Antibody arrays: an embryonic but rapidly growing technology

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Protein arrays are now an attractive proposition as they can measure a diverse range of protein interactions not possible with traditional DNA arrays. Antibody arrays are a specific subset of this technology. Originally conceived as multi-analyte detectors, antibody arrays are now used in a wide variety of applications. For instance, the potential of this technology to diagnose human diseases, such as leukemia, breast cancer and, potentially, heart failure, has stimulated much interest. Furthermore, identification of new protein targets in particular disease states will prove to be an invaluable tool in drug discovery and development. Patient prognosis and treatment are also potential applications of the technology. Antibody arrays have proved to be dynamic in response to these broad range of possibilities. This review examines variations in antibody array design and discusses current and potential applications of this novel and interesting technology.

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▼ Biomedical research has advanced rapidly in recent years with the sequencing of the human genome and the availability of technologies such as DNA arrays [1]. The success of DNA array technology lies in the ability of one strand (or part of a strand) of DNA to bind a second strand in a specific and complementary way. The representation of every human gene on a single chip would therefore enable gene expression to be quantified in any tissue.

Genomics is naturally linked to proteomics, and while DNA arrays depend on the reverse transcription of mRNA, protein arrays directly measure gene products and indirectly measure interactions, such as post-translational modification. There is a further need for protein arrays because not every transcribed mRNA is proportionally converted into its corresponding protein [2,3]. Use of proteins in this microarray format is relatively new (Fig. 1) and its application will increase as the technology is refined.

Protein arrays use the same robotics as initially defined by DNA arrays [4,5] and, superficially, the two look quite similar (Fig. 2). With further development, protein and DNA arrays will probably complement each other. For instance, DNA arrays can indicate the propensity of an individual to suffer from a particular disease, while protein arrays will indicate the actual existence of that condition.

The purpose of this review is to focus on a subset of protein microarray technologies – those involving antibody–antigen interactions.

The basics of an antibody array

Typically, antibodies are robotically delivered to the surface of a solid support and left to dry. The remaining surface is then blocked before the test sample is applied. Proteins 'captured' by an immobilized antibody can be identified by the addition of a second, labeled antibody that recognizes a different epitope. The appearance of a typical antibody array is shown in Fig. 2b. Kodadek [6] offers a more comprehensive review into the workings of this technology.

A technology that is opening eyes

Antibody arrays were originally conceived as multi-analyte detectors [7] but it is now clear they possess considerable potential for use in a variety of applications. Diagnosis of patient disease using this technology is stimulating much interest in, for example, leukemia [8], breast cancer [9] and preliminary studies into the diagnosis of congestive heart failure (http://www.biosite.com). Predicting the susceptibility of a patient for a particular disease is another potential clinical

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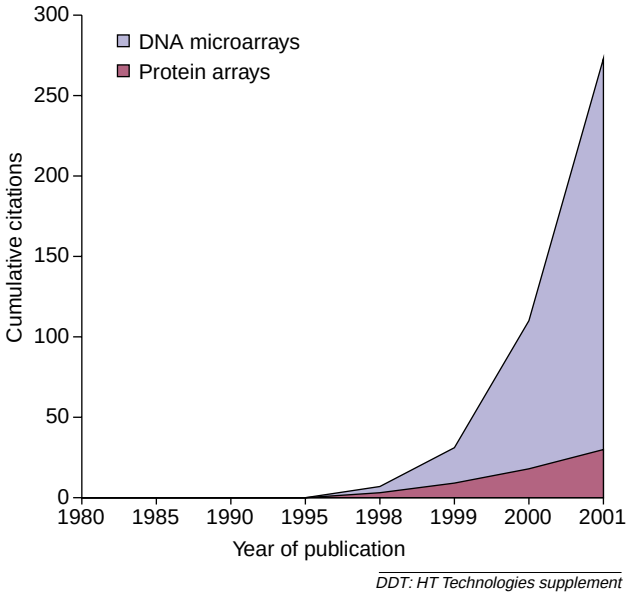

Figure 1. The rate at which citations for DNA and protein arrays have increased with time. 'DNA microarrays' and 'protein arrays' were searched as keywords in the online publication database Medline. Publications mentioning DNA microarrays are far more common than those of protein arrays, although this trend is beginning to change.

application, for instance, in cancer patients [10,11]. The high-throughput nature of antibody arrays suggests they can be used in large, centralized clinical pathology laboratories where efficient yet precise analysis is required. However, the certainty of diagnosis needs to be high (>80%), and negative predictive values must be very high (>98%) if these results are to be useful to the clinician (http://www.lsbc.com).

Antibody arrays will provide new lead-molecules and potential targets for drug discovery [12]. The key is the identification of altered protein expression levels in a particular disease state. Such proteins have already been identified in various forms of cancer [13–15] and other proteins could potentially be discovered using antibody arrays. Coupled with innovative protein chips containing potential drug candidates, this concept will improve drug specificity by providing structural information about the target protein [16].

The technology itself must be flexible if it is to meet a variety of applications. Probe and substrate design will be most important as these must reliably capture the protein of interest.

Antibodies: the ideal probe?

Antibodies appear to be the most appropriate receptor element in a protein detection array because they possess the specificity required to identify a target epitope. For commercial forms of this technology, recombinant antibodies are ideal because they

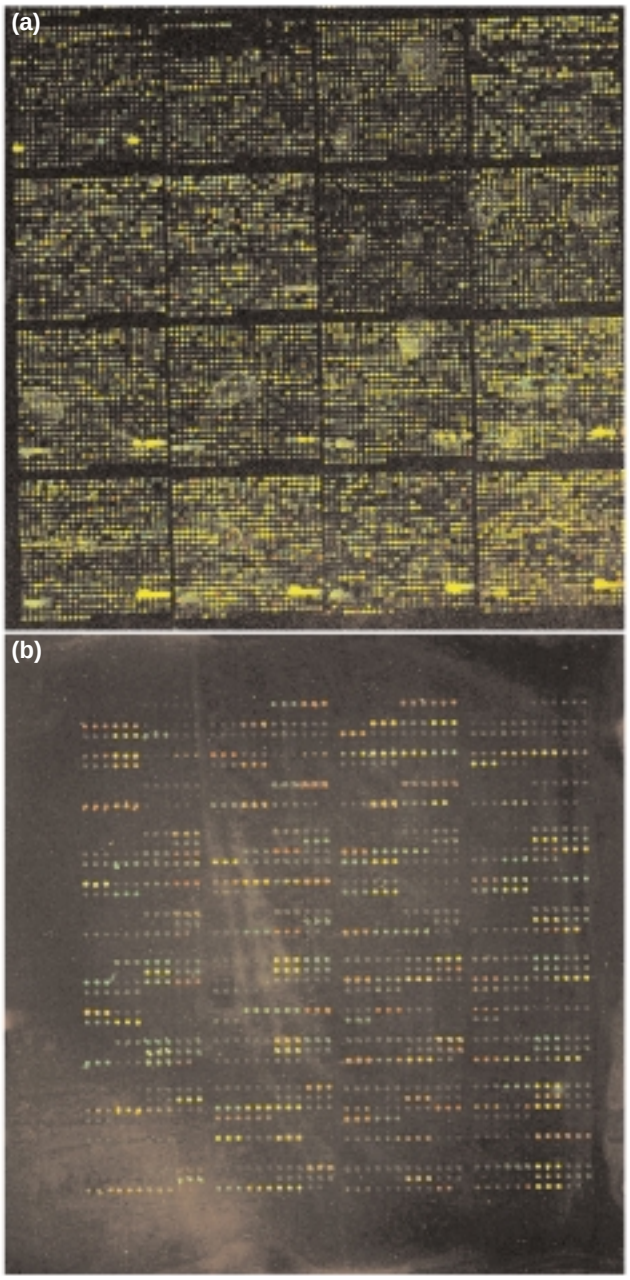

Figure 2. Comparison of a typical (a) DNA and (b) antibody array. At a first glance, DNA and protein arrays look quite similar. This is not surprising because protein arrays evolved from the same robotics as DNA arrays. However, in terms of targets and applications, the two technologies are very different.

are derived from cell lines that are essentially immortal. They can either be intact, or in fragments, such as Fabs (fragments having the antibody binding site) or the smaller, single-chain variable fragments (scFvs). The latter are used therapeutically because of their rapid uptake by cells, and have significantly improved tumor-targeting [17].

For high-throughput proteome-scale analysis, rapid isolation of antibodies to a particular protein is vital [18]. Phage display is commonly used to generate recombinant antibodies *en mass* [19] and provides a novel method for selecting the antibody–antigen interactions that might later constitute an array. This is being commercially exploited by expanding phage operations such as Dyax (http://www.dyax.com) and Morphosys (http://www.morphosys.com), which produce antibody fragments from large libraries in a multiplex format.

Although antibodies target specific epitopes, complementary binding with the intended protein cannot be guaranteed. Most antibodies are glycosylated and have large surface areas for binding. As a consequence, they might exhibit cross-reactivity among sample proteins [16]. In addition, the epitope could be inaccessible to the printed antibody, either through steric hindrance in the immobilization of the antibody, or because the target epitope itself could be masked from its antibody by a nonspecific interacting protein. This would be particularly true for patient sera.

Long-term storage of antibody arrays is necessary if the technology is to be convenient and readily available for use. Following printing and blocking, the arrayed antibodies might not retain optimal activity when stored for >8 months. Synthetic alternatives to antibodies have been developed that might overcome this problem [20]. Trinectin™-binding proteins (Phylos; http://www.phylos.com) and Affibodies™ (Affibody; http://www.affibody.com) recognize and bind to specific protein targets with high affinity and, because they are entirely synthetic, could be more stable.

A suitable surface

The effective operation of an antibody array depends on the surface onto which the antibodies are printed. Surfaces that retain antibodies via hydrophobic interactions include nitrocellulose [21–24] and glass slides [10,25–29]. Other less conventional surfaces that could act as potential substrates for this technology include gold [30], polystyrene [31] and poly(vinylidene fluoride) (PVDF) membranes [32]. High-affinity antibody–substrate binding minimizes antibody loss during exhaustive washing procedures and further downstream processing.

Nitrocellulose supports

Nitrocellulose film binds antibodies via non-polar, non-covalent bonds, and can retain large quantities of protein [22]. As an optimal surface for protein binding, the remaining area not occupied by the antibody must be blocked (e.g. with bovine serum albumin) before a test sample is applied. An increasing number of antibody arrays use this surface [8,21,24].

Huang *et al.* [21] constructed a 504-dot (18 × 28) antibody array on a Hybond membrane (Amersham Biosciences; http://www.apbiotech.com) and imaged the results using enhanced chemiluminescence (ECL). A template for the 504 dots defined each antibody location, and the delivered antibody bound to the surface with high affinity. A nitrocellulose film on a glass slide was also used to construct a 60-dot antibody array directed against a cluster of differentiation (CD) antigens [8]. This novel method captured whole cells (Fig. 3) rather than proteins in solution.

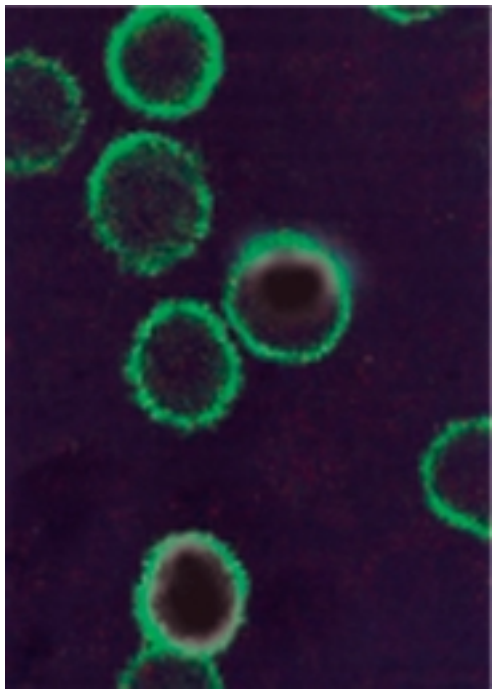

Figure 3. Confocal microscope image of captured cells on a cluster-of-differentiation (CD) antibody array. An antibody to CD5 was arrayed onto a nitrocellulose membrane which 'captured' CD5 positive leukocytes [8]. The captured cells were then observed using a fluorescein-labeled anti-CD3 (green) antibody. As expected, the observed CD antigen is localized at the cell membrane. This type of antibody array is restricted to cell suspensions but enables direct observation of the captured cells.

Although the nitrocellulose surface binds antibodies tightly [22], its permeability could complicate the kinetics of the protein–antibody interactions because of the slow diffusion of proteins through the nitrocellulose pores [23]. Cell capture would also be impeded as the pore sizes of these membranes are too small, resulting in the majority of binding occuring at the gross exposed surface of the membrane, rather than within it. Other disadvantages of nitrocellulose include light scattering and non-compatibility with mass spectrometry (MS) analysis (see later).

Glass supports and the importance of coupling

Glass is the most popular platform for antibody arrays [10,25–29]. However, it must be thoroughly cleaned with 1:1 methanol

and hydrochloric acid [29] and then chemically modified to facilitate covalent binding [27]. The latter involves coupling the antibody to the glass, which is vital for high-affinity probe–substrate interactions.

Silanization is a common coupling method. Silanized slides (containing amine attachment sites) can be obtained commercially (http://www.bdbiosciences.com/clontech) or can be individually silanized using 2% (3-mercaptopropyl)trimethoxysilane in toluene [29]. Antibodies also bind to silylated glass following treatment with an aldehyde-containing silane reagent [26]. They can also bind to glass slides coated with an agarose [28] or poly-L-lysine [25] film. A promising coupling method is the 'affinity-on-a-chip' technology, which uses the high-affinity capability of immobilized nickel ions for histidine tags, attached to expressed recombinant proteins (KPL; http://www.kpl.com). In theory, an antibody array could be constructed from histidine-tagged antibodies arrayed onto a nickel-coated surface.

As with nitrocellulose, glass–antibody interactions are hydrophobic (principally a dipole interaction) and could cause unfolding of the antibody with a subsequent loss in activity. Despite this possibility, glass platforms are still the most appropriate surface for use in high-throughput applications as they are widely available and cost-effective. Other less-established surfaces are currently under development and show promise for use in antibody array technology.

Innovative supports

Alternative surfaces to glass include gold [30], and PVDF membranes [32], which have both been used to investigate antibody–antigen interactions. Other innovative surfaces that could potentially be used in antibody arrays include polystyrene [31] and silica [34]. Microspheres also bind proteins and are now commercially available (Luminex; http://www.luminexcorp.com) [33].

All of these surfaces display high affinity for antibodies and, with further development (e.g. being able to withstand downstream processing without loss of activity), could easily be adapted for use in antibody arrays. Additionally, sophisticated surface chemistries involving chemical spacers, specific reaction chemistries, and reactive linker groups, will maximize the activities and orientation of the Fab domain of antibodies, making them compliant with a greater variety of surfaces. However, at present, modified glass remains the simplist vehicle for binding proteins [4].

Variations in form

In the vast majority of antibody arrays, each dot contains a single antibody. However, Wiese *et al.* [10] have developed a glass plate containing multiple wells, with each well containing an 8×8 element array of antibodies. This novel design enables high-throughput replication of samples or simultaneous analysis of multiple samples. As this array is a prototype, it remains to be seen whether it is more effective than conventional printing of all antibodies on a single, planar surface.

De Wildt *et al.* have developed an ingenious antibody array to screen bacterially expressed recombinant scFv antibody fragments [35]. Bacteria containing 18,342 antibody clones were robotically picked and arrayed at high density. The displayed bacterial clones were then transferred to filters coated with antigen, and ELISAs were performed. This array format enables HTS of antibody–antigen interactions in a multiplex, parallel format. For instance, it could be used to isolate antibodies against impure and complex antigens.

An interesting variation of antibody arrays is one that selectively captures cells. This concept was first described by Chang [36] but was limited to only four antibodies. Recently, a more extensive array of 60 antibodies was developed to capture leukocytes [8]. Both methods, however, are restricted to suspensions of isolated cells, fragments of cells or clusters of cells.

Arrays that capture proteins are more common. They have the potential to analyze vast numbers of different proteins and are likely to provide a simple and cost-efficient means of detecting changes in protein expression. Indeed, protein-detection technology could impact on proteomics in the same way as DNA microarrays impacted on genomics. However, achieving such a feat requires sensitive and dynamic detection.

The importance of detection

Array imaging provides a simple, qualitative confirmation of antigen–antibody binding. Detection of low-abundance proteins is more complicated. Until recently, it was accepted that amplification of protein binding, analogous to PCR in DNA arrays, was not possible using antibodies. However, signal-enhancement of bound proteins on a microarray has now been achieved using the so-called rolling-circle-amplification technique [37]. At present, proteins in antibody arrays are most sensitively detected by fluorescence, although chemiluminescent detection and MS are potential alternatives.

Fluorescence detection

Fluorescence visualization of an antibody array requires specific labeling of the protein antigen with a dye such as fluorescein. This dye is usually excited at 488 nm and the fluorescence emission is detected by fluorescence microscopy or confocal imaging. However, fluorescein is not an ideal probe. It photobleaches rapidly, has a low quantum efficiency, and its clear visualization can be confounded by auto-fluorescence. Now, new dyes are available, such as Cy (Amersham Biosciences) and Alexa (Molecular Probes; http://www.probes.com) probes,

which avoid most of these problems. For example, up to five Alexa dyes can be used simultaneously because their emission peaks have almost baseline separation.

Indocyanine green and its analogs (e.g. DBCy5) require biotinylation to achieve a high-affinity avidin–biotin interaction [31]. Cy5 emits at 710 nm (red), has a higher quantum efficiency than fluorescein, and does not readily photobleach. Good signal-to-noise detection can be achieved with only 10^5 Cy5 molecules per printed zone (200 μm diameter) [31].

In DNA arrays, the detection of Cy5 is commonly matched to Cy3 (green), and the ratio of these emissions is used to interpret expression levels. The exact same configuration has been used for protein arrays. Sreekumar *et al.* [38] showed that equivalent populations of proteins in a reference and a test sample can be labeled with Cy3 and Cy5, respectively, mixed, gel-filtered to remove the unbound dyes, and then incubated on a chip of pre-arrayed antibodies. Increased or decreased protein expression is assessed relative to a reference using a microarray scanner (Fig. 4).

Advances in scanner technology have improved the throughput nature of antibody array analysis by providing an efficient, automated means of detecting fluorescence and, hence, protein binding [25,26]. Several array scanners (PerkinElmer Life Sciences, http://www.perkinelmer.com/lifesciences; Axon Instruments, http://www.axon.com; Affymetrix, http://www.affymetrix.com) are now available for use with antibody arrays.

However, fluorescence imaging is not without its problems. The signal saturates when the concentration of captured protein exceeds ~1 mg ml^{-1} (~20 μM for the average protein) [26], although, at lower concentrations, there is a linear relationship between fluorescence intensity and protein concentration. The detection limit (approximately 150 fg μl^{-1}) is now comparable to the detection range for DNA arrays.

Non-fluorescence detection

An ECL-based method for imaging of antibody arrays has been used to improve detection sensitivity [21]. Biotin-conjugated anti-cytokine antibodies were combined with HRP-conjugated streptavidin and then linked to an ECL reaction. Emitted light was recorded on film and quantified by densitometry with high sensitivity (as little as 4 fmol μl^{-1} of protein was detected). Although this compares favorably with traditional fluorescence methods, its practicality and cost are yet to be evaluated. It also has the disadvantage of signal bleed onto adjacent areas, which therefore limits the density of spots that can be arrayed onto a unit surface area.

Mass spectrometry is another alternative. It could prove to be optimal for the detection of low-abundance proteins because it measures protein molecular mass (even in mixtures). A laser is applied directly onto the sample protein causing its evaporation, and the resulting particles are analyzed by a time-of-flight mass spectrometer. Mass spectrometry has already been used to increase the number of molecular targets for cancer diagnosis [39], and to improve sensitivity in the analysis of drugs in biological fluids [40]. This technology could easily be adapted to detect sample proteins captured on antibody arrays, especially those targeting drug discovery. Already, Ciphergen (http://www.ciphergen.com) is marketing

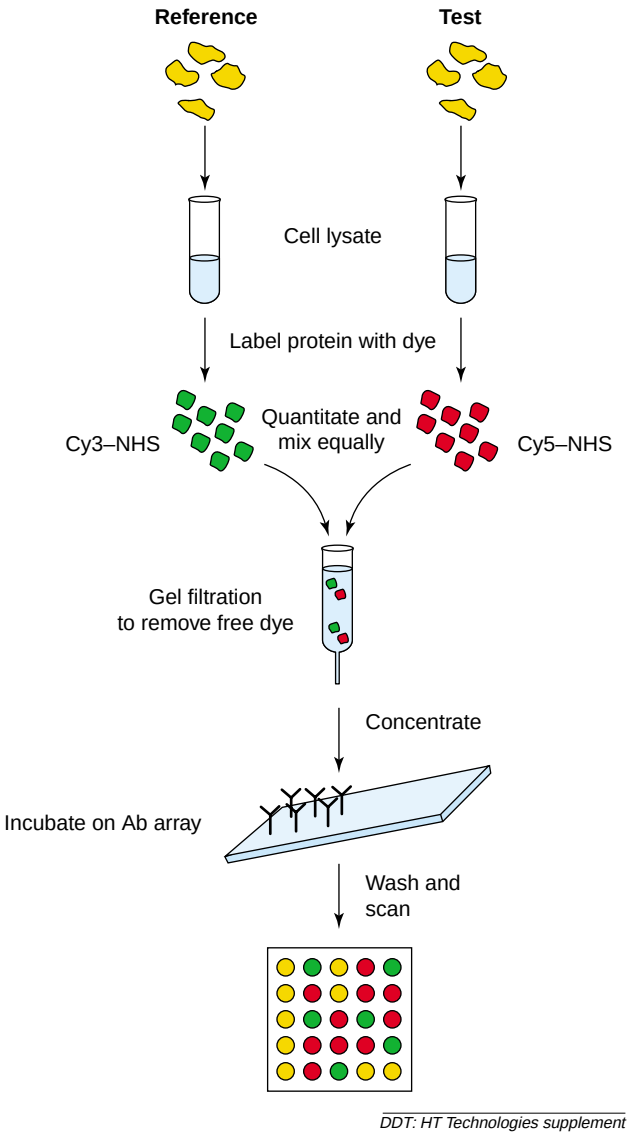

Figure 4. A novel adaptation of DNA array technology to antibody arrays (adapted from Sreekumar *et al.* [38]). Proteins from a test and reference sample are compared. Cells are lysed and separately labeled with Cy3 and Cy5. Protein concentrations are then adjusted, mixed equally, gel-filtered, concentrated, and applied to an antibody array. Changes in protein expression are determined from the ratio of the two dyes. Abbreviation: NHS, *N*-hydroxysuccinimidyl.

commercial protein microarrays that capture biological proteins by virtue of specific surface chemistries, such as their affinity for a particular ligand. Coupled with tandem-MS instruments, this technology was used to discover the structure of repifermin, a protein drug receptor, using only micrograms of sample protein [16].

Whatever the detection method, one significant obstacle is the vast range of concentrations over which proteins must be measured if they are to be clinically useful. Protein concentrations in a biological sample vary greatly (by up to a factor of 10^{14}), and so the simultaneous detection of both low- and high-abundance proteins on a single antibody array seems an unlikely prospect [16].

The future is still bright

Unlike DNA arrays, antibody arrays are well-designed for screening molecular interactions. Current screening of antibody–antigen interactions [41] can potentially be applied to any protein in the human proteome, provided there are corresponding antibodies. Differential proteome analysis for drug target discovery also seems attainable with antibody arrays. As proteins are commonly targeted by drugs, this technology will identify potential candidates for drug development. Furthermore, proteins are commonly post-translationally modified and this could vary with disease progression. Diverse antibody arrays could therefore conceivably monitor these modifications and identify different drug targets for different stages of a disease. Coupled with modern computational drug-design technology, the structural information gained from protein detection systems could be used to create virtual drug molecules that might later become a reality [42].

Concluding remarks

The advent of antibody arrays and their undoubted growth in the future will refine and complement existing methods of antibody–protein analysis. Some forms of this technology are already more sensitive and effective than traditional ELISA methods [21]. When the 'ideal' antibody array is produced, it will consist of a treated glass or silica surface that is strategically printed with different monoclonal antibodies directed against the same protein. This technology is likely to comprise the core of diagnostics, particularly if patient treatment involves monoclonal antibodies (that is, a theranostic). Antibody arrays will also serve as an invaluable tool in drug discovery and development.

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