High-Throughput Screen for Small Molecule Inhibitors of Mint1-PDZ Domains

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Abstract: Several hundred PDZ (postsynaptic density-95, Drosophila disks-large, ZO-1) domain-containing proteins have been identified in the human genome. PDZ domains play a critical role in organization and function of cellular signaling pathways. Thus, small molecule inhibitors of PDZ domain association with their targets have wide potential applications as research and therapeutic agents. PDZ domains typically bind to a carboxyl-terminal tail of the target protein. Here we describe a high-throughput screening (HTS) assay for small molecule inhibitors of association between Mint1-PDZ domains and N-type Ca2+ channel carboxyl-terminal peptide (NC peptide). The performance of a homogeneous time-resolved fluorescence resonance energy transfer (HTRF) and an amplified luminescent proximity homogeneous assay (ALPHA) were systematically compared in parallel pilot HTS experiments with glutathione S-transferase–Mint1-PDZ1/2 protein and biotinylated NC peptide. Both of the two assays showed similar sensitivities in our target protein assay. Using HTRF-based assay we screened a library of 100,000 small molecule compounds and identified a number of potential “hits.” The activity of isolated “hits” was confirmed by ALPHA assay. However, further evaluation revealed that isolated “hits” most likely act as “promiscuous binders,” not as specific Mint-PDZ inhibitors, and that additional screening will be required to identify the true Mint-PDZ inhibitors. The assays described provided an example of HTS for a small molecule inhibitor of Mint-PDZ domain that can be easily adapted to other PDZ domain-mediated interactions.

Introduction

Proper localization of signaling components in polarized cells is essential for their function. The proper targeting of signaling molecules in polarized cells is achieved and maintained via a complex network of protein–protein interactions, frequently mediated by PDZ (postsynaptic density-95, Drosophila disks-large, ZO-1) domain-containing adaptor proteins. PDZ domains bind to the last four to six carboxy-terminal amino acids in the target protein.1–4 Determination of PDZ domain structure by crystallography and nuclear magnetic resonance (NMR) methods5–7 revealed a compact globular module formed by six β strands and two α helices, which form a carboxyl-terminal peptide-binding groove. There are over 10,000 PDZ domains present in almost 7,000 proteins in the nonredundant genome sequence database. Despite similarities in secondary structure and the common preference for carboxyl-terminal ligands, PDZ domains display different binding specificity. Based on their ligand specificity, PDZ domains can be classified to multiple classes.8 Because of the important role played by...
PDZ domains in cellular signaling, small molecule inhibitors of PDZ-domain-mediated interactions are expected to have wide applications as research and therapeutic agents. For example, a recent report indicated that specific inhibitors of the second PDZ domain in postsynaptic density-95 protein may have significant neuroprotective effects in stroke and other excitotoxic disorders. \(^9\)

Chronic pain (neuropathic pain, inflammatory pain, cancer pain) is a major health problem. Sensation of pain is mediated by nociceptive neurons in the dorsal root ganglia. \(^10,11\) N-type voltage-gated Ca\(^{2+}\) channels (CaV2.2s) are abundantly expressed in dorsal root ganglia neurons\(^12–14\) and play a predominant role in synaptic transmission in the spinal cord. In agreement with the role of N-type Ca\(^{2+}\) channels in the pain pathway, pharmacological blockade of N-type Ca\(^{2+}\) channels by single injection or continuous infusion of synthetic \(\omega\)-MVIIA conotoxin SNX-111 inhibited phase II formalin response in the rat animal model of pain. \(^15,16\) The role of N-type Ca\(^{2+}\) channels in pain pathway was further supported by suppression of the phase II formalin response in CaV2.2 knockout mice when compared to wild-type mice. \(^17–19\)

An important role of a specific N-type Ca\(^{2+}\) channel splice variant in the pain pathway has been suggested by a recent small interfering RNA study. \(^20\) All these results pointed to N-type Ca\(^{2+}\) channels as a potential drug target for a treatment of persistent pain. Based on this idea, Elan Pharmaceuticals (South San Francisco, CA) developed a drug, Prialt\(^\text{®}\) (ziconotide, SNX-111, a synthetic version of \(\omega\)-MVIIA conotoxin), which has been recently approved by the Food and Drug Administration for treatment of severe chronic pain.

Although Prialt is highly effective for the treatment of chronic pain, there are a number of problems associated with its use. Prialt is difficult to synthesize, and thus manufacture, since it is a polypeptide with a complex chemical structure. Prialt does not pass the blood–brain barrier and has to be delivered by pump infusion directly into a spinal cord, greatly limiting its therapeutic applications. In addition, a number of severe side effects have been reported in patients undergoing Prialt treatment, such as dizziness, blurred vision, and lateral-gaze nystagmus. \(^21,22\) Because of Prialt’s shortcomings, two other drugs targeting N-type Ca\(^{2+}\) channels for pain are being developed. CNSBio (Melbourne, Australia) is developing AM336, a synthetic analog of \(\omega\)-CVID, which is a more selective and reversible inhibitor of N-type Ca\(^{2+}\) channels than Prialt. \(^23\) Neuromed Tech (Vancouver, BC, Canada) has a small molecule inhibitor of N-type Ca\(^{2+}\) channels (NMED-160) in phase II clinical trials for chronic pain. \(^23\) A high-throughput screen for small molecule N-type Ca\(^{2+}\) channel inhibitors has been recently developed using a scintillation proximity assay. \(^24\)

In our previous studies we discovered a specific association of CaV2.2 carboxyl-termini with the first PDZ domain of the modular adaptor protein Mint1. \(^25\) Consistent with our findings, the structure of Mint1-PDZ domain bound to CaV2.2 carboxyl-terminal peptide was recently solved by NMR. \(^26\) In additional experiments we demonstrated that carboxyl-terminal association of CaV2.2 protein with the Mint1-PDZ domain plays an important role in synaptic targeting and function of N-type Ca\(^{2+}\) channels. \(^27\) Thus, we reasoned that inhibitors of CaV2.2 association with Mint1-PDZ domains will selectively suppress synaptic function of N-type Ca\(^{2+}\) channels and may have a therapeutic potential for the treatment of pain. Because such molecules will only affect function of synaptic Ca\(^{2+}\) channels and not Ca\(^{2+}\) channels in the soma, it may minimize potential side effects. To identify such inhibitors, we set up HTS using Mint1-PDZ1/2 protein expressed in bacteria as glutathione S-transferase (GST)-fusion protein (GST-M15) and biotinylated (bio-) N-type Ca\(^{2+}\) channel carboxyl-terminal peptide (NC peptide) corresponding to the carboxyl-terminal tail of CaV2.2 protein.

In our HTS studies we utilized a time-resolved (TR) fluorescence resonance energy transfer (FRET) assay and an amplified luminescent proximity homogeneous assay (ALPHA). Both of the assays are widely used in HTS. \(^28–30\) TR-FRET has been developed by a few companies, including homogeneous TR-FRET (HTRF\(^\text{®}\), CisBio, Bagnols sur Cèze, France), LANCE\(^\text{®}\) (Perkin Elmer, Waltham, MA), and Lanthascreen\(^\text{®}\) (Invitrogen, Carlsbad, CA). The TR-FRET experiments are based on FRET between the caged donor fluorophore with a delayed emission and a neighboring acceptor fluorophore. \(^31\) The compound artifacts in this method have been discussed recently. \(^32\) The principle of ALPHA technology is based on the conversion of ambient oxygen to the singlet state by a photosensitizer in the donor beads upon illumination at 680 nm. \(^33\) The reaction of singlet oxygen with the acceptor beads is detected by optical methods. The association between GST-M15 protein and bio-NC peptide was measured in HTRF and ALPHA assays. The performance of HTRF and ALPHA was systematically compared in parallel HTS experiments with GST-M15 protein and bio-NC (or bio-polyethylene glycol [PEG]-G10-NC) peptide.

Materials and Methods

Materials and equipment

The HTRF assay reagents anti-GST monoclonal antibody labeled with europium cryptate (EuK) (EuK-anti-GST mAb) and streptavidin-XL665 (streptavidin-XL) peptide were purchased from CisBio (Cisbio-US, Bedford, MA). The HTRF assay was performed in black low-volume 384-well plates (ISCBioExpress, Kaysville, UT). ALPHA experiments reagents, anti-GST acceptor beads, and streptavidin donor beads for the ALPHA experiments were purchased from Perkin Elmer. The ALPHA was per-
formed in white 384-well plates from Costar (Costar, Bethesda, MD). Bio-GST protein was purchased from Perkin Elmer and was used for both HTRF and ALPHA experiments in 1 nM concentration. The pipetting of reagents in both HTRF and ALPHA HTS experiments was performed by using a Biomek® FX robotic pipetter with Biomek AP 384 P30 pipette tips (Beckman Coulter, Fullerton, CA). An EnVision™ 2102 plate reader (Perkin Elmer) was used to read the HTRF and ALPHA signals. The settings for HTRF experiments were: excitation wavelength, 320 nm; detection channel 1, 665 nm; detection channel 2, 590 nm; time between flashes, 2,000 μs; number of flashes, 200 per well; measurement height, 6 mm; delay, 50 μs; window reading time, 400 μs. The settings for ALPHA experiments were: excitation wavelength, 680 nm; detection channel, 570 nm; excitation duration, 180 ms; read time, 550 ms; number of flashes, one per well.

Biotin was purchased from Sigma (St. Louis, MO). The NC peptide RHSYHHPDQDHWC, which corresponds to amino acids 2327–2339 of the human CaV2.2 subunit, was chemically synthesized by the University of Texas Southwestern Medical Center Peptide Chemistry Core. Biotin was added to the NC peptide on the amino-terminus during synthesis to yield bio-NC (biotin-RHSYHHPDQDHWC). The bio-G5-NC (biotin-GGGGGRHSYHHPDQDHWC), bio-G10-NC (biotin-GGGGGGGGRHSYHHPDQDHWC), and bio-PEG-G10-NC (biotin-PEG24-GGGGGGGGRHSYHHPDQDHWC) peptides were also synthesized.

GST-Mint-PDZ1/2 protein expression and purification

A fragment of rat Mint1 encoding PDZ1/2 domains (amino acids 647–837) was cloned into pGEX-4T-3 expression plasmid (Amersham-Pharmacia, Piscataway, NJ) as previously described.25 The resulting GST-M15 protein was expressed in BL21 Escherichia coli strain by isopropyl β-D-1-thiogalactopyranoside induction at room temperature with shaking for 16–18 h. The bacteria were lysed in lysis buffer (50 mM imidazole [pH 6.8], 100 mM NaCl, 10 mM EDTA, 1 mM dithiothreitol [DTT], and 3 mg/ml lysozyme) with addition of protease inhibitors, and the GST-M15 protein was purified from bacterial lysates on glutathione agarose beads (Sigma). The GST-M15 protein was eluted in 10 mM glutathione buffer (50 mM Tris-HCl, pH 8.0) with shaking at 4°C and dialyzed against phosphate-buffered saline buffer at 4°C overnight. The final concentration of GST-M15 in phosphate-buffered saline was equal to 2.2 mg/ml (Protein Assay Dye Reagent, Bio-Rad, Hercules, CA).

Diversity of the compound library

An 8,000 test compound library and 100,000 HTS compound library in dimethyl sulfoxide (DMSO) were provided by the University of Texas Southwestern Department of Biochemistry HTS Laboratory. Compounds in the test library are representative of a larger set of drug-like small molecules purchased from ChemDiv Inc. (San Diego, CA) and Chembridge Corp. (San Diego).

HTRS data analysis

The analysis of obtained results was performed by Accord software from Accelrys (San Diego).

In vitro binding experiments

The in vitro binding experiments have been performed as we previously described.8,25 In brief, hemagglutinin
(HA)-tagged Mint1 protein was expressed in COS7 cells by the DEAE-dextran transient transfection method. The transfected COS7 cells were collected and solubilized for 30 min at 4°C in lysis buffer (0.5% Triton X-100, 20 mM imidazole [pH 6.8], 100 mM NaCl, 1 mM EDTA, and 1 mM DTT, with addition of protease inhibitors). Extracts were clarified by centrifugation for 10 min at 4°C. Bio-NC or bio-NCWA peptides (10 μg of each) and 15 μg of streptavidin agarose beads were added to 500 μl of lysis buffer and shaken at 4°C for 1 h. The beads were washed twice (5 min each) to remove the nonbinding peptides, and the clarified lysate containing HA-Mint1 was added to the beads. The competitive G10-NC peptide and FH69 and Ran1 compounds were premixed with HA-Mint1 lysate for 30 min prior to addition to the beads at concentrations indicated in the text. After the mixture of HA-Mint1 lysate and bio-NC beads was shaken for 2 h, the beads were washed three times with the lysis buffer by centrifugation. The attached protein samples were resolved by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, immobilized on HybondTM-C Extra nitrocellulose (Amersham Biosciences), and analyzed by western blotting with anti-HA monoclonal antibodies (1:1,000, Berkeley Antibody Co., Berkeley, CA) and horseradish peroxidase-conjugated secondary anti-mouse antibodies (Sigma). The Western Lightning Chemiluminescence Reagent Plus (catalog number NEL103, Perkin Elmer, Waltham, MA) was used for detection by blue x-ray film (Phenix, Chandler, NC).

Results

HTRF assay

In our HTRF experiments, the GST-M15 protein was labeled by EuK-anti-GST mAb, and bio-NC peptide was labeled by streptavidin-XL fluorophore. Association of bio-NC peptide with Mint-PDZ domain brings EuK and XL665 in proximity (Fig. 1A). Excitation of EuK with a brief flash of 320 nm light leads to FRET between these two fluorophores, and XL665 reemits a specific long-lived fluorescence at 665 nm (Fig. 1A). Photons emitted by EuK are absorbed by the nearby (within 10 nm) XL665 acceptor, which emits at 665 nm (Fig. 1A). HTRF signal is calculated as a ratio of channel 1 (665 nm) to channel 2 (590 nm) signals.

Bio-NC (or bio-G10-NC) peptide and GST-M15 protein were mixed in the binding buffer (25 mM HEPES, 100 mM NaCl, and 0.1% bovine serum albumin, pH 7.2) and incubated with EuK-anti-GST mAb and streptavidin-XL. In the negative control experiments biotin was used instead of bio-NC. The resulting HTRF reaction mixture was aliquoted at 20 μl per well to black low-volume 384-well plates by the Biomek FX robot. Following a 3-h incubation, KF was added to each well to yield 0.4 M final concentration, and HTRF signal in each well was read by the EnVision 2102 plate reader as described in Materials and Methods. In control experiments 1% DMSO was added to the final reaction mixture. The HTRF ratio of 665 nm/590 nm counts determined for each well indicates the degree of bio-NC peptide association with GST-Mint-PDZ protein.

ALPHA assay

The principle of ALPHA technology is based on the conversion of ambient oxygen to the singlet state by a photosensitizer in the donor beads upon illumination at 680 nm. The acceptor beads contain a thioxene derivative that reacts with the singlet oxygen to generate chemiluminescence at 370 nm. Energy transfer to fluorescent acceptors in the same beads shifts the emission wavelength to 520–620 nm. The half-life of the decay reaction is 0.3 s, which makes the AlphaScreen® (Perkin Elmer) fluorescence signal very long-lived and allows the technology to operate in a time-resolved mode. The short lifetime of singlet oxygen in aqueous solution (~4 μs) allows diffusion over a distance up to ~200 nm. In our experiments the GST-M15 protein was labeled by monoclonal anti-GST antibodies conjugated to acceptor beads, and bio-NC or another NC peptide was labeled by streptavidin conjugated to donor beads (Fig. 1B). Association of bio-NC peptide with the Mint-PDZ domain brings donor and acceptor beads in proximity to each other (Fig. 1B). The donor beads are excited by the 680 nm flash of light, and the ALPHA signal from the acceptor beads is detected at 570 nm wavelength (Fig. 1B). In our experiments an EnVision 2102 plate reader was used to measure the ALPHA signal as described in Materials and Methods.

The ALPHA assay experiments were performed under reduced lighting, and samples were protected from direct light exposure. The bio-NC, bio-G5-NC, bio-G10-NC, and bio-PEG-G10-NC peptides were mixed with GST-M15 protein in binding buffer (25 mM HEPES, 100 mM NaCl, and 0.1% bovine serum albumin, pH 7.2). The anti-GST acceptor beads and streptavidin donor beads were added as indicated in the text. In the negative control experiments biotin was used instead of bio-NC, bio-G5-NC, bio-G10-NC, or bio-PEG-G10-NC peptide. The resulting ALPHA reaction mixture was aliquoted at 20 μl per well to white 384-well plates by the Biomek FX robot. Following incubation for 1–48 h as indicated in the text, the ALPHA signal in each well was read by the EnVision 2102 plate reader as described in Materials and Methods. In control experiments 1% DMSO was added to the final reaction mixture. The ALPHA signal (570 nm) determined for each well indicates the degree of bio-NC peptide association with GST-Mint-PDZ protein.
Detection of Mint1-PDZ association with bio-NC by HTRF

In order to optimize the HTRF assay, we tested different concentrations of Mint-PDZ protein, bio-NC peptide, EuK-anti-GST mAb, and streptavidin-XL. As a result of HTRF assay optimization we obtained highly reproducible signal in each well (Fig. 2). We found that the optimal conditions for HTRF experiments correspond to 70 nM GST-M15, 120 nM bio-NC, 1.1 nM EuK-anti-GST mAb, and 26.7 nM streptavidin-XL (Table 1). The background signal was determined in parallel experiments by using 120 nM biotin instead of bio-NC. By comparing the HTRF signal (665/590 nm ratio) in wells containing bio-NC and biotin, we estimated signal-to-background ratio (S/B) equal to 4.8 and coefficient of variability (CV) equal to 2.8% when the assay was performed in the presence of 1% DMSO (Fig. 2 and Table 1). A recently developed simple statistical parameter ($Z'$ factor) is a better measure of HTS assay quality than S/B.34 Thus, we used a formula from Zhang et al.34 to calculate the $Z'$ factor from our data. The $Z'_{32/16}$ factor was calculated based on HTRF signal measured in the presence of 1% DMSO in 32 wells with bio-NC peptide (signal) and 16 wells with biotin control (background) (Fig. 2). We determined $Z'_{32/16} = 0.8$ from our data (Fig. 2 and Table 1) as being in the “excellent assay” range.34

**Table 1. Performance of HTRF and ALPHA Assays in Pilot Screen for Mint1-PDZ Inhibitors**

<table>
<thead>
<tr>
<th>HTS</th>
<th>Bio-NC or bio-NC peptide</th>
<th>Anti-GST (EaK or acceptor)</th>
<th>Streptavidin (XL665 or donor)</th>
<th>Assay volume</th>
<th>S/B</th>
<th>CV</th>
<th>$Z'$</th>
<th>$IC_{50}$ threshold</th>
<th>Z</th>
<th>Plate read time (min)</th>
<th>Number of dispensing steps</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTRF</td>
<td>70 nM</td>
<td>120 nM</td>
<td>1.1 nM</td>
<td>26.7 nM</td>
<td>20 μl</td>
<td>5</td>
<td>2.8%</td>
<td>0.8</td>
<td>40 μM</td>
<td>4 μM</td>
<td>0.5–0.9</td>
</tr>
<tr>
<td>ALPHA</td>
<td>80 nM</td>
<td>23 nM</td>
<td>20 μg/ml</td>
<td>20 μg/ml</td>
<td>20 μl</td>
<td>25</td>
<td>5.5%</td>
<td>0.7</td>
<td>4 μM</td>
<td>6 μM</td>
<td>0.6–0.8</td>
</tr>
</tbody>
</table>

**Pilot 8,000 compound HTRF screen**

The excellent S/B and $Z'$ factor obtained as a result of HTRF assay optimization (Fig. 2) enabled us to perform a pilot screen with the test library of 8,000 compounds. The test library, which was aliquoted to 25 barcoded 384-well plates, was provided to us by the University of Texas Southwestern HTS Laboratory (320 compounds per plate). Each tested compound (columns 3–22 on each test plate) was dissolved in DMSO and added to HTRF reaction mixture in the same time as bio-NC peptide and GST-M15 protein to yield 5 μM final concentration of each compound and 1% DMSO. The same amount of DMSO was included to two control columns (2 and 23, 32 wells per plate). Column 24 (16 wells per plate) contained biotin instead of bio-NC. The pilot HTRF screen yielded highly reproducible data (Fig. 3A). To evaluate the quality of our screen, we calculated the $Z'_{32/16}$ factor34 for each plate from the HTRF signal measured from columns 2 and 23 (DMSO control) and 24 (biotin control). We found that $Z'_{32/16}$ factors for all 25 plates were in the range from 0.5 to 0.9, with the mean $Z'_{32/16} = 0.77 ± 0.11$ (Fig. 3B and Table 1). To further evaluate the performance of our assay, we also calculated $Z_{320/16}$ factor for each plate by using results from 320 compound wells (columns 3–22) and column 24 (biotin control). We found that the $Z_{320/16}$ factor was in the range 0.6–0.8, with the mean $Z_{320/16} = 0.75 ± 0.08$ (Fig. 3C and Table 1). Thus, both $Z'_{32/16}$ and $Z_{320/16}$ factors were in the “excellent HTS screen” range.34

A number of compounds from the 8,000 compound library resulted in a drop in the HTRF signal below 3 SD from the mean (Fig. 3A). Are these compounds real “hits” that disrupt bio-NC association with GST-Mint-PDZ? To answer this question, we selected five compounds that gave the largest reduction in the HTRF signal (H1–H5) for further evaluation. Repeat of experiments with bio-NC peptide and GST-M15 confirmed four- to fivefold reduction of the HTRF signal in the presence of 5 μM compounds H1–H5 when compared to 1% DMSO control (data not shown). As an additional control we performed HTRF experiments with 1 nM bio-GST instead of bio-NC and GST-M15. We reasoned that if compounds H1–H5 indeed act by disrupting NC association with Mint-PDZ, these compounds should not affect HTRF signal measured with bio-GST. However, all five compounds caused four- to
fivefold reduction in the HTRF ratio measured with bio-GST, similar to their effects in experiments with bio-NC and GST-M15. Careful examination of obtained results indicated that compounds H1–H5 reduced the HTRF ratio not by reducing channel 1 (665 nm) signal but by increasing channel 2 (590 nm) signal (data not shown). Thus, we concluded that excitation of these compounds by 320 nm wavelength light leads to strong emission at 590 nm. The chemical structures of the H1–H5 "hits," which include multiple double bonds (data not shown), were consistent with autofluorescent behavior of these compounds in our HTRF experiments. These conclusions were further supported by results of fluorescence spectroscopy experiments performed with some of these compounds (data not shown). From these results we concluded that compound autofluorescence was an important source of false-positives in HTRF screen, which is consistent with data reported in the literature.32

Detection of Mint1-PDZ association with bio-PEG-G10-NC by ALPHA

In parallel with HTRF assay optimization, we also utilized the ALPHA method to detect association of GST-Mint-PDZ (GST-M15) protein with NC peptide. Different concentrations of GST-M15, bio-NC peptides, anti-GST acceptor beads, and streptavidin donor beads were tested to optimize in the ALPHA assay. We found that the optimal conditions for ALPHA experiments corresponded to 80 nM GST-M15, 23 nM bio-NC, 20 μg/ml anti-GST acceptor beads, and 20 μg/ml streptavidin donor beads (Table 1). The background signal was determined in parallel experiments by using 23 nM biotin instead of bio-NC. In contrast to HTRF experiments, we found that long incubation times are required to improve the S/B ratio in ALPHA experiments when bio-NC peptide was used. For example, in experiments with bio-NC we determined that S/B was <2 after a 4-h incubation but increased to 28 after a 48-h incubation (Fig. 4). To explain these findings, we reasoned that association of GST-M15 and bio-NC with ALPHA beads causes steric interference with bio-NC/GST-M15 complex formation. To alleviate this problem, we replaced bio-NC peptide with bio-G5-NC, bio-G10-NC, and bio-PEG-G10-NC peptides, which contain G5, G10, or PEG-G10 linker, respectively, between NC peptide and biotin. The kinetics of association between these four peptides and GST-M15 were compared by us-

FIG. 3. Pilot 8,000 compound HTRF screen. (A) The HTRF signals in each well were normalized to the mean DMSO signals (columns 2 and 23) on the same plate. (B) \(Z_{320/16}\) factors were calculated for each of 25 plates based on HTRF readings in columns 2 and 23 (bio-NC, 1% DMSO) and column 24 (biotin, 1% DMSO). (C) \(Z_{320/16}\) factors were calculated for each of 25 plates based on HTRF readings in 320 wells with test compounds and 16 wells with biotin (column 24).

FIG. 4. Kinetics of the ALPHA assay. The ALPHA signal is plotted against time for reaction mixtures composed of GST-M15 protein and biotin (○), bio-NC (△), bio-G5-NC (●), bio-G10-NC (▲), or bio-PEG-G10-NC (■) peptides as indicated. Streptavidin donor beads were added 0.5 h before reading to the mixture of GST-M15, NC peptides or biotin, anti-GST acceptor beads, and 1% DMSO. The ALPHA signals at each time point are shown as mean ± SE values (n = 3).
ing the ALPHA assay. We found that addition of G5 or G10 linker had a negligible effect on the kinetics of complex formation as detected by ALPHA (Fig. 4). In contrast, addition of PEG-G10 linker speeded up complex formation very significantly, with S/B equal to 25 within 4 h of incubation (Fig. 4). With the longer incubation time (48 h), all four peptides resulted in similar ALPHA signal and S/B (Fig. 4), consistent with the hypothesis that the underlying problem is kinetic in nature.

Even with bio-PEG-G10-NC peptide, the S/B in ALPHA experiments was only 1.5 (and Z’ = 0) after a 4-h incubation if donor and acceptor beads were added together with GST-M15, biotin, anti-GST-acceptor beads and streptavidin donor beads (Fig. 5A). However, we found that the quality of data was greatly improved when GST-M15, bio-PEG-G10-NC, and anti-GST acceptor beads were mixed first (in a 9-$\mu$l volume), and 11 $\mu$l of streptavidin donor beads was added to each well 3 h later. These “dual-addition” experiments yielded S/B = 25, CV = 5.5%, and Z’ = 0.8 for the bio-PEG-G10-NC and GST-M15 pair with a 4-h incubation time (Fig. 5B and Table 1).

Pilot 8,000 compound ALPHA screen

Good S/B and Z’ factors obtained as a result of ALPHA assay optimization (Fig. 5B) enabled us to perform a pilot screen with the same test library of 8,000 compounds. The format of the ALPHA screen was identical to the HTRF screen, with test compound included in columns 3–22, columns 2 and 23 used for DMSO control, and column 24 used for biotin control. Similar to the HTRF screen, 0.2 $\mu$l of each tested compound was dissolved in DMSO and mixed with 9 $\mu$l of ALPHA reac-

FIG. 5. Order of additions in ALPHA assay. ALPHA results are shown for 32 wells containing GST-M15, bio-PEG-G10-NC, anti-GST acceptor beads, and streptavidin donor beads (filled diamonds). Control results are shown for 16 wells containing GST-M15, biotin, anti-GST-acceptor beads and streptavidin donor beads (open squares). In all wells 1% DMSO was present. (A) All reagents were added together. S/B = 1.5, Z’ = 0. (B) GST-M15, bio-PEG-G10-NC (or biotin), and anti-GST acceptor beads were incubated for 3 h prior to addition of streptavidin donor beads. S/B = 27, Z’ = 0.8.

FIG. 6. Pilot 8,000 compound ALPHA screen. (A) The ALPHA signals in each well were normalized to the mean DMSO signals (columns 2 and 23) on the same plate. (B) Z’ factors were calculated for each of 25 plates based on ALPHA readings in columns 2 and 23 (bio-NC, 1% DMSO) and column 24 (biotin, 1% DMSO). (C) Z’ factors were calculated for each of 25 plates based on ALPHA readings in 320 wells with test compounds and 16 wells with biotin (column 24).
tion mixture that included GST-M15, bio-PEG-G10-NC, and anti-GST acceptor beads. After a 3-h incubation of plates at room temperature in the dark, 11 μl of streptavidin donor beads in the binding buffer was added to each well to yield a total reaction volume of 20 μl. The final concentration of each tested compound was 5 μM, and final concentration of DMSO was 1%. The ALPHA signal for each well was measured as described in Materials and Methods 1 h after addition of streptavidin donor beads. Analysis of results obtained with 25 tested plates yielded an S/B of 25–28 for most plates (Fig. 6A). The Z' factors calculated for each plate as described for the HTRF assay were in the range 0.6–0.8, with the mean Z' = 0.69 ± 0.11 (Fig. 6B), in the excellent range.34 The Z' factors calculated for each plate as described for the HTRF screen were in the range 0.1–0.6, with the mean Z' = 0.40 ± 0.15 (Fig. 6C and Table 1).

To evaluate a number of potential “hits” identified in ALPHA screen (Fig. 6A) we selected 10 compounds that gave the largest reduction in ALPHA signal (A1–A10). Repeat of experiments with bio-PEG-G10-NC and GST-M15 confirmed the reduction of ALPHA signal in the presence of 5 μM compounds A1–A8 and A10 when compared to 1% DMSO control (data not shown). However, the same nine compounds also caused a similar reduction in ALPHA signal measured with bio-GST control (data not shown). Thus, we concluded that the identified “hits” reduced ALPHA signal by a nonspecific mechanism and not by disrupting Mint-PDZ association with bio-PEG-G10-NC.

Comparison of HTRF and ALPHA assay performance

In the next series of experiments we compared the sensitivity of HTRF and ALPHA assays for Mint-PDZ inhibitors. These experiments were performed in the same conditions as described for pilot HTRF and ALPHA screens, but with addition of different concentrations of G10-NC peptide (0, 1 μM, 2 μM, 4 μM, 8 μM, 40 μM, and 120 μM) used as a competitive inhibitor of Mint-PDZ domain interactions. As expected, addition of G10-NC effectively reduced both HTRF and ALPHA signals (Fig. 7A). The concentration required to reduce the signal by 50% (IC50) for G10-NC was equal to 4 μM for ALPHA and 40 μM for HTRF (Fig. 7A). Analysis of 8,000 pilot screens revealed that the SD of the observed signals was equal to 0.207 for ALPHA and 0.0594 for HTRF (Figs. 3 and 6). The reduction of the signal by at least 3 SD is typically used as a criterion for selection of “hits” in an HTS screen (Figs. 3 and 6). The concentrations of G10-NC required to reduce the signal by 3 SD were equal to 6 μM for ALPHA and 4 μM for HTRF (Fig. 7A). Thus, the sensitivity of these two assays was similar if variability of observed signals is taken into consideration.

To further compare the performance of HTRF and ALPHA assays, we compared potential “hits” resulting from 8,000 HTRF and ALPHA screens. We found that 472 compounds out of 8,000 compounds tested reduced the ALPHA signal by more than 30% (Figs. 6A and 7B). On the other hand, 190 compounds out of the same 8,000 compounds reduced the HTRF signal by more than 15% (Figs. 3A and 7B). Interestingly, only five compounds were effective in both HTRF and ALPHA assays (Fig. 7B). When the same five compounds were retested, we found that only three compounds (FH85, FH86, and FH87) were active in both HTRF and ALPHA assays. However, the same three compounds showed similar activity in control HTRF and ALPHA experiments with

![FIG. 7.](image) Comparison of HTRF and ALPHA assays. (A) HTRF and ALPHA assays were performed in the presence of G10-NC competitive peptide inhibitor. The normalized HTRF (open circles) and ALPHA (solid circles) signals are shown as mean ± SE values (n = 3) at each concentration of G10-NC tested. The 3SD levels are shown for HTRF and ALPHA by solid lines. The significant-effect concentrations for HTRF (open star) and ALPHA (filled star) are shown. (B) Potential hits from the 8,000 compound pilot ALPHA (472) and HTRF (190) screens are represented by a Venn diagram. Only five compounds from the 8,000 tested were identified as potential hits in both assays.
bio-GST (data not shown), indicating that these compounds were not real hits and did not act by disrupting the association between Mint1-PDZ protein and NC peptide. We concluded that the library with much higher chemical diversity than the pilot 8,000 compound library needs to be screened in order to identify small molecule inhibitors of Mint-PDZ domains.

100,000 compound HTRF HTS

Excellent performance of our pilot 8,000 compound HTRF and ALPHA screens encouraged us to undertake a large-scale HTS for potential inhibitors of Mint1-PDZ association with CaV2.2-derived NC peptide. We used the HTRF-based approach to screen a small molecule library available at the University of Texas Southwestern HTS Laboratory. The HTRF screen was performed essentially as described above for the pilot 8,000 compound screen, except that bio-G10-NC peptide was used instead of bio-NC peptide. Each compound was tested at the final concentration of 5 μM in the presence of 1% DMSO and incubated with GST-M15 for 10 min at room temperature prior to addition of bio-G10-NC peptide and HTRF reagents. In our experiments we screened a total of 314 plates, which corresponds to 100,480 compounds (320 compounds per plate). The 100,000 compound HTS screen was run for 17 days, with 19 library plates tested each day. To verify stability of our assay, at the conclusion of each day we ran a single control plate with 1% DMSO included instead of test library compounds. The Z'_{320/16} for these 18 control DMSO plates was in the range of 0.7–0.9 (data not shown), confirming stability of our assay. The quality of the 100,000 compound screen was evaluated as described above for the pilot 8,000 compound screen. We found that Z'_{32/16} factors for 295 out of 314 plates were in the range from 0.5 to 0.9 (Fig. 8A), that is, in the “very good” to “excellent” assay range. The 19 compound plates with Z' < 0.5 were retested, and we found that 15 of these 19 plates yielded Z'_{32/16} in the 0.5–0.85 range in the repeat experiments (data not shown). The data from these 15 “repeat plates” were combined with results from 295 “good plates” from the first round of screening. Four out of the 19 retested plates yielded Z'_{32/16} < 0.5 in the repeat experiments (data not shown), and results with these plates were discarded. Thus, at the conclusion of initial and repeat screens we obtained HTRF results for 310 plates (99,200 compounds) with Z'_{32/16} factors in the 0.5–0.9 range. The Z_{320/16} factors were in the range 0.5–0.8 for most plates (Fig. 8B). The plates that yielded Z_{320/16} < 0.5 were highly enriched for autofluorescent compounds when compared to other plates in the library (data not shown).

From the 8,000 compound pilot HTRF screen we found that autofluorescent compounds are the main source of “false-positives” in our assay (see above). These compounds reduced the HTRF ratio not by reducing channel 1 (665 nm) signal but by increasing channel 2 (590 nm) signal. Taking this information into consideration, we utilized the following three criteria to select putative “hits”: (1) HTRF ratio must be reduced below the average HTRF ratio on the same plate by at least 4 SD from the mean; (2) channel 1 (665 nm) signal has to be reduced below average channel 1 signal on the same plate by at least 4 SD from the mean; and (3) channel 2 (590 nm) signal must be no more than twofold increased when compared to the average channel 2 signal on the same plate. By applying these three criteria, we identified 1,918 potential “hits” from 99,200 screened compounds, which were aliquoted (“cherry picked”) from master library plates to separate 384-well plates for further analysis.

To evaluate 1,918 selected compounds we repeated HTRF experiments with bio-G10-NC and GST-Mint-PDZ in the presence of each compound at 5 μM. In parallel, we performed HTRF experiments with bio-GST protein. As discussed above, experiments with bio-GST offered a simple way to identify “false-positives” isolated in our screen. To separate potential “hits” from “false-positives,” we focused on compounds that reduced HTRF signal by at least 30% in repeat experiments with bio-
G10-NC and GST-Mint-PDZ, but had less than 5% effect on HTRF signal in experiments with bio-GST. Out of 1,918 compounds isolated as potential “hits,” 52 compounds satisfied both of these criteria (Fig. 9), and these compounds have been selected for further evaluation.

Characterization of potential hits from the 100,000 compound screen

When the chemical structures of the 52 potential hits were reviewed it became apparent that many of identified compounds contain a maleimide core and likely to react with cysteine. The target NC peptide used in our screen contains a cysteine residue in the critical carboxyl-terminal position, and it is possible that maleimides reacted with this cysteine in our experiments. Moreover, cysteine residues present within the GST-M15 sequence may also be subject to covalent modification by maleimides. To test these possibilities, we repeated HTRF experiments with bio-G10-NC and GST-Mint-PDZ in the presence of 1 mM DTT. We reasoned that maleimides are less likely to react with cysteine in the reduced environment provided by 1 mM DTT. These 52 compounds were tested at 1.5 μM and 5 μM concentrations (for “high-affinity” hits, Fig. 10A) or at 5 μM and 15 μM concentrations (for “low-affinity” hits, Fig. 10B).

We discovered that the presence of 1 mM DTT rendered 43 compounds inactive in our HTRF assay, indicating that these compounds most likely act as “cysteine-modifiers” (Fig. 10). Although FH07, F53, FH62, and FH70 showed some inhibitory effect at 15 μM concentration in the HTRF assay performed in 1 mM DTT, they also showed nearly the same effect in the bio-GST control assay when tested at the same concentration. Only five compounds (FH01, FH11, FH40, FH69, and FH80) remained active in 1 mM DTT (Fig. 10), suggesting a potentially competitive mechanism of action.

We further reasoned that compounds that truly disrupt association of Mint-PDZ with bio-NC peptide should be effective in both HTRF and ALPHA assay formats. However, when five potential hits were tested for their ability to disrupt Mint-PDZ association with bio-PEG-NC in ALPHA assay, only two compounds (FH01, FH11, FH40, FH69, and FH80) remained active in 1 mM DTT (Fig. 10), suggesting a potentially competitive mechanism of action.

![Graph](image.png)

**FIG. 9.** Characterization of potential hits from the 100,000 compound HTRF screen: first screen. Normalized HTRF signals are shown for bio-NC/GST-M15 (solid bars) and bio-GST (open bars) for the 52 compounds isolated as putative “hits” in the 100,000 compound screen. The HTRF signals for each compound are normalized to signals obtained with 1% DMSO control (Con) and shown as mean ± SE values (n = 3). Each compound was tested at 5 μM concentration.
(data not shown), suggesting that activity was likely to be due to a low-level impurity in the FH11 sample. Moreover, we could not detect activity in the sample of FH11 that we had independently synthesized by the Chemistry Core Laboratory. Upon closer inspection it was determined that FH11 from the vendor was contaminated with minor amounts of sodium hydroxide that induced a retro-Michael reaction when dissolved in DMSO. This newly formed \textit{in situ} intermediate derived from FH11 (an $\alpha,\beta$-unsaturated ketone) we believe was responsible for the
observed activity (cysteine modification) analogous to the maleimide series discussed previously. In contrast to FH11, the FH69 compound purified by recrystallization retained its activity in both HTRF and ALPHA assays (data not shown). From analysis of the structure (Fig. 12A) and by collecting absorption and emission spectra (Supplementary Fig. S1; see online figure at www.liebertpub.com/adt) we confirmed that FH69 is not an autofluorescent compound. Furthermore, when FH69 compound and a number of FH69 analogs were synthesized by the Chemistry Core Laboratory, they retained activity in both HTRF and ALPHA assays (data not shown). In order to determine if FH69 is a true “hit” we analyzed association between FH69 and the Mint-PDZ domain by isothermal titration calorimetry assay.35 However, we could not detect strong association between FH69 and Mint-PDZ in this assay (data not shown). Moreover, we could not detect FH69 association with the Mint-PDZ domain in NMR experiments performed in collaboration with Dr. Mingjie Zhang (Hong Kong University of Science and Technology).26 Thus, we began to suspect that FH69 may act as a “promiscuous binder” and that the inhibitory effect of FH69 may be caused by the aggregation of this molecule in the detergent-free HTRF buffer used in our experiments. To test this hypothesis, we repeated HTRF experiments with FH69 in the presence of 0.01% Triton X-100. As previously reported,36 most “promiscuous binders” lose their activity in the presence of Triton X-100. The irrelevant compound Ran1 (Fig. 12A) was used as a negative control in these experiments. The G10-NC peptide served as a positive control. Consistent with previous results (Fig. 10B), addition of FH69 resulted in dose-dependent reduction of HTRF signal in the absence of Triton X-100 (Fig. 12B). Addition of G10-NC peptide caused a similar reduction in HTRF signal, whereas addition of Ran1 had no significant effect on HTRF signal (Fig. 12B). When the same experiments were repeated in the presence of 0.01% Triton X-100, we discovered that FH69 was no longer able to decrease the HTRF signal (Fig. 12B). The small remaining effect of FH69 on the HTRF signal was identical to the effect resulting from addition of Ran1 (Fig. 12B). In contrast, the ability of G10-NC peptide to reduce HTRF signal was not affected by addition of 0.01% Triton X-100 to the binding buffer (Fig. 12B). From these results we concluded that FH69 most likely acts as a “promiscuous binder” and not as a specific inhibitor of the Mint-PDZ domain.

To further evaluate the ability of FH69 to disrupt Mint1 association with NC peptide, we performed a series of biochemical in vitro binding experiments. These experiments have been performed by following procedures that we established in our previous studies of Mint1 association with Ca2+ channels.8,25 In these experiments re-

**FIG. 12.** Effect of Triton X-100. (A) Chemical structures of FH69 and Ran1. (B) Different concentrations of FH69 (circles), Ran1 (diamonds), and G10-NC (squares) were tested in HTRF assay in the standard buffer (open symbols) and in the presence of 0.01% Triton X-100 (filled symbols). The normalized HTRF signals at each concentration are shown as mean ± SE values (n = 3).
combinant HA-Mint1 protein was incubated with streptavidin agarose beads covered with bio-NC peptide. The HA-Mint1 protein associated with the beads was detected by western blotting with anti-HA monoclonal antibodies. Consistent with the earlier findings,25 we found that HA-Mint1 protein was efficiently precipitated by the agarose beads covered with bio-NC peptide, but not by the beads covered with control bio-NC-W2338A peptide (Fig. 13). Addition of 10 μM or 100 μM FH69 to the binding reaction had no significant effect on amount of HA-Mint1 precipitated by bio-NC beads (Fig. 13). Addition of 10 μM G10-NC also had no significant effect, but addition of 100 μM G10-NC prevented HA-Mint1 from associating with bio-NC beads (Fig. 13). These results further confirmed that FH69 is a “promiscuous binder” that cannot disrupt association between Mint1 protein and NC peptide in biochemical binding experiments.

Discussion

PDZ domains play important role in cellular signaling pathways. PDZ domain-mediated interactions produce a highly organized transduction complex that is necessary for proper signaling in vivo.37 PDZ domains have also recently emerged as central organizers of protein complexes at the plasma membrane.38 There are over 10,000 PDZ domains present in almost 7,000 proteins in the nonredundant genome sequence database. Thus, PDZ domains are very abundant and important drug targets.39 Although there are some published lower-affinity inhibitors (Kᵰ >100 μM) for a few PDZ domains based on NMR screening,40–42 developing additional and higher-affinity inhibitors is urgent for this important target. Here we described the development of two different HTS assays for small molecule inhibitors of Mint-PDZ domain association with NC peptide.

The performance of HTRF and ALPHA assays was compared side by side in our HTS experiments. Both assays were independently optimized, and the optimal conditions are summarized in Table 1. In the previous studies HTRF and ALPHA assays were used to test for inhibitors of FXR nuclear receptors association with the co-activator-derived peptide SRC1.28–30 When performed in 384-well format, the HTRF screen with FXR receptor resulted in S/B = 22, CV = 2.1%, and Z’ = 0.929 compared to S/B = 5, CV = 2.8%, and Z’ = 0.8 in our experiments (Fig. 2 and Table 1). The ALPHA screen with FXR receptors resulted in S/B = 350, CV = 5.2%, and Z’ = 0.8 compared to S/B = 5, CV = 5.5%, and Z’ = 0.8 in our experiments (Fig. 5B and Table 1). Thus, for both HTRF and ALPHA assays the FXR receptor screen resulted in higher S/B than the Mint-PDZ screen did. Nevertheless, Z’ factors for both HTRF and ALPHA assays are in the excellent range for Mint-PDZ assay.

Similar to FXR studies,28–30 we concluded that each assay had its advantages and disadvantages. The advantages of ALPHA are: (1) the S/B of 25–28 is five times higher than the HTRF’s S/B of 4–5; (2) autofluorescent compounds are not a big problem due to the extra long read times in ALPHA; and (3) reading time for the 384-well plate is 3.5 min, which is half of the reading time for HTRF (6.5 min). The advantages of HTRF are: (1) CV is only 2.8%, which is half of ALPHA’s CV (5.2%); (2) it is easier to optimize and not affected by steric effects; and (3) HTRF can be performed at normal light conditions. The main advantage of HTRF when compared to ALPHA is a lesser variability (CV) and better Z factors (Figs. 3C and 6C).34 Similar to FXR receptor studies,29 we found that ALPHA resulted in a higher S/B but more variability than HTRF did in the Mint-PDZ assay.

In conclusion, here we described HTS for small molecule inhibitors of Mint-PDZ domain association with NC peptide.

FIG. 13. In vitro binding assay. The recombinant HA-Mint1 protein was precipitated by streptavidin agarose beads covered with bio-NC peptide in the presence of FH69 or G10-NC peptide as indicated. The amount of precipitated HA-Mint1 protein was quantified by western blotting with anti-HA monoclonal antibodies. Beads covered with bio-NC-W2338A peptide were used as a control for specificity of the binding interaction.
to be an inhibitor of association between NC peptide and the Mint-PDZ domain (Figs. 10 and 11 and data not shown). However, we have not been able to confirm association between FH69 and Mint-PDZ by biophysical methods (isothermal titration calorimetry and NMR). Moreover, FH69 lost its ability to reduce the HTRF signal in the presence of 0.01% Triton X-100 (Fig. 12). Thus, we concluded that FH69 most likely acts as a “promiscuous binder”36 and not as a specific inhibitor of the Mint-PDZ domain. Thus, additional HTS will be required to identify true inhibitors of the Mint-PDZ domain. To minimize artefacts resulting from cysteine-reactive compounds and “promiscuous binder,” the HTS should be performed in the presence of 1 mM DTT and 0.01% Triton X-100 as described in our validation experiments. The described screens can be easily adapted to other PDZ domain targets.

Acknowledgments

The authors would like to thank Steve McKnight and Michael Roth for providing access to the HTS Laboratory and the generous gift of the test compound library. We are also thankful to Weihua “Wayne” Hao and Olga R. Jeter for helping develop the HTS assay and to Jeff Pfohl (Perkin Elmer) for helping with developing the ALPHAg assay and for comments on the manuscript. We also express our appreciation to the people in I.B.’s lab for technical help. This work was supported by National Institutes of Health grant R03NS050848 and U.S. Army Medical Research grant W81XWH0710278 (to I.B.). The HTS Laboratory and Chemistry Core laboratory (D.E.F.) are supported by P01 CA95471 (to S.M.), and some experiments were conducted in a facility constructed with support from Research Facilities Improvement Program grant C06 RR-15437.

References

24. Zhang SP, Kauffman J, Yagel SK, Codd EE: High-throughput screening for N-type calcium channel blockers using a


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FIG. S-1. Fluorescence spectrum of FH69 (dashed line). (A) Emission spectrum 330–700 nm) of FH69 (10 µM) in DMSO excited at 320 nm. (B) Absorption spectrum (300–690 nm) of FH69 (10 µM) in DMSO with emission at 700 nm. For comparison, the spectra of DMSO (solid light gray line) and an autofluorescent compound tested at 10 µM in DMSO (solid black line) are shown.