



Plasmodium falciparum calcium dependent protein kinase 1 (PfCDPK1): A novel target for the treatment of malaria

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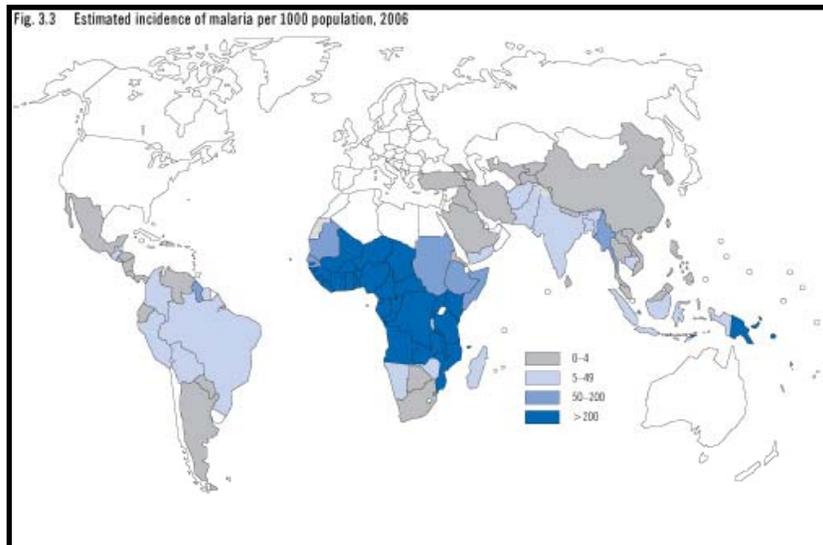


Why Malaria?



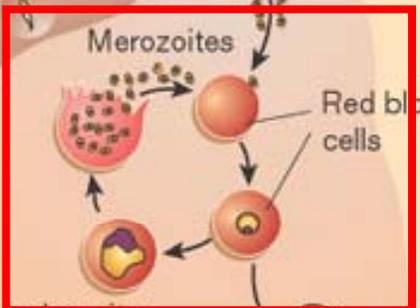
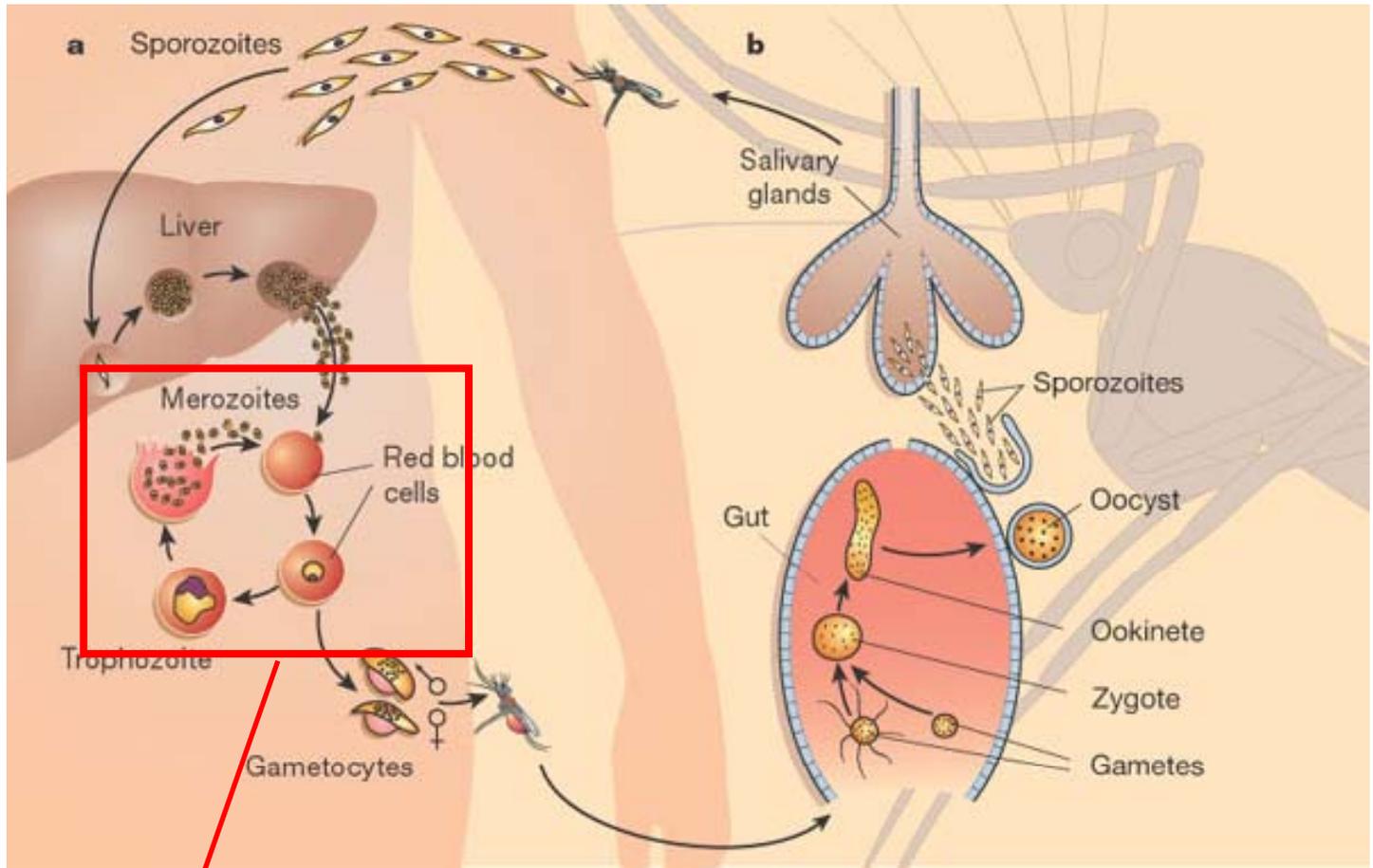
- Major public health problem in > 90 countries with combined population >2.5 billion people (~35% of the world's population)
- ~300-600 million clinical infections and a million deaths per year
- Mainly in Africa --- but incidence is growing in Asia and Latin America. Large potential for increased endemicity in India and China
- Also, malaria has been endemic in more northerly countries (e.g., UK, USA) and there is ongoing debate over whether climate change may see a return to endemicity in these countries

Global distribution of malaria



- Emergence of drug resistance has compromised the therapeutic efficacy of most anti-malarial drugs
- Artemisinin and derivatives, used in combination therapy, are currently the main line of defence against drug-resistant malaria but increasing concerns about decreasing effectiveness
- Humans can be infected by 5 species, but two account for vast majority (>95%) of cases: *Falciparum* (~70%) & *Vivax* (~25%)

The life cycle of *Plasmodium*

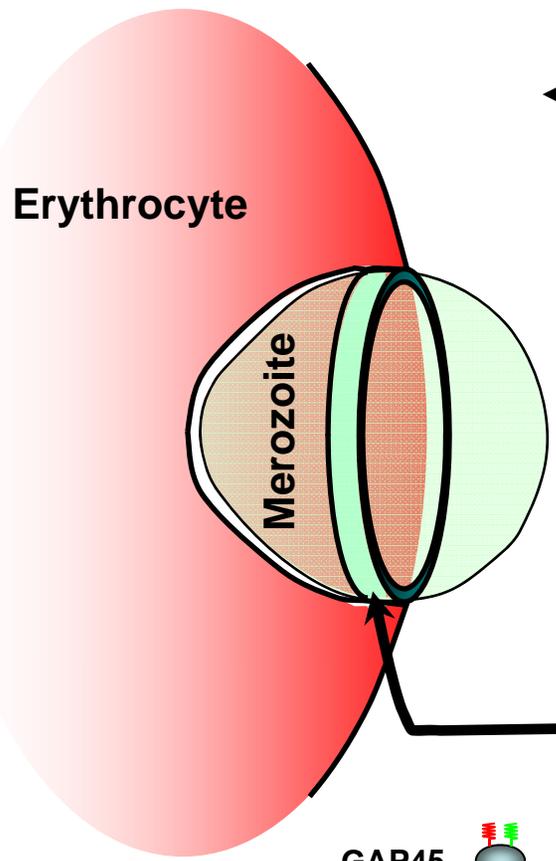


Blood stage

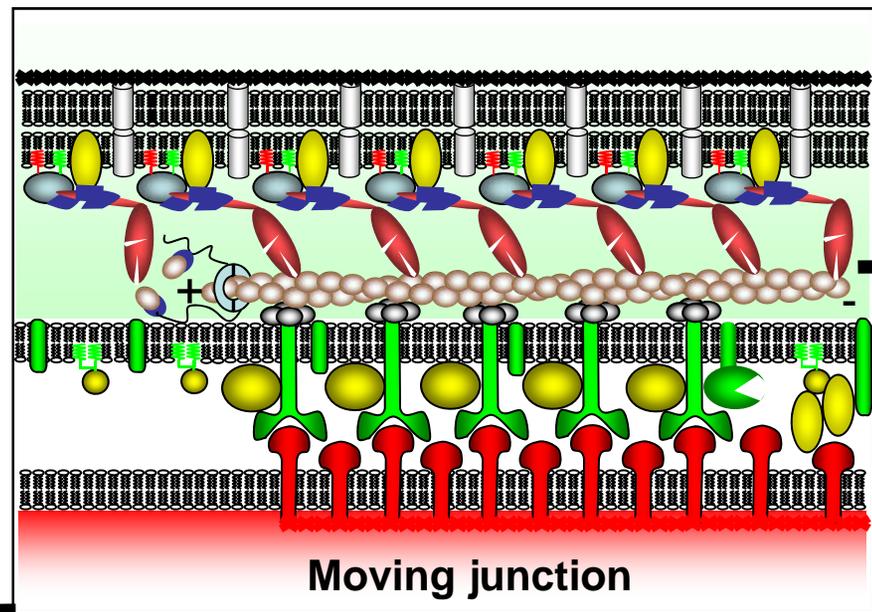
Targeting the blood cycle

- Parasite invasion of red blood cells and subsequent multiplication and release from erythrocytes is the stage of the parasite's life cycle that is responsible for manifestation of the disease.
- If erythrocyte invasion is prevented, the parasites die and the infection is cleared.
- Invasive half life of merozoites is very short (minutes).
- Parasite invasion of erythrocytes is driven by an actomyosin motor -- kinase activity and protein phosphorylation play a key role in this process.

Structure of the Motor

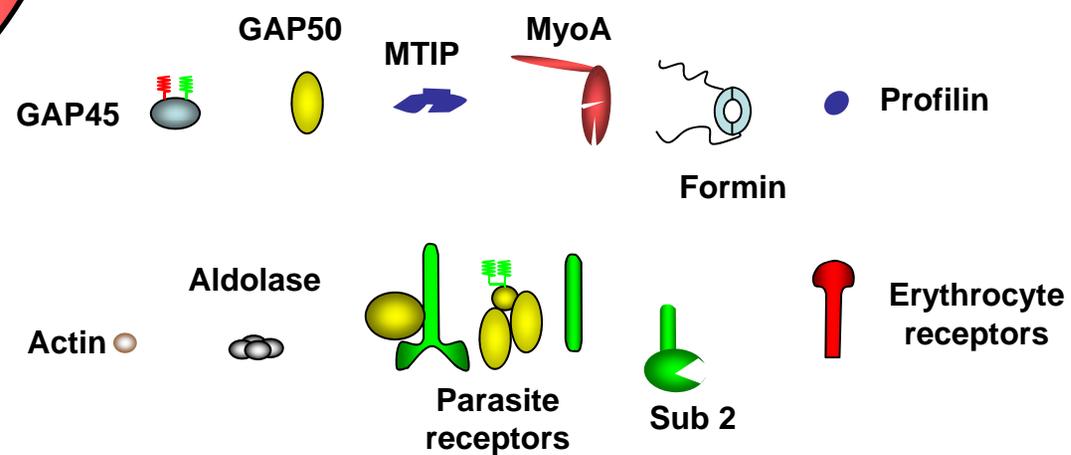


← Merozoite movement



Inner membrane complex
 Force
 Merozoite
 Erythrocyte

Moving junction

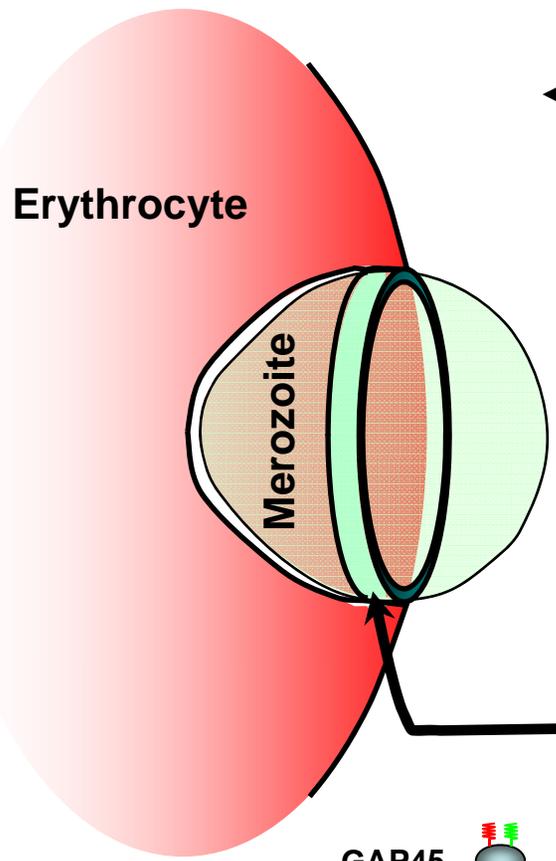


PfCDPK1: a novel target

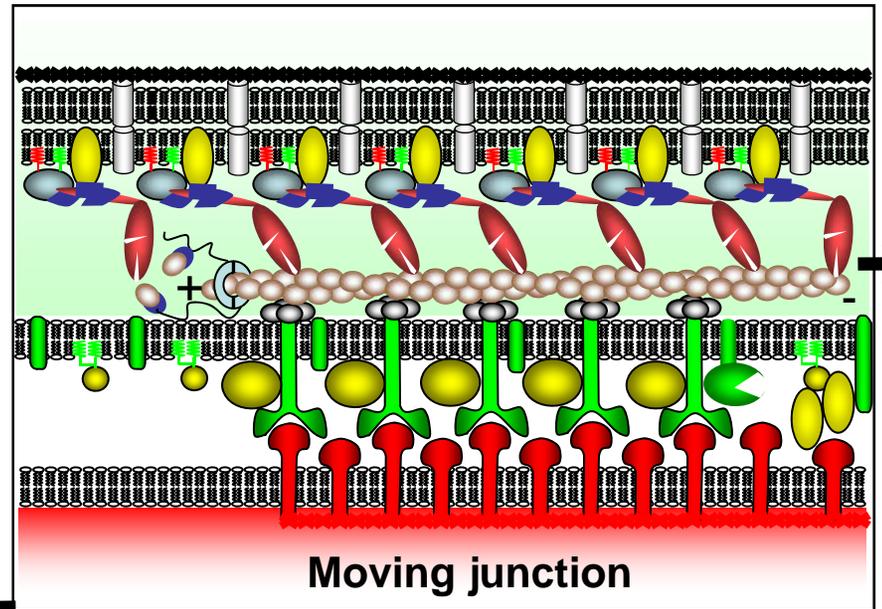


- One of 5 Ca-dependent ser/thr kinases of *Plasmodium* unique to plants and alveolates
- Pf CDPK1 is encoded by an essential gene
 - Expressed primarily in the merozoite (asexual blood stage)
 - Located at inner surface of parasite plasma membrane
- Attempts to knock out the CDPK1 gene have failed in both *Plasmodium falciparum* and the rodent parasite *Plasmodium berghei*
- PfCDPK1 phosphorylates Myosin Tail domain Interacting Protein (MTIP) and 45 kDa Glideosome Associated Protein (GAP45); 2 components of the motor complex
- Direct inhibition of the motor complex using compounds such as BDM (acting on MyoA), cytochalasin D (acting on actin) and myoA tail peptide (disrupting the MTIP-MyoA interaction) blocks parasite invasion of red blood cells and results in parasite death.

Structure of the Motor

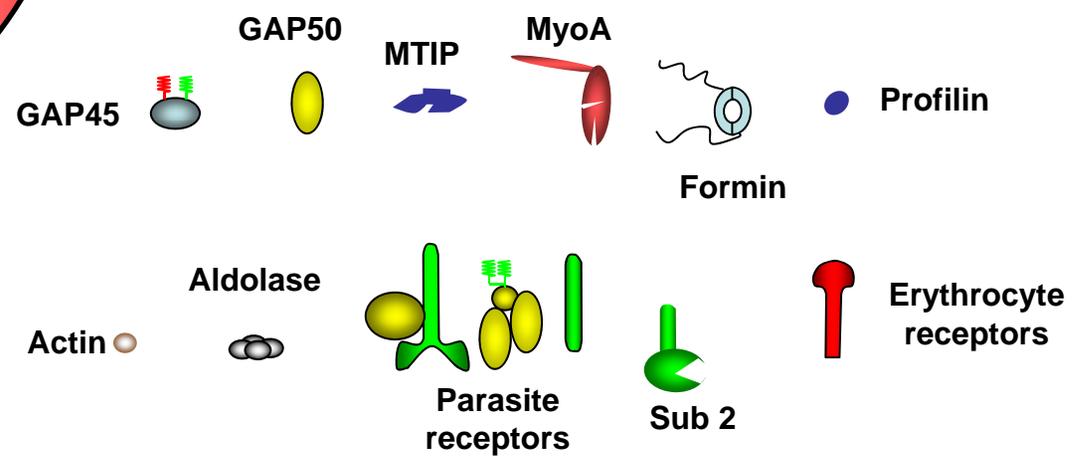


← Merozoite movement



Inner membrane complex
 Force →
 Merozoite
 Erythrocyte

Moving junction



PfCDPK1: a novel target



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- Hypothesis:
 - Small molecule inhibitor of PfCDPK1 will block cell invasion by merozoites, breaking blood-borne cycle
 - PfCDPK1 is a novel malaria target so inhibitors would be expected to be active against current drug resistant strains
 - High homology between PfCDPK1 and PvCDPK1 – can target both species



Initial aims of project:

- **Show that PfCDPK1 inhibitors can block parasite growth in erythrocytes by blocking merozoite invasion**
- **Provide tool compounds for further evaluation of role of PfCDPK1 in parasite life cycle**
- **Achieve *in vivo* PoC (mouse)**
- **Fulfil Medicines for Malaria Venture (MMV) criteria for Late lead – apply for MMV funding for further progression of project**



MRCT

Who are we?

The UK Medical Research Council's (MRC) technology transfer company
120+ staff, of which 70+ research staff doing early stage drug discovery
Generated >USD500m revenue in last 10 years

What do we do?

Protecting and commercialising IP
Antibody humanisation (since 1991)

Small molecule drug discovery (since mid-2006)

--in collaboration with MRC and (increasingly) non-MRC academic scientists
--not just in UK: also Germany, Canada and New Zealand.
Screening collaborations with GSK and AZ

Aim to bring small molecule projects to a partnering endpoint.

NIMR (National Institute for Medical Research)

NIMR is the largest research institute supported by the MRC that promotes research into all areas of medical and related science to improve health.

There are ~200 scientists, 100 post doctoral fellows, 100 PhD students and 200 support and ancillary staff

The Division of Parasitology at NIMR has 5 research groups with a principal focus on malaria.
Our mission is to understand the interaction between parasite and host and use the knowledge gained to promote the development of new therapeutics (drugs and vaccines) and diagnostics.



Projection initiation.

MRCT business managers (BM) review projects in MRC Institutes for possible drug discovery programs

PfCDPK1 identified as a project of interest

MRCT had existing contacts with NIMR (we're next door!) and had run programmes previously in collaboration with the Division of Parasitology.

Project reviewed and progressed into early stage drug discovery

Division of responsibilities:

MRCT

**Chemistry
Biochemical screening
ADMETOX**

NIMR

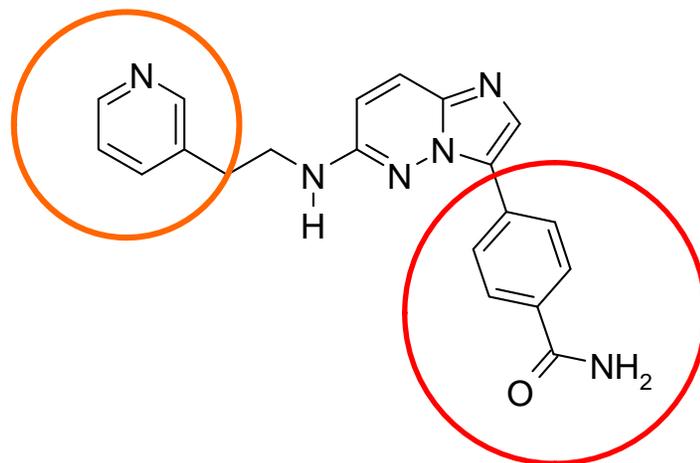
**Cell (parasite) work
Protein production
Mechanism of action studies**

Screen Triage



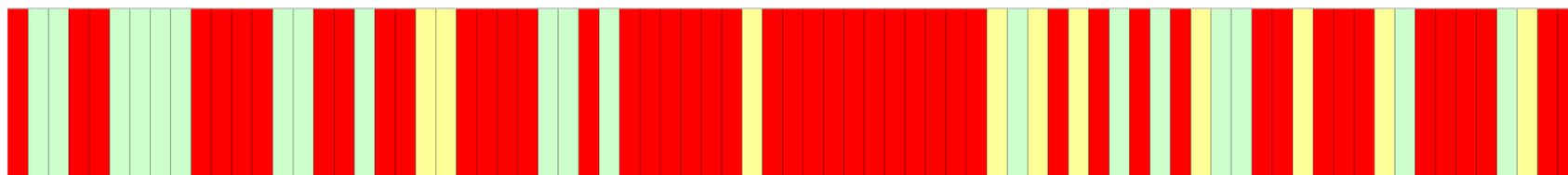
- 35k compounds screened at 10 μM (single point)
- 801 compounds confirmed hits unique to this target (v internal assays)
- 66 compounds with $\text{IC}_{50} < 5 \mu\text{M}$
- Triage on ligand efficiency, toxicophores, chemical tractability, IP, selectivity
- Casualties: oxindoles, diarylpyrazines, anilinoquinazolines.....
- Five series picked for initial investigations: two killed quickly (flat SAR) one halted (poor cellular efficacy)
- This talk will be about one series.

Imidazopyridazines



IC₅₀ = 60 nM

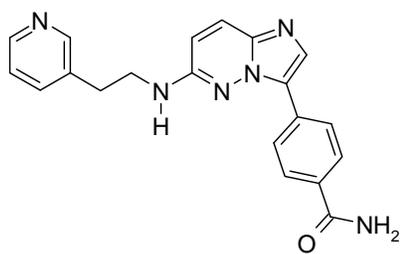
Very poor selectivity v human kinase panel



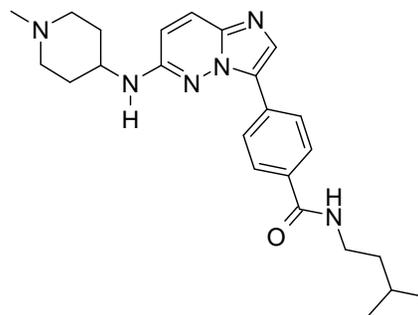
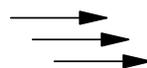
Can we improve kinase selectivity?

10 μM

Improved selectivity in human kinase panel



IC₅₀ 60 nM



IC₅₀ 23 nM

FACS EC₅₀ = 780 nM

LogD > 4

Cytotox IC₅₀ = 9 μM

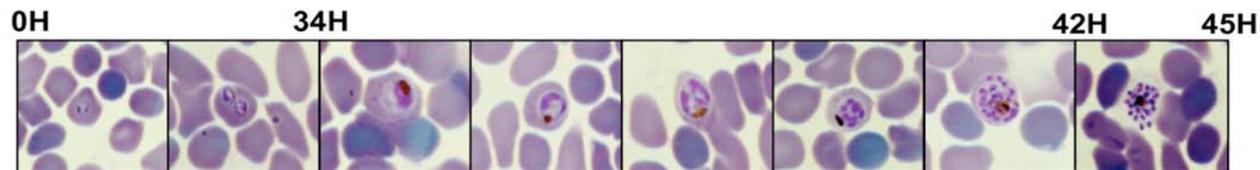
mlm 70% rem

FACS screening

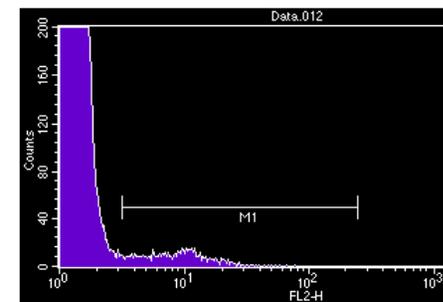
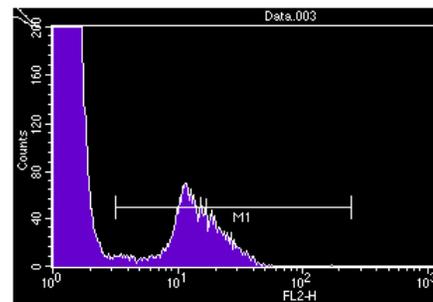
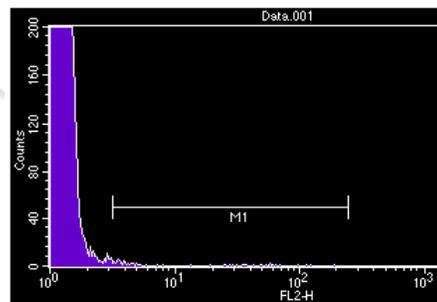
Fluorescence-activated cell sorting



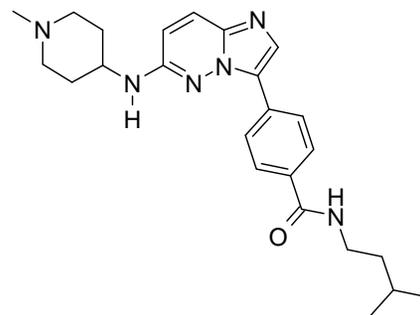
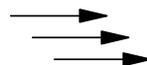
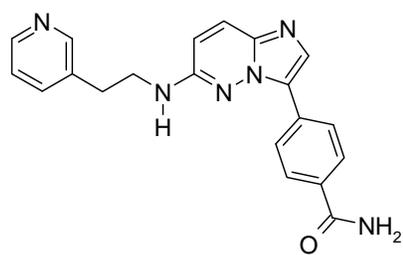
- Set up cultures at ~0.5% parasitaemia and 2% haematocrit
 - Add compound at about 30 h post-invasion. Merozoite release and reinvasion is at ~45 h.



- After ~42 h count parasitaemia using FACS
 - Hydroethidine staining
 - HE is oxidised in cells to ethidium. Ethidium increases in fluorescence when intercalated in DNA
 - In theory, counts only living cells, and more mature multinucleate parasites will give stronger signal



Improved selectivity in human kinase panel



IC₅₀ 60 nM

Selectivity



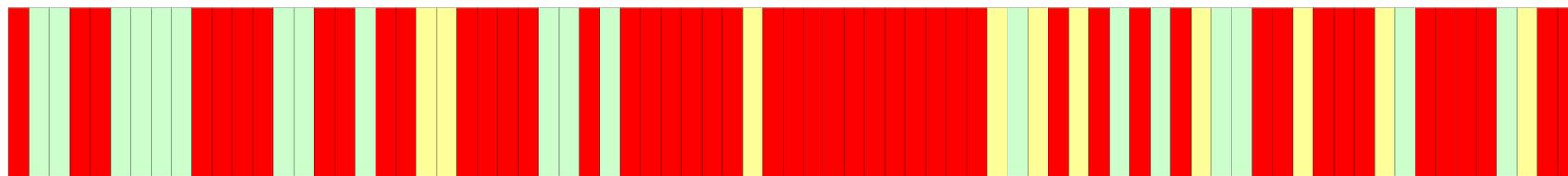
IC₅₀ 23 nM

FACS EC₅₀ = 780 nM

LogD > 4

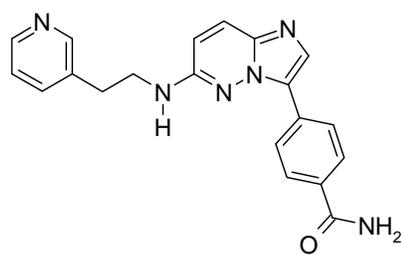
Cytotox IC₅₀ = 9 μM

mlm 70% rem

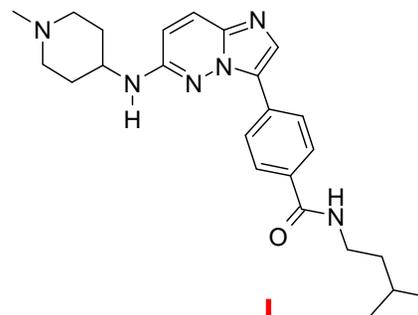
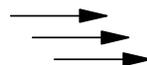


10 μM

Improved selectivity in human kinase panel



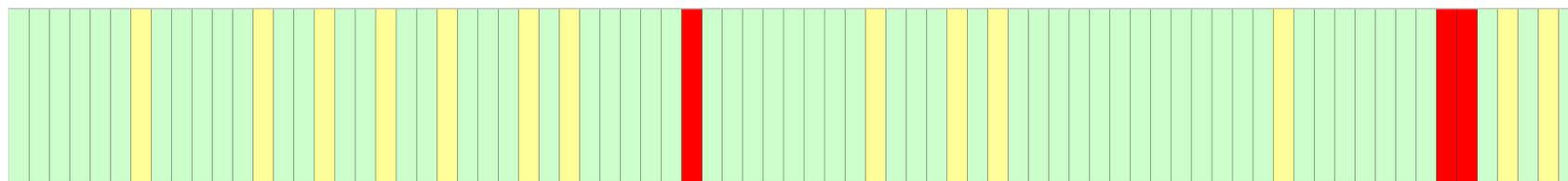
IC₅₀ 60 nM



IC₅₀ 23 nM
FACS EC₅₀ = 780 nM
LogD > 4
Cytotox IC₅₀ = 9 μM
mlm 70% rem

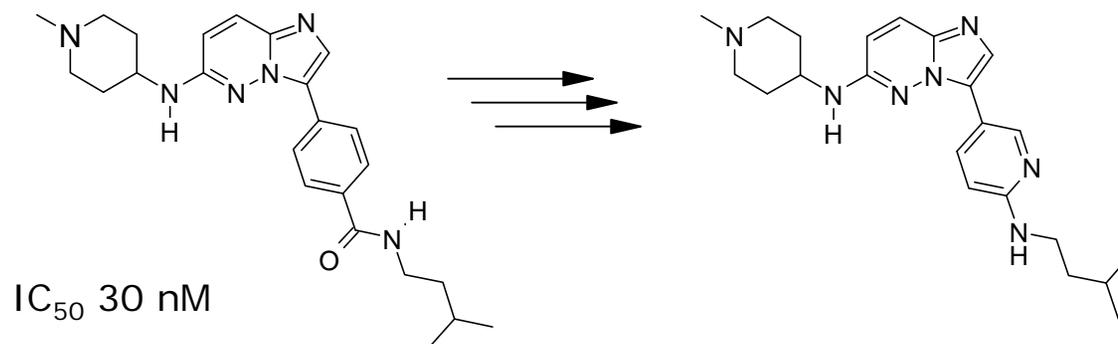


Improved selectivity.....



10 μM

Carboxamide to pyridine



IC₅₀ 30 nM

IC₅₀ 13 nM

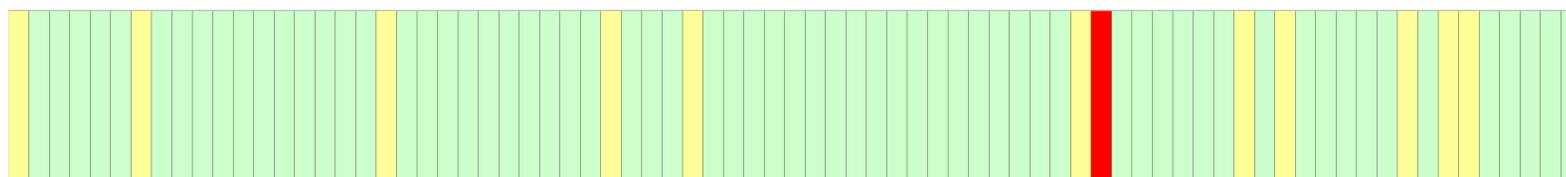
FACS EC₅₀ = 400 nM

Cytotox IC₅₀ = 9 μM

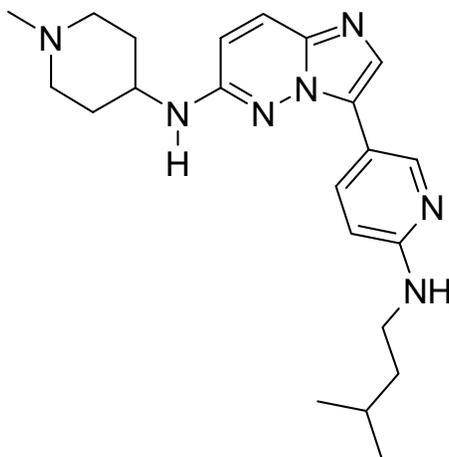
LogD = 3.4

mlm 85%

hlm 63%



1 μM



PAMPA – 81 nm/s

Mouse heps $t_{1/2}$ = 83 mins

hERG – clean at 10 μ M

Mppb – 90%

Stable in mouse plasma

iv PK (mouse)

Vd = 8.5 L/kg

Cl = 49 mL/min/kg

$t_{1/2}$ = 2 hr

Oral data shows good bioavailability

Dosed in mouse malaria model at 50 mg/kg (po)

In vivo testing



4-day standard Peters' test

- Mice are infected intravenously with red cells from a donor mouse infected with a rodent parasite, *Plasmodium berghei* (day 0)
- Treatment is carried out with a solution of the test compounds 2 hours post-infection (first dose), and once daily for a further 3 days.
- Parasitaemia is determined by microscopic examination of blood films taken on day 4 post infection.
- Microscopic counts of blood films from each mouse are processed and the percentage inhibition of growth calculated from the arithmetic mean parasitaemia of each group in relation to an untreated group.



In vivo results

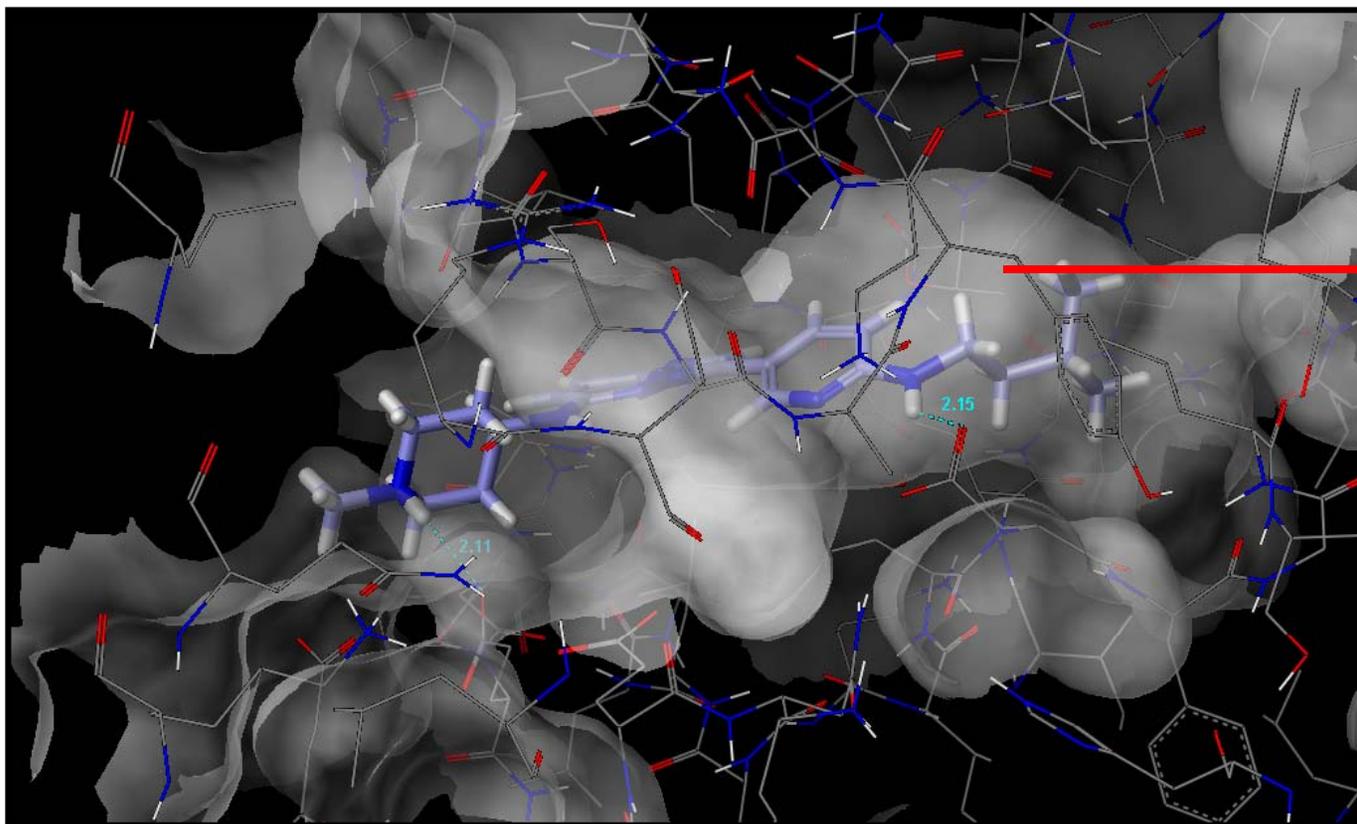
Reduction in parasitaemia = 46% v control (cf chloroquine at 100%)

Some signs of toxicity – abnormal behaviour in mice

Insufficient plasma exposure over 24 hr timeframe?

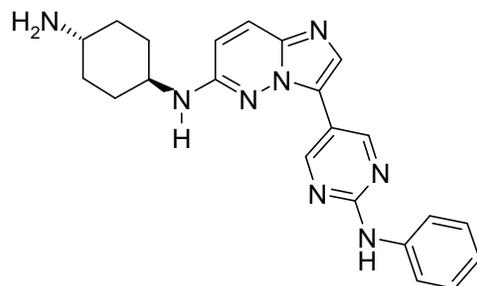
- Mouse plasma exposure data shows low plasma levels after 8 hours
- Need compound that is more potent/longer half life/better exposure.

Homology model constructed from X-ray structure of TgCDPK

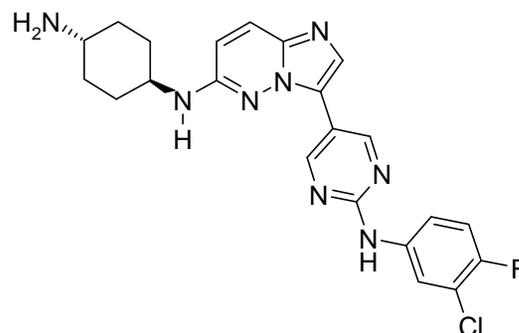


Does alkyl group fill this space optimally?

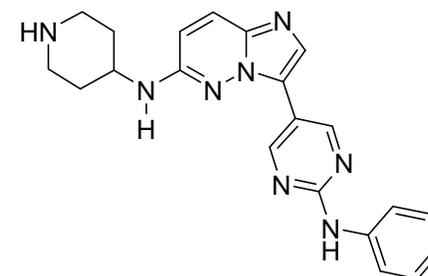
Alkyl to aryl



IC₅₀ 9 nM
EC₅₀ = 200 nM
Cytotox IC₅₀ = 6.6 μM
LogD = 1.6
mlm 92%
hlm 89%



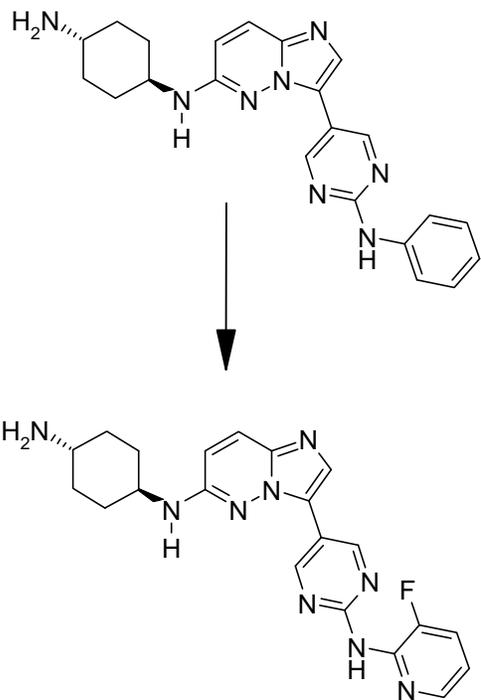
IC₅₀ 9 nM
EC₅₀ = 200 nM
Cytotox IC₅₀ = 3.6 μM
LogD = 2.7
mlm 81%
hlm 72%



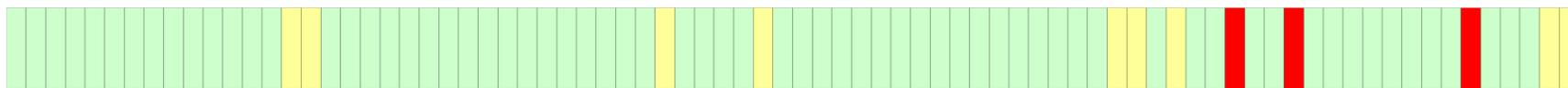
IC₅₀ 13 nM
EC₅₀ = 300 nM
Cytotox IC₅₀ = 9.6 μM
LogD = 1.2
mlm 79%
hlm 74%

Some improvement in cell potency.

Aryl to heteroaryl

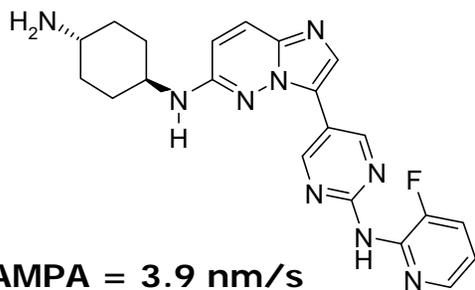


Enzyme IC ₅₀	8nM*
Parasite EC ₅₀	12nM
MW	419
cLogP	2.26
LogD (pH7.4)	0.2
Solubility (10% aq DMSO)	5.7mg/ml
Cytotox (HepG2) EC ₅₀	>20μM
MLM (% rem after 40 mins)	85%
HLM (% rem after 30 mins)	93%
Synthetic steps	5



Based on this and close analogues, successfully applied for MMV involvement in progressing project

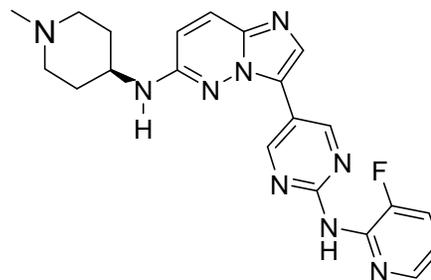
Improving permeability



PAMPA = 3.9 nm/s

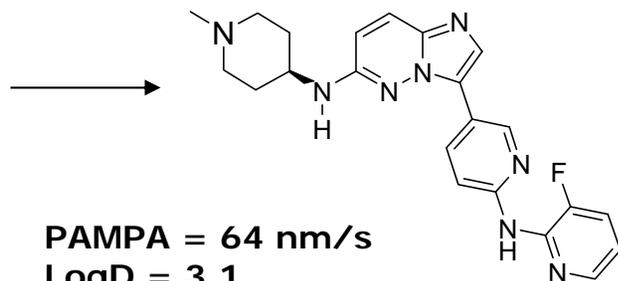
LogD = 0.2

Little *in vivo* activity (poor %F)



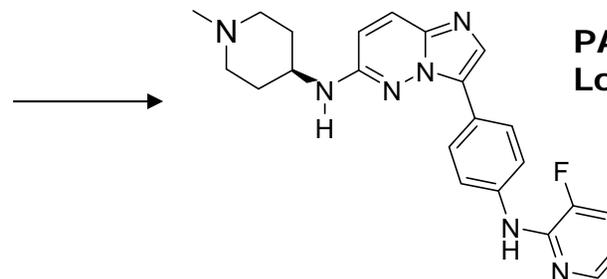
PAMPA = 1.0 nm/s

LogD = 1.4



PAMPA = 64 nm/s

LogD = 3.1

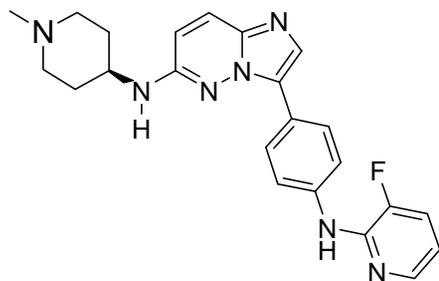


PAMPA = 171 nm/s

LogD = 3.2

Found pyrimidine needed for good efficacy, but always killed permeability

Further *in vivo* testing



IC₅₀ = 12 nM

FACS EC₅₀ = 350 nM

Rat PK

Cl = 14 mL/min/kg

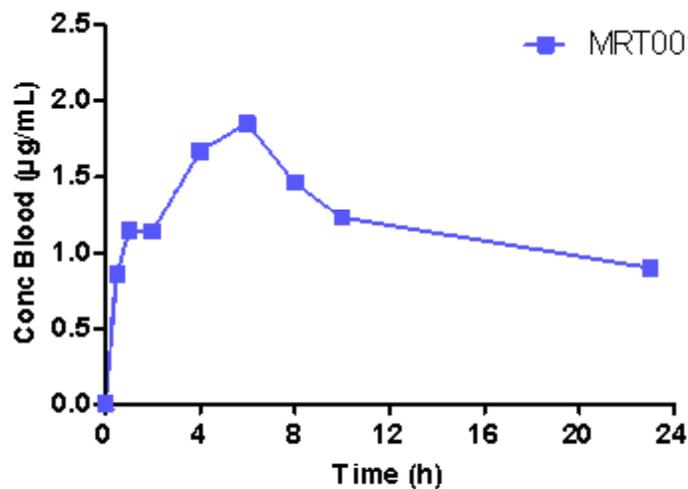
V_{ss} = 8 L/kg

iv t_{1/2} = 4 hrs

%F = 70

MRT00199987
50 mg/kg po; *P. falciparum*

Mouse dosing



1 µg/mL at 24 hrs = 2.4 µM

Reduction in parasitaemia only 51%



Plasma exposure high but *in vivo* effect only moderate

Proving very difficult to get highly efficacious compounds with good PK

➤ Decision reached that further progression was not possible.

However, several unanswered questions:

➤ Why are compounds with pyrimidine more efficacious in cell assay? (off target activity?) – would this be a potential target of interest?

➤ Why is reduction in parasitaemia not higher with apparently good plasma exposure? ---is CDPK1 not as important a target as originally thought?

➤ Are there other phosphorylation substrates?

Ongoing CDPK studies

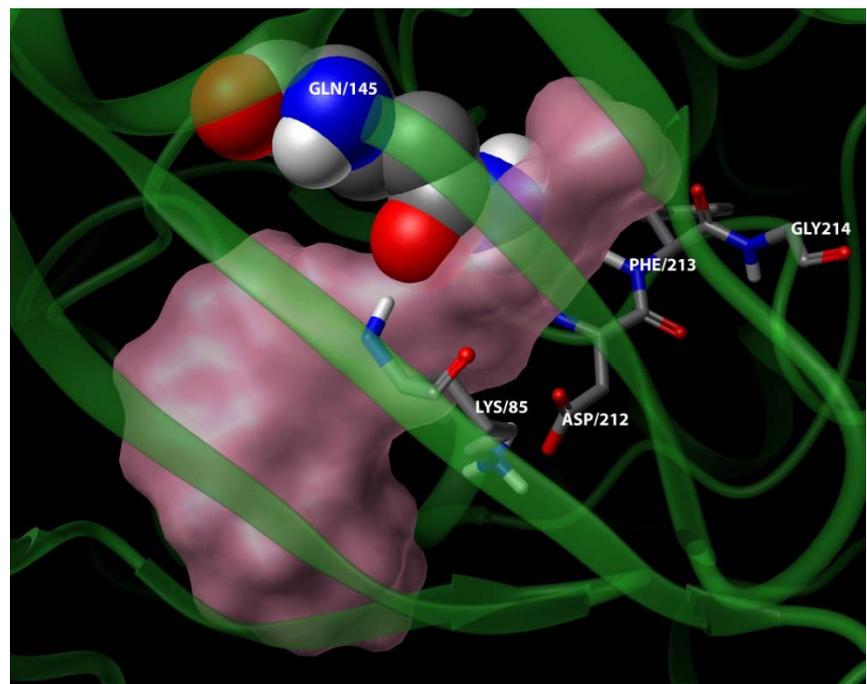
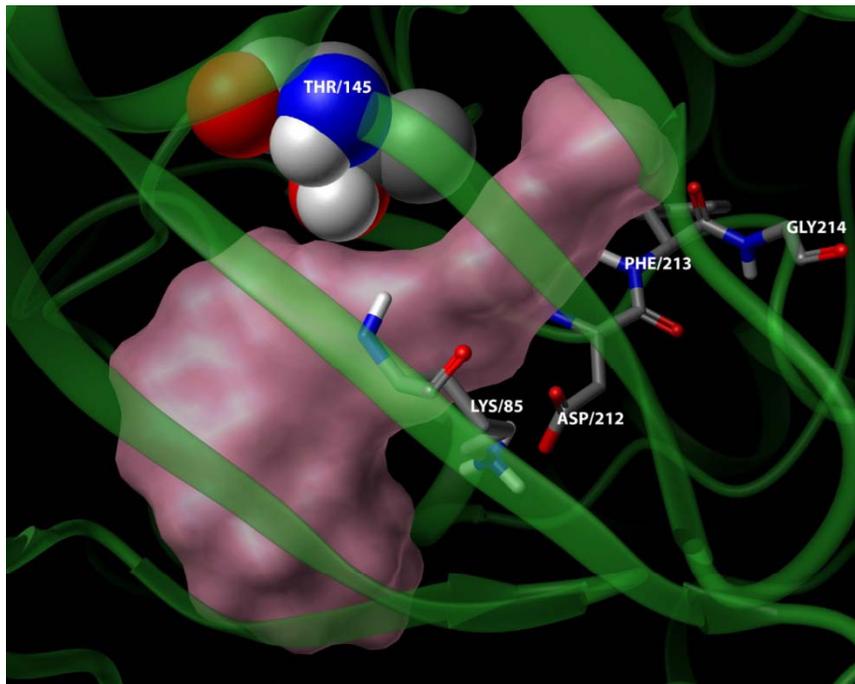
- are there off-target activities?
- substrates and mechanism of action



-
- **Knock-in of gatekeeper mutants (Thr145Gln and Thr145Gly)**
 - See if drug effects are off target
 - Thr145Gln should be drug-refractory
 - Identify additional CDPK1 substrates *in vivo*
 - Thr145Gly should allow use of large ATP analogues
 - **Phenotypic analysis of drug-treated parasites**
 - Identify precise point at which drugs act
 - Identify the protein phosphorylation affected by drug treatment
 - **Affinity labelled compounds to be used to identify alternative targets**

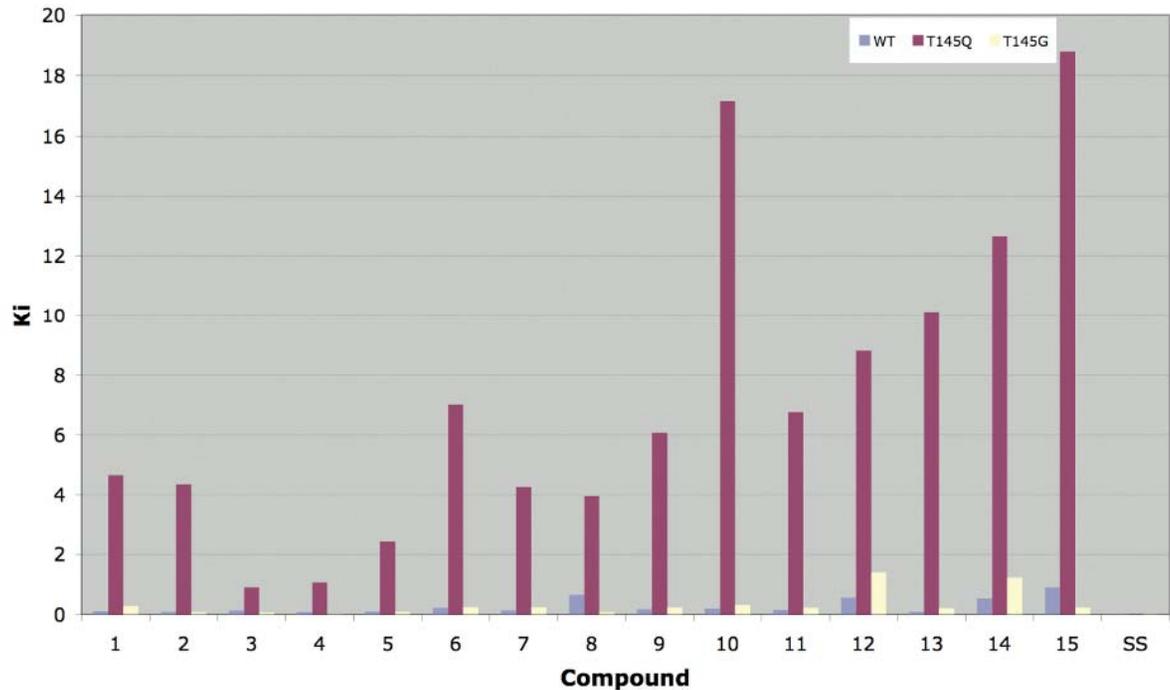
Influence of the gatekeeper residue on the docking of inhibitors

Substitution of gatekeeper threonine for glutamine should restrict access into ATP binding pocket for large inhibitors



PfCDPK1 model (based on TgCDPK1 structure)

Replacing gatekeeper with LARGE residue generates compound-refractory enzyme



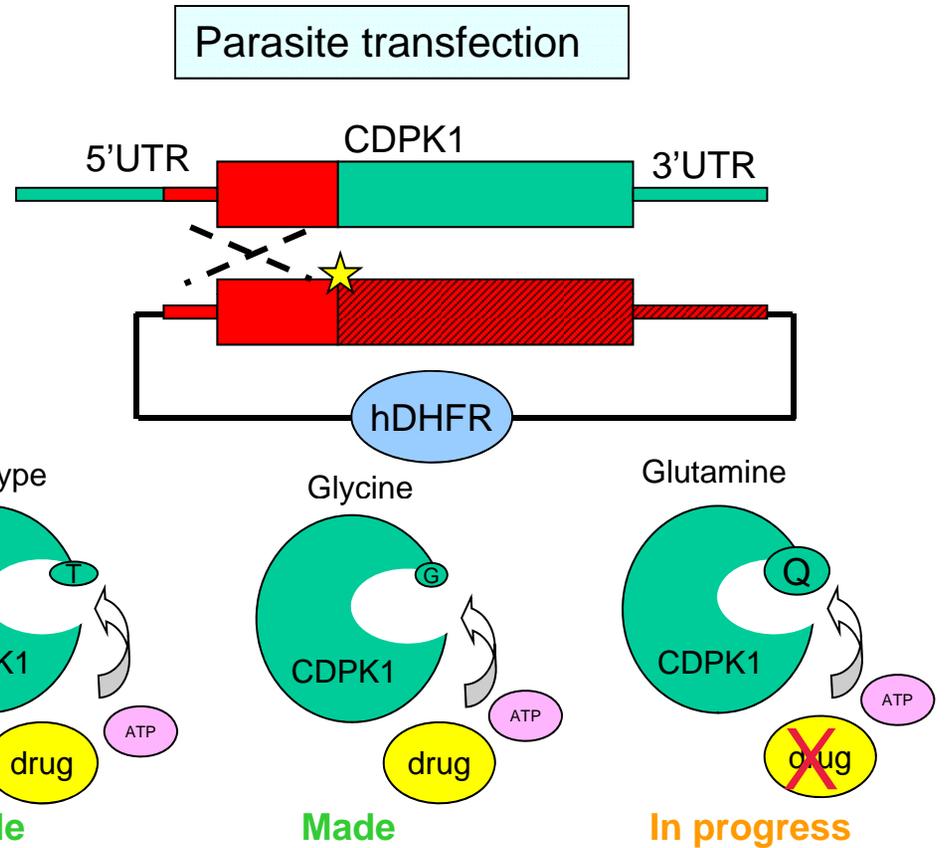
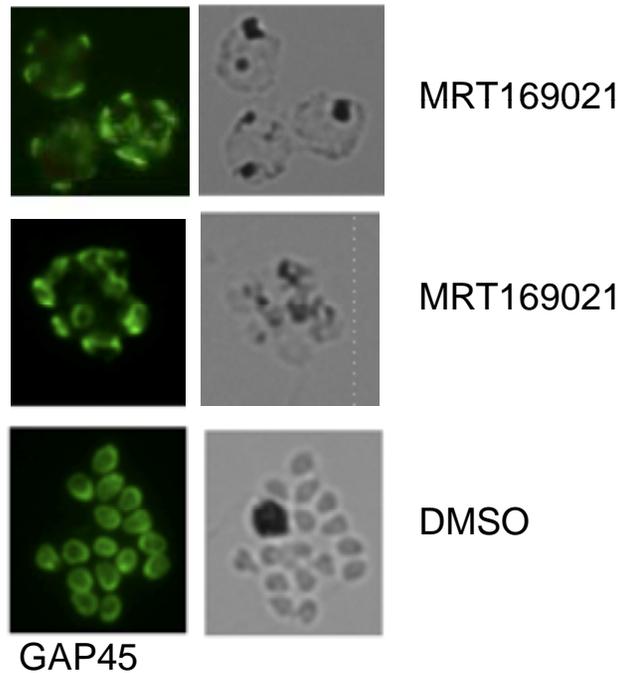
Inhibitor affinity was significantly reduced for T145Q compared to WT enzyme or T145G (4 -13 fold increase in Ki for T145Q).

Expressing this compound-refractory enzyme in *P. falciparum* will allow us to confirm that the effect of the inhibitors on parasite growth is due to CDPK1 inhibition, rather than off-target activity.

MoA studies – parasite:

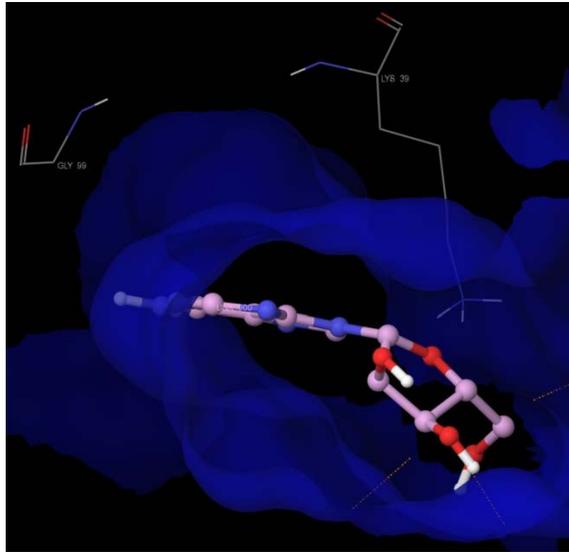
- Timing
- CDPK1 gate keeper mutants

100 nM MRT169021 (EC₅₀: 25 nM) added at ~30h and examined at 45h post-invasion

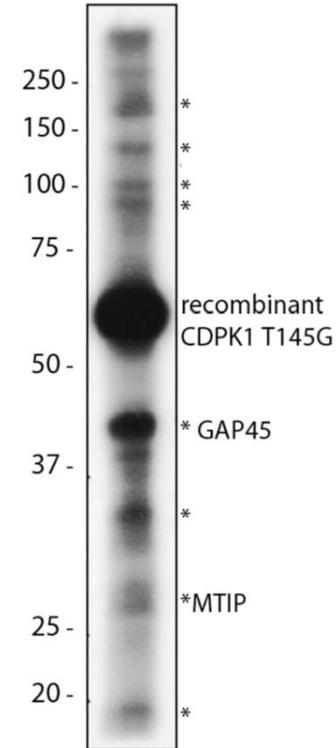
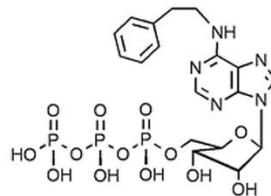
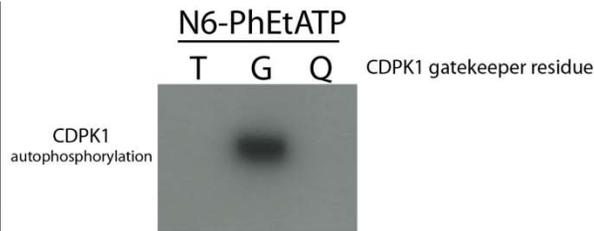


- T145Q - compound refractory
- T145G - identify new substrates/cell-permeable bumped kinase inhibitors should inhibit

Identify new substrates: replace gatekeeper with a SMALL residue



- Glycine at gatekeeper position allows the enzyme to use large ATP analogues



Labelling schizont lysates with CDPK1-T145G and $\gamma^{32}\text{P}$ -N6-PhEtATP
 Several proteins in *P. falciparum* schizont lysates (*) - new substrates for the enzyme?



Some lessons from running a two site academic drug discovery program.

- **Importance of target validation (gene knock out is not the same as Proof of Concept) as early as possible**
- **Regular face to face project meetings with all parties --- be aware of other resource/time commitments**
- **Management of Intellectual Property v Disclosure must strike the right balance as requirements are different for different groups**
- **Have a clear pathway to further development (in this case MMV involvement) that meets both parties expectations**
- **Extract the maximum benefit from any result—there is always something else to find out**
- **Don't let it distract from the main focus – but it may result in further targets being uncovered**



Conclusion

PfCDPK1 inhibitors show good anti-parasitaemia effects in a cellular assay but reduction of parasitaemia *in vivo* is disappointing

Compounds from the program are being used in MoA studies to determine the exact mechanism of action

Gatekeeper mutants to be used to determine extent of on-target activity

Affinity labelled compounds to be used to identify alternative targets that may be responsible for potent anti-parasite effects.

Acknowledgements



MRCT

Nathalie Bouloc

Tim Chapman

Jon Large

Ela Smiljanic

Claire Wallace

Kris Birchall

David Tickle

Sadhia Mahmood

Keith Ansell

Hayley Jones

Alison Levy

Michelle Raynor

NIMR

Judith Green

Barbara Clough

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