Methyl Gallate from *Galla rhois* Successfully Controls Clinical Isolates of *Salmonella* Infection in Both In Vitro and In Vivo Systems

Jang-Gi Choi¹,², Su-Hyun Mun³, Harendra S. Chahar², Preeti Bharaj², Ok-Hwa Kang¹, Se-Gun Kim¹, Dong-Won Shin⁴, Dong-Yeul Kwon¹*  

¹ Department of Oriental Pharmacy, College of Pharmacy, Wonkwang University, Wonkwang Oriental Medicines Research Institute, Institute of Biotechnology, Jeonbuk, Korea, ² Center of Excellence in Infectious Disease Research, Department of Biomedical Sciences, Paul L. Foster School of Medicine, Texas Tech University Health Sciences Center, El Paso, Texas, United States of America, ³ BK21 Plus Team, Professional Graduate School of Oriental Medicine, Wonkwang University, Iksan, Jeonbuk, Republic of Korea, ⁴ Department of Oriental Medicine Resources, Sunchon National University, Jeonnam, Republic of Korea

Abstract

*Galla rhois* is a commonly used traditional medicine for the treatment of pathogenic bacteria in Korea as well as in other parts of Asia. Methyl gallate (MG), a major component of *Galla Rhois*, exhibits strong antibacterial activity, but its mechanism of action against *Salmonella spp.* is unclear. In the present study, we investigated the antibacterial actions of MG against *Salmonella*. The antibacterial activity determined by broth dilution method indicated that the antibacterial activity of MG against *Salmonella* strains ranged from 3.9 to 125 μg/ml. *In vitro* bacterial viability test indicated that MG significantly decreased the viability of *Salmonella* over 40% when combined with ATPase inhibitors. The time-kill curves showed that a combined MG and ATPase inhibitors (DCCD and NaN3) treatment reduced the bacterial counts dramatically after 24 h. Oral administration of MG showed a strong anti-bacterial activity against WS-5 infected BALB/c mice. In contrast to the untreated *Salmonella* infected control animals, MG treated groups showed no clinical symptoms of the disease, such as lethargy and liver damage. It was observed that MG treatment significantly increased the survival of animals from *Salmonella* infection, while in untreated groups all animal succumbed to disease by the sixth day post infection. Thus, the present study demonstrates the therapeutic ability of MG against *Salmonella* infections.

Introduction

*Salmonella* enterica is a gram-negative bacterial pathogen capable of infecting animals and humans, causing significant morbidity and mortality worldwide [1]. *Salmonella* is a clinically important intracellular bacterial pathogen that leads to food poisoning and gastroenteritis in millions of people worldwide each year [2]. It is even a problem in industrialized nations, and the Centers for Disease Control (CDC) estimate that there are nearly 1.4 million foodborne *Salmonella* infections annually in the United States [3]. *S. Typhimurium*, *S. Enteritidis* and *S. Typhi* were the most frequent *Salmonella* serovars in foodborne diseases and diarrhea patients in Korea from 1998 to 2007 [4]. Similarly, serotypes that are highly adapted to poultry include *S. Gallinarum*, resulting in high morbidity and mortality [5].

Following oral acquisition, *Salmonella* infects the intestinal tract and can disseminate to cause systemic infection of various organs including the liver [6]. One major concern to public health has been the global dissemination of *Salmonella* Typhimurium definitive Type 104, which is commonly resistant to five or more antimicrobial agents [7–10]. Recent reports of infections related to strains of *Salmonella* with high-level resistance to antibiotics are therefore particularly worrying. The rise in antibiotic-resistant pathogens has led to the development of new therapeutic agents that are effective against these bacteria. Recently, there has been considerable interest in the use of plant materials as an alternative method to control pathogenic microorganisms [11], and many compounds of plant products have been shown to specifically target against resistant pathogenic bacteria [11].

*Galla rhois* is the term used for the gall caused by the Chinese sumac aphid, *Schlechtendalia chinensis* (Bell), on the nutgall sumac tree, *Rhus javanica* L. (*Anacardiaceae*) [12]. It has been used in traditional Korean medicine and other oriental medicine systems for years. It has long been used for the treatment of diarrhea, prolonged coughing, and spontaneous perspiration in Korea. It is a natural non-toxic traditional Korean medicine and contains several tannin-derived components, such as methyl gallate (MG) and gallic acid [12]. MG is a phytochemical compound with strong antioxidant properties found in various species including *Melia aerata*, *Galla Rhois*, *Rosa Rugosa* and it is also well known as an anti-oxidative beverage [13]. In addition, MG possesses other biological activities such as anti-platelet activity, protection of DNA damage against oxidative stress [14], protection of lung injury induced by phosgene [15], attenuation of diabetic oxidative...
stress and anti-apoptotic activity [15]. MG is known to perform a wide spectrum of biological activities, but its mechanism for antibacterial activity against Salmonella remains unclear. We hypothesized that there is synergy between MG and bacterial membrane permeabilization/binding agents such as tri(hydroxymethyl)aminomethane (TRIS), triton X-100 (TX) [16–18]. MG may be responsible for its increased antibacterial activity against Salmonella. Second, MG may exert its inhibitory effect on the bacterial replication by inhibiting cytochrome oxidase or by inhibition of proton-driven ATPases [16–18]. As a result, we decided to investigate the in vitro activities of MG separately or in combination with the bacterial membrane-binding agents TRIS and TX, and the ABC transporter-inhibiting agents NaN3 and an inhibitor of proton-driven ATPases N,N′-Dicyclohexylcarbodiimide (DCCD). In addition, we investigated the antimicrobial activity of MG against Salmonella in vivo.

Materials and Methods

Plant materials

Galla Rhois, purchased from the Oriental drug store Daehak Hanyak kak (Iksan, Korea), was authenticated by Dr. D.Y. Kwon. A voucher specimen (No. 06-021) was deposited in the Laboratory of Herbalogy, College of Pharmacy, Wonkwang University, Iksan, Korea.

Bacterial strains

Various Salmonella listed in Tables S1, were used in this study. We also tested the antimicrobial activity of MG against local isolates of S. Enteritidis, S. Gallinarum and S. Typhimurium, which were generously provided by the National Veterinary Research and Quarantine Service, Republic of Korea and kept as frozen glycerol stock. Cells in frozen stock were streaked onto nutrient agar medium to produce cell colonies, from which a single colony was transferred to Mueller-Hinton agar (MHA). For preparation of inocula, cells were grown for 20 h at 37°C in Mueller-Hinton broth (MHB). For mouse infection, cultured bacterial cells (WS-5) were recovered by centrifugation at 13,000 rpm for 30 s and then washed with and resuspended in PBS to the desired concentration of bacteria using a standard suspensions was measured. The cell suspensions were diluted with Mueller-Hinton broth (MHB) and the ABC transporter-inhibiting agents NaN3 and inhibitor of proton-driven ATPases N,N′-Dicyclohexylcarbodiimide (DCCD). In addition, we investigated the antimicrobial activity of MG against Salmonella in vivo.

Isolation of methyl gallate

The EtOH extracts were partitioned with organic solvents of different polarities to yield n-Hexane, EtOAc, n-BuOH and H2O fractions, in sequence (Figure 1). The EtOAc fraction of each plant was subjected to silica gel chromatography with CH2Cl2-MeOH-H2O (lower layers, by volume, 5:1:1→7:3:1) as the solvents to yield MG from Galla Rhois (Figure 1). The structure of the compound (Table 1) was determined by its physico-chemical and spectral data (1H-NMR and 13C-NMR) which were in agreement with those reported in literature [22,23].

Antimicrobial resistance testing

Resistance of the Salmonella strains to the different antimicrobial agents was determined using the disc-agar method based on interpretations according to Clinical and Laboratory Standards Institute (CLSI) [24]. The quality control strains were Enterococcus faecalis ATCC 29212 and Escherichia coli ATCC 25922 (Table S1).

Disc diffusion

The paper disc diffusion method was used to determine anti-bacterial activity [25]. Sterile paper discs (6 mm; Toyo Roshi Kaihsa, Japan) were loaded with 20 μl of MG (varying concentrations: 10, 50, and 100 μg) dissolved in 10% dimethyl sulfoxide (DMSO, Sigma, USA), and were left to dry for 12 h at 37°C in a sterile room. The bacterial suspensions were diluted to match the 0.5 McFarland standard scale (approximately 1.5×10⁸ CFU/ml), and were further diluted to obtain the final inoculum. The MHA was poured into petri dishes and inoculated with 100 μl of the suspension containing 1×10⁹ CFU of bacteria. The inhibition zone diameter around each of the discs was measured and recorded at the end of the incubation period. Ampicillin was included as positive control and 10% DMSO served as negative controls.

Determination of the minimum inhibitory concentrations (MICs)

The minimal inhibition concentration (MIC) values were determined for the microorganisms we found to be sensitive to MG during the disc diffusion assay. A preparation of the microorganism inocula was from 12 h broth cultures and the suspensions were adjusted to a 0.5 McFarland standard turbidity. Susceptibility tests were carried out by the standard broth microdilution method [26] in MHB with an inoculum of approximately 5×10⁸ CFU/ml. The MHB was supplemented with serial MG at.

Reagents and instruments

NMR spectra were measured with a JEOL Eclipse 500 FT-NMR spectrometer (1H, 500 MHz; 13C 125 MHz). Column chromatography was carried on silica-gel (Kieselgel 60, 70–230 mesh, Merck, Germany), a thin layer chromatography on silica-gel F254 (0.25 mm, Merck) and Sephadex LH-20 (25–100 μM, Sigma, U.S.A), Mueller-Hinton broth (MHB) and Mueller-Hinton agar (MHA) (Difco Laboratories, Baltimore, MD, USA). Ampicillin, amoxicillin/clavulanic acid, chloramphenicol, cephalothin, sulfisoxazole, nalidixic acid, norfloxacain, streptomycin, trimethoprim/sulfamethoxazole, ticarcillin, ciprofloxacin, N,N′-Dicyclohexylcarbodiimide, Sodium azide, Tris, Triton X-100 and solvents were purchased from Sigma Aldrich (St. Louis, USA).

Figure 1. Isolation of methyl gallate from Galla rhois. (A) Chemical structure of methyl gallate, (B) Procedures of extraction and fraction of the Galla Rhois, (C) Column chromatographic procedures of n-EtOAc fraction. doi:10.1371/journal.pone.0102697.g001
In vitro were diluted with fresh MHB to different time-points (0, 2, 4, 8 and 24 h) (12). Bacterial cultures using time-kill curves of bacterial growth in 96-well plates at five evaluated by time-kill assay as described previously [18]. The Synergy was defined as log10 CFU/ml (99.9%) for each of the indicated time-points [22]. Antimicrobial agents were considered to be bactericidal at the performed on plates and 30–300 colonies were counted. The lower free MHA plate following incubation for 24 h. Colony counts were time-point, the number of viable cells was determined on a drug-dilutions were prepared in saline. For samples obtained from each taking following 0, 2, 4, 8 and 24 h of incubation, and serial 10-fold incubated at 37°C for 24 h. Aliquots (0.1 ml) of the culture were then incubated overnight at 37°C on duplicate Salmonella–Shigella agar plates (Difco), which were fecal suspensions were serially diluted in PBS and then were plated bacteria per gram in feces were determined. Aliquots (100 μl) of the bacterial suspensions and the numbers of the administered to the animals in similar manner. Fecal samples were collected at 0, 1, 3, 4 and 5 days after administering the bacterial suspensions and the numbers of the bacteria per gram in feces were determined. Aliquots (100 μl) of fecal suspensions were serially diluted in PBS and then were plated on duplicate Salmonella–Shigella agar plates (Difco), which were then incubated overnight at 37°C, and typical colonies were counted for plates containing between 30 and 300 colonies. Confirmation of S. Typhimurium was performed by a PCR assay described by a previous report [20,21].

Table 1. 1H- and 13C-NMR spectral data of compound 1 (methyl gallate) isolated from Galla Rhois.

| Carbon NO. | 1H-NMR | 13C-NMR |
|------------|--------|---------|
| 1          | 6.93   | 119.8   |
| 2          | 109.1  |         |
| 3          | 146.1  |         |
| 4          | 138.9  |         |
| 5          | 146.1  |         |
| 6          | 109.1  |         |
| COOCH3     | 166.8  |         |
| COOCH3     | 52.1   |         |

Solvent: DMSO-d6. doi:10.1371/journal.pone.0102697.t001

concentrations from 0.97 to 1000 μg/ml. The data were reported as MICs, the lowest concentration of MG inhibiting visible growth after 24 hours of incubation at 37°C [26]. The MICs of ampicillin was also determined, and similarly defined as the lowest antibiotic concentration at which no visible bacterial growth was observed.

Anti-bacterial activity with detergents or ATPase-inhibitors
To elucidate whether anti-bacterial activity of MG was associated with the altered membrane permeability or antibacterial susceptibility, effect of MG was examined in the presence of detergents or ATPase-inhibiting agents. Minimum inhibitory concentrations (MICs) of the selected antimicrobial agents (detergents or ATPase-inhibiting agents) were determined by micro broth dilution assays. To increase the permeability of the outer membrane, the concentration of MG, (a fractional inhibitory concentration (FIC) determined in a combination assay with other therapeutic agents) was added to bacterial cells in the presence of 0.001% Triton X-100, and 125 μg/ml Tris, respectively. NaN3 and DCCD were used as inhibitors of ATPase [16–18]. The antibacterial susceptibility of MG in the presence of 0.005% NaN3 and 125 μg/ml DCCD, was also carried out at the same condition. The viability of bacteria was determined by a spectrophotometer (optical density at 600 nm, OD600) after incubation for 24 h. In vitro synergy between the agents was evaluated by time-kill assay as described previously [18]. The synergy between each pair of antimicrobial agents was determined using time-kill curves of bacterial growth in 96-well plates at five different time-points (0, 2, 4, 8 and 24 h) [12]. Bacterial cultures were diluted with fresh MHB to ~1.5×10⁵ CFU/ml, and incubated at 37°C for 24 h. Aliquots (0.1 ml) of the culture were taken following 0, 2, 4, 8 and 24 h of incubation, and serial 10-fold dilutions were prepared in saline. For samples obtained from each time-point, the number of viable cells was determined on a drug-free MHA plate following incubation for 24 h. Colony counts were performed on plates and 30–300 colonies were counted. The lower limit of sensitivity for the colony counts was 100 CFU/ml. Antimicrobial agents were considered to be bactericidal at the lowest concentration that reduced the original inoculum by 3 log10 CFU/ml (99.9%) for each of the indicated time-points [22]. Synergy was defined as ≥2 log10 decrease in the number of CFU/ml between the combination and the most active compound [27,28]. To confirm the results, time-kill assays for each experiment were performed in triplicate. Data are presented as the mean ± standard deviation [18].

In vivo assay using mice
Mice were obtained from Da Mool Science (Daejeon, Korea). All mice experiments in this study were approved by the Wonkwang National University Animal Ethics Committee in accordance with the guidelines of the Korean Council on Animal Care. Thirty male BALB/c mice (15–17 g) between 5 and 6 weeks old were used for all in vivo experiments. All animals were kept in a temperature-controlled room under a 12 h light ~12 h dark cycle. Animals had free access to commercial solid food (SCF Co., Ltd. Korea) and water ad libitum, and were acclimatized for at least 1 week prior to beginning the experiments. Mice were divided into the following groups: Salmonella-infected (SI), and Salmonella-infected + MG (SIMG). For survival and fecal shedding each treatment group contained 10 mice in SI and SIMG group. Each mouse was caged individually and subjected to infection as described below. For histopathologic examinations, a separate set of 5 mice per group (SI and SIMG) were infected and sacrificed consecutively for 3 days post infection. Tissue specimens of the liver were transferred into 10% buffered neutral formalin for histopathologic examinations and then processed using standard procedures. Sections of paraffin-embedded tissues were then stained with hematoxylin and eosin. Throughout each experiment, mice were provided with water that contained streptomycin (5 mg/ml) to reduce the level of facultative anaerobic bacteria that normally colonize the mouse intestine [28–30]. S. Typhimurium (WS-5) was grown overnight in Luria–Bertani broth (Difco), centrifuged, washed in phosphate-buffered saline (PBS), and then diluted to achieve a final concentration of 1×10⁴ CFU. Mice were orally infected with S. Typhimurium (1×10⁴ CFU) using a gavage needle; the suspensions were then diluted in 20% sucrose and fed to the SI and SIMG groups. One hour after infection, animals in the SIMG group were orally administered 50 mg/kg of the MG daily by gavage needle as described before [28], SI group treated with sterile PBS and administered to the animals in similar manner. Fecal samples were collected at 0, 1, 3, 4 and 5 days after administering the bacterial suspensions and the numbers of the bacteria per gram in feces were determined. Aliquots (100 μl) of fecal suspensions were serially diluted in PBS and then were plated on duplicate Salmonella–Shigella agar plates (Difco), which were then incubated overnight at 37°C, and typical colonies were counted for plates containing between 30 and 300 colonies. Confirmation of S. Typhimurium was performed by a PCR assay described by a previous report [20,21].
We investigated bacterial viability in the presence of MG with saccharide (LPS) [31,32]. These agents did not have any outer membrane in Gram negative bacteria by binding lipopolysaccharide reagents used 1/2 MIC) that can increase the permeability of the membrane-permeabilizing agents (all reagents used 1/2 MIC) disrupting electrochemical proton gradients in a bacterial environment [33,34]. MG in combination with NaN3 and DCCD significantly decreased the viability of WS-5 over 40% (Figure S1). Time–kill assays were performed for MG in combination with ATPase inhibitors (NaN3 and DCCD) for two strains S. Typhimurium (WS-5) and S. Typhi ATCC 19943 (WS-7) strains. Figure 2A and B shows that MG and DCCD alone at 1/2 MIC had very weak effect against both stationary-phase WS-5 and WS-7. NaN3 alone at 1/2 MIC reduced the CFU counts 3–5 log of bacteria 24 h of incubation. However, the combination of MG and DCCD or NaN3 reduced the CFU counts to zero at 24 h of incubation for WS-7 and also MG and DCCD or NaN3 reduced CFU counts 2–4 log the period of 24 h of incubation for WS-5. The time–kill assay demonstrated that there was a significant synergistic activity between MG and NaN3 or DCCD for the Salmonella strains tested (Figure 2).

Results

Anti-bacterial activity of MG against Salmonella

The antimicrobial efficacy of MG against the ten Salmonella strains was evaluated by the disc diffusion method via determination of the surrounding inhibition zones, as well as by evaluating the MIC using the broth micro dilution method. Table 2 shows the antimicrobial activity of MG determined by the disc diffusion method. The values of the inhibition zones produced against the tested bacteria ranged between 12 and 26 mm. The growth of all the tested strains was inhibited at 100 µg per disc. In addition, the response was dose-dependent, meaning the higher the dose the wider the inhibition zone. The MICs for MG and ampicillin against the 10 strains of Salmonella are shown in Table 3. The MICs determined using the broth dilution method confirmed the antimicrobial effects found through the disc diffusion method. MG showed antimicrobial activity against all the tested strains. The MICs of MG against the 10 Salmonella strains ranged from 3.9 to 125 µg/ml, and for ampicillin from 0.09 to 1000 µg/ml.

Anti-bacterial activity with detergents or ATPase-inhibitors

To investigate the effects of enhanced membrane permeability on the activity of MG using detergents, the anti-bacterial activity of MG under increased membrane permeability was examined using 125 µg/ml Tris, and 0.001% Triton X-100. Tris and Triton X-100 all of which are membrane-permeabilizing agents (all reagents used 1/2 MIC) that can increase the permeability of the outer membrane in Gram negative bacteria by binding lipopolysaccharide (LPS) [31,32]. These agents did not have any enhancing effect on the antibacterial activity of MG (Figure S1). We investigated bacterial viability in the presence of MG with 0.005% NaN3 and 125 µg/ml DCCD (all reagents used 1/2 MIC) as a metabolic inhibitor which can decrease ATP levels by disrupting electrochemical proton gradients in a bacterial environ-ment [33,34]. MG in combination with NaN3 and DCCD significantly decreased the viability of WS-5 over 40% (Figure S1). Time–kill assays were performed for MG in combination with ATPase inhibitors (NaN3 and DCCD) for two strains S. Typhimurium (WS-5) and S. Typhi ATCC 19943 (WS-7) strains. Figure 2A and B shows that MG and DCCD alone at 1/2 MIC had very weak effect against both stationary-phase WS-5 and WS-7. NaN3 alone at 1/2 MIC reduced the CFU counts 3–5 log of bacteria 24 h of incubation. However, the combination of MG and DCCD or NaN3 reduced the CFU counts to zero at 24 h of incubation for WS-7 and also MG and DCCD or NaN3 reduced CFU counts 2–4 log the period of 24 h of incubation for WS-5. The time–kill assay demonstrated that there was a significant synergistic activity between MG and NaN3 or DCCD for the Salmonella strains tested (Figure 2).

In vivo assay using mice

Barthel et al. [30] described a versatile animal model to understand the molecular mechanisms of enteric salmonellosis. We employed a similar in vivo model in the present study to determine the protective effects of MG against S. Typhimurium (WS-5) infection in mice. Briefly, mice were infected with 1 × 10^4 CFU of S. Typhimurium (SI). One hour later, the mice were orally administered MG (50 mg/kg). As shown in Figure 3A, treatment with the MG was found to have marked effects on mortality and in the MG treated group 70% of the animals survived while all animals in the untreated group infected with Salmonella died by the sixth day post infection. MG was found to have marked effects on the numbers of viable S. Typhimurium recovered from feces. At day 3 post-infection, seven of ten mice in the test group did not shed viable S. Typhimurium in feces, whereas all mice (1 mouse died) in the control group shed bacteria ranging 1.7 × 10^3–1.2 × 10^4 CFU/g in feces (Figure 3B). S. Typhimurium-infected mice that did not receive the MG were lethargic and showed signs of histological damage in the liver. In addition, the central and portal veins of the liver showed congestion with focal necrotic emboli-like materials (Figure 3C). Five different sections from liver of each mice were tested for necrotic lesions and neutrophil expression. There were multiple small necrotizing nodular lesions in the liver parenchyma with inflammatory cell infiltrate. Conversely, clinical signs and histo-

Table 2. Antimicrobial activity (as the inhibition zone diameter) of methyl gallate (MG) and ampicillin against Salmonella.

| Strains | Serotypes | Origin   | MG   | Ampicillin |
|---------|-----------|----------|------|------------|
|         |           |          | 10 µg| 50 µg | 100 µg | 10 µg |
| WS-1    | S. Gallinarum ATCC 9184 | Chicken | ND | 11 | 20 | ND |
| WS-2    | S. Gallinarum       | Chicken | ND | ND | 15 | ND |
| WS-3    | S. Gallinarum       | Chicken | ND | ND | 14 | 27 |
| WS-4    | S. Typhimurium | Cattle | ND | ND | 12 | 30 |
| WS-5    | S. Typhimurium | Pig | ND | 7 | 12 | ND |
| WS-6    | S. Enteritidis      | Human | 7 | 18 | 25 | 27 |
| WS-7    | S. Typhi ATCC 19943 | Human | 8 | 15 | 23 | 30 |
| WS-8    | S. Paratyphi A      | Chicken | 7 | 14 | 26 | 28 |
| WS-9    | S. Enteritidis      | Chicken | ND | 13 | 20 | 33 |
| WS-10   | S. Enteritidis      | Chicken | ND | 10 | 13 | 31 |

ND, No activity detected. * Positive control.
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logical damage were rarely observed in S. Typhimurium-infected mice fed with MG.

Discussion

Due to the recent appearance of the “Super Bacteria” of Salmonella DT 104 showing resistance to multiple antibiotics and the intractable bacterial types of Escherichia coli (E. coli O157, O111, O26) releasing stronger toxin due to the application of antibiotics, the development of new antibiotics is urgently required, which is even tendered as a social issue [35,36]. To overcome the emerging problem of bacterial antibiotic resistance, studies investigating plant extracts with antibiotics against clinical strains have been reported [7–9].

Galla Rhois is naturally found in Korea and China, where it is known as Chinese Sumac and has been used in the treatment of cold, fever, cough and malaria. It was previously reported that the methanol extract of Galla Rhois had significant growth-inhibitory activity towards both Clostridium perfringens and Escherichia coli [12]. MG is the main component of Galla Rhois, displaying several biological activities, and presents activity against Salmonella. It is observed that the anti-bacterial activity of MG described in this study is in agreement with results reported by others, showing that the activity of Galla Rhois, and of other species against Salmonella, is due to this compound [37,38]. The potential of MG to enhance the activity of antibiotics against Salmonella was studied previously [37,38] but the function of MG in Salmonella is still unclear. Therefore we investigated for the mechanism of MG against Salmonella. Antibacterial agents such as β-lactams antibiotics are inhibitor of cell wall synthesis [39], and fluoroquinolone antibiotics are inhibitor of DNA gyrase against both Gram-positive and Gram-negative bacteria [40]. In this study, MG was used in combination with detergents (TX and Tris) responsible for increasing the membrane permeability of bacterial strain. However, additional decrease in bacterial replication was not observed as determined by the OD value, where the bacterial culture was treated with MG along with cell of membrane-permeabilizing agents. Which indicates that may be MG does not interfere with cell wall synthesis. In contrast, MG in combination with ATPase inhibitors (NaN3 and DCCD) significantly decreased the viability of Salmonella WS-5 as determined by spectrophotometry. As determined in time-kill studies, MG in combination with ATPase inhibitors shows synergistic effect against WS-5 and WS-7. This finding indicates that the primary mechanism of MG action is via DNA gyrase or ATPase inhibition in the Salmonella but not via cell wall synthesis inhibition. In our previous study, we found that the combination of MG, at sub-MIC concentrations, with ciprofloxacin and nalidixic acid significantly improved the

Table 3. Antimicrobial activity of MG and ampicillin against 10 strains of Salmonella.

| Strains | Serotypes     | Origin   | *MIC (µg/ml) |
|---------|---------------|----------|--------------|
|         |               |          | MG          | bAmpicillin |
| WS- 1   | S. Gallinarum ATCC 9184 | Chicken  | 31.25        | 1000        |
| WS- 2   | S. Gallinarum  | Chicken  | 3.9         | 1000        |
| WS- 3   | S. Gallinarum  | Chicken  | 15.6        | 0.97        |
| WS- 4   | S. Typhimurium | Cattle   | 3.9         | 0.97        |
| WS- 5   | S. Typhimurium | Pig      | 15.6        | 1000        |
| WS- 6   | S. Enteritidis | Human    | 3.9         | 1.95        |
| WS- 7   | S. Typhi ATCC 19943 | Human | 3.9         | 0.97        |
| WS- 8   | S. Paratyphi A | Chicken  | 31.25       | 0.97        |
| WS- 9   | S. Enteritidis | Chicken  | 125         | 0.97        |
| WS- 10  | S. Enteritidis | Chicken  | 31.25       | 1.95        |

* MIC, Minimum inhibitory concentration. b Positive control.

Figure 2. Time-kill curves for (A) S. Typhimurium (WS-5) and (B) S. Typhi ATCC 19943 (WS-7) with MG and ATPase inhibitors (DCCD and NaN3). These results were confirmed in three independent experiments.

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Figure 3. Therapeutic effects of MG treatment on mice infected with Salmonella. (A) the survival of mice (n = 10, per group) treated or untreated with MG, infected with WS-5, (B) Effects of feeding MG on fecal shedding of S. Typhimurium (CFU/g) from mice. (C) Histopathological changes in liver of Salmonella-infected and Salmonella-infected treated MG. Salmonella-infected (SI) and Salmonella-infected + MG (SIMG). All data with P<0.05 were considered significant.

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activity of the antibiotic [37,38]. MG and fluoroquinolone antibiotics (ciprofloxacin and nalidixic acid) directly or indirectly attack the same target: DNA gyrase or ATPase inhibition in the Salmonella [39,41]. The additive or neutral effects of MG in combination with other agents strongly support this explanation. In the in vitro experiments using mice, the test group dosed with 50 mg/kg of MG, showed clear MG mediated protection which resulted in survival of 70% of mice during 6 days of experiment. However, all the mice in SI group died by 6 day of the experiment. In addition, the cytological identification of liver in MG dosed mice revealed less expression of neutrophil in SIMG group as compared to SI group by showing the treatment effect of MG against murine salmonellosis.

Hence the findings of our study clearly demonstrate the usefulness of MG in treatment of Salmonella infections and may provide the preliminary supportive data for in-depth studies regarding the mechanism of action of MG against bacterial pathogens. Therefore, it is possible to conclude that MG has the potential for use in the treatment of Salmonella infections in mice.

Supporting Information

Figure S1 The effects of membrane-permeabilizing agent and ATPase-inhibitor agent on Salmonella (WS-5) susceptibility to MG. The viability of bacteria was determined by a spectrophotometer (optical density at 600 nm, OD600) after incubation for 24 h with 1/2 MIC MG and the indicated concentration of Tris, TX, NaN3 and DCCD in WS-5 the data are Mean±S.D for triple-independent experiments. A(Tris), B(TX), C(NaN3), D(DCCD).

Table S1 List of Salmonella strains used in this study and growth inhibition zones produced by antibiotics.

Author Contributions

Conceived and performed the experiments: JC DK. Performed the experiments: JC SM OK SK DK. Analyzed the data: JC HG PB DS DK. Contributed reagents/materials/analysis tools: JC DK. Wrote the paper: JC DK.

References

1. Fink SL, Cookson BT (2007) Pyroptosis and host cell death responses during Salmonella infection. Cell Microbiol 9: 2562–2570.
2. Grasso GA, Valdez Y, Bergstrom KS, Vullance BA, Finlay BB (2008) Chronic enteric salmonella infection in mice leads to severe and persistent intestinal fibrosis. Gastroenterology 134: 768–780.
3. Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, et al. (1999) Food-related illness and death in the United States. Emerg Infect Dis 5: 607–625.
4. Kim S (2010) Salmonella serovars from foodborne and waterborne diseases in Korea, 1997–2000: total isolates decreasing versus rare serovars emerging. J Korean Med Sci 25: 1695–1699.
5. Barrow PA, Freitas Neto OC (2011) Pullorum disease and fowl typhoid—new paper: JC DK. Contributed reagents/materials/analysis tools: JC DK. Wrote the paper: JC DK.

3. Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, et al. (1999) Food-related illness and death in the United States. Emerg Infect Dis 5: 607–625.
4. Kim S (2010) Salmonella serovars from foodborne and waterborne diseases in Korea, 1997–2000: total isolates decreasing versus rare serovars emerging. J Korean Med Sci 25: 1695–1699.
5. Barrow PA, Freitas Neto OC (2011) Pullorum disease and fowl typhoid—new paper: JC DK. Contributed reagents/materials/analysis tools: JC DK. Wrote the paper: JC DK.

1. Fink SL, Cookson BT (2007) Pyroptosis and host cell death responses during Salmonella infection. Cell Microbiol 9: 2562–2570.
2. Grasso GA, Valdez Y, Bergstrom KS, Vullance BA, Finlay BB (2008) Chronic enteric salmonella infection in mice leads to severe and persistent intestinal fibrosis. Gastroenterology 134: 768–780.
3. Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, et al. (1999) Food-related illness and death in the United States. Emerg Infect Dis 5: 607–625.
4. Kim S (2010) Salmonella serovars from foodborne and waterborne diseases in Korea, 1997–2000: total isolates decreasing versus rare serovars emerging. J Korean Med Sci 25: 1695–1699.
5. Barrow PA, Freitas Neto OC (2011) Pullorum disease and fowl typhoid—new paper: JC DK. Contributed reagents/materials/analysis tools: JC DK. Wrote the paper: JC DK.
Europe 2000–2004: a report from the Enter-net International Surveillance Network. Microb Drug Resist 14: 31–35.

37. Choi JG, Kang OH, Lee YS, Oh YC, Chae HS, et al. (2009) Antibacterial activity of methyl gallate isolated from Galla Rhois or carvacrol combined with nalidixic acid against nalidixic acid resistant bacteria. Molecules 14: 1773–1780.

38. Choi JG, Kang OH, Lee YS, Oh YC, Chae HS, et al. (2008) In vitro activity of methyl gallate isolated from galla rhois alone and in combination with ciprofloxacin against clinical isolates of salmonella. J Microbiol Biotechnol 18: 1848–1852.

39. Perez-Capilla T, Baquero MR, Gomez-Gomez JM, Ionel A, Martin S, et al. (2005) SOS-independent induction of dinB transcription by beta-lactam-mediated inhibition of cell wall synthesis in Escherichia coli. J Bacteriol 187: 1515–1518.

40. Falconer SB, Czarny TL, Brown ED (2011) Antibiotics as probes of biological complexity. Nat Chem Biol 7: 415–423.

41. Mascellino MT, Farinelli S, Iegri F, Iona E, De Simone C (1998) Antimicrobial activity of fluoroquinolones and other antibiotics on 1,116 clinical gram-positive and gram-negative isolates. Drugs Exp Clin Res 24: 189–191.