Innovations

Variety is the spice of life

Affymax N.V.

Affymax N.V. in Palo Alto, California, is best known for its involvement in the field of combinatorial chemistry, the group of chemical methods that enable huge numbers of diverse compounds to be synthesized in a defined mixture. Although the company has not always been the clear leader in the field, it has developed a streamlined approach to determining the usefulness of the leads it produces. Glaxo Wellcome plc's decision in March 1995 to acquire the company for $533 million is seen as a testament to the value of their approach. "Obviously Glaxo thought they were one of the leaders in high-throughput screening," says Kim Janda of the Scripps Research Institute. "The best methodologies are all very well, but at the end of the day it's who gets the right drug....and Affymax have the horses to do it."

A new company

Affymax was founded in 1989 by Dr Alejandro Zaffaroni, a biochemist turned biotechnology entrepreneur. Increased cloning and expression of proteins in the latter half of the 1980s introduced a huge number of new targets for drug intervention, and Zaffaroni conceived the idea of an 'affinity matrix' (the origin of the company's name) to screen for new drugs. In essence, this is a way of arraying potential ligands in a defined format so that the identity of the bound ligand can be determined by its position in the matrix. "The idea was to let the targets find the molecules they wanted to interact with," says Mark Gallop, director of combinatorial chemistry at Affymax. "We could do this by developing technology that would allow us to make and screen a large number of compounds in a short amount of time."

Zaffaroni's original idea was to screen large numbers of marine natural products in this spatially addressable way. But Affymax scientists later decided, with the help of their scientific advisors (including Avram Goldstein, Stanford University, Peter Schultz, University of California, Berkeley and Joshua Lederberg, Rockefeller University), to use peptide synthesis to generate the molecules for screening.

Peptides

The idea that peptides could be synthesized using combinatorial techniques began with Mario Geysen and colleagues in Melbourne, Australia, who in 1984 synthesized a limited library with each peptide on a separate pin. In 1985, Richard Houghten (then at the Scripps Research Institute) developed a similar technique using mesh containers dubbed tea-bags. Like all combinatorial methods, these approaches used several sequential reactions, performed in parallel, to build up each peptide; each step adds one amino acid, but the amino acid added is different for each pin or teabag.

Two techniques were developed at Affymax to exploit these ideas. Phage display was developed simultaneously at Affymax, Cetus Corp., and the University of Missouri. In this technique, short oligonucleotides of random sequence are inserted into the gene for a phage coat protein, giving phage viruses that express a random peptide sequence, different for each phage, at the tip of the coat protein. The second technique, and Affymax's first foray into solid-phase chemical synthesis, began with an idea that was, says Gallop, "conceptually out of left field". Borrowing the concept of photolithography from the nearby semiconductor industry, they developed VLSIPS (very large scale immobilized polymer synthesis) chips, which can be covered with very dense arrays of peptides (up to 50 000 peptides cm^{-1}).

Synthesis of peptides on VLSIPS starts with glass slides covered with a linker group that has an amino function. The photolabile protecting group on the amino group is removed in a spatially controlled way by shining light through a mask that allows light to reach only some areas of the slide. Amino acid analogs (also with a photolabile group) can then be coupled to just those areas of the slide by standard chemistry. By arranging the position of the mask appropriately, a large number of different peptides can be made at known positions on the slide. After synthesis of the peptide array, the target protein is fluorescently labeled, allowed to bind to the slide, and the location (and therefore the structure) of tight binders are identified using an epifluorescence microscope.

Beyond peptides

By early 1992 it became apparent that diverse, polymeric building blocks could be made using unnatural substrates, such as the peptoids made at Chiron Corporation, and the protease-resistant polycarbamates that Affymax scientists made using activated amino alcohols in collaboration with Peter Schultz of the University of California, Berkeley.

Also in 1992, Jonathan Ellman and colleagues at the University of California, Berkeley, and Sheila DeWitt and the bioorganic group at Parke Davis showed that solid-phase synthesis could be used to generate 1,4-benzodiazepines, an example of the type of non-polymeric small organic molecules traditionally favored as drug leads by the pharmaceutical industry. (Similar heterocycles have proven to be ideal scaffolds, as different functionalities can be added radially instead of linearly.) Although previous workers had shown that solid-phase organic synthesis had utility beyond peptide and oligonucleotide chemistry, this work described optimized reaction sequences specifically designed to be useful in combinatorial synthesis.

Similar techniques were also being developed at Affymax. A common starting point for the formation of several heterocycles was an imine, which is synthesized by the condensation of an amino group (on a solid support) with aldehydes or ketones. Thiolate addition to imines

Innovations is a new monthly column that surveys the science done at biotechnology companies.

yields thiazolidinones, some of which interact with G-protein-coupled receptors. Libraries based on this chemistry have led to the discovery of novel inhibitors of cyclooxygenase (COX-1) and antagonists of opioid receptors. Imines were also the starting point for the generation (via ketene addition) of a library of β-lactams, potential antibiotics. Addition to imines also gives pyrrolidines and tetrahydropyridines, nuclei that are common in bioactive compounds, and a library of pyrrolidines has yielded a potent inhibitor of angiotensin converting enzyme. Finally, building on a hydroxyl rather than an amino group allows the synthesis of phosphonyl-acid-based libraries that may include novel metalloprotease inhibitors. Some compounds from these synthetic efforts are being tested in pre-clinical animal models, although none are yet in human trials.

The libraries

Combinatorial libraries of organic chemicals are generated by sequential reactions, each resulting in the addition of a small chemical building block to the compound. The diversity of the library depends on the number and combinations of the building blocks added at each step.

Affymax has focussed on solid-phase synthesis using beads 100–200 μm in diameter. The reactions used must therefore be restricted to those that will maintain the linkage to the bead, but the fact that the beads are easy to separate from the reagents allows each reaction in a multistep synthesis to be driven to completion by the use of vast excesses of reagents.

Libraries of compounds fall into two major categories. If there is no information as to what type of compound might bind the target protein a large, structurally diverse library of as many as 10^{10}–10^{11} peptides or 100 000–200 000 small organic molecules is searched. Once a lead has been found a local search will then be undertaken using a focussed library of a few hundred analogs made in parallel in 96-well plates, rather than on beads.

Screening is routinely done in solution after chemical or photocleavage. Researchers at Affymax have improved traditional nitrobenzyl photolinkers by alkyl and alkoxy additions, thereby shifting the cleavage wavelength out of the potentially damaging UV range, increasing the rate of photocleavage, and decreasing the reactivity of the products ~100-fold. Photocleavage is often preferable to chemical cleavage, as it is a mild technique that liberates a compound directly into an assay.

Decoding

Each bead has ~200–300 pmoles of compound bound to it, just enough for mass spectroscopy to be used to identify the compound after it has been cleaved from the bead. But in general the information gained from this technique is ambiguous, necessitating the development of other decoding techniques. Several researchers, including some at Affymax, suggested that the beads could be tagged at each synthetic step with a molecule whose structure was easier to determine. The initial approach at Affymax was to use oligonucleotides, which at the end of the synthesis could be amplified by the polymerase chain reaction (PCR) and sequenced, while other groups used amino acids and Edman sequencing. As oligonucleotides are susceptible to degradation in many of the reaction conditions used in combinatorial chemistry, Affymax has since developed more robust methods using secondary amine tags, which can be decoded by acid hydrolysis followed by HPLC.

Reaction progress can be rapidly monitored without cleavage from solid supports using solid-state NMR after incorporating ^{13}C-labeled building blocks into syntheses. Even more sensitive is ^{1}H NMR of resin-bound molecules using a Varian Nano-NMR probe, which allows structure determination for very small samples using magic-angle spinning. This technique, says Chris Holmes, a research fellow at Affymax, "brings protein NMR into the realms of solid-phase synthesis".

The future

Gallop sees the integration of chemistry, biology and engineering as the key to the continued success of Affymax. "Because we have been doing this for a long time we can move very fast," he says. For example, a generic expression system developed at Affymax allows the extracellular domains of receptors to be expressed, purified by cleavage of a lipid anchor, and immobilized using antibodies to a common tag for use in assays. "Their strength is in interfacing the libraries with the biology," says Mario Geysen, now the head of combinatorial chemistry at Glaxo.

As one major push for many combinatorial chemists is the automation of synthesis, the depth in engineering at Affymax is crucial. Engineering will also aid the current push towards miniaturization, which will not only conserve reagents, but could also stop potential hits from being overlooked. With current methods the solubilization of the contents of a single bead in the well of a microtitre plate gives a ~1 μM solution of the compound, so potential hits with an affinity of ~10 μM are missed.

New companies

Affymax is now big enough to be spawning younger companies. The VLSIPS technology is now solely developed by Affymetrix Inc., which was separately incorporated in 1992. Affymetrix has used VLSIPs to build DNA probe arrays that can be used for genetic tests, including the detection of single-base mutations. This technology is not, however, being used for small-molecule combinatorial chemistry, as the necessity for the attachment of photolabile groups at every step proved too restrictive on the types of functionalities that could be introduced.

A second spin-off company, Maxygen Inc., is being formed to focus on the potentials of the gene shuffling technique developed by Pim Stemmer at Affymax. In this technique, mutations in enzymes are recursively generated and recombined, after which the enzymes can be selected for novel properties such as the ability to degrade xenobiotics or synthesize novel small molecules.

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