Fragment-based drug discovery (FBDD) has become a powerful and versatile approach to generating high affinity small-molecule inhibitors of pharmacological targets [1-3]. In this approach small molecules representing fragments of drugs or biologically-active molecules are screened for binding to a target protein. Binding fragments are then elaborated or linked together to create ligands of successively higher affinity and specificity. The method is distinct from but complementary to high-throughput screening (HTS). The first drug molecule originating from fragment-based screening, Vemurafenib, was approved by the FDA in 2011. This example and several others in the literature demonstrate that fragment-based methods can be useful for the discovery of clinical candidates.

Fragment libraries and related collections available at KU. We are fortunate to have a number of well-known library collections available at KU. For starting out, we use the Zenobia Frag ment Library consisting of 288 organic compounds selected by experienced medicinal chemists for their diversity and flexibility with respect to chemical modification, linking and elaboration. This library contains 137 distinct scaffolds and the median MW, ClogP and tPSA for all 288 members are 165, 1.64 and 45.8, respectively. In addition we have access to the Prestwick Chemical Library of 1120 small molecules, the MicroSource-Spectrum collection of 2320 compounds and four major diversity sets comprising 33,000 unique compounds. Learn more at this link.

Detecting fragment binding. Fragments often exhibit relatively weak binding ranging from µM to mM affinities. Fortunately, two complementary methods for detecting such weak binding are available in the COBRE-PSF Core Labs, namely, STD-NMR spectroscopy and surface plasmon resonance (SPR). Both of these methods are now routinely being applied to screen our in-house 288 compound Zenobia Fragment Library against a number of proteins for our Core Lab clients. As described below, the results are very encouraging in terms of their reliability and the initial hits being detected.

Saturation Transfer Difference (STD) NMR spectroscopy is ideally suited to screen small molecules for binding to proteins in solution [4-8]. Typically two NMR spectra are recorded. In the first spectrum ("on-resonance"), selective irradiation of a protein resonance will result in rapid propagation of spin saturation over the entire protein (spin diffusion). Saturation is also transferred to a binding fragment at the fragment-protein interface; the fragment then dissociates back into bulk solution where its saturated state persists due to its slow, free-state, relaxation rate. On-off exchange of fragment while the selective irradiation pulse is applied increases the population of saturated fragments in the bulk solution. The second spectrum ("off-resonance") is recorded with irradiation such that no NMR resonances are affected. Subtracting the two spectra results in a saturation transfer difference (STD) spectrum showing only resonances that have experienced saturation, that is, those from the protein and the bound fragment. However, resonances from the protein are typically not visible because it is present in minimal concentration and the resonances are eliminated by R2 relaxation filtering prior to detection. Hence, the STD spectrum reveals only resonances from the binding fragments.

This is illustrated in Figure 1. Spectrum A shows the 1H-NMR spectrum of a mixture of human serum albumin (HSA, 10 µM) as the binding protein, salicylic acid (SA, 500 µM) as a binding ligand and sucrose (500 µM) as a non-binding molecule. When spectra recorded with and without applying saturating power...
to a protein resonance are subtracted, only the peaks from the SA appear, indicating that saturation was transferred from the protein to SA but not to sucrose. Hence SA interacts with HSA but sucrose does not. This experiment also indicates that several small molecules can be examined at the same time, as long as their NMR spectra do not overlap extensively.

**Surface Plasmon Resonance (SPR) for detecting binding “label-free”:** The phenomenon of surface plasmon resonance is related to the phenomenon of refraction of light by solids or solutions. In the case of SPR, proteins coupled to a special gold surface refract light in proportion to their abundance and molecular mass. When other molecules from solution bind to the attached proteins, the refractive index changes making the device a highly sensitive detector of binding interactions. The method is also “label-free” meaning that it does not depend on tags, radioactivity, fluorescence, etc.

The Biacore 3000 SPR instrument in the COBRE-PSF Core Lab uses a 4-channel flow cell. It can screen compounds presented in 96-well plates against up to three different proteins and a reference surface simultaneously to detect binding of the flowing solute molecules to one or more of the attached proteins. The flowing solute can be a small drug "fragment" (MW 100-200), a larger drug-like molecule (MW 200-500), or a macromolecule such as an oligonucleotide or another protein. The Biacore's automation features make it ideal for screening libraries such as our 288 compound Zenobia Fragment Library. Protein consumption is very low - often much less than 100 µg for an entire screening campaign.

Figure 2 below shows representative sensograms generated by flowing low concentrations (50 µM) of four different small molecules over a Biacore CM-5 chip loaded with carbonic anhydrase II. The first one, warfarin, is a negative control. The latter three are known inhibitors of differing binding potency, and they show a typical "top hat" shape: a rapid rise in the binding phase, a plateau indicating equilibrium binding (in this case saturation), and a rapid dissociation upon wash-out. By varying the concentration of the solute the on-rate, off-rate and equilibrium constant can be determined using very little material.

**Representative screening projects currently underway in our Core Labs.** Several investigators are now routinely using SPR, NMR or both to screen our 288 compound Fragment Library and others to identify fragments that bind specifically to their proteins. For example, Dr. Liang Xu studies RNA-binding proteins that are overexpressed in cancer cells. The RNA binding sites are very large and native RNA is easily degraded. To enable fragment library screening by SPR, our Protein Production Group expressed the RNA-binding protein HuR and two related proteins for Dr. Xu, and loaded them onto a CM-5 chip. Screening our fragment library revealed almost 20 fragments that gave a strong binding response to HuR but limited or no binding to the related proteins, indicating selectivity (Figure 3, right panel).

**Figure 2 - Sensograms for CAII inhibitors vs. a negative control compound.**

**Figure 3: Sensorgrams of the interaction between HuR protein and active (left) or inactive (center) fragments from the Zenobia library. X-axis: time scale in seconds, Y-axis: response scale. The fragments were injected for 100 seconds.**

**Dr. Roberto DeGuzman** studies bacterial Type III secretion systems. These needle-shaped multi-protein complexes are required for pathogenicity. Interrupting the protein-protein interactions critical to their formation with small molecules would disable the bacteria, rendering them no longer pathogenic and leaving them vulnerable to immune clearance and conventional antibiotic therapy. In this work DeGuzman loads three proteins onto the three lanes of a CM-5 chip. Their mutual interaction is confirmed and quantified by flowing various proteins as analytes over other proteins bounds on the
chip. In a similar fashion compounds from our fragment collection are being evaluated against all three proteins simultaneously using the automated injection capability of the Biacore 3000.

Dr. Mario Rivera studies iron metabolism in bacteria, with a focus on two redox-active proteins that interact specifically to facilitate iron storage and release. Fragment screening using STD-NMR identified a key fragment that bound to one partner. SPR confirmed the interaction and also allowed him to demonstrate that the small molecule blocked binding of the partner protein. Finally, X-ray crystallography, also conducted in our Protein Structure Core Lab, showed unequivocally that the fragment bound in a cavity that would normally accommodate the critical “bait” residue of the partner protein.

Once binding is detected, what comes next? After screening a library the apparent hits are usually first verified by re-screening them, often using a complementary method. SPR hits may be tested at several concentrations to determine their binding constants and kinetic parameters. Fragments rarely bind strongly enough to be biologically useful without further optimization of their structures. For this one would like to have more detailed information about their binding. For example: do all the hits interact at the same site on the target protein; what is the orientation of the fragment when bound; are simple analogs of a given hit bound better or worse.

The COBRE-PSF Core labs can address all of these questions and more. If the protein is expressed with N-15 labeling, 2D-NMR can be used to determine which residues of the protein are perturbed upon fragment binding, and thus whether all active fragments bind to the same or different sites on the protein. If the protein crystallizes, X-ray analysis of crystals soaked in solutions of fragment compounds can reveal not only the binding site on the protein but potentially the orientation of the bound fragment. Such information can be invaluable in moving hits forward to optimize them through synthetic medicinal chemistry. Even with this, other core labs at KU are equipped to help. We encourage you to peruse the COBRE-PSF Core lab Web sites for links to other core labs that provide consultation, collaboration and service in computational chemistry and synthetic medicinal chemistry.

References

Recent Core Laboratory Affiliated Publications
Biomolecular Core Laboratory

Protein Production Group Core Laboratory

Protein Structure Core Laboratory

The KU NIH Center of Biomedical Research Excellence in Protein Structure and Function (COBRE-PSF) is intended to i) synergize with other regional efforts in proteomics and protein chemistry and biology, ii) recruit, support and mentor new faculty intending to establish competitive research programs in protein structure and function, and iii) enhance the overall infrastructure for state-of-the-art research in protein structure and function through the establishment of Core Laboratories. The COBRE and its core research laboratories are supported by the NIH Institutional Development Award (IDeA) Program and the National Institute of General Medical Sciences (8P20GM103420 and P30 GM110761).