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On-bead combinatorial techniques for the identification of selective aldose reductase inhibitors

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Abstract—Aldose reductase (AKR1B1; ALR2; E.C. 1.1.1.21) is an NADPH-dependent carbonyl reductase which has long been associated with complications resulting from the elevated blood glucose often found in diabetics. The development of effective inhibitors has been plagued by lack of specificity which has led to side effects in clinical trials. To address this problem, a library of bead-immobilized compounds was screened against fluorescently labeled aldose reductase in the presence of fluorescently labeled aldehyde reductase, a non-target enzyme, to identify compounds which were aldose reductase specific. Picked beads were decoded via novel bifunctional bead mass spec-based techniques and kinetic analysis of the ten inhibitors which were identified using this protocol yielded IC_{50} values in the micromolar range. Most importantly, all of these compounds showed a preference for aldose reductase with selectivities as high as ~7500-fold. The most potent of these exhibited uncompetitive inhibition versus the carbonyl-containing substrate D/L-glyceraldehyde with a K_i of 1.16 μM.

1. Introduction

At normal physiological glucose concentrations, the majority of metabolic flux is directed through the glycolic pathway.[1] However, under the hyperglycemic conditions frequently found in diabetics, a significant portion is directed through the polyol pathway where aldose reductase (ALR2) is responsible for the NADPH-dependent reduction of glucose to sorbitol.[2] The subsequent re-oxidation of sorbitol to fructose by the NAD^+-dependent enzyme sorbitol dehydrogenase (SDH) is the rate-limiting step in this pathway leading to an accumulation of sorbitol in many cell types capable of insulin-independent glucose uptake[3] (Scheme 1a).

Either due to osmotic effects arising from the accumulated sorbitol or redox imbalances resulting from the different co-substrate specificities present in ALR2 and SDH, many different complications are associated with diabetic hyperglycemia.[4] Clinically, these are found to affect a wide range of tissues including nerve, kidney, retina, and eye lens among others.[3a,5] Although the physiological role of ALR2 remains unclear, strong in vivo evidence exists to associate this enzyme with the development of secondary diabetic complications and that inhibition of ALR2 will be useful in treating these.[6] For decades, much effort has been focused on the inhibition of ALR2 which has resulted in many compounds reaching various phases of clinical trials.[7] Currently, however, no ALR2-targeted drugs are commercially available in the United States. A major reason for this may be the non-selective inhibition of other members of the aldo–keto reductase (AKR) superfamily, a common characteristic associated with many of these compounds.[8] Human aldehyde reductase (ALR1; AKR1A1) which is 46% identical in sequence to ALR2 has previously been used to study the non-specific effects of ALR2 inhibitors although at least eight other AKRs are also believed to have catalytic function in humans aside from ALR1 and ALR2.[9] Comparison of human ALR1 and ALR2 structures demonstrates their similarity in both overall fold and architecture at the active site (Fig. 1).[10]

Knowledge of the atomic structure of an enzyme often provides insight for the rational design of inhibitors.[11] In the case of ALR2 and ALR1, structure-based inhibitor design for selectivity is difficult due to the significantly similar architecture of the two enzymes. This is

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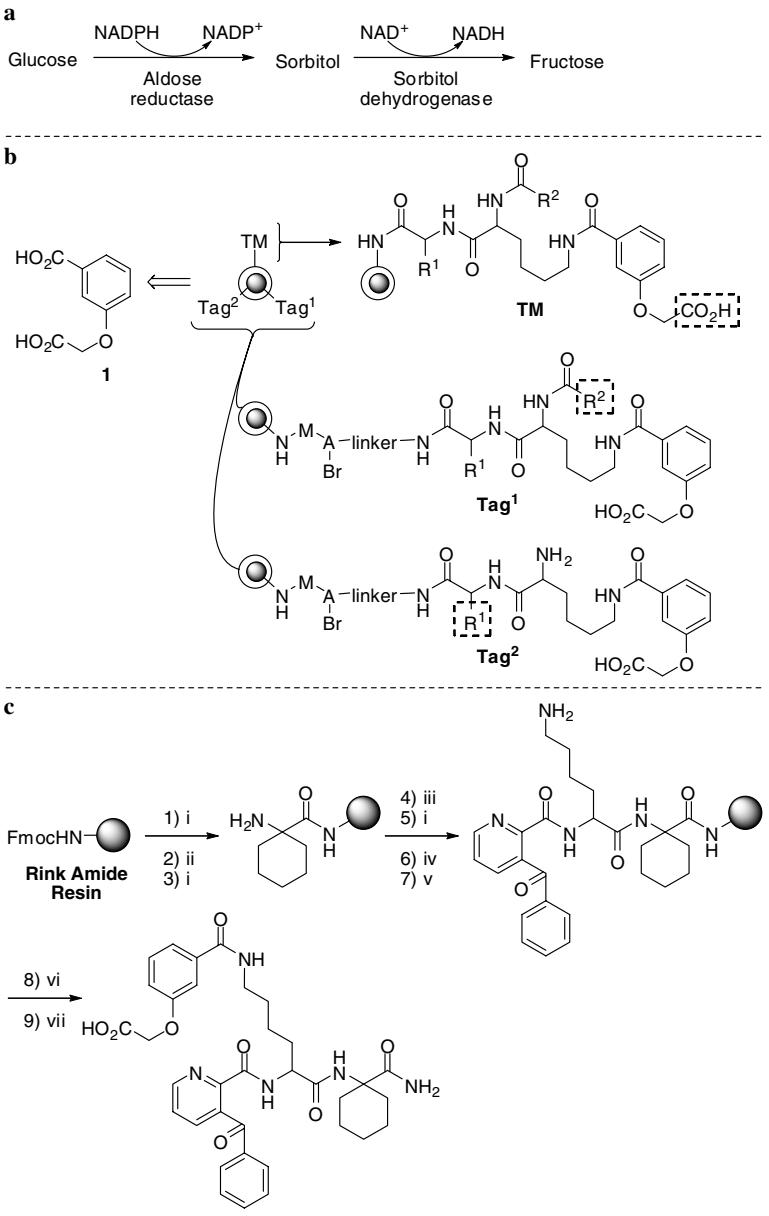

Scheme 1. (a) Reduction of glucose through the polyol pathway. (b) TentaGel bead with representative target molecule (TM), tag 1, and tag 2 illustrated. Reagents and conditions: (i) H_2O, 48 h; (ii) Alloc-OSu (0.1 equiv), DIEA, DCM/Et_2O (55:45, v:v), 30 min; (iii) Fmoc-Met-OH, HOBT, DIC; (iv) 20% piperidine in DMF, 2× 10 min; (v) *N*-Fmoc-3-(4-bromophenyl)-β-alanine, HOBt, DIC; (vi) Fmoc-Arg(Pmc)-OH, HOBt, DIC; (vii) *N*-Fmoc-2,2′-ethylenedioxy-bis(ethylamine)monosuccinamide, HOBt, DIC; (viii) $Pd(PPh_3)_4$ (0.24 equiv), $PhSiH_3$ (20 equiv), DCM; (ix) split beads into 42 columns; (x) library assembly (Fmoc-X_1-OH, HOBt, DIC); (xi) mix beads; (xii) Fmoc-Lys(Dde)-OH, HOBt, DIC; (xiii) H_2O, 24 h; (xiv) Fmoc-Osu (0.7 equiv), DIEA, DCM/Et_2O (55:45, v:v), 1 h; (xv) $(Boc)_2O$ (1 equiv), DIEA, DCM; (xvi) library assembly (X_2)-CO_2H, DIC, HOBt; (xvii) 2% hydrazine in DMF; (xviii) 3-(2-*tert*-butoxy-2-oxoethoxy)benzoic acid (**3a**), HOBt, DIC; (xix) TFA/phenol/TIS/H_2O/ethanedithiol (90:5:2:2:1, v/w/v/v/v). (c) Synthesis and reagents used for the resynthesis of ligand **9**. (i) 20% piperidine in DMF, 2× 10 min; (ii) Fmoc-1-amino-1-cyclohexanecarboxylic acid, HOBt, DIC; (iii) Fmoc-Lys(Dde)-OH, HOBt, DIC; (iv) 3-benzoyl-2-pyridinecarboxylic acid, HOBt, DIC; (v) 2% hydrazine in DMF; (vi) 3-(2-*tert*-butoxy-2-oxoethoxy)benzoic acid, HOBt, DIC; (vii) TFA/TIS/H2O (95:2.5:2.5, v/v/v).

further complicated by the fact that inhibitor binding has been shown to induce unpredictable conformational changes in the ALR2.[12] In consideration of these challenges presented by ALR2 and ALR1, we set out to identify compounds that selectively bind and inhibit ALR2.

Selectively targeting specific AKRs is not limited to ALR2 as the methods described here may be generally applied to the identification of other AKR targets. For example, by virtue of its ability to produce the proliferative compounds testosterone, 9α, and 11β-PGF2, prostaglandin F2 synthase (AKR1C3) may be a potential therapeutic target for malignancies of the prostate.[13] Other members of the AKR1A, B, and C families are responsible for activating various primarily steroidal ligands for nuclear receptors. Methods for the identification of selective inhibitors may be valuable for these enzymes as well.

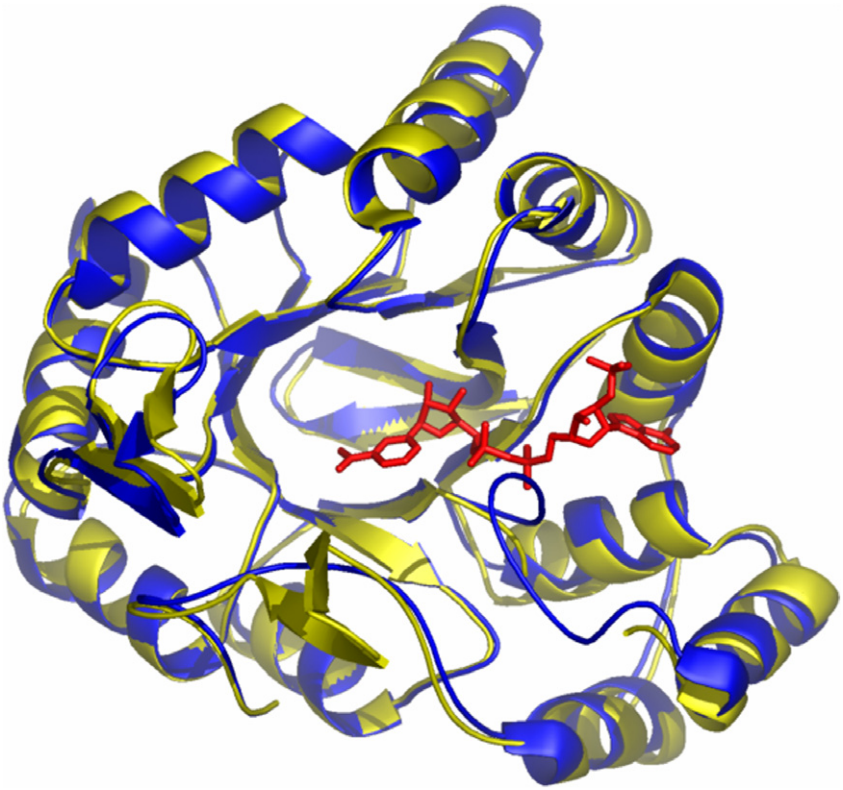

Figure 1. Human aldose reductase (blue) and human aldehyde reductase (yellow) with bound NADP (red) have similar folds but diverge somewhat in the substrate-binding site. This site is located on top of the nicotinamide ring (near the center of the β-barrel) which is formed by loops connecting the central β-strands with the external α-helices.

On-bead screening of one-bead one-compound combinatorial libraries has proven to be a powerful discovery tool[14] and the ability to screen large libraries and rapidly identify ligands makes one-bead one-compound libraries an attractive tool for our ALR2 studies. Herein, we describe techniques for the identification of ligands that selectively bind and inhibit ALR2 producing selective inhibition with micromolar IC_{50}s.

2. Results and discussion

2.1. Library synthesis

In previous work, we screened a 74,088 compound combinatorial library and identified ligands that selectively bound ALR2. However, attempts to study their inhibitory properties failed due to solubility problems.[15] To eliminate this drawback, we used a library that included constitutive components intended to enhance solubility. By virtue of an increased presence of polar and polarizable functional groups—principal among these was a carboxylic acid moiety (via **1**), their solubility in aqueous media should increase. In addition, a MALDI-TOF-based mass tag identification method was integrated into this library design to provide rapid and reliable hit identification. The library, synthesized following recently reported procedures,[16] is illustrated in Scheme 1b.

Briefly, bi-functionalization of TentaGel resin was accomplished by Alloc protecting the outer 10% of the free resin amines.[17] The free amines on the inner portion of the resin were then functionalized with Fmoc-protected methionine which subsequently served as the cleavable linker. After Fmoc deprotection, arginine followed by *N*-Fmoc-3-(4-bromophenyl)-β-alanine were coupled to the inner portion of the resin adding to the cleavable linker. The inner linker was completed by the addition of an Fmoc-protected ethylene glycol moiety. At this stage, removal of the Fmoc- and Alloc-protecting groups provided free amines on the inside and the outside of the resin, respectively. To these, the first diversity amino acid of the library was added—simultaneously to both the growing outer target molecule (TM) and the inner tag.

To create a tag for the second diversity element, the resin was bi-functionalized a second time and amine discrimination was accomplished by the addition of orthogonal protecting groups—Boc and Fmoc—on the inside and outside of the resin, respectively. Fmoc and Dde deprotections in DMF followed by acylation with the second diversity acid and **1** completed both the target molecule and the second tag. The final step, TFA cleavage, provided the novel one-bead one-compound library with the target molecule on the outer core and the encoding mass tags on the inner portions of the resin.

2.2. On-bead screening

With this library in hand, attention was focused on the on-bead screening of this combinatorial library using

differentially fluorescent-dye-labeled ALR2 and ALR1. AlexaFlor488-labeled ALR2 (green) and TexasRed-labeled ALR1 (red)[15] were incubated for 1 h with library beads that had been pre-blocked with buffer (pH ~7.4) containing gelatin, sodium azide, and Tween 20 (to prevent non-specific protein binding). The beads were then examined under a fluorescent microscope and individual beads were manually chosen. Ten beads that strongly fluoresced green (i.e., ALR2-bound) and not red (i.e., ALR1-bound) were collected as these indicated selective binding of ALR2. Following our protocols,[18] these beads were then stripped of protein by incubation with 8 M guanidine, cleaved with 0.25 M CNBr in 70% formic acid overnight (methionine → 2-amino-γ-butyrolactone), and decoded by MALDI-TOF to identify the target molecule (TM) presented by each bead.

Figure 2a shows a representative example of the mass spectrum obtained from a single bead. These data unambiguously identify ligand **10** (see Fig. 2b) as the target molecule built on this particular TentaGel bead (Fig. 2a). The nine unique ligands identified and decoded by this on-bead assay are depicted in Figure 2b. Interestingly, ligand **2** was identified twice. With these hits thus identified, the cleavable solid-phase synthesis of each ligand was undertaken. The targeted solid-phase synthesis procedure for ligand **9** is outlined in Scheme 1c. The remaining eight ligands were synthesized following similar protocols.

2.3. Inhibitor characterization

Briefly, Rink amide resin swollen in DMF was deprotected and coupled to Fmoc-1-amino-1-cyclohexanecarboxylic acid—the amino acid defined as the first diversity element—followed by Fmoc(Dde)-Lys-OH. Fmoc deprotection and 3-benzoyl-2-pyridinecarboxylic acid coupling followed by Dde deprotection and acid **1** coupling completed the solid-phase synthesis of ligand **9**. Cleavage from the resin under acidic conditions provided ligand **9** for kinetic studies.

After having thus prepared ligands **2**–**10**, inhibition assays were performed to distinguish whether they were simply ALR2-selective binders or whether they functioned as inhibitors. Initially, IC_{50} values were determined for each of the ligands in order to establish selectivity for ALR2 versus ALR1. The oxidation of NADPH was monitored continuously by a decrease in absorbance at 340 nm. Inhibitor concentrations for ALR2 ranged from a final concentration of 1 to 15 μM in the presence of saturating (100 μM) NADPH and K_m values of DL-glyceraldehyde (20 μM). Similar conditions for ALR1 were used with inhibitor concentrations between 1 and 15 μM. As shown in Table 1, seven out of the nine ligands were ALR2-selective inhibitors with three of these compounds showing exceptional selectivity: **2** with 256/1, **5** with 894/1, and **8** with 7495/1 in ALR2/ALR1 selectivity, respectively.

The identified ligands clearly show common elements with five of the nine (55%) containing the 3-benzoylpyridine-*s*-carboxamide moiety, two incorporating a cylcohexyl amino acid derivative, and two containing an isobutylene side chain. Interestingly, a cyclobutyl to isobutene exchange (ligands **8** and **10**) changes the selectivity for ALR2 by a factor of >300. This observation, coupled with the fact that ligand **8** exhibited the greatest selectivity, led us to probe this compound in further detail.

Complete inhibition analysis for ligand **8** is depicted in Figure 3. These data indicate uncompetitive inhibition versus both DL-glyceraldehyde and NADPH. Curve fitting of the uncompetitive inhibition equation indicates that the K_i is 1.6 μM versus DL-glyceraldehyde. In view of the fact that ALR2 in its apo form was used to screen the bead library, compounds that functioned as inhibitors were thought to most likely bind to the substrate or co-substrate-binding site. Although other ALR2 inhibitors exhibiting uncompetitive inhibition versus both NADPH and the carbonyl substrate—such as alrestatin and 7-hydroxy-4-oxo-4*H*-chromone-2-carboxylic acid—have been found,[19] it is difficult to reconcile the observed uncompetitive nature of the inhibitors versus both substrate and co-substrate. One possible explanation is derived from the kinetic mechanism of the enzyme which includes a necessary and relatively slow conformational change of a loop which fastens over the co-substrate upon formation of the ternary complex (Fig. 1). It is possible that this isomerization also takes place to some extent in the free enzyme and that it is this form which is recognized by compound **8**. In the course of assays, much of the enzyme is substrate-bound which promotes the conformational change. It is this form that can bind the inhibitor in a hypothetical quaternary complex. In order to further investigate the inhibitor binding, crystallographic studies are underway.

3. Conclusion

We have reported techniques that allow us to identify inhibitors via an on-bead screen which effectively eliminates beads (compounds) which are not selective for ALR2 by incubation of competing dye-labeled ALR2 and ALR1 with one-bead one-compound libraries. Conjugating small molecule dyes with distinct spectrophotometric properties to ALR2 and ALR1, respectively, enables this on-bead competition. The retention of >90% catalytic activity of both dye-labeled proteins ensures that these techniques are discriminating between active enzymes. Finally, coupled with the enhanced solubility of this library, we were able to establish that the identified ligands were, indeed, ALR2-selective inhibitors. The on-bead assay employed here has provided us with novel inhibitors with greater than 7000-fold selectivity for ALR2 over ALR1. With these methods now in hand, we are positioned to expand this novel selective assay procedure to include other members of the AKR family and to extend these techniques to on-bead small molecule libraries.

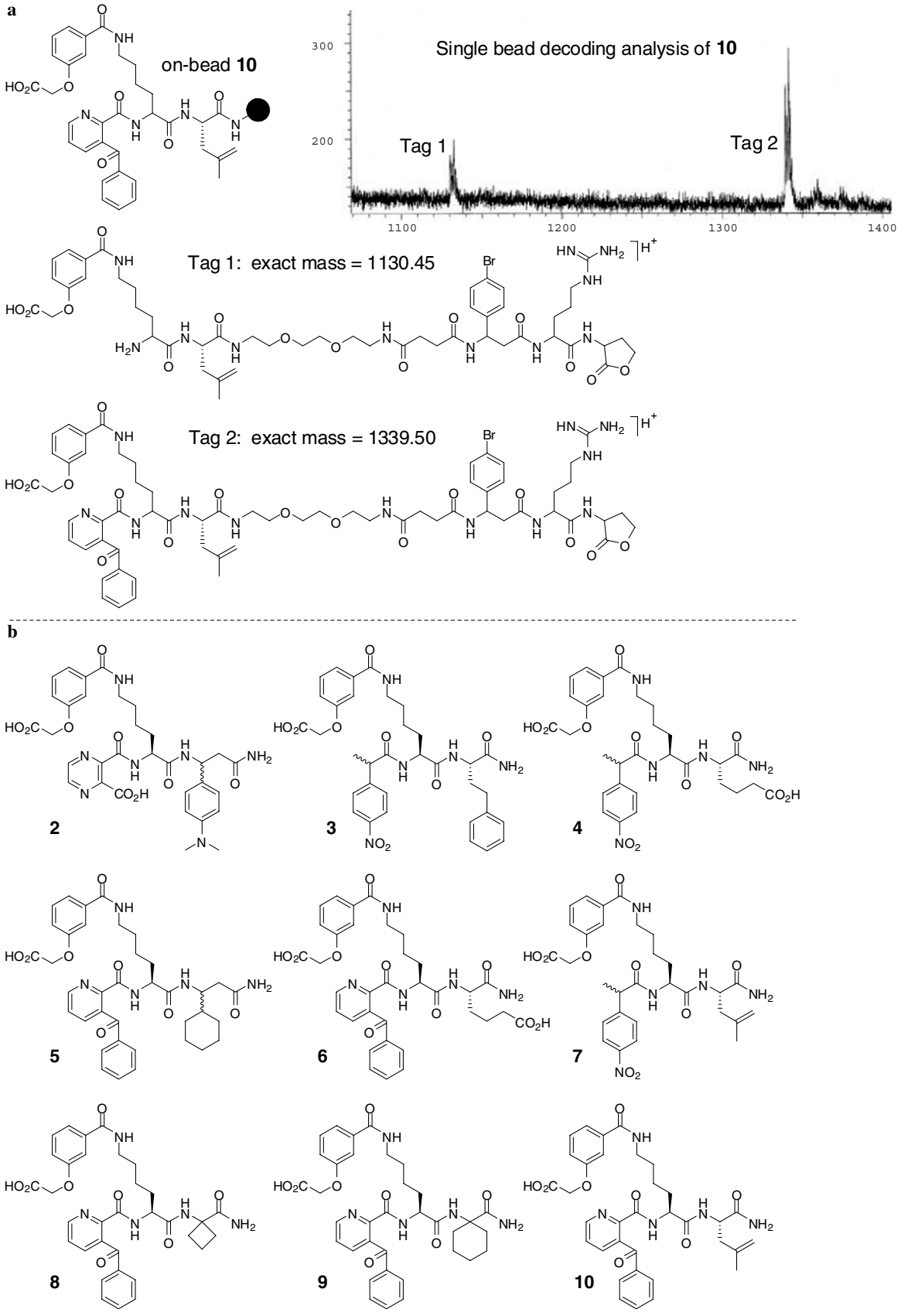

Figure 2. (a) Representative mass spectrum of decoded on-bead hit **10**. (b) ALR2-selective ligands identified through on-bead library screening.

Table 1. IC_{50} data for ALR2 and ALR1 inhibitors **2**–**10**

Compound	IC_{50} (μM)		Selectivity (ALR1/ALR2)
	ALR2	ALR1	
2	3.6	920	255.6
3[a]	—	—	—
4	46	418	9.1
5	6.2	5540	893.5
6[a]	—	—	—
7	15.8	196	12.4
8	2.1	15,740	7495.2
9	33.6	2510	74.7
10	6.1	123	20.2

[a] Solubility issues precluded these substrates from bioassay.

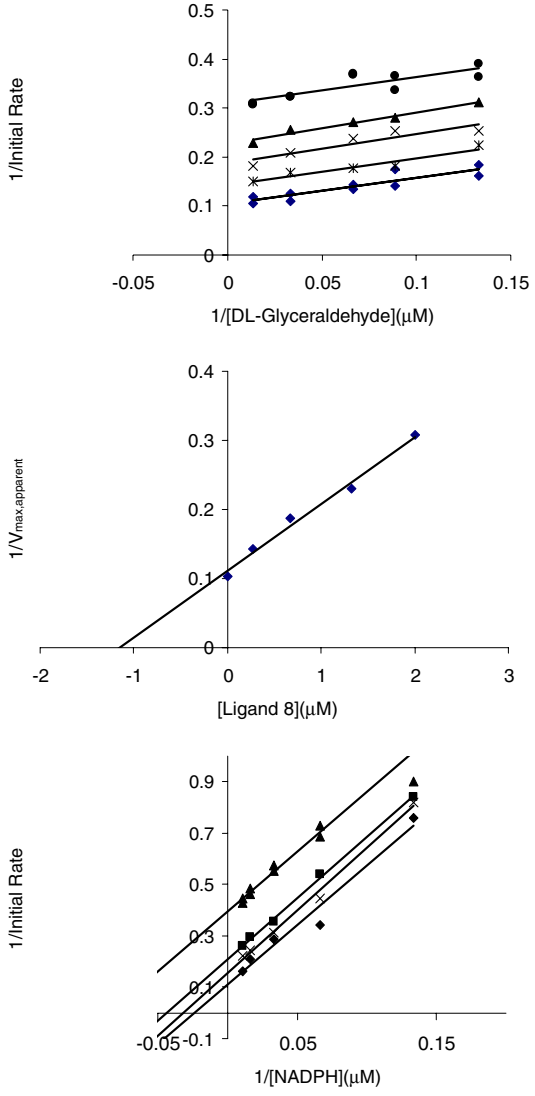

Figure 3. Double-reciprocal plots for ligand **8**. Top: Initial rate versus [DL-glyceraldehyde] with [ligand **8**]. (♦) 0.0 μM; (*) 0.27 μM; (×) 0.67 μM; (▲) 1.33 μM, and (●) 2 μM. Middle: a *y*-intercept replot used to determine the K_i of ligand **8** versus DL-glyceraldehyde. Bottom: initial rate versus [NADPH] with [ligand **8**]: (♦) 0.0 μM; (×) 0.27 μM; (■) 0.67 μM; and (▲) 1.33 μM.

4. Experimental

4.1. Enzyme preparation

Human ALR1 and ALR2 were cloned from a fetal cDNA pool using PCR primers which added an NdeI site to the 5′-end of the insert and a SmaI site to the 3′-end of the insert plasmid pTYB12 (New England Biolabs) to yield the final bacterial expression vector. Sequencing of the plasmid indicated no mutations were introduced by PCR. This plasmid was used to transform the *E. coli* expression strain ER2566. Recombinant ALR1 and ALR2 were produced in fusion with plasmid-derived intein- and chitin-binding domains. The wild-type protein was expressed in LB medium containing 100 μg/ml ampicillin and induced with 500 μM isopropyl-1-thio-β-D-galactopyranoside for 12 h at 15 °C. Cells were harvested and lysed using a microfluidizer at 15,000 psi. The lysate was clarified by centrifuging at 39,000*g* for 30 min.

The resulting cell extracts containing wild-type ALR1/ALR2-intein/chitin-binding domain fusion protein were passed over a column of chitin beads, which was washed with at least 20 column volumes of Buffer A (20 mM Tris, pH 8.0, 500 mM NaCl, and 0.1 mM EDTA) supplemented with 0.1% Triton X-100 followed by 20 column volumes of Buffer A alone. The ALR1/ALR2-intein fusion protein was then cleaved on the chitin column by incubation for 12 h in Buffer A with 50 mM 2-mercaptoethanol. The purified protein was then exchanged into PBS buffer, pH 7.4.

4.2. Protein labeling

Dye labeling was performed according to the manufacturer's instructions. All protein labeling and bead screening procedures were protected from light. The labeling reaction mixture of ALR2 consisted of 13 μL of 45 mg/mL of ALR2 in TEA buffer containing 0.2 mM DL-glyceraldehyde, 1 mM DTT, and 0.02 mM NADPH, and 0.2 mg Alexa Fluor 488 succinimidyl ester for 30 min with stirring. The labeling reaction mixture of ALR1 consisted of 13 μL of 29 mg/mL of ALR1 in TEA buffer containing 1.6 mM *p*-nitrobenzaldehyde, 1 mM DTT, and 0.02 mM NADPH, and 0.04 mg Texas Red for 30 min with stirring. The activity of the enzyme was monitored as a function of reaction time. The reaction was terminated and the enzyme purified from excess dye by passing the reaction solution over a Sephadex G-25 spin column equilibrated with TEA buffer.

4.3. Kinetic analyses of identified compounds

Compounds **2**–**10** were dissolved in PBS buffer, pH 8, to make 7–9 mM stock solutions. These solutions were clear and non-soapy except for compounds **3** and **6**, which were excluded from further studies. The remaining stock solutions required no co-solvents and were used for both ALR1 and ALR2 assays. IC_{50} data for the resynthesized compounds were collected using 100 μM NADPH, 20 μM DL-glyceraldehyde, and varying concentrations of inhibitors. It was necessary to

incubate ALR2 with DTT (1 mM final concentration) for 1 h before assaying to maintain activity. The activity was monitored by the decrease in NADPH at 340 nm. The K_i for inhibitor **8** was determined by varying the concentration of DL-glyceraldehyde and by varying the concentrations of inhibitor and fitting to the velocity equation describing uncompetitive inhibition.

4.4. Resynthesis and characterization of ligand 9

Larger-scale synthesis of the ligands identified as ALR2 binders was performed on 100 mg of Fmoc-Rink MHBA amide resin (capacity: 0.65 mmol/g) contained in a 1-mL plastic column. The beads were swollen in DMF for 60 min, drained, and then treated with 3 mL of 20% piperidine in DMF twice for 10 min. After Fmoc deprotection, the beads were washed with DMF (5× 0.8 mL) and the ligand sequence was constructed using Fmoc-1-amino-1-cyclohexanecarboxylic acid (3 equiv) along with HOBt and DIC (13 equiv each). Fmoc deprotection was completed with 20% piperidine in DMF, 2× 10 min. Fmoc-Lys(Dde)-OH (3 equiv) was coupled in the presence of HOBt and DIC (13 equiv each). The beads were washed with DMF (5× 0.8 mL). Fmoc deprotection was completed with 20% piperidine in DMF, 2× 10 min. 3-Benzoyl-2-pyridinecarboxylic acid (3 equiv) was coupled in the presence of HOBt and DIC (13 equiv each). The beads were washed with DMF (5× 0.8 mL). Dde removal was accomplished by 2% hydrazine in DMF, 2× 10 min. The beads were washed with DMF (5× 0.8 mL) and 3-(2-*tert*-butoxy-2-oxoethoxy)benzoic acid was coupled to the free amine with HOBt and DIC (13 equiv of each). Ligand **9** was cleaved from the resin using 0.9 mL of 95% TFA, 2.5% H_2O, and 2.5% TIS for 2 h. The cleavage solution was collected, concentrated by evaporation, and 5–10 mL ether was added until the product had completely precipitated. The filtrate was cooled to −80 °C for 14 h and centrifuged. The precipitated product was dried under vacuum for 24 h. The dried product was then purified by HPLC, collected fractions were lyophilized, and the resulting solid was subjected to HPLC and ESI-MS analysis.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2006.08.005.

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