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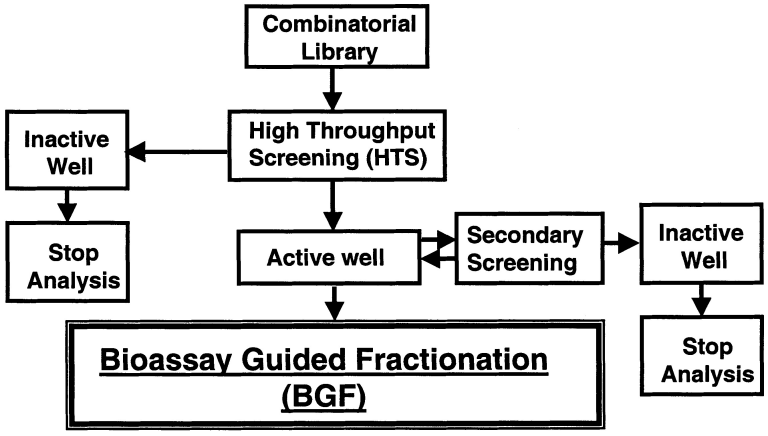

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High-Throughput Bioassay-Guided Fractionation: A Technique for Rapidly Assigning Observed Activity to Individual Components of Combinatorial Libraries, Screened in HTS Bioassays

Douglas W. Phillipson,* K. Eric Milgram, Alex I. Yanovsky, Linda S. Rusnak, David A. Haggerty, William P. Farrell, Michael J. Greig, Xiaobing Xiong, and Mark L. Proefke[†]

High-Throughput Discovery Division, Pfizer Global Research and Development−La Jolla/ Agouron Pharmaceuticals, Inc., 10770 Science Center Drive, San Diego, California 92121, and Pfizer Global Research and Development, Ann Arbor Laboratories, 2800 Plymouth Road, Ann Arbor, Michigan 48105

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In this paper, we describe an automated, high-throughput analytical tool for the unambiguous characterization of the active component(s) of a combinatorially derived reaction mixture. We call this technique high-throughput bioassay-guided fractionation (BGF). The novel aspects of this communication are the systematization of the BGF concept, the application of BGF to combinatorial chemistry, and the high-throughput nature of the identification technique. The identification of the active component in a well mixture is an essential step for subsequent resynthesis or isolation of the active component(s) or for removal of intractable wells from further consideration. We believe the technique described is also applicable to any mixture library, provided the expected component (or components) of each well is (are) known. Example mixture libraries would include collections of synthetic chemicals and collections of purified natural products. The mixture need not come from libraries produced using parallel synthesis. The BGF tool described herein allows full utilization of highly diverse combinatorial libraries, thereby obviating costly up-front purification or extensive prescreening characterization efforts.

Introduction

Generating large numbers of compounds by combinatorial chemistry-based synthesis strategies, then testing them in high-throughput screening (HTS) bioassays, is a widespread practice within the pharmaceutical industry. This practice can produce a larger number of active leads in a much shorter time than was possible prior to the advent of combinatorial methods.

Currently, HTS techniques permit testing of more than 100 000 wells/day for a given assay.[1] Leading combinatorial production groups are currently able to produce between 20 000 and 30 000 new samples/month. At this rate, a 1 000 000 compound library could be produced in ∼4 years. Although this hypothetical production rate may seem small relative to the numbers that can be accommodated by today's HTS assays, combinatorial methods are the best yet devised for generating a large number of structurally diverse compounds in a short time. The advantages of combinatorial chemistry to the drug discovery process have been described in many previous papers.[2−6]

The use of generalized reaction conditions, which usually results in mixtures, is one trait shared by all parallel-synthesis methods. Parallel-synthesis strategies are implemented by judiciously combining sets of reactants and subjecting them to a single set of generalized reaction conditions (e.g., reaction solvent, concentration of starting materials, reaction temperature, reaction time, and pH, just to name a few). Obviously, a set of generalized reaction conditions cannot be optimal for all of the starting materials to be used. In fact, the combinatorial synthesis protocol is often optimized in such a way that its scope is increased, with the sacrifice being that it is suboptimal for any given set of reactants.

A few examples of generalized reaction conditions affecting the final product are given here. For some reactants, the time or temperature is insufficient to allow the reaction go to completion. For others, the expected product might be formed initially but then react further to form a more thermodynamically stable product. In other reactions, one or more of the starting materials may be susceptible to partial or total hydrolysis, or even oxidation. Even more troublesome, the use of impure starting materials may affect the course of the reaction. The use of generalized reaction conditions could result in any combination of these unwanted effects; therefore, combinatorial synthesis methods often produce mixtures, not pure compounds.

Mixtures pose a serious impediment to the selection of lead compounds based on HTS assay data. The observed assay activity can be due to either one or multiple components present in the mixture. Therefore, two options exist in the modern synthesis/testing paradigm: screen mixtures and

[†] Pfizer Global Research and Development, Ann Arbor Laboratories.

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face the task of identifying the bioactive component after the well shows interesting activity in a HTS assay, or purify the expected product of each library reaction prior to screening.

If the screening of mixtures option is chosen, then some method for prioritizing samples based on their probability for success with follow-up efforts is needed. Otherwise, the number of positive assay responses yielded by combinatorial synthesis methods becomes unwieldy very quickly. If we could purify every expected reaction product and chose the second option, our experience suggests that we would eliminate valuable active compounds from the HTS assay. We would also eliminate the ability to observe a positive assay response from components of the mixture other than the expected reaction product. The ability to purify and characterize reasonable quantities of 20 000−30 000 mixtures/month has not yet been achieved, although various groups are attempting to purify thousands of compounds/month.[7]

To facilitate the identification of the active species in combinatorially produced well mixtures, we have developed an automated, high-throughput technique that determines the relative contribution of each component in a mixture to the total observed activity for that mixture. Furthermore, because we use a high degree of automation, our technique does not require operators to be highly specialized, and the inherent errors common to more manual methods are obviated. We call this experiment high-throughput bioassay guided fractionation (BGF).

Combinatorial Chemistry and Bioassay Guided Fractionation. In a typical synthesis-screening process, combinatorial parallel-synthesis methods are used to produce simultaneously large numbers of compounds in 96-well plates. The synthesis products are often mixtures of components. The synthesis products are generally stored frozen in DMSO rather than as a solid, because liquid-handling equipment is less expensive and less error prone than solids-handling equipment and substantially more amenable to automation. Additionally, the well mixtures are typically screened "as is" in HTS assays.

The "as is" synthesis-screening paradigm has been adopted for several reasons. First, as stated earlier, the purification and characterization of reasonable quantities of expected product from 20 000 to 30 000 wells synthesized per month is not feasible at present. Second, the screening of mixtures increases the number of compounds tested per unit time in an HTS assay and increases the diversity of the compound set under test. Third, because our compounds are stored in solution (rather than neat), there is increased potential for them to degrade over time. Even if effort was expended to obtain a purified compound from a mixture, the possibility exists that the purified compound could degrade. For all purified but unstable compounds in a screening file, a mixture will be assayed in the HTS assay. Fourth, screening putatively pure compounds can be misleading. At a typical HTS assay concentration, a 5−10% impurity in a sample could result in a positive assay response. Therefore, a purified compound that accounts for ∼90−95% of the test solution could be identified mistakenly as the active species in a given well, whereas the real species responsible for the activity could go unnoticed. Throughout the remainder of this paper, we will refer to a positive HTS assay response as a *well hit*, because most HTS assays are performed in 96-well plates (or some multiple thereof). The designation *well hit* indicates that some component in an HTS well produced a positive assay response, but that the identity of the activity-causing component is not known.

Finally, in addition to the four previously discussed reasons, we screen mixtures because we can! We are able to rapidly determine which component (or components) in the mixture is causing the desired assay response by application of our bioassay-guided fractionation technique. Currently, most analysts eschew screening of mixtures, because they have no facile method for quickly assigning the observed activity from a mixture to a single component. Without such a method, it becomes difficult to manage the large number of well hits produced using combinatorial synthesis methods that show a positive assay response.

Our inspiration for BGF came from the natural-products discipline. Natural-products chemists, who were faced with isolating and identifying the "bioactive" constituent(s) of a plant or animal extract, have developed a repertoire of specialized techniques for this purpose.[8,9] Starting with a classic technique and incorporating some modern improvements, mainly with respect to computing, automation and data reduction, we have developed a high-throughput method for assigning the activity from a mixture to one or more of its components.

In a paper by Cox et al.,[10] Professor Hunt's group described an experimental setup in which samples were separated by HPLC and collected as fractions while UV and MS data were obtained simultaneously. After the fractions were collected, they were tested for bioactivity, and those results were correlated with the analytical data. In their work, no description of high-throughput or automated data analysis was described, and the technique was applied to resolving "natural products" issues, not combinatorial issues.

Griffey et al. also described an approach that uses chromatographic deconvolution of active components in a mixture.[11] We report here the first combined use of sample fractionation for bioassay and MS analysis in the same experiment for "nonnatural product" isolation purposes.

The novel aspects of this communication are the systematization of the BGF concept, the application of BGF to combinatorial chemistry, and the high-throughput nature of the technique as described in the Results and Discussion Section. We believe this technique will find wide use wherever large numbers of mixtures are screened.

Experimental Section

Reagents. Water was filtered and deionized to better than 18 MΩ·cm with a Milli-Q laboratory purification unit (Millipore Corporation). HPLC grade acetonitrile was purchased from EM Science. All other reagents not explicitly designated were ACS grade. Samples subjected to BGF analysis (i.e., well hits) were produced by our parallel synthetic efforts and shown to give a positive assay response when tested in HTS assays. Aliquots were diluted for analysis and for controls, as described below.

Table 1. Mass Spectrometer Parameters

ionization mode: APCI (positive)	vaporizer 450 °C
capillary: 200 °C; 10 V	discharge current: 5.0 μA
scan range: *m*/*z* 160−1000	sheath gas: 80, Aux. gas: off
tube lens: 0 V	AGC on

Instrumentation. The major analytical instrumentation was composed of an HP1100 HPLC system (quaternary pump, inline vacuum degasser, and diode array detector), a Finnigan LCQ Duo ion trap mass spectrometer, and a Gilson 215 liquid handler, which had been fitted with a 17 (microtiter, deep well)-plate bed for fraction collection.

The chromatographic conditions employed in the analysis are given below. The total time between analyses for the fractionation experiment is 24 min.

prefilter: 0.5-μm Upchurch holder (A-316) and frit (A-102X)

column: Zorbax SB-C18, 150 × 4.6 mm, 5-μm particle

mobile phase: $t = 0$ min, 85/10/5 water/ACN/buffer linear ramp to

$t = 15$ min, 0/95/5 water/ACN/buffer then hold

$t = 20$ min, 0/95/5 water/ACN/buffer

$t = 20.1$ min, 85/10/5 water/ACN/buffer

$t = 24$ min, end reequilibration

buffer: 100 mM Ammonium Acetate (aqueous)

flow rate: 2 mL/min

column temp: 30 °C

inject volume: 22 μL (adjusted as described below, if needed, for dilutions)

detection: UV diode array, summed 205 to 450 nm; Digital resolution 2 nm

splitter: A Valco 3 way union with a 0.25-mm bore placed after the UV detector and appropriate lengths of peek tubing produced a 1:9 split to the MS/Fraction collector

Data collection commenced at the time of sample injection. Centroided data of scans from *m*/*z* 160 to 1000 were collected for the entire chromatographic run (20 min). The ion source and trap conditions are listed in Table 1.

Sample Preparations. Samples for BGF were typically received as a 35-μL aliquot of a 25 mM solution in DMSO. This aliquot was assigned a unique identifier (i.e., a database key) that was used for tracking purposes. After 1 μL of the neat solution was transferred to a control well (microtiter plate coordinate F11, see below), the remaining 34-μL volume of sample was diluted with 18 μL of acetonitrile and 16 μL of water.

Samples were then hand-shaken and checked for precipitation. Less than 10% of the samples showed a precipitate with this treatment. Up to two additional 68-μL aliquots of acetonitrile were added to dissolve samples if necessary. If additional solvent was added, the injection volume was increased accordingly (44 or 66 μL) to accommodate the increased total sample volume. Those samples that did not completely dissolve after the two additional acetonitrile additions were not pursued.

Once the samples were prepared as described above, they were loaded into the HP1100's autosampler. Additional controls were removed and transferred to the plate after fractionation (see below). Some samples were observed to precipitate and clog the column head, thereby causing a high backpressure and causing an instrument shutdown. Increasing the percentage of acetonitrile to 30% in the initial gradient conditions alleviated this problem if a subsequent analysis was deemed necessary.

Fraction Collection. Fractions were collected into 96 deep-well, 2-mL-capacity polypropylene plates (Beckman part no. 267006). Fraction collection commenced with the injection of the sample from the HP1100 autosampler, and fractions were collected at a rate of 1 well (500 μL) every 15 s.

Controls. Row 11 on the plate contained the analytical chemistry controls, and row 12 was reserved for controls used by the screening (biological assay) group. At the time of this writing, control samples were transferred to the fractionation plate manually, although this task and that of sample preparation could be automated. The analytical chemistry controls were prepared and pipetted into the row 11 wells at the time the fractionation plate was produced.

A11: 20 μL of the injected sample from the sample vial,

B11: 20 μL of the injected sample + 250 μL of ACN + 212 μL of water + 13 μL of buffer

C11: 50 μL of the B11 mixture (1:10 dilution)

D11: 5 μL of the B11 mixture (1:100 dilution)

E11: 1 μL of the B11 mixture (1:500 dilution)

F11: 1 μL of the original DMSO solution transferred earlier (described in the sample preparation section above)

G&H11: empty

Row 12 was left empty (reserved) for the screening group to add their assay controls.

Plate Drying and Reconstitution. Once the fractions were collected in the 96-well plate, for solvent removal, the plates were placed in a Genevac HT-12 evaporator that was fitted out to accommodate 24 plates. No sample heating was employed, but the Genevac case heaters were used. Drying of all solvent and volatile buffer removal typically required 6−8 h, and the plates were typically left in the Genevac overnight. Prior to dilution for biological assay, fractions were reconstituted in a minimum volume of DMSO. The exact volume of DMSO added to reconstitute the dried fraction was assay-dependent. Typically, 20−40 μL was added. A minimum of 20 μL of DMSO was required to reproducibly wet the entire surface of the well and dissolve the entire residue. The exact dilution was chosen such that the full-strength control wells (A11 and B11) gave the maximum possible HTS assay signal.

Screening. For a typical assay, a 10-μL aliquot of the reconstituted fractionation plate well contents was transferred to another plate and diluted to 100 μL with assay buffer. A second dilution of the 10% DMSO daughter plate was accomplished by removing 10-μL aliquots from these wells for the screens (run at 100 μL total volume). In some cases, an aliquot of the initial dilution was used directly in the assay. These volumes were based on the limitations of the robotics and the requirements of the screening assay selected. Once collected, the assay data was automatically posted to a proprietary database.

Table 2. Possible BGF Outcomes for Observed Activity of a Well Hit

possible BGF outcomes	action	example figure no.
1. corresponds to the expected synthesis product	follow up	1
2. corresponds to a starting material	follow up	2
3. corresponds to an expected side product	follow up	2
4. corresponds to an unexpected side product	follow up or discard	2
5. corresponds to multiple components	follow up or discard	3
6. elutes in a broad band	discard	4
7. does not elute from the HPLC column	discard	5

Data Analysis. Upon completion of the LC/MS run, the analytical data, which consisted of the mass spectrometer and diode array channels, was extracted from the raw data file and posted to a database. Upon completing a run, the Finnigan Xcalibur software triggered a proprietary program. This program extracted all masses from the entire 20-min MS data set, summed them, and sorted the resulting masses by abundance. For each 15-s block of data, which corresponds to a single collected fraction, the program identified the most abundant mass and all masses with at least 10% relative intensity of the most abundant mass ion. This MS data, along with the UV data, were then posted to the database.

Web-Enabled Viewing of Results. After assay of the fractionated plates, the screening results were correlated with the analytical (UV and MS) data in order to determine which components were responsible for the observed activity. To facilitate access to the correlated data, a proprietary program was written. Using a commercial Web browser and the viewer program, the experimental results can be viewed using the Web browser's graphical user interface (GUI). The viewer program integrates both the screening and analytical data by superimposing them in a single interactive plot. With this information, a user can readily ascertain which components were (or were not) responsible for the observed activity in the original mixture.

Results and Discussion

The main purpose of the BGF technique is the dichotomization of samples into those that are amenable to follow-up, with a reasonable expectation of success, and those that do not contain a discrete, identifiable component responsible for the observed activity. By allowing us to correlate swiftly the observed activity of a screened mixture with the individual components comprising the mixture, the BGF technique is an invaluable tool for assigning a priority to well hits for follow-up efforts. Eliminating well hits is usually not detrimental, as the number of well hits obtained from screening of libraries produced using parallel synthesis methodologies is usually large. Therefore, our challenge is to reduce this large number of well hits (thousands) in a rational manner to one that is more manageable (hundreds).

Using the systemized approach, the BGF experiment is capable of analyzing 400+ samples (wells)/month. One analyst using one instrument operating approximately 12 h/day, 5 days/week achieves this throughput. The Web viewer facilitates data analysis and significantly increases the throughput of the experiment.

From the BGF process, we are able to classify the well hits into one of seven groups. These groups are listed in order of ease of characterization in Table 2, which also contains a reference to the appropriate figure and our typical course of action for a given outcome.

We envisioned a single experiment that would provide sufficient information to allow us to quickly characterize the active component from a well hit. Table 2 describes all of the typical types of results for the samples we have seen. The figures illustrate how the viewer greatly facilitates the process of assigning the observed activity in a well mixture.

Occasionally, the activity results from one or more species eluting in the void volume. Activity is sometimes observed from species eluting as a very broad peak (or series of peaks). The activity may also result from species not eluting from the column or from component(s) possessing unrecognizable UV or MS signals or both. When any of these samples are encountered, the BGF experiment alone does not yield the identity of the active component. However, the appropriate follow-up action is still indicated for these classes of samples, based on the number of distinct chemical classes of hits that can be easily followed up.

The addition of LC NMR to the BGF instrumental set might provide more information than we currently gather. One possible advantage of such an addition would be for the confirmation of the presence of the correct isomer in the wells where multiple isomers are possible. The current embodiment of the BGF experiment does not distinguish isomers. In the event of an unexpected isomer's being produced in an active well, a clear understanding of the structure of the active species would ensue on resynthesis and full characterization of the resynthesis product. The intent of the experiment is to provide characterization of the active component of the well sufficient to decide to continue to follow up on the well hit or to drop it from further consideration.

LC NMR might aid our dichotomization efforts; however, the additional cost and throughput reduction that would ensue if such an addition were implemented cannot be justified at this time. The cases for which the data collected is insufficient for characterization of the active component(s) are (with the exception of the tractable unknowns) the least interesting cases from a hit evaluation perspective. As an example, we are not interested in the molecular weight of a polymeric component that exerts its activity by a nonspecific assay disrupting process. These well hits should be excluded from further consideration.

In Figure 6, an overview is presented of the decision processes we use to evaluate each well hit and to promote or drop the well hits from further consideration. To initiate the process, a combinatorially produced library is screened

Figure 1. Histogram of the biological activity correlates with the UV and MS data corresponding to the expected synthesis product. Information in the Web viewer screen is organized by assay and plate−well identifiers (the fields above the graph). The viewer also combines chemical information about the well, including structures, names, and molecular weights for the expected product and starting materials (shown to the left of the graph field and blocked out for proprietary reasons). The critical components of the viewer are the superimposed graphical representations of the UV (heavy yellow trace), MS (assorted colors corresponding to the extracted ion traces and associated *m/z* labels below the graph), and the bioassay (black histogram) chromatographic data found in the graph field. The horizontal axis usually labeled "time" would also be labeled fraction number (1−80) for the fractions assayed in the bioassay "detector channel". One important extracted ion trace corresponds to the *m/z* of the $[M + H]^+$ ion for the expected product. This graph always appears as the dark blue trace in the graph field. The experiment controls are also observable in the bioassay trace at well positions 81−86. Using the wealth of information provided in the viewer, the analyst can very rapidly classify the experimental result as one of the outcomes given in Table 2. In the case shown above, alignment of the activity histogram (black) with the dark blue expected product extracted ion chromatogram (dark blue) allows the immediate conclusion that the activity of this well is associated with the expected product.

in a suitable HTS assay. Inactive wells are of no further interest. Active wells are tested in a secondary assay, if available.

Confirmed active wells are subjected to BGF if we cannot be certain from other analytical techniques which well component was responsible for the observed activity. In other words, for a given reaction protocol that generates many well hits, subjecting a fraction of those hits to BGF is often sufficient to determine the compound structures causing the observed activities.

When the BGF results allow the identification of the active component, it is resynthesized. As observed in Figure 6, the BGF experiment is central to the decision-making process of triaging the well hits obtained from screening. The decision tree supposes the existence of a large screening library. HTS active wells are preferably subjected to a secondary assay that further limits the number of screening hits for follow-up. Wells confirmed to be active in the primary and secondary assays are evaluated to assess well quality.

When the active component of the well can be readily identified (as the expected product, a starting material or an expected side product of the reaction), which is the usual case, the next step in evaluating it is by obtaining a homogeneous sample. This is usually accomplished by resynthesis of the active component. When the BGF experiment indicates that the active component of the well is an identifiable unknown (a single component sufficiently resolved from other chemical constituents to allow facile isolation), isolation and structure elucidation of that active component from the carefully resynthesized mixture using the protocol used for the original well synthesis must be accomplished. In practice, this is done only when the number of chemically distinct well hits is small. Still, the process of identifying unexpected products can be a source of diverse lead compounds not anticipated to be in the screening deck. We therefore place a high value on these "tractable unknowns".

Control Wells. Because the control wells serve a very important analytical function, a full discussion of their utility is given here. Control wells are prepared at the time the well is put on test in the BGF experiment using the procedure given in the Experimental Section. Because they are produced along with the fractionation plate, these wells provide considerable insight into the reason or reasons for each of

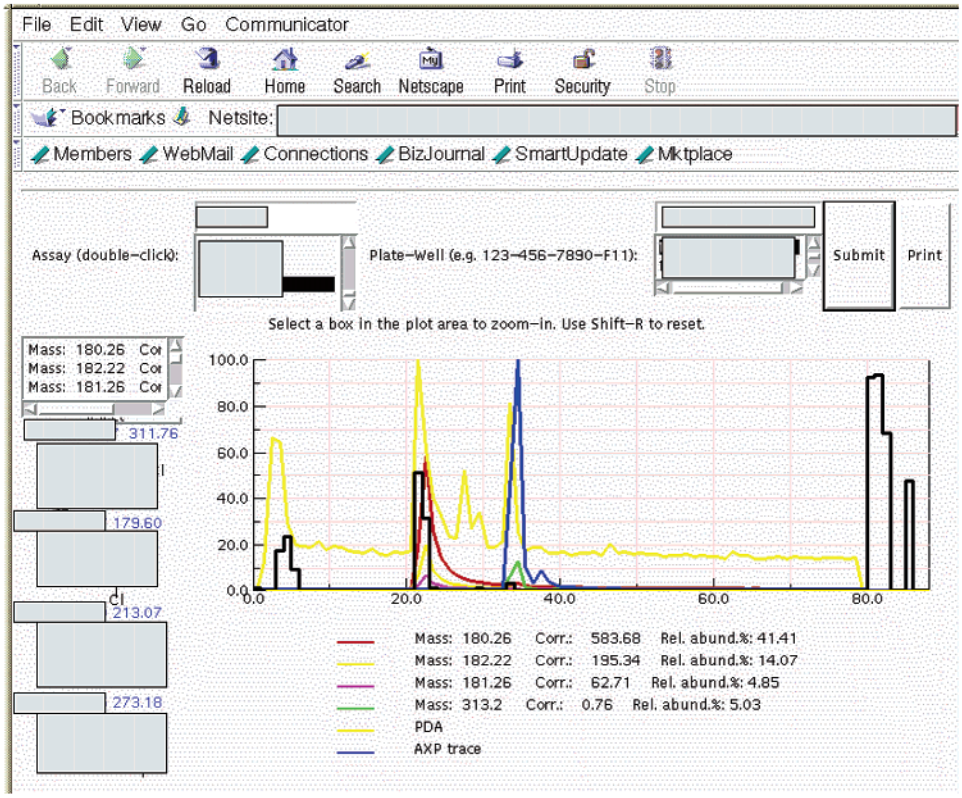

Figure 2. The biological activity of this well is due to a starting material. The utility of displaying the M_w information for the starting materials is demonstrated in this figure. The two major single ion chromatograms (with *m*/*z* 180 and 182 Da) correspond to the UV peak at fractions 21 and 22 and also correspond to the biologically active fractions. Examination of the starting material list reveals one of the starting materials as a mono-chlorinated structure with a M_w of 179 Da. This is the material yielding ions at *m*/*z* 180 and 182. The expected product is also present in the well, shown as the blue single ion chromatogram that overlaps the last major UV peak in the chromatogram, but is not active in the screening assay. If the activity were associated with a single discrete component that was not the expected product or one of the starting materials, MS extracted ions would still be associated with the black activity histogram. The full MS, UV, and possibly other chromatographic information would give an indication of the identity of the active component. Interpretation of these data allows us to assign the active species as an expected side product or intermediate (structure known) or an unexpected product. The assignment would correspond to outcomes 3 or 4, respectively, from Table 2. When a structure can be assigned for the active compound, (starting material, expected, or unexpected side product), these hits offer valuable additional chemical classes of hits for follow-up.

the possible experimental outcomes. The specific uses of these wells are explained in the paragraphs below.

Control well A contains ~10 times the amount of the well mixture that was observed to have produced a positive response in the screening assay. This well contains exactly the same volume of the same sample that was injected by the HP 1100 autosampler into the BGF instrument. If this well does not yield an assay response, there is no reason to believe any collected fraction will give a response either.

To rule out the unlikely (but possible) situation in which an activity-causing species reacts with a mobile phase component, control well B contains water, acetonitrile and buffer in approximately the same concentration experienced by those well components eluting toward the midpoint of the gradient run. Activity in control well A, but not in control well B is an indication that the activity was stable in the injection mixture but not stable under the chromatographic conditions used.

The amount of assay response for the control wells is also significant. Control wells C (1:10 of B), D (1:100 of B), and E (1:500 of B) allow the analyst to assess the degree to which the active component could be diluted before it would no longer be detected by the assay. Usually C is active and D and E provide an assay signal that is indistinguishable from the background signal.

Control well F, the 1-μL control well, is diluted for assay using DMSO such that it will give an assay response of between 50 and 90% of the full positive response. Well F should give the same assay response as control well C. If control well F is active but control wells A−E are not, the analyst can assume that the process of dilution for injection destroyed the activity.

If no analytical controls or chromatographic wells show activity in the assay, but the screening controls do, the analyst may presume there was a problem with the choice of the well for BGF. For example, the wrong sample may have been requested or "cherry-picked" from the assay plate. Another possibility is that the components responsible for the activity may have been unstable and, consequently, may have degraded during the time the sample spent at room temperature, in solvent, or exposed to air; or the active component might have been volatile and been removed from the BGF plate in the Genevac. If the appropriate control wells are active and no chromatographic wells show activity, we conclude that the active component(s) in the sample did not elute from the chromatographic column.

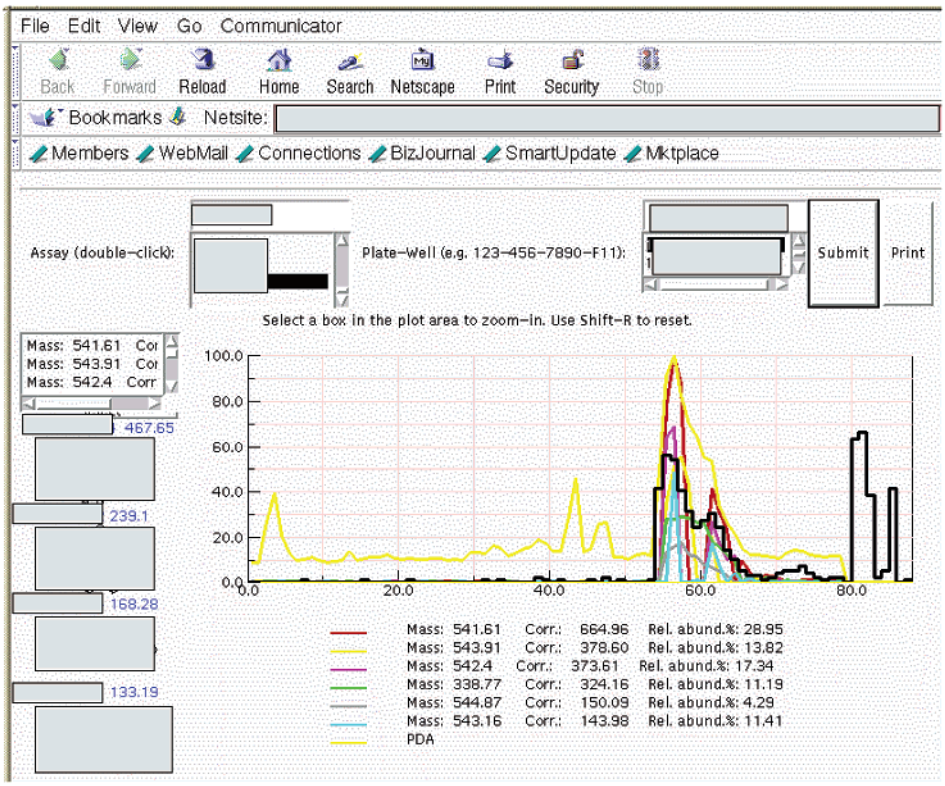

Figure 3. The activity of this well is due to multiple discrete components. The identification of these unexpected multiple active well constituents is optionally pursued. Follow-up efforts for this and similar wells is accordingly discretionary.

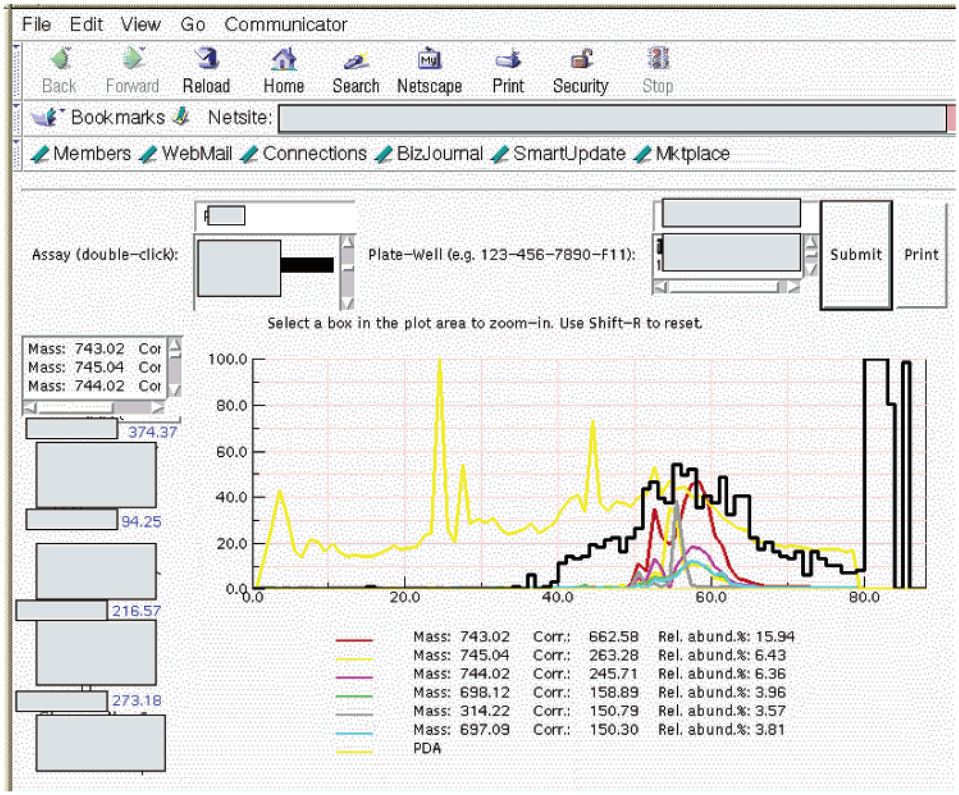

Figure 4. The activity of this well elutes from our fractionation column in a broad band. The corresponding UV and MS signals indicate that the active components of this well are not discrete drug-like molecules (e.g., aggregates or large-M_w or polymeric reaction products). Wells showing this activity pattern are not typically pursued.

Other Experimental Details. Collecting and analyzing every chromatographic fraction is a necessity. Sometimes, observed activity is due to a component that does not give a UV or an MS response, which is important information about the activity. We follow up on only those samples in which a correlation can be made between the assay activity

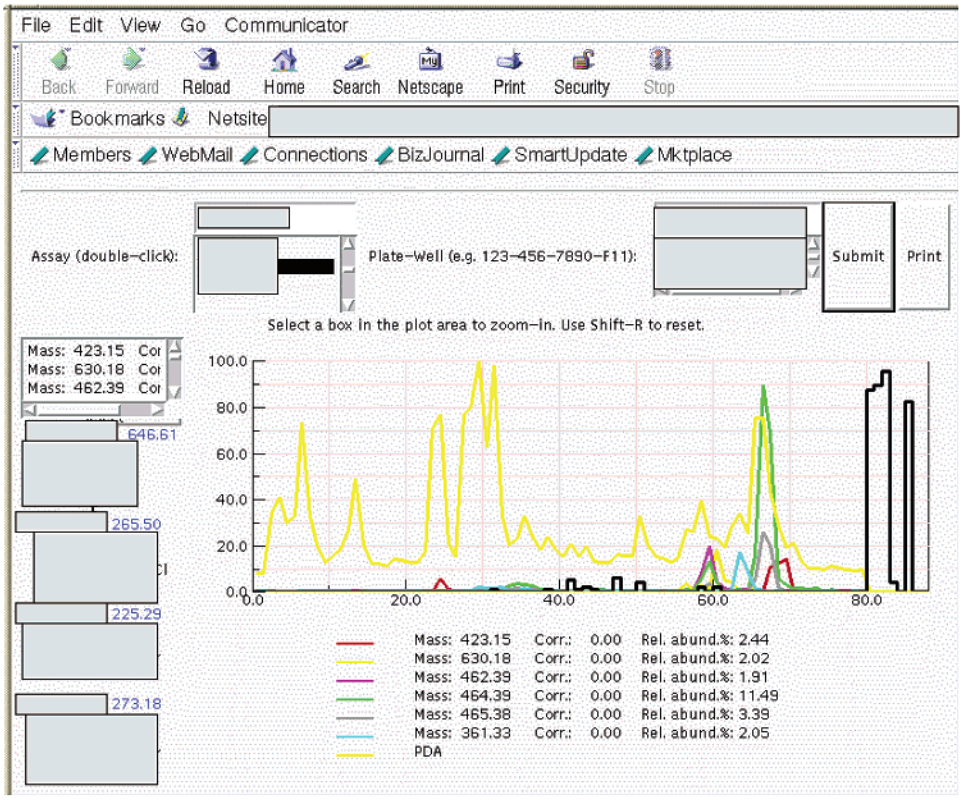

Figure 5. The active species from this well did not elute from the column. Occasionally, no active species elute from the reversed-phase HPLC column when aliquots of active wells are injected. Because virtually all small drug-like molecules elute as a single peak using our gradient conditions, we easily conclude that the activity associated with this well is not interesting from a drug discovery perspective, and the well is dropped from the follow-up list.

and some physicochemical parameter (such as the MS or UV response). This is because the correlation increases the probability of a successful follow-up effort. If no correlation can be made, the analyst concludes that the activity is not of interest and drops the well from further consideration.

In the pharmaceutical industry, just as in others, time is a very valuable (i.e., expensive) resource. To run the BGF experiment efficiently, the groups responsible for producing the BGF plates and for screening them must communicate on a daily basis. Some electronic means of gathering and displaying the data is a necessary component of the technique. The details of the various computing technologies required to accomplish this complex information gathering/communication process are proprietary. Gathering and evaluating the data on 400 wells/month is greatly facilitated by using the viewer shown in Figures 1−5.

When expected products, starting materials, or derivatives of starting materials are shown to be active, the resynthesis effort is easily accomplished. Similarly, when the activity is due to an expected side product, the library protocol reaction conditions usually can be altered in a manner that gives a higher yield of the active component. However, altering the reaction conditions is not necessary if the active component is easily separated from other well components. When the activity corresponds to a starting material, follow-up interest is usually lacking, although it can be accomplished easily if desired. If the activity is associated with a single unknown and the unknown was produced in high yield, we are usually able to resynthesize the active component by reproducing the initial synthetic protocol, followed by isolation and structure elucidation of the active component. When we are able to accomplish this process and identify the activity-causing species, these leads possess high value because they are typically unique and are of a chemical class different from other hits derived from the protocol design.

Occasionally, well hits are found to consist of multiple components that we are unable to chromatographically deconvolute. We have also observed situations in which the compound or compounds possessing activity either elute over many fractions in a chromatographic “broad band” or they do not elute from the column, even though the control wells are active. McGovern’s recent paper on compounds that form aggregates that yield false positive results in a variety of screening systems might explain these BGF results.[12] Although her work dealt with aggregation of screening compounds themselves, our work suggests that species other than the expected product might also aggregate to give a positive HTS assay result. Regardless of the exact mechanism of the interaction producing the positive screening result, the BGF experimental result demonstrates that follow-up efforts for these will not be straightforward. In these cases, we prefer to stop work on the well with the assumption that the component(s) causing the assay response would not make a reasonable lead compound or would have a low probability of affording a pharmaceutically interesting structure for medicinal chemistry.

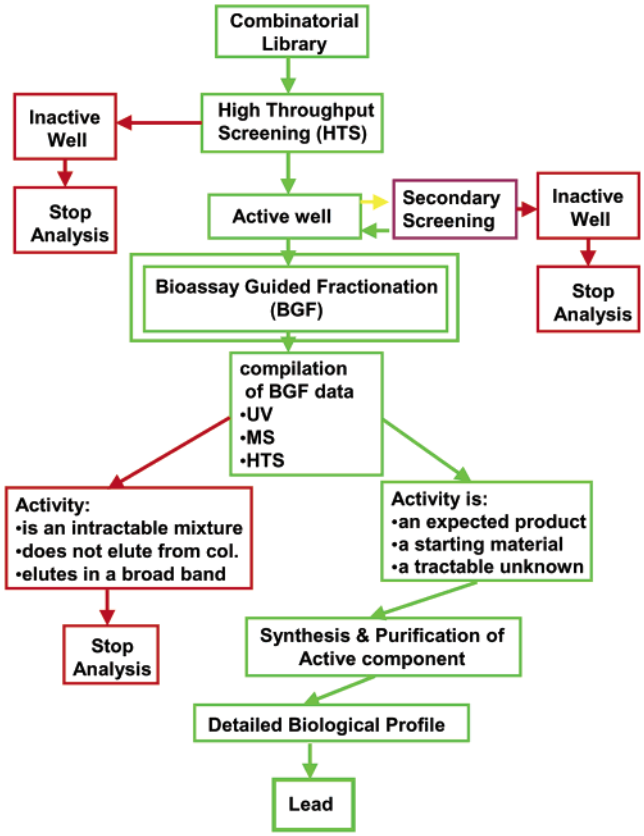

Figure 6. The role of BGF in lead determination. This scheme shows the role of BGF in shaping our understanding of the active constituent for a screening well hit on the basis of the dichotomization as "amenable to follow up" or "to be discarded". All combinatorial wells are typically screened using a primary high-throughput assay. Inactive wells are usually not considered further. Active wells from the primary assay are subjected to a secondary assay if one is available. Wells giving a positive response in the confirming assay can be grouped using chemical class and potency. Between 5 and 25% of the active wells in a group are typically examined using the BGF experiment. At the conclusion of the experiment, the data in the Web viewer is used to perform the dichotomization. Wells containing activity that correlates with an identifiable chemical component are followed up (green path). When the activity of a well is not attributed to a drug-like component, follow-up efforts are usually discontinued (red path).

Conclusions

Bioassay guided fractionation (BGF) is a useful technique for the characterization of species causing an observed biological activity in well mixtures produced by combinatorial synthesis. Identifying the active component(s) of a reaction mixture is a necessary first step for subsequent resynthesis and isolation of the active component(s). BGF is also an effective method for the rapid identification and removal of intractable wells from further consideration. The result is that unpurified libraries become a practical and economically efficient source of leads for further development, allowing efficient utilization of highly diverse combinatorial libraries.

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