Supplementary information for

Dimeric artesunate glycerophosphocholine conjugate nano-assemblies as slow-release antimalarials to overcome *Kelch 13*-mutant artemisinin resistance

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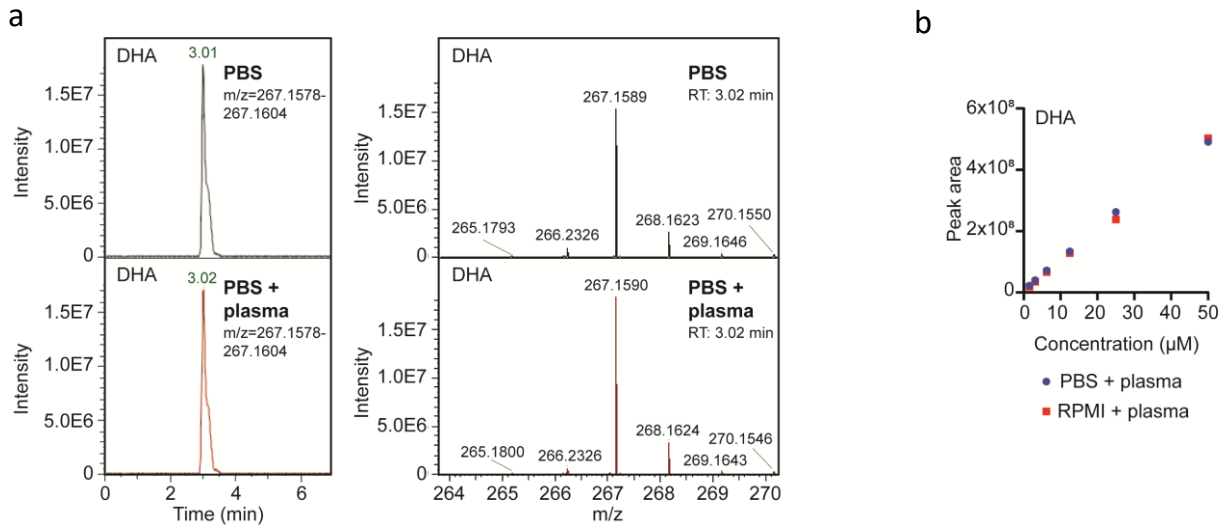

Figure S1. LCMS analysis of DHA under *in vitro* assay conditions. (a) Representative chromatogram (left) and mass spectrum (right) of DHA (m/z: 267.1591, positive ionisation mode) in PBS alone and PBS plus plasma. (b) LCMS peak areas of increasing concentrations of DHA in PBS plus plasma and RPMI plus plasma.

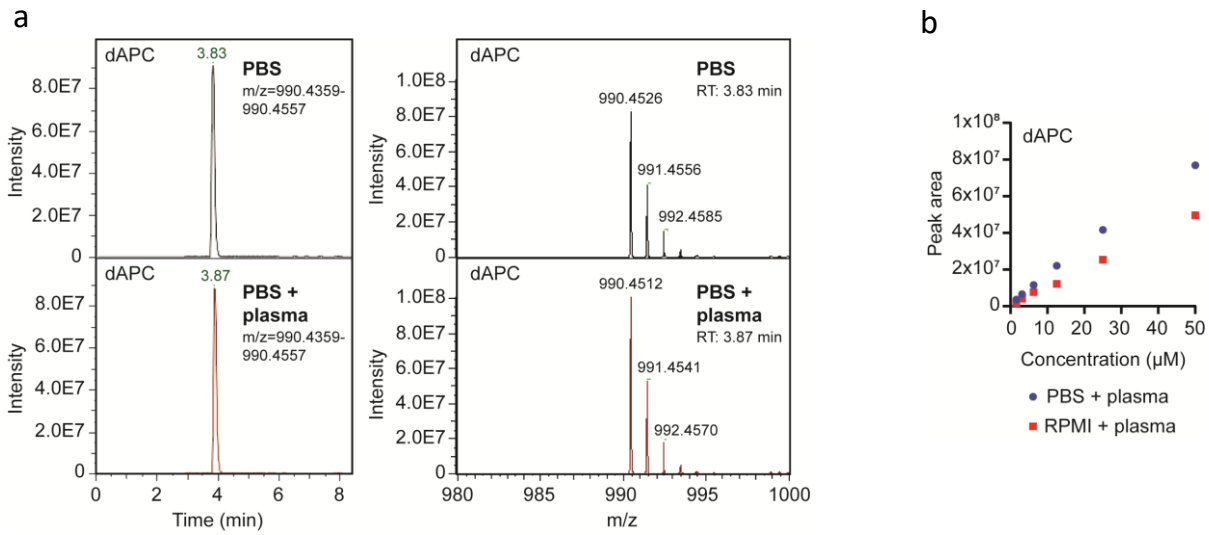

Figure S2. LCMS analysis of dAPC under *in vitro* assay conditions. (a) Representative chromatogram (left) and mass spectrum (right) of dAPC (m/z: 990.4458, positive ionization mode) in PBS alone and PBS plus plasma. (b) LCMS peak areas of increasing concentrations of dAPC in PBS plus plasma and RPMI plus plasma.

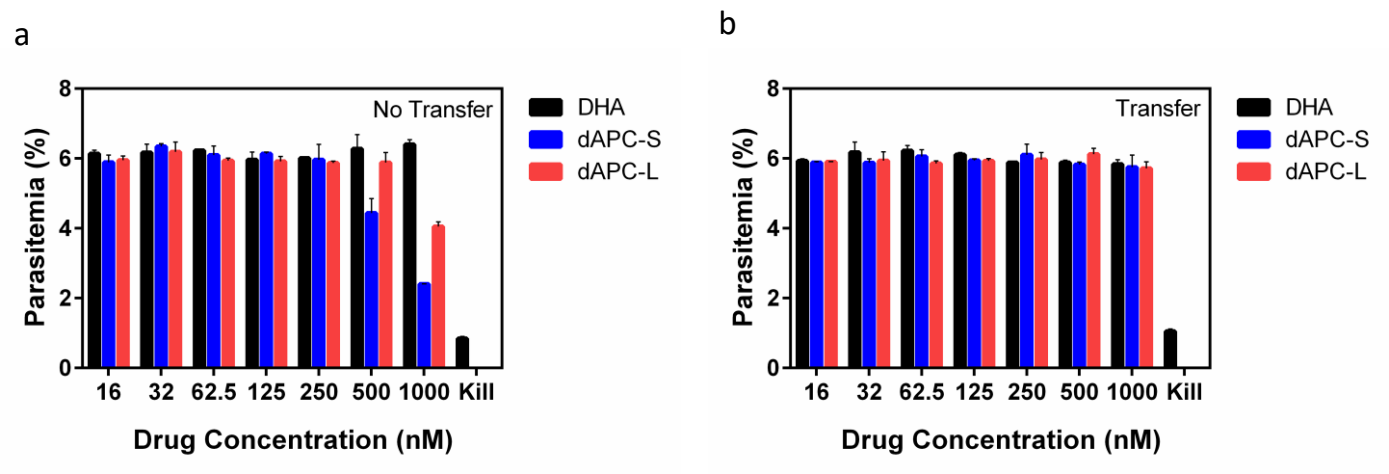

Figure S3. An enhanced wash protocol is required for pulse assays of dAPC. DHA, dAPC-S and dAPC-L (concentrations indicated) were incubated in media for 3 h in the presence of RBCs (0.2% hematocrit). RBCs were processed using either the standard - no transfer (a) or the modified - transfer (b) wash protocol, as defined in Materials and Methods. Data are typical of two independent experiments, each performed in duplicate. Error bars represent the range of values from one experiment.

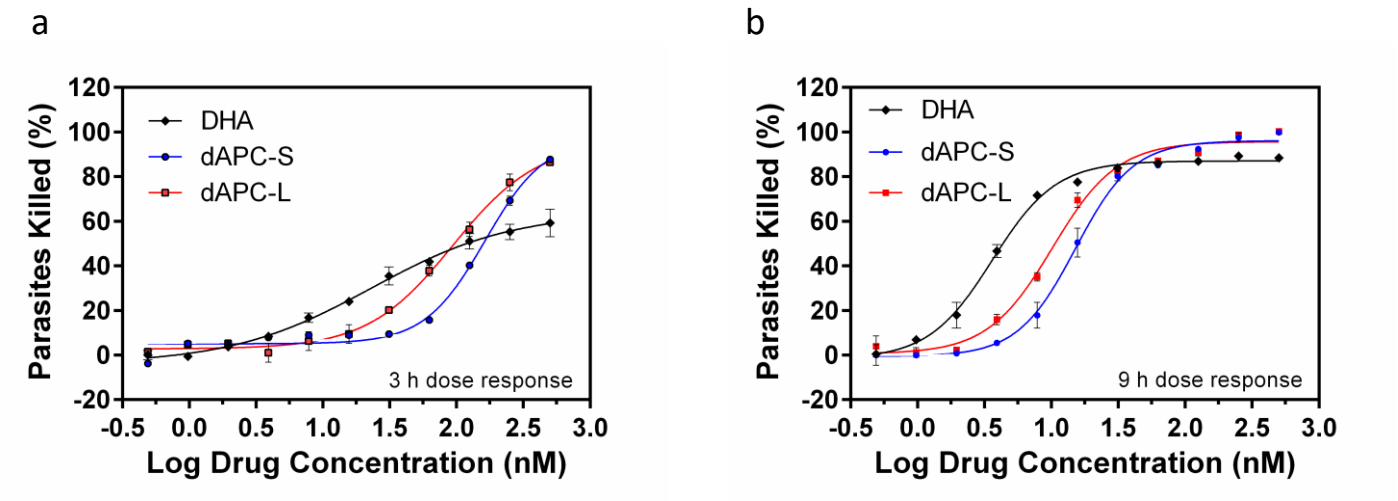

Figure S4. Antimalarial activity of DHA, dAPC-S and dAPC-L in different length exposure assays. Dose response curves of ring stage Cam3.II^{R539T} parasites following (a) 3-h and (b) 9-h exposure to DHA (black - diamonds), dAPC-S (blue - circles) and dAPC-L (red - squares). Data are typical of at least three independent experiments, each performed in duplicate. Error bars reflect the range of values from one experiment.

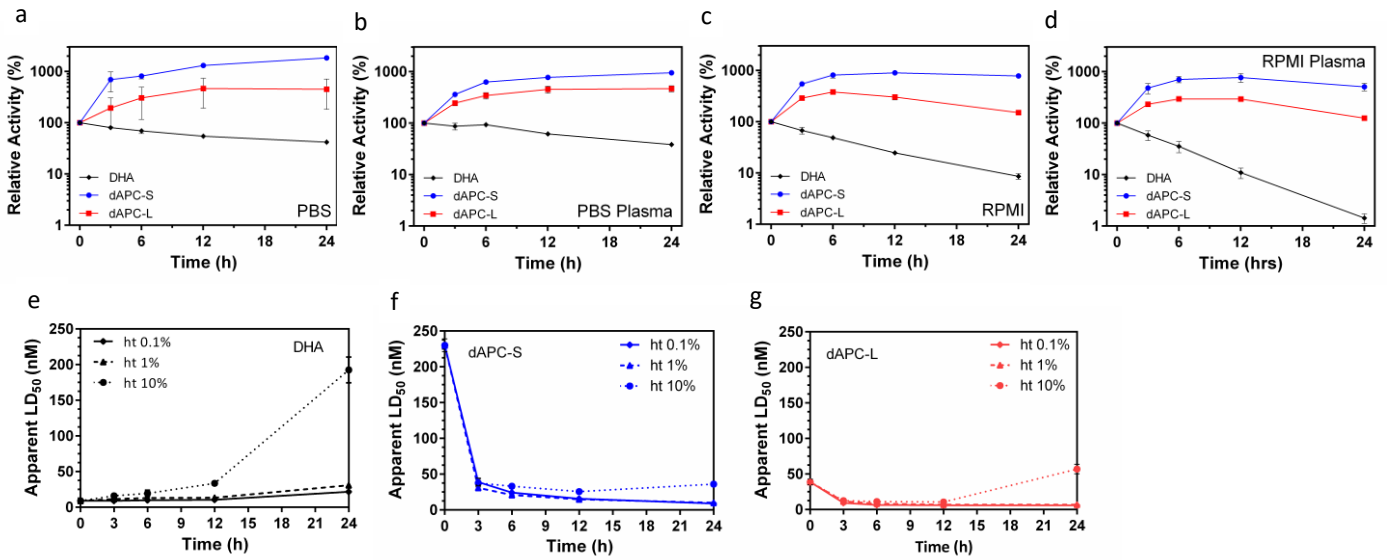

Figure S5. Effect of pre-incubation in different media and in the presence of RBCs on antimalarial activity of DHA, dAPC-S and dAPC-L. (a-d) DHA (black), dAPC-S (blue) and dAPC-L (red) were preincubated (a) PBS, (b) PBS + 10% plasma, (c) RPMI or (d) RPMI + 10% plasma for the indicated periods. (e) DHA (c), dAPC-S (f) and dAPC-L (g) were preincubated in the presence of RBCs at 0.1% ht (unbroken line), 1% ht (hatched line) and 10% ht (dotted line) for the indicated periods. Remnant activity was assessed by exposing infected RBCs the pre-incubated samples for 3 h, with viability assessed in the next cycle. Dose response curves were generated; and relative activity was estimated compared with activity prior to pre-incubation - defined as LD_{50} (time = 0)/LD_{50} (time = t) multiplied by 100. The relative activity or LD_{50} values are determined from a series of 10 different dilutions, in two independent experiments. Error bars represent range of values for the two experiments.

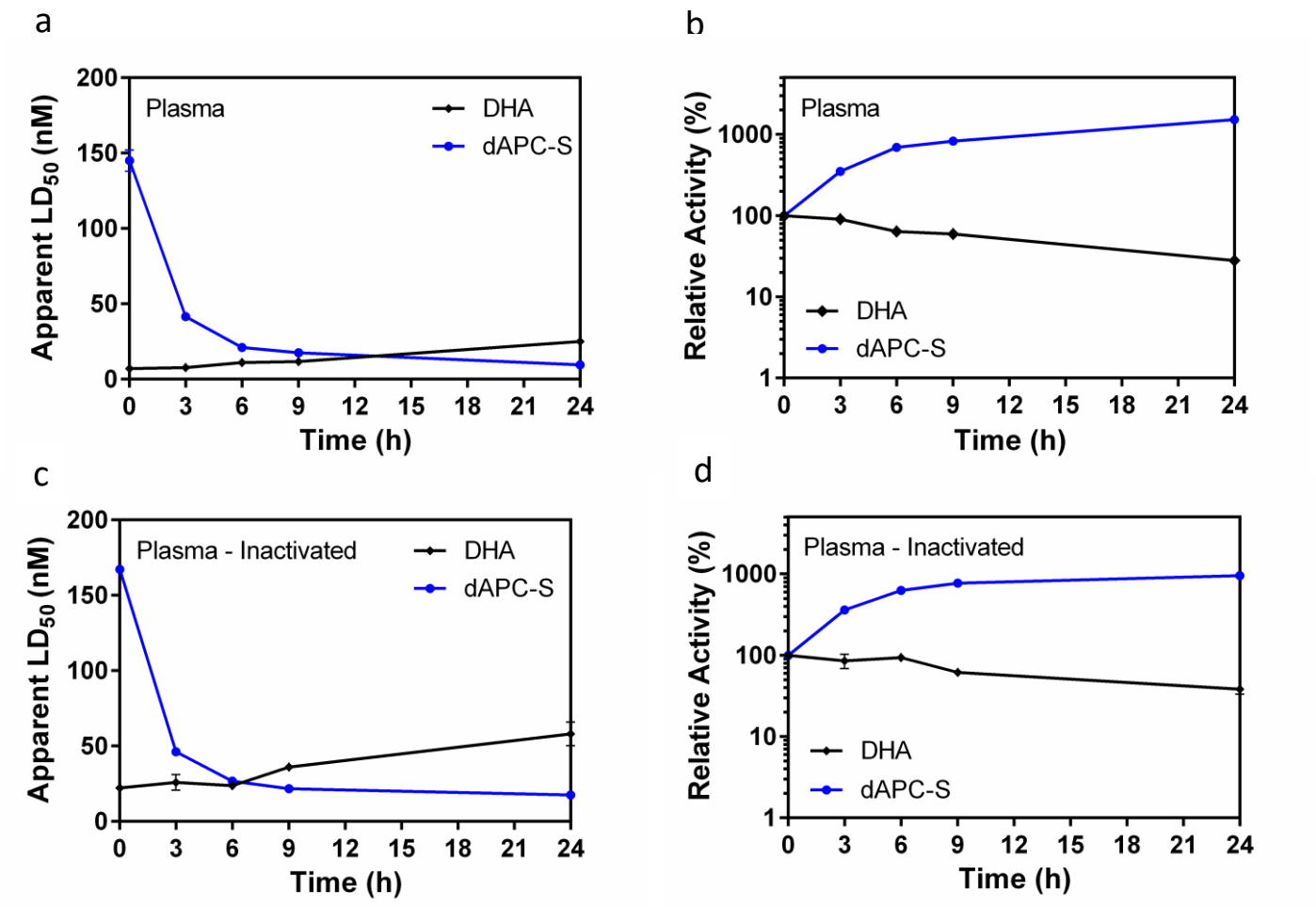

Figure S6. Effect of plasma inactivation on loss of antimalarial activity of DHA, dAPC-S and dAPC-L during pre-incubation. DHA and dAPC-S were preincubated with (a,b) PBS + 10% plasma or (c,d) PBS + 10% inactivated plasma for the indicated periods. Remnant activity was assessed by exposing the pre-incubated samples to infected RBCs for 3 h, with viability assessed in the next cycle. Dose response curves were generated; and are presented as apparent LD_{50} (a,c) or relative activity (b,d). The apparent LD_{50} values are determined from a series of 10 different dilutions, in two independent experiments. Error bars represent range of values for the two experiments.

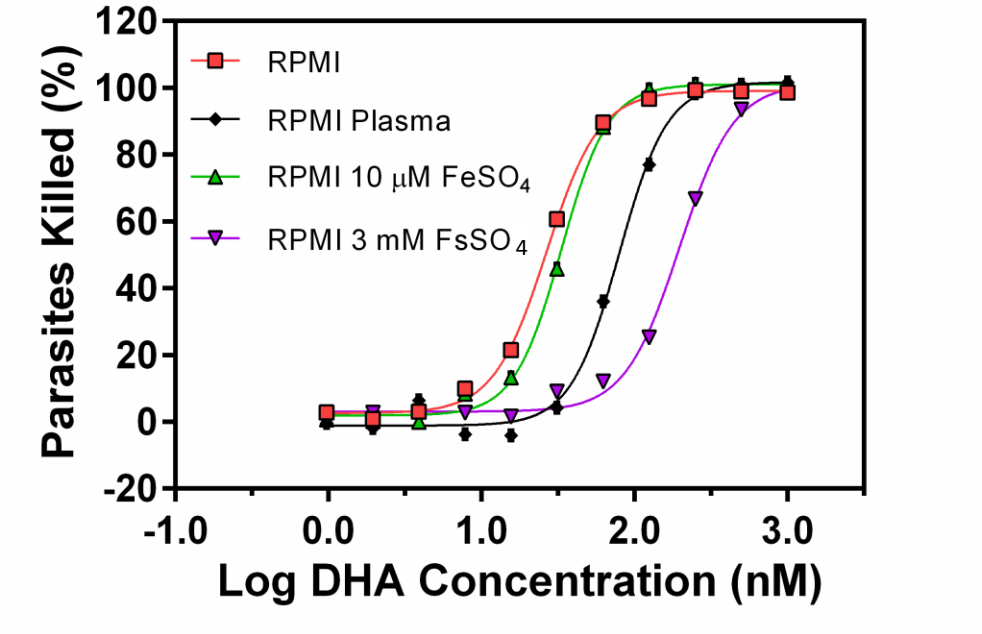

Figure S7. Effect of addition of ferrous iron on loss of activity of DHA during pre-incubation. Parasite viability (Troph stage Cam3.II^{rev}) was assessed in the cycle following a 3-h exposure to DHA (concentrations indicated) in RPMI (red - squares), RPMI + 10% plasma (black- diamonds), RPMI + 10 μM $FeSO_4$ (green - triangles) and RPMI + 3 mM $FeSO_4$ (purple – triangles). Dose response curves show parasite survival as a function of drug concentration.

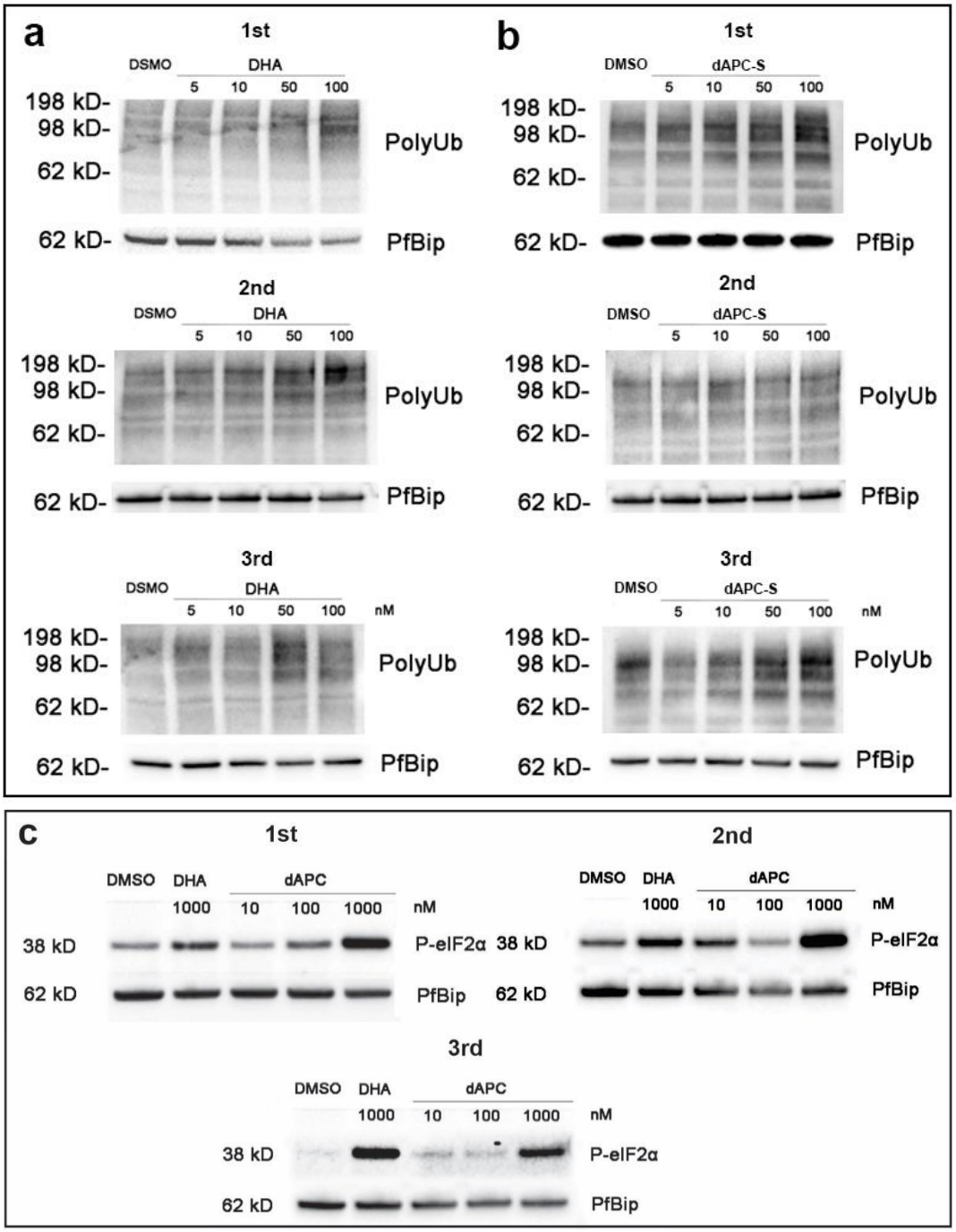

Figure S8. Replicate Western blots relating to Figure 6. (a,b) Trophozoite-stage infected RBCs (Cam3.II^{rev}) were incubated with carrier (0.1% DMSO) or increasing concentrations (indicated) of DHA (a) or dAPC-S (b) for 90 min. Extracts were probed with antiserum recognizing polyubiquitinated proteins. *Pf*BiP is a loading control. The three datasets represent matched blots (left and right sides) from the same day. The blots represent additional experiments relating to Figure 6 (a,b). (c) Lysate from trophozoite stage parasites exposed to carrier, DHA and dAPC (concentrations indicated), for 3 h, were analysed by Western blot for phosphorylated-eIF2α. *Pf*BiP is a loading control. The blots represent additional experiments relating to Figure 6c.

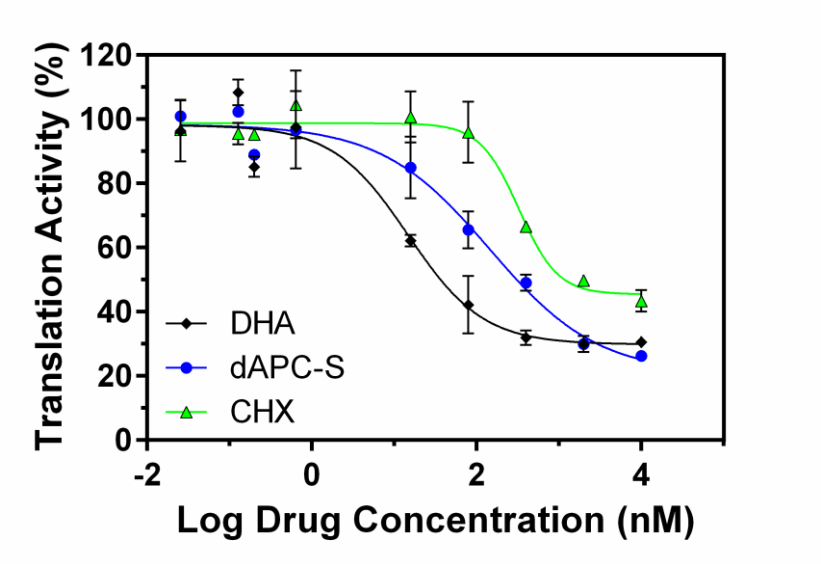

Figure S9. Replicate protein translation assay relating to Figure 6d. Protein translation was measured in trophozoite stage parasites (Cam3.II^{rev}) that were exposed to increasing concentrations of DHA (black - diamonds), dAPC-S (blue - circles), or cycloheximide (green - triangles) for 60 min before incorporation of o-propagyl-puromycin (OPP). Cultures were incubated for a further 120 minutes before harvesting parasites for determination of OPP incorporation. Error bars reflect the range from the average of one experiment performed in duplicate.

Table S1. Residual viability of ring stage Cam3.II^{R539T} following exposure to inhibitors.

Inhibitor Pulse Time (h)	V_{min} (%)[a]		
	DHA	dAPC-S	dAPC-L
3	43.0 ± 6.2	12.9 ± 0.3	14.3 ± 0.5
6	24.1 ± 2.2	< 1	< 1
9	11.6 ± 0.1	< 1	< 1
72	< 1	< 1	< 1

[a] The minimum viability, V_{min} is reported as the percentage of viable parasites following a 500 nM fixed dose inhibitor pulse for times indicated. Data are the average of three independent experimental measurements, each performed in duplicate ± SD.

Table S2. LD_{50} values of ring stage Cam3.II^{R539T} following exposure to inhibitors.

Inhibitor Pulse Time (h)	LD_{50} (nM)		
	DHA	dAPC-S	dAPC-L
3	45 ± 7*	161 ± 36*	57 ± 9*
6	14 ± 6*	49 ± 4	32 ± 7
9	6.2 ± 1.4*	18 ± 3	10 ± 1
72	2.9 ± 0.4	2.1 ± 0.1	1.9 ± 0.2

[a]Ring stage Cam3.II^{R539T} parasites were exposed to the inhibitors indicated (0.5 – 500 nM) for 3, 6, 9, and 72 h. LD_{50} values were determined from dose response curves shown in Figure 4, and Figure S4. Residual viability (V_{min}) values are reported in Table S1. The data represent the average of 3 or more independent experiments, each in duplicate. The error bars represent the S.D. *Indicates incomplete killing of parasites.