Novel Functional Assay Approaches for GPCR Ligand Discovery

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Drug targets for existing medicines

- GPCR the most studied membrane protein and amongst the most attractive drug targets
- GPCR’s déjà vu?
- GPCR’s renaissance: Novel pathways, molecular properties and assay technologies and HTS approaches are increasingly studied

Monitoring GPCR activation: some facts & basic principles

- **Functional & ligand binding GPCR assays**
  - The best approach to ligand discovery?
  - **Ligand binding assay**
    - in general straightforward but poor information content
    - In general 1st choice for medicinal chemists
  - **Functional assays**
    - Less simple to configure, high hit rates, false positives
    - Access to the GPCR biology, Uncovers novel compound mechanisms
    - May offer rich information output
- **Try to remain proximal to the receptor**
  - However reporter gene assays can be used in some circumstances
- **Imaging is used to enhance throughput**
  - One single screening platform for orphan receptors and for HTS
- **Use non–invasive assays**
  - Try to bring all receptors to signal through calcium
    - Assay multiplexing (selectivity, several pathways)
    - Allows to align more easily HTS data
  - Rich pharmacological data output including kinetic data analysis
    - Helps excluding non-specific compounds
GPCR signaling pathways and applied technologies is lead discovery

- $G_{12}$PCR
- $G_q$PCR
- $G_s$PCR
- $G_i$PCR

**RhoGEF**
- Translocation
- RhoGEF HCS
- FLIPR imaging

**Rho**
- ROCK
- JNK
- MLC-P
- Actin cytoskeleton

**CNG2**
- $35S$GTP
- $35S$GTP

**PLCβ**
- IP$_{1,2,3}$
- IP$_3$
- DAG
- PKC

**PKA**
- DNABP
- Reporter gene
- Transcription factors
- Promoters: CRE, SRE, NFAT

**Nucleus**
- Reporter gene assays

**Calcium imaging**
- Fluo4 /Fura-2/ AEQ

**Calcium signaling**
- ERK

**Adenyl cyclase**
- ATP
- cAMP accumulation
- HTRF, bioluminescent

**IP$_1$ HTRF imaging**

**ER**
- $Ca^{++}$

**ROCK**
- JNK
- MLC-P
- Actin cytoskeleton

**IP$_3$**
- DAG
- PKC

**GPCRs**
- $G_{12}$PCR
- $G_q$PCR
- $G_s$PCR
- $G_i$PCR
- $G_{12}$PCR
- $G_q$PCR
- $G_s$PCR
- $G_i$PCR

**GTPy$^{35}$S**
Inositol phosphate 1 for activation
Gq coupled receptors
IP1 accumulation HTRF assay

Inhibition by LiCl

Myo-inositol

(Courtesy of Cisbio, France)

fluorescent analog of IP1
monoclonal antibody against IP1
**IP1 accumulation: Histamine responses in CHOK1-H1R**

**Calcium FLIPR**

- Ext Ca\(^{++}\) = 1.26 mM

**IP1 HTRF**

- Cell # | IC\(_{50}\) (nM)
  - 10000 | 7.6
  - 30000 | 10.6
  - 60000 | 21.9

- Cell # | IC\(_{50}\) (nM)
  - 10000 | 12.1
  - 30000 | 16.3
  - 60000 | 30.3
**IP1 accumulation: Histamine H1 receptor in CHOK1 cells: antagonist effects**

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ (nM) Ca$^{++}$ (FLIPR)</th>
<th>IC$_{50}$ (nM) IP1 (HTRF)</th>
<th>IC$<em>{50}$, Ca$^{++}$/IC$</em>{50}$ IP1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>exp 1</td>
<td>exp 2</td>
<td>mean</td>
</tr>
<tr>
<td>Cetirizine</td>
<td>925</td>
<td>1450</td>
<td>1188</td>
</tr>
<tr>
<td>Ketotifen</td>
<td>6.4</td>
<td>6.6</td>
<td>6.5</td>
</tr>
<tr>
<td>Astemizole</td>
<td>460</td>
<td>458</td>
<td>459</td>
</tr>
<tr>
<td>Loratadine</td>
<td>3910</td>
<td>2350</td>
<td>3130</td>
</tr>
<tr>
<td>Clemastine</td>
<td>48.5</td>
<td>24.4</td>
<td>36</td>
</tr>
<tr>
<td>Doxepin</td>
<td>13.0</td>
<td>33.6</td>
<td>23</td>
</tr>
<tr>
<td>Mirtazapine</td>
<td>12.4</td>
<td>7.9</td>
<td>10</td>
</tr>
</tbody>
</table>

**Antagonism by Ketotifen**

- **Calcium**
  
  \[\text{IC}_{50} = 6.6 \text{ nM}\]

- **IP1**
  
  \[\text{IC}_{50} = 17.2 \text{ nM}\]
Alternative format for $G_{q}$-coupled receptors evaluated recently (see review June 06)
Homogeneous format (HTRF)
Applied for secondary screening of GPR40

![Diagram showing FLIPR/Ca++ and HTRF/IP1](image)

<table>
<thead>
<tr>
<th>Compound</th>
<th>CHOK1-C4-GPR40 Ca++</th>
<th>CHOK1-C4-GPR40 Ca**</th>
<th>CHOK1-C4-V1a Ca**</th>
<th>CHOK1-C4-GPR40 IP1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC$_{50}$ (µM)</td>
<td>Max. inh. (% of control)</td>
<td>IC$_{50}$ (µM)</td>
<td>Max. inh. (% of control)</td>
</tr>
<tr>
<td>RBAS1</td>
<td>&gt;30</td>
<td>20</td>
<td>&gt;30</td>
<td>20</td>
</tr>
<tr>
<td>RBAS2</td>
<td>&gt;30</td>
<td>0</td>
<td>&gt;30</td>
<td>0</td>
</tr>
<tr>
<td>RBAS3</td>
<td>3.6</td>
<td>100</td>
<td>3.0</td>
<td>100</td>
</tr>
<tr>
<td>RBAS4</td>
<td>6.8</td>
<td>100</td>
<td>2.6</td>
<td>100</td>
</tr>
<tr>
<td>RBAS5</td>
<td>2.1</td>
<td>60</td>
<td>4.8</td>
<td>62</td>
</tr>
</tbody>
</table>

Good correlation obtained between Ca++ and IP1 data
“Frequent hitters” from FLIPR screens readout can be excluded
CellKey™ System: Assay Principle

- Impedance based measurements
- Real-time, kinetic measurements across all 96 wells
- Automated fluid handling
- Simultaneous compound addition and read of all wells
- Monitors cell shape changes following receptor activation
- May constitute a phenotype assay reflecting cell migration
CellKey™ System: CHOm1  Muscarinic Agonist & Antagonist Effects

- **060329 CHOm1 Agonist CRC**
  - Monitors activity and kinetic shape
  - Shape determines GPCR coupling mode
  - Good correlation with FLIPR data

- **060329 CHOm1 Antagonists**
  - Monitors activity and kinetic shape
  - Shape determines GPCR coupling mode
  - Good correlation with FLIPR data
Different potencies with three technologies

Slow acting agonists in three GPCR assay formats

Calcium / FLIPR (3min)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Calcium (FLIPR)</th>
<th>IP1 (HTRF)</th>
<th>Impedance (CDS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBJ551</td>
<td>&gt;100</td>
<td>0.36</td>
<td>1.1</td>
</tr>
<tr>
<td>RBJ554</td>
<td>&gt;100</td>
<td>0.020</td>
<td>0.11</td>
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<tr>
<td>RBJ853</td>
<td>1.3</td>
<td>3.3</td>
<td>2.2</td>
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</table>

Impedance/CellKey (30 min)

IP1 / HTRF (60 min)

Novartis Institutes for BioMedical Research

9 March 2007
### Conclusions / summary

**Coupling**

<table>
<thead>
<tr>
<th>Coupling</th>
<th>Ligand binding</th>
<th>G-protein activation</th>
<th>Signaling</th>
<th>2\textsuperscript{nd} mess.</th>
<th>technology / Instrument</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>$G_q$</td>
<td>SPA</td>
<td>-</td>
<td>PLC-?</td>
<td>Ca\textsuperscript{++}</td>
<td>Fluo4 /FLIPR</td>
<td>multiplexing possible</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>PLC-?</td>
<td>IP1</td>
<td>HTRF / Viewlux imaging</td>
<td>Temporal multiplexing</td>
</tr>
<tr>
<td>$G_s$</td>
<td>SPA</td>
<td>SPA GTP?S</td>
<td>AC activation</td>
<td>cAMP</td>
<td>HTRF / Viewlux imaging</td>
<td>Temporal multiplexing</td>
</tr>
<tr>
<td>$G_s$</td>
<td></td>
<td></td>
<td>AC activation</td>
<td>cAMP / Ca\textsuperscript{++}</td>
<td>CNG2</td>
<td>Fluo4 /FLIPR</td>
</tr>
<tr>
<td>$G_s$</td>
<td></td>
<td></td>
<td>PLC-?</td>
<td>Ca\textsuperscript{++}</td>
<td>Fluo4 /FLIPR</td>
<td>Ca\textsuperscript{++} obtained via GPCR priming valid for ca 70 % Gi or Gs coupled receptors</td>
</tr>
<tr>
<td>$G_s$, 16 &amp; chimeric</td>
<td>SPA</td>
<td>SPA GTP?S</td>
<td>AC inhibition</td>
<td>cAMP</td>
<td>HTRF / Viewlux imaging</td>
<td></td>
</tr>
<tr>
<td>$G_i$</td>
<td>SPA</td>
<td>SPA GTP?S</td>
<td>AC inhibition</td>
<td>cAMP</td>
<td>HTRF / Viewlux imaging</td>
<td></td>
</tr>
<tr>
<td>$G_i$</td>
<td></td>
<td></td>
<td>PLC-?</td>
<td>Ca\textsuperscript{++}</td>
<td>Fluo4 /FLIPR</td>
<td>Ca\textsuperscript{++} obtained via GPCR priming</td>
</tr>
<tr>
<td>$G_{12/13}$</td>
<td></td>
<td></td>
<td>RHO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$G_s$, $G_q$, $G_i$, &amp; $G_{12/13}$</td>
<td></td>
<td></td>
<td>PKC / PKA ?</td>
<td>role unknown</td>
<td>Cell key</td>
<td>Non-invasive free-label technology</td>
</tr>
</tbody>
</table>

**FUNCTIONNAL ASSAYS**

- Novel generic assays being developed:
  - DiscoverX arrestin assays & sensigen assays