Guiding Structure Activity Relationship (SAR) Analysis using Phenotype MicroArrays

Introduction

Continual use of antibiotics has led to multiple drug-resistant microbes. These "super-bugs" leave the worldwide clinical community with fewer options for treating infectious disease. Researchers now need better biochemical and whole cell screening tools to help them discover novel pharmacophores that inhibit bacterial and fungal growth. By testing many derivatives of one pharmacophore, structure activity relationships (SARs) can be constructed to guide further medicinal chemistry efforts in producing viable antimicrobial drug candidates.

Despite their utility, SARs do not link drug candidates to their site of action within a cell. Four major approaches have been used to link chemicals within a SAR to their *in vivo* target:

1. Correlate the biological potency of a drug candidate with its level of target inhibition *in vitro*.
2. Create precise genetic changes to the presumed target to change the biological effect of the drug candidate.
3. Map resistance to a drug candidate to the gene encoding its target.
4. Use whole cells and radioactive tracers in experiments to identify the most sensitive biochemical pathway affected by the drug candidate.

Each of the above approaches is labor intensive and has its own limitation.

1. Correlative biochemical methods do not establish a link to the *in vivo* target.
2. Predicting which target to change and what change will alter its drug susceptibility is not likely to be successful.
3. Generating resistant mutants may be very rare if the target is multi-copy or genetically constrained.
4. Isotope experiments are difficult to interpret because their narrow focus ignores many other cellular biochemical pathways that may have been affected.

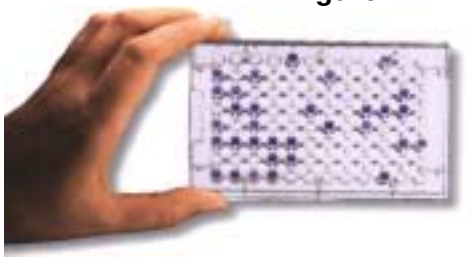
For SARs to be useful in drug development it is critical to link a drug candidate with its presumed *in vivo* target. Additional information indicating the specificity of this drug-target interaction will help guide the SAR, especially at branch points where chemical modification may have drastically altered the original pharmacophore.

Phenotype MicroArray Technology

Biolog has employed its Phenotype MicroArray (PM) technology to generate a unique view of target and drug interactions within a cell. The PM technology itself is a cellular analysis system that combines proprietary assays, high-throughput instrumentation, (the OmniLog™), and software. The assays are pre-filled and dried in 96-well MicroPlates and can monitor, either directly or indirectly, a variety of cell functions. Phenotypes analyzed include: 1) transport functions, 2) catabolism of carbon, nitrogen, phosphorus, and sulfur, 3) biosynthesis of small molecules, 4) cellular respiratory functions, 5) osmotic and pH stress, and 6) an array of chemical sensitivities.

The system is based on utilizing the exquisitely sensitive microbe (bacterial or yeast) as a readout of complex chemical interactions. The cellular response in each assay is determined by the amount of color development in the well produced by reduction of a tetrazolium compound (Figure 1).

Figure 1



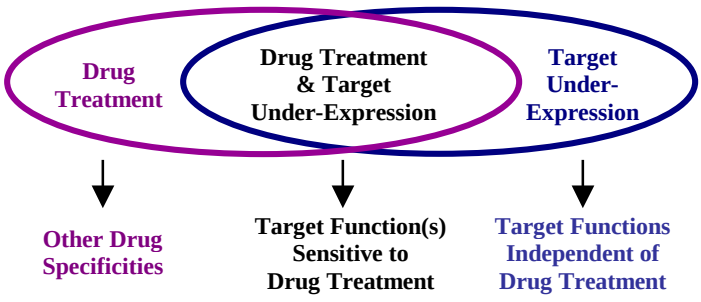
Cellular Response in a PM Plate

The accumulated color is measured over time in an OmniLog that can simultaneously track 4,800 phenotypic tests. Kinetic patterns of each cellular response create a unique PM fingerprint that essentially reports on interaction of the cell's biochemical machinery with its environment.

Basis of Guiding SARs

Target and drug interactions can be described and compared based on the phenotypes in a PM fingerprint that are altered either by drug treatment or by lowering the concentration of the cellular target protein using genetic means (Figure 2).

Figure 2



PMs can easily and efficiently link the biochemical potency of a drug *in vivo*, predict which drug will be effective at the target, and provide information on other biochemical pathways affected by the drug (i.e., side effects). Solving the limitations of other SAR approaches will lead to dramatic improvement in early stage drug discovery.

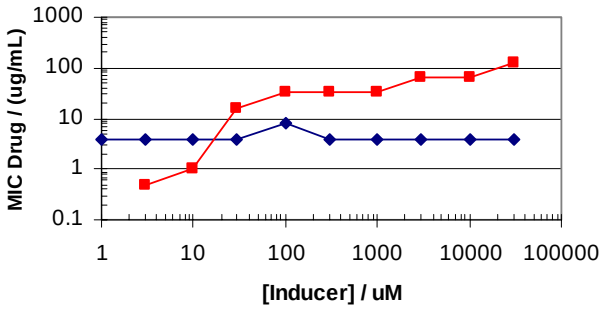
Altered phenotypes that arise upon different treatments can be placed into one of three groups:

1. Phenotypes affected by both drug treatment and target depletion comprise those target functions sensitive to drug and link the drug and target by their common effects. The number of phenotypes altered by drug treatment that overlap the number of phenotypes changed by target depletion is a measure of the drug candidate's specificity and would guide the SAR.
2. Phenotypes that change only upon target depletion may reflect additional biochemical functions of the protein or other physical interactions it makes within the cell. Such phenotypes may indicate that additional target domains are available for drug opportunities.
3. Phenotypes that change solely upon drug treatment may reflect other secondary or side effects of the drug.

EXAMPLE

Two strains of *E. coli* were used to demonstrate that PM technology could distinguish phenotypes altered by drug treatment and target depletion. One strain is the parental, which maintains normal levels of the target protein. The other strain is a derivative containing an essential target protein whose level of expression is regulated by the concentration of an inducer. The target protein activity is known to be inhibited by Drug *in vitro* (IC_{50} = 1.2 ug/mL).

Figure 3



Differential Drug Sensitivity Displayed by an *E. coli* Mutant Under- and Over-Expressing Target Protein

Mutant strain (Red) contains an essential target protein whose expression is controlled by inducer and is compared to its parental strain (Blue). Drug inhibits the activity of the inducible target protein *in vitro* at 1.2 ug/mL. Sensitivity of bacteria is measured by the minimal inhibitory concentration (MIC).

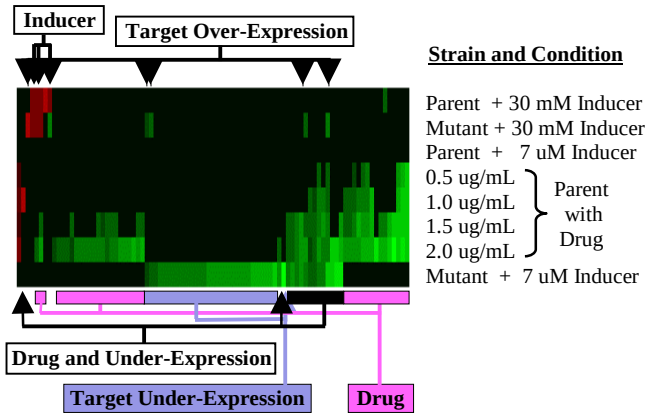
Inhibition of target function by this Drug *in vivo* is demonstrated by differential drug sensitivity of the two bacteria. Whereas the MIC value in the parental strain is constant, the MIC value in the target-depleted strain is determined by the concentration of the inducer chemical (Figure 3).

The mutant strain is dependent on the inducer for growth, needing greater than 3 ug/mL inducer. It displays both increased drug sensitivity at 7 uM inducer (MIC = 0.8 ug/mL) and increased drug resistance at 30 mM inducer (MIC = 124 ug/mL) which define states of under- and over-expression, respectively. The parental strain, as expected, maintained constant drug sensitivity at all inducer concentration (MIC = 4 ug/mL).

METHOD

Phenotype MicroArrays were used to analyze just the chemical sensitivity phenotypes of both parental and mutant strains in media containing 7 uM and 30 mM inducer. Target levels were verified in separate Drug sensitivity experiments for each PM data set collected for analysis to ensure that decreased and increased target protein concentration occurred only in the mutant (data not shown). Chemical sensitivity phenotypes that were altered in any given condition were determined by comparing their cellular responses with those of the parent at 7 uM inducer. The degree of stimulated or inhibited growth for each phenotypic response that was altered were then compared (Figure 4).

Figure 4



Phenotypes Altered by Drug Treatment and Under- and Over-Expression of Target Protein

Altered chemical sensitivity phenotypes determined with Biolog Panels PM11-PM20. All PM MicroArray data are expressed relative to the parental strain at 7 uM inducer. Increased, decreased or no change in the chemical sensitivity for each phenotypic test in the PM fingerprint of mutant and parental *E. coli* is indicated by green, red and black boxes, respectively. Darkness of red or green indicates magnitude of change. Mutant was shown to over- and under-express target protein at 7 uM and 30 mM inducer, respectively, by sensitivity to Drug (data not shown). Phenotypic groups are boxed.

Compared to the chemical sensitivity PM fingerprint of the parental strain at 7 uM inducer, many phenotypes were altered upon target protein under-expression in the mutant at 7 uM inducer (Figure 4; Blue and Black Boxes). Progressive numbers of phenotypes were also altered when the parent was treated with drug at increasing concentrations (Figure 4; Pink and Black Boxes). However, few phenotypes changed when the mutant over-expressed the target protein or when the parental strain was incubated at 30 mM inducer.

Five groups could be discerned based on the phenotypic changes observed for the mutant expressing different levels of target protein and parental strain treated with Drug that is known to inhibit target function:

1. Drug and under-expression altered phenotypes (Figure 4; Black box and arrows) allows the researcher to focus on cellular phenotypes that indicate drug and target interactions *in vivo*, which may guide the SAR. Members of a SAR group should maintain this common set of altered phenotypes that links drug treatment and target depletion.
2. Target under-expression altered phenotypes that are independent of drug altered phenotypes (Figure 4; Blue box) indicate other domains on the target that interact within the cell. These domains may be possible binding sites for other novel pharmacophores.
3. Drug altered phenotypes that are independent of target under-expression (Figure 4; Pink box) indicate other drug specificities. The number of drug altered phenotypes progressively increased with increasing drug concentration. This group of phenotypes may indicate multiple cellular drug interactions.
4. Inducer altered phenotypes were few in number and are defined as those changes that are common for both parental and mutant strain at 30 mM inducer. The PM technology sensitively scored the concentration change of the non-metabolized inducer.
5. Target over-expression altered phenotypes were also few in number. Four of five altered chemical sensitivity phenotypes caused by over-expression were also altered by under-expression. Of these four phenotypes, two were in common with drug exposure, again indicating a link between drug and target.

Lists can be generated of known inhibitors that comprise those chemical sensitivity phenotypes that were altered upon drug treatment. The lists will include those inhibitors that act synergistically (Figure 4; Green boxes) or antagonistic (Figure 4; Red boxes). These chemical interactions may be useful in downstream efforts of guiding clinical development decisions of the antimicrobial drug candidate.

SUMMARY

Phenotype MicroArrays can be used to identify traits of a cell that change when a drug target gene is under- or over-expressed, or when it is challenged with a drug. These sets of altered phenotypes can be compared rapidly to determine which phenotypes change in response to both target under-expression and drug treatment. Such common phenotypic changes support the hypothesis that the drug is interacting with the target *in vivo*. Moreover, the specificity of this interaction can be assessed based on the number of altered phenotypes caused by drug treatment that are in common with those altered by target under-expression.

Demonstrating the link between target and drug is just one benefit in anti-microbial and anti-fungal research afforded by PM technology. Medicinal chemistry efforts can be guided using PM technology. Members of a SAR series should consistently alter the same set of phenotypes. Those derivatives that induce different phenotypic changes indicate departures from the original pharmacophore.

The capture of growth kinetics by Biolog's OmniLog instrument, offers a very sensitive readout of the bacterial and fungal chemical environment. The change in sensitivity for a given chemical phenotype in a PM panel is actually a report on the interaction of that chemical with either the cell and Drug or the cell whose target is under- or over-expressed. By using PM technology to guide SARs, researchers can also collect chemical synergy and antagonism information. Such information may provide important advantages in formulating new clinical therapies.

PM technology can be used with a variety of systems to modulate gene expression, including promoters regulated by induction (arabinose and xylose) or antisense RNA. Additionally, targets whose function is temperature sensitive may also be used. Phenotype MicroArrays are highly sensitive, accurate, and efficient for documenting the common phenotypes that are affected upon drug treatment and target alterations. They are currently being used for *E. coli*, *Staphylococcus aureus, and Saccharomyces cerevisiae*.

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