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Quantitative assessment of the proliferation of the protozoan parasite *Perkinsus marinus* using a bioluminescence assay for ATP content

Surekha Shridhar [a,b], Kolaleh Hassan [a], David J. Sullivan [c], Gerardo R. Vasta [a], José A. Fernández Robledo [a,*]

[a] Department of Microbiology and Immunology, University of Maryland Baltimore, School of Medicine, Institute of Marine and Environmental Technology, 701 East Pratt Street, Baltimore, MD 21202-3101, USA

[b] The Community College of Baltimore County, Catonsville, 800 South Rolling Road, Baltimore, MD 21228-5317, USA

[c] W. Harry Feinstone Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, 615 North Wolfe Street, Baltimore, MD 21205-2103, USA

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ABSTRACT

Perkinsus marinus is a protozoan parasite that causes "Dermo" disease in the eastern oyster *Crasssostrea virginica* in coastal areas of the USA. Until now, intervention strategies against the parasite have found limited success, and Dermo still remains one of the main hurdles for the restoration of oyster populations. We adapted a commercial adenosine tri-phosphate (ATP) content-based assay to assess the *in vitro* proliferation of *P. marinus* in a 96-well plate format, and validated the method by measuring the effects of potential anti-proliferative compounds. The sensitivity ($1.5–3.1 \times 10^4$ cells/well), linearity ($R^2 = 0.983$), and signal stability (60 min) support the reliability of the assay for assessing cell proliferation. Validation of the assay by culturing *P. marinus* in the presence of increasing concentrations of triclosan showed a dose–response profile. The IC_{50} value obtained was higher than that reported earlier, possibly due to the use of different viability assay methods and a different *P. marinus* strain. The antibiotics G418 and tetracycline and the herbicide fluridone were active against *P. marinus* proliferation; the IC_{50} of chloramphenicol, ciprofloxacin, and atrazine was relatively high suggesting either off-target effects or inability to reach the targets. The validation of the ATP-based assay, together with significant advantages of the *Perkinsus* culture methodology (homogeneity, reproducibility, and high cell densities), underscores the value of this assay for developing high-throughput screens for the identification of novel leader compounds against *Perkinsus* species, and most importantly, for the closely-related apicomplexan parasites.

1. Introduction

Perkinsus marinus (Perkinsozoa) is a protozoan parasite of mollusks that causes "Dermo" disease in oysters and clams. Described in the early 1950s as associated with mass mortalities of eastern oysters (*Crassostrea virginica*) on the Gulf Coast (Mackin et al., 1950), the recent expansion of the *P. marinus* distribution range in the USA has been associated with global warming and the shellfish trade (Ford and Chintala, 2006; Ford and Smolowitz, 2007). Recently, *P. marinus* was identified in the Pacific coast of North America affecting the oysters *Crassostrea corteziensis* and *Saccostrea palmula* from México (Cáceres-Martínez et al., 2008, 2012). Additional *Perkinsus* species affect mollusks worldwide, thereby damaging the shellfisheries and the environment (Villalba et al., 2004).

Currently, there is no effective treatment for Dermo disease, and the intervention strategies for management of the resource, such as restricting transport of oyster stocks, have had limited success and failed to prevent the expansion of the parasite's distribution range (Ford, 1996; Pecher et al., 2008). Selective breeding of disease-resistant individuals from disease-exposed natural populations or from laboratory lines selected for resistance to the protozoan parasite *Haplosporidium nelsoni* (Haplosporidia) has resulted in partial resistance to Dermo (Debrosse and Allen, 1996; Ragone Calvo et al., 1997). The use of triploid oyster strains remains a promising alternative (Guo et al., 2000); although non-resistant to Dermo disease, triploid oysters reach commercial size before diploid oysters enabling oyster farmers to harvest them prior mortalities occur. Similarly, off-bottom oyster farming in Dermo endemic areas such as the Chesapeake Bay (Maryland, Virginia, USA) allows faster growth and earlier harvests, reducing exposure to *Perkinsus* spp. (Fincham, 2010). Because of the above-mentioned reasons, it is clear that there is an urgent need for the identification of effective anti-*Perkinsus* drugs. Although early studies evaluated the efficacy of *in vivo* and *ex vivo* chemo-

* Corresponding author. Present address: Bigelow Laboratory for Ocean Sciences, P.O. Box 380, 60 Bigelow Drive, East Boothbay, ME 04544-0380, USA. Tel.: +1 207 315 2567; fax: +1 207 315 2329.

E-mail addresses: jfernandez-robledo@som.umaryland.edu, jfernandez-robledo@bigelow.org (J.A. Fernández Robled.

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therapeutic approaches for Dermo (Ray, 1966; Calvo and Burreson, 1994), it was the development of *in vitro* culture methods for *P. marinus* (Gauthier and Vasta, 1993; Kleinschuster and Swink, 1993; La Peyre et al., 1993) that has greatly facilitated the search for anti-*Perkinsus* agents among drugs of well established antimicrobial, anti-parasitic, and herbicidal properties (Dungan and Hmailton, 1995; Delaney et al., 2003; Panko et al., 2006; Leite et al., 2011). Because the *P. marinus* genome was not available at the time, however, the molecular mechanisms involved in the inhibition observed remained elusive.

To address the need for identification of novel anti-*Perkinsus* drugs for both preventive and therapeutic use, we adapted and validated a commercial adenosine tri-phosphate (ATP) content-based assay in a 96-well plate format by testing *P. marinus* proliferation grown under shaking and static conditions, and in the presence of inhibitors of metabolic pathways likely to be conserved among parasites and microalgae. This rational target selection was based on the Chromalveolata hypothesis, under which *Perkinsus* and other non-photosynthetic relatives of both dinoflagellates and apicomplexans, share a photosynthetic ancestor *via* a secondary endosymbiosis with a red alga, raising the possibility that these lineages retain cryptic plastids (reviewed in Keeling, 2010). Enzymes involved in plastid pathways in apicomplexans have been recognized as promising drug targets (Fichera and Roos, 1997; Soldati, 1999; Aboulaila et al., 2012; Biot et al., 2012; Botte et al., 2012). Although the presence of a relic plastid in *Perkinsus* is still under debate, *Perkinsus* has key metabolic functions associated with this organelle (reviewed in Fernández Robledo et al., 2011). Accordingly, and based on the available information about plastid-related gene products currently being investigated as potential drug targets in apicomplexan parasites and the identification of related sequences in the *P. marinus* draft genome, we evaluated the effect(s) of herbicidal, anti-bacterial, and anti-parasitic drugs on *in vitro* proliferation of *P. marinus*. Among the possible drugs candidates, we selected triclosan, an anti-bacterial and anti-fungal agent previously reported to inhibit *Perkinsus* proliferation (Lund et al., 2005; Stelter et al., 2007), several anti-*Plasmodium* drugs (Goodman et al., 2007), and fluridone, a herbicide that targets phytoene desaturase (PDS), an enzyme involved in the indirect pathway for abscisic acid (ABA) synthesis and carotenoid biosynthesis, and already established as present in *P. marinus* (Fernández Robledo et al., 2011), and the widely-used herbicide atrazine (Jowa and Howd, 2011). The results support that the optimized ATP-based assay format will constitute a useful tool for the identification of novel effective chemicals for intervention against Dermo disease.

2. Materials and methods

2.1. Materials

The antibiotics chloramphenicol (CAM), ciprofloxacin, clindamycin, G418 (gentamicin), tetracycline, the herbicide atrazine (6-chloro-2,4-diamino-1,3,5-triazine), and the anti-bacterial/-fungal triclosan [5-chloro-2-(2.4 dichlorophenoxy)-phenol] were purchased from Sigma–Aldrich (St. Louis, MO); the herbicide fluridone (SC-235160)(1-Methyl-3-phenyl-5-[3-(trifluoromethyl)phenyl]-1,4-dihydropyridin-4-one Fluridone) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). We chose to carry out this study with *P. marinus* given that the draft genome (http://www.ncbi.nlm.nih.gov/genome/?term=perkinsus%20genome), and a transcriptome (Joseph et al., 2010) are available for this species, and that these resources should facilitate the interpretation and characterization of drug inhibition mechanisms. Additionally, the availability of fluorescent mutants (Fernández-Robledo et al., 2008) should facilitate both *ex vivo* and *in vivo* evaluation of the drugs in host-parasite interaction studies.

2.2. Characterization of P. marinus cultures by flow cytometry

Cultures of *P. marinus* PRA-240 (American Type Culture Collection, USA, http://www.atcc.org/) (*P. marinus* wild type; *Pm*WT) and *P. marinus* PRA-240 MOE[MOE]:GFP (green fluorescent protein) (Fernández-Robledo et al., 2008) (accession number ATCC PRA-393) (*P. marinus*-GFP; *Pm*GFP) were maintained as reported elsewhere (Gauthier and Vasta, 1995). Both *Pm*WT and *Pm*GFP cultures were seeded at 2×10^6 cells/ml (50 ml) either in 250 ml Erlenmeyer flasks and kept at 28 °C in an incubator fitted with orbital shaking (110–120 rpm), or in static cultures using 75 cm^2 (50 ml) vented flasks in a 28 °C microbiology incubator. Shaken and static cultures were sampled daily over a period of two weeks, and analyzed by flow cytometry [BD Biosciences AccuriC6™ Flow Cytometer (BD, Billerica, MA)] for SSC (side scatter, cell granularity or cell complexity) *versus* FSC (forward scatter, cell diameter).

2.3. ATP-based P. marinus viability assay in a 96-well plate format

*Pm*WT (100 µl, 2.0×10^6 cells/ml) cell suspension was serially diluted (twofold) in a 96-well plate (white OptiPlateTM-96, PerkinElmer Life Sciences, Boston, MA). The ATP contained in the samples was then quantified using ATPlite assay kit following manufacturer's recommendations (PerkinElmer). We also included wells with the standard ATP control (twofold serial dilution of the stock solution; 50 µM). Control wells included cells incubated in culture medium. Signal stability of ATPlite with cultured *P. marinus* cells was calculated at all the cell densities assayed over a 120-min period. In another experiment, both *Pm*WT and *Pm*GFP cultures were sampled each by directly taking 100 µl of the culture and by taking the volume corresponding to 200,000 cells based on OD_{600}. The resulting luminescence was quantified in a VersaMax ELISA Microplate Reader running SoftMax® Pro Microplate Data Acquisition & Analysis Software (Molecular Devices LLC, Drive Sunnyvale, CA).

2.4. Perkinsus marinus growth-inhibition assay

Biological triplicate cultures of *Pm*GFP were grown in sterile CulturPlate-96 (PerkinElmer) plates (100 µl; 2.0×10^6 cells/ml). *Perkinsus marinus* cells were exposed to the antibiotics ciprofloxacin; (75.2; 150; 300.8; 601.8; 1203; 2407; 4814 µM), clindamycin (0.173; 1.73; 17.3; 1733; 1733 µM), G418 (18; 36; 72.1; 144.2; 288.5; 577.4; 1154 µM), tetracycline (25.9; 51.9; 103.9; 207.9; 415.9; 831.8; 1663.5 µM), CAM (309.5; 619; 1238; 2476; 3095; 3714; 4950 µM), the herbicides atrazine (40; 80; 160; 320; 640; 1280; 2561 µM) and fluridone (18.9; 37.9; 75.9; 151.8; 303.7; 607.4; 1214.7 µM), which theoretically targets the *P. marinus* PDS (*PmPDS*) (GenBank XP_002765827.1 and XP_002772671.1), and triclosan (21.6; 43.2; 86.4; 172.7; 345.4; 690; 1381 µM). Stock solutions were prepared in water (ciprofloxacin, clindamycin, G418), 100% ethanol (CAM, tetracycline, fluridone), or DMSO (atriazine), and diluted in water to reach the concentrations indicated above (final concentration of ethanol and DMSO was 1% or less). Control wells included solvent alone (DMSO, ethanol, or water), the test drug with no cells, and solvent with cells. The effect of the drugs on *P. marinus* proliferation was evaluated using the ATPlite assay at days 2, 4, and 8 post-exposure to the selected drugs. Readings for each concentration were normalized to the control wells with each solvent (100% activity). The IC_{50} was calculated from the dose–response curve using Prims6 (sigmoidal) (Graphpad Software, Inc). In a replicate experiment, potential changes in *P. marinus* trophozoite morphology at the IC_{50} concentrations were monitored by fluorescence microscopy and flow cytometry.