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In vitro susceptibility of cultured human methanogens to lovastatin

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ABSTRACT

Lovastatin is a prodrug that is hydrolysed in vivo to β-hydroxy acid lovastatin, which inhibits 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-Co-A) reductase (HMGR), thereby lowering cholesterol in humans. A side effect of lovastatin is inhibition of isoprenoid synthesis and cell membrane formation in methanogenic Archaea, which are members of the human digestive tract microbiota and are emerging pathogens. In this study, the in vitro susceptibility of the human-associated methanogens *Methanobrevibacter smithii*, *Methanobrevibacter oralis*, *Methanobrevibacter massiliense*, *Methanobrevibacter arboriphilus* and *Methanomassiliicoccus luminyensis* to lovastatin (1–4 μg/mL) was tested in the presence of five gut anaerobes aiming to metabolise lovastatin into β-hydroxy acid lovastatin as confirmed by ultra-high-performance liquid chromatography. Five days of incubation with lovastatin had no measurable effect on the growth of the five gut anaerobes but significantly reduced CH_4 production and methanogen growth as measured by quantitative PCR ($P < 0.01$). Quantitative PCR analyses indicated that compared with controls, β-hydroxy acid lovastatin significantly increased the expression of the genes *mta* and *mcrA* implicated in methanogenesis and significantly decreased the expression of the *fno* gene implicated in methanogenesis. Expression of the HMGR gene (*hmg*) implicated in cell wall synthesis was significantly increased by β-hydroxy acid lovastatin ($P < 0.01$). These results strongly suggest that in the presence of gut anaerobes, lovastatin yields β-hydroxy acid lovastatin, which inhibits methane production and growth of methanogens by affecting their cell membrane biosynthesis. Lovastatin is the first licensed drug to exclusively affect the growth of methanogens whilst protecting the bacterial microbiota.

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1. Introduction

Archaea methanogens are the only confirmed biological sources of methane in humans but are still neglected micro-organisms forming parts of the intestinal, oral, vaginal and skin microbiota [1–6]. *Methanobrevibacter smithii* accounts for up to 94% of methanogens in the human intestinal microbiota, which also comprises *Methanobrevibacter oralis*, *Methanomassiliicoccus luminyensis*, *Methanosphaera stadtmanae* and *Methanobrevibacter arboriphilus* [4,7–9]. In the oral cavity, *M. oralis* and *Methanobrevibacter massiliense* have been convincingly linked to human periodontal diseases in modern and ancient populations [10–12]. These human-associated methanogens are broadly resistant to antibiotics, with only fusidic acid and metronidazole and its derivatives exhibiting in vitro activity against methanogens [13,14]. Lovastatin is a marketed serum cholesterol-lowering drug [15–18] that is also shown to reduce methane production in livestock [19]. This observation was linked to its activity against ruminal methanogens and more precisely interference with methanogen membrane biosynthesis, without affecting bacteria [19,20].

The prodrug lovastatin is converted into β-hydroxy acid lovastatin, the active form of the molecule, which inhibits 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (HMGR) [21,22]. In humans, this activation is mediated by intestinal anaerobes [23]. Surprisingly, biosynthesis of the isoprenoid cell membrane components in Archaea (archaeol) relies on the same HMGR enzyme that catalyses the biosynthesis of isoprenoid cholesterol in humans (mevalonate pathway). Despite the emerging role of methanogens in the physiology of the intestinal tract and their potential roles in gastrointestinal tract diseases [24–27] and extraintestinal diseases such as weight abnormalities [28,29], the impact of lovastatin on human-associated methanogens has never been studied.

Here we studied whether lovastatin has an inhibitory effect on the growth of human-associated methanogens following its activation by human anaerobes.

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Table 1
List of primers used in gene expression analyses.

Gene	Description	Primer sequence (5′→3′)	Size (bp)
16S rRNA	16S rRNA	Forward, GCTCAGTAACACGTGG Reverse, CGGTGTGTGCAAGGAG	343
mcrA	Methyl coenzyme M reductase, subunit A	Forward, TTCGGTGGATCDCARAGRGC Reverse, GBARGTCGWAWCCGTAGAATCC	140
fno	F420-dependent NADP reductase	Forward, GGGTTCAGCAGCAGAAAGG Reverse, CACATTCAATTGGGTCTGGA	118
hmg	HMG-CoA reductase	Forward, GGCTGTGAATTACCGCATATGG Reverse, TAACGGTCCGGCTACACCTACA	117
mta	Methanol:cobalamin methyltransferase	Forward, ATGTGGTGCAAAAGGACCTC Reverse, CAGAGTGTGCACAAACAGCA	112

2. Materials and methods

2.1. Micro-organisms

Methanobrevibacter smithii strain JMR02, *M. arboriphilus* strain ANOR1 and *M. luminyensis* strain B10 isolated from human faecal samples [8,9] and *M. oralis* strain N71 and *M. massiliense* strain N51 also isolated in our laboratory from dental plaque specimens [11] were used in this study. *Bacteroides thetaiotaomicron*, *Clostridium perfringens*, *Fusobacterium varium*, *Eubacterium limosum* and *Bifidobacterium longum* have also been isolated in our laboratory from stools as part of culturomics studies [30,31]. This study was approved by the Comité d'éthique de l'IFR 48 (Marseille, France).

2.2. Metabolism of lovastatin by human intestinal anaerobes

Lovastatin (25 mg) (Sigma-Aldrich, St Louis, MO) was dissolved in 1 mL of 70% ethanol to obtain a final stock concentration of 25 mg/mL. Then, 1 mL of methanogen medium (SAB medium) [9] was inoculated anaerobically in a Hungate tube (Dominique Dutscher S.a., Issy-les-Moulineaux, France) with 0.1 mL of each of the five bacterial cultures (ca. 18 h growth). Glucose (0.5%) was added as a substrate for bacteria and lovastatin was added at a final concentration of 100 μg/mL. This reaction mixture consisting of 1 mL of lovastatin and 1.5 mL of mixed bacteria in the SAB medium (pH 7.3) was incubated at 37 °C with continuous rotation (150 rpm) to allow bacterial hydrolysis of the lactone ring of lovastatin into β-hydroxy acid lovastatin, which is the active form of the molecule, thus mimicking the in vivo process of lovastatin activation in the gut. Lovastatin lactone form (Mevinolin, 98%, HPLC grade; Sigma-Aldrich) and β-hydroxy acid form (Cayman Chemicals, Ann Arbor, MI) were identified by ultra-high-performance liquid chromatography (UPLC) using an ACQUITY UPLC H-Class system (Waters, Saint-Quentin-en-Yvelines, France) as previously described [32].

2.3. In vitro susceptibility of methanogens to lovastatin

Following co-incubation for 48 h of mixed anaerobes and lovastatin, 0.1 mL of this mixture was added to 0.5 mL of one methanogen strain in a Hungate tube containing SAB medium to achieve final concentrations of 1, 2 and 4 μg/mL lovastatin. Commercial lovastatin contains 2% of the β-hydroxy acid form of lovastatin, thus the titrations of lovastatin β-hydroxy acid form at 1, 2 and 4 μg/mL represent 50, 100 and 200 μg/mL of lovastatin lactone form. Cultures were incubated with continuous rotation (150 rpm) at 37 °C for 5 days. In all experiments, control tubes without lovastatin, added as baseline growth controls, included five tubes containing SAB medium, bacteria mixture and one methanogen strain, one tube containing SAB medium without any addition and one tube containing SAB medium with 0.1 mL of 70% ethanol. Tubes containing each of the five bacteria at 10^4 CFU/mL and 10 mg/mL lovastatin were also included to study the effect of lovastatin on each bacterium. After 24 h of incubation, each mixture was inoculated on blood agar (bioMérieux, La Balme-Les-Grottes, France) for 48 h at 37 °C. Growth of methanogens was monitored by methane gas production in Hungate tubes as previously described [11]. In addition, real-time PCR was used to analyse the effect of lovastatin on the expression of the *hmg* gene involved in cell membrane biosynthesis as well as genes involved in methanogenesis including *mta* (methanol:cobalamin methyltransferase, catalysing the conversion of methanol into methyl-coenzyme M), *fno* (F420-dependent NADP reductase) and *mcrA* (last enzyme in the methanogenesis pathway) in the five tested methanogens [33,34] (see Table 1) as reported in the Supplementary material.

2.4. Statistical analysis

All experiments were performed in triplicate. Data were analysed as a completely randomised design using the general linear model procedure of SAS OnlineDoc 9.2 (SAS Institute Inc., Cary, NC). All multiple comparisons amongst means were performed using Duncan's new multiple range test ($\alpha = 0.05$).

3. Results

3.1. Identification of lovastatin metabolites

The chromatogram of lovastatin mixed with five human intestinal anaerobes was compared with those of the standards (lactone and β-hydroxy acid forms). Fig. 1 shows that commercial lovastatin was 99.7% in the lactone form and contained only traces of β-hydroxy acid form as previously reported [35]. Following incubation with intestinal anaerobes, the relative area of β-hydroxy acid lovastatin in the mixed culture of intestinal bacteria increased from 0.3% to 2.5%.

3.2. Micro-organism growth and CH_4 production

The five gut anaerobes grew as expected in the presence of 100 μg/mL lovastatin following 48 h of subculture on sheep blood agar. Also, the human-associated methanogens incubated for 5 days with the five bacteria without lovastatin yielded methane production in the presence of negative controls which yielded no methane. In contrast, the five methanogens under study yielded a 95% decrease in CH_4 production in culture tubes containing 1 μg/mL and 2 μg/mL lovastatin, whilst no CH_4 production was detected in cultures containing 4 μg/mL lovastatin ($P < 0.01$) (Fig. 2).

3.3. Effect of lovastatin on gene expression

The fact that there was no detectable methanogen growth and no CH_4 production in the presence of 4 μg/mL lovastatin and low

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