High-Content Screening of MMV Pathogen Box for *Plasmodium falciparum* Digestive Vacuole Disrupting Molecules Reveals Valuable Starting Points for Drug Discovery

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Running Head: High-content Screen for DV Disruption in *Plasmodium*

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Plasmodium falciparum infections leading to malaria have severe clinical manifestations and high mortality rates. Chloroquine (CQ), a former mainstay of malaria chemotherapy, has been rendered ineffective due to the emergence of wide-spread resistance. Recent studies, however, have unveiled a novel mode of action in which low micromolar levels of CQ permeabilized the parasite’s digestive vacuole (DV) membrane, leading to calcium efflux, mitochondrial depolarization and DNA degradation. These phenotypes implicate the DV as an alternative target of CQ and suggests that DV disruption is an attractive target for exploitation through the screening for DV-disruptive antimalarials. In the current study, high-content screening was performed on the Medicines for Malaria Venture (MMV) ‘Pathogen Box’ (2015) to select for compounds which disrupted the DV membrane as measured by the leakage of intravacuolar Ca^{2+} using the calcium probe Fluo-4 AM. The hits were further characterized by hemozoin biocrystallization inhibition assays and dose-response IC_{50} assays across resistant and sensitive strains. Three hits - MMV676380, MMV085071 and MMV687812 were shown to demonstrate lack of CQ cross-resistance in parasite strains and field isolates. Through systematic analyses, MMV085071 emerged as the top hit due to its rapid parasiticidal effect, low nanomolar IC_{50} and good efficacy in triggering DV disruption, mitochondrial degradation and DNA fragmentation in P. falciparum. These programmed cell death (PCD)-like phenotypes following permeabilization of the DV suggests that these compounds kill the parasite by a PCD-like mechanism. From the drug development perspective, the identification of MMV085071 as a potent DV disruptor offers a promising starting point for subsequent hit-to-lead generation and optimization through structure-activity relationships.
INTRODUCTION

With approximately 200 million cases of *Plasmodium falciparum* (*P. falciparum*) infections in 2016, malaria remains a global health burden. Although this striking figure represents a 17% decrease in the overall incidence rate as compared to 2010 (1), the spread of artemisinin-resistance had been progressively reported in the Southeast Asian regions since 2008 (2-4).

Coupled with chloroquine (CQ) resistance since the 1950s (5), there exists a dire going need for novel therapeutic antimalarials. In addition to the canonical mechanism of action in inhibiting hemozoin formation (6), administration of low micromolar levels of CQ were shown to permeabilize the parasite’s digestive vacuole (DV) membrane and led to Ca$^{2+}$ efflux (7), mediating downstream cellular events such as mitochondrial outer-membrane permeabilization (MOMP) and DNA degradation. These phenotypes were similar to the hallmark features of mammalian programmed cell death (PCD); indicating an alternative mode of action for the drug which can be repurposed despite drug resistance. Furthermore, given that the parasite’s DV is the major organelle for metabolic activities and its contents represent important and viable drug targets (8), the findings provide exciting possibilities for rational novel antimalarial drug design, targeting the DV.

Following the success of Malaria Box, Medicines for Malaria Venture (MMV) assembled the Pathogen Box (2015), consisting of 400 diverse compounds against malaria and a wide range of neglected tropical diseases. A high-content screening assay capitalizing on conventional flow cytometry capabilities alongside high-resolution single-cell imaging was developed and performed to select for Pathogen Box compounds that disrupted the DV membrane. Due to the abundance of calcium in the parasite’s DV (9), Ca$^{2+}$ release into the cytosol could serve as a surrogate indication for DV permeabilization (10) and DV disruption was thus quantified by the leakage of intravacuolar Ca$^{2+}$ using a fluorescent calcium-binding probe Fluo-4 AM.
Following the disruption of the DV, mitochondrial dysfunction and DNA fragmentation were assayed to validate the PCD-like features as consequences of DV membrane perturbation.

To narrow down the panel of primary hits, follow-on characterization studies were conducted to investigate their possible mechanisms of action and therapeutic potencies in a time- and dose-dependent manner across laboratory strains and field isolates. These characterization assays have led to the identification of a potent, non CQ and artemisinin cross-resistant, rapid parasiticidal and DV-permeabilizing hit MMV085071 against *P. falciparum* from the MMV Pathogen Box repository.
RESULTS

High-content DV permeabilization screen of MMV Pathogen Box. After gating for focused and circular singlets, the trophozoite population was determined in accordance to positive Hoechst staining and visual inspection of brightfield images (Fig. 1a). Given that the DV is a calcium ion store (9), compromisation of DV membrane integrity leading to subsequent Ca\(^{2+}\) efflux could be indicated by an increase in the area of green Fluo-4 fluorescence upon high affinity binding to Ca\(^{2+}\) (11). Quantification of Fluo-4 fluorescence redistribution (Fig. 1b–d) was measured by the application of an area feature mask built-in within the analysis software. To exclude non-specific background staining or staining of the erythrocyte, only areas above 250 pixels of intensity were incorporated for each single-cell analysis. To qualify as a DV-disrupting “hit” from the primary screen, the Pathogen Box compound had to display at least 70% efficiency in DV-disrupting phenotype relative to CQ. Fig. 1e shows the identity of the hits obtained from repeated rounds of screening. From the total of 400 Pathogen Box compounds, 10 of them had at least 70% increase in area of Fluo-4 fluorescence at 10\(\mu\)M, giving an overall hit rate of 2.5%. Of note, MMV676380 and MMV032967 permeabilized the parasite DV to a greater extent than that of CQ (Fig. 1f).

Since the mechanisms of action of the compound library are unknown, the phenotypic screen could offer preliminary mechanistic insights into their potential antimalarial activities.

Downstream induction of PCD-like features. To validate PCD-like parasitic cell death following DV disruption, parasites were treated with the panel of hits for 10 hours for detection of downstream cellular responses. To this end, ratiometric cationic JC-1 dye was used to characterize perturbation of mitochondrial integrity. After normalization to drug-free control (PBS), all the hits including CQ brought about the disintegration of mitochondrial transmembrane potential while CQ displayed the greatest degree of reduction in JC-1 red-to-green ratio (Fig. 2a). Corroborative results were obtained from the measurement of...
proportion of parasites with sub-G1 DNA content (Fig. 2b). Of particular note, MMV687248 demonstrated high efficacy in triggering DNA fragmentation similar to that of CQ while the remaining hits led to a minimum of threefold increase in the proportion of sub-G1 positive parasites. All in all, these assays also validated parasitic cell death after having compromised the parasitic vacuole and were in agreement with a novel mechanism of P. falciparum cell death following the disruption of DV integrity.

Spectrophotometric investigation of β-hematin biocrystallization. With the hits’ PCD-like features validated through repeated screens for MOMP and sub-G1 DNA assays, they were subjected to further characterization studies to increase understanding of the novel compounds. During the intra-erythrocytic stage of the malaria parasite, hemoglobin is degraded for amino acids and osmotic homeostasis as the parasite matures in size (12). As cytotoxic heme is generated alongside the degradation process, the parasites detoxify free monomeric heme into hemozoin biocrystals. While many theories have been developed to propose the antimalarial mechanism exerted by CQ, it is generally accepted that the drug acts by inhibiting the formation of hemozoin; thereby leading to heme accumulation and parasitic cell death (13). Despite its effectiveness and impact towards the ambitious goal of malarial elimination, resistance towards CQ had limited its administration. Against this backdrop, a de novo β-hematin assay was optimized to investigate the mode of action of the hits and to elucidate the novel non “CQ-like” hits from “CQ-like” activity indicated by low β-hematin present in the final pellet after washing off monomeric heme and heme oligomeric aggregates with an alkaline bicarbonate buffer (14). Pyrimethamine (PYR) was used as a negative mechanistic control to indicate non “CQ-like” effect as it is an established antimalarial drug which potency is attributed to the interference of DNA synthesis (15). Accordingly, absorbance readings resulted from unimpeded β-hematin polymerization were observed to be higher for PYR and non “CQ-like” hits compared to CQ, which impeded β-hematin
formation and thus resulted in lower absorbance readings. MMV676380, MMV085071 and MMV687812 emerged as hits which displayed high optical density measured at 405nm comparable to that of PYR after normalizing to drug-free PBS controls (Fig. 3). This preliminary finding suggested that the three compounds of the Pathogen Box are non CQ cross-resistant and thus potent against CQ-resistant \textit{P. falciparum} strains.

**Growth inhibition assay and potency across CQ-resistant strains.** As the Pathogen Box is an assembly of diverse novel compounds against neglected tropical diseases of interest, meaningful antimalarial activity at the low micromolar range had to be established prior to any subsequent hit-to-lead optimization. Laboratory strains CQ-sensitive 3D7 and CQ-resistant K1 were treated with a 11-point serial dilution of MMV676380, MMV085071 and MMV687812 for IC$_{50}$ determination and all the sigmoidal IC$_{50}$ curves obtained have goodness-of-fit above 0.97 (Fig. 4 and S1). Respective 48-hour incubation resistance index was then evaluated by the ratio of K1 IC$_{50}$ to 3D7 IC$_{50}$ (Table 2). The antimalarial efficacies of the hit compounds were determined to be at the nanomolar to low micromolar range with MMV085071 exhibiting the lowest IC$_{50}$. In particular, MMV676380 and MMV085071 were phenotypically validated be non cross-resistant with similar dosage required in 3D7 and K1; thereby substantiating preliminary findings obtained in Fig. 3 while 22 times the concentration of CQ was required against K1. MMV687812, however, showed slight significant difference in IC$_{50}$ with approximately 2- to 3-fold more of inhibitory concentration necessary in K1.

**Dose-dependent effects of DV destabilization and PCD-like phenotypes induction.** Having demonstrated the lack of CQ cross-resistance, parasites were then subjected to varying concentrations of hit compounds to quantitate the minimum dose required for induction of PCD hallmark features for evaluation of potencies. MMV085071 was shown to achieve notable DV-disruption, MOMP and increase in sub-G1 DNA at a comparable dosage
relative to CQ based on sigmoidal dose-response curves (Fig 5a-c). A calculation of EC\textsubscript{70} revealed the minimum doses required for 70% maximal phenotype induction (Table 3). CQ proved to display greatest potency in eliciting DV disruption and PCD-like phenotypes against 3D7 parasites. At least 1\mu M of CQ was required to be accumulated in the DV of parasites for potent permeabilization in 4 hours, causing mitochondrial dysfunction and DNA fragmentation in 10 hours. MMV687812, in contrast, required the highest dosage in all 3 investigated phenotypes. MMV676380 appeared to be most effective in compromising the parasite DNA integrity while MMV687812 targeted the mitochondria with most pronounced efficacy compared to its DV-disrupting and DNA degradation capacities.

**Assessment of parasite killing speed.** Using two-colour flow cytometry, the timing of action of the non “CQ-like” hits was characterized for viability and reinvasion capacity at the 24\textsuperscript{th}, 48\textsuperscript{th} and 72\textsuperscript{nd} hour timepoints after having treated the parasites with drugs of interest. 3D7 parasites were exposed to 10 times the pre-determined IC\textsubscript{50} (Table S1) to avoid sub-optimal drug treatment during the period of investigation (16). This concentration had been optimised by Sanz et al who demonstrated that concentrations corresponding to 10 times the IC\textsubscript{50} warranted maximal in vitro killing activity irrespective of the mode of action. As such, should delayed parasite death effect be observed, it would be associated with the timing of action and killing kinetics of the drug and not due to sub-optimal drug exposure. Optimization and validation of the assay was first achieved with 4 established antimalarials that exhibited distinctive killing rate profiles based on double CFDA-SE and Hoechst positive population (Fig. S2). The double positive population would correlate with viable parasites that were able to invade fresh uninfected CFDA-SE labelled erythrocytes. The killing rate profiles obtained were in agreement with the pharmacokinetics studies as atovaquone (ATV) and PYR have been known to exert their inhibitory effects slower than CQ and ART; the latter which are rapid-acting antimalarials (17). ATV collapses the mitochondrial membrane potential by
binding to parasite cytochrome \( b \) complex and despite its potent interference in parasite proliferation, it displays a delayed death phenotype where considerable decline in parasitemia could only be detected beyond the 72\textsuperscript{nd} hour of the \textit{P. falciparum} life cycle (18-20). In order to investigate parasite killing kinetics, flow cytometric analysis was repeated up to the 72\textsuperscript{nd} hour timepoint for dynamic coverage of the parasitic life cycle. Of the three non cross-resistant hits, MMV085071 demonstrated to be a fast-acting parasiticidal agent which lowered the parasitemia to 30\% within the first 24 hour while MMV687812 and MMV676380 displayed notable inhibitory action only after the 24\textsuperscript{th} to 48\textsuperscript{th} hour timepoint (Fig. 6).

**Dose-response assays on ART-sensitive and resistant strains.** Thus far, MMV676380, MMV085071 and MMV687812 have demonstrated to be DV-disruptive and effective across CQ-resistant and sensitive strains. In particular, MMV085071 had also shown to be a fast-acting compound that targeted the parasite growth within the first 24 hour of the life cycle. Due to artemisinin resistance from delayed parasite clearance associated with single nucleotide mutation of the parasite’s kelch 13 protein (21), it is pertinent to evaluate their applicability and efficacy on ART-sensitive and resistant strains. IPC 5202 is an ART-resistant strain with R539T polymorphism in the kelch 13 propeller domain. Isolated in 2011 from a Western Cambodia malaria patient, it showed delayed clearance and 40 to 49\% survival in \textit{in vitro} artemisinin-based susceptibility tests (22). Cam3.1\_rev is a revertant of IPC 5202 that is susceptible to ART. 48-hour re-invasion assay was performed and the 3 compounds demonstrated comparable IC\textsubscript{50} potencies with non-significant \( p \)-values regardless of delayed parasite clearance status (Fig. 7); thereby suggesting the lack of cross-resistance to ART. On the contrary, resistant strain IPC 5202 required an 8-fold increase in ART concentration for half maximal inhibitory effect as compared to sensitive strain Cam3.1\_rev.
DISCUSSION

Drug discovery campaigns often rely on 2 different approaches; target-based and phenotypic screening. While well-defined mechanisms of action may be elucidated and molecular hypotheses could be investigated through target-based screening, it also contributes to high attrition rates and low effectiveness in the subsequent drug development pipeline as specific molecular pathways and interactions increase in complexities in relation to disease pathogenesis and therapy (23). Phenotype-driven screenings, on the other hand, pose challenges in the optimization of drug candidates due to the lack of limited mechanistic. Yet first-in-class drugs with novel molecular entities and scaffolds could be developed due to the diverse screening strategy harnessed through a phenotype-centric approach. In the current study, we adopt a target- (DV-) based phenotypic screen, which leverages on the strengths of both approaches. As such, in the first-pass screening, the MMV Pathogen Box library was phenotypically screened for DV-permeabilization, MOMP and DNA degradation at 10µM (Fig. 1 and 2). Even though PCD in *P. falciparum* had been a contentious topic, growing evidence had substantiated the existence of apoptotic features during both the intra-erythrocytic (24-26) and the mosquito stage development (27, 28). Our work presents a robust combination of target-based phenotypic screening alongside downstream validation using MOMP and DNA degradation assays in accordance to the hallmark characteristics of mammalian apoptosis. Altogether, these findings corroborated with the emerging indication that mitochondrial and DNA degradation PCD phenotypes – incidental or genetically encoded – can be readily observed in *P. falciparum* through physiological stress, drug treatment and external stimuli.

MMV Pathogen Box contains 400 novel drug-like compounds active against a wide range of infectious tropical diseases and pathogenic helminths. With a hit rate of 2.5% (10/400), 6 of them were from the malaria disease cluster set while the remaining 4 were from the
tuberculosis disease set (Table 1). As these 4 hits exhibited activity against both *Mycobacterium tuberculosis* and *P. falciparum*, it is plausible that they exert overlapping mechanisms of action. Common enzymatic complexes of the type II fatty acid biosynthetic and/or the isoprenoid biosynthetic pathway(s) in both pathogens suggest possible putative drug targets. This notion is supported by emerging reports on the antimalarial and antimycobacterial activities of antibiotics inhibiting FabI and FabH enzymes of the type II fatty acid synthesis (FAS) pathway which is common in both microorganisms (29-32). Since the type II FAS pathway is the only means of *de novo* fatty acid biosynthesis in the malaria parasites (33), possible antimalarial mechanism of the 4 hits from the tuberculosis disease set may involve the common FAS pathway. This preliminary screen could henceforth promote the understanding of the pathogenesis of two deadliest infectious diseases. From the supporting information of MMV dataset, all of the 10 hits have cLogP values ranging from 1.9 to 3.4 and pKa values from 7.0 to 11.4. A high-content screen recently found that compounds with cLogP above 2 and a basic pKa from 6.5 to 11.0 are able to diffuse into lysosomes readily (34). These physiochemical properties are in agreement with the lysosomal accumulation of chemotherapeutics such as CQ and quinacrine due to their weak basicity and lipophilicity. Various anticancer drugs and tricyclic antidepressants with weak basicity and lipophilicity have also shown to exert their therapeutic effects through lysosomal sequestration (35). In light of these, it can be postulated that the panel of hits that permeabilized the parasite’s DV membrane, by this means resulting in downstream release of calcium ions and apoptotic-like cellular phenotypes.

Prior literature associates *P. falciparum* CQ resistance with *pfcrt* gene mutations which mitigate CQ’s antimalarial inhibitory effect on hemozoin biocrystallization by mediating its efflux from the DV (36). Consequently, it will be clinically relevant to discriminate novel hits which primary mode of action do not rely upon the parasite’s HDP. As β-hematin is
chemically and structurally equivalent to hemozoin, an optimised de novo β-hematin assay was adopted (37) distinguishing MMV676380, MMV085071 and MMV687812 as non “CQ-like” hits from the array of 10 hits. These three hits inhibited β-hematin formation to a significantly lower extent compared to CQ (Fig. 3) and in vitro resistance indices obtained via growth inhibition IC₅₀ assays (Table 2) supported such an observation. While K1 required a significantly greater dosage of MMV687812 than 3D7, (Fig. 4) MMV687812 remained as a promising hit relative to the resistance index exhibited by CQ. Furthermore, in vitro assays and sequencing analyses have identified a minimum threshold of 10-fold difference in IC₅₀ between sensitive and multidrug resistant *P. falciparum* strains to be a robust cut-off against false-positive resistance indication (38). With a resistance index of 2.58, MMV687812 thus continued to be categorized as non “CQ-like” together with MMV676380 and MMV085071. Overall, IC₅₀ values at the low micromolar and nanomolar range in 3D7, K1, IPC 5202 and Cam3.1_rev strains affirmed their antimalarial abilities even though the efficacies were lower relative to established antimalarials CQ and ART (Fig. 7), indicating the lack of in vitro cross-resistance.

Rapid-acting drugs are likely to eliminate circulating parasites faster and avoids recrudescence and resistance development from sub-optimal exposure to drug. *In vitro* killing rates revealed, unsurprisingly, that there was no association between antimalarial potency as indicated by IC₅₀ and killing rate given that compound-specific lag phase is independent of dose potency (39). Metabolism based assays often have limitations in that drugs such as ciprofloxacin, clindamycin and azithromycin have been reported to display a delayed death effect (40) and metabolic proxies such as parasite lactate dehydrogenase (pLDH) or histidine-rich protein 2 (HRP-2) as markers of parasite viability therefore do not always truly reflect the killing rates. For instance, bioconversion studies on a choline analog T3/SAR97276 developed by Sanofi-Aventis showed rapid metabolism of prodrug within 2 hours (41), but
data from metabolic based assays on pLDH and HRP-2 failed to correlate with the analog’s sub-nanomolar IC$_{50}$ efficacy and in vivo rapid onset of parasite clearance observed (42).

Parasite viability following intra-erythrocytic reinvasion capacity coupled with time point-specific drug removal is thus a more suitable read-out. By the 72$^{nd}$ hour of investigation, MMV085071 was able to reach low parasitemia levels similar to that of CQ and ART; thereby suggesting that even though the lag phase (time taken for a compound to exert its outcome of maximal parasite killing) was longer, MMV085071 could achieve an overall efficacious abrogation of *P. falciparum* growth (Fig. 6). Conversely, MMV676380, MMV687812, PYR and ATV were slower-acting where marked reduction in parasitemia could only be achieved after the first parasite cycle. In light of this, parasite recovery from the lack of complete clearance could be expected should the duration of investigation be extended over 2 developmental cycles when drug concentration falls below desired levels.

Nonetheless, moderate or slower acting drugs such as azithromycin had shown in vitro and in vivo synergistic efficacy with ART or quinine (43), while CQ treatment supplemented with doxycycline demonstrated significance in improving parasite clearance in patients (44). Since artemisinin resistance status is attributed to delayed clearance, therapeutically potent yet slow-acting drugs could be administered in combination to prevent recrudescence once the rapid parasiticidal action of artemisinin had worn off. Ring-stage assays with tightly synchronized ART-resistant parasites (45) could be performed with artemisinin-based compounds to better assess the potentiating effects of MMV676380 and MMV687812 against artemisinin resistance and reinvasion capacity; as implied by an enhanced susceptibility when pulsed in dose-specific combinations with ART or dihydroartemisinin.

According to compound report cards deposited on chEMBL (Neglected Tropical Disease archive), non cross-resistant hits MMV085071, MMV676380 and MMV687812 do not contain structural alerts nor any violations to Lipinski’s rule-of-5 – both of which are
important considerations to assess “drug-likeness” of uncharacterized compounds. The HepG2 CC$_{50}$ of MMV687812, however, was reported to be 3.9µM while the IC$_{50}$s against $P. falciparum$ 3D7 and K1 were in the high nanomolar to low micromolar range (Table 2 and Fig. S1). With an overall therapeutic index of 6.2, this may indicate inherent cytotoxicity and weak selectivity. MMV676380 offers an improved selectivity with a therapeutic index over 50. Notably, MMV085071 proved to be a top lead compound from the series of studies undertaken as the work here had revealed its rapid-acting parasticidal kinetics, comparable potency in PCD-like induction relative to CQ and its low nanomolar efficacy in 3D7 (IC$_{50}$ 76nM) and K1 (IC$_{50}$ 125nM). With therapeutic index over 125, it is reasonable to speculate a satisfactory safety profile. Surprisingly, a higher inhibitory dosage was observed for field isolates (Fig. 7) compared to laboratory strains (Fig. 4).

MMV085071 is categorized in the Malaria disease cluster set, potent against 3D7 and W2 (CQ-resistant) parasites likewise reported by Novartis (PubChem GNF-Pf-3843). Structurally, MMV085071, also known as 2-(5-methoxy-3-pyridinyl)-6-[4-(4-pyridinyl)-1-piperazinyl]pyrazine, contains pyridine, piperizine and pyrazine drug-like chemical moieties which are also basic heterocyclic amines. Pyrazine rings have long been involved in a range of antimycobacterial (in the form of pyrazinamide), anticancer and insecticidal effects (46).

Novel analogues with pyrazine substitutes were proposed to inhibit protein kinases while modulating cancer cells’ sensitivity towards apoptosis and anticancer chemotherapy (47).

Piperazine rings are popular constituents of pharmaceutical agents and specifically, pyridinylpiperazine and pyrimidinylpiperazine pharmacophores similar to that of MMV085071 are commonly recommended as medication for neurological disorders such as depression (mirtazapine) and Parkinson’s disease (piribedil). These chemical scaffolds also demonstrated anticancer and antiretroviral characteristics against Bcr-Abl tyrosine kinase and HIV respectively (48, 49).
Tracking along the database details in PubChem and chEMBL, MMV085071/GNF-Pf-3843 was identified from a high-throughput screen (1.7 million compounds) of the Novartis compound library in 2008. Substitution of the pyridine ring with an alkyl amine group in MMV085071/GNF-Pf-3843 gave rise to another compound GNF-Pf-1610, which showed a significant reduction of potency against 3D7 and CQ-resistant strain W2. More than 7-fold increase of dosage was required against both 3D7 and W2 parasites (50). Accordingly, this revealed preliminary insights of the SAR of MMV085071/GNF-Pf-3843, suggesting that the pyridine moiety is essential for the parasiticidal activity. The identification and characterization of MMV085071 thus presents an encouraging premise as a potential novel candidate for downstream hit-to-lead optimization.

In summary, the MMV Pathogen Box was subjected to a robust, high-content phenotypic screen for DV permeabilization followed by flow cytometric analysis of MOMP and DNA degradation. From here, 10 hits were subjected to further characterization studies. To narrow down the panel of PCD-like inducing hits, dose-response experiments on PCD phenotypes, CQ and ART resistance were performed and MMV676380, MMV085071 and MMV687812 were active in CQ-resistant K1 strain and ART-resistant IPC 5202 strain. MMV085071, however, exhibited the lowest IC₅₀ and had comparable efficacy to CQ in the induction of PCD-like phenotypes in *P. falciparum*. While its precise mode of action is not yet elucidated, low micromolar concentrations of MMV085071 was able to trigger DV disruption, induce downstream PCD-like features and parasite cell death within a short incubation time of 4 hours, suggesting that its mode of action is via a PCD-like mechanism. Additional studies on killing kinetics had uncovered that it was a fast-acting compound, further reinforcing it as a novel drug candidate against human malaria. Quantitative SAR analysis and hit-to-lead optimization of MMV085071 through analogues can be conducted, thus providing a strong basis for in depth mechanistic studies and drug development.
MATERIALS AND METHODS

*In vitro* culturing and synchronization of *P. falciparum* parasites. Laboratory CQ-sensitive strain 3D7 (MRA-102, MR4, ATCC, Manassas, Va, USA), CQ-resistant strain K1 (MRA-159, MR4, ATCC), field isolates artemisinin-sensitive strain Cam3.1_rev (MRA-1240, MR4, ATCC, contributed by David A. Fidock) and artemisinin-resistant strain IPC 5202 (MRA-1252, MR4, ATCC, contributed by Didier Ménard) were cultured and maintained in 25cm² or 75cm² non-vented cell culture flasks at 1.25% hematocrit and complete malaria culture medium (MCM) comprising of a filtered sterilized homogenous mixture of RPMI 1640 (Life Technologies, USA), 2.2g/l sodium bicarbonate, 0.5% w/v Albumax II (Gibco, Thermo Fisher Scientific, Massachusetts, USA), 0.005% w/v hypoxanthine (Sigma-Aldrich, Missouri, USA), 0.03% w/v L-glutamine (Sigma-Aldrich, Missouri, USA) and 25μg/l gentamycin (Gibco, Thermo Fisher Scientific, Massachusetts, USA). pH was adjusted to 7.4 using 1N NaOH. O+ human erythrocytes in CPDA-1 were obtained from the Interstate Blood Bank (Memphis, Tennessee, USA) and were tested negative for infectious agents by the FDA. The flasks were incubated at 37°C without light and gassed with 5% CO₂, 3% O₂ and 92% N₂. Routine PCR-based mycoplasma testing was conducted on a monthly basis to ensure the lack of contamination in cultures. Parasites were synchronized with 5% w/v sorbitol (Merck, Darmstadt, Germany) at 37°C for 10 min for the selection of ring-stage parasites, following which the cells were washed twice and resuspended to 1.25% hematocrit in MCM.

Drug preparation and MMV Pathogen Box library storage. CQ, pyrimethamine (PYR), atovaquone (ATV), artesunate (ART) and mefloquine (MQ) were all purchased from Sigma-Aldrich. CQ was dissolved in sterile 1× phosphate-buffered saline (PBS) while the remaining drugs were dissolved in sterile dimethyl sulfoxide (DMSO) to stock aliquots of 10mM, frozen at -20°C and protected from light. Fresh working drug concentrations were prepared after dilution with sterile PBS or cold MCM to ensure final assay concentration containing less
than 0.5% DMSO. MMV Pathogen Box was received from both Singapore University of Technology and Design, Singapore and MMV (Medicines for Malaria Venture, Switzerland) in Costar® 96-well round-bottom microtiter plates. Upon receipt, each plate was sealed with 96-well X-pierce™ adhesive film (Sigma-Aldrich, Missouri, USA) and all wells received in 10µl of 10mM were reconstituted with DMSO to a final stock concentration of 100µl at 1mM according to MMV’s instructions. Similarly, the plates were stored in -20°C and kept protected from light.

Parasite drug treatment and staining. 3D7 parasites were synchronized 2 cycles prior to the start of the assay for high parasitemia of over 10% consisting of mid-trophozoites (28 to 30 hour post invasion) and resuspended to 2.5% hematocrit in a 96-well flat-bottom plate. After drug treatment and a 37°C incubation with mixed gas in a humidified hypoxic chamber for 4 hours (for DV disruption experiments), cells were transferred to a 96-well v-bottom plate to concentrate the pellet and stained with 1µM Fluo-4 AM (Thermo Fisher Scientific, Massachusetts, USA) and 1µg/ml Hoechst 33342 (Sigma-Aldrich, Missouri, USA). Following incubation in the dark for 30 mins, cells were washed thrice with pre-warmed MCM and resuspended in 100µl of HyClone™ PBS (GE Healthcare, Chicago, USA) and sealed with X-pierce™ adhesive film to prevent evaporation of wells during data acquisition. For PCD-like validation assays on parasites’ mitochondria and DNA integrity, the trophozoites were incubated for 10 hours in the same atmospheric conditions and stained with 6µM JC-1 (Thermo Fisher Scientific, Massachusetts, USA) and 0.8µg/ml Hoechst 33342, subjected to the same washing steps.

DV permeabilization screen with ImageStream X MkII (Amnis, Darmstadt, Germany). ImageStream X MkII is a flow cytometer designed with imaging properties for high-content single-cell analysis. In addition to the analysis of cells populations based on fluorescence, brightfield and 2-D fluorescent images of each event could be captured and visualized.
Leveraging on its cell imaging capabilities, a high-content primary screen was developed and optimized (51) to detect and quantitatively assay for the increase in the area of Fluo-4 fluorescence as a consequence of drug-mediated DV disruption and Ca\(^{2+}\) redistribution from the DV to the parasite cytosol. Using a 60× objective and EDF element, each 96-well plate was assayed with the autosampler plate reader for high throughput data acquisition. Hoechst 33342 is a lipophilic DNA-binding fluorescent stain that was excited by the 375nm UV laser at 10mW, while Fluo-4 AM is a cell permeable calcium sensing dye which was excited by the argon ion 488nm laser at 200mW. Stringent gating was applied to select for focused, round Hoechst 33342 positive singlet parasites during data acquisition based on the area and aspect ratio of the cells in the brightfield channel and the fluorescence intensity of Hoechst 33342. Having acquired 2000 parasite events, data analysis was performed with the IDEAS Software (version 6.1). The gating strategy is elucidated in Fig.1. All of the acquired events were gated for focused cells on a gradient RMS (root mean square for image sharpness) histogram, followed by a scatter plot of aspect ratio against area for brightfield images (Fig. 1a). This ensures that the parasite images and events analyzed are sharp and focused singlets. Gating for trophozoites was based on fluorescence intensity and visual inspection of captured images. Single trophozoites which have matured to the schizont stage during the 4-hour duration of data acquisition would display multiple punctate forms of Hoechst-stained DNA-containing merozoites based on Hoechst intensity and as verified with visual images. Since schizonts mature with a larger DV during the parasite life cycle, they were not included in the analysis. Once applied, the gating for trophozoites was maintained in all subsequent analysis. Untreated drug-free PBS controls were included in all experimental runs and healthy PBS-treated parasites displayed intact DV with little Fluo-4 redistribution as Ca\(^{2+}\) remained sequestered in the DV (Fig. 1b). To quantify single-cell DV disruption, an area feature mask incorporated in the IDEAS Software was applied to measure for the increase in Fluo-4 fluorescence.
fluorescence area upon binding to the effluxed intravacuolar Ca\(^{2+}\) (Fig. 1c). Raw feature values were normalized and degree of DV disruption was compared relative to CQ. Single stain controls (Hoechst 33342 and Fluo-4 AM) were prepared fresh for every run of the experiment and compensation matrix was generated using the compensation algorithm of the software.

**PCD-like validation assays on MOMP and loss of DNA content.** The ratiometric JC-1 dye is dependent on the mitochondrial membrane potential and was used for the assessment of mitochondrial depolarization by flow cytometry. The shift of fluorescence emission from JC-1 green monomers (529nm) to red aggregates (590nm) is indicative of high membrane potential in healthy functional mitochondria while mitochondrial degradation is denoted by reduction in percentage of JC-1 red to JC-1 green. Using the Attune NxT flow cytometer (Invitrogen, California, USA), 405nm violet excitation laser fitted with 417LP 440/50BP filter was used to excite and detect Hoechst 33342 while blue excitation laser (Alexa Fluor® 488nm) fitted with 503LP 530/30BP filter and yellow excitation laser (561nm) fitted with 577LP 585/16BP filter were used to excite and detect JC-1. At least 100,000 events were acquired from each sample. Gating for sub-G1 DNA peaks as an indication of DNA degradation was determined relative to the untreated Hoechst positive controls.

**Spectrophotometric determination for inhibition of β-hematin formation.** To mature and proliferate, *P. falciparum* biocrystallizes toxic heme by-products into hemozoin crystals through the parasite’s heme detoxification pathway (HDP) during hemoglobin degradation. CQ had been widely proposed to inhibit parasite growth by upsetting the biocrystallization process. β-hematin had been established to be the chemical equivalent of hemozoin (52). As this study was interested in the discovery of novel potent antimalarials, the hits were subjected to a preliminary assessment of possible CQ cross-resistance based on β-hematin inhibition. To characterize the 10 hits, CQ was included as a control for a “CQ-like”
inhibitory mechanism of the HDP. PYR served as a non “CQ-like” mechanistic control because its mechanism of action primarily disrupts folate and DNA synthesis (53). In accordance to the rationale, a spectrophotometric assay was performed to select for hits which did not inhibit β-hematin formation. Nonidet P-40 (Roche, Basel, Switzerland) was used as a biological mimic of parasite lipid factors to catalyze the formation of β-hematin from hemin (54). Drug-free controls were treated with PBS while sample tubes were treated with compounds of interest (CQ, PYR and the 10 hits) at a final concentration of 20μM. Final reaction mixtures were maintained at 400μl with bovine hemin (Sigma-Aldrich, Missouri, USA) at 1mM, 30.5μM Nonidet P-40 in acetate buffer at pH 5.0 akin to the acidic pH of parasite DV (55). Reagents used for sodium acetate buffer (100mM, pH 5.0), Tris-HCl buffer (100mM, pH 8.0) with 2.5% w/v SDS and sodium bicarbonate buffer (100mM, pH 9.2) preparation were purchased from Sigma-Aldrich. After 14-hour incubation at 37°C and 195rpm, the tubes were centrifuged at 32000g for 10 mins at 4°C and resuspended with pre-warmed Tris-HCl buffer containing 2.5% w/v SDS for 30 mins at 37°C to remove unbound proteins, followed by pre-warmed alkaline sodium bicarbonate buffer to solubilize heme aggregates and precipitate β-hematin (37). The final β-hematin pellet was washed with distilled water and resuspended in 100μl 1N NaOH and 900μl 2/5% w/v SDS. The samples were then read with Tecan Infinite 200 PRO microplate reader at 405nm for detection of β-hematin. Relative absorbance readings were normalized to background (PBS-treated drug-free controls). Based on this spectrophotometric assay, CQ and hits which inhibited β-hematin formation would have lower absorbance readings while PYR and hits which did not inhibit β-hematin formation exhibited higher absorbance values.

**48-hour reinvasion assay for half maximal inhibitory concentration (IC$_{50}$) determination.**

Ring-stage parasites synchronized 1 cycle prior to the start of assay were incubated for 48 hours at 1.25% hematocrit and 0.7% parasitemia (for 3D7) or 1% parasitemia (for K1,
Cam3.I rev and IPC 5202) with a 11-point serial dilution of reference antimalarial drugs and compounds of interest. Parasite labelling was carried out with 1\(\mu\)g/ml Hoechst 33342 for 30 mins at 37°C and at least 100,000 events were collected by Attune Nxt flow cytometer as elaborated. IC\(_{50}\)s were then determined by GraphPad Prism 5 using a variable-slope logistic curve. Values were obtained from at least 3 separate triplicates.

**Parasite killing kinetics of hits on P. falciparum.** To determine if the compounds of interest were rapid or slower-acting, parasites were treated with the drugs of interest at concentrations that were 10x that of the IC\(_{50}\)s pre-determined prior to this assay (Table S1). This concentration ensured that any observed delayed killing effect was not due to sub-optimal exposure of the parasites to the drug treatment, but was attributed to the slower killing kinetics of the drug against *P. falciparum*. From the point of drug treatment (time 0), parasites were harvested at 24h, 48h and 72h. During parasite harvesting, drugs were washed off with MCM for at least 3 times. In this assay, parasites were treated with reference antimalarial drugs (CQ and ART for rapid-acting, PYR and ATV for slower-acting) and Pathogen Box hits (MMV676380, MMV085071 and MMV687812) at 5% parasitemia, 0.5% hematocrit at conditions similar to the IC\(_{50}\) assay. After harvesting and drug removal, 10\(\mu\)M of Vybrant® CFDA-SE (Thermo Fisher Scientific, Massachusetts, USA) pre-labelled erythrocytes at 2.5% hematocrit were added to the parasites and further incubated under the same conditions for an additional parasite cycle (48 hours) to allow for invasion into the labelled erythrocytes. Parasitemia in each well was determined with Hoechst 33342 labelling and acquired using Attune Nxt flow cytometer. Parasite viability and reinvasion capacity at all timepoints was indicated by double positive population (CFDA-SE and Hoechst 33342) and normalized to drug-free PBS controls at time 0. At least 3 separate experiments were performed at all timepoints (0h, 24h, 48h and 72h).
Statistical tests and analyses. Graphs, statistical tests and $p$ values were obtained with Graphpad Prism 5.0 two-sample Student’s $t$-tests. Error bars represent SEM from at least 3 separate independent experiments.
Acknowledgements. The authors are indebted to Medicines for Malaria Venture (MMV, Switzerland) and Rajesh Chandramohanadas of Singapore University of Technology and Design (Singapore) for providing the MMV Pathogen Box chemical library. Parasite strains were provided by Malaria Research and Reference Reagent Resource Center (MR4). K. SW. T. and J. X. T. would like to thank the Yong Loo Lin School of Medicine of National University of Singapore, and the National Medical Research Council, Singapore (NMRC/1310/2011 and NMRC/EDG/1038/2011) for support and grants.
REFERENCES


24


Fig. 1 High-content screening assay of *P. falciparum* 3D7 for DV permeabilization. a) Selection of focused and circular single cells based on gradient RMS, aspect ratio and area of cells in the brightfield channel. Representative fluorochrome plots of Fluo-4 against Hoechst 33342 and brightfield images were obtained in b) PBS-treated drug-free controls with intact DV of the trophozoite population and in treatment with c) 3µM of CQ displaying a redistribution of Fluo-4 fluorescence due to DV being breached. Ch01 and Ch02 correspond to fluorescence by DNA-binding dye Hoechst 33342 and Fluo-4 respectively while Ch03 corresponds to the brightfield channel. d) Histogram overlay showing the increase in area of Fluo-4 fluorescence when parasites were treated with CQ (blue) as compared to PBS (orange). e) Consolidated screening results of the Pathogen Box compound repository at 10µM. After normalization to the background (PBS), mean Fluo-4 fluorescence area was compared relative to CQ (positive control). Cut-off for hit selection was set at 70% relative to 3µM of CQ and the novel hits were subjected to analysis and further characterization. f) Identification of the 10 hits, which displayed DV disruption. 10µM mefloquine (MQ) was included as an internal negative control as it does not breach the parasite DV (56). 1% DMSO was included in the screening assays as the final concentration of screening compounds contained 1% DMSO diluted from 1mM to 10µM. Error bars are SEM obtained after performing at least 3 independent experiments. ***, *P* < 0.001, **, *P* < 0.01 and *, *P* < 0.05 compared to vehicle control (1% DMSO).
Table 1 Chemical structures, compound identification and disease set classification of the 10 hits acquired from whole-cell high-content screen of DV permeabilization. 4 of them are from the tuberculosis disease cluster as they were first assayed against *Mycobacterium tuberculosis* during the curation of MMV Pathogen Box while 6 of them were assayed against *P. falciparum*.

Fig. 2 Assessment of PCD-like features in malaria parasites at 10µM. Following DV disruption, 3D7 parasites were treated with the hits for 10 hours and assayed for a) mitochondrial depolarization normalized to drug-free control (PBS), and b) percentage of parasites with sub-G1 DNA content via flow cytometry. As shown from low JC-1 red:green ratio and increased percentage of parasites with sub-G1 DNA peaks, all the hits induced downstream cell death features after having disrupted the parasite’s DV. As previously reported (56), MQ was observed to induce a high percentage of sub-G1 DNA positive parasites but not mitochondrial dysfunction at 10µM. ***, *P* < 0.001, **, *P* < 0.01 and *, *P* < 0.05 compared to drug-free PBS control.

Fig. 3 Relative β-hematin formation using hemin and NP-40 detergent with 20µM of antimalarial controls and hits obtained from Fig. 1f. CQ served as the control for inhibition of β-hematin formation and PYR, an antifolate drug which does not target the parasite’s heme detoxification pathway (HDP), served as the mechanistic control of a non “CQ-like” feature. 3 compounds MMV676380, MMV085071 and MMV687812 were shown to be non “CQ-like”. Error bars represent SEM for at least 3 separate experiments. **, *P* < 0.01 and *, *P* < 0.05 compared to CQ.
Fig. 4 Phenotypic validation of non CQ cross-resistant hits’ antimalarial activities and potencies in CQ-sensitive (3D7) and CQ-resistant (K1) strains with in vitro sensitivity IC$_{50}$ evaluation. Error bars represent SEM for at least 3 separate experiments. ***, $P < 0.001$ between CQ-treated 3D7 and K1 parasites, while the IC$_{50}$s obtained for MMV676380 ($P = 0.235$) and MMV085071 ($P = 0.096$) were not significantly different between 3D7 and K1. * $P < 0.05$ indicated slight cross-resistance ($P = 0.016$) for MMV687812.

Table 2 Antimalarial efficacy of hits and determination of resistance index. Resistance index is expressed as the ratio of K1 IC$_{50}$ to 3D7 IC$_{50}$ (Fig. 4). The therapeutic indices of Pathogen Box compounds are expressed as the ratio of HepG2 CC$_{50}$ to 3D7 IC$_{50}$. HepG2 CC$_{50}$ was obtained from the Pathogen Box data sheet provided by MMV. The therapeutic index of CQ is expressed as the ratio of published HepG2 CC$_{50}$ (57) to 3D7 IC$_{50}$. Table shows mean ± SEM of at least 3 independent experiments.

Fig. 5 Dose-dependent assessment of PCD-like phenotypes. MMV676380, MMV085071 and MMV687812 were serially diluted from $10\mu$M while CQ was diluted from $3\mu$M and (a) area of Fluo-4 fluorescence, (b) JC-1 red:green ratio and (c) percentage of parasites with sub-G1 DNA content were normalized against PBS controls and plotted accordingly as shown. Error bars represent SEM for 3 separate experiments.

Table 3 Dosage required for 70% maximal DV disruption, MOMP and fragmentation of DNA. EC$_{70}$ was calculated for the evaluation of potency in inducing PCD-like phenotypes as a 70% cut-off was applied for the selection of hits during high-content primary screen (Fig. 1e).
Fig. 6 The timing of action of three non “CQ-like” hits and four reference antimalarials represented by consolidated profiles of parasite killing kinetics over 72 hours. Error bars represent SEM for 3-6 separate experiments.

Fig. 7 Evaluation of antimalarial potency on ART-sensitive (Cam3.1_rev) and resistant (IPC 5202) strains. ***, $P < 0.001$ between ART-treated Cam3.1_rev and IPC 5202 parasites, while IC$_{50}$s of MMV676380 ($P = 0.113$), MMV085071 ($P = 0.490$) and MMV687812 ($P = 0.117$) were not significantly different between the two strains.
Fig. 1e

Primary Screen of all 400 Compounds

Normalized Mean Fluorescence Area / %
Fig. 1f

DV-disrupting Capacity of Hits

Normalized Mean Fluorescence Area / %
Fig. 2b

Parasites with Sub-G1 DNA Content / %

DNA Degradation

MMV006901
MMV021375
MMV032967
MMV085071
MMV661713
MMV676380
MMV687248
MMV687749
MMV687812
Fig. 3

Relative β-hematin Biocrystallization

Normalized OD 405
Fig. 4

Half Maximal Inhibitory Concentration (IC$_{50}$) / nM

CQ
MMV676380
MMV085071
MMV687812

0
500
1000
1500
3D7
K1
***
nS
ns
*

Half Maximal Inhibitory Concentration (IC$_{50}$) / nM

CQ
MMV676380
MMV085071
MMV687812

0
500
1000
1500
3D7
K1
***
nS
ns
*
Dose-dependent DV Disruption Potency

Fig. 5a

-1 0 1 2 3 4 5
20
40
60
80
100
120
CQ
MMV676380
MMV085071
MMV687812

Normalized Area of Fluorescence / %

Log Concentration / nM
Dose-dependent Mitochondrial Depolarization

- CQ
- MMV676380
- MMV085071
- MMV687812

Normalized JC-1 red:JC-1 green / %
Log Concentration / nM

Fig. 5b
Fig. 5c
Dose-dependent DNA Degradation

-1 0 1 2 3 4 5
50
100
150
CQ
MMV676380
MMV085071
MMV687812

Log Concentration / nM
Normalized % of Parasites with Sub-G1 Content

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Fig. 6

Killing Rate Profile of Hits

Normalized Parasitemia / %

Timepoint

ART
CQ
MMV085071
MMV676380
MMV687812
PYR
ATV
Half Maximal Inhibitory Concentration (IC$_{50}$) / nM
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## Table 2

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<td>MMV687812</td>
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<td>1317 (1216 - 1428)</td>
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Table 3

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