Exploiting the information rich output of SPR biosensor technology in drug discovery – from fragment to lead

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Surface plasmon resonance (SPR) biosensor technology

**Diagram:**
- **Light source**
- **Prism**
- **Detector**
- **Sensor chip with gold film and dextran matrix**

**Graph:**
- **Response (RU)**
- **Time (s)**
- **Baseline**
- **Association**
- **Dissociation**
- **Injection start**
- **Injection stop**

**Text:**
- Ligand in buffer
- Biacore™ T200, 4000 etc
- Baseline
- Association
- Dissociation
The technology is well established
The high information content is recognized
Implementation is sometimes problematic

The user friendliness of commercial SPR biosensors is deceptive:
- Ease of use is not the same thing as ease of implementing the technology for actual projects
- Challenges in all steps from experimental design to interpretation of data

Why use it?
SPR biosensors are applicable throughout the lead discovery process

Target evaluation

Exploitability
Feasibility
Drugability

Hit identification & fragment lead generation

Fragment screening & hit characterization
Affinity and kinetics
Selectivity vs off-targets
Binding site specificity
Conditions/co-factor dependence

Lead generation & optimization

Interaction characteristics
Mode-of-action
High-resolution kinetics
Binding-site location
Structure-kinetic relationships
Thermodynamics
Chemodynamics
Serum protein binding
Understanding the regulation of synaptic signalling via protein-protein interactions and Ca$^{2+}$
Calcium binding proteins

![Diagram of calcium binding proteins](image-url)
Caldendrin - a calcium sensor in neurons

- Caldendrin/calbrain/calcium-binding protein 1
- Closest calmodulin homologue in the brain
- Hypothesized to interact with other synaptic proteins

Caldendrin

Calmodulin
A-kinase anchoring protein (AKAP) 79/150 is a master scaffolding protein in the postsynapse.

- Coordinates phosphorylation and dephosphorylation of receptors via anchoring kinases and phosphatases near their substrates.
- Calmodulin binds the B-domain of AKAP – does caldendrin too?

**Synaptic scaffolding proteins**
Caldendrin hypothesized to interact with AKAP79/150

Cell biological experiments revealed:
- Caldendrin is a binding partner of AKAP79
- Binding site mapped to B-domain
- Binding is competitive with calmodulin and dependent on Ca$^{2+}$
- Caldendrin and AKAP79/150 co-localize in dendrites
Caldendrin, calmodulin and AKAP interactions confirmed by SPR

Experimental design

B-domain of AKAP79

+ Calmodulin

+ Caldendrin

in Ca\(^{2+}\) buffer
Interaction between caldendrin and AKAP79/150 established

AKAP79/150 interacts with the neuronal calcium–binding protein caldendrin


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¶Anatomy, Westfälische Wilhelms University Münster, Germany

Molecular interpretation required more detailed mechanistic and kinetic study!
Caldendrin and calmodulin interact with different mechanisms

Different concentrations

Different injection times

Is the dissociation rate dependent on the injection time?
Yes, for caldendrin
Calmodulin and AKAP79 only interact at high $[\text{Ca}^{2+}]$.

Calmodulin interaction with AKAP79 is well described by a simple reversible interaction mechanism:

$$ CaM + AKAP \rightleftharpoons CaM - AKAP $$

where $k_1$ and $k_{-1}$ are the forward and reverse rate constants, respectively.
Determination of caldendrin interaction mechanism

Vary injection time and concentration

\[ A + B \rightleftharpoons AB \rightleftharpoons ABx \]

\[ A + B1 \rightleftharpoons AB1 \] \[ A + B2 \rightleftharpoons AB2 \]

Most logical model: \[ Ca^{2+}, k_1 \]

\[ CDD + AKAP \rightleftharpoons CDD - AKAP \rightleftharpoons "CDD - AKAP" \]
Caldmodulin only interacts with AKAP79 at high $[\text{Ca}^{2+}]$
Caldendrin and AKAP79 interact at high and low \([\text{Ca}^{2+}]\)

**A**

- **+ Ca\(^{2+}\)**
  - 16 nM
  - 31 nM
  - 63 nM
  - 125 nM
  - 250 nM

- Large amount of complex formed (250 nM)

**B**

- **No Ca\(^{2+}\)**
  - 63 nM
  - 125 nM
  - 250 nM
  - 500 nM

- Small amount of complex formed (500 nM)
Calmodulin and caldendrin compete for binding to AKAP79

Expected signal if no competition

- **Calmodulin+Calmodulin (theor.)**
  - 31 nM proteins
- **Caldendrin+Calmodulin**
  - 31 nM Calmodulin
  - 31 nM Caldendrin
- **Caldendrin+Calmodulin (theor.)**
  - 125 nM proteins
  - 125 nM Caldendrin
  - 125 nM Calmodulin
Caldendrin and calmodulin have different roles in synaptic function

Caldendrin and calmodulin interactions with AKAP79 differ:

- Kinetically and mechanistically
- Different calcium dependence

![Diagram showing interactions between Calmodulin, Caldendrin, and Ca\(^{2+}\)-dependent binding]
Caldendrin and calmodulin have different roles in synaptic function

Caldendrin and calmodulin interactions with AKAP79 differ:

- Kinetically and mechanistically
- Different calcium dependence

![Diagram showing Ca\(^{2+}\)-dependent binding and Ca\(^{2+}\)-independent conformational change between Calmodulin and Caldendrin]
Relevance of caldendrin interactions with AKAP 79/150

- Caldendrin may have a specific regulatory role in the synapse
- Potential effects by caldendrin on synaptic signalling via PKA and AKAP
- SPR biosensor analysis has provided a molecular understanding of caldendrin interactions with AKAP and how they differ from calmodulin
From fragment to lead

Identification and validation of fragment hits – membrane bound targets
Cys-loop receptors – pentameric ligand gated ion channels

Nicotinic acetylcholine receptor (2BG9.pdb)
Gated by GABA, major inhibitory neurotransmitter in CNS

Involved in neurological disorders, like anxiety and depression

Modulated by clinically relevant drugs

- Benzodiazepines
- Anaesthetics
Modular pentameric structure provides variable specificity

Diazepam

GABA

Histamine

β3

β3

Diazepam

GABA

β3

β3
Capture of solubilized GABA<sub>A</sub> receptor via His-Ab

- Homo-oligomeric β3 GABA<sub>A</sub> receptors with His8-tag expressed in baculovirus infected Sf9 cells
- Solubilized receptor membranes captured to Ab-surfaces
Screened 15 GABAergic and 51 histaminergic compounds

<table>
<thead>
<tr>
<th>Ligand</th>
<th>M [g mol⁻¹]</th>
<th>Ligand</th>
<th>M [g mol⁻¹]</th>
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<tbody>
<tr>
<td>4-Piperidine-sulfonic acid</td>
<td>165.2</td>
<td>(R)-α-Methylhistamine</td>
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<td>Etomidate</td>
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<td>4-Methylhistamine</td>
<td>125.2</td>
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<td>Flumazenil</td>
<td>303.3</td>
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<tr>
<td>Flurazepam</td>
<td>387.9</td>
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<td>Aminopotentidine</td>
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<td>Amthamine</td>
<td>157.2</td>
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<tr>
<td>Pentobarbital</td>
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<td>319.2</td>
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<td>Cimetidine</td>
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<td>Clemastine</td>
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<td>Conessine</td>
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<td></td>
<td>Fexofenadine</td>
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<td>Histamine</td>
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<td>ICI 162,846</td>
<td>306.3</td>
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</table>

GABAergic and histaminergic modulators are fragment-like
Interaction with histamine

$K_D = 100 \, \mu M$

MW 111.1
Sensorgrams for selected histaminergic ligands
## Affinities for GABAergic ligands

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$K_D$ [µM]</th>
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<tbody>
<tr>
<td>Etomidate</td>
<td>38</td>
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<tr>
<td>Propofol</td>
<td>42</td>
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<tr>
<td>PK-11195</td>
<td>61</td>
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<tr>
<td>Ro5-4864</td>
<td>69</td>
</tr>
<tr>
<td>Etazolate</td>
<td>79</td>
</tr>
</tbody>
</table>

**Assay useful for affinity determination and ranking**

- Etomidate
- Propofol
- PK-11195
- Ro5-4864
- Etazolate
## Affinities for histaminergic ligands

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$K_D$ [µM]</th>
<th>Histamine receptor activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thioperamide</td>
<td>13</td>
<td>H3/H4 antagonist</td>
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<tr>
<td>JNJ7777120</td>
<td>28</td>
<td>H4 antagonist</td>
</tr>
<tr>
<td>4-Methylhistamine</td>
<td>32</td>
<td>H4 agonist</td>
</tr>
<tr>
<td>Tiotidine</td>
<td>33</td>
<td>H2 antagonist</td>
</tr>
<tr>
<td>Burimamide</td>
<td>33</td>
<td>H2/H3 antagonist, H4 agonist</td>
</tr>
<tr>
<td>A-987306</td>
<td>46</td>
<td>H4 antagonist</td>
</tr>
<tr>
<td>Imetit</td>
<td>51</td>
<td>H3/H4 agonist</td>
</tr>
<tr>
<td>(S)-α-Methylhistamine</td>
<td>51</td>
<td>H3/H4 agonist</td>
</tr>
<tr>
<td>VUF 8430</td>
<td>54</td>
<td>H4 agonist</td>
</tr>
<tr>
<td>Clobenpropit</td>
<td>57</td>
<td>H3 antagonist, H4 agonist</td>
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<tr>
<td>Immepip</td>
<td>69</td>
<td>H3/H4 agonist</td>
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<tr>
<td>Famotidine</td>
<td>81</td>
<td>H2 antagonist</td>
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<tr>
<td>Histamine</td>
<td>98</td>
<td>endogenous H1/H2/H3/H4 agonist</td>
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<tr>
<td>Proxyfan</td>
<td>110</td>
<td>H3/H4 agonist</td>
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<tr>
<td>A-943931</td>
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<td>(R)-α-Methylhistamine</td>
<td>180</td>
<td>H3/H4 agonist</td>
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<tr>
<td>Iodophenpropit</td>
<td>300</td>
<td>H3/H4 antagonist</td>
</tr>
</tbody>
</table>
Competition analysis

Principle

- **Ligand 1**
- **Ligand 2**
- **Ligand 1 + Ligand 2 (no comp)**
- **Ligand 1 + Ligand 2**

Graph showing response over time: 
- **Response vs Time**
- **B**
- **R_{no-comp}**
- **±3 * σR**
- **Histamine**
- **Time [s]**
Mechanistic analysis

Alfaxalone interaction induces conformational change in receptor
Summary

- Unique histaminergic pharmacology of homopentameric β3 receptors identified
- Most ligands compete with histamine
- Ligand induced conformational changes detectable

- SPR assay for Cys-loop receptors has adequate sensitivity and throughput for fragment screening and lead optimization
Identification and validation of fragment hits – combining creative experimental design and data analysis with a SAR by catalogue strategy
Fragment screening and evolution – an iterative process

- Fragment library/designed compounds
- Experimental design
  - Molecular modelling, design & synthesis
  - Selection of hits/leads
  - Mechanistic interpretation
- Sensor surface(s)
  - Screening/characterization
  - Data analysis
Step 1: Fragment library screening

- 85 active site binders identified
  - $K_D$ values: 0.5–500 $\mu$M
  - LE values: 0.25–0.84 kcal mol$^{-1}$
  - ~25 chemical scaffolds identified amenable for medicinal chemistry exploration
- 3 allosteric binders identified
  - 3 unique scaffolds targeting a non-precedented allosteric binding site
- Assay development, screening and affinity determinations completed in less than 3 months
SAR by catalogue 1\textsuperscript{st} iteration

\begin{itemize}
  \item Fragments of interest selected
  \item Build an ‘active substructure’ hypothesis to search for analogues
  \item Use identified analogues for constructing pharmacophore hypotheses to prioritize among commercial analogues
  \item Refine hypotheses by testing Sprint\textsuperscript{™} analogues and purchased fragments
\end{itemize}

→ 83 fragments purchased
Step 2: SAR by catalogue 1st iteration

- 31 new fragment hits identified
  - $K_D$ values: 0.2–400 µM
  - LE values: 0.37–0.85 kcal mol$^{-1}$
  - Series specific SAR starting to emerge

- 18 new chemical scaffolds identified
  - Structural diversity significantly improved

- First iteration completed in 6 FTE weeks
  - Molecular modelling, purchase campaign, and interaction analysis
Fragment library screening using SPR

- Current instruments have required throughput and sensitivity
- Promiscuous binders easily detected
- Creative experimental design useful for selection of suitable hits
- Kinetic information typically not obtained
- Affinity estimates sometimes possible
- Ranking and selection of hits requires filters based on qualitative features
Advanced characterization of leads

Influence of model systems and conditions – from mechanisms and kinetics to chemodynamics
BACE1: $\beta$-secretase, $\beta$-site amyloid precursor protein cleaving enzyme

BACE1
(alias Asp2 or memapsin 2)

Amyloidogenic APP processing
Poor correlation between inhibition of BACE and APP cleavage in cells

- No discernible trend
- Why different?
  - Ectodomain vs. full length enzyme?
  - Conditions (pH 4.5 vs. 7.4)
  - Ca$^{2+}$?

<table>
<thead>
<tr>
<th>Compound</th>
<th>Enzyme assay$^a$</th>
<th>Cell assay$^b$</th>
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<tbody>
<tr>
<td>1</td>
<td>8</td>
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<td>2</td>
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<td>5</td>
<td>5</td>
<td>900</td>
</tr>
<tr>
<td>6</td>
<td>30</td>
<td>?</td>
</tr>
</tbody>
</table>
Interaction analysis of inhibitors with BACE1

- Full length BACE1 immobilized in a lipid membrane via antibody capture

- Ectodomain BACE1 immobilized directly (no transmembrane region)
Inhibitor interactions with BACE1

Same $K_D$ for truncated and full length BACE at pH 4.5 (for all compounds)

Different $K_D$ for truncated and full length BACE at pH 7.4 (for some compounds)

Different $K_D$ at pH 4.5 and 7.4 for both truncated and full length BACE (for some compounds)

What is relevant?
Inhibitor interactions with BACE1

Correlation between IC$_{50}$ and $K_D$ at 7.4
Interpreting the data

pH effect understood via modelling of protonation of inhibitors and Asp dyad

Hypothesis

- Inhibitors bind BACE1 at the cell surface (neutral pH)
- BACE1 is internalized into endosomes for cleavage (acidic pH)
- Inhibitors need to bind BACE1 at neutral and acidic pH!


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Interactions understood. Leads improved.
Thermodynamic analysis of interactions using SPR biosensors

- Profiling of melagatran analogues interacting with thrombin

1. Determination of enthalpic contributions to binding by ITC at multiple temperatures
Thermodynamic analysis of interactions using SPR biosensors

Relationships between $k_{on}$ and $k_{off}$ over a range of temperatures

Thermodynamic profiles from SPR at 25 °C

ΔH (blue), -TΔS (red), and ΔG (green)

Thremodynamic interpretation is based on high resolution X-ray structures