Minireview

Cytological profiling: providing more haystacks for chemists' needles

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Published: 29 July 2005

Genome ***Biology*** 2005, **6:**228 (doi:10.1186/gb-2005-6-8-228)

The electronic version of this article is the complete one and can be found online at http://genomebiology.com/2005/6/8/228

Abstract

Conventional high-throughput 'chemical genetic' screening seeks to identify small-molecule inhibitors of a specific protein or pathway. A recent study describes how unbiased screening of cellular morphology, followed by affinity purification to identify targets of compounds with interesting effects, can lead to the identification of novel inhibitors.

Scientists in the pharmaceutical industry are in constant search of new drugs that can activate or inhibit their target molecule of interest. Combinatorial chemistry has allowed the synthesis of huge numbers of new compounds by combining sets of building blocks, and researchers are busily sorting through this haystack of drugs for the perfect needle - a drug specific for the intended target. The usual approach to this search is high-throughput screening of a large library of compounds (500,000 to 1,000,000 compounds is typical) in an assay for a single, specific effect, such as inhibition of an enzyme. Although this strategy allows large numbers of molecules to be screened, it measures only a limited range of biological effects. The vast majority of compounds screened will fail to show the desired effect and will be discarded as useless. Thus, molecules that may have interesting and useful characteristics will be missed if they do not have the specific effect that is being measured in the screen.

Screening strategies that measure the effects of compounds on biological pathways, rather than single proteins, cover a broader subset of biological space. As a result, a larger number of active compounds may be identified from a given chemical library. Several enzymes or proteins could be targeted in such an assay. For example, targeting of a receptor, adaptor protein, or transcription factor in one pathway could all be detected in a single assay for activation of a reporter construct that responds to the transcription factor. Porter and colleagues [1] performed such a screen for agonists of the pathway downstream of the extracellular signaling molecule Hedgehog, by looking for small molecules that caused up-regulation of a Hedgehog-responsive promoter. The agonist identified in this screen acts by binding Smoothened, a Hedgehog activator protein related to G-protein-coupled receptors, but agonists of any of the components of this signaling pathway could have been identified in the screen [1].

Cytological screening for active compounds

A recent paper by Adams, Shokat, and colleagues [2] goes a step beyond screening that uses a specific pathway to broaden further the biological spectrum of activities that can be detected in a single screen. In their study [2], a relatively small number (107) of compounds that are structurally similar to known kinase inhibitors were selected for screening. Rather than screening each of these molecules for inhibition of a specific enzyme or pathway, or for a specific phenotype produced, the authors instead searched for small molecules that perturbed any measurable aspect of cell morphology using a cytological profiling approach. Five cell types (four cancer cell lines plus endothelial cells) were treated with each compound at a range of doses, and the effects on cell morphology were analyzed using an automated imaging and analysis system (CytoMetrix™ by Cytokinetics, San Francisco, USA). Cells were stained with reagents

that detected DNA, the Golgi apparatus, and microtubules (see Figure 1). The Cytometrix system was then used to measure a large number of different parameters, including the morphology of the cell and organelles (nuclei, microtubules, and Golgi), staining intensity, and localization of organelles in each cell type for each treatment condition [2].

The large number of images collected and measurements made in this system yielded a large volume of data, which was condensed using a method called principle component analysis. This statistical-analysis method converts the many variables measured into a smaller number of 'principle components', which represent the majority of the variability in the dataset with a small number of parameters. The principle component analysis grouped the active compounds into three 'phenotypes', two of which were characteristic of compounds that stabilized microtubules or inhibited protein kinases. The final phenotype was produced by only one compound, called hydroxy-PP, and was distinct from that of compounds with known mechanisms of action. Although structurally related to known kinase inhibitors, hydroxy-PP caused a phenotype distinct from other kinase inhibitors, suggesting that hydroxy-PP is likely to act through a distinct mechanism [2].

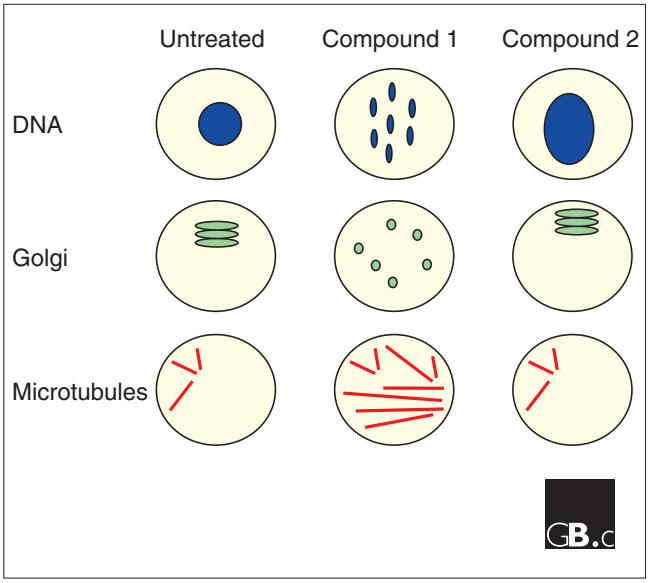

Figure 1
The principle of cytological profiling. Cells are treated with test compounds at varying concentrations and then stained with reagents that detect various cellular proteins or organelles. In this example, cells are treated with reagents that detect cellular DNA, the Golgi apparatus, or microtubules. Compound 1 shows a profile characteristic of a microtubule stabilizer, which leads to longer microtubules but dispersed DNA and Golgi apparatus as a result of the mitotic arrest that is a secondary consequence of microtubule stabilization. Compound 2 has more subtle effects, inducing changes in nuclear size and shape, with little effect on microtubules and only a small shift in the position of the Golgi. In an actual experiment (such as in [2]), cytological changes are measured at a variety of different drug concentrations, and a variety of measurements are made on each image. This complex dataset is then reduced using various statistical approaches to identify the key parameters that change as a function of drug concentration.

To determine how hydroxy-PP induced this unique phenotype, an affinity-purification approach was pursued to identify the biochemical target of the compound. Affinity chromatography using small molecules can often be very challenging if a compound binds its target weakly or if the compound binds many proteins nonspecifically. To circumvent these problems, the authors [2] synthesized a pair of structurally related compounds that could be attached to a solid-phase matrix. One compound retained activity, whereas a highly structurally related compound (differing only by the absence of a hydroxy group) was used as an inactive control. Cell lysates were applied to both columns, and many different wash conditions were tested until proteins were retained specifically on the matrix containing the active compound and not the other matrix. This approach yielded only a few potential binding proteins, one of which was identified as carbonyl reductase 1 (CBR1), an NADPH-dependent oxidoreductase that has been implicated in detoxification of foreign compounds and the metabolism of cellular messengers containing ketone groups, such as prostaglandin E.

To confirm that hydroxy-PP is indeed an inhibitor of CBR1, the authors [2] showed that the compound was capable of inhibiting CBR1 in a biochemical assay *in vitro*, with a concentration that gives 50% inhibition (IC_{50}) of 788 nM. The compound was also found to be a potent inhibitor of cellular kinases such as the c-Src family member Fyn (IC_{50} = 5 nM), however, limiting the usefulness of hydroxy-PP for further studies. Structure-based design was therefore used to design a more specific inhibitor, termed hydroxy-PP-Me, which was shown to lack kinase-inhibitory activity. This inhibitor was then used to characterize more closely the biological role of CBR1 and to evaluate the potential therapeutic utility of the inhibitor.

Why would an inhibitor of CBR1 be medically useful? The oxidoreductase activity of CBR1 is thought to be responsible for the cardiotoxicity of anthracycline anti-cancer drugs such as daunorubicin. CBR1 metabolizes daunorubicin into daunorubicinol, which lacks anti-cancer activity and has adverse affects on the heart. A specific inhibitor of CBR1 could be useful in cancer therapy to maintain daunorubicin in its active form and to prevent the toxicity associated with treatment with anthracycline-type drugs. Tanaka *et al.* found [2] that treatment of cancer cells with hydroxy-PP-Me was able to strengthen the cell-killing effect of daunorubicin. No enhancement of cell killing by daunorubicin was observed when a related molecule PP-L, which does not inhibit CBR1, was used. This suggests that CBR1 inhibition enhances killing of cancer cells by blocking metabolism of daunorubicin. Thus, not only may CBR1 inhibition have the potential to block daunorubicin-induced cardiotoxicity, it may also potentiate

cancer-cell killing by maintaining daunorubicin in its active form. Further investigation of the compound's effects in cells revealed that inhibition of CBR1 by hydroxy-PP-Me protects cells against the apoptosis induced by serum withdrawal [2]. This result was confirmed by knocking down the levels of CBR1 using RNA interference, thus identifying a previously unknown role for CBR1 in apoptosis.

The classical method of high-throughput chemical screening covers a broad subset of chemical space but a very narrow subset of biological space. The work of Shokat and colleagues [2] demonstrates that the converse strategy - screening a small chemical library using an assay that measures a large number of potential biological outcomes - can also lead to the identification of novel and specific inhibitors. As described earlier, these researchers [2] also found that compounds with related mechanisms of action, such as kinase inhibition, had similar profiles or phenotypes in the screen. This suggests that it might be possible to use cytological profiling to generate 'fingerprints' characterizing the potential mechanism of action of novel compounds.

Multidimensional profiling

A paper by Altshuler, Mitchison, and colleagues [3] demonstrated that it is indeed possible to generate specific fingerprints of many biologically active compounds through immunofluorescent measurement of the abundance and localization of specific protein markers in cells. In this study [3], HeLa cells were treated with 100 different compounds, all with a known biological target, at a wide range of concentrations. Cells were then fixed and stained with antibodies or dyes that reported on 11 distinct proteins or cellular processes. Automated microscopy was used to collect images of the cells, and computer algorithms were then used to analyze the images and generate cytological dose-response profiles of each drug. These profiles describe both the phenotypic changes induced by each drug and the dose at which these changes occur.

Of the 100 compounds tested in this study [3], 61 showed measurable effects, indicating that a broad range of biological effects can be detected with a limited number of markers. The fact that 39 compounds appeared to have no measurable effect suggests, however, that additional markers beyond the 11 used in this study will be required in order to increase further the sensitivity of the approach. This raises the interesting question of how many markers would be required to detect a perturbation of any biological pathway. To minimize the total number of markers required, it will also be important to identify markers specific to the effects of only one biological pathway. Further optimizing the set of markers will also require a better understanding of how different biological pathways interact with one another.

Of the drugs that did show a response in the Altshuler study [3], those with common targets and mechanisms generally showed similar profiles. Drugs with similar chemical structures but distinct mechanisms of actions showed dissimilar profiles. Thus, this approach is capable of grouping drugs with similar mechanisms of action together, suggesting that it may be useful for predicting the potential mechanisms of action of new compounds and identifying the targets of compounds identified in phenotypic or pathway-based screens. Cellular profiling may also provide useful insights into the potential toxicities of compounds of interest identified in conventional single-target high-throughput screens. In this case, markers that report on activation of cell stress pathways may be especially valuable.

A variety of other profiling approaches have been developed for characterizing the activity and specificity of small molecules at the cellular, protein, or mRNA level [4-10]. How does cytological profiling complement these approaches? First, it provides details of the intracellular localization of marker proteins, making it a sensitive indicator of the many pathways that regulate protein localization. Second, cellular organelles such as the Golgi apparatus or the nucleus, which depend on the activity of many pathways for proper organization and function, may provide very sensitive readouts of functional perturbations. This property may make cytological profiling useful for detecting toxic or off-target effects of molecules of interest. Finally, imaging-based approaches provide information on a cell-by-cell basis; they do not average effects over entire populations of cells as is the case for RNA-based or protein-based (proteomic) approaches. This may enable more sensitive detection of effects in subpopulations of cells, and it may enable effects to be detected at lower doses of compounds.

Most small-molecule screening has been performed with large chemical libraries tested against specific biological pathways or targets. This 'biology-centric' view of screening serves an important purpose: to identify small-molecule probes or compounds of interest to biologists or those working to cure a specific disease. This approach leaves much to be desired, however, if you are a chemist who has recently designed a novel chemical library and you want to understand how each molecule in your library might perturb biological function. In this case, 'chemistry-centric' approaches are needed, which can identify the compounds in the library that have any biological activity at all. Cytological profiling, which takes an unbiased approach to discovery of biological activity, provides an excellent starting point for discovering novel biological activities or therapeutic potential in a collection of novel molecules. The results of such profiling studies may be especially useful in helping to guide which areas of chemistry space need to be more fully developed and explored [11].

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