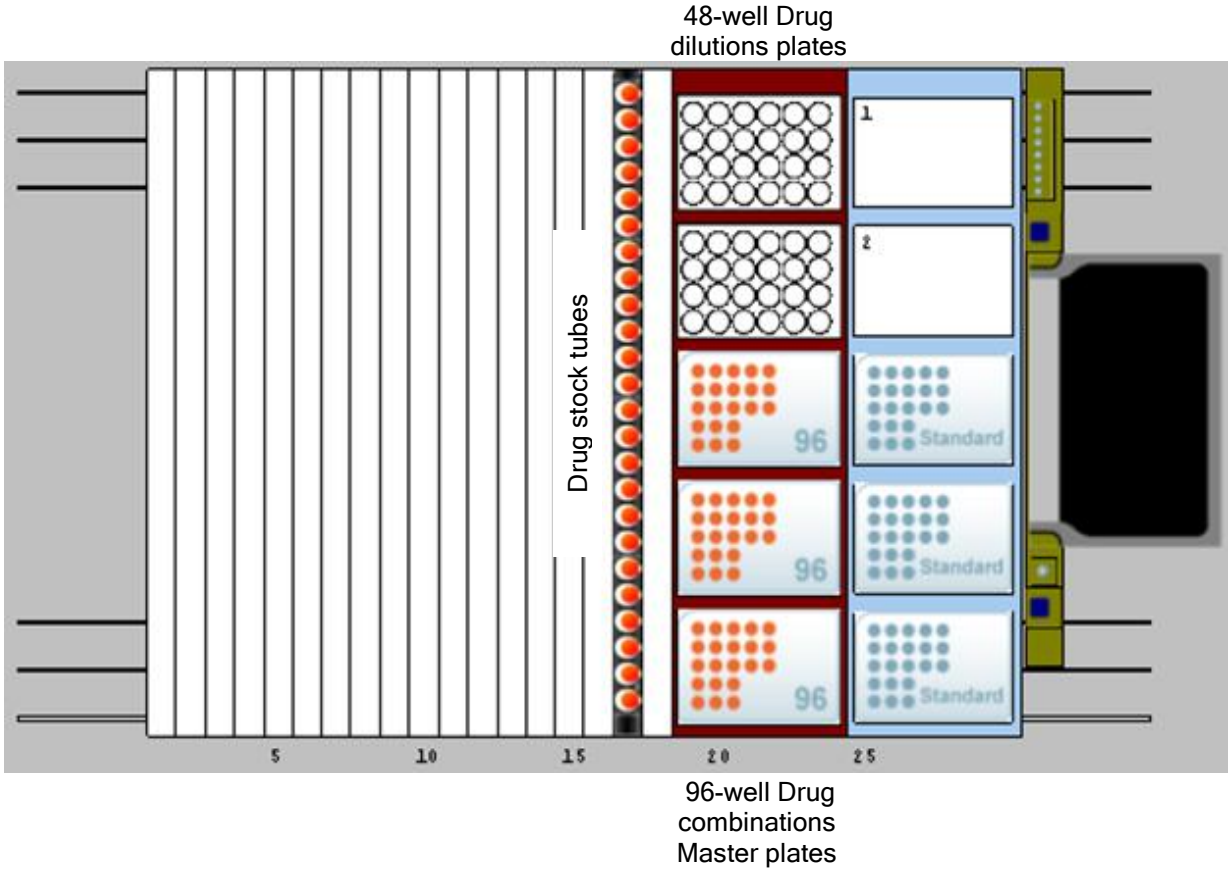


Fig. 8. View of Hamilton Robot setup.

For the drug dilutions preparation, each drug was diluted from a stock 15-mL Falcon tube, into 2 to 5 dilutions located on a either a 48-well plate or 24-well plate (Fig.8). Then, on a second sequence each drug at its specific concentration is added to a 96-well plate well, where

each individual well is a 14 drug combination designed according to the selected design. These plates are called master plates, where each drug combination contains at least three times the volume required per data point. Then, each plate is manually replicated three times, aliquoting the contents of each well into three different cell culture wells.

II.VI. Experimental design

Among the wide array of possibilities of different experimental designs for factor screening and response surface modeling, the designs selected for this project are based on the new class of composite designs that possess both a two-level factorial design and a three-level orthogonal array proposed by Xu et. Al. These new composite designs have been proved to perform in-depth analyses, which can be used in either a single or a sequential experiment [63].

II.VII. Drug dosage selection

Initial drug dosages were selected based on the individual drug achieved inhibitions. These measurements are found on Appendix X.

II.VIII. Data analysis

All the data was analyzed and used for generating the linear regression models with MATLAB®.

II.IX. FSC Iterations

A summary of the FSC Iterations is shown on table 1. A detailed explanation of the steps involved and the results obtained will be covered in the following section.

Iteration	Design	Drugs	Runs	Concentration Levels	Concentrations
1	OA	14	128	2	25%, 10%
2	OA	14	128	2	15%, 5%
3	OA	14	128	2	10%, No Drug
4	OACD	9	128	3	15%, 7.5%, No Drug
5	OACD	6	50	3	20%, 10%, No Drug
6	OA	5	50	5	20%, 15%, 10%, 5%, No Drug

Table 1. FSC iterations summary. Concentrations are expressed as the concentration that achieves a percentage of inhibition for each individual drug.

III. Results & discussion

According to the design of experiments theory, the first FSC iteration is intended to be a screening test, that is, an experiment designed to study the factors involved with the intention of eliminating those that are insignificant; the objective is to reduce the candidate variables so that subsequent experiments will be more efficient [61]. In terms of our application, this means we are looking for those drugs that seem to have a strong positive impact over the inhibition of TB.

II.I. Screening test: Iterations 1, 2 and 3

Figure 9 shows the experimental results of the initial screening test. The results shown most of the combinations achieve a 100% inhibition. Since the system was saturated, this data would not be useful to construct a proper linear model. Thus, a second and third experiments where the dosage levels were reduced were needed to find enough variation between the results obtained for each run.

The third screening test was used to derive a linear first order model through a stepwise regression function. A stepwise regression is a systematic method for adding or removing terms from a generalized linear model, where the criteria to do so is the statistical significance of each of these terms in explaining the response variable.

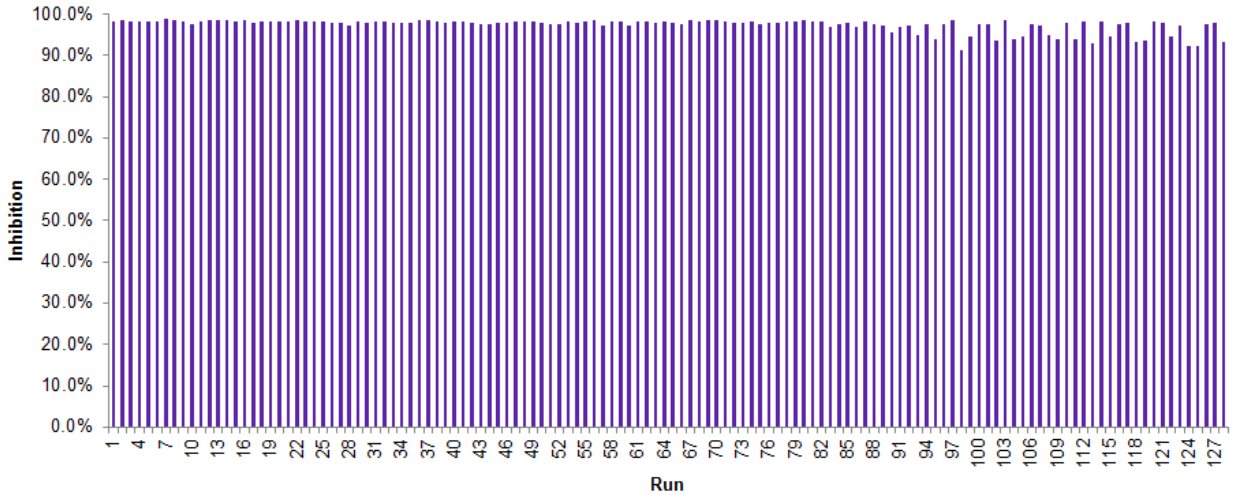


Fig. 9. FSC Iteration 1. Most of the runs tend to hit the 100% inhibition, thus the system is saturated. A propel model cannot be generated based on this information.

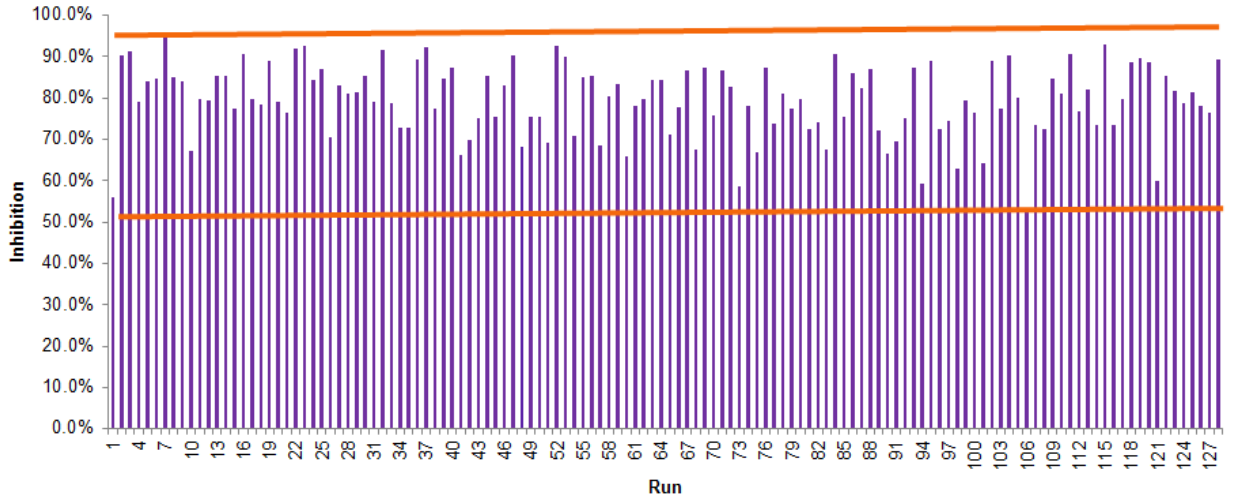


Fig. 10. FSC Iteration 2. The inhibition rate achieved by the different runs varies in between ~52% to ~96%. A bigger variation is needed to construct a proper linear regression model.

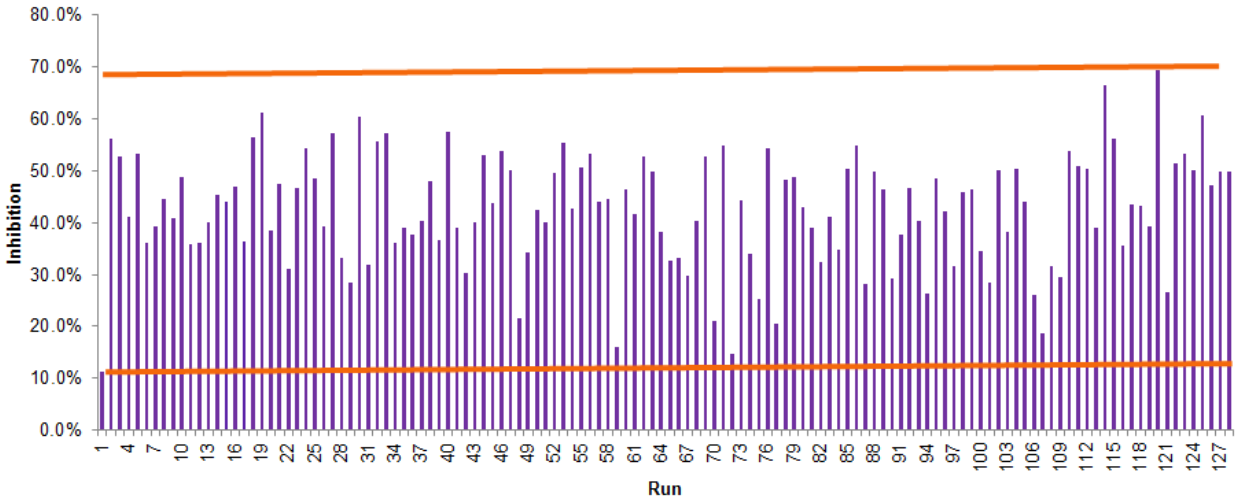


Fig. 11. FSC Iteration 3. The experiment runs are now varying in between ~11% and ~68%. This would give enough information to construct a linear regression model.

The software used to do the fitting, MATLAB®, uses forward and backward stepwise regression to determine the terms included in the final model. For each step, the method looks into terms to be added or removed from the model based on the p-values of an F-statistic to test the model with and without the term being examined (Fig. 12). If a term is not currently in the model, then the null hypothesis to test is that the term would have a coefficient of zero if it were added to the model; if there is enough evidence to reject this null hypothesis, then the term is included in the model. On the other hand, if a term is already included in the model, then the null hypothesis to test is that the term has a coefficient different than zero; if there is enough evidence to reject this null hypothesis, then the term is removed from the model. These steps are performed as followed:

1. A generalized linear model is fitted.

2. If any terms that are not included in the model have a p-value smaller than an entrance tolerance (in other words, the probabilities of the term having a zero coefficient if added to

the model are too small), then the term with the smallest p-value is added to the model and step 2 is repeated; otherwise, the method advances to step 3.

3. If there are any terms in the model that have a p-value larger than an exit tolerance (in other words, there is enough evidence to reject the hypothesis of a zero coefficient), then the term with the largest p-value is removed and the model goes back to step 2; otherwise, end.

The method terminates when no single step improves the model.

```
1. Removing D1^2, FStat = NaN, pValue = NaN
2. Removing D2^2, FStat = NaN, pValue = NaN
3. Removing D3^2, FStat = NaN, pValue = NaN
4. Removing D4^2, FStat = NaN, pValue = NaN
5. Removing D5^2, FStat = NaN, pValue = NaN
6. Removing D6^2, FStat = NaN, pValue = NaN
7. Removing D7^2, FStat = NaN, pValue = NaN
8. Removing D8^2, FStat = NaN, pValue = NaN
9. Removing D9^2, FStat = NaN, pValue = NaN
10. Removing D10^2, FStat = NaN, pValue = NaN
11. Removing D11^2, FStat = NaN, pValue = NaN
12. Removing D12^2, FStat = NaN, pValue = NaN
13. Removing D13^2, FStat = NaN, pValue = NaN
14. Removing D14^2, FStat = -Inf, pValue = NaN
15. Removing D4:D6, FStat = 8.0457e-05, pValue = 0.99292
16. Removing D1:D10, FStat = 0.00089315, pValue = 0.97642
17. Removing D5:D9, FStat = 0.0011545, pValue = 0.97318
18. Removing D2:D8, FStat = 0.0034609, pValue = 0.95356
19. Removing D10:D12, FStat = 0.0045808, pValue = 0.94656
20. Removing D7:D14, FStat = 0.0087377, pValue = 0.92622
21. Removing D10:D14, FStat = 0.010288, pValue = 0.91993
22. Removing D3:D11, FStat = 0.011509, pValue = 0.9153
23. Removing D7:D11, FStat = 0.01571, pValue = 0.90109
24. Removing D3:D10, FStat = 0.016653, pValue = 0.89816
25. Removing D9:D12, FStat = 0.017683, pValue = 0.89504
26. Removing D8:D11, FStat = 0.025561, pValue = 0.87395
27. Removing D8:D13, FStat = 0.027622, pValue = 0.86899
28. Removing D9:D14, FStat = 0.029545, pValue = 0.86452
29. Removing D6:D11, FStat = 0.034915, pValue = 0.85282
30. Removing D7:D12, FStat = 0.038188, pValue = 0.84614
31. Removing D5:D14, FStat = 0.048623, pValue = 0.82666
32. Removing D2:D6, FStat = 0.062716, pValue = 0.80357
33. Removing D5:D13, FStat = 0.064797, pValue = 0.80037
34. Removing D5:D7, FStat = 0.068545, pValue = 0.79478
35. Removing D4:D9, FStat = 0.071492, pValue = 0.79049
36. Removing D7:D10, FStat = 0.074592, pValue = 0.78607
37. Removing D2:D13, FStat = 0.081339, pValue = 0.77683
38. Removing D7:D9, FStat = 0.094467, pValue = 0.75999
39. Removing D5:D8, FStat = 0.10875, pValue = 0.74307
40. Removing D2:D4, FStat = 0.11119, pValue = 0.74027
41. Removing D11:D13, FStat = 0.12592, pValue = 0.72426
42. Removing D4:D7, FStat = 0.14603, pValue = 0.70401
43. Removing D2:D5, FStat = 0.16998, pValue = 0.68189
44. Removing D9:D13, FStat = 0.18708, pValue = 0.66718
45. Removing D3:D7, FStat = 0.19566, pValue = 0.66008
46. Removing D8:D14, FStat = 0.24994, pValue = 0.61919
47. Removing D5:D10, FStat = 0.28785, pValue = 0.5938
48. Removing D3:D8, FStat = 0.3008, pValue = 0.5856
49. Removing D6:D9, FStat = 0.34108, pValue = 0.56155
50. Removing D3:D5, FStat = 0.36018, pValue = 0.55079
51. Removing D4:D8, FStat = 0.38751, pValue = 0.53605
52. Removing D3:D14, FStat = 0.41317, pValue = 0.52286
53. Removing D4:D5, FStat = 0.43509, pValue = 0.51203
54. Removing D2:D14, FStat = 0.49113, pValue = 0.48609
55. Removing D10:D13, FStat = 0.49574, pValue = 0.48401
56. Removing D1:D9, FStat = 0.51634, pValue = 0.47506
57. Removing D4:D12, FStat = 0.54605, pValue = 0.46264
58. Removing D4:D11, FStat = 0.58411, pValue = 0.44747
59. Removing D5:D11, FStat = 0.66246, pValue = 0.41862
60. Removing D7:D13, FStat = 0.66594, pValue = 0.41736
61. Removing D2:D11, FStat = 0.70622, pValue = 0.40365
62. Removing D8:D10, FStat = 0.75246, pValue = 0.3887
63. Removing D6:D14, FStat = 0.84773, pValue = 0.36036
64. Removing D3:D4, FStat = 0.86121, pValue = 0.35654
65. Removing D6:D13, FStat = 0.92914, pValue = 0.33831
66. Removing D3:D12, FStat = 0.93533, pValue = 0.33668
67. Removing D3:D6, FStat = 0.94431, pValue = 0.33434
68. Removing D4:D13, FStat = 0.98382, pValue = 0.32445
69. Removing D13:D14, FStat = 0.99772, pValue = 0.32103
70. Removing D5:D6, FStat = 1.0439, pValue = 0.31011
71. Removing D1:D12, FStat = 1.0665, pValue = 0.30492
72. Removing D1:D8, FStat = 1.0796, pValue = 0.30196
73. Removing D11:D14, FStat = 1.2637, pValue = 0.26432
74. Removing D4:D14, FStat = 1.4877, pValue = 0.22612
75. Removing D6:D10, FStat = 1.5599, pValue = 0.21524
76. Removing D6:D12, FStat = 1.648, pValue = 0.2028
77. Removing D2:D7, FStat = 1.6401, pValue = 0.20384
78. Removing D1:D5, FStat = 1.7581, pValue = 0.18841
79. Removing D1:D2, FStat = 1.765, pValue = 0.18751
80. Removing D9:D10, FStat = 1.8744, pValue = 0.1745
81. Removing D3:D9, FStat = 1.9029, pValue = 0.17125
82. Removing D2:D3, FStat = 2.0179, pValue = 0.15895
83. Removing D12:D13, FStat = 2.1092, pValue = 0.1499
84. Removing D2:D10, FStat = 2.2938, pValue = 0.13336
85. Removing D1:D7, FStat = 2.3618, pValue = 0.12777
86. Removing D1:D11, FStat = 2.4728, pValue = 0.11923
87. Removing D9:D11, FStat = 2.4396, pValue = 0.12167
88. Removing D10:D11, FStat = 2.5102, pValue = 0.11643
89. Removing D3:D13, FStat = 2.753, pValue = 0.10033
```

Fig. 12. Stepwise removal of coefficients during iteration 3.

Once all the unnecessary coefficients are removed, we obtain the final model for our screening experiment. Figure 13 shows the results obtained. These include the estimated coefficients along with their standard errors, t-statistic values and their p-values. Notice the standard error is the same for all the coefficients, and that is a result of choosing an orthogonal array as mentioned previously. The display contains R-squared, adjusted R-squared, and F-statistics. The fitting correlation is also reported.

The p-value is the probability that a hypothesis is true, and it expresses the probability of rejecting the null hypothesis.

The F-statistic is the test statistic for the analysis of variance approach to test the significance of the model.

R-squared is the proportion of the total sum of squares explained by the model; it measures the proportionate amount of variation in the output explained by the independent variables; An R-squared of 1.0 indicates that the regression line perfectly fits the data. The ordinary R-squares is calculated as:

$$R^2 = \frac{SSR}{SST} = 1 - \frac{SSE}{SST} \tag{9}$$

where is SSE is the sum of squared error, SSR is the sum of squared regression, SST is the sum of squared total. Since R-squared increases with added predictor variables in the regression model, an adjusted R-squared is calculated accounting for the number of coefficients that the model has:

$$R^2_{adj} = 1 - \left(\frac{n-1}{n-p}\right)\frac{SSE}{SST} \tag{10}$$

where n is the number of observations, and p is the number of regression coefficients (including the intercept).

All these information is presented to verify the regression model obtained is acceptable.

```
    Inhibition ~ [Linear formula with 31 terms in 14 predictors]

Estimated Coefficients:
                    Estimate       SE           tStat       pValue
    (Intercept)        0.42505    0.0040688     104.47    1.7388e-101
    D1                0.011922    0.0040688       2.93      0.0042255
    D2                0.021398    0.0040688     5.2591     8.6341e-07
    D3               -0.0052364   0.0040688     -1.287         0.20117
    D4                0.018654    0.0040688     4.5847     1.3564e-05
    D5               0.0065456    0.0040688     1.6087         0.11092
    D6                0.011191    0.0040688     2.7504      0.0071023
    D7               0.0029515    0.0040688    0.72541         0.46995
    D8                0.041372    0.0040688     10.168     5.8088e-17
    D9                0.021311    0.0040688     5.2378      9.446e-07
    D10                0.01799    0.0040688     4.4215     2.5601e-05
    D11              0.0067715    0.0040688     1.6643       0.099287
    D12               0.014923    0.0040688     3.6676     0.00039985
    D13               0.062366    0.0040688     15.328     1.1671e-27
    D14               0.022765    0.0040688     5.5951     2.0399e-07
    D1:D3            -0.0072003   0.0040688     -1.7697       0.079926
    D1:D4             0.010079    0.0040688     2.4773        0.01497
    D1:D6             0.009051    0.0040688     2.2245       0.028432
    D1:D13           -0.0075934   0.0040688     -1.8663       0.065026
    D1:D14            0.0083355   0.0040688     2.0487        0.043197
    D2:D9            -0.0077197   0.0040688     -1.8973       0.060762
    D2:D12            -0.015298   0.0040688     -3.7598     0.00029077
    D4:D10            0.0070397   0.0040688     1.7302        0.086779
    D5:D12           -0.0073061   0.0040688     -1.7956       0.075664
    D6:D7             0.0096242   0.0040688     2.3654        0.020001
    D6:D8            -0.0070775   0.0040688     -1.7395       0.085123
    D7:D8            -0.0079007   0.0040688     -1.9418       0.055064
    D8:D9             -0.007387   0.0040688     -1.8155       0.072533
    D8:D12            -0.014318   0.0040688      -3.519     0.00066119
    D11:D12           -0.023157   0.0040688     -5.6915     1.3385e-07
    D12:D14          -0.0080474   0.0040688     -1.9778       0.050784


Number of observations: 128, Error degrees of freedom: 97
Root Mean Squared Error: 0.046
R-squared: 0.863,  Adjusted R-Squared 0.821
F-statistic vs. constant model: 20.4, p-value = 5.33e-30
Fitting Correlation is: 0.92915
```

Fig. 13. Finalized model achieved after following a stepwise regression.

The model can also be graphically represented as shown in figure 14.

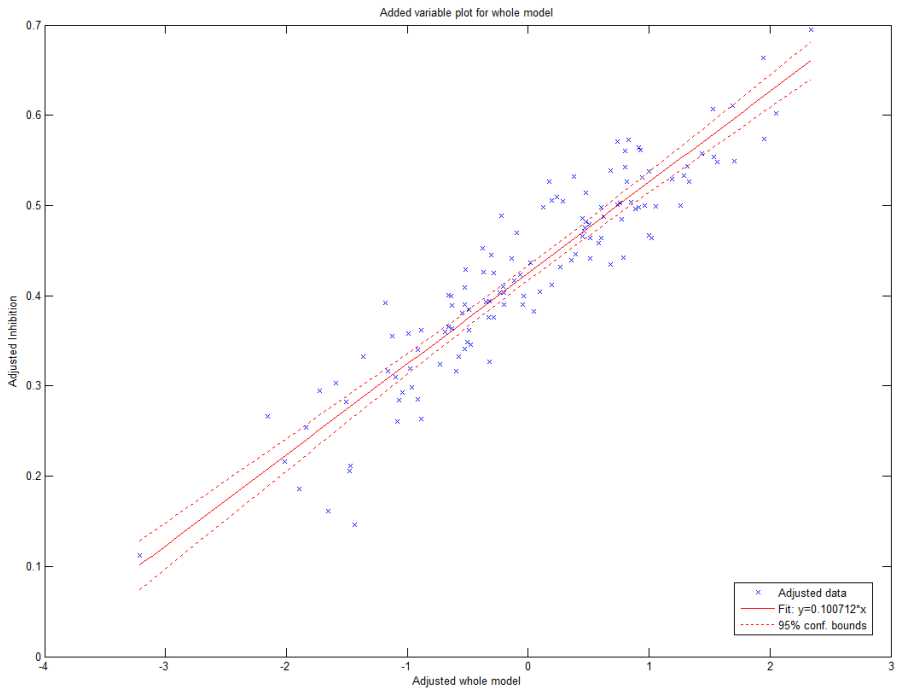


Fig. 14. Final regression model achieved after following a stepwise regression, graphic representation.

A more complete evaluation of the model can be done by studying the residuals obtained for the regression model, and there are several diagnostic plots that help identifying problems in the fitting. They can also help identify outliers.

Outliers are observed data points that lie far from the regression line; this means that they have large "errors", where this "error" or residual is the vertical distance from the line to the point. Outliers must be examined closely; some of them can be the result of erroneous data, and thus they should be dismissed from the analysis to build a better model. In some other cases,

these outliers may hold valuable information about the population under study, and must be included in the model.

The first one is the Plot of residuals vs. fitted values; it is a scatter plot of residuals on the *y* axis and fitted values, or estimated responses, on the *x* axis. This plot is commonly used to find any non-linearity is present, unequal error variances, and possible outliers. A desirable residual vs. fitted values plot have the following characteristics: a) The residuals randomly appear around the 0 line; this suggests that the relationship is linear; b) the residuals form a horizontal band without around the 0 line (no observable trends), which means that the variances of the error terms are equal; c) None of the residuals stands out from the random pattern of residuals, suggesting that there are no possible outliers.

The next plot is the Cook's distance plot, which is also helpful to identify outliers. The Cook's distance is defined as the normalized change in the vectors of coefficients given a deletion of an observation, and it is calculated as:

$$D_i = \frac{\sum_{j=1}^{n}\left(\widehat{y_j} - y_{j(i)}\right)^2}{p\ MSE} \tag{11}$$

Where y_j is the j^{th} fitted response value, $y_{j(i)}$ is the j^{th} fitted response value, where the fit does not include observation *i*. *MSE* is the mean squared error, and *p* is the number of coefficients in the regression model. As a rule of thumb, any observation that possesses a Cook's distance that is larger than three times the mean Cook's distance might be an outlier.

The normal probability plot presents the theoretical percentiles of the normal distribution versus the observed sample percentiles. If the data is normally distributed with mean μ and variance σ^2, then the plot should appear approximately linear. This also means that the error terms are normally distributed as well, which can also be shown with a histogram of residuals plot.

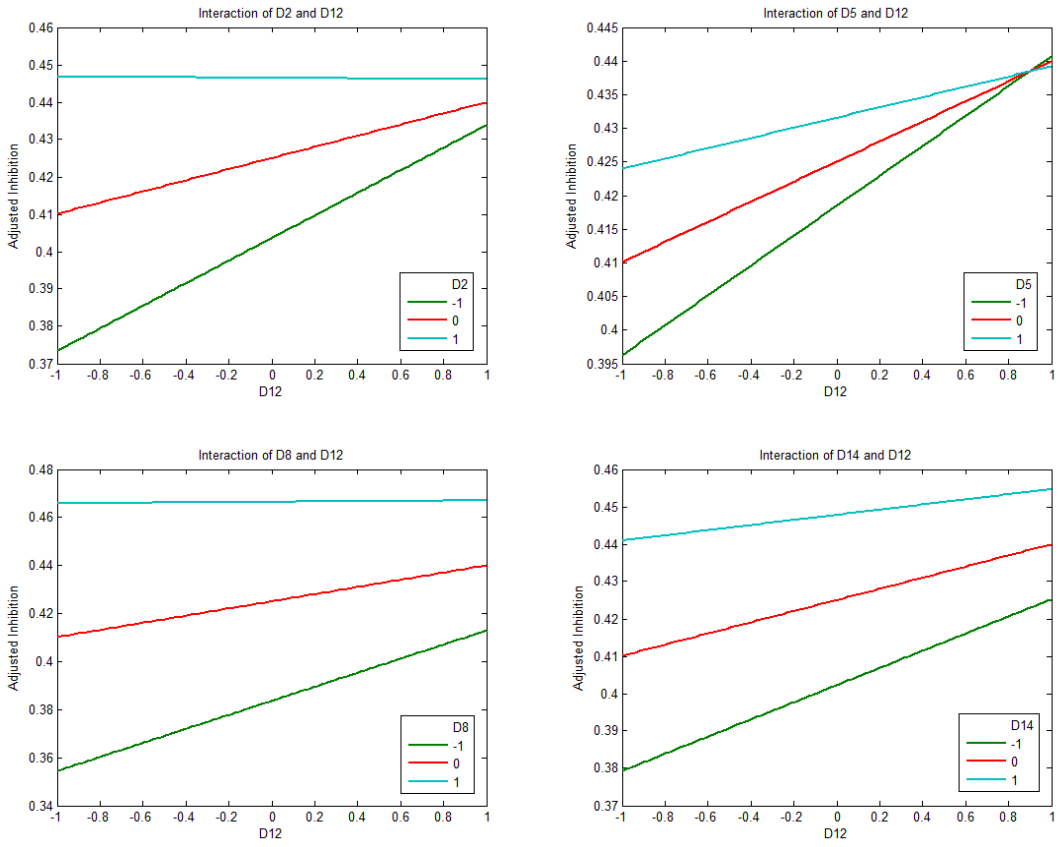


Fig.15. Antagonistic interactions between D12 and several other drugs.

We are also interested in comparing our results with the standard regimens from the 1960s and 1980s. In this iteration, we compared the best inhibitions obtained in our experimental results to those achieved by the 1960s and 1980s combinations, as seen on table 2. It is important to notice that, since the experimental design is defined as an orthogonal array, any given combination tested has at least six drugs. Thus, we present the best achieved result, in this case a nine drug combination, and the best inhibition achieved by the minimum number of drugs tested (six drugs). Both cases are superior compared to those of 1960s and 1980s regimens. These results do not reflect any optimized inhibition.

Comb/Drug	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14	D15	Inhibition
9 drug	1	1	0	1	1	1	0	1	0	1	0	0	1	1	0	69.44%
6 drug	0	1	0	0	1	0	0	1	0	1	1	0	1	0	0	61.07%
1980s	0	0	0	1	1	0	0	0	0	0	1	1	1	0	0	28.94%
1960s	0	0	0	1	1	0	0	0	0	0	0	1	0	0	1	27.40%

Table 2. Comparison of the inhibition obtained by the best nine drug and six drug combinations vs. 1960s and 1980s regimens.

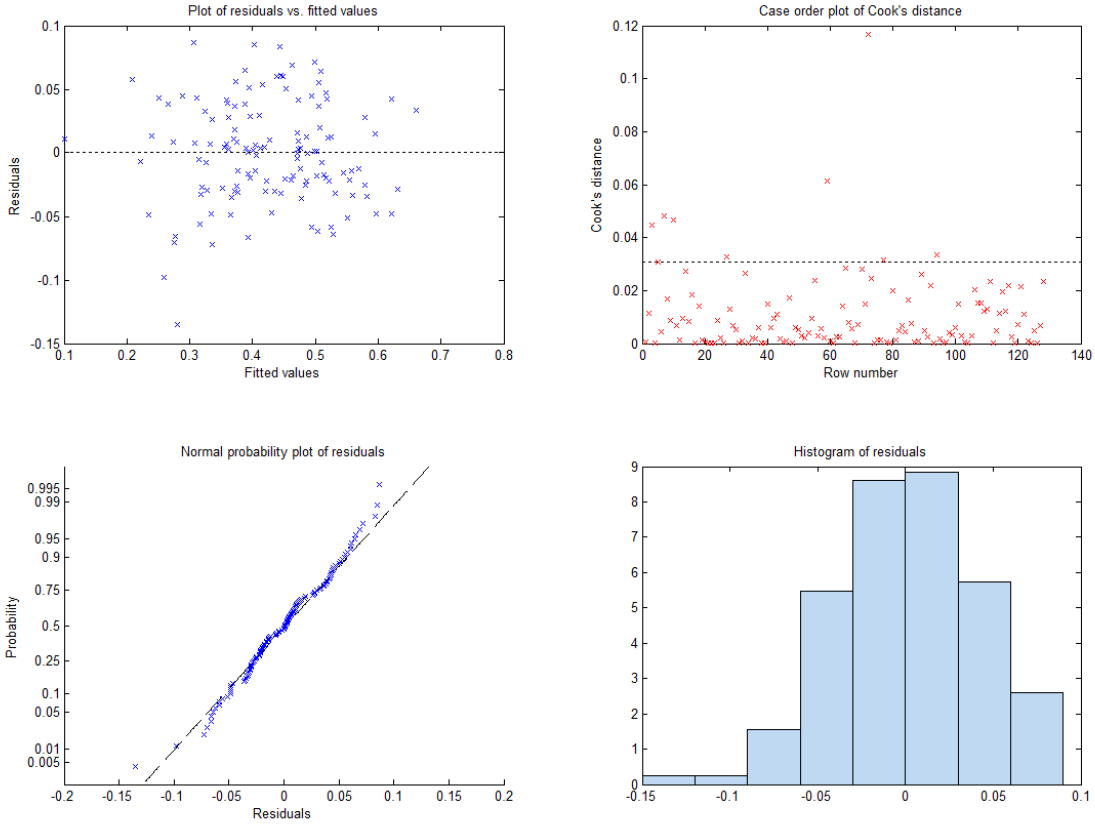


Fig. 16. Residuals analysis for iteration 3.

Based on all these information from the first iteration, we conclude that the derived model has been validated and can be used to screen out the drugs that we will be using for the next iteration. From the list of estimated coefficients in figure 13, it is noted that D3, D5 and D11

can be dropped out based on the fact that they have a p-value larger than 0.05. At a level of significance of 0.01, D1, D6 and D7 would also be removed. Also, it was noted that D12 seems to perform well as an individual drug, but it has negative interactions with D2, D5, D8, D11 and D14 (Fig. 15). Despite this information, both D11 and D12 were kept for the next iteration.

II.II. Iteration 4

For the next iteration, a pool of 9 drugs at three different concentrations was tested. Results are shown in figure 18.

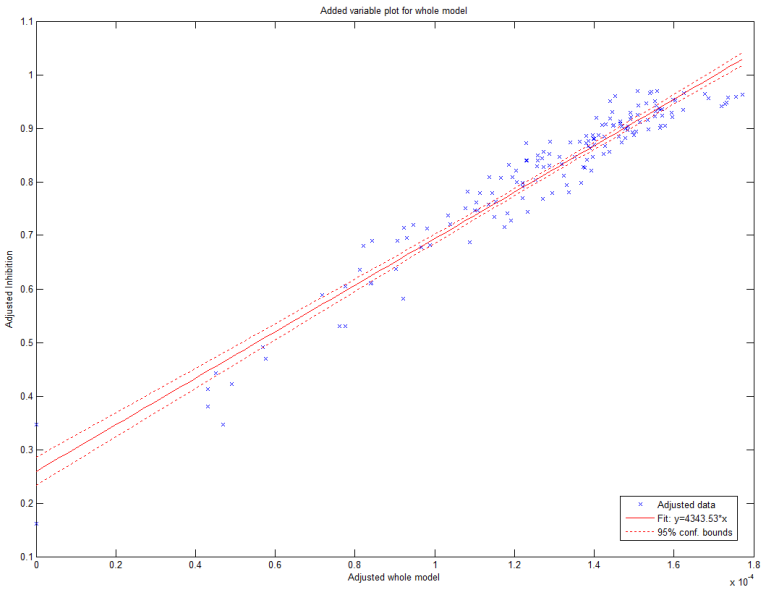


Fig. 17. Graphic representation of regression model for iteration 4.

```
Linear regression model:
    Inhibition ~ [Linear formula with 28 terms in 9 predictors]

Estimated Coefficients:
                    Estimate      SE           tStat      pValue
    (Intercept)      0.25938     0.016474      15.745    1.2149e-31
    D2                4.6459      0.58289      7.9704    7.8595e-13
    D4                1.1386     0.063828      17.839    2.1229e-36
    D8                 11.42       1.9344      5.9039    3.0411e-08
    D9               0.47828        0.193      2.4781      0.014519
    D10               8.3802      0.67464      12.422    1.1125e-23
    D11             0.007657    0.0009043      8.4673    5.2645e-14
    D12               106.35       15.438      6.8885    2.3362e-10
    D13               1.4522     0.072374      20.065    3.3716e-41
    D14               3.9357       7.1457     0.55077       0.58276
    D2:D4            -1.8844       0.5528     -3.4088    0.00087472
    D2:D10           -21.288       4.9118      -4.334    2.9494e-05
    D2:D12           -462.39       132.67     -3.4852    0.00067553
    D2:D13           -4.3266      0.52979     -8.1667    2.7165e-13
    D4:D10            -7.107       2.1373     -3.3252     0.0011553
    D4:D11         -0.013257    0.0038483      -3.445    0.00077439
    D4:D13           -3.0967       0.2299      -13.47    3.1041e-26
    D8:D10           -179.68       102.54     -1.7522      0.082151
    D9:D13            -2.743       1.1034      -2.486      0.014219
    D10:D11        -0.091127     0.033974     -2.6823     0.0082842
    D10:D13          -13.074       2.0411     -6.4055    2.6601e-09
    D10:D14          -149.44       60.108     -2.4863      0.014208
    D11:D12          -2.2641      0.91751     -2.4677       0.01493
    D12:D13          -133.46       55.443     -2.4071      0.017516
    D12:D14          -4086.4       1622.6     -2.5184      0.013032
    D13:D14          -13.867       6.4921     -2.1359      0.034605
    D2^2             -21.113       5.4267     -3.8906    0.00016048
    D14^2             1366.8       818.68      1.6695      0.097477


Number of observations: 155, Error degrees of freedom: 127
Root Mean Squared Error: 0.0406
R-squared: 0.936,  Adjusted R-Squared 0.922
F-statistic vs. constant model: 68.7, p-value = 4.99e-63
Fitting Correlation is: 0.96745
Optimal Output of Y is 1.0462
```

Fig. 18. Regression model for iteration 4.

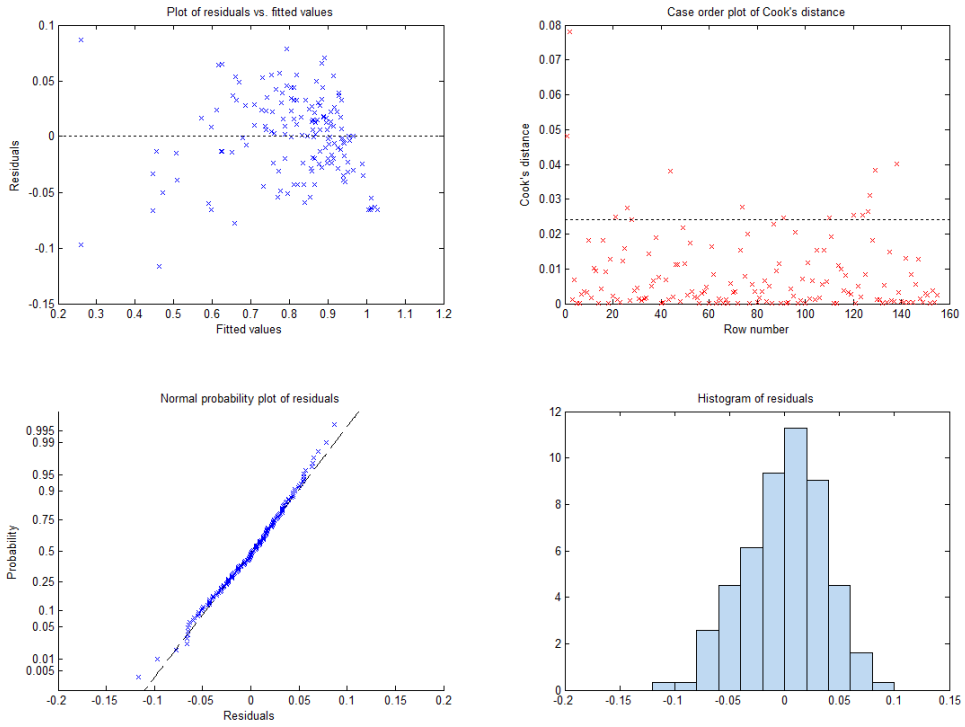


Fig. 19. Residuals analysis for iteration 4.

The obtained model was validated as seen on figure 19. Based on these results, D9 has a small effect on the model and according to the level of significance it is also dropped out. D8 is a drug that seems to perform well, but it is also left out of the next iteration given the fact that it is still being tested as an experimental drug. D12 still shows several negative interactions, but it is kept for the next iteration. Moreover, plots of the different drug-drug interactions were created, and it was noticed that D4 and D13 seem to have very similar effects on the remaining drugs (Fig. 20). This suggests that these drugs could have a similar mechanism of action, and thus they are interchangeable. Since D13 is still being evaluated as an experimental drug, it was decided to leave it out on the next iteration.

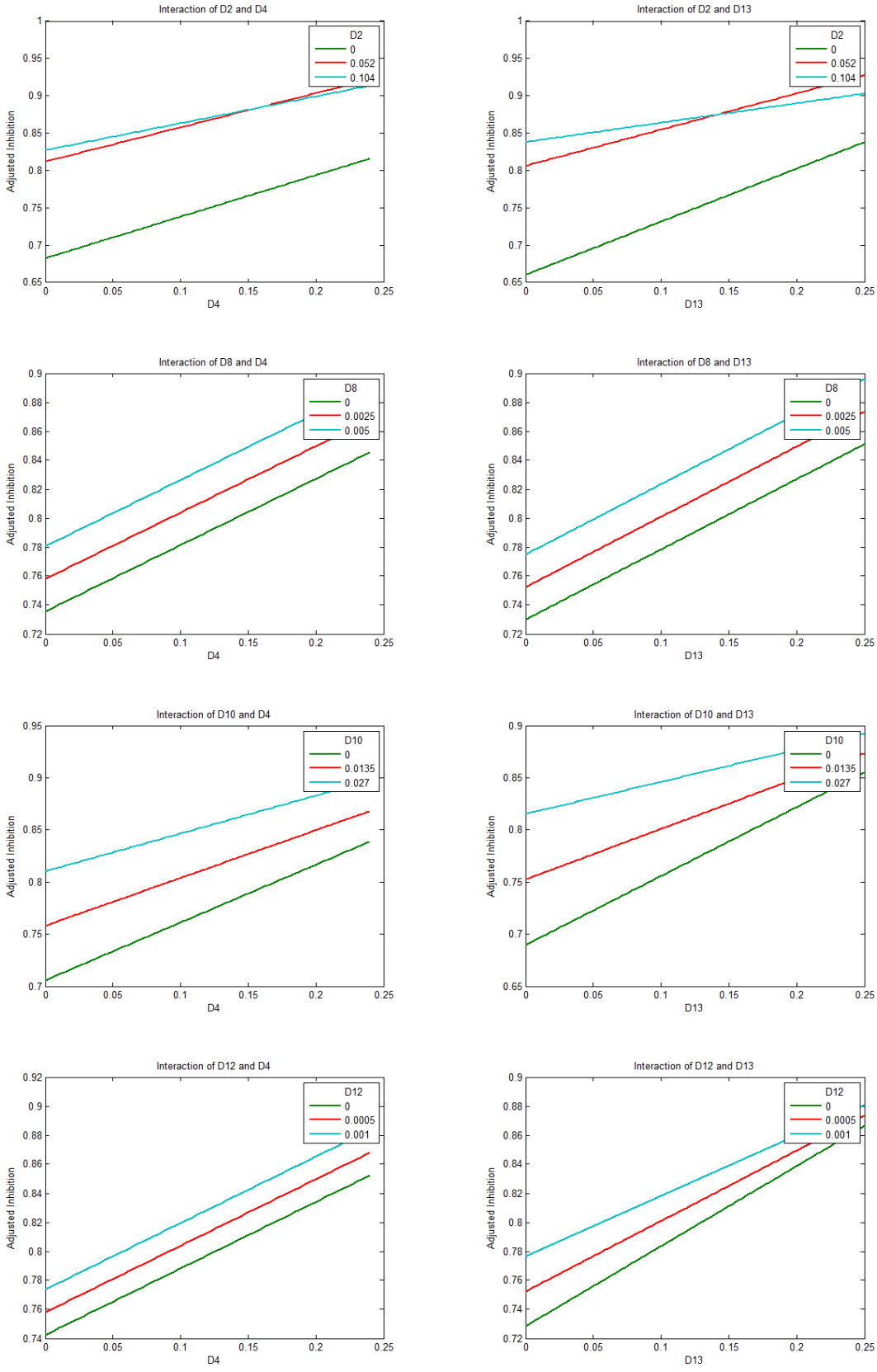


Fig. 20. Similarities in between D4 and D13 interactions with other drugs.

The comparison between the best inhibitions obtained by the experimental results is summarized on table 3. Again, these results do not reflect any optimized inhibition. When optimized, the optimal drug ratios achieve 100% Inhibition.

Comb/Drug	D2	D4	D5	D8	D9	D10	D11	D12	D13	D14	D15	Inhibition
Top Comb	1	1	-1	1	1	1	1	1	1	1	-1	96.9%
5 drug	1	1	-1	1	-1	-1	-1	-1	1	1	-1	96.5%
5 drug	1	1	-1	1	-1	1	-1	-1	-1	1	-1	95.6%
5 drug	1	1	-1	1	1	-1	-1	-1	-1	1	-1	95.4%
4 drug	-1	-1	-1	-1	-1	1	1	-1	1	1	-1	92.9%
1980s	-1	1	1	-1	-1	-1	1	1	-1	-1	-1	85.0%
1960s	-1	1	1	-1	-1	-1	-1	1	-1	-1	1	88.1%

Table 3. Comparison of the inhibition obtained by the best nine drug and six drug combinations vs. 1960s and 1980s regimens.

III.III. Iteration 5

During iteration 5 a pool of 6 drugs at 5 different concentrations was tested. Results are shown in figure 21. The model was also statistically validated according to the residuals analysis shown in figure 23.

```
Linear regression model:
    Inhibition ~ 1 + D2*D12 + D4*D11 + D10*D11 + D12*D14 + D4^2

Estimated Coefficients:
                    Estimate      SE           tStat       pValue
    (Intercept)       0.11457     0.026444      4.3324     0.00010409
    D2                  1.736      0.24092       7.206     1.2968e-08
    D4                -0.33217       1.0317    -0.32195        0.74926
    D10                 6.1777      0.99144       6.231     2.7451e-07
    D11               0.014241    0.0017851      7.9779     1.2182e-09
    D12                  165.5       20.579      8.0419     1.0039e-09
    D14                   23.4       2.9248      8.0007     1.1368e-09
    D2:D12              -809.04       234.94     -3.4436      0.0014127
    D4:D11            -0.036791     0.020971     -1.7544        0.08743
    D10:D11            -0.21582     0.084889     -2.5424       0.015205
    D12:D14              -12418       2836.3     -4.3782     9.0547e-05
    D4^2                 17.652       9.9769      1.7693       0.084863


Number of observations: 50, Error degrees of freedom: 38
Root Mean Squared Error: 0.0558
R-squared: 0.914,  Adjusted R-Squared 0.889
F-statistic vs. constant model: 36.6, p-value = 8.26e-17
Fitting Correlation is: 0.95586
Optimal Output of Y is 0.87959
```

Fig. 21. Regression model for iteration 5. The linear regression equation is expressed in Wilkinson notation.

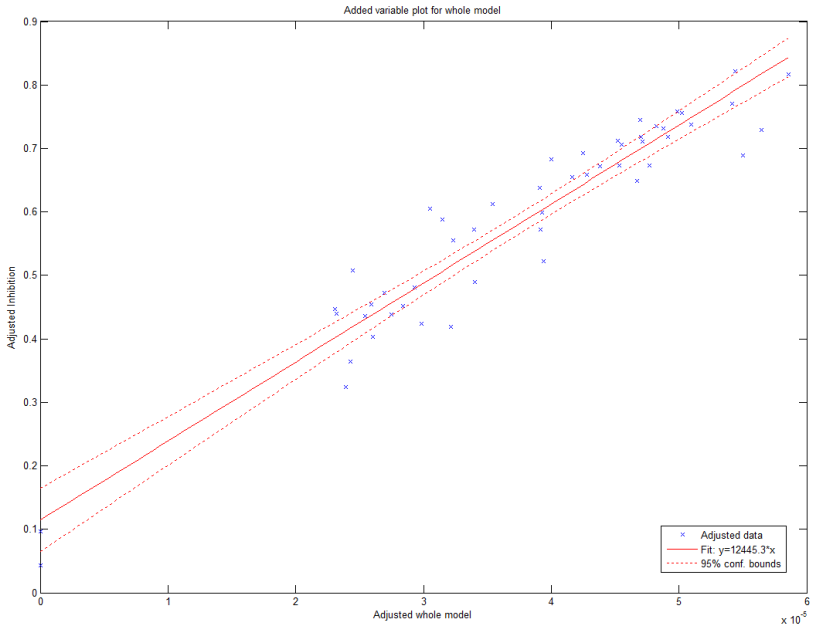


Fig. 22. Graphic representation of regression model for iteration 5.

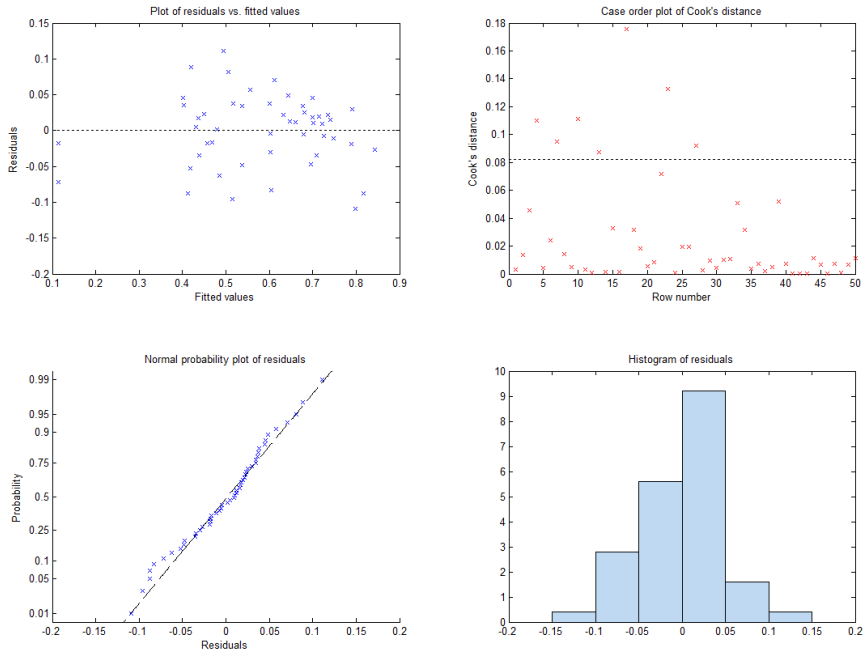


Fig. 23. Residuals analysis for iteration 5.

The comparisons between the best inhibitions obtained by the experimental results are summarized on table 4. Again, these results do not reflect any optimized inhibition. Optimized combinations within the same drug concentrations reach a similar maximum inhibition.

Comb/Drug	D2	D4	D5	D10	D11	D12	D14	D15	Inhibition
Top 1	1	1	-1	1	-1	-1	1	-1	82.2%
Top 2	1	1	-1	1	1	1	1	-1	81.6%
1980s	-1	1	1	-1	1	1	-1	-1	61.8%
1960s	-1	1	1	-1	-1	1	-1	1	70.9%

Table 4. Comparison of the inhibition obtained by the best four drug combinations vs. 1960s and 1980s regimens.

This iteration also showed ho D12 keeps having negative interactions with the remaining tested drugs; thus, it is decided that it should not be included in the next iteration.

II.IV. Iteration 6

II.IV.I. Overall results

Iteration 6 is the last iteration performed for this optimization problem, where a pool of 5 drugs at 5 different concentrations were tested. Results are summarized in figure 24.

```
Linear regression model:
    Inhibition ~ 1 + D4 + D2*D14 + D10*D11 + D10^2 + D11^2

Estimated Coefficients:
                   Estimate       SE           tStat       pValue
    (Intercept)      0.037389     0.034031      1.0987       0.27484
    D2               0.062312     0.009113      6.8376     9.3692e-10
    D4               0.021948    0.0051976      4.2226     5.7634e-05
    D10             -0.0070426     0.019772    -0.35619       0.72253
    D11               0.01079     0.020002     0.53944       0.59092
    D14              0.070967     0.009113      7.7874     1.1157e-11
    D2:D14          -0.0065853    0.0037427     -1.7595       0.081892
    D10:D11         -0.0081842    0.0036753     -2.2268       0.028455
    D10^2            0.015524    0.0044009      3.5275     0.00066259
    D11^2            0.012151    0.0044654      2.7211      0.0078117


Number of observations: 100, Error degrees of freedom: 90
Root Mean Squared Error: 0.0735
R-squared: 0.809,  Adjusted R-Squared 0.79
F-statistic vs. constant model: 42.5, p-value = 1.23e-28
Fitting Correlation is: 0.89964
Optimal Output of Y is 1.2436
```

Fig. 24. Regression model for iteration 5 expressed in Wilkinson notation.

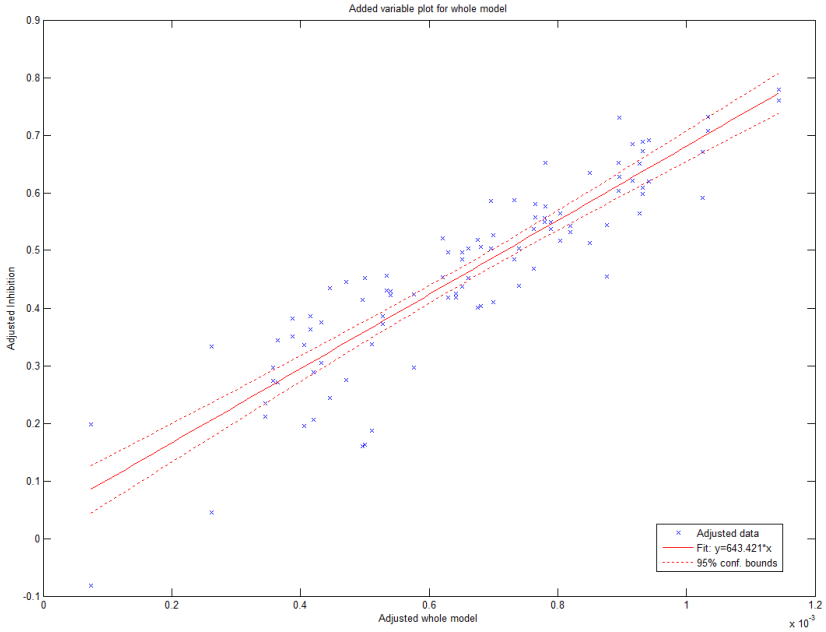


Fig. 25. Graphic representation of regression model for iteration 6.

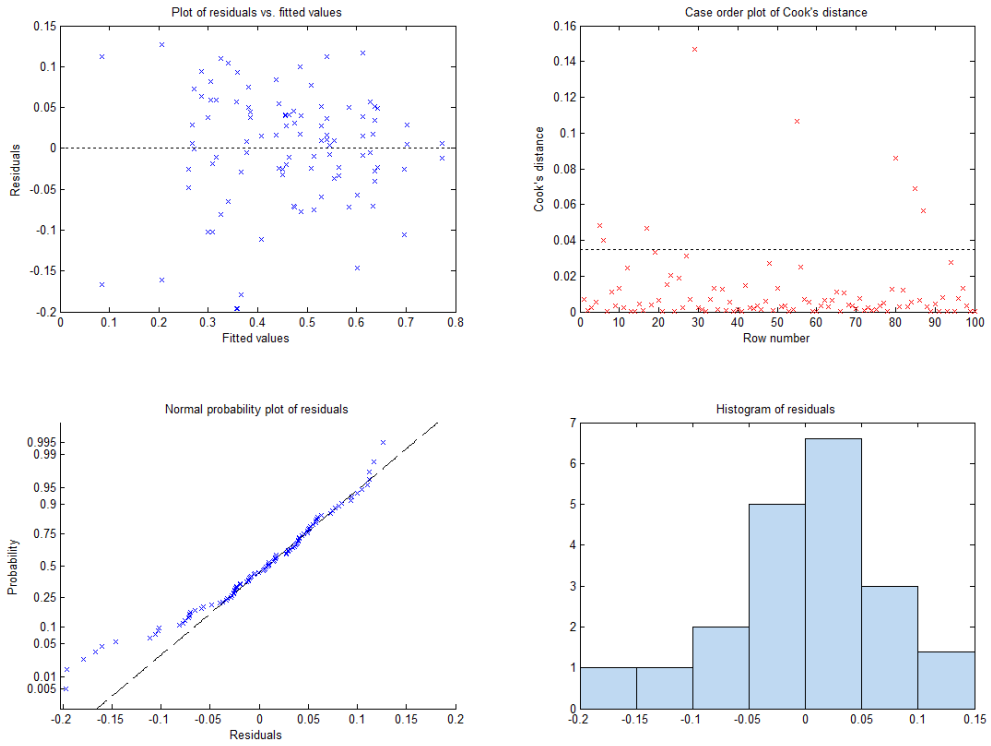


Fig. 26. Residuals analysis for iteration 6.

The previous residuals analysis suggests that the residuals (and hence the error terms) may not be normally distributed in their entirety, since the distribution of residuals seems to be tailed. Thus, the selection of best combinations takes into consideration the overall performance of these drugs along the different iterations.

For this last iteration, the heat maps of the drug-drug interactions were analyzed (fig. 27). The plots show that in all cases using the highest drug concentration possible always yields to the highest inhibition; this can be explained by the fact that the drug concentrations are too low to be toxic to the cells. Also, the plots show that despite having several good combinations that can achieve good results, drug-drug interactions show very low synergistic effects.

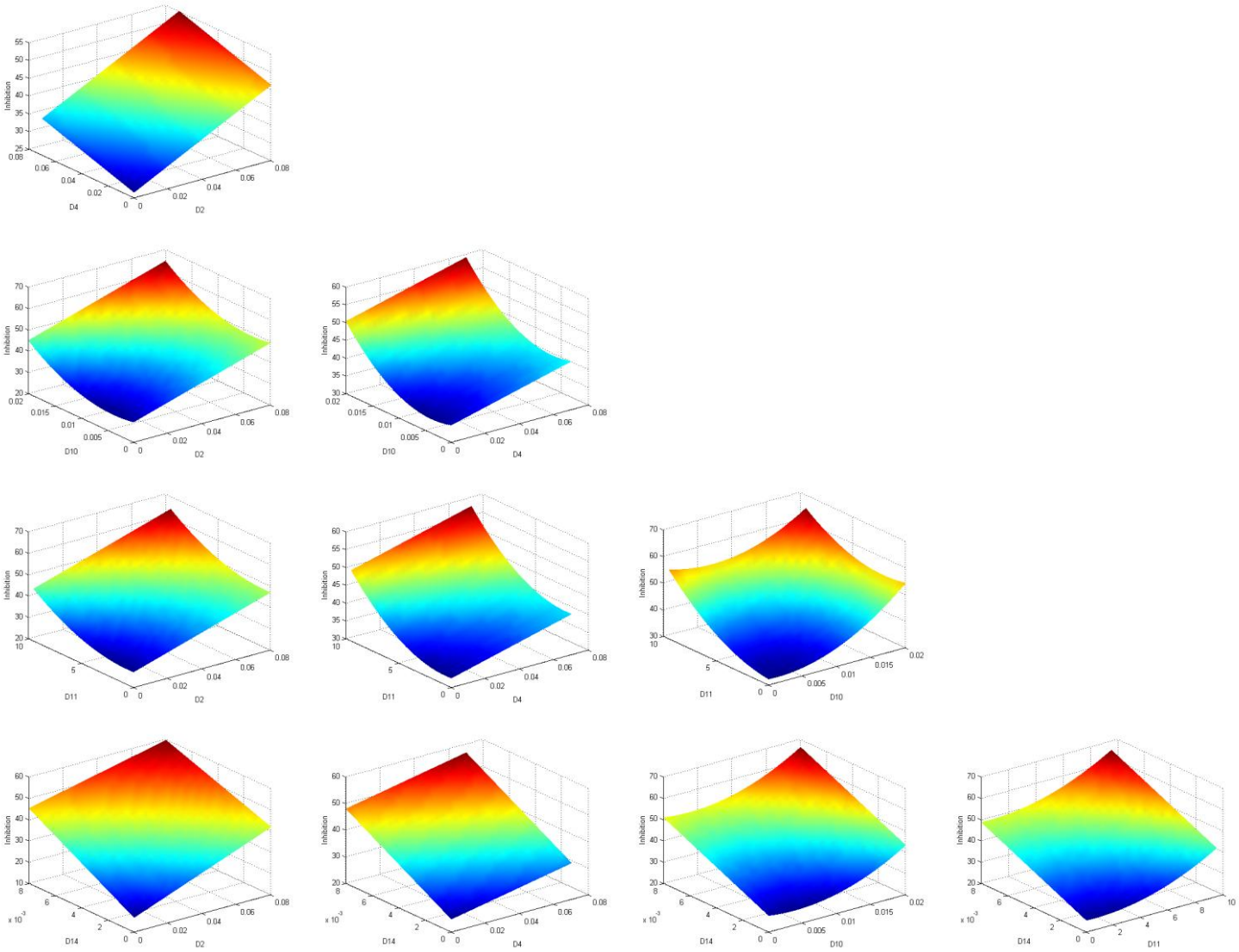


Fig 27. Heat maps of drug-drug interactions for iteration 6.

The comparison between the best inhibitions obtained by the experimental results are summarized in table 5. Again, these results do not reflect any optimized inhibition. When optimized, the optimal drug ratios achieve ~86% Inhibition.

Comb/Drug	D2	D4	D5	D10	D11	D12	D14	D15	Inhibition
Top 1	4	4	0	2	4	0	4	0	77.00%
Top 2	4	0	0	4	4	0	2	0	71.99%
1980s	2	2	0	0	2	2	0	0	32.52%
1960s	2	2	0	0	0	2	0	2	36.95%

Table 5. Comparison of the inhibition obtained by the best five and four drug combinations vs. 1960s and 1980s regimens.

The following table summarizes the step by step elimination of drugs across the different iterations.

			FSC 3	FSC 4	FSC 5	FSC 6
D1	A/C	Amoxicilin/Clavulanate	✓			
D2	CLZ	Clofazimine	✓	✓	✓	✓
D3	CYC	Cycloserine	✓			
D4	ETH	Ethambutol	✓	✓	✓	✓
D5	INH	Isonazid	✓			
D6	LNZ	Linezolid	✓			
D7	MXF	Moxifloxacin	✓			
D8	PA824	Nitroimidazopyran	✓	✓		
D9	PAS	Para-aminosalicylic acid	✓	✓		
D10	PRO	Prothionamide	✓	✓	✓	✓
D11	PZA	Pyrazinamide	✓	✓	✓	✓
D12	RIF	Rifampicin	✓	✓	✓	
D13	SQ109	SQ109	✓	✓		
D14	TMC207	Bedaquiline	✓	✓	✓	✓

Table 6. Drug selection through each FSC iteration.

The list of drug combinations that yield the best results are listed on table 11, along with their projected inhibitions.

II.IV.II. Drug ratio sensitivity analysis

An important lesson learnt from observation on the projected inhibitions calculated from the derived models is the effect of the ratio over the final outcome. We were interested to study these ratios on the top combinations found on our experiments; the results are shown on tables 7, 8 and 9. Each table shows a drug combination for which there are several possible ratios, and each combination shows the drug concentration for every drug expressed as mg/ml. In table 7, combination 1 shows the maximum drug concentrations used. Combinations 2-4 take one of the drugs and reduce its concentration by 50%. Combinations 5-7 take two drugs and reduce their concentration by 50%.

Combo	D2 CLZ	D4 ETH	D10 PRO	D11 PZA	D14 TMC	Projected Inhibition
1	0.1	0	0.025	0	0.01	0.892047
2	0.05	0	0.025	0	0.01	0.818584
3	0.1	0	0.0125	0	0.01	0.61857
4	0.1	0	0.025	0	0.005	0.796946
5	0.05	0	0.0125	0	0.01	0.545107
6	0.05	0	0.025	0	0.005	0.682325
7	0.1	0	0.0125	0	0.005	0.523469

Table 7. Drug ratio analysis example.

Combination 1 achieves the highest projected inhibition as expected. Surprisingly, reducing one drug down to 50% its original concentration (combination 2) would only reduce the projected inhibition by ~7.4%. If a different drug is reduced by 50% (combination 3), then the projected inhibition would be severely affected and dropping ~27.4%. Unexpectedly, it is possible to achieve a better projected inhibition by reducing two drugs concentrations by 50% (combination 6).

A similar situation can be found on table 8, this time for a 4 drug combination.

Combo	D2 CLZ	D4 ETH	D10 PRO	D11 PZA	D14 TMC	Projected Inhibition
1	0.1	0.0875	0.025	0	0.01	1.001785
2	0.05	0.0875	0.025	0	0.01	0.928322
3	0.1	0.04375	0.025	0	0.01	0.946916
4	0.1	0.0875	0.0125	0	0.01	0.728308
5	0.1	0.0875	0.025	0	0.005	0.906684
6	0.05	0.04375	0.025	0	0.01	0.873453
7	0.05	0.0875	0.0125	0	0.01	0.654845
8	0.05	0.0875	0.025	0	0.005	0.792063
9	0.1	0.04375	0.0125	0	0.01	0.673439
10	0.1	0.04375	0.025	0	0.005	0.851815
11	0.1	0.0875	0.0125	0	0.005	0.633207

Table 8. Drug ratio analysis example.

In this example, combination 1 achieves a 100% projected inhibition. When reducing a single drug by 50%, the projected inhibition varies between 94% and 72% (combinations 3 and 5, respectively). Similarly to last example, it is possible to obtain a better projected inhibition if the concentration of two drugs are reduced by 50%, when compared to the projected inhibition achieved by reducing the drug concentration of a single drug (combinations 6, 10 and 8).

A third example is provided on table 9.

Combo	D4 ETH	D10 PRO	D11 PZA	D14 TMC	Projected Inhibition
1	0.0875	0	11.875	0.01	1.006601
2	0.0875	0	11.875	0.01	0.933138
3	0.04375	0	11.875	0.01	0.951732
4	0.0875	0	5.9375	0.01	0.751802
5	0.0875	0	11.875	0.005	0.9115
6	0.04375	0	11.875	0.01	0.878269
7	0.0875	0	5.9375	0.01	0.67834
8	0.0875	0	11.875	0.005	0.796879
9	0.04375	0	5.9375	0.01	0.696933
10	0.04375	0	11.875	0.005	0.856631
11	0.0875	0	5.9375	0.005	0.656702

Table 9. Drug ratio analysis example.

Here, we compare the best achieved inhibition (100% in combination 1), against a range of ~95.1% to ~75.1% inhibition when reducing a single drug concentration by 50%

(combinations 3 and 4, respectively). When the concentration of two drugs are reduced (for example, combinations 6, 10 and 8), it is possible to achieve a better inhibition than when only a single drug concentration is reduced (combination 4).

III.IV.III. Colony Forming Unit verification assay

Despite the exciting results obtained during the FSC process, it is important to recognize that the GFP essay performed to obtain the readings may only assess bacteria inhibition rates and not precisely bacteria killing rates. Thus, a colony forming unit (CFU) assay was used to study the top combinations found during the FSC iterations. These combinations were tested at their optimal ratios found on previous iterations, and then these concentrations were tested at 1x, 4x, and 16x. These combinations were evaluated 1 day and 3 day after drug exposure. Moreover, one combination was tested at different ratios (treatments M, L, X).

Table 10 summarizes the results obtained. The results show that all combinations except for treatment Q outperform the 80's regimen control (treatment V). It was also found that, for treatment M, the ratio of the drugs does affect the performance: according to the results obtained, reducing the concentration of two drugs by 50% (treatment L) yields a better killing rate than reducing one of them (treatment X).

The two assays used to assess the drug cocktail performances were expected to have different results. Table 11 summarizes the projected inhibition achieved by the same combinations according to the GFP inhibition essay. The only noticeable difference is that treatment Q was expected to be one of the top combinations, whereas it performed worse than the 80's regimen in the killing CFU assay (treatment V).

Treatment	Drugs	Code	1X-1D	4X-1D	16X-1D	1X-3D	4X-3D	16X-3D	SCORE
M	CLZ/ETH/PRO	D2/D4/D10 (High/High/High)	0.016524	0.176493	0.277732	0.027904	0.258428	0.414926	1.172008
G	PRO	D10	0.029556	0.142274	0.317048	0.01587	0.178495	0.421604	1.104847
S	CLZ/ETH/PRO/TMC	D2/D4/D10/D14	0.010289	0.12167	0.148718	0.031195	0.373817	0.408954	1.094642
T	CLZ/ETH/PZA/TMC	D2/D4/D11/D14	0.026324	0.130331	0.148718	0.050899	0.326318	0.404357	1.086948
O	CLZ/PRO/TMC	D2/D10/D14	0.032055	0.083391	0.116719	0.04916	0.331158	0.400808	1.013292
L	CLZ/ETH/PRO	D2/D4/D10 (Low/High/Low)	0.005073	0.131291	0.307457	0.011091	0.153982	0.338111	0.947005
W	CLZ/PZA/RIF/TMC	D2/D11/D12/D14	0.027926	0.092536	0.14671	0.063356	0.267067	0.341741	0.939336
X	CLZ/ETH/PRO	D2/D4/D10 (High/Low/High)	-0.00107	0.128597	0.179658	0.05747	0.190777	0.374217	0.929649
J	SQ109	D13	0.039546	0.104339	0.167945	0.053395	0.247078	0.291406	0.903708
E	MXF	D7	0.003812	0.097801	0.149105	0.019453	0.099084	0.477883	0.847139
U	CLZ/PRO/PZA/TMC	D2/D10/D11/D14	0.0144	0.121524	0.089595	0.036976	0.249985	0.303111	0.815591
N	CLZ/PRO/PZA	D2/D10/D11	-0.02132	0.13934	0.099164	0.033461	0.204268	0.301763	0.756672
R	CLZ/ETH/PRO/PZA	D2/D4/D10/D11	-0.03678	0.12674	0.146994	0.02396	0.232879	0.225096	0.718886
P	ETH/PRO/TMC	D4/D10/D14	0.003188	0.102424	0.060761	0.051605	0.182397	0.286932	0.687307
K	TMC207	D14	-0.00107	0.0714	0.100432	0.027904	0.154997	0.309857	0.663522
V	INH/ETH/PZA/RIF	D5/D4/D11/D12 (80's regimen)	0.047616	0.13226	0.017822	0.047793	0.198096	0.198746	0.642332
Q	ETH/PRO/PZA/TMC	D4/D10/D11/D14	0.0144	0.118107	0.066658	0.043495	0.177164	0.212739	0.632564
D	INH	D5	0.00635	0.026483	0.204337	0.002064	0.013334	0.37769	0.630258
C	ETH	D4	0.008958	0.081189	0.097239	0.009556	0.123855	0.153905	0.474701
B	CLZ	D2	-0.00107	0.013702	0.039181	0.016811	0.030084	0.150189	0.248899
H	PZA	D11	-0.00683	-0.01121	0.016524	0.011313	0.045123	0.076022	0.130943
F	PAS	D9	-0.01174	0.01301	-0.0169	0.004285	0.002463	0.023192	0.014307
A	No drug	No treatment	-0.01589	-0.00739	0.012322	0.003876	-0.0003	-0.00931	-0.01669
I	RIF	D12	0.010289	-0.01121	0.013702	-0.02211	-0.03598	0.019453	-0.02585

Table 10. Killing essay results

Treatment	Drugs	Code	Projected inhibition
Q	ETH/PRO/PZA/TMC	D4/D10/D11/D14	1.055000066
T	CLZ/ETH/PZA/TMC	D2/D4/D11/D14	1.053631468
S	CLZ/ETH/PRO/TMC	D2/D4/D10/D14	1.048815507
U	CLZ/PRO/PZA/TMC	D2/D10/D11/D14	1.045157318
R	CLZ/ETH/PRO/PZA	D2/D4/D10/D11	1.011724101
P	ETH/PRO/TMC	D4/D10/D14	0.901889624
O	CLZ/PRO/TMC	D2/D10/D14	0.892046875
M	CLZ/ETH/PRO	D2/D4/D10 (High/High/High)	0.858613658
N	CLZ/PRO/PZA	D2/D10/D11	0.854955468
X	CLZ/ETH/PRO	D2/D4/D10 (High/Low/High)	0.780229342
L	CLZ/ETH/PRO	D2/D4/D10 (Low/High/Low)	0.429357679
H	PZA	D11	0.395102989
K	TMC207	D14	0.392222676
G	PRO	D10	0.390287028
B	CLZ	D2	0.34894671
C	ETH	D4	0.194157345

Table 11. Projected inhibition of top combinations according to the model generated during iteration 6.

IV. Future directions

IV.I Understanding drug cocktail's mechanism through phospho flow

As mentioned before, FSC is a powerful tool to drive biological systems to achieve a desired phenotype. it does so by treating the system in question as a black box, ignoring its dynamics; however, in order to develop a trustworthy therapy, is it imperative to understand how the newly developed drug cocktails work. In this context, FSC is also a helpful scheme to debunk the complex cell dynamics: it is easier to try to understand drug combination kinetics once this combination is known.

It is well known that cell response to a stimulus involves several actions, particularly protein modification through post-translational processes. These include protein cleavage, protein splicing, acetylation, and coupling to small peptides such as glutathione, and phosphorylation, this last one being a significantly important regulator of signal transduction pathways [79].

Phosphorylation is the addition of a phosphate group to a protein, a transient and reversible event that indicates activation status of signaling proteins. Therefore, by measuring the phosphorylation state of proteins, it is possible to determine which signaling cascades are used in response to specific stimuli, the elements such as cytokines or growth factors involved, the kinetics associated to this signaling activity, and the downstream targets that are transcribed. Furthermore, by comparing diseased cells to healthy samples, it is possible to identify aberrant signaling events, a useful trait to characterize cancers and immune disorders [80].

To measure phosphorylation events, antibodies that are specific to the phosphorylated form of the protein of interest must be raised; these phosphor specific antibodies are coupled to fluorophores that can be further detected and analyzed by flow cytometry. In general, a heterogeneous sample of cells is treated with two different stimuli, A and B, to induce distinct signaling cascades, resulting in phosphorylation of two target proteins. A third sample is treated with both stimuli. The three samples are then fixed, permeabilized and stained with the fluorophore-conjugated phospho-specific forms of the proteins. Then the samples are ready to be analyzed with flow cytometry, where an increase in fluorescence reading is correlated with an increase in phosphorylation [80].

Depending on the flow cytometer used, more than 13 parameters can be simultaneously analyzed in a single cell. These readings can also be performed in 96-well plates in parallel, making it a suitable option for high throughput experiments [81].

Phospho flow has successfully been used to give new insights on the cell dynamics of diseased cells based on the results delivered by the FSC drug cocktail optimization method: new discoveries in the herpes virus infection mechanism were made after infected cells treated with an optimized drug cocktail were compared to an untreated control (Unpublished data). Following this example, we expect to do an important contribution regarding the understanding of drug resistance treatment.

IV.II. Animal tests

Translating drug cocktails, which are designed at the cellular level into clinical applications, is a challenging task. The discontinuities in the hierarchical complex system, spanning from the cell to the human body as a system, impede the direct application of these

drug combinations [82]; for example, some drugs used in the combinatorial experiments work at a systemic level rather than at the cell level. Once again, a top-down approach, such as the FSC platform, would serve as a good candidate to solve this problem.

For this project, the results obtained in the inhibition and CFU enumeration essays can be used to guide a drug cocktail optimization search in animal tests; thus, the following step is to optimize a 3- and 4- drug combination, based on our previous top combinations, using a mice model. Since the FSC allows for dramatic reduction in the number of subjects needed to find these optimized combinations in animal tests, each experimental design will only require 10 groups of mice. For these experiments, once mice are infected with MTB by aerosol, there are two weeks of waiting time for the bacteria to grow before starting a 5-day per week treatment that lasts for 4 weeks. At the end of the 4-week treatment period, mice are euthanized for CFU enumeration. Overall, this animal experiment takes 12 weeks.

IV.III. Clinical Trials

There are several factors contributing to the long duration of TB drug clinical development; some of them are the limited number of biomarkers available to track drug efficacy in early clinical development, the long doubling time of TB, the lengthy treatment periods, the requisite of long patient follow-up periods, and the need of a large number of patients [10].

In this regard, the FSC scheme can serve to minimize the required effort needed to achieve this endeavor. Once an optimal combination is found in cell culture, it serves as an initial cue to develop a tailored cocktail in clinical trials. Moreover, FSC can still guide an effective search of drug cocktails in clinical trials, and the cost function can also be adjusted to

include different considerable factors, such as secondary effects, or drug availability. A new FSC.III technique is currently being developed, a powerful tool that has the capacity to optimize drug combinations for single patients; it holds the potential of becoming a true personalized medicine tool.

These clinical trials can be designed under the guidance of the adaptive design clinical trials for drugs and biologics FDA guidelines, which are intended to provide a reference when designing clinical trials with adaptive features that may make the studies more efficient, and more likely to demonstrate an the effects of the drugs used [83].

Moreover, the results obtained from this experiment align with the Qualified Infectious Disease Product (QIDP) program, an FDA program intended to facilitate and expedite development and review of new drugs to address unmet medical needs in the treatment of serious or life threating diseases: fast track designation, breakthrough therapy designation, accelerated approval, and priority review designation [84].

V. Concluding remarks

In summary, this project aimed to identify drug combinations that could serve as a potential, more efficacious alternative treatment for TB. By using FSC.II, we identified eleven combinations that outperform the standard regimen, and these results were validated in two different cell culture based assays.

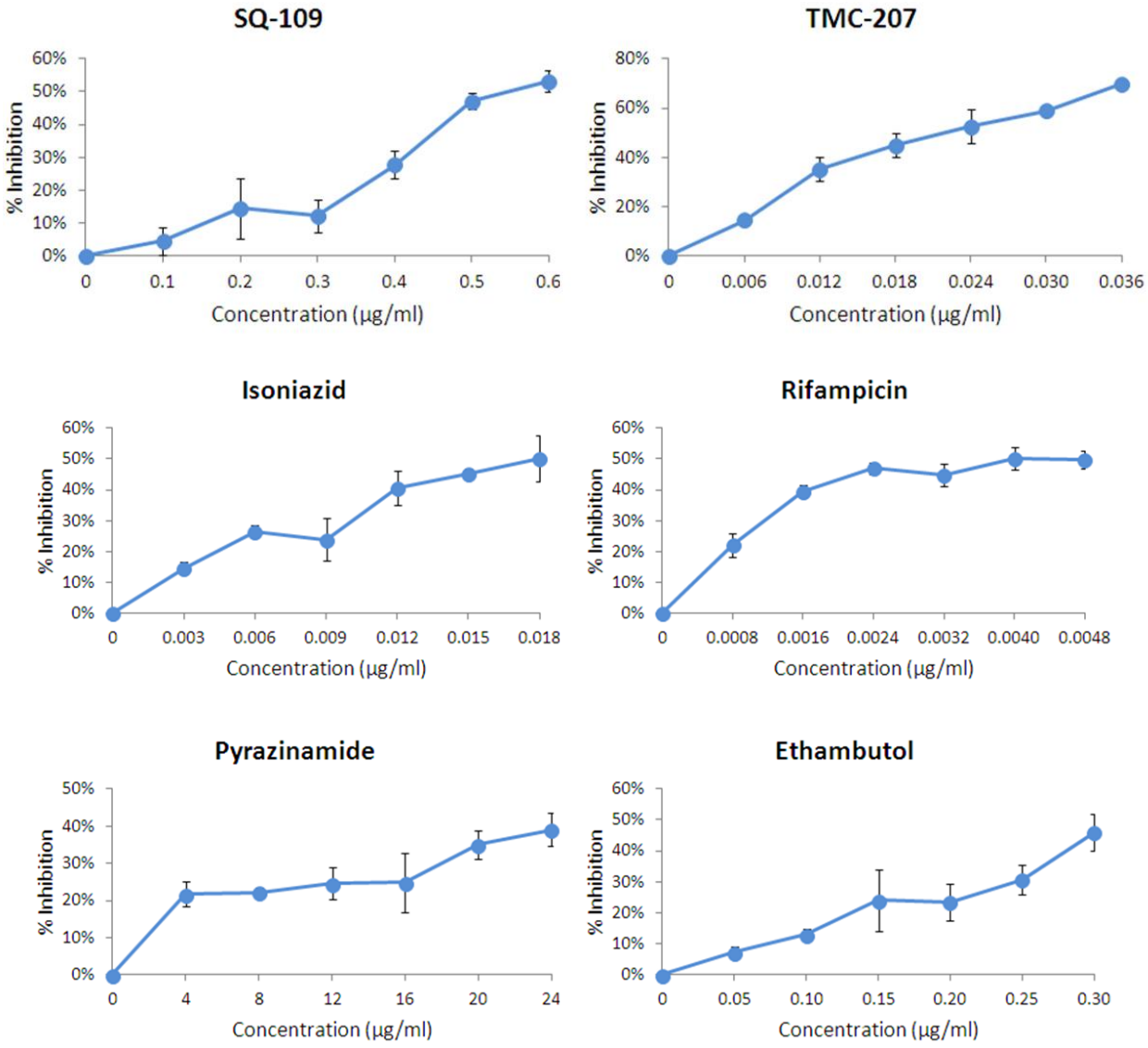
By achieving this, we accomplished our goals of reducing the labor, time and costs associated to drug cocktail design experiments, and reduce the potential side effects of the drug by penalizing the use of aggressive and/or highly concentrated drugs.

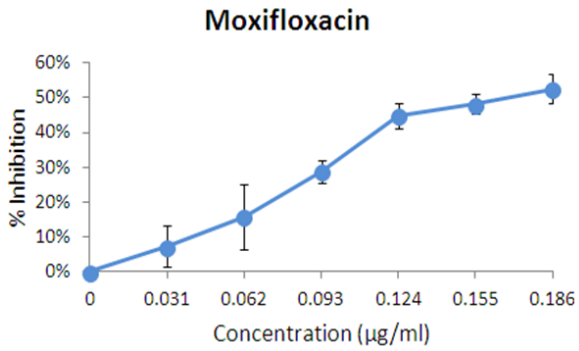
With our findings, we expect to facilitate the design and execution of animal tests and the transition of these results into clinical trials.

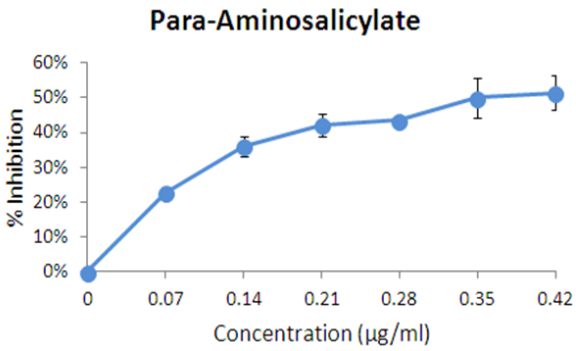
Overall, the outcome of this project has a potential impact on the drug development current procedures. An optimized drug cocktail developed using less resources, faster and cheaper than how is traditionally done means higher profits for pharmaceutical companies, and more importantly means access to better and more affordable drugs to patients in need. Furthermore, the development of optimized therapies that successfully avoid drug resistance is now feasible, as well as reduction of the total treatment time for TB. In conclusion, this project has an important relevance and will positively impact the field of translational medicine.

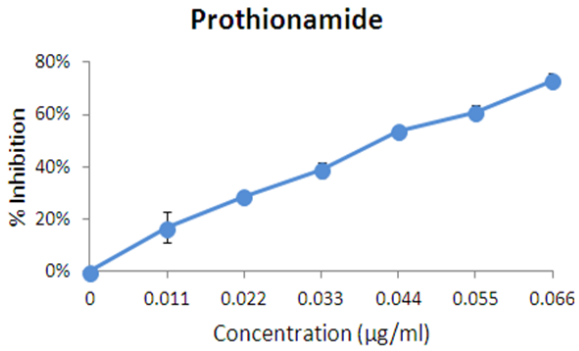
VI. Appendices

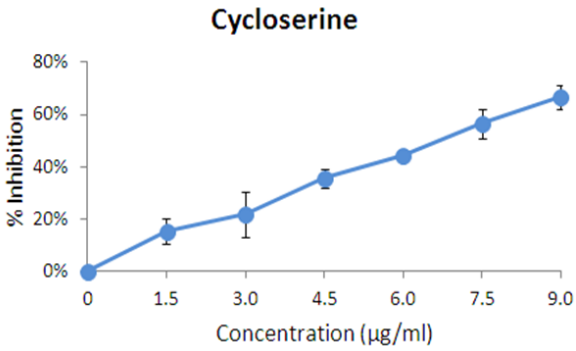
VI.I. Appendix A. Dose-response curve for single drugs

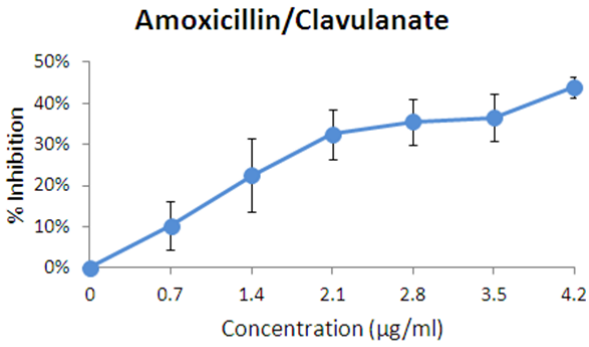


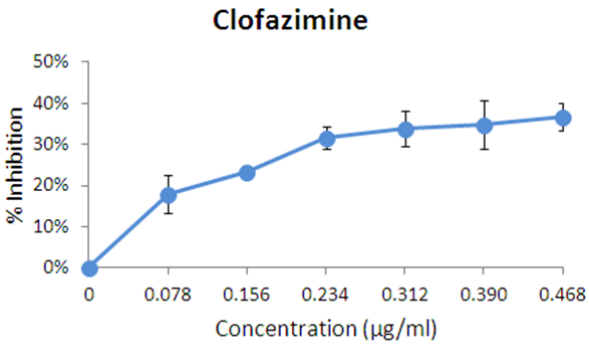

Moxifloxacin
% Inhibition
60%
50%
40%
30%
20%
10%
0%
0
0.031
0.062
0.093
0.124
0.155
0.186
Concentration (µg/ml)

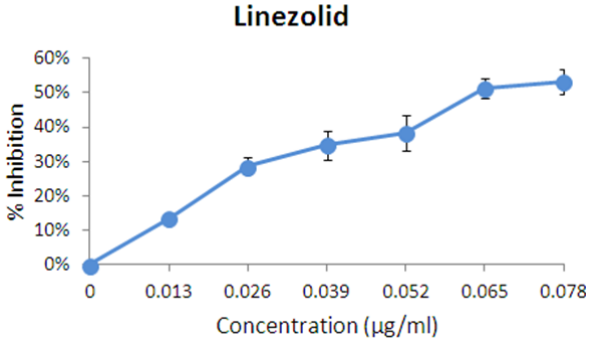

Para-Aminosalicylate
% Inhibition
60%
50%
40%
30%
20%
10%
0%
0
0.07
0.14
0.21
0.28
0.35
0.42
Concentration (µg/ml)

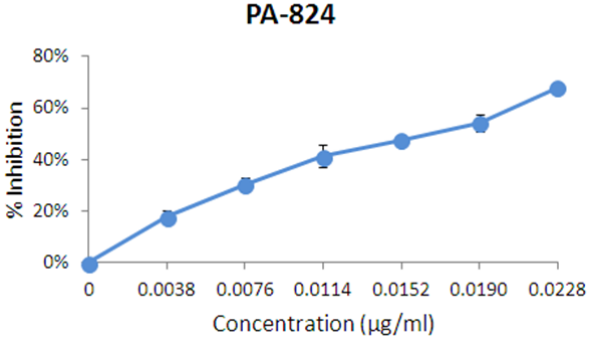

Prothionamide
% Inhibition
80%
60%
40%
20%
0%
0
0.011
0.022
0.033
0.044
0.055
0.066
Concentration (µg/ml)


Cycloserine
% Inhibition
80%
60%
40%
20%
0%
0
1.5
3.0
4.5
6.0
7.5
9.0
Concentration (µg/ml)


Amoxicillin/Clavulanate
% Inhibition
50%
40%
30%
20%
10%
0%
0
0.7
1.4
2.1
2.8
3.5
4.2
Concentration (µg/ml)


Clofazimine
% Inhibition
50%
40%
30%
20%
10%
0%
0
0.078
0.156
0.234
0.312
0.390
0.468
Concentration (µg/ml)


Linezolid
% Inhibition
60%
50%
40%
30%
20%
10%
0%
0
0.013
0.026
0.039
0.052
0.065
0.078
Concentration (µg/ml)


PA-824
% Inhibition
80%
60%
40%
20%
0%
0
0.0038
0.0076
0.0114
0.0152
0.0190
0.0228
Concentration (µg/ml)

VI.II. Appendix B. FSC experimental design tables

Experimental design for iteration 3

Run/Drug	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14
1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1
2	-1	-1	-1	-1	-1	1	1	1	1	1	1	1	1	-1
3	-1	-1	-1	-1	1	-1	1	-1	-1	1	-1	1	1	1
4	-1	-1	-1	-1	1	1	-1	1	1	-1	1	-1	-1	1
5	-1	-1	-1	1	-1	-1	-1	-1	1	-1	1	1	1	1
6	-1	-1	-1	1	-1	1	1	1	-1	1	-1	-1	-1	1
7	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	-1	-1
8	-1	-1	-1	1	1	1	-1	-1	1	1	-1	-1	1	-1
9	-1	-1	1	-1	-1	-1	-1	1	-1	1	1	1	-1	1
10	-1	-1	1	-1	-1	1	1	-1	1	-1	-1	-1	1	1
11	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	-1	-1
12	-1	-1	1	-1	1	1	-1	-1	-1	-1	1	1	1	-1
13	-1	-1	1	1	-1	-1	1	-1	-1	1	1	-1	1	-1
14	-1	-1	1	1	-1	1	-1	1	1	-1	-1	1	-1	-1
15	-1	-1	1	1	1	-1	-1	1	-1	-1	-1	-1	1	1
16	-1	-1	1	1	1	1	1	-1	1	1	1	1	-1	1
17	-1	1	-1	-1	-1	-1	1	-1	1	1	1	-1	-1	1
18	-1	1	-1	-1	-1	1	-1	1	-1	-1	-1	1	1	1
19	-1	1	-1	-1	1	-1	-1	1	-1	1	1	-1	1	-1
20	-1	1	-1	-1	1	1	1	-1	1	-1	-1	1	-1	-1
21	-1	1	-1	1	-1	-1	1	1	1	-1	-1	-1	1	-1
22	-1	1	-1	1	-1	1	-1	-1	-1	1	1	1	-1	-1
23	-1	1	-1	1	1	-1	-1	1	1	1	-1	1	-1	1
24	-1	1	-1	1	1	1	1	-1	-1	-1	1	-1	1	1
25	-1	1	1	-1	-1	-1	-1	-1	1	1	-1	1	1	-1
26	-1	1	1	-1	-1	1	1	1	-1	-1	1	-1	-1	-1
27	-1	1	1	-1	1	-1	1	1	1	-1	1	1	1	1
28	-1	1	1	-1	1	1	-1	-1	-1	1	-1	-1	-1	1
29	-1	1	1	1	-1	-1	1	-1	-1	-1	-1	1	-1	1
30	-1	1	1	1	-1	1	-1	1	1	1	1	-1	1	1
31	-1	1	1	1	1	-1	-1	-1	1	-1	1	-1	-1	-1
32	-1	1	1	1	1	1	1	1	-1	1	-1	1	1	-1
33	1	-1	-1	-1	-1	-1	-1	1	1	1	-1	-1	1	1
34	1	-1	-1	-1	-1	1	1	-1	-1	-1	1	1	-1	1
35	1	-1	-1	-1	1	-1	1	-1	1	-1	1	-1	1	-1
36	1	-1	-1	-1	1	1	-1	1	-1	1	-1	1	-1	-1
37	1	-1	-1	1	-1	-1	1	-1	1	1	-1	1	-1	-1
38	1	-1	-1	1	-1	1	-1	1	-1	-1	1	-1	1	-1
39	1	-1	-1	1	1	-1	-1	-1	-1	1	1	-1	-1	1
40	1	-1	-1	1	1	1	1	1	1	-1	-1	1	1	1
41	1	-1	1	-1	-1	-1	1	1	-1	-1	-1	1	1	-1
42	1	-1	1	-1	-1	1	-1	-1	1	1	1	-1	-1	-1
43	1	-1	1	-1	1	-1	-1	-1	1	-1	-1	1	-1	1
44	1	-1	1	-1	1	1	1	1	-1	1	1	-1	1	1
45	1	-1	1	1	-1	-1	1	1	1	-1	1	-1	-1	1
46	1	-1	1	1	-1	1	-1	-1	-1	1	-1	1	1	1
47	1	-1	1	1	1	-1	-1	1	1	1	1	1	1	-1
48	1	-1	1	1	1	1	1	-1	-1	-1	-1	-1	-1	-1
49	1	1	-1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1
50	1	1	-1	-1	-1	1	1	-1	-1	1	-1	-1	1	-1
51	1	1	-1	-1	1	-1	1	1	-1	-1	-1	-1	-1	1
52	1	1	-1	-1	1	1	-1	-1	1	1	1	1	1	1
53	1	1	-1	1	-1	-1	1	1	-1	1	1	1	1	1
54	1	1	-1	1	-1	1	-1	-1	1	-1	-1	-1	-1	1
55	1	1	-1	1	1	-1	-1	-1	-1	-1	-1	1	1	-1
56	1	1	-1	1	1	1	1	1	1	1	1	-1	-1	-1
57	1	1	1	-1	-1	-1	-1	-1	-1	-1	1	-1	1	1
58	1	1	1	-1	-1	1	1	1	1	1	-1	1	-1	1
59	1	1	1	-1	1	-1	1	-1	-1	1	1	1	-1	-1
60	1	1	1	-1	1	1	-1	1	1	-1	-1	-1	1	-1
61	1	1	1	1	-1	-1	-1	1	-1	1	-1	-1	-1	-1
62	1	1	1	1	-1	1	1	-1	1	-1	1	1	1	-1
63	1	1	1	1	1	-1	1	-1	1	1	-1	-1	1	1
64	1	1	1	1	1	1	-1	1	-1	-1	1	1	-1	1

Experimental design for iteration 3 cont.

Run/Drug	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14
65	-1	-1	-1	-1	-1	-1	1	1	1	-1	-1	1	-1	1
66	-1	-1	-1	-1	-1	1	-1	-1	-1	1	1	-1	1	1
67	-1	-1	-1	-1	1	-1	-1	-1	1	1	1	1	-1	-1
68	-1	-1	-1	-1	1	1	1	1	-1	-1	-1	-1	1	-1
69	-1	-1	-1	1	-1	-1	-1	1	-1	1	-1	1	1	-1
70	-1	-1	-1	1	-1	1	1	-1	1	-1	1	-1	-1	-1
71	-1	-1	-1	1	1	-1	1	1	1	1	1	-1	1	1
72	-1	-1	-1	1	1	1	-1	-1	-1	-1	-1	1	-1	1
73	-1	-1	1	-1	-1	-1	-1	1	1	-1	1	-1	1	-1
74	-1	-1	1	-1	-1	1	1	-1	-1	1	-1	1	-1	-1
75	-1	-1	1	-1	1	-1	1	-1	-1	-1	1	-1	-1	1
76	-1	-1	1	-1	1	1	-1	1	1	1	-1	1	1	1
77	-1	-1	1	1	-1	-1	-1	-1	1	1	-1	-1	-1	1
78	-1	-1	1	1	-1	1	1	1	-1	-1	1	1	1	1
79	-1	-1	1	1	1	-1	1	-1	1	-1	-1	1	1	-1
80	-1	-1	1	1	1	1	-1	1	-1	1	1	-1	-1	-1
81	-1	1	-1	-1	-1	-1	1	-1	-1	-1	1	1	1	-1
82	-1	1	-1	-1	-1	1	-1	1	1	1	-1	-1	-1	-1
83	-1	1	-1	-1	1	-1	-1	-1	1	-1	-1	-1	1	1
84	-1	1	-1	-1	1	1	1	1	-1	1	1	1	-1	1
85	-1	1	-1	1	-1	-1	-1	1	-1	-1	1	-1	-1	1
86	-1	1	-1	1	-1	1	1	-1	1	1	-1	1	1	1
87	-1	1	-1	1	1	-1	1	-1	-1	1	-1	-1	-1	-1
88	-1	1	-1	1	1	1	-1	1	1	-1	1	1	1	-1
89	-1	1	1	-1	-1	-1	1	1	-1	1	-1	-1	1	1
90	-1	1	1	-1	-1	1	-1	-1	1	-1	1	1	-1	1
91	-1	1	1	-1	1	-1	-1	1	-1	-1	-1	1	-1	-1
92	-1	1	1	-1	1	1	1	-1	1	1	1	-1	1	-1
93	-1	1	1	1	-1	-1	1	1	1	1	1	1	-1	-1
94	-1	1	1	1	-1	1	-1	-1	-1	-1	-1	-1	1	-1
95	-1	1	1	1	1	-1	-1	-1	-1	1	1	1	1	1
96	-1	1	1	1	1	1	1	1	1	-1	-1	-1	-1	1
97	1	-1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	-1
98	1	-1	-1	-1	-1	1	-1	-1	1	-1	-1	1	1	-1
99	1	-1	-1	-1	1	-1	-1	1	-1	-1	1	1	1	1
100	1	-1	-1	-1	1	1	1	-1	1	1	-1	-1	-1	1
101	1	-1	-1	1	-1	-1	1	-1	-1	-1	-1	-1	1	1
102	1	-1	-1	1	-1	1	-1	1	1	1	1	1	-1	1
103	1	-1	-1	1	1	-1	-1	1	1	-1	-1	-1	-1	-1
104	1	-1	-1	1	1	1	1	-1	-1	1	1	1	1	-1
105	1	-1	1	-1	-1	-1	1	-1	1	1	1	1	1	1
106	1	-1	1	-1	-1	1	-1	1	-1	-1	-1	-1	-1	1
107	1	-1	1	-1	1	-1	-1	-1	-1	1	-1	-1	1	-1
108	1	-1	1	-1	1	1	1	1	1	-1	1	1	-1	-1
109	1	-1	1	1	-1	-1	-1	-1	-1	-1	1	1	-1	-1
110	1	-1	1	1	-1	1	1	1	1	1	-1	-1	1	-1
111	1	-1	1	1	1	-1	1	1	-1	1	-1	1	-1	1
112	1	-1	1	1	1	1	-1	-1	1	-1	1	-1	1	1
113	1	1	-1	-1	-1	-1	-1	-1	-1	1	-1	1	-1	1
114	1	1	-1	-1	-1	1	1	1	1	-1	1	-1	1	1
115	1	1	-1	-1	1	-1	1	1	1	1	-1	1	1	-1
116	1	1	-1	-1	1	1	-1	-1	-1	-1	1	-1	-1	-1
117	1	1	-1	1	-1	-1	-1	-1	1	1	1	-1	1	-1
118	1	1	-1	1	-1	1	1	1	-1	-1	-1	1	-1	-1
119	1	1	-1	1	1	-1	1	-1	1	-1	1	1	-1	1
120	1	1	-1	1	1	1	-1	1	-1	1	-1	-1	1	1
121	1	1	1	-1	-1	-1	1	-1	1	-1	-1	-1	-1	-1
122	1	1	1	-1	-1	1	-1	1	-1	1	1	1	1	-1
123	1	1	1	-1	1	-1	-1	1	1	1	1	-1	-1	1
124	1	1	1	-1	1	1	1	-1	-1	-1	-1	1	1	1
125	1	1	1	1	-1	-1	-1	1	1	-1	-1	1	1	1
126	1	1	1	1	-1	1	1	-1	-1	1	1	-1	-1	1
127	1	1	1	1	1	-1	1	1	-1	-1	1	-1	1	-1
128	1	1	1	1	1	1	-1	-1	1	1	-1	1	-1	-1

Experimental design for iteration 4

Run/Drug	D2	D4	D8	D9	D10	D11	D12	D13	D14
1	-1	-1	-1	-1	-1	-1	-1	-1	-1
2	-1	-1	-1	-1	-1	-1	1	-1	1
3	-1	-1	-1	-1	-1	1	-1	-1	1
4	-1	-1	-1	-1	-1	1	1	-1	-1
5	-1	-1	-1	-1	1	-1	-1	1	-1
6	-1	-1	-1	-1	1	-1	1	1	1
7	-1	-1	-1	-1	1	1	-1	1	1
8	-1	-1	-1	-1	1	1	1	1	-1
9	-1	-1	-1	1	-1	-1	-1	1	-1
10	-1	-1	-1	1	-1	-1	1	1	1
11	-1	-1	-1	1	-1	1	-1	1	1
12	-1	-1	-1	1	-1	1	1	1	-1
13	-1	-1	-1	1	1	-1	-1	-1	-1
14	-1	-1	-1	1	1	-1	1	-1	1
15	-1	-1	-1	1	1	1	-1	-1	1
16	-1	-1	-1	1	1	1	1	-1	-1
17	-1	-1	1	-1	-1	-1	-1	1	1
18	-1	-1	1	-1	-1	-1	1	1	-1
19	-1	-1	1	-1	-1	1	-1	1	-1
20	-1	-1	1	-1	-1	1	1	1	1
21	-1	-1	1	-1	1	-1	-1	-1	1
22	-1	-1	1	-1	1	-1	1	-1	-1
23	-1	-1	1	-1	1	1	-1	-1	-1
24	-1	-1	1	-1	1	1	1	-1	1
25	-1	-1	1	1	-1	-1	-1	-1	1
26	-1	-1	1	1	-1	-1	1	-1	-1
27	-1	-1	1	1	-1	1	-1	-1	-1
28	-1	-1	1	1	-1	1	1	-1	1
29	-1	-1	1	1	1	-1	-1	1	1
30	-1	-1	1	1	1	-1	1	1	-1
31	-1	-1	1	1	1	1	-1	1	-1
32	-1	-1	1	1	1	1	1	1	1
33	-1	1	-1	-1	-1	-1	-1	1	1
34	-1	1	-1	-1	-1	-1	1	1	-1
35	-1	1	-1	-1	-1	1	-1	1	-1
36	-1	1	-1	-1	-1	1	1	1	1
37	-1	1	-1	-1	1	-1	-1	-1	1
38	-1	1	-1	-1	1	-1	1	-1	-1
39	-1	1	-1	-1	1	1	-1	-1	-1
40	-1	1	-1	-1	1	1	1	-1	1
41	-1	1	-1	1	-1	-1	-1	-1	1
42	-1	1	-1	1	-1	-1	1	-1	-1
43	-1	1	-1	1	-1	1	-1	-1	-1
44	-1	1	-1	1	-1	1	1	-1	1
45	-1	1	-1	1	1	-1	-1	1	1
46	-1	1	-1	1	1	-1	1	1	-1
47	-1	1	-1	1	1	1	-1	1	-1
48	-1	1	-1	1	1	1	1	1	1
49	-1	1	1	-1	-1	-1	-1	-1	-1
50	-1	1	1	-1	-1	-1	1	-1	1
51	-1	1	1	-1	-1	1	-1	-1	1
52	-1	1	1	-1	-1	1	1	-1	-1

Experimental design for iteration 4 cont.

Run/Drug	D2	D4	D8	D9	D10	D11	D12	D13	D14
53	-1	1	1	-1	1	-1	-1	1	-1
54	-1	1	1	-1	1	-1	1	1	1
55	-1	1	1	-1	1	1	-1	1	1
56	-1	1	1	-1	1	1	1	1	-1
57	-1	1	1	1	-1	-1	-1	1	-1
58	-1	1	1	1	-1	-1	1	1	1
59	-1	1	1	1	-1	1	-1	1	1
60	-1	1	1	1	-1	1	1	1	-1
61	-1	1	1	1	1	-1	-1	-1	-1
62	-1	1	1	1	1	-1	1	-1	1
63	-1	1	1	1	1	1	-1	-1	1
64	-1	1	1	1	1	1	1	-1	-1
65	1	-1	-1	-1	-1	-1	-1	1	1
66	1	-1	-1	-1	-1	-1	1	1	-1
67	1	-1	-1	-1	-1	1	-1	1	-1
68	1	-1	-1	-1	-1	1	1	1	1
69	1	-1	-1	-1	1	-1	-1	-1	1
70	1	-1	-1	-1	1	-1	1	-1	-1
71	1	-1	-1	-1	1	1	-1	-1	-1
72	1	-1	-1	-1	1	1	1	-1	1
73	1	-1	-1	1	-1	-1	-1	-1	1
74	1	-1	-1	1	-1	-1	1	-1	-1
75	1	-1	-1	1	-1	1	-1	-1	-1
76	1	-1	-1	1	-1	1	1	-1	1
77	1	-1	-1	1	1	-1	-1	1	1
78	1	-1	-1	1	1	-1	1	1	-1
79	1	-1	-1	1	1	1	-1	1	-1
80	1	-1	-1	1	1	1	1	1	1
81	1	-1	1	-1	-1	-1	-1	-1	-1
82	1	-1	1	-1	-1	-1	1	-1	1
83	1	-1	1	-1	-1	1	-1	-1	1
84	1	-1	1	-1	-1	1	1	-1	-1
85	1	-1	1	-1	1	-1	-1	1	-1
86	1	-1	1	-1	1	-1	1	1	1
87	1	-1	1	-1	1	1	-1	1	1
88	1	-1	1	-1	1	1	1	1	-1
89	1	-1	1	1	-1	-1	-1	1	-1
90	1	-1	1	1	-1	-1	1	1	1
91	1	-1	1	1	-1	1	-1	1	1
92	1	-1	1	1	-1	1	1	1	-1
93	1	-1	1	1	1	-1	-1	-1	-1
94	1	-1	1	1	1	-1	1	-1	1
95	1	-1	1	1	1	1	-1	-1	1
96	1	-1	1	1	1	1	1	-1	-1
97	1	1	-1	-1	-1	-1	-1	-1	-1
98	1	1	-1	-1	-1	-1	1	-1	1
99	1	1	-1	-1	-1	1	-1	-1	1
100	1	1	-1	-1	-1	1	1	-1	-1
101	1	1	-1	-1	1	-1	-1	1	-1
102	1	1	-1	-1	1	-1	1	1	1
103	1	1	-1	-1	1	1	-1	1	1
104	1	1	-1	-1	1	1	1	1	-1

Experimental design for iteration 4 cont.

Run/Drug	D2	D4	D8	D9	D10	D11	D12	D13	D14
105	1	1	-1	1	-1	-1	-1	1	-1
106	1	1	-1	1	-1	-1	1	1	1
107	1	1	-1	1	-1	1	-1	1	1
108	1	1	-1	1	-1	1	1	1	-1
109	1	1	-1	1	1	-1	-1	-1	-1
110	1	1	-1	1	1	-1	1	-1	1
111	1	1	-1	1	1	1	-1	-1	1
112	1	1	-1	1	1	1	1	-1	-1
113	1	1	1	-1	-1	-1	-1	1	1
114	1	1	1	-1	-1	-1	1	1	-1
115	1	1	1	-1	-1	1	-1	1	-1
116	1	1	1	-1	-1	1	1	1	1
117	1	1	1	-1	1	-1	-1	-1	1
118	1	1	1	-1	1	-1	1	-1	-1
119	1	1	1	-1	1	1	-1	-1	-1
120	1	1	1	-1	1	1	1	-1	1
121	1	1	1	1	-1	-1	-1	-1	1
122	1	1	1	1	-1	-1	1	-1	-1
123	1	1	1	1	-1	1	-1	-1	-1
124	1	1	1	1	-1	1	1	-1	1
125	1	1	1	1	1	-1	-1	1	1
126	1	1	1	1	1	-1	1	1	-1
127	1	1	1	1	1	1	-1	1	-1
128	1	1	1	1	1	1	1	1	1
129	-1	-1	-1	-1	-1	-1	-1	-1	-1
130	-1	-1	0	0	-1	1	0	1	1
131	-1	-1	1	1	-1	0	1	0	0
132	-1	0	-1	0	1	0	-1	0	1
133	-1	0	0	1	1	-1	0	-1	0
134	-1	0	1	-1	1	1	1	1	-1
135	-1	1	-1	1	0	1	-1	1	0
136	-1	1	0	-1	0	0	0	0	-1
137	-1	1	1	0	0	-1	1	-1	1
138	0	-1	-1	0	0	0	0	-1	0
139	0	-1	0	1	0	-1	1	1	-1
140	0	-1	1	-1	0	1	-1	0	1
141	0	0	-1	1	-1	1	0	0	-1
142	0	0	0	-1	-1	0	1	-1	1
143	0	0	1	0	-1	-1	-1	1	0
144	0	1	-1	-1	1	-1	0	1	1
145	0	1	0	0	1	1	1	0	0
146	0	1	1	1	1	0	-1	-1	-1
147	1	-1	-1	1	1	1	1	-1	1
148	1	-1	0	-1	1	0	-1	1	0
149	1	-1	1	0	1	-1	0	0	-1
150	1	0	-1	-1	0	-1	1	0	0
151	1	0	0	0	0	1	-1	-1	-1
152	1	0	1	1	0	0	0	1	1
153	1	1	-1	0	-1	0	1	1	-1
154	1	1	0	1	-1	-1	-1	0	1
155	1	1	1	-1	-1	1	0	-1	0

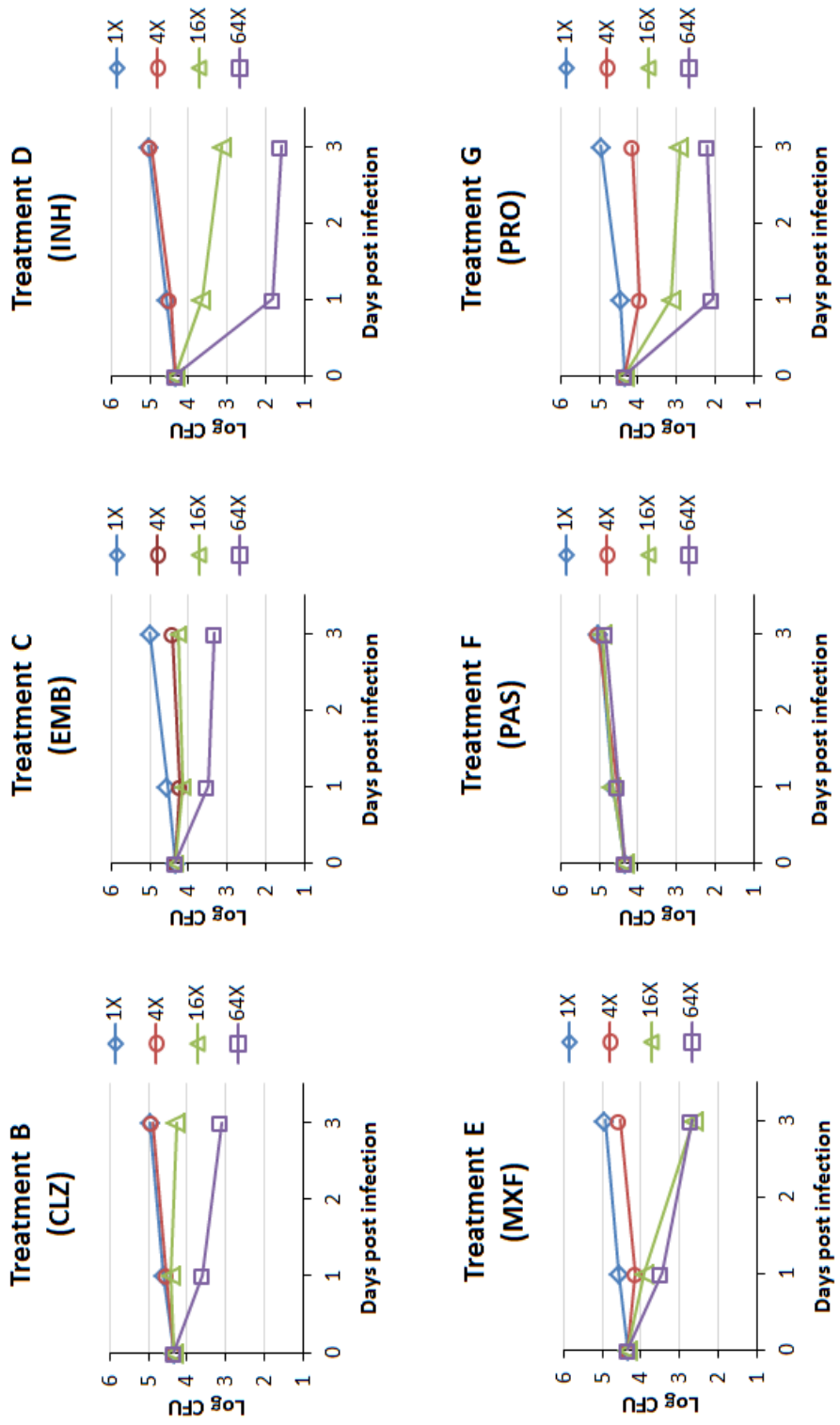
Experimental design for iteration 5

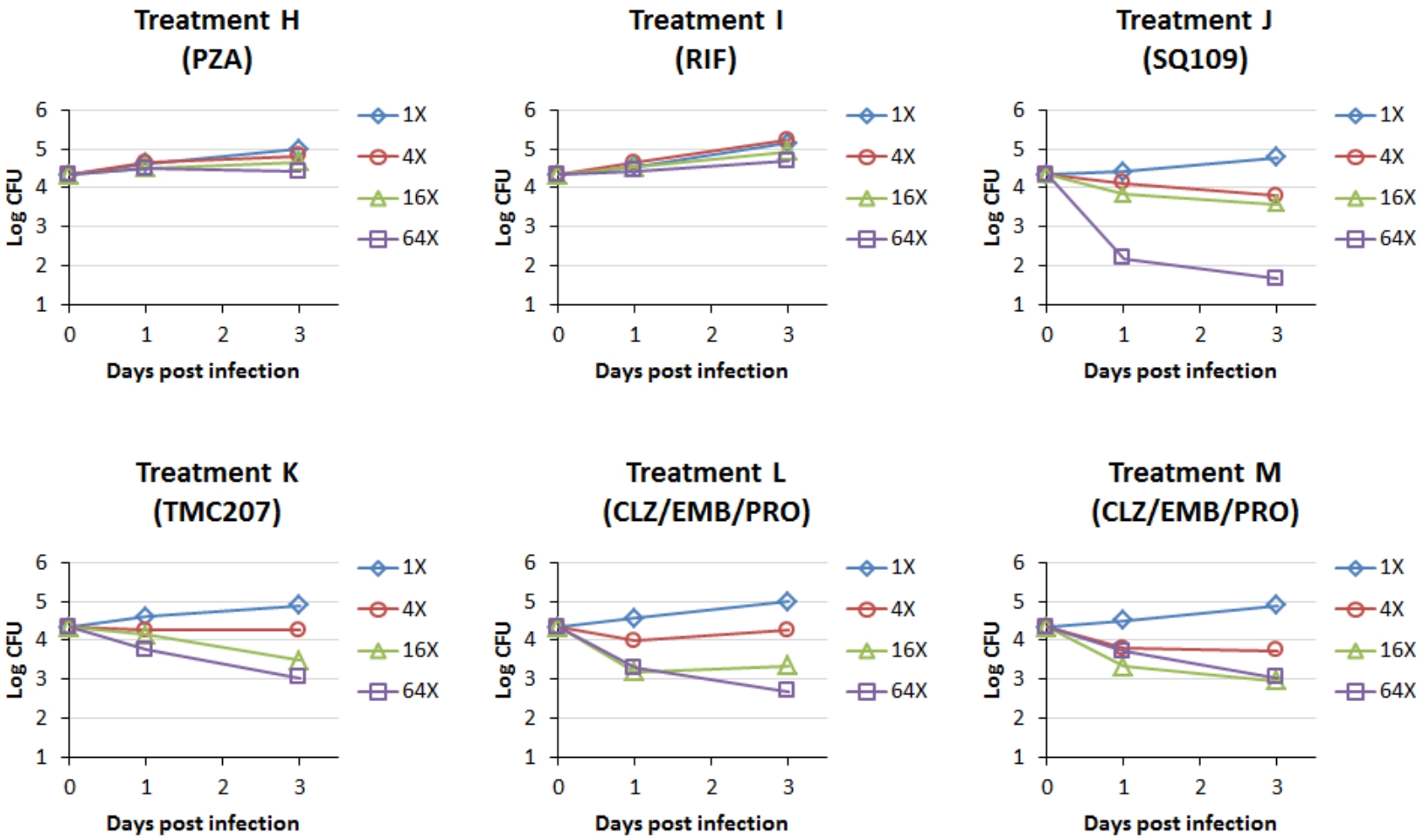
Run/Drug	D2	D4	D10	D11	D12	D14
1	-1	-1	-1	-1	-1	-1
2	-1	-1	-1	-1	1	1
3	-1	-1	-1	1	-1	1
4	-1	-1	-1	1	1	-1
5	-1	-1	1	-1	-1	1
6	-1	-1	1	-1	1	-1
7	-1	-1	1	1	-1	-1
8	-1	-1	1	1	1	1
9	-1	1	-1	-1	-1	1
10	-1	1	-1	-1	1	-1
11	-1	1	-1	1	-1	-1
12	-1	1	-1	1	1	1
13	-1	1	1	-1	-1	-1
14	-1	1	1	-1	1	1
15	-1	1	1	1	-1	1
16	-1	1	1	1	1	-1
17	1	-1	-1	-1	-1	1
18	1	-1	-1	-1	1	-1
19	1	-1	-1	1	-1	-1
20	1	-1	-1	1	1	1
21	1	-1	1	-1	-1	-1
22	1	-1	1	-1	1	1
23	1	-1	1	1	-1	1
24	1	-1	1	1	1	-1
25	1	1	-1	-1	-1	-1
26	1	1	-1	-1	1	1
27	1	1	-1	1	-1	1
28	1	1	-1	1	1	-1
29	1	1	1	-1	-1	1
30	1	1	1	-1	1	-1
31	1	1	1	1	-1	-1
32	1	1	1	1	1	1
33	-1	-1	-1	-1	-1	-1
34	-1	0	0	0	0	0
35	-1	1	1	1	1	1
36	0	-1	-1	0	0	1
37	0	0	0	1	1	-1
38	0	1	1	-1	-1	0
39	1	-1	0	-1	1	0
40	1	0	1	0	-1	1
41	1	1	-1	1	0	-1
42	-1	-1	1	1	0	0
43	-1	0	-1	-1	1	1
44	-1	1	0	0	-1	-1
45	0	-1	0	1	-1	1
46	0	0	1	-1	0	-1
47	0	1	-1	0	1	0
48	1	-1	1	0	1	-1
49	1	0	-1	1	-1	0
50	1	1	0	-1	0	1

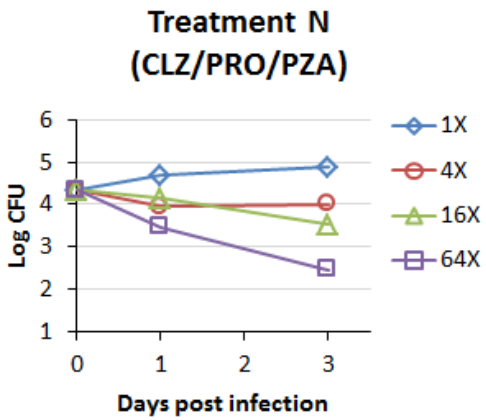
Experimental design for iteration 6

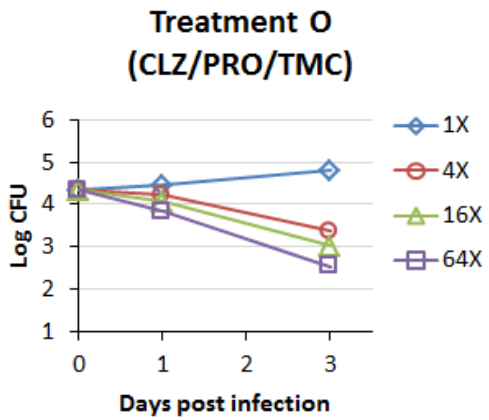
Run/Drug	D2	D4	D10	D11	D14
1	1	1	3	4	4
2	2	2	4	3	3
3	3	3	0	2	2
4	4	4	1	1	1
5	0	0	2	0	0
6	1	2	1	2	0
7	2	3	2	1	4
8	3	4	3	0	3
9	4	0	4	4	2
10	0	1	0	3	1
11	1	3	4	0	1
12	2	4	0	4	0
13	3	0	1	3	4
14	4	1	2	2	3
15	0	2	3	1	2
16	1	4	2	3	2
17	2	0	3	2	1
18	3	1	4	1	0
19	4	2	0	0	4
20	0	3	1	4	3
21	1	0	0	1	3
22	2	1	1	0	2
23	3	2	2	4	1
24	4	3	3	3	0
25	0	4	4	2	4
26	1	2	2	0	3
27	2	3	3	4	2
28	3	4	4	3	1
29	4	0	0	2	0
30	0	1	1	1	4
31	1	3	0	3	4
32	2	4	1	2	3
33	3	0	2	1	2
34	4	1	3	0	1
35	0	2	4	4	0
36	1	4	3	1	0
37	2	0	4	0	4
38	3	1	0	4	3
39	4	2	1	3	2
40	0	3	2	2	1
41	1	0	1	4	1
42	2	1	2	3	0
43	3	2	3	2	4
44	4	3	4	1	3
45	0	4	0	0	2
46	1	1	4	2	2
47	2	2	0	1	1
48	3	3	1	0	0
49	4	4	2	4	4
50	0	0	3	3	3

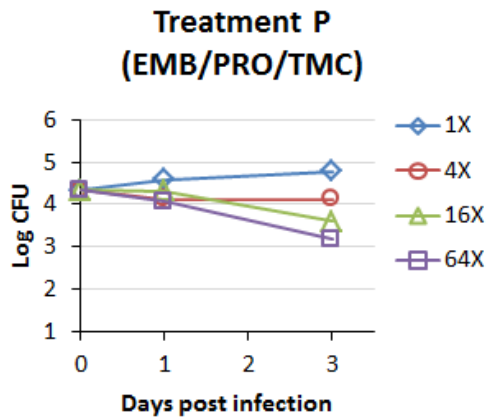
VI.III. Appendix C. CFU Experimental data

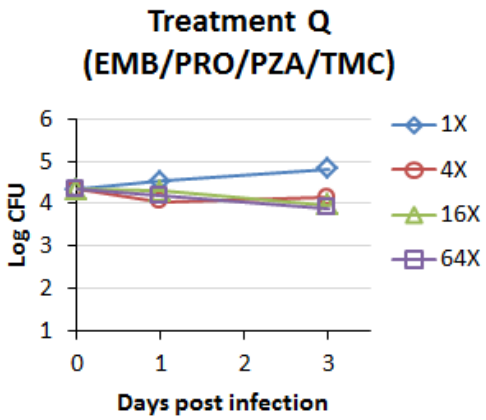


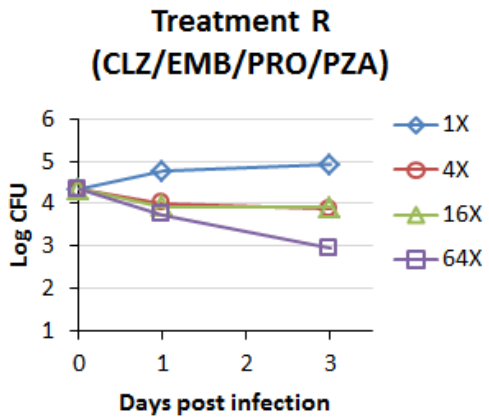

Treatment H
(PZA)
Treatment I
(RIF)
Treatment J
(SQ109)
Treatment K
(TMC207)
Treatment L
(CLZ/EMB/PRO)
Treatment M
(CLZ/EMB/PRO)
Log CFU
Days post infection
1X
4X
16X
64X

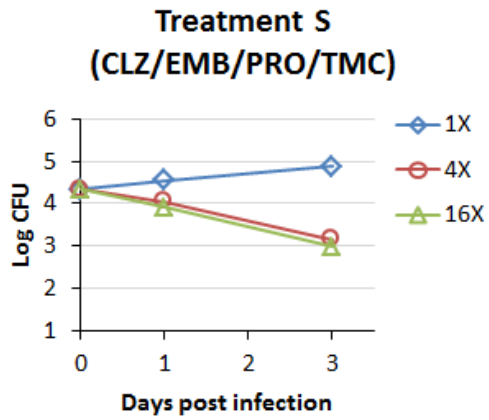

Treatment N
(CLZ/PRO/PZA)
Log CFU
Days post infection
1X
4X
16X
64X

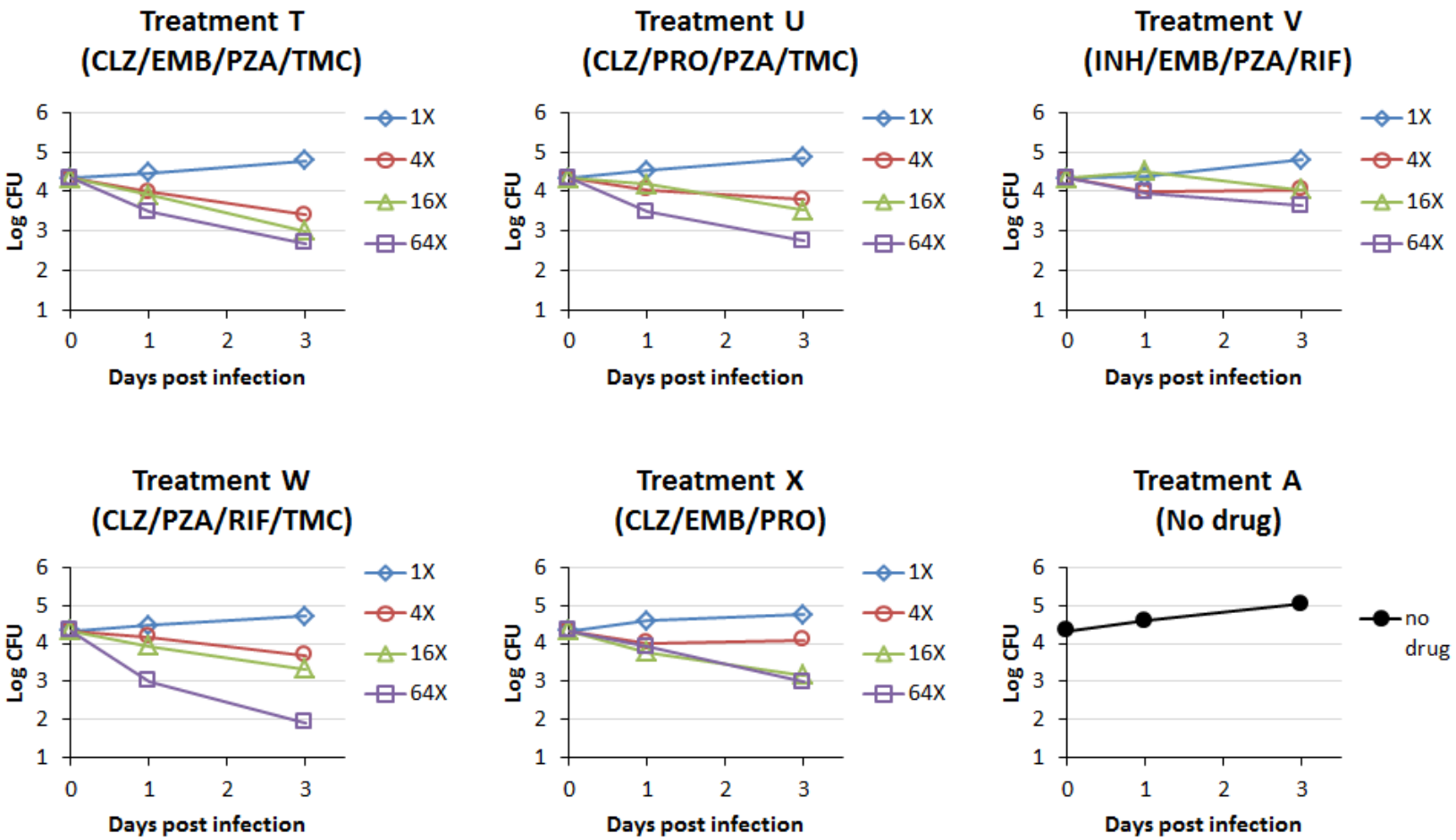

Treatment O
(CLZ/PRO/TMC)
Log CFU
Days post infection
1X
4X
16X
64X


Treatment P
(EMB/PRO/TMC)
Log CFU
Days post infection
1X
4X
16X
64X


Treatment Q
(EMB/PRO/PZA/TMC)
Log CFU
Days post infection
1X
4X
16X
64X


Treatment R
(CLZ/EMB/PRO/PZA)
Log CFU
Days post infection
1X
4X
16X
64X


Treatment S
(CLZ/EMB/PRO/TMC)
Log CFU
Days post infection
1X
4X
16X


Treatment T
(CLZ/EMB/PZA/TMC)
Treatment U
(CLZ/PRO/PZA/TMC)
Treatment V
(INH/EMB/PZA/RIF)
Treatment W
(CLZ/PZA/RIF/TMC)
Treatment X
(CLZ/EMB/PRO)
Treatment A
(No drug)
Log CFU
Days post infection
1X
4X
16X
64X
no drug

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