In vitro and in vivo efficacies of amlodipine against *Listeria monocytogenes*

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**Abstract** *Listeria monocytogenes* causes suppurative gastritis in BALB/c mice. We investigated the effect of the antihypertensive drug amlodipine (Aml) on the growth of *L. monocytogenes* in vitro and in vivo. Aml showed noteworthy inhibitory action (minimum inhibitory concentration, MIC<sub>90</sub> 32 μg/ml) against *Listeria* strains and demonstrated cidal (minimum bactericidal concentration, MBC 64 μg/ml) activity. Aml administered orally at 2.5 μg/g in female BALB/c mice for 7 days, commencing 4 days before oral challenge (1×10<sup>8</sup> CFU/ml with *L. monocytogenes* ATCC 51774), significantly reduced bacterial counts in the stomach (*P*<0.01), liver (*P*<0.01), and spleen (*P*<0.05), and decreased (*P*<0.05) gastric lesions, neutrophilic infiltration, edema, vascular degeneration, and necrosis of gastric tissues. It caused the down-regulation of expression of inflammatory cytokines (IFN-γ, IL-1β, and TNF-α) compared to drug-free control. Aml may be used in the presence of an antibiotic as adjunct therapy that boosts the host immunity against *Listeria*. Further, QSAR studies might contribute in manipulating it as a lead compound for the synthesis of new, more effective non-antibiotics (helper compounds), perhaps devoid of side-effects, that could be recommended as compassionate therapy for listeriosis.

*L. monocytogenes* is a Gram-positive, motile, ubiquitous bacterium, capable of growing at −4°C to 50°C. It is the causative agent of listeriosis. This organism has been recognized as an animal pathogen as early as the 1920s, but its role in several outbreaks of food-borne illnesses in humans has been speculated only in the past two decades. The main acquisition route of listeriosis has been established to be through food, but the ingestion of foods having sparse populations of *L. monocytogenes* does not significantly affect most healthy humans. However, existing chronic illness, immuno-suppression, pregnancy, or extreme youth or age (less than 1 year or over 60 years) can make some groups of the population predisposed to the development of listeriosis. This translates to a major public health problem, because in such sections of the population, listeriosis is fatal in up to 30% of cases. Earlier, patients diagnosed with listeriosis were typically treated with penicillin or ampicillin in combination with an aminoglycoside, although the use of tetracycline, erythromycin, or chloramphenicol, singly or in conjunction, has also been reported. Ampicillin and gentamicin used together is the current therapy of choice for all forms of listeriosis [1].

In general, species of *Listeria* are sensitive to antibiotics that are inhibitory to other Gram-positive bacteria. However, recent reports suggest a tendency of antibiotics resistance in this class of bacteria. Such instances are in line with a global incidence of escalating antibiotics resistance, including multiple drug resistances among several bacterial classes. The cause of this phenomenon has been attributed to the excessive exploitation of antibiotics in animals and humans ever since their discovery several decades ago. Furthermore, many antibiotics-resistant saprophotrophic or commensal food-borne species might transfer their resistance genes to some pathogenic food-borne bacteria, including
those within the gastrointestinal tract, leading to adverse clinical implications for the host.

Under such challenging circumstances, there is an urgent need for the discovery of antilisterial compounds possibly acting through mechanisms different from those of existing drugs, namely, the 'non-antibiotics' [2, 3], acting by the enhancement of antibiotic activity or the reversal of antibiotic resistance, as well as by the induction and control of efflux pumps.

Non-antibiotics have been defined as a variety of compounds that are employed in the management of pathological conditions of a non-infectious etiology, but additionally exhibit broad-spectrum antimicrobial activity in vitro as well as in vivo against a variety of Gram-positive and Gram-negative bacteria [2, 3]. Research on such unconventional antibacterial agents over the last few decades has revealed that the anti-hypertensive calcium channel blocker amlodi- pine (Aml) possesses noticeable in vitro and in vivo antibacterial activity [4, 5]. Aml has been found to synergistically enhance the efficiency of the aminoglycoside streptomycin [5] against specific bacteria.

*L. monocytogenes* is common worldwide and patients with listerial infection are often administered an antihypertensive drug (Aml) to treat cardiovascular disorders. We undertook this study to evaluate the effects of Aml (at a conventional anti-hypertensive dose) on the treatment of suppurative gastritis caused by *L. monocytogenes* in BALB/c mice [6–8], with a special emphasis on the mechanism of action of this drug.

Nine strains (including seven serotypes) were chosen for this study, as described previously [9]. Aml was obtained from Sigma. Determination of the minimum inhibitory concentration (MIC) and the bactericidal/bacteriostatic nature of this drug were performed according to procedures described previously [2, 9]. Animal experiments were performed following the guidelines [8] for the care and use of laboratory animals. The characteristics of the gastritis caused by oral challenge with *L. monocytogenes* ATCC 51774 infection in BALB/c mice along with the time course of infection has been reported earlier [6–8]. In the present study, the drug (2.5 mg/kg/day) was administered for 7 days, commencing 4 days before oral challenge with *L. monocytogenes* ATCC 51774 in order to neutralize gastric acid [8]. Aml was administered for 7 days, commencing 4 days before oral challenge at a dose of 2.5 mg/kg body weight per day to all animals in Group 3. Group 2 was administered sterilized PBS instead of Aml for those 7 days. During all experimental periods, food was removed from the cages 4 h prior to drug administration. Bacterial enumeration in organs, histology, RNA extraction, and cytokine mRNA expression were analyzed as previously described [8].

Aml at 32 µg/ml resulted in significantly lower OD₆₂₀ values for all nine strains of *L. monocytogenes* (from 4 h onwards); *L. monocytogenes* ATCC 51774 was the most sensitive strain compared to the positive controls (without Aml) (Fig. 1a).

By the agar dilution method, the MIC₉₀ of Aml was found to be 32 µg/ml for all strains of *L. monocytogenes*. The MIC₉₀ values of each of gentamicin, ciprofloxacin, and ceftriaxone an...
d) Fundus | NGR | Pylorus

Negative control

Positive control

Drug treated

e) TNF-α | IFN-γ | IL-1β | GAPDH

| Negative control | Positive control | Drug treated |
rifampicin were 1.0 μg/ml, tetracycline 2.0, polymyxin B 64.0, and penicillin G 0.5 μg/ml, with respect to the nine strains.

Aml showed bactericidal action, since the addition of 64 μg/ml (2×MIC) at the logarithmic phase resulted in a marked reduction of CFU count at 12 h. Further, it showed bactericidal action (99.9% killing) compared to the control (without Aml) (Fig. 1b).

Treatment with Aml significantly reduced bacterial counts in the stomach (P<0.01), liver (P<0.01), and spleen (P<0.05) of infected mice, as compared to the positive control (saline-treated). Bacteria were not detected in the stomach, liver, and spleen samples of the non-treated (negative control) animals (Fig. 1c).

In the stomachs of the untreated (negative control) mice, no or mild neutrophilic infiltration was observed in the submucosa of the non-glandular region (NGR), fundus, or pylorus (Fig. 1d).

The stomach of mice infected with L. monocytogenes ATCC 51774 revealed marked infiltration of neutrophils in the mucosa, submucosa, lamina muscularis, and serosa of the NGR, fundus, and pylorus (Fig. 1d). Necrosis and degeneration of the gastric glands, edema, and congestion were seen in the lamina propria of the fundus and pylorus. In the submucosa of all parts of the stomachs, connective tissue was loose and expanded due to edema. Vacular degeneration and massive necrosis of muscle cells was observed in lamina muscularis. Aml diminished llisterial infection in the stomach, liver, and spleen. The mean ± SD of the severity of the drug-treated group was significantly (P<0.05) decreased compared to the positive control group.

The expression of inflammatory cytokines was assessed by analyzing the mRNA levels in mouse liver samples. Host resistance to L. monocytogenes infection is controlled by cell-mediated immunity that is regulated by endogenous cytokines, such as IFN-γ (TH1 cytokine), IL-1β, and pro-inflammatory cytokines like TNF-α. Independent, essential functions for TNF in murine responses to L. monocytogenes most likely reflect the role of TNF in facilitating the early recruitment of neutrophils, which are crucial for the containment of early listerial infection. As shown in Fig. 1c, the mRNA levels of all (IL-1β, TNF-α, and IFN-γ) these cytokines increased (P<0.05) in the liver samples of positive control mice compared to the non-treated group (negative control), whereas there was a tendency of down-regulation (IL-1β, TNF-α, and IFN-γ) in the drug-treated group compared to the positive control group.

To better understand the activity of Aml and its mechanism of action against L. monocytogenes, nine strains, including seven serotypes, have been used. More specifically, L. monocytogenes strain ATCC 51774 is a serotype 1/2a (genomic division I) that was one of the most responsible pathogens for the outbreak of listeriosis and successfully caused gastritis in a murine model.

In the current study, the MIC of Aml against L. monocytogenes was found to be 52 μg/ml and the minimum bactericidal concentration (MBC) was 64 μg/ml. These results are in accordance with data from other workers. The MIC of Aml was reported to be 10-50 μg/ml against 12 standard reference strains, including Staphylococcus aureus NCTC 8530, 8532, ML 123, 324, Bacillus pumilus NCTC 8241, B. licheniformis NCTC 10341, Salmonella typhimurium NCTC 74, Shigella dysenteriae NCTC 519/6, Sh. boydii NCTC 254/66, and Vibrio cholerae ATCC 14033, 14035, 865. Aml is cidal against Gram-positive bacteria [4, 5].

Aml could offer significant protection to mice challenged with a virulent bacterium. Rats and mice treated with Aml in the diet for up to two years, at concentrations calculated to provide daily dosage levels of 0.5, 1.25, and 2.5 mg/kg/day, showed no evidence of a carcinogenic effect of the drug. In accordance with such reports, it was found in this study that mice could tolerate Aml for the entire period of the experiment [4, 5].

Here, protection was achieved at 2.5 mg/kg/day×7 days, which is the conventional dose (10 mg/day) of Aml as an anti-hypertensive agent. An uncommon dose of 50 mg/L for an adult human with 5 L of blood translates to a 10-mg/L plasma concentration, which is close to the 32 μg/ml microbial inhibitory concentrations observed in our in vitro studies. This could be possible because Aml is rapidly and completely absorbed after oral administration. Oral bioavailability ranges from 52 to 88%. Peak plasma concentrations are achieved between 6 to 9 h post-dose, and maximum hypotensive effects are correspondingly delayed. The drug is approximately 93% bound to plasma proteins. Aml is extensively metabolized to inactive compounds, and 10% of the parent compound and 60% of the inactive metabolites are excreted in the urine. The mean terminal half-life of Aml is 35 h following a single-dose administration, which is significantly longer than dihydropropyridines, which are currently available. The fact that the mice can stand this drug better than humans is because of the faster heart rate of mice (800 beats/ min), which results in rapid distribution/alteration of the drugs through the liver and, hence, detoxification by the cytochrome system is faster and more extensive. Moreover, non-antibiotics may be concentrated more than 10-fold by macrophages that have phagocytosed bacteria. Our results reflect the findings by Martin et al. that the curative activity of murine tuberculosis by another non-antibiotic phenothiazine thioridazine is significant in the pulmonary systems but not in the liver and spleen [10].

Aml is a well-known immunoregulator. RT-PCR studies in this experiment showed that the mRNA levels of cytokines like TNF-α, IFN-γ, and IL-1β decreased in the
liver samples of Aml-treated mice compared to the non-treated ones (Fig. 1). It has been reported that the drug significantly inhibits nitrite production both in LPS [Escherichia coli (serotype O127:B8)] and IFN-γ-stimulated rat aortic smooth muscle cells and in the rat model of septic shock accompanied by the suppression of iNOS induction. Similarly, in the rat endotoxic model, the serum NOX, TNF-α, and IL-1β levels, as well as the iNOS expression of lungs, were also suppressed by the administration of Aml. The drug has been shown to prevent a decrease in the vascular responsiveness induced by the injection of Salmonella typhosa LPS.

Aml has been able to cure a highly virulent bacterial infection in mice. This indicates a similar potential of this drug for the management of specific multi-drug-resistant bacterial infections of humna. Further, Aml could be employed as a ‘lead compound’ to synthesize more active novel agents that might be free of side-effects.

Aml structurally correlates with other non-antibiotics in consisting of a benzene ring attached to another that may be considered as an incomplete phenothiazine ring. Moreover, the presence of a halogen (chlorine) moiety may be crucial in conferring antimicrobial property.

Detection of the moiety responsible for antibiotic property may be possible by QSAR studies in Aml. Such studies are likely to contribute to the generation of a novel adjuvant to existing therapies of problematic bacterial infections, like listeriosis.

Recently, phenothiazines, as possible resistance modifiers, were shown to co-micellize a wide variety of poorly soluble antifungal, antibacterial, and anticancer drugs. This feature also promises neurotropic/antibiotic efficacy for antimicrobe and anticancer treatment. The combination of the non-antibiotics, as helper compounds, with classical antibiotics holds intriguing promise for forthcoming new therapies and may allow the management of listerial infections.

References