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Quinazoline-based anti-virulence compounds selectively target Salmonella PhoP/PhoQ signal transduction system

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Running Head: anti-virulence drug discovery for salmonellosis

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The rapid emergence of multi-drug resistance among bacterial pathogens has become a significant chal-

lenge to human health in our century. Therefore, development of next-generation anti-bacterial com-

pounds is an urgent need. Two-component signal transduction systems (TCS) are stimulus-response
coupling devices that allow bacteria to sense and elaborate adaptive responses to changing environ-

tmental conditions, including the challenges that pathogenic bacteria face inside the host. The differential

presence of TCS, present in bacteria but absent in the animal kingdom, makes them attractive targets in

the search for new antibacterial compounds. In *Salmonella enterica*, the PhoP/PhoQ two-component

system controls the expression of crucial phenotypes that define the ability of the pathogen to establish

infection in the host. We now report the screening of 686 compounds from the GlaxoSmithKline Pub-

lished Kinase Inhibitor Set in a high throughput whole-cell assay that targets *Salmonella* Typhimurium

PhoP/PhoQ. We identify a series of quinazoline compounds that show selective and potent down-

regulation of PhoP/PhoQ-activated genes and define structural attributes required for their efficacy. We
demonstrate that their bioactivity is due to repression of the PhoQ sensor autokinase activity by intera-
c tion with its catalytic domain, acting as competitive inhibitors of ATP binding. While being non-
cytotoxic, the hit molecules exhibit anti-virulence effect by blockage of *S.* Typhimurium intramacro-

phage replication. Together, these features make these quinazoline compounds stand out as exciting

leads to develop a therapeutic intervention to fight salmonellosis.

**INTRODUCTION**

Infection by bacteria of the genus *Salmonella* constitutes a major global burden of human morbidity and

mortality (1). The most recent estimates suggest that nontyphoidal *Salmonella serovars* is the cause of

155,000 deaths each year (1). One in every four diarrheal diseases can trace its origin back to *Salmonel-

la serovars* (1). Non-typhoidal *Salmonella serovars* generally cause a 2-7 day self-limiting illness in

immunocompetent individuals. In high-income countries, these infections are characterized by acute

fever, abdominal crumps, diarrhea, nausea and vomiting. However in low-income, underdeveloped re-

gions, non-typhoidal *Salmonella* are the most common bacterial bloodstream isolates and are fatal in

20–25% cases of infection (2). Successful treatment of these infections has increasingly been hindered

by the emergence of strains that are resistant to multiple antibacterial drugs, including the last line of
defense, extended-spectrum cephalosporins and fluoroquinolones (3, 4). *Salmonella* is a growing global

health risk with increasing prevalence and continuous emerging resistance. Efforts to identify novel an-
timicrobial agents and mechanisms to counter *Salmonella*-borne infections are a key priority to combat

infection (5).
There are a number of alternatives to already exploited bacterial targets. One promising strategy consists in interfering with bacterial pathogenicity traits using pathoblockers, anti-virulence compounds that target crucial mechanisms in the development of disease. As these agents would not exert bactericidal or bacteriostatic effects, it is thought that, depending on the importance of the chosen target for bacterial survival throughout the infection path, anti-virulence strategies might allow the host to mount an immune response that would favor the clearance of the pathogen (6-8).

In *Salmonella enterica*, the PhoP/PhoQ TCS orchestrates the expression of phenotypes that allow the pathogen to thrive in the mammalian host. PhoP/PhoQ consists of a canonical signal transduction (TCS), which couples environmental stimuli to tailored bacterial adaptive responses. This TCS is comprised by PhoQ, a transmembrane sensor with bifunctional (histidine-kinase/phosphatase) activity, and PhoP, a cytoplasmic response regulator. PhoQ and PhoP communicate each other by a phospho-relay mechanism (9). The PhoP/PhoQ regulon comprises more than 100 genes with expression that are directly or indirectly controlled by PhoP action. These genes include those that are required for Mg$^{2+}$ homeostasis (10), resistance to acidic pH, and determination of bacterial susceptibility to cationic antimicrobial peptides produced by the host (11, 12). The system is also involved in controlling the bacterial entry mechanism into intestinal epithelial cells. Once inside either non-phagocytic or phagocytic host cells, PhoP-modulated genes expression define the intracellular survival and proliferation capacity of *Salmonella* (13, 14). Host phagocytes act as *Salmonella* dispersal vehicles to vital organs such as liver and spleen (15) and therefore play a major role in the development of systemic infection.

To identify new starting points to target this mechanism, we screened the first and second generations of the GlaxoSmithKline (GSK) Published Kinase Inhibitor Set (PKIS) (16) for compounds that could inhibit *S. Typhimurium* PhoP/PhoQ activity. The PKIS set was designed to include a range of chemotypes and eukaryotic kinase inhibition profiles. Compounds in PKIS were originally prepared in lead optimization efforts to target human serine, threonine, and tyrosine kinases. In fact, scaffolds of the PKIS library are also present in chemotypes that include FDA-approved drugs such as lapatinib (17) and erlotinib (18), tyrosine kinase inhibitors that target the epidermal growth factor receptor.

Histidine kinases (HK), which include PhoQ, are members of the GHKL family, that also comprises GyrB, Hsp90, and MutL. The HK catalytic domain is characterized by a unique ATP-binding Bergerat fold, which topologically differs from the fold of Ser/Thr/Tyr sensor kinases (19). The Bergerat fold has been previously explored for the generation of HK inhibitors. They include the GHKL inhibitors radicicol, thienopyridone, a modified Gyrase B ligand, and a thiophene containing scaffold (Fig. 1) (19-21). These scaffolds were each shown to inhibit HK activity by competing with ATP in the autophosphorylation reaction. To our knowledge these series have not been investigated further (22-25).
Despite the structural differences between HK and Ser/Thr/Tyr sensor kinases, we expected PKIS to be a useful screening set to discover lead molecules able to target PhoQ HK activity, resulting in the identification and development of useful anti-virulence agents to treat *Salmonella* infections.

**RESULTS AND DISCUSSION**

**Primary screening and lead compounds selection.** PKIS, a library containing 686 compounds from kinase drug discovery programs, was screened based on our previously established format to identify inhibitors of the *Salmonella* Typhimurium PhoP/PhoQ signal transduction system (26, 27). In a first round of screening, isogenic *Salmonella* Typhimurium MS14028s strains carrying transcriptional lacZ fusions to *virK*, a representative PhoP-activated gene, were grown in Lysogeny broth (LB) supplemented with 50 µM of each compound. The resulting percentage of β-galactosidase activity repression relative to the value obtained without compound was determined (Dataset 1). Simultaneously, we monitored bacterial growth and eliminated those compounds that exerted alterations in growth curves from further analysis as described under Materials and Methods (Dataset 1). We set >35% inhibition of *virK* reporter activity levels as the threshold for compound progression and performed a second round of selection including a second PhoP-dependent reporter (*pagC-lacZ*) and a PhoP/PhoQ-unrelated transcriptional reporter (*tppB-lacZ*), whose expression is regulated by the EnvZ/OmpR TCS (28, 29), to exclude compounds that might exert indiscriminate inhibition upon HKs (Dataset 1).

Next we selected molecules that showed ≥50% down-regulation of the *virK* and *pagC* reporters’ activity levels and no significant alteration of *tppB* reporter activity levels. This step resulted in the identification of two hit compounds, GI261520A and GI262866A, differing in the substitution of the 6-position of the quinazoline scaffold, having a methoxy or an alcohol substituent, respectively (Fig. 2A). To verify the significance of this selection towards the PhoP/PhoQ activity, we added four additional reporters from the PhoP/PhoQ regulon (*pagK, pcgM, pcfF* and *pipD*) and reinforced the selectivity criterion by addition of another unrelated transcriptional reporter from the CpxAR TCS regulon, *cpxP-lacZ*, to our β-galactosidase assays. We also added stringency to the test by lowering the compounds concentration to 25 µM (Fig. 2B). To obtain preliminary hints for chemical moieties responsible for the inhibitory effect of the selected leads, we searched the PKIS library for additional, related quinazoline analogs and found GI261590A and GW320571X. These two compounds were included for comparison in subsequent assays (Fig. 2B). These results further substantiated the selection of compounds, showing that the two hit compounds exerted 36-80% repressive effect over the β-galactosidase activity levels measured from the six PhoP-controlled reporters tested. This result indicated that substituents in positions R¹, R², R³ and R⁴ were relevant for the core quinazoline moiety to confer capacity to down-regulate PhoP/PhoQ.
Dose-dependent inhibition was verified by performing β-galactosidase assays from virK and pagC reporters using a final concentration of the compounds in the 0-50 µM range (Fig. 3A and B). Consistently, although EnvZ/OmpR and CpxAR belong to the same “OmpR sub-family” as PhoP/PhoQ (30), the two selected compounds showed negligible action on the β-galactosidase activity expressed from the EnvZ/OmpR or CpxAR-dependent reporter genes (Fig. 3C and D); indicating high selectivity of these pharmacophores towards the PhoP/PhoQ TCS.

Each PhoP-controlled gene has been shown to display differential PhoP-binding sequence features, including orientation, co-regulatory cis-acting motifs located at different distances of the DNA PhoP-box and/or the need to overcome silencing by nucleoid-associated proteins such as H-NS (31). These differences imply distinct requirements of activated PhoP levels required for induction or repression and also a characteristic expression timing for each PhoP-dependent gene, upon the presence of a given PhoP/PhoQ input signal (32, 33). This accounts for the different extent of β-galactosidase activity levels decrease exerted by the compounds over each one of the six PhoP-regulated reporters, in spite of the fact that all of them harbor a conserved PhoP-recognition site (27, 33, 34). Moreover, although the down-regulation of the activity levels driven from the six reporters strongly suggest an inhibitory action over the TCS, we cannot at this point rule out that the compounds might exert an indirect effect over PhoP/PhoQ.

GI262866A and GI261520A target PhoQ HK activity. Our results indicate that GI262866A and GI261520A were the most effective compounds in the down-regulation of the activity levels of PhoP-dependent transcriptional reporters. To examine whether these compounds exert a direct effect over PhoP/PhoQ and taking into account their original design as kinase inhibitors, we sought to explore whether PhoQ autophosphorylation activity, the first phospho-transfer checkpoint in the PhoP/PhoQ signal transduction cascade, was targeted by the selected quinazolines. We isolated PhoQ-enriched membrane vesicles derived from the S. Typhimurium phoQ strain that expresses PhoQ from the pUHE-21–2::phoQ plasmid, as we described previously (27) (also see Materials and Methods). Salmonella-derived vesicles were prepared from bacteria grown in LB with or without the addition of the indicated concentration of each compound. To determine PhoQ autophosphorylation activity, vesicles were incubated with a [γ-32P]ATP-containing reaction medium, samples were analyzed by SDS-PAGE followed by autoradiography, and the intensity of the bands was quantified by densitometric analysis as described (27). As shown in Fig. 4 and S2, PhoQ autophosphorylation was inhibited by either GI262866A or GI261520A in a dose-dependent manner over the 6-50 µM concentration range tested, demonstrating that the chosen compounds target PhoQ autokinase activity. Consistent with the PhoP-regulated report-
ers results, compound GI261590A showed a reduced inhibitory effect when compared with the selected
hits, while GW320571X exerted no effect as repressor of PhoQ autokinase activity (Fig. 4 and S2).

**Structure-activity relationship analysis.** Additional quinazolines were designed to define structure-
activity relationships. We synthesized a series of compounds (1-21) based on GI261520A and
GI262866A through nucleophilic aromatic displacement of 4-chloroquin(az)olines. We were able to
furnish products in excellent yields (55-91%) consistent with previous reports and without protection of
the alcohol substituted quinazoline starting material (please see supplemental material at http://www.ibr-conicet.gov.ar/wp-content/uploads/2019/10/Carabajal-et-al-AAC01744-19-Supplementary-Information-Combined.pdf): Fig. S1, Supplementary Information-Compound characterization and Supplementary
Methods) (35-38).

GI262866A from the screening was re-synthesized, tested, and verified to produce virK and pagC activity
level repression consistent with our previous results (Fig. 5). Moving the alcohol to the 7-position (1)
reduced inhibition by 3-fold on both virK and pagC repression. This repression was not rescued by re-
storing the alcohol to form the 6,7-diol (2), which showed similar activity to (1). The switch to the quin-
oline of GI262866A (3) yielded a compound with toxicity to the bacteria rather than inhibition of the
TCS.

As shown in Fig. 5, a fluorine substitution at either the para- (4) or meta-position (5) of the pendant
benzyl of GI262866A reduced activity on both virK and pagC repression up to one half. The fluorine
substitution at the ortho-position (6) had the most defined effect, showing very diminished action as
repressor over either virK or pagC reporters’ expression levels. The combination of para-fluorine and
the alcohol at the 7-position (7) as well as the addition of the alcohol to form the 6,7-diol (8) did not
change the activity of the respective unsubstituted benzyl compounds, (1) and (2). Methylation of the
alcohol of GI262866A to form GI261520A had equipotent virK or pagC reporters’ activity repression.
The 7-methoxy position analog (9) resulted in diminished levels of the two reporters’ activity repression,
similar to (1). The most intriguing result was that the dimethoxy substitution (10) was able to rescue the
repression back to the original levels, something not observed with the 6,7-diol of (2) or (8).

**Assessment of structural boundaries.** With these observations in hand we sought to explore the limits
of the pharmacophore with additional structural alterations. GI261590A and GW320571X (Fig. 2A)
were structurally related to lapatinib and were likely generated in the same medicinal chemistry pro-
gram. We incorporated fragments from this clinical inhibitor into our quinazoline series, along with sub-
structures from erlotinib (Fig. 5 and S3). Both of these inhibitors showed inhibition levels lower than
12.2% on virK or pagC expression. Modification of the erlotinib quinazoline to the 6-position alcohol
(11) yielded a 15-fold rise in virK repression and a slight increase in pagC repression (Fig. S3). There
was no loss in repression after relocating the alcohol from the 6- to 7-position (12). However, the 6,7-diol derivative (13) showed up to 5-fold decrease over virK or pagC repression compared to (12). In accordance to the importance of a 6-position alcohol, while the quinoline analog with the alcohol in the 7-position (15) was inactive, a switch to the 6- position quinoline (14) restored a modest repressive activity. Capping the 6-position alcohol of (11) to produce the methoxy (16) yielded up to a 10-fold repression levels reduction. Both the 7-methoxy (17) and 6,7-dimethoxy analogs (18) as well as the the switch to the quinoline (19) were inactive (Fig. S3). Interestingly, the clipped aniline of GI262866A with a simple para-methoxy (20) showed good repression of β-galactosidase activity levels driven from the virK (33%) or pagC (45%) reporters, about two thirds of GI262866A. The truncated aniline of GI261520A with a para-methoxy group (21) showed a >5-fold drop of repression potency (Fig. S3). All these results point towards the pendant benzyl being an important contributor to activity, and the partial inhibition recovery in compounds (20) and (21) support the involvement of the 6-position quinazoline alcohol.

We performed dose-response experiments on pagC reporter expression levels with GI262866A, GI261520A, (10), (20) and (21) to assess the IC₅₀ values for these compounds (Fig. 6 A and B). In accordance to our previous results (Fig. 4 and 5), GI262866A, GI261520A, and (10) all showed IC₅₀ values ≤10μM, indicating that an alcohol or a methoxy group are required at the 6-position quinazoline for the inhibitory action. (20) showed an estimated 7-fold increase relative to GI262866A, while (21) showed a 64-fold increase relative to GI261520A in the IC₅₀ values, substantiating the importance of the pendant benzyl group. In sum, the analysis of these structural analogs helped confirm that GI262866A and GI261520A were indeed the most active analogs and further defined the activity of this chemotype.

**Molecular mechanism of action on PhoQ HK activity.** We sought to assess whether GI262866A and GI261520A directly interfered with the activity of the PhoQ catalytic domain. An autokinase assay was performed by using a soluble purified protein combined the maltose binding protein (MBP) with PhoQ cytoplasmic domain that harbors the HK catalytic domain and was previously demonstrated to retain both PhoQ autokinase and PhoP-phosphotransfer activities (PhoQc) (39). The cytoplasmic domain of EnvZ, purified as a histidine-tagged fusion protein (EnvZc) was used as control. GI261520A and GI262866A inhibited 80% and 94% PhoQc autokinase activity, while they did not significatively affect EnvZc autophosphorylation capacity, reinforcing the specificity of the compounds towards PhoQ (Fig. 7A and B). Consistent with their failure to down-regulate PhoP-activated reporters’ expression, neither (20) or (21) were able to alter PhoQc or EnvZc autokinase activity (Fig. 7A and B). Either GI262866A or GI261520A inhibited PhoQc autokinase activity in a dose-dependent manner, within the 12.5–400 μM concentration range (Fig. 7C).
To test whether GI262866A or GI261520A act as allosteric or competitive inhibitors of PhoQc, we compared the IC\textsubscript{50} values obtained for each compound at two ATP concentrations (20 and 100 μM). The IC\textsubscript{50} of an allosteric inhibitor should remain unchanged and independent of the ATP concentration, while in the case of a competitive inhibitor, the IC\textsubscript{50} value will be influenced by the ATP concentration used. The reduction of the calculated IC\textsubscript{50} values when substrate ATP was lowered from 100 to 20 μM in the reaction medium indicate that both molecules act as competitive inhibitors of ATP in the PhoQ auto-phosphorylation reaction (Fig. 7C and D).

To rationalize the molecular basis of binding, as shown in Fig. 8 A and B, we docked the two key compounds (GI261520A and GI262866A) into the ATP-binding domain of PhoQ (PDB:3CGY) (19). The compounds were able to act as ATP mimics in a non-traditional hinge binder conformation where a key water mediated bridge with Asp416 forms the strongest interaction with the quinazoline nitrogen. This locks the molecule in place with an additional direct interaction with the alcohol and Gly420. We also found the benzyl was required for extended pi-pi aromatic interactions, supporting our observation of the pronounced loss of functional activity of the benzyl clipped compounds (20) and (21). To further investigate the binding, we employed WaterMap (see Materials and Methods) and located the key water network interactions confirming that a water bridge was formed between the quinazoline nitrogen and Asp416 on PhoQ (Fig. 8C) (40). We also solved the small molecule crystal structure of GI262866A as a monoclinic structure with each unit found to be in a similar conformation to that predicted for the bound inhibitor (Fig. 8D, Cambridge Crystallographic Data Centre, deposition number 1953813 and Supplemental Information). These results demonstrate the key interactions occurring with the bound ligand and the wider protein structure and reinforce the mechanistic action of the quinazoline molecules that resemble substrate binding and hinder ATP interaction precluding PhoQ autocatalytic phosphorylation reaction.

**Effect on Salmonella intramacrophage survival.** PhoP/PhoQ governs the capacity of *Salmonella* to survive and replicate inside macrophages, and the control of bacteria within these cells is critical to overcome infection. Therefore, we examined the effect of the selected compounds as anti-virulence agents (41). To first investigate their potential cytotoxicity, we performed a tetrazolium dye (MTT) reduction assay in cultured RAW264.7 macrophages (42). This assay measures NAD(P)H-dependent cellular oxidoreductase activity (43). Neither GI261520A nor GI262866A affected the viability of the cultured cells (Fig. 9A). Additionally, (20) and (21) were included and similarly showed no cytotoxicity in RAW264.7 cells (Fig. 9A).

Next, we measured *S. Typhimurium* intramacrophage survival and replication capacity by performing a conventional gentamycin protection invasion assay (41). In the same media used for the MTT reduction
We have identified two key quinazoline-based lead compounds, GI262866A and GI261520A, which down-regulate PhoP-activated genes in S. Typhimurium by the selective inhibition of PhoQ histidine kinase activity (see Fig. 10 for a model that depicts the mechanistic action of the compounds on the...
Salmonella PhoP/PhoQ signal transduction system. Profiling a focused series of designed analogs allowed initial definition of the pharmacophore and highlighted that small structural changes had a significant impact on antibacterial activity. These quinazolines have significantly improved properties over currently available anti-virulence compounds directed against Salmonella identified in the literature.

The components of the PhoP/PhoQ signal transduction system reside inside the bacteria. By employing a whole-cell screening approach, we were able to circumvent or exclude potential problems derived from accessibility of the drugs to the desired bacterial target. The strategy allowed us to select only those molecules that were competently permeable and that were not completely extruded by efflux pumps nor consumed by inactivating enzymes. The selectivity towards PhoQ over other HKs suggests the possibility of a therapeutic intervention directed to inhibit Salmonella pathogenic traits with minimal impact on beneficial microbiota.

The ability of the compounds to block intramacrophage survival and replication capacity of S. Typhimurium without cytotoxic effects supports their capacity to reach and exert their effect on intravacuolar Salmonella, being innocuous for host cells. Moreover, the hit molecules were able to inhibit the intramacrophage replication of Salmonella enterica serovars different from S. Typhimurium, indicating an anti-virulence effect useful for potential treatment of salmonellosis in diverse warm-blooded vertebrates.

The pathoblocker compounds identified here could be standalone therapeutic agents to treat Salmonella-borne infections. Alternatively, they might be employed as adjuvants, administered in conjuction with classic antimicrobials, lowering the required dose of usage and reducing the prospects for development of resistance. The in vitro and in vivo activity of the compounds demonstrates an exciting possibility for the development of a new generation of anti-virulence drugs against Salmonella.

MATERIALS AND METHODS

Bacterial strains, cell culture and growth conditions. Bacterial strains used in this work are listed in Table S1. Overnight cultures of bacteria were inoculated with a single colony and routinely grown in LB media (10 g L\(^{-1}\) NaCl, 10 g L\(^{-1}\) Tryptone, 5 g L\(^{-1}\) yeast extract) supplemented with antibiotics as appropriate (kanamycin, 50 \(\mu\)g mL\(^{-1}\), ampicillin, 100 \(\mu\)g mL\(^{-1}\)) and 0.7 mM isopropyl-\(\beta\)-D-thiogalactopyranoside (IPTG) when required, and/or the addition of the compound of interest at the concentrations indicated in each assay.

Compounds screening and dose-response inhibition assays. The potential inhibitory effect of the PKIS library was evaluated by growing S. Typhimurium in LB broth containing either the compounds’ vehicle DMSO 0.5 % (v/v) as control or the corresponding compound solution at a final concentration of 25 or 50 \(\mu\)M, dissolved in DMSO. The assays were carried out in sterile 96-well microtiter plates,
incubated for 18 h with agitation at 37 °C, and optical density at 600 nm was measured every 60 min with the BioTek Synergy 2 multimode microplate reader. β-galactosidase activity from PhoP-modulated reporter genes was determined for the compounds that did not exhibit alterations in growth curves (44). Dose-dependent inhibition was performed using final concentration of the compounds in the 0-50 µM range. Samples were processed by duplicates and proper blank controls were used in each microplate.

**General procedure for the synthesis of 4-anilinoquin(az)olines**: 4-chloroquin(az)oline derivative (1.0 equivalent), aniline derivative (1.1 equivalent.), and tPr2NEt (2.5 equivalent) were suspended in ethanol (10 mL) and refluxed for 18 h. The crude mixture was purified by flash chromatography using EtOAc:hexane followed by 1-5 % methanol in EtOAc; After solvent removal under reduced pressure, the product was obtained as a free following solid or recrystallized from ethanol/water. SMILES and Labbook codes for the compounds are provided in Table S2. The chemical structure, the mass spectrometry method, the charts spectra for the compounds and crystal data and experimental for G1261520A (2017ncs0878q) is provided in Supplementary Information-Compounds Characterization and Supplementary Methods.

**Preparation of PhoQ-enriched membranes**. To assess the autokinase activity of sensor PhoQ, membranes were prepared from overnight cultures of *S. Typhimurium* strains that express PhoQ from the pUHE-21-2::phoQ, as described previously (27). Briefly, overnight culture of *S. Typhimurium* strain PB4663 was used to inoculate LB containing the corresponding compound at the final concentration indicated in each experiment. These were then grown at 37°C to logarithmic phase (A630 nm = 0.6), and protein expression was induced by the addition of 0.7 mM IPTG for an additional 3 h with shaking. Cells were collected and treated as detailed in (27). Cells were collected and resuspended in a solution containing 20 mM Tris-HCl (pH 8.0), 20 % sucrose, 5 mM EDTA, and 150 mg µl-1 lysozyme. After 40 min incubation at 4°C, 20 mM MgCl2 was added and the pellet was resuspended in ice-cold 10 mM Tris-HCl (pH 8.0) and subjected to sonication. Membrane fraction was recovered and washed subsequently with 10 mM Tris-HCl (pH 8.0), 2 M KCl, 10 mM Tris-HCl 5 mM EDTA, and 10 mM Tris HCl by 40-min centrifugations rounds at 21,000 x g. Each time the supernatant was discarded. Finally, the membranes were resuspended in 25 mM Tris-HCl (pH 8.0), 50 mM KCl. All procedures were carried out at 4°C. Protein concentration was determined by the bicinchoninic acid assay (Sigma) using bovine serum albumin as standard.

**Autokinase activity assays and immunodetection analysis**. To test the autokinase activity of the sensor, PhoQ-enriched membranes (50 µg of total protein) were obtained as described previously (27). Membranes were incubated 10 min at 37°C in a 30 µl mixture containing 25 mM Tris-HCl (pH 8.0), 50 mM KCl, 1 mM MgCl2, 50 µM non-labeled ATP and 0.16 µCi µl-1 of [γ-32P]ATP. For the identification
of the molecular mechanism of action on PhoQ, non-labeled ATP (20 and 100 µM) and trace (0.03 and 0.32 µCi µl⁻¹) amounts of [γ⁻³²P] ATP were incubated with purified PhoQc (10 µg) or EnvZc (2 µg) fusion proteins and increasing amounts of compounds GI261520A, GI262866A, (20) and (21), as indicated. Reactions were started by addition of the reaction mixture, and stopped by addition of 6 µl of 5X SDS-PAGE sample buffer (2.5% β mercaptoethanol, 9% glycerol, 10% SDS, 600 mM Tris-HCl (pH 6.8), 0.006% bromphenol blue). All reactions were analyzed by SDS-PAGE (12% polyacrylamide), transferred to nitrocellulose, and then subjected to western blot or autoradiography analysis. Western blot membranes were incubated with rabbit anti-PhoQCyt or anti-EnvZCyt polyclonal antibodies (27), and developed by incubation with protein A conjugated with phosphatase, coupled to a chromogenic reaction using nitro blue tetrazolium and 5 bromo-4-chloro-3-indolyl phosphate as substrates. Autoradiographies and western blot membranes were scanned for densitometry using Adobe Photoshop CS to perform quantitative determinations (27).

**Gentamicin protection assay.** *Salmonella* invasion of RAW264.7 macrophages was tested by gentamicin assays as previously described (45). Briefly, monolayers of RAW264.7 macrophages cultured in 48-well tissue plates in DMEM + 10% FCS medium were infected at a multiplicity of infection (MOI) of 10:1 (bacteria to eukaryotic cells). The cells were incubated for 30 minutes at 37°C and rinsed with gentamicin-containing medium 100 µg mL⁻¹ and the selected compound up to 25 µM or DMSO 0.5% v/v as control (compounds’ vehicle). After 1.5 h, cells were incubated at a lower gentamicin concentration (30 µg mL⁻¹) for a total of 18 h post infection. The cells were washed with gentamicin-free medium and lysed with 0.1% Triton X-100. Intracellular bacteria were collected and enumerated on Luria broth agar plates and CFU mL⁻¹ was calculated. The results for each experiment are the average of at least three independent assays performed in duplicate.

**Data analysis.** Data were prepared and analyzed in GraphPad Prism (version 6.01, GraphPad Software, San Diego, California, U.S.A., www.graphpad.com). For all dose-response curves (β-galactosidase and autokinase activity assays), data were fit the following equation:

\[
y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10^\left(\frac{(\log IC_{50} - x) \times \text{HillSlope}}{HillSlope}\right)}
\]

Some compounds exhibited incomplete dose-response curves because going to higher concentrations would have solubility problem, the IC₅₀ values were estimated for purposes of comparison to other compounds, by constraining the bottom of the curve to “0”, as described (25).

**Statistical analysis.** Statistical analysis was performed using one-way ANOVA and Kruskal-Wallis multiple comparisons test with an overall significance level of 0.05. Asterisks in the plots denote the values among the treatment groups in which a statistically significant difference was determined.
Molecular modelling. Molecular modelling was performed using Schrödinger Maestro software package. Structures of small molecules were prepared using and the LigPrep module of Schrödinger suite employing OPLS3 force for all computations. X-ray crystal structure for PhoQ (PDB ID: 3CGZ and 3CGY (19)) was pre-processed using the protein preparation wizard of Schrödinger suite in order to optimize the hydrogen bonding network. Prior to Glide docking, the grid box was centered using corresponding X-ray ligand as template. The ligand docking was performed using default SP settings of Schrödinger Glide with additional hydrogen bond constraints to NH of CYS126 (hinge residue). Graphical illustrations were generated using MOE software, Maestro (WaterMap) or PyMOL (The PyMOL Molecular Graphics System, ver. 1.8 Schrödinger, LLC).

Hydration site analysis. Hydration site analysis calculated with WaterMap (Schrödinger Release 2016-3: WaterMap, Schrödinger, LLC, New York, NY (USA), 2016.). The (PDB ID: 3CGZ and 3CGY (19)) structures were prepared with Protein Preparation Wizard (as above). Waters were analysed within 5 angstroms of the co-crystallized ligand, and the 2 nS simulation was conducted with OPLS3 force field (46).

Additional details for Materials and Methods are provided in the Supplementary Information material.

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FIGURE LEGENDS

FIGURE 1. Chemical structure of previously reported histidine kinases inhibitors.

FIGURE 2. Inhibitory action of selected compounds from the primary screen of the PKIS set molecules. (A) Chemical structures of four molecules selected in the screening of the Published Kinase Inhibitor Set (PKIS) from GlaxoSmithKline (GSK). Numbers correspond to the reference code for each molecule assigned by GSK. (B) Inhibition action was calculated with the β-galactosidase activity from lacZ transcriptional fusions to six different PhoP-activated genes reporters (virK, pagC, pagK, pcgM, pcgF, and pipD) and two PhoP-unrelated control reporters (tppB and cpxP). Cells were grown overnight either in LB supplemented with 0.25 % DMSO (compounds’ vehicle) or in LB plus 25 µM of the indicated compound. Relative activity was calculated taking the values obtained with the sole addition of 0.25% DMSO as 100%. Results are the average of three independent assays performed in duplicate, and error bars correspond to S.D.

FIGURE 3. Dose-response inhibition action of selected molecules on the activity of S. Typhimurium PhoP-activated reporter genes. β-galactosidase activity from (A) virK::lacZ, (B) pagC::lacZ, (C) tppB::lacZ or (D) cpxP::lacZ transcriptional fusions was measured in cells grown overnight in LB with either the indicated final concentration of the corresponding compound. β-galactosidase activity was measured as described under Materials and Methods. Results are the average of three independent assays performed in duplicate, and error bars correspond to S.D.

FIGURE 4. Autophosphorylation inhibitory activity of PhoQ of candidate molecules. (A) Increasing concentrations of GI261520A, GI262866A, GI261590A or GW320571X, were added to the Salmonella growth medium. Membranes obtained by cell fractionation, harboring PhoQ, were incubated for 10 min at 37 °C in a reaction medium containing [γ-32P] ATP, as described in Materials and Methods. The phosphorylation levels and the protein expression levels of PhoQ in each condition assayed were determined by densitometry, and the ratio (labelled against immunodetected) was plotted, taking the values obtained with the sole addition of DMSO 0.5% v/v (compounds’ vehicle) as 100%. Data shown represents results from three independent experiments, and error bars correspond to S.D. All groups were compared with Kruskal-Wallis test. *P<0.05, **P<0.01, ***P<0.001.

FIGURE 5. Initial optimization around GI262866A and GI261520. The chemical structure shows X, R1, R2, R3 and R4 positions that were substituted by the functional groups indicated below. The inhibition action was calculated with the β-galactosidase activity from lacZ transcriptional fusions to two different PhoP-activated genes (virK and pagC) and one PhoP-unrelated control reporter (tppB). Cells were grown overnight in LB plus DMSO 0.25% v/v or 25 µM of the indicated compound. Repression
percentages were calculated taking the values obtained with the sole addition of DMSO 0.25% v/v as 100%. Results are the average of three independent assays performed in duplicate. * compounds that show bacterial growth inhibition. ** Lapatinib chemical structure is provided in Table S2.

FIGURE 6. Assessment of the IC$_{50}$ values for the compound optimization of GI262866A and GI261520A. (A) IC$_{50}$ values of compounds GI262866A, GI261520A, (10), (20) and (21) were calculated with the $\beta$-galactosidase activity from $pagC::lacZ$ transcriptional fusion measured in cells grown in LB supplemented with 0.25% DMSO or with the corresponding compound. (B) Dose-response curve of inhibitory action of GI261520A, GI262866A on the expression of $S$. Typhimurium $pagC$ gene. IC$_{50}$s were calculated by Prism 6.1 (GraphPad Software). $\beta$-galactosidase activity was measured as described under Materials and Methods. Results are the average of three independent assays performed in duplicate.

FIGURE 7. Autophosphorylation inhibitory activity of PhoQ cytoplasmic domain by GI262866A and GI261520A. (A) Autophosphorylation of catalytically active PhoQc or EnvZc in the presence of compounds 200 µM of GI261520A, GI262866A, (20) or (21). Purified protein was incubated for 10 min at 37 °C in a reaction medium containing $[\gamma^{32}P]ATP$. The autophosphorylation reactions were analyzed by SDS-PAGE (12% polyacrylamide) and transferred to nitrocellulose, followed by autoradiography (top) or by immunodetection analysis developed with anti-PhoQC$_{cyt}$ or anti-EnvZC$_{cyt}$ polyclonal antibodies, respectively. (B) The phosphorylation levels and the protein expression levels of PhoQc or EnvZc in each condition assayed were determined by densitometry (as described under “Materials and Methods”), and the ratio (labelled against immunodetected) was plotted, taking the values obtained with the sole addition of 0.5% DMSO as 100%. All groups were compared with Kruskal-Wallis test. *P<0.05, ***P<0.001. (C) Relative activity of catalytically active PhoQc in the presence of increasing amounts of compound GI261520A and GI262866A were measured in a one time-point (10 min) experiments using ATP 20 µM (■) or ATP 100 µM ATP (▲). (D) IC$_{50}$s were calculated by Prism 6.1 (GraphPad Software). Data shown represents results from five independent experiments, and error bars correspond to S.D.

FIGURE 8. Docking of GI261520A and GI262866A compounds into the PhoQ binding site. Docking of (A) GI262866A, (B) GI262866A in PDB:3CGY using Schrödinger glide suite, highlighting key binding interactions. (C) WaterMap simulation of GI262866A in PhoQ binding site proving that there is pivotal interaction with a bridging water and the quinoline nitrogen. (D) Small molecule crystal structure of GI262866A including Cl$^-$ counterion and solvent water. ADP ellipsoids are shown at 50% probability.
FIGURE 9. Inhibitory action on *S. Typhimurium* intracellular growth. (A) Cytotoxicity effect assay. RAW264.7 macrophages were incubated with 25 µM of either GI261520A or GI262866A, and either (20) or (21) as controls for 18 h, cells were washed and MTT was added. After incubation, violet formazan crystals were dissolved and absorbance was read at λ = 570 nm (+: cells incubated with or 0.01 % Triton X-100 as positive control, -: cell incubated with DMSO 0.25% v/v as negative control). Results are the average of five independent assays performed in triplicate, and error bars correspond to S.D. (B) Recovery of intracellular *Salmonella* at 1.5 and 18 h post infection from RAW264.7 macrophages incubated with 12 µM of GI261520A, GI262866A, (20) or (21). (C) Dose-response effect on intracellular *Salmonella* recovery at 1.5 and 18 h post infection from RAW264.7 macrophages incubated with GI262866A (left) or GI261520A (right). All groups were compared with one-way ANOVA test. **P<0.01, ****P<0.0001.

FIGURE 10. Model of action for the quinazoline-based anti-virulence compounds. Compounds GI262866A or GI261520A (orange hexagon) bind to the catalytic site of PhoQ and compete for the binding of substrate ATP, precluding the phosphotransfer reaction from ATP to the conserved His (H) residue located in the DHp domain of PhoQ (protein domain in red). This inhibition hinders the downstream steps in the signal transduction reaction: His (H)-Asp (D) phosphotransfer from phosphorylated PhoQ to the response regulator PhoP, and its subsequent binding to the target promoters of PhoP-regulated genes. This blockage prevents the induction of virulence genes expression, including those required for *Salmonella* intramacrophage replication and disease progression in the infected host.

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