Screensavers: trends in high-throughput analysis

Meeting report

Even, and perhaps especially, in the post-genomic era, the search for new drugs begins with the detection and (hopefully) validation of a novel target, and with the development of an assay for the interaction of an artificial agonist or antagonist – and of its natural ligand, if known – with that target.

As the race to develop new chemical entities with novel pharmacological activity hots up, the major pharmaceutical companies have invested heavily in automation and robotics, and in the high-throughput assaying of large compound libraries for pharmacological activities against drug receptors. The current state of the art in this area was the theme of a recent international conference*, which reflected the intense applied interest in this area by the fact that some 98% of the 1256 delegates were from industrial corporations.

Cruising the post-genomic orphanage

Both the results of the whole-genome-sequencing programmes and the contents of the extensive expressed sequence tag (EST) databases have made it abundantly clear that many gene products have no close relatives in the databases (they are 'orphans') and are of unknown function, that many are cell-membrane receptors (especially G-protein-coupled receptors), that their natural ligands are also unknown, and that they consequently represent important novel targets for both agonists and antagonists.

The newly discovered orexins A and B and their receptors are a case in point (Masashi Yanagisawa, University of Texas Southwestern Medical Center, Dallas, TX, USA), and are involved in the control of food intake. Direct binding assays are possible but measurements based on the agonist-dependent production of Ca^{2+} transients in transfected cells provide a more pertinent, functional assay. It is recognized that there are some 10 000–40 000 different mRNAs in the 500 000 molecules expressed in a typical mammalian cell. This means that, of the many novel approaches to functional genomics, expression profiling of these mRNAs using oligonucleotide arrays has enormous power for providing clues to the function of orphan genes (Eugene Brown, Genetics Institute, Cambridge, MA, USA). Modern systems of this type are linear from 0.5 to 500 pM target RNA and are reproducible and quantitative. Such arrays, preferably prepared using bacterial artificial chromosomes, allow the facile detection of huge numbers of single-nucleotide polymorphisms (Janice Kurth, Genset Corporation, La Jolla, CA, USA). These can be exploited in screening patients for their likelihood of acquiring particular illnesses and for their suitability for chronic drug therapy.

What you see is what you get – novel optical methods for high-throughput screening

Fluorescent methods are probably the methods of choice for high-throughput screening (HTS) assays, and their repertoire continues to increase. Those based on variants of time-resolved fluorescence (which allows the discrimination of the signal of interest from other fluorescent background signals) have particular merit, especially as the drive towards miniaturization means that, because of contributions from the assay plates, the signal decreases more quickly than the background as the assay volume is reduced (Jack Owicki, LJL Biosystems, Sunnyvale, CA, USA).

The current move is away from the traditional 96-well plate to 384- and especially 1536-well versions, where reagent costs are typically 100 times lower and assay volumes drop from some 400 μl to 5–10 μl (Jonathan Burbaum, Pharmacopoeia, Princeton, NJ, USA). Technical issues become significant here, such as the use of conical rather than square wells to avoid wicking and the importance of measures to stop evaporation, but the great benefit is cost reduction, with typical costs for a screening campaign being reduced from US$35 million to US$1.1 million. Best of all is if there are no reagents. The use of infrared spectroscopy in HTS is a novel, reagentless and generic technique requiring at most a few μl of sample; as an example from titre-improvement programmes, the measurement time may be reduced to 1 sec from the 15 min required for the traditional HPLC analysis (Douglas Kell, University of Wales, Aberystwyth, UK).

Classical analysis of optical assays in microtitre plates used scanning methods in which the results were read sequentially by a single detector. This represented a substantial bottleneck in the speed of the overall screening process and thus a major trend is towards imaging methods in which, by coupling a telecentric (non-parallax) lens and a CCD camera, an entire plate may be imaged and read simultaneously (Ronald Barrett, Affymax Research Institute, Palo Alto, CA, USA; Neil Cook, Amersham Pharmacia Biotech, Cardiff, UK). To achieve these levels of sensitivity (at which the photon flux may be a hundredth to a ten-thousandth of that of starlight), improvements are required in all areas, with reagents, hardware and software all contributing to the achievement of the required sensitivity.

Fibre-optic arrays provide another means of interrogating many assays in parallel; etching microwells onto the end of such optical fibres allows assays to be performed in volumes as low as 90 fl (David Walt, Tufts University, North Grafton, MA, USA). More accessibly, confocal methods exploiting fluorescence-correlation spectroscopy (FCS) can interrogate a volume of 1 μm^3 (i.e. 1 fl), in which a 10 nM solution of a fluorophore contains on average six molecules. Analysis of the time course of fluctuations in their number density provide much information on their molecular environment and, in particular, on whether they are bound or free; any 'traditional' fluorescence assay may be configured for FCS (Keith Moore, SmithKline Beecham, Harlow, UK).

The numbers game; tracking chemical diversity

Imagine that there are just ten crucial and independent parameters ('explanatory variables') that can contribute to a drug's activity and

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that obtaining a lead compound with a binding constant of 1 μM or better requires that each of them is within ±10% on a linear scale of the 'correct' or optimal value. This means that we are looking for a single entity in 5^{10} possible sets of properties (and for a precision of just twice this per explanatory variable, there are 10^{10} possibilities). Considerations such as this have led to the explosion of libraries of candidate drug compounds, typically containing 10^5–10^6 pure substances. To produce these is therefore as significant as the need to analyse their pharmacological behaviour, and to assess and maximize the chemical diversity within a library is of particular importance.

However, using the methods of combinatorial chemistry combined with evolutionary algorithms means that a potential library of 160 000 (from a four-step chemical synthesis in which there are 10, 10, 40 and 40 reagents of each type) can be decreased to just 400 experiments in which after a 'random' 20 mixtures, each reagent type is optimized over 19 further generations (Klaus Gubernator, Combichem, San Diego, CA, USA). Indeed, the combination of chemical diversity with intelligent computer analysis is crucial to this type of enterprise. Both active and inactive compounds contribute useful information as one seeks to decrease the search space for useful pharmacophores, especially if structural (rather than biophysical) parameters are used as the inputs (Susan Bassett, Bioreason, Santa Fe, NM, USA). In terms of finding metrics for assessing diversity (a generic problem that may also be applied to biodiversity metrics), good metrics for library design must always cluster molecules with similar biological activities together (Dora Schnur, Pharmacopeia, Princeton, NJ, USA), and yet diversity metrics should seek to minimize the proximity of molecules in descriptor space (Adrienne Tymiak, Bristol-Myers Squibb Pharmaceutical Research Institute, Buffalo, NY, USA). A similarity index based on the average number of shared atom-pairing descriptors over the total number of such descriptors provides a robust metric for such analyses.

Both chemical libraries and the results of assays using them produce huge amounts of data, which many workers in a large organization may wish to access. The design and construction of appropriate databases is thus another great need, and the interface must be constructed in a way that both makes navigation easy for the bench scientist and permits the facile deployment of powerful query and report tools. Only hierarchical methods permit this with any convenience, and allow the user to organize data from thousands of drug screens. The *Discovery explorer* tool is one such implementation, which provides decision support via a scalable, robust, flexible and enterprise-wide architecture (Anthony Kreamer, SmithKline Beecham, King of Prussia, PA, USA).

Given that the human genome probably contains some 70 000 genes, that one might wish to assay some 10% of these and that half may be amenable to direct binding assays, the big pharmaceutical companies will certainly need to be looking at the results of several thousand screens (Mario Geysen, GlaxoWellcome, Research Triangle Park, NC, USA). If combinatorial chemistry is to be the main source of leads (as well as natural products), only the split-and-mix strategy of synthesis on solid supports (beads) is appropriate; discrimination between beads may be carried out by encoding them via a linker labelled with stable isotopes in various ratios.

Small is beautiful: miniaturization in ultra-HTS

A common, if arbitrary, definition of a system for ultra-HTS is one in which 100 000 assays are run per day. This is slightly more than one per second and requires careful integration of the necessarily robotic systems, which deploy compound libraries, run the assays and analyse the data. With primary hit rates typically running at 0.1%, it is critical to minimize both false positives and false negatives, and to ensure that the miniaturized assays in 1536-well plates with volumes under 10 μl behave exactly like those carried out in the test tube or the 96-well plate. Even the 1536-well plate has its competitors, as laboratory-on-a-chip systems (in which reagents, cells and drug candidates are mixed by electrokinetic forces operating in microfluidic channels of 10–100 μm) allow complex assays such as those for Ca^{2+} release to be operated at rates of 2000 cells min^{-1} and allow several thousand replicates to be analysed in a total volume of less than 20 μl (Michael Knapp, Caliper Technologies, Palo Alto, CA, USA).

However, analyses done under these ultra-HTS conditions must be considerably more robust than those initially developed by the scientists studying novel targets. The optimization of such assays represents a combinatorial problem as intractable as that described above regarding the statistical difficulty of optimizing a drug lead [even the question of whether to include one of 16 buffer components – never mind optimizing its concentration – gives 2^{16} (or 65 536) possibilities requiring 683 96-well plates if all are to be assessed]. Modern methods of experimental design can reduce this to just two or three such plates, and their preparation may be integrated into a laboratory robotics system (Frances Stewart, SmithKline Beecham, King of Prussia, PA, USA).

As assays become smaller, we enter the field of nanotechnology; nm-sized gold microspheres possess unusual optical properties (such as a molar extinction coefficient of some 10^9 M^{-1} cm^{-1}) that change dramatically upon ligand binding. They have great potential for exploitation in different types of binding measurements based on surface-plasmon resonance (Michael Natan, Pennsylvania State University, University Park, PA, USA) and in the highly selective detection of DNA. In this technique, the chromophoric changes attending nucleic-acid binding to gold microspheres derivatized with complementary nucleic acids are both unusually temperature dependent and permanent (Chad Mirkin, Northwestern University, Evanston, IL, USA).

New ways to analyse cellular properties may also be greatly assisted by modifying the biology. The yeast two-hybrid system is a well-known method of detecting protein–protein interactions *in vivo* but traditional versions can be rather tedious, as cells need to be cotransfected with both putative partners of the binding event. A new variant has, however, been developed in which an entire cDNA library containing the 'prey' is held in one yeast mating type and cells of the other mating type are transformed with the 'bait' (Yiwu He, GlaxoWellcome, Research Triangle Park, NC, USA). Coincubation of the cells followed by a chemiluminescent β-galactosidase reporter assay

means that one person can supervise the automated performance of 50 full assays per month, each typically producing 20 binding partners that might provide novel drug targets.

Interrogating single molecules is clearly the ultimate in miniaturization and, although such methods cannot yet easily be parallelized, scanning-probe methods such as atomic-force microscopy with functionalized probes allow the direct and elegant measurement and discrimination of the interaction between single molecules (Saul Tendler, Nottingham University, Nottingham, UK).

And was it all worth it?

Is the modern marriage of biochemical-, genomic- (and intuition-) based target development and HTS leading to new and useful drugs? Two examples suffice to indicate that the answer is resoundingly in the affirmative.

Viramune is a novel, nonnucleosidic drug active against the reverse-transcriptase enzyme of the human-immunodeficiency-virus 1 (the major causative agent of AIDS), and was approved for use in 1996 (John Proudfoot, Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT, USA). In 1988, it was known only that the target enzyme contained two subunits (an X-ray structure became available in 1992) and a drug screen against it was initiated (at this time, HTS meant 100–200 compounds per week!). After testing approximately 1600 molecules, of which 1% gave some kind of 'hit', a lead compound was discovered with an IC_{50} of 6 μM. Optimization of this compound led to a novel chemical entity with excellent pharmacological properties and an IC_{50} of 35 nM, low enough that therapeutic doses (giving a blood concentration of some 25 μM) were active even against 'resistant' strains.

It is now well established that the many complications of diabetes, such as nephropathy, neuropathy and, in particular, retinopathy leading to blindness, are exacerbated when glucose levels are not well controlled. What has been much less clear is the mechanism by which chronic hyperglycemia actually causes these complications. Following detailed work at Sphinx Pharmaceuticals, which developed assays specific for the many kinds of protein kinase, it was hypothesized that it was, in fact, an excessive activity of the β isoforms of protein-kinase C (PKC) induced by the higher levels of diacylglycerol formed under conditions of glucose excess that might be the major culprit (William Heath, Lilly Research Laboratories, Indianapolis, IN, USA).

Despite management opposition (because non-selective inhibitors of the PKC family were known to be highly cytotoxic), a series of screens was developed against each of the eight human PKC isozymes. An indolecarbazole natural product related to staurosporine led to the development of LY 333531, a highly selective molecule with an IC_{50} of 5 nM against the β isoforms but several hundred nM against the rest. Interestingly, even though the screen was intended to find molecules acting on the regulatory site of the PKC enzyme, it was actually the catalytic (ATP-binding) site that is the target, and the competitive inhibition was only observed when the assay was run at ATP levels significantly lower than those thought to be prevalent *in vivo*! 'The ATP level in the cell is 1 mM, but not everywhere in the cell.'

Assay I say

In conclusion, it is evident that the trend towards miniaturization, the intelligent generation and deployment of chemical libraries, the innovative hardware and software, and the robust automation now available are major forces in the drive to develop new pharmaceuticals with novel targets, high efficacy and, of course, substantial commercial potential. However, for these hopes to be realized, good assays will remain paramount.

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Meeting report

The sheep and the goats

Communication between the makers and the users of laboratory equipment is always a problem. The user wants to know what is available, and to tell the maker the problems with their equipment; the maker wants to make sure that the user knows about the latest products, and to make sure their own products are as good as they can be. A recent meeting* gave an excellent opportunity for this sort of exchange of information.

The speakers were a varied mixture – some from the manufacturers of different types of separation equipment, extolling the virtues of their latest devices, others from the sharp end, reporting their own experience of various techniques, yet others discussing issues arising from the procedures involved.

Products

There are many different sorts of separation equipment out there, including classical chromatography columns, packed and fluidized beds, and membrane-based systems. Harvey Brandwin (Pall Filtron, Northborough, MA, USA) discussed some of the problems of membrane-based systems, including the need to optimize each system individually, but emphasized their advantages, especially in virus removal. He also reported that Pall Filtron have developed new filters capable of 3-log removal of viruses down to 20 nm. When using filters with such narrow pores, it is essential to use prefilters, to prevent the filter rapidly clogging up, and they will also help when using larger-pore-size filters.

W-D. Schleuning (Schering, Berlin, Germany) introduced new surrogate systems for evaluating the biological activity of new agents to replace the standard animal models. These included the use of hormone-sensitive promoters in yeast and the two-hybrid system, and also touched on issues of high-throughput-screening systems, which are already moving from 96-well to 384-well plates, and may in the future move to 864- and even 1536-well plates.

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