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Synthetic Compound Libraries Displayed on the Surface of Encoded Bacteriophage

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Summary

We describe a technology for attaching libraries of synthetic compounds to coat proteins of bacteriophage particles such that the identity of the chemical structure is encoded in the genome of the phage, analogous to peptides displayed on phage surfaces by conventional phage-display techniques. This format allows a library of synthetic compounds to be screened very efficiently as a single pool. Encoded phage serve as extremely robust reporters of the presence of each compound, providing exquisite sensitivity for identification of active compounds engaged in complex biological processes such as receptor-mediated endocytosis and transcytosis. To evaluate this approach, we constructed a library of 980 analogs of folic acid displayed on T7 phage, and demonstrated rapid identification of compounds that bind to folate receptor and direct endocytosis of associated phage particles into cells that express the targeted receptor.

Introduction

Screening conventional phage-displayed peptide libraries is a powerful method for identification of ligands for a wide variety of receptors with no known lead structures [1, 2].

Recently, libraries of phage expressing peptides and antibodies have been employed in selections even more challenging than the discovery of ligands against purified, immobilized target proteins. These include in vitro assays of cellular internalization, translocation across polarized epithelial cell monolayers, and localization in explanted tissue [3–10]. Phage display has also found use in in vivo applications that include screens for homing to specialized sites in vascular endothelium, and targeting to tumor cells and other diseased tissues [11–17]. Phage-based methods provide unique advantages for these types of applications. Selections for functions in animals, for example, can produce high volumes of distribution of the particles, resulting in high dilutions and low numbers of particle recovery. In vitro selections of phage internalized by single cells, or even into specific subcellular compartments, can also present a challenge in particle recovery. The enormous amplification potential of phage enables the facile recovery and identification of active clones.

A significant limitation of phage display for these and other applications is the restriction to biologically expressed proteins and peptides. While this chemical space is rich with molecules interactive with a wide variety of receptors, it is a space often avoided for pharmaceutical purposes because of the generally poor pharmacokinetic properties of L-peptides. Advances in combinatorial synthetic chemistry have provided large numbers of molecules representing chemical structures deemed more desirable for lead discovery; but these are usually cleaved from the solid state synthesis support prior to assay and generally cannot be pooled in large numbers for efficient screening [18]. Bead-based libraries displaying synthetic molecules permanently tethered to the beads have occasionally been used effectively for ligand discovery; and encoded versions of these libraries are a useful tool for sorting through large pools of synthetic compounds for molecules binding to a protein of interest [19–22]. However, the large size of the beads (tens to hundreds of microns) required to carry sufficient quantities of the tag molecules for detection and decoding, limits their usefulness for in vitro studies of cellular uptake and transport, and for most in vivo applications.

We have created a hybrid of phage display and combinatorial synthetic chemistry that retains some of the most desirable attributes of both approaches. This method is particularly useful for the discovery of effector molecules for a variety of important biological activities via assays requiring small particle size, multivalent display of the compounds, and the exquisite sensitivity for decoding provided by the amplification potential of the phage, but requiring synthetic molecules for subsequent lead development and drug delivery applications. In addition to their role as linked identifiers of the compounds, the phage particles also serve as extremely sensitive reporters of the attached compounds.

In the method we describe here, compartmentalization of the compounds is required prior to attachment to the phage clones; but following this step, the encoded, compound-decorated phage may be mixed in large pools for selection of positives. Following the selection step, the identities of recovered phage clones are rapidly deduced by hybridization of probes prepared from the pool of recovered phage to archive filter arrays of all the phage library members. The location of selected clones on the arrays also specifies the compounds with which those phage are associated, permitting the rapid identification of active compounds (Figure 1).

We utilized two phage species for this method, filamentous phage fd, approximately 6 nm in diameter and about 1 μm in length, and icosahedral phage T7, spherical particles of 60 nM diameter. As a model of library construction and selection, we chose to assemble a collection of folate analogs for display on T7 phage. Folic acid is absorbed by cells via endocytosis following binding to folate receptor at the cell surface [23]. Folate

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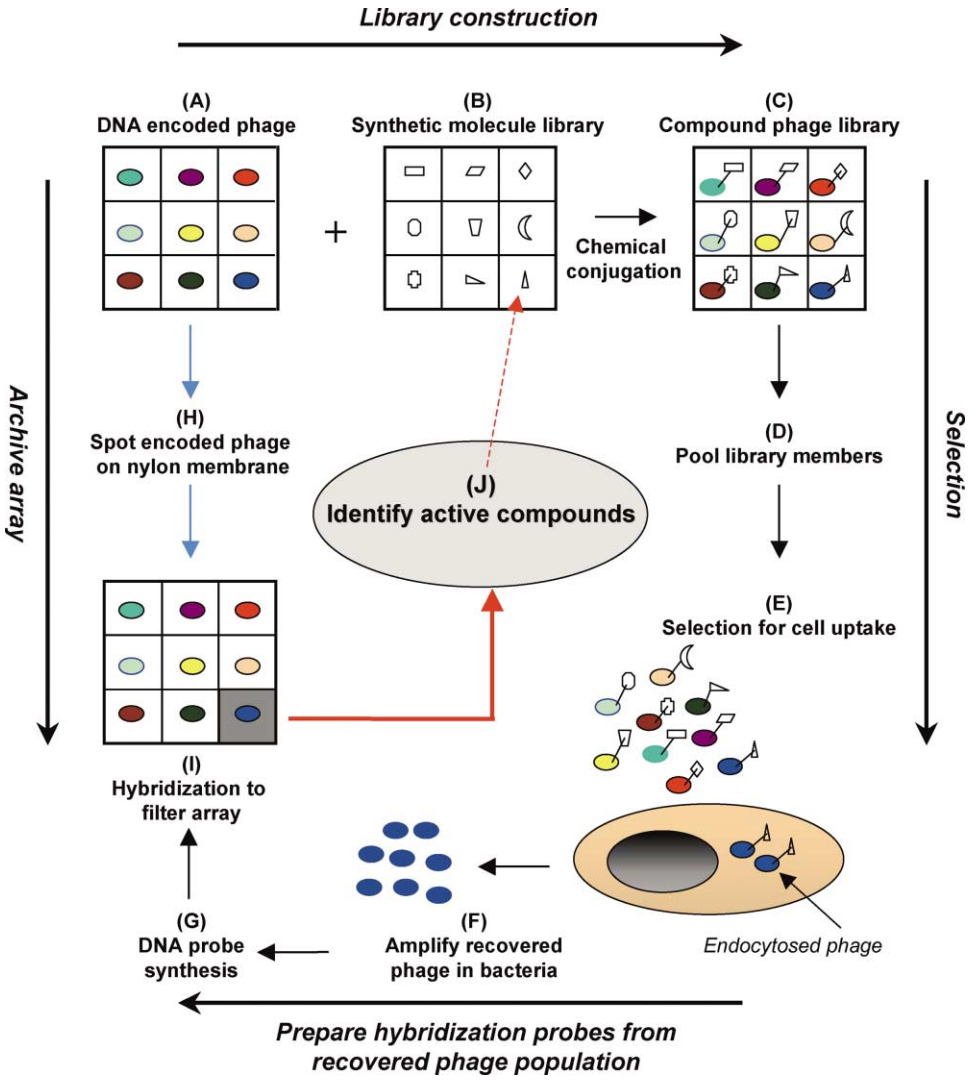

Figure 1. Schematic Diagram Illustrating the Process of Constructing and Screening a Library of Synthetic Compounds Displayed on Phage

(A) A library of phage is produced, each uniquely encoded by the insertion of a DNA tag in a nonexpressed region of the genome; and the clones are arrayed and grown in wells of microtiter plates.

(B) A library of synthetic compounds is produced, and each compound placed in a well of an archive plate.

(C–G) The display library is then assembled by bringing together the compounds and phage—one compound, one clone—retaining the special addressability of each pair; and the reaction coupling the compounds to the phage is allowed to proceed. In this example of selecting for endocytosis, (D) the compound phage library members are pooled and (E) screened for phage taken up into cells. Phage recovered from cell lysate are (F) amplified in *E. coli*, and (G) a labeled DNA probe is prepared from the population.

(H) In preparation for the decoding step, an archive array of the encoded phage is prepared by spotting a small amount of each phage culture onto a nylon membrane, again retaining the spatial relationship of the clone storage plates.

(I) The identities of phage recovered after a single round of selection are deduced by hybridizing the probes to the archive clone array representing all the encoded library members.

(J) Detection of the positive clones immediately reveals the location of the wells of the compound archive that contain active compounds.

receptors are abundant on cells of certain tumors and have been proposed as a route of targeting chemotherapeutic compounds to diseased tissue. Previous work has demonstrated that proteins and liposomes conjugated to folic acid are internalized by cells expressing folate receptor [24, 25]. To evaluate the ability of cells to internalize phage displaying folate, and to select folate analogs imparting endocytotic activity, we constructed a library of encoded phage clones, each displaying one of 980 compounds, and screened for those clones internalized by KB cells, a cell line with high levels of expression of folate receptors. The T7 phage species employed in this experiment was chosen because its vesicular-like size and shape may provide advantage in identifying ligands directing cellular endocytosis.

Results

Chemical Attachment of Small Molecules to Phage

We investigated several strategies to display synthetic molecules on both species of phage. Features we

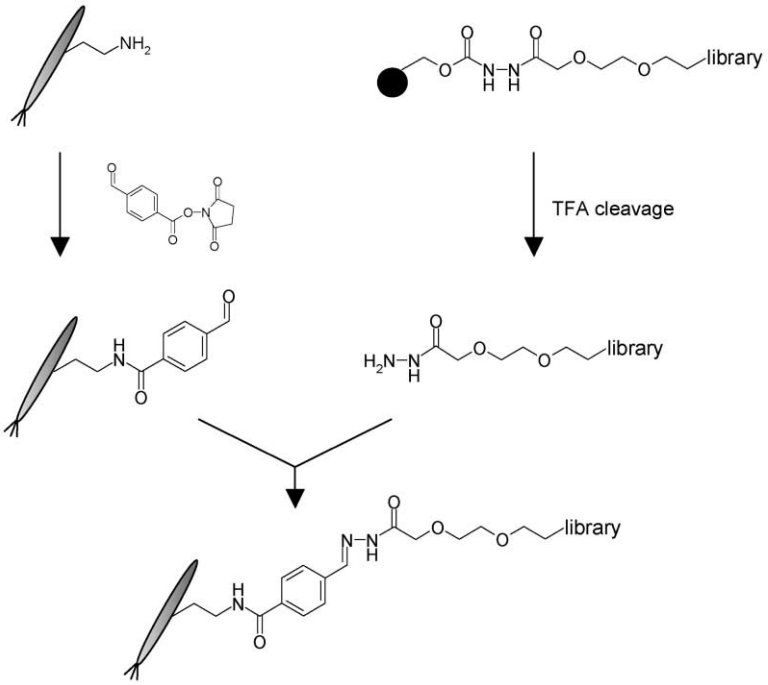

Figure 2. Hydrazone Attachment Chemistry to Display Compounds on Phage

Phage are incubated with succinimidyl 4-formylbenzoate to introduce an aldehyde at exposed primary amines present in the coat proteins. Members of a synthetic compound library are cleaved from solid support to reveal a hydrazide group, which is then reacted with aldehyde-bearing phage to covalently attach the compound to the coat protein via a hydrazone linkage.

sought in a reliable attachment chemistry included aqueous compatibility, relatively fast kinetics, compatibility with endogenous functionality present in phage coat proteins, facile adaptability to a solid phase synthesis strategy, capability to vary density of compound display on the phage surface, and a reaction quenchable under mild conditions to allow for pooling of library members prior to screening. We chose a chemical strategy in which the phage surface is first modified to display aldehydes, then reacted with synthetic molecules containing a hydrazide functional group, thus attaching compounds to phage via a hydrazone linkage (Figure 2) [26–28]. Unreacted aldehydes remaining on the phage surface after exposure to the hydrazide-containing compounds are quenched by addition of excess hydroxyethylhydrazine. This attachment chemistry is well suited to a solid phase synthesis strategy that utilizes a masked hydrazide linkage to solid support that is revealed upon cleavage. We included a PEG spacer between the synthetic compounds and the hydrazide group to extend the presentation of the compounds from the phage surface and enhance their ability to interact with protein receptors. Because the hydrazide and PEG spacer are a constant library feature, we expect the attachment kinetics to be similar for most library members.

We used hydrazone attachment chemistry to display fluorescein on filamentous phage to determine which coat proteins are modified, and to characterize the density of compound display. Fluorescein derivatized with a hydrazide functional group and PEG spacer was prepared using our masked hydrazide linkage to solid support. Phage ($\sim 10^{12}$ TU) were treated with 300 μM succinimidyl 4-formyl benzoate at 0°C for 2 hr, then incubated with 300 μM fluorescein-PEG-hydrazide at room temperature. Reactions were stopped at various times by addition of an excess of hydroxyethylhydrazine (3 mM) followed by overnight dialysis into PBS. Phage samples were run on a denaturing protein gel, and the fluorescence intensity of the proteins was measured to determine the amount of fluorescein associated with each coat protein. This analysis showed pVIII to be the major site of attachment and that increasing reaction time increases the amount of attached fluorescein (data not shown). Quantitative comparison of the fluorescence intensity of the decorated phage proteins to a calibration curve of fluorescein-labeled neutravidin showed that $\sim$25 molecules of fluorescein per phage particle are displayed after a 2 hr reaction. This increases to $\sim$100 molecules per phage particle after a 22 hr incubation (data not shown). Titering the labeled phage samples showed no reduction in infectivity, at either density of labeling.

Similar experiments with T7 phage, purified by CsCl density centrifugation, showed the gene 10 coat protein to be the major site of attachment. By comparison to a calibration curve of fluorescein-labeled neutravidin we estimated that $\sim$50 fluorescein molecules per phage particle were present after a 1.5 hr reaction, and $\sim$330 fluorescein per particle after an 18 hr reaction (Figure 3). The highest density of attachment, corresponding to almost one fluorescein molecule per copy of gene 10 coat protein, did result in a drop in titer of $\sim$10-fold.

Selection of Fluorophore-Labeled Target Phage

To test the efficiency of recovering phage decorated with a synthetic molecule, fluorescein was displayed at a density of $\sim$100 molecules per phage particle on a DNA-tagged filamentous phage clone. The target clone was spiked at levels of $1/10^4$ and $1/10^6$ into a background of undecorated phage, and selected with immobilized anti-fluorescein monoclonal antibody. Representation of target phage in the recovered population was determined by hybridizing a DNA probe specific for the target's DNA tag to a colony lift of recovered phage clones.

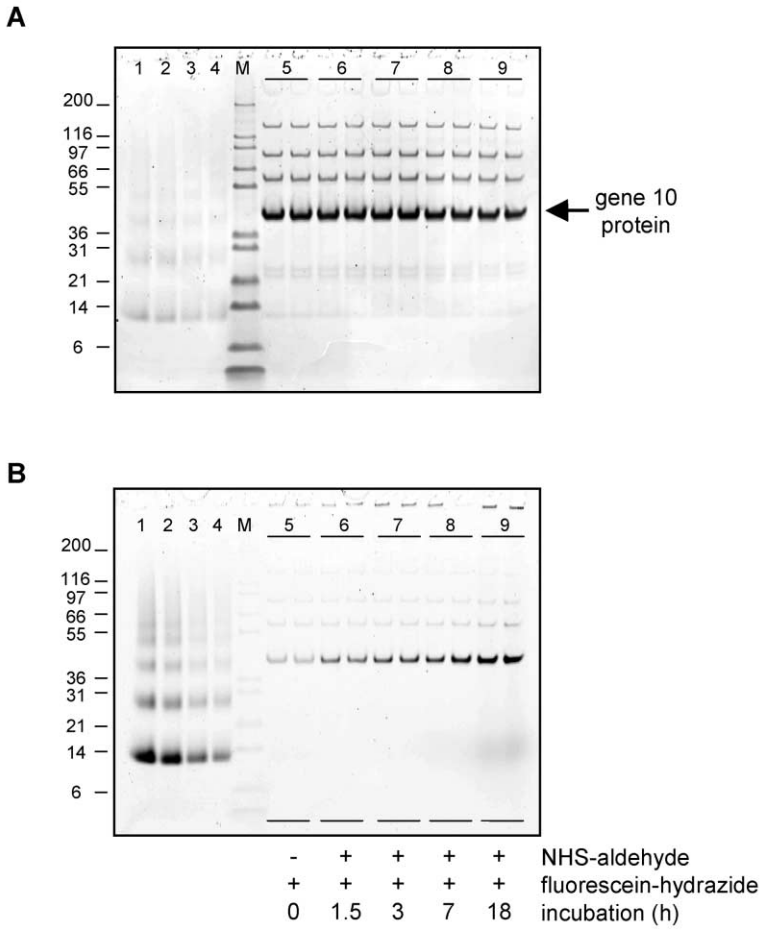

Figure 3. Chemical Attachment of Fluorescein Hydrazide to Bacteriophage T7

Aliquots of density gradient purified T7 phage (10^{12} pfu) were treated with 300 μM succinimidyl 4-formylbenzoate at 0°C for 2 hr, and incubated with 300 μM fluorescein-PEG-hydrazide at room temperature for varying times. Reactions were quenched by the addition of an excess of hydroxyethylhydrazine. Samples from each reaction were then electrophoresed on a 4%–12% NuPAGE gel to resolve individual phage coat proteins.

(A) Gel stained with SYPRO Ruby to detect total proteins shows equivalent amounts of phage coat proteins in all samples (lanes 5–9, duplicate wells). Molecular mass standards (kDa) are shown in lane M.

(B) The amount of fluorescein associated with the major coat protein was measured by scanning the gel with a Typhoon Imager (532 nm excitation laser and 526 nm short-pass emission filter). Dilutions of fluorescein-labeled Neutravidin were included in lanes 1–4 (40, 30, 20, and 15 pmol fluorescein, respectively) to prepare a calibration curve for quantitiation.

Results showed that 90% of recovered phage from the $1/10^4$ population was target phage, an enrichment of 9×10^4-fold (enrichment determined by dividing the ratio of target phage to background phage in eluant population by the ratio of target phage to background phage in input population). A similar experiment with the $1/10^6$ population yielded a 2×10^6-fold enrichment of target phage. No enrichment of target phage occurred when selected on immobilized BSA. These high levels of enrichment suggest that rare binding events can be detected in a single round of selection, and that phage particles provide a suitable scaffold for effective presentation of synthetic compounds for binding to protein receptors.

To determine whether phage displaying other synthetic molecules could also be recovered from a background of nontarget phage, we generated a small library of fluorophore-displaying phage. Fluorescein, BODIPY, dansyl, and Texas red, each of which can be captured by a corresponding anti-fluorophore antibody, were displayed on four unique DNA-tagged filamentous phage clones. The four clones were pooled by spiking each at a level of $1/10^4$ into background undecorated phage and then selected against the four anti-fluorophore antibodies. Each of the four fluorophore-displaying clones was efficiently selected by its corresponding antibody, resulting in substantial levels of enrichment in a single round (Table 1). Selection against the anti-Texas red antibody resulted in enrichment of both Texas red and fluorescein display phage, consistent with ELISA results showing the antibody binding to both fluorophores (data not shown).

Cell Uptake of Phage Displaying Folic Acid

Folate-PEG-hydrazide prepared by solid phase synthesis was attached to T7 phage particles using aldehyde-hydrazide chemistry. In an ELISA format, these phage were captured by immobilized anti-folate antibody, demonstrating that folate is present on the surface of

Table 1. Selection of Fluorophore-Display Phage Library on Immobilized Anti-Fluorophore Antibodies

	% Fluorophore-Labeled Target Phage			
Capture Antibody	BODIPY	DANSYL	Fluorescein	Texas Red
Anti-BODIPY	90	0	0	0
Anti-DANSYL	0	56	0	0
Anti-fluorescein	0	0	80	0
Anti-Texas red	0	0	20	50

Input population consisted of 0.01% of each fluorophore-labeled phage mixed in a background of unlabeled wild-type phage. Data shown are percent of target phage in the recovered population from a single round of selection against each antibody.

phage and is displayed in an orientation that allows it to bind the antibody.

To test for endocytosis, folate-displaying T7 phage (2 hr attachment reaction) were incubated under various conditions with KB cells, a human nasopharyngeal carcinoma cell line that expresses high levels of folate receptor. Folate-displaying and undecorated phage were incubated separately with cells for 1 hr at 37°C. Cells were then washed extensively with PBS and once with a low pH stripping buffer to distinguish phage that were internalized from those simply bound to the cell surface. We found that brief exposure to stripping buffer inactivates nearly all T7 particles and does not perturb the cells (data not shown). Cells were lysed with PBS/1% SDS, and the lysate was titered to determine the number of internalized phage. Approximately 100-fold more folate-displaying phage were recovered compared to undecorated phage. This enhanced recovery of folate-displaying phage was blocked by first treating KB cells with phosphatidylinositol-specific phospholipase-C (PI-PLC), which removes the phosphatidylinositol glycan-linked folate receptor from the cell surface [29]. Incubating phage and KB cells in the presence of free folic acid or at 4°C also lowered the recovery of folate-displaying phage to background levels of undecorated phage (Figure 4A).

To examine the influence of ligand affinity and density of display on phage endocytosis, methotrexate was displayed on T7 phage, and their uptake in KB cells was compared with folate-displaying phage. We determined the binding affinity of methotrexate-PEG-hydrazide for folate receptor to be 1–2 μM, and the affinity of folate-PEG-hydrazide to be 1–2 nM, which match values reported in the literature for unmodified folate and methotrexate [30]. As described above, we found that display of folate at low density (2 hr labeling reaction) on phage led to cellular endocytosis. In contrast, recovery of methotrexate-decorated phage was observed only at the highest density of display (24 hr labeling reaction, Figure 4B). This result demonstrates that recovery of low-affinity ligands (micromolar range) is possible if a high density of compound display is achieved.

To determine the enrichment achievable in the KB cell uptake assay, a DNA-tagged T7 phage clone was labeled with folic acid (2 hr labeling reaction), mixed at levels of 1/100 and $1/10^4$ into a background of undecorated phage, and incubated with KB cells for 2 hr at 37°C. Cells were washed extensively with PBS, once with stripping buffer, then lysed with 1% SDS/PBS and the recovered phage plated onto LB plates. Plaque lifts probed with a radiolabeled oligonucleotide specific for the DNA tag of the folate-labeled clone revealed a 30-fold enrichment of folate-displaying phage relative to control phage when mixed at 1/100, and 300-fold when mixed at $1/10^4$. These experiments provide evidence that synthetic compounds may be attached to phage with an appropriate orientation and density to allow effective interaction with the folate receptor and engagement of the endocytotic mechanisms mediated by the receptor.

Folate Analog Library Displayed on Phage

We constructed a library of 980 analogs of folic acid using parallel combinatorial synthesis. The library consisted of 960 exploratory compounds and 20 compounds predicted to have affinities for folate receptor similar to that of folic acid. To design this library, we viewed folic acid as consisting of three modules: glutamate, p-aminobenzoic acid, and 2-amino-4-hydroxypteridine. The 960 exploratory compounds were synthesized by combining 19 amino acids (plus a null position) at the glutamate position, 8 aminobenzoic acids, and 6 nicotinic acids at the pteridine position (Figure 5). Nicotinic acids were chosen to replace the pteridine portion of folic acid to greatly simplify the synthesis of this library. The 20 compounds predicted to be high-affinity ligands were constructed by substituting only the glutamate portion of folic acid with 19 other amino acids consisting of natural and unnatural amino acids, and a null position (no amino acid). Building blocks that change the negative charge of glutamate to a positive charge, and the neutral isostere glutamine were included to probe the charge requirement for binding folate receptor. Binding affinities of these 20 putative ligands, obtained by TFA cleavage of the solid phase synthesis support with no further purification, ranged between 1 and 10 nM when measured in competition for binding to immobilized folate receptor (data not shown). These results are consistent with previous studies showing that the glutamate moiety of folic acid is not a major determinant of the binding interaction with folate receptor [31].

T7 phage containing unique DNA sequence tags were prepared by cloning a collection of 100-base random oligonucleotides generated by standard mixed-base synthesis (each randomized position contained an equimolar mixture of all four deoxynucleotides) into a nonexpressed portion of the phage genome. The recombinant DNA was packaged in vitro, and the phage produced were titered. In constructing the initial library of $\sim 10^6$ tagged phage, we utilized only a small fraction of the available 4^{100} ($>10^{60}$) possible encoded clones. Even in the case where as few as 20 consecutive bases of the sequence determine the specificity of hybridization, only about $1/10^{10}$ tags would be expected to cross hybridize. To further ensure specificity the hybridizations were done under high-stringency conditions commonly used to identify unique gene sequences. Therefore, this system has the capacity to unambiguously encode libraries much larger than that described here.

It is not necessary to know the actual DNA sequence of the tags at any point in the screening process, nor is it necessary that each phage culture contain only a single tagged phage clone. Taking advantage of the convenience of automated liquid handling over picking single plaques, we inoculated each of 980 individual log phase cultures of *E. coli* BL21 cells grown in deepwell microtiter plates with media containing several encoded T7 clones, and incubated until cell lysis occurred. Phage cultures were prepared for compound attachment by centrifugation to remove cellular debris, followed by precipitation of phage particles with PEG 8000. Phage were efficiently precipitated in the multiwell format, and titers of individual stocks were within 2-fold of one another. Protein gel analysis of ten samples revealed protein profiles with nearly equivalent amounts of phage coat proteins (data not shown). Portions of these phage

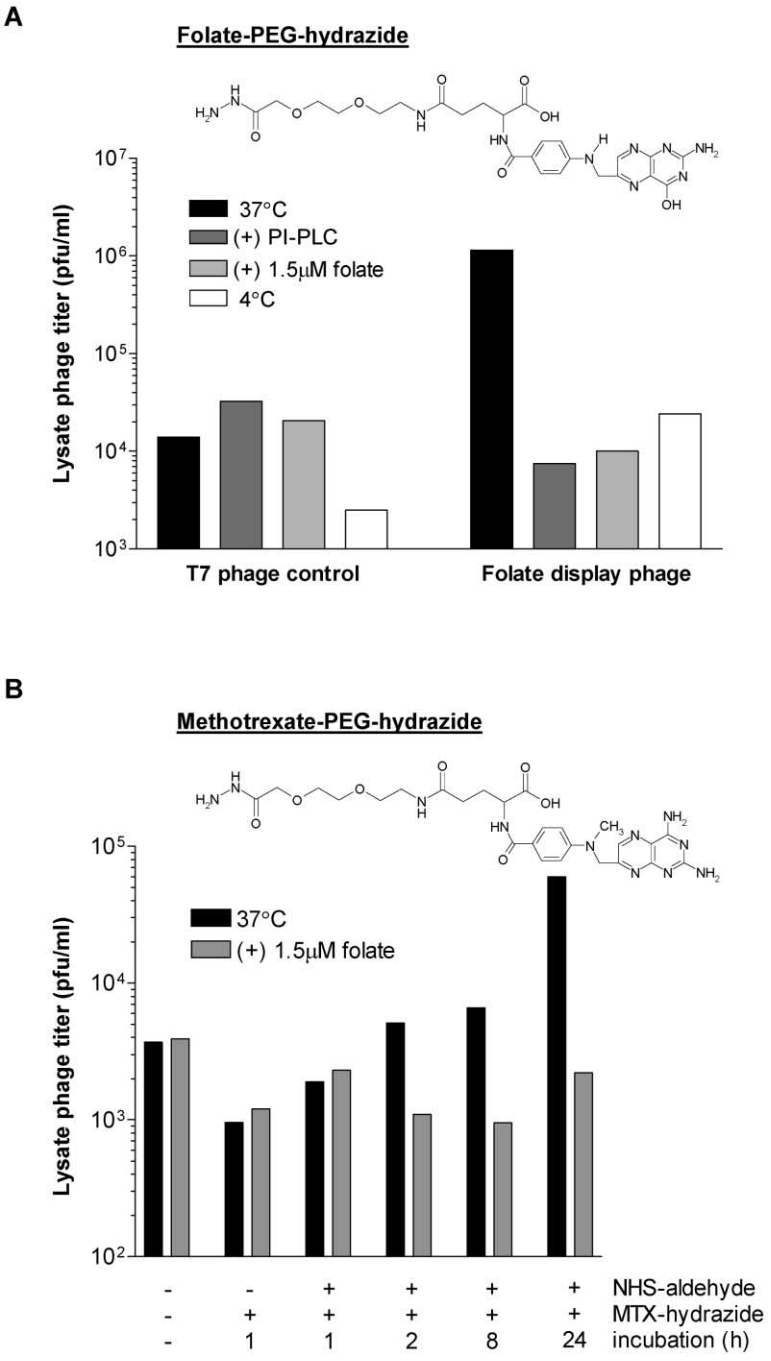

Figure 4. Uptake of Folate- and Methotrexate-Labeled T7 Phage by KB Cells

(A) Phage titers of lysates from KB cells incubated with folate-labeled phage or unlabeled phage for 2 hr at 37°C. Folate receptor-dependent endocytosis of phage into cells is blocked by adding 1.5 μM folic acid to the medium, pretreating cells with phosphatidylinositol-specific phospholipase C (PI-PLC) to remove the folate receptor from the cell surface, or incubation at 4°C.

(B) Phage titers of lysates from cells incubated with methotrexate (MTX)-labeled phage for 2 hr at 37°C. Uptake of methotrexate-labeled phage increases with labeling reaction time (conditions that favor a greater density of ligand display on the phage). Nonspecific uptake was measured by addition of folate to the medium.

stocks were used to create archive filter arrays for subsequent hybridizations by spotting them onto nylon membranes with a 96-pin replicator. The remaining phage were treated with 300 μM succinimidyl 4-formylbenzoate to introduce a reactive aldehyde at available primary amines, followed by treating each well with one of the hydrazide-containing folate analog library members for either 2 or 24 hr prior to quenching with an excess of 2-hydroxyethylhydrazine. Quenching with hydroxyethylhydrazine was critical to prevent cross-reaction between phage clones and other members of the chemical library upon pooling for purification by CsCl density centrifugation and dialysis in phosphate buffered saline.

Screening Phage-Displayed Folate Analog Library on KB Cells

Aliquots of ~10^{10} folate-analog-displaying phage from the 2 hr and overnight reactions were added to tubes containing 10^7 KB cells and incubated for 2 hr at 37°C. These aliquots represent ~10^7 copies of each compound bearing phage in the library and ~1000 infective phage particles per KB cell. In control experiments, aliquots of the library were added to cells in media that

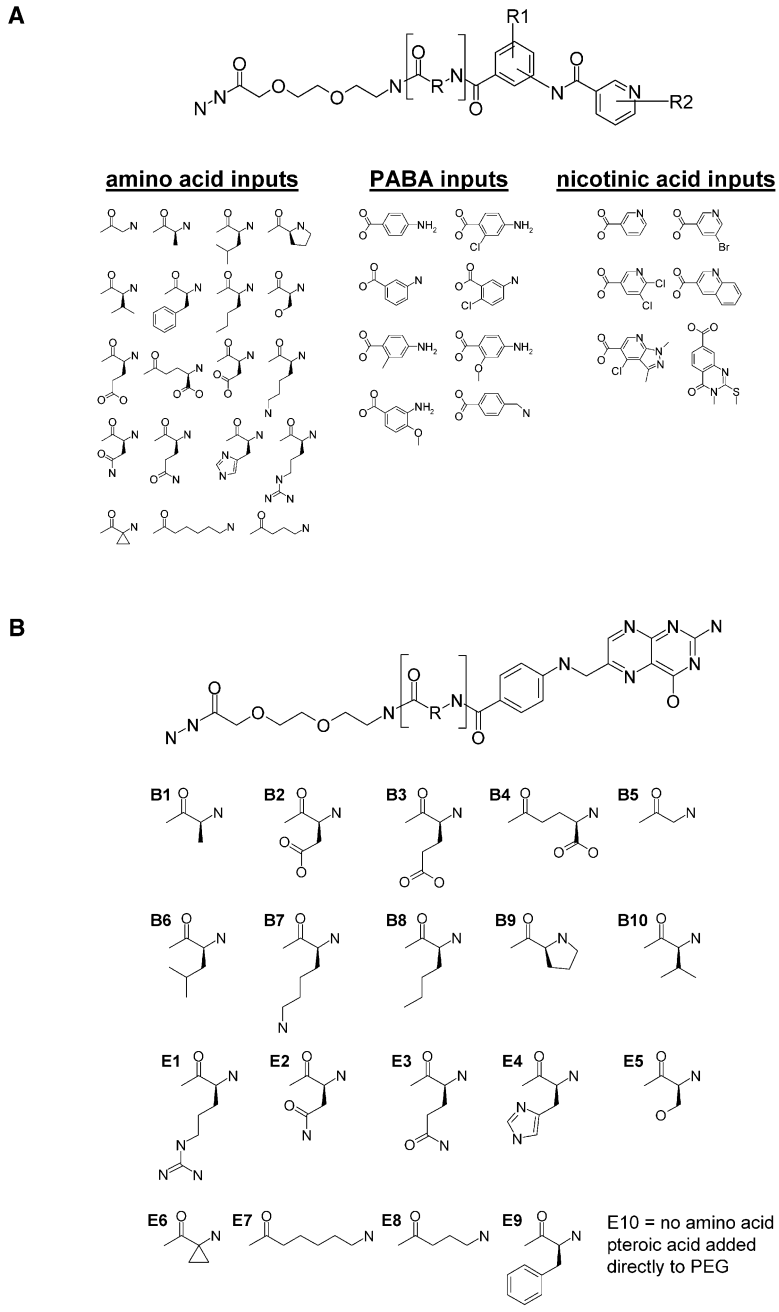

Figure 5. 980 Member Folate Analog Library

(A) 960 folate analogs were prepared by solid phase synthesis by combining 20 amino acids at the glutamate position (R) of folic acid, 8 p-aminobenzoic acids (R1), and 6 nicotinic acids at the 2-amino-4-hydroxypteridine position (R2) to resin modified with an acyl hydrazide and a PEG linker.

(B) 20 folate analogs predicted to be high-affinity ligands were synthesized by substituting only the glutamate portion of folic acid with a collection of 19 natural and unnatural amino acids, as well as a null position (no amino acid). Labels B1-10 and E1-10 refer to the encoded phage clones to which these compounds were attached in the library.

contained folate (10 μM), or were incubated at 4°C to block endocytosis. Following the incubation period, cells were washed with PBS, once with low pH stripping buffer, and extracted with PBS/1% SDS to recover internalized phage. We typically recovered 5×10^5 to 1×10^6 infective phage from cells incubated at 37°C, about 0.005%–0.01% of the total input. Approximately 10-fold fewer phage were recovered from cells incubated in the presence of folate or at 4°C (data not shown).

Phage recovered from KB cell lysates were amplified by infecting log phase cultures of *E. coli* BL21 cells. To identify library members that were enriched, we isolated the DNA tags from the amplified phage stocks by PCR. Radiolabeled probes representing these tags were then prepared and hybridized to archive filter arrays. A probe derived from lysate of KB cells incubated at 37°C with the 24 hr conjugated folate-analog library hybridized strongly to 9 of the 20 clones corresponding to phage carrying high-affinity folate analogs (IV-B3, 4, 6, 7, 8 and IV-E2, 3, 5, 9) (Figure 6A). Quantitative analysis of the array filters revealed five additional clones (IV-E1, 4, 6, 7, and 10) of the 20 displaying high-affinity folate analogs, with signal intensities exceeding 2 standard deviations from the average of all 960 exploratory clones (Figure 6B). We prepared hybridization probes from three positives (IV-B6, E2, and E9) to hybridize to plaque lifts from the input population and from the KB cell lysates, and verified that all three were recovered at 10-

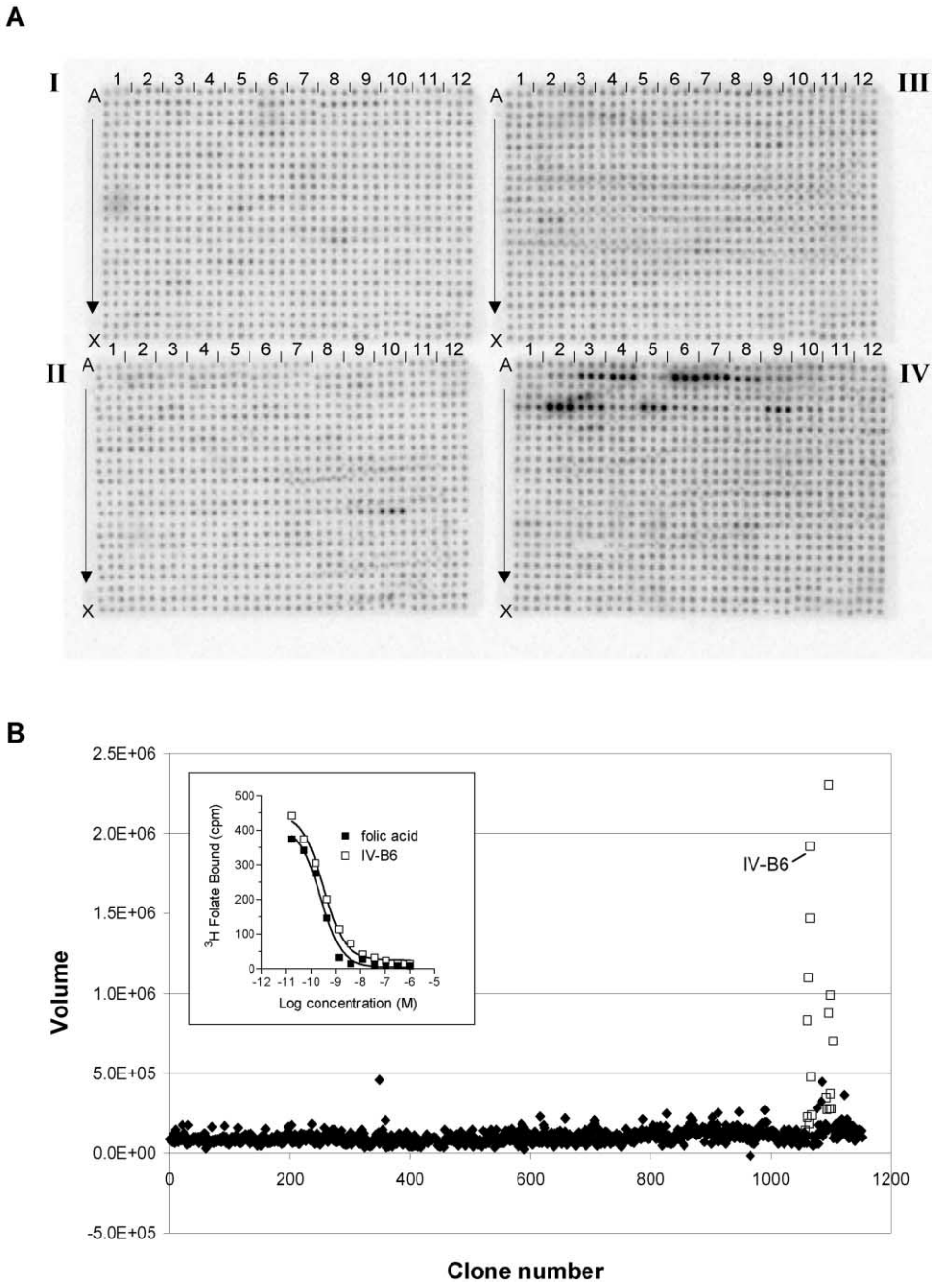

Figure 6. Identification of Folate Analog Phage Library Members Recovered from Selection on KB Cells

(A) A radiolabeled DNA probe was prepared from phage that were recovered from a lysate of KB cells incubated with the folate analog phage library for 2 hr at 37°C. This probe hybridized strongly to clones displaying known high-affinity analogs spotted in triplicate on archive filter IV (B1-10, E1-10), indicating that these members were enriched in the recovered population.

(B) The stored phosphorimage obtained from hybridized archive array filters was quantified using ImageQuant (Amersham Biosciences). The integrated intensity of all pixels within a defined area surrounding each clone (triplicate spots) was calculated and is displayed in volume units. Values for the 20 known high-affinity folate analogs are represented by open squares. (Inset) Inhibition of binding of ^{3}H folate to immobilized human folate receptor by compound IV-B6 and folic acid control. In this assay the median inhibitory concentration of both compounds was ~0.5 nM.

to 30-fold greater frequency than their starting representation in the library. Sufficient material remained in the archive plate well representing clone IV-B6 to purify the compound to greater than 75% purity, as determined by peak integration of the extracted ion chromatograph of the LC/MS trace. The material exhibited a mass of 585.38, identical to the predicted mass of the B6 target compound. The concentration of the compound was determined by chemiluminescent nitrogen detection. Compound B6 was tested for competition with ^{3}H folate

in binding to immobilized folate receptor, and showed a median inhibitory concentration (IC50) of 0.5 nM, essentially identical to that of folate (inset to Figure 6B). Eight clones displaying exploratory compounds produced hybridization signals that were slightly over the 2 SD threshold; however, when tested in a competition binding assay, these compounds did not block ^{3}H folate binding to receptor at the highest concentration tested (estimated to be $\sim$50 μM, based on the average yield of the compounds tested during quality control analysis of the library), indicating that these were false positives. Probes derived from control cell lysates did not bind to any of the clones labeled with a high-affinity folate analog, indicating that no enrichment of these clones had occurred in selections conducted in the presence of excess folate or at 4°C.

Discussion

Compounds generated by solid-state synthesis in parallel or split-and-pool formats are usually cleaved free of the supports prior to assay. With compounds in this form, many types of assays can be performed, but testing of large pools is often not feasible. Alternatively, compounds can be screened for target binding while still tethered to the synthesis beads. Identification of compounds selected in this configuration is greatly facilitated by encoding the compound structures with molecular tags; however, the mass of tag molecules needed for decoding typically requires that beads larger than cells be used. In contrast, utilizing phage particles for the display and screening of synthetic compounds provides a very compact vehicle for screening and selection of tethered and encoded synthetic compounds, coupling many of the advantages of the conventional phage system to the selection of synthetic molecules.

To develop a generally useful compound-on-phage display system, we explored several means of associating the compounds with the phage, including both covalent and noncovalent linkage. We chose aldehyde-hydrazide attachment chemistry as robust, compatible with the biological functions of the phage, adjustable in compound density, and synergistic with solid-state compound synthesis. The addition of the aldehyde installs unique functionality on the surface of the phage to receive the library compounds. Unveiling of the hydrazide group upon cleavage from the synthesis support renders the compound immediately activated for attachment to the phage, an important feature necessary to extend our method to very large libraries of compounds produced by split-and-pool synthesis. Reactive hydrazide groups on the library compounds efficiently couple to aldehydes and yield a compound density controllable over a 10-fold range by the duration of reaction. A high density of compound display, possible with either of the two phage species, should allow the formation of multivalent interactions with the targets. This arrangement has been previously shown for peptide display to greatly enhance the sensitivity for detection of low-affinity ligands [32]; and based on our results with the low-affinity folate analog methotrexate, appears to enhance the avidity for ligands in the compound-on-phage display format as well.

In this initial work, we chose to utilize primary amines (lysines, N-terminal amines) present in the phage coat proteins for attachment of synthetic compounds; however, there exists precedent for the introduction of non-natural constituents into peptide side chains, providing alternative, perhaps multiple, orthogonal attachment sites on the phage surface [33–35]. Specific attachment points may also be used to display libraries of synthetic compounds in the context of a random peptide library displayed on phage to select modified peptides with novel biochemical properties. Li and Roberts have described such an approach in which a peptide library, random but for a fixed cysteine, was generated. The penicillin derivative 6-bromoacetyl penicillanate was then appended to each thiol side chain. Selection on *Staphylococcus aureus* penicillin binding protein 2a retrieved peptide-penicillin conjugates with significantly improved inhibitory activity compared with the drug alone [36].

To fully realize the potential chemical diversity that is available for these types of biosynthetic libraries, we sought to develop an encoding strategy that would allow one to screen a library in a single pool and identify members of the selected population using a method of clone decoding based on hybridization. Evaluation of several encoding schemes showed 100-base oligonucleotide tags to provide high fidelity and reliability of identification of the correct clones. For libraries made by parallel synthesis, as in the example we report, the identity of active compounds is immediately revealed upon identification of the positive clones. For libraries made by stochastic means—a split-and-pool synthesis with synthesis supports randomly distributed to the wells—an additional step is required to determine the chemical structures of the positive compounds. This may be done by mass spectrometric analysis of the compounds in the positive wells [37].

The compound-on-phage system differs from conventional phage display in an important way. The compound-displaying phage recovered from a selective round cannot be amplified for a subsequent round of screening. There are several means of circumventing this limitation. In one approach, a large excess of compound-phage may be used in the initial round to provide sufficient recovery of positives to take directly into the next round. A more flexible approach requires rapid identification of putative positives from the initial round, pooling of these compound phage from archives withheld from the first screening, and rescreening this enriched pool. The hybridization method of clone identification we describe here quickly provides information on the location of the wells containing positive compounds in the archive plate, allowing subsequent selection rounds to be performed at daily intervals.

Our initial studies of the selective enrichment of phage-displayed fluorophores binding to antibodies demonstrated a reasonably efficient capture of target phage and remarkably robust enrichment of positives over background phage. From a starting ratio of 1 positive per 10^4 background phage, a single round of selection produced a recovery of 60% of the input positives and a population containing 90% positive phage—an enrichment of 90,000-fold. While an actual library of

structurally related synthetic compounds might be expected to present a higher background of nonspecific interactions with components of the capture medium, results from the test screen reveal the potential of the system to effectively recover rare positive events.

We next evaluated the method with the much more stringent test of a complex library of compounds selected for activity in a multistep cell-based process. The selection required the compound-displaying phage to engage and activate cell surface folate receptors, and to undergo internalization into cells. A critical obstacle to selections of this type is a potentially high background of nonspecific interactions of phage with the many different structures available on the cell surface. Methods were developed for effectively removing phage-associated superficially with the cells, and for efficiently recovering phage taken into the cells. Test selections conducted with folate-decorated phage produced enrichments of up to 300-fold over background phage, indicating that folate is displayed in a configuration permitting interaction with the folate receptor and engagement of the endocytotic mechanisms directed by this receptor. In our screening of a 980 member library of folate analogs, the recovery of library members, comparable in affinity to the natural ligand folate, serves to validate the utility of the technique for display of synthetic molecules linked to bacteriophage coat proteins to allow interaction with external structures, and selected for activity in certain cellular processes, followed by rapid identification of their chemical structures.

Significance

Among the advantages of conventional phage display methods are marked sensitivity for low-affinity interactions, an unparalleled capability to detect extremely rare events, and the efficiency of pooled screening of large libraries. The principal disadvantage is the availability of only biologically expressed proteinaceous compounds as library members. The compound-on-phage approach we describe brings many of the intrinsic advantages of the phage-display format to the screening and selection of diverse molecular types available only through synthetic chemistry.

One of our purposes in developing the compound-on-phage technology is to discover compounds engaged in cell targeting, uptake, and translocation, with the goal of targeted delivery of therapeutic agents into cells and across tissue barriers. Phage are particularly suited for this purpose since they are small enough to enter cells and subcellular compartments and are detectable and decodable at the single particle level. With compounds displayed on phage, and information on a compounds' identity encoded in the genome of each particle, processes such as those listed above can be studied both in vitro and in vivo. The high information capacity and enormous amplification potential of phage provide exquisite detection sensitivity and reliable identification of active compounds associated with the phage. Furthermore, molecules that emerge from this tethered selection format all possess a site that is available for attachment to a therapeutic compound or nanovessel capable of encapsulating many drug molecules. In essence, the decorated phage serve as a nanoparticle surrogate in a selection process designed to discover "tetherable" targeting molecules. Because of the pooled nature of the selection process, and the extraordinary sensitivity of detection (and identification) of single phage particles, we foresee an important role for compound-on-phage technology for cell-based, tissue-based, and in vivo selection strategies aimed at the discovery of nonpeptide ligands targeting specific cell types and intracellular and transcellular transport mechanisms.

Experimental Procedures

Phage Display Vectors and Host Strains

T7Select 415-1 phage and *E. coli* BL21 were obtained from Novagen (Madison, WI). The filamentous phagemid vector (p8XENO) was derived from pBAD18, which was obtained from American Type Culture Collection (Manassas, VA). Helper phage M13KO7 was purchased from Invitrogen (Carlsbad, CA).

Preparation and Selection of Fluorophore-Labeled Phage

A filamentous phagemid clone containing a unique DNA sequence tag was chemically labeled with fluorescein by incubating $\sim 10^{12}$ infective phage particles in 600 μl phosphate-buffered saline (PBS) containing 30 μM fluorescein-5-EX succinimidyl ester (Molecular Probes, Eugene, OR) for 3 hr at 4°C. Fluorescein-labeled phage were separated from free dye by adding 0.1 vol 1 N acetic acid to the reaction, incubating for 10 min on ice, followed by centrifugation at 16,000 $\times$ g for 10 min to pellet phage. Supernatant was removed and the phage pellet was resuspended in PBS. Three additional encoded phage clones were labeled with either BODIPY-FL-X succinimidyl ester, dansyl-X succinimidyl ester, or Texas red-X succinimidyl ester (Molecular Probes) by the same procedure.

Anti-fluorophore antibodies (Molecular Probes) were immobilized in microtiter plates by adding 2.5 μg antibody per well and incubating for 1 hr at 37°C. Wells were then blocked with PBS/1% bovine serum albumin (BSA). Fluorophore-labeled phage mixed in a background of wild-type phage were added to antibody-coated wells and incubated overnight at 4°C. Following multiple washes with PBS, antibody-bound phage were recovered by adding acid elution buffer (0.1 M glycine-HCl, pH 2.2, 0.1% BSA) and incubating at room temperature for 10 min. Eluates were collected and neutralized with Tris base. Log phase *E. coli* K91 recA cells were infected with the recovered phage, plated on L-agar plates containing 100 μg/ml ampicillin, and incubated overnight at 37°C. The resultant colonies were transferred to nitrocellulose filter circles, and probed with radioactively labeled 29-base oligonucleotides complementary to each of the four flouorphore-labeled encoded phage clones. Filters were hybridized overnight at 62°C with 3×10^5 cpm/ml of probe, washed in 2$\times$SSPE/0.1% SDS for 20 min at room temperature, and developed using a phosphorimager (Amersham Biosciences Typhoon, Piscataway, NJ).

Cell Uptake of Folate-Labeled Phage

Adherent KB cells (obtained from ATCC) were grown in folate-free RPMI with 5% fetal bovine serum. Cells were removed from flasks using PBS containing 0.53 mM EDTA, washed one time with PBS, and suspended in folate-free RPMI/0.1% BSA. Folate-labeled T7 phage were incubated with cells for varying amounts of time at 37°C with gentle agitation. Cells were washed two to three times with PBS, once with stripping buffer (0.1 M glycine-HCl, pH 2.5, 500 mM NaCl), and then extracted with PBS/1% SDS to recover internalized phage.

Construction of DNA-Encoded T7 Phage Library

Synthetic oligonucleotides (5′-CCCAAGCTTAACTAGATAAGCGG CCGCACTCGAGCGGCTA-3′ and 5′- TAGCCGCTCGAGTGCG-3′) obtained from Operon (Alameda, CA) were annealed, extended with Sequenase enzyme (Amersham Biosciences), and cloned into the

T7Select 415-1 phage display vector with HindIII and XhoI to insert stops codons in all three reading frames upstream of the DNA tag insertion site, thus preventing translation and expression of the DNA tag. The stop codon-modified T7 phage vector was used to construct a library of clones containing unique 100-base oligonucleotide tags. Two synthetic oligonucleotides (5′-TTAAGCGGCCGC$(N)_{100}$CACTTCTATAGTGTC-3′ and 5′-TAGTTACTCGAGATTTAGGTGACACTATAGAAGTG-3′; N represents an equimolar mixture of all four nucleotides) were annealed and extended with Sequenase enzyme to create a double-stranded DNA insert. DNA was digested with NotI and XhoI, purified by spin column gel filtration, and ligated to the T7 vector at a ratio of 0.06 pmol insert to 0.02 pmol vector with T4 DNA ligase. Ligated phage DNA was packaged with a T7 in vitro packaging extract obtained from Novagen according to the manufacturer's protocol [38]. To prepare "clonal cultures" for compound attachment, *E. coli* BL21 cells grown to $OD_{600} = 0.8$ in M9TB medium (1.2% bacto tryptone, 2.4% yeast extract, 0.4% glycerol, 0.4% glucose, 0.1% NH_4Cl, 0.3% KH_2PO_4, 0.6% Na_2HPO_4, 1 mM $MgSO_4$) were dispensed into 11 deepwell microtiter plates (1 ml per well) and inoculated with 100 μl of diluted phage library (~50 pfu/well). Plates were incubated in a high-speed oxygenated shaker (HiGro; Gene Machines) for 2–3 hr at 37°C until cell lysis occurred. One tenth volume 5 M NaCl was added to each well, and plates were centrifuged for 15 min at 2200 × g to remove cell debris. Cleared supernatants were transferred to new deepwell plates, 1/6 volume 50% PEG 8000 was added, and plates were incubated on ice for a minimum of 1 hr. Plates were then centrifuged at 2200 × g for 15 min at 4°C, supernatants were removed, and phage pellets resuspended in PBS.

Preparation of Archive Filter Arrays

50 μl of each encoded phage culture was mixed with an equal volume of denaturation buffer (0.5 N NaOH/100 mM NaCl) in 96-well microtiter plates and incubated for 10 min at room temperature. Each sample was then spotted on Nytran filters (Schleicher & Schuell, Keene, NH) in triplicate using a 96-pin replicator (Nalge Nunc, Rochester, NY). Filters were incubated 5 min at room temperature in high-salt denaturation buffer (1.5 M NaCl, 0.5 N NaOH), followed by a 5 min incubation in neutralization buffer (1.5 M NaCl, 0.5 M Tris-Cl, pH 7.5), and then baked 2 hr at 80°C.

Synthesis of 980 Member Folate Analog Library and Attachment to Encoded T7 Phage

Wang resin modified with a PEG linker and acyl hydrazide attachment point was first reacted with nitrophenyl chloroformate to form a nitrophenyl carbonate, followed by displacement of nitrophenol from this mixed carbonate with hydrazine. The acyl hydrazine was acylated with 8-azido-3,6-dioxo-octanoic acid, followed by Staudinger reduction of the azide to an amine.

For attachment of the 20 amino acids, ~500 mg of resin was distributed into each of 20 reaction vessels. Two equivalents of FMOC-protected amino acid succinimidyl ester in N-methylpyrrolidinone were added into each vessel. Following the coupling step, extensive rinsing of the resin and removal of the FMOC protecting group with 20% piperidine in N-methylpyrrolidinone (NMP) prepared the resin for the next round of coupling reactions.

Resin from each amino acid reaction was subdivided into eight reaction vessels for coupling aminobenzoic acid analogs, consisting of four para-aminobenzoic acids, three meta-aminobenzoic acids and one para-aminomethylbenzoic acid. The meta-aminobenzoic acids and para-aminomethylbenzoic acid required FMOC protection of the amine to avoid oligomerization on solid support. Diisopropyl carbodiimide and hydroxybenzotriazole were used to couple the aminobenzoic acids to available amines on solid support, using 5 equivalents of all reagents for the meta-aminobenzoic acids and para-aminomethylbenzoic acid, and 10 equivalents of all reagents for the para-aminobenzoic acids. Extensive rinsing of the resin, and deprotection of the FMOC-protected inputs with 20% piperidine/NMP prepared the resin for the final coupling step.

Resin from each aminobenzoic acid reaction was subdivided into six vessels for attachment of nicotinic acid derivatives. Nicotinic acid analogs were attached to the amine of the aminobenzoic acids using 2 equivalents each of nicotinic acid, HATU, and diisopropylethylamine in N-methylpyrrolidinone. Extensive rinsing of the resin prepared each aliquot for cleavage of compounds from solid support. Each compound was cleaved into one well of a deep well microtiter plate using 1 ml neat trifluoroacetic acid, which was then removed under vacuum, and the dried compounds were dissolved in 1 ml DMSO. The maximal theoretical yield from each aliquot of resin at the chosen synthesis scale was 10 μmol for each of the 960 library members. Therefore, the final concentration of each compound in DMSO was ≤10 mM.

The folate-analog library was attached to phage by adding 5 μl of 4-formylsuccinimidyl benzoate (6 mM in DMSO) to microtiter wells containing 100 μl of each encoded phage clone (~10^{10} pfu) in PBS and incubating at 0°C for 2 hr. To each aldehyde-derivatized phage clone we then added 5 μl of one of the compounds and incubated the reaction plates at room temperature for 2 or 24 hr. Reactions were quenched by adding 10 μl of 30 mM hydroxyethylhydrazine and incubating at room temperature for 1 hr. Reactions were pooled and phage were precipitated using PEG, and purified by CsCl density centrifugation [38].

DNA Probe Synthesis and Hybridization

DNA tags from encoded phage clones were amplified by PCR. Amplification reactions were carried out in a final volume of 50 μl containing 3 μl phage supernatant, 1 μM forward (5′-GGGCACTACTGGTCAGAAGCACG-3′) and reverse (5′-AACTGACGGGAAGCCTTGGTGAC-3′) primers, 200 μM dNTPs, 1× Herculase Buffer, and 2.5 U Herculase DNA polymerase (Stratagene). The PCR product from 20 cycles of amplification was digested with XhoI and NotI, electrophoresed on a 1.5% agarose gel, and the fragment containing the DNA tag was excised and recovered using QIAquick columns (Qiagen). Approximately 0.15 pmol template DNA was denatured and added to a DNA synthesis reaction containing a 16-base oligonucleotide primer (5′-TGACACTATAGAAGTG-3′), dNTPs, α^{32}P dCTP, and Klenow fragment to generate radiolabeled DNA probes. Archive array filters were hybridized overnight at 65°C with 10^6 cpm/ml of probe, stringently washed at 65°C in 0.1×SSPE (0.9 mM NaCl, 50 mM NaH_2PO_4, 5 mM Na_2EDTA, pH 7.0)/0.1% SDS, and developed using a phosphorimager. Hybridization signals were quantified using ImageQuant software (Molecular Dynamics).

Folate Receptor Competition Binding Assay

Full-length human folate receptor was cloned by PCR using gene-specific primers and human kidney cDNA obtained from Clontech. An HA epitope tag was inserted immediately upstream of the naturally occurring signal sequence that directs the attachment of a phosphotidylinositol glycan (PI-G) anchor. The gene construct was cloned into pCDNA3 (Invitrogen) and transfected into CHO-K1 cells. Phosphotidylinositol-specific phospholipase C treatment of transfected cells cleaved PI-G-linked folate receptor from the cell surface, producing a soluble HA-tagged form for binding assays. 2.5 μg anti-HA antibody 12CA5 (Boehringer Mannheim) in PBS was added to each well of a microtiter plate (HB Isoplate; Perkin Elmer) and incubated for 1 hr at 37°C. Wells were blocked with PBS/1%BSA, and 50 μl of soluble HA-tagged folate receptor was added and incubated for 1 hr at 4°C. Wells were washed with PBS and serial dilutions of test compounds in PBS/0.1% BSA were mixed with 50,000 cpm ^{3}H folic acid (Moravek) and added to folate receptor-coated wells. Following overnight incubation at 4°C, wells were washed with PBS and the amount of ^{3}H folate bound was measured in a liquid scintillation counter (Wallac MicroBeta TriLux).

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