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Development of a polyvalent assay system for lead identification

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Abstract—In an effort to identify new approaches to lead discovery a polyvalent assay was developed to allow identification of weak inhibitors. This approach involves the polyvalent display of a protein binder off a Tenta-gel® scaffold and the generation of a polyvalent display of protein by biotinylation followed by complexation with fluorescently labeled streptavidin. Subsequent exposure of the streptavidin complexed protein to Tenta-gel® beads with active protein binders results in fluorescent beads, which are easily viewed under a fluorescent microscope.

Lipinski's Rule of 5 suggests that compounds with larger molecular weights and lipophilicity are not ideal starting points for drug design as compounds with these physical properties have reduced chances of good oral bioavailability.[1,2] More recently Veber et al. illustrated the deleterious effect that increased conformational flexibility, as measured by the number of rotational bonds, as well as increased polar surface area can have on oral bioavailability.[3] As leads with properties of larger molecules will have correspondingly larger parameters (e.g., MW, Log P, # rotational bonds, polar surface area), they do not leave the investigator much room to try to optimize other important drug like properties, such as potency, specificity and permeability. In response to these concerns, a number of reports have discussed what types of molecules are appropriate for leads and the odds of obtaining lead like compounds with appropriate affinities for their targets.[4–6] Recently there has been a movement toward identifying low molecular weight compounds with correspondingly lower affinity, and then building in the desired drug-like properties from these 'well behaved' lead like molecules.[7–10]

A concern with identifying low molecular weight compounds is the lower affinity (100 μM–1 mM) of these compounds. Identification of low affinity compounds can be problematic due to the need to screen for these at correspondingly higher concentrations. At these higher concentrations solubility and aggregation can become issues. DMSO is often added to help dissolve the compounds, but many proteins do not respond favorably to higher concentrations of DMSO (5–10%). We wish to report an approach to lead identification that allows one to circumvent these problems by multiplying the binding events. If one investigates the potential ligands in a polyvalent manner and thus multiplies the interactions, then one would have an interaction worth up to $n \times \Delta G$ or more[11] where n is the number of simultaneous interactions and ΔG is the energy of the interaction. This would allow a single interaction of −4 kcal (millimolar) to become −8 kcal (micromolar) for two simultaneous interactions and up to −12 kcal (nanomolar) for four simultaneous interactions.[12]

The last decade has seen expanding interest in polyvalent interactions due to their pivotal role in cell adhesion,[13,14] cholera toxin and associated AB_5 toxins[15–17] and viral infection.[18–20] Most studies have centered on how to utilize multivalent interactions to mimic nature's ability to obtain high affinity interactions in an effort to identify analogs of known multivalent ligands. Scaffolds used to display the ligand in a polyvalent manner include polymers,[21–23] dendrimers[24–27] as well as liposomes.[17,19,20] There have also been reports of structure-based efforts wherein the exact number of complementary ligands were tethered in an appropriate framework.[15,16] Herein we report our progress in the development of a polyvalent assay to identify low affinity compounds.

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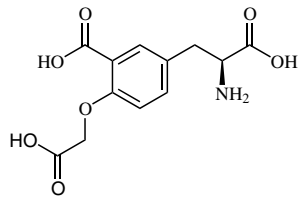
Our target protein was Protein Tyrosine Phosphatase-1B (PTP1B), a phosphatase that plays a crucial role in insulin sensitivity by the dephosphoralation of the insulin receptor.[28] The relationship between obesity, type 2 diabetes and insulin resistance suggests that PTP1B would be an ideal target for the treatment of these indications.[29] The peptide inhibitors were based on the phosphotyrosine mimetic **X** (Fig. 1).[30] We chose to display multiple copies of the small molecules by synthesizing them on solid phase using Tenta-gel® resin, which has been shown to be compatible with on-resin assays (see Fig. 2).[31,32] This approach not only allows a multivalent presentation of the ligand, but should also circumvent solubility and aggregation, issues associated with trying to identify weak inhibitors by screening at higher concentrations. With the wealth of information in solid phase organic synthesis a large variety of scaffolds with various functional groups displayed should be accessible.[33,34] We chose to use streptavidin as the scaffold from which to display the protein in a polyvalent manner. Streptavidin is an ideal scaffold as it has four binding sites that strongly bind biotin. Biotinylation of the protein, followed by complex formation with fluorescently labeled (Texas Red®) streptavidin affords the fluorescently labeled protein complex.



X

Figure 1. Phosphotyrosine mimetic **X** used in PTP1B binding peptides.

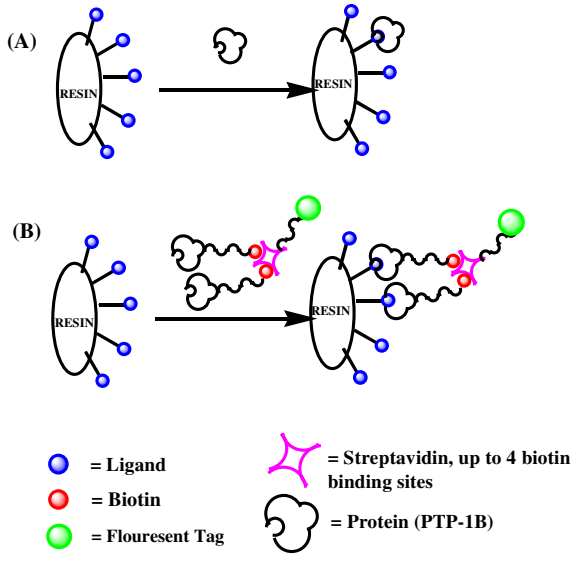


Figure 2. (A) On-bead assay wherein the polyvalent ligand is exposed to the monovalent protein. (B) On-bead assay wherein polyvalent ligand is exposed to polyvalent protein. While illustrated as bivalent for simplicity, note that streptavidin can cross link up to four proteins simultaneously by binding four biotins.

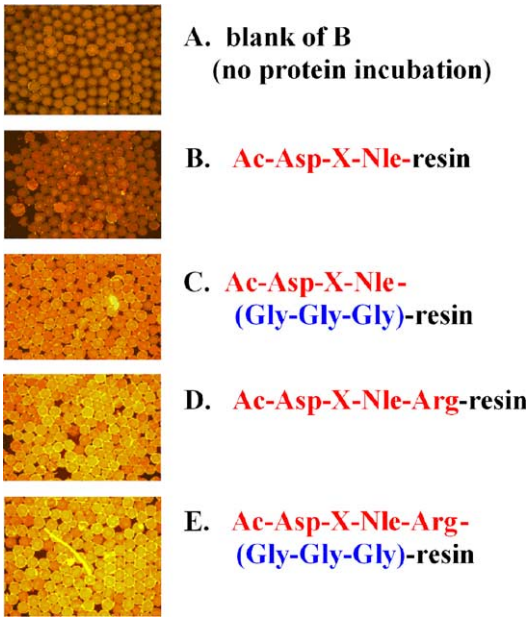


Figure 3. Texas Red-labeled Streptavidin precomplexed with Biotinylated PTP1B and exposed to Tenta-gel® bound peptides visualized by fluorescence microscopy.

Exposure of the resin bound ligand to the protein complex followed by filtration and washing gave the PTP1B exposed beads, which were visualized by fluorescence microscopy (Fig. 3).[35,36] The PTP1B/streptavidin complexed beads were quite fluorescent indicating protein binding to the beads. To confirm that the fluorescently labeled streptavidin is not binding to the beads directly we exposed the Texas Red® labeled streptavidin to the beads using the same protocol and observed no fluorescence activity (data not shown). While the protein requires a spacer (Gly-Gly-Gly) for the shorter peptide (**C** vs **B**) in order to bind to the resin-bound ligand, the spacer is not required for the longer tetramer. The compounds were also prepared in the free soluble form to confirm their PTP1B activity, as well as to determine what role, if any, the spacer plays in ligand binding to protein. As illustrated in Table 1, the trimer **B** is 41 μM compared with 11 μM for the tetramer **D**. As the addition of the Gly-Gly-Gly spacer to the tetramer (**E**) slightly reduces the affinity (18 μM) it appears that it does act solely as a spacer in the on-resin assay and does not participate in any adventitious binding.

Herein we have demonstrated an approach to lead identification with a novel polyvalent assay. The goal of this approach is to increase the signal of moderate inhibitors significantly, allowing them to become detectable. Other advantages to this approach include the lack of solubility and aggregation issues. Future efforts will focus on

Table 1. K_i values of isolated peptides against PTP1B[37,38]

B. N-acetyl-Asp-X-Nle-NH_2	41 μM
C. N-acetyl-Asp-X-Nle-(Gly-Gly-Gly)-NH_2	ND
D. N-acetyl-Asp-X-Nle-Arg-NH	11 μM
E. N-acetyl-Asp-X-Nle-Arg-(Gly-Gly-Gly)-NH_2	18 μM

identifying weak binders (~1 millimolar) and demonstrating their signal amplification.

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12. Note: Recall the exponential relationship between ΔG, K and K_i. For $R = 1.99 \times 10^{-3}$ kcal/mol K, $T = 297\,^{\circ}C$, $\Delta G = -RT\ln K = -1.3 \log K$, $K = 10^{-\Delta G/1.36}$ kcal/mol $K_i = 1/K$.
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35. Assay conditions: 75 μL of Texas Red-labeled streptavidin ($c = 1$ mg/mL) was premixed with 150 μL biotinylated-PTP1B ($c = 0.82$ mg/mL) and allowed to form the complex for one hour at 4 °C, then the complex was diluted to a final PTP1B concentration of 0.01 mg/mL for incubation with the resin-bound peptides. The resin-bound peptides were incubated with the complex for one hour at rt, filtered, washed with Casein blocker in TBS, taken up in 0.1% Tween in TBS buffer and transferred to a microtiter plate for visualization. Resin-bound peptides binding to fluorescently tagged protein were visualized with an Olympus 1X51 Inverted fluorescent microscope with a Texas Red® filter and 100 W mercury bulb. The images were captured with a Q Imaging QICAM monochrom and color high performance digital CCD camera.
36. We tried to quantitate the binding using a fluorescent plate reader (Tecan, Saphire (Austria)). However due to heterogeneric nature of the beads the reader could not accurately measure the fluorescence.
37. Experimental: All compounds were prepared by standard solid phase synthesis on a Argonaut Quest-210 using 1-hydroxybenzotriazole (HOBT) and 1,3-diisopropylcarbodiimide (DIC) as the coupling agents. Tenta-Gel® S-NH2 was used for solid phase peptides that would be used for on-bead assays. Tenta-Gel® S-RAM resin was used for peptides B–D that would be cleaved from the resin. All isolated peptides were characterized by ^{1}H NMR and mass spectrometry.
38. PTP1b_Ki: The enzymatic assay was carried out at room temperature in 96-well plates. The buffer used was 50 mM 3,3-dimethylglutarate, pH 7.0, containing 1 mM EDAT, 10 mM DTT, 0.1% TRITON-100, 5% DMSO with an ion strength of 0.15 M adjusted with NaCl. The initial rate of PTP1B-catalyzed hydrolysis of *p*-nitrophenol phosphate (pNPP) was measured by following its optical density change at 405 nm IC 50 value was determined under fixed pNPP concentration of 1 mM. All the assays were carried out in triplicate and the average results are presented. K_i is derived from IC50 based on competitive inhibition $K_i = IC50\,K_m/(K_m + [substrate])$.