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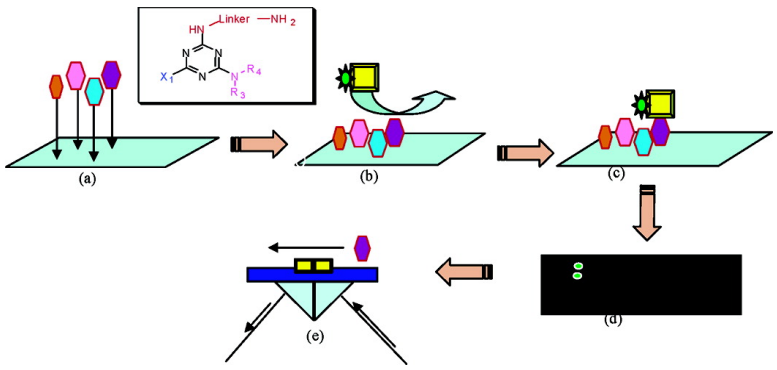

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Articles

Microarrays of Tagged Combinatorial Triazine Libraries in the Discovery of Small-Molecule Ligands of Human IgG

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A novel and highly diverse tagged triazine library incorporating a triethylene glycol-based linker was synthesized using an orthogonal combinatorial approach on the solid phase and covalently immobilized on a glass substrate as a small molecule microarray (SMM). The SMM was screened with a fluorophore-conjugated human IgG, and 4 novel binders from a library of 2688 compounds were identified from the fully spatially addressable array without the need for compound decoding. Using surface plasmon resonance (SPR) analysis, binding seen on the array was confirmed, and a binding constant as low as $K_d = 2.02 \times 10^{-6}$ M was measured.

Introduction

The study of small molecule−protein interactions has become biologically relevant not only for the functional understanding of protein modulation but also in the discovery of novel protein ligands.[1] Traditional chemical genetics approaches often involve time-consuming screening procedures in which library members are screened individually and complex pull-down experiments are required.[2] To accelerate ligand discovery, a robust method incorporating small-molecule immobilization on microarrays in which thousands of small molecules are covalently immobilized on glass substrate platforms that provide for miniaturized, high-throughput screens against any potential binder was thereby conceived.[3]

Along with the need for sufficiently large, highly pure compound libraries, the vast potential of microarray technology intrinsically hinges on array fabrication that demands creative immobilization and surface modification strategies in order to ensure uniform slide printing, thereby increasing the veracity and reproducibility of downstream results. Herein, we describe the rapid generation of a small-molecule microarray for the efficacious discovery of ligands of a candidate protein, human IgG, (with K_d values as low as 10^{-6} M, as measured by surface plasmon resonance, SPR), thereby highlighting the value of a truly addressable small-molecule microarray (SMM) and lead identification. The array produced herein is fully addressable in that our strategy synthesized the small-molecule library through a parallel route in which each compound's unencoded identity was known before printing and easily identifiable, post-screening, merely from its position within the array. Schreiber et al. recently presented a microarray-based strategy using the so-called diversity oriented synthesis (DOS) in the discovery of calmodulin[4] ligands and a transcriptional factor inhibitor.[5] However, with the encoded, split-and-mix DOS library strategy adopted, every hit generated from the microarray screening necessitates a further deconvolution effort to determine its identity, thus limiting the aforementioned throughput, as promised by microarray-based technologies, especially where many putative hits arise.

Results and Discussions

Triazines (1,3,5-triazine) possessing 3-fold symmetry were chosen in our library as the molecular scaffold because they allow for versatile modifications uncomplicated by regiochemcial worries and have proven themselves to be useful biological targets.[6] To further ameliorate the task of developing purposeful leads, members of the library were engineered with one of a range of amino-functionalized linkers that serve as a uniform, rapid, covalent attachment point to glass slides derivatized with *N*-hydroxysuccinimide esters.[7]

We had previously reported the synthesis of libraries of triazine compounds through our orthogonal solid-phase synthesis pathway using various synthetic strategies.[8] Herein, we report a robust, tagged triethylene glycol (TG)-based amino linker triazine library (TG library) that incorporates

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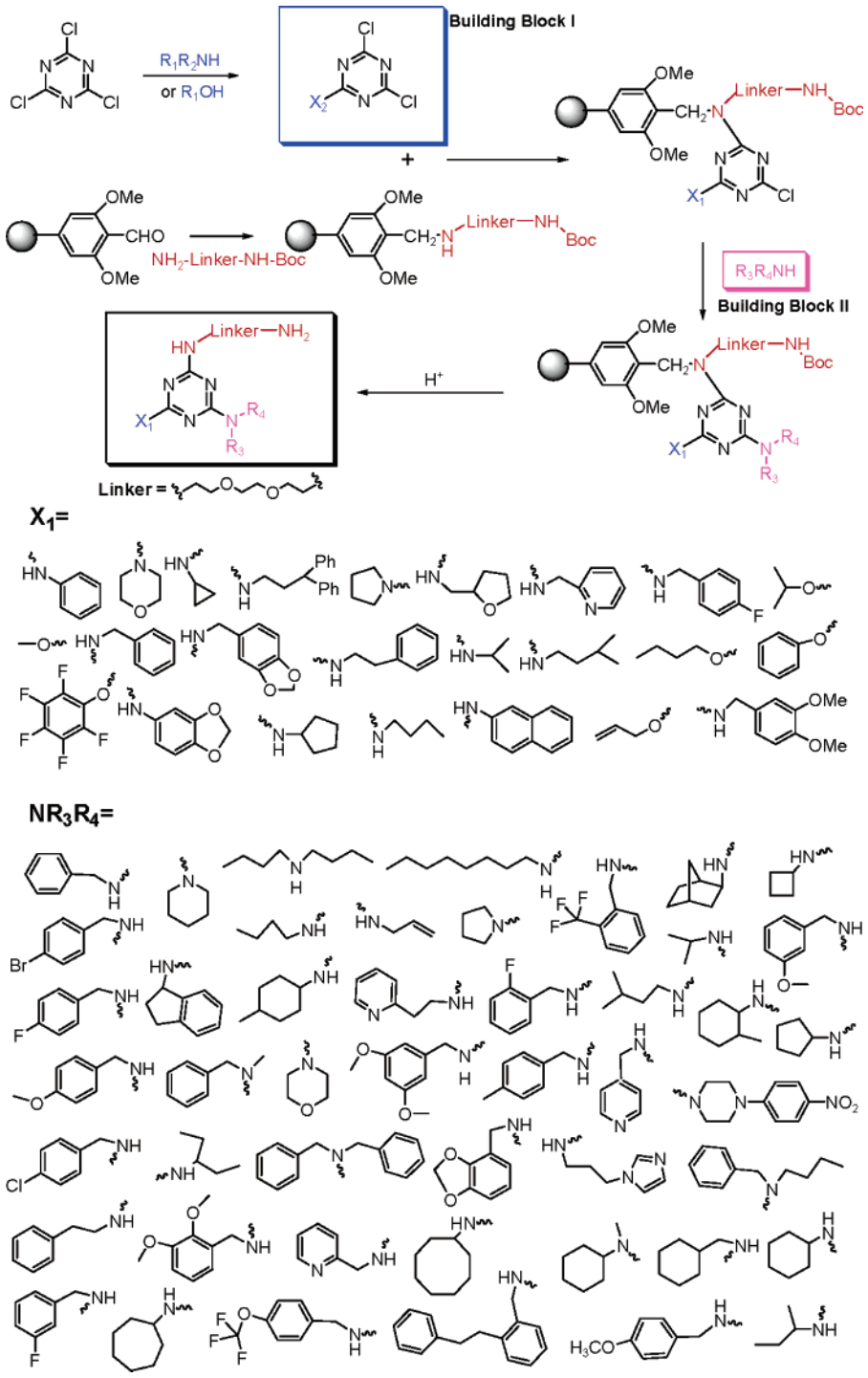
Scheme 1. General Synthetic Scheme for Construction of TG Triazine Library

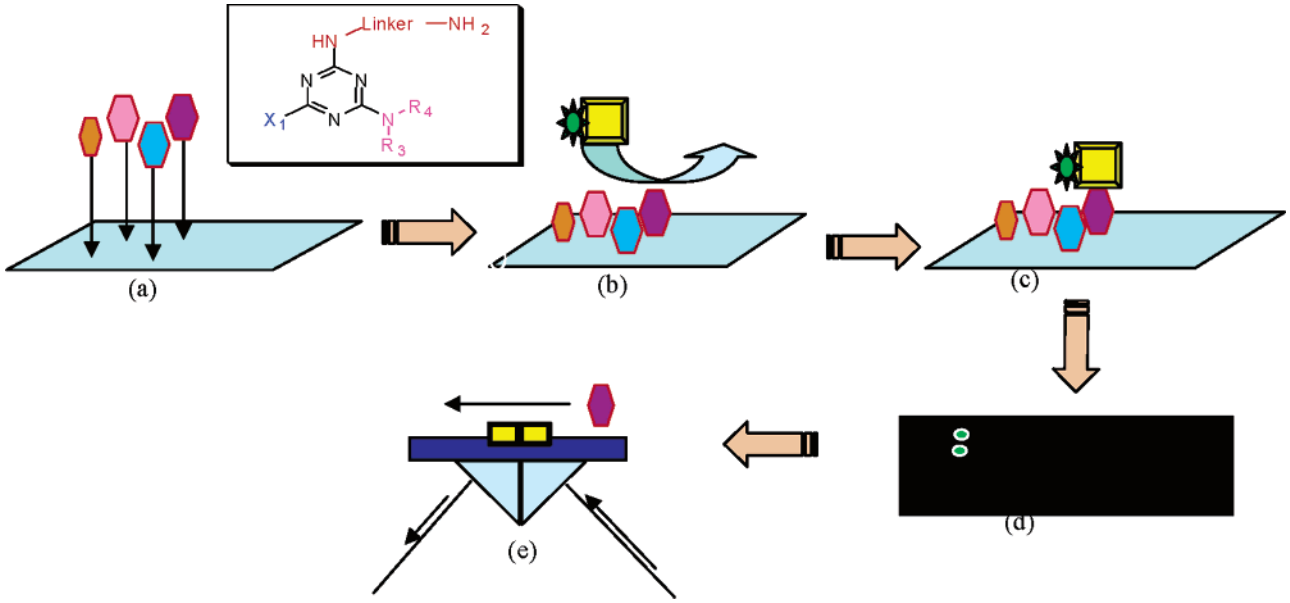


a new orthogonal synthetic pathway, affording much greater functional group diversity over our previous tagged library efforts[9] (Scheme 1). The tagged linker strategy is desirable because incorporation of the linker from the beginning, before biological screening and as an intrinsic part of the compound, eliminates worries about any downstream tether effect, thus providing a straightforward means for target isolation without any detrimental effect to the compound's activity or the need for further structure activity relationship (SAR) experiments.[9]

This TG library compliments and offers advantages over our previously reported efforts[8,9] in a couple of ways. The synthetic scheme differs in that a mono (amino or hydroxy)-substituted cyanuric dichloride moiety was loaded onto a TG-linker-functionalized resin, whereas previously, a linker mono-substituted (the linker as the first substituent) cyanuric dichloride was loaded onto the resin as the first step in the solid-phase synthesis (Scheme 1).[9] This TG-linker-functionalized resin allows for rapid library diversification through simple splitting of the resin. As a consequence of the altered scheme, the second and more important improvement is the addition of primary alcohols to the library building block palette that were unattainable with our previous approach.

Primary alcohols may only be efficiently and cleanly added to the cyanuric chloride scaffold as the first (of three) substitutions. This is due to the drastic decrease in reactivity

Scheme 2. General Experimental Scheme[a]



[a] (a) Directed immobilization of triazine libraries to generate a high-density microarray. (B) Incubation with a fluorescently labeled protein. (C) Removal of the unbound protein through washing. (D) Detection with instantaneous deconvolution of positive hits. (E) Assessing efficacy of hits using SPR.

seen with substituted cyanuric chloride analogues. Introducing an alcohol moiety as the first substituent, thus forming a building block II which can be subsequently loaded to a TG-linker-functionalized resin, is a very useful addition to our chemical toolbox and allows for N versus O atom substitution comparisons with hit compounds in later studies.

The general tagged linker strategy is advantageous for a number of additional reasons. The basic linker used in all cases, 2,2′-[1,2-ethanediyl-bis(oxy)]bisethanamine, is commercially available and affordable and is easily mono-protected (*N*-Boc) in one step. Compound cleavage from the resin and linker deprotection is accomplished simultaneously in one step. The linker provides a sufficient space between the compounds and the microarray surface, at the same time allowing for greater conformational flexibility in the immobilized compounds. Furthermore, its hydrophilic character may provide a more protein-friendly environment during subsequent microarray screening processes. Last, the amino functional group allows for facile small-molecule immobilization and for a rapid transition to further downstream studies, such as affinity matrix pull-down experiments, without the need for any hit compound modification.

The compounds were spotted, in duplicates, as an SMM on a modified glass substrate derived from standard microscope slides in a deterministic fashion that ensures immediate high-fidelity locus-based identification (Scheme 2). In total, 5376 spots corresponding to 2688 triazine-based library compounds were printed; 1152 of those were TG compounds and were synthesized as reported herein, and 1536 compounds were synthesized as reported previously by our group.[9] In addition, we included in our arrayed grids a dye reference to not only validate the slide derivatization process, but also appropriately home in the software in the subsequent data acquisition.

Immunoglobulins have seen numerous applications spanning immunoassays, diagnostics, and immunotherapeutics.[1c] The production of immunoglobulins, for example, valuable humanized variants, for therapeutic applications requires stringent purification measures before being administered as approved drugs. However high molecular weight ligands, such as staphylococcal protein A and streptococcal protein G, are unfavorable for medicinal applications for their potential pyogenic effect as well as for other problems, including low biological stability, leakage from solid support, and difficulty in large-scale production and purification, contributing to high overall cost.[10] Recent literature has shown that triazines may prove useful small-molecule ligand alternatives to IgG. For example, Li et al. used computer-aided molecular modeling to successfully identify triazene analogues that bind to IgG with affinity constants of 10^5–10^6 M^{-1}.[1c] We thus hypothesized valuable potentials in screening human IgG against our arrays not only as proof of our overall concept but also in the discovery of efficacious ligands with direct relevance to industry.

Human IgG was fluorescently labeled with Cy3−NHS to allow for sensitive visualization of small molecule−IgG interactions on the array. Spotted slides alone, without incubation with labeled IgG, were also scanned to ensure that the fluorescence did not originate from the spotted compounds themselves. The resulting scans were typical to that seen in Figure 1. Cases in which only one of the two duplicate spots displayed a substantive signal were dismissed as artifacts, and only hits that corroborated well in repeated experiments were deemed true positives. Three of the strongest hits on the array, based on intensity, were chosen for further validation, namely **AMD10**, **AMD3**, and **K28**. A faint positive, **K42**, and a negative, **APF29**, were also used as comparative benchmarks. In separate control experiments, other fluorescently labeled proteins (unrelated to human IgG) were used to screen against the same slide: none of the hits (e.g. **AMD10**, **AMD3**, **K28**, and **K42**) showed any positive binding, indicating that their binding toward human IgG is, indeed, highly specific. The spot intensities

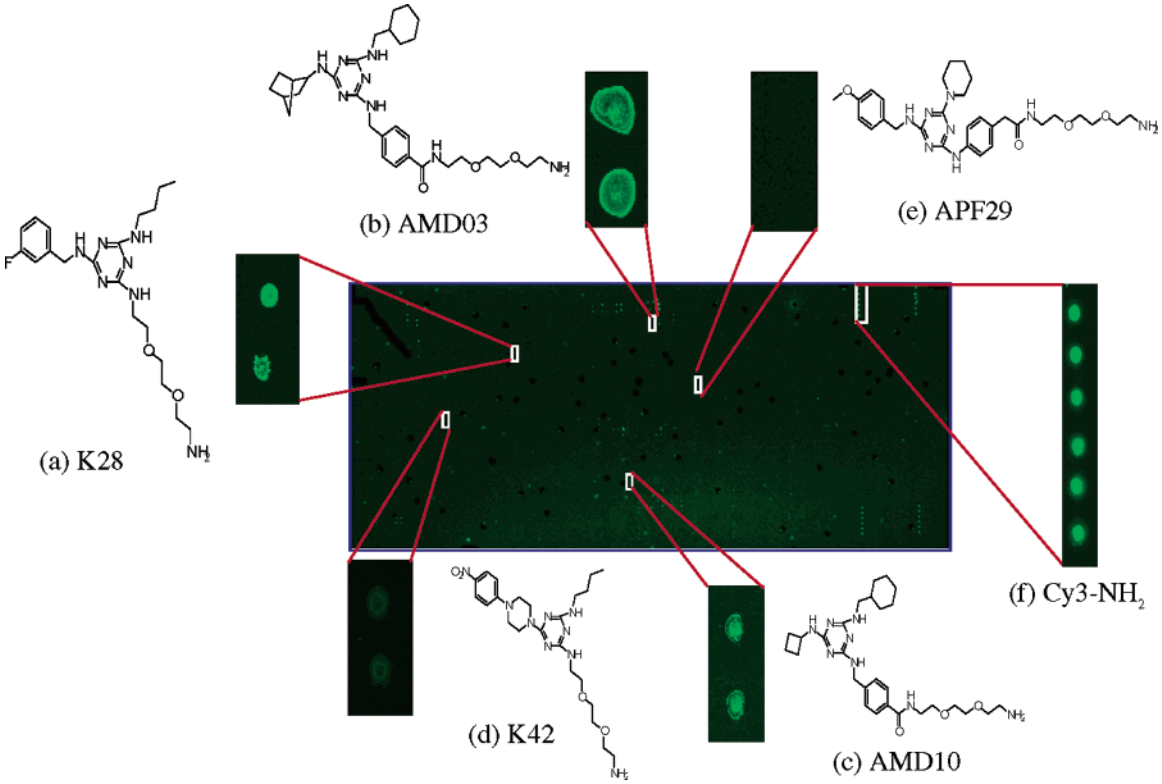


Figure 1. NHS-derivatized slides with 2688 triazine compounds spotted in duplicates and probed with human IgG−Cy3. The actual-sized array is enclosed in a blue box, with blow-ups describing the loci and the corresponding molecules that were selected for further assessments. (a−c) Correlated with strongly positive molecules, producing spot intensities at least two times that of the background. An intermediate (d) and a negative control (e) were also picked for comparative assessment. The reference control (f) is shown, and four sets of the Cy3−NH_2 dye were printed at the ends of the grids.

Table 1. Microarray and SPR Results Obtained with Five Selected Triazines

small molecule	array signal (fluorescence units)	K_d/M	χ^2
AMD10	179 (++)	4.35×10^{-6}	3.42
AMD03	185 (++)	2.02×10^{-6}	2.32
K28	143 (++)	2.54×10^{-6}	0.917
K42	65 (+)	6.02×10^{-5}	2.19
APF29	<10 (−)	1.51×10^{-4}	4.02

of these molecules are given in Table 1, with the background subtracted accordingly.

Dissociation constants were determined for each of the compounds selected using SPR on a Biacore X system with BiaEvaluation software. Competitive binding experiments were performed with differentially proportioned mixtures of a small molecule and protein A on a CM-5 chip immobilized with human IgG (see Supporting Information) (Table 1), which also ensures the small molecule binding to the same site of IgG as protein A. An averaged dissociation constant of 1.25×10^{-9} M was obtained for protein A with IgG alone. Further assessments made by passing 2.5 μM of a small molecule against the IgG surface were also performed to give measurable association levels that correlate with binding affinities (Table 1). Immobilizing the small molecules and applying IgG in the solution phase obtained equivalent binding measurements; however, by employing the competitive binding method described, a single chip surface may be used for screening against multiple potential small-molecule ligands, economizing the process. A χ^2 value of <10 was obtained for all K_d measurements, denoting good statistical validity of the results obtained.

All three of the strong hits defined by the microarray screening were shown to give significant dissociation constants in the micromolar region with IgG. This relationship was further confirmed with a strong increase in response units (RU) when these three molecules were passed across an IgG-derivatized surface. **AMD10**, **AMD3**, and **K28** gave the strongest results with the lowest K_d values of 4.35×10^{-6}, 2.02×10^{-6}, and 2.02×10^{-6} M, respectively. These values were more than an order of magnitude lower than that of the secondary binder, **K42**, which was only weakly positive on the microarray screen. Expectedly, the negative control gave the weakest binding signals. These results further validate that tagging of the target protein with the dye for array applications did not perturb its binding properties with the small molecules. It is also interesting to note that the dissociation constant (e.g., K_d), as well as K_{off} (Supporting Information), of the hits as determined by SPR correlated well with their fluorescence intensity obtained from the microarray screening, with tight-binding compounds consistently giving stronger fluorescence spots. Overall, the dissociation constants obtained from the best hits identified in our experiments compare favorably with what was reported previously with other triazine-based small molecules.[1c]

Conclusion

In conclusion, we have developed a high-throughput screening system to detect small-molecule ligands for virtually any target and have shown its efficacy in discerning

targets of a model protein, human IgG. The SMM used libraries of tagged triazine compounds, one of which is a novel library possessing a high degree of diversity and synthetic versatility. The tagged libraries intrinsically factor the linker in the screen, thus eliminating potential false negatives and increasing throughput. Further, we have developed a fully addressable microarray containing a few thousand compounds, with each compound, once being displayed as a positive, becoming immediately identifiable solely by its position within the grid without the need for additional assessment. The IgG ligands discovered herein may soon find potential applications in the large-scale purification of immunoglobulins and would be useful alternatives to existing protein A-based isolation and purification systems. Studies are underway to establish the utility of the hits in this respect as well as work to identify further triazine ligands for other candidate proteins and DNA targets.

Experimental Section

Materials Used. Unless otherwise noted, materials and solvents were obtained from commercial suppliers (Acros and Aldrich) and were used without further purifications. PAL-aldehyde (4-formyl-3,5-dimethoxyphenoxymethyl) resin from Midwest Bio-Tech (Catalogue No. 20840, Lot no. SY03470, loading level 1.10 mmol/g) was used for the generation of library compounds. Building block II compounds, made by solution phase chemistry, were purified by flash column chromatography on Sorbent Technologies silica gel, 60 Å (63−200 mesh). TLC was performed on SAI F_{254} precoated silica gel plates (250-μm layer thickness). All library products were identified by an LC−MS at 250 nm (Agilent Technology, HP1100) using a C18 column (20 × 4.0 mm) with a gradient of 5−95% CH_3CN-H_2O (containing 0.1% acetic acid) as an eluent over 4 min.

Preparation of Triazine Libraries. The parallel syntheses of triazine libraries, excluding the TG library reported herein, and the synthesis of Boc-linker (2-[2-aminoethoxyethoxyethyl]carbamic *tert*-butyl ester), were previously published.[9]

Preparation of TG Libraries. General Procedure for Coupling of the Linker onto the Resin (Scheme 1). To a solution of PAL-aldehyde resin (1.0 g, 1.1 mmol) was added Boc-linker (2-[2-aminoethoxyethoxyethyl]carbamic *tert*-butyl ester) (1.36 g, 5.5 mmol, 5 equiv) in THF (50 mL, containing 2% of acetic acid) at room temperature. The reaction mixture was stirred for 1 h at room temperature, followed by the addition of sodium triacetoxyborohydride (1.63 g, 7.7 mmol, 7 equiv). The reaction mixture was stirred for 12 h and filtered. The resin was washed with DMF (3 times), dichloromethane (3 times), methanol (3 times), and dichloromethane (3 times). The resin was dried in vacuo.

General Procedure for Building Block I. Cyanuric trichloride (1 equiv) was dissolved in THF with DIEA (10 equiv) at 0 °C. The desired amine (1.2 equiv) in THF was added dropwise. For addition of alcohols to cyanuric chloride, the same reaction conditions were followed, except 2.5 equiv of K_2CO_3 was used instead of DIEA. The reaction mixture was stirred and monitored by TLC. Reaction time was 45 min to 1 h. A solid precipitate slowly formed. Upon completion of the reaction, the reaction mixture was quickly filtered through a plug of flash silica and washed with EA. The filtrate was evaporated in vacuo. The resulting products were purified using flash column chromatography (particle size 32−63 mm) and characterized by LC−MS.

General Procedure for Coupling Building Block I with the Resin. Building block I (0.44 mmol) was added to the resin (0.11 mmol) in DIEA (1 mL) and anhydrous THF (10 mL) at room temperature. The reaction mixture was heated to 60 °C for 3 h and filtered. The resin was washed with DMF (5 times); alternatively with dichloromethane and methanol (5 times); and finally, with dichloromethane (5 times). The resin was dried in vacuo.

General Procedure for the Final Amination on the Resin and Product Cleavage Reaction. Desired amines (4 equiv) were added to the resin (10 mg), coupled with building block I and Boc linker, in DIEA (8 μL) and 1 mL of NMP/*n*-BuOH (1:1). The reaction mixture was heated to 120 °C for 3 h. The resin was washed with DMF (5 times); alternatively with dichloromethane and methanol (5 times); and finally, with dichloromethane (5 times). The resin was dried in vacuo. The product cleavage reaction was performed using 10% trifluoroacetic acid (TFA) in dichloromethane (1 mL) for 30 min at room temperature and washed with dichloromethane (0.5 mL). The purity and identity of all the products were monitored by LC−MS at 250 nm (Agilent 1100 model); more than 90% of compounds demonstrated >90% purity.

AMD03: ESI-MS $(M + H)^+$ calcd, 580.4; found, 581.6. **AMD10:** ESI-MS $(M + H)^+$ calcd, 540.4; found, 541.5. **TGK28:** ESI-MS $(M + H)^+$ calcd, 421.3; found, 422.5. **TGK42:** ESI-MS $(M + H)^+$ calcd, 503.3; found, 504.5. **APF29:** ESI-MS $(M + H)^+$ calcd, 578.3; found, 579.5.

Preparation and Analysis of Small-Molecule Arrays. Preparation of Slides Activated with *N*-Hydroxysuccinimide Esters[2] Briefly, 25 mm × 75 mm slides (Fisher Scientific) were cleaned in piranha solution (sulfuric acid/hydrogen peroxide, 7:3). An amine functionality was incorporated onto the slides by silanization using a solution of 3% (aminopropyl)triethoxysilane in 2% water and 95% ethanol. After 1−2 h of soaking, the slides were washed with ethanol and cured at 150 °C for at least 2 h. Subsequently, the amine slides were incubated in a solution of 180 mM succinic anhydride in DMF for 30 min thereafter were transferred to a boiling water bath for 2 min. The slides were washed again in ethanol and dried under a stream of nitrogen. The carboxylic acid moieties now created on the slide surface were activated with a solution of 100 mM of TBTU (*O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate), 200 mM DIEA, and 100 mM *N*-hydroxysuccinimide in DMF, thus generating the NHS-derivatized slides. The slides once generated were stored in a desiccator at −20 °C and used within 3 months.

Microarray Printing. By individually weighing the solid compounds, triazine stock solutions were prepared to 2.5 mM in DMF, and 40-μL preparations were distributed across seven 384-well plates to give a total of 2688 distinct and pure compounds. Slides were spotted on an ESI SMA arrayer (Ontario, Canada) with the printhead installed with eight ArrayIt Chipmaker 7 Microspotting pins (Telechem, U.S.A.).

Printing was performed in duplicate, and the pins were washed and sonicated in ethanol between samples. A prespotting blotting process was also performed on plain slides to ensure spot uniformity. An additional solution of 0.2 mM Cy3−NH_2 [11] reference was spotted at the ends of the grids using a final eighth plate.

After spotting, the slides were allowed to sit for at least 12 h in situ and then were quenched by washing in an 1% ethanolamine (in DMF) bath for 2 h. After rinsing with ethanol and drying, the arrayed slides were stored in a desiccator at 4 °C. The slides were stable for extended periods and, when required, were simply brought to room temperature.

Protein Screening with Labeled Human IgG. Proteins were tagged with Cy3−NHS by incubating 5 μL of 20 mM Cy3−NHS (Amersham Biosciences, U.K.) with 200 μg of human IgG (Calbiochem, U.S.A.) in a sodium bicarbonate buffer at pH 8. After 1 h of incubation, the labeled protein was separated from the free dye by a NAP-5 Sephadex G-50 column (Amersham Biosciences, U.K.). Before incubation with the labeled protein, the slides were preblocked to remove any nonspecific binding by soaking in 1% BSA in PBS for 1 h. After a brief rinsing with water, the slides were incubated with the labeled IgG.

A 1000-fold dilution of the above protein preparation was used as the incubation solution in a PBS buffer containing 1% BSA with the small-molecule arrays using the coverslip method for 30 min in a humid incubation chamber. Excess IgG was then removed by washing with distilled water. The background was further reduced using repeated washes with PBST. Control screening experiments were performed with unrelated, fluorescently labeled proteins to ensure spots identified from the IgG experiment were highly specific.

Slide Scanning and Analysis. Slides were scanned on an ArrayWoRx scanner (Applied Precision, U.S.A.). Excel sheets were prepared to assign various compounds to specific numberings that could be readily tallied with reference numbers generated by the program. The ArrayWoRx software allows generation of reference files in which the spotting arrangement and the program overlay the spots on the results obtained, allowing compounds to be assigned in a rational fashion to every position. The software also provides large-scale analysis of hits, which rapidly analyzes the entire array, further enhancing throughput.

Surface Plasmon Resonance (SPR) Determination of Dissociation Constants. Maintenance. SPR measurements were made on a Biacore X system (Biacore AB, Uppsala, Sweden). Various maintenance steps were performed to ensure that the instrument was kept in good working conditions. The integrated flow cell was washed, sanitized, and maintained using standard cleansing reagents on a weekly basis. Calibration checks were performed quarterly to ensure that the signal was of good quality, and the instrument was kept separate from other equipments to prevent interference. When not in use, the system was docked with a spare chip and flushed with water at a low flow rate of 5 μL/min to prevent clogging. The system was primed at least twice before use or for the purpose of initiating a new buffer type. A desorb process was performed every 2 days during periods of active use to remove proteins or other contaminating compounds that may have accumulated within the flow cell.

Procedures. Various approaches were conceived to assess the K_d values of the interactions. One successful method immobilized the small molecule on the CM-5 sensor chips and ran them through varying concentrations of IgG. This was found to be a suitable but costly method, because it required multiple chips for analyzing the binding interactions of different small molecules. We conceived that it would be easier to immobilize IgG on the surface and apply differing proportioned mixtures of the "hit" small molecule and protein A (Amersham Biosciences, U.K.). The Heterogeneous Analyte Module of the BiaEvaluation software using this method worked efficiently in providing the required K_d values of the small molecules with IgG, allowing a single chip to be used repeatedly to assess different binding constants. Checks showed that up to 200 injections could be delivered on a single chip with negligible loss in signal output, with a regeneration buffer of dilute HCl, pH 2 (used throughout). Additionally, protein A was found not to bind to any of the small molecules that were tested. The presence of DMF in our small-molecule preparation was problematic in SPR measurements, because it perturbed the refractive index of the buffer, causing anomalous results. In our case, we overcame this problem by using a reference flow cell, thereby negating the effect of differing refractive indexes of the sample and buffer during sample introduction.

Immobilizing Samples on CM-5 Chips. The standard protocol supplied by the manufacturers was employed. The system was set to 25 °C and equilibrated with degassed HBS buffer (comprising 10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.005% v/v P20 surfactant). One flow cell was activated with 1:1 NHS/EDC for 8 min with a flow rate of 5 μL/min, while the other was kept as a reference. After coupling IgG to give an immobilization increase of 5000 RU, the surface was quenched using 1 M ethanolamine, pH 9, for 7 min.

Determination of Association Levels of Small Molecule with IgG. Small-molecule preparations of 2.5 μM were passed through the IgG-activated flow cell with the reference automatically negating bulk effects. The flow rate used was 30 μL/min using PBS buffer, and 50 μL of each small molecule was applied over 1 min. The increase in response units observed directly tallied with small molecules that actively bound to the IgG-activated surface, thus providing a semiquantitative method to intercompare putative binders.

Determination of Dissociation Constants of Small Molecule with IgG. Twenty-five-microliter preparations of the small-molecule analytes (250 nM, 1 μM, 2 μM, 5 μM) were premixed with an equal volume of 238 nM of protein A and injected to the flow cell. The flow rate used was 30 μL/min with degassed PBS buffer. The results were entered into the BiaEvaluation module where the Heterogeneous Analyte Module was applied to obtain the binding and association constants required. Again, the reference cell was used to eliminate any bulk effects arising from the differing buffer composition.

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Supporting Information Available. Compound identity tables and SPR results. This material is available free of charge via the Internet at http://pubs.acs.org.

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