Chemical biology of Glycosylphosphatidylinositol (GPI) anchors

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Topology of biological membrane

PC, PE, PS (major) and PI (minor)
Key building blocks of plasma membranes

(Glycerophospholipids)

Phosphatidyl-ethanolamine
Phosphatidyl-choline
Phosphatidyl-serine
Phosphatidyl-Inositol
Two major cell signaling pathways are mediated by Phosphatidylinositolos and Inositol phosphates
Regulation of PI3K activity by phosphatases
(PTEN and SHIP)

PTEN and SHIP2 expressed in wide variety of cells
SHIP is expressed only in blood cells
sSHIP is expressed in stem cells

Approx 50% of human cancers contain PTEN mutations
Relationship of PI and GPI anchors
(biosynthesis and topology)
Novel mode of attachment of cell surface proteins and carbohydrate antigens

Protein

trans-amidation
GPI signal sequence

(different from common trans-membrane peptide anchors)

Plasma Membrane
GPI molecules are abundant in protozoan parasites
(Leishmania, Trypanosoma and Malaria)
GPI anchors and cholesterol organize functional micro-domains (lipid rafts) in plasma membrane

A GPI-anchored protein is attached to the exoplasmic leaflet

A doubly acylated Src-kinase to the cytoplasmic leaflet

A model of a lipid raft with two intercalated proteins
Chemical biology of GPI anchors

- Organic synthesis, biosynthesis and cell biology of PI/GPI molecules
- Design and synthesis of structural and functional mimics of PI/GPIs to probe biological questions
- Targeting PI3K/AKT/mTOR pathway for anticancer drug discovery
- Role of PI3K isoforms (α, β, γ, δ) in stem cell differentiation
Synthesis and biosynthesis of GPI-anchored Lipophosphoglycan (LPG) of Leishmania parasite

Unique GPI-anchor
Galα-containing Glycan core
Variable Phosphoglycan repeats

GPI anchor is made in ER and PG assembled in Golgi
(MPT and GDP-Man transporter)

Synthesis of GPI-anchor of LPG

Fully protected GPI anchor

Gal-Gal-Gal$_f$ Segment-C

Man-Man Segment-B

GlcN-Ino Segment-A

RETROSYNTHETIC SCHEME
Synthesis and biosynthesis of GPI-anchors of L. donovani, T. cruzi and E. histolytica

Infection and Immunity, 73, 8381-8392 (2005)
Synthesis of a full-length GPI anchor of malaria parasite (*Plasmodium falciparum*)

The Enzymes, 26, 181-227 (2009)
Retro-synthetic strategy

1: Malarial GPI

2

3a: R₁=Ac, R₂=Ac
3b: R₁=H, R₂=H
3c: R₁=TBDPS, R₂=Bn

5

8

A

B

C

D

E

F

- O=P-O-CH₂-NH-Cbz

- O=P-O-CH₂-NH₂⁺
Glucosamine-Inositol intermediate
Resolution of GlcN-Inositol

Tetra-mannose Block
Synthesis of Upper Mannobiose Segment

1. **All-OH**
   - Reagent: BnBr, NaH
   - Products: 22, Allyl-α-Mannose

2. **22**
   - Reagent: PhCH(OMe)₂, PTSA
   - Product: 24

3. **tBuOK, DMSO, H⁺, CCl₃, DBU**
   - Reagent: Bu₂SnO, BnBr
   - Products: 11, 12
25b \xrightarrow{\text{Ac}_2\text{O, Py}} 25c

25c \xrightarrow{\text{Me}_2\text{NH}, -20 ^\circ\text{C}} 25d

\text{Mannobiose Donor
Synthesis of Lower Mannobiose Segment

1. **HBr, AcOH**
   - Reaction with **BzCl, Py** results in
   - **All-OH**
   - **TrCl, Py**
   - **BnBr, NaH**
   - **PTSA, MeOH**

2. **Pentenol Lutidine**

3. **D-Mannose**

4. **NaOMe, MeOH**

5. **BnBr, NaH, DMF**
Construction of Tetra-mannose Block

Upper Mannobiose

TMSOTf
DCM, -5 °C

PdCl₂, AcOH

CCl₃CN, DBU

Lower Mannobiose

Tetramannose

8b

8c

HN
CCl₃
Synthesis of GPI-Glycan

Tetramannose

GlucN-Inositol

GPI-glycan

3d
Selective acylations

![Diagram of selective acylations process]
Palmitic Acid
DCC, DMAP

4 days

TBAF, THF
Final Assembly

Key questions of PI/GPI biology

- Topology of GPI biosynthetic pathway in Endoplasmic reticulum (ER) membrane
- Stereo-chemical recognition PI in biogenic ER membrane
- Role of GPIs in the organization of membrane micro-domains (lipid rafts) at the Plasma membrane
- Golgi specific GDP-Man transporter in *Leishmania* species
- Phosphatidylinositol pathway as key anticancer drug target
Evidence for protein-mediated, ATP-independent Trans-bilayer movement of GPI intermediates
Fluorescent GPI probes with label on lipid domain

Fluorescent GlcNAc-PI

Fluorescent GlcN-PI

NBD =

Biochemical Reconstitution of ER Flippase activity

SWER
↓
TE

0.3 mole % of the NBD-PC probe

Triton X-100 extract of ER or other biogenic membrane
OR
lipid extract of biogenic membrane dissolved in Triton X-100

+ egg phosphatidylcholine

remove detergent by adsorption using SM2 Bio-Beads

centrifuge to collect vesicles

GlcNAc-PI and GlcN-PI both flip in proteoliposomes reconstituted from ER

Evidence for protein-mediated, ATP-independent Trans-bilayer movement of GlcN-PI intermediates
Trans-bilayer movement of PI Across the ER
Is stereochemistry important?
Bishop and Bell (Cell, 1985)
BIOGENIC MEMBRANE
“PC flipping appears to be stereo-specific in ER”

Pagano (JBC, 1987)
PLASMA MEMBRANE (ABC transporters)
“Trans-membrane movement of PE and PS was Inhibited by their analogues, suggesting that the Process is stereo-specific”
All four PI diastereo-isomers synthesized in fluorescent forms

1 (D-Ino-D-Glycero-PI)

2 (D-Ino-L-Glycero-PI)

3 (L-Ino-D-Glycero-PI)

4 (L-Ino-L-Glycero-PI)

5 (D-Ino-D-Glycero-Myristoyl-PI)

6 (D-Ino-L-Glycero-Myristoyl-PI)
Dithionite based Flippase assay

liposome or inactive proteoliposome → dithionite → 50% reduction of NBD-PL

flippase-equipped proteoliposome → dithionite → 100% reduction of NBD-PL

NBD-PL → red. NBD-PL → PL flippase → other protein

New fluorescent probes revealed that flip-flop of PI across the ER does *not* depend on the stereochemistry of the lipid.
Alternative Assays

1. BSA Back-extraction Assay after Reconstitution in Proteoliposomes
2. BSA Back-extraction Assay in Intact ER
3. Kinetics Measurements

The rate constants were identical for these two myr-PI's, and similar to those for a myr-PC sample measured alongside

Role of GPI anchored proteins in Plasma Membrane Microdomain Organization

A GPI-anchored protein is attached to the exoplasmic leaflet.

A doubly acylated Src-kinase to the cytoplasmic leaflet.
Chemical basis of GPI structure recognition

Fluorescent GlcN-PI probe

Fluorescent Man-GlcN-PI probe


*Chem. Commun.*, 5852-5854 (2011)
Synthesis of head group labeled GPI probes

Nanoclusters of GPI-anchored proteins are formed by cortical actin-driven activity

*Cell, 135, 1085-1097 (2008)*
GDP-Man transport in Leishmania during GPI biosynthesis

Mammalian cells have no requirement for a Golgi localised GDP-Man transporter. This makes Leishmania transporter an ideal target for drug-design and glycobiology.
Higher Organism

GDP-Man → GDP

Leishmania

GDP-Man → GMP
Synthesis of novel GDP-Man probes

GDP-Man

6,6-difluoro-6-deoxy-GDP-Man

6-fluoro-GDP-Man

4,6-difluoro-4,6-dideoxy-GDP-Man

6-oxo-(hemihydrate)-GDP-Man

6-fluoro-6-deoxy-GDP-Man

(L-manno)-GDP-Man (CHIRAL ANTIPODE)
GDP-Man transporter assay (using Golgi vesicles)

Leishmania lysate (Membrane proteins)

Sucrose gradient fractionation
(0.25 to 2.5 M gradient, 80000 rpm)

Golgi Vesicles
(evaluated by GalT assay)

1M-1.5M fractions had maximum GalT activity

[3H]-GDP-Man
(0.16 μCi)

Reaction mix contained 300 μg protein, 10 mM Tris-HCl, sucrose, TLCK, leupeptin, PMSF and DTT

Incubation at 28 °C for 6 min
and snap-frozen

Filtered through HA filter, washed
and bound radioactivity counted

transport rate =16 pmole GDP-Man/mg protein

Vesicles broken with CM treatment and applied to TLC along with standard GDP-Man and Bioscan
Transport of GDP-Man analogues

GDP-Man Transport Assay
Using Leishmania Golgi Vesicles
Transport rate 16 pmole/mg

1. Control (8 μM GDP-Man)
2. DIDS (8 μM)
3. [6-deoxy-6-fluoro]-GDP-Man (8 μM)
4. [6-deoxy-6-fluoro]-GDP-Man (32 μM)
5. [6-deoxy-6,6-difluoro]-GDP-Man (8 μM)
6. [L-Man]-GDP-Man (8 μM)
7. [L-Man]-GDP-Man (32 μM)

Unpublished Results
Phosphatidylinositol 3-kinase (PI3K) pathway as clinically validated anticancer target

- Inhibition of PI-3K kinase (isoform specific inhibitors)
- PI3Kα for cancer and PI3Kγ for inflammation
- Up-regulation of PTEN phosphatase
- Inhibition of AKT activity
- Inhibition of mTOR
Medicinal chemistry of natural products and clinically validated candidates for cancer drug discovery

**PI3K Inhibitors: Scaffolds selected for Med Chem Prog**

- **Liphagal Wyeth (Preclinical)**
  - \( p_{110\alpha} \text{IC}_{50} \sim 100 \text{ nM} \)
  - \( p_{110\beta} \text{IC}_{50} \sim \text{ND} \)
  - \( p_{110\gamma} \text{IC}_{50} \sim 1 \)
  - \( p_{110\delta} \text{IC}_{50} \sim \text{ND} \)

- **BEZ235 as PI3K/mTOR inhibitors (Novartis, Phase II)**
  - \( p_{110\alpha} \text{IC}_{50} \sim 4 \text{ nM} \)
  - \( p_{110\beta} \text{IC}_{50} \sim 76 \text{ nM} \)
  - \( p_{110\gamma} \text{IC}_{50} \sim 7 \text{ nM} \)
  - \( p_{110\delta} \text{IC}_{50} \sim 5 \text{ nM} \)

- **CAL-101, PI3K inhibitor (Calistoga, Phase I)**
  - \( \text{IC}_{50} \sim 2.5 \text{ nM in PI3K} \delta \)

- **GSK2126458 (Developed by GSK)**
  - \( \text{PI3K} \alpha \text{IC}_{50} \sim 0.04 \text{ nM} \)

- **XL147, PI3K inhibitor (Exelixis, Phase I)**
  - \( p_{110\alpha} \text{IC}_{50} \sim 39 \text{ nM} \)
  - \( p_{110\beta} \text{IC}_{50} \sim 383 \text{ nM} \)
  - \( p_{110\gamma} \text{IC}_{50} \sim 25 \text{ nM} \)
  - \( p_{110\delta} \text{IC}_{50} \sim 36 \text{ nM} \)

- **XL765 as PI3K inhibitor (Exelixis, Phase I)**
  - \( p_{110\alpha} \text{IC}_{50} \sim 13 \text{ nM} \)
  - \( p_{110\beta} \text{IC}_{50} \sim 113 \text{ nM} \)
  - \( p_{110\gamma} \text{IC}_{50} \sim \text{ND} \)
  - \( p_{110\delta} \text{IC}_{50} \sim 43 \text{ nM} \)

- **GDC0941 as PI3K inhibitor, (GenetechPhase I)**
  - \( \text{PI3K} \alpha \text{IC}_{50} \sim 3 \text{ nM} \)
  - 1 log unit sensitive against \( \beta/\gamma \)

Scaffolds in blue color: Synthesis completed at IIIM-Jammu
Patent search have been completed and analogs have been synthesized
Medicinal chemistry of Liphagane scaffold
(First isoform selective PI3K inhibitors)

A meroterpenoid isolated from marine sponge *Aka coralliphaga*
Inhibitory activity against PI3Kα (10 fold selectivity)

More selective than the synthetic LY294002 and Wortmanin.

IC<sub>50</sub>: 100 nM

Org. Lett. 2006, 8, 321 (Isolation, synthesis)
*Tetrahedron Lett.* 2009, 50, 5260-5262
Org. Lett., 2010, 12, 2394–2397
Org. Lett., 2010, 12, 4450-4453
*J. Med. Chem.,* 2010, 53, 8523-8533
(60 nM IC<sub>50</sub> and 27 fold selectivity)
*Synth. Commun.*, 2011, 41, 177-183
*Wyeth* WO 2006/081659 A1
Our synthesis of Liphagal

\[
\begin{align*}
&\text{MeO-CHO} \quad \xrightarrow{\text{BBR}_3, \text{DCM}} \quad \text{MeO-CHO} \quad \xrightarrow{\text{2-Butanone}} \quad \text{MeO-Br} \\
&\text{MeO-CHO} \quad \xrightarrow{\text{K}_2\text{CO}_3, \text{70\%}} \quad \text{MeO-CHO} \\
&\text{MeO-CHO} \quad \xrightarrow{\text{tBuOK, Toluene}} \quad \text{MeO-CHO} \\
&\text{MeO-CHO} \quad \xrightarrow{\text{Pd/CaCO}_3, \text{MeOH}} \quad \text{MeO-CHO} \\
&\text{MeO-CHO} \quad \xrightarrow{\text{Ph}_3\text{P}^+ \text{CH}_3 \text{I}^-} \quad \text{MeO-CHO} \\
&\text{MeO-CHO} \quad \xrightarrow{\text{n-BuLi, THF}} \quad \text{MeO-CHO} \\
&\text{MeO-CHO} \quad \xrightarrow{\text{ClSO}_3\text{H, Nitropropane}} \quad \text{MeO-CHO} \\
&\text{MeO-CHO} \quad \xrightarrow{\text{AlCl}_3, \text{DMS}} \quad \text{MeO-CHO} \\
&\text{MeO-CHO} \quad \xrightarrow{\text{0 °C - rt}} \quad \text{MeO-CHO} \\
\end{align*}
\]

+ Liphagal
NCEs based on Liphagal scaffold (first series)
NCEs Liphagal scaffold (second series)

IC$_{50}$ of two best compounds from Liphagal series

Control IC$_{50}$ 100 nM

<table>
<thead>
<tr>
<th>Events or Targets</th>
<th>Sample code</th>
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<tbody>
<tr>
<td><strong>SIP-1003</strong></td>
<td><strong>SIP1004</strong></td>
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<tr>
<td><strong>In vitro</strong></td>
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</tr>
<tr>
<td>enzyme assay IC$_{50}$</td>
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<tr>
<td><strong>PI3K</strong>$\alpha$</td>
<td>140 nM</td>
</tr>
<tr>
<td>$\beta$</td>
<td>100 nM</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>ND</td>
</tr>
<tr>
<td>$\delta$</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Cell based assay</strong></td>
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</tr>
<tr>
<td>2.6 $\mu$M (MCF-7)</td>
<td>3.1 $\mu$M (MCF-7)</td>
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<tr>
<td><strong>Annexin-V</strong></td>
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<tr>
<td>35-57% (1-9 $\mu$M)</td>
<td>50-58% (1-9 $\mu$M)</td>
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<tr>
<td><strong>Cell Cycle</strong></td>
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<tr>
<td>50-60% GI (1-9 $\mu$M)</td>
<td>64-70% GI (1-9 $\mu$M)</td>
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<tr>
<td><strong>Wound Healing</strong></td>
<td></td>
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<tr>
<td>Effective</td>
<td>Highly effective</td>
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<tr>
<td><strong>Phospho-Akt</strong></td>
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<tr>
<td>50-55% Down-regulation</td>
<td>60-68% Downregulation</td>
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IP Filed
Medicinal chemistry of Imidazo-quinoline scaffold

BEZ-235 as PI3K/mTOR inhibitor

- BEZ235 is effective against advanced solid tumors, advanced breast cancer.
- Potent, orally active dual PI3K/mTOR inhibitor under phase II clinical trial.
- Inhibits PI3K and mTOR Kinase activity by binding to the ATP binding cleft of these enzymes
Synthesis of reference candidate BEZ-235

In house
IC$_{50}$ 23 nM
Modeling of BEZ-235 with PI3K structure

<table>
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<tr>
<th>S.NO</th>
<th>Molecules</th>
<th>Dockscore</th>
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<tbody>
<tr>
<td>1</td>
<td>SDS-23</td>
<td>-11.40</td>
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<tr>
<td>2</td>
<td>NVPBEZ235</td>
<td>-7.57</td>
</tr>
<tr>
<td>3</td>
<td>WORTMANIN</td>
<td>-7.79</td>
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</table>
Key NCEs from this scaffold
Medicinal chemistry of purinyl-quinazolinone scaffold

- Patent space identified and NCEs have been prepared

<table>
<thead>
<tr>
<th>Compd</th>
<th>P110α(nM)</th>
<th>P110β(nM)</th>
<th>P110γ(nM)</th>
<th>P110δ(nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAL101</td>
<td>200,000</td>
<td>16,000</td>
<td>61,000</td>
<td>2.5</td>
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</table>
NCEs synthesized

Two compounds shown potent IC\textsubscript{50} value in \textit{in vitro} and cell based assay

<table>
<thead>
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<th>Events or Targets</th>
<th>Sample code</th>
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<tbody>
<tr>
<td>\textit{In vitro} IC\textsubscript{50}</td>
<td>SIP-1107</td>
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<tr>
<td>PI3K(\alpha)-</td>
<td>57 nM</td>
</tr>
<tr>
<td>(\beta) -</td>
<td>ND</td>
</tr>
<tr>
<td>(\gamma) -</td>
<td>ND</td>
</tr>
<tr>
<td>(\delta) -</td>
<td>98 nM</td>
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</table>

<table>
<thead>
<tr>
<th>Cell based assay IC\textsubscript{50}</th>
<th>Colo-205=200nM</th>
<th>A549=7.3(\mu)M</th>
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</thead>
<tbody>
<tr>
<td>Colo-205-2=9.8 (\mu)M</td>
<td>HCT-115=7.2(\mu)M</td>
<td>HCT-115=7.2(\mu)M</td>
</tr>
</tbody>
</table>
Summary: Functional Diversity *within* PI class

- PKC signaling
- Phosphorylation
- PI-3 Kinase signaling
- Phosphorylation
- Glycosylation
- Lipidation/glycosylation
- Membrane anchoring of proteins
- Microdomain Organization (Lipid Raft)
- Virulence factors of parasites
- PI-PLC inhibition

**Phosphatidylinositol**
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