Applications of NMR in (Fragment-Based) Drug Discovery

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Fragment Based Lead Discovery at Vernalis

- Vernalis - biotech in Cambridge, UK
  - Founded in 1997, spin-out from LMB Cambridge
  - Developing FBLD approaches since 1998: RNA, proteins
- Collaborations across many therapeutic areas
  - Academics, large & small pharma
  - Eight development candidates generated in the past eight years
- Focus on “challenging” targets
  - Protein-protein interactions
    - Bcl-2, Mcl-1 programmes in Phase I
- FBLD is key part of overall SBDD strategy
  - Biophysics and structural biology
Early Stage Drug Discovery (and Chemical Biology)

- Hit Identification
  - High Throughput Screening
  - Fragment-based Lead Discovery
  - Virtual Screening
- Hit-To-Lead
  - Modify chemotypes & scaffolds
  - Affinity, specificity, physchem

Target Hypothesis  Hit Identification  Lead Optimisation
Pre-Clinical  Hit-To-Lead  Target Validation

Design  Test  Make
Why fragments?


Fragments & ligand efficiency

- Ligand efficiency
  - Key concept for fragments
  - Binding energy per heavy atom
- Low MW startpoint will have lower affinity because of small size
- Defining feature of FBLD
  - Fragments are no different to any other hit; just small
  - Low affinity is purely a result of size
  - Each fragment represents a large area of chemical space
- Low affinity will have major implications for Hit ID and evolution
  - Careful experimental design
    - Robust assays, reliable validation – low error rate
  - Strategies for fragment evolution
    - Transition from low affinity “fragment” to more potent “hit”

\[ LE = \frac{(-2.303RT)}{HAC} \log K_D \]

Intrinsic LE of target

- “Intrinsic” ligand efficiency of a binding site varies from protein to protein
  - LE varies from at least 0.6 to 0.15
    - Low intrinsic LE (0.2-0.35)
    - Medium intrinsic LE (0.3-0.45)
    - High intrinsic LE (> 0.4)
- Predict expected $K_D$
  - Assay must be robust and reliable over this range

<table>
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<th>1mM</th>
<th>100uM</th>
<th>10uM</th>
<th>1uM</th>
<th>100nM</th>
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</table>

480 target-assay pairs with more than 100 compounds covering 329 human drug targets

Hopkins et al. (2014) Nat Rev Drug Disc. 13 1474-1776
Detecting Fragment Binding

- Fragments typically 10-18 HAC
- Predicted $K_D$s in the region of 10mM – 10nM
  - Typically 1mM – 10µM
- Choice of assay will depend on expected $K_D$
  - Reliability range of assay
  - High LE targets: e.g. $K_D$ 10µM
  - Low LE targets: e.g. $K_D$ 1-10mM
- Biophysical binding assays
  - Widely used, robust and generic
  - Direct observation of bound species
  - Information rich data
Artefacts and Errors

• Expected $K_D$'s in the region of 10mM – 10nM
  • Most typically 1mM – 10µM
• Ligand concentrations typically 1-10x $K_D$
  • 100µM – 10+ mM
• Pushing most assays to their limits

• Easy to mistake artefacts for weak binding
  • At [L]=1mM a 1% contaminant is 10 µM
  • Assay interference from high concentrations of compounds
    • pH, redox behaviour, DMSO, metal chelation, detergents, fluorescence or absorption, interference with secondary/coupled detection system
  • Compound solubility & aggregate formation

Learning from our mistakes: the 'unknown knowns' in fragment screening
Davis & Erlanson (2013) Bioorg Med Chem Lett. 23(10):2844-52
Artefacts when characterising low affinity interactions

• Need to identify and characterise interaction between ligand and protein with a high degree of confidence
  • Particularly an issue with FBLD – easy to mistake artefacts for binding
  • Subsequent work (particularly medicinal chemistry & biology) hinges on understanding this interaction

• Need to be sure of:
  • Is the protein what I think it is?
  • Is it folded correctly and relevantly?
  • Is it stable over the required timescale?
  • Is the ligand what I think it is?
  • Is the ligand stable over the required timescale?
  • Do the ligand and protein actually interact to any significant extent in the relevant conditions?

• What's the structural basis for this interaction?

• Confidence to focus on and progress a hit series into a lead
Multiple soaks are often required to obtain a crystal structure of a fragment

<table>
<thead>
<tr>
<th></th>
<th>Fragments which gave xtal structure</th>
<th>Av. Number of attempts /fragment (total)</th>
<th>Av. Number of attempts /fragment (to get structure)</th>
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<tr>
<td>HSP90</td>
<td>79%</td>
<td>1.6</td>
<td>1.3</td>
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<tr>
<td>Kinase A</td>
<td>55%</td>
<td>1.9</td>
<td>1.9</td>
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<tr>
<td>Kinase B</td>
<td>30%</td>
<td>2.0</td>
<td>2.5</td>
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<tr>
<td>Allosteric Target A</td>
<td>52%</td>
<td>1.6</td>
<td>1.8</td>
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<tr>
<td>PPI Target A (occluded active site)</td>
<td>0%</td>
<td>n/a</td>
<td>n/a</td>
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</table>

- Vary:
  - Soak duration 16hr - 7 days
  - Temperature 4C, 20C, 30C
  - [Ligand] Start high & reduce
  - Ligand preparation

- If these don’t work:
  - Crystal form, Space group, Packing, Construct

- Protein engineering

- Highly resource intensive - confidence
Role of NMR in Drug Discovery

- **Solution NMR**
  - Large amounts of material
  - Not high throughput
  - Quantitation poor compared to other methods
  - Expensive & specialised
  - But…
  - Allows direct observation of (most) species present in solution
  - With care, very low false positive and false negative rates
  - High levels of confidence in the data
  - Characterisation of molecular interactions by NMR
    - Ligand, receptor and putative complex
    - Integrate with other biophysical and biochemical methods

Reality check - kick the tyres
Fragment Based Lead Discovery

Characterised Target → Curated Library → Robust Assay → Preliminary Hits → Validation → Characterisation

Fragment Based Screening (FBS) → Fragment Hits → Structure
Characterised Target Protein QC

- Simple $^1$H 1D of every batch of protein
  - Focus on amides and shifted aliphatics
- Batch-to-batch variation
  - Co-factors (e.g., Zn$^{2+}$)
  - Expression levels; handling; ...
- Estimate $\tau_c$ from 2 point spin echo
  - J. Biomol. NMR (1993) 3, 121-6
- Sample degradation over time
- Thermal stability & reversibility

Ratio 0.56
$\tau_c \approx 23$ ns
$\text{Mw}_{(\text{eff})} \approx 46$ kDa
(expected 50 kDa)
Characterised Target Protein interactions

DMSO & pH controls
- Titrate simple acid or base
- Buffer components
- Phosphate buffer
- Reducing agents
- Metal ions

Compound MOA

Tween-20 KD 20mM (0.025%)
Fragment Based Lead Discovery

Characterised Target → Preliminary Hits → Validation

Curated Library → Robust Assay → Characterisation

Fragment Based Screening (FBS) → Fragment Hits

Structure
Curated Library

- **Correct compound?**
  - Vendors & chemists do make mistakes
  - Correct isomer (bosutinib, TIC10)
- **Impurities**
  - Low levels of potent impurities
  - Metals
- **Compound stability**
  - Long term DMSO, 24 hour aqueous
- **Reactive molecules**
  - PAINS (pan-assay interference compound)
  - Redox cyclers
- **Aggregators & self-associators**
  - Particulate formation

![Chemical structures](image1)

**Water-LOGSY**

**zgesgp**

**DMSO**
Fragment Based Lead Discovery

- Characterised Target
- Curated Library
- Robust Assay
- Fragment Based Screening (FBS)
- Preliminary Hits
- Validation
- Characterisation
- Structure
Fragment Screening Methods

• NMR, SPR, TSA, MST, X-ray, biochemical assays ...
  • All suffer from artefacts – no assay is perfect
• Which technique to use?
  • Availability, expertise, throughput, resource, sensitivity, accuracy & precision
  • Primary vs orthogonal methods
• If the experiment is well configured, and the library is good, all techniques can give robust results
  • Quality and completeness of data will vary
  • Understand limitations of technique and validate preliminary hits carefully

• Examples of recent workflows at Vernalis:
  • Ligand observed NMR or SPR as primary screen
    • Protein observed NMR, MST or X-ray as orthogonal validation
  • X-ray as primary screen, SPR as secondary validation
    • Target readily crystallised, protein production challenging
  • Biochemical assay followed by ligand and protein observed NMR
    • High intrinsic LE, expecting $K_D < 10\mu M$
Fragment Screening by NMR

- Sensitive - detect binding at [L] below $K_D$
- Robust – low false positive & false negative rates
- Generic - little optimisation required, no chemical modification or labelling

Observe Protein
- **Chemical shift perturbations (CSP)**
- Direct indication of binding site ($^{13}$C HSQC)
- Size restricted
  - ~ 50 kDa; Labelling strategies
- Quantity of material
  - Large amounts of isotopically labelled protein

Observe Free Ligand
- **Modulation of ligand spectrum by interaction with receptor in bound state**
- Usually observe the free state of the ligand
- Less demanding on receptor supply and properties
- Infer binding site
- **COMPETITION STEP**
Widely used ligand observed NMR experiments

**STD**

\[ I_{\text{obs}} = f(P_{\text{bound}}) \]

**Water-LOGSY**

\[ I_{\text{obs}} = f(P_{\text{bound}} - P_{\text{free}}) \]

**Relaxation Edited \(^1\text{H}\) or \(^{19}\text{F}\)**

\[ I_{\text{obs}} = f(P_{\text{free}}) \]

Dalvit et al. (2001) J. Biol. NMR 21, 4, 349-359
Hajduk et al. (1997) JACS 119, 50, 12257-12261

**Robust test sample** (Davis (2013) MiMB 1008 389-413)

- 10 μM avidin
- 500 μM octanoic acid
- 500 μM 2-imidazolidinone
- 500 μM sucrose
- 20 mM potassium phosphate pH 7.5
- 10 % D2O
- ± 20uM biotin
Compound b binds and is displaced by competitor in all experiments ("class 1" hit)
NMR “Binding class”

- Empirical indication of confidence levels
  
  *(Not distinguishing between experiment types; this varies from protein to protein)*
  
  - Class 1  binds *(and displaced)* in all three experiments
  - Class 2  binds *(and displaced)* in two of three experiments
  - Class 3  binds *(and displaced)* in one of three experiments

- Success in crystallographic follow-up
  
  *(averaged across projects with routine crystallography)*
  
  - Class 1  75%
  - Class 2  52%
  - Class 3  41%

- More consistent behaviour across multiple experiments increases chance of obtaining crystal structure
  
  - But cannot ignore class 2 and class 3 hits – valuable information
Inconsistent results from orthogonal methods

• Inconsistencies observed between results from different NMR experiments
  • Same sample, same conditions, same time
• Consider role of orthogonal validation
  • “Hard” filter
• Soft filter required to assess overall data package
  • Class used for prioritisation, not exclusion
• More generally ...
  • What is the best way to combine output from orthogonal validation?
• Why are assay results from orthogonal methods inconsistent?
  • Compound issues
  • Differences in conditions
  • Experimental error
  • Confidence levels
  • Different measured parameters

• Synergy between techniques
Examine inconsistencies between techniques
“Kin1” Case Study

- Collaboration with Genentech
- SPR and biochemical assay consistent
  - Wild type, single phosphorylation site (Kin1-1P)
  - Low expression levels
- Kin1-1P expression poor
  - Use Kin1-DN mutant for NMR and X-ray
  - Expresses at high levels
- NMR inconsistent with SPR
  - Extremely high hit rate cf SPR and biochemical
- Anomalous water-LOGSY spectra
  - “inversion” on addition of staurosporine (red)
- System not behaving as expected
  - Low success rate in crystallography
- SPR systematic deviation wt vs mutant
Protein construct and buffer optimisation

- Alternative protein construct designed
  - Kin1-TA
  - Expresses readily
- Behaviour more consistent
  - More heat on binding potent compound
- Larger STD signal for adenine control
  - Anomalous water-LOGSY still observed
- Optimise buffer conditions
  - NMR: titrate MgCl₂
  - Anomalous LOGSY reduced
- FBS under these conditions
  - NMR:SPR show >90% correlation
- Tractable crystallography
- 34 bound fragment structures
Fragment Based Lead Discovery

- Characterised Target
- Curated Library
- Robust Assay
- Fragments Based Screening (FBS)
- Preliminary Hits
- Validation
- Characterisation
- Structure
Setting up an NMR Fragment Screen

- Ideally, we have a known low affinity ligand (“probe”)
  - Positive control
  - Substrate or product analogue, literature compound, binding partner

- Can we observe binding of the probe to the target protein?
  - Do related compounds bind?
  - Is this binding stable?
  - What timescale is the protein stable over?

- Can we displace the probe with a known potent ligand (“competitor”)?
  - How much competitor do we need?
  - Is the competitor stable?
Testing the binding assay:
Probe binding and competition

- Known low affinity ligand
- Known potent ligand
- Clear binding
- Clean competition
- Structurally related compounds also show the same behaviour

500 µM probe, 10 µM protein
+25 µM mid nM competitor
Displacement of probe by competitor

500 μM probe, 10 μM protein
25 μM competitor
50 μM competitor
100 μM competitor
Separate samples. STD spectra, competitor added after:

<table>
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<tr>
<th>Time (hr)</th>
<th>Spectra</th>
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<tr>
<td>0</td>
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- Bulk sample of protein + probe prepared, split into four samples
- 50 µM competitor added after specified time
- Stability of binding and competition monitored at specific times
Competitor compound stability

- Competitor decarboxylates on standing in DMSO (stable in aqueous solution)
- Literature compound
  - Crystal structure of protein:ligand complex in PDB, density missing for carboxylate
• Competitor has limited stability in DMSO
  • Degraded material does not bind to protein
• Fragment screen as usual
  • Make up fresh competitor stock immediately prior to use and QC
• Initially ~100 compounds (“trial library”) to troubleshoot
  • 2 days
• Scale up to screen full library
  • ~1500 compounds
  • Mixtures and singletons
  • 3% validated hit rate
Fragment Based Lead Discovery

Characterised Target

Curated Library

Robust Assay

Preliminary Hits

Validation

Characterisation

Structure

Fragment Based Screening (FBS)
**Fragment validation and characterisation**

- **Validation** of preliminary hits
  - Initially “data are consistent with binding” ...
  - Orthogonal screening methods
    - MST, SPR, crystallography if routine
  - Protein vs ligand observed NMR
    - Single point HSQC
      - Check ligand solubility first

- **Characterisation** of validated hits
  - Affinity
    - $K_D$ [$k_{on}$, $k_{off}$, $\Delta G$, $\Delta H$ & $T\Delta S$]
  - Structure of protein:ligand complex
    - Crystal [solution, dynamics]
    - NMR [full structure or NMR guided model]
1D $^1$H Protein observed CSP

- $^{15}$N-$^1$H ($^{13}$C-$^1$H) HSQC “gold standard” for low affinity interactions
  - Isotopic labelling, slow, size limitations
- $^1$H resonances shifted below ~ 0.5ppm typically arise from hydrophobic core
  - Frequently perturbed by ligand binding, particularly for proteins < ~ 35 kDa
- Determine KD from HPC CSP
  - Correlates well with $^{15}$N-$^1$H HSQC KD
- Magnitude of HPC single point shift correlates with HPC KD
  - Use single point HPC CSP to validate compounds prior to HPC KD titration
- Rapid, inexpensive, robust
- For recent early stage project
  - 735 single point CSP in 6 months
  - 171 KD determinations
- Widely used for early stage projects

Increasing [compound]
NMR derived eKᵢ

- **¹⁹F containing ligand**: “probe”
  - -CF₃ works well
  - Known Kₛ
- Displace with test molecule
  - Reduces [protein]ₑff, R₂
  - Calculate $\%_{\text{inhib}}$ and eKᵢ
- ~ 30 compounds/night
- Simple analysis

Example conditions
- 8uM protein
- 25uM probe ligand
- 100uM test ligand
- 400ms 19F CPMG

Linear dependency of $^{19}$F R₂ on [protein]

Evolving Fragments without Crystal Structures

- Can find & validate fragments that bind
  - Evolution requires robust model of fragment binding
- Guide medicinal chemistry with structural model
- Best model is from X-ray structure
  - X-ray structures not always available
  - Don’t rule out a target just because crystallography is challenging
- NMR structures
  - Full structure is time consuming, too slow for routine use
  - Data is incremental – NMR guided modelling
- NMR guided models often good enough to guide chemistry
  - Ligand observed
    - STD-GEM, ILOE & trNOE
  - Protein observed
    - Chemical shift perturbations (CSP), filtered edited NOESY
- Track binding models with NMR data and SAR
NMR Guided Models
Proof of concept

- Low success rate for crystallography with fragments for Bcl2
  - Require alternative methods to steer medicinal chemistry
- NMR guided models
  - Assign ligand in bound state using 13C,15N purged 1Ds & NOESYs
  - Acquire 3D 13C-edited, 13C15N-filtered NOESY (X-Filtered NOESY)
    - Identify intermolecular NOEs between ligand and protein
  - Generate ensemble of protein conformations
    - Experimental or computational
  - Dock into ensemble
  - 26 NOEs observed between Bcl-2 and VER-00155493
  - Use NOEs as filter of docking poses
- Excellent agreement with crystal structure
NMR Guided Models
Project Applications

- In absence of routine crystallography, steer chemistry with robust models
  - Sparse NMR data, modelling and SAR
  - > 60 NGMs determined over recent PPI projects
- 1.5 weeks from submission to model
  - NMR solubility determination
  - HPC single point; KD
  - 15N-1H HSQC (binding site, KD)
  - NGM titration (0, 0.5:1, 1:1)
  - NGM acquisition (2.5 days)
  - Modelling (1 week)
- Models suitable for purpose
  - Protein conformation characterised
  - Binding site identified
  - Ligand orientation determined
  - Vectors orientated correctly
  - RMSD 2.5-4Å
    - Where crystal structure later determined
- Confidence to make difficult molecules
Summary and Conclusions

- FBLD is a well validated, robust method of identifying ligands for drug discovery
  - Fragments are inherently low affinity due to small size
  - Care must be taken with low affinity ligands in order to avoid artefacts

- NMR is a powerful technique for identifying, validating and characterising low affinity ligands
  - “Reality check”
  - Identify issues before investment of resource

- Integration with other biophysical methods can reveal valuable insights

- Fragment evolution can be guided by NMR in absence of crystal structures
  - Generation of structural data in timely manner
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