Evaluation of selective inhibitors of the malarial cyclic GMP-dependent protein kinase (PKG)

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Talk summary

• An overview of the *P. falciparum* cGMP dependent protein kinase (PfPKG)

• Why we think that PfPKG is a good drug target

• The strategy we are using to evaluate selective inhibitors of PfPKG
PF14_0346: cGMP dependent protein kinase

Ser/Thr kinase: Transfers phosphate group from ATP to protein target(s)
3 cGMP binding sites (and a fourth pseudo-site)
Catalytic domain (ATP binding pocket)

- Recombinant enzyme and parasite lines bearing this mutation have been created within the Baker lab over several years
- Using these molecular tools we are aiming to develop improved inhibitors that are both selective and specific for the ATP binding pocket of PfPKG

Allosteric activation by cGMP

ATP binding pocket – catalyses transfer of phosphate to substrate peptide/protein

PfPKG has a small gatekeeper residue
Addition of a large amino acid at the entrance to the ATP binding pocket will confer insensitivity to some inhibitors
Threonine 618 replaced by glutamine (T618Q)
The cGMP-dependent protein kinase of *P. falciparum* performs critical roles throughout the life-cycle.

In the absence of PKG, rupture of blood stage schizonts is blocked.
In the absence of PKG rupture of bloodstage schizonts is blocked

- Asexual stage parasites, in the presence of PKG inhibitors, form mature schizonts that are unable to rupture (left).
- Untreated cultures proceed progress normally (right)

**PKG inactivated** –
- Schizont rupture blocked

**Active PKG** –
- Schizont rupture to continue asexual cycle

- PKG inhibition at this stage could provide a blood-stage schizonticide
- This phenotype is not shown in cell lines bearing the T618Q mutant PfPKG
PKG inhibition blocks rupture of late stage schizonts in < 1hr

Parasites with PKG inhibited proceed through the bloodstage but do not rupture. This assay measures how quickly invasion of new red blood cells is prevented.

- Within 1 hour rupture and re-invasion events are blocked by the PKG inhibitor (compound 2)
- Even over the course of 3 hours artemisinin treated parasites are rupturing and re-invading
The cGMP-dependent protein kinase of *P. falciparum* performs critical roles throughout the life-cycle.

**Transmission blocking potential**

In the absence of PKG rounding up of gametocytes is blocked.
Compound 1 inhibits gamete formation at the first step

XA-activated gametocytes

Compound 1-treated gametocytes cannot round up

Wild type
T618Q mutant

The effects of compound 1 on rounding up are abolished in the T618Q mutant confirming that PKG is the primary target of the inhibitor and that PKG is essential for gametogenesis
Strategy to develop potent and selective inhibitors of PfPKG

In all of this work we use the gatekeeper mutation to confirm PKG is the primary target

Recombinant proteins: WTPfPKG and T618Q PfPKG gatekeeper mutant both produced in *E. coli*

*Parasite lines: P. falciparum* line bearing T618Q mutation in *PfPKG* as well as parent line
  Drug resistant lines against common anti-malarials

Screen against recombinant PfPKG produced in *E. coli* → Test against cultured *P. falciparum* → Test against *P. berghei* mouse model
A fluorescence shift assay for PKG

- This assay tests activity against purified protein (WT PfPKG and T618Q PfPKG)
- Runs on EZ Reader platform
- Use a 6-carboxyfluorescein labelled peptide (FAM) substrate and microfluidic detection system to detect phosphorylation due to PKG.
- Product will migrate more quickly than substrate in capillary
- Measure area under curve to determine degree of reaction with different concentrations of inhibitors

A single reaction could look like this:

Fluorescence intensity over time

Product: More Negative
(-)
Electrophoresis of Analytes
(+)
LED Detection

Pressure-driven Flow

Substrate:
More Positive

(Vacuum)
(Sipper)
PF14_0346: cGMP dependent protein kinase

- **cGMP** catalytic domain

ATP binding pocket – catalyses transfer of phosphate to substrate peptide/protein

Allosteric activation by cGMP

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*Threonine 618 replace by glutamine (T618Q)*
Evaluation of WT PfPKG and T618Q PfPKG (gatekeeper mutant)

<table>
<thead>
<tr>
<th></th>
<th>Km cGMP (nM)</th>
<th>Km ATP (µM)</th>
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<tbody>
<tr>
<td>WT PfPKG</td>
<td>315 +/- 55</td>
<td>30 +/- 12</td>
</tr>
<tr>
<td>T618Q PfPKG</td>
<td>501 +/- 57</td>
<td>66 +/- 26</td>
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</table>
cGMP analogues can also activate PfPKG

PET-cGMP has similar effectiveness to cGMP. May present options to control activation of cGMP in parasite culture

Analogues with substituents in the 8 position are much less effective
Single point discontinuous assay employed for inhibitor screening

Discontinuous assay for inhibitors of WT and mutant PfPKG.
- Use 1.25 nM enzyme, 1.5 μM peptide substrate, 10 μM cGMP, varied inhibitor
- Pre incubate for 30 min
- Initiate with ATP (at Km for each enzyme)

<table>
<thead>
<tr>
<th>Compound</th>
<th>wt PfPKG IC50 (uM)</th>
<th>T618Q PfPKG IC50 (uM)</th>
<th>Selectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound 1</td>
<td>0.00392 +/- 0.0018</td>
<td>11.27 +/- 0.55</td>
<td>2871</td>
</tr>
<tr>
<td>Compound 2</td>
<td>0.00178 +/- 0.0011</td>
<td>6.02 +/- 1.08</td>
<td>3207</td>
</tr>
</tbody>
</table>
An activator of the T618Q PfPKG mutant

- In the course of screening compounds that appear to activate the gatekeeper mutant of PfPKG have been identified.
- Kinetic analysis revealed increase Vmax but no change to Km cGMP.
Benefits of the EZ Reader based assay

- Excellent sensitivity and no radiation
  - Inhibitor testing has identified inhibitors with ca. 200 pM IC$_{50}$ values
- Multiple sampling from single well allows easy simple kinetic analyses
- Endpoint screening assay suitable for HTS
- Good agreement with GFC assay
Strategy to develop potent and selective inhibitors of PfPKG

Screen against recombinant PfPKG produced in *E. coli* → Test against cultured *P. falciparum* → Test against *P. berghei* mouse model

In all of this work we use the gatekeeper mutation to confirm PKG is the primary target

**Recombinant proteins**: WTPfPKG and T618Q PfPKG gatekeeper mutant both produced *in E. coli*

**Parasite lines**: *P. falciparum* line bearing T618Q mutation in *PfPKG* as well as parent line
Drug resistant lines against common anti-malarials

- $^3$H hypoxanthine incorporation assay
- Flow cytometry based assay
**P. falciparum** replication assay also shows reduced sensitivity in mutant line

- Culture parasites in the presence of compounds for 48 hours
- Add $^3$H hypoxanthine for a further 24 hours
- Measure radiation incorporated as a percent of uninfected controls

- Absolute IC$_{50}$ values are **ca. 100-fold greater** in parasite assay compared to recombinant protein assay (2-3 nM vs enzyme, 200-300 nM vs parasite)
- The IC$_{50}$ shift observed with mutant is much smaller than in the enzyme assay
  - **Ca. 20-fold vs 3000 fold**
- It is likely there are additional targets in the parasite but shift suggests that PKG is the main target.
*P. falciparum* replication can also be assessed by flow cytometry

PFPKG inhibition blocks rupture of schizont: Accumulation of DNA rich cells

- Using cultures labelled with SYBR green it is possible to visualise the quantify of DNA in any given cell
- Mean fluorescence intensity shifts as parasite DNA replication occurs
Compound 2
50 µM
Cell death

Compound 2
5 µM
Schizont accumulation

Compound 2
500 nM
Schizont accumulation

Compound 2
50 nM
No effect, some schizonts but many ring stages

Plot mean fluorescence of infected cells at each concentration

DNA rich population accumulates when PfPKG is inhibited

Concentration (nM)
Comparison of mean fluorescence (DNA) with infected cells: Artemisinin

Infected cells (black bar) can be used to determine IC₅₀ whilst blue bars represent mean fluorescence of cell

No change in mean fluorescence irrespective of replication status
Comparison of mean fluorescence (DNA) with infected cells: Compound 2

Infected cells (black bar) can be used to determine IC₅₀ whilst blue bars represent mean fluorescence of cell.

Mean fluorescence increase at high dose while number of infected cells does not/ Consistent with continued development within cycle but not rupture and progression to the next.
Parasite testing of PfPKG inhibitors

- Selectivity of PfPKG inhibitors can be measured using the parasite line bearing the T618Q mutant as well as by phenotype analysis using flow cytometry.

- PfPKG inhibitors are generally 100-fold less active against the whole parasite than they are against the wild type enzyme.

- Selectivity of WT PfPKG P. falciparum over the T618Q PfPKG line is greatly reduced compared to the recombinant enzyme.
  - Likely due to non-PfPKG targets in the parasite.
**In vivo testing (mouse model)**

- Compound 2 treatment gives a small reduction in parasitemia in the mouse model.
- Half life is ca. 50 minutes.
- Inhibition of PKG is effective for only a small window during parasite egress.
- It is likely that insufficient compound levels were maintained throughout the experiment.
Conclusions

• Selective inhibition of PfPKG may have potential to block the parasite lifecycle at multiple stages:
  • Escape from liver cells (in human host)
  • Escape from red blood cells (in human host)
  • Rounding up of gametocytes (in mosquito)

• Gatekeeper mutants of PfPKG have been used to demonstrate selectivity and confirm parasite phenotypes
  • T618Q gatekeeper provides ca. 2000-fold reduction in sensitivity to compounds
  • T618Q PfPKG provides ca. 20-fold reduction in compound sensitivity in culture

• **Primary screen**: Enzyme specificity and selectivity using kinase assay on EZ Reader
  • Lower limit of assay sensitivity not yet reached (currently IC50 ~ 200 pM)
• **Secondary screen**: Anti-parasite activity in cultured *P. falciparum* using gatekeeper mutants and phenotype analysis
• **Tertiary screen**: *Plasmodium berghei* malaria model
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