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Original Article

Repurposing the tyrosine kinase inhibitor nilotinib for use against intracellular multidrug-resistant *Salmonella* Typhimurium

Shih-Hsiu Chou [a,1], Tsai-Wen Wan [a,1], Chung-Wai Shiau [b,1], Ling-Han Chen [a], Hsueh-Chun Lin [a], Hao-Chieh Chiu [a,c,*]

[a] *Department of Clinical Laboratory Sciences and Medical Biotechnology, College of Medicine, National Taiwan University, Taipei 10048, Taiwan*
[b] *Institute of Biopharmaceutical Sciences, National Yang Ming Chiao Tung University, Taipei 11221, Taiwan*
[c] *Department of Laboratory Medicine, National Taiwan University Hospital, College of Medicine, National Taiwan University, Taipei 10021, Taiwan*

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Abstract *Background/purpose:* The increasing incidence of infections caused by multidrug-resistant *Salmonella enterica* has become a serious threat to global public health. Here, we found that the tyrosine kinase inhibitor nilotinib exhibits antibacterial activity against intracellular *S. enterica* serovar Typhimurium in RAW264.7 macrophages. Thus, we aimed to pharmacologically exploit the anti-intracellular *Salmonella* activity of nilotinib and to elucidate its mechanism of action.
Methods: The antibacterial activity of the compounds was assessed by high-content analysis (HCA) and intracellular CFU, minimum inhibitory concentration (MIC), and bacterial growth assays. The cytotoxicity of the compounds was evaluated by HCA and a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) cell viability assays. The levels of cellular AMPK, phospho-AMPK, Atg7 and β-actin were determined by immunoblotting.
Results: The screen identified two small molecule compounds (SCT1101 and SCT1104) with potent activity against intracellular *S.* Typhimurium. Moreover, SCT1101 and SCT1104 enhanced the efficacy of ciprofloxacin and cefixime against intracellular *S.* Typhimurium. However, only SCT1101 exhibited activity against intracellular MDR and fluoroquinolone-resistant *S.* Typhimurium isolates. Subsequent mechanistic studies showed that neither of these nilotinib

* Corresponding author. Department of Clinical Laboratory Sciences and Medical Biotechnology, College of Medicine, National Taiwan University, Taipei 10048, Taiwan.
E-mail address: hcchiu@ntu.edu.tw (H.-C. Chiu).
[1] Shih-Hsiu Chou, Tsai-Wen Wan, and Chung-Wai Shiau contributed equally to this work.

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derivatives increased the phospho-AMPK level in RAW264.7 cells. Neither the AMPK inhibitor compound C nor SBI-0206965 reversed the inhibitory effects of SCT1101 and SCT1104 on intracellular *Salmonella*. Furthermore, neither blockade of autophagy by 3-MA nor shRNA-mediated knockdown of Atg7 protein expression in RAW264.7 cells affected the antibacterial activity of SCT1101 and SCT1104.

Conclusion: The structure of nilotinib could be used to develop novel therapeutics for controlling MDR S. Typhimurium infections.

Introduction

Salmonella enterica is a leading cause of gastroenteritis. It is estimated that tens of millions of cases and hundreds of thousands of deaths are associated with *S. enterica* infection each year worldwide.[1] Among the more than 2600 *S. enterica* serovars, *S. enterica* serovar Typhi (hereafter, *S.* Typhi) causes disease only in humans, while infection with *S. enterica* serovar Typhimurium (hereafter, *S.* Typhimurium) often leads to gastroenteritis in humans but a typhoid-like disease in animals.[2] However, in immunocompromised patients, infection with *S.* Typhimurium can lead to fatal invasive nontyphoidal *Salmonella* (iNTS) disease, which has a mortality rate of up to 14.5%.[3] During infection, *Salmonella* can penetrate the intestinal mucosa and be phagocytosed by macrophages in Peyer's patches and lymph nodes. In macrophages, *Salmonella* employs various virulence factors to evade cellular innate defense mechanisms, proliferate inside *Salmonella*-containing vacuoles (SCVs) and cause secondary infections in the liver, spleen, and bone marrow after hematogenous spread.[4,5] The host cytoplasmic membrane and the SCV membrane protect intracellular *Salmonella* from host immune defenses as well as antibiotics with poor membrane permeability. In addition, the emergence and spread of resistance genes have rendered first-line antibiotics ineffective against more than two-thirds of *Salmonella* isolates,[6] and the increasing incidence of nalidixic acid-resistant or ciprofloxacin-resistant isolates further limits antibiotic options for treating *Salmonella* infections.[7,8] Thus, the World Health Organization (WHO) has included antibiotic-resistant *S. enterica* on its list of pathogens in great need of new therapeutics.[9]

AMP-activated protein kinase (AMPK) is an energy-sensing enzyme that plays a key role in regulating cellular energy metabolism.[10] AMPK is composed of a catalytic α-subunit and regulatory β and γ subunits. When intracellular ATP levels decrease, AMPK inhibits energy-consuming processes and activates energy-producing pathways, thereby increasing the AMP/ATP ratio. Additionally, AMPK can activate the autophagy pathway by phosphorylating Atg1/ULK1.[11] Recently, Truc et al. showed that bacterial outer membrane vesicles can increase AMPK activity to induce autophagy-mediated eradication of invading *S.* Typhimurium, suggesting that AMPK is a potential target for the control of intracellular bacteria.[12] Here, we assessed the anti-intracellular bacterial activity of three different pharmacologic AMPK activators—5-amino-4-imidazolecarboxamide riboside (AICAR), metformin and nilotinib—and found that only nilotinib exhibited a significant suppressive effect on intracellular *S.* Typhimurium in macrophages. Accordingly, this study aimed to pharmacologically exploit the anti-intracellular *Salmonella* activity of nilotinib by structural modification and to elucidate the roles of AMPK and autophagy in the antibacterial activity of nilotinib derivatives.

Materials and methods

Cells

The murine macrophage cell line RAW264.7 was purchased from the Bioresource Collection and Research Center (Taiwan) and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, USA) supplemented with 10% heat-inactivated FBS (Gibco-BRL). Cells were cultured in T-75 flasks at 37 °C in a 5% CO_2 atmosphere and stored in DMEM containing 10% dimethyl sulfoxide (DMSO; Sigma—Aldrich, USA) in liquid nitrogen vapor.

Bacterial strains

S. Typhimurium ATCC 14028 was purchased from the American Type Culture Collection (ATCC). The multidrug-resistant (MDR) *S.* Typhimurium strain 0911R and ciprofloxacin-resistant strain SB10 were obtained from the Taiwan Centers for Disease Control. Red fluorescent protein (RFP)-expressing *S.* Typhimurium was obtained by transforming pBR-RFP.1 into bacterial cells.[13] Bacteria were grown in Luria—Bertani (LB) broth (Athena Enzyme Systems, USA) and stored in LB broth supplemented with 15% glycerol at −80 °C.

Reagents

Dorsomorphin (compound C) (Abcam Inc., Canada), SBI-0206965 (Cayman Chemicals, USA), metformin (Cayman Chemicals), AICAR (Cayman Chemicals), and nilotinib (Cayman Chemicals) were dissolved in DMSO (Sigma—Aldrich) as stock solutions. Gentamicin (Bio Basic, Canada), ampicillin (Bio Basic), ciprofloxacin (Sigma—Aldrich), streptomycin (Bio Basic), ofloxacin (Bio Basic), and vancomycin (Cayman Chemicals) were dissolved in deionized water and filtered through a 0.45-μm filter. Tetracycline (Bio Basic) and chloramphenicol (Amresco, USA) were dissolved in 95% ethanol as stock solutions.

Synthesis of nilotinib derivatives

The starting materials for the synthesis of the nilotinib-focused compound library were purchased from Sigma–Aldrich, Acros Organics, or Tokyo Chemical Industry (TCI). The purity of all tested compounds was determined to be 95% by proton nuclear magnetic resonance (^{1}H NMR) spectroscopy with Bruker DPX400 (400 MHz) instruments. All chemicals were dissolved in DMSO as stock solutions. The details of the synthesis of nilotinib derivatives will be described elsewhere.

High-content analysis (HCA)

RFP-expressing *S.* Typhimurium was cultured in LB broth supplemented with 100 mg/L ampicillin for 18 h and was then diluted 1:100 in fresh LB broth prior to incubation for 2 h at 37 °C with agitation. Bacteria were then collected by centrifugation at 6200 g for 3 min and suspended in phosphate-buffered saline (PBS; pH 7.2) to an optical density of 0.6 at 600 nm, equivalent to approximately 1.4×10^8 colony-forming units (CFUs)/mL. RAW264.7 macrophages were seeded in 96-well black clear-bottom plates (CELLSTAR, Greiner Bio-One, Austria) and incubated for 20 h prior to infection with RFP-expressing *S.* Typhimurium at a multiplicity of infection (MOI) of 50 for 1 h at 37 °C in a 5% CO_2 atmosphere. Infected cells were washed and treated with 100 mg/L gentamicin, a membrane-impermeable bactericidal antibiotic, for 1 h to eliminate extracellular bacteria and were then treated with test compounds combined with gentamicin (20 mg/L) at 37 °C in a 5% CO_2 atmosphere. After 24 h, the cells were washed and stained with CellTracker Green 5-chloromethylfluorescein diacetate (Thermo Fisher Scientific, USA) in serum-free DMEM for 30 min. Then, the cells were cultured in DMEM supplemented with 10% FBS for 30 min and fixed with 3.7% formaldehyde (Sigma–Aldrich) for 20 min prior to staining with 0.2 mg/L 4',6-diamidino-2-phenylindole (DAPI; AAT Bioquest, Sunnyvale, CA) in PBS for 30 min. Images of individual wells were then acquired using a high-content imaging system (ImageXpress Micro 4, Molecular Devices, USA) and analyzed with MetaXpress software (Molecular Devices). The half-maximal cytotoxic concentration (CC_{50}) in cells and half-maximal effective concentration (EC_{50}) against intracellular *S.* Typhimurium of each compound was determined using CalcuSyn software (Biosoft, UK).

Intracellular CFU assay

RAW264.7 cells were seeded in a 6-well plate (5.0×10^5 cells/well) and infected by bacteria at MOI = 10 for 30 min. After infection, the cells were washed and were then exposed to 100 mg/L gentamicin for 1 h to kill non-invaded bacteria. Then, the cells were treated with gentamicin (20 mg/L) alone or in combination with SCT1101 or SCT1104. After 24 h, the culture medium was collected and serially diluted in PBS. Infected cells were washed, lysed with 0.1% Triton X-100 for 10 min at 37 °C and 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 bacterial colonies grown on the plates were counted, and the values were expressed as CFUs.

Bacterial growth assay

Overnight cultures of *S.* Typhimurium in LB medium were inoculated into fresh cation-adjusted Mueller–Hinton broth (CAMHB) or DMEM supplemented with 10% FBS to a final concentration of 5×10^5 CFU/mL prior to exposure to increasing concentrations of nilotinib (0–10 μM) in a flat-bottom 96-well plate. The plate was incubated at 37 °C, and bacterial growth was monitored by measuring the absorbance at 600 nm using the VersaMax Microplate reader (Molecular Devices) at the designated times over a 24 h period.

Immunoblot analysis

Cells were washed with cold PBS, suspended in IGEPAL-CA630 lysis buffer (Sigma–Aldrich), and incubated on ice for 10 min. After centrifugation at 11,000 g for 10 min at 4 °C, the lysate supernatants were mixed with 4 × Laemmli buffer (Thermo Fisher Scientific) and incubated at 95 °C for 10 min. Proteins were then separated on a sodium dodecyl sulfate–polyacrylamide gel and transferred onto 0.2 μm PVDF membranes (Merck Millipore, USA). The membranes were blocked with 3% bovine serum albumin (BSA) in Tris-buffered saline (TBS) for 1 h. After two washes with 0.5% Tween 20 in TBS (TBST), the membranes were incubated for 18 h at 4 °C with antibodies specific for AMPK alpha (GeneTex, Taiwan), phospho-AMPK (Cell Signaling, USA), Atg7 (GeneTex) and β-actin (GeneTex) at the appropriate dilutions in TBST for 18 h at 4 °C. The membranes were then washed three times with TBST and incubated with a horseradish peroxidase-conjugated goat IgG secondary antibody in TBST containing 3% BSA. The immunopositive bands were visualized by enhanced chemiluminescence (GE Amersham, USA) and detected using a LAS-4000 chemiluminescence imaging system (Fujifilm, Japan).

ShRNA-mediated knockdown of Atg7 expression

Knockdown of Atg7 was performed by transfecting macrophages with plasmids expressing short hairpin RNA (shRNA) targeting *ATG7*. Three plasmids containing different shRNA sequences were obtained from The RNAi Consortium (TRC), National RNAi Core Facility, Academia Sinica, Taiwan, and prepared using an EndoFree plasmid purification kit (QIAGEN, Germany). Cells were transfected with Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's instructions. In brief, RAW264.7 cells were seeded in 6-well plates (2.5×10^5 cells/well) for 24 h, and the culture medium was then replenished with fresh medium. The shRNA expression plasmids and Lipofectamine 3000 were complexed in OptiMEM (Thermo Fisher Scientific) and were then gently added to each well. Macrophages transfected with empty vector (pLKO_TRC005) served as controls. Cells were harvested 48, 72, and 96 h after transfection, and the knockdown efficiency of each plasmid was assessed by immunoblotting with antibodies specific for Atg7 (GeneTex) and β-actin (GeneTex). The plasmid that induced the maximal repression of Atg7 was selected for use in

subsequent experiments. Stably transfected clones were selected using medium supplemented with puromycin (2.5 mg/L; Thermo Fisher Scientific), and knockdown of target gene expression was evaluated by immunoblotting. The selected clones were maintained in the presence of 1 mg/L puromycin to retain clonal homogeneity.

Statistical analysis

The data are expressed as the means ± standard deviations (SDs). Differences between group means were analyzed using a two-tailed *t* test or one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test for independent samples. Differences with a p value of <0.05 were considered significant. Statistical analysis of the data was performed using GraphPad Prism 8.0 software (GraphPad Software, USA).

Results

Nilotinib suppresses intracellular S. Typhimurium in macrophages

To investigate whether AMPK activation can suppress intracellular S. Typhimurium, we evaluated the effect of three AMPK activators—AICAR, metformin and nilotinib—on the viability of intracellular S. Typhimurium and infected RAW264.7 cells simultaneously using image-based HCA. Infected cells were treated with increasing concentrations of AICAR, metformin and nilotinib in the presence of 20 mg/L gentamicin to eliminate bacteria in the medium. As shown, AICAR and metformin increased the level of phospho-AMPK in RAW264.7 cells at concentrations of 0.5 mM and 10 mM, respectively (Fig. 1A and B, right panel). However, neither drug showed suppressive activity against intracellular S. Typhimurium (Fig. 1A and B, left panel). In contrast, nilotinib at 3 μM appreciably reduced the viability of intracellular S. Typhimurium to 60% of that in mock (DMSO)-treated cells but did not exhibit significant cytotoxicity toward infected macrophages even at concentrations of up to 10 μM (Fig. 1C, left panel). Additionally, the level of phospho-AMPK in infected cells was significantly increased after treatment with 3 μM and 10 μM nilotinib (Fig. 1C, right panel). Furthermore, we did not observe any significant difference in the growth of S. Typhimurium in either CAMHB or cell culture medium (CCM), suggesting that nilotinib was only active against intracellular *Salmonella* in macrophages (Fig. 1D). Collectively, our results showed that nilotinib can suppress the growth of intracellular *Salmonella* at AMPK-activating concentrations. Thus, the structure of nilotinib can be further developed as a new antibacterial agent with activity against intracellular *Salmonella*.

SCT1101 and SCT1104 exhibit potent antibacterial activity against intracellular S. Typhimurium in macrophages

The above findings indicated that nilotinib exhibits activity against intracellular *Salmonella* in macrophages. To

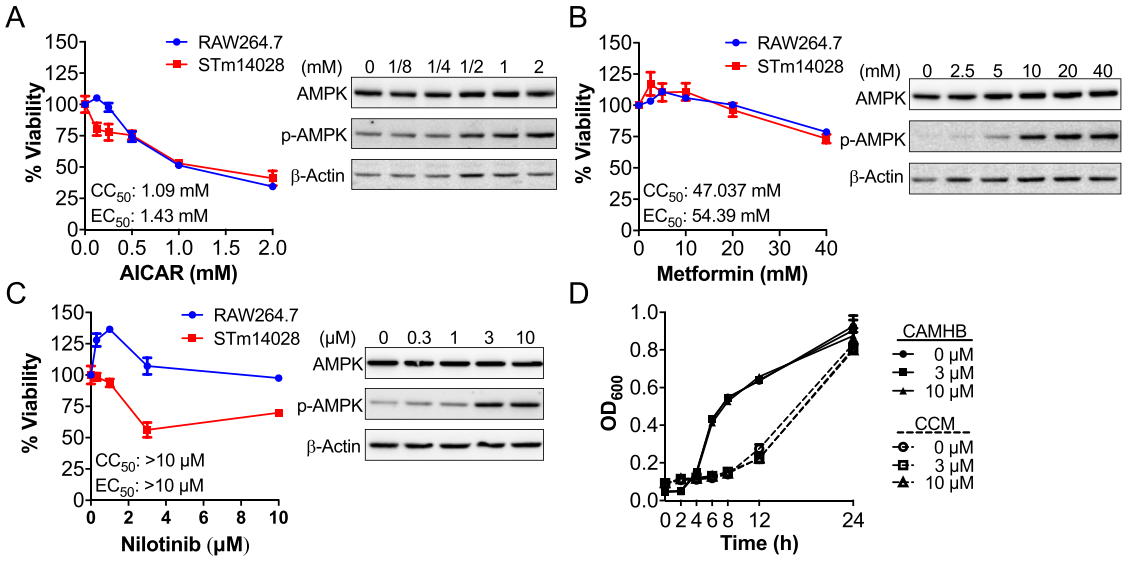

Figure 1. Nilotinib suppresses intracellular S. Typhimurium in RAW264.7 macrophages (A, B, C) S. Typhimurium ATCC 14028 (STm14028)-infected RAW264.7 cells were treated with various concentrations of AICAR (A), metformin (B) and nilotinib (C) combined with 20 mg/L gentamicin for 24 h. Left panel, the viability of infected RAW264.7 cells and intracellular S. Typhimurium were assessed by HCA. The data are expressed as percentages relative to the untreated control and are presented as the means ± SDs (n = 3/group). Right panel, the levels of AMPK, phospho-AMPK and β-actin in RAW264.7 cells treated with individual AMPK activators for 2 h were determined by immunoblotting (D) S. Typhimurium ATCC 14028 was cultured in CCM (DMEM supplemented with 10% FBS) or CAMHB in the presence of various concentrations of nilotinib. Bacterial growth was monitored by measuring the optical density at 600 nm of each well at designated times over a period of 24 h and is presented as the means ± SDs (n = 3/group).

exploit the antibacterial activity of nilotinib, we designed and synthesized 24 nilotinib derivatives and assessed their activity against intracellular *S.* Typhimurium (EC_{50}) as well as their cytotoxicity (CC_{50}) by HCA (Fig. 2A). The screen identified SCT1101 and SCT1104 (see structures in Fig. 2B) as having the best selectivity (CC_{50}/EC_{50}) among the 24 nilotinib derivatives (data not shown). Moreover, both SCT1101 and SCT1104 at 3 μM reduced the abundance of intracellular *S.* Typhimurium in RAW264.7 cells to close to 20% of that in control-treated cells (Fig. 2C and D). Like nilotinib, these two compounds did not inhibit bacterial growth in MIC assays at concentrations up to 128 μM (Table S1). Thus, we demonstrated that the structure of nilotinib can be further optimized for increased antibacterial activity against intracellular *Salmonella*.

SCT1101 and SCT1104 sensitize intramacrophage Salmonella to ciprofloxacin and cefixime

Using combinations of more than one antibiotic to reduce the dosage requirement of a single antibiotic is an alternative method to decrease the usage of antibiotics and mitigate resistance.[14] To observe whether SCT1101 and SCT1104 can increase the sensitivity of intracellular *Salmonella* to two first-line antibiotic classes, i.e., fluoroquinolones and cephalosporins, we infected RAW264.7 cells with RFP-expressing *S.* Typhimurium ATCC 14028 and treated the infected cells with ciprofloxacin or cefixime alone and in combination with SCT1101 or SCT1104 for 24 h. Both antibiotics inhibited the survival of intracellular *Salmonella* in macrophages in a dose-dependent

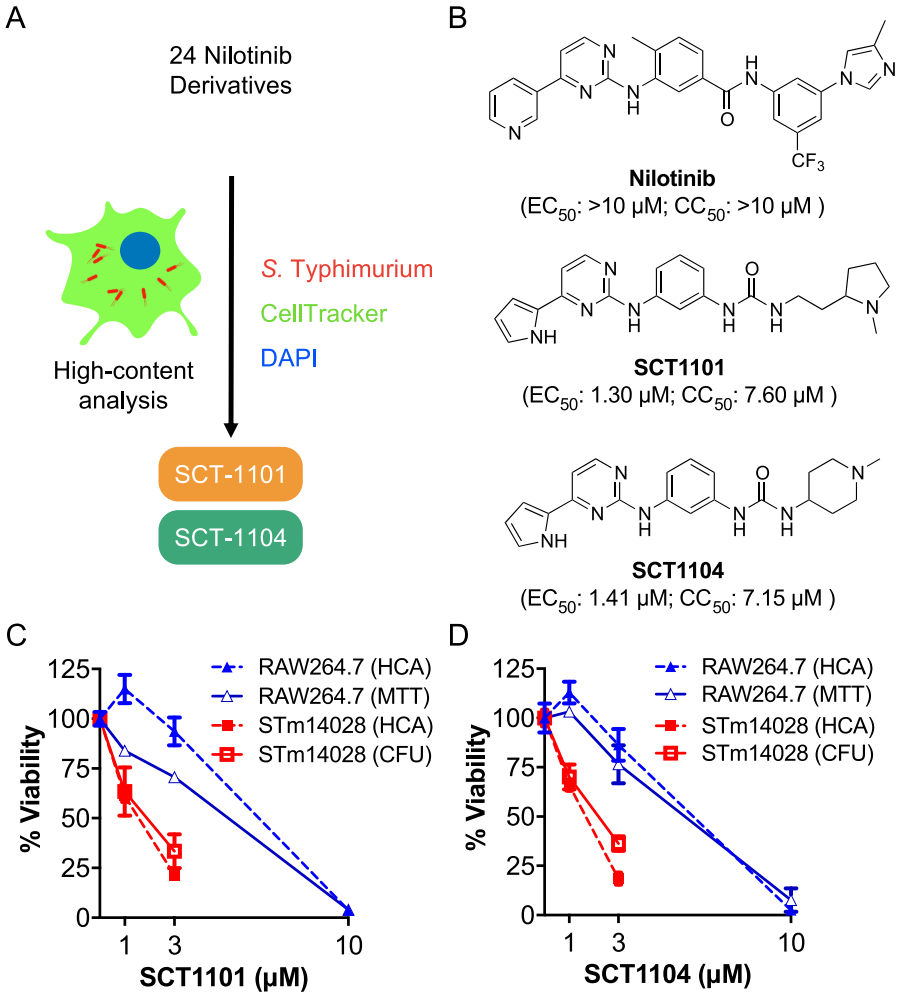

Figure 2. Identification of new nilotinib derivatives, SCT1101 and SCT1104, with potent antibacterial activity against intracellular *S.* Typhimurium (A) Flowchart of the image-based HCA procedure (B) Structures, CC_{50} in RAW264.7 macrophages, and EC_{50} against intracellular *S.* Typhimurium of nilotinib, SCT1101 and SCT1104. The data are representative of two independent experiments (C, D) *S.* Typhimurium ATCC 14028 (STm14028)-infected RAW264.7 cells were treated with various concentrations of SCT1101 (C) and SCT1104 (D) combined with 20 mg/L gentamicin for 24 h. RAW264.7 cell viability was evaluated by HCA or an MTT assay, and the viability of intracellular *Salmonella* was evaluated by HCA or an intracellular CFU assay. The data are expressed as percentages relative to the untreated control and are presented as the means ± SDs ($n = 3$/group).

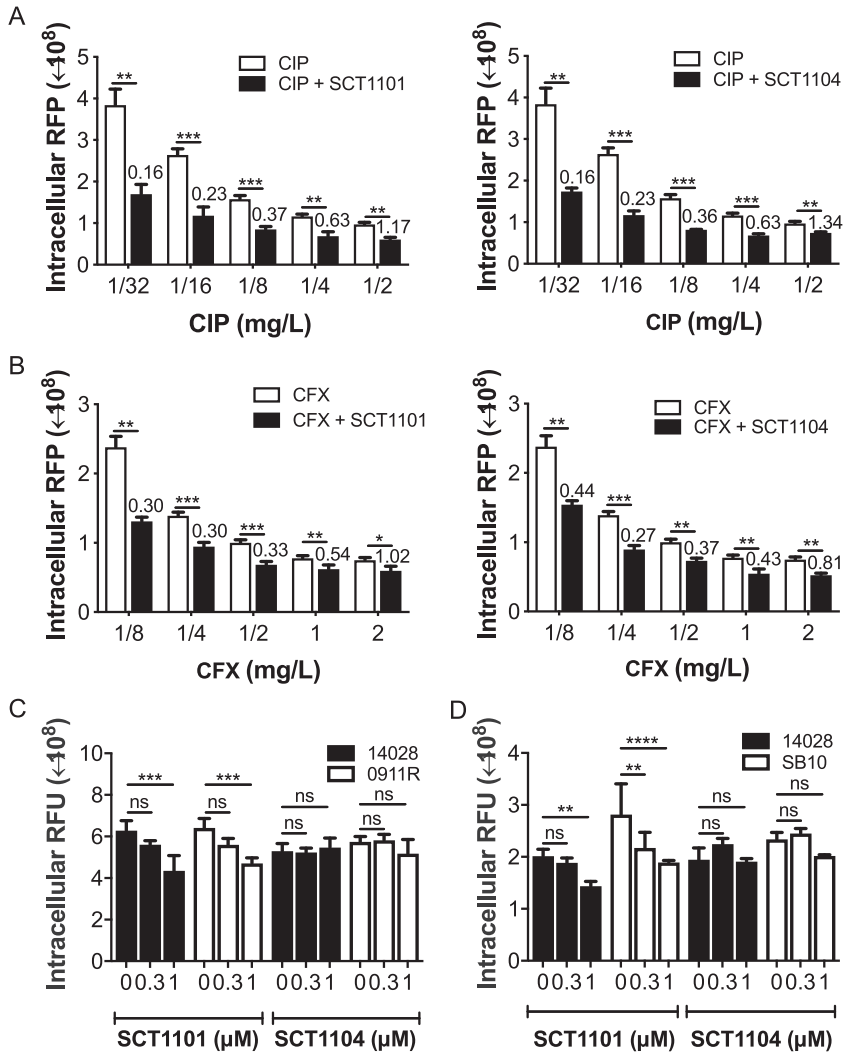

Figure 3. SCT1101 and SCT1104 sensitize intracellular *S.* Typhimurium to ciprofloxacin and cefixime (A, B) *S.* Typhimurium ATCC 14028-infected RAW264.7 macrophages were treated with ciprofloxacin (CIP; A) or cefixime (CFX; B) in combination with 1.5 μM SCT1101 (left panel) or SCT1104 (right panel) for 24 h. The RFU values of intracellular bacteria were determined by HCA. The data are presented as the means ± SDs (n = 3/group). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. The combination index of individual combination was calculated using CompuSyn (ComboSyn, Inc.) Synergism (CI <1), additive-effect (CI = 1) and antagonism (CI>1) (C, D) RAW264.7 macrophages were infected with the *S.* Typhimurium reference strain ATCC 14028, MDR strain 0911R (C) or ciprofloxacin-resistant strain SB10 (D) and were then treated with SCT1101 or SCT1104 at increasing concentrations for 24 h. The relative fluorescence unit (RFU) values of intracellular bacteria were determined by HCA. The data are presented as the means ± SDs (n = 3/group). ns, nonsignificant; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ for the difference between the drug-treated group and the corresponding control group.

manner (Fig. 3A and B). Moreover, the efficacy of both individual antibiotics was improved when they were used in combination with 1.5 μM SCT1101 or SCT1104, as demonstrated by the significant decreases in the relative numbers of intracellular *Salmonella* in macrophages treated with the drug combinations to amounts equal to or less than those in cells treated with a 2-fold concentration of the corresponding antibiotic alone. In addition, the combination index calculated using CompuSyn also indicated that SCT1101 and SCT1104 have synergistic effects with low doses of ciprofloxacin or cefixime against intracellular *Salmonella* (Fig. 3A and B). Therefore, our findings indicated that the two nilotinib derivatives can enhance the suppressive effect of these first-line antibiotics against intracellular *Salmonella*.

SCT1101 is active against intramacrophage MDR S. Typhimurium

The emergence and spread of MDR *S.* Typhimurium with resistance to ampicillin, chloramphenicol, streptomycin,

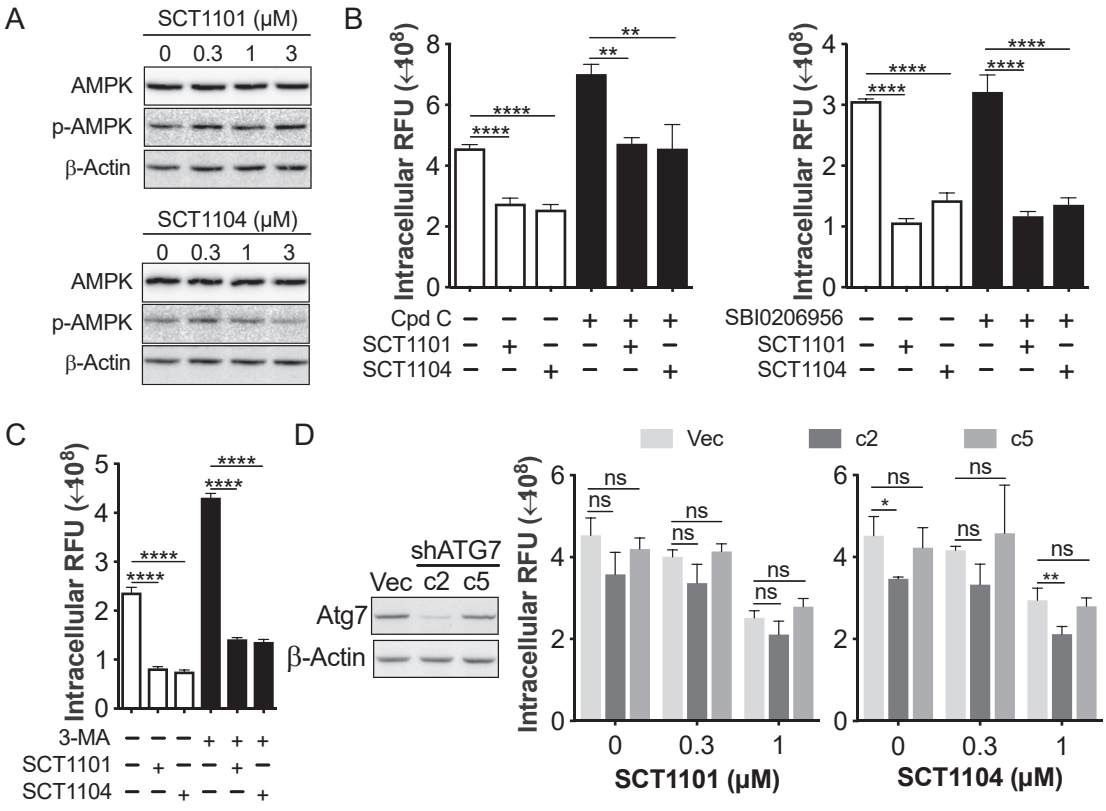

Figure 4. The anti-intracellular *Salmonella* activity of SCT1101 and SCT1104 is independent of cellular AMPK and autophagy (A) The levels of AMPK, phospho-AMPK and β-actin in RAW264.7 cells treated with SCT1101 (*upper*) or SCT1104 (*lower*) for 2 h were determined by immunoblotting (B) S. Typhimurium ATCC 14028-infected RAW264.7 cells were treated with mock (DMSO), 1 μM compound C (left) or 1 μM SBI-0206965 (right) for 1 prior to treatment with 1.5 μM SCT1101 or SCT1104 for 24 h. The RFU values of intracellular bacteria were determined by HCA. The data are presented as the means ± SDs ($n = 3$/group). **, $P < 0.01$; ****, $P < 0.0001$ (C) S. Typhimurium ATCC 14028-infected RAW264.7 cells were treated with 3-MA (10 mM) for 1 h prior to mock (DMSO), SCT1101 (1.5 μM) or SCT1104 (1.5 μM) treatment for 24 h. The RFU values of the intracellular bacteria were assessed using HCA. The data are presented as the means ± SDs ($n = 3$/group). ****, $P < 0.0001$ (D) RAW264.7 cells were stably transfected with a plasmid expressing shRNA targeting *ATG7* (shATG7; c2, clone 2; c5, clone 5) or with empty vector (Vec). The left panel shows the levels of the Atg7 protein and β-actin in the stable transfectants as determined by immunoblotting. The data are representative of two independent experiments. The right panel shows intracellular bacterial survival in SCT1101-or SCT1104-treated RAW264.7 cells with shRNA-mediated knockdown of Atg7 expression. The data shown are the means; the error bars show the SDs ($n = 3$). ns, nonsignificant; *, $P < 0.05$; **, $P < 0.01$.

and tetracycline has become a serious threat to public health worldwide.[7,15,16] To investigate whether SCT1101 and SCT1104 can suppress intracellular MDR *Salmonella* infection, we transformed the RFP expression plasmids into the MDR clinical isolate of S. Typhimurium (0911R) and evaluated the effects of both compounds on infection of this MDR isolate in macrophages. Despite its antibiotic resistance (Table S1), the intracellular replication of this MDR S. Typhimurium isolate is susceptible to inhibition by SCT1101 but not SCT1104 (Fig. 3C). Next, we extended the investigation to the S. Typhimurium isolate SB10, which is resistant to ciprofloxacin (Table S1). As shown in Fig. 3D, the intracellular proliferation of this ciprofloxacin-resistant isolate was also susceptible to inhibition by only SCT1101. Thus, our findings indicated that the antibacterial activity of SCT1101 is not affected by the resistance mechanisms of *Salmonella* to common antibiotics.

The anti-intracellular Salmonella activity of SCT1101 and SCT1104 are independent of AMPK and autophagy

To shed light on the mechanisms of action of SCT1101 and SCT1104, we first investigated whether their inhibitory activity against intracellular *Salmonella* is AMPK dependent. Unlike the findings in cells treated with nilotinib, we did not observe a significant increase in the level of phospho-AMPK in cells treated with SCT1101 or SCT1104 (Fig. 4A). Moreover, the efficacy of these two nilotinib derivatives against intracellular *Salmonella* was not influenced by treatment with the AMPK inhibitor compound C or SBI0206965 that have distinct structures and inhibitory profiles (Fig. 4B).[17] The finding indicating that the suppressive effects of SCT1101 and SCT1104 on intracellular *Salmonella* are not mediated by AMPK activation.

Next, we further investigated the role of autophagy in the anti-intracellular *Salmonella* activity of SCT1101 and SCT1104. As shown in Fig. 4C, the presence of the autophagy inhibitor 3-methyladenine (3-MA) increased the bacterial load in infected RAW264.7 cells. However, both nilotinib derivatives remained active in reducing the viability of intracellular *Salmonella* in 3-MA-treated cells. Moreover, we utilized shRNA expression plasmids to specifically knock down the autophagy-related gene *ATG7* in RAW264.7 cells and then assessed the efficacy of SCT1101 and SCT1104 against intracellular *Salmonella* in the Atg7-depleted cells (Fig. 4D, left panel). No significant differences in the anti-intracellular *Salmonella* activity of either SCT1101 or SCT1104 in Atg7 knockdown cells compared with vector-transfected cells were observed (Fig. 4D, right panel). Thus, our data indicated that the effects of SCT1101 and SCT1104 on intracellular bacteria are not mediated through autophagy.

Discussion

Infection with nontyphoidal *Salmonella* occasionally leads to invasive disease, especially in developing countries due to unsound public health systems.[3,18] Recently, the concepts of controlling intracellular bacterial infection through host-targeted or virulence-targeted mechanisms have attracted considerable attention.[19] Here, we showed that the tyrosine kinase inhibitor nilotinib can suppress *S.* Typhimurium infection in macrophages but has no inhibitory effect on bacterial growth in broth, implying that it might act via a non-bacteria-targeted mechanism. Subsequent pharmacologic exploitation led to the discovery of SCT1101, which exhibited potent antibacterial activity against intracellular MDR *S.* Typhimurium. The findings provide proof-of-concept that the structure of nilotinib is a promising scaffold for the development of new antibacterial agents with activity against intracellular MDR *Salmonella*.

Though AICAR, metformin and nilotinib are all capable of activating cellular AMPK activity, the mechanism of action of individual compound is different. Only AICAR acts by direct binding to the γ subunit of AMPK and allosterically activate the enzyme.[20] In contrast, metformin binds PEN2 which interacts with ATP6AP1 to activate AMPK via the lysosomal glucose-sensing pathway.[21] Nilotinib suppresses the activity of the protein phosphatase 2A (PP2A), leading to the activation of AMPK and autophagy.[22] The difference in the mechanism of actions of these AMPK agonists might explain why only nilotinib exhibits suppressing effect on intracellular *Salmonella*. On the other hand, nilotinib has been shown to induce autophagic eradication of intracellular *Mycobacterium tuberculosis* by activating AMPK.[23] However, our data indicated that neither AMPK nor autophagy is involved in the anti-intracellular *Salmonella* activity of the two nilotinib derivatives SCT1101 and SCT1104. Given that nilotinib not only was originally designed to treat chronic myeloid leukemia through the inhibition of BCR-ABL tyrosine kinase[24] but also has been shown to impair cellular mitochondrial function and increase ROS levels.[25] Thus, PP2A, the BCR-ABL tyrosine kinase, or ROS might play a role in the antibacterial activity of nilotinib, SCT1101 and SCT1104. Currently, we are using transcriptomic analysis to elucidate the pathways altered by SCT1101 and SCT1104 in *Salmonella*-infected RAW264.7 cells as well as a proteomic approach to assess the interaction of these two compounds with cellular proteins. Revealing the detailed antibacterial mechanism of nilotinib derivatives will foster the development of more potent antibacterial agents for treating infections with MDR *Salmonella*.

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Declaration of competing interest

We declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2023.01.005.