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Original Article

Loxapine, an antipsychotic drug, suppresses intracellular multiple-antibiotic-resistant *Salmonella enterica* serovar Typhimurium in macrophages



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Abstract *Background:* The emergence of multiple-antibiotic-resistant (MAR) *Salmonella* has been a serious threat worldwide. *Salmonella* can invade into host cells and evade the attacks of host humoral defenses and antibiotics. Thus, a new antibacterial agent capable of inhibiting intracellular *Salmonella* is highly needed.

Methods: The anti-intracellular activity and cytotoxicity of drugs on intracellular bacteria and macrophages were assayed using intracellular CFU assay and MTT cell viability assay, respectively. The uptake of gentamicin into macrophage and the effect of autophagy inhibitor on loxapine's anti-intracellular *Salmonella* activity were assessed by using image-based high-content system. The expression of bacterial genes was measured by real-time PCR. The efflux pump activity of bacteria was measured by Hoechst accumulation assays.

Results: With our efforts, an antipsychotic drug, loxapine, was identified to exhibit high potency in suppressing intracellular MAR *S. Typhimurium*, *Staphylococcus aureus*, *Shigella flexneri* or *Yersinia enterocolitica*. Subsequent investigations indicated that loxapine's anti-intracellular bacteria activity was not associated with increased penetration of gentamicin into bacteria and macrophages. Loxapine didn't inhibit bacterial growth in broth at concentration up to 500 μ M and has no effect on *Salmonella*'s type III secretion system genes' expression.

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Blockage of autophagy also didn't reverse loxapine's anti-intracellular activity. Lastly, loxapine suppressed bacterial efflux pump activity in all bacteria tested.

Conclusion: Altogether, our data suggested that loxapine might suppress intracellular bacteria through inhibiting of bacterial efflux pumps. In light of its unique activity, loxapine represents a promising lead compound with translational potential for the development of a new antibacterial agent against intracellular bacteria.

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Introduction

Salmonella enterica, a Gram-negative facultative intracellular bacterium, is one of the leading causes of gastrointestinal infection worldwide.^{1,2} *S. enterica* causes diseases from minor diarrhea to life-threatening typhoid fever, gastro-intestinal bleeding or perforation.² Among its six subspecies and more than 2500 serotypes, *S. enterica* serovar Typhimurium (hereafter *S. Typhimurium*) infects both humans and animals, and causes typhoid-like symptoms in mice, making it a useful model in studying *Salmonella* pathogenesis and evaluating new treatments for salmonellosis.³ After invading host cells, *Salmonella* resides and proliferates in endosome-like vesicles, called *Salmonella*-containing vacuoles (SCVs).⁴ Membranes of host cells and SCVs can protect the intracellular *Salmonella* from the attacks of host defenses and antibiotics with poor membrane permeability, such as aminoglycosides.² Moreover, the emergence and spread of *Salmonella* strains with resistance to multiple antibiotics, including amoxicillin, chloramphenicol, streptomycin, sulfamethoxazole, tetracycline, cotrimoxazole, azithromycin, fluoroquinolones, imipenem, ceftriaxone and colistin, have further limited the choice of effective treatment.^{5–11} Thus, WHO has included antibiotic-resistant *Salmonella* on the list of priority pathogens with urgent need for new antibiotics.¹²

Phenothiazines are antipsychotic drugs with the ability to antagonize dopamine D2 receptor and serotonin 5-HT2A receptor.¹³ In addition to the antipsychotic activity, phenothiazines have also been reported to possess antibacterial activity against a variety of Gram-positive, Gram-negative bacteria and Mycobacteria, including those resistant to multiple antibiotics.^{14–17} For example, chlorpromazine, the first neuroleptic compound for psychotic disorders, was shown to exhibit suppressive activity against *Mycobacterium tuberculosis* as well as to improve the antibacterial activity of several antibiotics at *in vitro* and *in vivo*.¹⁸ A similar complementary effect is observed in another phenothiazine drug, thioridazine.¹⁹ It is noteworthy that a clinical report indicated that thioridazine can act synergistically with antibiotics to eradicate tuberculosis from patients infected with extensively drug-resistant *M. tuberculosis*.²⁰ In light of its unique synergistic activity with antibiotics, the phenothiazine structure has been proposed as a potential scaffold for developing novel antibacterial agents against MAR bacteria.¹⁴

Previously, we demonstrated a celecoxib-derived compound, AR-12, exhibited potent antibacterial activities against intracellular *S. Typhimurium* and *Francisella*

tularensis via induction of autophagy and inhibition of Akt kinase in macrophages.^{3,21,22} In this study, we identified an antipsychotic, loxapine, which is clinically similar to phenothiazine, having potent inhibitory activity against intracellular MAR *S. Typhimurium* in macrophages without appreciable cytotoxic effect toward the host cells. Our data indicate that loxapine has no direct effect on the growth and virulence of *S. Typhimurium*. In addition to *Salmonella*, intracellular proliferation of methicillin-resistant *Staphylococcus aureus* (MRSA), *Shigella flexneri* and *Yersinia enterocolitica* were also inhibited by loxapine. Together, these findings suggested that loxapine possesses a broad-spectrum antibacterial activity.

Material and methods

Bacteria

S. Typhimurium ATCC14028 was obtained from American Type Culture Collection (Manassas, VA). The MAR *S. Typhimurium* (type ACSSuT) strains 0911R and NL-08.10 were obtained from Dr. Chien-Shun Chiou, Centers for Disease Control, Taiwan, and strains BN9181, CG618 were obtained from Dr. Chi-Shih Chu, National Chiayi University, Taiwan. The MRSA USA300 was obtained from Dr. Lee-Jene Teng, National Taiwan University, Taiwan. The *Y. enterocolitica* and *S. flexneri* were obtained from Dr. John S. Gunn, the Ohio State University, Columbus, Ohio. Bacteria were cultured in Luria Bertani (LB) broth (Athena Enzyme Systems, Baltimore, MD) at 37 °C and stored in LB broth supplemented with 15% glycerol at –80 °C.

Cells

Murine macrophage cell line RAW264.7 was obtained from Bioresource Collection and Research Centre (Hsinchu, Taiwan) and maintained in Dulbecco's Modified Eagle Medium (DMEM, GIBCO-BRL, Invitrogen Corp., Carlsbad, CA) supplemented with 10% heat-inactivated FBS (GIBCO-BRL) and 4.5 g/L D-glucose. Cells were cultured in 75-T flask at 37 °C in a 5% carbon dioxide atmosphere.

Intracellular CFU assay

RAW264.7 cells were seeded in a 6-well plate at 5.0×10^5 cell/well and infected by bacteria at the multiplicity of infection (M.O.I) = 10 for 30 minutes. After infection, cells were washed and then exposed to 100 µg/

mL gentamicin (USB, Santa Clara, CA) for 1 hour to kill non-invaded bacteria. Afterwards, cells were treated with gentamicin (20 µg/mL), or a combination with loxapine (Sigma–Aldrich, St. Louis, MO). After 24h, cell culture medium collected and serially diluted in PBS. Infected-cells were washed, lysed with 0.1% Triton X-100 for 10 min at 37 °C and then serially diluted in PBS. Diluted culture medium and cell lysates were spread on LB agar plates and incubated at 37 °C for 18 h. The bacteria colonies grown on plates were counted and expressed as CFU.

Cell viability assay

A total of 1.0×10^4 cells was seeded into each well of a 96-well plate and treated with varying concentrations of drugs for 24h followed by the addition of MTT substrate (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide) to a final concentration of 0.5 mg/mL. Cells were incubated at 37 °C for 1h, and the reduced MTT dye in each well was dissolved with 100 µL DMSO. The absorbance of each well at 570 nm was measured with a microplate reader (VersaMax, Molecular Devices, CA, USA). The IC_{50} of individual drug was determined from dose–response curve using CalcuSyn software (Biosoft, Cambridge, UK).

MIC assay

The minimum inhibitory concentration (MIC) of gentamicin was determined by the broth microdilution method following the guidelines of Clinical and Laboratory Standards Institute.²³ The bacteria suspension was diluted in cation-adjusted Müller-Hinton (CAMH) broth (Difco Laboratories, Detroit, MI) to a final concentration of 5.0×10^5 CFU/mL and then treated with gentamicin at escalating doses ranging from 0.125 to 64 µg/mL in a U-shape 96-well plate. After incubation at 37 °C for 20h, the MIC was determined as the lowest concentration with no visible growth.

Bacteria growth assay

Bacteria suspension was diluted in CAMH broth or cell culture medium to a final concentration of 5.0×10^5 CFU/mL followed by exposing to different concentrations of loxapine in a flat-bottom 96-well plate. The plate was incubated at 37 °C, and the bacterial growth was monitored by measuring the absorbance at 600 nm at designated times for a total of 96h.

Gentamicin uptake

RAW264.7 cells seeded in a 96-well black plate were treated with 100 µg/mL of gentamicin for 1h, washed with PBS and then treated with 20 µg/mL of gentamicin alone or in combination with different concentrations of loxapine for 24h. After treatment, cells were washed and fixed with 4% formaldehyde for 30 min at 37 °C. To reduce auto-fluorescence, cells were quenched with 0.1 M glycine in PBS for 15 min followed by incubation in blocking buffer (1% BSA in PBS) for 1h. Cells were then permeabilized with 0.1% Triton-X for 1h at room temperature. Intracellular

gentamicin was detected by using fluorescein isothiocyanate (FITC) conjugated anti-gentamicin antibody (GeneTex, Hsinchu City, Taiwan) for 1h. Cell nuclei were stained with 0.5 µg/mL DAPI for 20 min. Cells were photographed using a high-content system (Cellomics ArrayScanR, Thermo Fisher Scientific), and fluorescence signals were quantified using HCS Studio® image analysis software (Thermo Fisher Scientific).

Image-based high content assay

RAW264.7 cells seeded in a 96-well black plate with clear bottom (Greiner Bio-one, Germany) were infected by pBR-RFP transformed *S. Typhimurium* at M.O.I = 50 for 1h, washed with PBS and then exposed to 100 µg/mL gentamicin and 3-MA (10 mM, TCI) for 1h. Then cells were washed and treated with loxapine (4 µM) or mock for 24h. Afterwards, cells were wash and stained with CellTracker Green (Thermo Fisher Scientific) followed by fixation in 4% formaldehyde for 20 min at 37 °C and stained with 0.1 µg/mL DAPI (4,6-diamidino-2-phenylindole; AAT Bioquest, Sunnyvale, CA) for 20 min. The fluorescence signals of DAPI, CellTracker Green and RFP were captured by ImageXpress Micro 4 High-Content System (Molecular Devices) at wavelength of 447 nm, 536 nm and 624 nm, respectively, and quantified using MetaXpress image analysis software (Molecular Devices) to analyze the viability of intracellular bacteria.

Gene expression assay

S. Typhimurium ATCC14028 was treated with 4 µM of loxapine or mock for 4h at 37 °C, collected by centrifugation and washed with DEPC-treated water twice. The total RNA of bacteria was extracted using Trizol LS reagent (Thermo Fisher Scientific), treated with DNase I (Qiagen, Germany) at 37 °C for 30 min and then converted to cDNA using SuperScript III First Strand Synthesis system (Thermo Fisher Scientific). The quantitation of individual gene's expression was performed using LabStar SYBR qRT-PCR kit (LabTurbo, Taipei, Taiwan) with primer pairs (Table 2) on a 7500 real-time PCR system (Applied Biosystems, CA). The expression level was normalized by subtracting the cycle threshold (Ct) of individual gene with that of the housekeeping gene, and expressed as ΔCt .

Hoechst accumulation assay

Salmonella cultures were diluted to an OD600 of 0.1 in PBS supplemented with 2.5 µM Hoechst 33342 (Thermo Fisher Scientific) and loxapine or CCCP (Sigma–Aldrich), a known efflux pump inhibitor. Fluorescence was monitored on a SpectraMax M5 (Molecular devices) with a 355 nm excitation filter and 460 nm emission filter.

Statistical analysis

Data are expressed as means \pm the standard deviation (SD). Differences between group means were calculated using a two-tailed Student's t-test for independent samples and were considered significant at $P < 0.05$.

Results

Loxapine exhibited potent activity against intracellular *S. Typhimurium* without cytotoxic effects on the host cells

Salmonella can invade and proliferate inside macrophage where it can evade the attack of humoral immune system and become less susceptible to antibiotics. To identify a new antibacterial agent with anti-intracellular *Salmonella* activity, the effect of a collection of non-antibiotic drugs in combination with gentamicin on intracellular *S. Typhimurium* ATCC14028 in RAW264.7 murine macrophages was evaluated. The screening identified an antipsychotic drug, loxapine, exhibiting potent activity to suppress intracellular *S. Typhimurium* in macrophages. As shown, the number of bacteria inside macrophages (intracellular bacteria) and in the medium (extracellular bacteria) were both reduced by loxapine in a dose-dependent manner. The concentration of loxapine required for 50% reduction (EC50) of intracellular bacteria was 4.6 μM (Fig. 1A–C). This suppressive effect was not associated with reduced

macrophage viability, as our data indicate that loxapine was not cytotoxic to RAW264.7 cells at concentrations up to 64 μM (Fig. 1D). Thus, loxapine can suppress intracellular *Salmonella* without causing cytotoxic effect on host cells.

Loxapine is active against MAR or fluoroquinolone-resistant *S. Typhimurium*

The emergence and spread of MAR *S. Typhimurium* strains (ACSSuT type), with resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline, has been a serious threat to public health.²⁴ As data shown in Fig. 2A, the infectivity and intracellular proliferation ability of these MAR *S. Typhimurium* strains, except strain BN9181, were higher than the reference *S. Typhimurium* strain ATCC14028, as demonstrated by a 2- to 3-fold increase in the total bacteria load in mock treated groups. Despite differences in bacterial infectivity, the intracellular growth of these MAR *S. Typhimurium* isolates was all suppressed by loxapine (Fig. 2A).

Next, we extended the test on *S. Typhimurium* isolates with resistance to ciprofloxacin, which is a

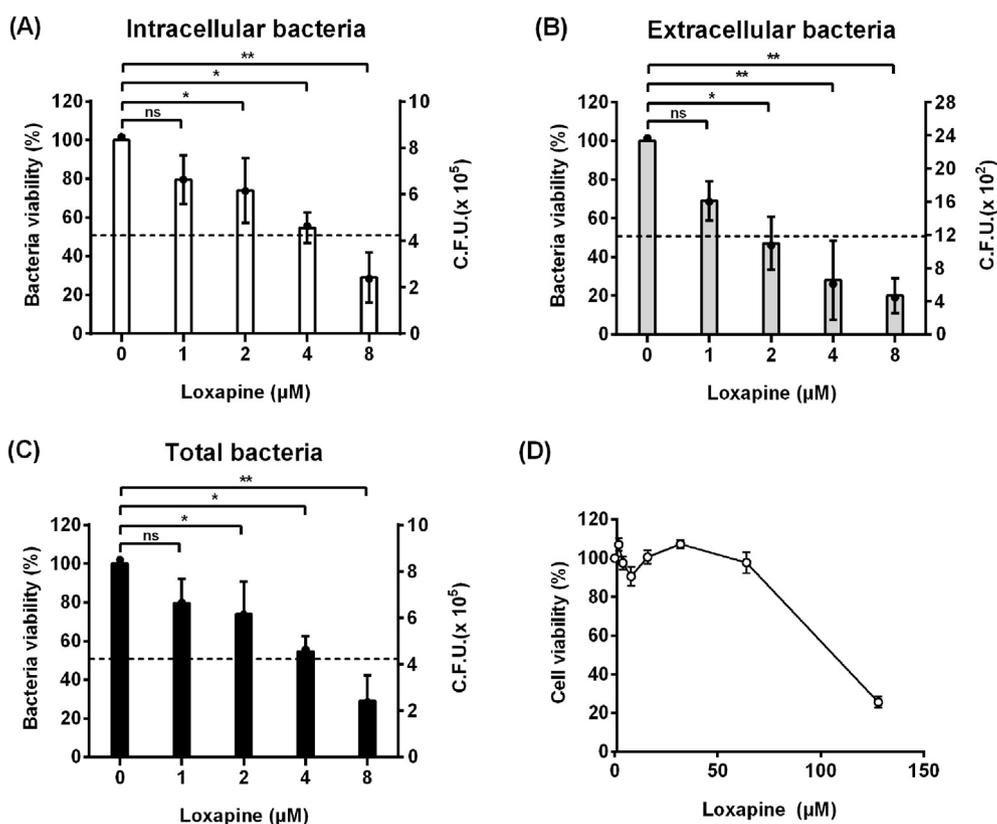


Figure 1. Loxapine suppressed intracellular *S. Typhimurium* in RAW264.7 macrophages. *Salmonella*-infected RAW264.7 cells were treated with various concentrations of loxapine in combined with 20 $\mu\text{g}/\text{mL}$ of gentamicin for 24 h. The number of (A) intracellular, (B) extracellular, and (C) total bacteria (sum of intracellular and extracellular bacteria) were analyzed and presented as CFU (right Y axis). The left Y axis presented the percentage (%) of viable bacteria in cells treated with loxapine comparing to that treated with gentamicin-only (100%). Data are presented as means \pm SD ($n = 3$). Statistical significance was measured with a two-tailed t -test. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). (D) The cytotoxic effect of loxapine on RAW264.7 cells was assessed using MTT cell viability assay. Cells were treated with various concentrations of loxapine for 24 h followed by the addition of MTT reagent to measure the viability of cells. Data are presented in percentage of viability comparing to that of mock treated cells (100% of viability). Points indicated means, and bars indicated SD ($n = 6$).

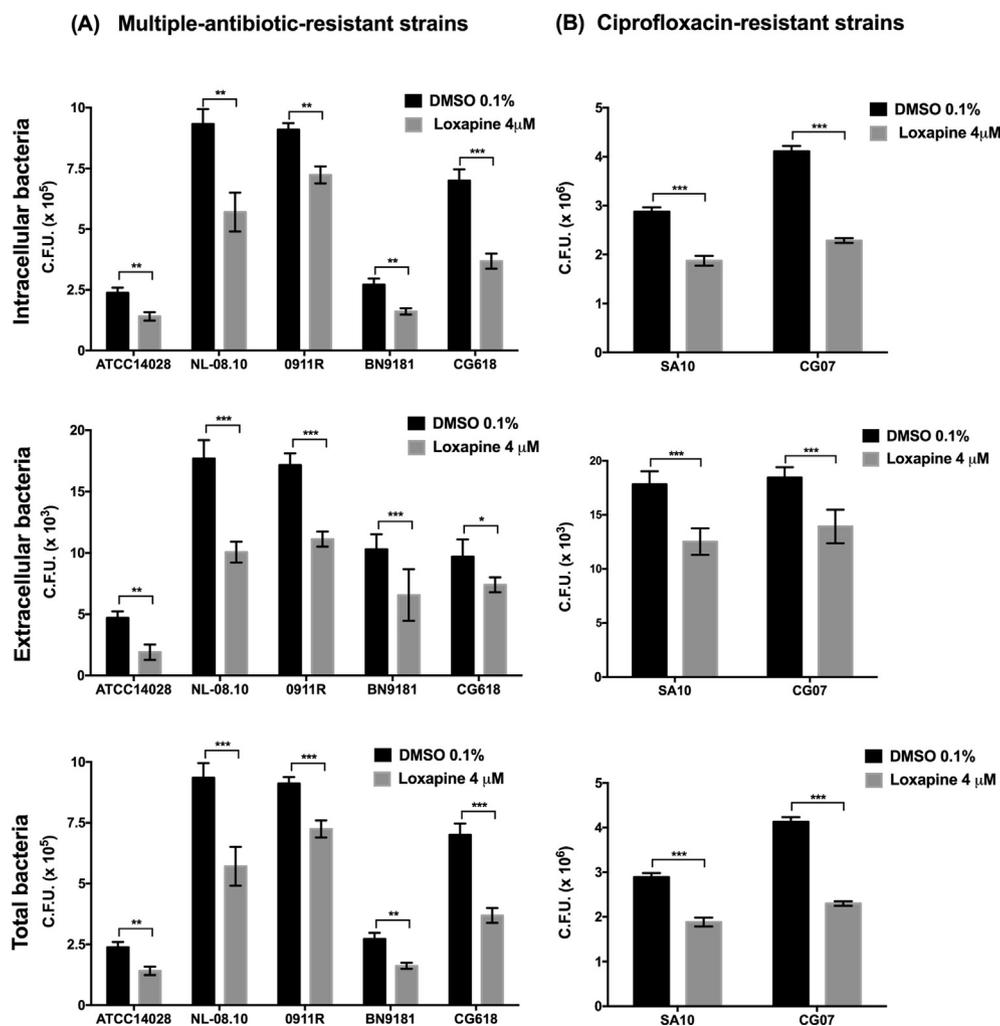


Figure 2. Loxapine is active against intracellular MAR or ciprofloxacin-resistant *S. Typhimurium*. RAW264.7 cells were infected with (A) *S. Typhimurium* ATCC14028, MAR isolates (type ACSSuT; 0911R12362, NL08.10, BN9181, and CG618) or (B) ciprofloxacin-resistant isolates (SA10 or CG07) followed by the exposure to gentamicin (20 $\mu\text{g}/\text{mL}$) alone or a combination of gentamicin and loxapine (4 μM) for 24 h. The number of intracellular, extracellular, and total bacteria were analyzed and presented as CFU. Data are presented as means \pm SD ($n = 3$). Statistical significance was measured with a two-tailed *t*-test. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

fluoroquinolone antibiotic and often used to treat salmonellosis.²⁵ As data shown in Fig. 2B, we observed a significant reduction of intracellular *Salmonella* in the drug combination group compared to that treated with gentamicin alone, indicating that loxapine is also active against intracellular fluoroquinolone-resistant *S. Typhimurium*. These results indicated that loxapine was active against intracellular *S. Typhimurium* isolates with resistance to a variety of antibiotics, and its antibacterial activity was not affected by bacterial infectivity or antibiotic resistance.

Loxapine suppressed intracellular methicillin-resistant *S. aureus* (MRSA), *Y. enterocolitica* and *S. flexneri*.

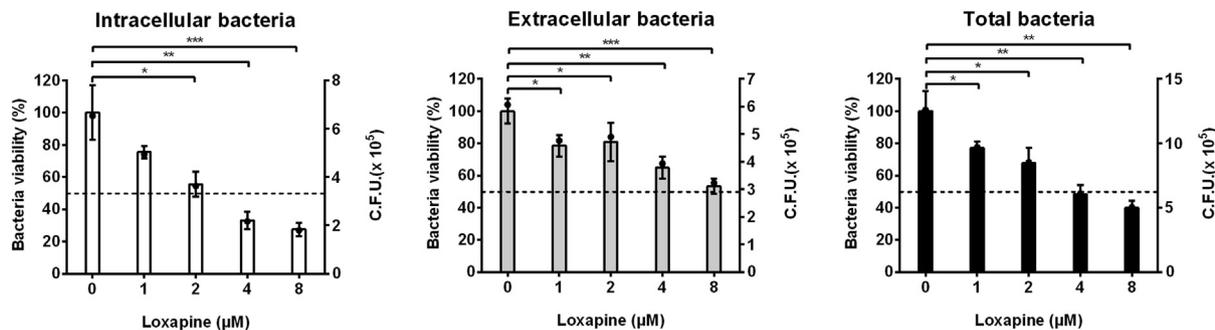
To investigate loxapine's antibacterial spectrum, we evaluated its antibacterial activity against a variety of

pathogenic bacteria, including MRSA, *Y. enterocolitica* and *S. flexneri*. As result shown, the intracellular proliferation of these bacteria was also suppressed by loxapine to a level similar with that of *S. Typhimurium* (Fig. 3). The data indicated that loxapine might possess a broad-spectrum antibacterial activity against intracellular bacteria in macrophages.

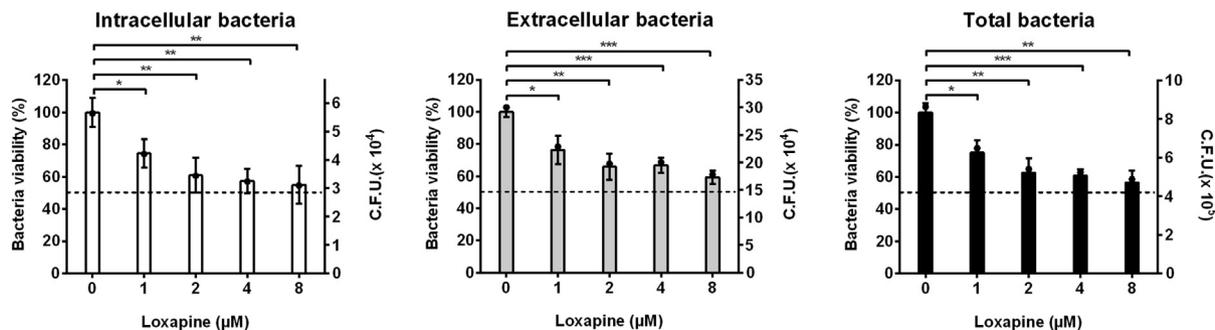
Loxapine does not enhance gentamicin's antibacterial activity or accumulation in macrophages

Our results showed that loxapine can suppress intracellular *Salmonella* in the presence of gentamicin. This raised a possibility that loxapine may act via potentiating the antibacterial activity of gentamicin. Pursuant to this issue, we evaluated the MICs of gentamicin against *S. Typhimurium* in the presence of loxapine or vehicle control in CAMH broth

(A) *Shigella flexneri*



(B) *Yersinia enterocolitica*



(C) MRSA USA300

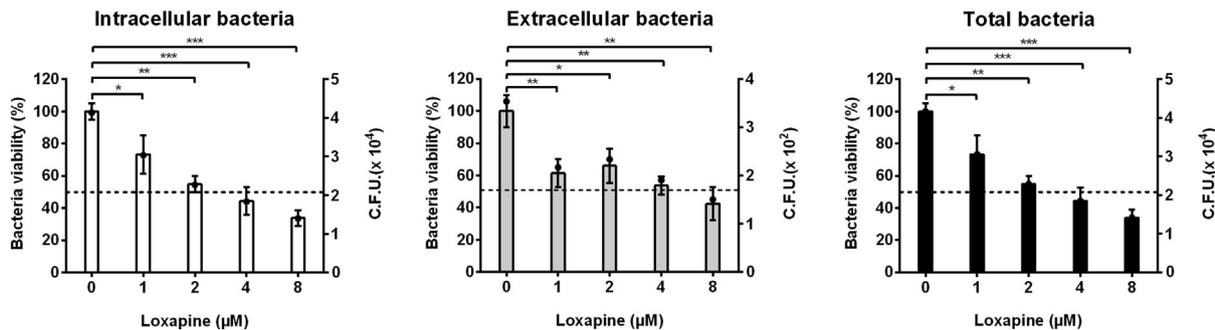


Figure 3. Loxapine suppressed intracellular *Shigella*, *Yersinia* and MRSA in macrophages and in broth. The proliferation of (A) *S. flexneri*, (B) *Y. enterocolitica* and (C) MRSA USA300 in RAW264.7 cells treated with various concentrations of loxapine was assessed. Data are presented as means ± SD (n = 3). Statistical significance was measured with a two-tailed *t*-test. (ns, *P* > 0.05, *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001).

Table 1 Antibacterial activity of gentamicin against *S. Typhimurium*.

Assay media	<i>S. Typhimurium</i> ATCC14028	
	Gentamicin MIC (μg/mL)	
	DMSO	Loxapine (4 μM)
CAMH Broth	2	2
DMEM + 10% FBS	2	2

and the RAW264.7 cell culture medium. As shown, the MICs of gentamicin is not affected by loxapine in both media (Table 1), indicating that loxapine doesn't potentiate gentamicin's antibacterial activity.

Despite a poor ability to cross cellular membrane, recent reports indicated that gentamicin could enter into phagocytes and macrophages via endocytosis and pinocytosis.²⁶ Thus, it is possible that loxapine might enhance uptake of gentamicin into macrophage to kill intracellular bacteria. To investigate this issue, intracellular gentamicin in mac-

Table 2 Sequence of primers used in this study.

Primers	Sequence
<i>sseA</i> sense	5'-CTTGCTGAAAGGGCAGAGAG-3'
<i>sseA</i> antisense	5'-TTCTTTAAATCCTTCTCGGCC-3'
<i>sseC</i> sense	5'-GAGGTGCAAAATGCATTACGTA-3'
<i>sseC</i> antisense	5'-CTTTACGCGCTTTATCCTCT-3'
<i>steC</i> sense	5'-GACGGATGCATCACAACCAG-3'
<i>steC</i> antisense	5'-CATGTAACCGATAGACTGTCCCA-3'
<i>sopD2</i> sense	5'-CATGGATGTGAACGTGAAAATC-3'
<i>sopD2</i> antisense	5'-AAGCGGTCTTGTGAGATGG-3'
<i>ssrB</i> sense	5'-GGAAGCTATCCTGGCTGAATTA-3'
<i>ssrB</i> antisense	5'-ATTCATCCGGTGTGTTTCG-3'
<i>hilD</i> sense	5'-GTACGTCAACGCTCAAACG-3'
<i>hilD</i> antisense	5'-GAAACATTGAATGAAGTAGGACG-3'
<i>sopE2</i> sense	5'-TATCAGCCTGTCGACGAGTCTG-3'
<i>sopE2</i> antisense	5'-CTGGTTAATACCGCCTACCCT-3'
<i>phoP</i> sense	5'-AAGAGGTAATGGCGCGTATG-3'
<i>phoP</i> antisense	5'-GATGACCTTTCATTGACGGA-3'

rophages treated with gentamicin alone or in combination with various concentrations of loxapine was assessed using FITC-conjugated anti-gentamicin antibody. In line with previous reports, we found that gentamicin could be internalized into macrophages (Fig. 4A). However, no significant difference in the level of intracellular gentamicin between gentamicin-treated and combination treated cells was observed (Fig. 4B), indicating that loxapine did not affect the uptake of gentamicin into macrophages.

Loxapine has no effect on bacterial growth in broth

Above results showed that both intracellular bacteria and extracellular bacteria were suppressed by loxapine treatment. To examine whether loxapine could directly inhibit bacterial growth in medium, bacteria was exposed to

escalating concentrations of loxapine in CAMH broth and in cell culture medium followed by monitored OD₆₀₀ of bacteria culture at different time points for a total of 96 h. As showed, no significant inhibitory effect of loxapine on the growth of bacteria in both media was observed at concentrations up to 500 μ M (Fig. 5A), suggesting that loxapine has no inhibitory activity directly on bacteria, and its suppressive activity is specifically targeting to bacteria in macrophages.

Autophagy is not involved in loxapine's anti-intracellular activity

The intracellular-*Salmonella* specific inhibitory effect of loxapine suggested that its activity may act through a host-targeted or virulence-targeted mechanism. Report indicate that trifluoperazine, a drug with structure similar to loxapine, can suppress intracellular *Salmonella* via activating autophagy.²⁷ To test whether autophagy plays a role in loxapine's anti-intracellular bacteria activity, infected cells were treated with 3-MA (an autophagy inhibitor) for 1h followed by treatment of mock or loxapine for 24h. As result shown in Fig. 5C, intracellular bacteria were significantly increased in 3-MA treated cells, suggesting that the autophagic defense was inhibited. However, 3-MA didn't affect loxapine's anti-intracellular bacteria activity, as demonstrated by the viability of bacteria in 3-MA treated and none-treated cells to be $49 \pm 1.8\%$ and $48 \pm 7.7\%$, respectively. The data indicated that autophagy is not involved in loxapine's anti-intracellular bacteria activity.

Loxapine has no effect on type III secretion system (T3SS) in *S. Typhimurium*

To elucidate whether loxapine acts on bacteria virulence, we first evaluate its effect on the T3SS in *Salmonella*. During infection, *Salmonella* uses T3SS as a molecular

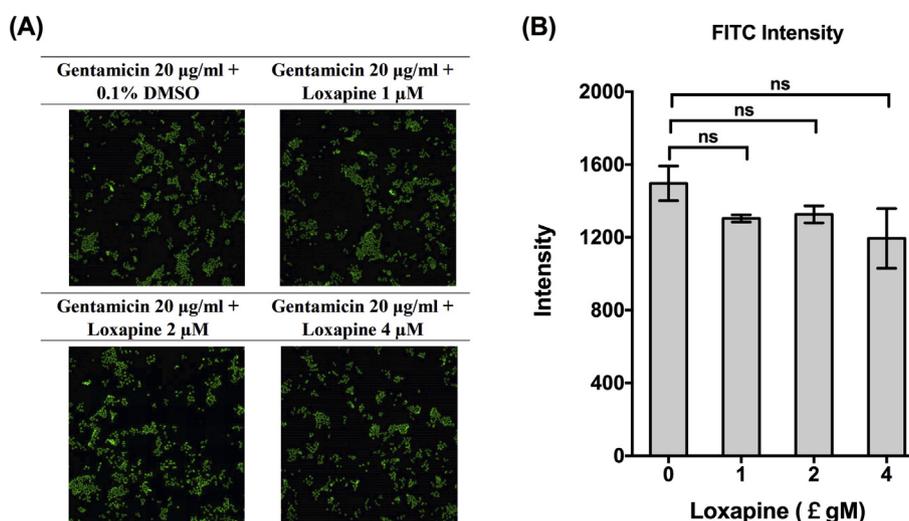


Figure 4. Loxapine has no effect on the accumulation of gentamicin in RAW264.7 cells. Cells were treated with gentamicin (20 μ g/mL) alone or in combined with different concentrations of loxapine (1, 2 or 4 μ M) for 24 hours. After treatment, intracellular gentamicin was detected with FITC-conjugated anti-gentamicin antibody. The fluorescence signals were (A) photographed with a high-content system (HCS) and (B) quantified with an image analysis software. Data are presented as means \pm SD ($n = 6$).

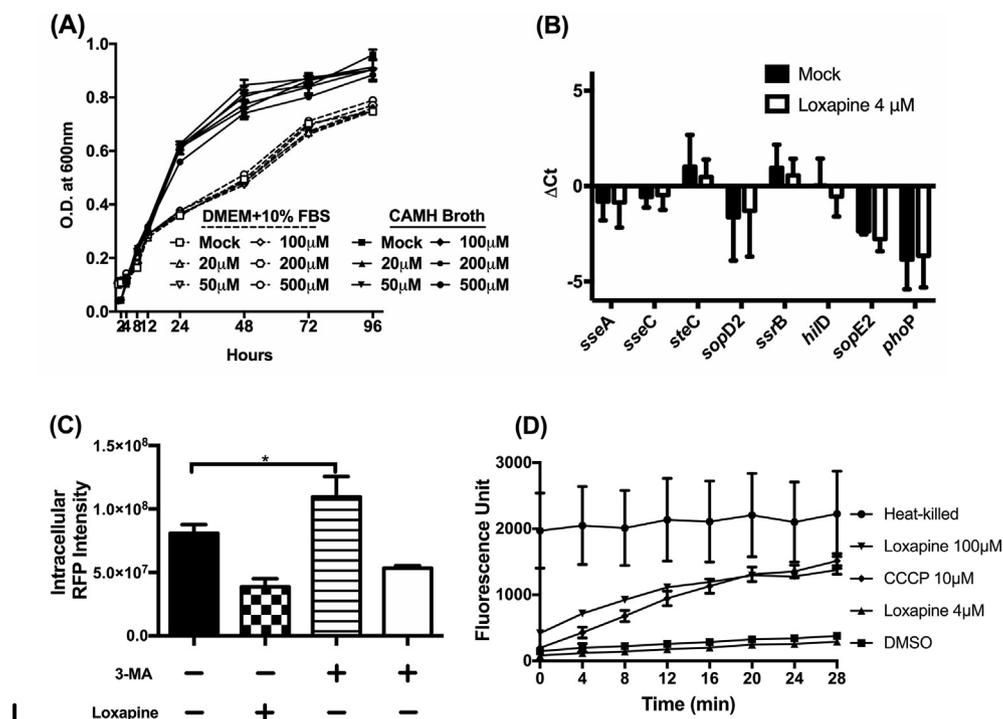


Figure 5. Loxapine suppresses bacterial efflux pump activity and has no effect on bacterial growth, bacterial T3SS or host autophagic defense. (A) Loxapine does not affect the growth of *S. Typhimurium* in broth. Bacteria were cultured in CAMH broth or cell culture medium in the presence of various concentrations of loxapine. The growth of bacteria was monitored by measuring the OD₆₀₀ of each well at designated times for a total of 96h. Points indicated means, and bars indicated SD (n = 6) (B) Loxapine has no effect on the T3SS expression of *Salmonella*. Bacteria were cultured in cell culture medium containing mock or loxapine (4 μM) for 4 hours. The expression level of individual T3SS associated gene was measured using real-time PCR approach. Data represent means; error bars represented the SD (n = 3). (C) Autophagy is not involved in loxapine's antibacterial activity. Infected cells were treated with 3-MA for 1h followed by loxapine or mock treatment. The viability of intracellular bacteria was assessed using a high-content system. Data represent means; error bars represented the SD (n = 3) Statistical significance was measured with a two-tailed *t*-test (*, *P* < 0.05). (D) Loxapine suppress efflux pump activity of *Salmonella*. The accumulation of Hoechst 33342 in *Salmonella* treated with loxapine was measured using a microplate reader at designated times for a total of 60 min. Points indicated means, and bars indicated SD (n = 8).

syringe to inject effector proteins into host cells for invasion and intracellular proliferation.⁴ To investigate whether loxapine affects bacterial T3SS, the expression level of several T3SS associated genes, including *sseA*, *sseC*, *steC*, *sopD2*, *ssrB*, *hilD*, *sopE2*, *phoP*, and a housekeeping gene, *gyrB*, was quantitated by real-time PCR approach. As result showed in Fig. 5B, no significant difference between loxapine-treated and mock-treated bacteria was observed, indicating that loxapine does not affect T3SS associated genes in *Salmonella*.

Loxapine inhibits efflux pump activity of bacteria

In addition to T3SS, *Salmonella* requires several efflux pumps to survive in host cells.^{28,29} To investigate whether loxapine suppress bacterial efflux pump activity, the accumulation of Hoechst 33342, an efflux pump substrate, in *Salmonella* was assayed. The result showed that loxapine has a dose-dependent inhibiting activity on efflux pump of *Salmonella* (Fig. 5D). In addition, the efflux pump activity of MRSA, *S. flexneri*, and *Y. enterocolitica* were all

suppressed by loxapine (Fig. S1), suggesting that loxapine's anti-intracellular bacteria activity might act through inhibition of bacterial efflux pump.

Discussion

The rapid emergence and spread of MAR bacteria highlight the urgent need for an innovative strategy to combat antibiotic resistance. Among the new proposed strategies, the virulence-targeted approach is particularly notable. Unlike common antibiotics that kill or inhibit bacterial growth, virulence-targeted intervention blocks bacterial infection via targeting to bacterial pathogenic factors important for infection. Due to their unique action mechanism, virulence-targeted agents can suppress bacterial infection without exerting selective pressure. Therefore, bacteria are less likely to develop resistance to virulence-targeted antibacterial agents. In this study, we demonstrated that an antipsychotic drug, loxapine possesses potent anti-intracellular bacteria activity against

intracellular *S. Typhimurium*, MRSA, *Y. enterocolitica* and *S. flexneri* in macrophages. Loxapine didn't affect bacterial growth in broth, but exhibited suppressive activity on bacterial efflux pump activity which is required for bacteria survive in host cells. Thus, loxapine could exert its anti-intracellular bacteria activity via a virulence-targeted mechanism.

In addition to loxapine, several phenothiazine antipsychotics have also been reported to possess potent antibacterial activity against *Salmonella* infection. For instance, thioridazine, a typical antipsychotic drug, has been demonstrated to inhibit intracellular *S. Typhimurium* at the concentration of 10 µg/ml, which is 50-fold lower than its MIC against bacteria in broth.³⁰ Moreover, intraperitoneal administration of thioridazine at 200 µg/mouse (approximately 10 mg/kg) not only reduce the bacterial burden in organs but also cure the salmonellosis in infected mice.³⁰ Another two phenothiazines, fluphenazine, and prochlorperazine, have also been showed to suppress the *Salmonella* infection in mice by intraperitoneal administration of individual drug at 3 mg/kg and 1.5 mg/kg, respectively.^{31,32} Moreover, a recent report indicated that amoxapine, a metabolite of loxapine, was able to suppress the infection of *Yersinia pestis* at both *in vitro* and *in vivo*.¹⁵ The reports together with our findings provide evidences to support the translational potential of these antipsychotics as a novel therapeutic for treating bacterial infection.³³ Subsequent structure optimization to dissociate the antibacterial activity from antipsychotic activity of loxapine and other antipsychotics may generate a new class of antibacterial agent against intracellular multiple-antibiotic bacteria.

Author contributions

CYY, CYH, CSF, and HCC conceived and designed the experiments. CYY, CYH, and CSF performed the experiments. CYY, CYH, CSF, and HCC analyzed the data. CYY, CWS, CSC and HCC wrote the paper.

Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

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