Communication

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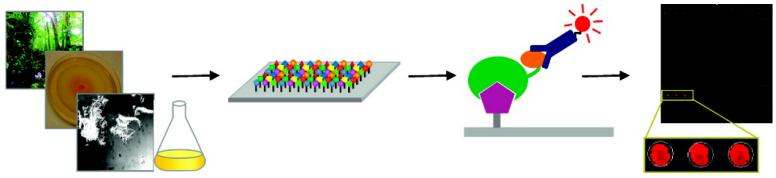

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Detecting Binding Interactions Using Microarrays of Natural Product Extracts

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High-throughput screening's (HTS) emergence as the dominant discovery tool linking small molecules to biological effects has had the unintended consequence of diminishing the role of natural products, a valuable source of structurally diverse biologically active small molecules.[1] Natural product researchers have responded by developing methods to make natural product extracts more compatible with HTS. A crucial decision is the degree to which crude natural product extracts need be purified. Purification to the stage of a single molecule requires substantial resources and leads to the selection of the most abundant compounds, while too little purification leads to unwanted effects from dilution and interference by compounds with pleiotropic activity and poor pharmacokinetic properties. Several laboratories have reported methods to purify crude extracts so that they are compatible with HTS.[2] Most studies have focused on multiwell HTS formats. Herein we report initial studies on binding assays in the small-molecule microarray (SMM) format, which we call natural product-extract microarrays (NPEMs).

SMMs have emerged as an efficient method to screen libraries of synthetic compounds for binding to proteins.[3] Over 10 000 compounds can be printed on a slide, and as little as 0.01 nmol of each compound and 1−5 μg of protein is needed to perform a binding assay in triplicate. The method does not require a known protein function and facilitates discovery of ligands for uncharacterized proteins that can be purified or expressed recombinantly. Among the immobilization methods developed for different purposes, isocyanate-coated slides have been shown to be capable of capturing pure compounds, both natural and synthetic, containing amines, alcohols, thiols, carboxylic acids, and amides.[4]

We chose *Streptomyces hygroscopicus* (subspecies *hygroscopicus*) as a model microorganism for initial studies on microarray preparation and performance. *S. hygroscopicus* originally isolated from soil on Easter Island produces rapamycin, an immunosuppressant natural product that binds to the immunophilin FKBP12 with very high affinity (0.12 nM).[5] *S. hygroscopicus* was grown in different culture media, and the rapamycin content of extracts was determined by analytical high-performance liquid chromatography (HPLC; see Supporting Information). Crude extracts from different growth time points and samples of pure rapamycin were printed as described previously.[7] NPEMs were then incubated with recombinant FKBP12-glutathione-*S*-transferase (GST) fusion protein followed by an Cy5-labeled anti-GST antibody. Protein bound to the NPEM was detected using a laser scanner with an excitation wavelength of 635 nm and an emission wavelength filter of 655−695 nm. An increase in sample concentration leads to an increase in fluorescence intensity and in feature size; therefore the total intensity corrected for background intensity was chosen for quantitative evaluation. Extracts of rapamycin-producing cultures exhibited a fluorescent signal, and the total intensity increased in correspondence to the measured rapamycin content (Figure 1). Signal intensities from the extracts were generally lower than the signal intensities from an equal amount of pure rapamycin. Lower intensities were also observed for rapamycin added to pools of *S. hygroscopicus* extracts lacking rapamycin (see Supporting Information). It is likely that this effect is a result of competition with other metabolites for binding to the isocyanate surface, lowering the amount of immobilized rapamycin. Alternatively, dense packing of metabolites on the NPEM surface may reduce detection by FKBP12 due to steric interactions.

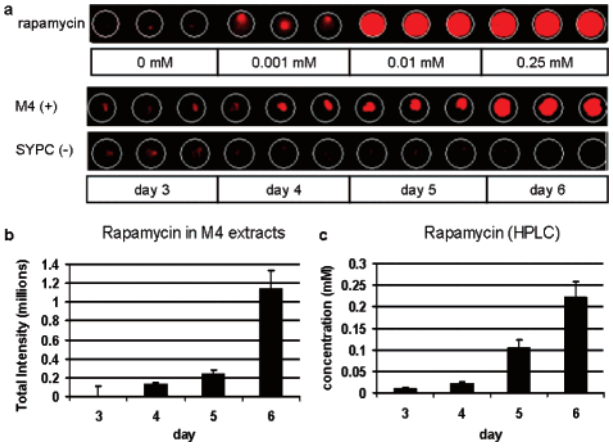

Figure 1. Detecting rapamycin production in *S. hygroscopicus* cultures using NPEMs. All slides were incubated with FKBP12−GST fusion protein and with Cy5-labeled anti-GST antibody for detection. (a) Signal intensity increases over time in extracts from cultures grown in M4(+) medium. No significant signal is observed for cultures in SYPC(−) undefined medium. Features of pure rapamycin printed from different concentration stocks are shown for comparison. (b) Signal intensities for crude extracts printed on an NPEM as a function of culture time. The total intensity is corrected for background intensity and (c) corresponds to the rapamycin content as determined by HPLC. The identity of the rapamycin in the crude extracts was confirmed as described in the Supporting Information.

Crude extracts contain a large number of uncharacterized compounds, some of which may interact nonspecifically with the protein or antibody and give rise to false positive signals. To evaluate the specificity of the protein interaction with the array features, NPEMs were incubated with the GST tag alone followed by detection with Cy5-labeled anti-GST antibody and with Cy5-labeled anti-GST antibody alone. While the antibody alone did not bind to pure rapamycin or crude extracts, a weak signal was observed when GST incubated with the printed extracts. However, this signal is weaker than that observed for the FKBP12-GST.

In an effort to distinguish specific and nonspecific interactions, we incubated free ligand in solution with cognate protein. NPEMs

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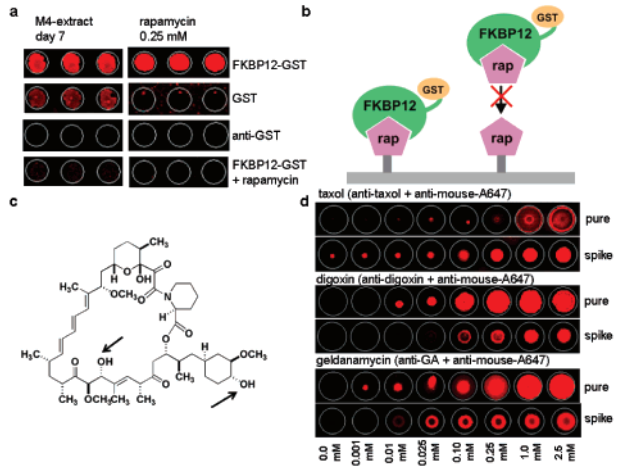

Figure 2. Extracts from *S. hygroscopicus* cultures exhibit specific and nonspecific interactions with FKBP12−GST. (a) Crude extract of *S. hygroscopicus* culture and pure rapamycin incubated with FKBP12−GST/Cy5-anti-GST and control incubations with GST/Cy5-anti-GST, Cy5-anti-GST alone, and FKBP12−GST with 10 equivalents of rapamycin/Cy5-anti-GST. (b) Free rapamycin binds to its specific binding site on FKBP12 and prevents the detection of rapamycin bound to the array, but not nonspecific interactions. (c) Structure of rapamycin. The arrows indicate hydroxyl groups involved in binding to the isocyanate slide. (d) Spiked extracts with taxol, digoxin, and geldanamycin and the corresponding pure compounds printed in different concentrations and detected with the corresponding labeled antibodies.

were incubated with FKBP12−GST supplemented with a 10-fold excess of free rapamycin (Figure 2b). Indeed, spots of pure rapamycin exhibited no fluorescence when incubated with a mixture of FKBP12 and rapamycin. Likewise, the signal intensity from *S. hygroscopicus* extracts was strongly attenuated when incubated with the FKBP12−rapamycin complex as compared to FKBP12 alone, suggesting that the signal results from FKBP12 binding to immobilized rapamycin (Figure 2a). While the signal obtained from incubation with proteins represents all interactions (both specific and nonspecific), incubation with protein and excess ligand allows the detection of nonspecific interactions. Thus, the difference in signal intensities between both experiments provides a semiquantitative measure of interaction specificity.

To show that additional molecules can be detected on NPEMs, extracts from *S. hygroscopicus* were spiked with various amounts of the natural products taxol, geldanamycin, and digoxin and printed as for rapamycin described above. Incubation with the corresponding antibodies[6] detected the extracts with high concentrations of the respective compounds (Figure 2d). Nonspecific interactions were observed in controls with selected antibodies in complex with their ligands (see Supporting Information). These results further demonstrate that NPEMs can be used to detect natural products from crude extracts. They also suggest that antibodies may be used to screen for classes of compounds in crude extracts on NPEMs.

The large number of functional groups that are reactive toward the isocyanate slides is essential for the immobilization of extracts. As the reactivity of individual functional groups toward isocyanates differs greatly, molecules that are more reactive toward the surface may compete with molecules with less reactive groups.[4] Printing defined compound mixtures in varying dilutions can improve the detection of individual components (see Supporting Information). Natural products with many functional groups will be immobilized in different orientations, increasing the potential binding modes accessible to a protein. Although we have not determined the concentration of a ligand from spot intensity, the comparison of intensities obtained from incubation with free protein and with the protein−ligand complex gives an estimate of the contribution of nonspecific interactions to the overall signal intensity.

NPEMs may be used to screen natural product extracts for biologically active metabolites since high-throughput methods for the preparation of large sample collections have been developed.[2] NPEMs may be used for the rapid credentialing of extracts for further investigation and to monitor the production of metabolites of interest in high-throughput culture experiments. Advances in the determination of structures of active components in extracts will be required to take full advantage of the increased throughput that NPEMs afford. Ongoing efforts include preparing and screening microarrays of a fungal extract collection. These screens will allow us to further evaluate the potential of NPEMs for use in high-throughput screening.

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Supporting Information Available: Culture conditions for *S. hygroscopicus*, HPLC traces for extracts, list of chemicals, antibodies, proteins, printing protocols, results from experiments with spiked extracts, and txt data files that lead to the graphs shown. This material is available free of charge via the Internet at http://pubs.acs.org.

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