Whole Cell Phenotypic Screening Of MMV Pathogen Box identifies Specific Inhibitors of *Plasmodium falciparum* merozoite maturation and egress.

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Abstract

We report a systematic, cellular phenotype-based antimalarial screening of the MMV Pathogen Box collection, which facilitated the identification of specific blockers of late stage intraerythrocytic *Plasmodium falciparum* maturation. First, from standard growth inhibition assays, we discovered 62 additional antimalarials ($EC_{50} \leq 10\mu\text{M}$) over previously known antimalarial candidates from Pathogen Box. A total of 90 potent molecules ($EC_{50} \leq 1\mu\text{M}$) were selected for evaluating their stage-specific effects during the intra-erythrocytic development of *P. falciparum*. None of these molecules had significant effect on ring-trophozoite transition, 10 molecules inhibited trophozoite-schizont transition, and 21 molecules inhibited schizont-ring transition at $1\mu\text{M}$. These compounds were further validated in secondary assays by flow cytometry and microscopic imaging of treated cells to prioritize 12 molecules as potent and selective blockers of schizont-ring transition. Seven of these were found to strongly inhibit calcium ionophore induced egress of *Toxoplasma gondii*, a related apicomplexan parasite, suggesting that the inhibitors may be acting *via* similar mechanism in the two parasites, which can be further exploited for target identification studies. Two of these molecules, with previously unknown mechanism of action, MMV020670 and MMV026356, were found to induce fragmentation of DNA in developing merozoites. Further mechanistic studies would facilitate therapeutic exploitation of these molecules as broadly active inhibitors targeting development and egress of apicomplexan parasites relevant to human health.

Keywords – Phenotypic screening, *Plasmodium falciparum*, Medicines for Malaria Venture, Pathogen Box, stage-specific inhibition, Egress

Introduction

Despite technological advancements and improved knowledge on disease manifestation and pathogen biology, progress in therapeutic development has been slower than expected for human infectious diseases affecting the developing world. In addition to limited financial prospects, pharmaceutical companies remain under significant pressure to address high attrition rates. In this context, repurposing of old drugs is becoming a viable alternative through the exploitation of known compounds with established mechanisms thereby shortening the time window between efficacy testing to pharmacophore optimization¹⁻³ to eventual application. In recent efforts to speed up the process of drug development against various pathogenic diseases, a collaborative model of public-private partnerships (PPPs) facilitated through Medicines for Malaria Venture (MMV: mmv.org) was formed. This resulted in well-characterized drug-like small molecule libraries being made accessible to academics worldwide, for the purpose of identifying novel bioactivities against various tropical disease pathogens and conducting focused mechanistic studies. In 2015, the consortium launched the open access “Pathogen Box” consisting of 400 drug-like small molecules, included 26 reference compounds, with confirmed bioactivity against at least one of the neglected disease pathogens (*Plasmodium*, *Mycobacterium*, Kinetoplastids, *Schistosoma*, *Cryptosporidium*, helminths and dengue virus),⁴ as a way to facilitate open-ended drug discovery.

Open access to the Pathogen Box has led to the discovery of newly identified activities against various pathogens, for example, MMV688934 (Tolfenpyrad) showed activity against the helminth parasite and barber’s pole worm⁵ while MMV688768 was shown to demonstrate inhibitory activity against *Candida albicans* biofilm formation⁶. Previously, these molecules were established as inhibitors of kinetoplastid and schistosomiasis⁵⁻⁶ respectively. However, the latter molecule has been recently documented to have no activity against *S. mansoni*⁷. Dual activity against *Giardia* and *Cryptosporidium* were reported for MMV010576, MMV028694 and MMV676501 that showed $\geq 75\%$ inhibitory efficacy⁸. Several such studies have accelerated the identification of small molecules that are now re-profiled, repurposed and reclassified to show broader inhibitory activities on more than one pathogenic agent⁹⁻¹².

Plasmodium falciparum is the most virulent human malaria species and is widespread, accounting for 99.7% of estimated malaria cases in the African Region, 62.8% in South-East Asia, 69% in Eastern Mediterranean region and 71.9% in Western Pacific region¹³⁻¹⁵. Within the human host, *Plasmodium* undergoes vastly different developmental changes in liver cells and red blood cells. However, intra-erythrocytic (blood stage) development is most obvious during infection and is responsible for malaria-associated pathological outcomes. Over the past several decades, many attempts have been made to eradicate malaria but with limited success due to challenges in the form of drug resistance, changes in vector distribution and density, and improper regulatory framework in many malaria endemic areas. Malaria-endemic regions are rapidly losing affordable and effective therapeutic options, making it necessary to develop new drugs with novel modes of action and molecular targets.

We have exploited the MMV Pathogen Box to dissect the blood stage specific antimalarial activity of the molecules in this library. To document the stage-specific activity of MMV Pathogen Box molecules against *P. falciparum*, a phenotype-based screening approach was used. First, growth inhibition assays were carried out to assess the antimalarial potential of all 400 molecules in the library. This revealed the previously known antimalarial compounds as well as 62 additional novel antimalarial compounds from MMV Pathogen Box. Further evaluations on 90 most potent molecules ($EC_{50} \leq 1\mu M$) revealed their stage specific inhibitory effect during the course of blood stage *P. falciparum* development. This identified 10 molecules that inhibited trophozoite development and 21 molecules that inhibited schizont-ring transition. Based on drug-induced phenotypes, 12 of these molecules were found to selectively impair maturation and egress of merozoites. Interesting, 7 of these molecules were potent inhibitors of calcium ionophore induced egress of tachyzoite stage *Toxoplasma gondii*, suggesting a likely conserved mechanism of action across species. We demonstrate that two of these molecules affect DNA integrity in developing *Plasmodium* merozoites while the others appear to interfere directly with the egress process. These findings provide starting points for subsequent mechanistic studies on these molecules and further development of new therapies to treat malaria and toxoplasmosis.

Materials and Methods

MMV Pathogen Box Chemical Library: Stock solutions and Storage

The MMV Pathogen Box was received in 96-well plates containing 10µl of 10mM stock solution in 100% dimethyl sulfoxide (DMSO). The stocks were immediately diluted and aliquoted to 5 subsets with a final drug concentration of 1mM in DMSO (20µl of stock solution and 180µl of filtered DMSO), as recommended by MMV. These plates were stored at -80° C. Care was taken to avoid multiple freeze-thaw of the plates during the course of this study.

P. falciparum in vitro culturing and maintenance

Blood for culturing *P. falciparum* was purchased from Interstate Blood bank (USA). All experiments reported in this study used the 3D7 strain of *P. falciparum* and infected RBCs were maintained in sodium bicarbonate/Hepes-buffered RPMI-1640 culture medium (Sigma Aldrich, Singapore) at pH 7.4, supplemented with hypoxanthine and 2.5 mg/mL Albumax II (Gibco) ^[12]. Ring stage parasites were synchronized by treatment with 5% sorbitol at 37° C. Parasites used for the schizont-ring transition assays were enriched using MACs separation (Miltenyi Biotech, Bergisch Gladbach, Germany), as reported in prior work¹⁶. Parasitemia was scored using flow cytometry (Accuri C6 sampler, BD biosciences Singapore) after staining with Hoechst dye (Hoechst 33342, Thermo Fisher). In parallel, Giemsa-stained thin smears were microscopically examined to match with flow cytometry results and to record phenotypic characteristics arising from drug treatment, as established previously¹⁷⁻¹⁸.

EC₅₀ determination of Pathogen Box molecules against *P. falciparum*

Antimalarial efficacy screening was performed in 96 well plate format. The molecules were plated by 2-fold serial dilution, from 10 µM to 0.01 µM, and the parasite culture (200 µl per well) was maintained at 2.5 % hematocrit and ~ 2 % starting parasitemia. As positive controls, the antimalarial drugs atovaquone and/or chloroquine were used at 1 µM each, while 1 % DMSO treatment served as the negative control. Parasite growth inhibition was estimated after 60 h incubation of assay plates under optimal growth condition. For assay readout, 25 µl of staining reagent (10X SYBR Green dye and 0.5% Triton X-100 in phosphate buffered saline (PBS), pH 7.4) was mixed into each well of assay plate and fluorescence emitted by DNA bound dye was quantified, using excitation and emission wavelengths of 498 nm and 522 nm respectively, in a GloMax plate reader (Promega). Data processing and statistical analysis were performed using Microsoft Excel software.

In vitro assessment of stage-specific inhibition.

A total of 90 MMV Pathogen Box molecules with nanomolar potency ($EC_{50} \leq 1\mu M$), were chosen for testing their inhibitory effect on the development and progression of ring stage (6-8 hours post invasion (hpi)), trophozoite stage (22-24 hpi) and schizont stage (40-42 hpi) *P. falciparum*. The parasites were treated with the inhibitors for 12-14 h in each stage, following which the cultures were stained with Hoechst 33342 and analyzed by flow cytometry to estimate parasitemia and determine the parasite stage¹⁷. Briefly, 50 µl of culture was aliquoted from the master assay plates and fixed with

0.1 % glutaraldehyde in PBS at 4° C overnight. The cells were then washed once with PBS and permeabilized with 0.25 % Triton X-100 for 10 min at room temperature and washed twice with PBS before staining with Hoechst 33342. Flow cytometry was performed using the Accuri C6 instrument (BD Biosciences), and 100,000 events was recorded and analyzed for deriving parasitemia and parasite stage transition data. Statistical analysis of the data was performed using GraphPad Prism (GraphPad Software, Inc.)¹⁹.

Characterization of drug-induced phenotypes and rupture50 (R₅₀) determination

Following inhibitor exposure, thin blood smears were prepared on glass slides which were then fixed using 100% methanol and stained with 1:10 ratio filtered Giemsa (Merck Millipore) in distilled water. A standard phase-contrast light microscope (Leica DM750, Leica Microsystem) was utilized for visualising the phenotypic changes of the cell and image capturing. For R₅₀ determination, synchronized schizont stage parasites (~40 to 42 hpi) were treated with selected inhibitors at 10, 3, 1, 0.3 and 0.1 µM concentration for 12 h, at which point ring stage parasites can be observed in DMSO-treated control cultures. Blood smears were taken for staining with Giemsa at various time points. In parallel, cells were harvested for flow cytometry analysis for further validation. DMSO and E-64 (a broad spectrum cysteine protease inhibitor known to completely block *P. falciparum* egress were used as controls for all egress studies²⁰.

Measurement of Intracellular ROS.

Magnet purified schizont stage parasites (~42-44 hpi) were treated with selected Pathogen Box molecules at a concentration of 1 µM for 1 h. Following incubation, 5 µM CellROX® Green reagent (Thermo Fisher Scientific, Inc.) was added to the cells and incubated for 30 min in a 5% CO₂ incubator at 37° C. The cells were co-stained with Hoechst 33342 during the last 15 min of incubation in MCM at 37° C in dark. The cells were then washed with PBS three times and visualized using a fluorescent microscope (Olympus CKX53 inverted microscope).

Assessment of DNA damage using Comet Chip.

Alkaline Comet Chip assay was performed as described²¹⁻²². Briefly, schizont stage *P. falciparum* parasites were enriched on a percoll gradient and resuspended in complete RPMI at 0.5% v/v. Cell suspensions were incubated with different concentration of drugs or mock solution (0.05% DMSO) in 96-well plate for 1 h at 37 °C. Treated cells were transferred to Comet Chip, lysed overnight at 4°C in alkaline lysis buffer and electrophoresis at 4°C for 30 min at 1 V/cm and ~300 mA with alkaline electrophoresis buffer as previously described²¹. The chip was then washed twice at room temperature with neutralization buffer containing 0.4M Trizma® HCl (Sigma-Aldrich, USA). The Comet Chip was stained with 1X of SYBR™ Gold (Invitrogen, USA) for 30 min at room temperature in dark. Fluorescent images of the comets were captured at 40X magnification using epifluorescence microscopes, Olympus IX83, with a 480-nm excitation filter. Images were analysed using Guicometanalyzer, a customized software developed in MATLAB (The MathWorks Inc., USA) as previously described²². The median of comets in each well was calculated from results generated in Guicometanalyzer and converted into excel file using Comet to excel, a program developed in Python.

Egress inhibition studies in *Toxoplasma gondii*.

Tachyzoite stage RH strain *T. gondii* were cultured in the laboratory as previously reported¹⁸. When exposed to the calcium ionophore A23187, intracellular tachyzoites are known to rapidly rupture the parasitophorous vacuole and egress out of the host cells²³⁻²⁵. Here, the ionophore induced egress of *T. gondii* tachyzoites was performed after treating the parasites with selected inhibitors (at 10 μ M for 24 h) to assess their effect on the egress process. The time required for vacuole rupture and parasite egress was monitored using a 40X objective fitted to an inverted bright-field microscope (Primo Vert, Zeiss) for a period of 10 min and images were captured at 30 sec intervals.

Results and Discussion

Antimalarial activity profiling of MMV Pathogen Box Molecules.

To first identify all MMV Pathogen Box compounds that possess antimalarial activity, a standard end point growth inhibition assay was performed on blood-stage *P. falciparum* parasites. Sorbitol synchronized ring stage parasites were treated with all 400 Pathogen Box compounds at 10 μ M for 60 h and those which inhibited parasite growth by $\geq 80\%$ were selected for EC_{50} determination. In the dose assay, the compounds were tested at concentrations ranging from 10 μ M to 1 nM. Out of 400 Pathogen Box compounds, 162 molecules (~40% hit rate) were found to inhibit growth of blood stage *P. falciparum* parasites by $\geq 80\%$. Out of the 125 Pathogen Box compounds with previously reported antimalarial activity, only 100 showed $\geq 80\%$ inhibition of parasite growth (**Fig. 1A**), and the remaining 25 compounds had varying levels of inhibitory activity, presumably due to differences in the assay conditions¹⁰ or parasitic stages tested. A compilation of screening data is provided in the supplementary information (**Supplemental Dataset, S1**). Interestingly, 62 compounds, previously shown to be active against other pathogens, were also found to have potent antimalarial activity (**Fig. 1B**). For instance, anti-kinetoplastid molecules like MMV688362 and MMV688271 were found to inhibit *P. falciparum* growth with nanomolar potency ($EC_{50} \leq 0.4 \mu$ M). These molecules are predicted to bind to the minor groove at AT-rich regions of DNA in other organisms²⁶⁻²⁷, which may render them inhibitory to multiple pathogens. Furthermore, MMV652003 and MMV688283, which are benzamide 4-quinolinamine class of compounds and predicted to target leucyl-tRNA synthetase and β -hematin formation respectively²⁸⁻²⁹, were identified positively as antimalarial hits. Another compound, MMV675968, which has chemical features similar to inhibitors of dihydrofolate reductase (DHFR) in *Cryptosporidium* and *Toxoplasma*^{8, 11}, displayed potent antimalarial activity with an estimated EC_{50} of 0.07 μ M. Therefore, it is likely that this compound may be targeting the DHFR enzyme in *P. falciparum*³⁰. MMV671636, a Quinolinone class molecule with anti-filarial activity, showed very potent antimalarial activity with an estimated EC_{50} of 0.01 μ M. This compound is predicted to target mitochondrial cytochrome bc1 complex, making it a promising candidate for chemoprophylaxis treatment of malaria³¹⁻³². Compounds previously shown to be active against *Mycobacterium tuberculosis*, such as MMV021660 (guanidine) and MMV687765 (pyrimidine) with EC_{50} value of 0.07 and 0.47 μ M, were found to be inhibiting *P. falciparum*. Although, their mechanism of action against *P. falciparum* remains unknown, these molecules are known to disrupt the folate pathway and tyrosine kinase activities in *M. tuberculosis*³³⁻³⁴. Several other compounds such as MMV688943, MMV688371, MMV688274, MMV688754, MMV688273, MMV688550, MMV023969, MMV495543, MMV637229, MMV668727, MMV676063, MMV688761, MMV688763, MMV688552, MMV688509 belonging to kinetoplastids, tuberculosis, hookworm infections, filariasis, schistosomiasis, toxoplasmosis disease sets in Pathogen Box, showed submicromolar to micromolar efficacy against *P. falciparum* and warrant further consideration.

Segregation of Pathogen box molecules by stage-specific inhibition of *P. falciparum*.

Conventional growth inhibition assays on *P. falciparum* do not capture parasite stage specific effects of small molecule inhibitors. This remains a challenge since drug sensitivity could vary substantially

during the 48 h blood stage development lifecycle of *P. falciparum* and can be further influenced by metabolic fate of the drugs under prolonged *in vitro* conditions leading to alterations to the pharmacodynamic properties³⁵⁻³⁶. Therefore, to further categorize the Pathogen Box molecules by stage-specific inhibitory potential, the 90 most potent molecules ($EC_{50} \leq 1 \mu M$) were analyzed by phenotype screening. Progressive developmental stages of parasites - rings (6-10 hpi), trophozoites (20-22 hpi) and schizonts (40 to 42 hpi) - were treated with 1 μM inhibitor and assessed after ~12h treatment for their ability to block stage transition using flow cytometry based detection of the parasite stages (**Fig. 2A**). None of the molecules showed any significant inhibition of ring-trophozoite transition [R→T], which is in line with previous reports suggesting that ring stage parasites are rather resilient to antimalarial compounds³⁷⁻³⁸. 20 newly identified molecules with EC_{50} value $\leq 1 \mu M$ (**Fig. 2A, highlighted in blue**) were part of the stage-transition inhibition assay which showed that these molecules affect different developmental stages of the parasite. We observed varying degree of inhibition for trophozoite-schizont [T→S] and schizont-ring [S→R] transitions for the molecules. For instance, we observed that MMV688271 (guanidine) and MMV671636 (quinolinone) had no effect on [T→S], although they are known to target mitochondria cytochrome bc-1 complex and AT rich sites on the DNA, respectively. Another two molecules, MMV688754 and MMV675968, for which mechanistic insights are limited, were found to block [T→S] and [S→R] transitions. Since trophozoites are engaged in a variety of metabolic activities essential for parasite survival and proliferation, they may offer more target options when compared to the metabolically more quiescent ring stage parasites. Furthermore, trophozoites are also transcriptionally more active and are engaged in preparation of the parasite cell for daughter cell formation and cell division, which makes them more “target-rich” for small molecule inhibitors³⁹⁻⁴⁰. Interestingly, we identified 21 molecules, which had significant effect on [S→R] transition. Among them, MMV021660 is known to inhibit folate pathway in *Mycobacterium*⁴¹⁻⁴². It is noteworthy that although EC_{50} values for some of the newly identified antiplasmodial molecules (MMV637229, MMV024311, MMV687812, MMV687765, MMV652003, MMV676600, MMV688271, MMV688279, MMV688362) are in nanomolar range, their stage specific inhibitory efficiencies are quite varied and distinct. Our observations highlight that for most inhibitors, the overall potency of the inhibitor (expressed as EC_{50}) as determined from 60 h growth inhibition assays, is poorly correlated to their effect on each stage of malaria parasite. The ability of ring stage parasites to tolerate drug exposure and facilitate emergence of drug resistance is documented³⁹. Thus, it is necessary to carefully study the progressive developmental response following inhibitor treatment.

Validation of molecules affecting schizont to ring transition.

From the stage transition assays, we observed that a 22 of the screened molecules were inhibiting the [S→R] transition. In order to avoid missing out on other potential [S→R] inhibitors from the antimalarial set of Pathogen Box, we screened all 162 molecules against schizonts. In order to obtain a more detailed insight on the inhibitory activity of the molecules on egress and invasion processes, we employed two levels of validation using flow cytometry and microscopic examination. Synchronized parasites were allowed to develop into schizont stage (40 to 42 hpi) parasites and then treated with three different concentrations (1, 3, and 10 μM) of the inhibitors in the initial screen. After

12 h treatment (i.e., ~55 hpi) the number of parasites present as newly formed rings and un-ruptured schizonts were scored using flow cytometry. DMSO and E64 were included as negative and positive controls respectively. To observe the parasite cell phenotypic following inhibitor treatment, microscopic examination of Giemsa-stained smears was performed. We observed that 50 of the 162 pathogen box molecules showed inhibitory effect of $\geq 50\%$ in [S→R] transition at 10 μM , which dropped to 38 molecules at 3 μM and 29 molecules at 1 μM (**Fig. 2B**). Although 29 molecules inhibited [S→R] transition at 1 μM , microscopic analysis of phenotype indicated that some of them were parasitocidal (and thus unlikely to have any direct effect on the egress/invasion process). Molecules inhibiting [S→R] transition only at 3 μM and 10 μM were not consider for further study since at higher concentrations the observed inhibition can be due to general cytotoxicity on merozoite viability⁴³⁻⁴⁴.

From our experiments, 12 molecules were short-listed as potent [S→R] transition inhibitors at $\leq 1 \mu\text{M}$ (**Fig. 3A**). The following molecules: MMV020520, MMV020710, MMV020391, MMV006239, MMV020623, MMV675958, MMV010576, MMV020670, MMV026356, MMV085071, MMV024443, MMV020081 where efficient inhibitors of schizont maturation at sub micromolar concentrations (**Fig. 3B**). Microscopy revealed that treatment with the molecules MMV020081 and MMV006239 resulted in cellular phenotypes reminiscent of E64 treatment, indicating specific inhibition of egress process. However, parasites treated with MMV026356 or MMV020670 appeared to be unhealthy and dying, indicating that there may be no direct inhibition of egress or invasion process (**Fig. 3C**). We then performed a concentration-dependent [S→R] transition assay for the top 12 molecules as indicated in **Fig. 3D**. With respect to egress inhibition, MMV020081 appeared to be the most potent with a R_{50} value of 90 nM while its actual estimated EC_{50} is 1.7 μM , indicating good selectivity (**Table.1**). Similarly, MMV024443 showed substantially higher potency on egress inhibition with an R_{50} of 0.3 μM in contrast to its EC_{50} value of 1.65 μM . MMV675968, which was shown to inhibit thymidylate biosynthesis by targeting the dihydrofolate reductase enzyme in *M. tuberculosis* and *T. gondii*, showed comparable values for EC_{50} and R_{50} . All of these molecules belong to diverse chemical classes indicating unique mechanism of action (**Supplemental Figure. S1**).

MMV020623, MMV020520, MMV020081, MMV020710 and MMV020391, which are reported to target PfATP4 and disrupt cellular cation homeostasis, were also found to strongly inhibit [S→R] transition suggesting that PfATP4 activity maybe important for parasite egress⁴⁵⁻⁴⁶. MMV024443 (an indole-2-carboxamide) which has been shown to target *P. falciparum* calcium-dependent protein kinase 1 (PfCDPK1)⁴⁷⁻⁴⁹ showed excellent egress inhibition in our studies. Previous studies in *P. falciparum* have shown the importance of PfCDPK1 in parasite development and invasion⁴⁷⁻⁵⁰. Interestingly, we found that this molecule can also inhibit calcium ionophore induced egress of *T. gondii* (discussed in below section). The *T. gondii* calcium-dependent protein kinase 1 (TgCDPK1) is an essential regulator of calcium-dependent parasite egress and invasion⁴⁹. PfCDPK1 and TgCDPK1 are orthologous and share a sequence identity of 53.85%, suggesting that they may have conserved functions in both parasites. We have determined the Rupture 50 (R_{50}) values, defined as the

concentration of the molecule at which there is a 50% reduction in newly formed rings compared to the DMSO control. We found that all the 12 molecules tested had R_{50} values in sub-micromolar range.

Effect of *Plasmodium* schizont inhibitors on toxoplasma egress. Being an obligate intracellular parasite, *T. gondii* actively invades and proliferates inside host cells, within the parasitophorous vacuole. At the end of an intra-vacuolar replicative cycle (or prematurely in response to external perturbations), the parasites will have to egress out and again invade a new host cell to survive and proliferate. Thus, egress and invasion are two critical aspects which are essential for continuity of the replicative cycle. We were interested in finding out whether any of the Pathogen Box molecules, which affected the maturation and egress of *P. falciparum* merozoites, might inhibit egress of tachyzoite stage *T. gondii*. For this we used a calcium ionophore mediated egress assay as previously reported²⁵. Intracellular tachyzoite stage parasites were treated with 10 μ M concentration of inhibitors for 24 h following they were induced to egress using the calcium ionophore (A23187). As control, DMSO (1%) treated cells were used to estimate the normal egress time taken by parasites to egress out. Of the 12 molecules tested, 9 were capable of inhibiting *T. gondii* egress in ionophore induced egress assay. Average egress time for DMSO-treated parasites was ~1.07 min. For inhibitor-treated parasites, we considered an average egress time delay of at least 4 min to assign them as potential egress inhibitors in *T. gondii* (Fig. 4A). Interestingly, we identified 7 molecules (MMV020081, MMV085071, MMV024443, MMV026356, MMV020670, MMV006239 and MMV020710) with significant egress-blocking activity, and at least in case of 3 molecules (MMV020670, MMV020081 and MMV026356) egress block extended beyond 7 minutes (Fig 4A, highlighted in blue). Only two of the molecules tested, MMV020623 (average egress time, ~4.06 min) and MMV020520 (average egress time, ~3.26 min) showed modest inhibition of *T. gondii* egress.

Mechanistic insights into parasite death when treated with MMV020391 and MMV010576.

Although we identified several molecules with inhibitory potential against late state parasite development and egress, it is reasonable to expect that these molecules have different targets and operate differently at the molecular level, given their structural diversity. Furthermore, microscopy of cellular phenotypes revealed both genuine egress inhibition (MMV020391 and MMV010576) as well as unhealthy and dying merozoites (MMV026356 and MMV020670). Molecules that are cytotoxic could kill parasites by producing ROS and damaging parasite proteins and DNA^{51,51}. To validate such a possibility, we evaluated ROS generation in late-stage schizonts treated with the 7 molecules that were effective against both *Plasmodium* and *Toxoplasma* (MMV020081, MMV085071, MMV024443, MMV026356, MMV020670, MMV006239 and MMV020710). All 7 molecules were positive for ROS generation, albeit to varying degrees (Fig. 5A). Following this observation, we performed a quantitative flow cytometry-based ROS assay on these molecules, which indicated that at least 5 of these molecules (MMV006239, MMV020670, MMV026356, MMV024443 and MMV085071) were potent ROS generators (Fig. 5B).

This was an interesting observation, and since increased intracellular ROS can cause DNA damage, we tested this using a comet assay that allows visualization of the extent of DNA damage/break-down following inhibitor treatment. From these experiments, $\geq 50\%$ DNA damage was observed following treatment with the molecules MMV020670 and MMV026356 (**Fig. 5C**), as indicated by comet tail moment at 5 μM drug exposure (**Fig. 5D**). Evidently, the increased levels of ROS production generated by MMV020670 and MMV026356 has caused considerable DNA damage. A variety of cellular mechanisms can be responsible for ROS production, including oxidation of lipids, protein and other cellular regulatory mechanisms⁵²⁻⁵⁴.

Targeting [S $\rightarrow$ R] transition offers an excellent opportunity for chemotherapy development against *Plasmodium* infection. First, by blocking newly formed invasion ready merozoites from egressing out of infected RBC, rapid increase of the parasite load in clinical cases can be prevented. Second, [S $\rightarrow$ R] transition involves sequential preparatory and remodelling events in the parasite, which offers numerous targets for inhibitors. Further mechanistic studies on selected Pathogen Box molecules is needed to fully realize the therapeutic potential of these molecules. Studies on altered gene and protein expression, combined with proteome modification studies can be good starting points for obtaining further insights on the mechanism of these molecules.

Conclusions.

Growth inhibition studies on blood stage *P. falciparum* using the MMV Pathogen Box chemical library identified 162 antimalarial molecules, out of which 62 molecules are newly identified. Further, we segregated these molecules based on stage-selectivity, inhibitory efficacy and cellular phenotypes that allowed us to prioritize 12 molecules that interfere with late stage parasite development and egress. We demonstrate that two of the schizont inhibitors, MMV020670 and MMV026356 induce DNA break-down in segmented schizonts leading to parasite arrest. Identifying molecules that are inhibiting egress in both *Plasmodium* and *Toxoplasma* is very exciting, as this can facilitate target identification and other mechanism studies. This also opens up the possibility of exploiting these molecules a therapeutic agent against multiple apicomplexan parasites.

Author Contributions

ATP: Involved in study design, conducted *Plasmodium* experiments, analysed results, prepared figures and assisted with manuscript preparation, **TH:** Performed antimalarial screening for all Pathogen Box molecules, analysed results and assisted with manuscript preparation, **MB:** Performed *T. gondii* egress assays and analysed results, **AX:** Performed Comet assay analysed results and assisted with manuscript preparation, **GS:** Assisted with plasmodium experiments and assisted with manuscript preparation, **ZB:** Involved in study design, provided tools/reagents and assisted with manuscript preparation, **PRP:** Involved in study design, provided tools/reagents and assisted with manuscript preparation, **DS:** Involved in study design, analysed data, verified results and assisted

with manuscript preparation, **RC**: Designed the study, coordinated research, verified results and wrote the manuscript.

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Figure Legends

Figure 1: Identifying Pathogen Box molecules inhibiting growth of blood stage *P. falciparum* parasites. (A) Segregation of molecules based on their antimalarial activity. From the 400 molecules tested (indicated in grey), 162 molecules inhibited parasite growth by $\geq 80\%$ at 10 μM concentration (indicated in blue pattern). Out of these, 100 molecules were previously documented for antimalarial activity (indicated in black) and 62 molecules were newly identified in this study. 25 molecules previously reported to have antimalarial activity showed moderate potency ($\geq 50\%$) in our assays (indicated in white). (B) EC_{50} values for the 162 molecules with antimalarial activity are presented to show their antimalarial potency distribution. Newly identified antimalarial molecules from this study are indicated in blue colour.

Figure 2: Stage-specific segregation of antimalarial activity for selected pathogen box molecules. (A) Flow cytometry data showing % of parasites which have successfully transitioned from ring to trophozoite [R $\rightarrow$ T], trophozoite to schizont [T $\rightarrow$ S], and schizont to ring [S $\rightarrow$ R]. Highlighted in blue are the newly identified molecules with antimalarial activity from this study. (B) Pie chart showing inhibition of *P. falciparum* schizont to ring transition upon treatment with 10, 3 and 1 μM of drug concentration (showing inhibitory efficiency $<25\%$, 25 to 50%, $>50\%$ and no inhibition). At 10 μM nearly 40% of molecules affected [S $\rightarrow$ R] transition, which was reduced to 10 % and 5% at 3 and 1 μM respectively, indicating progressive increase in selectivity of the hits identified from higher to lower concentration of the molecules.

Figure 3: Characterization of schizont inhibitors. (A) (Left panel) Showing heatmap for 12 potent schizont inhibitors tested at 1 μ M for their stage specific inhibitory effect. While molecules such as MMV675968 and MMV020623 appeared to affect both trophozoite and schizont growth transition, all other molecules were selective against schizont-ring transition **(B)** Late-stage schizonts (42-44 hpi) incubated with the inhibitors were monitored for schizont inhibition through counting newly formed rings by flow cytometry. All molecules tested showed a profound, yet selective activity against [S $\rightarrow$ R] transition in similar manner to E64 which was included as a positive control for egress inhibition. **(C)** Giemsa-stained smears upon microscopic investigation revealed arrest of late stage parasite development and egress. In case of MMV026356 and MMV020670, unhealthy daughter merozoites were observed indicating cytotoxic activity of these molecules. **(D)** Dose–response curves of potential schizont inhibitors from Pathogen Box library against *P. falciparum* schizonts. DMSO and E64 were included as negative and positive controls respectively. A majority of the Pathogen Box molecules showed remarkable schizonticidal activity.

Figure 4: *P. falciparum* [S $\rightarrow$ R] blockers inhibit calcium ionophore-mediated egress of *T. gondii* tachyzoites (A) Whisker plots showing the timing of ionophore-induced egress following 24 h of treatment for Pathogen Box molecules at 10 μ M. Red, 1% DMSO-treated control cells; blue, inhibitors for which the median egress time was >6 min.

Figure 5: ROS production and DNA fragmentation by Pathogen Box molecules which inhibit parasite egress. (A) Evaluation of ROS production visualized by staining with CellROX Green dye of schizonts after 3 h treatment with 1 μ M and 5 μ M inhibitor concentration. Varying degrees of ROS production is evident from the observed green fluorescence. (B) Quantification of ROS production using flow cytometry revealed high extent of ROS production for MMV020670, MMV026356 and MMV024443. (C-D) Results from comet assay showing DNA damage in late-stage parasites treated with MMV020670 and MMV026356. None of the other molecules tested showed detectable DNA fragmentation.

Declaration of conflict of interest

There are no conflicts to declare.

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Figure 1

A

B

Figure 2

A

B

Figure 3

Figure 4

A

Figure 5

Table 1: Molecules from other disease sets exhibiting antimalarial activity

Pathogen Box ID	Disease Set	Chemical Class	Trophozoite Inhibition (%)	Schizont Inhibition (%)	DNA Damage (%)	EC ₅₀ Value (μM) (this study)	Toxo. Ionophore Egress Assay	Function and Possible Targets*
MMV024443	Malaria	Indole Derivatives	NA	58.6	30%	1.66	7.00 min	CDPK1 - Calcium-dependent protein kinase 1; lactate dehydrogenase ⁵⁰⁻⁵² .
MMV020623	Malaria	Indole Derivatives	14.15	70.6	30-40%	0.22	4.10 min	PfATP4 dysregulation affecting Na ⁺ efflux ⁴⁹ .
MMV010576	Malaria	Pyridines and derivatives	20.57	60.4	30%	0.19	6.50 min	Phosphatidylinositol 4-kinase (PI4K) activity ⁸ based on the elucidated target for clinical candidates MMV390048.
MMV020520	Malaria	Benzoxazines	35.84	84.6	30%	0.11	3.30 min	PfATP4 dysregulation affecting Na ⁺ efflux ⁴⁹ .
MMV085071	Malaria	Indole Derivatives	36.89	47.2	30%	0.03	6.50 min	Rapid parasitocidal effect and programmed cell death (PCD)-like phenotypes ¹² .
MMV020670	Malaria	6-naphthyridine-2-carboxamide	37.03	64	>50%	0.07	7.00 min	NA
MMV020081	Malaria	Benzene and substituted derivatives	38.23	43.4	30%	1.7	7.00 min	PfATP4 dysregulation affecting Na ⁺ efflux ⁴⁹ .
MMV026356	Malaria	2-methylpyrazole-3-carboxamide	40.27	61.4	>50%	1.56	7.00 min	NA
MMV006239	Malaria	Alkaloid Derivatives	42.32	90	25-30%	0.16	7.00 min	NA
MMV020710	Malaria	Azoles	43.63	84	25-30%	0.06	7.00 min	PfATP4 dysregulation affecting Na ⁺ efflux ⁴⁹ .
MMV020391	Malaria	Benzene and substituted derivatives	44.04	90.4	25-30%	0.22	NA	PfATP4 dysregulation affecting Na ⁺ efflux ⁴⁹ .
MMV675968	Cryptosporidiosis	Diazanaphthalene	77.48	41.6	25-30%	0.07	NA	Inhibits Dihydrofolate reductase (Toxoplasma and Cryptosporidiosis) ¹¹ .

Fig S1

Chemical Structure of 12 Schizont Inhibitors

MMV024443

MMV020623

MMV010576

MMV020520

MMV085071

MMV020391

MMV006239

MMV026356

MMV020670

MMV020710

MMV020081

MMV675968

Fig S1: Structure of the 12 most effective compounds identified by *in vitro* screening using 1 μ M as the highest concentration, as late stage parasite inhibitors. These compound structures are obtained from PubChem (<https://pubchem.ncbi.nlm.nih.gov>) and MolView (<http://molview.org/>)

Fig S2

Fig S2: Heat Map showing parasite growth progression data of 96 molecules testing against *P. falciparum*. The data shown are mean values of two biological replicates.

Table 2: Molecules from other disease sets exhibiting antimalarial activity

ChEMBL ID	Pathogen Box ID	Compound Class	Disease Set	Pfal IC50 (µM) (this study)	Cytotoxicity Data (Pathogen Box dataset)	Predicted Cellular Target	Literature References
CHEMBL227240	MMV688514	Benzenecarboximidamide	Kinetoplastids	3.66	2.64	No Hits	1
CHEMBL466426	MMV688943	Difenoconazol	Kinetoplastids	6.18	0	No Hits	NA
CHEMBL564829	MMV676602	Milciclib	Kinetoplastids	6.06	<0.26	No Hits	3
CHEMBL2441383	MMV652003	Benzamide	Kinetoplastids	0.11	0.413	Leucyl-tRNA synthetase	34
CHEMBL1981047/ CHEMBL384575/ CHEMBL402548	MMV676600	Benzamide	Kinetoplastids	0.25	4.83	No Hits	5
CHEMBL1836562	MMV688410	Acetamide	Kinetoplastids	3.85	16.4	No Hits	6
CHEMBL1197423	MMV688547	1H-1,2,3-Triazole	Kinetoplastids	1.78	>80	DNA Targeted agent	7,10
CHEMBL3637895	MMV688283	4-Quinolinamine	Kinetoplastids	0.50	8	Potentially target Beta-hematin formation	35
CHEMBL394241	MMV676057	1-Piperazinecarboxylic acid	Kinetoplastids	6.51	4.03	No Hits	9
CHEMBL45843	MMV688179	2,2'-[2,5-Furandiylbis(3-chlor-4,1-phenylen)] diguanidin	Kinetoplastids	0.74	6.75	No Hits	10
CHEMBL227667	MMV688361	Isoxazole	Kinetoplastids	1.45	4.51	No Hits	1
CHEMBL3431105	MMV595321	Benzamide	Kinetoplastids	5.59	0	No Hits	11
CHEMBL3637896	MMV688371	Benzamide	Kinetoplastids	5.85	0	No Hits	NA
CHEMBL474899	MMV659004	Pyrimidine	Kinetoplastids	2.39	10.2	Methionine aminopeptidase	13
CHEMBL3637894	MMV688274	4-Morpholineacetamide	Kinetoplastids	3.48	10.9	No Hits	NA
CHEMBL1197424	MMV688407	1H-1,2,3-Triazole	Kinetoplastids	2.92	22.9	No Hits	7

CHEMBL520654	MMV688362	1H-Imidazole	Kinetoplastids	0.42	14.3	Binds to the DNA minor groove at AT-rich regions of DNA	15
CHEMBL1230468	MMV688180	Benzenesulfonamide	Kinetoplastids	5.31	0.0454	No Hits	2,4,8
CHEMBL1836611	MMV688279	4-Quinazolinol	Kinetoplastids	0.22	10	No Hits	6
CHEMBL238442	MMV687706	Piperazine	Kinetoplastids	7.65	6.43	No Hits	9
CHEMBL413331	MMV688271	Guanidine	Kinetoplastids	0.40	13.5	Binds to the DNA minor groove at AT-rich regions of DNA	10
CHEMBL3104375	MMV689243	3-Pyridinamine	Kinetoplastids	2.00	25.8	Cytochrome P450 inhibitors	16
CHEMBL1897483/ CHEMBL3183941/ CHEMBL3637899	MMV688754	Benzeneacetic acid	Kinetoplastids	0.01	0	No Hits	NA
CHEMBL3637893	MMV688273	4,6-Quinazolinediamine	Kinetoplastids	1.61	9.72	No Hits	NA
CHEMBL472881	MMV658988	4-Pyrimidinamine	Kinetoplastids	2.84	11.3	Methionine aminopeptidase	13
CHEMBL3637898	MMV688550	Imidazo[1,2]purine	Kinetoplastids	3.05	44.3	No Hits	NA
CHEMBL2097846	MMV688889	pyrimidin-7-amine	Tuberculosis	8.24	NA	No Hits	12
CHEMBL2098256	MMV688888	Pyrimidine	Tuberculosis	0.95	NA	No Hits	12,14
CHEMBL204432	MMV661713	4-Pyrimidinamine	Tuberculosis	0.60	NA	Glutaminyl cyclase	12,17
CHEMBL2109905	MMV688936	2-Furancarboxamide	Tuberculosis	6.60	NA	No Hits	12
CHEMBL1622353	MMV676401	4-Quinazolinamine	Tuberculosis	5.99	18.59	No Hits	18
CHEMBL2098478	MMV676449	1H-Benzimidazole-1-ethanol	Tuberculosis	8.51	43.2	No Hits	12
CHEMBL2098184	MMV676477	Benzamide	Tuberculosis	0.76	1.34	No Hits	12,14
CHEMBL2098293	MMV676512	1H-Imidazole-5-carboxamide	Tuberculosis	3.66	3.78	No Hits	12
CHEMBL2109740	MMV687703	1H-Benzimidazole	Tuberculosis	4.03	24.98	No Hits	12

CHEMBL535116	MMV023969	Isoquinoline	Tuberculosis	0.19	8.02	No Hits	NA
CHEMBL531893	MMV021660	Guanidine	Tuberculosis	0.07	6.46	Folate Pathway	12,19,20
CHEMBL2098162	MMV688122	2-pyrimidin-4-amine	Tuberculosis	5.53	14.26	Methionine aminopeptidase	12,21
CHEMBL2109766	MMV687749	Pyrimidine	Tuberculosis	0.85	29.03	Tyrosine Kinase	12
CHEMBL2109769	MMV687248	1H-Benzimidazol-2-amine	Tuberculosis	1.41	31.5	No Hits	12
CHEMBL2109877	MMV688125	Benzenesulfonamide	Tuberculosis	4.04	17.69	No Hits	12
CHEMBL561057	MMV687273	SQ-109 , In clinical trials 2012 TB	Tuberculosis	3.29	7.1	HMG-CoA reductase pathway?	22,23,20,24, 25
CHEMBL580032	MMV024311	1H-Indole	Tuberculosis	0.45	12.71	No Hits	12
CHEMBL2313136	MMV687807	Benzamide	Tuberculosis	2.47	0.65	No Hits	12,26,27
CHEMBL3637864	MMV687812	2-Pyrazinecarboxamide	Tuberculosis	0.26	3.86	No Hits	28
CHEMBL1397830	MMV676411	Propanamide	Tuberculosis	5.02	29.29	No Hits	12
CHEMBL530275	MMV021013	4-pyrimidinamine	Tuberculosis	0.72	NA	Methionine aminopeptidase	12
CHEMBL2109928	MMV687765	Pyrimidine	Tuberculosis	0.47	14.08	Tyrosine Kinase	12
CHEMBL3637867	MMV495543	Benzamide	Tuberculosis	0.26	47.64	No Hits	NA
CHEMBL1242290	MMV675993		Cryptosporidiosis	3.93	1.63	No Hits	29
CHEMBL88430	MMV675968		Cryptosporidiosis	0.07	3.44	Dihydrofolate reductase	30,31,32
CHEMBL1626/315 157/1620822	MMV637229	2-pyrimidin-4-amine	Hookworm Trichuriasis	0.01	9.28	No Hits	NA
CHEMBL3637906	MMV668727	Terpyridin-2'-amine	L. Filariasis- Onchocerciasis	3.64	>80	No Hits	NA
CHEMBL3637912	MMV676063	oxy-phenyl urea	L. Filariasis- Onchocerciasis	3.16	5.77	No Hits	NA

CHEMBL3637903	MMV671636	quinolinone	L. Filariasis-Onchocerciasis	0.01	>80	Mitochondria cytochrome bc ₁ complex	NA
CHEMBL3637922	MMV688761	Benzamide	Schistosomiasis	3.46	6.94	No Hits	NA
CHEMBL3637921	MMV688763	pyridazinone	Schistosomiasis	2.17	1.55	No Hits	NA
CHEMBL3637924	MMV688766	5-methoxyphenol	Schistosomiasis	1.71	>80	No Hits	36
CHEMBL3637923	MMV688552	1H-Pyrazole-1-carboxamide	Schistosomiasis	4.49	30.7	No Hits	NA
CHEMBL3637929	MMV688509	1-Piperidinecarboxylic acid	Toxoplasmosis	5.55	>50	No Hits	NA
CHEMBL190228	MMV688703	Pyridine	Toxoplasmosis	1.39	>50	cGMP-dependent protein kinase	33