Directed evolution of a \textit{G} protein-coupled receptor for expression, stability, and binding selectivity

Casim A. Sarkar, Igor Dodevski, Manca Kenig, Stefan Dudli, Anja Mohr, Emmanuel Hermans, and Andreas Plückthun


Speaker: I-Lun Chiang
Advisor: Ching-Tsan Huang
Date: 2009.12.14
Importance of G protein-coupled receptors

• GPCRs are the largest family in the human genome
• A lot of drugs on the market target GPCRs
• Crystal structures available only for a few of GPCRs.

5 structures solved since 2000
GPCR-related diseases

Chapter 4  G Protein-Coupled Receptors as Cardiovascular Drug Targets
Mika Scheinin and Amir Snapir

Chapter 5  G Protein-Coupled Receptors and Cancer
Martine J. Smit and Remko A. Bakker

Chapter 6  G Protein-Coupled Receptors in Metabolic Disease
Regina M. Reilly and Christine A. Collins

Chapter 7  G Protein-Coupled Receptors in CNS Drug Discovery
Rita Raddatz and Deborah S. Hartman
GPCRs as Targets for New Drugs

(Class A)

(Adapted from Chalmers, D.T. and Behan, D.P. Nat. Rev. Drug Disc. 2002)
Flowchart of GPCR drug discovery research

1. **Bioinformatics**
   - Candidate cDNAs selected

2. **Full length cloning**

3. **Expression system**

4. **High Throughput Screening (HTS)**

5. **Compound library screening**

6. **Functional assays**

7. **Lead optimization**

---

**Expression system**

**High Throughput Screening (HTS)**

**Lead optimization**
Hosts for GPCR production

- E. coli
- Yeast
- Natural sources
- Insect cells
- Mammalian cells
- Cell-free system
## Overexpression of GPCR

<table>
<thead>
<tr>
<th>Host</th>
<th>Yield</th>
<th>GPCR</th>
<th>Strategy</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>~0.15-0.20 mg/L</td>
<td>Adenosine $\text{A}_{2A}$ receptor</td>
<td>N-terminal MBP fusion</td>
</tr>
<tr>
<td>Yeast</td>
<td>~4 mg/L</td>
<td>Adenosine $\text{A}_{2A}$ receptor</td>
<td>GFP fusion</td>
</tr>
<tr>
<td>Insect cells (baculovirus)</td>
<td>~2 mg/L</td>
<td>Adenosine $\text{A}_{2A}$ receptor</td>
<td>baculovirus infected insect cells</td>
</tr>
<tr>
<td>Mammalian cells</td>
<td>~1.7 mg/L</td>
<td>$\beta_2$-adrenergic receptor</td>
<td>tetracycline-inducible HEK293 cell line</td>
</tr>
<tr>
<td>Cell-free system</td>
<td>~1 mg/mL reaction mixture</td>
<td>$\beta_2$-adrenergic receptor</td>
<td>N-terminal thioredoxin fusion</td>
</tr>
</tbody>
</table>

Heterologous expression was achieved in a few of GPCRs.
What’s other approaches to enhance GPCR expression level?
Approaches for enhancing GPCR surface expression
Approaches for enhancing GPCR surface expression

Addition of sequence
- promote more efficient receptor targeting through the ER and membrane insertion

ex: Cannabinoid Receptor 1 (CB1)
Andersson, H. et al. (2003)

Deletion of sequence
- relieve ER retention and enhance surface expression

ex: GABA$_B$ receptor
Calver, A.R. et al. (2001)
Approaches for enhancing GPCR surface expression

Co-expression of receptor-interacting partner

ex: Olfactory receptor surface expression is driven by association with the $\beta_2$-adrenergic receptor


translocation to the plasma membrane
Approaches for enhancing GPCR surface expression

Treatment with Pharmacological chaperone
Fusion of GPCR to well-expressing protein

Biotinylated oxaloacetate decarboxylase (BAD)

Glutathione-S-transferase (GST)

Maltose binding protein (MBP)

The highest reported levels of expression using MBP fusion method:
rat neurotensin receptor ~0.15-0.20 mg/L

Thioredoxin (TrxA)

(Advances and Challenges in Membrane Protein Expression. 2007 AIChE)
Neurotensin Receptor Study

✓ Major study group: Grisshammer

Expression of a rat neurotensin receptor in *Escherichia coli*

Reinhard GRISSHAMMER,* Robin DUCKWORTH* and Richard HENDERSON†

*Cambridge Centre for Protein Engineering/MRC Centre, Hills Road, Cambridge CB2 2QH, U.K., and †MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, U.K.

Purification of a rat neurotensin receptor expressed in *Escherichia coli*

Julie TUCKER and Reinhard GRISSHAMMER*

Centre for Protein Engineering/MRC Centre, Hills Road, Cambridge CB2 2QH, U.K.

The conformation of neurotensin bound to its G protein-coupled receptor

Sorin Luca*, Jim F. White†, Awinder K. Sohal‡‡, Dmitri V. Filippov†, Jacques H. van Boom†, Reinhard Grisshammer‡‡, and Marc Baldus*
Expression levels still need to improve

The highest reported levels of expression using MBP fusion method: rat **neurotensin receptor** \~0.15-0.20 mg/L
Motivation of the authors

The expression levels using other approaches seems not able to acquire sufficient amount of NTR-1

Directed evolution in *E. coli*

Enhance expression, stability and ligand selectivity

Apply in other GPCRs as a platform

GPCR drug discovery and structure-based drug design
Framework

Directed Evolution

mutagenesis
epPCR  StEP

screening
FACS

Evolved receptors

Expression

Western blot
P. pastoris
HEK293T cells

Purification
E. coli

Stability

Thermal stability test
E. coli

Ligand selectivity

Radioligand binding assay
E. coli

Ca^{2+} signaling patterns
HEK293T cells
Neurotensin Receptor-1 (NTR-1)

*Agonist: Neurotensin(8-13)*

Mediates most effects of neurotensin (NT)

Involved in CNS as neuromodulator
Random mutagenesis: error-prone PCR

- Taq polymerase: low fidelity
- MgCl₂: stabilize noncomplementary pairs
- MnCl₂: mutagenic to polymerase

Gene recombination: Staggered Extension Process (StEP)

cross hybridization of growing gene fragments during polymerase-catalyzed primer extension

Directed Evolution

NTR-1

mutagenesis

epPCR

screening

StEP

FACS

Evolved receptors

Isolation of high affinity ligand-binding proteins by periplasmic expression with cytometric screening (PECS)
Nature Biotechnology, 2001
Strategy of Directed Evolution

4x4 rounds FACS

Fluorescent-labeled ligand: BODIPY-NT

expression level

ligand selectivity
Summary of Directed Evolution

NTR-1 \rightarrow \text{mutagenesis} \rightarrow \text{expression} \rightarrow \text{screening} \rightarrow \text{Evolved receptors}

- epPCR
- StEP
- E. coli periplasma
- FACS

(a few) random mutations \rightarrow \text{select/screen} \rightarrow \text{expression level} \rightarrow \text{NO} \rightarrow \text{repeat} \rightarrow \text{YES} \rightarrow \text{Evolved gene (= evolved protein)}

Parent gene (= parent protein)

ligand selectivity

(Bloom J D, Arnold F H PNAS 2009;106:9995-10000)
Directed Evolution

- wild type receptors
  - epPCR
  - StEP
- mutagenesis
  - Western blot
  - Purification
- screening
  - FACS
- Evolved receptors
  - E. coli
  - P. pastoris
  - HEK293T cells

Expression

- E. coli
  - HEK293T cells

Stability

- Thermal stability test
  - E. coli

Ligand selectivity

- Radioligand binding assay
  - E. coli
- Ca²⁺ signaling patterns
  - HEK293T cells
Ligand Selectivity: Ligand binding site of NTR-1

Agonist: Neurotensin (NT)  
Antagonist: SR 48692

Competitively inhibits NT binding to NTR-1
Radioligand binding assay
radioactive agonist $[^3H]-NT$

expression level
Radioligand binding assay

radioactive agonist \(^{3}\text{H}\)-NT

Altering ligand selectivity

G10
Isolation of gene encoding for improved variants: G10

Key selectivity mutation in G10

F358S: Phe → Ser
D03 retains biochemical and pharmacological properties of WT

G10 abolish binding to SR48692
Framework

Directed Evolution

wild type receptors → mutagenesis → screening → Evolved receptors

epPCR → StEP → FACS

Expression

Western blot

Purification

E. coli

Stability

Thermal stability test

E. coli

Ligand selectivity

Radioligand binding assay

Ca$^{2+}$ signaling patterns

E. coli

HEK293T cells
NTR-1 trigger PLC cascade and release intracellular Ca$^{2+}$

DAG=Diacylglycerol
PIP=Phosphatidylinositol-biphosphate
IP=inositol-phosphate
Ca\textsuperscript{2+} signaling patterns in HEK293T cells expressing D03 changed.

D03 contains a substitution of Arg\textsuperscript{167} with Leu.

Mutations in the highly conserved DRY motif in helix III of class A GPCRs might affect ligand binding and G-protein coupling.

Back mutant: D03-L167R  \(ightarrow\) DRY motif restored
Isolation of gene encoding for improved variants: D03

- **Non-silent mutations**
  - His103 (CAT) → Asp (GAT)
  - His105 (CAC) → Tyr (TAC)
  - Ala161 (GCC) → Val (GTC)
  - **Arg167 (CGC) → Leu (CTC)**
  - Arg213 (CGC) → Leu (CTC)
  - Val234 (GTC) → Leu (CTC)
  - His305 (CAC) → Arg (CGC)
  - Ser362 (TCC) → Ala (GCC)
  - Ser417 (AGC) → Cys (TGC)

- **Silent mutations**
  - Leu72 (CTG) → Leu (CTA<sup>rare</sup>)
  - Thr231 (ACA) → Thr (ACT)
  - Leu247 (CTG) → Leu (CTA<sup>rare</sup>)
  - Phe346 (TTC) → Phe (TTT)
  - Ala418 (GCC) → Ala (GCT)
Directed Evolution

- wild type receptors
- mutagenesis
  - epPCR
  - StEP
- screening
  - FACS
- Evolved receptors

Expression
- Western blot
- Purification
- HEK293T cells
- E. coli
- P. pastoris

Stability
- Thermal stability test
- E. coli

Ligand selectivity
- Radioligand binding assay
- Ca^{2+} signaling patterns
- HEK293T cells
- E. coli
Western blot analysis of NTR1

**E. coli**

**P. pastoris**

Whole-cell Western blot of receptors expressed in *E. coli* and *P. pastoris*
Expression level comparison in *E. coli*

![Expression level comparison in *E. coli*](image1)

Comparison of purification yield

![Comparison of purification yield](image2)

Immobilized metal ion affinity chromatography (IMAC)

Expression level comparison in *P. pastoris*

![Expression level comparison in *P. pastoris*](image3)

Expression level comparison in HEK293T cells

![Expression level comparison in HEK293T cells](image4)
Framework

Directed Evolution

- wild type receptors
- mutagenesis: epPCR, StEP
- screening: FACS
- Evolved receptors: E. coli, P. pastoris, HEK293T cells

Expression
- Western blot: E. coli
- Purification: P. pastoris, HEK293T cells, E. coli

Stability
- Thermal stability test: E. coli

Ligand selectivity
- Radioligand binding assay: E. coli
- Ca²⁺ signaling patterns: HEK293T cells
• Solubilized and purified receptors were incubated at 45°C
• The remaining activity was measured after cooling

![Graph showing thermal stability test results](image)

- D03
- D03-L167R
- Better stability
- NTR-1
Directed evolution of NTR-1 successfully enhance the expression, stability and ligand selectivity.
G protein-coupled receptors (GPCR)

Hope to apply to other GPCR expression structure-based drug discovery

G-protein-coupled receptor (GPCR) family classification of known and orphan GPCRs.
THE END

Thanks for your attention