

Introduction

Carbonic anhydrase (CA) catalyzes the reversible reaction $\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{HCO}_3^- + \text{H}^+$. CA is especially well-suited as a model enzyme for biophysical studies of protein / ligand binding (*Chem Rev.* 2008 108(3): 946–1051), because it is inexpensive, well-characterized, and medically relevant (in glaucoma, obesity, and altitude sickness). CA inhibitors, primarily aryl-sulfonamides, are also well understood and readily available.

Here we use CA as a model enzyme for protein / ligand binding study with high-resolution native mass spectrometry (nMS). There are a number of technologies for protein / ligand screening, including fluorescent or radioactive labeling, surface plasmon resonance spectroscopy, circular dichroism, and isothermal titration calorimetry. Relative to these methods, nMS offers some advantages: no need for chemical tags, observation of multiple and nonspecific binding, and direct identification of ligands in competitive binding experiments.

Experimental Methods

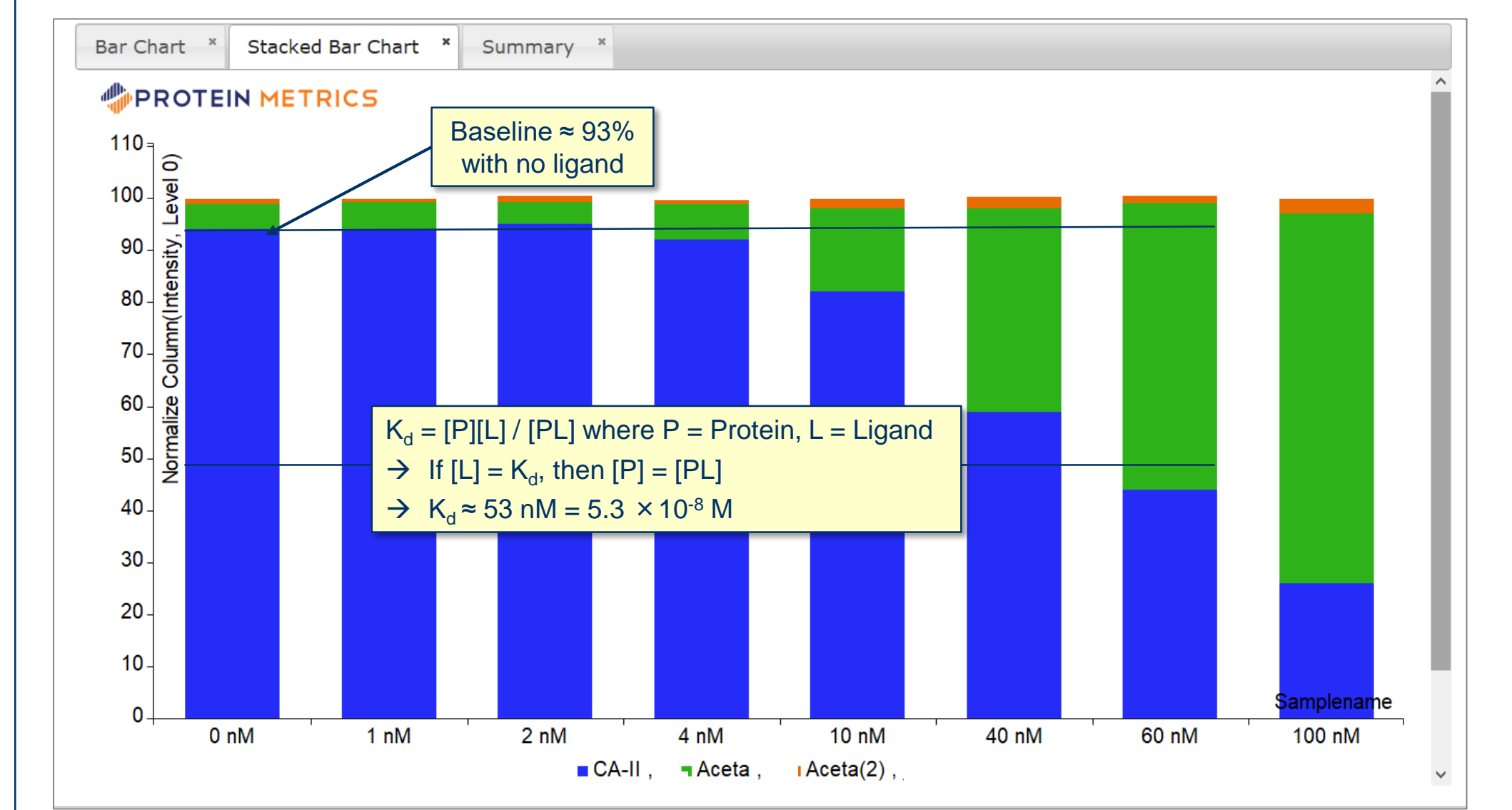
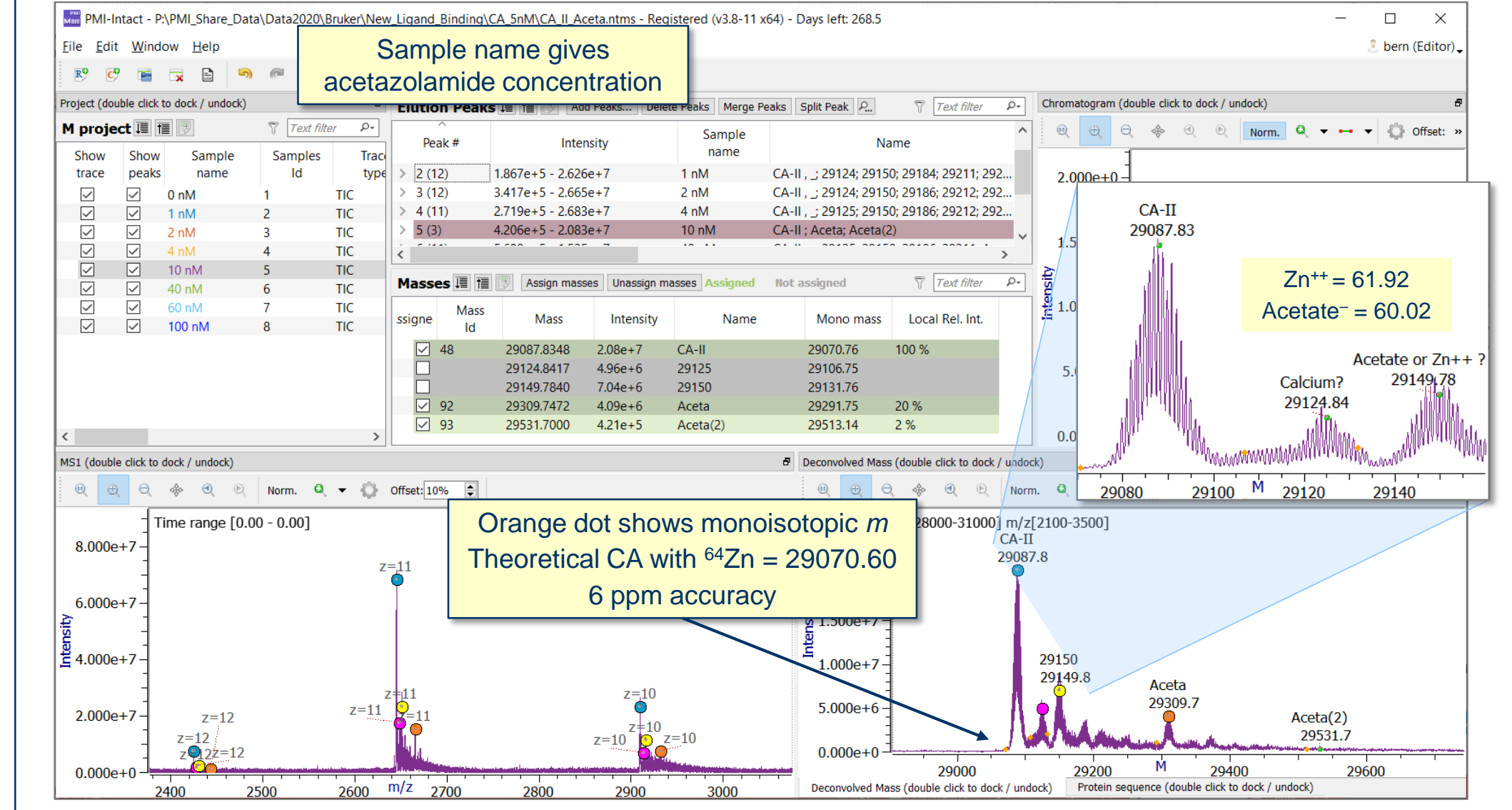
Bovine carbonic anhydrase II (CA) was purchased from Sigma (C-3934) and purified by buffer exchange with 10 mM ammonium acetate. Final concentration of CA was 3 μM for spray solution in 10 mM ammonium acetate. The following drugs (200 mM in DMSO) were tested with 3 μM CA: **Zonisamide**, **Acetazolamide**, **Methazolamide**, **Topiramate**, **Dorzolamide**, and **Brinzolamide** with protein : ligand molar ratios from 1 : 0.07 up to 1 : 30. (Not all drugs were tested at all ratios.) In addition, **Acetazolamide** was also tested with **low protein concentrations** of 5 nM and 20 nM.

The samples were measured with a scimaX MRMS system using an ESI source in positive mode. Sample solutions were infused at a flow rate of 5 $\mu\text{l}/\text{min}$ for high concentration and 2 $\mu\text{l}/\text{min}$ for low concentration. Precursors of $z=8+$ to 11+ were isolated in the quadrupole for high concentration, and $z=10+$ to 12+ for low concentration, with resolving power 70,000 at m/z 2620. 200 single scans were added for the final m/z spectrum, and the total time to acquire one spectrum was ~ 7 min.



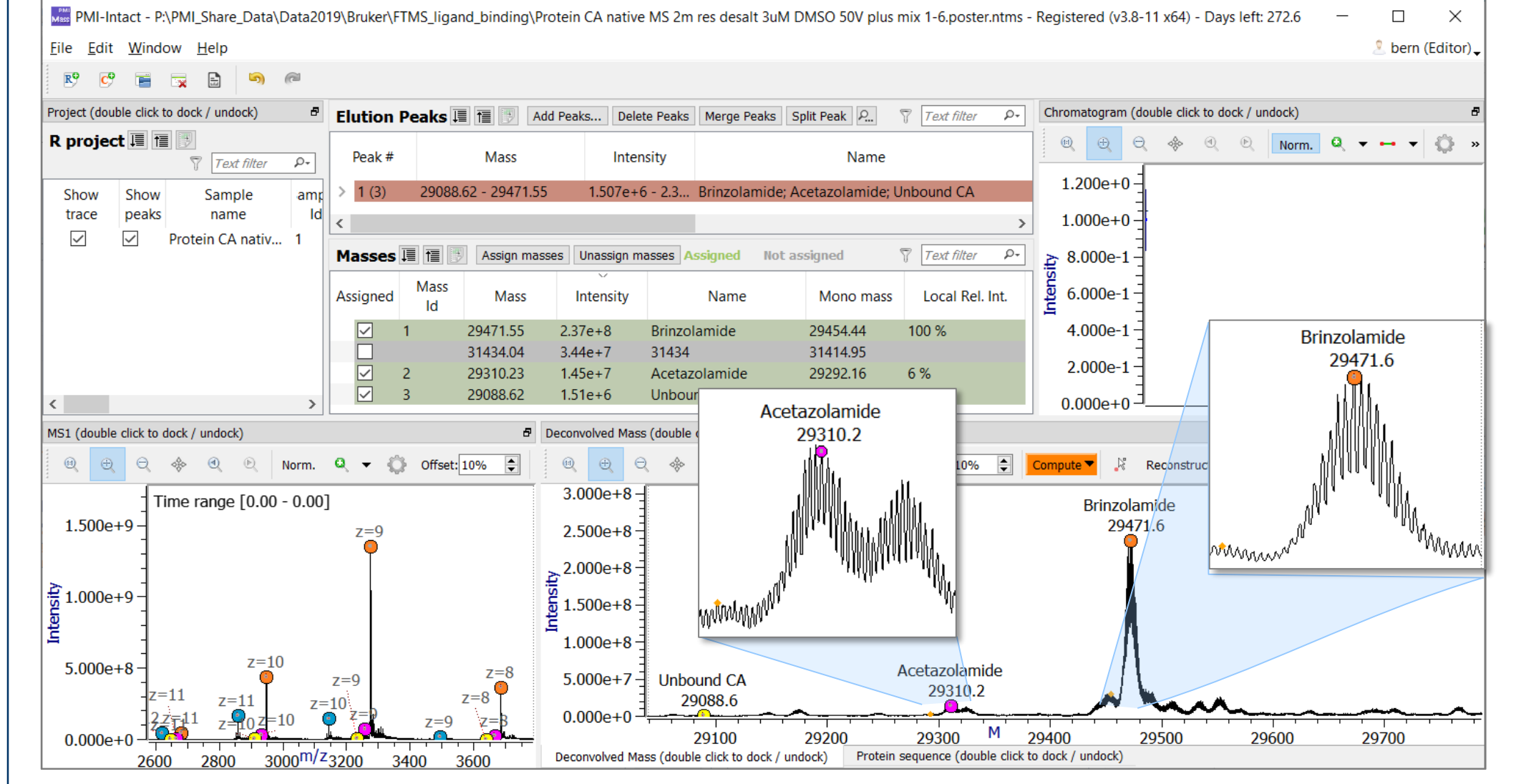
Binding Affinity

Intact Mass™ software includes several features useful for protein / ligand binding studies: (1) “Three-channel” charge deconvolution using multiple charge states, isotope spacing, and known mass deltas (e.g., ligand mass); (2) Observed monoisotopic mass determined with user-defined average atomic formula for isotope peak ratios; (3) Automated peak matching of expected masses, and quantitation of both expected and unexpected masses; (4) Pivot tables and graphics for highly customizable reporting. Below we show titrated acetazolamide binding with 5 nM CA.



Competitive Binding

In this experiment 3 μM CA was combined in 1 : 1 molar ratio with all 6 CA inhibitors. CA bound with brinzolamide was about 16x more intense than CA with the second-best binder, acetazolamide.



Discussion and Conclusions

Shanghai Institute of Materia Medica has adopted high-resolution nMS to test compounds from traditional Chinese medicine (which gave the world artemisinin!) against protein targets (and off-targets). For these experiments, Bruker high-resolution native MRMS offers advantages:

- Direct observation and accurate-mass identification of bound forms, including stoichiometry (e.g., binding may occur only with dimers);
- Detection of unexpected masses, e.g., adducts;
- Isotopic resolution gives monoisotopic mass, which is especially helpful for PTMs and ligands with similar masses;
- Dilution series can be used to compute dissociation constant K_d either by fitting a theoretical curve or— the route taken here— by interpolating to determine the concentration at which $[P] = [PL]$;
- 7 min per well gives medium throughput (~ 200 experiments / day), improvable by faster acquisition and/or multiplexing ligands.

Protein Metrics Intact Mass software is well-suited to this application, because it seamlessly handles isotope resolved and unresolved data, works with few (or even single) charges, and gives customized reports.