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# Affinity-based ranking of ligands for DPP-4 from mixtures

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**Abstract**—Affinity-based selection strategies have recently emerged as a complement to traditional high throughput screening for the rapid discovery of lead compounds for the large number of protein targets emerging from—omics technologies. Herein, we describe a method for the ranking of mixtures of ligands by affinity selection and apply it to rank order a set of inhibitors for the enzyme dipeptidyl peptidase IV.


Affinity selection screening has emerged as a complementary strategy to traditional high throughput screening for small molecule lead discovery.[1,2] For target validation, affinity selection offers the advantage of requiring, once purified protein is obtained, minimal method development in order to screen large libraries of compounds. Screens can be run against multiple forms and activation states of proteins and protein complexes (i.e., inactive and activated forms of kinases)[3] as well as multiple binding pockets on each protein.[4,5] To date, several affinity selection (AS) technologies have been developed which combine mass spectrometry (MS) as a detector with various strategies to separate protein-bound ligands from free small molecules. These separation strategies include frontal affinity chromatography,[3,5] ultrafiltration,[6,7] affinity capillary electrophoresis,[8,9] and size exclusion chromatography (SEC).[10–12] Recently automated systems which integrate SEC separation with LC–MS detection into a single platform provide a technology capable of rapidly screening large compound libraries against a given target.[13–16]

Utilizing an integrated SEC–LC–MS platform, an approach to rank order ligands by affinity selection has been reported where relative binding affinities for members of small mixtures of compounds are determined through competition with a known ligand.[4] Herein, we describe an alternative method to rank order mixtures of ligands by affinity selection that does not require a well-characterized ligand and present a proof-of-concept by ranking a series of active-site inhibitors for dipeptidyl peptidase IV (DPP-4). As a serine protease that rapidly degrades two incretin hormones that enhance glucose stimulated insulin secretion, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), the inhibition of DPP-4 has emerged as an attractive target for the treatment of type II diabetes.[17]

An integrated AS/MS platform outlined in Figure 1 was applied to the rank ordering of a set of DPP-4 inhibi-

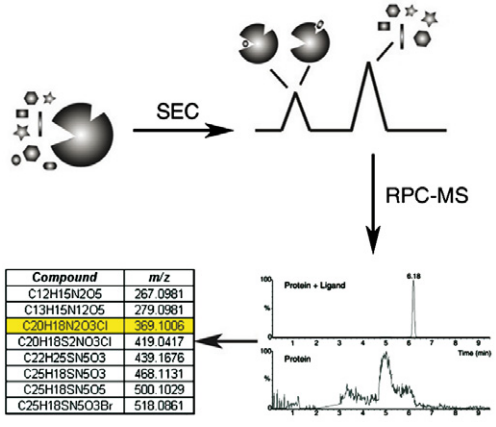


**Figure 1.** Diagram of ASMS method.

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tors. For a standard affinity selection experiment,[18] a mixture of compounds is pre-incubated with the protein target at 4 °C and then injected into a two-step LC–MS system where a fast size exclusion chromatography column rapidly separates the protein and protein-bound ligands from unbound compounds. The excluded protein peak is then trapped and diverted to a reverse phase column (RPC) where low pH (~pH 2) aids in the dissociation of the ligands from the protein. The ligands are subsequently separated and detected with MS, then correlated to a list of exact masses for each mixture to identify the bound ligands.

The enzyme DPP-4 was selected as a model system for the affinity-based rank ordering method due to the availability of purified protein and a wide array of chemical tools.[19] A set of 20 DPP-4 inhibitors (Fig. 2) with $IC_{50}$ values ranging from 2 nM to 2209 nM were selected for which the synthesis and potency of many have been disclosed previously.[20–22] All ligands were detected individually and as members of small libraries by standard LC–MS prior to screening by affinity selection (data not shown).

To confirm that ligands for DPP-4 can be detected with the AS/MS system, 10 μM of each compound was pre-incubated with 10 μM of DPP-4 and then injected in the affinity selection system. Ligands are detected by comparing the extracted ion chromatogram (XIC) for the $M+H^+$ of the protein incubated with buffer alone (Fig. 3a, top panels) to the XIC for the sample containing ligand and protein (Fig. 3a, bottom panels). The spectral data for the detection of four DPP-4 ligands, **1** ($IC_{50}$ = 2 nM), **17** ($IC_{50}$ = 1397 nM), **18** ($IC_{50}$ = 1460 nM), and **20** ($IC_{50}$ = 2209 nM), are displayed in Figure 3a. A peak corresponding to the retention time of the small molecule is detected for the expected $M+H^+$ of each ligand but absent in the control sample. As a system control, all ligands detected as binders to DPP-4 were subsequently screened against β-lactoglobulin, an unrelated protein with low observed small molecule binding capacity, in order to detect any false positives.

After confirmation that all compounds are detected as ligands for DPP-4, the compounds were divided into libraries containing equimolar concentrations of 10 components, with ligand mixture DP4LM1 composed of ligands **1**, **3**, **4**, **6**, **10**, **12**, **14**, **16**, **18**, and **20** and DP4LM2 composed of ligands **2**, **5**, **7**–**9**, **11**, **13**, **15**, **17**, and **19**. To determine whether larger mixtures hinder ligand recovery, 10 μM DPP-4 and 1 μM DP4LM2 were incubated in the presence or absence of 1 μM of a library of 250 compounds containing no known DPP-4 inhibitors for a total ligand concentration of 260 μM and analyzed with the AS/MS system (Fig. 3b). Detection of DPP-4 ligands in mixture DP4LM2 was not hindered by the presence (Fig. 3b, violet bars) or absence (Fig. 3b, blue bars) of the mixture of 250 compounds. Larger mixtures of compounds did not cause ligand breakthrough with the system control β-lactoglobulin (Fig. 3b, yellow and turquoise bars) or with human serum albumin (HSA) (Fig. 3b, green and orange bars)

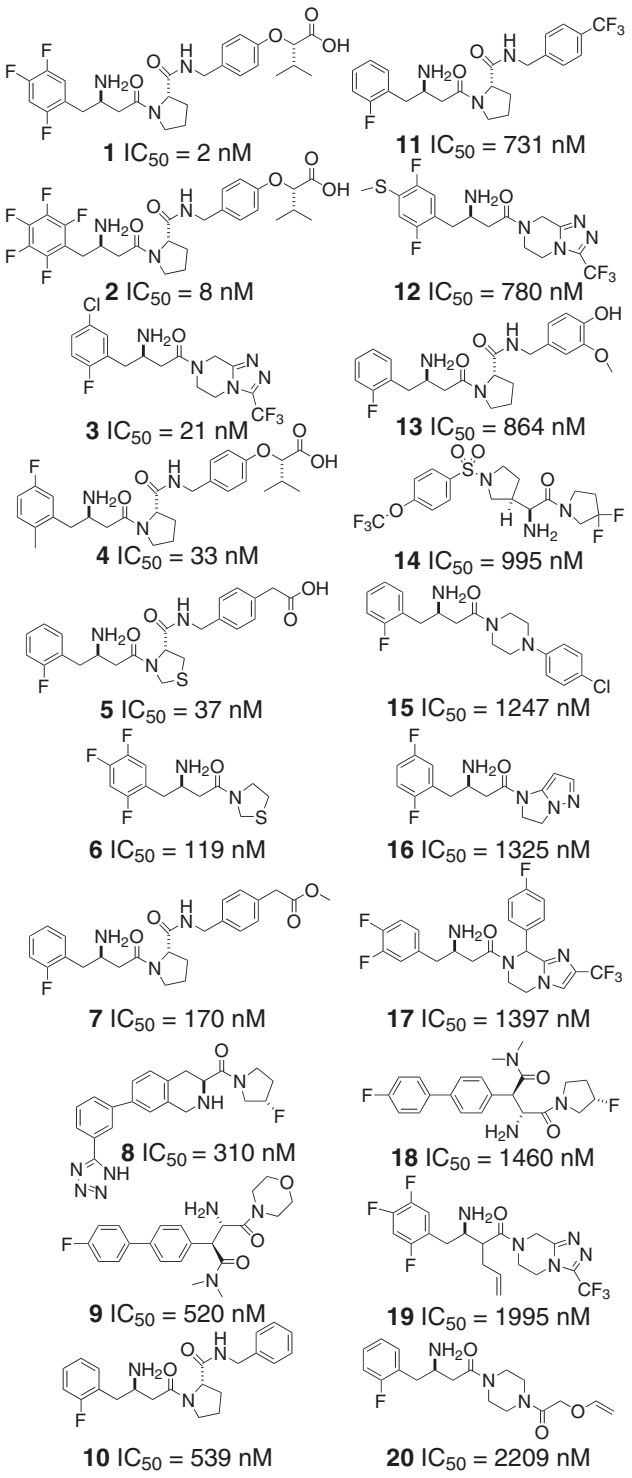


**Figure 2.** DPP-4 inhibitors with their corresponding $IC_{50}$ values used in the affinity selection rank ordering experiments. Several compounds have been reported previously: **1**, **2**, **4**, **5**, **7**, **10**, and **11**,[20] **6**,[21] and **9**.[22]

indicating the feasibility of this technology for screening large mixtures of compounds. As a negative control, two known ligands for HSA, phenylbutazone and warfarin, were not detected with either DPP-4 or β-lactoglobulin but were identified as ligands for HSA further highlighting the applicability of this method to identifying selective ligands for different proteins.

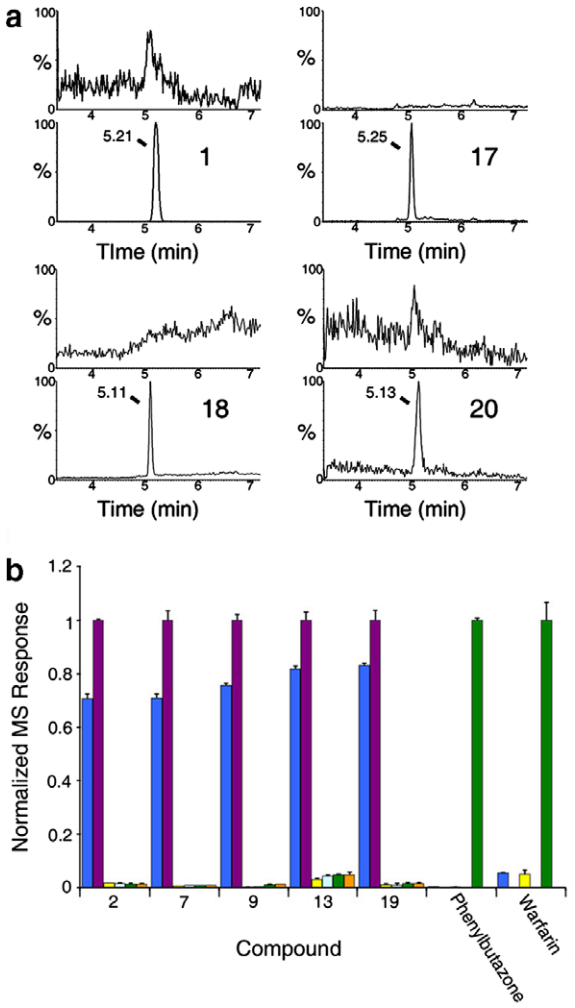


**Figure 3.** DPP-4 ligands can be detected with ASMS. (a) ASMS recovery of each ligand **1**, **17**, **18**, and **20** (10 μM) incubated individually with DPP-4 (10 μM) by detection of XIC of $M+H^+$ (bottom panels) but not found in protein incubated with buffer alone (top panels). (b) ASMS recovery of ligands in DP4LM2 (1 μM) incubated with DPP-IV (10 μM) in the presence (violet bars) or absence (blue bars) of a 250-member small molecule library (1 μM). DP4LM2 was also screened with β-lactoglobulin (10 μM) in the presence (turquoise bars) or absence (yellow bars) of the library and with human serum albumin (10 μM) in the presence (orange bars) and absence (green bars) of the library. Phenylbutazone and warfarin (1 μM) were screened against each protein in the absence of the library. Due to the variation in MS ionization efficiency between each library member, the MS response for each ligand is normalized with the highest response for each ligand set to 1.

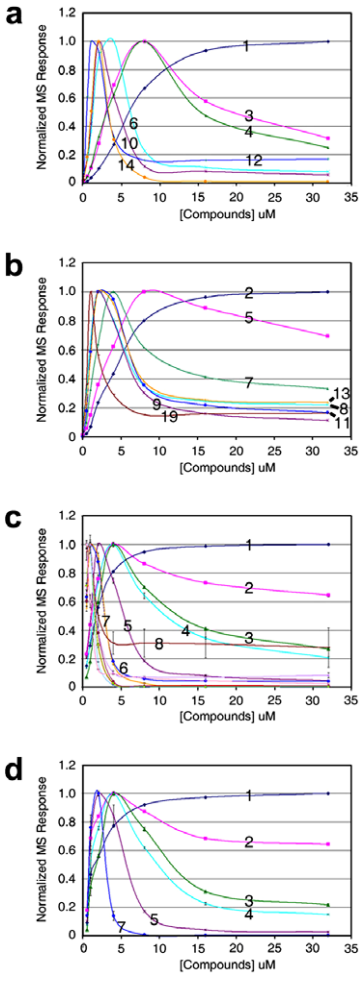


**Figure 4.** Rank ordering mixtures of DPP-4 inhibitors by ASMS. Graphs show normalized MS responses for each ligand recovered from mixtures (0.5 μM to 32 μM) after pre-incubation with DPP-4 (10 μM) and analysis by ASMS. (a) Normalized MS responses for compounds in mixture DP4LM1: **1** (♦), **3** (■), **4** (▲), **6** (X), **10** (✱), **12** (|), and **14** (●). (b) Normalized MS responses for compounds in mixture DP4LM2: **2** (♦), **5** (■), **7** (▲), **8** (X), **9** (✱), **11** (●), **13** (|), and **19** (▬). (c) Normalized MS responses for compounds in mixture DP4LM3: **1** (♦), **2** (■), **3** (▲), **4** (X), **5** (✱), **6** (●), **7** (|), **8** (▬), **9** (▬), **10** (♦), **11** (■), **13** (▲), **14** (X), and **19** (✱). (d) Normalized MS responses for compounds in mixture DP4LM4: **1** (♦), **2** (■), **3** (▲), **4** (X), **5** (✱), **7** (●).

To rank order ligands for DPP-4 by affinity, the ligand mixtures were pre-incubated with 10 μM DPP-4 at concentrations ranging from 0.5 to 32 μM per library member, then analyzed with the AS/MS platform. The integrated MS response ($M+H^+$) for each library member was averaged ($n = 4$) and normalized with the highest averaged MS response for each molecule set to 1. At lower ligand concentrations (0.5 and 1 μM) the protein is in excess. With increasing ligand concentration, conditions change from ligand limited to protein limited creating a competitive environment with the higher affinity ligands competing the lower affinity ligands off of the protein. With library DP4LM1 (Fig. 4a), the lowest affinity ligands **16**, **18**, and **20** were not detected in the rank ordering experiments due to ligand recovery below the limits of detection. In Figure 4a, the ligands are ranked from highest affinity to lowest affinity by the order the normalized MS signal for each compound reaches its maxima from right to left. With increasing concentration of ligands, the highest affinity ligand in the mixture **1** ($IC_{50} = 1.6$ nM) approached its maximum at 1 with increasing ligand concentration. The medium affinity ligands **3** and **4** ($IC_{50} = 21$ nM and 33 nM, respectively) reach their normalized MS response maxima around 10 μM with the lower affinity ligands **6**, **10**, **12**, and **14** reaching their maxima from 2.5 to 5 μM. Similar results were achieved with mixture DP4LM2 where the highest affinity ligand as determined by affinity selection correlated with the most potent inhibitor in the mixture, compound **2** ($IC_{50} = 8$ nM, Fig. 4b). The AS/MS ranking of the ligands,

**2** > **5** > **7** > **8** ~ **9** > **11** ~ **13** > **19**, correlated closely with the relative $IC_{50}$'s of the compounds. Due to weak MS signals at low ligand concentrations, two of the weaker ligands, **15** and **17**, were not detected in the rank ordering experiment.

To demonstrate the applicability of the AS/MS rank ordering method to the analysis of larger mixtures of compounds, all 20 DPP-4 inhibitors were combined and analyzed (Fig. 4c). With an increase in library size, the affinity-based ranking of DPP-4 inhibitors was similar to the activity-based ranking of ligands indicating that library size for ranking experiments is not limited by the concentration of protein. To find strong ligands for high hit rate proteins, larger mixtures of compounds could be screened under protein limited conditions to find the highest affinity ligands, which can then be recombined for further ranking. In Figure 4d, the three highest affinity ligands as determined by rank ordering from DP4LM1 (**1**, **3**, and **4**) were combined with the three highest affinity ligands from DP4LM2 (**2**, **5**, and **7**) and analyzed by AS/MS with 10 μM DPP-4. The affinity-based ranking of the ligands, **1** > **2** > **3** > **4** > **5** > **7**, correlated with the relative $IC_{50}$'s for the six DPP-4 inhibitors demonstrating the utility of applying an iterative approach to the affinity ranking of molecules within a larger set of ligands.

With the rapid influx of new targets requiring validation, affinity selection techniques may be utilized to rapidly identify ligands for a given protein target which can be further developed into proof-of-concept molecules. Herein we have presented a method to rank mixtures of ligands by affinity for their given target and provided proof of concept with a set of inhibitors for the enzyme DPP-4. Future studies will focus on refining the methodology to identify allosteric inhibitors as well as to rank compounds to aid in the rapid identification of inhibitors for new protein targets.

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