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Mode of action uncovered for the specific reduction of methane emissions from ruminants by the small molecule 3-nitrooxypropanol

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Within the last 200 years the concentration of the atmospheric greenhouse-gas methane has tripled and ruminants such as cows, sheep and goats, of which there are several billions raised by humans, have contributed significantly to this increase. Therefore, the recent finding that 3-nitrooxypropanol (3-NOP) can persistently decrease enteric methane emission from dairy cows with no negative effect on milk production, may help mitigate anthropogenic climate change. To ascertain that 3-NOP action is specific, we now studied the yet unknown mechanism of methane inhibition and found that the drug, at µM concentrations, specifically inhibits methanogenic archaea in the rumen by inactivation of the nickel-enzyme methyl-coenzyme M reductase (MCR) that is unique to methanogens. Upon MCR inactivation, 3-NOP is converted to nitrite, nitrate and 1,3-propanediol that at low concentration are also not toxic to animals.

Since the agricultural- and industrial revolution two hundred years ago the methane concentration in the atmosphere has increased from less than 0.6 ppm to now 1.8 ppm. The

present concentration is only 0.45% of that of CO_2 but since methane has a greenhouse gas potential on a 100 year horizon more than 25 fold higher than that of CO_2, it contributes significantly to global warming(*1*). The short atmospheric lifetime of methane, relative to that of CO_2, allows a rapid climate response to emission reductions which is why measures targeting methane emissions are considered very important to mitigate climate change(*2*).

One of the main anthropogenic sources of atmospheric methane are ruminants (cows, sheep, goats), the number of which has increased in parallel with the world population. In their rumen, plant material is fermented by anaerobic bacteria, protozoa, fungi and methanogenic archaea in a trophic chain to predominantly yield acetate, propionate, butyrate, CO_2 and methane with H_2 as intermediate(*3*, *4*) (fig. 1). Whereas the organic acids are absorbed and metabolized by the animals, methane escapes the rumen into the atmosphere via belching and breathing of the animals, up to 500 l methane per day in the case of a dairy cow, accounting for up to 12% of the gross energy content of the feedstock(*5*).

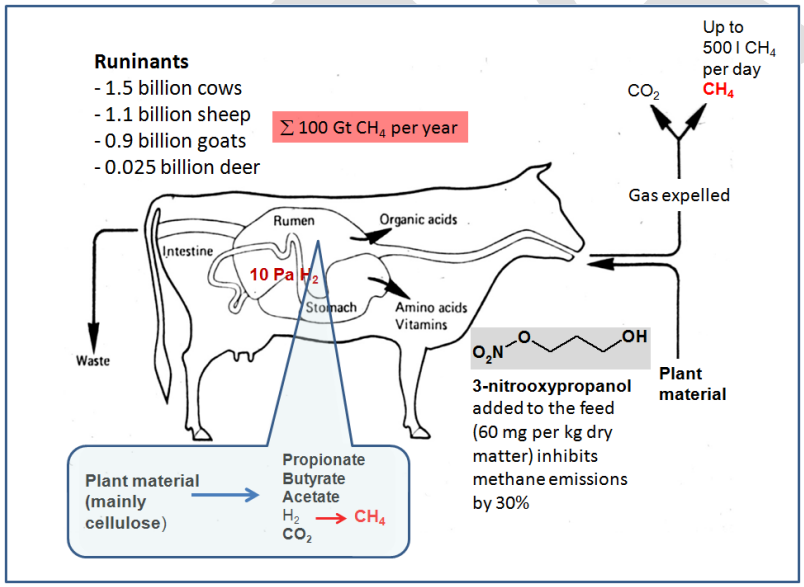

Fig. 1. Methane formation in the rumen of a dairy cow and its inhibition by 3-nitrooxypropanol (3-NOP). The H_2 partial pressure in the rumen is 10 Pa (≙ 0.01% in the gas phase at 10^5 Pa)

Methane (CH_4) is the main H_2 sink in the rumen. It is formed by methanogenic archaea at the bottom of the trophic chain mainly from carbon dioxide (CO_2) and hydrogen

(H_2) (fig. 1). However, the methane belched by ruminants contains only minute amounts of H_2 (H_2 partial pressure is only 10 Pa) indicating that in the rumen H_2 is consumed by the methanogens more rapidly than it is formed by the other microorganisms. The H_2 concentration increases noticeably only when methanogenesis is inhibited to more than 50%, and this also depends on the inhibition strategy(*6*). Already a small increase in the H_2 concentration leads to a down-regulation of the H_2-generating pathways(*7*) and to an up-regulation of H_2-neutral pathways such as propionate formation resulting in more energy supply to the host animal(*8, 9*). Thus, the H_2 concentration is kept constant when methane formation is inhibited. This can explain why methane formation can significantly differ between individual animals per unit feedstuff and that the amount formed is a heritable trait(*10*). It is also the basis for the search for specific inhibitors of methanogenesis that are not toxic for the animals(*11*, *12*). However, a compound that can both substantially decrease CH_4 and increase propionate productions in the rumen without compromising animal performance and health had not yet been described.

Recently, 3-nitrooxypropanol (3-NOP) (for structure see fig. 1) was found to persistently decrease enteric methane emission from sheep(*13*), dairy cows(*14*) and beef cattle(*15*) without apparent negative side effects(*16*). 3-NOP, applied at about 60 mg/kg feed dry matter, to high-producing dairy cows not only decreased methane emissions by 30% but also increased body weight gain significantly without negatively affecting feed intake or milk production and composition. However, the mechanism of methane inhibition by the drug has remained elusive, despite the fact that the nitrate ester was designed by us to specifically inhibit methyl-coenzyme M reductase (MCR).

MCR catalyzes the methane forming reaction in methanogenic archaea, namely the reduction of methyl-coenzyme M with coenzyme B to methane and the heterodisulfide formed from coenzyme M and coenzyme B (fig. 2A). MCR is a nickel enzyme in which the nickel is ligated in a tetrapyrrolic compound named cofactor F_{430}(*17, 18*). The nickel-containing

cofactor has to be in the Ni(I) oxidation state for the enzyme to be active. Since the redox potential $E^{o'}$ of the $F_{430}(Ni^{2+})/ F_{430}(Ni^{1+})$ couple is - 600 mV, the enzyme is very susceptible to inactivation by oxidants (*17, 18*). MCR has been well characterized by high resolution X-ray(*19, 20*) and EPR structures(*21*) with either substrates or products bound.

Based on the structure and properties of MCR we developed 3-NOP as inhibitor by 3D-pharmacophore-based virtual screening and molecular docking focusing on analogues of methyl-coenzyme M as lead structure. The inhibitor should be non-charged allowing cell penetration by diffusion and a moderate oxidant thereby facilitating the oxidation of Ni(I) in the active site of MCR. This resulted in a series of potential candidates that best fit into the active site of MCR. From these the binding pose of 3-NOP into the active site (fig. 2B) was found to be very similar to that of the natural ligand methyl-coenzyme M (fig. 2C and D). The nitrate group of 3-NOP, that can easily be reduced, is positioned in electron-transfer distance to the Ni(I).

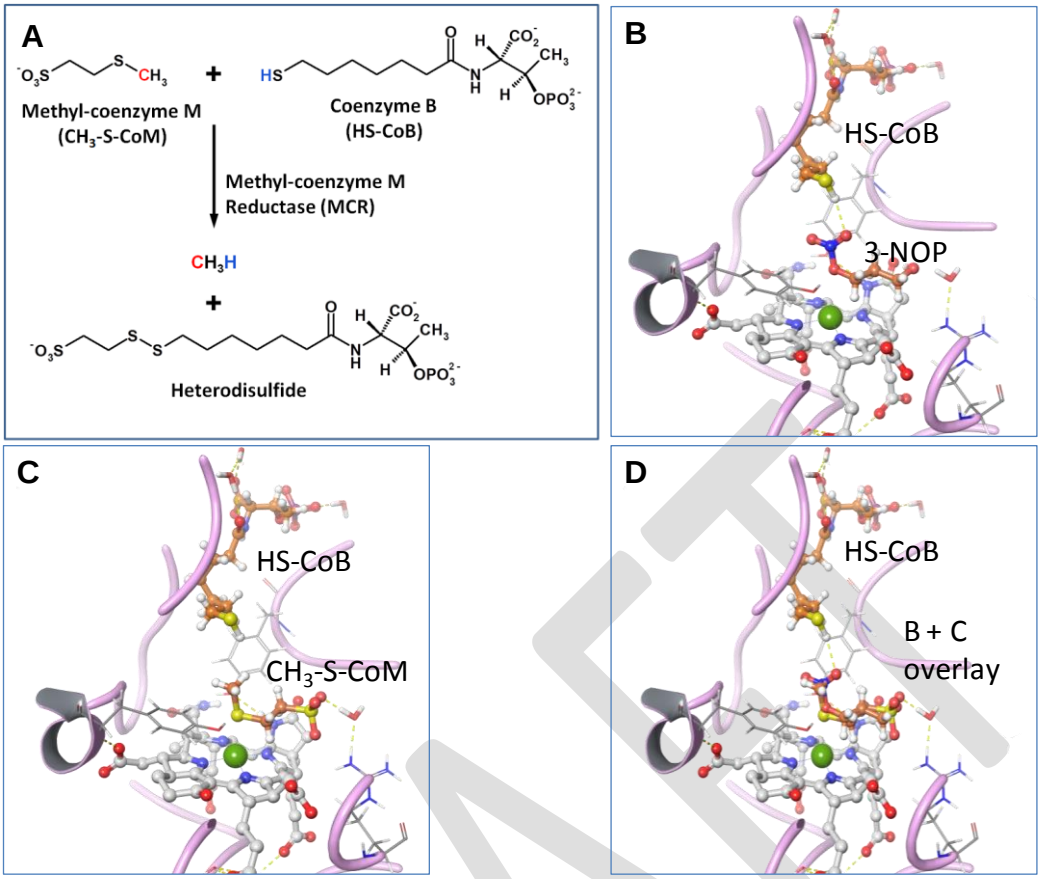

Fig. 2. Methyl-coenzyme M (MCR) catalyzed reaction(A) and docking studies with 3-nitrooxypropanol (3-NOP) (B) and methyl-coenzyme M (CH_3-S-CoM) bound in the active site. A 3-NOP/CH_3-S-CoM overlay is shown in fig.2D. 3-NOP**,** CH_3-S-CoM and HS-CoB are drawn as ball-and-stick models in orange and F_{430} in light gray highlighting nitrogen in blue, oxygen in red, sulfur in yellow and nickel(I) as a green sphere. The position of methyl-coenzyme M obtained via docking is almost identical to that found via EPR measurements(*21*).

Does 3-NOP really inhibit MCR *in vitro* and *in vitro* as predicted theoretically? Indeed, purified MCR was found to be inhibited by (at?) very low concentrations of the nitrate ester (fig. 3A). From the time course of inhibition it is evident that inhibition occurs by inactivation of MCR. Only 0.1 µM of 3-NOP were required to completely inactivate MCR within several minutes of exposure.

The mechanism of MCR inactivation was studied by looking at the effect of 3-NOP on the Ni(I) EPR signal MCR_{red1} of MCR(*22*) (fig. 3B). Prior to inactivation, the EPR spectrum corresponded to 95% to that of MCR_{red1} (the active Ni(I) form of the enzyme) and to 5% to that of MCR_{ox1} (an inactive Ni(III) form)(*22*). After inactivation by 3-NOP only the signal

corresponding to the 5% MCR_{ox1} were detected. Apparently, the MCR_{red1} signal was completely quenched, implying that Ni(I) in MCR_{red1} was oxidized to an EPR silent Ni(II) (see Methods).

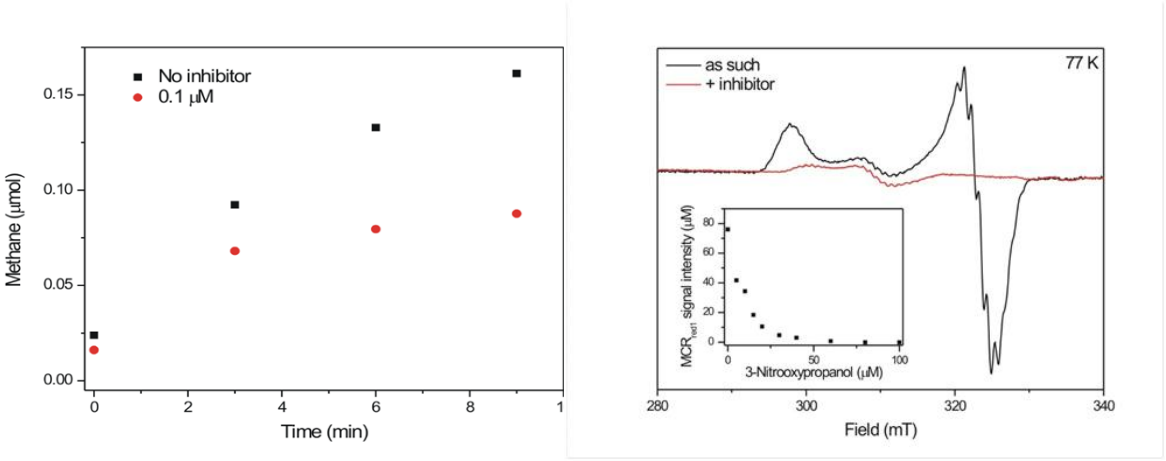

Fig. 3. Effect of 3-NOP on the activity (A) and EPR signals (B) of purified methyl-coenzyme M reductase (MCR) from *M. marburgensis*. MCR activity was measured by following methane formation from methyl-coenzyme M and coenzyme B. The reaction was started by the addition of enzyme. The EPR spectrum is from a sample that contained 190 µM Ni(I) (MCR_{red1}) and 10 µM Ni(III) (MCR_{ox1})(*22*). The spectrum remaining after MCR inactivation is that of MCR_{ox1} (see text). Fig.3B, insert: Quenching of the EPR signal MCR_{red1} by 3-NOP at different concentrations. The sample contained 78µM Ni(I) (MCR_{red1}

The insert in fig. 3B displays the change of EPR signal intensity over the course of titrating active MCR with 3-NOP. The EPR signal decreased with increasing concentrations of 3-NOP. Less than 20 µM 3-NOP were required for 50% quenching of the 78 µM MCR_{red1} signal. After complete inactivation, about 0.2 mol nitrite and 0.7 mol nitrate per mol MCR_{red1} quenched were found in the samples (fig. S1) indicating that 3-NOP was at least partly reduced to nitrite and 1,3-propanediol. Interestingly, nitrite was also found to inactivate isolated MCR at very low concentrations (fig. S2). The inactivation of MCR by nitrite explains why less than 1 mol 3-NOP was required to oxidize 1 mol of Ni(I) to Ni(II) in MCR (Fig. 3B, insert). 3-NOP therefore can be considered as double warhead(*23*) inhibitor. Sodium nitrate (fig. S2) and 1,3-propanediol, up to 10 mM, had no effect on the EPR spectra of MCR.

We were curious to see whether the products of 3-NOP reduction could be identified in the crystal structure of MCR inactivated by 3-NOP *in vivo*. Indeed, as structural comparison of active MCR and of 3-NOP inactivated enzyme revealed differences that can be interpreted to suggest that the reduction products of 3-NOP, namely nitrite and 1,3-propanediol, were trapped in the active site where they are not bound rigidly enough to be fully resolved by X-ray diffraction (fig.3S and table 1S).

After having shown that 3-NOP inactivates MCR *in vitro* we determined whether the nitrate ester is also effective *in vivo*. We first tested the effect of 3-NOP on growth with the model organism *Methanothermobacter marburgensis*. Upon addition of 3-NOP (final concentration 10 µM) to cultures of *M. marburgensis*, growth and methanogenesis almost immediately stopped (fig. 4). At a tenfold lower concentration of 3-NOP (1 µM), complete inhibition was also observed, but after five hours, growth and methane formation resumed again. It is known that methanogens contain a repair system that can reactivate MCR in a H_2-, ATP- and chaperone-dependent reduction process(*24-26*). Inhibition of methanogenesis is thus reversible.

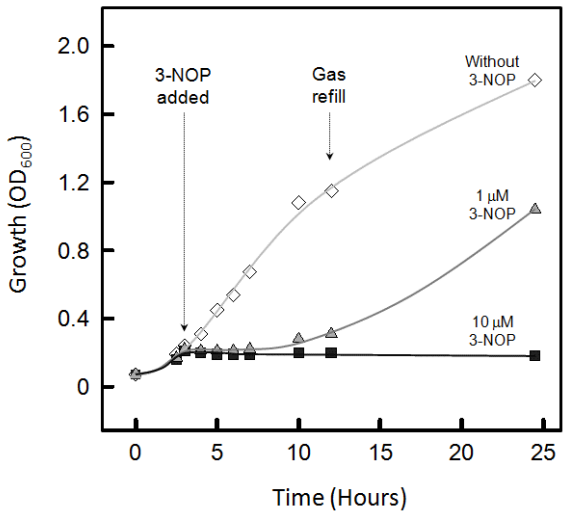

Fig. 4. Inhibition of growth of *M. marburgensis* on H_2 and CO_2 in the presence of 3-NOP.

Inhibition of methane production by 3-NOP was also observed with methanogens from the rumen and from other environments (table S2): *Methanobrevibacter ruminantium* ($[3\text{-NOP}]_{50\%}$ < 1 µM), *M. smithii* (1 µM), *M. millerae* (1 µM), *Methanobacterium bryantii* (1 µM), *Methanothermobacter wolfeii* (<1 µM), *Methanomicrobium mobile* (>50 µM), *Methanosphaera stadtmanae* (5 µM) *and Methanosarcina barkeri* (250 µM). At the 3-NOP concentrations given in brackets inhibition was only transient (as shown in fig. 4 for 1 µM 3-NOP). As a control, the effect of 3-NOP (100 µM) on the growth of non-methanogenic rumen bacteria such as *Ruminococcus albus, R. flavefaciens, Selenomonas ruminantium, Streptococcus bovis, Fibrobacter succinogenes, Anaerovibrio lipolytica, Prevotella. bryantii, P. ruminicola Megasphaera. elsdenii, Butyvibrio fibriosolvens, Clostridium aminophilum,* and *Escherichia coli* was tested (table S2). Growth of none of these cultures was negatively affected by the nitrate ester. Inhibition by 3-NOP is thus highly specific for methanogenic archaea in the rumen.

Since nitrate, nitrite and 1,3-propanediol were formed associated with MCR inactivation by 3-NOP, we also tested the effect of these compounds on the growth of *M. marburgensis*. At 10 µM concentration none of them were found to be growth inhibitory. At this low concentration they also appear not to be toxic to animals(*16, 27, 28*). 1,3-Propanediol(*29*) and nitrite(*30*) are normally occurring intermediates in the rumen.

In the past, two other specific inhibitors of MCR have been found, namely bromoethane sulfonate (BES) and bromopropane sulfonate (BPS). Both compounds exert their inhibitory effect *in vitro* at low concentrations by inactivation of MCR, BES at IC_{50} (concentration required for 50% inhibition) of 4 µM and BPS at IC_{50} of 0.05 µM(*31, 32*). The mechanism of inactivation has been shown to be an electrophilic attack of the bromo compounds on the Ni(I) resulting in its alkylation and oxidation(*33*). Because of the negatively charged sulfonate group of BES and BPS, both inhibitors cannot freely diffuse through the cytoplasmic membrane of methanogens and are therefore generally poor

inhibitors of methanogenesis *in vivo*. E. g., for growth inhibition of *M. marburgensis* more than 10 mM BES or BPS are required. However, for *M. ruminantium* an *in vivo* IC_{50} for BES of 1 µM was reported(*34*). This rumen methanogen is an exception in requiring coenzyme M as vitamin(*35*, *36*) and in containing a coenzyme M transporter(*37*), by which most probably also BES is actively taken up by the cells(*38*). However, the unfavorable toxicological profile of BES, because of its alkylating potential, prevents it from being authorized as a feed additive for ruminants(*34*).

In conclusion, 3-NOP specifically inhibits enteric methane emission from ruminants by inactivation of the enzyme MCR. The mode of action of this – so far unique – type of double warhead inhibitor was uncovered.

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The authors working at Auburn University and at the Max Planck Institute in Marburg declare no competing financial interests. The authors with DSM addresses have filed patent applications for nitrate esters as inhibitors of rumen methanogenesis.

All authors discussed the results and commented on the manuscript.

SUPPLEMENTAL MATERIALS

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Material and Methods

Figs. S1-S3; Tabls. S1 and S2

References (39-43)