Identification of MMV Malaria Box Inhibitors of *Perkinsus marinus* Using an ATP-Based Bioluminescence Assay

Yesmalie Aleman Resto, Jose A. Fernández Robledo

1 Bigelow Laboratory for Ocean Sciences, Boothbay, Maine, United States of America, 2 Research Experiences for Undergraduates (REU) NSF Program - 2013 - Bigelow Laboratory for Ocean Sciences, Boothbay, Maine, United States of America

Abstract

“Dermo” disease caused by the protozoan parasite *Perkinsus marinus* (Perkinsozoa) is one of the main obstacles to the restoration of oyster populations in the USA. *Perkinsus* spp. are also a concern worldwide because there are limited approaches to intervention against the disease. Based on the phylogenetic affinity between the Perkinsozoa and Apicomplexa, we exposed *Perkinsus* trophozoites to the Medicines for Malaria Venture Malaria Box, an open access compound library comprised of 200 drug-like and 200 probe-like compounds that are highly active against the erythrocyte stage of *Plasmodium falciparum*. Using a final concentration of 20 μM, we found that 4 days after exposure 46% of the compounds were active against *P. marinus* trophozoites. Six compounds with IC50 in the μM range were used to compare the degree of susceptibility in vitro of eight *P. marinus* strains from the USA and five *Perkinsus* species from around the world. The three compounds, MMV666021, MMV658507 and MMV666102, displayed a uniform effect across *Perkinsus* strains and species. Both *Perkinsus marinus* isolates and *Perkinsus* spp. presented different patterns of response to the panel of compounds tested, supporting the concept of strain/species variability. Here, we expanded the range of compounds available for inhibiting *Perkinsus* proliferation *in vitro* and characterized *Perkinsus* phenotypes based on their resistance to six compounds. We also discuss the implications of these findings in the context of oyster management. The *Perkinsus* system offers the potential for investigating the mechanism of action of the compounds of interest.

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Introduction

*Perkinsus marinus* and *Perkinsus chesapeaki* cause Dermo disease in oysters and clams in the USA. Described in the early 1950s, Dermo disease is associated with mass mortalities of eastern oysters (*Crassostrea virginica*) in the Gulf Coast [1]; now it is under surveillance by the World Organization for Animal Health (OIE; http://www.oie.int/); Aquatic Animal Health Code, Section 11: Diseases of Molluscs). The Chesapeake Bay (Maryland, Virginia, USA) is a clear example of where *P. marinus* has contributed to the decimation of the oyster industry (today’s production in Maryland is 4.2% of the production in the mid-1960s). The expansion of the *P. marinus* distribution range in the USA has been associated with global warming and the shellfish trade [2,3]. Dermo remains an important obstacle to the restoration of oyster populations in numerous eastern states [3,4]. Interestingly, *P. marinus* has also been reported with high prevalence in oysters from eastern states with no noticeable mollusk mortality [5], and recent records of *P. marinus* in oysters from the West Coast of North America were not associated with mortalities [6]. The presence of *P. marinus* phenotypes and genotypes might account for differences in virulence [7–9]. In the USA, *P. chesapeaki* displays a high preference for infecting clams and it appears to be better adapted to lower salinities and temperatures than *P. marinus* [5] and recently it has been detected in cockles (*Cerastoderma edule*) in Europe [10]. Worldwide, seven *Perkinsus* spp. have been described, most of them in the last decade with five of them available in *in vitro* culture (reviewed in [11]).

Compared to parasites of human and veterinary relevance, the pharmacopoeia for marine protozoan parasites is still very limited, and some of these compounds are toxic to the marine environment [12,13]. *Perkinsus* and other non-photosynthetic relatives of both dinoflagellates and apicomplexans lineages have lost the ability to perform photosynthesis; still, they have retained a cryptic plastid and its pathways (Chromalveolata hypothesis), which are recognized as promising drug targets [14,15]. Government agencies, drug companies, and non-profit organizations have screened multiple compound libraries against *Plasmodium falciparum* resulting in the Medicines for Malaria Venture (MMV) Malaria Box (http://www.mmv.org/malariabox). [16]. This compound library is being used to find inhibitors of defined parasite life stages [17,18], to describe mechanisms of action [19], and to find active compounds against other protozoan parasites [20,21]. Here, we followed similar approach and tested the MMV Malaria Box for...
the discovery of novel hits against *Perkinsus* using an adenosine tri-phosphate (ATP) content-based assay to test *P. marinus* proliferation growth [13].

**Materials and Methods**

**Materials**

The MMV Malaria Box constitutes 200 drug-like and 200 probe-like compounds with activity against the blood-stage of *Plasmodium falciparum* 3D7 (http://www.mmv.org/research-development/malaria-box-supporting-information). Stock solutions (20 mM) (Batch April2013; Table S1) in dimethyl sulfoxide (DMSO) were diluted in water and tested in the primary screening at a final concentration of 20 μM. The compounds were not repurchased nor re-synthetized; consequently, the results should be considered as primary unconfirmed hits until the identification of these compounds is followed up by a proper confirmation.

**Parasite strains and in vitro culture**

Experiments were carried out with eight *P. marinus* strains and five *Perkinsus* species (Table 1). Cultures were maintained in Dulbecco modified Eagle’s Ham’s F12 (1:2) supplemented with 5% fetal bovine serum in 25 cm2 (5 ml) vented flasks in a 26–28°C microbiology incubator fitted with orbital shaking (70–80 rpm). Cells were exposed once to the MMV Malaria Box (20 μM; final concentration of DMSO point dose-response curve (10 μM to 0.156 μM) using Prim6 (sigmoidal) (Graphpad Software, Inc.). Eight *P. marinus* strains and five *Perkinsus* spp. (Table 1) were tested to compare their relative sensitivity using 2 μM day 2 post-exposure. *In vitro* cultures *Perkinsus olseni*, *P. chesapeaki*, and *P. mediterraneus* are characterized by either sporulating, making the culture medium acid or remaining in clumps, or having very large trophozoites [24–27]. Consequently, to standardize the assay, aliquots from the cultures in the exponential phase were used for ATP measurement and then the experimental-well plates seeded with cells- ATP activity equivalent to *P. marinus* PRA240 2.0–5.0×106 cells/2000–4000 relative fluorescence units, RFU) [13]. The effect of the compounds on *P. marinus* strains and *Perkinsus* spp. proliferation was evaluated as above.

**Results and Discussion**

**MMV Malaria Box screen**

In this study, we screened the MMV Malaria Box for compounds that might inhibit *P. marinus* proliferation *in vitro*, an approach that has been successfully used to identify compounds against other protozoan parasites [19–21]. In our previous study, the effect of the drugs on *P. marinus* proliferation was evaluated at days 2, 4, and 8 post-exposure; however, it was at day 4 post-exposure when the inhibitory effect(s) of most drugs tested became apparent [13]. Consequently, for the MMV Malaria Box

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**Table 1. Perkinsus spp. and Perkinsus marinus strains used in the study.**

| Perkinsus sp.* | Strain | ATCC # | Location/year of isolation | Host | Reference |
|---------------|--------|--------|---------------------------|------|-----------|
| *P. marinus*  | C13-11 | 50896  | Cotuit, MA (USA)/1998      | Crassostrea virginica |           |
|               | LICT-1 | 50508  | Long Island Sound, CT (USA)/1998 | Crassostrea virginica |           |
|               | DBNJ-1 | 50509  | Delaware Bay, NJ (USA)/1993 | Crassostrea virginica |           |
|               | CBSD4  | PRA240 | Bennett Point, MD (USA)/2008 | Crassostrea virginica | [23]      |
|               | CBSD4  | PRA393 | GFP mutant derived from PRA240 | Crassostrea virginica | [13,23]   |
|               | H Cedar2 | 50757 | Cedar Keys, FL (USA)/1998  | Crassostrea virginica |           |
|               | HTIP14 | 50763  | Fort Pierce, FL (USA)      | Crassostrea virginica |           |
|               | TXsc   | 50983  | Galveston Bay, TX (USA)/1993 | Crassostrea virginica | [28]      |
| *P. chesapeaki* (= andrewsi) | A8-4a | 50807  | Fox Point, MD (USA)/2001   | Macoma balthica | [25]      |
| *P. olseni* (= atlanticus) | ALG1 | 50984  | Ria Formosa, Algarve (Portugal)/2002 | Tapes decussatus | [24]      |
| *P. mediterraneus* | G2 | PRA238 | Menorca (Spain)/2003 | Ostrea edulis | [47]      |
| *P. honshuensis* | Mie-3G/H8 | PRA177 | Gokasho Bay, Mie Pref. (Japan)/2002 | Venerupis philippinarum | [56]      |

*Perkinsus marinus* PRA240 was used for the primary screen. A total of eight *P. marinus* strains isolated from oysters from the East and Gulf Coast of the USA and five *Perkinsus* spp. from around the world were used for the secondary screen. In all the cases cultures were maintained in Dulbecco modified Eagle’s Ham’s F12 (1:2) supplemented with 5% fetal bovine serum.

*Perkinsus qugwadi* and *Perkinsus behaiensis* have never been available in culture [57,58].

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screening we measured cell viability at day 4 post-exposure. We found that 46% of the compounds active against the *P. falciparum* erythrocyte trophozoites (Table S2). A total of 58 compounds (31.8%) resulted in at least 50% inhibition; from these compounds, 13 (7.1%) resulted in at least 90% inhibition (Figure 1). The repertory of available compounds against *Perkinsus* drugs has gradually increased over the past two decades thanks to the establishment of the culture methodologies for *Perkinsus* spp. [28–30] (Figure 2A). Still, prior to this study, the number of available compounds against *Perkinsus* spp. was very limited (Figure 2B) compared to compounds against protozoan parasites of medical and veterinary relevance [31–34]. Previous screenings for compounds inhibiting *Perkinsus* proliferation have been based on the strong line of evidence for the presence in *Perkinsus*, like those in apicomplexan parasites, of pathways linked to a relic plastid [12,13,35,36]. Here we have shown that the MMV Malaria Box offers a promising alternative way of finding compounds effective against *Perkinsus* spp.

**Table 2.** List of compounds active against *Plasmodium falciparum* selected for the MMV Malaria Box (http://www.mmv.org/malarialbox) for secondary *Perkinsus marinus* growth-inhibition screen (IC50) and *Perkinsus marinus* strain and *Perkinsus* species sensitivity.

| HEOS Compound ID | Target | Smiles | EC50 (μM)** | Set | MW (KDa) | EC50 (μM)** |
|-----------------|--------|--------|-------------|-----|----------|-------------|
| MMV665941       | Unknown| CCNC(1ccc(1)c(2)N(3)c(2)C(4)cc(1)c5cccc(OC)c5) | 0.255 | Probe-like | 389.53 | 5.35 |
| MMV666021       | Yes, 29| Cc1ccc(1)c2c3c(3)c1Nc2ccc(2)c(C)F(F)c2F & 0.094 | Probe-like | 272.30 | 1.05 |
| MMV665807       | TM protease serine 4 | Oc1ccc(1)c(2)c(3)Nc2ccc(2)c(C)F(F)F | ND | Drug-like | 315.67 | 2.00 |
| MMV666102       | Functional 17 | CN(c1ccc(1)c2c3c(3)c1Nc2ccc(2)c(C)F(F)F & 1.150 | ND | Drug-like | 252.31 | 1.77 |
| MMV396719       | Functional 11 | n1c2ccc(2)c3c4c(4)c4c5Nc2ccc(2)c(C)F(F)F & 1.50 | ND | Drug-like | 341.40 | 2.08 |
| MMV006522       | Functional 19, Cytotoxic | CCO1ccc2cnc(1)c(2)cNc3ccc(Br)c3c2c1 & 0.480 | ND | Drug-like | 357.24 | 35.61 |

*Plasmodium falciparum* 3D7; **Perkinsus marinus** PRA240 primary screen. doi:10.1371/journal.pone.0111051.t002

Secondary *Perkinsus marinus* growth-inhibition screen (IC50)

Three drug-like and three probe-like of the 13 compounds with the highest inhibitory effect on *P. marinus* (Table 2) were randomly selected for calculating the IC50 in an 8-point dose-response curve (10 μM to 0.156 μM). We found that the IC50 varied between 1.05 μM for MV66602 and 5.35 μM for MMV665941; for MV006522 the IC50 was 35.6 μM a high concentration or leaving the compound longer time would have resulted in a fitted sigmoidal curve. In this study the IC50 for the selected compounds (Figure 3) was in the lower μM range and much lower than for the compounds tested in our previous study [13], still it is higher than the corresponding *P. falciparum* IC50 values (Table 2); consequently, without knowing the mechanism of action of the compounds, we cannot rule out off-target effects due to non-specific cytotoxic agents, including detergent effects, multitargeting and oxidative effects. The nature of the assays (*Plasmodium falciparum* relies on infected erythrocytes and the *P. marinus* screen is performed in the absence of the host cells) and culture medium can also account for the differences in the IC50 values. With a direct life cycle, *P. marinus* trophozoites are phagocytized by the oyster hemocytes [37,38] where they resist oxidative killing [39]. Interestingly, MMV666021 has been involved in the inhibition of glutathione-S-transferase (GST) activity of prostaglandin D2 synthase (PGDS) [40]. GST are involved in parasite survival by protecting them against oxidative stress from the host or from products derived from their own metabolism [41], and in *P. falciparum* it has been associated with chloroquine-resistance [42]. We grow *Perkinsus* in a host cell-free culture medium; hence, if the MMV666021 is indeed affecting the oxidative stress from the host or from products derived from their own metabolism. *Perkinsus marinus* trophozoites have an expanded transporter repertoire, which is useful not only for transporting nutrients but also for secreting extracellular products (ECP) intended to inactivate the host defense and to break down host tissues [43,44]. Protease activity variations significantly decrease the migration of hemocytes [44] and have been associated with differences in the average cell size and growth rate [45]. MMV665807 is believed to target transmembrane serine protease activities in the spent medium of cultured *P. marinus* and *P. mediterraneus* [46,47] and mutations in the promoter region of serine protease inhibitors (SPIs) in *C. virginica*
which confer resistance to Dermo disease [48,49]. The parasite proteases could be the target of MMV665807; however, to prove this hypothesis would require further experiments outside the scope of this study.

**Perkinsus marinus** strains and Perkinsus species sensitivity

Our results comparing the effect of a single compound concentration (2 μM) against seven *P. marinus* strains indicate that for MMV666021, MMV665807 and MMV666102, the inhibition of the different strains was within an equivalent range (Table 3). Interestingly, for MMV665941, MMV396719 and MMV006522, there was a high degree of variability among the *P. marinus* strains. The presence of *Perkinsus* in low salinity estuaries and the sudden spread of the parasite between oyster beds is often seen as indicating the presence of strains adapted to low salinity and strains of variable virulence; parasites isolated from the Atlantic coast are more virulent than Gulf isolates [50,51]. Indeed, *P. marinus* "races" and genetic strains have been documented along the Atlantic and Gulf coasts of the USA [7–9]. *Perkinsus marinus* strains from Maryland and Virginia appeared to be more susceptible to treatment with the antimalarial drug Quinine (130 μM) [52]. We also found strain variability to the compounds tested; *P. marinus* HCedar 2 from Florida appears to be much more sensitive to MMV006522 than the other *P. marinus* strains. On the other hand, MMV665941 appears to be less effective against *P. marinus* LICT-1 [CT-1] and DBNJ-1 [NJ-1], isolated from oysters from Connecticut and Delaware respectively. *Perkinsus marinus* LICT-1 [CT-1] also appears to be the strain less sensitive to MMV396719. There is a genetic base beneath *P. marinus* strains [8,9] and recent microsatellite analyses suggest that *P. marinus* utilizes both sexual and asexual reproduction and, over the short-term, selection acts upon independent parasite lineages rather than upon individual loci in a cohesive, interbreeding population [53]. Drawing a parallel to other protozoan parasites with markedly clonal population structure and variable degree of virulence [54], it is possible that the observed variability respond to a clonal population structure (strains derived from one original single clone) with variable virulence. Indeed, Reece et al. [8] grouped 76 *P. marinus* isolates in 12 different composite genotypes with >88% of isolates possessing one of three predominant genotypes.
We also compared all five Perkinsus spp. in culture using the panel of six drug-like and probe-like compounds at a concentration within the range of the determined IC$_{50}$ (2 μM). We found that MMV666021, MMV665807 and MMV666102 were active against all five Perkinsus spp. Interestingly, some compounds were not equally effective against all the Perkinsus spp. P. chesapeaki was not sensitive to MMV665941, MMV396719 and MMV006522 at the concentration tested. Perkinsus chesapeaki affects both oysters and clams along the East Coast of the USA [5] and recently it was detected in cockles from Europe [10]. Compared to other Perkinsus spp., P. chesapeaki A8-4a is characterized by making the culture medium acidic [25,26], which could affect the potency or the uptake of the compounds tested. Both P. mediterraneus G2 and P. honshuensis Mie-3G/H8 were not sensitive to MMV665941 at the concentration tested; interestingly, they were also less sensitive to MMV666102, a compound that showed a high degree of inhibition in most P. marinus strains and in P. chesapeaki. This study highlights an unexpected degree of variability between Perkinsus spp. A plausible explanation could be variations in the propagation rates and strategies in the in vitro culture affecting the uptake of the compounds. For example, some Perkinsus spp. in culture appear to be “locked” at the trophozoite stage while other Perkinsus spp. continuously zoosporulate [26,55]. P. mediterraneus cell density only increases two- to sixfold over a 6-week period compared to ten- to thirtyfold in P. marinus [47]. With the culture medium indicated above, both P. mediterraneus G2 and P. honshuensis Mie-3G/H8 are characterized by forming large in clumps in culture; whether this phenotype conditions the uptake of the compounds remains to be demonstrated. To answer these
### Table 3. Activity of antimalarial drug-like and probe-like (2 μM) compounds on *P. marinus* strains and *Perkinsus* spp. determined at day 2 post-exposure.

| Species          | Strain       | HEOS Compound ID | MMV65941 Mean | SDV | MMV66021 Mean | SDV | MMV665807 Mean | SDV | MMV666102 Mean | SDV | MMV396719 Mean | SDV | MMV006522 Mean | SDV |
|------------------|--------------|------------------|---------------|-----|---------------|-----|---------------|-----|---------------|-----|---------------|-----|---------------|-----|
| *P. marinus*     | C13-11 [MA-2-11] Massachusetts | MMV665941 | 34.9 | 1.9 | 97.1 | 0.8 | 74.5 | 1.6 | 39.7 | 7.7 | -10.3 | 0.2 |
|                  | LICT-1 [CT-1] Connecticut | MMV666021 | -20.7 | 10.2 | 79.6 | 1.6 | 95.6 | 0.2 | 64.6 | 1.9 | 36.7 | 10.2 | -46.7 | 7.7 |
|                  | DBNJ-1 [NJ-1] Delaware | MMV665807 | -6.8 | 11.0 | 71.6 | 3.2 | 92.4 | 1.1 | 72.6 | 1.7 | -1.3 | 8.6 | -47.4 | 3.3 |
|                  | CBSD4 Maryland | MMV666102 | 34.3 | 2.3 | 848.4 | 2.4 | 96.4 | 0.7 | 64.4 | 1.7 | 44.4 | 3.6 | 4.5 | 2.9 |
|                  | CBSD4-GFP Maryland | MMV396719 | 41.8 | 3.0 | 858.1 | 1.7 | 95.2 | 1.6 | 64.7 | 3.7 | 48.1 | 2.8 | -14.3 | 6.9 |
|                  | HCDar2 Florida | MMV006522 | 39.7 | 5.6 | 865.1 | 1.8 | 97.5 | 0.1 | 75.0 | 0.3 | 54.4 | 7.6 | 20.9 | 3.0 |
|                  | HTP14 [FL-6] Florida | MMV011051 | 44.0 | 3.1 | 823.1 | 5.1 | 95.0 | 0.9 | 59.1 | 4.1 | 53.9 | 2.2 | -1.7 | 5.0 |
|                  | TXsc Texas | MMV011051 | 10.7 | 6.8 | 725.3 | 3.8 | 91.5 | 0.6 | 68.1 | 2.3 | 7.9 | 10.5 | -22.4 | 8.3 |
| *P. chesapeki*    | A8-4a Maryland | MMV011051 | -47.8 | 15.6 | 55.7 | 6.8 | 84.1 | 2.0 | 88.0 | 3.3 | -12.0 | 8.2 | -96.0 | 9.0 |
| *P. olseni*      | ALG1 Portugal | MMV011051 | 14.3 | 14.7 | 80.4 | 1.4 | 94.9 | 0.7 | 51.1 | 1.3 | 22.5 | 1.4 | 10.5 | 3.2 |
| *P. mediterraneus* | G2 Spain | MMV011051 | -41.9 | 27.4 | 78.2 | 1.3 | 92.6 | 0.4 | 57.0 | 7.5 | 36.5 | 10.9 | 1.2 | 21.7 |
| *P. honshuensis* | Mie-3G/H8 Japan | MMV011051 | -38.5 | 15.4 | 86.2 | 3.3 | 95.4 | 1.5 | 8.8 | 14.1 | 64.6 | 4.5 | 38.0 | 5.9 |

Data expressed as inhibition mean (%).

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questions would require fine-tuning cultures and dedicated experiments outside the scope of this preliminary large screening.

Conclusions

By taking full advantage of both the open access Malaria Box and having *Perkinsus marinus* in culture, we have identified numerous compounds that affect the *in vitro* proliferation of the parasite. These primary “hits”, if confirmed, would expand the number of available compounds against *P. marinus* fivefold. To determine whether the drugs tested in this study will be effective in treating or preventing *P. marinus* infections in bivalves, we first must find a delivery method that administers an effective dose to the oyster tissue and toxicity to the bivalve hosts and other organisms in the environment [13]. In this study, we have taken an indirect approach for identifying and characterizing geographic phenotypes on the basis of resistance to selected compounds from the Malaria Box. At this point, we do not have an explanation for this variability. Most of the compounds tested have a very low Malaria Box. At this point, we do not have an explanation for this types on the basis of resistance to selected compounds from the

Moreover, these findings should also make the scientific community aware that the conclusions may be limited or can change depending on the particular strain used in the study. What the

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Supporting Information

Table S1 List of Medicines for Malaria Venture Malaria Box compounds developed against *Plasmodium falciparum* 3D7 and used in this study (http://www.mmv.org/research-development/malaria-box-supporting-information). (XLSX)

Table S2 Percentage of inhibition of *Perkinsus marinus* trophozoites growth using drug-like compounds and probe-like compounds (20 μM) at day 4 post-exposure. (XLSX)

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Author Contributions

Conceived and designed the experiments: JAFR. Performed the experiments: YAR JAFR. Analyzed the data: YAR JAFR. Contributed reagents/materials/analysis tools: JAFR. Wrote the paper: JAFR.
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