The Effect of the Potential PhoQ Histidine Kinase Inhibitors on Shigella flexneri Virulence

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Abstract

PhoQ/PhoP is an important two-component system that regulates Shigella virulence. We explored whether the PhoQ/PhoP system is a promising target for new antibiotics against S. flexneri infection. By using a high-throughput screen and enzymatic activity coupled assay, four compounds were found as potential PhoQ inhibitors. These compounds not only inhibited the activity of SF-PhoQc autophosphorylation but also displayed high binding affinities to the SF-PhoQc protein in the Surface Plasmon Resonance response. A S. flexneri cell invasion assay showed that three of these potential PhoQ inhibitors inhibit the invasion of HeLa cells by S. flexneri 9380. In a Mouse Sereny test, mice inoculated with S. flexneri 9380 pre-treated with the potential PhoQ inhibitors 1, 2, 3 or 4 displayed no inflammation, whereas mice inoculated with S. flexneri 9380 alone displayed severe keratoconjunctival inflammation. All four potential PhoQ inhibitors showed no significant cytotoxicity or hemolytic activity. These data suggest that the four potential PhoQ inhibitors inhibited the virulence of S. flexneri and that PhoQ/PhoP is a promising target for the development of drugs against S. flexneri infection.

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Introduction

Shigella is a gram-negative facultative intracellular pathogen with enhanced cell invasion, intracellular growth and intracellular spreading capabilities. The bacteria are transmitted fecal-oral and will invade the mucosa of the colon. Infection by only 10 to 100 organisms will cause shigellosis. Because of the overuse of antibiotics, Shigella drug resistance in clinical settings is increasing [1,2,3]. Therefore, new therapeutic targets and drugs are needed to reduce the incidence of shigellosis worldwide. Understanding the regulation of Shigella virulence may lead to the development of new drugs that can inhibit or reduce the virulence of Shigella as well as provide new strategies for treating shigellosis.

PhoQ/PhoP is a two-component system (TCS) that governs virulence, monitors extracellular Mg2+, and regulates several cellular activities in many gram-negative species [4]. The PhoQ/PhoP TCS consists of the transmembrane sensor PhoQ and the cytoplasmic regulator PhoP. PhoQ is a transmembrane histidine kinase with a functional kinase domain that binds ATP. It responds to environmental signals by phosphorylating itself as well as PhoP. PhoP has a functional domain, which when phosphorylated influences virulence by activating a phosphorylation cascade that regulates a series of downstream effecter genes in several bacterial species, including Shigella flexneri, Salmonella enterica, and Escherichia coli [5,6]. In Shigella, a functional phoP gene is important for virulence [7]. It has been proven that PhoP regulates Shigella’s susceptibility to polymorphonuclear leukocytes (PMNs) and antimicrobial molecules. A phoP Shigella mutant is highly sensitive to killing by neutrophils [7]. Furthermore, infection of a mouse eye with a wild-type Shigella strain will cause keratoconjunctivitis, whereas infection by a phoP Shigella mutant was resolved more quickly relative to wild type infections [7]. The research of PhoQ/PhoP TCS in Salmonella showed that mutants in the PhoQ/PhoP system can greatly reduce bacterial virulence and intracellular survival in macrophages [8]. This prompted us to investigate whether PhoQ/PhoP in Shigella would be an appropriate target for the design of novel antibacterial agents.

In the present study, we chose the PhoQ protein of S. flexneri as the target for screening by a chemical library, and four potential PhoQ inhibitors were identified. Both the cell invasion assay and Mouse Sereny test showed that these potential PhoQ inhibitors abate the virulence of S. flexneri. These potential PhoQ inhibitors displayed low cytotoxicity on mammalian cells and had no hemolysis effect. Our data indicate that PhoQ may be a promising target for the development of new antibiotics to treat S. flexneri infection.
Results

Sequence analysis of PhoQ HK and structure-based discovery of potential PhoQ inhibitors in silico

Alignment of PhoQ amino acid sequences of the Shigella strains S. flexneri 301, S. dysenteriae 197, S. boydii 227 and S. sonnei 046 show that the proteins share 100% identity. An optimized 3D model of the PhoQ HK domain of Sf301 was constructed based on the crystal structure of PhoQ HK from E. coli, which is highly homologous to S. flexneri (more than 99.8%). The ATP binding pocket consists of two different cavities connected by a gorge-like channel (Figure 1). The 3D model was used to identify potential PhoQ inhibitors of S. flexneri PhoQ HK by high-throughput virtual screening (HTVS). Using the drug-selection filter from the SPECS database, 85,000 potential drug-like molecules, constituting an in-house database (named SPECS_1), were searched for potential binding molecule structures, using the program DOCK4.0 as a primary screening. Then, the most optimal 200 structures were rescoped with the programs FlexX and CSCORE. Finally, according to molecular diversity, shape complementarity, and potential for forming hydrogen bonds in the binding pocket of the PhoQ, 100 molecules were manually selected as inhibitor candidates, and 64 compounds were purchased from the SPECS Company for further bioassays.

Effect of the potential PhoQ inhibitors on enzymatic activities of SF-PhoQc

To measure the interaction of the 64 compounds with PhoQ, a prokaryotic expression plasmid containing the Sf301 PhoQ intracellular domain was constructed, the recombinant His-tagged SF-PhoQc was purified and analyzed by SDS-PAGE (Figure S1A), and western blot analysis (Figure S1B) was performed with a mouse anti-His monoclonal antibody. The molecular mass of the recombinant SF-PhoQc was confirmed by MALDI-MS as 27.914 kDa (Figure S1C), which is in agreement with the theoretical molecular mass of 27.914 kDa.

The enzymatic activity of SF-PhoQc was determined using the Pyrophosphate Reagent, in which ATP initiates the reaction and ADP generated by ATP is accompanied by the reduction of NADH to NAD+. The rate of NADH reduction to NAD+ is detected by a change in absorbance readings at 340 nm. The activity of SF-PhoQc is measured by detecting a decrease in NADH at A_{340}. Recombinant SF-PhoQc (25 µmol/L) was added to initiate the reaction, and the A_{340} value was recorded by SoftMax vision 4.8 each minute for 30 minutes. The A_{340} value reduced from 1.47 to 0.65 in the presence of SF-PhoQc (Figure 2).

The effect of the 64 compounds on SF-PhoQc enzymatic activity was measured by using Pyrophosphate Reagent (Sigma-Aldrich, MO, USA). At a concentration of 100 mmol/L, four compounds (1, 2, 3 and 4) (Figure 3) inhibited the enzymatic activity of SF-PhoQc. Compounds 1, 2, 3 and 4 decreased SF-PhoQc catalyzed consumption of ATP by 100%, 82.3%, 67.1% and 65.8%, respectively (Figure 4). Then, the IC_{50} of each compound was determined by adding 4 µg of purified SF-PhoQc to different concentrations of each compound. The IC_{50} values of compounds 1–4 were 12.03±3.91 µmol/L, 56.99±1.04 µmol/L, 27.77±10.70 µmol/L and 27.75±5.59 µmol/L, respectively (Table 1).

Luminescent Kinase Assay was used to confirm the effects of compounds 1, 2, 3 and 4 on the enzymatic activity of SF-PhoQc. In the Luminescent Kinase Assay, the putative kinase activity of SF-PhoQc protein was measured by quantifying the amount of ATP that remained in solution after the reaction. A direct relationship between the luminescence measured and the amount of ATP was observed, indicating that the purified SF-PhoQc protein possessed ATPase activity. Next, the effect of each compound on the enzymatic activity of SF-PhoQc was measured. The IC_{50} values of compounds 1–4 were calculated as 69.37±0.56 µmol/L, 48.9±14.7 µmol/L, 7.99±5.61 µmol/L and 27.2±15.44 µmol/L, respectively (Table 1).

Binding affinities of potential PhoQ inhibitors to SF-PhoQc

The binding affinities of compounds 1–4 to the SF-PhoQc were determined by Surface Plasmon Resonance (SPR). All four compounds displayed significant binding affinities to the SF-PhoQc protein in the SPR response (Figure 5). The data were fitted to a steady-state affinity model for potential PhoQ inhibitors, and a K_{D} value was determined using the Biacore 3000 evaluation software. The dissociation constants of compounds 1–4 to SF-PhoQc were 4.50, 10.6, 7.56 and 9.40 µmol/L, respectively, and they were named as potential PhoQ inhibitors (Table 1).

Evaluation of potential PhoQ inhibitors on the virulence of S. flexneri and S. typhimurium in vitro

At concentration of 200 µmol/L (highest concentration in the present study) the four potential PhoQ inhibitors had no obvious effect on growth of Sf3080. The growth curve of Sf3080 treated with or without potential PhoQ inhibitors at concentration 200 µmol/L were tested. The growth curves of bacteria treated with or without potential PhoQ inhibitors treatment were similar, suggesting that four potential PhoQ inhibitors have no effects on...
the growth of Sf9380 (Figure S2). The gentamicin protection assay was used as a cellular model to evaluate the inhibitory effect of the four potential PhoQ inhibitors [9]. The bacteria were treated with each of the four potential PhoQ inhibitors in a serial dilution and grown for 8 hours. Next, the bacteria were inoculated and incubated with HeLa cells for 60 min prior to the addition of gentamicin to kill extracellular bacteria. For testing intracellular growth and spreading to adjacent cell, cells were further incubated for 290 min [9]. The results of the assays were expressed as the number of bacteria recovered from gentamicin-treated cells divided by the number of inoculated bacteria added to the cell. After lysates of HeLa cells were plated, the number of CFU that formed on LB agar were counted. Treated with 12.5 μmol/L of potential PhoQ inhibitor 1, invasion of HeLa cells by Sf9380 was reduced to 1.36 \( \times \) 10^{-2}, 4.3 times lower than that of untreated Sf9380 (5.87 \( \times \) 10^{-2}). A concentration of 25 μmol/L of potential PhoQ inhibitor 1 had a similar effect, whereas lower concentrations had no obvious effects. At a concentration of 25 μmol/L, potential PhoQ inhibitor 2 reduced cell invasion of the bacterium to 3.6 \( \times \) 10^{-1}, 16.3 times lower than the control, concentrations lower than 25 μmol/L had no obvious effects. Potential PhoQ inhibitor 3, at 100 μmol/L, reduced invasion of HeLa cells by Sf9380 to 1.95 \( \times \) 10^{-2}, concentrations lower than 100 μmol/L had no obvious effects. Potential PhoQ inhibitor 4, at 100 μmol/L, had

![Figure 2. The activity of recombinant SF-PhoQc.](image)

![Figure 3. The chemical structures of four compounds as potential PhoQ inhibitors.](image)
no effect on the bacterial invasion of HeLa cells (shown in Figure 6A). For intracellular growth and spreading to adjacent cell, potential PhoQ inhibitor 1 (12.5 μmol/L) reduced cell invasion of bacterium to 0.061, 1.96 times lower than that of untreated S9380 (0.12), potential PhoQ inhibitor 2 (25 μmol/L) was reduced to 0.036, 3.3 times lower than that of untreated S9380, PhoQ inhibitor 3 (100 μmol/L) was reduced to 0.046, 2.6 times lower than that of untreated S9380, while PhoQ inhibitor 4 (100 μmol/L) have no obvious effect on the cell intracellular growth (0.12) (shown in Figure 6B).

Table 1. Biological effects of the four potential PhoQ inhibitors.

| Potential PhoQ inhibitors No. | IC50 (μmol/L) for SF-PhoQc(a) | IC50 (μmol/L) for SF-PhoQc(b) | Kd value (μmol/L)(c) | CC50 (μmol/L) on Vero cell(d) | Hemolysis (%) |
|------------------------------|-------------------------------|-------------------------------|----------------------|-----------------------------|----------------|
| 1                            | 12.03±3.91                    | 69.37±0.56                    | 4.50                 | >200                        | <0.1(m) (<0.1(f)) |
| 2                            | 56.99±1.04                    | 48.90±14.7                   | 10.6                 | >200                        | <0.1 (<0.1) |
| 3                            | 27.77±10.70                   | 7.99±5.61                    | 7.56                 | >200                        | <0.1 (<0.1) |
| 4                            | 27.75±5.39                    | 27.20±15.44                  | 9.40                 | >200                        | <0.1 (<0.1) |

(a)The Pyrophosphate Reagent detects autophosphorylation. IC50 represents the concentration of the potential PhoQ inhibitor needed to inhibit 50% of SF-PhoQc’s autophosphorylation. Values are means ± standard deviations from 3 independent wells.
(b)The Luminescent Kinase Assay detects autophosphorylation. IC50 represents the concentration of the potential PhoQ inhibitor needed to inhibit 50% of SF-PhoQc’s autophosphorylation. Values are means ± standard deviations from 3 independent wells.
(c)Kd: Equilibrium Dissociation constant.
(d)CC50 represents the concentration of a potential PhoQ inhibitor that results in 50% cytotoxicity of Vero cells. The highest concentration tested is 200 μmol/L.
(e)Healthy human erythrocytes were used for the hemolysis assay, and the hemolytic activities of the four potential PhoQ inhibitors are shown at 25 μmol/L and 100 μmol/L.

Figure 4. Dose response curve of recombinant SF-PhoQc enzyme inhibition by potential PhoQ inhibitors 1–4. The inhibition effects of potential PhoQ inhibitors on SF-PhoQc at different concentrations (0–100 μmol/L) detected by using the Pyrophosphate Reagent, and the dose-dependent curve of each compound were measured by using Origin 7.0 software (OriginLab, Northampton, USA). Relative activity in Y-axis was calculated by 100% – Rp, indicating the enzyme activity of SF-PhoQc after potential PhoQ inhibitor treatment.

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The gentamicin protection assay shows the inhibitory effect of the four potential PhoQ inhibitors on S. flexneri 2a 301 phoQ/phoP knock-out mutant (Sf301 ΔphoQ/phoP) HeLa cells invasion. The results of the HeLa cell invasion showed that the cell invasion rate of Sf301 ΔphoQ/phoP treated with potential PhoQ inhibitor 1 (12.5 μmol/L), 2 (25 μmol/L), 3 (100 μmol/L), 4 (100 μmol/L) were 0.064, 0.102, 0.083 and 0.053, respectively. The cell invasion rate of Sf301 ΔphoQ/phoP was 0.069 (shown in Figure 6C).

The effects of potential PhoQ inhibitors on cell invasion of Salmonella were determined by gentamicin protection assay. It showed that the cell invasion rate of Salmonella enterica Typhimurium SL1344 treated with potential PhoQ inhibitor 1 (12.5 μmol/L), 2 (25 μmol/L), 3 (100 μmol/L) were 0.89×10⁻² (6.74 times lower than the control), 1.56×10⁻² (3.84 times lower than the control) and 0.036×10⁻² (166 times lower than the control), respectively, while potential PhoQ inhibitor 4 (100 μmol/L) had no obvious effect on the cell invasion (the cell invasion rate was 5.7×10⁻²). The cell invasion rate of Salmonella was 6×10⁻² (shown in Figure 7).

Effects of the potential PhoQ inhibitors on S. flexneri virulence in vivo

A Mouse Sereny test was used to investigate the inhibitory effect of the four potential PhoQ inhibitors on the virulence of S. flexneri Sf9380. The mice were infected with 1×10⁸ CFUs per eye. Mice inoculated with Sf9380 showed severe keratoconjunctivitis without purulence at 24 hours post-infection, which developed to keratoconjunctivitis with purulence after 48 hours, and the condition was sustained for 96 hours (Figure 8, Table 2). Mice inoculated with bacteria pre-treated with potential PhoQ inhibitors 1 (12.5 μmol/L) or potential PhoQ inhibitors 2 (25 μmol/L) or potential PhoQ inhibitors 3 (100 μmol/L) or potential PhoQ inhibitor 4 (100 μmol/L) displayed a slight conjunctival inflammation at 24 hours after infection, but keratoconjunctivitis was resolved by 48 hours (Figure 8, Table 2). Mice inoculated with bacteria pre-treated with potential PhoQ inhibitor 3 (100 μmol/L) or potential PhoQ inhibitor 4 (100 μmol/L) displayed a slight conjunctival inflammation 24 hours post-infection, with keratoconjunctivitis resolved by 72 hours (Figure 8, Table 2). Mice infected with S. flexneri Sf9380 treated, with the same amount of DMSO (0.1%, v/v) as the potential PhoQ inhibitors, displayed severe conjunctival inflammation 24 hours post-infection, with keratoconjunctivitis resolved by 72 hours (Figure 8, Table 2). Mice inoculated with E. coli ATCC 25922 did not develop conjunctival inflammation as a negative control (Figure 8, Table 2).

Cytotoxicity assay and erythrocyte hemolysis assays of potential PhoQ inhibitors

The cytotoxicity of each potential PhoQ inhibitor towards a Vero cell line was analyzed by the traditional MTT assay. The
Figure 6. The effects of potential PhoQ inhibitors on virulence of *Shigella*. The gentamicin protection assay shows the inhibitory effect of the four potential PhoQ inhibitors on *S. flexneri* HeLa cells invasion. The bacteria pre-treated with potential PhoQ inhibitor were added to HeLa cells at a multiplicity of infection of 100. Then gentamicin was added to kill extracellular bacteria and cell lysates were plated onto LB plates, and the colonies were counted. The results of the assays were expressed as the number of bacteria recovered from gentamicin-treated cells divided by the number of inoculated bacteria added to the cell. *S. flexneri* Sf9380 (treated without DMSO), a clinical strain with strong virulence, served as the positive control for the cell invasion assay; *E. coli* ATCC 25922 (treated without DMSO), an avirulent strain, served as the negative control for the cell invasion assay; bacteria treated with 0.1% DMSO (the solvent of the potential PhoQ inhibitor) also served as a control for the cell invasion assay. Values are means ± standard deviations from 6 independent wells. *p < 0.01 vs. Sf9380. (A) The effects of potential PhoQ inhibitors on cell invasion of *S. flexneri* Sf9380.
CC₅₀ values of all four compounds were higher than 200 μmol/L, the highest tested concentration of potential PhoQ inhibitors. As a control, an equal concentration of DMSO (0.1%) was added to a well of Vero cells, and no obvious cytotoxicity was observed (Table 1).

To determine whether the four potential PhoQ inhibitors would induce hemolysis of mammalian erythrocytes, the hemolytic activities of each potential PhoQ inhibitor were tested at concentrations of 25 μmol/L and 100 μmol/L. All four potential PhoQ inhibitors showed no hemolytic activity (Table 1).

**Discussion**

Currently, there is an increase in antibiotic resistance among *Shigella* isolates [1], and this drug resistance phenomenon is causing complications and difficulties for clinical treatment. Several virulence regulator factors, such as two-component signal systems [10,11,12], quorum sensing systems [11,13,14], type III secretion systems [15], and the assembly of adhesive organelles [16], have been recognized as interesting targets to reduce bacterial infection. Bacterial two-component systems have gained increasing interest as novel antibacterial targets because these systems are required for virulence of pathogenic microorganisms [17,18]. In the present study, we found that the PhoQ/PhoP two-component system of *Shigella* may be a promising target for developing new antibiotics against *S. flexneri* infection.

PhoQ/PhoP is a two-component system that governs virulence [4], monitors the extracellular Mg²⁺, and regulates several cellular activities in many gram-negative species. The system also helps bacteria resist antibiotic peptides by regulating lipid A [7,19,20,21,22]. Bivalent cations and antibiotic peptides can competitively bind to the acidic structural domain on the cytoplasmic surface of PhoQ [23]. In addition to the concentration of Mg²⁺ or Ca²⁺ cations in the cytoplasm, it has been shown that the concentration of antibiotic peptides in the external environment [24], in addition to an acidic environment, will mediate the activation of PhoQ [25]. In *Salmonella*, PhoQ/PhoP can change the structure of the external cell membrane by regulating the remodeling of lipid A to strengthen a bacterium’s resistance to the environment [26]. In *Shigella*, the PhoQ/PhoP two-component system is necessary for virulence, as demonstrated by an infection of mice with a *phaP* mutant of *Shigella* that resulted in milder keratoconjunctivitis than a wild type strain [7].

PhoQ is an attractive target for an antibiotic because it is absent in mammals [10,27]. In this study, we have explored the possibility of using the PhoQ as a potential target by performing a screen for inhibitors. After constructing a 3D model of the PhoQ HK domain of S301, 64 compounds were selected as inhibitor candidates based on their molecular diversity, shape complementarities, and potential for forming hydrogen bonds in the binding pocket of PhoQ. To confirm the interaction of the compounds and PhoQ, a prokaryotic expression plasmid containing the S301 PhoQ intracellular domain which contains HK domain was constructed, because the main biology activity of PhoQ is depends on its HK domain [28]. To confirm whether these inhibitor candidates targeted the PhoQ HK domain, enzymatic activities of PhoQ were determined in the presence or absence of four compounds. The enzymatic activity of SF-PhoQc was measured using both a Pyrophosphate Reagent and a Luminescent Kinase Assay. The Pyrophosphate Reagent can reflect the reaction of HK and ATP at real time, but not sensitive. The Luminescent Kinase Assay is more sensitive than Pyrophosphate Reagent for kinase reaction but cannot reflect the reaction of HK and ATP at real time. Therefore, in the present study we used two assays to evaluate the enzymatic activity of PhoQ.

Figure 7. The effects of potential PhoQ inhibitors on cell invasion of *Salmonella*. The gentamicin protection assay shows the inhibitory effect of the four potential PhoQ inhibitors on *Salmonella enterica typhimurium* SL1344 HeLa cells invasion. The bacteria pre-treated with potential PhoQ inhibitor were added to HeLa cells at a multiplicity of infection of 100. Then gentamicin was added to kill extracellular bacteria and cell lysates were plated onto LB plates, and the colonies were counted. The results of the assays were expressed as the number of bacteria recovered from gentamicin-treated cells divided by the number of inoculated bacteria added to the cell. *Salmonella enterica typhimurium* SL1344 served as the positive control for the cell invasion assay; *E. coli* ATCC 25922 (treated without DMSO), an avirulent strain, served as the negative control for the cell invasion assay; bacteria treated with 0.1% DMSO also served as a control for the cell invasion assay. Values are means ± standard deviations from 6 independent wells. *p<0.01 vs. SL1344.

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Potential PhoQ inhibitors can reduce the degree of keratoconjunctivitis produced by Shigella in mice. A Mouse Sereny test shows the virulence of S. flexneri with or without potential PhoQ inhibitor treatment. Mice inoculated with 1 x 10^8 CFUs of S. flexneri SF9380 show severe keratoconjunctivitis without purulence at 24 hours after infection, which develops to keratoconjunctivitis with purulence after 48 hours and continues for 96 hours. SF9380 cultured with a serial dilution of potential PhoQ inhibitors were also inoculated into mouse eyes at 1 x 10^8 CFUs. Mice inoculated with the bacteria pre-treated with potential PhoQ inhibitors 1 (12.5 μmol/L) or potential PhoQ inhibitors 2 (25 μmol/L) display a slight conjunctival inflammation at 24 hours post-infection, and keratoconjunctivitis is resolved by 48 hours. Mice inoculated with the bacteria pre-treated with potential PhoQ inhibitors 3 (100 μmol/L) or potential PhoQ inhibitors 4 (100 μmol/L) display a slight conjunctival inflammation at 24 hours post-infection, and keratoconjunctivitis is resolved by 72 hours. Mice inoculated with the negative control, E. coli ATCC 25922, did not develop conjunctival inflammation. SF9380 treated with the same amount of DMSO in which the potential PhoQ inhibitors were dissolved served as a control.

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| Table 2. Keratoconjunctivitis in mice inoculated with potential PhoQ inhibitors treated SF9380. |
|-------------------------------------------------------------|
| **24 h** | **48 h** | **72 h** | **96 h** |
| 1 | 2 | 3 | 4 | 5 | 6 | 1 | 2 | 3 | 4 | 5 | 6 | 1 | 2 | 3 | 4 | 5 | 6 |
| SF9380(a) | ++ | ++ | + | ++ | ++ | ++ | +++ | + | +++ | ++ | + | ++ | ++ | +++ | + | ++ | + | + |
| ATCC 25922(b) | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| DMSO(c) | ++ | ++ | + | ++ | ++ | ++ | +++ | + | +++ | ++ | + | ++ | ++ | +++ | + | ++ | + | + |
| I 1 (12.5 μmol/L) | + | + | - | + | - | - | + | - | - | - | - | - | - | - | - | - | - | - |
| I 2 (25 μmol/L) | + | + | + | + | - | - | + | - | - | - | - | - | - | - | - | - | - | - |
| I 3 (100 μmol/L) | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| I 4 (100 μmol/L) | + | + | + | + | + | + | - | - | - | - | - | - | - | - | - | - | - | - |

A Mouse Sereny test shows the virulence of S. flexneri SF9380. Mice inoculated with 1 x 10^8 CFUs display severe keratoconjunctivitis without purulence 24 hours post-infection, developed keratoconjunctivitis with purulence after 48 hours, and sustained the infection for 96 hours. SF9380 cultured with a serial dilution of potential PhoQ inhibitors was inoculated into mice, at 1 x 10^8 CFUs per eye. The degree of keratoconjunctival inflammation in each of the six mice infected with SF9380, ATCC 25922, DMSO, and SF9380 pre-treated with different concentrations of potential PhoQ inhibitors 1, 2, 3 or 4 at 24, 48,72 and 96 hours (n = 6). Mouse Keratoconjunctivitis was rated as follows: -, no disease; ±, little keratoconjunctivitis; +, mild keratoconjunctivitis; ++, keratoconjunctivitis with some purulence; ++++, fully developed keratoconjunctivitis with great purulence.

(a)Inoculation of SF9380 alone, served as the positive control for the Mouse Sereny.
(b)ATCC 25922, an avirulent E. coli strain, served as a negative control for the Mouse Sereny test.
(c)DMSO, SF9380 treated with DMSO, the solvent of the potential PhoQ inhibitors, served as a control for the Mouse Sereny test.

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confirm the results. The different IC50 values of potential PhoQ inhibitors 1 and 3 determined by the two assays may be the sensitivity difference between the two assays.

By using cell invasion assays, the features of cell invasion process including penetration into epithelial cells and spreading to adjacent cells were tested [29]. The *S. flexneri* (SF9380) were treated with four potential PhoQ inhibitors (100 μmol/L) for 4, 6 or 8 hours, respectively. Compared with cell invasion of the positive control SF9380 alone, the potential PhoQ inhibitors (100 μmol/L) treated for 8 hours had obvious inhibition effects on the bacteria cell invasion by using gentamicin protection assay, while potential PhoQ inhibitors treated for 4 or 6 hours had no significant inhibition effects on SF9380 cell invasion (Figure S3). Therefore, *Shigella* cell invasion assay and Mouse Sereny test were carried out by the bacteria treated with potential PhoQ inhibitors for 8 hours.

To confirm these four potential PhoQ inhibitors were affecting PhoQ histidine kinase, we made a *S. flexneri* phoQ/phoP knock-out mutant (SF301 AphoQ/phoP) and the cell invasion ability was tested. The results indicated that potential PhoQ inhibitors 1, 2, 3 can inhibit HeLa cell invasion ability of SF301 (Figure S4) but have no obvious effects on SF301 phoQ/phoP knock-out mutant (Figure 6C). It indicated that these potential PhoQ inhibitors can affect PhoQ histidine. The results also suggested that the cell invasion ability of SF301 AphoQ/phoP decreased significantly compared to the wild type strain. It indicated that phoQ/phoP could regulate the cell invasion of *S. flexneri*.

The PhoQ of *Salmonella* is high homology to that of *Shigella* and in *Salmonella* PhoP/PhoQ regulates virulence including cell invasion [4,6]. So it was curious for us to evaluate whether these four potential PhoQ inhibitors have similar effect on *Salmonella*. The results of cell invasion suggested that cell invasion of *Salmonella* SL1344 was inhibited by the potential PhoQ inhibitors 1, 2, 3 while potential PhoQ inhibitor 4 has no obvious effect on cell invasion which were similar to the results of *Shigella* (Figure 7). It indicates that the potential PhoQ inhibitors can inhibit the cell invasion of *Salmonella*.

In the present study, we chose the PhoQ protein, a transmembrane sensor of the PhoQ/PhoP TCS in *S. flexneri* 2a 301, as the target and found that three potential PhoQ inhibitors can inhibit the bacterial ability to invade HeLa cells. Further, we found that with phoQ/phoP knock-out mutants, the cell invasion ability of SF301 AphoQ/phoP decreased significantly, compared to the wild type strain, and no obvious effects of potential PhoQ inhibitors on SF301 AphoQ/phoP were observed. However, Moss et al has reported that there were no significant differences between wild-type and phoP mutant of *S. flexneri* serotype 5 strain M90T in HeLa cell invasion [7]. In gram-negative pathogen, there are many cross-talks between two-component systems (TCSs) [30,31,32,33], in which one HK (Histidine Kinase) can regulate several RR (Response Regulator), and one RR’s phosphorylation can be regulated by several HKS to gather several signal pathways of TCSs and induce expression of a battery of downstream genes. This difference between phoQ/phoP knock-out mutant of *S. flexneri* 2a 301 and phoP knock-out mutant of *S. flexneri* 5 M90T may due to the cross-talks between TCSs in the bacterium, although in *Shigella* it remains poorly understood. Involvement of the PhoQ/PhoP cascade on *Shigella* virulence across strains, serotypes and species need to be investigated in the future.

In the present study, four potential PhoQ inhibitors, at 200 μmol/L, showed no effect on *Shigella* growth (Figure S2). This was expected because the PhoQ/PhoP signaling system does not directly regulate bacterial growth. With increasing knowledge about bacterial virulence, several researchers have found that bacterial virulence genes are essential to mount a harmful infection, but they are usually dispensable for growth of bacteria *in vitro*. These results indicate that inhibition of microbial virulence without inhibiting their growth may be a promising strategy [34]. In contrast, currently available antibiotics either kill bacteria or prevent their growth. Drugs that block disease without killing the pathogen bacteria [35,36] may cause less selective pressure for the generation of drug resistance [34,36,37]. These alternative drug strategies would presumably induce pathogen resistance at a much slower rate because the targeted non-essential genes or functions are under less selective pressure to mutate. The host will be subjected to intact avirulent bacteria, allowing the host to develop an adequate immune response against the pathogen. This would allow the host to efficiently respond to and eradicate an invader upon re-exposure. Therefore, the strategy to target bacterial virulence factors has become an attractive approach for the development of new therapeutic agents [35].

In addition to novel drug targets, the use of small organic molecules is gaining interest more than gene-based drugs [38, 39]. Small organic molecules that target specific proteins may be used for the prevention or treatment of infections caused by a wide variety of gram-negative bacteria species, including *Escherichia coli* [17,40], *Salmonella typhimurium* [18] and *Yersinia pseudotuberculosis* [41], as well as gram-positive bacteria such as *Staphylococcus epidermidis* [42]. In this study, four promising potential PhoQ inhibitor candidates were evaluated using enzymatic activity assays and binding affinities.

In previous studies, some potential PhoQ inhibitors displayed side effects, such as membrane damage or excessive protein binding, which would be an obstacle for their further development [43]. In this study, we found four potential PhoQ inhibitors that reduce the virulence of *Shigella* that also have low cytotoxicity and hemolysis of mammalian cells at their effective concentrations. We demonstrated that PhoQ/PhoP is a promising target for the development of new drugs against *S. flexneri* infection and proved that four potential PhoQ inhibitors can inhibit the virulence of *Shigella*. In future work, we will modify the compound structure to increase the efficacy of the potential PhoQ inhibitors and identify which stage of infection is inhibited by these potential inhibitors which is important to the therapy of shigellosis.

**Materials and Methods**

**Ethics Statement**

All procedures performed on mice were conducted according to relevant national and international guidelines (the Regulations for the Administration of Affairs Concerning Experimental Animals, China, and the NIH Guide for the Care and Use of Laboratory Animals) and were approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Medical College, Fudan University (IACUC Animal Project Number: 20090601-qu).

**Bacterial strains, growth conditions and reagents**

*S. flexneri* 2a 301 (*SF901, Genbank Accession number for chromosomes is AE005674*) was kindly provided by Pr. Jianguo Xu (Chinese Center for Disease Control and Prevention, Beijing, China). A clinical strain of *S. flexneri, SF9380*, was isolated from an epidemic in the Hebei province by Dr. Xuanyi Wang from our laboratory. *E. coli* ATCC 25922 was kindly provided by Dr. Bijie Hu (Zhejiang Hospital, Shanghai, China). *S. enterica* typhimurium SL1344 and plasmid pSB890 were kindly provided by Dr Daoguo Zhou (Purdue University, USA). *E. coli* JM109 and plasmid pQE30 were purchased from Qiagen (Germany). *E. coli* JM109 was used for both recombinant DNA manipulation and protein production. *S. flexneri* and *E. coli* were grown at 37°C in...
Luria–Bertani medium (LB; Oxoid, Wesel, Germany). All compounds used as inhibitor candidates were purchased from the SPECS Company (Netherlands). Stock solutions (10 mmol/L) of the potential PhoQ inhibitors were prepared in dimethyl sulfoxide (DMSO).

3D structure modeling of the PhoQ histidine kinase of *S. flexneri* SF301

The PhoQ histidine kinase sequence of *S. flexneri* 2a strain 301 was retrieved from GenBank (accession number AE005674). To identify potential inhibitors of PhoQ histidine kinase (PhoQ HK) by virtual screening, we obtained the crystal structure of the PhoQ HK domain (PDB entry code: 1ID0) from the Brookhaven Protein Database (PDB http://www.rcsb.org/pdb). The missing residues in the PhoQ HK were added and subjected to energy minimization in Sybyl 6.8 [43] using the steepest descent method up to the gradient tolerance of 0.05 kcal/(mol Å) to relieve possible steric clashes and overlaps of side chains. The potential of the 3D structure of PhoQ HK domain was assigned according to the Amber force field with Kollman-undated-atom charges encoded in Sybyl 6.8 [43].

Structure-based virtual screening of potential PhoQ HK inhibitors

The ATP-binding pocket formed by residues within a radius of 7 Å around the ATP site of the PhoQ HK catalytic domain was used as the target site for high-throughput virtual screening (HTVS). The optimized 3D model of PhoQ HK was used as the target for virtual screening with the SPECS database (http://www.specs.net). In the first step, 85,000 potential drug-like molecules were selected from the SPECS database, creating an in-house database (named SPECS_1). Next, the SPECS_1 database was searched for potential binding molecule structures using the program DOCK4.0 [44,45]. The most optimal 10,000 structures were subsequently re-scored using the FlexX [46] and CSCORE, a consensus scoring method that integrates five popular scoring functions. Two hundred molecules passed this highly selective filter. Finally, 100 molecules were manually selected as inhibitor candidates, according to their molecular diversity, their shape complementarities, and their potential for forming hydrogen bonds in the binding pocket of the PhoQ HK domain.

Expression and purification of recombinant SF-PhoQc

A DNA segment encoding the cytoplasmic domain of PhoQ HK was amplified by PCR from the S/f301 genomic DNA with primers PhoQc5’ (5’-CGCCGATCCGTAACACCTGAACCGATT-3’) and PhoQc3’ (5’-CCGAAAGTTTTTATTGACTCTTGGGCGGAG-3’), which have BamHI and HindIII restriction sites, respectively. The amplified DNA segment encodes PhoQc, 25 mmol/L KCl, 2.5 mmol/L MgCl 2, 2 mmol/L ATP, 1 mmol/L phosphoenolpyruvate, 0.35 mmol/L NADH, 1.5 U/ml PK, 1.25 U/ml LDH and 50 mmol/L Tris–Cl (pH 8.0). The enzyme activity was determined in the presence of various concentrations of potential PhoQ inhibitors (0–100 mmol/L) to investigate dose–dependence inhibition effects. The IC50 value was recorded by SoftMax vision 4.8 every minute, for 30 minutes. The rate of SF-PhoQc enzymatic activity inhibition by a compound was calculated by the following formula:

\[
Rp = \frac{A_{405}(\text{PhoQc} + \text{compound} + \text{ATP} + \text{Reagent}) - A_{405}(\text{PhoQc} + \text{ATP} + \text{Reagent})}{A_{405}(\text{ATP} + \text{Reagent}) - A_{405}(\text{PhoQc} + \text{ATP} + \text{Reagent})} \times 100%
\]

Rp (Ratio of Phosphorylation) was defined as the ratio of the decrease activity of SF-PhoQc treated with compound vs the activity of SF-PhoQc treated without compounds. Rp was stand for the percentage of decrease activity of SF-PhoQc.

IC50 is the concentration of inhibition of 50% values of potential PhoQ inhibitors were obtained by fitting the data to a sigmoid dose–response equation using Origin 7.0 software (OriginLab, Northampton, USA).

In the Luminescent Kinase assay, reactions were performed in solid black, flat-bottomed 96-well plates. To determine the IC50 of potential PhoQ HK inhibitors, each compound underwent 2-fold serial dilutions with DMSO (0.1%) and was added into the reaction mixture. The final reaction mixture contained 25 μmol/L purified SF-PhoQc, 1 mmol/L NaCl, 2.5 mmol/L MgCl 2, 2 mmol/L ATP, 1 mmol/L phosphoenolpyruvate, 0.35 mmol/L NADH, 1.5 U/ml PK, 1.25 U/ml LDH, 50 mmol/L Tris–Cl (pH 8.0).
before recording the luminescence reading on a Fluoroskan Ascent FL machine (Thermo Scientific, USA). The rate of phosphorylation inhibition by a compound was calculated with the following formula:

\[
Rp = \frac{RLU(\text{PhoQc} + \text{compound} + \text{ATP} + \text{Reagent}) - RLU(\text{PhoQc} + \text{ATP} + \text{Reagent})}{RLU(\text{ATP} + \text{Reagent}) - RLU(\text{PhoQc} + \text{ATP} + \text{Reagent})} \times 100%
\]

The IC_{50} value of each compound was determined using the Origin 7.0 software (OriginLab, Northampton, USA).

**Binding affinities of potential PhoQ inhibitors to SF-PhoQc**

The binding affinities of potential PhoQ inhibitors to SF-PhoQc were determined in vitro using Surface Plasmon Resonance (SPR) technology with the dual flow cell Biacore 3000 instrument (Biacore AB, Uppsala, Sweden). The standard primary amine coupling reaction wizard immobilized SF-PhoQc to the hydrophilic carboxymethylated dextran matrix of the sensor chip, CM5 (Biacore). The SF-PhoQc covalently bound to the matrix was diluted in 10 mmol/L sodium acetate buffer (pH 4.2) to a final concentration of 0.035 mg/ml. Equilibration of the baseline was completed by a continuous flow of HBS-EP running buffer (10 mmol/L HEPES, 150 mmol/L NaCl, 3 mmol/L EDTA, and 0.005% (v/v) surfactant P20, pH 7.4) through the chip for 1–2 hours. All the Biacore data were collected at 25 °C with HBS-EP as the running buffer at a constant flow of 30 μl/min. All the sensorgrams were processed by using automatic correction for nonspecific bulk refractive index effects. The equilibrium constants (K_D values) evaluating the protein-ligand binding affinities were determined by the steady-state affinity fitting analysis of the Biacore data.

**Construction of the S. flexneri phoQ/phoP deletion mutant**

The plasmid for phoQ/phoP deletion in S. flexneri 2a 301 was constructed. The upstream and downstream of the phoQ/phoP were amplified by using PCR with the primers listed in the Table 3: phoQ/phoP U-F and phoQ/phoP U-R were used to amplify upstream fragment; phoQ/phoP D-F and phoQ/phoP D-R were used to amplify downstream fragment. The amplified upstream fragment of phoQ/phoP was digested with NotI and XbaI restriction endonucleases, ligated into NotI and XbaI-cut pSB890 vector (a suicide vector [47]), resulting in a plasmid name as pSB890up. The amplified downstream fragment of phoQ/phoP was digested with XbaI and SmaI restriction endonucleases and ligated into XbaI and SmaI-cut pSB890up vector, resulting in a plasmid pSB890 phoQ/phoP with the upstream and downstream fragments of phoQ/phoP. The plasmid pSB890 phoQ/phoP was transferred into E. coli SM10pir cells, then pSB890 phoQ/phoP was introduced into S. flexneri 2a 301 by conjugation. Equal volumes (5 ml) of S. flexneri 2a 301 wild-type recipient and E. coli SM10pir contain pSB890 phoQ/phoP were mixed, and 100 μl was spotted onto an LB agar plate. After incubation (37 °C for 24 h), the bacteria were recovered, resuspended in 1 ml of Luria-Bertani (LB) broth, and cultured on LB agar plate containing ampicillin and streptomycin at 37 °C. Individual ampicillin-resistant S. flexneri colonies were inoculated into LB broth, grown overnight at 22 °C, and plated onto LB agar supplemented with 5% sucrose. Sucrose-tolerant colonies were screened using PCR with primers 5’-CCCC-CGCCGTGGTTTATTTAATGGTTTATA-3’ and 5’-ACCACCGCCTG-TATATTTCTTGTGTC-3’. A ST301 PhoQ/phoP was screened out and identified by PCR and sequencing.

**S. flexneri cell invasion assay**

Bacterial ability to invade HeLa cells was tested with a gentamicin protection assay [9]. HeLa cells were grown in six-well tissue culture plates to semi-confluent monolayers (DMEM, 10% FCS, 5% CO_2). S. flexneri 2a 301 was treated with potential PhoQ inhibitors for 3 hours in LB at 37 °C (final concentrations of 100, 50, 25, 12.5 or 6.25 μmol/L) prior to infection. The bacteria pre-treated with potential PhoQ inhibitor without washing, were added to semi-confluent HeLa cells at a multiplicity of infection of 100, at the same time the bacteria were diluted and plated onto LB agar plates. The CFUs were counted as initial bacterial number before added into HeLa cells. Then the plates were centrifuged at 900 ×g for 5 minutes. After incubating at 37 °C for 60 minutes (For testing intracellular growth and spreading to adjacent cell, cells were further incubated for 290 min [9]), the cells were washed three times with PBS, and gentamicin was added to the medium to a final concentration of 10 μg/ml for 20 minutes at 37°C. After the incubation, the HeLa cells in each well were lysed in 1 ml of PBS containing 0.1% Triton X-100 for 10 minutes at room temperature. The lysates were diluted and plated onto LB agar plates in triplicate. Colonies grown on LB plates were counted. The results of the assays were expressed as the number of bacteria recovered from gentamicin-treated cells divided by the number of inoculated bacteria added to the cell. The cells were inoculated with untreated S. flexneri 2a 301 PhoQ/phoP (treated without DMSO) as a positive control and E. coli ATCC 25922, an avirulent strain treated without DMSO, as a negative control. DMSO is the solvent of the potential PhoQ inhibitor compounds; therefore, S. flexneri 2a 301 was also treated with the same amount of 0.1% DMSO as the other groups to control for any DMSO-specific effects. The cell invasion assay was performed in triplicate for each potential PhoQ inhibitor, and the assay was repeated twice.

**Salmonella cell invasion assay**

The gentamicin protection assay was used as a cellular model to evaluate the inhibitory effect of the four potential PhoQ inhibitors. *Salmonella enterica typhimurium* SL1344 was treated with each of the four potential PhoQ inhibitors (final concentrations: potential PhoQ inhibitor 1 was 12.5 μmol/L, 2 was 25 μmol/L, 3 was 100 μmol/L, and 4 was 100 μmol/L) and grown for 8 hours. The bacteria pre-treated with potential PhoQ inhibitor without washing, were added to HeLa cells at a multiplicity of infection of 100, at the same time the bacteria were diluted and plated onto LB agar plates. The CFUs were counted as initial bacterial number before added into HeLa cells. Next, the bacteria were inoculated to HeLa cells and incubated with for 60 minutes prior to the addition of gentamicin to kill extracellular bacteria. After the incubation, the HeLa cells in each well were lysed in 1 ml of PBS containing 0.1% Triton X-100 for 10 minutes at room temperature. The lysates were diluted and plated onto LB agar plates in triplicate. Colonies grown on LB plates were counted. The results of the assays were expressed as the number of bacteria recovered from gentamicin-treated cells divided by the number of inoculated bacteria added to the cell. After lysates of HeLa cells were plated, the number of CFU that formed on LB agar were counted. The cells were inoculated with untreated SL1344 (treated without DMSO) as a positive control and E. coli ATCC 25922, an avirulent strain treated without DMSO, as a negative control. SL1344 was treated with the same amount of 0.1% DMSO as the other groups to control for any DMSO-specific effects. The cell invasion assay was performed in triplicate for each potential PhoQ inhibitor, and the assay was repeated twice.
Table 3. Primers used in homologous recombinations to construct knockout strain.

| Primers       | Sequences                      | Length (bp) | Restriction sites |
|---------------|--------------------------------|-------------|------------------|
| phoQ/phoP U-F | 5’ ATAAAGAT GCGCCGCG GCGTGACCTGACCGACTCCA 3’ | 528         | NotI             |
| phoQ/phoP U-R | 5’ TGC TCTAGA TACGCCATTTTTATTTCTCGCTT 3’ |             | XbaI             |
| phoQ/phoP D-F | 5’ TGC TCTAGA GATGAATAAATATGTCCTTTTACC 3’   | 555         | XbaI             |
| phoQ/phoP D-R | 5’ TCC CCCCCG CCTCAATCGTGACGGAGA 3’         |             | Smal             |

The underlined sequences represent the restriction sites. Primers phoQ/phoP U-F and phoQ/phoP U-R were used to amplify upstream fragment of phoQ/phoP; phoQ/phoP D-F and phoQ/phoP D-R were used to amplify downstream fragment of phoQ/phoP.

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S. flexneri Mouse Sereny test

A Mouse Sereny test was used to evaluate the virulence of S. flexneri Sf9380 [48]. A single red colony of Sf9380 on Congo Red agar (tryptic soy broth provided by Oxford Co. with 1.5% agar and 0.01% Congo red) [48], was inoculated into LB and grown with or without potential PhoQ inhibitors (final concentration 100,50,25,12.5 or 6.25 μmol/L) at 37°C for 8 hours with constant shaking. The female BALB/c mice (about 18 g) were infected with 1 x 10⁶ CFUs per eye. The eye of the mouse was inoculated with the bacterium with or without potential PhoQ inhibitor treatment (n = 6 mice in each group). Keratoconjunctivitis in the mice infected with bacteria was observed at 24, 48, 72 and 96 hours after inoculation. In the experiment, mouse eyes inoculated with untreated Sf9380 (treated without DMSO) served as the positive control for keratoconjunctivitis, and mouse eyes inoculated with E. coli ATCC 25922 (treated without DMSO) served as the negative control. Because DMSO is the solvent of the potential PhoQ inhibitor, Sf9380 was treated with the same amount of DMSO (0.1%) as the other groups. The invasiveness of the bacteria was scored: no disease or mild irritation as “−”, + as mild conjunctivitis or late development and/or rapid clearing of symptoms, ++ as keratoconjunctivitis without purulence, and +++ as fully developed keratoconjunctivitis with purulence [49].

Cytotoxicity assay of the potential PhoQ HK inhibitors

Cytotoxicity of the potential PhoQ inhibitors on cultured Vero cells was measured by using the Cell Proliferation Kit I (MTT) (Roche, Indianapolis, USA). An equal concentration of DMSO (0.1%) in the medium was used as a negative control. The cytotoxicity assay was performed in triplicate for each potential PhoQ inhibitor, and the assay for each potential PhoQ inhibitor was repeated twice. The results were converted to percentage of the control (cells only containing 0.1% DMSO) and CC₅₀ (concentrations that produce a 50% cytotoxicity effect on Vero cell) of each potential PhoQ inhibitors was calculated using the Origin v7.0 software (OriginLab, Northampton, USA).

Erythrocyte hemolysis assays of potential PhoQ inhibitors

The hemolytic activity of the potential PhoQ inhibitors was determined using human erythrocytes [50]. The erythrocytes were washed three times with sterile saline and resuspended to 5% cell concentration prior to assay. Then, 200 μl erythrocyte suspensions containing 25 μmol/L or 100 μmol/L concentrations of each potential PhoQ inhibitors were added in triplicate to the wells of the 96-well microtiter plates. Cells without potential PhoQ inhibitors treatment or with 1% Triton-100 treatment were used as the zero or 100% hemolysis control, respectively. The cell suspensions were incubated for 1 hour at 37°C and centrifuged for 10 minutes at 1200 xg. Supernatants (100 μl) were transferred to another sterile plate, and the released hemoglobin was measured absorbance readings at 570 nm. An equal concentration of DMSO (0.1%) in the medium did not affect the integrity of the erythrocyte membrane. The hemolysis assay was repeated twice.

Supporting Information

Figure S1 Identification of purified recombinant SF-PhoQc. A Purified recombinant SF-PhoQc was analyzed by SDS-PAGE. The theoretical molecular mass calculated according to the amino acid sequence of SF-PhoQc is 27.9 kDa. (B) The purified recombinant protein was confirmed by Western blot with a monoclonal antibody against the His tag. (C) The molecular mass of the protein was determined by mass spectrometry. The MS spectral data shown gives a 27.914 kDa molecular mass of the recombinant SF-PhoQc, which matches the theoretical molecular mass of 27.914 kDa calculated from the amino acid sequence.

Figure S2 The potential PhoQ inhibitors have no effects of on Shigella growth. The growth curves of Sf9380 which treated with four potential PhoQ inhibitors were tested. Sf9380 inoculated with potential PhoQ inhibitors (final concentration 200 μmol/L), and Bacterial Concentration (OD 600) of Sf9380 in the culture were counted every one hour, for 12 hours. X-axis was the hours of bacterial growth, Y-axis was the Bacterial Concentration (OD 600). These result shows that four potential PhoQ inhibitors can not affect the growth of S. flexneri.

Figure S3 Duration of treatment influences the effects of potential PhoQ inhibitors on cell invasion of Shigella. The gentamicin protection assay shows the inhibitory effect of the four potential PhoQ inhibitors on Shigella HeLa cells invasion. The S. flexneri Sf9380 were treated with each of the four potential PhoQ inhibitors (100 μmol/L) grown for 4, 6 or 8 hours. Then the bacteria were inoculated with HeLa cells for 60 minutes prior to the addition of gentamicin to kill extracellular bacteria. After the incubation, the HeLa cells in each well were lysed in 1 ml of PBS containing 0.1% Triton X-100. The lysates were diluted and plated onto LB agar plates in triplicate. Colonies grown on LB plates were counted. The results of the assays were expressed as the number of bacteria recovered from gentamicin-treated cells divided by the number of inoculated bacteria added to the cell. Result indicated that compared with cell invasion of the positive control Sf9380 alone, the potential PhoQ inhibitors (100 μmol/L) treated for 8 hours had obvious inhibition effects on the bacteria cell invasion, while potential PhoQ inhibitors treated for 4 or 6 hours had no significant inhibition effects on Shigella cell invasion.

Figure S4 The effects of potential PhoQ inhibitors on cell invasion of Shigella Sf301. The gentamicin protection
that potential PhoQ inhibitors 1, 2, 3 can inhibit HeLa cells. The assay shows the inhibitory effect of the four potential PhoQ inhibitors on S. flexneri 2a 301 HeLa cells invasion. The bacteria were treated with each of the four potential PhoQ inhibitors grown for 9 h. Then the bacteria were inoculated with HeLa cells for 60 minutes prior to the addition of gentamicin to kill extracellular bacteria. After the incubation, the HeLa cells in each well were lysed in 1 ml of PBS containing 0.1% Triton X-100. The lysates were diluted and plated onto LB agar plates in triplicate. Colonies grown on LB plates were counted. The results of the assays were expressed as the number of bacteria recovered from gentamicin-treated cells divided by the number of inoculated bacteria added to the cell. Values are means ± standard deviations from 6 independent wells. *p<0.01 vs. S/301. The results indicated that potential PhoQ inhibitors 1, 2, 3 can inhibit HeLa cell invasion ability of S/301 while potential PhoQ inhibitor 4 had no effect on S/301 invasion of HeLa cells.

(TIF)

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Author Contributions

Conceived and designed the experiments: XS DQ HLJ. Performed the experiments: XJ ZJ MLC YJC JQZ. Analyzed the data: XJ ZJ MLC WQX JVC JQZ. Contributed reagents/materials/analysis tools: XJ ZJ MLC WQX JVC JQZ. Wrote the paper: XJ ZJ MLC WQX DQ YW.

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