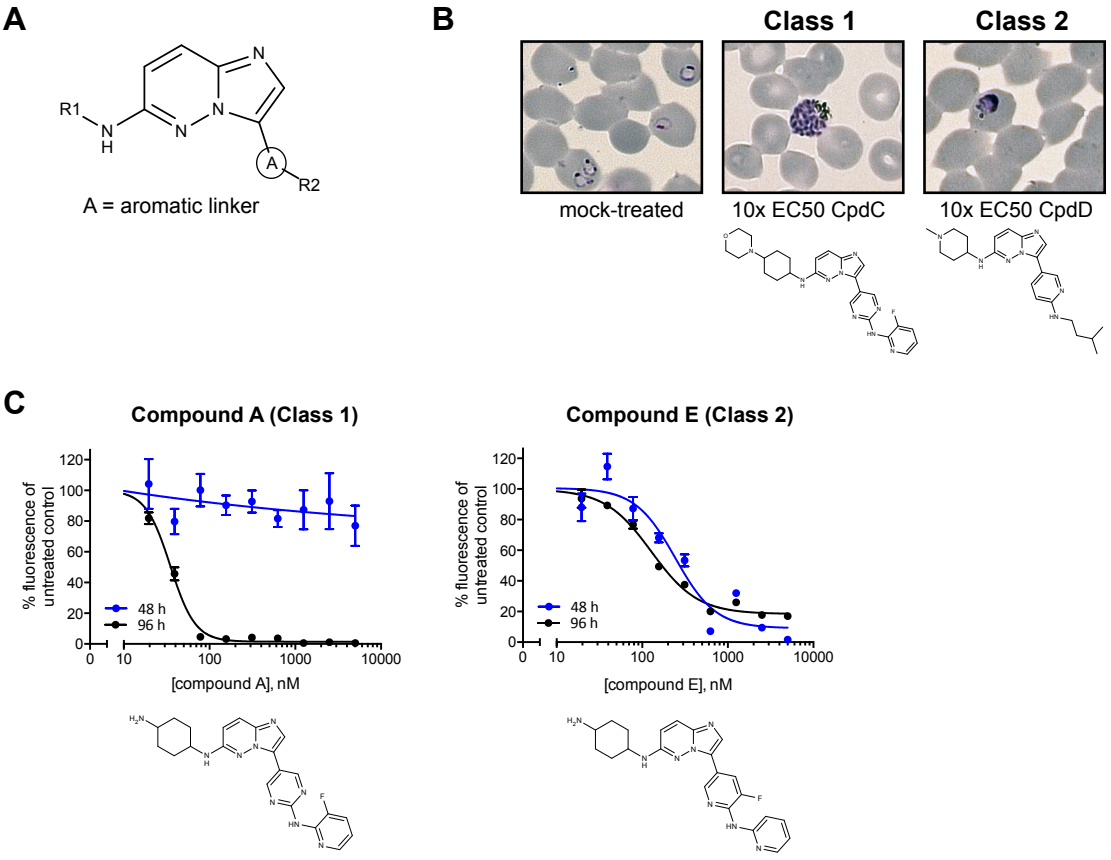


Figure 1. **Imidazopyridazine compounds have two modes of action.**
A. Imidazopyridazine compounds in this study are characterised by an invariant core, an aromatic linker group (A) and variable R1 and R2 groups.
B. *P. falciparum* 3D7 approximately 21 hours after merozoite invasion of erythrocytes were incubated for 48 h with a concentration equal to 10-times the EC_{50} of each compound (determined by FACS (23)). In mock-treated cultures (0.05 % DMSO) newly invaded erythrocytes with ring-stage parasites are seen. In the presence of compound C (0.3 µM) parasites developed until very late schizogony, then arrested prior to merozoite egress, whereas with compound D (4 µM), parasites failed to develop beyond late rings/early trophozoites.
C. EC_{50} determination using a SYBR Green I assay. *P. falciparum* cultures were incubated for either 48 h or 96 h with serial dilutions of inhibitors. For compound A, the EC_{50} with 48 h incubation was not able to be determined with any accuracy, whereas with 96 h incubation the EC_{50} was 0.02 µM (SD = 0.007). The EC50 for compound E was 0.298 (SD = 0.006) or 0.142 µM (SD = 0.024) with 48 h or 96 h incubation respectively. The graphs shown are representative examples of triplicate experiments. Error bars represent the standard error of the mean for duplicate samples.

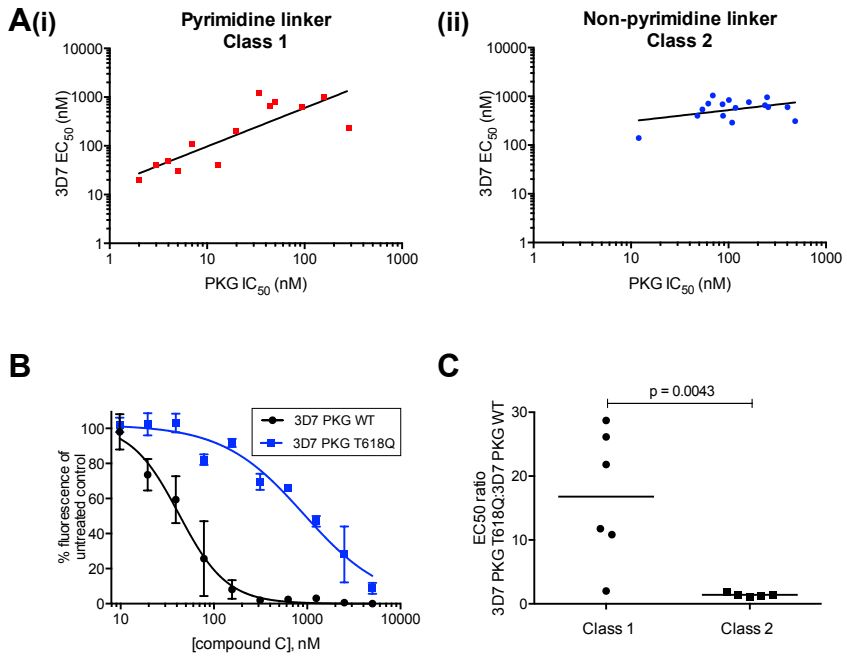


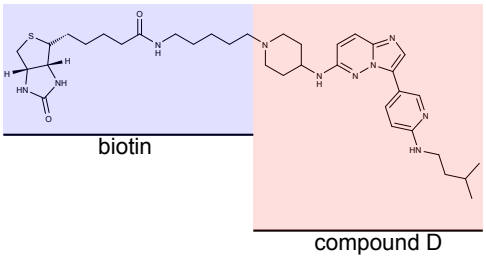
Figure 2. Parasite killing activity of class 2 compounds can be attributed to inhibition of cGMP-dependent protein kinase.

A.(i) Correlation between *P. falciparum* EC_{50} and PKG IC_{50} for 14 class 1 compounds. The calculated coefficient of determination (r^2) value of 0.71 from a linear least squares regression indicates a good correlation between the two data sets. A p value of 0.0002 indicates a significant relationship between the two measurements. **(ii)** Correlation between *P. falciparum* EC_{50} and PKG IC_{50} for 16 class 2 compounds. $r^2 = 0.17$ indicates little correlation between the 3D7 EC_{50} and the PKG IC_{50} for class 2 compounds. In addition, a p value of 0.114 indicates thatthere is no significant relationship between the two measures.

B. Parasites expressing a large gatekeeper variant PKG (3D7 PKG T618Q) are insensitive to compound C compared to WT parasites (3D7 PKG WT). SYBR Green assays were used to measure the parasitaemia of cultures treated with serial dilutions of compound C for 96 h. The experiment was performed twice; data from a single experiment are shown. The EC_{50} values are 0.923 µM (SD = 0.226) for 3D7 PKG T618Q and 0.042 µM (SD = 0.018) for 3D7 PKG WT parasites. Error bars show the standard error of the mean of duplicate samples.

C. Mann-Whitney test to compare the ratio of EC_{50} values for 3D7 PKG T618Q:3D7 PKG WT treated with class 1 and class 2 compounds. Horizontal bars show the median value for each class of compounds (16.8 for class 1, 1.4 for class 2). The calculated p value is 0.0043.

A

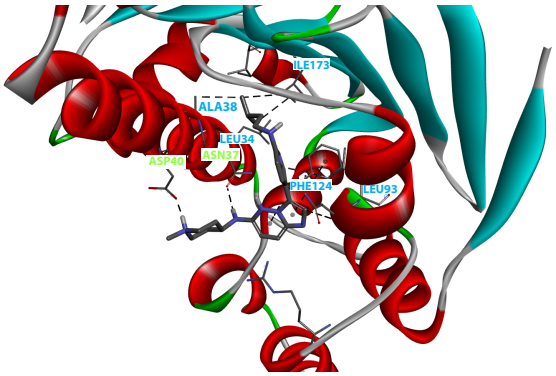


B

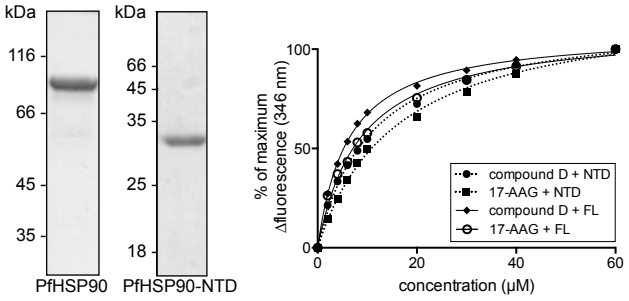
Predicted interactions between compound D and PfHSP90 (kcal/mol)

Hydrogen bonds	Hydrophobic
ASN37 (-1.8386)	PHE124 (-1.4225)
ASP40 (-0.3766)	LEU34 (-0.5926)
	LEU93 (-0.5256)
	ALA38 (-0.4258)
	ILE173 (-0.406)

C



D



	Kd (μM) [SE]	
	compound D	**17-AAG**
PfHSP90-NTD	10.73 [0.62]	15.84 [0.93]
PfHSP90	6.17 [0.18]	8.70 [0.69]

Figure 3. Affinity purification of cellular targets of compound D.

A. Biotin linked to the R1 group of compound D. This was bound to streptavidin-agarose and used to affinity purify proteins from a trophozoite cell lysate. The only significant hit identified by LC-MS/MS was HSP90 (Table S3).

B. Predicted interactions between compound D and PfHSP90.

C. Modelling of the most likely binding orientation of compound D to the ATP-binding site of HSP90 was carried out using DockingServer. Residues predicted to form hydrogen bonds with compound D are labelled in green, while those predicted to form hydrophobic interactions are labelled in blue.

D. Recombinant PfHSP90 binds to compound D. Purified recombinant PfHSP90 and PfHSP90-NTD used in subsequent experiments are shown in the Coomassie stained gels to the left of the figure. Changes in the tryptophan fluorescence (346 nm) of HSP90 were monitored in the presence of increasing amounts of compound D or 17-AAG. K_d values were calculated for both full length PfHSP90 and PfHSP90-NTD. Values in brackets show the standard error of the mean of triplicate measurements.

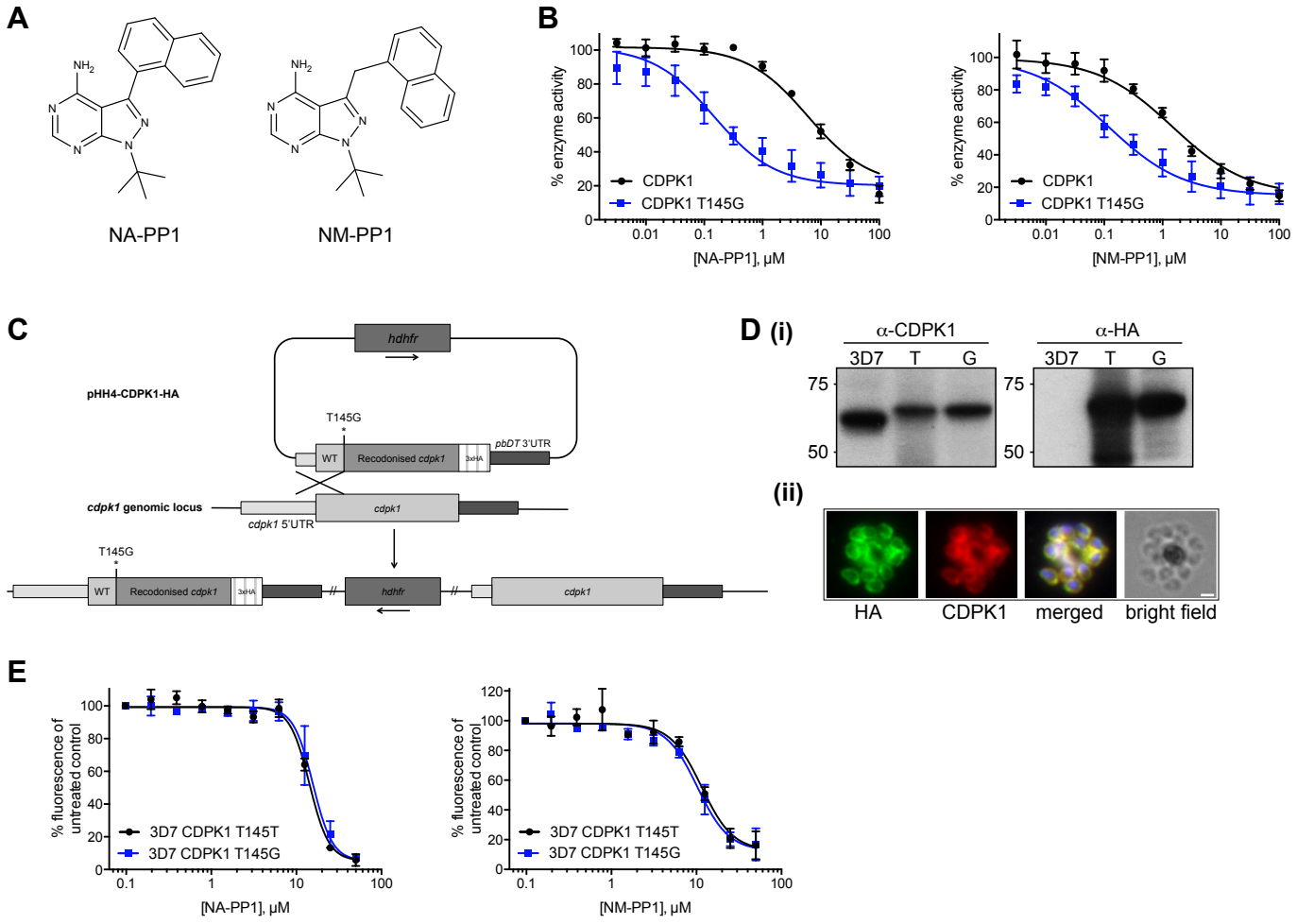


Figure 4. Inhibition of PfCDPK1 has no effect on asexual parasite growth.

A. Chemical structures of the bumped kinase inhibitors (BKIs) NA-PP1 and NM-PP1.

B. Recombinant CDPK1 with a glycine at the gatekeeper residue (amino acid 145) shows increased sensitivity to both BKIs. A ParM ADP biosensor assay to measure ATPase activity of CDPK1 enzymes was carried out in the presence of serial dilutions of BKIs. The IC_{50} of CDPK1 T145G for NA-PP1 decreased 41-fold compared to WT CDPK1, with IC_{50} values of 0.15 µM [95% confidence intervals (CI) 0.08 to 0.26 µM] and 6.01 µM [95% CI 3.4 to 10.5 µM] respectively. The IC_{50} of NM-PP1 was 0.12 µM [95% CI 0.06 to 0.23 µM] for CDPK1 T145G compared to 1.5 µM [95% CI 0.77 to 2.90 µM] for WT CDPK1, a 13-fold decrease. Graphs show mean values from five independent experiments, with error bars indicating the standard error of the mean.

C. Scheme for generating a *P. falciparum* 3D7 CDPK1 T145G-HA parasite line by single crossover homologous recombination at the *cdpk1* genomic locus. Crossover upstream of the T145 codon is forced by recodonising sequences downstream of and including the T145 codon. Integration at the locus results in a chimaeric cdpk1 gene with a modified gatekeeper residue. An identical method was used in which the gatekeeper residue was left as a threonine to generate the parasite line 3D7 CDPK1 T145T-HA.

D.(i) Whole cell lysates from *P. falciparum* 3D7, 3D7 CDPK1 T145T-HA (T) and 3D7 CDPK1 T145G-HA (G) were probed with antibodies against CDPK1 (left panel), and against the HA epitope tag (right panel).

(ii) Immunofluorescence using anti-HA and anti-CDPK1 antibodies confirms that CDPK1 T145G-HA localizes correctly to the plasma membrane of parasites. Scale bar is 1 µm.

E. SYBR Green assays to measure EC_{50} values of *P. falciparum* 3D7 CDPK1 T145T-HA and 3D7 CDPK1 T145G-HA lines to NA-PP1 and NM-PP1. The EC_{50} for NA-PP1 in 3D7 CDPK1 T145T-HA was 14.26 µM [95% CI 12.3 to 16.5] and in 3D7 CDPK1 T145G-HA was 15.65 µM [95% CI 13.4 to 18.3]. The EC_{50} for NM-PP1 was 11.55 µM [95% CI 9.1 to 14.6] and 10.28 µM [95% CI 8.1 to 13.0] for 3D7 CDPK1 T145T-HA and 3D7 CDPK1 T145G-HA respectively. The experiments were performed three times, with at least six replicate samples per experiment. Graphs show data from three independent experiments. Data points are mean values, with error bars indicating the standard error of the mean.