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Screening of an OBOC combinatorial library for beta-actin identifies molecules active toward Ramos B-lymphoma cells

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Abstract

The search for small molecules that specifically recognize protein targets is a laborious process if conducted in a one protein – one compound manner. A high throughput antibody based screening of "one-bead one-compound" (OBOC) combinatorial small molecule libraries is described here, whereby libraries contain thousands of different small molecule ligands are synthesized on individual TentaGel beads and simultaneously screened for protein binding to individual beads, each with a different compound. The use of "OBOC" libraries greatly facilitates this simultaneous screening of thousands of compounds. Now, through the use of monoclonal or affinity purified antibodies, we identified small molecules that bind a particular protein contained in a complex mixture of biological molecules. This method identified small molecule ligands that bound beta-actin present in cytoplasmic cell extracts of Ramos B-lymphoma cells. These small molecule ligands were resynthesized in immobilized and soluble forms and tested for binding of beta-actin present in Ramos B-cell extracts and for activity against Ramos lymphoma cells. This high throughput screening immunoassay method has great promise for improving our ability to find relevant, bioactive small molecules that target a specific native protein in a complex protein mixture without purification of the protein.

Keywords

combinatorial library; small molecule; immunoassay; high throughput screening; antibody; proliferation; Ramos B-lymphoma

Introduction

One-bead one-compound (OBOC) combinatorial libraries are traditionally screened with tagged purified protein [1–7]. More recently, we reported the development of an enzyme-linked colorimetric subtraction screening method to probe OBOC libraries with complex protein mixtures [8]. Here we report on the application of the two-stage subtraction screening method, in conjunction with anti-actin antibody to screen an encoded OBOC small molecule combinatorial library for ligands that bind to actin or actin-associated proteins present in total

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cell lysate of Ramos B-lymphoma cell. One of these ligands selected was found to have anti-proliferative activity on Ramos cells.

A mouse monoclonal antibody to beta actin (Sigma) was selected for this screening method. Beta actin in Ramos cell extracts is an abundant protein and was chosen as a target for several reasons; 1) because it is an abundant protein a large number of small molecules would be expected to interact with it; 2) actin is cytoskeletal protein with biological functions important for cell shape, motility and signaling often through the presence of actin binding proteins, which can be regulated by cellular signals [9], so a small molecule interacting with it might possess biological activity; and 3) the actin cytoskeleton may be important for oncogenesis [10,11] and could be a target for treatment of cancer. Specificity of the beta actin monoclonal antibody was assessed by Western blot analysis of Ramos cell extract using a goat anti-mouse IgG conjugated with horseradish peroxidase followed by incubation with a chemiluminescent substrate. A single strong reacting protein band was detected at approximately 42 kDa.

Generating combinatorial small molecule libraries with a large number (e.g., >10000 compounds) of different molecules has been challenging, but the use of the OBOC method has greatly facilitated the construction of large diverse encoded combinatorial libraries [2,3,12–14]. Thousands to millions of unique chemical molecules are synthesized directly on individual TentaGel beads that are then screened in numerous ways to discover ligands or substrates against various biological targets [6,12]. For instance, a fluorescent quench method can be used to detect efficient peptide substrates for a specific protease [15,16] and ³³P-radiolabeling method can be used to identify efficient substrate for protein kinases [17–19]. Whole cell binding assay with living cells has been used to discover ligands that bind to cell surface receptor of Jurkat T-leukemia cells [20] or ovarian cancer cells [21]. For soluble biological targets, the proteins can be tagged with biotin [1,8], fluorescent dyes [22,23] or enzymes [1, 24] to detect protein-ligand interaction on the beads. Although these methods could identify ligands that bind specific protein, these proteins needed to be purified and derivatized before the screening could take place. Any chemical derivatization, however, could potentially impair or alter the function of the target protein. This is also true for the enzyme-linked colorimetric subtraction screening method [8] we recently reported for screening OBOC libraries with complex protein mixture as the molecular probes. In this method, the OBOC library was screened against and colored by a biotinylated protein mixture in the first step, followed by screening with another biotinylated protein mixture in the second step. Beads that interact with the protein mixture in the second step but not first step could be identified through image subtraction analysis.

Here we report on a method that enables us to use underivatized complex biological mixtures, such as whole cell lysate, to screen OBOC combinatorial libraries for specific target protein or protein complexes present within the complex mixture of biological molecules. Positive beads can be detected by an enzyme-linked colorimetric method using alkaline phosphatase (AP) conjugated anti-target protein antibody directly, or by an AP-linked secondary antibody that recognizes the primary antibody. In order to eliminate all the false positive beads, the library screening will be performed in a two-step approach followed by image subtraction as previously described [8]. In principle this method can be performed serially on one single OBOC library with whole cell lysate but using antibodies against a series of target proteins, many of which are commercially available.

Material and Methods

Rink amide MBHA resin (loading 0.65 mmol/g) was purchased from GL Biochem (Shanghai, China). *N*-Hydroxybenzotriazole (HOBt) and 1,3-diisopropylcarbodiimide (DIC) were purchased from Advanced ChemTech (Louisville, KY). Fmoc-protected amino acids were

obtained from SynPep Corporation (Dublin, CA), Chem-Impex International, Inc (Wood Dale, IL) and NeoMPS, Inc (San Diego, CA). *N,N*-Dimethylformamide (DMF) was purchased from VWR (Brisbane, CA). Dichloromethane (DCM), Methanol (MeOH), diethyl ether and acetonitrile (CH₃CN) were purchased from Fisher (Houston, TX). All other chemical reagents were purchased from Aldrich (Milwaukee, WI). Analytical reversed-phase high performance liquid chromatography (RP-HPLC) analyses and semi-preparative RP-HPLC purification were performed on a Beckman System Gold HPLC system (Fullerton, CA). Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) analysis was performed on a Bruker BIFLEX III mass spectrometer (Billerica, MA). Amino acid sequencing was performed on an Applied Biosystems Procise 494 Protein Sequencer. Anti-beta actin monoclonal antibody other chemicals (glycerophosphate, sodium vanadate, sodium chloride, glycerol, Trizma base, Tween 20) came from Sigma (St. Louis, MO). Bead library screenings were performed in disposable polypropylene columns from Perkin-Elmer Life Sciences. All buffer reagents were from Sigma unless otherwise noted. Phosphate buffered saline (PBS), Tris buffered saline (TBS), and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) buffers were as described in [8,25]. The anti-mouse IgG-alkaline phosphatase conjugate came from Santa Cruz Biotech (Santa Cruz, CA). Cell reagents (RPMI 1640, penicillin/streptomycin) came from Invitrogen (Carlsbad, CA). Bead immobilization was performed in Sea-Plaque agarose from BioWittaker (Walkersville, MD). Scanned images were generated on an Umax Astra 2400S flatbed transparency scanner (see [8] for details).

Synthesis of a small molecule “OBOC” combinatorial library

A peptide-encoded small molecule OBOC combinatorial library (Figure 1) was synthesized on bilayer TentaGel beads (Rapp Polymere, Tubingen, Germany) (loading 0.26 mmol/g) using the procedure described by Liu *et al.* [26]. Briefly, Fmoc-amino acids (Aa₁) were first anchored to the beads, followed by Fmoc deprotection with 20% piperidine in DMF. Then, Fmoc-4-nitrophenyl-β-alanine, a tri-functional scaffold, was coupled to the N-terminus of Aa₁. The nitro group was reduced with 2M of SnCl₂ in DMF and the resulting free aniline was acylated by the second building block (R₂COOH, R₂COCl or R₂SO₂Cl). Fmoc was removed from the scaffold, and the third building block (R₃COOH or anhydrides) was incorporated. The side-chains of X₁ were deprotected with trifluoroacetic acid (TFA)/triisopropylsilane (TIS)/H₂O (95:2.5:2.5, v/v/v) and washed with *N,N*-diisopropylethylamine (2% in DMF), DMF, DCM, MeOH, DMF, H₂O and 70% EtOH. The library beads were stored in 70% EtOH.

Preparation of Ramos B-lymphoma cell extract

Ramos B-lymphoma cells (ATCC CRL-1596) were grown in RPMI 1640 with 10% FBS, 1% glutamine, 100 u/ml penicillin/streptomycin, 37°C, 5% CO₂. To prepare cell extracts, Ramos cells were washed with PBS and then lysed with PBS containing 1% Triton X-100, 1 mM dithiothreitol (DTT), glycerophosphate, sodium vanadate, 10% glycerol, leupeptin, aprotinin, on ice for 15 min then centrifuged (Eppendorf centrifuge, 14,000 rpm, 15 min, 4°C). The cell extracts (supernatant) were frozen at -70°C.

Screening of a small molecule combinatorial library

Subtractive screening of the small molecule OBOC combinatorial library was performed using whole cell lysate derived from Ramos cells (lysed with 1% Triton X-100 in PBS). Screening of combinatorial libraries was conducted in small disposable chromatography columns (Perkin Elmer). Each screen used 25,000–30,000 beads that were first blocked with 0.1 gelatin in phosphate buffered saline with 0.1% Tween 20 (PBST). Stage 1 of the procedure was conducted by incubating the beads with an appropriate dilution of anti-actin antibody, washed, incubated with anti-mouse IgG conjugated with alkaline phosphatase (anti-mouse IgG-AP), washed and incubated with the phosphatase substrate, BCIP. Beads that had bound anti-actin antibody and/

or anti-mouse IgG-AP became blue and remained colored beyond this screening step, while the rest of the ligand beads were clear. An appropriate dilution of the anti-mouse IgG-AP, generally 1:50,000 to 1:100,000 dilution in PBSTG, was one that produced few colored beads. Stage 2 of the procedure was performed by recycling the whole bead-library after Stage 1 with washing of the library with PBST, followed by re-blocking of the library with 0.1% gelatin in PBST (PGSTG) and then incubation with Ramos B-lymphoma cell extract (approximately 250 ng/ml diluted with PBSTG). After washing (with PBST) the library beads were mixed with low melt agarose (molten at 37–40°C), transferred to Petri dishes (35 × 10 mm) and cooled at room temperature. The Petri dishes were then transported to the flatbed scanner and substrate (BCIP) added to the plate. The plates were scanned at times 0 and 2 h. The resultant images were subtracted by visual inspection using a stereo-microscope and the color difference used to determine the positive reactions. Those beads with the greatest difference in color change at $t = 2$ h of incubation, with little or no blue color at $t=0$ were judged to be true positives. The positive beads were removed, deproteinated with 8M guanidine-HCl, the peptide coding tag sequenced (ABI Procise 494) and the chemical structures of small molecule ligands determined based on the corresponding sequence of the coded peptide tag [26].

Resynthesis of small molecule ligands on PEGA beads or in soluble form

Using the similar synthetic approach described in the library synthesis, small molecule ligands were resynthesized on PEGA bead [bis(2-acrylamidoprop-1-yl) poly(ethylene glycol) cross-linked dimethyl acrylamide and mono-2-acrylamidoprop-1-yl [2-aminoprop-1-yl] poly(ethylene glycol)] (Polymer Laboratories, Amherst, MA) for use in affinity chromatography. PEGA beads were chosen because they are more porous than TentaGel bead and therefore more suitable for affinity chromatography. In addition, the ligands were also synthesized in soluble form for biological test. In this case, the compounds were synthesized on Rink amide resin and then cleaved off the resin with TFA-based cleavage cocktail (TFA/TIS/H₂O). After evaporation of TFA and the solvents, the concentrated cleavage product was precipitated with cold ether and purified by semi-preparative RP-HPLC.

Binding of Ramos proteins to ligand-PEGA beads

Ligand PEGA beads were hydrated in PBST and then blocked with PBSTG for 1 h. These beads were then incubated with Ramos cell extract (diluted in PBST) for 1 h (RT or 4 °C), washed with PBST, proteins eluted with SDS PAGE sample buffer and analyzed by 10% SDS PAGE. Gels were stained with colloidal Coomassie blue (Invitrogen, Carlsbad, CA).

Bioactivity assays of soluble ligands

Small molecule ligands were solubilized in 100% dimethyl sulfoxide (DMSO) at 100 mM and stored at –20°C. The ligands were initially diluted in ethanol or DMSO because of the low solubility of some of these compounds before dilution in culture media and addition to the cells. Final concentration of the ligand stock solution was 50 mM, unless otherwise noted. Cell counting was conducted on a Coulter counter and the proliferation assay was performed using [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, MTS (CellTiter, 96 AQuous One Solution Cell Proliferation Assay, Promega). The assay was read at 490 nm (Molecular Dynamics Microtiter Plate Reader).

Protein identification

Confirmation of beta-actin in the SDS PAGE gels was performed by excising the suspected protein band from colloidal Coomassie stained gels, in-gel digest the protein with trypsin and analyze the peptide fragments by MALDI-TOF mass spectrometry (Bruker Biflex III) [Qin, 1997 #655]. The protein was identified from mass spectrometry data using MASCOT

(<http://www.matrixscience.com>) and confirmed by Western blotting with a specific beta-actin antibody.

Results

Synthesis of an OBOC small molecule library

A peptide-encoded OBOC small molecule library with three diversities was synthesized using 4-nitrophenyl β -alanine as a scaffold (Figure 1). The library was generated employing the bilayer beads [26,27] and the split-mix synthesis method [2,28,29]. In this library, the small molecule displayed on the surface of the bead, and the tri-peptide coding tag resided in the inner of bead, which eliminated its interference with the screening. At the Aa₁ position, 96 amino acids, which were encoded by Aa₂, were used including 20 natural L-amino acids and 19 D-isomers, 15 β -amino acids and 42 other unnatural amino acids. At the R₂ position, 33 carboxylic acids, acyl chlorides or sulfonyl chlorides were employed. At the R₃ position, a total of 49 different carboxylic acids or anhydrides were used. Altogether 155,232 different permutations of small molecule compounds were created.

Immuno-screening of the small molecule ligand OBOC library for small molecule ligands that bind beta-actin

An OBOC encoded small molecule library was screened using an immuno-detection system that employed a monoclonal antibody to actin, an abundant cytosolic protein in Ramos cells (Figure 2 and see methods for details). This assay is dependent on the use of a monoclonal antibody to the protein target of interest; which in this study is cytoplasmic (beta) actin. Our "subtraction assay" is a sandwich assay that uses a two-stage process. Beads that interact with either the primary and secondary antibodies without the protein mixture are colored by the anti-mouse IgG- alkaline phosphatase enzyme conjugate and its substrate (BCIP) during the first stage and are not considered positive reactions. The second stage adds the protein mixture, followed by the primary and secondary antibody-AP conjugate incubations, and finally the BCIP substrate again after the beads are immobilized in agar. The beads with a significant change in color between t=0 and t=2 h are considered true positive reactions. It is important for this method to have a low background in the first step so that the actual assay with total cell extract during the second step would yield strong and true positive results that can easily be distinguishable from the background binding of the assay reagents.

Screening results from this assay are shown in the Petri dish scanned at t=0 and after t=2 hr of incubation (Figure 3). The insert shows the same expanded region of the plate that is imaged at t=0 and then the 2 hr scan. The red spots are red marker beads added to the bead mixture just before plating the beads in agarose and the addition of substrate. The inclusion of the red marker beads facilitate the picking of the beads by enabling the scans to be aligned with the beads in the Petri dish so accurate selection of positive beads is possible. Arrows are used to identify beads with positive results. Only those beads with no color at t=0 and a big color difference at 2 hr were selected for decoding. An amplified region of the plate corresponding to the scanned image as observed through a light inverted microscope shows the presence of a red marker bead and a positive blue bead among many non-colored beads. The true positive ligand beads were picked from the plate (see methods for details) and decoded [26].

Identification of the small molecule ligands after sequencing of the coding tag on each ligand beads

Decoding result of the 12 selected ligand beads is shown in Table 1. The amino acid (Aa₁) and two R groups (R₂ and R₃) attached to the scaffold for each small molecule ligand are listed (Table 1). The majority of ligands identified (7 out of 12) contained arginine in the Aa₁ position. Several carboxylic acids such as benzoic acid, 2,5-dimethoxy phenyl acetic acid, or 5-

hydantoin acetic acid were identified in the R₂ position. There were 2 duplicates (4-dimethylamino) phenylacetic acid and 4-hydroxyphenyl acetic acids in the R₃ position. No repetitive sequences were observed.

Resynthesis and test of ligands on PEGA beads or in a soluble form for further evaluation

Eleven small molecule ligands from the beta-actin screen were resynthesized in an immobilized form covalently linked to PEGA beads for affinity binding studies. Concurrently, these small molecule ligands were resynthesized on Rink resin and then cleaved from the resin to produce a soluble form of the ligand that could be tested in cell-based assays. Complex protein cell extracts were prepared from Ramos lymphoma cells and used in affinity binding studies with the small molecule ligand-PEGA beads. After binding of the Ramos cell proteins, these proteins were eluted with SDS sample buffer, separated by SDS PAGE and the gels stained with Coomassie blue (Figure 4A). Most ligand PEGA beads bound multiple proteins. Ligands LML39 and 42 bound little protein. Interestingly, LML32, 33, 34, 36, 37, 38 and 40 bound similar proteins, whereas other ligands identified through different screening methods did not show the same proteins bound (data to be published). A protein band with an approximate molecular weight of 42 kDa was extracted from the gel and trypsin digested. The peptide fragments were analyzed by mass spectrometry and identified as actin by peptide mass fingerprinting (see methods). A duplicate set of ligand bound proteins were analyzed by immunoblotting with an anti-beta-actin monoclonal antibody (Figure 4B) and the presence of actin was confirmed in proteins bound to LML37, 38 and 41.

The same small molecule ligands were resynthesized on Rink amide resin and cleaved from the resin for testing as soluble ligands. The soluble ligands were dissolved in solvent (DMSO) and further diluted in aqueous media (cell culture media) for testing on Ramos lymphoma cells. We examine the bioactivity of these ligands by testing their effect on the proliferation of Ramos lymphoma cells by Coulter counting and by MTS (Promega) assay. Results from the Coulter counting experiment (Figure 5) show LML33 and 41 reduced the proliferation of Ramos lymphoma cells. Interestingly LML41, which binds actin, reduced proliferation of Ramos lymphoma cells as well as LML33, which may only weakly or indirectly binds actin, also reduced proliferation, suggesting the two small molecules may be affecting different protein targets. To show the variability in the assay, the control (C) samples (three separate sets of 3 samples each) contained Ramos B-lymphoma cells and the DMSO containing diluent used to dilute the small molecule compounds for their final concentration in the assay. Values from these controls were themselves averaged (Avg) and a standard deviation calculated. Further investigation into the bioactivity and mechanism of action of these ligands is presently being conducted.

Effect of dose on Ramos B-lymphoma cell proliferation

An analysis of the effective dose of LML33 on Ramos B-lymphoma cells was performed (Figure 6). It was determined that the effect of LML33 on Ramos cell proliferation started at 1–10 μ M, increased at 25 μ M and had the greatest effect at 50 μ M concentration. LML33 was relatively insoluble at higher concentrations and the concentration of DMSO, the solvent used to solubilized the compound, had to be kept low so that it does not affect cell proliferation; consequently, testing this compound at higher concentrations was not possible at this time. The IC₅₀ of LML33 was estimated to be approximately 50 μ M.

Discussion

The screening method reported in this paper enabled us to screen a 155,232 diversity encoded OBOC library for small molecule ligands that bind actin or an actin-containing protein complex in a relatively short period of time. This assay is dependent on the use of a monoclonal antibody

to the protein target of interest; which in this study is cytoplasmic (beta) actin. Our "subtraction assay" uses a two-step process whereby the first step is the identification and marking of the ligand beads that recognize the assay reagents, namely the primary monoclonal antibody and/or the secondary antibody enzyme conjugate used to detect the primary antibody. The actual screening assay with the Ramos cell extract was performed in the second step. It is important for this method to have a low background so that the actual assay would yield strong positive results that could easily be distinguished from the background binding of the assay reagents. The chemical structure of the small molecule compound on each positive bead was determined by Edman sequencing of the peptide encoded tags [26], which actually took the longest time since only 8 beads could be sequenced a day. OBOC encoded libraries are now being produced that contain encoded tags that can be determined by mass spectrometry [30,31]. We expect this will greatly reduce the amount of time needed for chemical decoding.

This proof of concept study demonstrates that our immuno-detection method works well for the identification of small molecule ligands that bind beta-actin or actin-containing protein complexes. There are a number of reasons we selected actin as our first protein target. Actin is a highly abundant protein in hematopoietic cells. Specific antibodies to actin are available. Small molecule ligands that bind cytoplasmic actin may interfere with its cellular function and cause these lymphoma cells to become apoptotic. Finally, actin is an abundant protein that because of its high abundance often interferes with proteomic studies in cells. An affinity matrix that could bind actin selectively could be used for depleting actin from a complex mixture of proteins thereby allowing the detection of other, lesser abundant proteins. More importantly, actin might be a suitable target for reagents that interfere with its cellular function and cause apoptosis or reduce cell proliferation. This type of reagent might have inhibitory properties for actin function and be useful for basic and applied research projects as already demonstrated in this report.

As hundreds of monoclonal antibodies against a large number of cell signaling proteins are commercially available, one can easily apply this two-step subtraction method to identify small molecule ligands against other proteins or protein complexes within a complex mixture of biological molecules such as the total cell lysate. Since small molecule library is used, ligands identified through such screening will have a good chance to be able to enter intact cells and exert biological effects on cellular functions mediated by these target proteins.

In this study, binding of beta actin in Ramos B-lymphoma cell extracts to immobilized LML small molecule ligands was assessed by Western blot analysis with the anti-beta actin monoclonal antibody. Not all LML small molecules bound actin suggesting that these molecules may have been indirectly identified as a member of a protein complex. Biological activity was assessed by the ability of the small molecule ligands to reduce proliferation of Ramos B-lymphoma cells. After screening approximately 25,000 beads of a 155,000 permutations library, eleven positive beads were identified and further studied. Three of these eleven ligands (LML33, 34 and 41) were found to exert some additive anti-proliferative effects on Ramos B-lymphoma cells. LML32 – 42 (50 μ M) when combined with 1 nM paclitaxel, an amount that was not cytotoxic, did not cause a significant reduction in Ramos B-cell proliferation (data not shown). However, LML33 (50 μ M) when tested with adriamycin did reduce Ramos proliferation more than adriamycin by itself (data not shown). Although showing promising early results, these small molecules will need to be optimized and the mechanism of action determined. Activity of these small molecules could be improved using a "focused" OBOC library approach. This type of approach was recently used successfully to develop a lymphoma targeting peptide ligand LLP2A (IC_{50} = 2pM)[20,32]. In a mouse xenograft model, LLP2A was able to image alpha4beta1-expressing lymphomas with high sensitivity and specificity when conjugated to a near infrared fluorescent dye [20].

The modified method described in this report involves the use of a specific monoclonal antibody for beta-actin, but would also be suitable for any affinity-purified antibody with good specificity. This antibody-based screening method for OBOC libraries has several advantages: 1) screening for a specific target can be preformed with a complex mixture of biological molecules; 2) it is not necessary to purify the protein; 3) the method enables one to detect ligands that bind to native protein or protein complexes, which could sometimes be difficult if not impossible with proteins cloned and expressed in *E. coli*; 4) chemical derivatization such as bioitnylation of the target protein(s) is not needed, and 5) many monoclonal antibodies against a large number of target proteins are commercially available. The time and effort needed to identify small molecules that bind specific proteins is significantly reduced with this approach because there is no need to perform time-consuming protein purification by affinity and/or separation chromatography or generating recombinant proteins. Furthermore, recombinant proteins produced in *E. coli* may not be correctly folded or post-translationally modified and may not reproduce the actual native protein. Also, proteins often exist in protein complexes within living cells so targeting native protein complexes (as opposed to a purified protein) with small molecules might produce lead compounds that interfere with functions that are more relevant physiologically.

Conclusion

The two-step immunodetection method described in this report is simple, efficient, robust, and highly versatile. When used in conjunction with encoded OBOC combinatorial small molecule or macrocyclic libraries, one should be able to rapidly identify chemical molecules that can interact with cellular targets of biological importance.

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Figure 1.
Chemical structure of the OBOC small molecule library.

Figure 2.
Subtractive screening of OBOC libraries using anti-beta actin antibodies

Figure 3. Screening for actin binding small molecules with anti-beta actin antibodies identifies Ramos lymphoma proteins bound to small molecule ligands attached to OBOC ligand beads
 OBOC beads with small molecule ligands attached were incubated in small columns with cell extracts prepared from Ramos B-lymphoma cells (see methods and Figure 2 for details). After washing, the beads were incubated with anti-beta actin monoclonal antibodies and then with anti-mouse IgG-alkaline phosphatase (anti-mouse IgG-AP). Beads were plated in low melt agarose and incubated with BCIP substrate for two hours with images taken at $t=0$ (A) and $t=2$ h (B). A small region of a bead with no color at 0 h, but a positive reaction at 2 h is expanded and noted by arrows. (C) Shows a photograph of this small expanded region with the positive

reacting bead. Included in this photo is a red marker bead used to orientate the scans with the position of the bead on the plate.

Figure 4. Binding of Ramos B-lymphoma cell extract proteins to small molecules resynthesized on PEGA beads identified small molecule ligands that bound actin, which was confirmed by Western blotting with an anti-beta actin antibody

A) Coomassie staining of SDS PAGE gel; B) Western blot analysis using an anti-beta actin antibody.

Figure 5. Anti-proliferative effect of LML small molecules identified in the actin antibody screen Ramos B-lymphoma cells were incubated for 3 days with small molecules ligands resynthesized in a soluble form after which the number of cells were counted by Coulter counting (duplicate samples). The control samples contained Ramos cells and the DMSO diluent used to dilute the small molecules. This experiment was repeated twice with similar results.

Figure 6. Dose response curve of LML33 on proliferation of Ramos B-lymphoma cells
LML33 (1 μl) was added to 15,000 Ramos B-lymphoma cells in 100 μl per well (96 well plate) to a final concentration of 0.1, 1, 10, 25 and 50 μM LML33. The plate was incubated for 3 days at 37°C, 5% CO₂. MTS reagent (see methods) was added and the plate read at 490nm after 2 h. The experiment was performed in triplicates (n= 3).

R₃
Acylation reagent
Butyric acid

4-
(Dimethylamino)
phenylacetic acid
Phenoxyacetic
acid

Structure

R₃
Acylation reagent
L-Pyrroglutamic
acid

Structure

Structure

R_3

Acylation reagent

trans-4-Cotinine
carboxylic acid

Structure

Structure

R_3

Acylation reagent

(S)-(+)-Oxo-4-phenyl-3-oxazolidineacetic acid

Structure

R_3

Acylation reagent

(-)-2-Oxo-4-
thiazolidine
carboxylic acid

Structure

Structure

R₃
Acylation reagent4-
(Dimethylamino)
phenylacetic acid

Structure

Structure

4-Bromophenyl
acetic acid

Structure

R₃

Acylation reagent

4-Hydroxyphenyl
acetic acid

Structure