Molecular Interactions on Microarrays

Vehary Sakanyan at ProtNeteomix highlights the advantages of using small molecule microarrays in drug discovery programmes

The spiralling cost of drug development calls not only for political and fiscal decisions, but also for the integration of cost-effective technological innovations into discovery programmes. The emergence of miniaturised arrays designed to detect numerous small molecule interactions with target proteins in a single assay opens up promising prospects for the pharmaceutical industry. This article reviews some recent successful attempts to develop and apply small molecule microarrays (SMMs) to drug discovery.

SMALL MOLECULES AND DIVERSITY OF HTS

Small molecules, which specifically recognise and bind to druggable sites on proteins, appear to be the most promising substances for developing a new generation of effective drugs against many diseases. High-throughput screening (HTS) of chemical compound libraries is primordial for the successful discovery of small molecule drug candidates. Until recently, HTS has been primarily used to detect compounds that modulate a given function of a target protein. This was a rather laborious task that required an expensive screening infrastructure that had to be adapted to each drug discovery project. In recent years, the methodology of cell-based and biochemical screens has been enriched by the advent of genomic, proteomic and metabolomic approaches (see Figure 1). The virtual screening of compound libraries has become almost indispensable for drug development, as it predicts the interactions of small molecules with druggable sites in proteins of which the 3D structure had been resolved, and thereby helps to reduce the number of molecules that have to be screened. However, straightforward screening of large numbers of molecules is still desirable in order to compare their ability to bind to target proteins, and to select the most appropriate compounds in a quick and cost-effective fashion. This new approach is reflected in spatially-addressable SMMs, which can replace hundreds of thousands of individual reactions by a single binding assay that is able to detect numerous interactions with target proteins.

SMALL MOLECULE MICROARRAY TECHNOLOGY

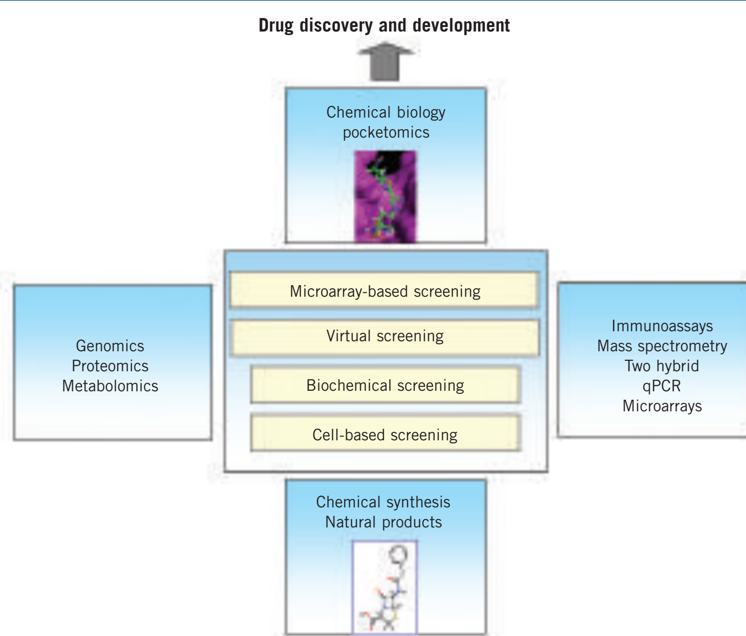
The format of a SMM on a glass microscope slide is perfectly amenable for HTS; it involves robotic printing of molecules onto the support, and a sensitive readout of signals from the small molecule-protein complexes formed in microspots (see Figure 2a, page 76). Following a pioneering study of Schreiber *et al* (1), diverse chemical and photoreactivation methods have been developed to immobilise small molecules onto appropriately activated surface on glass slides (2). Using uniform linker-oriented strategy appears to improve the accessibility of target sites in proteins to small molecules. Since these immobilisation approaches rely on specific functional moieties in molecules during activation, they can consequently result in the formation of by-products, which can affect the homogeneity of the surface and lead to false-positives in the spots. This means that covalent coupling requires time-consuming procedures to protect sensitive groups from unpredicted modifications. Sensitive, one-colour, fluorescence detection is typically used to identify the small molecules bound to targets in spots by supplying labelled proteins, secondary antibodies or tag-mediated approaches (see Figure 2, page 76).

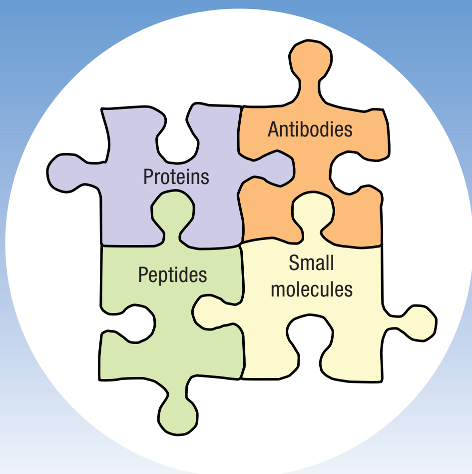
USING MICROARRAYS TO SCREEN COMPOUND LIBRARIES

SMMs prepared by chemoselective immobilisation have been used to screen large compound libraries, and good affinity ligands have been detected for proteins of therapeutic importance, such as caspase (3), tumour necrosis factor α (4) and cathepsin (5).

The utility of microarrays for HTS is limited by the lack of a generally applicable approach to immobilising various chemical structures onto the support. This

Figure 1: Various HTS approaches developed for drug discovery





✓ Compound library screening
✓ Lead discovery
✓ Lead optimisation
✓ Target validation
✓ Drug toxicity

Cost-Effective Microarray-Based Solutions for Drug Discovery & Development

The main challenge facing the pharmaceutical industry is the extremely high overall cost and time consuming nature of drug discovery and development. ProtNeteomix provides faster and cheaper solutions based on innovative miniaturized arrays for obtaining valuable information about target proteins, and generating effective drugs to combat human diseases.

Our microarrays could be used at different steps of DDD programs, including high-throughput screening, leads identification, selecting the most appropriate candidates for pre-clinical trials. They could also be used to evaluate the safety and efficacy of leads in clinical trials. This new philosophy to DDD should make it possible to speed up drug design, replace expensive in vivo tests by in vitro assays, cut the huge expenditure wasted on evaluating "useless" compounds, as well as demonstrating the strengths of the best drug candidates.

Proprietary Technologies

- Small Molecule Microarrays to screen chemical compound libraries (non-covalent immobilization of molecules)
- Chemically synthesized and phage displayed Peptide Microarrays to detect high-affinity binders to desired targets
- Multiplexed Antibody Microarrays to assess protein expression and phosphorylation
- In vitro assembled Protein Microarrays to evaluate immune response

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Antibodies
Peptides
Small molecules

Compound library screening
Lead discovery
Lead optimisation
Target validation
Drug toxicity

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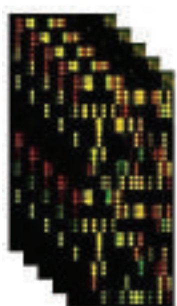
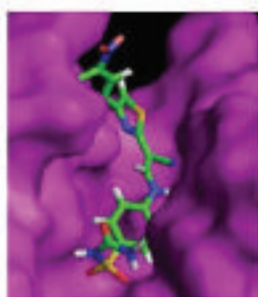
High sensitivity
Low consumption



High-throughput
Multiplex
High sensitivity
Low consumption



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shortcoming can be partially overcome by coupling nucleophilic functional groups of different molecules onto the isocyanate-functionalised surface without forming any deleterious by-products (6). A single-slide SMM has been prepared by printing several libraries, consisting of molecules originating from a diversity-oriented synthesis, natural products and commercial bioactive compounds. Such microarrays representing 10,000 features have been probed with cell lysates bearing an overexpressed target protein. Broad affinity ligands have been detected for the protein FKBP12 that recognises rapamycin, an immunosuppressant agent produced by *Streptomyces hygroscopicus*. The use of cell lysates appears to be appropriate if the protein of interest cannot be purified, or if it undergoes misfolding and loses its binding properties during purification (3,6,7).

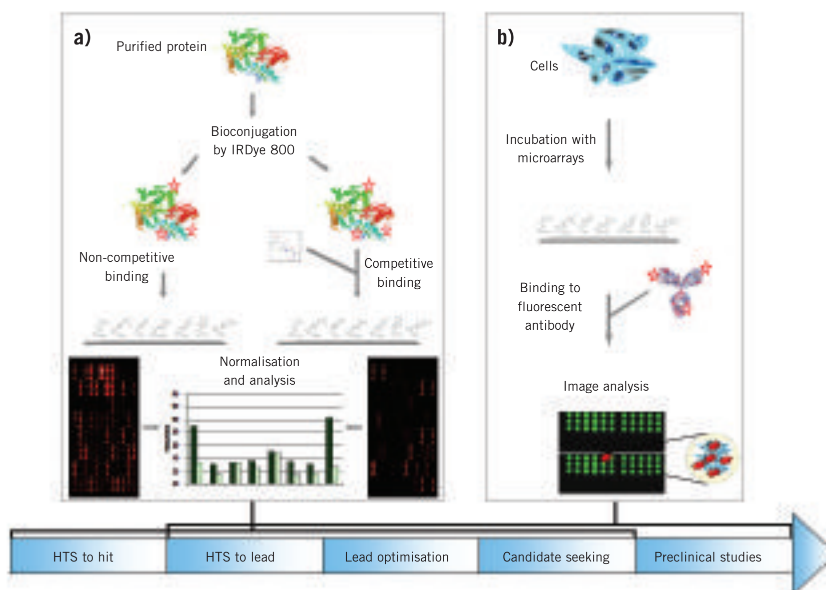
The isocyanate-mediated capture strategy has been extended to complex biological mixtures by coupling the nucleophilic groups of molecules produced in extracts of *S hygroscopicus*, and determining the level of expression of rapamycin and related products (8). The relative contribution of non-specific interactions to the overall interaction response from spots has been estimated by comparing the signal intensity of spots after incubating with free FKBP12 and with the FKBP12-rapamycin complex.

The distinction between specific and non-specific interactions of the molecules selected by screening with SMMs requires downstream confirmation by conventional methods. Recently, the two-colour detection strategy has been applied to immediately distinguish between specific and non-specific signals (9). Both the active and denatured forms of a target protein were reciprocally labelled with two spectrally-distinct fluorescent dyes, and used as probes to assess small molecule-protein interactions in two parallel assays. Using the denatured probe as an internal control simplifies the elimination of non-specific signals. The feasibility of the approach has been demonstrated by ligand fingerprinting of metalloproteases in a synthetic hydroxamate peptide library. The authors estimated the approximate affinity of some molecules by measuring the signal intensity of microarrays after incubating them with metalloproteases at different concentrations. Several binders with dissociation constant of the order of less than 1µM have been selected as promising candidates for developing high-affinity inhibitors of thermolysin and anthrax lethal factor.

Figure 2: Screening small molecules with microarrays, and the potential use of SMMs at different stages of drug discovery

a) Non-competitive and competitive binding assays with a target protein labeled with near-infrared fluorescence dye (for example)

b) Cell assay using a fluorescent antibody to detect the target protein



NON-COVALENT IMMOBILISATION OF COMPOUNDS

Several strategies have been proposed for non-covalent immobilisation of small molecules to fabricate microarrays composed of compounds with a wide range of chemical structures on a single support. One of the first approaches used took advantage of a two-step immobilisation procedure to increase the high-throughput capacity, and to make this screening method quicker to perform than methods that make use of microtitration plates. Small molecules dissolved in dimethyl sulfoxide were first printed on a polystyrene sheet to prepare an addressable microarray. The dried compounds were then diffused into an agar gel sheet (10) or blotting paper (11) containing the embedded target protein, making it possible to assess the effect of small molecules on binding or enzymatic reactions on these supports. Using this method, new inhibitors of HIV integrase, p56lck tyrosine kinase and other proteins have been picked out from libraries consisting of several hundred thousand compounds (10,12).

Another approach exploits the specific fluorous affinity between two fluorocarbon structures: one attached to the fluoroalkylsilanised glass slide; and the other containing small tagged molecules, such as carbohydrates, to be recognised by a protein of interest (13). Fluorous-based SMMs have been used to detect histone deacetylase inhibitors when the orientation of the compounds displayed is important (14). It is noteworthy that this strategy offers the possibility of dissociating non-covalently bound fluorocarbons, and therefore of reusing the slides to immobilise other tagged compounds (15).

In an effort to overcome the limitations of covalent coupling, we turned to improving the physical absorption of molecules onto a gel-covered surface (16). The effectiveness of this simple, non-covalent immobilisation of compounds has been demonstrated in proof-of-concept experiments with β-lactam antibiotics that specifically attack the transpeptidase catalytic site of penicillin binding proteins (PBPs) involved in bacterial wall synthesis. We used near-infrared fluorescence detection, which allowed us to increase the signal-noise ratio and decrease the impact of auto-fluorescence from compounds that emit in the visible fluorescence range (17). Most of the antibiotics tested bound to various ampicillin-sensitive PBPs, whereas no binding to proteins carrying mutations of the catalytic site was observed. Moreover, ampicillin markedly reduced the signal intensity in competition assays for sensitive

PBPs, thereby confirming the specificity of the recognition of the transpeptidase site by the arrayed β-lactam antibiotics.

Next, several hundred molecules, which had been preselected by virtual screening from various compound libraries, were printed on a single slide and incubated with sensitive and resistant PBPs in parallel assays. New molecules belonging to different structural classes and devoid of the β-lactam ring have been identified in these discrete libraries. Some of the molecules selected exhibited an approximate dissociation constant within the micromolar range for both sensitive and resistant PBPs, as estimated from real-time binding experiments using surface plasmon resonance. Furthermore, several molecules inhibited the growth of gram-positive and gram-negative bacteria tested, including some ampicillin-resistant strains (16).

MICROARRAYS AND CELL-BASED ASSAYS

Once the candidate molecule has been selected, its activity in living cells and in animal models is investigated. Different type of microarrays, including antibodies, cells or tissues, can be used to do this. In this context, discrete microarrays composed of pre-selected small molecules are of particular interest for the high-throughput evaluation of their behaviour by incubating with mammalian cell lines (see Figure 2b).

A cell-based assay has been developed for screening large numbers of chemical compounds with microarrays. Small, 200μm diameter discs of poly-(D),(L)-lactate/glycolide impregnated with chemical compounds are printed on a glass slide, and covered with a layer of cells (18). Compounds are slowly released from the spots in contact with the proximate cells, and their effects can be observed using different approaches, including immunofluorescence microscopy. In particular, staining with phospho-specific antibody has revealed a decrease in the fluorescence response from a spot area exposed to rapamycin, indicating the inhibition of signalling pathways in the lung cancer cells used for testing.

REMAINING CHALLENGES?

The kinetics of the binding constants of different spots varies considerably depending on the structural and organoleptic properties of the small molecules and proteins used as probes. In addition, various external factors attenuate the interactions between small molecules and proteins on the interface of solid and liquid phases in microspots, and thereby impair the performance of microarrays. The signal intensity of the spots reflects a whole complex of intervening parameters that makes it unrealistic to attempt a quantitative determination of the affinity of all arrayed molecules. An algorithm that takes into consideration the complexity of a given situation might help to solve the problem. Nevertheless, the operational efficiency of SMMs has recently been remarkably enhanced by: using high-quality chemical libraries, by using a homogenous surface for immobilisation; by optimising the solubility of compounds; by printing equimolar quantities of molecules from higher concentrations; and by performing binding assays under appropriate conditions based on structural data and the location of target proteins in the cell compartments.

Another challenge is that of determining the specificity of the molecular interactions between arrayed compounds

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and target proteins. Using native versus denatured proteins, or competition assays with a molecule with a known mechanism of action, appears to help distinguish between compounds that bind specifically to a druggable site and those that interact with non-druggable or decoy sites on the target protein. It is noteworthy that 'non-specific signals' detected from small molecules bound to decoy sites can suggest potential new drugs if this interaction abolishes an undesirable function of the target; in particular, the high phosphorylation rate of proteins implicated in cancer progression.

Small-molecule microarrays prepared by a non-covalent immobilisation approach look particularly promising for use in drug discovery, as they create favourable conditions for quick, high-throughput and multiplexed screening of different compound libraries. Combining the advantages of high-throughput and high-content screenings in a powerful assay blurs the distinctions between microarray and cell-based concepts. Indeed, new microarray-based cell and/or *in vitro* assays, accompanied by internal quality controls, are in great demand for the reliable assessment of the toxicity of leads in preclinical studies.

CONCLUSION

Recent advances highlight the unprecedented advantages of using small molecule microarrays for the cost-effective screening of large compound libraries. There is no doubt that a range of microarrays combined in a single platform could provide valuable complementary information about the therapeutic potential of small molecules. Such platforms can be expected to become an integral part of discovery programmes intended to identify and validate the most promising drugs.

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