IDENTIFYING ANTIMICROBIALS AND THEIR MECHANISM OF ACTION USING PHENOTYPE MICROARRAYS

INTRODUCTION

The advent of multiple drug-resistant microbes has prompted renewed interests in finding novel pharmacophores to treat infectious disease. Biochemical and whole cell screening are currently being used to discover novel anti-microbials from natural products and chemical libraries. After an inhibitor is found, two immediate questions arise:

1. Is the natural product a novel antimicrobial?
2. What is the mechanism by which the novel inhibitor prevents microbial growth?

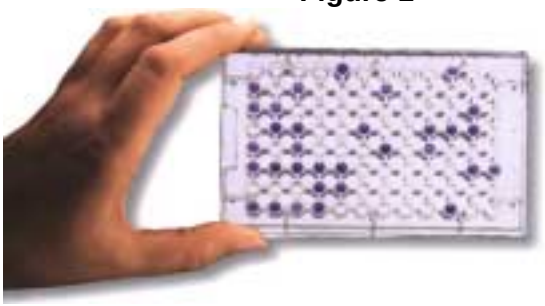
Determining the identity of an active natural product can be a lengthy process. Repeated cycles of time consuming chemical fractionation and bioassays are needed to purify a chemical moiety for physical identification methods. Such time investments are wasted if the natural product is already described.

For novel antimicrobials, a large effort is also needed to determine their mechanism of action (MOA). Discovering an inhibitor by biochemical screening does not guarantee that it will inhibit its presumed target *in vivo*. Genetic and biochemical approaches are labor intensive and may fail to discover a MOA. Biolog's Phenotype MicroArray (PM) technology offers a unique way to identify natural product hits and to infer a MOA of an inhibitor.

PHENOTYPE MICROARRAY TECHNOLOGY

PM technology is a cellular analysis system that combines proprietary assays, high-throughput instrumentation (OmniLog™), and software. The assays are pre-filled and dried in 96-well microplates that can monitor chemical sensitivities. Cell response in each assay well is determined by the amount of color development produced by reduction of a tetrazolium compound (Figure 1).

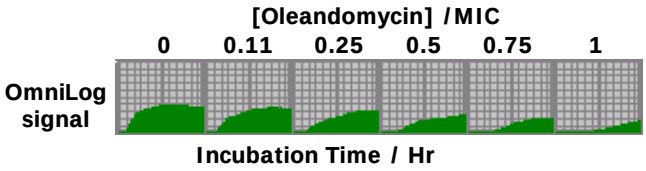
Figure 1



Cellular Response in a PM Plate

The accumulated color is measured over time in an OmniLog that can simultaneously track up to 4,800 independent assays. A kinetic response curve, which parallels microbial growth, is generated so that a growth parameter can be extracted (Figure 2).

Figure 2



OmniLog Response Curves

S. aureus ATCC29213 incubated with oleanomycin and monitored in an OmniLog at 36°C for 48 hours. Minimal inhibitory concentration (MIC), in this example, is defined as the absence of growth in a microtiter well after 18 hours at 36°C in the dark.

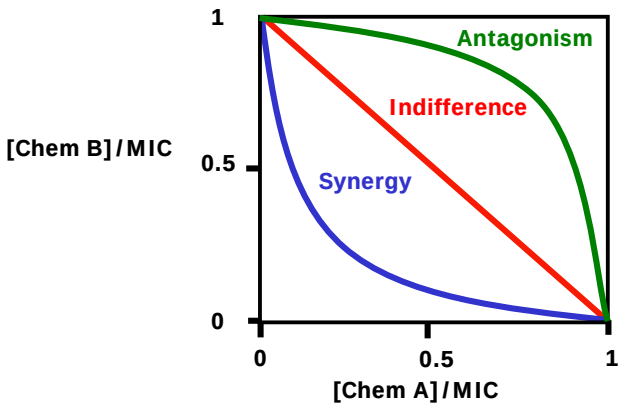
The ability to measure microbial growth kinetics at a variety of inhibitor concentrations allows researchers to quantify biological effects to an unparalleled degree. Biolog PM technology takes advantage of OmniLog growth data to identify known chemicals and to infer MOA for inhibitors.

PRINCIPLES OF CHEMICAL IDENTIFICATION

The ability to identify chemicals and infer their MOA rests on Biolog's ability to generate high quality isobolograms. Isobolograms are graphs that display the interactions that two inhibitors have on microbial growth. Typically, isobolograms are generated by mixing two chemical inhibitors in different proportions at fractional minimal inhibitory concentrations (MICs) and then determining if those combinations prevent microbial growth. A line is then drawn to separate those combinations of chemical concentrations that allow growth from those that completely inhibit growth.

Depending on the chemicals, one of three interactions can be observed based on the shape of the line drawn in the isobologram. Indifference (or additivity) is recognized when the inhibition by one chemical can be added to the inhibition caused by the other. Synergy is recognized when one chemical increases the inhibitory effects of the other. Antagonism, its converse, occurs when one chemical lessens the inhibition caused by the other. These effects need not be symmetrical. In addition, the degree of synergy and antagonism can be quantified (Figure 3).

Figure 3

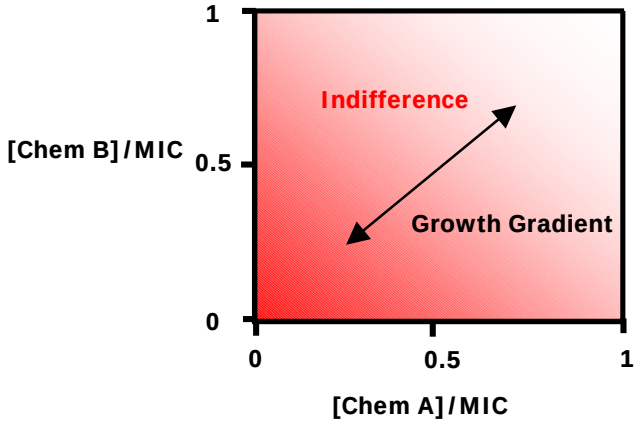


Classical Isobolograms and Chemical Interactions

Lines indicate minimum concentrations at which the combination of chemical inhibitors A (Chem A) and B (Chem B) have completely inhibited bacterial growth. Chemical inhibitors are present as a fraction of their minimal inhibitory concentration (MIC).

Although useful, classic isobolograms ignore the effects inhibitors have on the rates of microbial growth. Biolog's PM technology captures these growth rate changes over a range of different chemical combinations. This enables the construction of detailed isobolograms that map gradients of microbial growth as a function of chemical concentrations (Figure 4).

Figure 4



Biolog Isobolograms use Microbial Growth Rates to Describe the Interactions between Two Antimicrobials

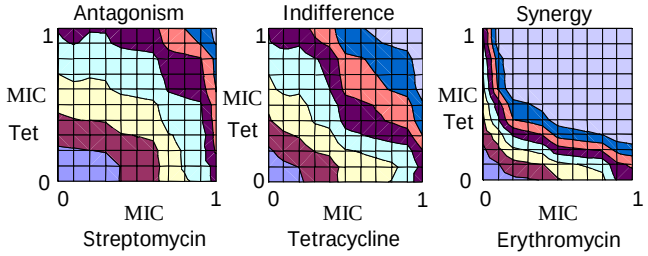
Gradient of high (red) and low (white) levels of microbial growth observed at different concentration combinations of chemical inhibitors A (Chem A) and B (Chem B). Chemical inhibitors are present as a fraction of their minimal inhibitory concentration (MIC), defined as a maximal growth rate observed at 24 Hr. Normal maximal growth rates occur at 7 Hr.

The magnitude of synergy, or antagonism, between any two inhibitors can be quantified. Using an array of chemicals, a scoring matrix based on isobologram data is generated for each inhibitor. These matrices are then used in clustering algorithms to group the inhibitors based on their pattern of synergy or antagonism. A chemical (or natural product) would be identified based on matching its isobologram matrix score to one of the entries in a database. Novel inhibitors that cluster within a group of inhibitors are inferred to have the same MOA.

METHOD

S. aureus (or another model cell type) is mixed with known antibiotics at fractional MICs (0, 0.25, 0.5, 0.75 and 1). The treated cells are added to one chemical sensitivity PM plate that contains an array of inhibitors also at fractional MICs. The growth of the bacteria at each combination of inhibitor concentration is recorded in an OmniLog over 48 Hr. Gradient isobolograms are then constructed using the growth data (Figure 5 is an example of representative data).

Figure 5

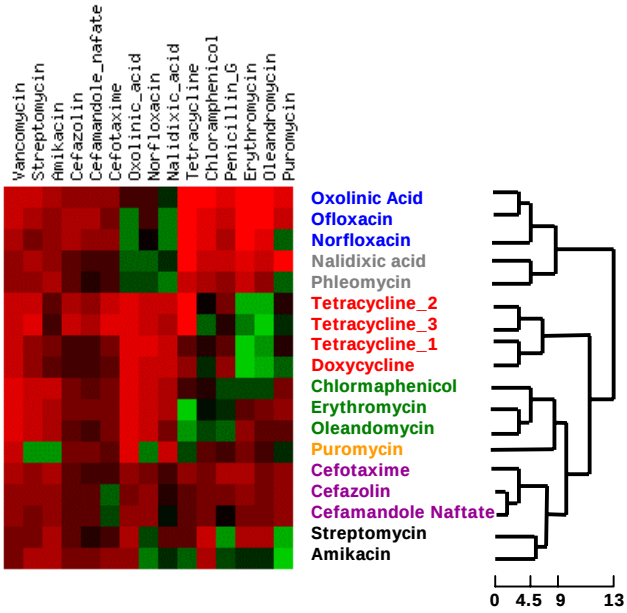


Biolog OmniLog PM Isobolograms

S. aureus strain was ATCC29213. Protein inhibitors streptomycin, tetracycline and erythromycin displayed antagonism, indifference and synergy, respectively, in combination with tetracycline. Lines in graphs represent inflection times extracted from OmniLog growth data (in Hr): 6-10 (blue), 10-14 (maroon), 14-18 (yellow), 18-22 (light green), 22-26 (purple), 26-30 (salmon). 30-34 (dark blue) and 34-38 (light purple). MIC is defined as a maximal growth rate occurring at 24 Hr.

Using Biolog isobologram data, a synergy or antagonism magnitude is calculated. These values are used in generating a matrix that describes the chemical interaction between the chemicals in the PM plate and the added inhibitors being tested. The matrix is imported into a standard clustering program to generate the following grouping (Figure 6).

Figure 6



Biolog Isobologram Data used to Group Known Antibacterials against *S. aureus*

Synergy (green), antagonism (red) and indifference (dark red, black or dark green) observed between inhibitors in the PM plate (top row) was used to differentiate known inhibitors (right side). Synergy and antagonism values were derived from Biolog isobolograms and clustered using the manhattan distance complete linkage (maximal distance) algorithm. *S. aureus* strain ATCC29213 was used. Discernable groups of inhibitors are color-coded. The number following tetracycline refers to the day it was tested.

The chemicals in the PM plate (Figure 6; top row) were used to differentiate known inhibitors (Figure 6; chemicals in the column). Based solely on the matrix provided by the discriminatory chemicals in the PM plate, Biolog isobologram data were able to cluster the inhibitors by MOA. Indeed, chemicals with identical or nearly identical structures group together as demonstrated by the seven groups:

1. All the topoisomerase IV inhibitors (ofloxicin and nofloxacin and oxolinic acid).
2. DNA gyrase (nalidixic acid) and DNA nicking agent (phleomycin).
3. Tetracyclines (tetracycline and doxycycline).
4. Macrolides (oleandomycin and erythromycin) that may be separated from chloramphenicol.
5. Puromycin.
6. All of the cephalosporins (cefamandole naftate, cefazolin and cefotaxime).
7. Aminoglycosides (streptomycin and amikacin).

In *S. aureus*, the separation of norfloxacin, oflaxacin and oxolinic from nalidixic acid may be due to differences in their MOA. Fluoroquinones like norfloxacin inhibit topoisomerase IV and have a different cellular MOA than nalidixic acid which inhibits DNA gyrase. The four groups of protein synthesis inhibitors observed are not unexpected. These inhibitors have diverse MOAs, including mis-incorporation of amino acids into proteins, chain initiation and chain termination.

Reproducibility of this analysis is demonstrated by the clustering of tetracycline, each tested on a different day. All three of the tetracycline entries grouped together along side its structural analog doxycycline.

Summary

Bacteria are exquisitely sensitive to their chemical environment. Thus, the interaction of the bacteria's biochemical machinery and inhibitory chemicals will dictate its growth characteristics. Biolog uses OmniLog data to capture growth information, which can be exploited to generate a high-resolution isobologram. The synergy and antagonism magnitudes taken from such isobolograms can be used to cluster chemicals with known mechanisms of action (Figure 6). Such chemical information can be used to assign MOA by inference and can be used to identify chemicals whose synergy/antagonism pattern (or fingerprint) is already in a database.

Even greater sensitivity can be obtained with Biolog gradient isobologram data. Expansion of the chemical library will enable more resolving power between and within inhibitor classes. Additional resolving power may be obtained considering the detailed shapes of the isobologram curves. Using different subsets of chemicals in clustering will further increase resolution within classes of inhibitors.

Phenotype MicroArray technology can be used with a variety of bacteria and fungal species, including chemical sensitive mutant strains (*tolC* or *acrAB*) that can reduce the amount of material needed for testing.

A database is being generated using *Staphylococcus aureus* to report on antibacterial activity. A variety of other bacterial species and yeast could also be employed to make similar databases.

For more information visit our website www.biolog.com or contact Biolog, Inc.