Novartis Institute for Tropical Diseases

Latent vivax malaria drug discovery

Erika Flannery on behalf of the team

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Joint International Tropical Medicine Meeting

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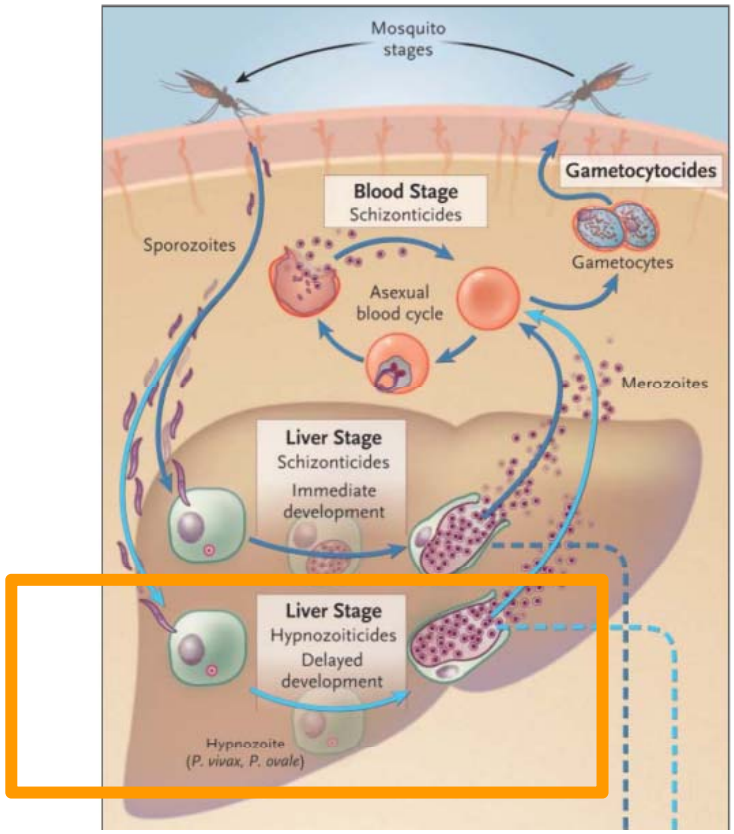
P. vivax latent liver forms cause relapsing malaria

Hypnozoites

- Clinically silent phase
- No diagnostic for detection
- Can activate months to years later

Challenge to eradication and elimination

- 90-96% of blood stage infections are due to relapse
- Mass Drug Administration (MDA) is a strategy
- In many countries, as Pf incidence declines, Pv incidence increases



***Plasmodium vivax* life cycle (hypnozoite stage is unique compared to *P. falciparum*)**

Wellems and Miller, *NEJM*, 2003

Antimalarials are stage specific

- There are 5 species of *Plasmodium* that cause infection in humans.
- *P. vivax* forms dormant hypnozoites and can therefore relapse.

Blood stage active

Chemotherapeutics

- Cure symptoms
- Kill the parasite and prevent recrudescence

Liver stage active

Chemoprophylactics

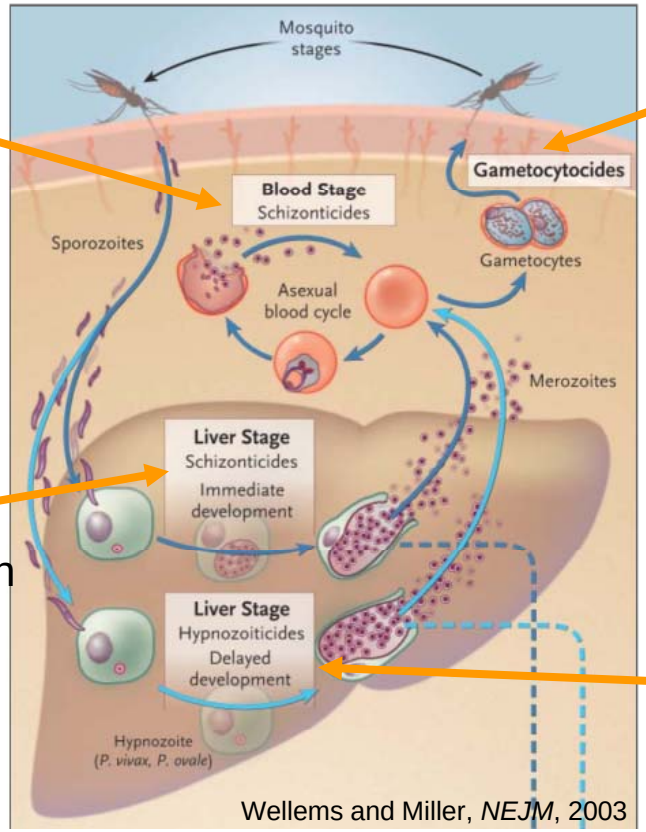
- Prevent reinfection

Causal prophylactics

- Prevent sporozoite infection
- Prevent early liver stage development
- Prevent late liver stage development

Suppressive prophylactics

- Kill parasites very early after egressing from the liver



Wellems and Miller, *NEJM*, 2003

Sexual blood stage active

Transmission blocking

- Kill the gametocyte in the patient blood
- Kill mosquito stages (gamete, ookinete, oocyst, sporozoite) in the mosquito

Latent liver stage active

Radical cure

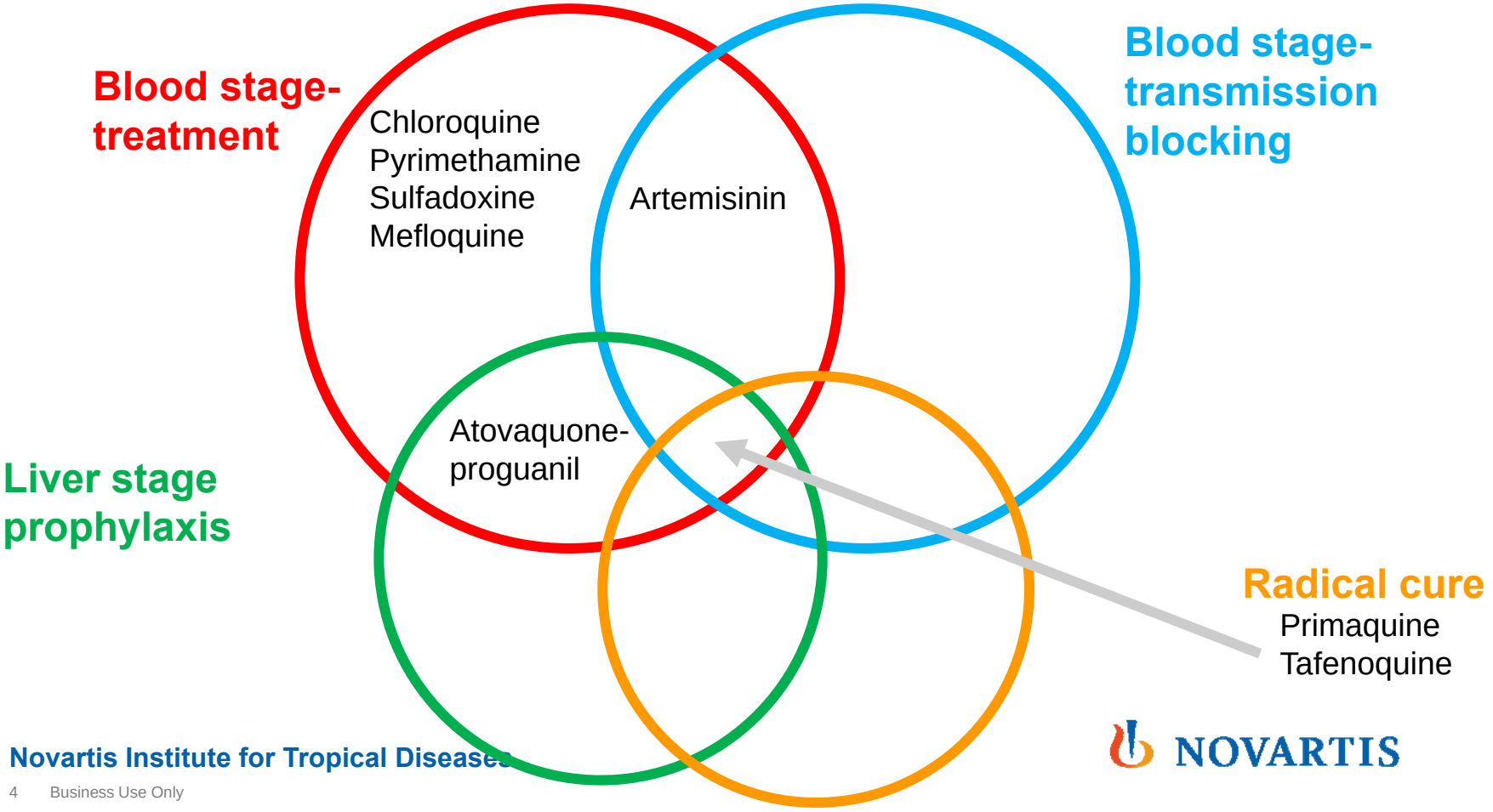
- Prevent *P. vivax* relapse

Pv – ***P. vivax*** **(human, relapsing)**
Pc – ***P. cynomolgi*** **(monkey, relapsing)**
Pf – *P. falciparum* (human, non-relapsing)
Pb – *P. berghei* (rodent, non-relapsing)

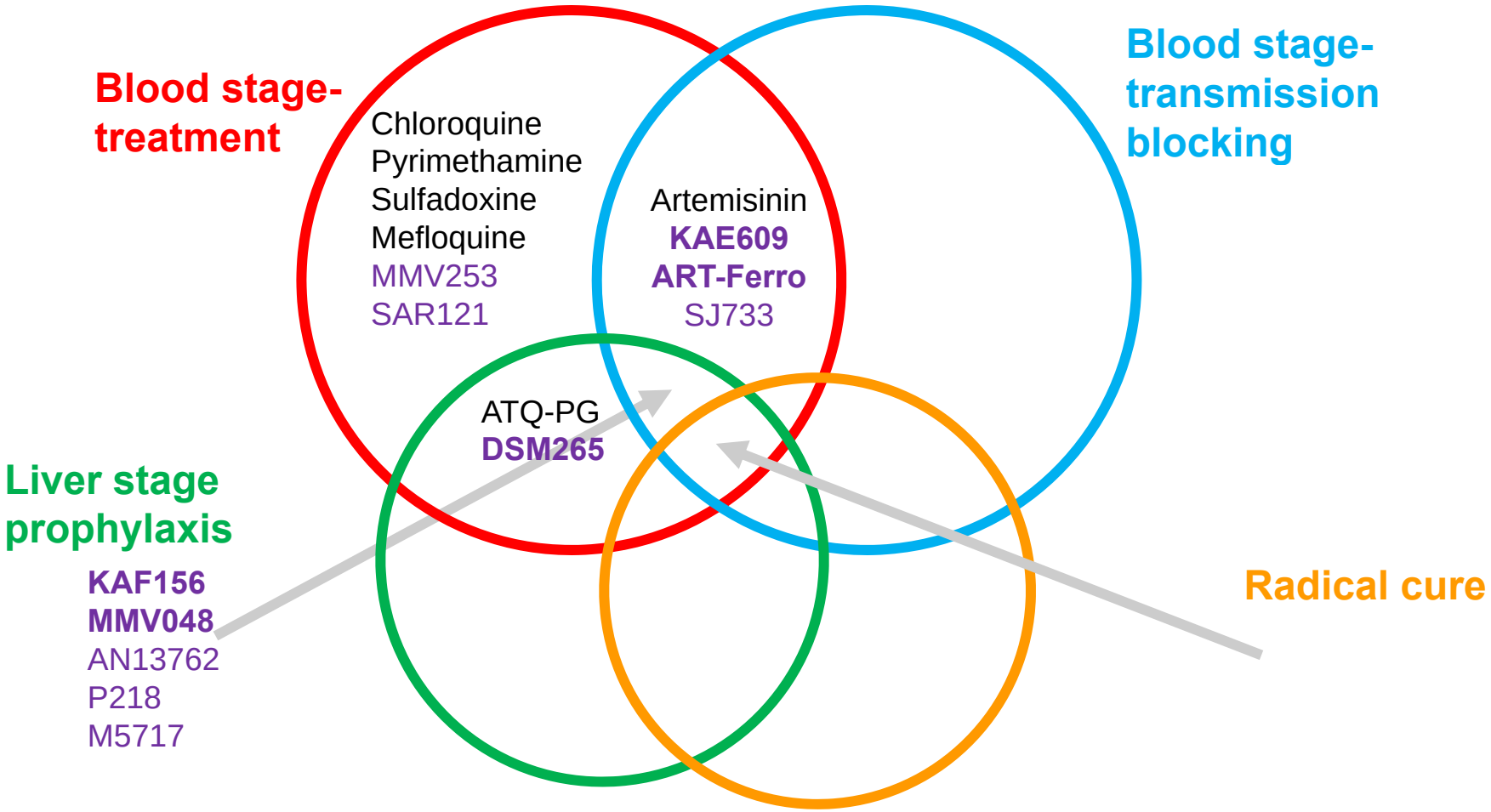
Current antimalarials

Representative from each class listed

- Choice of drug depends on country, resistance profile, formulation needed
- Standard of care: Artemisinin combination therapies (*P. falciparum*)
  Chloroquine + Primaquine (*P. vivax)*

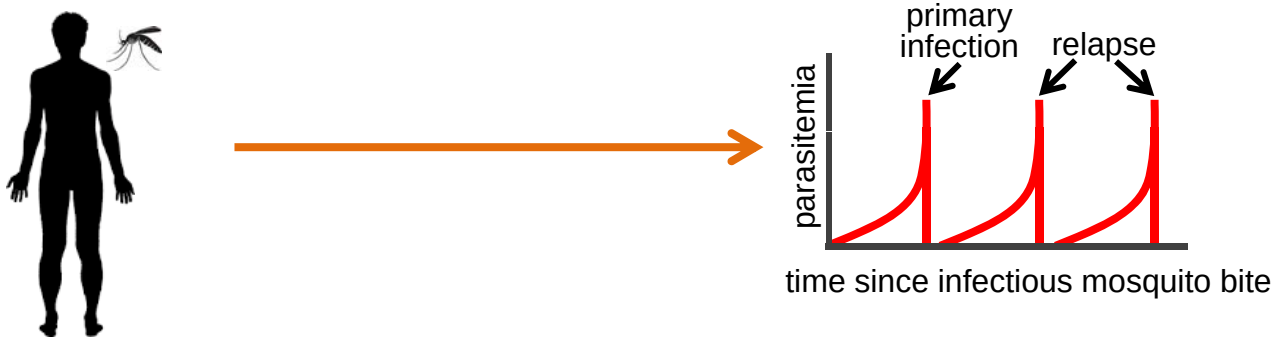


Antimalarial drugs in product development



8-aminoquinolines are the only anti-relapse treatments

- Relapsing malaria occurs >30d after primary infection
- Standard of Care
  - **14 day treatment (compliance issues)**
  - 8-aminoquinoline, requires metabolism for activity, active metabolite unknown
  - Unknown MOA
  - **Contraindicated in persons with G6PD deficiency (causes hemolysis) and pregnant women**
    - **Tafenoquine is not approved in children**
  - **Mutations in CYP450 affect efficacy of PQ**
  - Discovered in the 1950s in a 'medium throughput screen' of over 600 analogs in rhesus macaques



Malaria radical cure drug discovery

Goal: Identify a low molecular weight inhibitor for *P. vivax* latent in fection

Assay development, screening, hit finding

- *P. cynomolgi* relapsing monkey parasite

Lead optimization

- PK optimization
- ***P. vivax* human liver chimeric huHep mouse**

Preclinical profiling

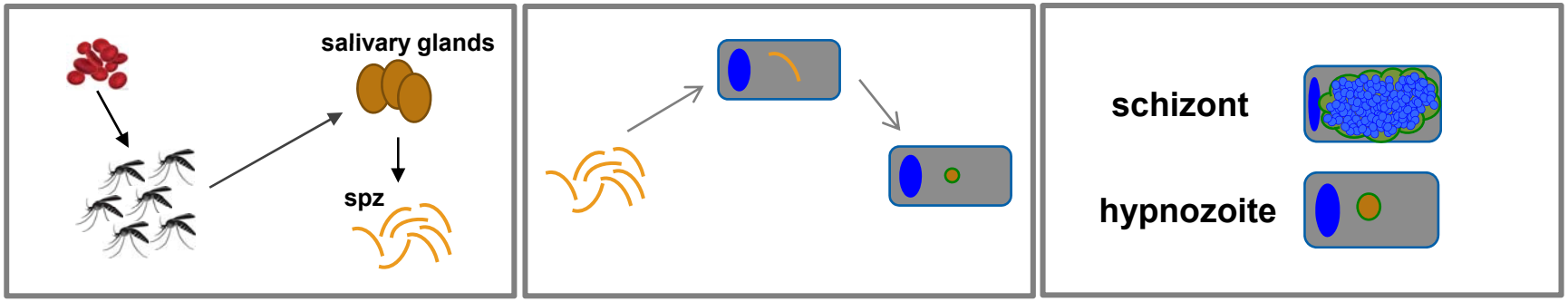
- Rat and dog *in vivo* tox
- Developability and formulation
- **Optional: *P. cynomolgi* monkey model**

New development candidate for radical cure

Additional on-going liver stage activities

- Cross-species activity: *P. falciparum* human liver chimeric huHep mouse
- Cross-stage activity: asexual blood stage and transmission blocking
- Clinically relevant optimizations: mosquito bite infection

Development of a radical cure assays

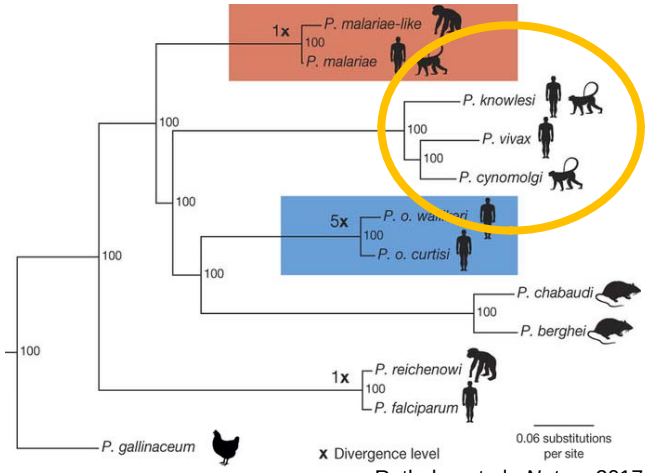


Sporozoites	Hepatocytes	Detection

• High level of infectiousness to mosquitoes (spz/mosquito)
• Propagation of blood stage sexual forms
  • in vitro
  • in host
• Hypnozoite-forming *Plasmodium* species
	• Susceptible to sporozoite infection
	• Support liver stage development and hypnozoite formation
	• Survive 2-3 weeks to assess radical cure efficacy
		• Identifies parasite (0.1% infection rate)
		• Differentiates between schizonts and hypnozoites
		• Differentiates between hypnozoites and dead/arrested parasites

Determining the best parasite model for radical cure drug discovery

	P. berghei/yoelli	*P. cynomolgi*	*P. vivax*
host	mouse	monkey	human
Liver incubation period	2 days	8 days	8 days
Relapsing (forms hypnozoites)	no	yes	yes
Strain	Laboratory	Laboratory	Clinical
in vitro culture	no	yes	no
Genetic modification possible	yes	yes	no



Rutledge et al., *Nature* 2017

Continuous *P. cynomolgi in vitro* culture established

In vitro blood stage culture conditions:

- NO antibiotics in *P. cynomolgi in vitro* culture or donor NHPs.
- Serum and RBCs from malaria naive NHPs.
- Serum and RBCs can be pooled.
- 10% Serum is sufficient.

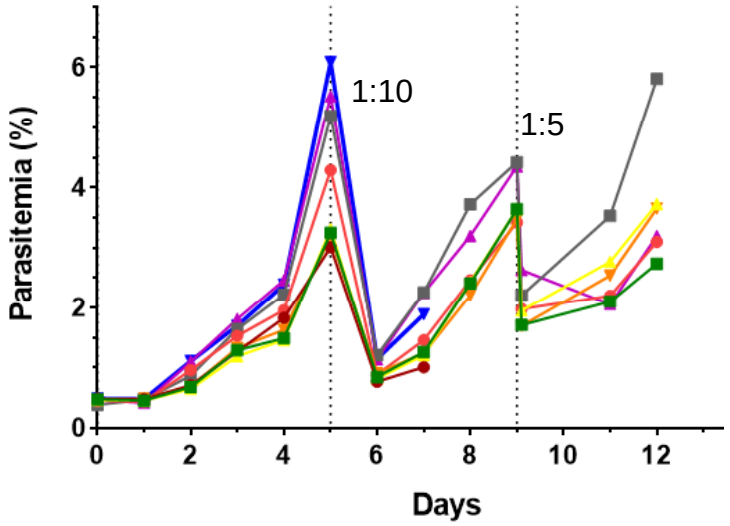
Next step:

- Pc gametocyte induction
- Passage through monkeys to increase gametocyte number

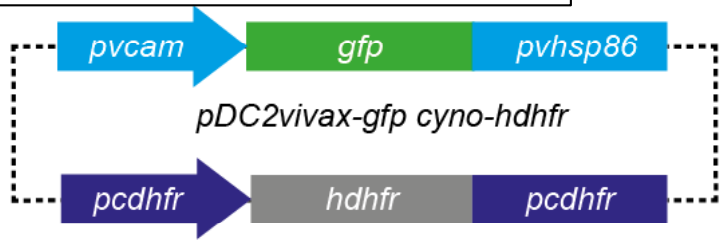
Plans to generate transgenic parasite

- First step: episomal GFP transfection
- Optimize selectable marker and cassette
- Optimize electroporation protocol
- Design liver stage reporter lines

***P. cynomolgi* growth curve in 8 NHP sera/RBCs**



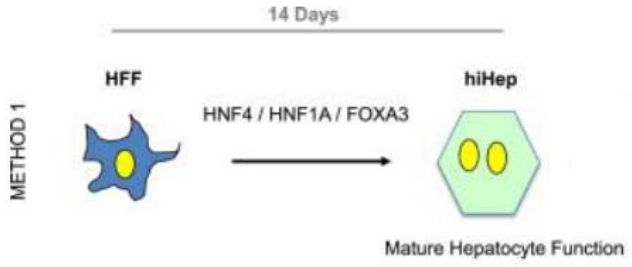
***P. cynomolgi* transfection plasmid**

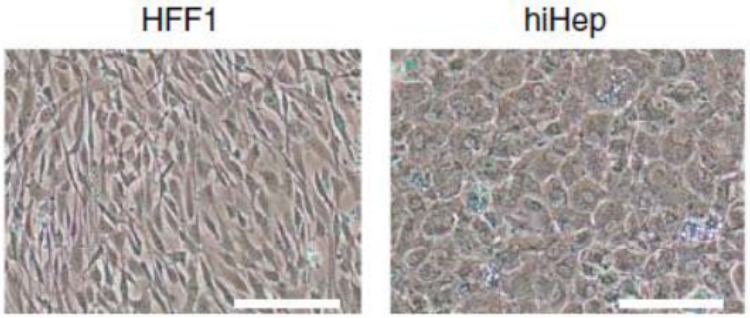


Induced pluripotent stem cells to generate long-lived hepatocytes

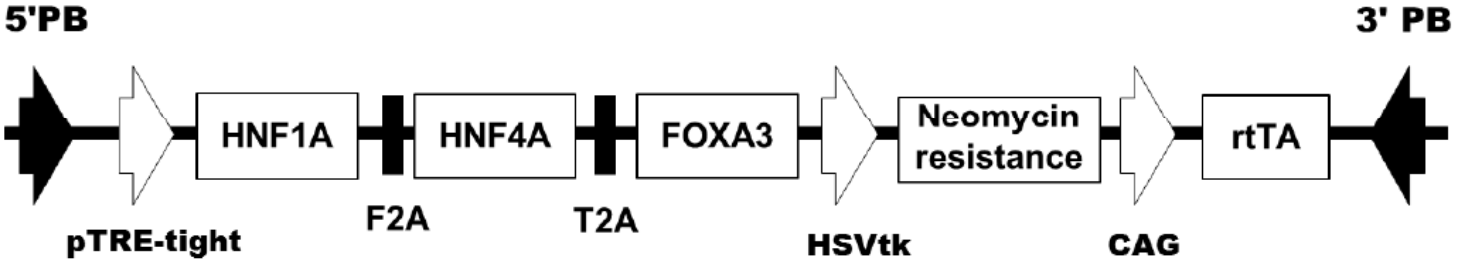
Direct reprogramming & Dox inducible system

Direct reprogramming into hepatocytes:



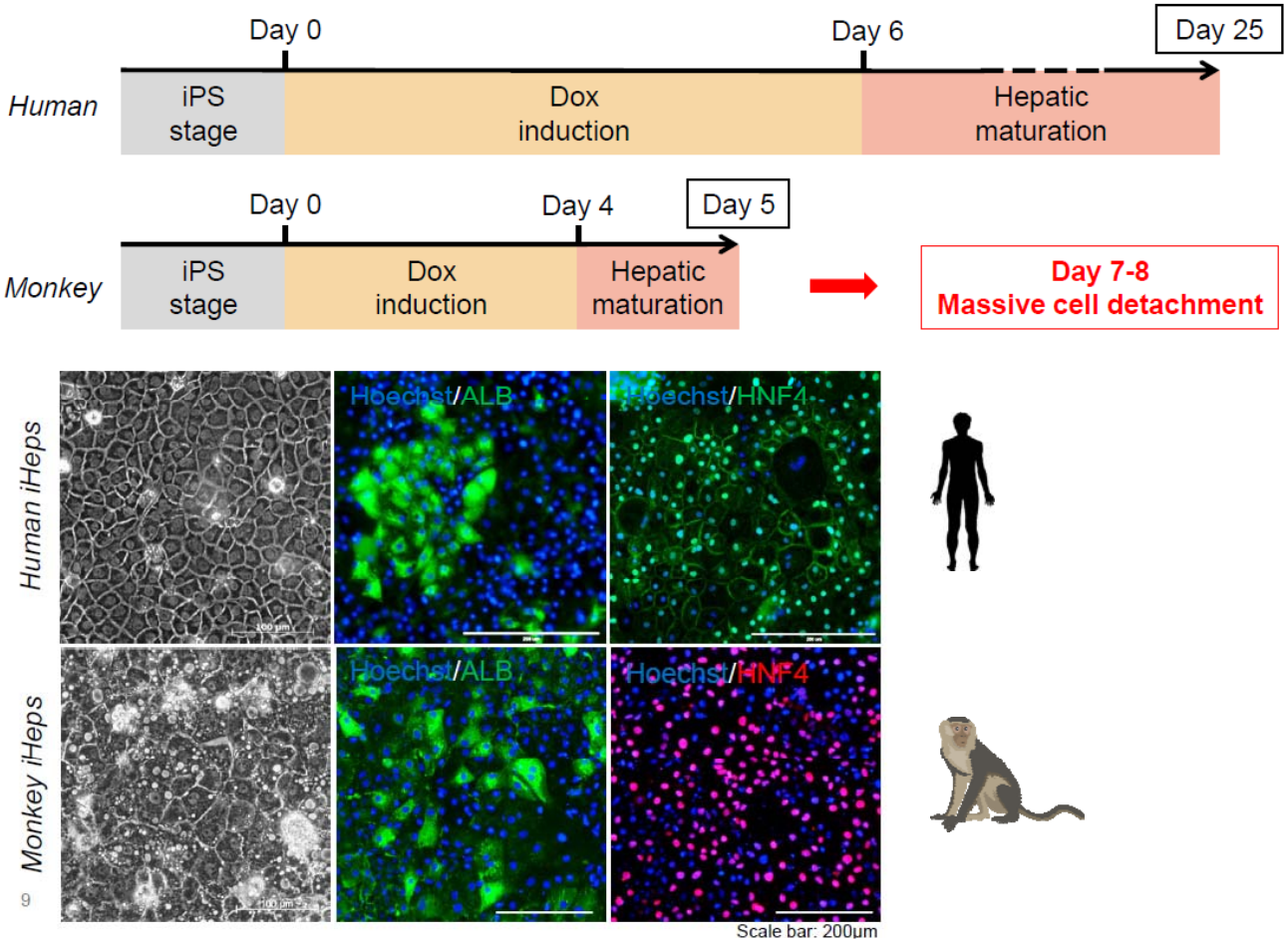


Dox-induced construct (cloning realized by Carole Manneville):



Melanie Pellisson & Matthias Rottman (STI)
Matthias Mueller (CBT)

Induced hepatocyte-like cells



MOA box screen for inhibitors that increase hepatocyte longevity

Preliminary screen

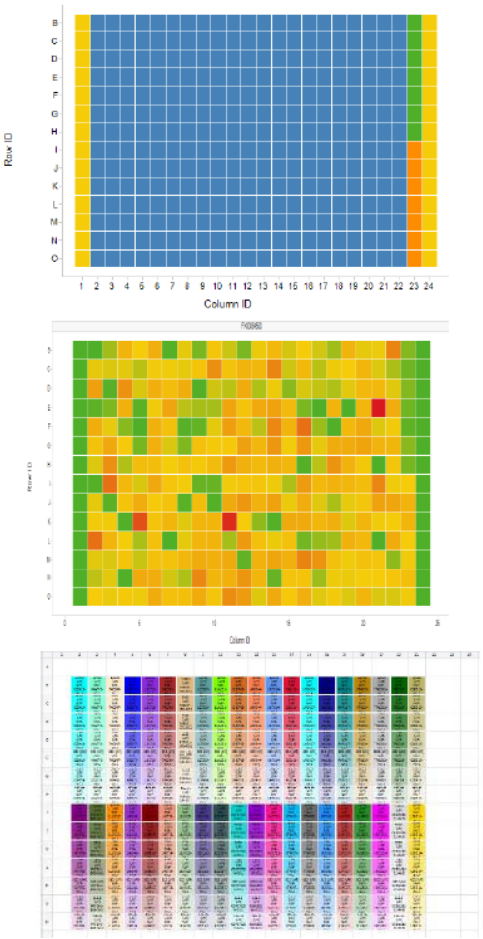
- 3800 tested compounds (5µM)
  → **80 hits selected** (5 overlap with human iHeps maturation screen)

Validation screen

- 7 points dose response (10µM → 0.16µM)
- Triplicates (1 replica per plate)
  → **18 hits validated**

Validation in 96-well plate

- IFA with ALB and HNF4
- ALB secretion (ELISA)
  → **3 identified hits for monkey hepatocyte survival**

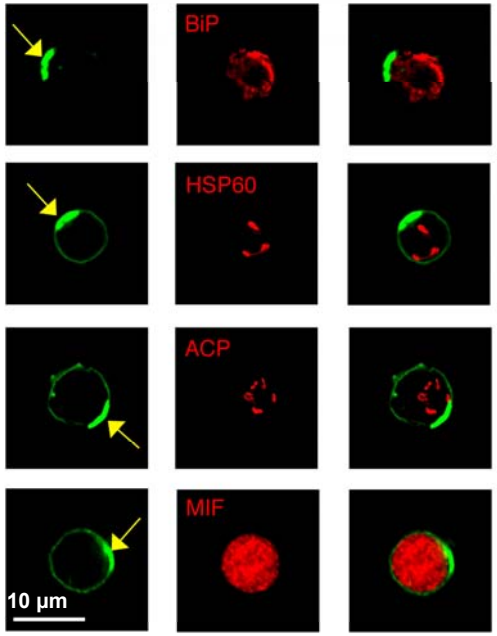


Screening support: Olaf Galuba, Isabelle Fruh

Imaging: optimization of high content imaging algorithms for automated hypnozoite identification

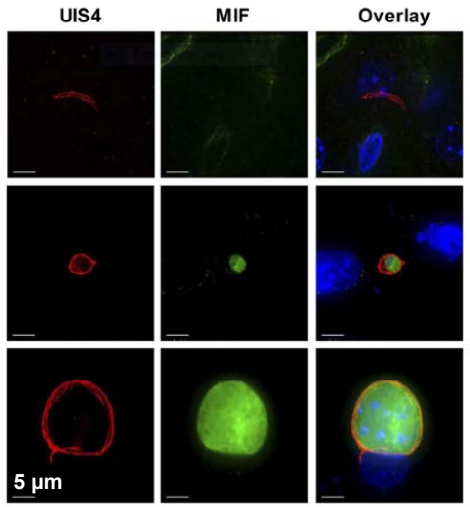
- Creation of novel *P. cynomolgi* monoclonal antibodies

P. vivax hypnozoites 8 days post-infection



Mikolajczak et al., *Cell Host Microbe* 2015

rodent *Plasmodium* trophozoites 2, 16, 30 hours post-infection



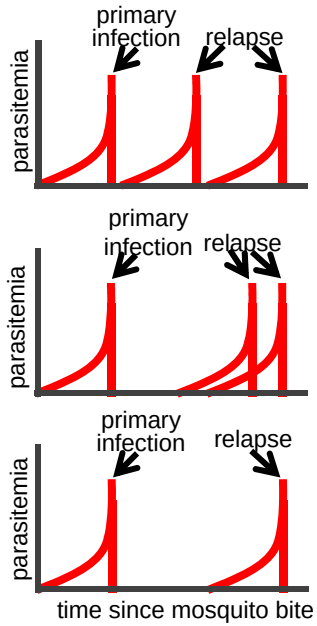
Miller et al., *Infect Immun* 2011

Vida

Nont

Measuring relapse in man and monkey

- In humans, only relapse in the blood can be measured
- Time to patency is the endpoint
- In monkeys, livers can be harvested but logistically and ethically challenging and only a handful of hypnozoites have ever been observed



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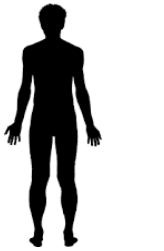
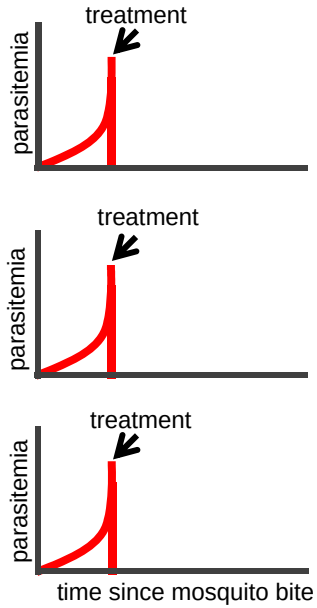
Measuring relapse in man and monkey

- In humans, only relapse in the blood can be measured
- Time to patency is the endpoint
- In monkeys, livers can be harvested but logistically and ethically challenging and only a handful of hypnozoites have ever been observed
- The current drug primaquine was discovered using these models



+primaquine



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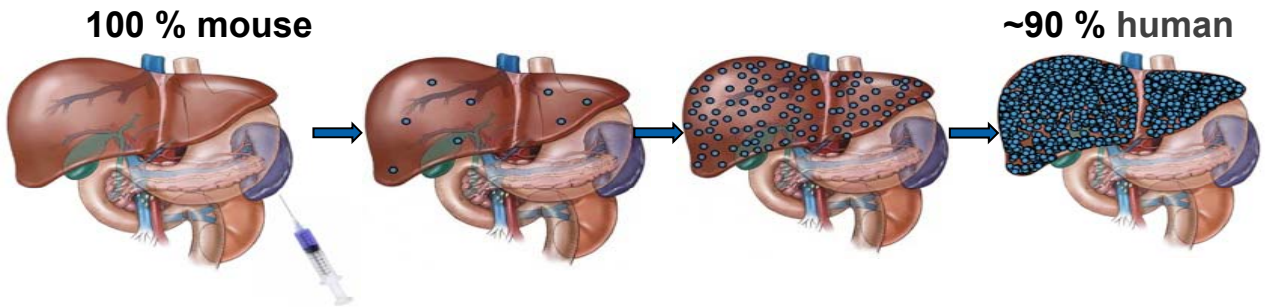
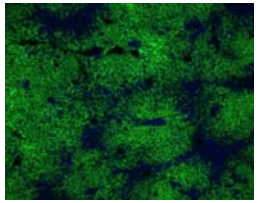
The human liver chimeric mouse is an advanced small animal model

Scale matters

Humanization of mouse livers





hFAH

Sebastian Mikolajczak & Ashley Vaughan

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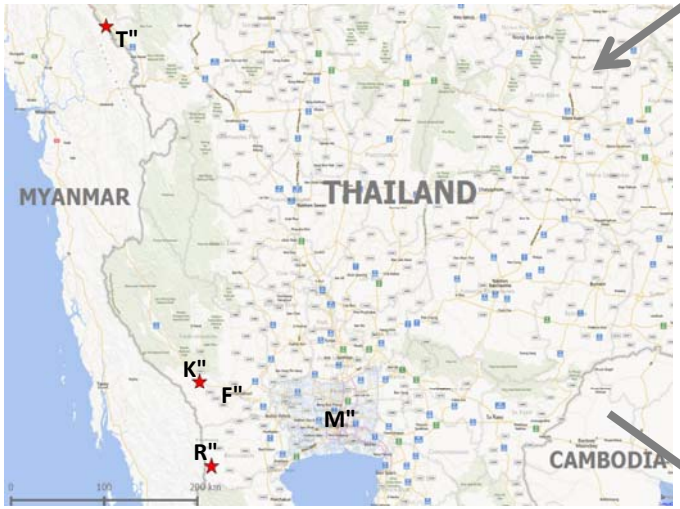
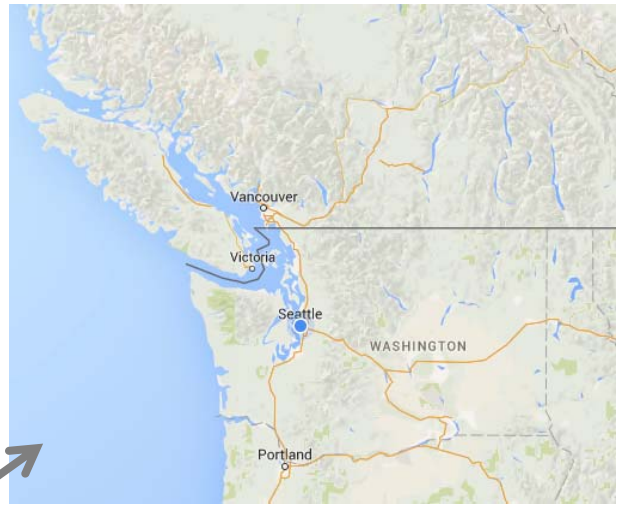
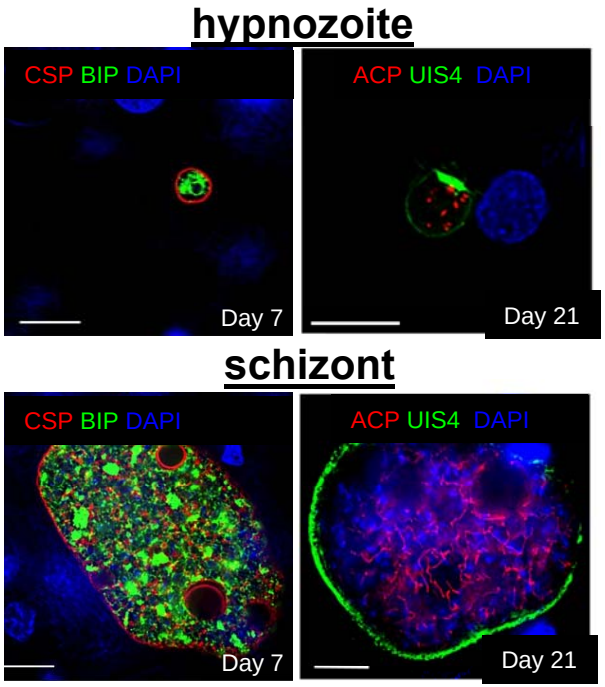
Sporozoite supply: workflow between CIDR (Seattle) and MVRU (Bangkok)





Liver and blood samples

Hypnozoites can be identified and characterized in the huHep liver



Scale bar: 10 um

<u>Factors differentiating hypnozoites from schizonts</u>

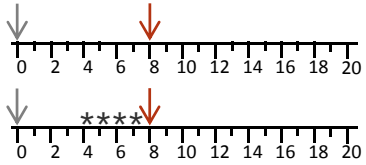
- Size
- UIS4 reactivity
- DAPI staining

<u>Structures and organelles that can be localized</u>

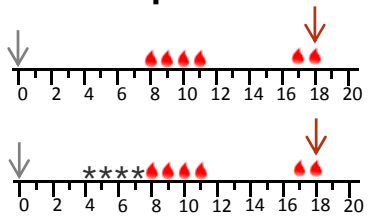
- Endoplasmic reticulum, parasite plasma membrane, parasitophorous vacuole membrane, apicoplast, mitochondria, histone acetylation

Relapse can be observed in the FRG huHep mouse

Primary infection



Latent/relapse infection

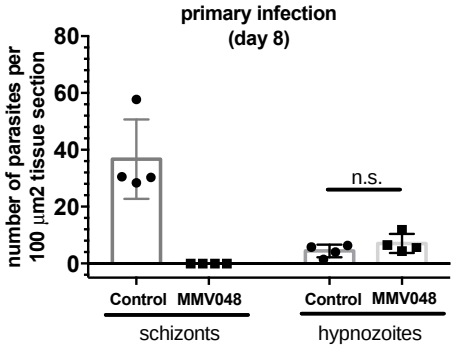


infection with 0.6M spz i.v. (VTTY-111)

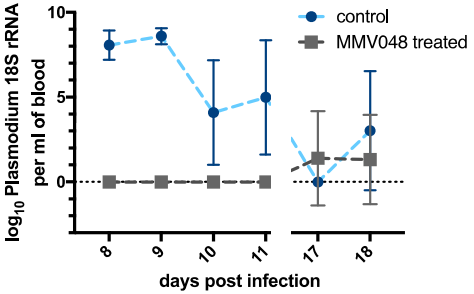
liver harvest for IFA and rtPCR

* 30 mg/kg MMV048 p.o.

Presence of schizonts in the liver (primary infection)



Presence of merozoites in the blood (primary infection)



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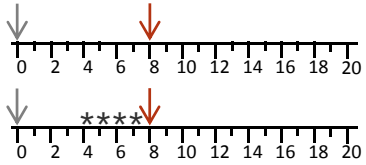
Relapse can be observed in the FRG huHep mouse

Primary infection



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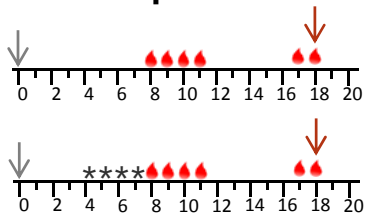

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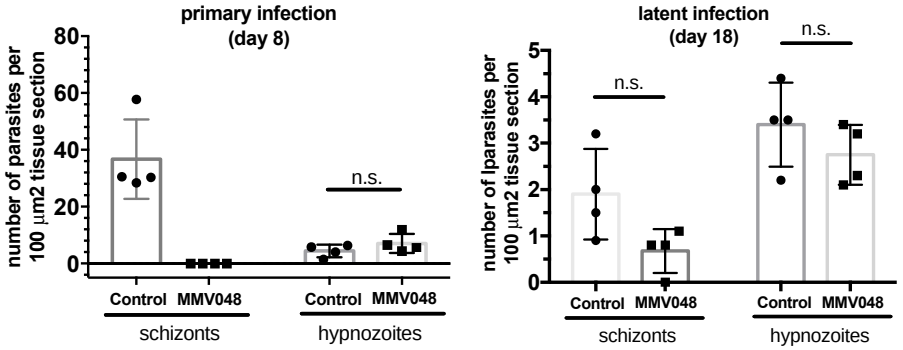
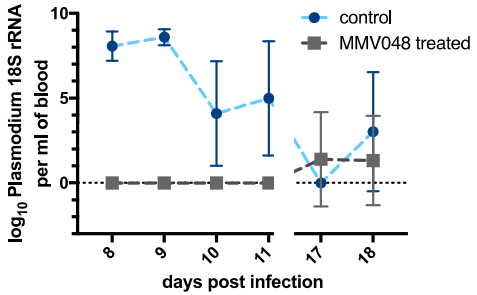


Presence of merozoites in the blood (latent infection)



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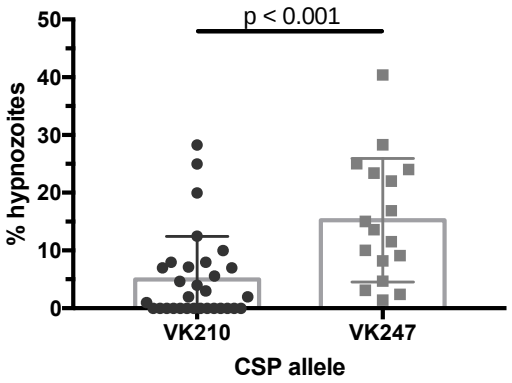
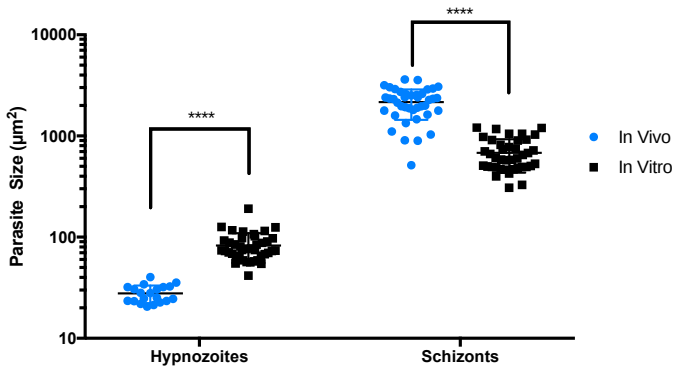
Frequency of hypnozoite formation is strain and platform dependent

CSP allele type correlates with %hypnozoites formed



Mean frequency: VK210 = 5.0, VK247 = 15.2
Each dot represents one mouse.
Number of isolates used: VK210 = 9, VK247 = 9

A greater number of hypnozoites are formed in vitro than in vivo



Hypnozoite formation frequency
- in vitro: 43.4%
- in vivo: 10.7%

- Hypnozoite formation frequency varies by isolate
- Genes playing a role in hypnozoite formation may be linked to CSP
- Using the same isolate, hypnozoite formation frequency is great in vitro than in vivo
- Stress may induce the formation of hypnozoites
- Schizonts in vitro may be smaller due to limitations on available nutrients

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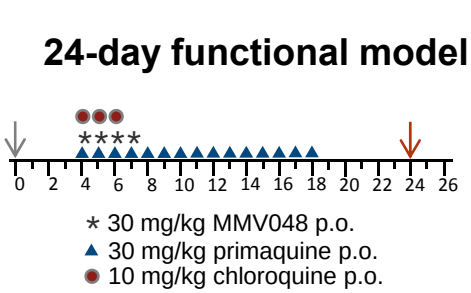
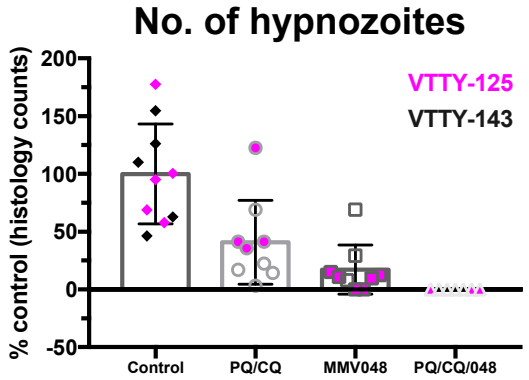
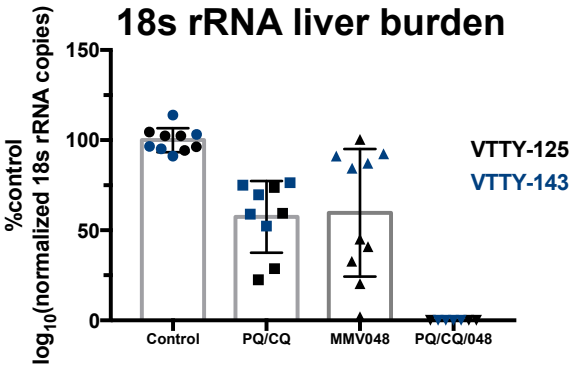
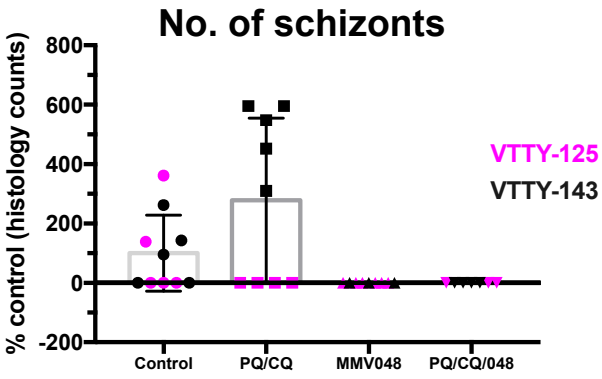
Using the FRG huHep mouse to test efficacy of novel potential radical cure molecules







*Each symbol represents one animal



- PQ + CQ reduces the number of hypnozoites and schizonts
- CQ-treated schizonts are arrested in one experiment
- May be due to strain-to-strain variability or host clearance mechanisms
- Triple therapy clears all forms of parasites from the liver

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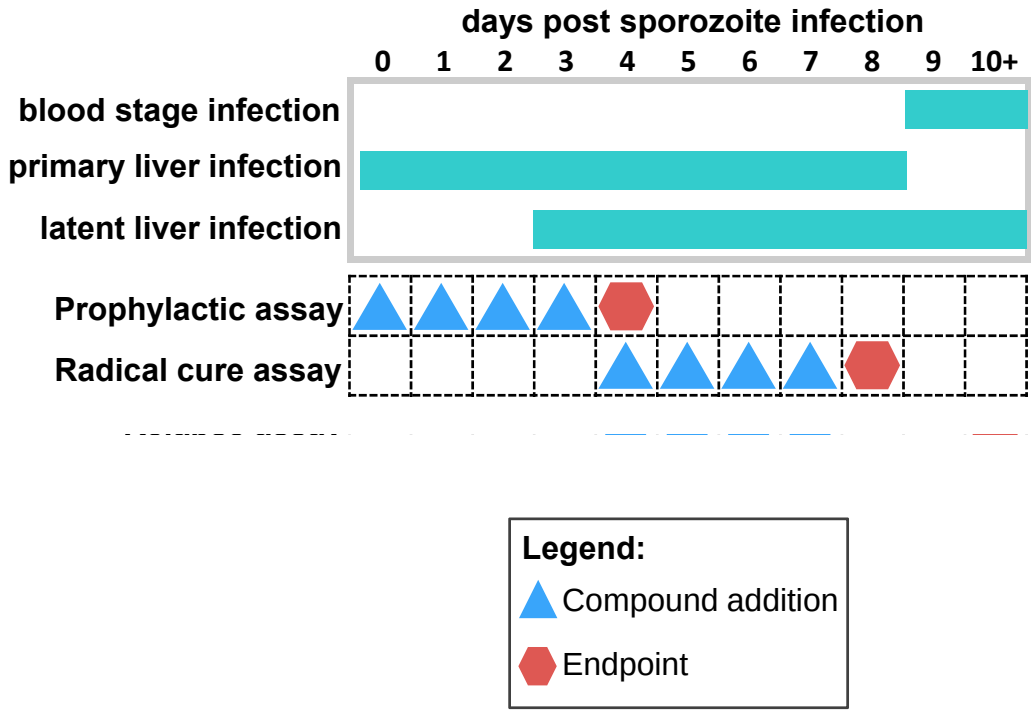
Cellular based assays assess dormant parasite disappearance and functional inhibition of relapse

Timing of dosing effects efficacy



- Several novel liver stage active drugs recently identified
- Active in prophylactic dosing, but **not radical cure** against relapsing parasites
  - PI4K inhibitors (MMV048, KAI407)
  - DHODH inhibitors (DSM265, P218)
  - Unknown target (KAF156)
  - eEF2 (DDD107498)

Parasite clearance is not necessary for efficacy

- Single dose treatment prevents transmission
- 24 hours post treatment gametocytes there is no reduction in the presence of gametocytes by blood smear
- At the same time, there is a large reduction in transmission

Single dose PQ:

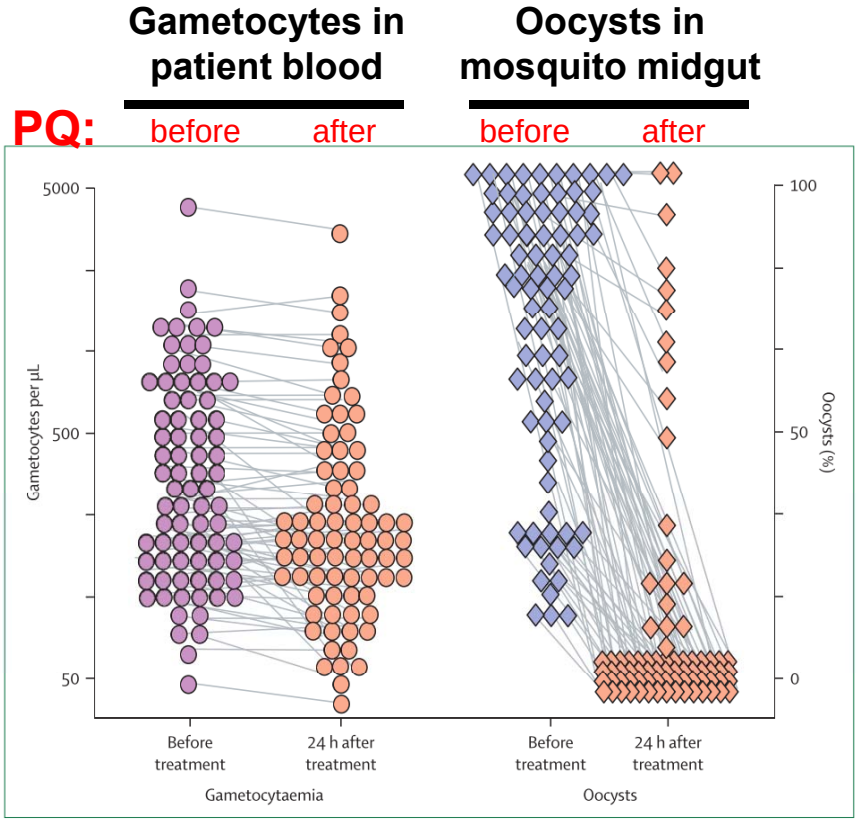
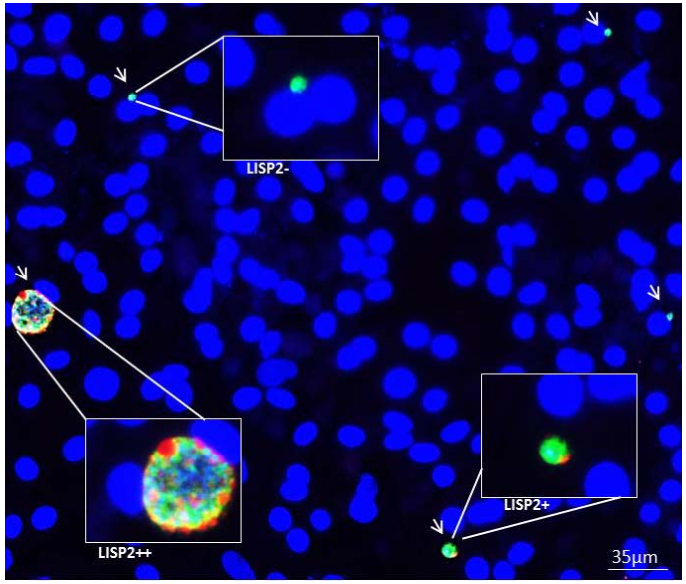


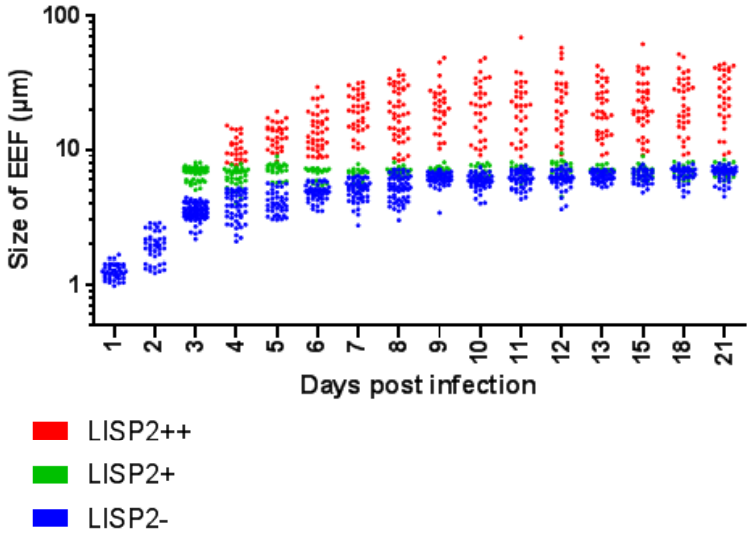
Figure 3: **Infectivity to mosquitoes that fed 24 h after patients with falciparum malaria and gametocytaemia were treated with plasmoquine or primaquine**
Oocysts were typically assessed in ten to 20 mosquitoes 6–7 days after they had fed. Each pair of circles or diamonds represents one patient.[21–38] Gametocytaemia changed little in 24 h, although it generally declined rapidly thereafter, but oocyst numbers fell rapidly to zero in most mosquito batches. When assessed later in parallel batches, sporozoites were correspondingly absent.

White, 2012 *Lancet Infectious Diseases*

Detection Plan B: LISP2 as an activation marker

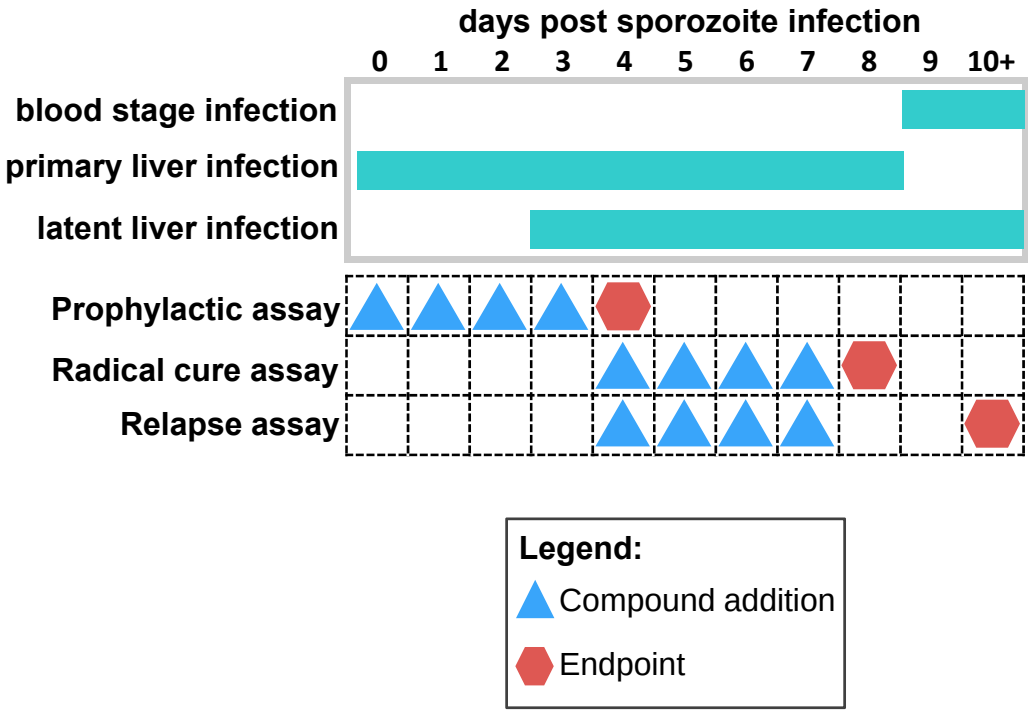
- LISP2 protein is a marker of hypnozoite activation
- Could be used to assess functionality of hypnozoites





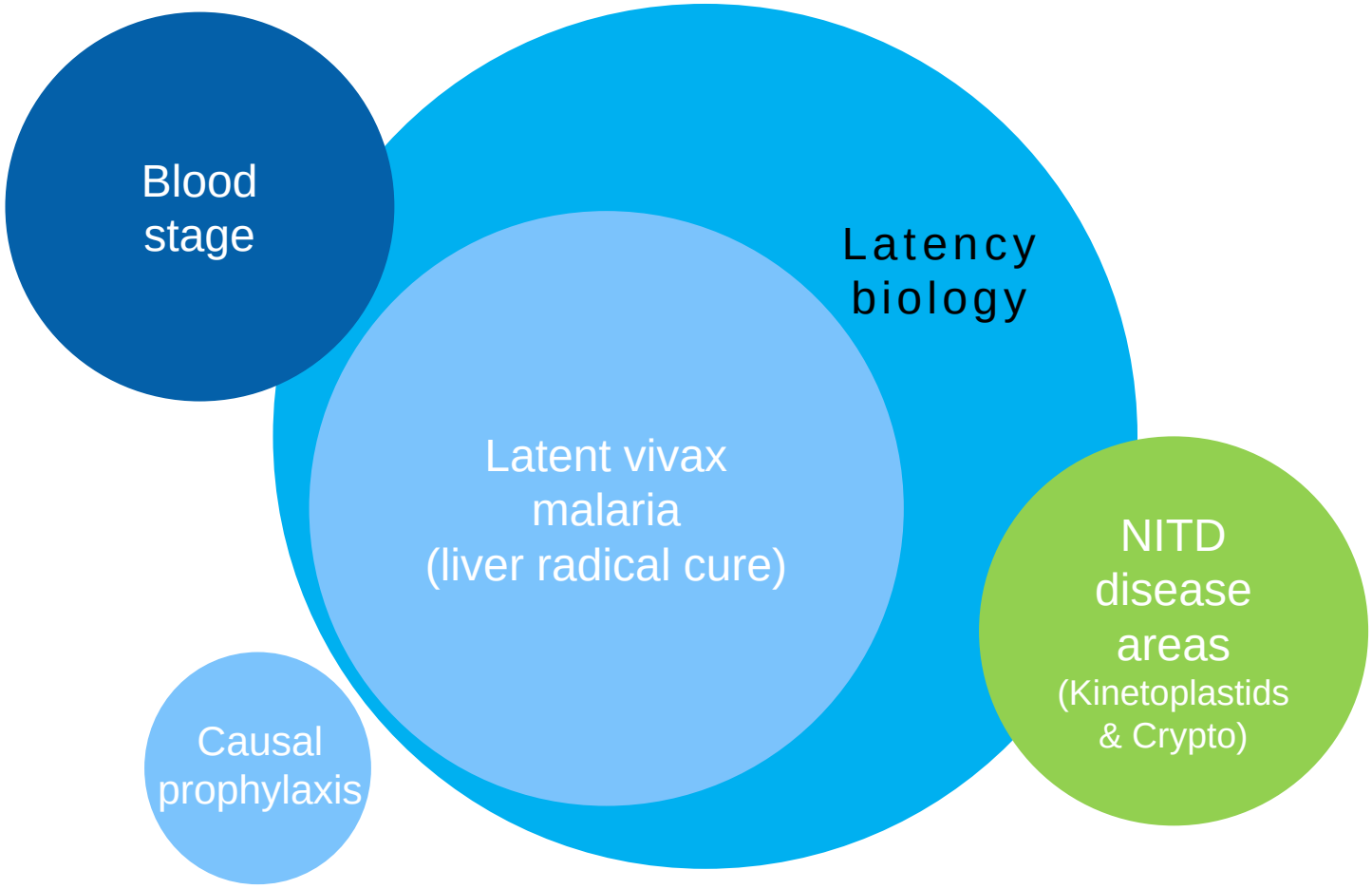
Cellular based assays assess dormant parasite disappearance and functional inhibition of relapse

Timing of dosing effects efficacy



- Several novel liver stage active drugs recently identified
- Active in prophylactic dosing, but **not radical cure** against relapsing parasites
  - PI4K inhibitors (MMV048, KAI407)
  - DHODH inhibitors (DSM265, P218)
  - Unknown target (KAF156)
  - eEF2 (DDD107498)

An important theme of study at NITD: latency



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•UW: Sean Murphy

•BMGF: Omar Vandal and Richard Elliott

•MMV: Brice Campo

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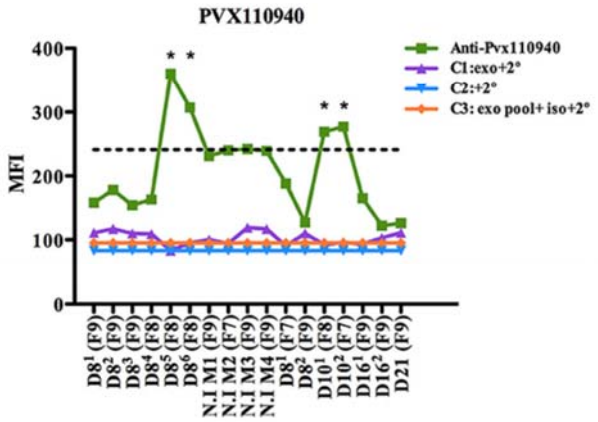
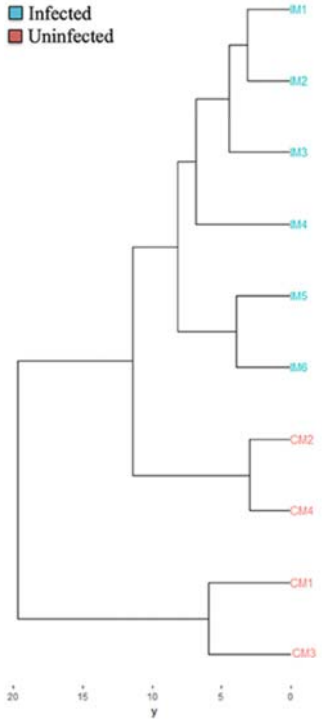
Back up

Using the FRG huHep mouse to identify biomarkers of latent liver infection

- Parasite proteins are consistently detected in exosomes from FRG huHep mouse serum
  - HSP70 and histone 2A are present in all infected samples



- PVX_110940 is syntenic with *P. falciparum* LISP1 although low sequence identity
- It is detected in a subset of infected mice



Human proteins associated with infection

- Clustering of proteins present in exosomes differentiates infected and uninfected animals

- 19 of 23 proteins are also present in *P. vivax* infected patient samples

Human proteins specific to liver-derived exosomes

- Markers of liver specific exosomes can be discovered using the FRG huHep mouse

In collaboration with Hernando del Portillo
Gualdron-Lopez, Flannery, et al. 2018

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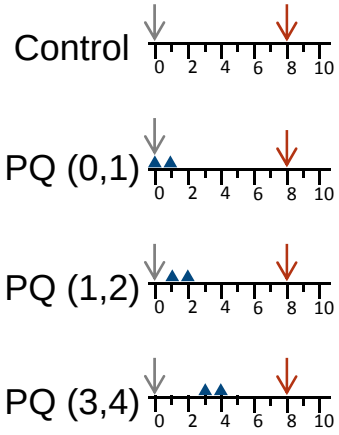
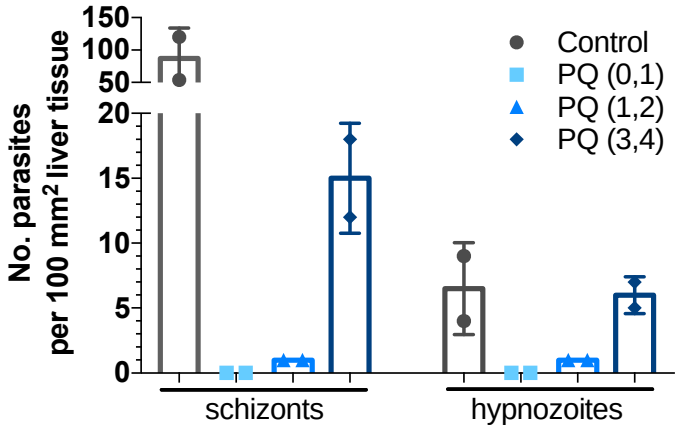
Hypnozoite ‘maturity’ is differentiated by primaquine 3 days post infection



infection with 1.0M spz i.v. (VTTY-125)

liver harvest for IFA and rtPCR

30 mg/kg PQ p.o.



- Early treatment prevents hypnozoite formation
- Treatment beginning three days post infection has no effect on the number of hypnozoites present

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Malaria elimination requires different strategies for each species

P. vivax

- High morbidity
- Asymptomatic infections are common
- 8.5M estimated cases in 2016
- 2.5 billion people live in endemic areas
- **Relapse contributes to disease incidence**

P. falciparum

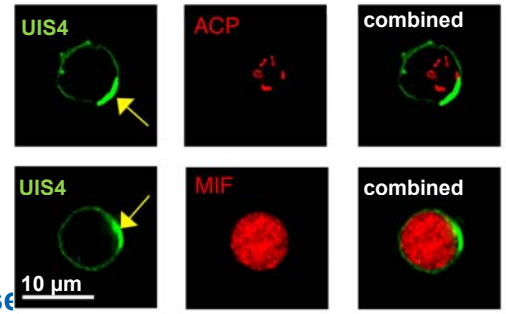
- High mortality
- Few asymptomatic cases
- 99% of cases in sub-Saharan Africa are *P. falciparum* (214 million)
- 91% of all malaria deaths are in sub-Saharan Africa (405,000)
- **Does not relapse**

World Malaria Report, WHO 2016
Bhatt et al., Nature 2015

Production of large-scale monoclonal antibodies for high content imaging

	ACP (acyl carrier protein)	UIS4 (up-regulated in infectious sporozoites 4)	MIF (macrophage inhibitory factor)
Protein expression and purification	complete	complete	upcoming
immunization	complete	in progress	upcoming
ELISA B cell screen	complete	Mid-July expected	upcoming
Secondary fixation screen	in progress	upcoming	upcoming
Hybridoma fusion	in progress	upcoming	upcoming
Overexpression and validaton	upcoming	upcoming	upcoming

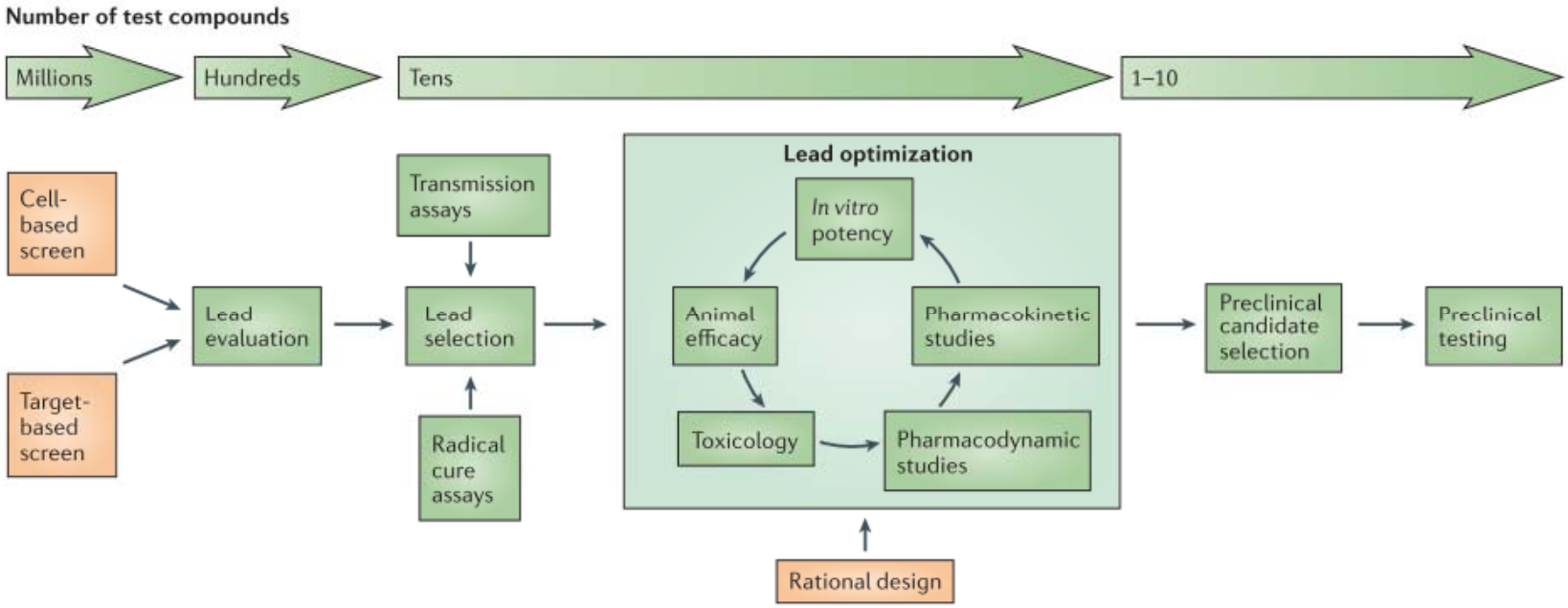
Proteins are characteristically expressed in *P. vivax* hypnozoites



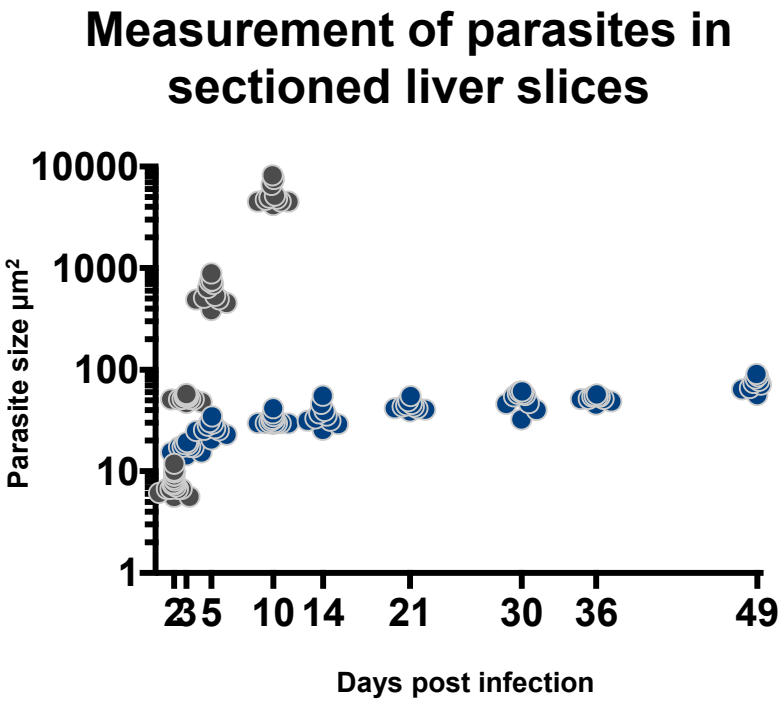
Mikolajczak et al., *Cell Host Microbe* 2015

In collaboration with Qianting Zhai, Tiffany Tsang, Rasika Kapoor, Mikias Woldegiorgis, Hassan Issafras and Isabel Zazor (Emeryville Protein Sciences)

High throughput screening to discover radical cure drugs



Hypnozoites are persistent in the FRG huHep mouse liver



- We have observed hypnozoites in the mouse liver 49 days post infection
- The number of hypnozoites declines over time

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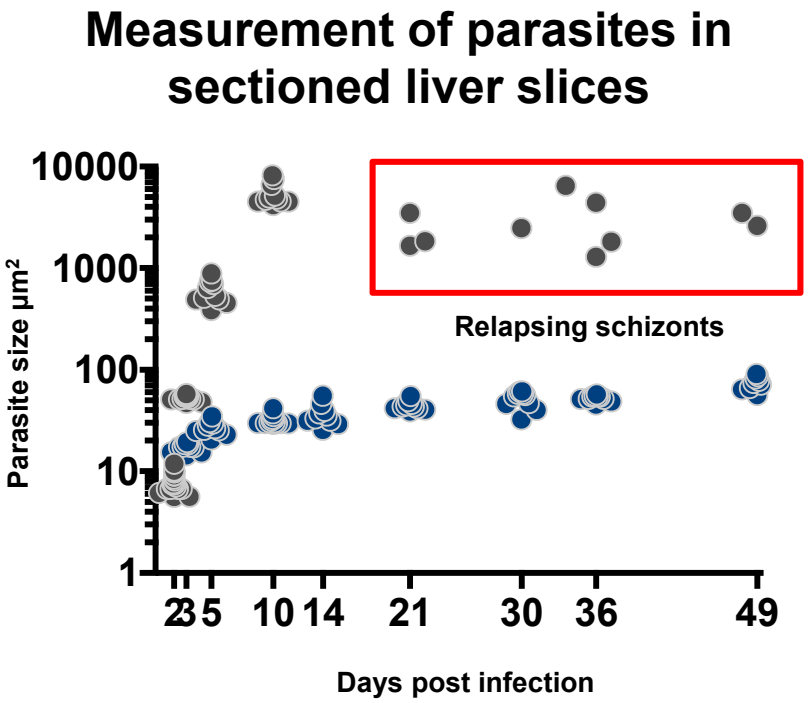
LMW molecule for prevention of latent vivax malaria relapse

- Latent *P. vivax* hypnozoite stages cause relapsing malaria in patients
- Establishment of an in vitro platform to assay efficacy of molecules against latent malaria
- Strategy: Use the monkey relapsing parasite, *P. cynomolgi*
  - Phylogenetically similar to *P. vivax* and greater potential for high-throughput screening

Two sequential phases to establishing the gold standard, cellular assay at NITD

	First latent infection assay	Future latent infection assay
Purpose (Timeline)	Begin working (9 months)	Optimize for throughput, efficiency and cost (15 months)
Sporozoites	Ship Pc infected mosquitoes from AFRIMS (Thailand)	In house Pc infected mosquitoes
Hepatocytes	Primary simian hepatocytes	iPSC-derived simian hepatocytes
Detection	High content imaging (antibodies)	Plate reader (Pc GFP-luc reporter strain)

Hypnozoites are persistent in the FRG huHep mouse liver



- We have observed hypnozoites in the mouse liver 49 days post infection
- The number of hypnozoites declines over time
- The number of schizonts observed at >12 days post infection is small

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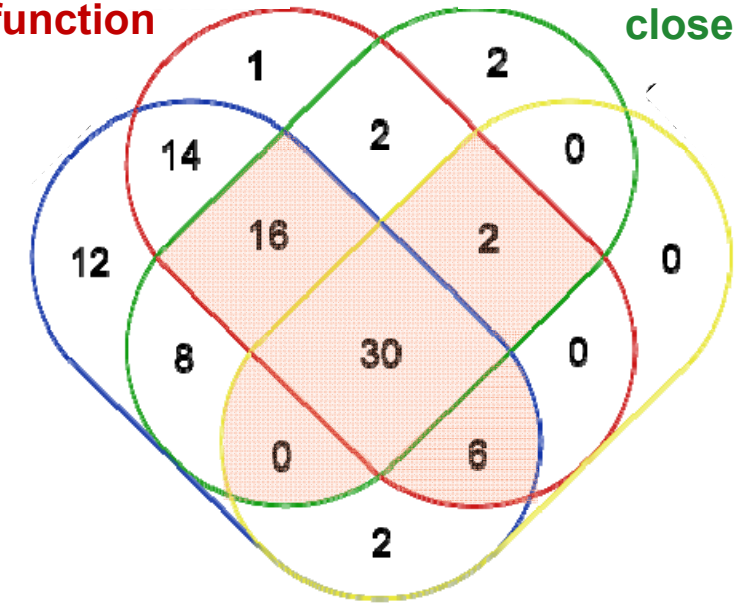
Selection of promising druggable candidates

Essential / known biological function

Conserved in *P. vivax*, no close homology to human

Expressed in hypnozoites

With protein structure /Inhibitors available



54 genes in >3 conditions

Exclusion criteria: Proteins of the Proteasome / Ribosomal proteins

Basel Team
Guglielmo Roma
Nicole Bertschi
Sven Schuierer
Florian Nigsch

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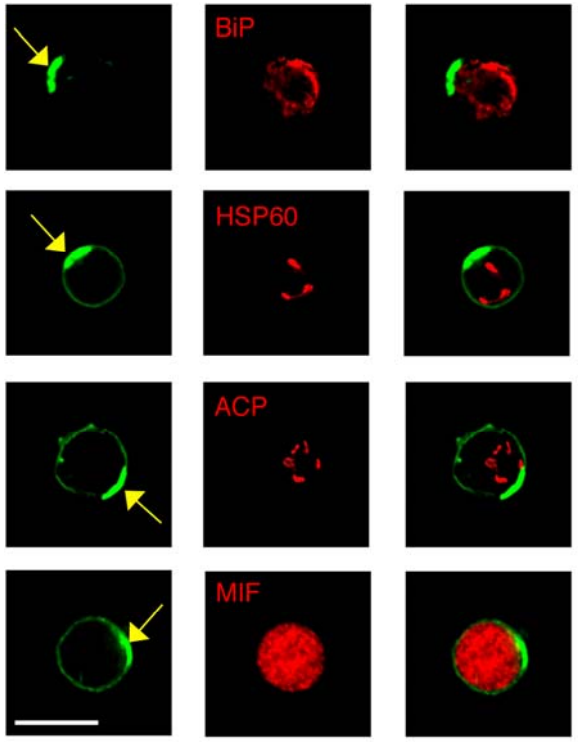
P. cynomolgi organelle antibody markers

P. vivax:



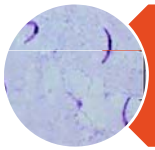
P. cynomolgi:

BiP	•Cross-reactive with *Pc*
HSP60	•Cross-reactive with *Pc*
HSP70	•Developed at NITD (polyclonal)
ACP	•To be developed
MIF	•To be developed
UIS4	•To be developed

In collaboration with Qianting Zhai, Tiffany Tsang, Rasika Kapoor, Mikias Woldegiorgis, Hassan Issafras and Isabel Zazor (PS)

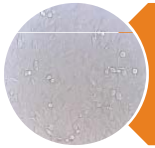
Detection: use *P. berghei* early liver trophozoites to optimize imaging of small hypnozoite-like parasites

Experimental Design:

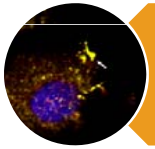


Receive *P. berghei* infected salivary glands

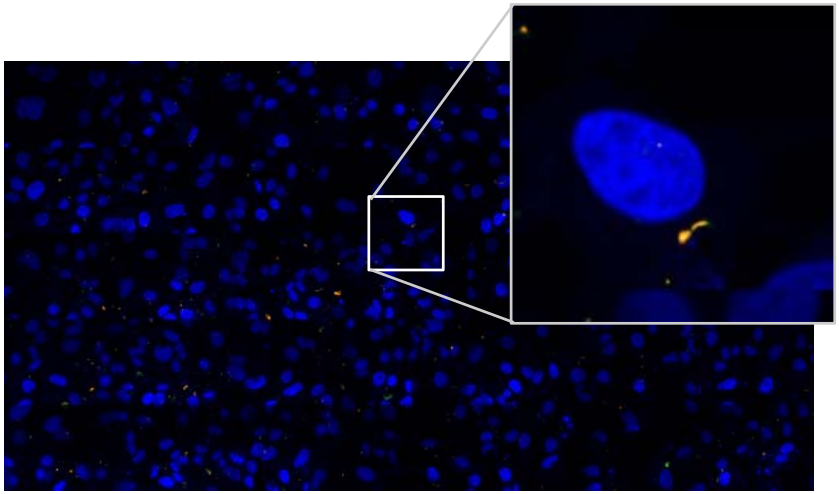
Harvest sporozoites



Infect HepG2 cells



Fix & stain at 2,4,6,8hpi



- Infections were successful, small rounded trophozoites were detected
- Image analysis pipelines established for gridding images, quantification with CellProfiler
- Next step: improve staining, establish automation, prepare for larger screens

Pv – ***P. vivax*** **(human, relapsing)**
Pc – ***P. cynomolgi*** **(monkey, relapsing)**
Pf – *P. falciparum* (human, non-relapsing)
Pb – *P. berghei* (rodent, non-relapsing)

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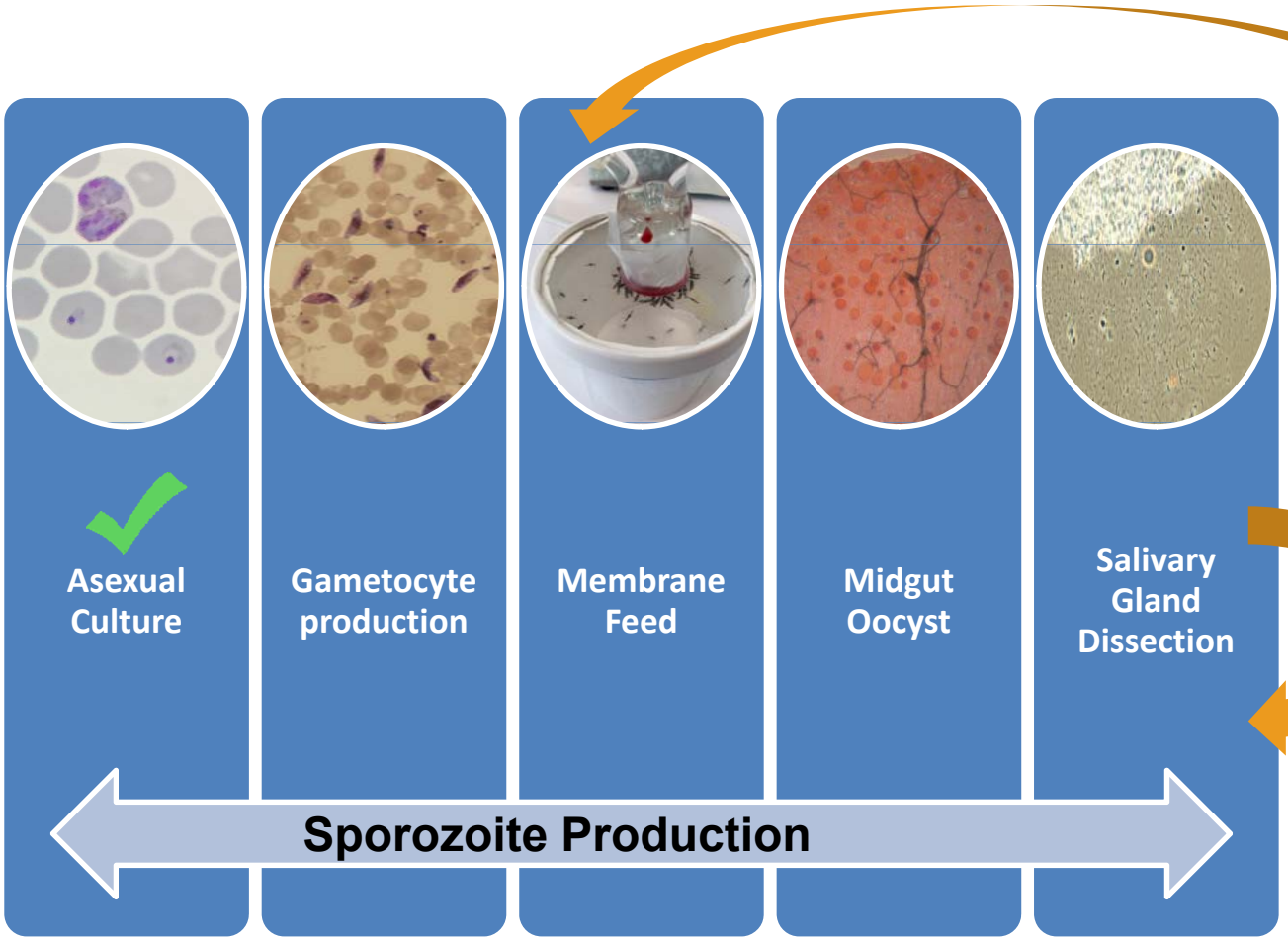
Optimization of first in vitro Pc mosquito infections



- High capacity production
- 2000 day 4 adults weekly
- Genetic selection of Pc strain

- Passage sporozoites through monkey
- Selection of high relapsing strains

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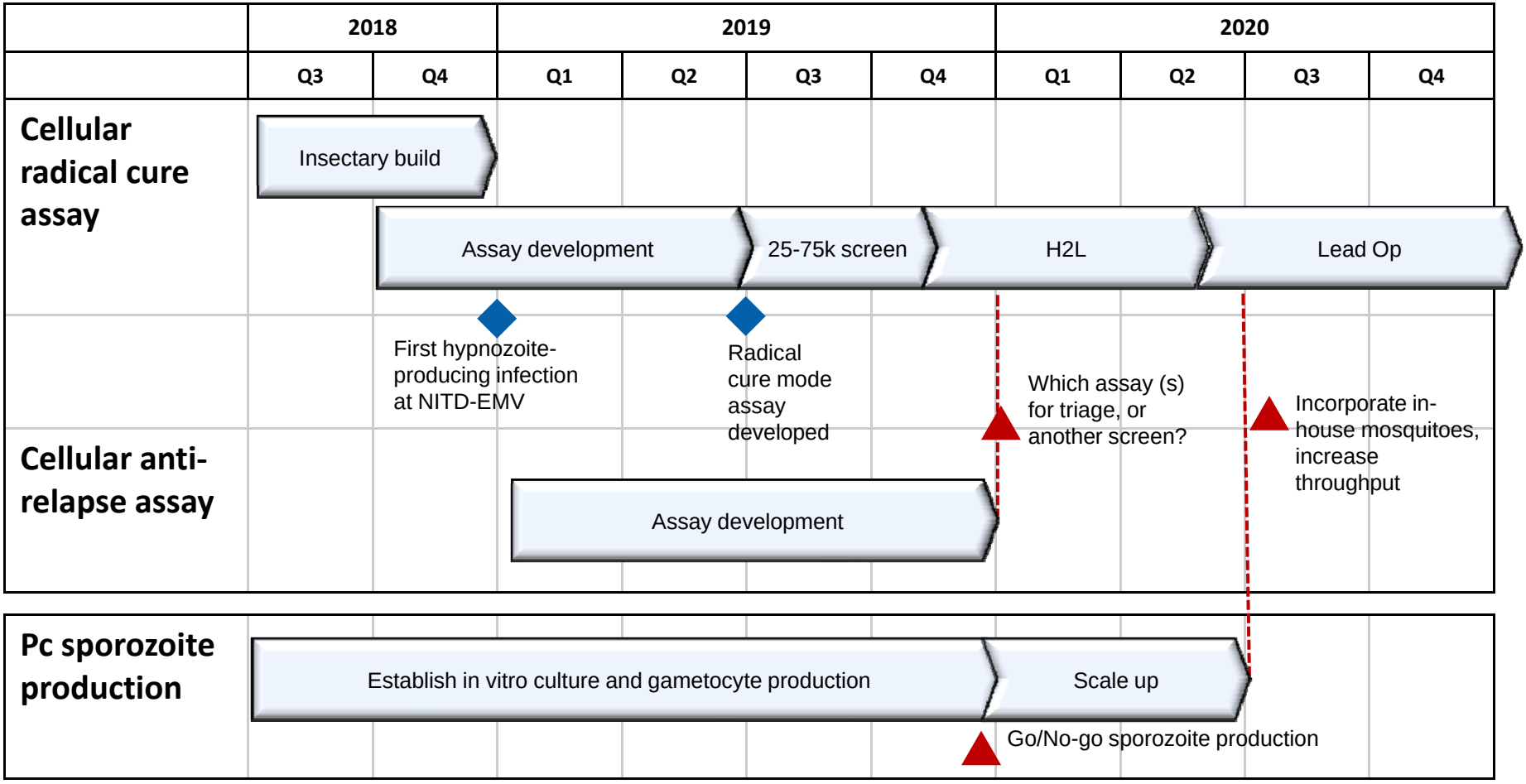
Cellular-based strategy:

assay development and initial screening timeline



milestone decision point

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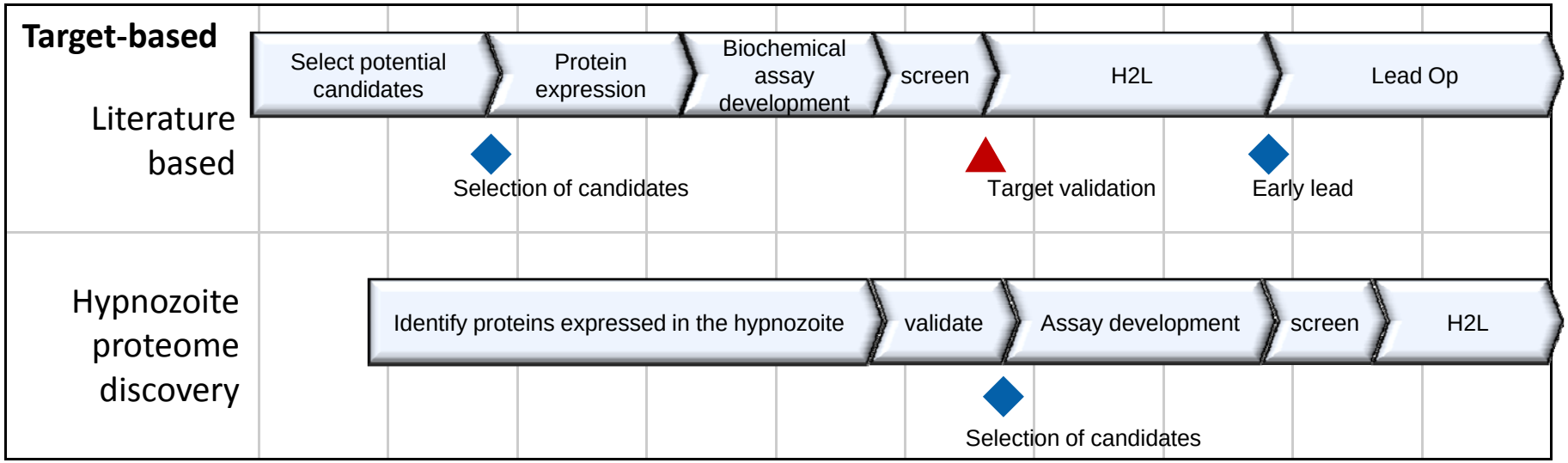
Target-based strategy:

assay development and initial screening timeline

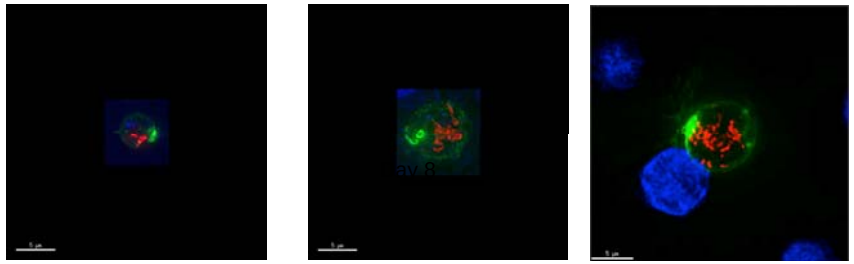


milestone decision point

Efforts to develop a target-based screen

- No known targets
  - Including PQ
- Pathways hypothesized to be important
  - Post-translational repression
  - Phosphorylation of eIF2alpha
  - Apicoplast and mitochondria are morphologically changing in hypnozoites
- Exclusive expression in hypnozoite is not necessary
- Learn from other latent malaria stages (or even other dormant pathogens)

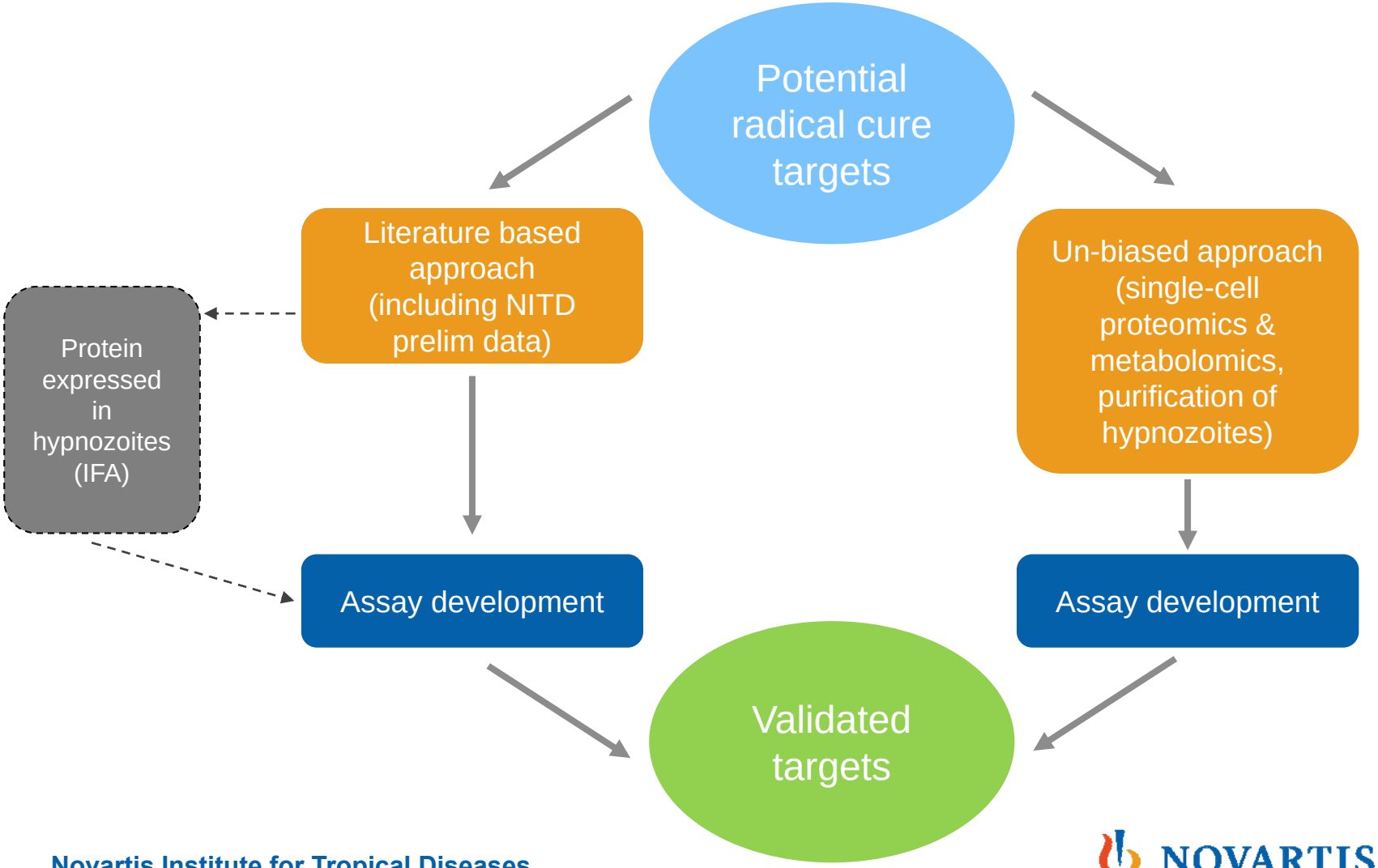
Apicoplast and mitochondrial structure changes



Day 8 Day 24 Day 49

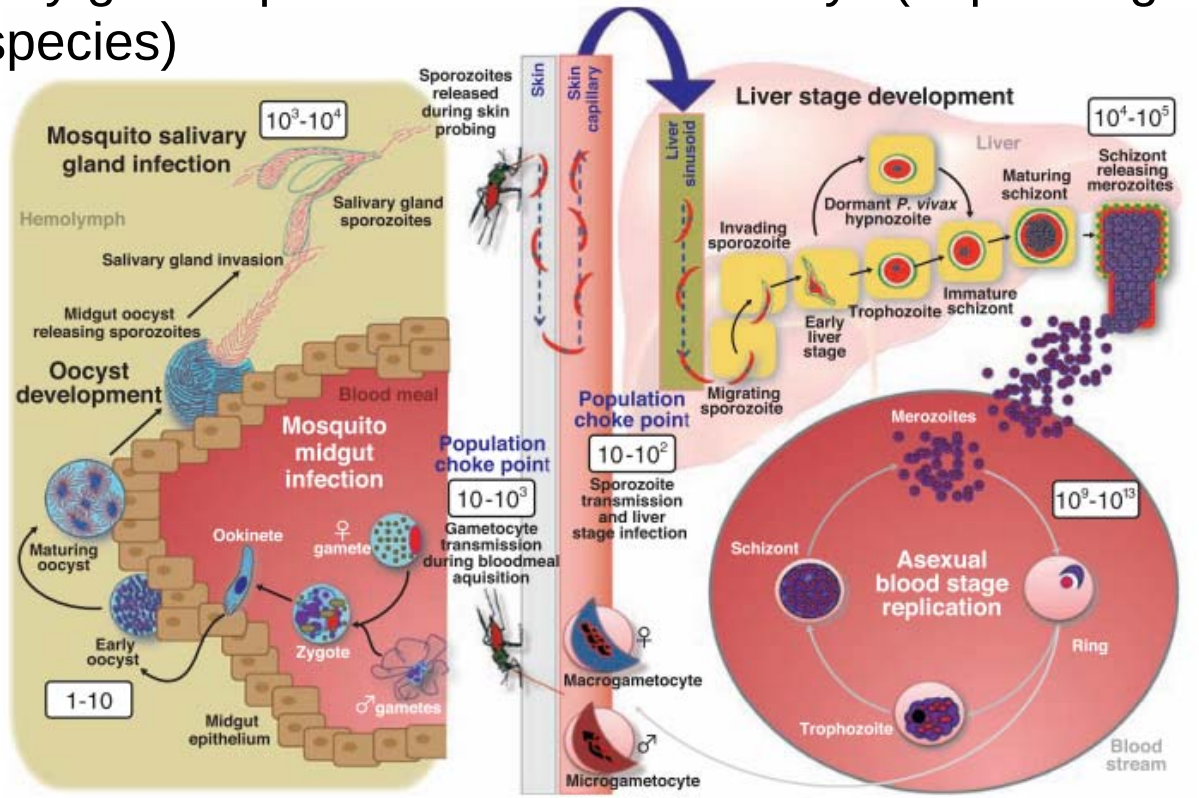
UIS4 parasitophorous vacuole membrane marker
ACP apicoplast marker
DAPI DNA

Exploratory biology to identify hypnozoite proteins



Reagent sourcing for liver stage malaria work

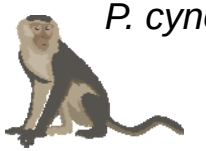
- Sporozoites are necessary to conduct liver stage malaria research
- Production period from host blood infection to mosquito salivary gland sporozoites is 17 – 28 days (depending on the species)



Kappe et al., *Science* 2010

Models available for cross-species and multi-stage testing

- Models used at NITD-EMV in collaboration with partners

	Direct Feed (mosquitoes feed directly on host) 	**Direct Membrane Feed** (host infected blood in feeder) 	**Membrane feed** (*in vitro* continuous culture, infected Blood in feeder) 
 *P. falciparum*	Yes (clinical trials)	Yes	Yes
P. vivax	Yes (clinical trials)	**Yes**	No
 *P. cynomolgi*	Yes (depends on facility)	**Yes**	**In development**
 *P. berghei* *P. yoelli*	Yes Yes	Unnecessary	Unnecessary

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Next steps – induced hepatocytes

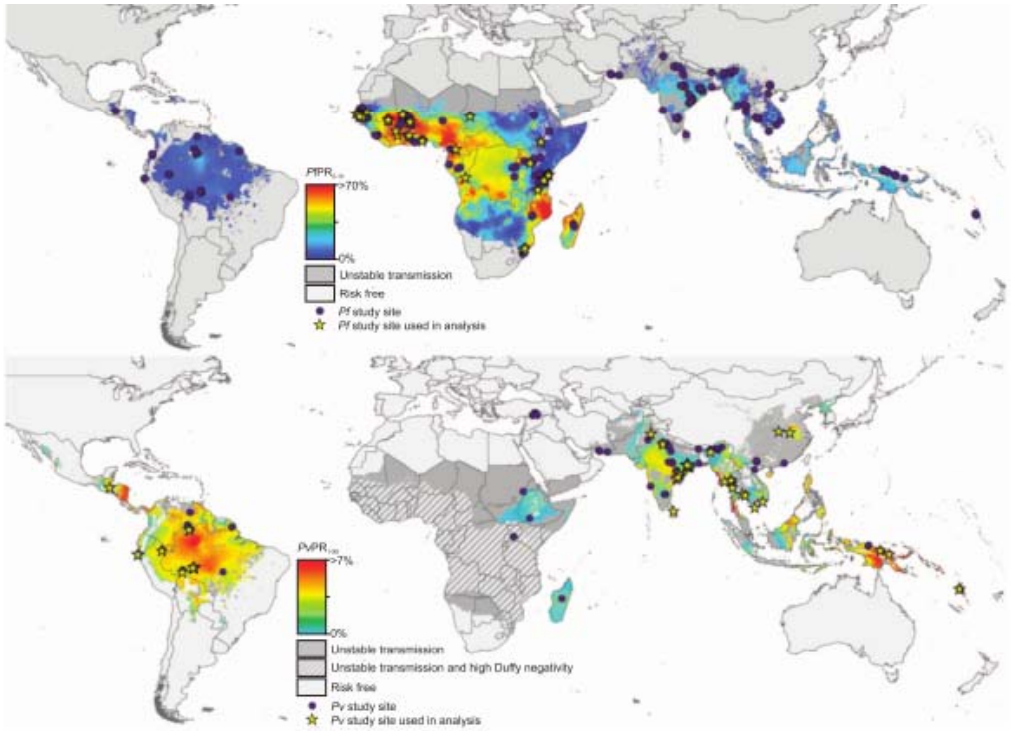
- *P. cynomolgi* infection is successful
  - Schizonts and hypnozoites observed
  - Infection rate may be greater than primary simian hepatocytes
    - High content imaging algorithm needs validation
- Compare cell function to primary simian hepatocytes
- Validate tool compounds in the platform
- Verify parasite life cycle

Malaria is predominantly caused by *P. vivax* and *P. falciparum*

Malaria global distribution by species

P. falciparum

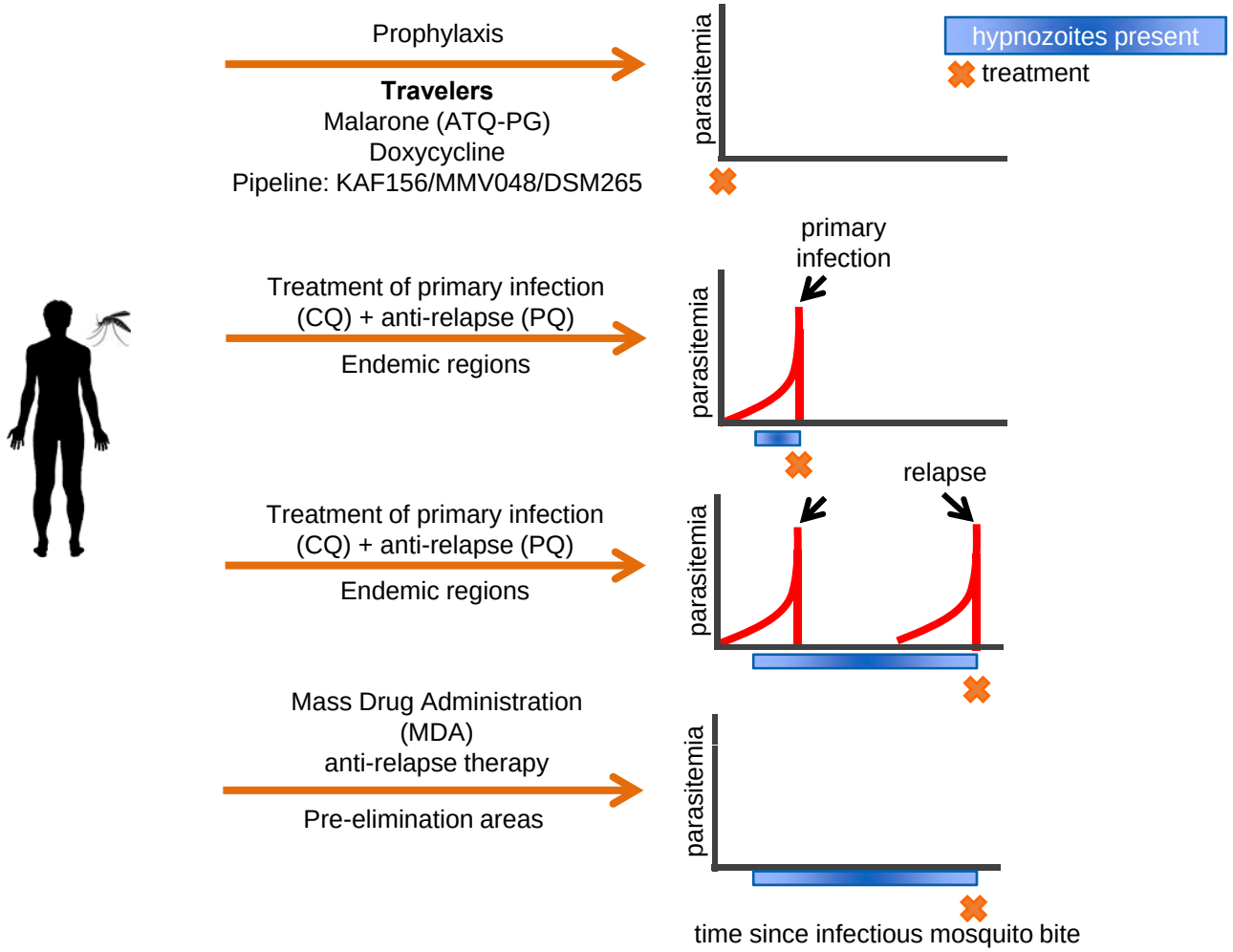
P. vivax



Battle et al., *Scientific Data*, 2015

- 44 countries with fewer than 10,000 cases
- 21 E-2020 countries (elimination by 2020)

Goal: identify new radical cure molecules and protein targets



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MMV Tareget Candidate Profiles for liver stage malaria

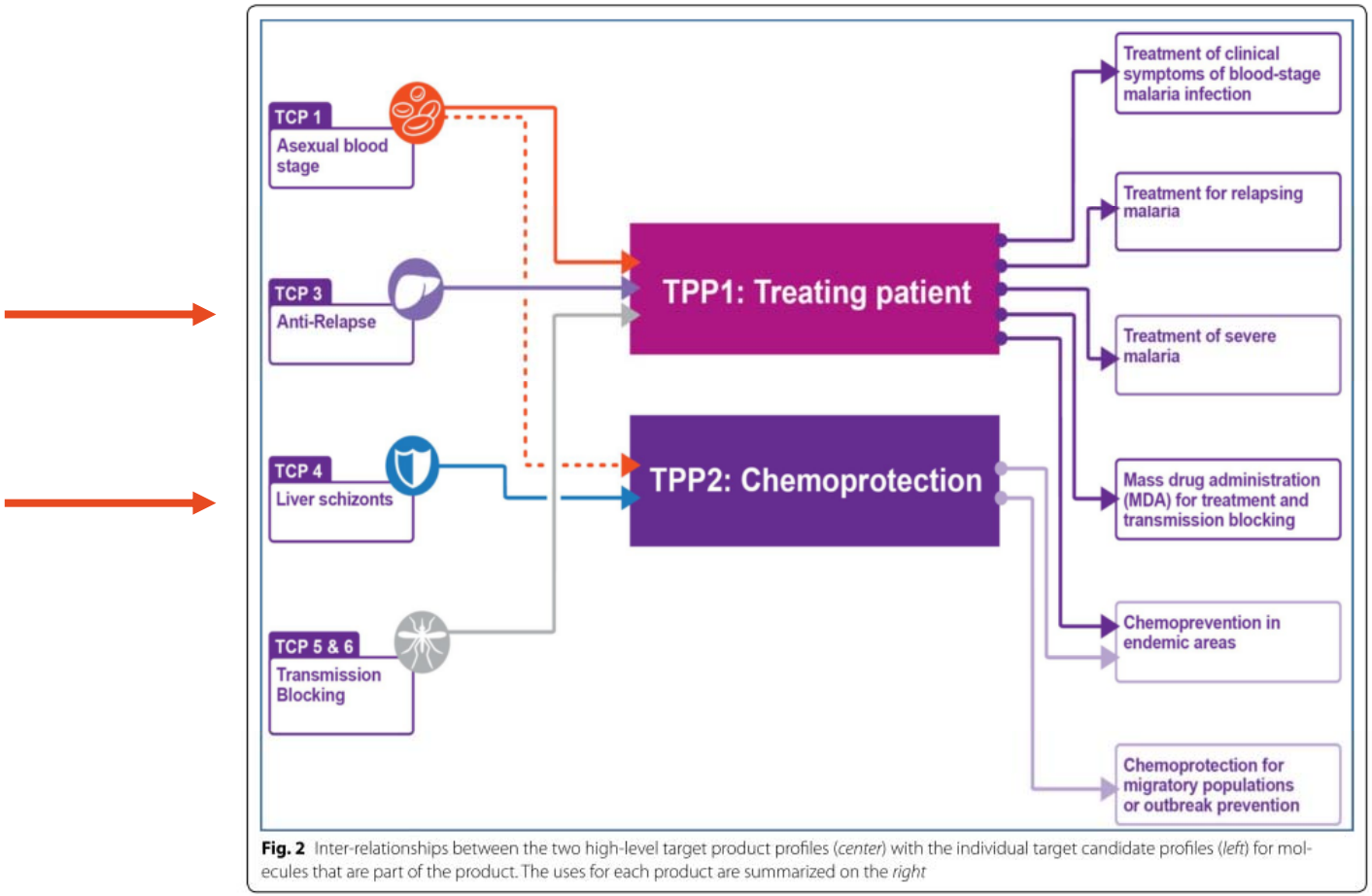

Fig. 2 Inter-relationships between the two high-level target product profiles (*center*) with the individual target candidate profiles (*left*) for molecules that are part of the product. The uses for each product are summarized on the *right*

Past liver stage screens did not identify radical cure molecules

P. yoelli/P. berghei screen (2011, 2015)

- Hepatoma cells
- HCI, luciferase
- 5K, 500K compounds

Meister et al., *Science*, 2011
Swann et al., *ACS Inf Dis*, 2016

P. cynomolgi (2011)

- Primary simian hepatocytes
- HCI
- Tool compounds (10)

Dembele et al., *PloS One,* 2011

P. vivax (2018)

- Primary human hepatocytes
- HCI
- 10K compounds (unpublished)

Roth et al., *Nat Commun*, 2018