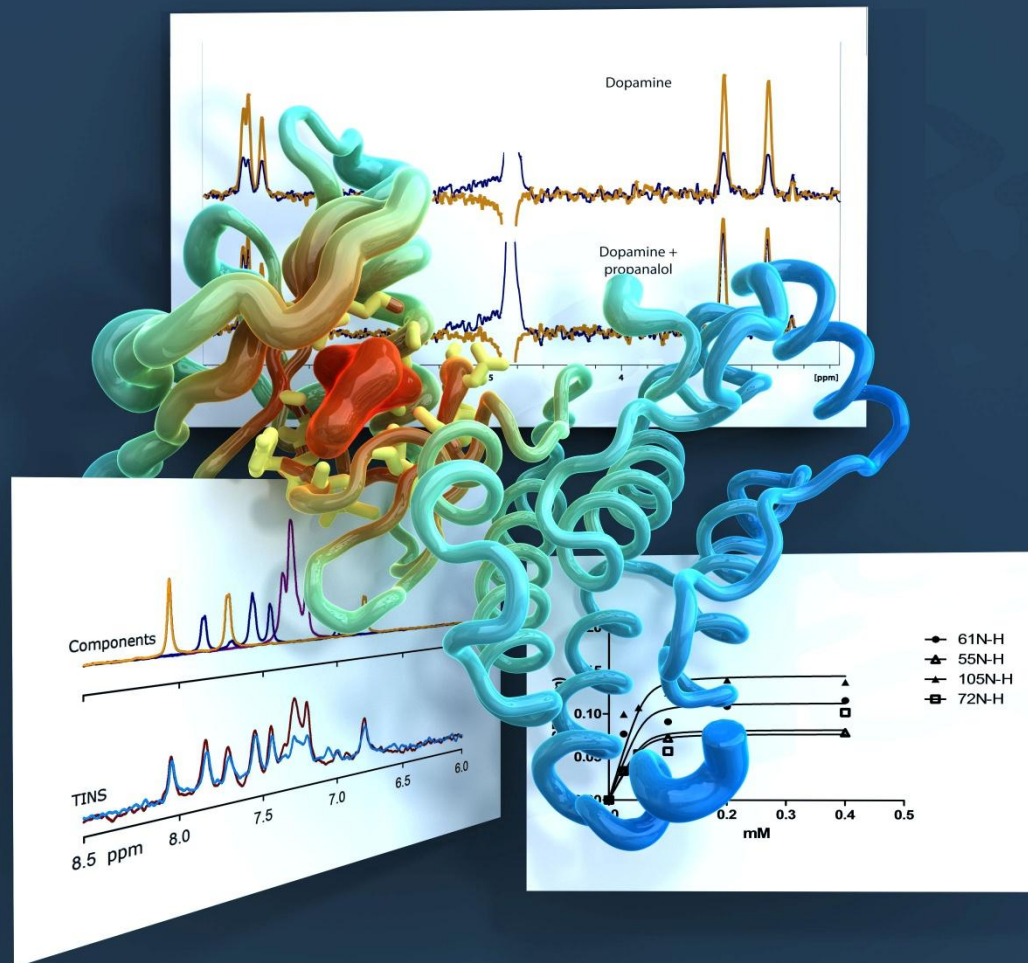


Case Studies

2011 ZoBio



ZoBio Corporate Overview

- Founded as Dutch BV 11/2004
- Full access to all lab facilities of UL
- Self funded (grants and commercial activities)
- Doubled income 5 consecutive years
- 9 employees
- Customers: 5 of 15 largest pharma
- Business model:
 - Primarily Fee-for-Service
 - Limited internal discovery via academic collaboration
- Projects typically completed in 1-2 months

www.zobio.com

ZoBio Service overview

Services offered for Fragment Library screen

1. Protein Production – See 7 below
2. Fragment library screening using TINS
 - Our Library
 - Customer's library
3. Fragment Hit validation
 - TINS
 - selectivity
 - binding site identification by competition
 - Protein Observed NMR (HSQC)
 - SPR
4. Analoging of validated Hits
5. Large scale drugability studies

Services offered for Follow-up of screen

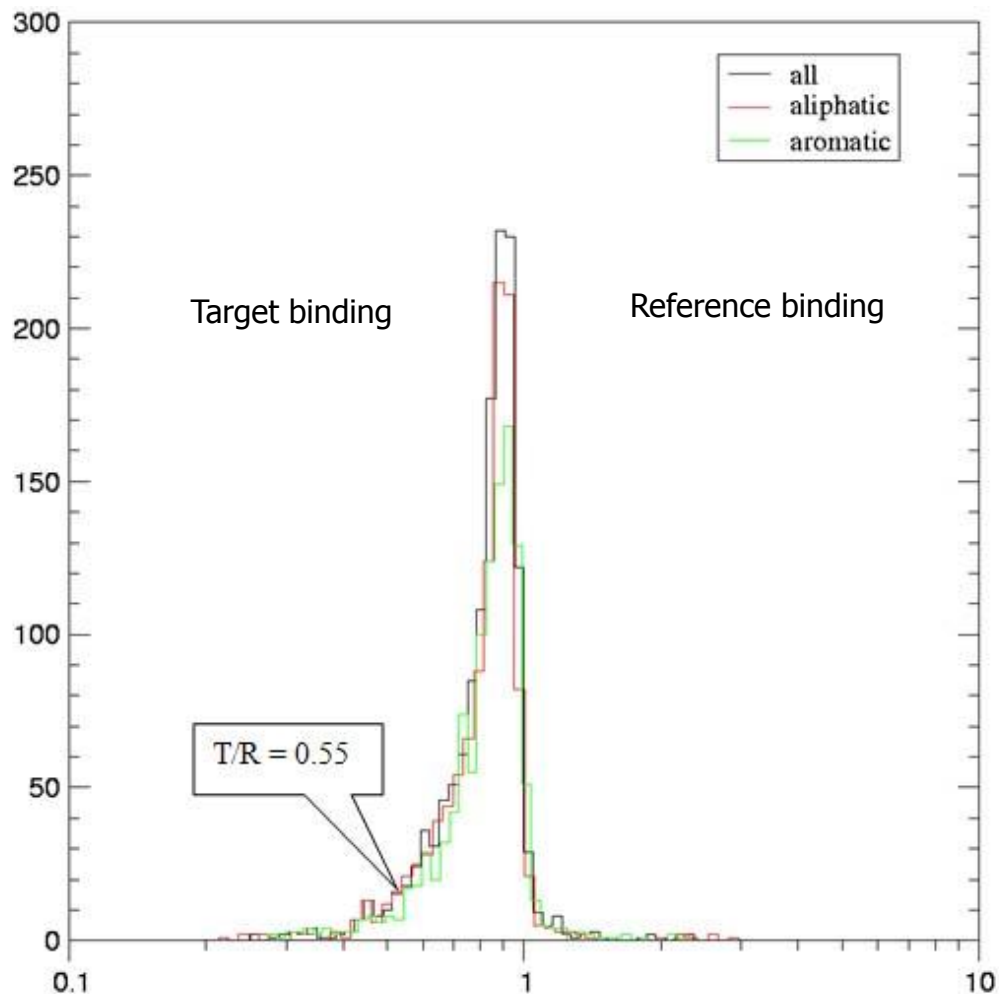
7. Hit triaging
8. NMR-based structure elucidation
 - NMR assignment and NOE based structure of protein
 - NOE-based protein-ligand complex
 - Low resolution binding site mapping e.g. Spin labelling

“Always flexible in arranging our services according to the customers needs”

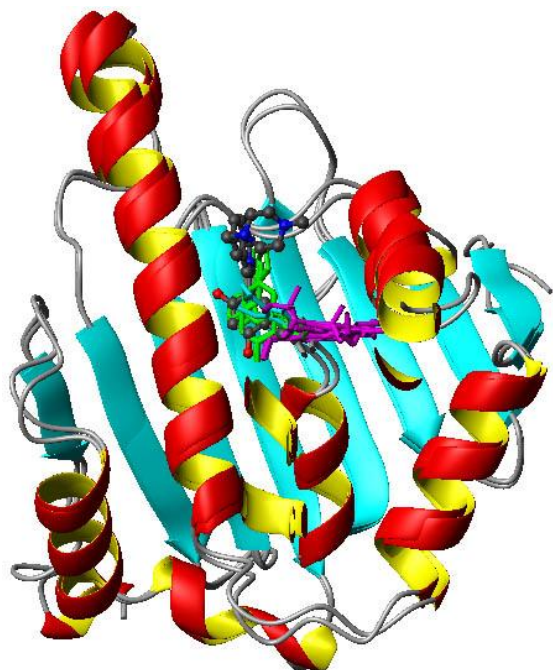
Case Study 1: HSP90

- 1.5 mg protein
- 1,393 compounds screened
- 91 hits
- Competition binding
- Biacore X-ray crystallography

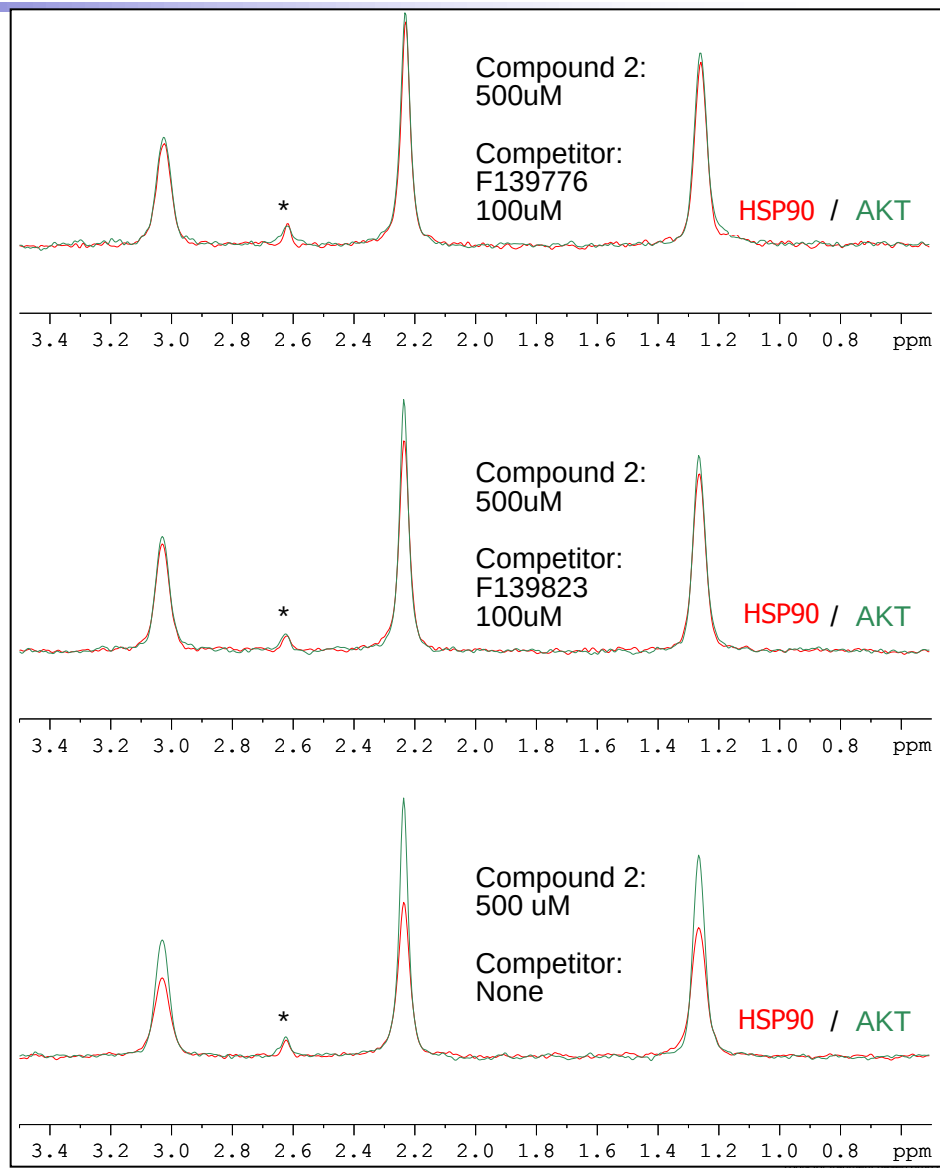
ZoBio has worked on HSP90 for multiple customers. The first project was for UCB, which has subsequently dropped oncology from its portfolio. The figure at right shows the result of the TINS screen of the ZoBio fragment library. The height of each bar shows the number of compounds with the indicated preferential binding ratio ($T/R < 1$ indicates preferential target binding). Based on this profile, 93 hits were selected for the follow on studies shown below.



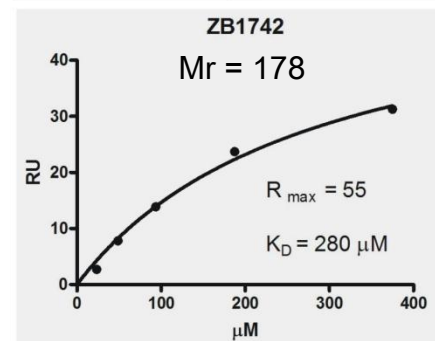
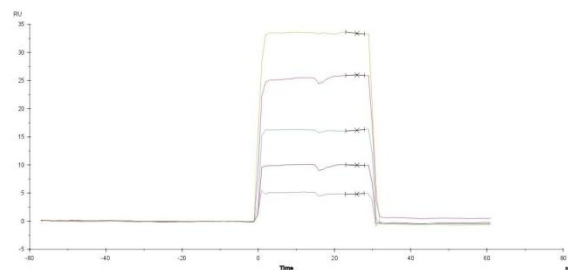
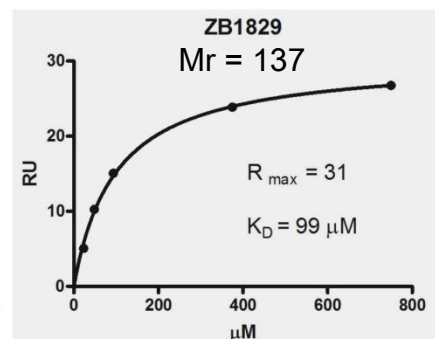
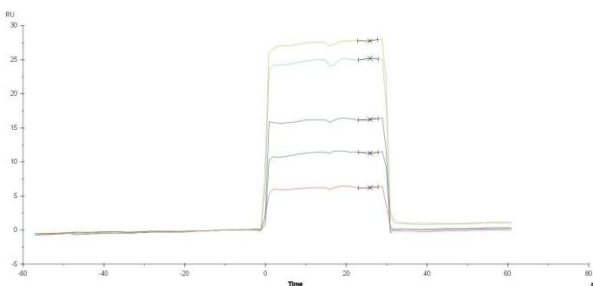
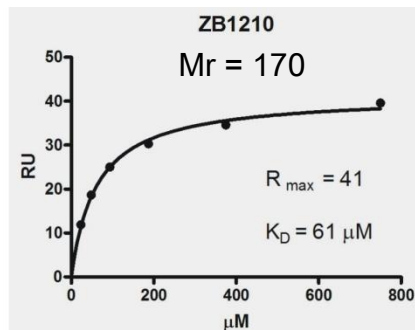
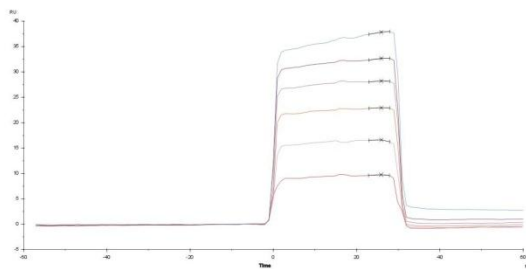
HSP90 Competition Binding Studies



Based on crystal structures, tool compounds were selected for competition binding studies to a) confirm the screening data and b) define the binding site. Competition for 1 fragment (Compound 2) vs 2 different tool compounds is shown at right. F139823 nearly blocks all fragment binding while F139776 completely abolishes fragment binding (the difference between the green and orange spectra disappear).

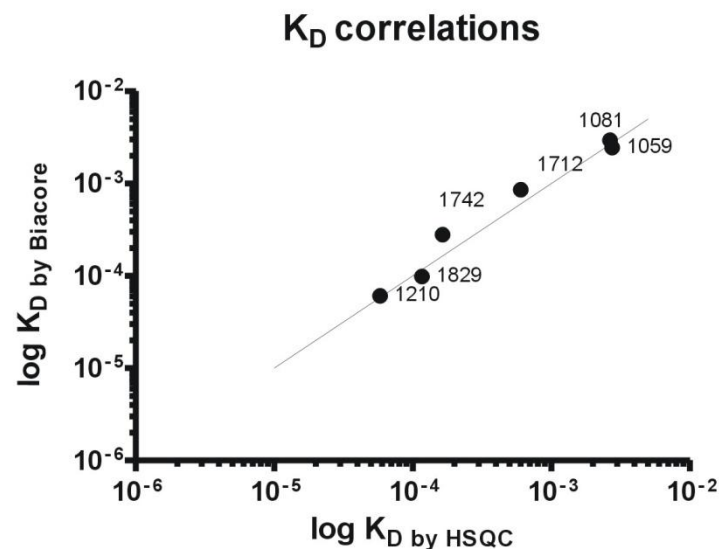


HSP90 Fragment Hit validation via SPR



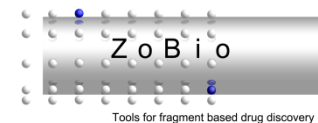
HSP90 N-term

Gregg Siegal
ZoBio



Fragments positive in the competition binding assay were analyzed by SPR to obtain the binding affinity. Fragments with excellent ligand efficiency (LE) were found. The SPR data correlates very well with protein observed NMR data (HSQC, above).

Ligand	LE
ZB1210	0.51
ZB1829	0.54
ZB1742	0.29



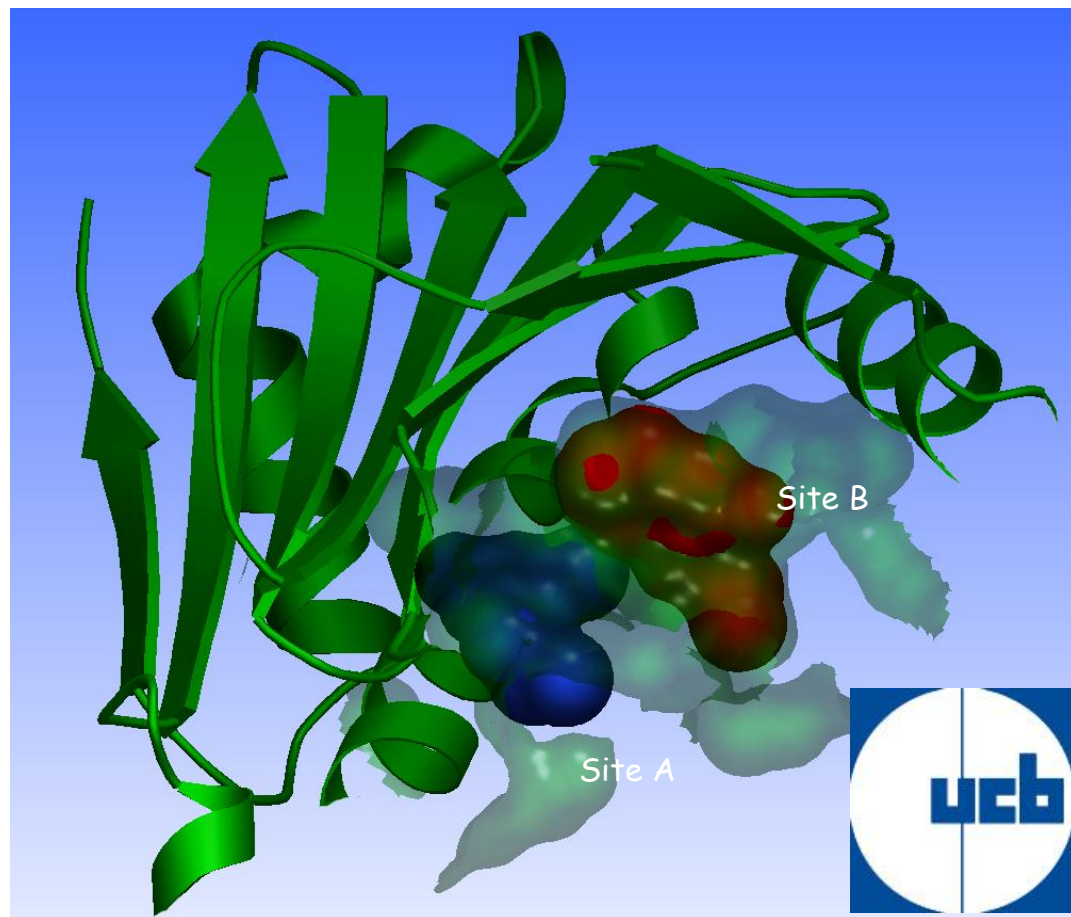
HSP90 Crystallography of ZoBio fragments

Simple soaking
1 attempt 17/49
 K_D of ligands 0.06-12 mM!

Crystallography summary

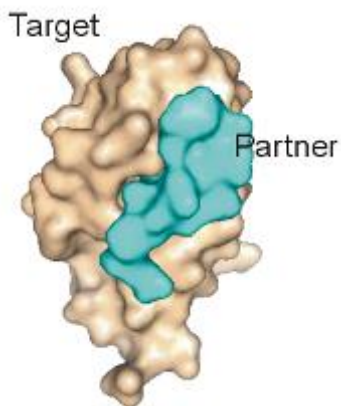
Site A	5
Site B	11
Site A & B	1

49 validated fragments were selected for X-ray crystallography using the “soaking” approach. In a single attempt, 17 compounds yielded crystal structures (right). UCB was able to evolve these fragments to single digit μ M potency in 1 round of chemistry before the project was terminated.



Case Study 2: Protein – Protein Interactions in Oncology

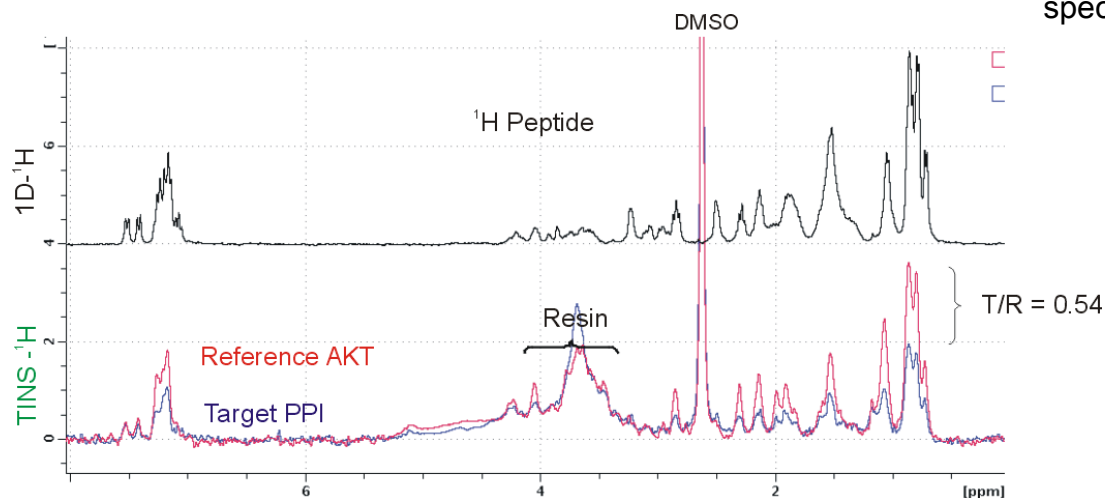
Target: MDM4



TINS screening condition

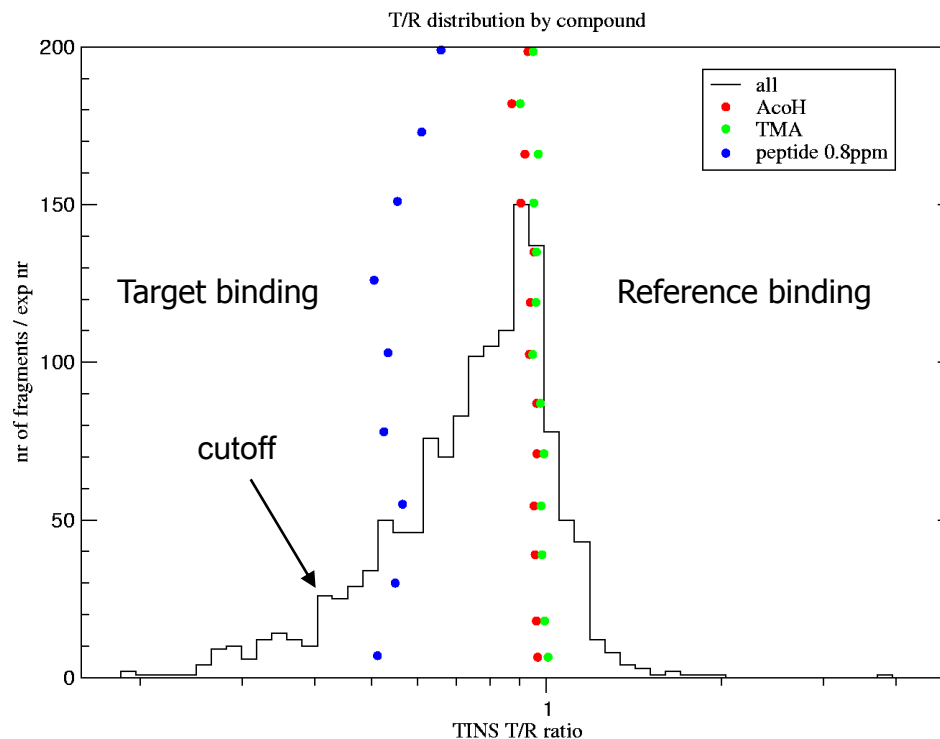
Immobilization	Schiff-base 1° amine coupling
Target /Reference AKT	75 μ M (0.6mg of 15 kDa)
Control peptide	250 μ M
ZoBio fragments	500 μ M
Buffer	dPBS

ZoBio was tasked with finding hit matter for MDM4 after both HTS and fragment based projects had failed. The functionality of immobilized protein was demonstrated by successfully showing binding of the p53 peptide (native ligand) as evidenced by the difference between the height of peaks in the red and blue spectra below.



Immobilized target is functionally active in peptide binding

TINS screen MDM4



The histogram represents the number of fragments in the screen with a T/R ratio in the indicated bucket values. The green and red dots indicate the T/R ratio of two small, non-binding molecules as assayed at various points during the screen. The blue dots are the average T/R ratio of the p53 peptide during the screen and indicate the residual activity of the immobilized PPI target.

1414 fragments screened

89 hits identified

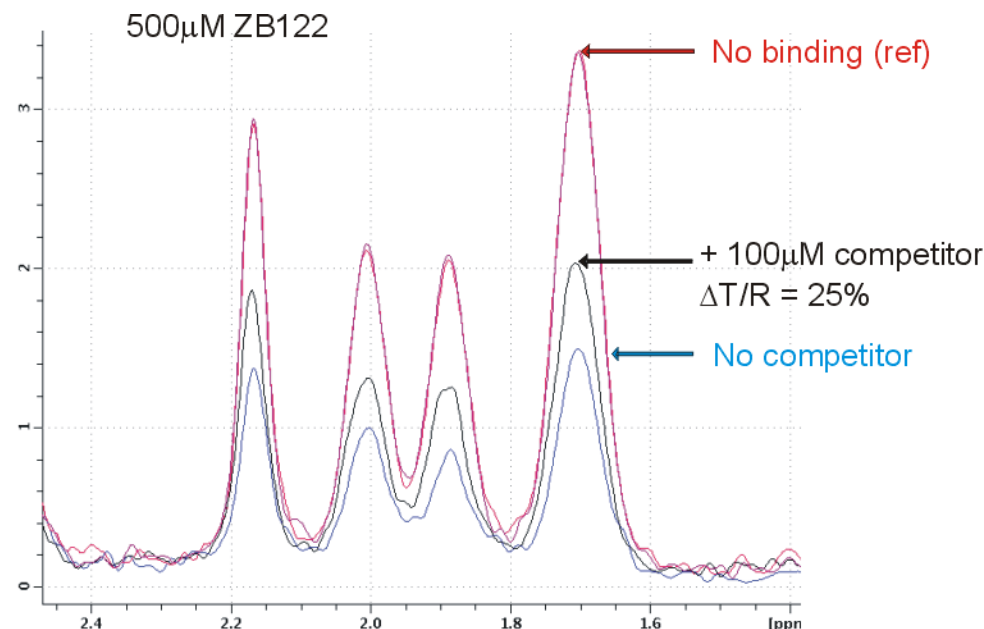
Binding site characterization by TINS Competition

Competition condition

Target / Reference 20 μ M
TINS delay parameter 80ms
ZB fragment 500 μ M
Nutlin 100 μ M

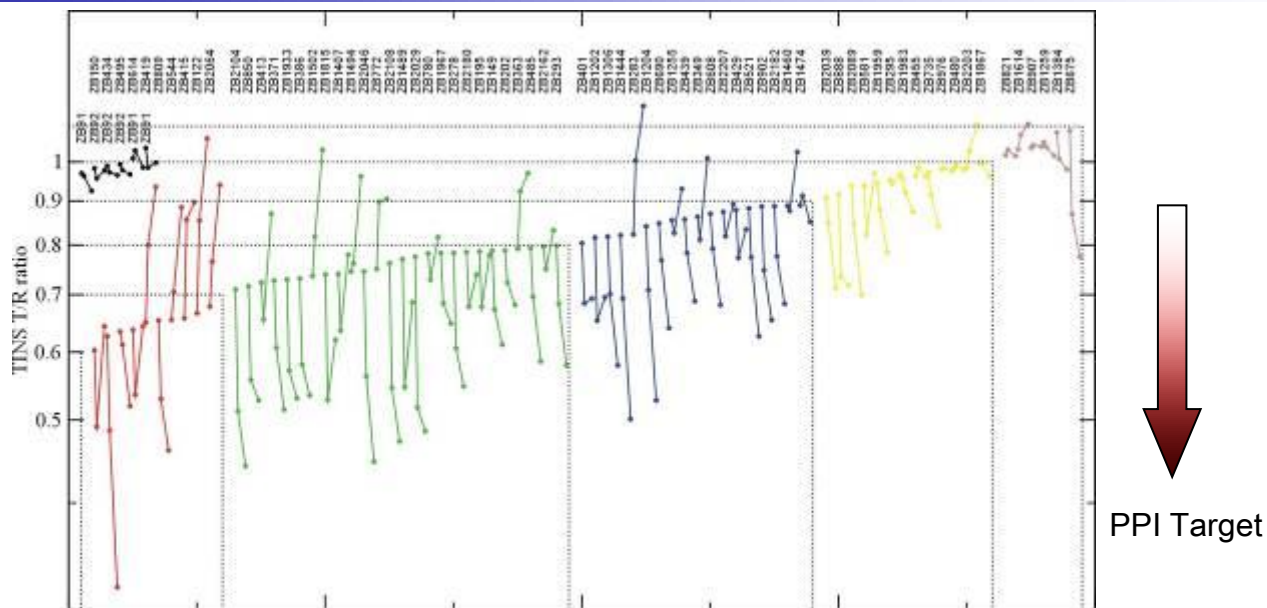
($K_D = 25 \mu M$)

Competitive criteria $\Delta T/R$ ratio >10%
Competitive ZB hits 27 out of 78



With the success of the screen the customer requested ZoBio to perform a number of validation and follow-up services. Competition binding with Nutlin, a small molecule inhibitor of the closely related MDM4 protein was the first. Nutlin is not an ideal tool compound since the ratio of its solubility to affinity is rather low. Nonetheless 27 of 78 hits from the screen that were assayed were shown to be competitive.

Target Specificity TINS Hits



ZoBio was then asked to determine the binding preference of the fragment hits for MDM4 vs MDM2. This was accomplished in a single experiment by immobilizing each protein in a cell of the dual-cell sample holder (see Marquardsen et al, J. Mag. Res., 2006, **182**, p. 55) and measuring the TINS response. In the figure above, 3 different TINS measurements were performed for each hit and the T>R value for each is connected by a line. Lines that go down indicate compounds that strongly preferentially bind MDM4, lines that remain roughly constant have weak preferential binding to MDM4 while those that go upwards have no or even slightly preferential binding for MDM2.

Summary Timeline of PPI

ZoBio Fragment Library Screen (1.5 month)

Feasibility (2 ~ 3 weeks upon arrival of the sample)

- 0.65mg of 15kDa protein delivered
- Immobilization via 1° amine to the resin by Schiff-base chemistry
- Functionality check on the immobilized target

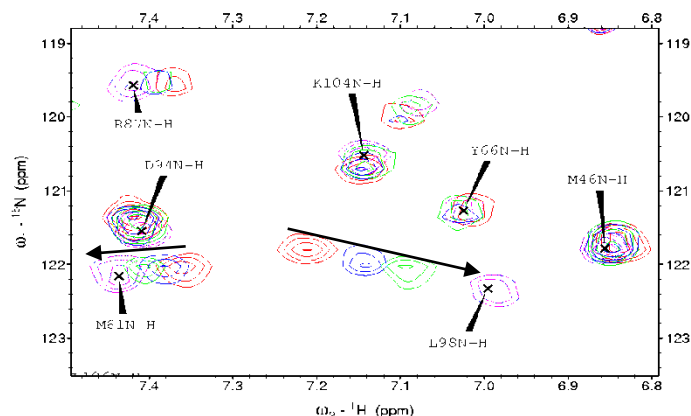
Library screen (2 weeks)

- 1414 fragments screened
- Data analysis
- 89 hits

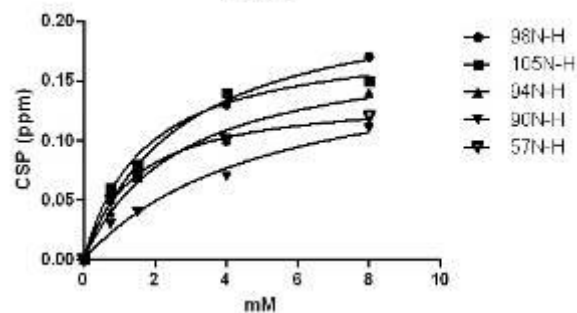
Validation (1.5 month)

- Singleton affinity ranking (2 weeks including data analysis)
- Selectivity among homolog (2 weeks including data analysis)
- Competition (2 weeks including data analysis)

K_D and Binding Site Characterization of ZB Fragment Hits



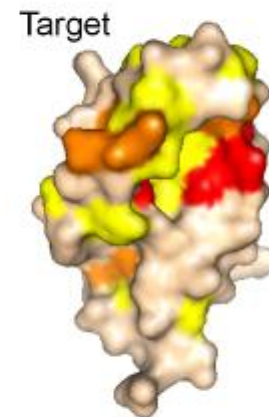
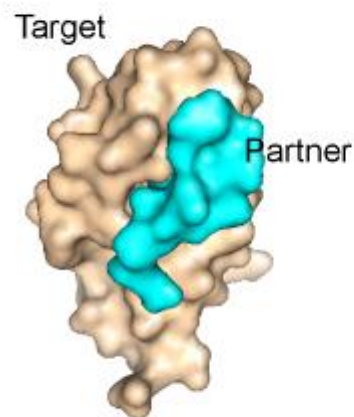
DMSO, 0.75, 1.5 & 4 mM
ZB122



In order to provide both affinity and binding site information, 86 of the TINS hits were titrated into isotope (¹⁵N) labeled protein and a 2D NMR spectrum of the protein was acquired (this is similar to the “SAR by NMR” approach). 84 of the hits were positive and by mapping the changes in the spectra to amino acids in the crystal structure of MDM4, the binding site of each was determined. All fragments bound at the p53 peptide binding site.

CSPs observed for 84/86 TINS Hits

Low resolution binding site determination



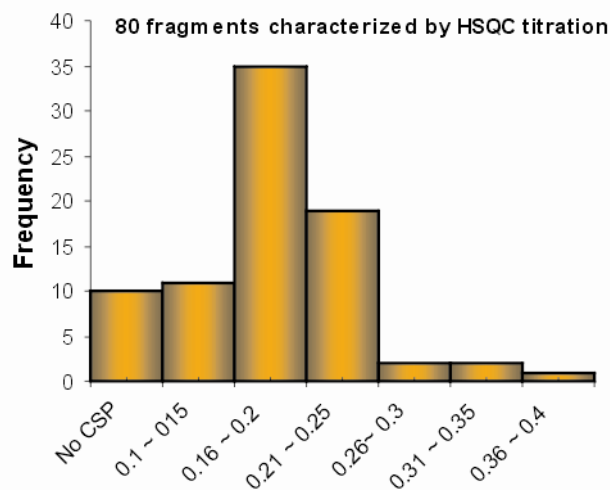
CSP: ■ > ■ > ■

All hits bind in the vicinity of PPI site

Characteristics of Fragment Hits from TINS

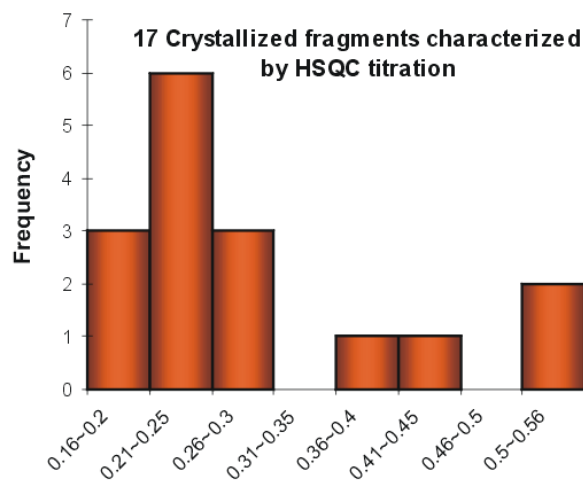
All TINS hits were assessed by [^{15}N , ^1H]-HSQC titration

Against MDM4

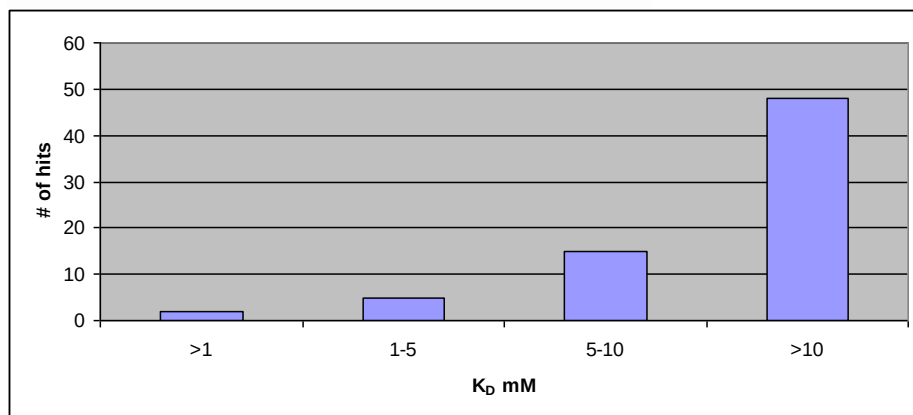


LE of initial fragment hits

Against HSP90



LE of crystallized hits

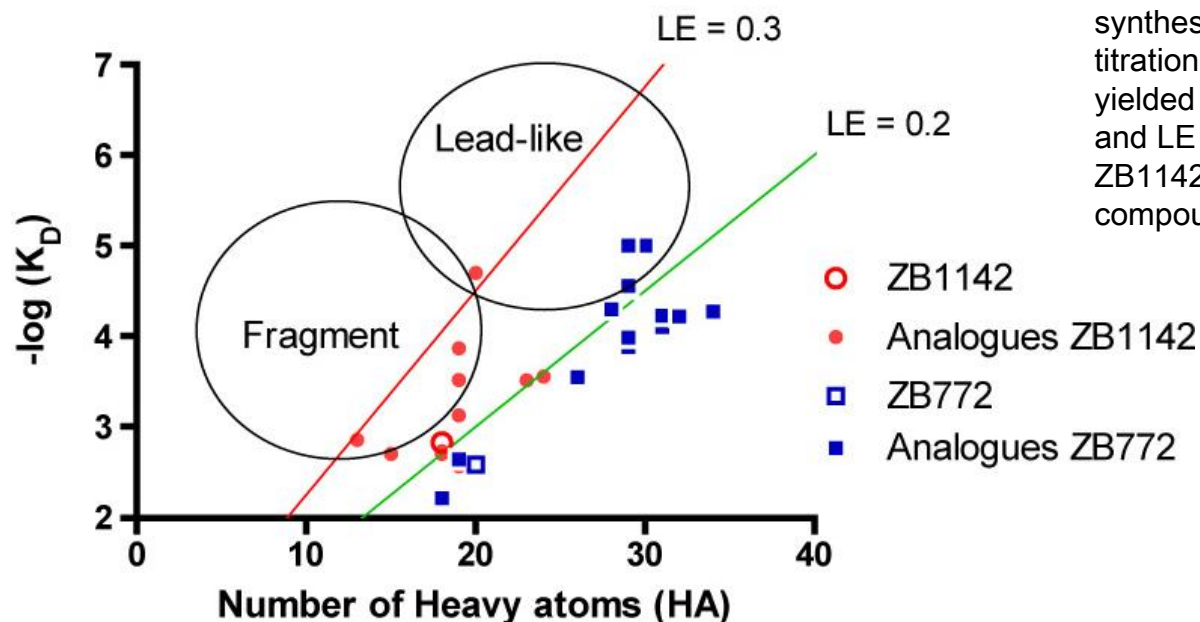


The validated hits from MDM4 are compared to the 17 fragments for which crystal structures were obtained with HSP90. Note the typically lower LE for the protein-protein interaction target and the very low affinities of the initial hits (below left). Using screening methods other than TINS, more than 90% of the hits would have been missed.

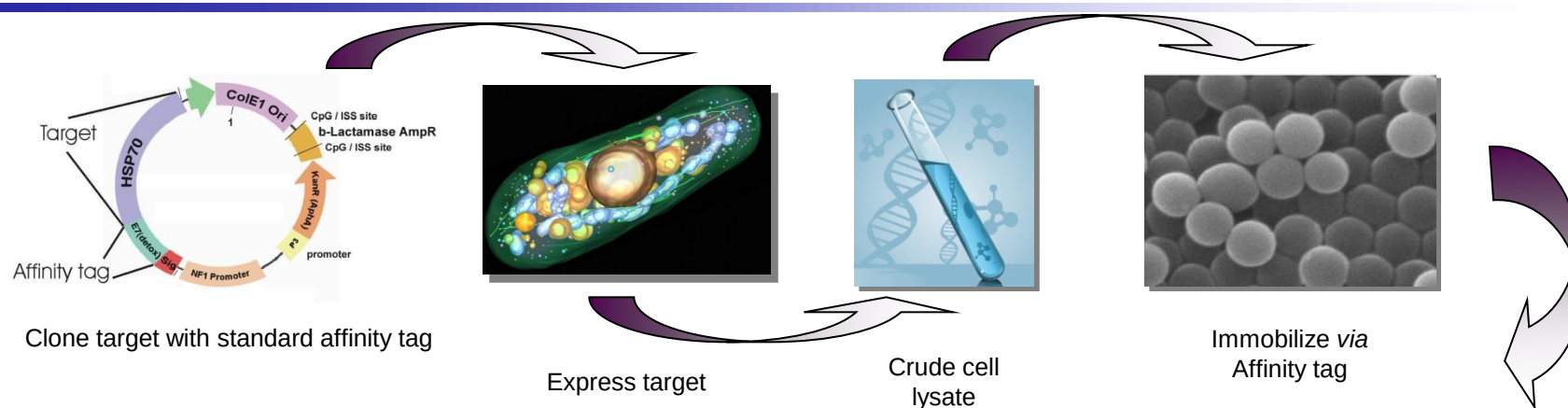
Elaboration of TINS hits guided by K_D from NMR titration

- X-ray crystallography failed to obtain protein – fragment complex
- Binding site and K_D determined by HSQC titration supported ligand-target docking

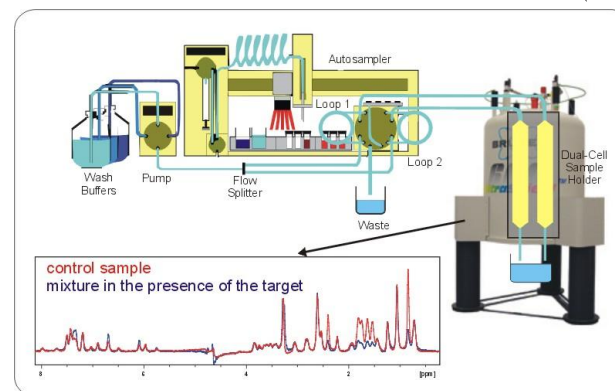
Analoging of TINS hit against PPI



Case Study 3 – Large Scale Druggability Screening

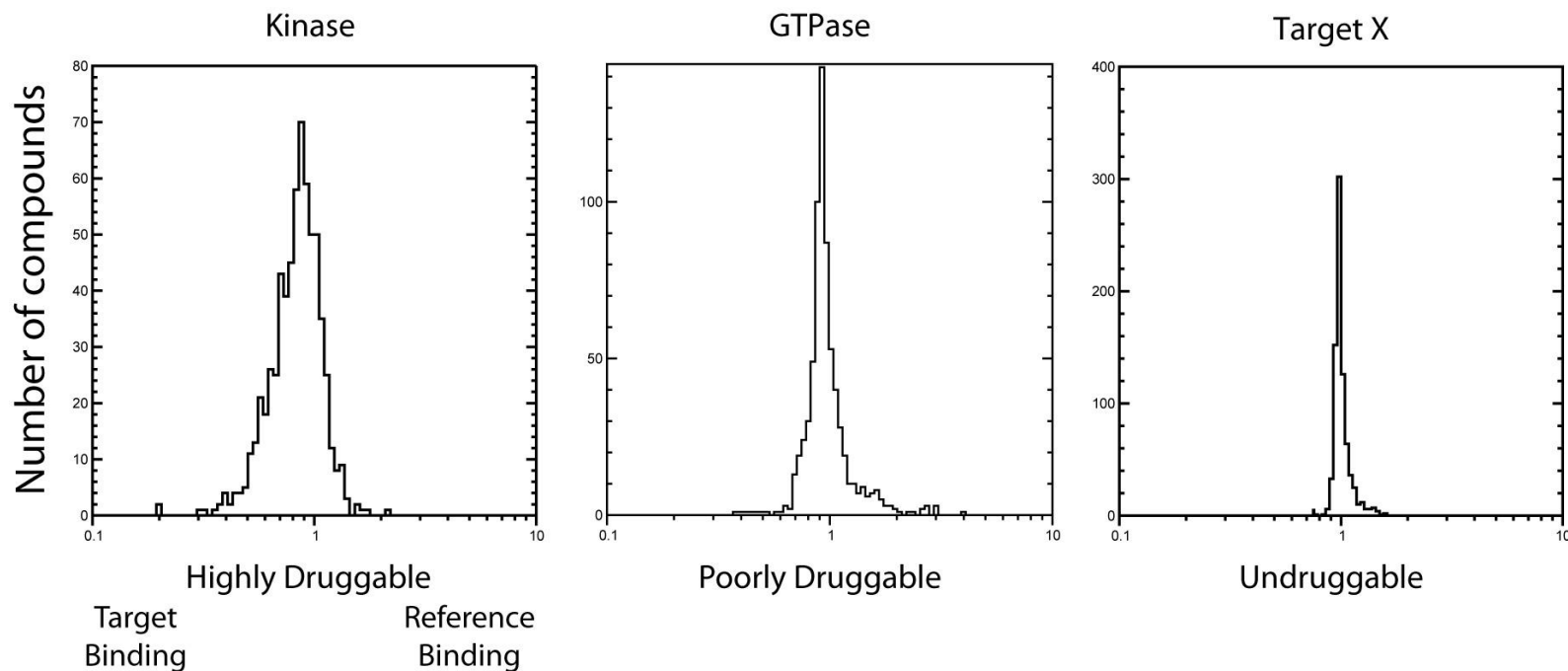


We (and others e.g. Hajduk *et al*, 2005, J. Med. Chem, 48, p. 2518) have noted a strong correlation between the hit rate (and hit diversity) in NMR fragment screening and the “druggability” of a target. The scheme presented here will allow large scale (15-20 targets/yr), efficient fragment screening on targets at a very early stage in the drug discovery process (e.g. before HTS and even before a purification strategy has been developed). In this way early prioritization based on data can be made, HTS results can be more intelligently interpreted and FBDD can have an early start.



TINS fragment screen

5 – Large Scale Drugability Screening



It has been noted (Hajduk *et al*, 2005, J. Med. Chem, 48, p. 2518, Edfeldt *et al.*, Drug Discovery Today, 2011,**16**, p 284-287) that there is a strong correlation between the hit rate (and hit diversity) in NMR fragment screening and the “druggability” of a target. ZoBio has developed its own correlation between TINS and druggability as shown above. In TINS, binding is best represented by the ratio of the heights of each peak of a compound in the presence of the reference and the target (the T/R ratio). Each of these graphs presents the number of compounds with a T/R in each of the bins presented on the x-axis. This gives a profile of the screen that is unique for each target. The figure above demonstrates how the profile varies with the known druggability of three different targets.

ZoBio Recommended workflow

- Primary TINS Screen
- Orthogonal validation/quantitation
 - NMR
 - SPR
 - Biochemistry (when available)
- Computational Studies
- Analog studies
- Structural Biology

Project Structure

- Typically, client provides target and known ligands or enzymatic assay.
- ZoBio performs feasibility study to show functional, immobilized target. ~2-3 weeks
- Client approves feasibility study and commits to screen. ~2-3 weeks
- Validation/characterization stages. ~4-6 weeks each

ZoBio Collaborators & Customers



NMR Hardware



Fragment Library



Abbott Laboratories

TINS testing



UNIVERSITY of VIRGINIA

John Bushweller DsbB



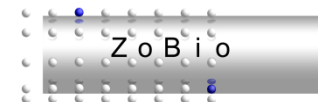
GlaxoSmithKline

BioFocus DPI
A Galápagos Company



Galápagos

Johnson & Johnson Pharmaceutical Research
& Development,
a division of Janssen Pharmaceutica NV



Tools for fragment based drug discovery