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Note

A high-throughput screening assay for identification of inhibitors of the A_1A_O-ATP synthase of the rumen methanogen *Methanobrevibacter ruminantium* M1

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ABSTRACT

We report the development of a high-throughput screening platform to identify inhibitors of the membrane-bound A_1A_o-ATP synthase from the rumen methanogen *Methanobrevibacter ruminantium* M1. Inhibitors identified in the screen were tested against growing cultures of *M. ruminantium*, validating the approach to identify new inhibitors of methanogens.

Methane (CH_4) accumulation in the atmosphere is considered to be contributing to climate change and global warming. Globally, CH_4 production from agriculture represents around 40% of the emissions produced by human-related activities, with enteric fermentation from ruminants the largest source (Steinfeld et al., 2006). CH_4 is produced in the rumen of domesticated ruminants (such as cattle, sheep and goats) by a group of microorganisms known as methanogens. During normal rumen function, plant material is broken down by fibre-degrading microorganisms and fermented to volatile fatty acids (VFAs), ammonia, hydrogen (H_2) and carbon dioxide (CO_2) (Janssen and Kirs, 2008). Hydrogenotrophic rumen methanogens use H_2 to reduce CO_2 to CH_4 in a series of reactions coupled to ATP synthesis by the membrane-bound A_1A_o-ATP synthase via a chemiosmotic mechanism (Thauer, 2011).

Several strategies have been proposed to control CH_4 production in ruminant animals, including breeding for low-CH_4 emitting animals, developing vaccines, and supplementation with dietary fatty acids (Buddle et al., 2011). Another strategy is to develop novel small molecule inhibitors that target specific enzymes in rumen methanogens, and this has been employed recently, from lead compounds identified using screening programmes (Martínez-Fernández et al., 2014). Small molecule inhibitors have the potential to provide sustained knockdown of CH_4 emissions from ruminants by targeting essential enzymes that are conserved across all rumen methanogens, without affecting the enzymes for normal digestive functions in other rumen microbes (Leahy et al., 2010). The rumen houses a range of different methanogen species, but *Methanobrevibacter* spp. are the most abundant, accounting for over 60% of rumen methanogens (Janssen and Kirs, 2008). The genome of *Methanobrevibacter ruminantium* M1 has recently been sequenced and was subjected to comparative genomic and metabolic pathway analyses, identifying conserved and methanogen-specific genes that may be suitable targets for the discovery of novel inhibitors of rumen methanogens (Leahy et al., 2010). The majority of these potential target genes encode enzymes within the methanogenesis or cofactor biosynthesis pathways, but also include enzymes involved in energy production, such as the archaeal membrane-bound A_1A_o-ATP synthase.

We have recently purified and biochemically characterized the A_1A_o-ATP synthase from *M. ruminantium* M1 (McMillan et al., 2011). The A_1A_o-ATP synthase in archaea differs from the V_1V_o and H^+ ATP synthases, which might be exploited to serve as an archaeal-specific target in inhibitor screening assays (Cross and Muller, 2004; Gruber et al., 2001, 2014). In this communication, we report the development of a high-throughput screening assay using the ATP hydrolysis activity of the A_1A_o-ATP synthase as a target to screen for inhibitors that slow or inhibit the growth of *M. ruminantium* strain M1.

The ATP hydrolysis activity of the A_1A_o-ATP synthase was determined using the DiscoveRx ADP Hunter™ Plus Assay kit (DiscoveRx, Fremont, CA). The assay measures the accumulation of ADP from an

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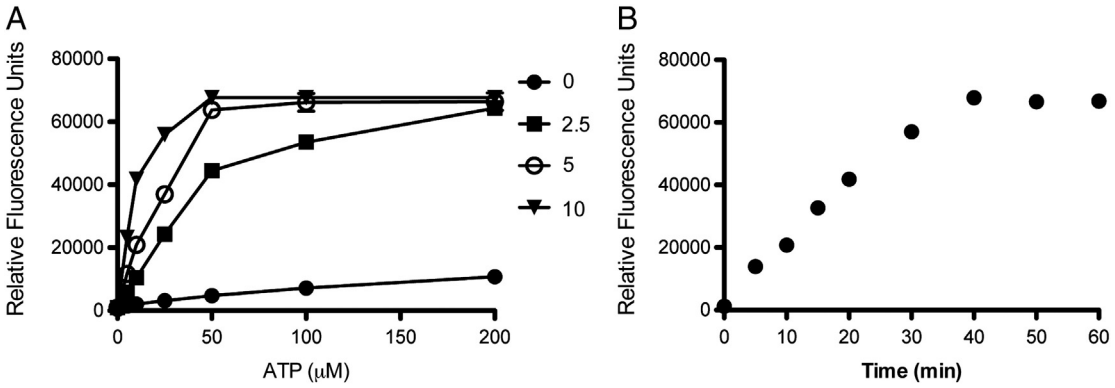

Fig. 1. MbbrA_1A_o-ATP synthase assay optimization. (A) Membrane-bound MbbrA_1A_o-ATP synthase was titrated from 0 to 10 μg and ATP was titrated from 0 to 200 μM. (B) Time course experiment. 5 μg membrane-bound MbbrA_1A_o-ATP synthase was incubated with 50 μM ATP. Reactions were incubated for 30 min in (A) or for a specific time in (B) followed by the addition of 10 μl reagent A and 20 μl reagent B. After 15 min development at room temperature, the fluorescence was measured on a Modulus™ Microplate Multimode reader. Results shown are as means ± SD of two replicates.

A_1A_o-ATP synthase-catalyzed ATP hydrolysis reaction. Hydrogen peroxide is then generated from ADP as a result of a coupled enzyme reaction. Hydrogen peroxide in combination with ADHP (fluorescent dye precursor) in the presence of peroxidase generates the fluorescently active resorufin dye that is detected by a fluorescent plate reader. Two forms of the A_1A_o-ATP synthase (membrane-bound and purified) were used in this study. The A_1A_o-ATP synthase from *M. ruminantium* M1 (MbbrA_1A_o-ATP synthase) was first recombinantly expressed in an *Escherichia coli* DK8 (Δ*atp*) strain and inverted membrane vesicles (membrane-bound A_1A_o-ATP synthase) were prepared as described previously (Klionsky et al., 1984). This inverted membrane vesicle preparation was then subjected to further purification as described previously (McMillan et al., 2011). To determine optimal enzyme and substrate concentrations for the assay, a cross-titration of the MbbrA_1A_o-ATP synthase in inverted membrane vesicles from *E. coli* DK8 (Δ*atp*) and ATP was performed at room temperature for 30 min. Inverted membrane vesicles (5 μg) and 50 μM ATP were selected for further experiments (Fig. 1A). There was no significant difference in the ATPase activity at room temperature or at 37 °C and the reaction rate was constant for 30 min (Fig. 1B). The final assay contained 5 μg MbbrA_1A_o-ATP synthase in inverted membrane vesicles from *E. coli* DK8 (Δ*atp*) in a reaction buffer consisting of 50 mM HEPES (pH 7.4), 20 mM NaCl, 1 mM EGTA (ethylene glycol tetraacetic acid), 0.02% Tween 20, 10 mM $MgCl_2$ and 0.1 mg/ml BGG (bovine-γ-globulins) in a final volume of 20 μl. The reaction was initiated by addition of 50 μM ATP, incubated at room temperature for 30 min and terminated by the addition of 10 μl reagent A and 20 μl reagent B, provided by the ADP Hunter™ Plus Assay kit. After 15 min incubation at room temperature, fluorescence was measured (λ_{Ex}535 nm and λ_{Em}590 nm) on a Modulus™ Microplate Multimode reader (Turner BioSystems, Sunnyvale, CA).

Using the assay conditions described above, the LOPAC 1280 library (Sigma-Aldrich, St. Louis, MO), comprising 1280 biologically active compounds, was screened. The library was prepared as 1.0 mM stocks in dimethyl sulfoxide (DMSO) and assessed for inhibition of A_1A_o-ATP synthase activity at a final compound concentration of 20 μM. Each tested microplate contained negative control wells (MbbrA_1A_o-ATP synthase + DMSO) and positive control wells (MbbrA_1A_o-ATP synthase + 5.0 mM amiloride (positive control) + DMSO) (McMillan et al., 2011). The LOPAC screen was characterized by an average Z-factor of 0.67, indicating that this is an excellent screening assay (Zhang et al., 1999).

Eleven compounds that caused ≥50% inhibition were identified (Fig. 2). We further validated these against a purified MbbrA_1A_o-ATP synthase (100 ng), prepared as described previously (McMillan et al., 2011) (Fig. 2). Resveratrol, hydroquinone, and morin were further selected to test their effect on the growth of *M. ruminantium* strain M1 as described previously (Wedlock et al., 2010). All inhibitors were dissolved in DMSO to a final concentration of 100 μM. In contrast to hydroquinone, resveratrol and morin inhibited the growth of *M. ruminantium* M1 (Fig. 3).

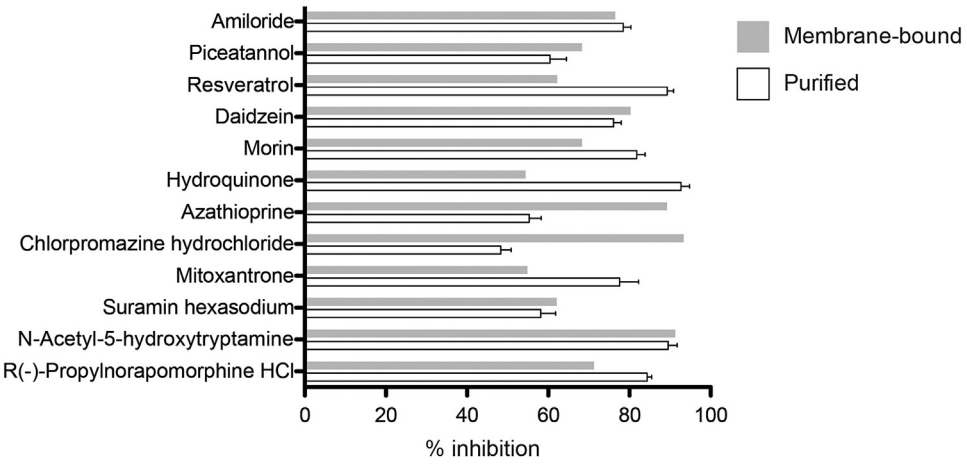

Fig. 2. Inhibition profile of the compounds identified in the LOPAC screen on the activity of membrane-bound MbbrA_1A_o-ATP synthase in inverted membrane vesicles from *E. coli* DK8 (Δ*atp*) (5.0 μg) and purified MbbrA_1A_o-ATP synthase (100 ng). Results for purified MbbrA_1A_o-ATP synthase are presented as means ± SD of three replicates.

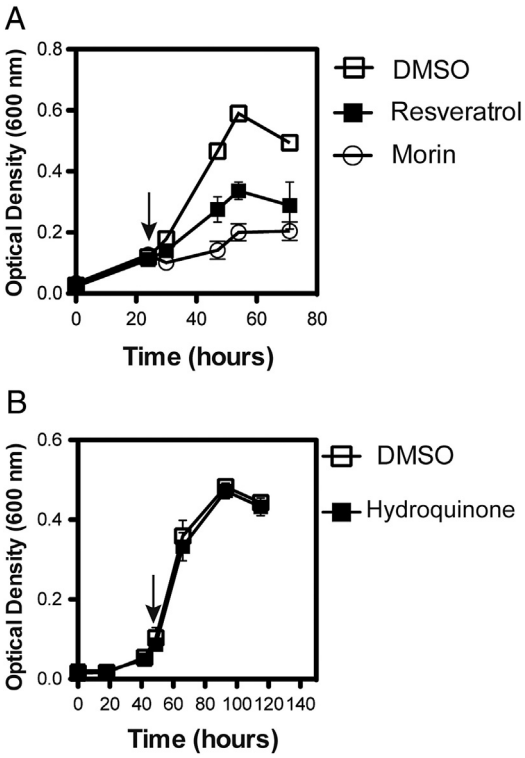

Fig. 3. Effect of resveratrol, morin, and hydroquinone on the growth of *M. ruminantium* M1. Inhibitors were added (final concentration of 100 μM) at an OD_{600} of 0.1 as indicated by the arrow. Results are shown as means ± SD of three biological replicates.

In conclusion, the high-throughput screen developed in this study provides a biochemical platform to accelerate discovery of novel inhibitors of rumen methanogens to mitigate methane emissions.

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