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Discovery of selective aldo-keto reductase ligands—an on-bead assay strategy

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Abstract—An enzyme labeling and screening strategy for the discovery of ligands selective in binding two structurally similar members of the aldo-keto reductase family of enzymes is reported. The resulting fluorescence microscope data obtained by screening a 74,088 member library led to the identification of selective ligands for aldose reductase (ALR2) and aldehyde reductase (ALR1). Resynthesis results validate the selectivity of these ligands.

Selective binding of proteins by synthetic receptors provides insights useful in a wide range of studies, including protein–protein interaction dynamics,[1] noncovalent ligand–substrate interactions,[2] development of diagnostic sensors,[3] selective protein sensing,[4] discovery of therapeutics,[5] and, when the synthetic receptor is immobilized on a solid support, protein separation, and isolation.[6] With these studies in mind, small molecules capable of differentiating the 13 known human members of the aldo-keto reductase (AKR) family[7] would constitute a potentially useful tool. Aldose reductase (ALR2; systematic name AKR1B1) is a 36 kDa enzyme that catalyzes the NADPH-dependent reduction of glucose to sorbitol. Although the biochemical role of ALR2 is not completely understood, it is believed that excessive flux of glucose through ALR2, a clinically relevant AKR, leads to diabetic complications such as neuropathy and nephropathy.[8] Thus, finding compounds that selectively engage ALR2 may, ultimately, lead to strategies which reduce diabetic complications.[9,10] While inhibitors of aldose reductase have previously been identified, attempts to selectively inhibit ALR2 without hindering the functions of other members of the aldo-keto reductase family—such as ALR1—have been less successful.[11] Since ALR1 and ALR2 (Fig. 1) are structurally similar (46% identical by sequence) and yet have distinct biological functions, we set out to develop an on-bead binding assay capable of differentiating these two aldo-keto reductases.

To realize this objective, three components were necessary: a library of potential ligands, dye-labeled ALR1

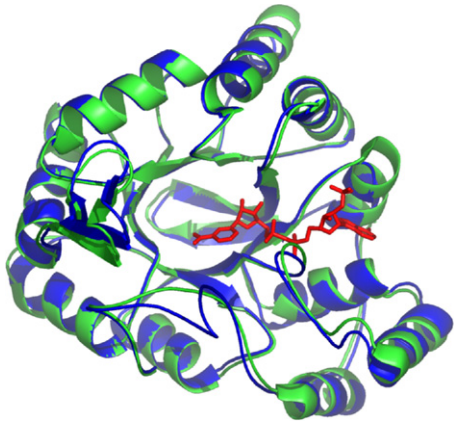

Figure 1. Alpha carbon traces of human aldose reductase (green) superimposed on porcine aldehyde reductase (blue) with NADP bound (red).

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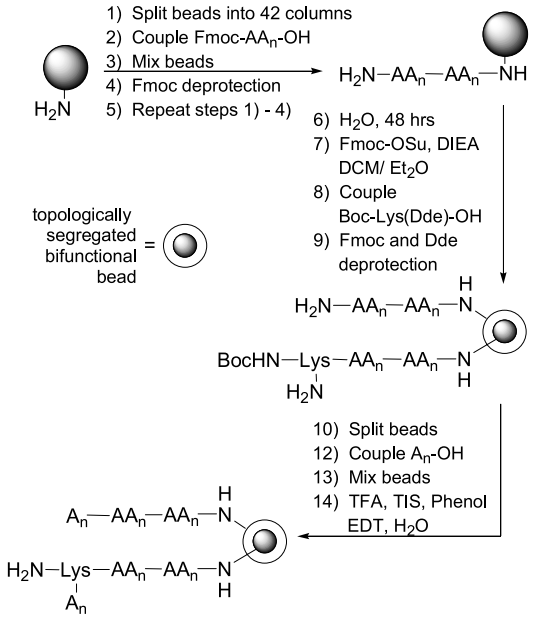
and ALR2 enzymes, and a working assay. Although several methods exist for the identification of inhibitors with high-throughput screening,[12,13] nonfluorescent methods tend to be complicated and tedious in their applications. For our assay, we elected to employ a fluorescent method due to the relative ease of bead selection with a fluorescence microscope. Although fluorescent labeling of enzymes is well established, their application in selective ligand identification among structurally similar enzymes has not, to the best of our knowledge, been reported.

To begin this work, a demonstration peptidomimetic library was synthesized on TentaGel® resin as outlined in Scheme 1. Standard methods for solid-phase peptide synthesis were employed for the introduction of the first two amino acid diversity elements (see Fig. 2). The resin beads were topologically segregated into bifunctional beads where the outer layer contains the testing peptidomimetic and the inner sphere contains an Edman degradation-ready coding tag which allows for sequencing after the isolation of identified beads.[14] In order to code for carboxylic acid diversity (step 12 in Scheme 1), an orthogonally protected lysine (Nα-Boc/Nε-Dde) was introduced to the inner tagging sphere of the beads.[15]

The Nε-Dde protecting group of the tagging lysine was removed and the carboxylic acid diversity elements (see Fig. 3) were introduced by *N*-acylation. Finally, all remaining protecting groups were cleaved by TFA treatment to deliver the demonstration library.

With the library in hand, we turned our attention to fluorescent labeling of the target protein. Employing standard protocols, Human ALR1 and ALR2 were over



Scheme 1. Synthesis scheme for the preparation of a 74,088 member peptidomimetic library.

expressed in *Escherichia coli*, [11] isolated, and purified by the IMPACT fusion system,[16] and found to match literature activity values.[17] In order to differentiate the two proteins, Alexa Fluor 488 and the Texas Red dyes, each having unique fluorescence properties, were selected for protein conjugation to surface lysine residues. Each protein was independently incubated in triethanolamine buffer with NADPH, substrate (DL-glyceraldehyde for ALR2 and 4-nitrobenzaldehyde for ALR1), and 20 equiv of the respective activated succidimidyl ester dye (ALR1 + Texas Red, ALR2 + Alexa Fluor 488) for 1 h.

The resulting conjugates were purified by size exclusion chromatography using a G-50 sephadex column to remove the excess dye. The pure, labeled proteins were analyzed by UV spectroscopy in order to determine the extent of labeling by relative concentrations of dye and protein (Fig. 4).[18] Kinetic studies both prior to and after the labeling process showed k_{cat}/K_m values were virtually unchanged (<10%).

With the model library and labeled proteins in hand, we turned our attention to assay development. The nonselective hydrophobic sites on the beads were 'preblocked' by incubation with TEA buffer containing tween, sodium azide, and gelatin.[19] After incubation for 1 h, the pre-blocking solution was drained and TEA buffer containing the labeled proteins with a final total concentration of 3.6×10^{-5} mM was added. After incubation for 1 h, the resulting mixture was thoroughly washed to remove unbound protein and immediately examined under the fluorescence microscope. Under FITC Green and Texas Red broad-band filter cubes, beads that bound ALR1 fluoresced red while beads that bound ALR2 fluoresced green (see Fig. 5). Beads that bound both ALR1 and ALR2 fluoresced both red and green. In the event, microscopic examination allowed for the identification of each type of binding ligand. The resulting picked beads (one each of red, green, and red/green) were then washed with 8 M guanidine to remove the bound protein and decoded by Edman degradation with an ABI automatic microsequencer.[15] The identified ligands (Fig. 6A) were then resynthesized on TentaGel resin in order to test, by reincubation, the validity of the assay: indeed, peptidomimetic **1** selectively binds ALR2, peptidomimetic **2** selectively binds ALR1, and peptidomimetic **3** binds both ALR2 and ALR1.

Each re-synthesized resin-bound peptidomimetic was separately subjected to the initial assay conditions and visualized under the fluorescence microscope in both the green and the red regions. Figure 6B illustrates the selective binding characteristics of each of the peptidomimetics when incubated with the ALR1 and ALR2 protein mixture and then extensively washed to remove unbound protein. The results of this experiment show that these three resin-bound peptidomimetics produce no fluorescence in the absence of labeled protein (pictures #1–#3). However, incubating these peptidomimetics with labeled proteins followed by extensive washing led to selective binding such that peptidomimetic **1** binds

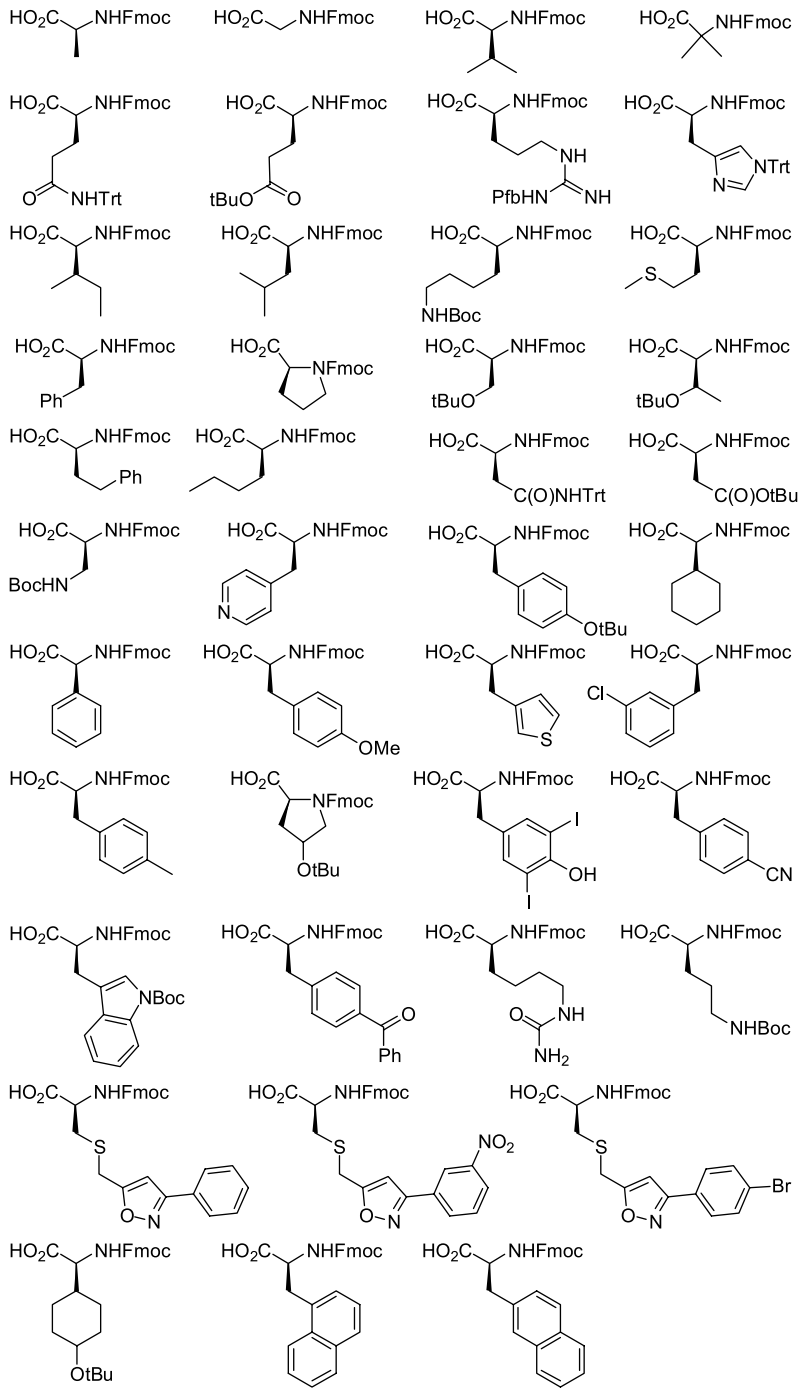


Figure 2. Amino acids employed in Scheme 1, step 2.

ALR1 and fluoresces only in the red region (picture #10 vs picture #4) while peptidomimetic **2** binds ALR2 and fluoresces only in the green region (picture #5 vs picture #11). In contrast, peptidomimetic **3** binds both ALR1 and ALR2, which leads to both green (picture #6) and red (picture #12) fluorescence.

Solution phase kinetic studies were performed to determine the potential inhibitory effects of resynthesized peptidomimetics **1**–**3**. However, the insolubility of these compounds under inhibition study conditions (TEA buffer) proved problematic. In future studies, solubilizing linkers will be developed for these compounds to improve their water solubility.

In conclusion, we report a strategy for the identification of peptidomimetic ligands capable of selectively binding the ALR1 and ALR2 enzymes—two structurally similar members of the aldo-keto reductase family. These methods should be useful for the identification of synthetic receptors for the study of proteins in a wide range of applications.

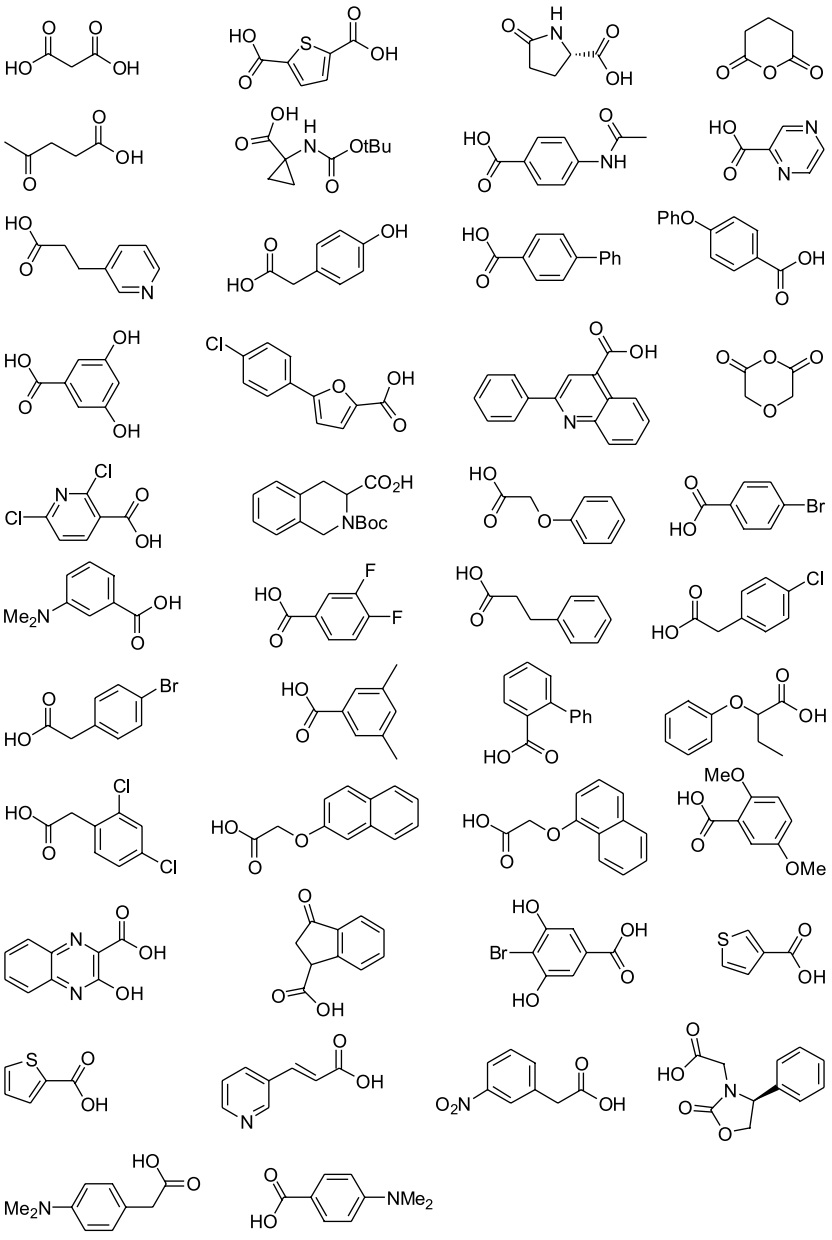


Figure 3. Carboxylic acids employed in Scheme 1, step 12.

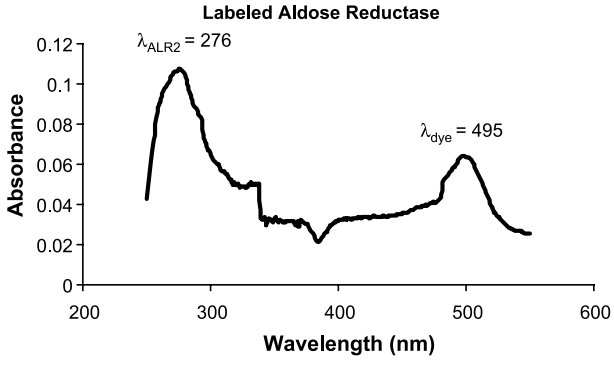


Figure 4. UV spectrum of ALR2–Alexa Fluor 488 conjugate.

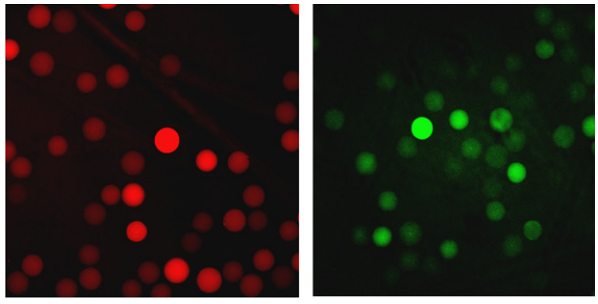


Figure 5. Red (ALR1) and green (ALR2) fluorescent beads in the 74,088 member peptidomimetic library.

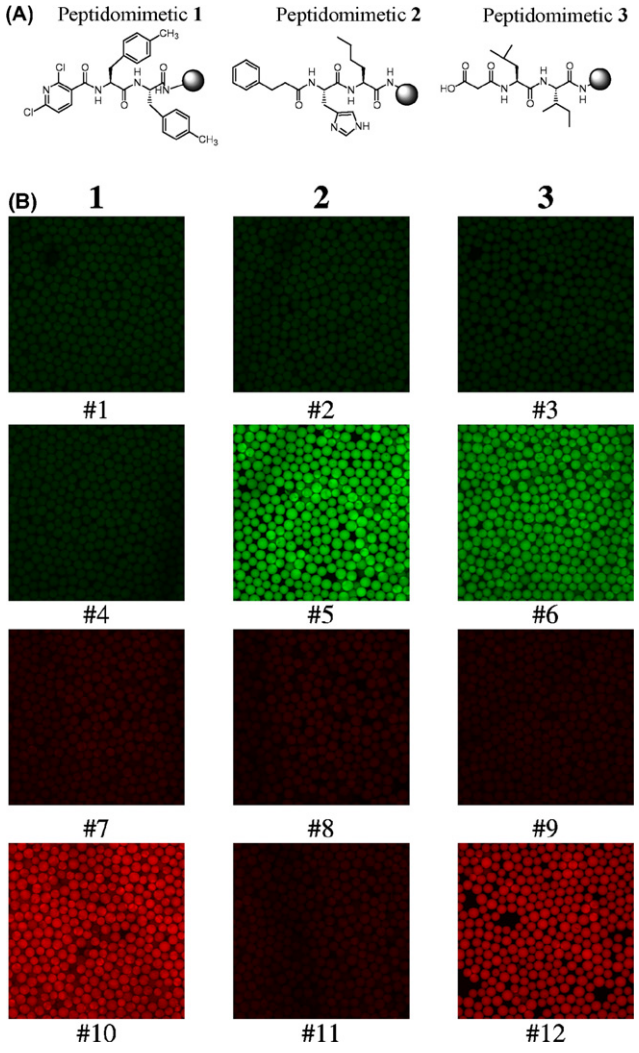


Figure 6. (A) Peptidomimetics **1**–**3**. (B) Digital images of the ALR1-(red)/ALR2(green)-bound beads where rows 1 and 3 are negative controls (no labeled protein incubation) under both green (row 1) and red (row 3) fluorescent light; row 2 (ALR1/ALR2 incubation followed by washing; under green light) and row 4 (ALR1/ALR2 incubation followed by washing; under red light) depict these single peptidomimetic beads subjected to our on-bead assay.

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