Design of small molecule inhibitors of type III secretion system ATPase EscN from enteropathogenic *Escherichia coli*

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Enteropathogenic *E. coli* (EPEC) is a human pathogen using type III secretion system for delivery of proteins directly into the human host. The system contains a single ATPase, EscN, which is essential for uncoupling of proteins from their complexes with chaperones before the delivery. The structure of EscN ATPase (PDB code: 2obm) was used to screen computationally for small molecule inhibitors blocking its active site. Two lead candidates were examined but only one, Compound 54, was selected for further optimization. After extended QSAR optimization, two derivatives were found to be competitive inhibitors of EscN capable of blocking ATPase activity with a $K_i$ below 50 µM. One candidate, WEN05-03, with a $K_i$=16±2 µM, was also minimally toxic to mammalian cells as determined by other assays. In the cell infection model of HeLa cells with EPEC, Compound WEN05-03 completely blocked actin cluster formation at 100 µM concentration, when analyzed by confocal microscopy. The second best inhibitor of EscN ATPase activity was WEN04-34 with a $K_i$=46±2 µM. However, the compound was highly toxic to the BALB/3T3 cell line. In summary, the work identifies a compound blocking bacterial ATPase in its active site without causing cellular toxicity to the host cells. It is the first report showing feasibility of using bacterial virulence system ATPase as a target for safe, non-toxic compounds and offering a proof-of-concept for non-antibiotic alternatives.

**Key words:** Escherichia coli (E. coli), type III secretion system (T3SS), enzyme inhibitor, small molecule

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**Table I.** List of primers used for PCR amplifications.
**Figure S1.** *EPEC* escN gene disruption by insertion of kanamycin resistance cassette.

Panel A: amplification strategy, Panel B: final construct. DNA sequences: black – kanamycin, red – loxP, green – nucleotides introduced to preserve the open reading frame of escN gene, yellow and red – escN gene sequence. Selected restriction sites are shown for information purpose only.
**Figure S2. Kinetics of EPEC infection in the HeLa cell model.**

HeLa cells were incubated with EPEC at MOI 20:1 for 5 h and cells were fixed with PFA every hour. To visualize EPEC-induced actin pedestals F-actin was labeled with phalloidin Atto-488. Propidium iodide was used to label DNA in HeLa cells and in EPEC. Images were taken on a Zeiss Axio Vert.A1 fluorescent inverted microscope. **Panel A:** Time points of infection from 0 to 5 hrs. Labels indicate time post-infection. **Panel B:** Enlargement of a section from 2 hrs time point showing formation of actin pedestals. **Panel C:** Enlargement of a section from 3 hrs time point showing formation of more actin pedestals. **Panel D:** The same enlargement stained for DNA only.
Bacterial culture of EPEC was grown in LB medium at 37°C in a 96-well plate in the presence or absence of 100 µM inhibitor. DMSO was included to account for residual DMSO added with inhibitors.

Figure S3. Bacterial growth inhibition by derivatives of Compound 54 derivatives.
Figure S4. Cell cycle analysis on HeLa and BALB/3T3 cells treated with Compound 54 derivatives.

Panel A: HeLa cells, Panel B: BALB/3T3 cells. Inhibitors were added to the cells to a final concentration of 100 μM and the cells were incubated at 37°C for 24 hrs as described under Materials and Methods. Cell cycle analysis was performed using MUSE (Merck) flow cytometer as described. DMSO-cells treated with DMSO, HeLa, BALB/3T3- untreated cells. DMSO was included to account for residual DMSO added with inhibitors.
Figure S5. Analysis of cell viability and apoptosis of BALB/3T3 cells treated with Compound 54 derivatives.

Panel A: Cell count and viability, Panel B: Apoptosis induction by Annexin V marker. Cells were incubated with 100 μM inhibitors at 37°C for 24 hrs as described under Materials and Methods. Cell count and viability as well as apoptosis were measured using MUSE (Merck) flow cytometer. DMSO was included to account for residual DMSO added with inhibitors.
Figure S6. Analysis of mitochondrial potential in BALB/3T3 cells treated with Compound 54 derivatives.

Cells were incubated with 100 μM inhibitor concentration at 37°C for 24 hrs as described under Materials and Methods. Mitochondrial potential interference was measured using MUSE (Merck) flow cytometer. DMSO was included to account for residual DMSO added with inhibitors.
Figure S7. Analysis of EPEC-induced pedestal formation in the HeLa cell infection model.

HeLa cells were infected with EPEC at MOI 20:1 in the presence or absence of inhibitor for 2 hrs. The cells were fixed and stained with DAPI for DNA (blue) and phalloidin-Atto-488 for actin (green).

Panels A, D, G and J: Maximum intensity projection images acquired on a confocal microscope (contrast enhanced for presentation). Panels B, E, H and K: Quantification of pedestal formation with identified actin clusters based on intensity segmentation of the original phalloidin channel. Clusters are color-coded according to size. Panels C, F, I and L: Quantification of the clusters for each size range after cell area normalization.
Figure S8. Type III secretion system ATPases for selected human pathogens.