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Characterization of the Binding of Hydroxyindole, Indoleacetic acid and Morpholinoaniline to the *Salmonella* Type III Secretion System Proteins SipD and SipB

Andrew C. McShan^a, Asokan Anbanandam^b, Sikta Patnaik^a, and Roberto N. De Guzman^{a,*}

^aDepartment of Molecular Biosciences, University of Kansas, Lawrence, Kansas USA 66045

^bBio-NMR Core Facility, University of Kansas, Lawrence, Kansas USA 66045

Abstract

Many Gram-negative bacteria require the type III secretion system (T3SS) to cause infectious diseases in humans. A looming public health problem is that all bacterial pathogens that require the T3SS to cause infectious diseases in humans have developed multi-drug resistance to current antibiotics. The T3SS is an attractive target for the development of new antibiotics because of its critical role in virulence. An initial step in developing anti-T3SS based therapeutics is the identification of small molecules that can bind to T3SS proteins. Currently, the only small molecules that are known to bind to the *Salmonella* T3SS proteins SipD and SipB are bile salts to SipD; and sphingolipids and cholesterol to SipB. Here, we show results from a surface plasmon resonance screen of 288 compounds, which identified the binding of 4-morpholinoaniline to SipD, 3-indoleacetic acid to SipB, and 5-hydroxyindole to both SipD and SipB. We also identified by NMR the SipD surfaces involved in binding. These 3 compounds represent new molecules that can bind to T3SS tip and translocon proteins that can be potentially used for future drug design.

Graphical Abstract

*Corresponding author: Roberto N. De Guzman, Department of Molecular Biosciences, University of Kansas, 1200 Sunnyside Ave., Lawrence, KS 66045 USA. Phone: (785) 864 4923, rdguzman@ku.edu.

Keywords

type III secretion system; tip; translocon; SipD; SipB; NMR; STD NMR; small molecules; surface plasmon resonance

Introduction

Salmonella, a causative agent of food borne illness and typhoid fever that is responsible for millions of infections each year worldwide,^[1] and other Gram-negative bacteria belonging to the *Pseudomonas*, *Shigella*, *Chlamydia*, and *Yersinia* genera assemble the type III secretion system (T3SS) to initiate infectious diseases in humans.^[2] The rise of antibiotic resistance among these pathogens coupled with the dearth of new antibiotics necessitates the development of new anti-bacterials.^[3] The T3SS is an attractive target for developing new anti-bacterials because of its essential role in virulence, its exposure on the bacterial surface, and its presence only among pathogens.^[3]

The function of the T3SS is to inject effector proteins into the host cytoplasm to manipulate host signaling pathways for the benefit and survival of bacteria.^[2] The structural component of the T3SS is a mega-Dalton assemblage of over 20 proteins that form the needle apparatus, which consists of a base, an extracellular needle, a tip complex and a translocon.^[4] In *Salmonella*, the tip protein is SipD and the translocon proteins are the membrane proteins SipB and SipC. The tip protein SipD is exposed to the extracellular environment prior to contact with host cells.^[5] Upon contact with the host cell, the translocon assembles on the tip complex to form a pore on the host cell membrane to allow the passage of effectors into the host cell cytoplasm.^[5] The crystal structure of SipD^[6] and other tip proteins^[7] are known. SipD contains an N-terminal α -helical hairpin, an elongated central coiled-coil and a domain of mixed α -helices and β -strands.^[6] The crystal structures of the N-terminal ectodomains of the *Shigella* IpaB^{74–224} and *Salmonella* SipB^{82–226} translocon proteins show coiled-coils of three anti-parallel α -helices,^[8] and the IpaB and SipB ectodomains interact directly with their tip proteins.^[9]

Currently, the only known small molecules that bind to the tip protein and translocon proteins are bile salts (to SipD and its *Shigella* homolog, IpaD)^[10] and sphingolipids and cholesterol (to SipB).^[11] To identify other molecular fragments that could bind to SipD and SipB that could potentially be used as potential scaffolds for development of novel T3SS inhibitors, we screened a library of 288 compounds from a Zenobia library. We identified that 5-hydroxyindole (structure **1**, Figure 1) binds to both SipD and SipB^{82–240}, 3-indoleacetic acid (**2**) binds to SipB^{82–240}, and 4-morpholinoaniline (**10**) binds to SipD. Our results revealed new small molecule scaffolds based on indole and morpholinoaniline that can bind to SipD and SipB.

Results

SPR screening

The expression and purification of SipD and SipB^{82–240} yielded milligram amounts of proteins and their solubility allowed for SPR screening followed by NMR characterization.

SPR is a label free method of screening for small molecule fragments that bind proteins.^[12] The SPR screen of 288 compounds from a structurally diverse library of fragment-like compounds (Zenobia Library 2) identified the binding of 5-hydroxyindole (**1**, Fig 1) to both SipD and SipB^{82–240}; 3-indoleacetic acid (**2**) to SipB^{82–240}; and 4-morpholinoaniline (**10**) SipD (Figure 2). Results of the SPR screen also identified the important functional groups within the scaffolds in binding to SipD and SipB^{82–240}. Regarding the binding of the indole scaffold to SipD and SipB^{82–240}, other functional groups such as hydroxyl and acetyl groups were needed for binding, as the indole ring (**3**) alone was incapable of binding. Replacing the 5-hydroxy group with a 5-cyano group (**4**) abrogated the interaction suggesting that the 5' hydroxyl group in (**1**) might be needed for hydrogen bonding with SipD or SipB^{82–240}. The acetate methylene group (in **2**) was needed for binding SipB^{82–240} as its removal (in **5** and **6**) resulted in loss of binding. Likewise, the carboxylic acid group (in **2**) was important for binding as its removal (in **7**, **8**, **9**) resulted in loss of binding. Regarding the binding of the morpholinoaniline (**10**) scaffold to SipD, hydroxylation or removal of the amino group (in **11**) or introducing a methylene group between the two rings (in **12**) resulted in loss of binding.

NMR titrations of SipD with 5-hydroxyindole

NMR was used to characterize the interaction of SipD with 5-hydroxyindole. SipD uniformly labeled with both ¹⁵N and ILV was titrated with 5-hydroxyindole at increasing molar ratios of 1:0, 1:25, 1:50 and 1:100 and the titration was monitored by acquiring 2D ¹H-¹⁵N TROSY and 2D ¹H-¹³C HSQC datasets. Titration of SipD with 5-hydroxyindole resulted in chemical shift perturbations of specific SipD amide peaks in a concentration dependent manner (Figure 3). SipD residues that were affected by binding (*e.g.* E133, M174, G175, L179, N211 and W234e) showed progressive changes in their peak positions indicating interaction in fast exchange NMR time scale. In addition to the ¹⁵N resonances of SipD, the ILV ¹³C methyl groups of SipD were also used as probes of the interaction (Figure 4). Like the results of ¹⁵N titration, titration with 5-hydroxyindole resulted in chemical shift perturbations of specific SipD ILV peaks in a concentration dependent manner (Figure 4). Based on the chemical shift deviations of ¹⁵N data (for E133, M174, G175 and L179, Figure 3B) and ILV data (for L48, L178 and V187, Figure 4B), the dissociation constant for the binding of 5-hydroxyindole to SipD was estimated to be 32 ± 8 mM.

The chemical shift deviation for each non-overlapped amide (Figure 5A) and ILV (Figure 5B) peak of SipD identified the residues that were strongly affected by binding of 5-hydroxyindole. Results of the ¹⁵N titration showed that the SipD residues affected by 5-hydroxyindole ranged from hydrophobic (L97, A115, I134, L171, G175, L179, V187) to polar charged (R106, R126, E133, K188, K302) residues (Figure 5A). The ILV titrations confirmed the results of the ¹⁵N titration as many residues such as I134, L171 and V187 identified in the ¹⁵N titration also showed significant chemical shift deviations in the ILV titration with 5-hydroxyindole. Additionally, the ILV titrations identified other residues (I45, V253, V323, Figure 5B) that were not previously identified in the ¹⁵N titration that showed significant chemical shift deviation upon binding to 5-hydroxyindole. The methyl side chains of I45, V253, and V323 were likely involved in hydrophobic contacts with 5-hydroxyindole.

The residues that showed significant chemical shift deviations clustered on two surfaces on SipD (Figure 6). One surface maps at the mixed α/β domain (involving residues G175, L178, and V187). The second larger surface maps near the bottom of the coiled-coil and is formed by S110, S114, and R126 in the 23-residue loop spanning residues 110–132 that connects the α -helical hairpin to the coiled-coil. I45 in the α -helical hairpin and I134 in the coiled-coil show strong chemical shift deviations. Our results suggest that 5-hydroxyindole binds at the bottom of the coiled coil and the loop 110–132 because most of the residues affected by interaction are in this region. The other site that showed chemical shift deviations perhaps indicate transient, weaker interactions with 5-hydroxyindole.

STD NMR of 5-hydroxyindole with SipD

Results of ^1H STD NMR experiments identified which parts of 5-hydroxyindole were needed for binding to SipD. As expected, the off-resonance reference spectrum shows NMR peaks from both SipD and 5-hydroxyindole (Figure 7A). The resulting ^1H STD NMR spectrum shows all the NMR peaks corresponding to 5-hydroxyindole remained above noise level (Figure 7B). This indicated that, with the exception of the $-\text{OH}$ group for which there was no NMR peak in the STD NMR, all the protons in 5-hydroxyindole were affected by the interaction with SipD. This suggested that the entire molecule of 5-hydroxyindole was embedded in SipD upon binding. As control experiment, irradiation of 5-hydroxyindole using the same conditions in the absence of SipD did not result in STD peaks, indicating that the observed STD peaks were not due to indirect saturation of 5-hydroxyindole (Figure 7C).

SipD binds to 4-morpholinoaniline

In addition to 5-hydroxyindole, SPR identified the binding of another scaffold, 4-morpholinoaniline, to SipD (Figure 2B). Titration of ^{15}N /ILV-labeled SipD with 4-morpholinoaniline at molar ratios of 1:0, 1:25, 1:50 and 1:100 resulted in distinct chemical shift deviations of specific SipD peaks in a concentration dependent manner as monitored by ^{15}N TROSY and ^{13}C HSQC (Figure 8 and Figure S1). Representative panels from the ^{15}N titration (S110, A111, F117, E133, V187 and K188, Figure 8A) and ILV titrations (L138, L171, L178, V187 and L194, Figure 8B) showed peaks in fast exchange NMR time scale. Plots of the weighted chemical shift deviations (Figure 8C,D) identified the SipD residues that were strongly affected by 4-morpholinoaniline. These residues ranged from hydrophobic (G94, A111, F117, V138, L150, V156, L179 and V213) to polar charged (D131, E133, K188, K279) residues (Figure 8C). The dissociation constant, however, could not be estimated as plots of chemical shift deviations vs. concentrations of 4-morpholinoaniline did not reach saturation. STD NMR signals were not observed because SipD could not be selectively saturated as there are proton resonances of 4-morpholinoaniline near the STD NMR irradiation frequency. Nevertheless, mapping the SipD residues affected by 4-morpholinoaniline showed two distinct surfaces: the top of the mixed α/β domain and near the bottom of the coiled-coil (Figure 9), similar to the surface maps of SipD interaction with 5-hydroxyindole (Figure 6). Results with 5-hydroxyindole and 4-morpholinoaniline suggest that these surfaces in SipD could be hotspots for binding small molecule fragments.

SipB⁸²⁻²⁴⁰ binds to 5-hydroxyindole and 3-indoleacetic acid

We also screened for small molecules that will bind to the major translocon protein SipB using the N-terminal soluble domain, SipB⁸²⁻²⁴⁰. SPR identified the binding of 5-hydroxyindole (Figure 2C) and 3-indoleacetic acid (Figure 2D) to SipB⁸²⁻²⁴⁰. NMR titrations of SipB⁸²⁻²⁴⁰ with 5-hydroxyindole at protein to compound ratios of 1:0, 1:25 and 1:50 and monitored by 2D ¹H-¹⁵N TROSY resulted in distinct chemical shift perturbations of specific SipB⁸²⁻²⁴⁰ peaks in a concentration dependent manner (Figure S2A). Likewise, stepwise titration SipB⁸²⁻²⁴⁰ with 3-indoleacetic acid at protein to compound ratios of 1:0, 1:4 and 1:8 resulted in distinct chemical shift perturbations of specific SipB⁸²⁻²⁴⁰ peaks in a concentration dependent manner (Figure S2B). Likewise, the ILV methyl resonances of SipB⁸²⁻²⁴⁰ were affected by titration with 5-hydroxyindole (Figure 10A) and 3-indoleacetic acid (Figure 10B). There are currently no NMR assignments available for SipB⁸²⁻²⁴⁰, thus, it is not possible to use NMR chemical shift perturbation methods to identify which parts of SipB⁸²⁻²⁴⁰ are involved in the interaction with 5-hydroxyindole or 3-indoleacetic acid. NMR assignments of SipB⁸²⁻²⁴⁰ would have been challenging and not carried out because only ~50% of the expected amide resonances were observed (Figure S2)

STD NMR confirmed the binding of 5-hydroxyindole and 3-indoleacetic to SipB⁸²⁻²⁴⁰. Further, STD NMR identified how 5-hydroxyindole and 3-indoleacetic acid bound to SipB⁸²⁻²⁴⁰. The off-resonance reference spectra show both compound and SipB⁸²⁻²⁴⁰ signals present (Figure 11A, D). The resulting ¹H STD NMR spectra show many of the signals corresponding to 3-indoleacetic acid (Figure 11B) and 5-hydroxyindole (Figure 11E) remained above noise level. This suggests that most of 5-hydroxyindole or 3-indoleacetic acid interacting with or in close proximity to SipB⁸²⁻²⁴⁰. In contrast, irradiation of compounds using the same conditions in the absence of SipB⁸²⁻²⁴⁰ resulted in no observable STD signals, indicating that SipB⁸²⁻²⁴⁰ must be present to observe the STD signals of the compounds (Figure 11C, F).

Discussion

There are currently only a few small molecules that are known to bind to the T3SS tip and translocon proteins. Bile salts, such as deoxycholate, are sterol-like compounds that bind to SipD and its *Shigella* homolog IpaD.^[6a, 6b, 10, 13] Cholesterol and sphingolipids bind to the translocon protein SipB and its *Shigella* homolog IpaB.^[11, 14] Finally, 2,2'-thiobis-(4-methylphenol) binds to the translocon protein YopD of *Yersinia pestis*.^[15] Here, we identified new small molecule scaffolds (Figure 1) that bind to SipD (5-hydroxyindole and 4-morpholinoaniline) and SipB⁸²⁻²⁴⁰ (5-hydroxyindole and 3-indoleacetic acid).

Comparison of the results of NMR titrations (Figure 3, Figure 5, Figure 8) suggests that 5-hydroxyindole (Figure 6) or 4-morpholinoaniline (Figure 9) perturbed similar surfaces on SipD. Both compounds affected SipD residues near the mixed α/β domain and the bottom region of the coiled-coil (Figure 6 and Figure 9). The bottom of the coiled-coil of SipD was previously identified as an important surface for the interaction with the needle protein PrgI.^[16] Therefore, it may be possible to block the SipD:PrgI interaction by developing compounds that bind to SipD with higher affinity. Our efforts at co-crystallization of SipD or SipB⁸²⁻²⁴⁰ with their interacting compounds has so far failed. Development of tighter

binding compounds should allow co-crystallization to characterize binding by X-ray diffraction.

The observation that two surfaces on SipD were affected by the compounds could be because both compounds have weak affinity to SipD (~30 mM for 5-hydroxyindole) and are therefore not very selective for a specific binding surface. The perturbations of methyl ILV resonances of SipD with both 5-hydroxyindole (Figure 4) and 4-morpholinoaniline (Figure 8B, D) suggest that the protein-small molecule interaction included hydrophobic contacts. However, the small molecules also bound to SipD by polar interactions (perhaps by hydrogen bonding or ionic contacts) because many charged SipD residues were affected as well (Figure 2B and Figure 3B). This interaction with hydrophobic and hydrophilic surfaces is possible because the compounds contain hydrophobic rings as well as hydrophilic groups that are available for hydrogen bonding, such as OH and NH (Figure 1).

Indole-like chemical scaffolds have not been identified as inhibitors of T3SS.^[3] In contrast, molecules that are somewhat chemically related to the aniline scaffold have been previously shown to inhibit type III secretion, such as salicylideneaniline and the 8-hydroxyquinoline derivative INP1750.^[3] Salicylideneanilines (salicylidene + aniline like scaffold) have been suggested to function as a transcriptional inhibitor of the *E. coli* T3SS,^[17] although the specific molecular target has not been identified, while the mechanism of INP1750 (quinoline + piperazine + aniline like scaffold) is currently unknown.^[18] Because the tip and translocon proteins are conserved in many bacterial pathogens, it may be possible that more potent binding versions of indole and morpholinoaniline scaffolds might interact with homologs of SipD, such as IpaD from *Shigella*, BipD from *Burkholderia*, LcrV from *Yersinia* and PcrV from *Pseudomonas*. Indeed, some T3SS inhibitors, such as thiazolidinones^[19] and salicylidene acylhydrazides,^[20] have been shown to be active against a broad range of bacteria, suggesting their target is a conserved T3SS protein.^[3] Efforts are currently underway to determine if 5-hydroxyindole, 3-indoleacetic acid or 4-morpholinoaniline interact with proteins from other bacterial T3SS systems.

To summarize, the significance of this work is that we have identified new small molecules based on the indole and aniline scaffolds that interact with the *Salmonella* translocon protein SipB and the tip protein SipD. These scaffolds could be used as potential starting structures in the rational design of inhibitors of type III secretion.

Experimental Section

Expression and Purification of SipD and SipB^{82–240}

The expression and purification of SipD (residues 39–343 C244S) has been described previously.^[6a] The construct SipB^{82–240} was designed based on the crystal structure of SipB^{82–226}.^[8] SipB^{82–240} was PCR amplified from *Salmonella typhimurium* LT2 strain and subcloned in the NdeI/XhoI sites of pET-22b. The SipD construct contained an N-terminal solubility tag of His₆-GB1 domain and a tobacco etch virus (TEV) protease cleavage site whereas the SipB^{82–240} construct retained an 8-residue non-cleavable C-terminal his tag (LEH₆). Plasmids for protein expression were transformed into *E. coli* DNAY BL21(DE3) and culture media contained kanamycin (30 µg/mL) and carbenicillin (100 µg/mL).

Unlabeled proteins for SPR screening were obtained by cell growth in 1 liter of LB medium. Proteins for NMR were labeled simultaneously with ^{15}N and ILV (where the methyl groups of isoleucine, leucine, and valine were ^{13}C -labeled) by cell growth in M9 minimal medium supplemented with 1 g/L ^{15}N -ammonium chloride (Sigma). Cells were grown at 37°C , when OD_{600} reached ~ 0.4 , the growth medium was supplemented with 60 mg/L of 2-ketobutyric acid-4- ^{13}C sodium salt hydrate (#571342, Sigma) and 100 mg/L of 2-keto-3-(methyl- ^{13}C)-butyric-4- ^{13}C acid sodium salt (#571334, Sigma). When OD_{600} reached ~ 0.8 , cells were induced with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) and cell growth was continued overnight at 15°C to a final OD_{600} of ~ 2.6 (for LB) and ~ 2.1 (for M9).

Cells were harvested by centrifugation (4000 rpm, 10 min), resuspended in buffer (500 mM NaCl, 20 mM Tris-HCl, 5 mM imidazole, pH 8.0, 1 mM phenylmethanesulfonyl fluoride) and sonicated. Cell lysate was centrifuged (13,000 rpm, 10 min), and 700 μL of 5% polyethyleneimine was added to the supernatant to precipitate the nucleic acid, followed by centrifugation (13,000 rpm, 10 min). The supernatant was loaded on a 5 mL Ni^{2+} -affinity resin (Gold Biotechnology) and washed with 150 mL of binding buffer (500 mM NaCl, 20 mM Tris-HCl, 5 mM imidazole, pH 8.0). SipD or SipB^{82–240} was eluted from the Ni^{2+} column with 40 mL of elution buffer (500 mM NaCl, 20 mM Tris-HCl, 250 mM imidazole, pH 8.0). To remove the His₆-GB1 tag in the SipD construct, fractions were pooled and incubated overnight with 250 μL of 0.04 mM recombinant TEV protease^[21] in buffer (50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 1 mM DTT, 20 mM NaCl) at room temperature and the digest was passed through a 5 mL Ni^{2+} -affinity column, which retained the His₆-GB1 tag. Proteins were dialyzed into either 1.05 $\times$ phosphate-buffered saline (PBS, pH 7.4) or NMR buffer (20 mM NaCl, 10 mM sodium phosphate, pH 7.4) and concentrated using Amicon Ultra 3K (Millipore) filtration columns. Protein concentrations were determined by absorbance at A_{280} .

SPR Screening

Surface Plasmon Resonance (SPR) was used for screening using a Biacore 3000 instrument (GE Healthcare). The amine coupling kit (#BR-1106-33) and CM5 sensor chip (#BR100399) were purchased from GE Healthcare. The target proteins SipD and SipB^{82–240} were covalently immobilized to the sensor chip surface by standard amine coupling chemistry with 1.05 $\times$ PBS buffer as the running buffer. There were four flow cells available for immobilization of target protein per CM5 chip. Flow cell 1 was kept as reference flow cell without immobilized protein. The remaining 3 flow cells were loaded with target proteins. All four flow cells were activated for 7 minutes with a mixture of N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) at 1:1 ratio and at a flow rate of 15 $\mu\text{L}/\text{min}$ at 25°C . Proteins at a concentration of 50 $\mu\text{g}/\text{mL}$ in 10 mM sodium acetate (pH 4.3) were injected for 7 minutes resulting in 2,727 response units (RU) for SipB^{82–240} and 10,110 RU for SipD. All four flow cell surfaces were treated with a 7 minute injection of 1M ethanolamine (pH 8.0) to eliminate all the unbound proteins and unreacted esters of NHS/EDC from the flow cells.

A library of 288 compounds (Fragment Library 2, Zenobia, San Diego) was obtained in three 96-well microplates, with each compound at a concentration of 100 mM stock in 100%

DMSO. Compounds were diluted to final concentration of 1 mM in 5% DMSO and 1.05× PBS. Biacore 3000 allowed the automated injection of compounds from 96-well plates. The compounds were injected over the chip surface for 60 sec at a flow rate of 60 µL/min and the dissociation was monitored for 60 sec. Running buffer (1.05× PBS) was used as negative control. The surface was not regenerated between sample injections. The flow system, except sensor surface, was washed with 1:1 mixture of DMSO and water to remove any unbound compound from the flow system. Running buffer was injected between each compound run to monitor carryover effects. Eight DMSO calibration solutions with varying concentrations (4–6%) were injected sequentially at the beginning and end of the experiments, using the same flow rate as for the compounds.

NMR Spectroscopy

NMR data were acquired using a Bruker Avance 800 MHz spectrometer equipped with a cryogenic triple resonance probe and were processed using NMRPipe^[22] and analyzed using NMRView.^[23] Saturation transfer difference (STD) NMR data were acquired using a Bruker Avance 600 MHz spectrometer equipped with a TXI-RT probe and processed using Topspin. Two-dimensional ¹H-¹⁵N TROSY and ¹H-¹³C HSQC spectra were acquired at 30°C using 0.3 mM of ¹⁵N/ILV SipD or 0.5 mM of ¹⁵N/ILV SipB^{82–240} in buffer (10 mM sodium phosphate pH 7.4, 20 mM NaCl, and 10% D₂O). For NMR titrations, the compounds were dissolved in d₆-DMSO (Cambridge Isotope Laboratories, Inc., Andover, MA). All titration samples for NMR contained 1.5% (v/v) d₆-DMSO for SipD or 2.5% (v/v) d₆-DMSO for SipB^{82–240}. Typical 2D ¹H-¹⁵N TROSY acquisition parameters were 16 scans at 30 ppm ¹⁵N sweep width centered at 118 ppm, whereas typical 2D ¹H-¹³C HSQC acquisition parameters for ILV-labeled samples were 8 scans, 18 ppm ¹³C sweep width centered at 18 ppm and 10 ppm ¹H sweep width centered at 4.69 ppm. Chemical shift deviation (δ) was calculated using $\delta_{\text{HN}} = [\frac{1}{2} (\delta_{\text{H}}^2 + \delta_{\text{N}}^2/25)]^{1/2}$ ^[24] for backbone amides and $\delta_{\text{ILV}} = [\frac{1}{2} (\delta_{\text{H}}^2 + \delta_{\text{C}}^2/4)]^{1/2}$ for ILV labels. Dissociation constants were estimated from plots of chemical shift deviation vs. compound concentration for multiple residues and curve fitting using GraphPad Prism Version 5 (GraphPad Software, Inc., USA). The R² values for the fits were 0.99.

One-dimensional ¹H Saturation Transfer Difference (STD) NMR^[25] data were acquired at 30°C on samples containing protein plus compound at a 1:100 molar ratio (40 µM protein, 4000 µM compound, 10% D₂O, 0.4% d₆-DMSO) or compound only (4000 µM compound in 100% d₆-DMSO). The protein saturation pulse (Gaussian) was 50 ms over a period of 2 sec with the center of the on-resonance pulse varied from –0.2 to 0.1 ppm to give an optimal STD signal, while the off-resonance center was kept at 40 ppm. Typical acquisition parameters were 128 scans, 16 ppm ¹H sweep width centered at 4.701 ppm, and 2 sec recycle delay.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

NMR	nuclear magnetic resonance
CSD	chemical shift deviation
SPR	surface plasmon resonance
STD	saturation transfer difference
T3SS	type III secretion system

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Figure 1.

Structure of small molecule scaffolds. SPR identified the binding of 5-hydroxyindole (**1**) to SipD and SipB^{82–240}, 3-indoleacetic acid (**2**) to SipB^{82–240}, and 4-morpholinoaniline (**10**) to SipB^{82–240}. The non-binding or relatively weaker binding of other similar scaffolds revealed the important functional groups needed for binding to SipD or SipB^{82–240}.

Figure 2.

SPR screening of SipD and SipB⁸²⁻²⁴⁰. SPR sensorgrams showing the binding of A) 5-hydroxyindole to SipD, B) 4-morpholinoaniline to SipB⁸²⁻²⁴⁰, C) 5-hydroxyindole to SipD, and D) 3-indoleacetic acid to SipB⁸²⁻²⁴⁰.

Figure 3.

^{15}N NMR titration of SipD with 5-hydroxyindole. A) Overlay of five 2D ^1H - ^{15}N TROSY spectra of ^{15}N /ILV-labeled SipD titrated with increasing molar ratios of 5-hydroxyindole, with expanded sections for selected SipD residues affected by the interaction with 5-hydroxyindole. B) Plot of chemical shift deviation (CSD) vs concentration of 5-hydroxyindole for selected residues.

Figure 4.

ILV titration of SipD with 5-hydroxyindole. A) Overlay of five 2D ^1H - ^{13}C HSQC spectra of SipD titrated with increasing mole ratio of 5-hydroxyindole. B) Plots of chemical shift deviation (CSD) vs concentration of 5-hydroxyindole for selected ^{13}C methyl peaks.

Figure 5. Plots of chemical shift deviations of SipD with 5-hydroxyindole. Chemical shift deviations from A) ^{15}N and B) ILV titrations of SipD with 5-hydroxyindole (σ , average CSD).

Figure 6. SipD surfaces affected by binding of 5-hydroxyindole. Ca of SipD colored according to ^{15}N and ILV chemical shift deviations as follows 1σ (yellow), 2σ (orange), 3σ (red); where σ is the standard deviation from average CSD in Figure 5.

Figure 7. STD NMR of 5-hydroxyindole with SipD. A) Off-resonance, B) on-resonance STD NMR spectra of 5-hydroxyindole with SipD, and C) STD NMR spectrum of 5-hydroxyindole in the absence of SipD.

Figure 8.

NMR titrations of SipD with 4-morpholinoaniline. Selected SipD peaks from A) ^{15}N and B) ILV titrations of SipD with 4-morpholinoaniline. Plots of chemical shift deviation (CSD) of SipD with 4-morpholinoaniline based on C) ^{15}N and D) ILV titrations (σ , average CSD).

Figure 9. Surface of SipD affected by binding of 4-morpholinoaniline. Ca of SipD colored according to ^{15}N and ILV chemical shift deviations as follows 1σ (yellow), 2σ (orange), 3σ (red); where σ is the standard deviation from average CSD in Figure 8.

Figure 10.

ILV titration of SipB⁸²⁻²⁴⁰ with A) 5-hydroxyindole and B) 3-indoleacetic acid.

Figure 11. STD NMR of SipB with (A-C) 3-indoleacetic acid and (D-F) 5-hydroxyindole. (A, D) Off-resonance and (B, E) on-resonance STD NMR spectra of SipB with the small molecules. (C, F) STD NMR of the small molecules in the absence of SipB⁸²⁻²⁴⁰.