# A validated bioluminescence-based assay for the rapid determination of the initial rate of kill for discovery antimalarials.

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Running title: Relative rate of kill for antimalarials

***Abstract***

***Objectives:*** A future treatment for uncomplicated malaria will contain at least one component that exerts a rapid rate of kill (RoK). We describe here the validation and application of a simple, robust and rapid bioluminescence-based assay for the determination of the initial RoK in intraerythrocytic asexual stages of *Plasmodium falciparum*.

***Methods:*** A modification to the concentration-response bioluminescence (here termed bioluminescence relative rate of kill, BRRoK) assay, utilizing exposure to fold-$IC_{50}$ concentrations (0.33x to 9x), is used to monitor the immediate cytocidal effect of 372 open source compounds for antimalarial drug discovery available through the Medicine for Malaria Venture's Malaria Box.

***Results:*** Antimalarial drugs that exert a rapid cytocidal effect produce a concentration dependent loss of bioluminescence signal that correlates with available *in vitro* and *in vivo* estimates of parasite clearance time and parasite reduction ratio. Following the measurement of $IC_{50}$ for the Malaria Box compounds in $Dd2^{luc}$, the BRRoK assay was used to identify and rank 372 compounds for their initial cytocidal activity. Fifty three compounds in the Malaria Box show an initial relative RoK greater than that of chloroquine, with 17 of these with an initial relative RoK greater than that of dihydroartemisinin.

***Conclusion:*** The BRRoK assay provides a rapid assay format for the estimation of a key pharmacodynamic property of antimalarial drug action. The simplicity and robustness of the

assay suggests it would be readily scalable for high throughput screening and a critical decision-making tool for antimalarial drug development.

***Keywords***

Malaria, rate of kill, pharmacodynamics, bioluminescence, drug screening, MMV Malaria Box

## *Introduction*

The past decade has seen, for the first time in generations, a decline in mortality and morbidity from malaria, largely on account of the use of effective antimalarials and the widespread coverage of insecticide-treated bed nets and other transmission preventative measures.[1] However, the estimated number of deaths is still very high, estimated at 438,000 in 2015,[2] the majority of which are among African children under 5 years of age. In the same period, the malaria parasite has assembled more counter measures than ever before to overcome chemotherapy with some parts of the world reporting clinical failures to artemisinin and artemisinin combination therapy (ACTs), the last effective antimalarial drug class currently available.[3-7] The need to develop new drugs with novel modes of action, which circumvent current parasite resistance mechanisms, therefore remains an urgent priority.[8,9]

Towards this aim, in the past 5 years, the Medicines for Malaria Venture (MMV) has coordinated the screening of more than 5 million compounds against *P. falciparum in vitro*. This has generated nearly 30,000 compounds inhibiting in the submicromolar range.[10-12] However, there is currently no clear and informed path to rationally triage the 20-30,000 hits that are now at our disposal. At best, the pharmaceutical and academic malaria research communities will be able to progress 20-30 chemotypes down the traditional discovery and development pathway within the next decade. Technology platforms able to identify the most effective and tractable chemotypes for progression into the drug development pipeline are therefore urgently needed. Key features of the sought-after next generation antimalarial for the treatment of uncomplicated malaria, termed Single Exposure Radical Cure and Prophylaxis (SERCaP), have been rationalized and have resulted in the

recommendation of a series of Target Candidate Profiles (TCPs) for the component parts of this drug combination therapy. TCP-1 emphasises the requirement for a component part of SERCaP to rapidly eliminate the initial parasite burden, ideally as fast as artesunate, but with a minimal essential requirement to do so as fast as chloroquine.[9]

A current bottleneck in antimalarial discovery projects is that the current gold standard in the determination of killing dynamics for new inhibitors is slow, necessitating between three to four weeks of recrudescence parasite growth following drug exposure.[13] To address this bottleneck, we describe here the validation of a microplate-based bioluminescence assay that provides, within 6 hours, a determination of a compound's initial cytocidal action. Benchmarking these data against existing antimalarial compounds, for which rate of kill (RoK) pharmacodynamics are known, provides a relative rate of initial kill and thus a means to triage a compound's activity against the minimal essential and ideal criteria as TCP1 candidates. The utility of this rapid, simple and robust assay format is demonstrated in a relative rate of kill screen of the MMV Malaria Box, an open access resource provided for drug discovery in malaria and neglected tropical diseases.[14] The MMV Malaria Box contains 400 compounds distilled from the initial large chemical screens performed by St. Jude Children's Research Hospital, Novartis and GlaxoSmithKline.[10-12] These compounds reflect a cross section of the chemical diversity available in the 20-30,000 hits, providing 200 starting points for oral drug discovery (termed drug-like) and 200 compounds to explore malaria parasite biology (termed probe-like). Our findings suggest this bioluminescence relative rate of kill (BRRoK) assay provides the required throughput and discrimination necessary to assist in the decision making process to prioritise leads in the 20-30,000 antimalarial compound set for further development.

*Materials and methods*

**Drug stocks**

Antimalarial drugs were sourced from Sigma-Aldrich and prepared as follows: atovaquone (ATQ, 10mM in dimethyl sulfoxide [DMSO]), artemether (ARM, 50mM in ethanol), chloroquine (CQ, 100mM in deionised water), dihydroartemisinin (DHA, 50mM in methanol), mefloquine (MQ, 50mM in DMSO) piperaquine (PPQ, 100mM in ethanol), pyronaradine (PYN, 100mM in deionised water), quinine (QN, 100mM in ethanol) and tafenoquine (TFN, 100mM in DMSO) and stored at -20$^{o}$C. The Malaria Box was provided by the Medicine for Malaria Ventures (www.mmv.org) and was provided as 20 µL solutions of 10 mM concentration in DMSO and stored at -20$^{o}$C. In all experiments, the final maximum final concentration of solvent was 0.6% v/v.

***Plasmodium falciparum*** **cell culture**

The transgenic Dd2 *P. falciparum* clone (Dd2$^{luc}$) expresses luciferase under the control of *Pfpcna* flanking sequences to produce a strong peak of temporal reporter expression during S-phase in trophozoite stage parasites.[15,16] Dd2$^{luc}$ were cultured using standard continuous culture conditions (RPMI1640 medium supplemented with 37.5 mM HEPES, 10 mM D-glucose, 2 mM L-glutamine, 100 µM hypoxanthine, 25µg mL$^{-1}$ gentamycin, 4% v/v human serum, 0.25% v/v Albumax II, 5nM WR99210 and 2.5 µg/mL blasticidin S) at a 2% haematocrit in an atmosphere of 1% $O_2$, 3% $CO_2$, and 96% $N_2$. WR99210 and blasticidin S drug selection media were removed 48 h prior to initiation of fluorescence and bioluminescence assays. Staging and parasitaemia of the *in vitro* culture were assessed by light microscopy of Giemsa-stained thin blood smears. Synchronization of cultures was

attained using sequential sorbitol lysis treatment,[17] with experiments carried out at least one intraerythrocytic cycle later.

**Concentration-response assays using bioluminescent and fluorescent assay formats**

Trophozoite-stage (20-26 h post-infection) cultures of $Dd2^{luc}$ (100 µL, 2% parasitaemia, 4% haematocrit, n = 3) were added to 96-multiwell plates containing 100 µL of pre-dosed (a final five-fold dilution series) complete culture medium. On each assay plate, three wells containing 200 µL of 2% parasitaemia cell culture (2% haematocrit) in the absence of drugs served as a positive control (100%), whereas the same culture mix in the presence of a 10 µM concentration of chloroquine served as a negative growth control (0%). The outermost wells on each plate contained 200 µL of incomplete medium (complete culture medium without human serum or Albumax II supplements) to minimize edge effects from evaporation during incubation.

To determine estimates of the $IC_{50}^{48h}$, the parasite inoculum was incubated continuously in the presence of the compound/drug for 48 h prior to assay. Estimation of the $LC_{50}^{6h}$ was carried out using a modification of the protocol originally described by Pagiuo et al.[18] Here the parasite inoculum was incubated for 6 h in the presence of compound/drug. The infected erythrocyte cultures were collected by centrifugation (3000g, 5 min at room temperature) and the medium supernatant discarded. Three repeat washes with 10 volumes of complete cell culture medium and centrifugation were completed before re-suspending the infected erythrocytes in 200 µL of 37°C complete culture medium for an additional 42 h in the absence of compound/drug prior to assay. Luciferase and Malaria Sybr Green I fluorescent (MSF) assays were also carried out immediately following the 6 h

incubation in drug/compound, this data used to estimate the $EC_{50}^{6h}$. Experiments were carried out as technical triplicates on the same plate, with three independent biological repeats of each plate performed.

For bioluminescent assays, relative light units were measured using the luciferase assay system (Promega, UK). A standard single-step lysis procedure was used throughout.[19] 40 µL samples of *P*. *falciparum* culture were transferred to a white 96-multiwell plate (Greiner, UK) and 10 µL of passive lysis buffer (Promega, UK) added and homogenized by pipetting. An equal volume, 50 µL, of the supplied luminogenic substrate was mixed with the lysed parasites and the bioluminescence was measured for 2 sec in a Glomax Multi Detection System (Promega, UK).

Fluorescent signals were measured using a standard MSF assay[20] as modified.[16] MSF lysis buffer (100 µL of 20 mM Tris (pH 7.5), 5 mM EDTA, 0.008% (w/v) saponin and 0.08% (v/v) Triton X-100) containing SYBR green I (1× final concentration, from 5000x stock supplied by Invitrogen, UK) was added to 100 µL of $Dd2^{luc}$ aliquoted onto a black 96-multiwell plate (Greiner, UK). Well contents were homogenized by pipetting and incubated for one hour in the dark at room temperature. The fluorescent signal was measured using the blue fluorescent module (excitation 490 nm: emission 510–570 nm) in a Glomax Multi Detection System (Promega, UK).

Irrespective of the assay, growth was expressed as a proportion of the untreated control (i.e. 100%) and calculated as follows: $100x[\mu_{(S)} - \mu_{(-)}/\mu_{(+)} - \mu_{(-)}]$ where $\mu_{(S)}$, $\mu_{(+)}$ and $\mu_{(-)}$ represent the means for the sample in question and 100% and 0% controls, respectively. Note, as a complete kill could not be technically demonstrated to be achieved within 6 h,

the 0% control for the $LC_{50}^{6h}$ and $EC_{50}^{6h}$ determination was established as a 48 h continual exposure to 10µM of chloroquine. The % growth was plotted against $log_{10}$-transformed drug concentration and the parameters described above determined using a nonlinear regression (sigmoidal concentration–response/variable slope equation) in GraphPad Prism v5.0 (GraphPad Software, Inc., San Diego, CA).

**Bioluminescence relative rate of kill (BRRoK) assay**

Trophozoite-stage (20-26 h post-infection) cultures of $Dd2^{luc}$ (100 µL, 2% parasitaemia, 4% haematocrit, n = 3) were added to 96-multiwell plates containing 100 µL of pre-dosed (final three-fold $IC_{50}$ dilution series) complete culture medium and mixed by pipetting. The multiwell plate was incubated for 6 h at 37°C. 40 µL samples from each well were removed and the bioluminescent signal measured using the luciferase single-step lysis protocol described above. Controls in each biological replicate consisted of trophozoite stage culture with no drug added (100%) or uninfected erythrocytes (0%). The mean and standard deviation (SD) of bioluminescence data from three independent biological repeats was expressed as a proportion of the untreated control (100%) using the calculation shown above. For the screening of the MMV Malaria Box (6 h assays using a 9x to 0.3x $IC_{50}$ series), a Principle Components Analysis was performed on the bioluminescence endpoints (expressed as a percentage of untreated control) using the KNIME analytics platform to reduce the dimensionality of these data set.[21] As the first principle component captured 89% of the variance in the data, a zero-meaned PC1 value was used to represent the BRRoK parameter. The Z' score of the BRRoK assay was calculated as follows: $Z' = 1 - [(3\sigma_{(+)} +$

176 $3\sigma_{(-)})/ \mu_{(+)} - \mu_{(-)}]$, where $\mu_{(+)}$ and $\sigma_{(+)}$ are the mean and SD of the no-drug (untreated) positive
177 control, respectively; $\mu_{(-)}$ and $\sigma_{(-)}$ are the mean and SD from uninfected erythrocytes
178 (negative control), respectively.[22] The signal to background ratio was calculated as follows:
179 $[\mu_{(+)} - \mu_{(-)}]/\sigma_{(-)}$.

180 ***Results***

181 ***A microplate-based bioluminescence assay monitors a concentration- and time dependent***
182 ***loss of parasite viability immediately following drug perturbation***

183 *P. falciparum* genetically modified to express a luciferase reporter gene ($Dd2^{luc}$) show a time
184 and concentration-dependent loss of bioluminescence signal immediately, i.e. within 6 h,
185 following exposure to the rapid acting cytocidal drugs chloroquine (CQ), dihydroartemisinin
186 (DHA) and artemether (ARM).[16] By contrast, exposure to the cytostatic drug atovaquone
187 (ATQ) results in no apparent loss in bioluminescence signal over the same timeframe.[16] To
188 validate that this drug-induced loss of bioluminescence reports a loss in parasite viability,
189 $Dd2^{luc}$ parasites were subjected to a lethality assay adapted from that originally described by
190 Paguio et al.[18] Early trophozoite stage parasites were exposed to a serial dilution of
191 antimalarial drug in a 6 h bolus, the drug was then washed off before replacing the parasites
192 into culture for 42 h to complete a cycle of intraeythrocytic development. The principle here
193 is that parasites killed during the drug bolus will not divide after removal of the drug,
194 whereas those inhibited by the drug will recommence growth upon removal of drug
195 pressure. A range of antimalarial drug classes were tested to explore the applicability of this
196 approach, including; the 4-aminoquinoline chloroquine, the 8-aminoquinoline tafenoquine,
197 the 4-methanolquinolines mefloquine and quinine, the bisquinoline piperaquine, the

198 sesquiterpene lactones dihydroartemisinin and artemether and a napthoquinone
199 atovaquone.

200 Three log concentration-response curves, normalised against untreated controls, were fitted
201 using data derived from either a bioluminescence assay (Luc) or a Malaria Sybr Green I
202 fluorescence (MSF) assay of DNA content (see Figure S1 for an experimental schema). These
203 curves (Figure 1 and Figure S2) report the following parameters (Table 1); a 50% effective
204 concentration recorded immediately following the drug bolus but prior to drug wash-out
205 ($EC_{50}^{6h}$), a 50% lethal concentration estimate following drug wash out and re-culture ($LC_{50}^{6h}$)
206 and a 50% inhibitory concentration estimate following 48 h of continuous culture in the
207 presence of the drug ($IC_{50}^{48h}$).

208 The majority of drugs show a right-shift in the lethal concentration curve compared to that
209 of the inhibitory concentration, reflecting the higher concentration of drug required to
210 affect a kill within a 6 h window of exposure (Figure 1 and Figure S2). As expected, the right
211 shift was more pronounced for the quinolone drugs than for the artemisinins.[18,23] The minor
212 shift in lethal concentration for dihydroartemisinin and artemether likely reflects their
213 formation of covalent adducts with their target(s), rendering them resistant to the wash
214 steps, the use of trophozoite stage parasites in this assay as well as their profoundly rapid *in*
215 *vitro* rate of kill.[13,23,24] As previously shown, $IC_{50}^{48h}$ data developed using either MSF or
216 bioluminescence assays are essentially identical.[16] The same observation for the $LC_{50}^{6h}$ assay
217 data, whilst not unexpected, is shown here for the first time. Clear differences between the
218 MSF and bioluminescence assay immediately following the 6 h drug bolus ($EC_{50}^{6h}$) are,
219 however, evident. Here, the intrinsic instability of the luciferase reporter protein ($t_{½}$ of
220 approximately 1.5 h)[16] compared to that of the far more stable DNA biomarker (reported in

the MSF assay), offers an apparently more dynamic report of immediate drug action during this 6 h period. Importantly, the $EC_{50}^{6h}$ values determined using the bioluminescence assay (without drug wash and reculture) are almost identical to the $LC_{50}^{6h}$ estimated using either assay format. The observation that the 6 h bioluminescence curve closely fits that of the lethal concentration curve for the majority of drugs, indicates that the loss of bioluminescence not only apparently reports loss of viability, but also that this 6 h assay provides a rapid determination of the immediate cytocidal action of these drugs. The sole exception, atovaquone, shows no reduction in the bioluminescence signal within 6 h, a reflection of its previously reported pharmacodynamic killing lag time.[13]

To compare the relative concentration-dependent effects of different antimalarial drugs, a revised bioluminescence assay was devised that utilised fold-changes in $IC_{50}$ concentrations to ensure exposure to equipotent concentrations of drug. Here, $Dd2^{luc}$ parasites were exposed to a three-fold serial dilution (81 to 0.33x$IC_{50}$) for 6 h, with the bioluminescent signal, normalised to an untreated control at the same timepoint, plotted against drug concentration (Figure 2). These data illustrate an apparent saturation in the immediate lethal effect of drug concentrations greater than 9x$IC_{50}$ for all, except atovaquone, of the drugs tested. This observation is in agreement with the findings of Sanz et al,[13] who suggest that at a 10x$IC_{50}$ concentration the maximal rate of *in vitro* kill was achieved. Direct comparison between the data shown in figure 2 indicates an apparent relative ranking order of artemisinin > chloroquine > 4-methanolquinolines > atovaquone, that is identical to the relative order of RoK described both *in vivo* and *in vitro* for the same drugs.[25-29] To explore this correlation further, linear regression analysis was performed between the normalised bioluminescent signals produced for each drug concentration of drug tested and the *in vitro*

parasite reduction ratio (a Log ratio between parasitaemia at the onset of treatment and that after 48 h exposure, Log PRR) and parasite clearance time (time of drug exposure to elicit a 99.9% reduction in parasitaemia, PCT) reported in Sanz et al.[13] (Figure 3 and Figures S3 and S4). Comparison of bioluminescence against the PCT shows a strong, and significant, correlation at higher concentrations of drug, with the slope and intercept of the regression analysis essentially unchanged at concentrations greater than $9xIC_{50}$ reflecting an apparent saturation in the rate of kill achieved at these concentrations (Figure 3c and Figure S3). Comparisons with the Log PRR show a strengthening trend with higher concentrations of drug (Figure S4), although these just fail to reach a level of significance ($\alpha = 0.05$). Here, the available shared data (artemisinins are excluded as Log PRR only reported as >8) and the limited distribution in Log PRR for the quinoline drugs used would appear to be the likely limitations in achieving a significant correlation.

***Screening the Malaria Box for compounds that exert a rapid cytocidal effect***

The application of a microplate-based bioluminescence assay to quickly identify compounds that exert an immediate cytocidal effect was explored using the compound set available in the MMV Malaria Box. Using a 48 h MSF assay, $IC_{50}$ data was developed here in the $Dd2^{luc}$ clone for 396 compounds, the remaining 4 omitted as insufficient material was available. These data have been deposited in the ChEMBL - Neglected Tropical Disease Open Access repository (CHEMBL3392923). Of these 396 compounds, sufficient material was available for 372 to complete a 6 h bioluminescence cidality assay using a serial three-fold dilution of compounds between 9 to $0.33xIC_{50}$; this range selected to monitor the range of initial cytocidal action without the saturation effects observed at higher concentrations (Figure 3).

We term this the Bioluminescence Relative Rate of Kill assay, as the initial cytocidal activity of each compound is compared against a set of benchmark antimalarials for which *in vitro* rates of action are known. Log concentration-normalised bioluminescence signal plots for all 372 compounds are reported in the online supplementary materials (Figures S5 and S6. See also Table S1 for compound positions in these figures). Ten assay plates (each with *n*=3 replicates) of $Dd2^{luc}$ exposed for 6 h to either no drug (100% growth) or uninfected erythrocytes (0%) allow the *Z'* score, signal to background (S/B) ratio and coefficient of variation (%CV) assay parameters to be determined. The 95% confidence intervals for *Z'* score (0.9-0.97), maximum %CV (0.96-2.98%) and S/B (806-993) indicate a robust and sensitive microplate-based assay format.[22]

Using the mean and standard deviation of the normalized bioluminescence signal for each $IC_{50}$-fold concentration in the BRRoK assay, a Principle Components Analysis was carried out to capture the concentration-dependent effects in a single parameter (see Figures S7, Tables S2 and S3 in online supplementary materials). The first Principle Component (PC1) accounts for 89% of the variance in the 6 h assay dataset with the majority of the loading contributions provided by the 9x and $3xIC_{50}$ data ($0.63x[9xIC_{50}] + 0.62x[3xIC_{50}] + 0.41x[1xIC_{50}] + 0.22x[0.33xIC_{50}]$). Zero-meaned PC1 values provide a sequential order of the initial cytocidal effect exerted by the 372 MMV compounds screened (see Table S4 in the online supplementary materials), with lower value PC1 representing a greater cytocidal effect. This order of initial cytocidal effect is then informed by comparison against the initial cytocidal effect provided for each of the benchmark antimalarial compounds, providing the necessary surrogate information regarding the initial relative rate of kill for the MMV compounds. As such, the BRRoK assay indicates that 53 MMV compounds exert an initial

290 rate of kill at least the same as that for chloroquine (PC1=-73.7), with 17 (Figure S8) of these
291 compounds showing an initial rate of kill at least as good as that of dihydroartemisinin
292 (PC1=-97.4). Support for the surrogacy of PC1 in informing a compound's initial RoK is
293 provided by comparing PC1 with the *in vitro* estimates of Log PRR and PCT for the
294 benchmark antimalarial drugs tested (Figure 4).[13] This comparison shows significant
295 correlations between both PCT ($r^2$=0.91, $p$=0.003) and Log PRR ($r^2$ = 0.78, $p$=0.05) with the
296 PC1 values determined in the BRRoK assay.

297 Plotting PC1 against the $IC_{50}$ for the MMV compounds screened provides a simple means to
298 explore the interplay between the $IC_{50}$ potency and relative initial RoK against a background
299 of known antimalarial drug benchmarks (Figure 5). Potent, low $IC_{50}$, compounds with an
300 initial rapid rate of kill, exemplified by artemisinins (i.e. dihydroartemisinin), occupy the
301 bottom left quadrant of this plot. Atovaquone, whilst potent in terms of its $IC_{50}$, is a slow
302 acting drug and occupies the upper left hand quadrant. This analysis reveals that the 372
303 Malaria Box compounds tested display a wide range of apparent initial rates of kill, but also
304 that, as would be expected from previous *in vitro* assays of rate of kill, that there is no
305 correlation between $IC_{50}$ and rate of kill ($r^2$=0.1).[13,30] Also, as would be expected from the
306 MMV Malaria box, where compounds have been selected on their basis as starting points
307 for drug discovery programmes rather than $IC_{50}$ potency, no compound tested falls within
308 the optimal lower-left quadrant of this plot.

309

## *Discussion*

The development of the next generation of medicines for the treatment of uncomplicated malaria recognises the need for a combination of small molecule drugs that meets a series of challenging targets around efficacy, cost and safety.[8,9,31] Amongst these targets is the requirement to quickly clear the parasite burden through the action of (at least one) rapidly acting cytocidal drug within this combination therapy.[9] Here we describe the development of a rapid and robust assay for the screening of fast-acting cytocidal compounds against the intraerythrocytic trophozoite stages of *P. falciparum.* Moreover, the microplate-based format and minimal experimental manipulations required to complete the assay offers the potential for it to be readily scaled for high throughput screening of compound libraries such as the Tres Cantos Antimalarial Set (TCAMS) to triage hits with this rapid killing kinetic.

The utility of this assay was demonstrated through a medium throughput screen of 372 compounds sourced from the Malaria Box, an open access resource provided by the MMV to pump-prime antimalarial drug development.[14] Here the concentration-dependent loss of bioluminescence signal for these compounds was compared against those for a range of benchmark antimalarial drugs, providing a simple relative assessment of the initial rate of kill for each MMV compound. Thus, 53 compounds in the MMV Malaria Box were shown to have an initial rate of kill at least as good as chloroquine, with 17 of these compounds showing an initial rate of kill better than that for dihydroartemisinin. This relative description of the initial rate of kill relates directly to the minimum essential and ideal criteria, respectively, specified for a TCP 1 component of a future SERCaP drug by MMV.[9] Thus, whilst BRRoK assay doesn't provide a direct measurement of the rate of kill in terms of the currently defined PRR and PCT parameters, it readily meets the challenge of identifying,

and discriminating between, fast acting cytocidal drugs – and does so within one working day.

The development of this assay followed from the observation that the bioluminescent signal in parasites genetically modified to express a luciferase reporter protein is reduced in a concentration and time dependent way immediately following drug exposure when exposed to known cytocidal drugs.[16] This inherent dynamic response, a result of the short half-life of the luciferase, provides an apparent indirect report of the viability of the parasite. That is, dead and dying parasites do not synthesise new luciferase, the remainder being rapidly turned over. Here, using an adaptation of a lethality assay,[18] the concentration-dependent loss of bioluminescence over a 6 h drug bolus was shown to relate with that of the apparent lethal effect of a range of antimalarial drugs monitored following a drug wash and regrowth into a subsequent intraerythrocytic cycle of growth.[18]

To facilitate a relative comparison of cytocidal activity between different antimalarial drugs, the bioluminescence protocol was adapted to use fold-$IC_{50}$ concentrations of drug bolus. Comparisons between the loss of bioluminescent signal at 6 h when exposed to between 0.33 to 81x$IC_{50}$ concentrations correlated significantly with *in vitro* PCT at 3x$IC_{50}$ or greater. Interestingly, the slopes for these correlations remained unchanged at concentrations greater than 9x $IC_{50}$, mimicking the observation of Sanz et al., that suggests a 10x$IC_{50}$ concentration is generally sufficient, irrespective of mode of action of a drug, to achieve its maximal rate of *in vitro* kill.[13] Whilst the losses of bioluminescence signal at 6 h failed to consistently provide a significant correlation with *in vitro* PRR, the coefficient of variation are consistently greater than 0.69 for concentrations of 3x$IC_{50}$ and more. Given these observations, the BBRoK assay was adapted to screen the MMV Malaria Box using

concentrations between 0.33 to 9x$IC_{50}$. This choice balancing the apparent saturation in *in vitro* cytocidal action at concentrations higher than 9x$IC_{50}$ and the observation that concentrations as low as 0.33x$IC_{50}$ still elicited a measurable loss of bioluminescence from profoundly rapidly acting drugs such as dihydroartemisinin. A distinction between the BRRoK and *in vitro* PRR assays is the use of chloroquine resistant and sensitive strains, respectively. However, given the approach of using $IC_{50}$-fold concentrations of drug the effect on the interpretation of the relative rates of kill was considered minimal, and appears so from the correlation of benchmark drugs using both assays. The use of the $IC_{50}$-fold concentration approach in the analysis of drug-action in chloroquine sensitive and resistance strains is supported by the observation that the temporal accumulation of haemozoin shows no difference between these strains when the appropriate 1x or 2x $IC_{50}$-fold concentration of chloroquine is added.[32]

Potential modes of action have been attributed to 135 compounds in the MMV Malaria Box, although for most the target association is considered tentative.[33] Using these data, compounds exhibiting three modes of action were correlated with the BRRoK assay data to explore the validity of the assay format (Figure 6). Specifically; inhibition of (i) the $bc_1$ complex of the mitochondrial electron transport chain, (ii) dihydroortate dehydrogenase (DHODH), a key step in the *de novo* synthesis of pyrimidines in a parasite that otherwise lacks a pyrimidine salvage pathway and (iii) PfATP4, a $Na^+$-ATPase located in the parasite's plasma membrane. These modes of action were selected as *in vitro* PRR data are available for drugs/compounds that share these modes of action, with at least ten MMV compounds annotated for each.[33-38] Fifteen compounds are annotated as inhibitors of the parasite's $bc_1$ complex and are all reported in the BRRoK assay as having a slow initial cytocidal action (PC1

between 25.2 and 100.9), comparable to the exemplar drug, atovaquone, sharing the same mode of action (PC1= 55.4, log(PRR)=2.9 and 99.9% PCT=90 hr).[13] Twelve compounds are annotated as DHODH inhibitors, with five of these being structural analogues of the triazolopyrimidine early clinical trial candidate DSM265.[34] BRRoK data reports these compounds as sharing a slow initial cytocidal action (PC1 between 23.1 and 68) with this apparent slow rate of kill correlating with the atovaquone-like *in vitro* PRR data available for DSM265 due to a lag-phase of between 24 to 48hr before eliciting its cytocidal activity *in vitro*.[34] Of the 28 potential PfATP4 inhibitors in the MMV Malaria Box,[35] 26 were screened here. *In vitro* PRR data are available for the exemplar PfATP4 inhibitors (+)-SJ733,[36] a dihydroisoquioline with a slow to moderate (between pyrimethamine and atovaquone) rate of kill, and KAE609/NITD609,[37] a spiroindolone with a moderate to fast (between pyrimethamine and artesunate) rate of kill. The majority of the potential PfATP4 inhibitors in the MMV Malaria Box were reported in the BRRoK assay (PC1 between -47.4 and -114.9) as having an initial rate of kill between the moderate mefloquine (PC1=-42.4, log(PRR)=3.7 and 99.9% PCT= 43 hr), comparable to the *in vitro* PRR reference pyrimethamine (log(PRR)=3.5 and 99.9% PCT= 55 hr),[13] and the fast-acting dihydroartemisnin (PC1=-97.4). The fastest acting PfATP4 inhibitor in the BRRoK assay was the spiroindolone MMV396749. The Malaria Box also contains five structural analogues of (+)-SJ733.[35] Of these, four reported BRRoK data (between -16.4 and 49.1) falling between mefloquine and atovaquone, supporting the moderate to slow rate of kill of these dihydroisoquinolines. These data, taken together with the expected relative order of the benchmark antimalarial drugs tested, validates the application of the BRRoK assay in determining initial cytocidal activity across a diverse range of chemotypes.

Comparison of BRRoK with other available *in vitro* assays of rate of kill suggests a number of advantages to our approach. Two alternative assays offer an indirect measurement of cytocidal activity, either by monitoring mRNA levels or through the use of a modified $^{3}$H-hypoxanthine incorporation assay.[25,26] The two remaining alternatives offer a direct measurement of viability, essentially through the monitoring of erythrocyte re-invasion (reinvasion into fluorescently labelled erythrocytes) and growth post-drug challenge – i.e. the current "gold" standard offered through monitoring parasite recrudescence after limiting dilution.[13,27] Apart from the recrudescence assay, these formats offer readouts of parasiticidal activity within 3-10 days, with the recrudescence assay taking between 21-28 days. The advantage of the recrudescence assay, however, is based on the fact that it provides a direct measurement of rate of kill, albeit *in vitro* conditions generate PRR values that are not always directly comparable to *in vivo* PRR measurements.[13,28,36,39] The erythrocyte invasion viability assay, like the BRRoK assay, offers a relative measure of RoK, with classifications of slow (atovaquone-like), moderate (pyrimethamine-like) and fast (artemisinin-like).[27] Comparison of the PC1 values for the 30 MMV compounds and benchmark antimalarials similarly tested in the erythrocyte-invasion viability assay shows good correlation (Figure S9), with significant differences between PC1 values for fast and slow RoK drugs as defined in the invasion assay.[27,30] Moderate RoK compounds, however, showed no significance difference to slow and fast RoK compounds when compared to the BRRoK assay, but the sample size is small and there are outliers in both groups. These outliers may be potentially interesting as they likely represent differences between compounds being exposed for 6 h to trophozoites (BRRoK) or for between 24 to 48 h (invasion assay), encompassing most developmental stages of the intraeythrocytic asexual cycle, and may provide some guide to lag in cytocidal activity or stage-specific action.

The key limitation to the recrudescence assay is that it is only available as a low throughput tool, due to the technical and time consuming challenge of the assay protocol – but it does provide the ability to discriminate between whether a compound meets the minimal essential or ideal TCP1 criteria for RoK. This discrimination between TCP1 candidates is similarly provided by the BRRoK assay, although here it is provided in 6 h from a microplate format assay requiring minimal manual intervention beyond a single-step lysis and readout protocol. The simplicity of this assay format offers a significant opportunity to scale up for high throughput screening through semi-automation and denser microplate formats. Z' and S/B rations comparable to those from the 96-well microplate assay can be achieved using 30 µl of intraerythrocytic culture (2% haematocrit and 2% parasitaemia) indicating that this robust assay can be readily adapted into a 384- or 1536 - well microplate format. Whilst offering advantages in terms of speed, simplicity, scalability and discrimination, the BRRoK assay in its current format defines RoK only in terms of the relative immediate cytocidal action against trophozoite stage parasites. This assay appears best placed in the early drug discovery pipeline, with priority hits from BRRoK assay to be subsequently confirmed using the *in vitro* PRR assay.

The requirement for transgenic parasites expressing the luciferase reporter gene limits the genetic backgrounds available for this assay, and does represent a distinct limitation of this *in vitro* method when compared to the PRR and erythrocyte invasion assay.[13,27] However, these transgenics can be reasonably readily generated using stable episomally maintained reporter plasmids, with the opportunity to extend the utility of the assay, beyond the current limitation of trophozoite stage parasites, to explore stage-specific rate of kill dynamics through the selection of gene flanking sequences that would offer temporal

449 expression at other intraerythrocytic stages. The potential for a revised reporter construct in
450 developing a cytocidal activity assay in ring stage parasites for the exploration of artemisinin
451 action and resistance being a particularly relevant target of interest. One disadvantage
452 evident from this study was that it took significantly longer to generate the $IC_{50}$ data for the
453 MMV Malaria Box compounds than to perform the BRRoK assay. Typically, $IC_{50}$ data from
454 large high throughput screens are available for the 3D7 strain of *P. falciparum*, and were
455 provided with the Malaria Box resource. Given that multiples of $IC_{50}$ are used in the BRoK
456 assay, and that the $9xIC_{50}$ assay data best correlated with available *in vitro* PRR and PCT
457 data, we examined whether Dd2-specific $IC_{50}$ data was actually needed to triage rapidly
458 acting cytocidal compounds or whether a similar outcome could be achieved using the more
459 widely available $IC_{50}$ data from the 3D7 strain. Using the available 3D7 data to provide for a
460 $9xIC_{50}$ concentration against $Dd2^{luc}$, the loss of bioluminescence signal in a single
461 concentration/single timepoint (6 h) triage assay was carried out with 396 of the MMV
462 compounds and compared to the same data derived using our Dd2 $IC_{50}$ values (Figure 7a).
463 The loss of bioluminescence measured using the 3D7 $IC_{50}$ concentrations significantly
464 correlate with those determined using the Dd2 $IC_{50}$ values ($r^2$=0.88 $p$<0.0001). Looking
465 specifically at the 53 compounds that the BRRoK assay define as having an initial rate of kill
466 at least as good as chloroquine, setting a threshold of discovery based on a 50% loss of
467 bioluminescence signal using the 3D7 $IC_{50}$ concentration would ensure >95% of these TCP1
468 candidates would be identified in this simplified assay format using the more readily
469 available 3D7 data (Figure 7b). The discovery rate using 3D7 $IC_{50}$ data falls as a more
470 stringent bioluminescence signal threshold is applied, although the same is similarly true
471 when using the actual Dd2 $IC_{50}$ data. This simple triage assay, utilising the opportunities
472 afforded through denser microplate formats and available 3D7 $IC_{50}$ data, provides the

means for a quick and robust exploitation of the available chemical libraries in our search for rapid acting cytocidal antimalarial drugs to meet the challenge of malaria control and eradication in a post-artemisinin era.

**Acknowledgements**

The authors thank the Medicine for Malaria Venture for the assembly and supply of the Malaria Box.

**Funding**

Authors wish to acknowledge support from the Medical Research Council (MR/L000644/1, MC_PC_13069 and MC_PC_14111 , Keele University (ACORN PhD scholarship award to IU) and the Charles Wallace Pakistan trust (to IU).

**Transparency Declaration**

None to declare.

**Supplementary data**

Supplementary data are available at JAC Online (http://jac.oxford journals.org/).

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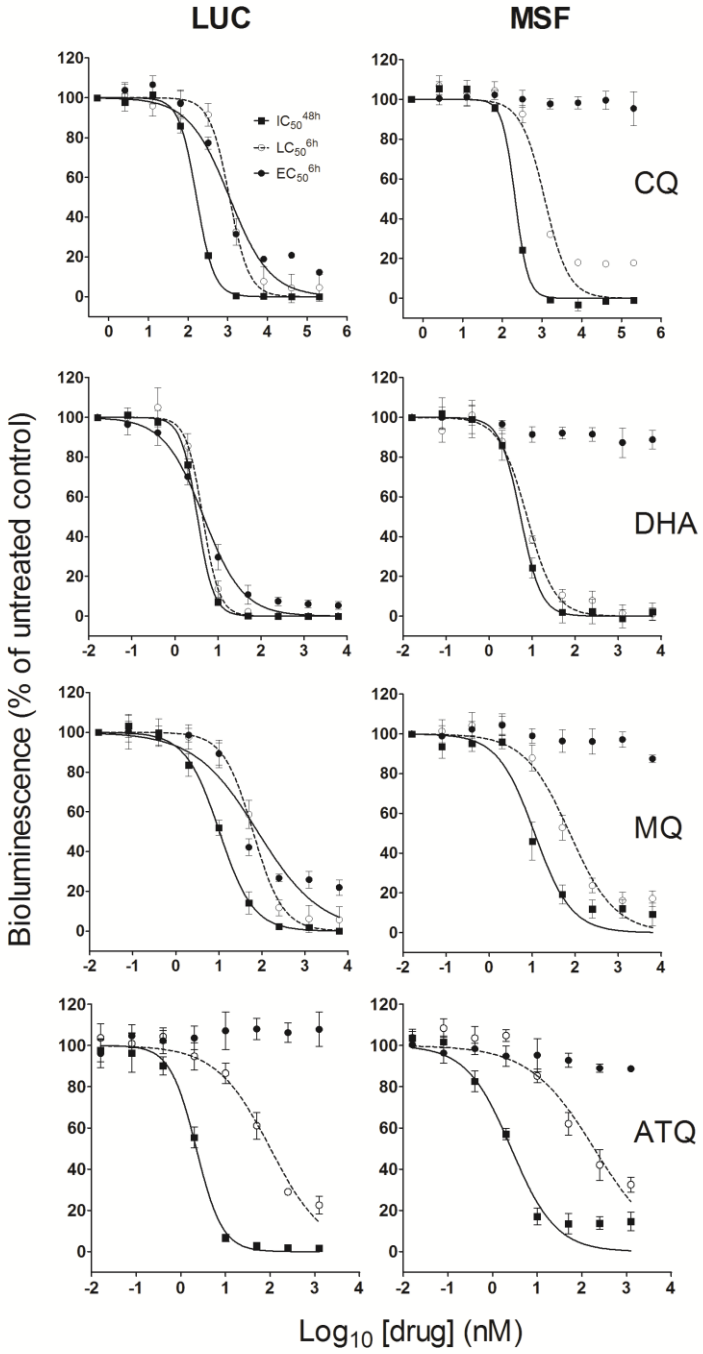


575

576 **Figure 1**. Monitoring drug-induced loss in parasite viability using a bioluminescence assay.
577 Panels illustrate log-concentration response curves following exposure to the indicated drug
578 using either a bioluminescence (LUC) or fluorescence assay (MSF) format. From these
579 curves, estimates of the $IC_{50}^{48h}$, $LC_{50}^{6h}$ and $EC_{50}^{6h}$ (see main text for definition and key for
580 symbols used) were determined using each assay format (reported in Table 1). Data
581 represents the mean of three biological replicates, with SD indicated by error bars. ATQ,
582 atovaquone; CQ, chloroquine; DHA, dihydroartemisinin; MQ, mefloquine. See Figure S1 for
583 same charts for artemether, quinine, piperaquine and tafenoquine. Note that non-linear
584 regression for $EC_{50}^{6h}$ estimates using the MSF assay were not possible.

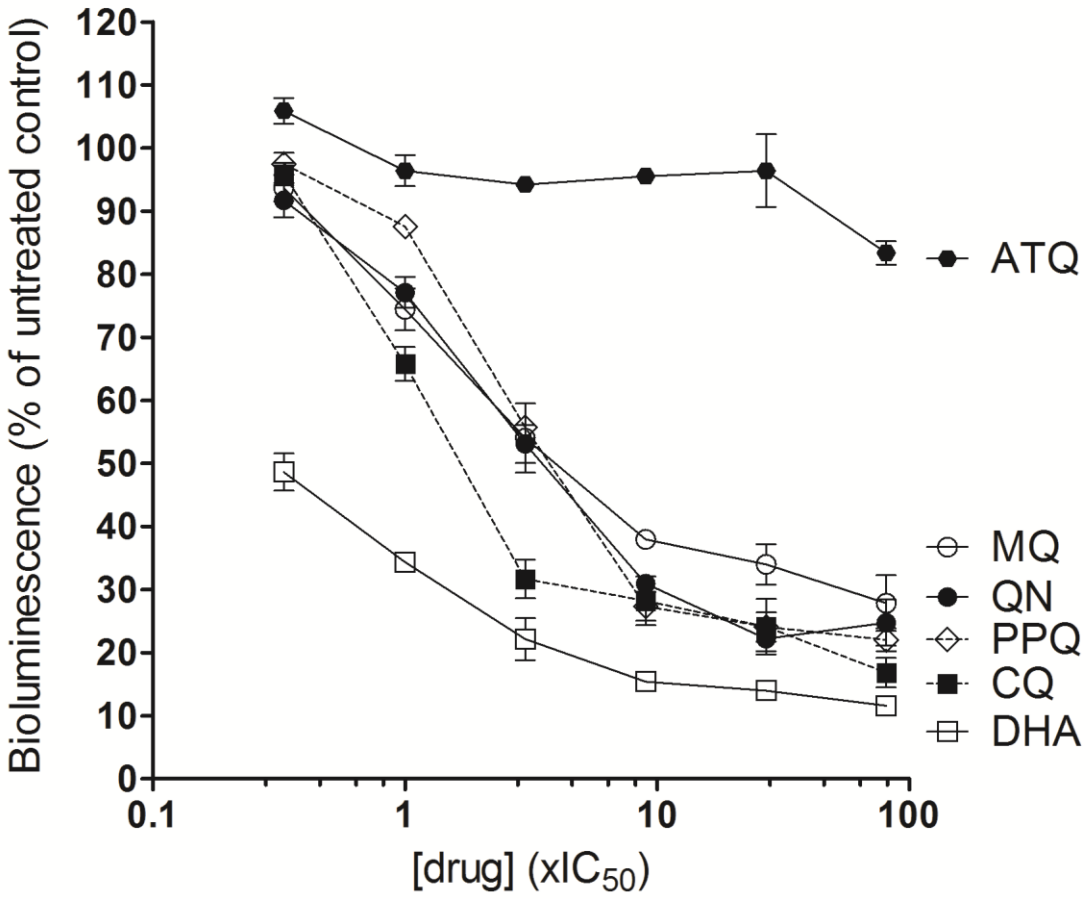


**Figure 2.** Equipotent-$IC_{50}$ concentration-dependent loss of bioluminescence for standard antimalarial drugs. The mean (error bars represent ±SD from three biological replicates) bioluminescence signal, normalised against an untreated control, remaining after a 6 h exposure to the indicated fold-$IC_{50}$ concentration of drug (see key to right of chart). A serial three-fold dilution from 81x$IC_{50}$ to 0.33x$IC_{50}$ is reported. ATQ, atovaquone; CQ, chloroquine; DHA, dihydroartemisinin; MQ, mefloquine; PPQ, piperaquine; QN, quinine.

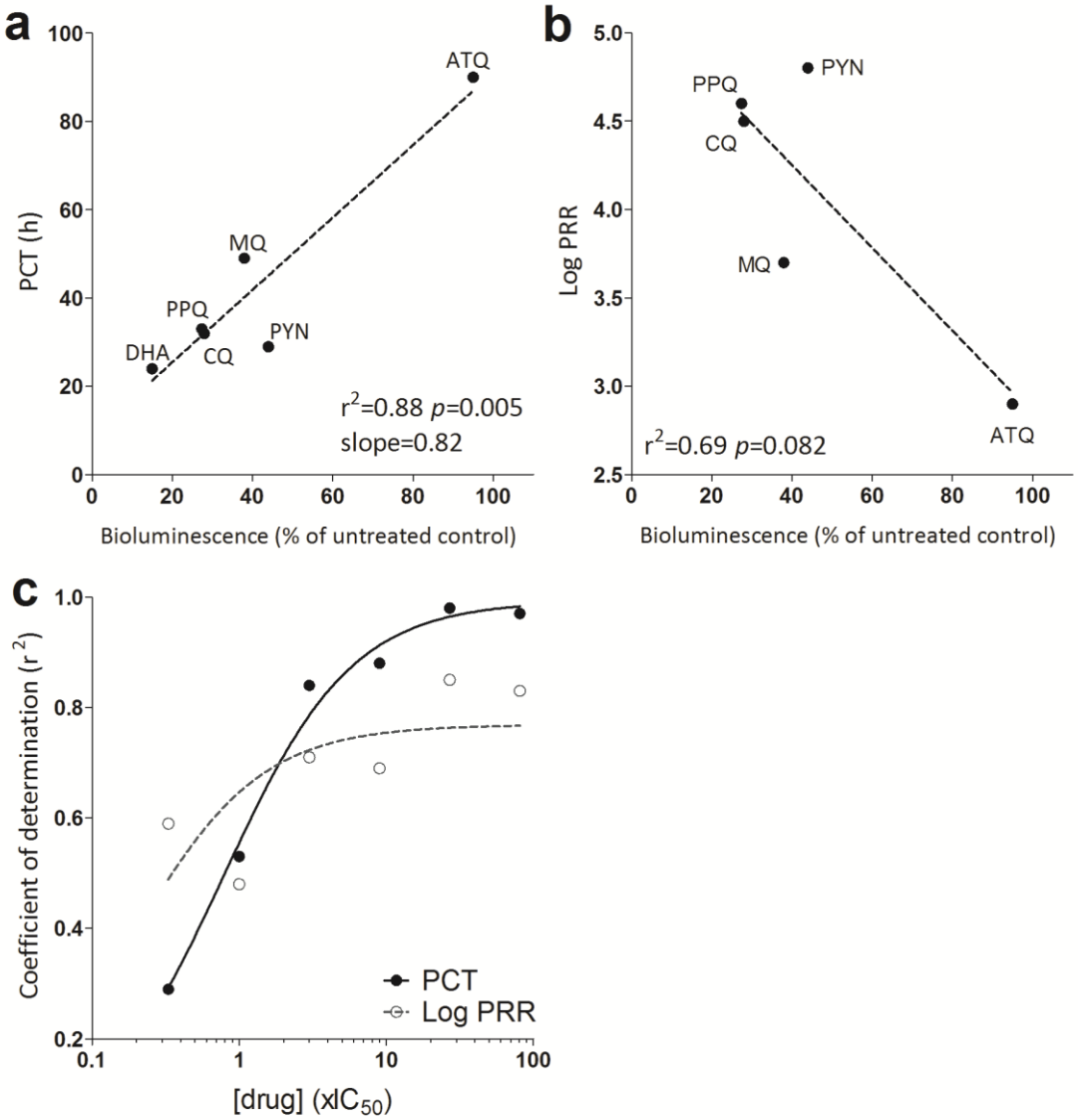


**Figure 3.** Correlating the concentration-dependent loss of bioluminescence signal with *in vitro* (**a**) Parasite Clearance Time (PCT, h) and (**b**) Log Parasite Reduction Ratio (PRR). Each panel represents on the x-axis the bioluminescence signal, as a % of an untreated control, following exposure to a 9 x $IC_{50}$ concentration of drug for 6 h. *In vitro* PCT and Log PRR data from the Sanz *et al.,*[13] study are plotted on the y-axis. Linear regressions are indicated with a dotted line, with parameters reported on each chart. Charts representing the effect at 81, 27, 3, 1 and 0.33 x $IC_{50}$ are shown in Figures S3 and S4. (**c**) Plot illustrating the apparent saturation in coefficients of determination ($r^2$) derived when correlating *in vitro* PCT and Log PRR data against the loss of bioluminescence signal at higher fold-$IC_{50}$ drug concentrations tested. ATQ, atovaquone; CQ, chloroquine; DHA, dihydroartemisinin; MQ, mefloquine; PPQ, piperaquine; PYN, pyronaridine.

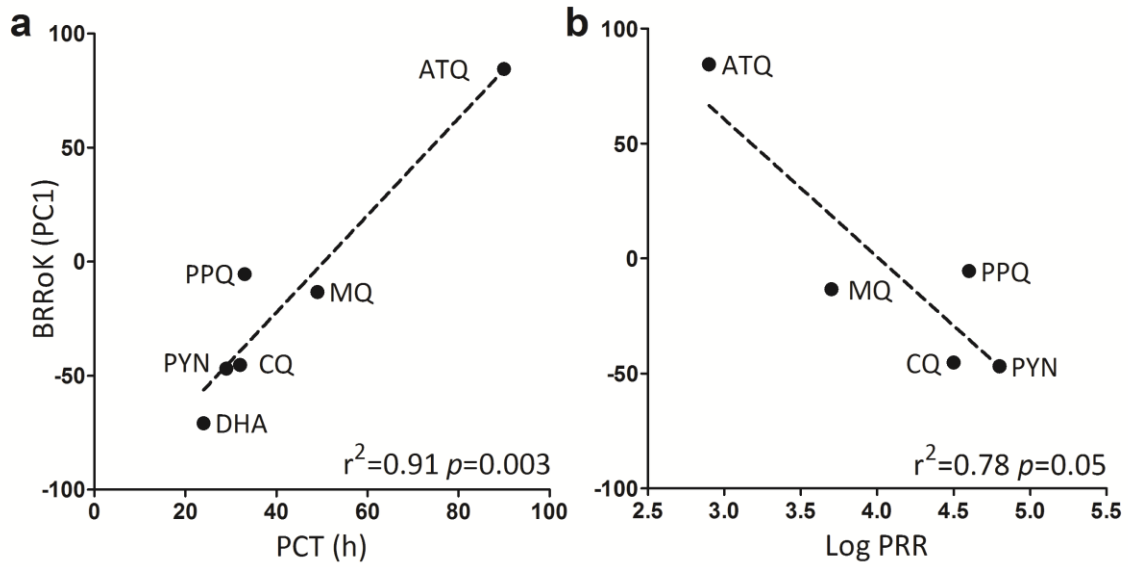


**Figure 4.** Correlating the Bioluminescence Relative Rate of Kill (BRRoK, PC1) with *in vitro* (**a**) Parasite Clearance Time (PCT, h) and (**b**) Log Parasite Reduction Ratio (PRR). Zero-meaned PC1 data are plotted on the y-axis for antimalarial drugs where *in vitro* PCT and PRR data are available from Sanz *et al*.[13] Linear regressions are indicated with a dotted line, with parameters reported on each chart. ATQ, atovaquone; CQ, chloroquine; DHA, dihydroartemisinin; MQ, mefloquine; PPQ, piperaquine; PYN, pyronaridine.

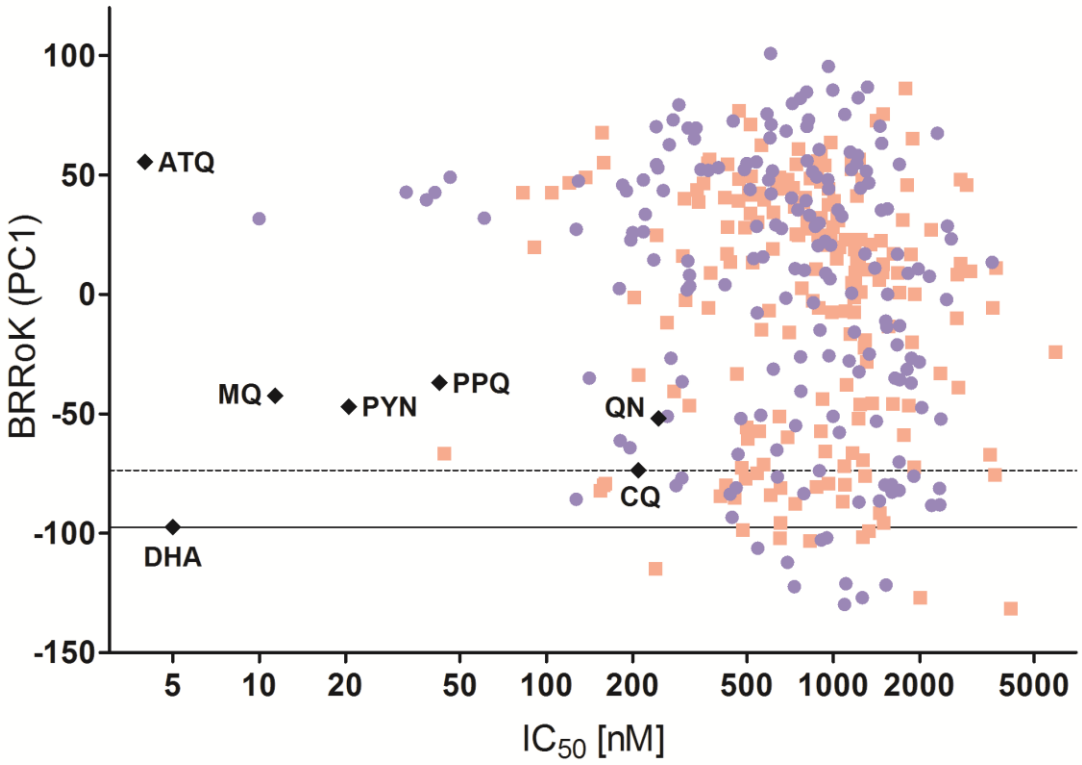


**Figure 5.** Distribution of Bioluminescence Relative Rate of Kill (BRRoK, PC1) against $IC_{50}$ for the MMV Malaria Box compounds. Zero-meaned PC1 data for 372 compounds in the MMV Malaria Box (drug-like in red squares and probe-like in blue circles) and 7 benchmark antimalarial drugs (black diamonds) are plotted against their $IC_{50}$ (note: faster initial rates of cytocidal activity are represented with lower PC1 values). See Table S4 in online supplementary materials for PC1 and $IC_{50}$ data for individual compounds. The minimum essential threshold (dotted line) and ideal threshold (solid line) for TCP1 candidates are indicated based on the BRRoK assay data for CQ and DHA, respectively. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC.* ATQ, atovaquone; CQ, chloroquine; DHA, dihydroartemisinin; MQ, mefloquine; PPQ, piperaquine; PYN, pyronaridine; QN, quinine.

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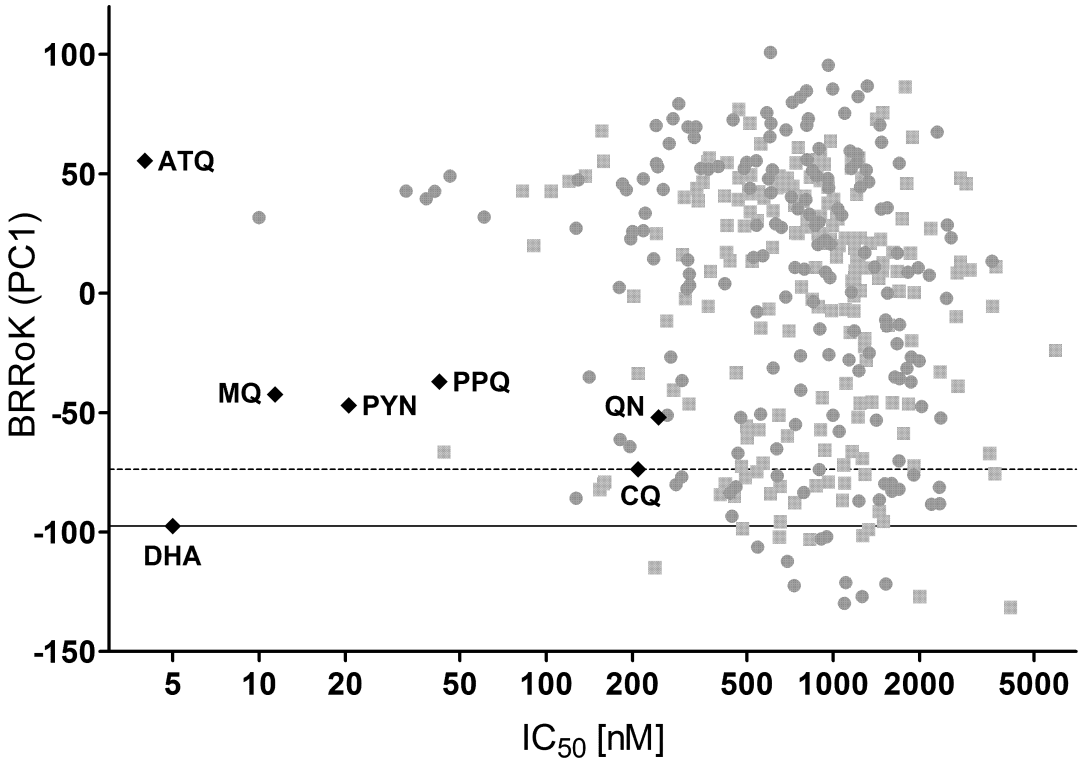


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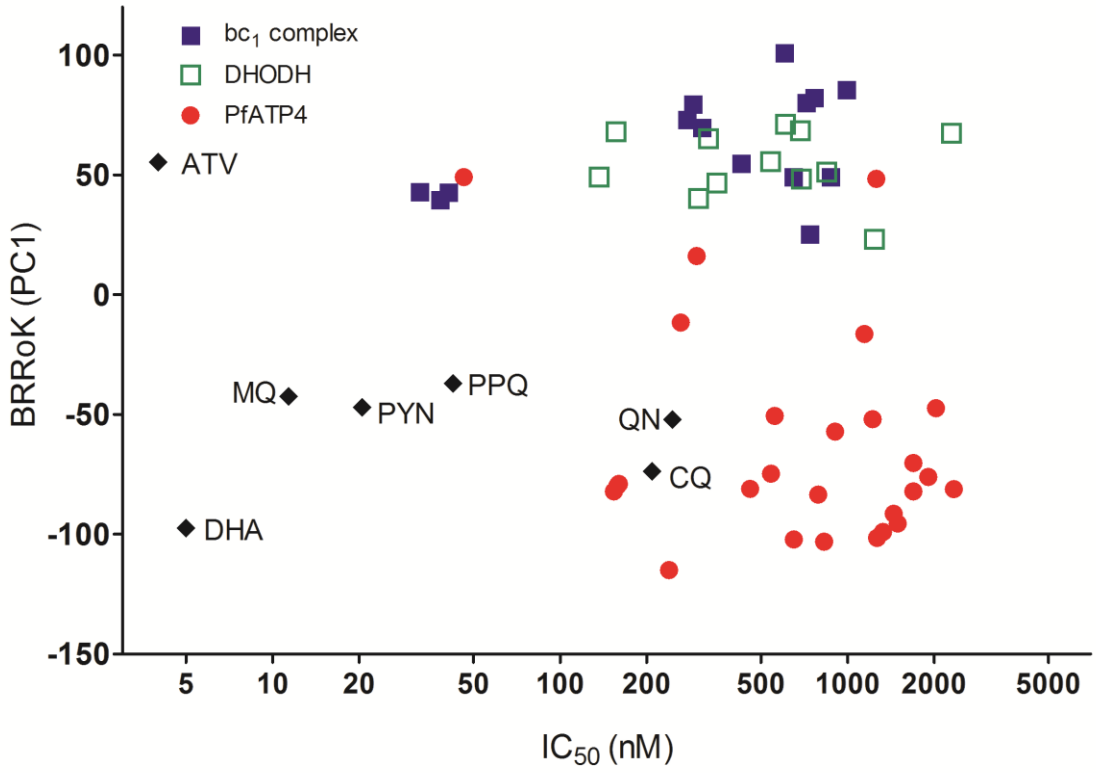
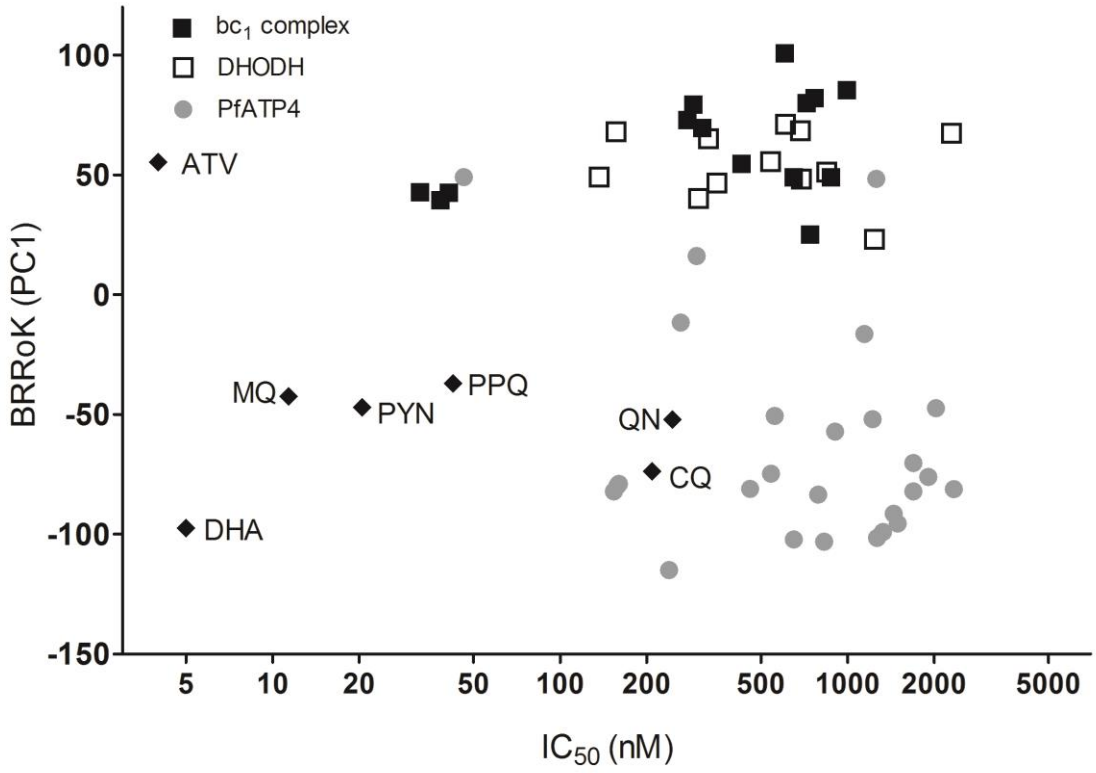



**Figure 6.** Correlating mode of drug action with the BRRoK assay. Zero-meaned PC1 data for MMV compounds with predicted modes of action that target (i) $bc_1$ complex (blue filled square), (ii) DHODH (green unfilled square) and (iii) PfATP4 (red filled circles) are plotted against their $IC_{50}$.[32] This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*. ATQ, atovaquone; CQ, chloroquine; DHA, dihydroartemisinin; MQ, mefloquine; PPQ, piperaquine; PYN, pyronaridine; QN, quinine.

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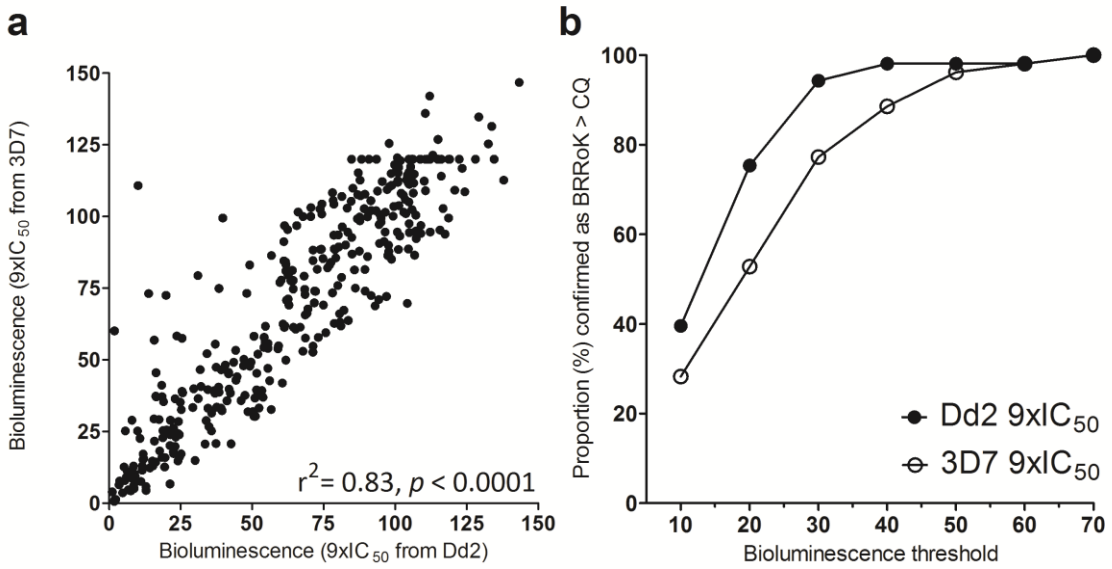


**Figure 7.** Scaling the BRRoK assay – the utility of a single concentration /single time point assay. (**a**) Chart illustrating the correlation between the bioluminescence signals (as a % of untreated control) when $Dd2^{luc}$ is exposed to a $9xIC_{50}$ concentration of MMV Malaria Box compound for 6 h – the x-axis representing data using Dd2 $IC_{50}$ data derived in this study, and the y-axis $IC_{50}$ data from a 3D7 clone (values provided with the Malaria Box resource)[14]. (**b**) Plot representing the proportion of the 53 compounds from the full BRRoK assay that show an initial rate of cytocidal activity at least as good as that of chloroquine (CQ) that would be identified when the indicated thresholds of bioluminescence signal post-treatment are chosen. The two curves represent the bioluminescence signal thresholds when $9xIC_{50}$ concentrations of either the Dd2 or 3D7 strains are used in this assay. Note that selection of a 50% bioluminescence threshold, irrespective of the source of the $IC_{50}$ data used in the single concentration/single time point assay, identifies >95% of the MMV compounds shown in this report to have an initial cytocidal activity that would meet the minimal essential requirement for a TCP1 candidate.

670

671 **Table 1.** $IC_{50}^{48h}$, $LC_{50}^{6h}$ and $EC_{50}^{6h}$ estimates determined using bioluminescence (Luc) and
672 fluorescence (MSF) assay formats.

673

| | $IC_{50}^{48h}$ | | $LD_{50}^{48h}$ | | $ED_{50}^{6h}$ | |
|---|---|---|---|---|---|---|
| | Luc[1] | MSF[2] | Luc | MSF | Luc | MSF |
| Chloroquine[3] | 162 | 209 | 1093 | 1163 | 1091 | nd[4] |
| | (150-174)[5] | (156-232) | (895-1336) | (880-1431) | (906-1350) | |
| Piperaquine | 37.0 | 42.5 | 339 | 376 | 351 | nd |
| | (34.5-39.6) | (34.1-52.8) | (273-391) | (224-547) | (232-436) | |
| Mefloquine | 10.3 | 11.4 | 62.3 | 74.7 | 79.8 | nd |
| | (8.9-11.9) | (8.3-16.6) | (51.2-75.8) | (59.3-87.5) | (52.4-92.1) | |
| Quinine | 306 | 246 | 1532 | 2031 | 1865 | nd |
| | (271-346) | (205-295) | (1142-1953) | (1681-3053) | (1462-2055) | |
| Tafenoquine | 354 | 373 | 2356 | 3207 | 3169 | nd |
| | (285-441) | (308-506) | (1950-2846) | (2215-4365) | (2465-3847) | |
| Dihydroartemisinin | 3.3 | 4.1 | 4.4 | 6.8 | 5.6 | nd |
| | (3.2-3.5) | (3.9-4.8) | (3.9-5.0) | (5.8-8.7) | (4.6-6.2) | |
| Artemether | 5.5 | 10.0 | 6.7 | 12.7 | 7.8 | nd |
| | (5.0-6.0) | (9.1-10.9) | (5.8-7.6) | (10.8-14.9) | (5.7-10.8) | |
| Atovaquone | 2.2 | 2.6 | 101 | 187 | nd | nd |
| | (1.9-2.6) | (1.7-3.7) | (73.1-142) | (91.9-248) | | |

[1]Bioluminescence luciferase assay [2]Malaria Sybr Green Fluoresence assay

674 [3]Mean (n≥6 measurements) in nM [4]not determined [5]95% Confidence intervals