Deconvolution of Combinatorial Libraries for Drug Discovery: Experimental Comparison of Pooling Strategies

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An experimental evaluation of several different pooling strategies for combinatorial libraries was conducted using a library of 810 compounds and an enzyme inhibition assay (phospholipase A_2). The library contained compounds with varying degrees of activity as well as inactive compounds. The compounds were synthesized in groups of three and pooled together in various formats to realize different pooling strategies. With one exception, all iterative deconvolution strategies and position scanning resulted in identification of the same compound. The results are in good agreement with the predicted outcome from theoretical and computational methods. These data support the tenet that active compounds for pharmaceutically relevant targets can be successfully identified from combinatorial libraries organized in mixtures.

Introduction

New synthetic methods in combinatorial chemistry have enabled the preparation of large numbers of samples for drug screening. With some advanced chemical methods,[1−5] the same synthetic effort can produce a unique compound or a mixture of 10, 100, 1000, or more compounds. A combinatorial synthesis group is more limited by the number of screening samples it can produce than the number of total compounds. Thus, the number of compounds to screen becomes more of a strategic than resource-driven decision.

Screening mixtures of compounds allows much greater screening throughput than testing individual compounds. The historical success of natural product screening in drug discovery proves that active compounds can be discovered from compound mixtures.[6−10] However, mixture screening is more complex than testing individual compounds, and a number of caveats are introduced. One of them is the potential for missing active compounds because of interference from other compounds in the mixture (antagonism). Active compounds can also be missed because they are not present in high enough concentration in the screening sample. In addition, the potential exists for a screening sample to appear to have an interesting compound, but upon purification, a suitably active compound is not found. This may be due to a synergistic activity from two or more compounds or simply to an additive effect for many weakly active compounds. Though seemingly daunting, these caveats have not prevented many interesting compounds from being discovered from natural product extracts.

In contrast to natural product extracts, the composition and complexity of the combinatorial library can be controlled. In order to take advantage of this new capability, it is important to determine how to optimally organize combinatorial mixtures for successful screening. The controllable variables include the mixture complexity and the choices of which compounds to group in the same mixture (pooling strategy). Because there are so many choices available to the experimentalist, it is important to determine the most effective strategies.

We have previously developed theoretical and computational methods to gain some insights into how to best organize combinatorial libraries for screening. In a previous paper[11] and in an accompanying report,[12] we used computer simulations of library deconvolutions to test the success of various mixture complexities and pooling and deconvolution strategies. We calculated that, even in the presence of many molecules with suboptimal activity, iterative deconvolution by fixed position almost always selected the compound with the greatest activity. The calculations also predicted that, in the presence of realistic experimental error, either the most active molecule or one with activity close to it will be selected and that the order of deconvolution did not significantly influence the results.

In addition to fixed position pooling, we also calculated what might happen if the compounds were pooled in a completely random fashion (random pooling) or if the most active compound was mixed with the least active compounds and the best suboptimal binders were kept together (hard pooling). We also calculated the performance of position scanning,[13,14] a noniterative deconvolution strategy.

We now report an experimental evaluation of these different strategies using a library of 810 compounds and a human type II phospholipase (PLA_2) inhibition assay. We determined that the library contained a number of active compounds that inhibited PLA_2 to varying degrees as well as many inactive compounds, which make it a good experimental system to test the theoretical predictions. The results showed that all pooling strategies except hard pooling resulted in identification of the same compound and that the experimental results were in general agreement with predictions from theory.

Results

Chemical Library of 810 Compounds. In order to test the variations of iterative deconvolution, a library

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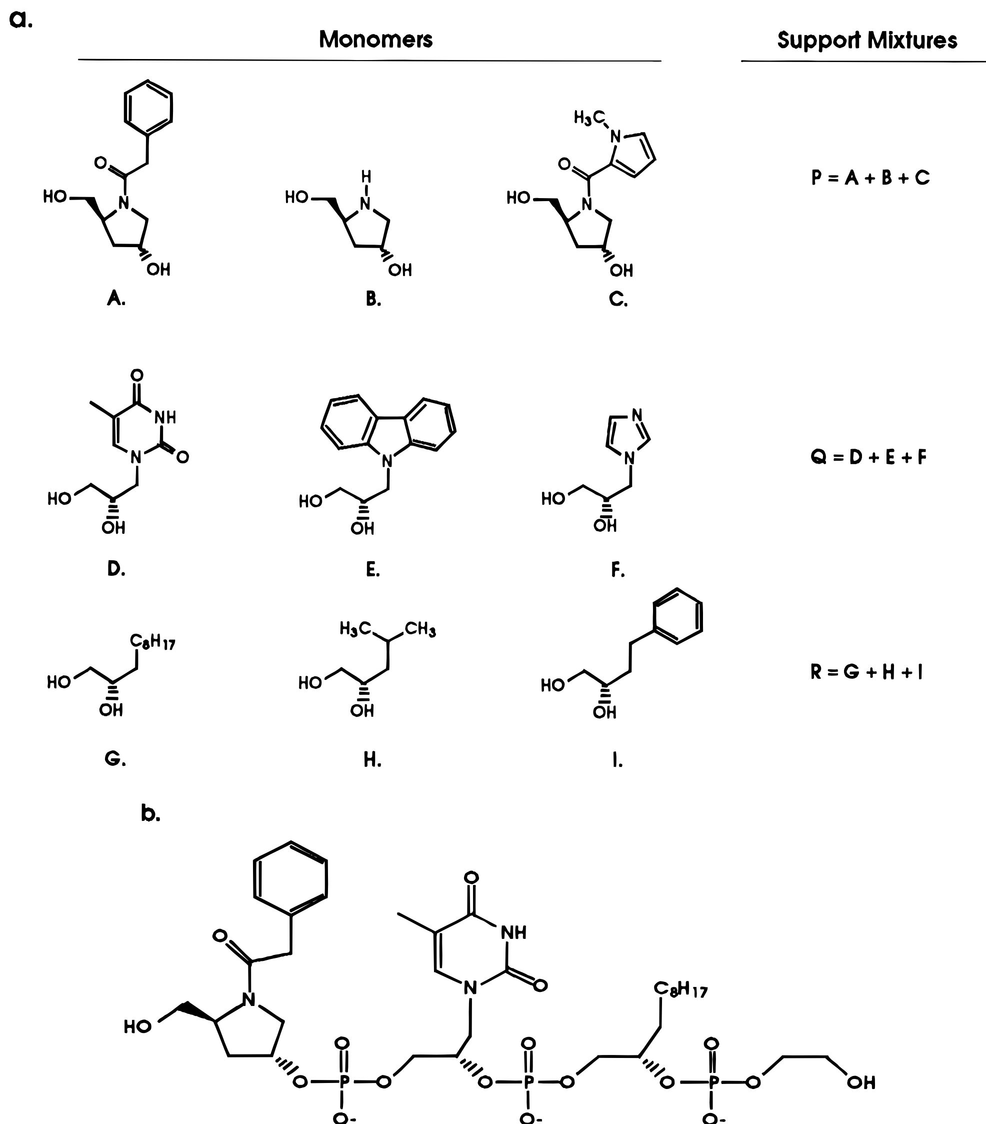

Figure 1. Chemical components of the library of 810 compounds. The library consisted of phosphate diester-linked trimers and dimers composed of the nine monomers shown in panel a. All permutations of trimers (729) and dimers (81) were made in mixtures of three compounds per sample by pooling support mixtures as indicated in panel a. The structure of a trimer compound (ADG) from the library is shown in panel b.

of 810 compounds was synthesized using nine chemical monomer units (Figure 1a). Each of the monomers was attached to a solid support, and three sets of support mixtures (P, Q, and R) were prepared by pooling equal amounts of support-bound monomers as indicated in Figure 1a. The support mixtures were distributed on the automated synthesizer, and two additional monomers were sequentially linked to each of the support mixtures by phosphate linkages in all permutations creating 243 screening samples of three compounds per sample. In total, 729 trimers such as the one in Figure 1b were synthesized. An additional 81 dimer compounds were made by coupling one monomer to each of the support mixtures in all permutations creating 27 more screening samples of three compounds per sample.

PLA_2 Assay. Using a robotic pipetting station, the 270 screening samples were pooled in various formats to recreate the same types of mixture compositions used in iterative deconvolutions. Samples were tested in parallel for inhibitory activity of PLA_2 at a fixed concentration, and activity is reported as the percent of activity relative to an untreated control. The concentration reported for each experiment is the total concentration of all compounds in the mixture. After each round of assays, the most active mixture was "resynthesized" by robotic pooling from the master plates, simulating resynthesis of simpler subsets in an iterative deconvolution.

Order of Deconvolution. Iterative deconvolution is a process of dividing a library into nonoverlapping subsets. The subsets are tested separately, and the one with the greatest activity is identified. This subset is resynthesized as a collection of less complex subsets which are tested for activity. The process is repeated until a unique molecule is identified. There are many ways of organizing subsets or "pooling strategies" for iterative deconvolution. The most common is pooling by fixed position, where, at each round, the functionality at a single position is fixed and the others are randomized. For example, in round 1, the subsets could consist of all molecules NXN, where X is a single functionality unique to each subset and N is an equimolar mixture of all functionalities in that position. Strategies based on pooling by fixed position can differ from one another in which positions are fixed at each round.

In our mixtures of trimers, either the left or middle

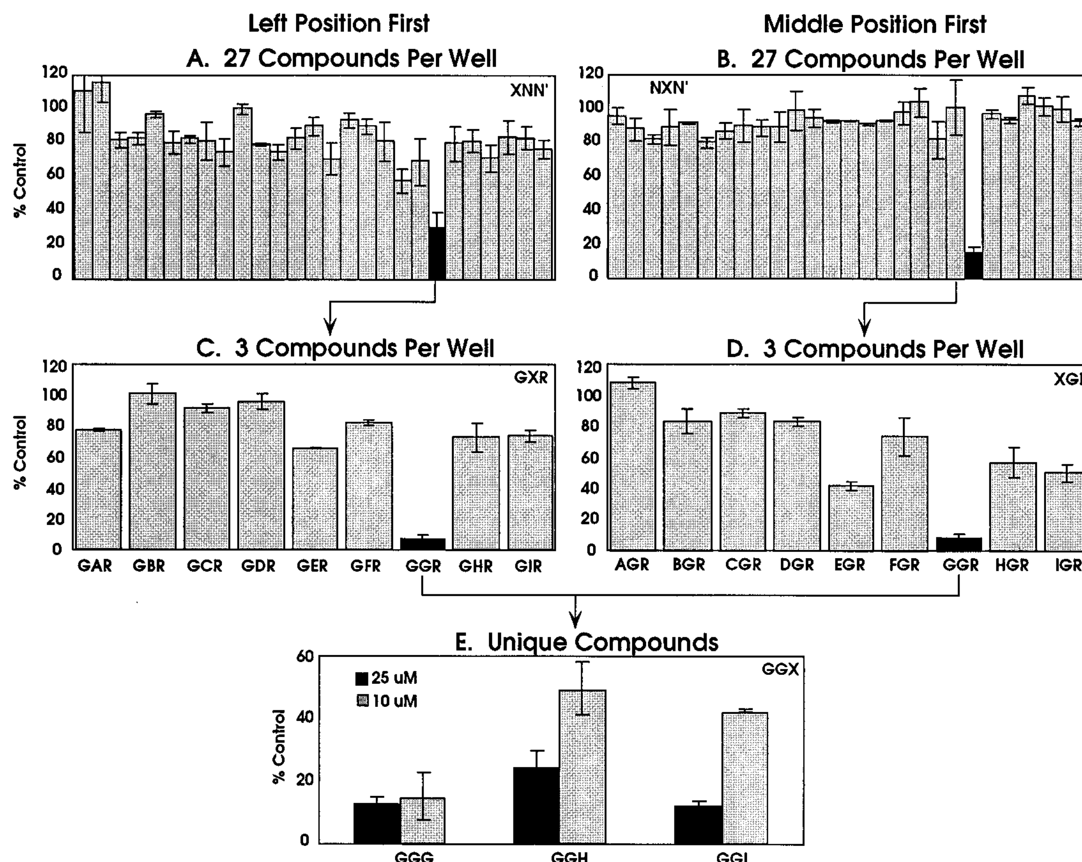

Figure 2. Effect of deconvolution order for pooling by fixed position. In the first round, the 729 trimers were pooled into 27 subsets with 27 compounds per subset. Molecules in each XNN′ subset (panel A) contained a single monomer in the first position, a mix of all nine monomers in the second position, and a mix of three monomers (group P, Q, or R, see Figure 1) in the third position. From left to right, the bars represent ANP, ANQ, ANR, BNP, BNQ, BNR, CNP, etc. The NXN′ subsets (panel B) were similar except position 2 contained a fixed monomer and position 1 was a mix of all nine monomers. From left to right, the bars represent NAP, NAQ, NAR, NBP, NBQ, NBR, NCP, etc. For these first round subsets, inhibition of PLA_2 was measured at 67.5 μM (total concentration). In the second round, the 27 molecules from the most active round 1 subset were divided into nine subsets with three compounds per subset. Molecules in each GXR subset (panel C) contained G in position 1, a single monomer in position 2, and a mix of three monomers in the third position. Molecules in each XGR subset (panel D) contained a single fixed monomer in position 1, G in position 2, and a mix of three monomers in the third position. Activity of the round 2 subsets was measured at 15 μM. Both deconvolution orders identified GGR as the most active subset in round 2. In the third round (panel E), the three compounds in GGR were synthesized and tested as unique compounds. Activity was measured at 25 and 10 μM.

position could be "fixed" first. The right position was always derived from one of three support mixtures (P, Q, or R) and therefore was "partially fixed" and represented as N′. Each library consisted of 27 samples of 27 trimers per sample. In the library with the left position fixed first, the subset with the GNR structure showed the greatest activity in round 1 (Figure 2A). In the second round, GGR was most active (Figure 2C). To complete the deconvolution, the individual compounds from GGR (GGG, GGH, and GGI) were synthesized and tested. GGG showed the greatest activity (Figure 2E), suggesting that GGG was the most active compound in the library.

When the middle position was fixed first, deconvolution proceeded from NGR to GGR and GGG (Figure 2B,D,E, respectively), resulting in selection of the same compound as when the left position was fixed first. This result agrees with the theoretical analysis, suggesting that order of deconvolution is unimportant in iterative deconvolution.[12] In addition, the selected compound showed clearly detectable activity in a mixture of related compounds in two different groupings. As expected, at each round of deconvolution, the activity of the winning subset increased due to progressive enrichment of the winning compound in the more simplified subsets.

Mixture Complexity. In order to determine the effect of mixture complexity on deconvolution, the 729 trimers were pooled into 9 subsets of 81 compounds per subset. Under these pooling conditions, subset GNN resulted in greatest activity in the first round followed by GGN, GGR, and GGG in the subsequent rounds (Figure 3). This result suggests that a 3-fold further increase in subset complexity, compared with the Figure 2 libraries, did not preclude finding the same GGG winning compound. Moreover, when all 729 trimers were pooled together in a single mixture (Figure 3A), significant activity was observed in the assay demonstrating that activity could be detected in a more complex mixture.

Pooling Randomly. In order to determine if there is an advantage to pooling by fixed position over random pooling, the 270 screening samples were pooled randomly into 30 samples of 27 compounds per sample. In the first round of deconvolution, a most active pool of 27 compounds was clearly identified (Figure 4A). When the compounds were tested in groups of three (Figure 4B), the GGR mixture was clearly most active, which is the same result obtained from fixed position pooling.

Because fixed position pooling tends to keep similar compounds together more than random pooling, a slight

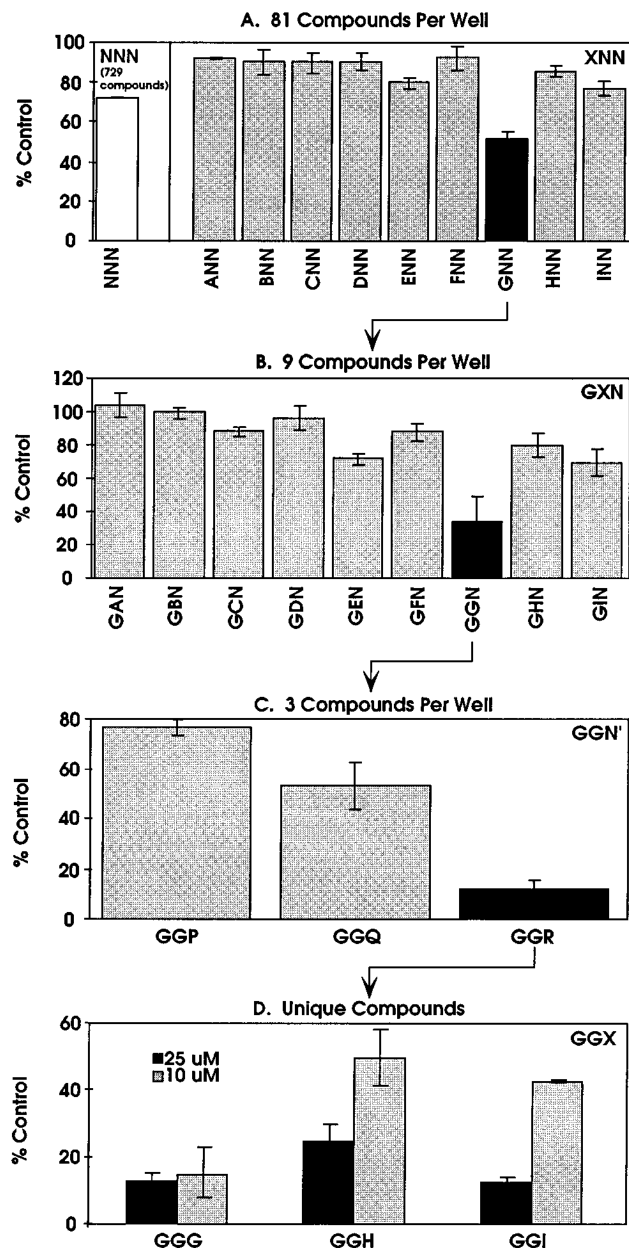

Figure 3. Effect of increased mixture complexity on deconvolution profile. In the first round, the 729 trimers in the library were pooled into nine subsets with 81 compounds per pool. Molecules in each round 1 subset (XNN, panel A) contained a single monomer in the first position and a mix of all nine monomers in positions 2 and 3. Activity of each round 1 subset was measured at 90 μM (total concentration). The open bar in panel A represents the activity of a mix containing all 729 trimers at 90 μM (total concentration). In the second round, the 81 molecules from the most active round 1 subset were divided into nine subsets with nine compounds each. Molecules in each GXN subset (panel B) contained G in position 1, a single monomer in position 2, and a mix of all nine monomers in the third position. Activity of round two subsets was measured at 22.5 μM. In the third round (panel C), the nine molecules from the most active round two subset were divided into three subsets with three compounds per subset. Molecules in each GGN′ subset contained G in positions 1 and 2 and a mix of three monomers (group P, Q, or R, see Figure 1a) in the third position. Activity of the round 3 subsets was measured at 15 μM. In the final round (panel D), the three compounds in GGR were synthesized and tested as unique compounds. Activity was measured at 25 and 10 μM.

advantage in its success rate was observed in the simulated deconvolutions.[12] There is some evidence

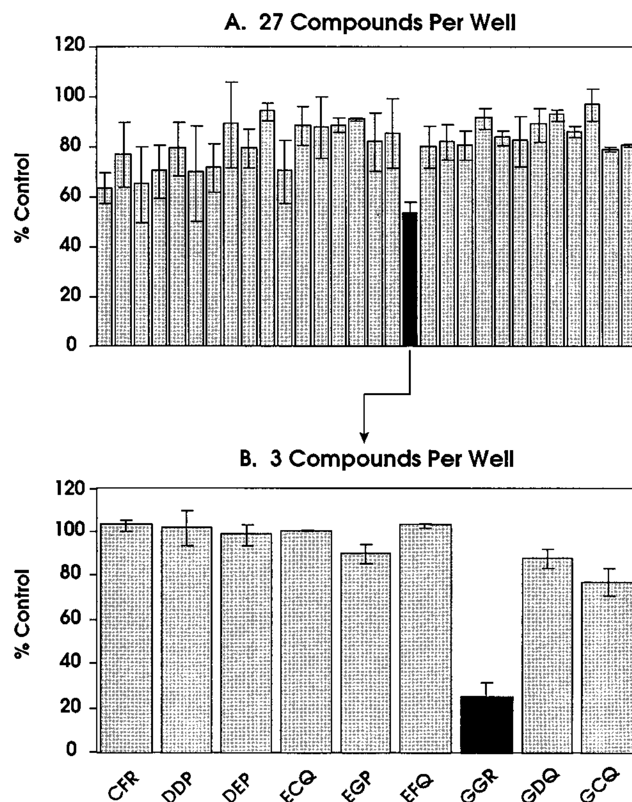

Figure 4. Effect of random pooling on deconvolution. The 810 compounds were randomly pooled into 30 subsets of 27 compounds per sample. The last position was not completely random because three compounds were synthesized together. (A) The 30 round 1 subsets were tested at 67.5 μM. (B) The most active round 1 subset was partially unrandomized in round 2 to nine pools containing three compounds per pool. These subsets were screened at 15 μM total concentration.

that this also occurred experimentally. In both fixed position poolings (left and middle positions fixed, Figure 2A,B), the winning subsets were more active than the winning subset in random pooling.

Hard Pooling. Hard pooling is a exercise to try to make it as difficult as possible to find the best compound by hiding it in a pool with the least active compounds in the library. The next best compounds are pooled together to make the second subset, and the process is continued until all the compounds are assigned to a subset. Since all of the earlier deconvolutions resulted in selection of GGG as the most active compound, we reasoned that G is important in all three positions and compounds without G in any position are likely to be the least active in the library.

We experimentally tested this worst-case scenario by grouping the GGG-containing mixture GGR with eight other sets of three compounds which did not contain G in any position. We then grouped sets of compounds all with two Gs to create the second and third subsets of hard pooling. As might be expected, the subset containing GGG did not have the greatest activity (Figure 5A). The second and third subsets showed the best activities. The most active two subsets and the one containing GGG were deconvoluted further. In round 2, it was apparent that GGR was indeed grouped with essentially inactive compounds (Figure 5B) and that the next two subsets contained multiple active compounds (Figure 5C,D). The compound that would have been selected from hard pooling (either from GGQ or IGR) was severalfold less active than GGR.

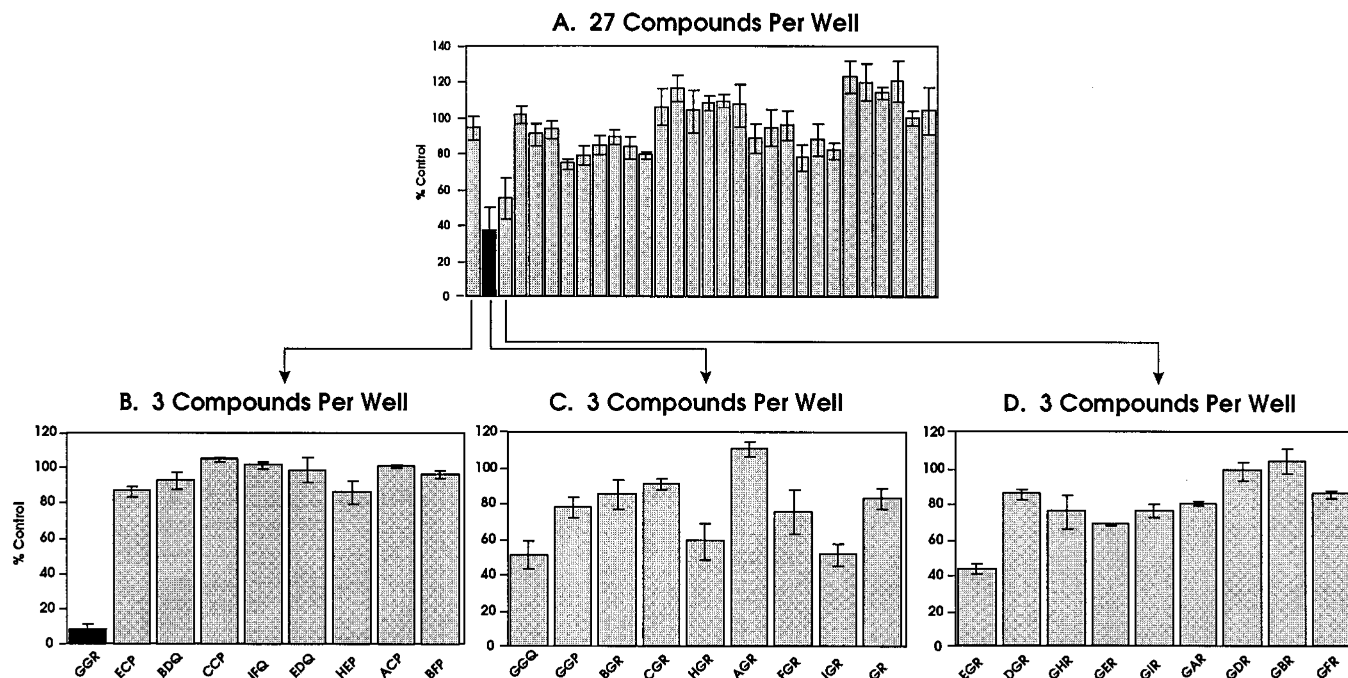

Figure 5. Deconvolution with hard pooling. The 270 sets of three compounds were grouped according to their activities as described in the text. The first subset contained GGR and no other active compounds. Other active molecules were in the second and third subsets. (A) The 30 round 1 subsets were assayed at 67.5 μM total concentration. (B–D) Each of the first three subsets was partially unrandomized in round 2 to nine pools containing three compounds per pool. These subsets were screened at 15 μM total concentration.

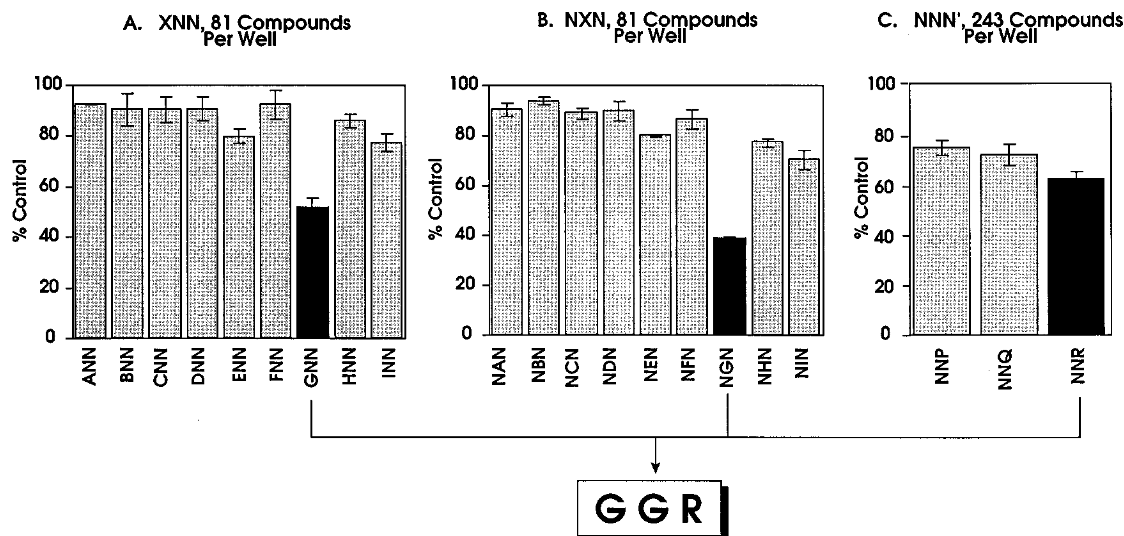

Figure 6. Position scanning was evaluated using three sets of mixtures. Subsets in which position 1 (panel A) or 2 (panel B) was fixed contained 81 compounds per subset. When position 3 was fixed (panel C), each subset contained 243 compounds because monomers in groups P, Q, and R were pooled together. All subsets were tested at a total concentration of 90 μM.

Position Scanning. Position scanning is a noniterative selection strategy. A set of mixtures is synthesized for each position with a single monomer fixed in each position. The most active compound is deduced by selecting the monomer from the most active subset in each position.[13,14] We pooled our mixtures in a fashion that replicates a position scanning experiment, except that we partially fixed the third position as indicated in Figure 6.

In position scanning there is only one round of synthesis and screening, which is the potential advantage of the method. The results showed that position scanning gave the same result, GGR, as the iterative deconvolutions.

Effect of Experimental Error on Deconvolution. In the theoretical studies, 2-fold error in subset activity decreased the likelihood of selecting the best compound. In these experimental studies, activity was typically measured at a single dose, and thus error in IC_{50} could not be determined. To compare the errors in percent control observed at a single dose with a 2-fold error in activity, binding curves were simulated for compounds with K_D values that differed by 2-fold, and differences were calculated at each point on the curve. A 2-fold error in K_D correlated with a 7% error in signal at 85% activity and with a 16% error in signal at 50% activity. Errors reported in Figures 2–6 are comparable to these values, suggesting the observed errors were consistent with a 2-fold error in subset activity.

Discussion

The success of screening compound mixtures from natural product extracts suggests that active compounds should be discovered if present in combinatorial library mixtures. However, there are some significant differences between combinatorial libraries and natural product extracts. The composition and complexity of the natural product extracts from which useful drugs have emerged were largely unknown, but the extracts certainly contained highly diverse compounds. Combinatorial libraries have better defined compositions and complexities, and the diversity of the compounds in chemical libraries is probably less than that of natural product extracts. All combinatorial library compounds have some common feature, either a scaffold or a linking reaction, which relates the compounds to each other. In addition, in most strategies, functional groups appear in more than one compound in a mixture. It is not clear how the relatedness of compounds will affect screening results in various pooling strategies.

To address this issue experimentally, we screened a combinatorial library of 810 compounds for inhibitors of the enzyme PLA_2. The compounds were synthesized in mixtures of three using a parallel-array synthesizer. Using a robotic pipetting station, we mixed the compounds to create several pooling scenarios and then pursued deconvolution based upon the experimental screening results in each round as one would in a deconvolution of a complex library.

In a previous report[11] and in an accompanying paper,[12] we developed theoretical and computational methods to evaluate pooling strategies for combinatorial libraries. The results showed that, even in the presence of many molecules with suboptimal activity and in the presence of realistic experimental error, iterative deconvolution by fixed position was a very successful strategy. Additionally, the order of deconvolution was relatively unimportant to success, but keeping active molecules together in early rounds enhanced success somewhat. Noniterative methods, such as position scanning, had mixed success depending upon the library.

The experimental results in this paper are consistent with these theoretical results. Each of the pooling strategies except hard pooling succeeded in finding the same compound. The two different orders of deconvolution showed some difference in how much the most active pool compound stood out compared with the other pools (Figure 2A,B). Screening individual compounds (or groups of three) showed that monomer “G” in the middle position was somewhat more important than in the left- or right-most position. This was reflected in greater relative activity in the first round with the middle position fixed first (Figure 2B).

Within the relatively modest range possible in this library, increasing mixture complexity had no effect on the outcome. Increasing complexity from 27 compounds per pool to 81 resulted in finding the same compound. Even when all 729 trimers were pooled, activity could be detected relative to the control or to pools of 81 compounds not containing the most active compound (Figure 3A).

The simulations suggested a small advantage to keeping active molecules together in the early rounds and separating them in the later rounds when the pools are less complex. This has also been suggested by others.[15] Fixed position pooling tends to keep like molecules together relative to random pooling because all the molecules in the pool have a common unit in the fixed position. This may have been responsible for the relative ease in selection of the correct active pool in fixed position strategy (Figure 2B) compared to random pooling (Figure 4A). However, the same compound was selected using random pooling.

Although these results are supportive of the iterative deconvolution strategy, a contrived worst-case scenario shows that it is possible to miss the most active compound by intentionally hiding it within a pool with inactive compounds and grouping all the next best compounds in a pool together. Although this scenario precluded selection of the pool with the most active compound (Figure 5A, first bar), it resulted in selection of one of the more active suboptimal inhibitors (Figure 5C).

Position scanning produced mixed results in the simulations, depending upon whether or not register complexity confused assembly of the correct sequence. In our experiment, positional scanning succeeded in finding the same compound as the iterative methods. However, because the most active compound was a trimeric repeat, register complexity could not cause an error in this system as it did in the theoretical simulations.

The attraction of synthesis and screening of combinatorial mixtures is the relatively rapid rate with which a unique compound can be identified. For example, in Figure 3, only 24 subsets were prepared and tested. If these compounds had been prepared one at a time, 729 samples would have been synthesized and assayed. Data from mixture testing cannot conclusively prove that the most active compound was identified. However, position scanning and all iterative strategies tested, with the exception of hard pooling, identified the same inhibitor of PLA_2. The selected compound has an IC_{50} of 5 μM and is a trimeric repeat of monomers with hydrophobic alkyl chains linked via phosphate diester groups. The compound resembles the natural substrate of the enzyme. The library also contained several other somewhat less active compounds with different hydrophobic groups and many inactive compounds. Although the selected inhibitor may not be of therapeutic interest, this library of diverse but related compounds coupled with the PLA_2 assay provided a good system to test the effects of suboptimal inhibitors in different scenarios of iterative deconvolution. The experimental results confirmed the theoretical predictions for iterative deconvolution; all strategies except hard pooling identified the same compound. For position scanning, the experimental results were more successful than the theoretical predictions, probably because register complexity did not cause an error in this system.

Experimental Section

Library Synthesis. Compounds were synthesized on solid support using phosphoryl-linking chemistry as previously described,[16–18] except that synthesis was conducted on an automated instrument capable of independent parallel syntheses in 96 reaction vessels.[19]

Enzyme Purification. Human type II PLA_2 cloned from human placental cDNA was expressed in a baculovirus expression system as previously described.[20] The frozen

growth media were thawed overnight and filtered to remove any precipitates (Whatman 3MM paper), and the pH was adjusted with acetic acid to pH 4.5. The enzyme solution was pumped onto a Pharmacia SP-Sepharose column (2.9 × 17 cm, bed volume 110 mL) equilibrated in 50 mM acetate buffer (pH 4.5), 200 mM NaCl at 5 mL/min. The column was washed with approximately 250 mL of the acetate buffer and then washed with 200 mL of 50 mM sodium borate buffer (pH 9.0). The column was washed with a further 100 mL of acetate equilibration buffer and finally the enzyme eluted with 2 M NaCl, 50 mM acetate buffer (pH 4.5). The enzyme was pooled and dialyzed (3500 MW cutoff) against 50 mM acetate buffer (pH 4.5), 200 mM NaCl, 40% (v/v) glycerol at 4 °C. The enzyme was assayed for activity, BSA was added, and 0.5 mL aliquots were frozen at −70 °C. The enzyme was diluted 1/200 in 1× PLA_2 buffer (100 mM Tris, pH 7.5, 1 mM $CaCl_2$) before each use.

PLA_2 Assay. PLA_2 activity was measured using a standard assay[21] adapted for use with a 96-well microtiter plate in a final reaction volume of 100 μL, using [^{3}H]arachidonate-labeled *Escherichia coli* cell suspension (DuPont NEN, NET-1062) as substrate. Compound was incubated with PLA_2 for 5 min in 1× PLA_2 buffer. Labeled *E. coli* suspension (50 000 cpm) was added, and the reaction mixtures were incubated for 15 minutes at 37 °C. The reactions were terminated by the addition of 50 μL of 2 M HCl followed by 50 μL of 20 mg/mL fatty acid free BSA. The microtiter plates were centrifuged at 1200*g* for 5 min to remove bacterial debris. [^{3}H]Arachidonate release was determined by scintillation counting of aliquots (165 μL) of the supernatant. Data are the average of three or more experiments and are reported as percent activity relative to control with no compound present. Error bars represent the standard deviation of these replicate measurements.

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