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Formulation and Target Identification of D8, a Host-Directed Antibiotic

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Honors Thesis
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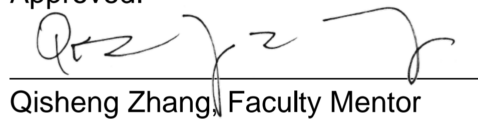


Qisheng Zhang, Faculty Mentor

Abstract

Introduction

Host-directed therapeutics present a new strategy to combat antibiotic resistance. We discovered a molecule, D8, that inhibits intracellular growth of *Francisella tularensis* in cell culture and mouse studies. However, the limited solubility of D8 leads to variability in dosing and the mechanism of action of D8 is unknown. Consequently, the objectives of this study are to increase the solubility of D8 utilizing salt formation and β-cyclodextrin derivatives, as well as, identification of proteins that interact with D8 through chemoproteomics experiments.

Methods

Absorbance-based solubility assay. An absorbance-based solubility assay was developed based on our observation of increased absorbance above 400 nm for D8 aggregates. When D8 is completely soluble, the absorbance at 400 nm is close to the baseline value. However, once D8 reaches saturation state, the absorbance intensity increases linearly with concentration.

Identification of D8-interacting proteins. D8-33 and D8-34 are D8 and D8-05 (inactive analog of D8) derivatives with a diazirine group and a terminal alkyne. J774 cells were incubated with either D8-33 or D8-34. After incubation, the cells were illuminated at 365 nm to crosslink D8-33 or D8-34 with its interacting proteins. After cell lysis, the cell lysate was conjugated with biotin-N_3 through click reaction and the resulting labeled proteins were subsequently enriched with streptavidin-coated resins for proteomics studies.

Results

Methyl-β-cyclodextrin substantially increases the solubility of D8 from 0.03 mM to 2.9 mM, enabling the delivery of D8 within 100 μl PBS to reach dosage of 5 mg/kg for mouse studies. Captisol® and (2-hydroxypropyl)-β-cyclodextrin increase the solubility of D8 to 0.19 mM and 0.61 mM, respectively. In contrast, salt formations do not significantly increase D8's solubility. We also optimized the conditions for click reactions to couple D8-interacting proteins with biotin-N_3.

Conclusion

We developed a formulation that enhances D8's solubility by 100 times, enabling accurate dosing for animal studies. To identify D8-interacting proteins, we modified "click" reaction protocols. Further chemoproteomic studies should be completed to provide a list of potential D8-interacting proteins to provide not only more insight into the mechanism of D8's antibacterial activity, but also new therapeutic targets for future drug discovery.

Introduction

Antibiotic resistance has posed an economical and health burden to the U.S. population and health care system, accounting for $20 billion and approximately 70,000 deaths each year.[1,2] This issue is even more pressing because new targets for antibacterial growth are lacking and the economic appeal of antibiotic development is low due to inevitable emergence of resistance.[1] An issue with traditional antibiotics is that these drugs target pathogen specific properties such as cell wall biosynthesis and bacterial specific ribosomes for protein biosynthesis inhibition, making them active against limited groups of pathogens and susceptible to antibiotic resistance arising from bacterial mutations. Due to the rapid proliferation rate of bacteria, mutations are common in bacterial infections, with an average of 1 thousand mutations in 10^{10} bacteria, making them highly susceptible to developing a phenotypic mutation that renders a bacterium resistant against the antibiotic treatment.[3] The resistant bacterial strain proliferates in the absence of antibiotic susceptible bacteria, resulting in subsequent antibiotic resistant bacterial infections.

Various strategies have been implemented to combat antibiotic resistance. Specific examples include: *i*) modification of compound moieties that are targets of bacterial enzymes and efflux pumps; *ii*) development of adjuvant antibiotics to target antibiotic-destroying hydrolases; *iii*) screening for small molecules that are active against resistant strains; *iv*) development of new structural classes of antibiotics; *v*) identification of new bacteria specific targets for antibacterial growth; and *vi*) inhibition of bacterial gene targets that encode for enzymes that are essential for virulence and survival.[3,4,5,6] These strategies are only temporarily effective because they are highly susceptible to bacterial resistance due to the focus on targeting bacterial components and high incidence of bacterial mutations. Host-directed therapies offer immunity to antibiotic resistance due to shifting from pathogen targets to host-pathogen interactions.

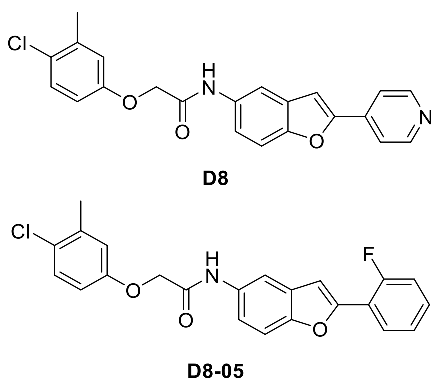
Host-directed therapies modulate immune responses and host defense mechanisms to enhance cellular responses to pathogens and improve therapeutic outcomes.[7] The ability of host-directed therapies to suppress or enhance immune function make them potential strategies for prevention and treatment of infections through inhibition of pathogen invasion, replication, and proliferation. This approach for antibiotic development has many advantages, including prevention of antibiotic resistance and development of an antibiotic with broad-spectrum antimicrobial activity. Through targeting the host rather than pathogen mechanisms, there is no selective pressure for the development of antibiotic resistance.[8, 9] In addition, it fosters a broad range of protection against various classes of microbes due to the non-specificity of innate and adaptive immunity.[8] This enables prophylactic antibiotic use in high-risk groups, and empiric treatment, resulting in better therapeutic outcomes due to timeliness of administration of therapy.[8] These host-directed antimicrobial approaches provide new targets for improved treatment of drug resistant pathogens.

In collaboration with Dr. Tom Kawula's lab at Washington State University, we have developed a small molecule, D8 (Fig. 1A), that inhibits the intracellular growth of *Francisella tularensis* in mouse bone marrow derived macrophages (Fig. 1B), with an IC_{50} value of 0.21 μM. A structurally related analog, D8-05 (Fig. 1A), has no inhibitory effect and serves as a negative control. Importantly, D8 does not show toxic effect on host cells alone or inhibit bacteria growth outside host cells suggesting that it functions through mediating host-specific pathways. Intracellular growth of two other bacterial pathogens, *Listeria monocytogenes* and

Campylobacter, is also inhibited by D8. Furthermore, mice infected with *F. tularensis* showed up to 10% weight loss and rapid bacterial growth in lung, liver, and spleen three days after inoculation. In contrast, those treated with D8 in phosphate buffered saline (PBS) with 5% DMSO maintained their weight although the bacterial growth is only inhibited in the liver. The lack of efficiency to control bacterial growth is likely due to the poor solubility of D8 in PBS. Additionally, the poor solubility of D8 contributes to variability of mice study results. Mice 2 and 3 reached clinical cure indicated by inhibition of bacterial growth (Fig. 1C). However, mouse 1 exhibited significant bacterial growth. A factor contributing to the variability in mouse study results is the variation in the concentration of D8 each mouse received in each dose, due to the precipitation of D8 in the syringe prior to intravenous injection. Consequently, there is a need for methods to enhance the solubility of D8.

Figure 1. D8 is a novel host-targeting antibiotic compound.
A. Chemical structures of D8 and its analog D8-05. D8-05 is the negative control for D8. It is structurally similar with no biological activity. **B. Activity of D8 in macrophage cells post infection.** Inhibition of bacterial growth in mice macrophage cells is represented by the absence of green fluorescence seen in the treatment group that received D8. **C. Bacterial growth in mice infected with *F. tularensis* and treatment with either D8 in PBS or PBS (control).** Mouse 2 and 3 exhibited significant reductions in bacterial burden. However, this effect was not seen in mouse 1.

A



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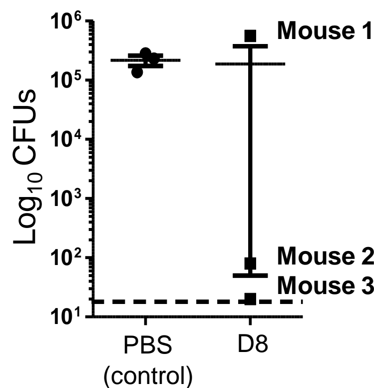

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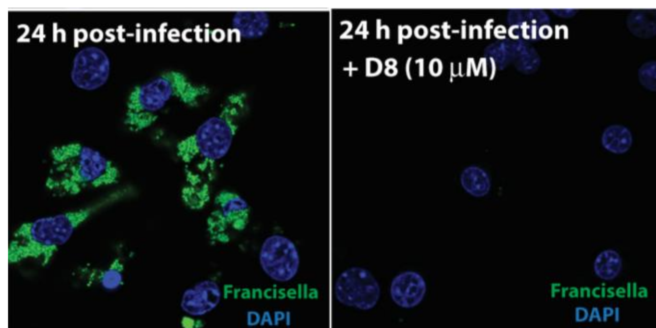


Despite the promise of further developing D8 as a host-targeting antibiotic, its mechanism of action is still unknown. Further research needs to be conducted to explore other potential targets for host-directed therapeutics and to investigate the potential benefits of these therapies in combination with traditional antibiotics for more effective infectious disease treatment. This highlights the importance of identifying the protein that D8 interacts with through pulldown experiments, as further investigation of D8 can provide additional information regarding new potential targets for host-directed therapies.

Herein we report the development of a new formulation to increase D8's solubility and a chemoproteomics approach to identify D8-interacting proteins.

Methods

β-cyclodextrin Formulations

(2-Hydroxypropyl)-β-cyclodextrin (HBC). To measure the solubility of D8 in HBC-containing buffer, 50% HBC in H_2O (600 µL) was mixed with D8 in DMSO (50 µL) at various concentrations, 10x PBS (100 µL), and water (250 µL) at room temperature, with 2 cycles of vortexing for 1 min and sonication for 5 min. Each sample contained 30% HBC, 5% DMSO, and various concentrations of D8 (0.3, 0.6, 0.8, 1, and 1.5 mM). The absorbance of each sample was measured at the wavelength of 400 nm utilizing *SmartSpec™ 3000* Spectrophotometer manufactured by *Bio-Rad Laboratories*. The absorbance values were plotted against D8 concentration to obtain the calibration curve used to determine the solubility of D8 in 30% HBC, 5% DMSO in PBS. The maximum solubility of D8 for the formulation was determined by calculating the D8 concentration at the intersection point of the two linear functions obtained from the concentration versus absorbance data prior to and after saturation of D8 in the vehicle. This absorbance-based solubility assay was developed based on our observation of increased absorbance at 400 nm for D8 aggregates, which are observed when the D8 concentration exceeded saturation of D8 in the vehicle. When D8 is completely soluble, the absorbance at 400 nm is close to the baseline value. Likewise, the solubility of D8 in PBS containing 20% HBC and 10% DMSO or 5% DMSO only (without HBC) was measured. Samples of 20% HBC, 10% DMSO in PBS were prepared with various D8 concentrations (0.04, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.5 mM). Similarly, samples of 0% HBC, 5% DMSO in PBS were prepared with D8 concentrations of 0.001, 0.01, 0.02, 0.03, 0.1, and 0.2 mM to determine the effects of HBC concentration on the solubility of D8. To determine the effects of DMSO on the solubility of D8, samples of 30% HBC, 10% DMSO in PBS with 1 mM or 1.5 mM D8 and samples of 20% HBC, 5% DMSO in PBS with 0.5 mM or 0.6 mM D8 were prepared in a similar manner.

Methyl-β-cyclodextrin (MBC). To measure solubility of D8 in MBC-containing buffer, 50% MBC in water (600 µL) was mixed with D8 in DMSO (50 µL) at various concentrations, 10x PBS (100 µL) and water (250 µL) at room temperature, with 2 cycles of vortexing for 1 min and sonication for 5 min. Each sample contained 30% MBC and 5% DMSO, and various concentrations of D8 (0.6, 0.8, 1.0, 1.2, 1.5, 1.78, 2.0, 3.0, 4.0, and 5.0 mM). The absorbance of each sample was measured, and the calibration curve was obtained by the same methods as stated above.

Captisol® (sulfobutylether-β-cyclodextrin). To measure solubility of D8 in Captisol®-containing buffer, 50% Captisol® in H_2O (600 µL) was mixed with D8 in DMSO (50 µL) at various concentrations, 10x PBS (100 µL), and water (250 µL) at room temperature, with 2 cycles of vortexing for 1 min and sonication for 5 min. Each sample contained 30% Captisol®,

5% DMSO, and various concentrations of D8 (0.1, 0.2, 0.3, 0.6, 0.8, and 1 mM). The absorbance of each sample was measured, and the calibration curve was obtained by the same methods as stated above.

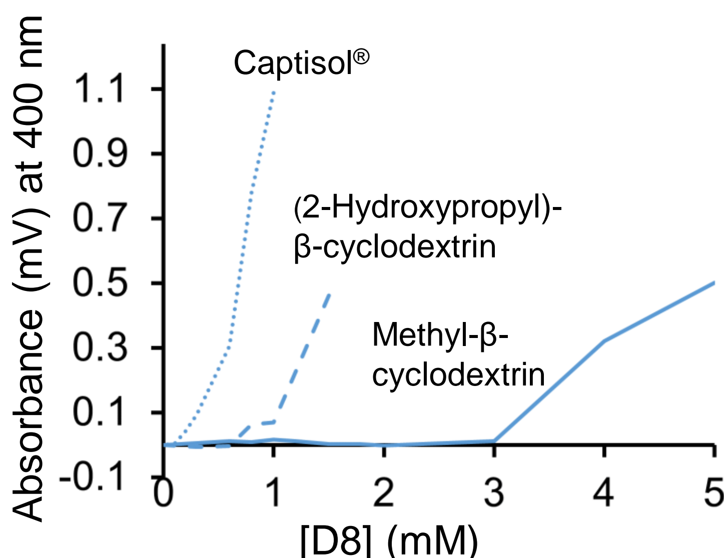
Salt Formation

Starting with 500 μL of 2.5 mM D8 in 25% DMSO and 75% water, 1 M concentration of sulfuric acid, hydrobromic acid, phosphoric acid, citric acid, L-tartaric acid, and L-lactic acid were added in 3 increments of 500 uL to determine the effect of various acids of on the solubility of D8. The absorbance of each sample was measured by the same method as stated above.

Identification of protein targets of D8 utilizing click reaction and pull down experiments

J774 cells were treated with either D8-33 (100 μM) or D8-34 (100 μM) in serum-free Dulbecco's Modified Eagle Medium (DMEM) at 37 °C in an incubator with 5% CO_2 for 30 min. D8-33 is a D8 derivative incorporated with a photoactivatable diazirine group and a terminal alkyne. Likewise, D8-34 is the corresponding derivative of D8-05, a structurally similar but inactive analog of D8. After removal of the culture medium, the cells were illuminated with light at 365 nm for 15 min to crosslink D8-33 or D8-34 with its interacting proteins. The cells were then washed with cold phosphate-buffered saline (PBS) twice and harvested by scratching off the plate with cold PBS. The cell pellets were collected by centrifugation at 14,000g for 5 min. To lyse the cells, PBS with 0.1% NP-40 and protease inhibitors was added to re-suspend the pellet by passing the suspension through a 27G needle 10 times. The supernatant was collected after the mixture was centrifuged at 14,000g for 10 min as cell lysate fraction 1. The resulting cell pellets were then resuspended with PBS with 1.0% NP-40 and the corresponding supernatant is cell lysate fraction 2. Each fraction of cell lysate (500 μL) was conjugated with biotin-N_3 through click reaction (0.1 mM BTTAA, 0.1 mM biotin-N_3, 1 mM $CuSO_4$, 1 mM ascorbic acid, 1 mM TCEP, flushed with N_2 for 1 min, incubated at 37 °C for 4.5 h) and the proteins were subsequently precipitated with 4:1 methanol/chloroform (2500 μL) and PBS (1000 μL) to form a

Figure 2. Absorbance of D8 in formulations with various β-cyclodextrins (30%). The maximum solubility of D8 in 30% Captisol, 30% HBC, and 30% MBC are 0.19 mM, 0.61 ± 0.06 (SD) mM, and 2.9 ± 0.13 (SD) mM respectively. The maximum solubility of D8 in each formulation was determined by calculating the D8 concentration at the intersection point of the two linear functions obtained from the concentration versus absorbance data prior to and after saturation of D8 in the vehicle. The standard deviations were determined by measuring the absorbance values of the samples at 2-3 additional time points. Number of replicas: 1.



protein disc. The organic and aqueous layers were pipetted out and discarded after centrifugation (10 min). The protein disc was washed 3 times with 1:1 methanol/chloroform (1000 µL) and subsequently, 4:1 methanol/chloroform (3000 µL) was added and the sample was sonicated (1 min) and centrifuged (10 min). Then, the supernatant was discarded and PBS (800 µL) with 1% SDS and 1x protease inhibitor was added and subsequently incubated with Dynabead (streptavidin column) at 4 °C for 1.5 h. After immobilization of the streptavidin column with DynaMag™-spin magnet, the supernatant was pipetted off as flow through. The beads were then washed with 0.5% NP-40 PBS (400 µL) three times. The interacting proteins were eluted by adding 3 mM biotin-N_3 0.5% NP-40 PBS (200 µL) and heating the beads at 95 °C for 20 min (repeated for elution 2).

Results

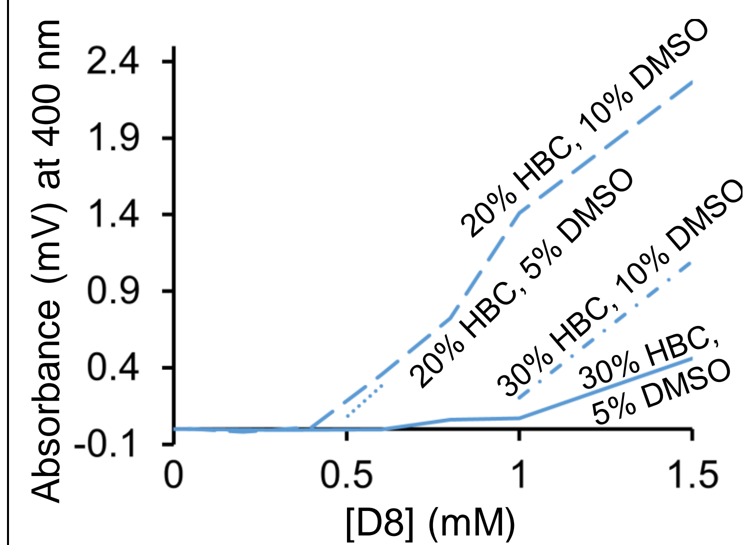
<u>β-cyclodextrin Formulations</u>

The absorbance values were plotted against D8 concentration to obtain the calibration curve used to determine the solubility of D8 in each β-cyclodextrin formulation. Based on the calibration curves, the maximum solubility of D8 in 0% HBC, 30% Captisol, 30% HBC, and 30% MBC are 0.03 mM, 0.19 mM, 0.61 $\pm$ 0.06 mM, and 2.9 $\pm$ 0.13 mM respectively (Fig. 2). Based on this data, 30% MBC has shown to increase the solubility of D8 by 100 fold, from 0.03 mM to 2.9 $\pm$ 0.13 mM.

Additionally, the effect of DMSO concentration on D8 and HBC were analyzed (Fig. 3). Comparing 0.6 mM D8 in 20% HBC, 5% DMSO and 20% HBC, 10% DMSO, the absorbance values are 0.28 and 0.36, respectively. Comparing 1.5 mM D8 in 30% HBC, 5% DMSO and 30% HBC, 10% DMSO, the absorbance values are 0.46 and 1.10, respectively. The increase in absorbance at 10% DMSO compared to 5% DMSO indicates that as the concentration of DMSO increases, the solubility of D8 in HBC decreases.

Additional analysis was conducted to determine the effect of HBC concentration on the solubility of D8 (Fig. 4). The maximum solubility of D8 in 0% HBC, 5% DMSO; 20% HBC, 10% DMSO; and 30% HBC, 5% DMSO are 0.03 mM, 0.41 mM and 0.61 $\pm$ 0.06 mM respectively. This indicates that increasing the

Figure 3. Absorbance of D8 in formulations with various concentrations of (2-Hydroxypropyl)-β-cyclodextrin and DMSO formulations. DMSO negatively impacts the solubility of D8. An increase in DMSO concentration decreases the solubility of D8 in HBC formulations. This is represented by the increase in absorbance values when the concentration of DMSO is increased. Number of replicas: 1.



concentration of HBC significantly increases the solubility of D8.

Salt Formation

The effect of salt formation on the solubility of D8 was evaluated qualitatively and quantitatively utilizing spectrophotometry following each incremental addition of 500 µLof 1 M acid to 500 µL of 2.5 mM D8 in 25% DMSO and 75% water. Both strong and weak acids were used in this set of experiments and the corresponding absorbance of various acids with the addition of 0 µL, 500 µL, 1000 µL, and 1500 µL of various acids were measured (Fig. 5). Addition of 1500 µL of 1 M sulfuric acid, hydrobromic acid, phosphoric acid, citric acid, L-tartaric acid, and L-lactic acid changed the absorbance from 1.09 to 1.69, 1.23 to 1.50, 1.56 to 0.55, 1.98 to 0.49, 1.85 to 0.68, and 1.92 to 0.66, respectively. As the volume of acid added increased, the absorbance values decreased, but did not reach the baseline of 0.

Identification of protein targets of D8 utilizing click reaction and pull down experiments

Optimization of click reaction

"Click" reaction is the copper-catalyzed [3+2]-cyclization between an azide and an alkyne and has been widely used in biological applications. To identify D8-interacting proteins, we developed a D8 analog, D8-33 (Fig. 6A), with a diazirine and an alkyne group incorporated. The diazirine group was introduced to

Figure 4. Absorbance of D8 in formulations with various concentrations of (2-Hydroxypropyl)-β-cyclodextrin. Increasing the concentration of HBC increases the solubility of D8. The maximum solubility of D8 in 0% HBC, 5% DMSO; 20% HBC, 10% DMSO; and 30% HBC, 5% DMSO are 0.03 mM, 0.41 mM and 0.61 ± 0.06 (SD) mM respectively.
Number of replicas: 1.

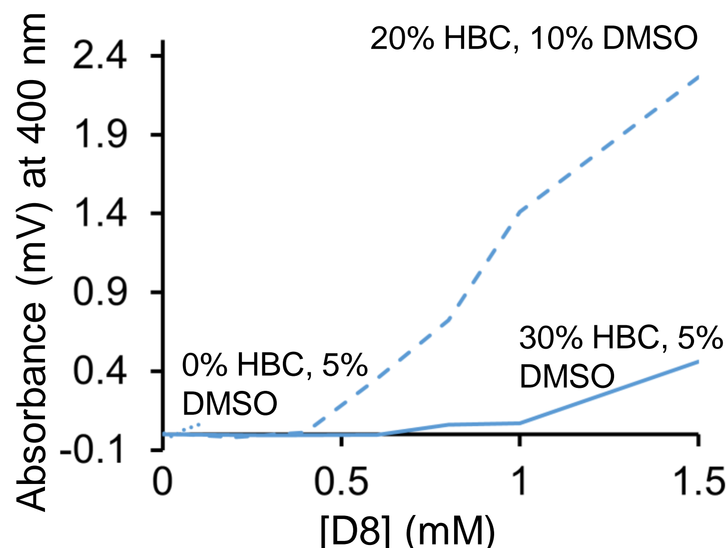
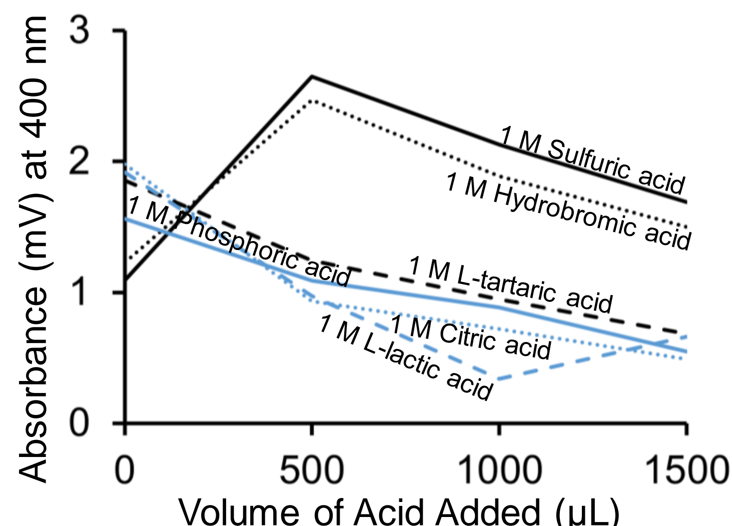


Figure 5. Absorbance at various volumes of various acids added to 500 µL of 2.5 mM D8 in 25% DMSO, 75% water. Salt formulations were ineffective in significantly enhancing the solubility of D8, as evidenced by the presence of visible precipitation in the samples, and absorbance > 0, even with substantial volumes of acid added. Number of replicas:1.



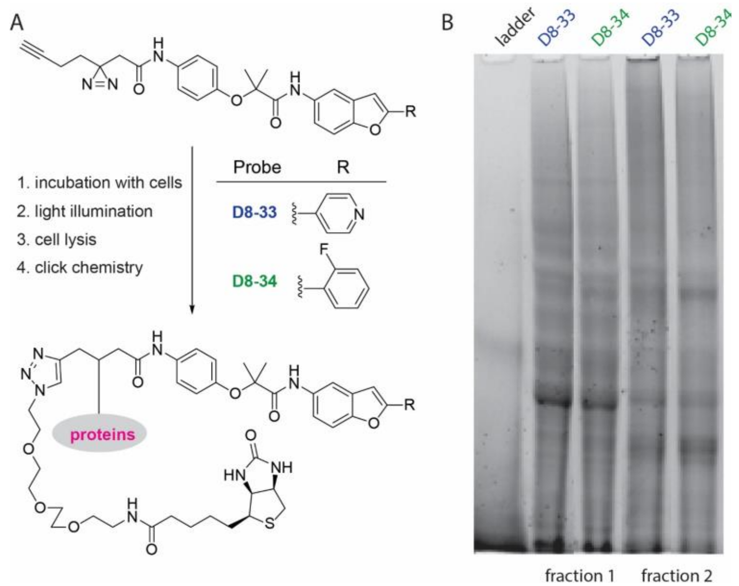
crosslink with D8-interacting proteins in live cells upon light illumination. The alkyne group is used for “click” reaction, which leads to the addition of the biotin-N_3 functional group used for pull down experiments. The negative control, D8-34 is a derivative of D8-05.

To optimize the conditions for “click” reaction between crosslinked D8-interacting proteins and 6-FAM-N_3, we varied the concentration of various components involved in the reaction including $CuSO_4$, reducing agent, and 6-FAM-N_3. In addition, we also investigated the effects of reaction time and temperature. 6-FAM- N_3 was used instead of biotin-N_3 in optimization of click reaction experiments due to its ability to be visualized on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) without dye. The reaction efficiency was measured by fluorescence intensity of 6-FAM-N_3-labeled proteins on a gel. The optimal reaction conditions were determined. Briefly, D8-crosslinked proteins were mixed with 0.1 mM BTTAA, 0.1 mM 6-FAM-N_3, 1 mM $CuSO_4$, 1 mM ascorbic acid, and 1 mM TCEP at room temperature. The resulting mixture was flushed with N_2 for 1 min and incubated at 37°C for 4.5 h. The proteins in the reaction were then resolved through SDS-PAGE and detected by fluorescence. One representative image is shown in Fig. 6B.

Identification of D8 interacting proteins

Utilizing the optimized “click” reaction conditions, the pulldown experiment was next conducted. In this experiment, biotin-N_3 was used in place of 6-FAM-N_3 to label crosslinked D8-interacting proteins. The biotinylated proteins were then bound to streptavidin-coated Dynabead. The non-specific interacting proteins were first washed out. The D8-interacting proteins were then eluted with biotin (3 mM) at 95 °C. As shown in Fig. 7, specific bands of proteins

Figure 6. Target identification of D8.
A. Crosslinking and affinity tag labeling of D8-interacting proteins. To identify D8-interacting proteins, we developed a D8 analog, D8-33, and the negative control, D8-34, with a diazirine and an alkyne group incorporated. The diazirine group is utilized to crosslink with D8-interacting proteins in live cells upon light irradiation. The alkyne group then underwent “click” reaction, which led to the addition of the biotin-N_3 functional group used for pull down experiments.
B. SDS-PAGE of the cell lysates labeled with 6-FAM-N_3. Fraction 1 represents cell lysates with 0.1% NP-40 PBS while fraction 2 represents cell lysates with 1% NP-40 PBS. Optimal click reaction conditions were identified and supported by high fluorescence intensity visualized via SDS-PAGE. High fluorescence intensity shown in both fractions of samples D8-33 and D8-34 correlates with high reaction efficacy and significant 6-FAM-N_3 labeled proteins. Number of replicas: 1.



are visualized in the sample resulting from D8-33, but not D8-34, suggesting that affinity chromatography is effective in eluting D8 interacting proteins. The eluted proteins were sent to the proteomics core for identification.

Discussion

β-Cyclodextrin Formulations

β-cyclodextrins are effective in enhancing solubility through incorporation of hydrophobic drugs inside the cylindrical shape and the presence of a hydrophilic exterior enabling it to be soluble in water. Based on the results, absorbance values are in the order of tenths of thousandths to hundredths of thousandths when D8 is completely solubilized. This indicates an absorbance of essentially 0 (baseline) when D8 is completely solubilized. This is further supported by the absence of visible precipitation in solution. However, as the concentration of D8 increases and the maximum solubility of D8 in the vehicle is reached, the absorbance values increase drastically and D8 forms visible aggregates. Additionally, when samples were centrifuged to remove the precipitation, the absorbance returned to baseline, confirming that absorbance at 400 nm corresponds to D8 aggregation.

Figure 7. Pulldown experiment results using optimized click reaction conditions. Specific bands of proteins are visualized in the sample resulting from D8-33, but not D8-34, suggesting that affinity chromatography is effective in eluting D8 interacting proteins. Number of replicas: 1.

All three cyclodextrin derivatives substantially increase the solubility of D8. Most strikingly, MBC formulation effectively increased the solubility of D8 by 100-fold from 0.03 mM to 2.9 $\pm$ 0.13 mM. The low solubility of D8 in PBS leads to the precipitation of D8 in 2.7 mM D8 in the PBS formulation used for animal studies. This precipitation of D8 in PBS was attributed to the variation in animal study data through variations in IV dosing. Therefore, effective solubilization of 2.7 mM D8 with the MBC formulation provides accurate IV dosing for animal studies. Elimination of variations in animal study data will provide further insight on D8's biological effect in inhibition of *F. tularensis* growth.

The effect of DMSO concentration on HBC formulations were investigated by comparing 5% and 10% DMSO concentrations in 30% and 20% HBC. The results indicate that increasing the DMSO concentration impacts D8-β-cyclodextrin interactions, decreasing the solubility of D8 in β-cyclodextrin formulations (Fig. 3). While DMSO cannot be eliminated in the formulation, the concentration of DMSO should be minimized. DMSO in the formulation cannot be eliminated due to difficulty in solubilizing solid D8 in 30% HBC and can be attributed to a limitation of this study. In order to easily prepare formulations of D8 in β-cyclodextrin, D8 in DMSO was added to 50% β-cyclodextrin stock solution, 10x PBS, and water to obtain a formulation of D8 in 30% β-cyclodextrin, 5% DMSO in PBS.

The effect of HBC concentration on the solubility of D8 was also investigated by comparing 0%, 20%, and 30% HBC concentrations. Increase in HBC concentration significantly increased the solubility of D8. Therefore, further increase in HBC concentration will likely further increase the solubility of D8. However, the concentration of β-cyclodextrin is limited by renal toxicity.[10,11] The LD_{50} of β-cyclodextrin for IV dosing in rats is 788 mg/kg.[12] In comparison, the β-cyclodextrin concentration in 2 mL of 30% β-cyclodextrin D8 formulation is 600 mg. Further increasing the concentration of β-cyclodextrin could lead to toxicity in animal studies.

An advantage of this approach is that enhanced solubility is achieved through specific interactions rather than changes to the structural properties of the small molecule, thereby preserving the biological activity of D8. Limitations include limits on the concentration of β-cyclodextrin in the formulations and the inconsistent concentrations of DMSO between the 30% HBC, 5% DMSO and 20% HBC, 10% DMSO formulations. Due to this inconsistency, we are not able to quantitatively determine the degree of increase in solubility between 20% HBC and 30% HBC formulations (Fig 4). However, the conclusion that increasing the concentration of HBC increases the solubility of D8 is valid based on the increase in the solubility of D8 in 30% HBC, 5% DMSO compared to 20% HBC, 5% DMSO from 0.5 mM to 0.6 mM (Fig 3).

These results were consistent with a study investigating the solubility of aromatic small molecules: hydrocortisone, diazepam, digitoxin, and indomethacin in various modified β-cyclodextrins. MBC formulations resulted in the greatest increase in solubility of these small molecules.[13] Future research should focus on conducting animal studies with MBC-D8 formulation to accurately evaluate the effect of D8 for inhibition of *F. tularensis* facilitated by accurate IV dosing of D8.

<u>Salt Formation</u>

Salt formations were utilized to enhance the solubility of D8 due to the preservation of D8's structure and the cost effectiveness of this approach. We hypothesized that the pyridine functional group (pKa 5.25) of D8 will easily form salts with the selected acids, thereby increasing solubility.

Salt formulations were ineffective in significantly enhancing the solubility of D8, as evidenced by the presence of visible precipitation in the samples, and absorbance > 0, even with substantial volumes of acid added (Fig. 5). The decrease in absorbance with each 500 µL increment of acid added to the samples is attributed to the effect of dilution because increasing the volume of acid did not decrease the amount of visible precipitation in the samples. These results differed from findings in other salt formation solubility studies of aromatic small molecules. A study conducted by Bastin, et al. investigated salt formations for increasing the solubility of a weak base with a pKa of 5.3 and a poor aqueous solubility of 10 µg/mL. This compound formed stable salts with hydrochloride and hydrobromide counterions, with solubility values of 16.68 mg/mL and 3.29 mg/mL respectively.[14]

Strengths of this approach include the cost effectiveness and preservation of the chemical structure of the parent molecule. Limitations include unacceptable high acidity in formulations for IV dosing, breakdown of parent molecule in high acidity, and dilution effect of adding large volumes of acid to D8. The dilution effect limits the ability to accurately quantify the effect of various acids on the solubility of D8. However, due to absorbance values > 0 and the presence of visible precipitation, we can conclude that salt formation did not significantly increase the solubility of D8.

Identification of protein targets of D8 utilizing click reaction and pull down experiments

Optimization of click reaction

Optimal click reaction conditions (0.1 mM BTTAA, 0.1 mM 6-FAM-N_3, 1 mM $CuSO_4$, 1 mM ascorbic acid, and 1 mM TCEP, flushing with N_2 for 1 min, incubated at 37^oC for 4.5 h.) were identified and supported by high fluorescence intensity visualized via SDS-PAGE (Fig.6B). High fluorescence intensity correlates with high reaction efficacy and increased 6-FAM-N_3 labeled proteins. Successful optimization of click reaction conditions enables efficient generation of biotin-N_3 labeled D8 interacting proteins for high specificity elution of D8 interacting proteins utilizing streptavidin beads.

The optimal click reaction conditions were determined by investigating the different components involved (6-FAM-N_3 concentration, ascorbic acid concentration, reaction time, reaction temperature, and with or without flushing with N_2 for 1 min). A reaction time of 4.5 h was chosen because longer reaction times lead to increased protein degradation likely due to the generation of reactive oxygen species and shorter reaction times lead to decreased intensity of proteins labeled with 6-FAM-N_3. Degassing with N_2 is suspected to decrease the formation of reactive oxygen species and therefore decrease protein degradation. A potential limitation to this study is that this hypothesis was not formally tested to quantify the effects of degassing on protein degradation. Another method to decrease reactive oxygen radical species during the reaction is to use a reducing reagent, ascorbic acid. However, the click reaction experiments investigating 0 mM vs 1 mM ascorbic acid and 37^oC vs room temperature were inconclusive. Differences in 6-FAM-N_3 labeled protein intensity could not be determined because the fluorescence intensity of 6-FAM-N_3 labeled protein in the all samples were too low to detect differences in fluorescence intensity between samples. Therefore, the results were confounded by inefficiency in 6-FAM-N_3 labeling. The effects of ascorbic acid and reaction temperature on protein degradation cannot be visualized due to low protein yield. Therefore, this is considered a limitation. Increased 6-FAM-N_3 concentration (0.025 mM vs 0.1 mM) yielded higher fluorescence intensity in 6-FAM-N_3 labeled proteins due to more complete reaction. Further studies need to be conducted to quantify the impact of 1 mM ascorbic acid, degassing with nitrogen, and reaction temperature on protein degradation during click reaction.

These results differed from click reaction results in other studies. Click reaction conditions utilized by Parker et al (0.1 mM TBTA, 1 mM $CuSO_4$, 25 µM TAMRA-N_3, and 1 mM TCEP) and Höglinger et al (2.5 mM TBTA, 25 mM $CuSO_4$, 25 mM biotin-N_3, and 25 mM ascorbic acid) were effective for affinity tag labeling of proteins.[15,16] However, these click reaction conditions are not optimal for our small molecule.

Identification of D8 interacting proteins

Affinity chromatography with streptavidin-coated Dynabead is effective in enriching biotinylated D8-interacting proteins, indicated by visualization of specific bands of proteins in the sample derived from D8-33, but not that from D8-34 (Fig. 7). D8-34 is the negative control because it was determined to have no inhibitory activity. Proteins that interact with both D8-33 and D8-34 are considered non-contributory to inhibition of bacterial growth by D8. Eluted D8-interacting proteins were sent to proteomics core for identification. These results were consistent with experiments conducted by Kimble et al, which showed successful elution of biotin-N_3 labeled proteins using streptavidin beads.[17] Strengths of this approach include tight interaction between biotin and streptavidin (K_d ~10^{-15} M) enabling stringent washing and

resulting in low amounts of non-interacting proteins. Furthermore, light-induced photo-affinity labeling forms a covalent bond between D8 and its interacting protein targets. This crosslinking enables elution of both strong and weak D8 interacting proteins. One limitation of this approach is the potential for inefficient elution of D8 interacting proteins from the streptavidin affinity column due to the tight interaction between biotin-N_3 and streptavidin. Therefore, further research needs to be conducted to compare the efficiency of D8 interacting protein elution using 3 mM biotin-N_3 in buffer with 0.5% NP-40 PBS compared to the utilization of trypsin digestion to dissociate D8 interacting proteins from the streptavidin column. Identification of a list of potential D8-interacting proteins will provide more insight into the mechanism of D8's antibacterial activity, and new therapeutic targets for future drug discovery.

Conclusion

In conclusion, we developed a formulation that enhances D8's solubility by 100 times, thereby, enabling accurate dosing for animal studies. Future animal studies with accurate D8 dosing will provide less data variability and more insight on the efficacy of D8 as a host-directed antibiotic. To identify D8-interacting proteins, we optimized click reaction protocols. This enables efficient generation of D8-interacting proteins labeled with biotin-N_3, which will be subsequently enriched utilizing streptavidin beads. Further chemoproteomic studies should be completed to provide a list of potential D8-interacting proteins to not only provide more insight into the host-directed mechanism of D8's antibacterial activity, but also new therapeutic targets for future drug discovery. Future development of host-directed antimicrobials provides a new strategy for improved treatment of drug resistant pathogens and sustainable prevention of antibiotic resistance.

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Report Addendum

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Conflicts of Interest
The authors have no conflicts of interest to disclose.