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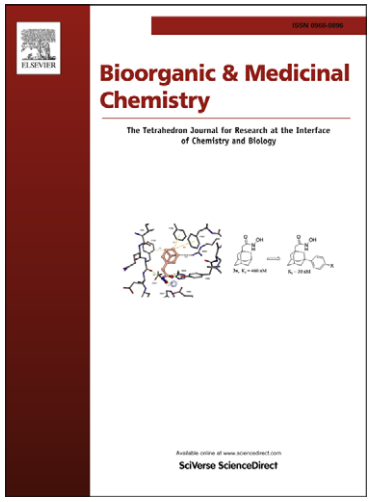

Benzimidazole-Based Antibacterial Agents Against *F. tularensis*

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PII: S0968-0896(13)00260-5
DOI: http://dx.doi.org/10.1016/j.bmc.2013.02.059
Reference: BMC 10695

To appear in: *Bioorganic & Medicinal Chemistry*

Received Date: 7 December 2012
Revised Date: 11 February 2013
Accepted Date: 19 February 2013

Please cite this article as: Kumar, K., Awasthi, D., Lee, S-Y., Cummings, J.E., Knudson, S.E., Slayden, R.A., Ojima, I., Benzimidazole-Based Antibacterial Agents Against *F. tularensis*, *Bioorganic & Medicinal Chemistry* (2013), doi: http://dx.doi.org/10.1016/j.bmc.2013.02.059

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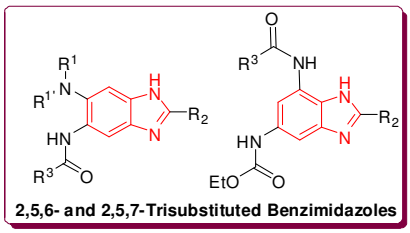

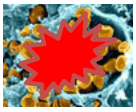

F. tularensis

Preliminary Screening: 23 compounds with 90 % growth inhibition at 1 µg/mL

21 compounds
MIC_{90} ~ 0.35-48.6 µg/mL

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Benzimidazole-Based Antibacterial Agents Against *F. tularensis*

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ARTICLE INFO

Article history:
Received
Revised
Accepted
Available online

Keywords:
Antibacterial
Benzimidazole
Francisella tularensis

ABSTRACT

Francisella tularensis is a highly virulent pathogenic bacterium. In order to identify novel potential antibacterial agents against *F. tularensis*, libraries of trisubstituted benzimidazoles were screened against *F. tularensis* LVS strain. In a preliminary screening assay, remarkably, 23 of 2,5,6- and 2,5,7-trisubstituted benzimidazoles showed excellent activity exhibiting greater than 90 % growth inhibition at 1 μg/mL. Among those hits, 21 compounds showed MIC_{90} values in the range of 0.35-48.6 μg/mL after accurate MIC determination. In *ex-vivo* efficacy assays, four of these compounds exhibited 2-3 Log reduction in colony forming units (CFU) per mL at concentrations of 10 and 50 μg/mL.

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1. Introduction

Bacterial infections due to pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA),[1] vancomycin-resistant *enterococci* (VRE),[2] *F. tularensis,*[3] *Y. pestis*[4] and others have re-emerged as major health concerns throughout the world. *F. tularensis* is one such pathogen which has attracted widespread attention as a potential bioterrorism weapon.[5] It is a highly contagious gram-negative bacteria which is the causative agent of tularemia or "Rabbit fever", a zoonotic disease.[6] Symptoms of tularemia generally depend on the mode of infection. Predominantly the symptoms are fever, ulcers, dyspnea, and others.[5, 7] Normally the bacteria reside in rabbit or rodents but it can infect humans through vectors such as mosquitos and fleas.[5] Bacteria primarily infect macrophages of the host organism where it multiplies and later invades the lungs, spleen, liver, and kidneys.[5, 8] Among the four known subspecies of *F. tularensis*, Type A is the most virulent and is associated with most lethal pulmonary infections.[9] It is highly virulent as it can cause a fatal infection on exposure to doses as low as 10 colony forming units.[10,11] Due to its high infectivity that enables airborne transmission, difficult diagnosis and a very low infection dose, *F. tularensis* has been classified as category A list of select agents by NIAID.[5, 8] Airborne infection by *F. tularensis* could be potentially used as a biological weapon.[3, 5, 8] Once people inhale the bacteria, they can develop life threatening respiratory illness, including fatal systemic infection.[12] It has been also estimated that if the infection is left untreated, the mortality rate can be as high as 30 to 60% of the cases.[11, 13] According to studies done in 1970, the WHO predicted that the release of 50 kg of *F. tularensis* in air over a city with a population of 5 million would result in 250,000 cases of disease.[14]

Treatment against this pathogen still relies on a rather limited number of aging antibacterial drugs which target cell wall biosynthesis, nucleic acid synthesis, protein synthesis, etc.[15] The infection is largely treated by antibiotics like aminoglycosides, streptomycin, tetracycline, chloramphenicol, and quinolones.[12, 16] However, these antibiotics have either shown high toxicity or a high relapse rate.[5, 17] As an alternative treatment option, use of vaccines has acquired considerable interest and is in a very early phase of development. *Live vaccine strain* (LVS) was successful enough in decreasing the incidence rates of laboratory-acquired tularemia.[18] However, due to the unknown role of the vaccine, FDA rejected applications to license the LVS.[5] Additionally, the widespread bacterial resistance to existing antibiotics due to poor drug compliance has been another crucial obstacle in finding suitable and more efficient treatments.[17]

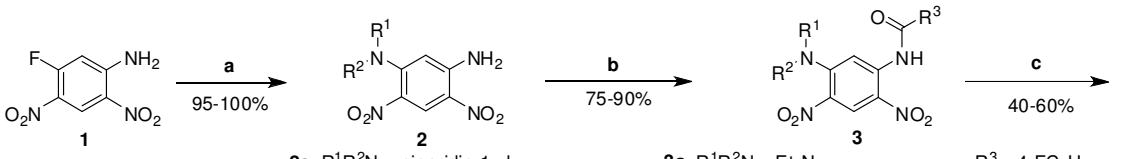
About 10 years ago, we launched our research program on the discovery and development of new antibacterial agents for *M. tuberculosis* (*Mtb*), which would overcome bacterial drug resistance by exploiting a drug target that had not been extensively studied. Then, we chose FtsZ, an essential protein for bacterial cell division, as the target since the disruption of cell division should lead to inhibition and arrest of bacterial growth. Since FtsZ is a homolog of tubulin/microtubules in eukaryotes, known tubulin inhibitors were screened against *Mtb*, and several potent pyridopyrazine- and pteridine-based FtsZ inhibitors with anti-TB activity were identified.[19-21] We found that novel C-seco-taxoids exhibited significant antibacterial activity against *Mtb* by blocking the FtsZ depolymerization.[22] Also, thiabendazole and albendazole, known tubulin inhibitors, were found to interfer and delay the *Mtb* cell division processes.[23] Based on the structural similarity of the pyridopyrazine moiety, pteridine moeity, albendazole and thiabendazole,[19-21] we selected the benzimidazole scaffold for the development of novel FtsZ inhibitors. Then, we investigated novel trisubstituted-benzimidazoles for antibacterial activity against *Mtb*, and found a series of highly active compounds against drug resistant *Mtb* strains. Also, the target of these benzimidazoles was validated by *Mtb* FtsZ polymerization inhibition by light scattering and TEM as well as GTPase assay.[24] For the *Mtb* project, we created a library of ca. 1,100 novel trisubstituted benzimdazoles. Accordingly, it was very logical for us to screen this compound library against *F. tularensis* since it had been shown that FtsZ was highly conserved and ubiquitous cell division protein among a wide range of bacteria,[25-28] and the validity of FtsZ as a novel antibacterial drug target has been confirmed by the work of various groups including ours.[19-22, 24, 29-37]

Thus, we have screened the in-house libraries of 2,5,6- and 2,5,7-trusubstituted benzimidazoles in the hope of discovering novel antibacterial agents against *F. tularensis,* and in fact found a dozen promising hit compounds. We describe here the screening, identification of hit compounds, resynthesis, MIC90 determination, ex-vivo efficacy evaluation of selected hit compounds against RAW 264.7 macrophages infected by *F. tularensis* LVS.

2. Chemistry

Screening of in- house libraries against *F. tularensis* LVS presented 23 hits at MIC <1 µg/mL. A general procedure for the synthesis of these libraries of 2,5,6- and 2,5,7-trisubstituted benzimidazoles have been reported in the previous work.[219] Chemically pure 2,5,6-trisubstituted benzimidazole hit compounds were synthesized following the synthetic protocol as outlined in **scheme 1**. The aromatic nucleophilic substitution of commercially available 2,4-dinitro-5-fluoroaniline (**1**) with various amines afforded 5-amino-2,4-dinitroanilines **2a-2e** in 94-98 % yield as yellow solids. The acylation of intermediates **2a-2e** with different acyl chlorides afforded the corresponding *N*-acylanilines **3a-3i** in 75-95% yield. One-pot reduction and subsequent cyclization in the presence of stannous chloride dihydrate and 4 *M* hydrochloric acid gave 5-aminobenzimida-zoles **4a-4i** in 65-79% yield. The derivatization of the 5-aminobenzimidazoles **4a-4i** using various acyl chloride, acids, sulfonylchlorides, chloroformates or corresponding *N*-hydroxysuccinimide ester generated the desired analytically pure hit compounds **5a-5k** in 42-62% yields.

Chemically pure 2,5,7-trisubstituted benzimidazole hit compounds were synthesized as outlined in **scheme 2**. Commercially available 5-amino-2,4-dinitrobenzamide (**6**) was hydrolyzed to give 4-amino-3,5-dinitrobenzoic acid, which was converted to acyl chloride **7**. The reaction of **7** with sodium azide afforded the corresponding acyl azide, which was subjected to the Curtius rearrangement to give the corresponding isocyanate. The isocyanate was treated with corresponding alcohol to form intermediate **8a-8b** as bright yellow solid in 85 % yield. The reduction of **8a-8b** followed by cyclocondensation with the bisulfite salts of different aldehydes afforded 7-aminobenzimidazoles **9a-9e**. The derivatization of the 7-aminobenzimidazole using various acyl chloride, chloroformates or the corresponding *N*-hydroxysuccinimide esters generated the desired hit compounds **10a-10i** in analytically pure form in 41-53% yields.

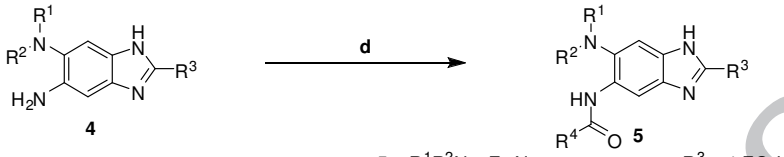


1

2a: R^1R^2N = piperidin-1-yl
2b: R^1R^2N = Et_2N
2c: R^1R^2N = $Me_2NCH_2CH_2(Et)N$
2d: R^1R^2N = $Me_2NCH_2CH_2(Me)N$
2e: R^1R^2N = pyrrolidin-1-yl

2

3a: R^1R^2N = Et_2N,	R^3 = 4-FC_6H_4
3b: R^1R^2N = Et_2N,	R^3 = 2-$MeOC_6H_4$
3c: R^1R^2N = Et_2N,	R^3 = 4-MeC_6H_4
3d: R^1R^2N = Et_2N,	R^3 = Ph
3e: R^1R^2N = piperidin-1-yl,	R^3 = 2-thienyl
3f: R^1R^2N = piperidin-1-yl,	R^3 = 2-furyl
3g: R^1R^2N = pyrrolidin-1-yl,	R^3 = c-C_6H_{11}
3h: R^1R^2N = $Me_2NCH_2CH_2(Et)N$,	R^3 = c-C_6H_{11}
3i: R^1R^2N = $Me_2NCH_2CH_2(Me)N$,	R^3 = c-C_6H_{11}



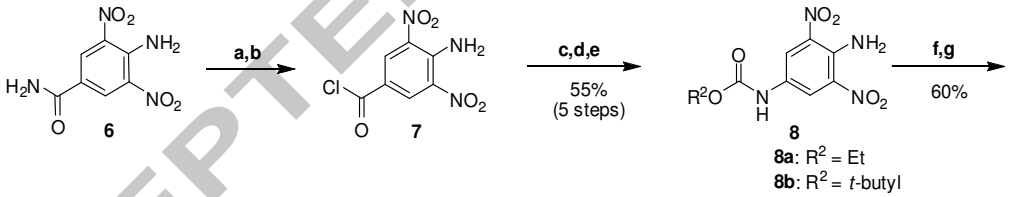
4

5

4a: R^1R^2N = Et_2N,	R^3 = 4-FC_6H_4
4b: R^1R^2N = Et_2N,	R^3 = 2-$MeOC_6H_4$
4c: R^1R^2N = Et_2N,	R^3 = 4-MeC_6H_4
4d: R^1R^2N = Et_2N,	R^3 = Ph
4e: R^1R^2N = piperidin-1-yl,	R^3 = 2-thienyl
4f: R^1R^2N = piperidin-1-yl,	R^3 = 2-furyl
4g: R^1R^2N = pyrrolidin-1-yl,	R^3 = c-C_6H_{11}
4h: R^1R^2N = $Me_2NCH_2CH_2(Et)N$,	R^3 = c-C_6H_{11}
4i: R^1R^2N = $Me_2NCH_2CH_2(Me)N$,	R^3 = c-C_6H_{11}

5a: R^1R^2N = Et_2N,	R^3 = 4-FC_6H_4,	R^4 = c-Bu
5b: R^1R^2N = Et_2N,	R^3 = 2-$MeOC_6H_4$,	R^4 = c-Bu
5c: R^1R^2N = Et_2N,	R^3 = 4-MeC_6H_4,	R^4 = c-Bu
5d: R^1R^2N = Et_2N,	R^3 = 2-$MeOC_6H_4$,	R^4 = 2-$MeOC_6H_4$
5e: R^1R^2N = piperidin-1-yl,	R^3 = 2-thienyl,	R^4 = $(C_6H_4)CH_2O$
5f: R^1R^2N = piperidin-1-yl,	R^3 = 2-thienyl,	R^4 = 2-$MeOC_6H_4$
5g: R^1R^2N = piperidin-1-yl,	R^3 = 2-furyl,	R^4 = 2-$MeOC_6H_4$
5h: R^1R^2N = piperidin-1-yl,	R^3 = 2-furyl,	R^4 = 1-naphthyl
5i: R^1R^2N = $Me_2NCH_2CH_2(Et)N$,	R^3 = c-C_6H_{11},	R^4CO = $PhSO_2$
5j: R^1R^2N = $Me_2NCH_2CH_2(Et)N$,	R^3 = c-C_6H_{11},	R^4CO = $PhSO_2$
5k: R^1R^2N = pyrrolidin-1-yl,	R^3 = c-C_6H_{11},	R^4 = c-Pr
5l: R^1R^2N = Et_2N,	R^3 = Ph,	R^4 = 4-MeC_6H_4

Scheme 1. Synthesis of 2,5,6-trisubstituted benzimidazoles. Reagents and conditions: (a) R^1R^2NH, DIPEA, THF, 2 h, room temperature (RT); (b) R^3COCl, pyridine, reflux, overnight; (c) $SnCl_2{\cdot}2H_2O$, 4 N HCl, EtOH, reflux,4 h; (d) $R^4COCl/R^4COOCl/R^4COOH/R^4SO_2Cl$, CH_2Cl_2, overnight, RT.

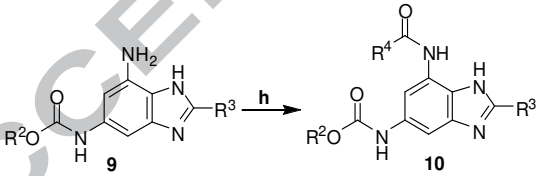


6

7

8

8a: R^2 = Et
8b: R^2 = t-butyl



9

10

9a: R^2 = Et,	R^3 = 4-BrC_6H_4	
9b: R^2 = Et,	R^3 = 3,5-$Me_2C_6H_4$	
9c: R^2=Et,	R^3 = 2-furyl	
9d: R^2=Et,	R^3 = 4-$MeOCOC_6H_4$	
9e: R^2=t-butyl, R^3 = Ph		

10a: R^2 = Et,	R^3 = 4-BrC_6H_4,	R^4 = 2-$MeOC_6H_4$
10b: R^2 = Et,	R^3 = 4-BrC_6H_4,	R^4 = c-Pr
10c: R^2 = Et,	R^3 = 4-BrC_6H_4,	R^4 = C_6H_5
10d: R^2 = t-butyl,	R^3 = Ph,	R^4 = $C_6H_4CH_2O$
10e: R^2 = Et,	R^3 = 4-$MeOCOC_6H_4$,	R^4 = c-C_6H_{11}
10f: R^2 = Et,	R^3 = 4-$MeOCOC_6H_4$,	R^4 = 4-$MeOC_6H_4$
10g: R^2 = Et,	R^3 = 4-$MeOCOC_6H_4$,	R^4 = Ph
10h: R^2 = Et,	R^3 = 2-furyl,	R^4 = 4-FC_6H_4
10i: R^2 = Et,	R^3 = 2-furyl,	R^4 = Ph

Scheme 2. Synthesis of 2,5,7-trisubstituted benzimidazoles. Reagents and conditions: (a) 6 M HCl, reflux, 36 h; (b) $SOCl_2$, reflux, 12 h; (c) NaN_3, acetone, 0 °C, 30 min; (d) toluene, reflux, 4 h; (e) R^2OH, RT, 24 h; (f) 10% wt Pd/C, HCO_2NH_4, EtOH, 1 h, RT; (g) $R^3CH(OH)SO_3Na$, EtOH:H_2O (1:1), 14 h; (h) $R^4COCl/R^4OCOCl/R^4COOH$, DMF, 12 h, RT.

3. Results and discussions:

3.1 *In-vitro* preliminary screening of libraries of 2,5,6- and 2,5,7-trisubstituted benzimidazoles against *F. tularensis*

The libraries of 2,5,6- and 2,5,7-trisubstituted benzimidazoles (~1100 compounds) were screened for their activity against *F. tularensis* LVS strain using the "Microplate Alamar Blue assay (MABA)" in a 96-well format (single point assay in triplicates). Remarkably, 23 compounds showed significant activity exhibiting greater than 90 % growth inhibition at 1 µg/mL (**Fig. 1**). Of these hit compounds, 7 compounds exhibited 40-50 % growth inhibition at 0.2 µg/mL (**Fig. 1**). It is interesting to note that none of the hit compounds against *Mtb*, showed appreciable activity against *F. tularensis*. The absence of apparent cross activity between *Mtb* and *F. tularensis* is discussed later.

In order to confirm their activity, these compounds were synthesized in analytically pure form following the synthesis protocol outlined in **scheme 1** and **scheme 2**. These compounds were then re-tested for their activity against *F. tularensis* LVS to determine MIC values. Among the hits, 21 compounds showed MIC_{90} values in the range of 0.35-48.6 µg/mL with no appreciable cytotoxicity (IC_{50} >200 µg/mL) against Vero cells, except **10h** (IC_{50} <50 µg/mL) (**Table 1**). It should be noted that two compounds **10f** and **10g** did not show any activity, even though they were found to be active in preliminary screening. As **Table 1** shows, the most active compounds (MIC_{90} <1.0 µg/mL) are **5e**. **10b**, **10c** and **10d** (most potent: MIC_{90} 0.34 µg/mL).

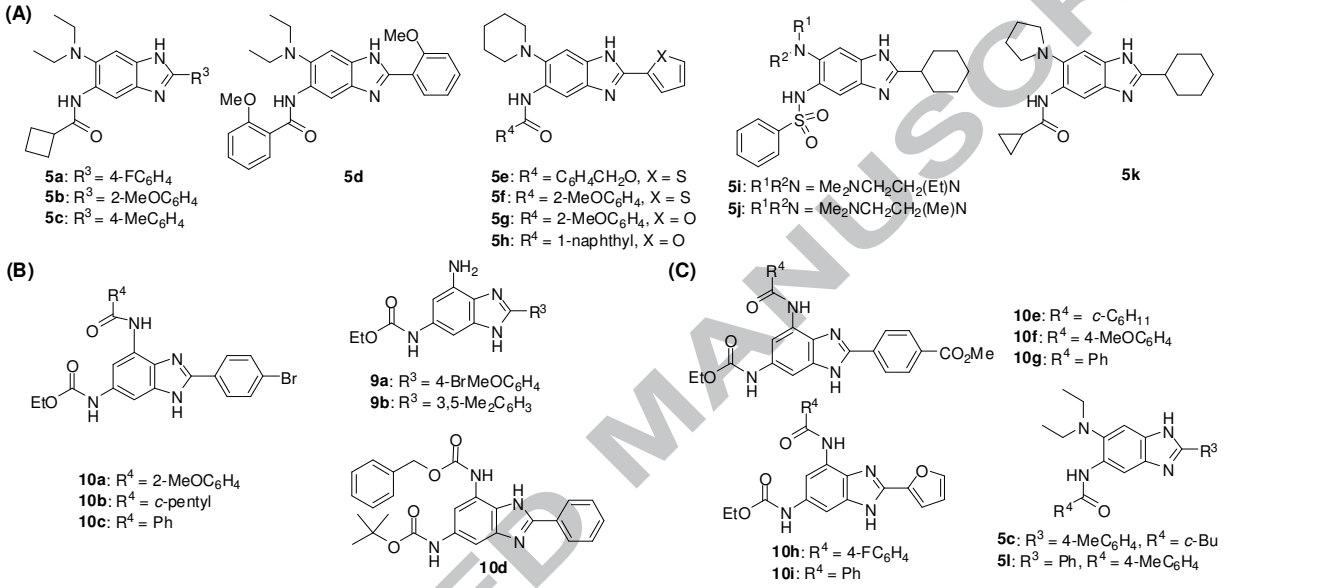


Figure 1. Hit trisubstituted benzimidazoles against *F. tularensis*.

(A) Structures of 2,5,6-trisubstituted benzimidazoles against *F. tularensis* LVS at 1 µg/mL with >90 % growth inhibition. (B) Structures of 2,5,7-trisubstituted benzimidazoles against *F. tularensis* LVS at 1 µg/mL with >90 % growth inhibition. (C) Structures of hit benzimidazoles against *F. tularensis* LVS with 40-50 % growth inhibition at 0.2 µg/mL

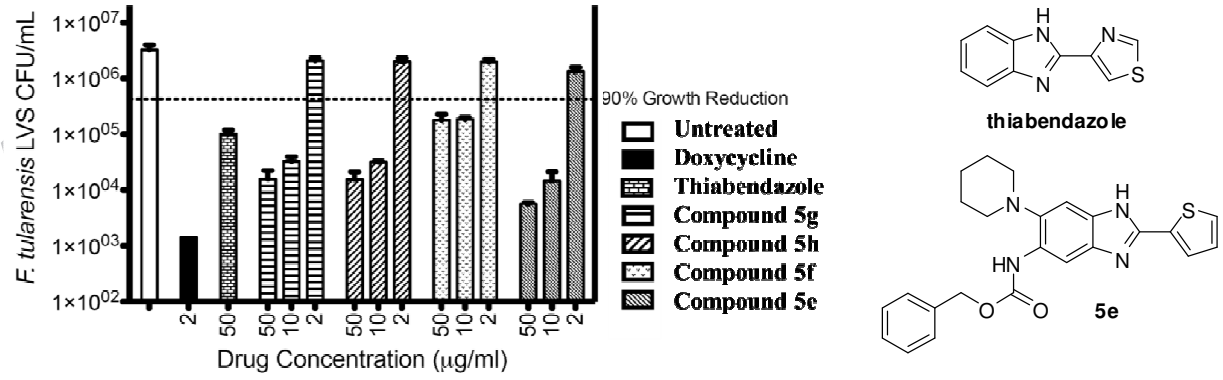
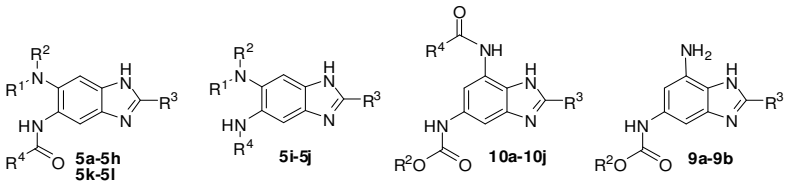


Figure 2. Effect of benzimidazoles on *F. tularensis* LVS infection in RAW 264.7 macrophages *ex vivo*.

RAW macrophages infected with *F. tularensis* LVS were treated with various benzimidazoles at different concentrations. After the treatment the cells were lysed and lysate transferred to CHAB plates for CFU enumeration.

Table 1
MIC_{90} of hit compounds against *F. tularensis*



Compound	R^1R^2N	R^3	R^4	MIC_{90} (µg/mL) *F. tularensis* LVS	Cytotoxicity (µg/mL) Vero cells
5a				4.84	> 200
5b				7.59	> 200
5c				1.16	> 200
5d				4.85	> 200
5e				0.98	>200
5f				1.6	>200
5g				2.1	>200
5h				2.4	>200
5i				35.8	>200
5j				48.6	> 200
5k				2.1	>200
5l				12.9	>200

Compound	R^2	R^3	R^4	MIC_{90} (µg/mL) *F. tularensis* LVS	Cytotoxicity (µg/mL) Vero cells
10a				1.4	>200
10b				0.75	>200
10c				0.48	>200
9a				2.99	>200
9b				4.33	>200
10d				0.35	>200
10e				1.5	>200
10f				>256	>200
10g				>256	≥200
10h				2.8	≤ 50
10i				3.8	>200

3.2 Activity of lead benzimidazoles against *F. tularensis* LVS in the *ex vivo* model of efficacy.

To assess the efficacy potential of active compounds identified with potency against *F. tularensis* LVS, selected lead compounds were further evaluated for activity in the *ex vivo* efficacy model. The lead compounds **5e, 5f, 5g** and **5 h** were subjected to *ex vivo* efficacy assay against *F. tularensis* LVS infection of RAW macrophages. With the exception of **5f**, the lead compounds were highly effective in reducing bacterial numbers in a dose-dependent manner over the concentration range of 10 and 50 µg/mL (**Fig. 2**). There was a 2-3 Log reduction in the colony forming units (CFU) per mL which is considered to be significant. Among the four lead compounds examined, **5e** was found to be the most potent, which is consistent with its smallest MIC_{90} value as compared to other three. Doxycycline and thiabendazole, which are known to be effective against *F. tularensis*, were used as reference compounds for this study. The assay indicates that these compounds are very effective in killing intercellular bacteria in macrophages. Given the high activity of the trisubstituted benzimidazole compound class against the bacteria cells, ability to permeate mammalian cell membranes as well as maintain that activity *ex vivo*, and low to no associated toxicity to mammalian cells make these lead compounds attractive for further evaluation in mouse models for their efficacy *in vivo*.

As mentioned above, none of the hit compounds for *Mtb* exhibited appreciable activity against *F. tularensis*. This apparent lack of cross activity implies a couple of possibilities: (i) the target of the hit compounds for *F. tularensis* is not FtsZ of this bacteria although two groups of trisubstituted benzimidazoles are structurally very similar to each other; (ii) the hit compounds for *F. tularensis* target FtsZ, but not at the highly conserved GTP binding catalytic domain, i.e., bind an allosteric site of FtsZ. In fact, the exact binding site of the lead benzimidazoles active against *Mtb* in FtsZ has not been identified yet, although it has been verified that *Mtb* FtsZ is the target of these lead compounds. Accordingly, we expressed and purified *F. tularensis* FtsZ for target verification. However, unfortunately, the *F. tularensis* FtsZ, thus obtained, has not shown appreciable polymerization in the presence of GTP, to date, under the conditions similar to those for *Mtb* FtsZ. Thus, at present, we do not have evidence to claim that these hit/lead benzimidazoles target *F. tularensis* FtsZ.

Nevertheless, this work has clearly demonstrated that at least several novel trisubstituted benzimidazoles exhibit highly promising activity against *F. tularensis* cells as well as macrophages infected by *F. tularensis* in *ex-vivo* evaluation, which warrants further active investigation.

4. Conclusion

In summary, in the present investigation, libraries of trisubstituted benzimidazoles were screened using high throughput screening method against *F. tularensis* LVS strain to identify novel anti-bacterial compounds. In a preliminary screening assay, 23 of 2,5,6- and 2,5,7-trisubstituted benzimidazoles were identified exhibiting greater than 90 % growth inhibitory activity at 1 µg/mL. Upon resynthesis of hit compounds, 21 compounds showed MIC_{90} values in the range of 0.35-48.6 µg/mL. Subsequently, in *ex-vivo* efficacy assays, selected lead compounds exhibited 2-3 Log reduction in colony forming units (CFU) per mL at concentrations of 10 and 50 µg/mL. Further investigation on the mechanism of action as well as the proof of concept study against animal models *in vivo* are actively underway in these laboratories.

Experimental

The chemicals were purchased from Sigma Aldrich Co., Synquest Inc., Alfa Aesar and purified before use by standard methods. Tetrahydrofuran was freshly distilled from sodium metal and benzophenone. Dichloromethane was also distilled immediately prior to use under nitrogen from calcium hydride. ^{1}H and ^{13}C NMR spectra were measured on a Varian and Brucker 300, 400 or 500 MHz NMR spectrometer. Melting points were measured on a Thomas Hoover Capillary melting point apparatus and are uncorrected. TLC was performed on Merck DCalufolien with Kieselgel 60F-254 and column chromatography was carried out on silica gel 60 (Merck; 230-400 mesh ASTM). High-resolution mass spectra were obtained from Mass Spectrometry Laboratory, University of Illinois at Urbana-Champaign, Urbana, IL. Purity was determined by Agilent 1100 series HPLC assembly.

Compounds, **2a-2e, 3a-3d, 3g-3i, 4a-4d, 4g-4i**, **8** and **9a-9d** were prepared by using the procedures reported earlier by these laboratories.[28]

2,4-Dinitro-1-(2-thiophenyl)carboxamido-5-piperidinyl-benzene (3e)

A solution of **1** (800 mg, 3 mmol) and 2-thiophenecarbonyl chloride (529 mg, 3.61 mmol) in pyridine (8 mL) was refluxed overnight. The reaction mixture was diluted with dichloromethane, washed with water (30 mL x 3) and dried over anhydrous magnesium sulfate. The solvent was removed to give yellow oil which was dried under vacuum to afford yellow solid. The solid was washed with methanol to give **3e** as a yellow solid (959 mg, 85 % yield): mp 178-180 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.72 (m, 6 H), 3.30 (t, 4 H, *J* = 4.8 Hz), 7.19 (dd, 1 H, *J* = 0.8 Hz, 4 Hz), 7.67 (d, 1 H, *J* = 4.8 Hz), 7.75 (d, 1 H, *J* = 4 Hz), 8.66 (s, 1 H), 8.86 (s, 1 H), 11.7 (s, NH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 23.60, 25.36, 52.03, 108.1, 125.5, 127.5, 127.6, 128.3, 129.5, 132.7, 133.0, 138.6, 139.0, 150.6, 160.6; MS (ESI) *m/z* 377.3 $(M+1)^+$**.**

A similar procedure was used for the synthesis of **3f**.

2,4-Dinitro-1-(2-furyl)carboxamido-5-piperidinylbenzene (3f)

Yellow solid; 81 % yield; mp 196-198^{o}C;^{1}H NMR (400 MHz, $CDCl_3$) δ 1.72 (m, 6 H), 3.30 (t, 4 H, *J* = 4.8 Hz), 6.61 (dd, 2 H, *J* = 1.2 Hz, 2.4 Hz), 7.30 (d, 1 H, *J* = 3.6 Hz), 7.63 (m, 1 H), 8.68 (s, 1 H), 8.85 (s, 1 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 23.60, 25.34, 52.01, 108.4, 112.9, 116.9, 125.8, 127.4, 132.7, 138.7, 145.7, 146.9, 150.5, 156.7; MS (ESI) *m/z* 361.3 $(M+1)^+$**.**

5-Amino-6-piperidinyl-2-thiophenyl-1*H*-benzo[d]imidazole (4e)

To a solution of **3e** (800 mg, 2.12 mmol) in dioxane (50 mL) and ethanol (50 mL) was added ammonium formate (3.5 g) and 10 wt. % Pd-C (340 mg) with stirring. The reaction mixture turned from yellow to red and then to colorless in 60 min at room temperature. The catalyst and excess ammonium formate were filtered off to give the product solution. Concentrated hydrochloric acid was added to this solution and adjusted to 4 M HCl concentration. Then, the mixture was refluxed for 6 h. The reaction mixture was basified by addition of ammonium hydroxide, extracted with ethyl acetate (40 mL x 3) and dried over anhydrous magnesium sulfate. The crude product was purified by flash chromatography on silica gel using ethyl acetate/hexanes (gradient: AcOEt/hexanes = 20/80–40/60) as eluent to give pure **4e** as a pale yellow solid; 50 % yield; mp 145-148^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.64 (m, 6 H), 2.73 (broad s, 4 H), 6.80 (s, 1 H), 6.94 (t, 1 H, *J* = 4.8 Hz), 7.19 (s, 1 H), 7.24 (d, 1 H,*J* = 5.2 Hz), 7.64 (d, 1 H,*J* = 3.6 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 24.17, 26.87, 53.44, 98.05, 107.4, 125.9, 127.0, 127.9, 133.4, 134.5, 139.0, 139.2, 145.6; HRMS (ESI) *m/z* calcd for $C_{16}H_{18}N_4SH^+$: 299.1330, Found: 299.1330 (Δ = 0.0 ppm).

A similar procedure was used for the synthesis of **4f**.

5-Amino-2-furyl-6-piperidinyl-1*H*-benzo[d]imidazole (4f)

Dark brown solid; 55 % yield; mp 124-126 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.68 (m, 6 H), 2.78 (broad s, 4 H), 6.45 (m, 1 H), 6.85 (s, 1 H), 7.09 (m, 1 H), 7.24 (s, 1 H), 7.40 (s, 1 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 24.19, 26.88, 53.48, 98.04, 107.5, 109.4, 112.1, 133.3, 133.9, 139.2, 139.3, 142.1, 143.0, 145.6; HRMS (ESI) *m/z* calcd for $C_{16}H_{18}N_4OH^+$: 283.1559, Found: 283.1561 (Δ = 0.7 ppm).

4-Amino-3,5-dinitro-1-(*tert*-butoxycarbonylamino)benzene (8b)

A suspension of 4-amino-3,5-dinitrobenzamide (**6**) (543 mg, 2.4 mmol) in 4 N HCl (20 mL) was refluxed overnight. The reaction mixture was cooled and the precipitate was filtered off to give 4-amino-3,5-dinitrobenzoic acid as a yellow solid, which was dissolved in thionyl chloride (4 mL) and refluxed overnight. The reaction mixture was cooled down to room temperature and excess thionyl chloride was distilled off to give compound **7**. The crude product **7** was immediately dissolved in acetone (2.4 mL) in an ice-bath. To this solution was added dropwise NaN_3 (448 mg, 5.94 mmol) in ice-water (0.88 mL). The mixture was stirred for 20 min at 0 ^{o}C until a solid precipitated out. After dilution with ice-water (12 mL), the reaction mixture was extracted with dichloromethane (6 mL x 2), dried over anhydrous magnesium sulfate at 0 ^{o}C for 1 h, and filtered. The filtrate was concentrated on a rotary evaporator (below room temperature), and the residue dissolved in toluene (15 mL). After refluxing for 2 h, the reaction mixture was cooled down to room temperature, and *tert*-butanol (10 mL) was added. After stirring overnight at room temperature, the reaction mixture was concentrated *in vacuo* and purified by flash chromatography on silica gel (hexane/ethylacetate = 1/1) to afford 4-amino-3,5-dinitro-1-(*tert*-butoxycarbonylamino)benzene (**8b**) (84% yield) as a bright red solid: mp 178-180 ^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.48 (s, 9 H), 8.19(s, 2 H), 8.65 (s, 2 H), 9.69 (s, 1 H); ^{13}C NMR (100 MHz, DMSO-d_6) δ28.02, 79.97, 123.3, 126.6, 134.3, 136.9, 152.8; MS (ESI) *m/z* 299.2 $(M+1)^+$**.**

7-Amino-2-phenyl-5-(*tert*-butoxycarbonylamino)-1*H*-benzo-[d]imidazole (9e)

To a suspension of **8b** (324 mg, 1.08 mmol) in ethanol (24 mL), was added ammonium formate (1.8 g) and 10 wt. % Pd-C (170 mg) under nitrogen. The mixture was stirred at room temperature overnight. The catalyst and excess ammonium formate were filtered off on a Celite pad. The filtrate was treated with the sodium bisulfite adduct of benzaldehyde (228 mg, 1.08 mmol) at 0 °C. After the solution was stirred for 12-16 h at room temperature under nitrogen, a trace of insoluble material was removed by filtration and the filtrate was concentrated on a rotary evaporator until approximately 60-70% of the solvent was removed. To the residue was added an equal volume of ethyl acetate and the mixture was transferred to a separatory funnel. The organic layer was separated, and the water layer was extracted with ethyl acetate (40 mL). The combined organic layers were washed with brine, dried over anhydrous sodium sulfate. The crude product was purified by flash chromatography on silica gel (hexane/ethyl acetate = 1/1) to afford **9e** (169 mg, 48% yield) as brown solid: mp >200 °C; ^{1}H NMR (400MHz, CD_3OD) δ 1.51 (s, 9 H), 6.49 (s, 1 H), 7.15 (s, 1 H), 7.48 (m, 3 H), 8.00 (d, 2 H, *J* = 8.4 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ28.94, 80.71, 93.64, 101.0, 127.4, 130.1, 130.8, 131.4, 137.4, 139.1, 151.2, 155.7; HRMS (ESI) *m/z* calcd for $C_{18}H_{20}N_4O_2H^+$: 325.1665, Found: 325.1669 (Δ = 1.2 ppm).

5-(Cyclobutanamido)-6-*N,N*-diethylamino-2-tolyl-1*H*-benzo-[d]imidazole (5c)

To a solution of **4c**[28] (115 mg, 0.39 mmol) in dichloromethane (5 mL) was added a solution of cyclobutanecarbonyl chloride (0.45 ml, 0.39 mmol) in dichloromethane (4 mL) dropwise at room temperature. After the addition, the mixture was stirred at room temperature overnight. After the completion of the reaction, the reaction mixture was washed with a saturated solution of sodium bicarbonate. The organic layer was separated and dried over anhydrous magnesium sulfate, filtered and concentrated under reduced pressure. The crude product was purified by flash chromatography on silica gel (gradient: AcOEt/hexanes = 20/80–40/60) to give **5c** as a white solid (85 mg, 58 %): mp >200 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.92 (t, 6 H, *J* = 7.2 Hz), 1.83 (m, 1 H), 1.95 (m, 1 H), 2.22-2.37 (m, 7 H), 2.92 (q, 4 H, *J* = 7.2 Hz), 3.29 (m, 1 H), 7.17 (d, 2 H, *J* = 8 Hz), 7.52 (s, 1 H), 7.99 (d, 2 H, *J* = 8 Hz), 8.79 (s, 1 H), 9.40 (s, 1 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 12.90, 17.93, 21.27, 25.69, 41.44, 50.30, 101.6, 112.7, 126.6, 127.4, 129.3, 132.0, 133.1, 135.5, 139.6, 152.6, 173.1; HRMS (ESI) *m/z* calcd for $C_{23}H_{28}N_4OH^+$: 377.2341, Found: 377.2341 (Δ = 0.5 ppm).

A similar procedure was used for the synthesis of **5a, 5b, 5d, 5f, 5g, 5h, 5k** and **5l**. For the synthesis of **5i** and **5j**, benzenesulfonyl chloride was used.

5-Cyclobutanamido-6-*N,N*-diethylamino-2-(4-fluorophenyl)-1*H*-benzo[d]imidazole (5a)

White solid; 48 % yield; mp 170-172 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.93 (t, 6 H, *J* = 7.2 Hz), 1.81 (m, 1 H), 2.02 (m, 1 H), 1.35-2.19 (m, 4 H), 2.94 (q, 4 H, *J* = 7 Hz), 3.30 (m, 1 H), 7.06 (t, 2 H, *J* = 8.8 Hz), 7.55 (s, 1 H), 8.08 (m, 2 H), 8.78 (s, 1 H), 9.41 (s, 1 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 12.91, 17.96, 25.71, 41.44, 50.32, 101.7, 112.9, 115.6, 115.9, 126.4, 128.7, 132.2, 133.2, 135.9, 139.3, 162.6, 164.8, 173.3; HRMS (ESI) *m/z* calcd for $C_{22}H_{25}N_4FOH^+$: 381.2091, Found: 381.2090 (Δ = -0.3 ppm).

5-Cyclobutanamido-6-*N,N*-diethylamino-2-(2-methoxyphenyl)-1*H*-benzo[d]imidazole (5b)

White solid; 42 % yield; mp 110-112 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.95 (t, 6 H, *J* = 7.2 Hz), 1.93 (m, 1 H), 2.01 (m, 1 H), 2.28 (m, 2 H), 2.35 (m, 2 H), 2.96 (m, 4 H, *J* = 7 Hz), 3.23 (m, 1 H), 4.07 (s, 3 H), 7.05 (d, 1 H, *J* = 8 Hz), 7.10 (t, 1 H, *J* = 8 Hz),), 7.39 (m, 1 H), 7.59 (s, 1 H), 8.51 (d, 1 H, *J* = 7.6 Hz), 8.69 (s, 1 H), 9.26 (s, 1 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 12.90, 18.01, 25.53, 41.40, 50.35, 55.94, 100.4, 111.4, 113.1, 117.9, 121.6, 129.6, 130.8, 132.6, 135.5, 149.8, 156.6, 172.8; HRMS (ESI) *m/z* calcd for $C_{23}H_{28}N_4O_2H^+$: 393.2289, Found: 393.2291 (Δ = -0.5 ppm).

6-*N,N*-Diethylamino-5-(2-methoxybenzamido)-2-(2-methoxy-phenyl)-1*H*-benzo[d]imidazole (5d)

White solid; 54 % yield; mp >200 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.96 (t, 6 H, *J* = 7.2 Hz), 3.10 (q, 4 H, *J* = 7 Hz), 4.04 (s, 3 H), 4.06 (s, 3 H), 7.04 (t, 2 H, *J* = 7.6 Hz), 7.09 (q, 2 H, *J* = 7.2 Hz), 7.39 (t, 1 H, *J* = 3.2 Hz), 7.45 (t, 1 H, *J* = 3.2 Hz), 7.65 (s, 1 H), 8.35 (d, 1 H, *J* = 8 Hz), 8.54 (d, 1 H, *J* = 8 Hz), 9.00 (s, 1 H), 11.50 (s, 1 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 12.32, 50.54, 55.62, 55.89, 101.8, 111.2, 11.5, 113.3, 117.9, 121.2, 121.6, 122.6, 129.6, 130.8, 132.4, 132.7, 133.9, 135.9, 149.8, 155.6, 157.2, 162.8; HRMS (ESI) *m/z* calcd for $C_{26}H_{28}N_4O_3H^+$: 445.2240, Found: 445.2240 (Δ = -0.0 ppm).

5-Benzyloxycarbonylamino-6-piperidin-1-yl-2-thiophen-1*H*-benzo[d]imidazole (5e)

To a solution of **4e** (200 mg, 0.67 mmol) in dichloromethane (10 mL) was added a solution of *N*-benzyloxycarbonyloxy-succinimide (167 mg, 0.67 mmol) in dichloromethane (5 mL) dropwise at room temperature. After the addition, the mixture was stirred at room temperature overnight. After the completion of the reaction, the reaction mixture was concentrated under reduced pressure. The crude product was purified via flash chromatography on silica gel (gradient: AcOEt/hexanes = 20/80 – 40/60) to give **5e** as white solid (206 mg, 48 % yield): mp 105-107 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.69 (m, 6 H), 2.72 (broad s, 4 H), 5.22 (s, 2 H), 6.93 (t, 1 H, *J* = 3.9 Hz), 7.27 (d, 1 H, *J* = 4.8 Hz), 7.37 (m, 6 H), 8.21 (s, 1 H), 8.27 (s, 1 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 24.12, 25.80, 27.03, 54.60, 66.97, 101.2, 109.9, 126.5, 127.8, 128.1, 128.2, 128.4, 128.7, 130.1, 133.0, 136.5, 139.8, 147.0, 153.9, 171.3, 173.6; HRMS (ESI) *m/z* calcd for $C_{24}H_{24}N_4O_2SH^+$: 433.1698, Found: 433.1698 (Δ = -0.0 ppm).

5-(2-Methoxybenzamido)-6-piperidinyl-2-thiophen-1*H*-benzo-[d]imidazole (5f).

White solid; 52 % yield; mp 165-167 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.65 (m, 6 H), 2.74 (broad s, 4 H), 4.01 (s, 3 H), 6.71 (t, 1 H, *J* = 3.6 Hz), 7.01 (m, 2 H), 7.17 (d, 1 H, *J* = 4.8 Hz), 7.37 (d, 1 H, *J* = 3.2 Hz), 7.43 (m, 2 H), 8.89 (s, 1 H), 10.70 (s, 1 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 23.87, 26.65, 54.39, 56.16, 104.0, 109.6, 111.5, 121.3, 122.6, 126.1, 127.1, 127.5, 129.5, 132.1, 132.9, 133.6, 139.6, 140.8, 147.6, 157.3, 164.0; HRMS (ESI) *m/z* calcd for $C_{24}H_{24}N_4O_2SH^+$: 433.1698, Found: 433.1699 (Δ = 0.2 ppm).

2-Furyl-5-(2-Methoxybenzamido)-6-piperidinyl-1*H*-benzo[d]-imidazole (5g)

White solid; 56 % yield; mp 155-157 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.66 (m, 6 H), 2.74 (broad s, 4 H), 4.06 (s, 3 H), 6.32 (d, 1 H, *J* = 3.6 Hz), 6.97 (d, 2 H, *J* = 3.6 Hz), 7.06 (m, 2 H), 7.42 (s, 1 H), 7.51 (t, 1 H, *J* = 8.4 Hz), 9.01 (s, 1 H), 10.81 (s, 1 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 23.86, 26.62, 54.33, 56.20, 104.1, 109.6, 111.5, 111.8, 121.3, 122.6, 126.1, 129.8, 131.4, 132.5, 132.9, 139.2, 140.9, 142.9, 144.1, 145.7, 157.3, 163.9; HRMS (ESI) *m/z* calcd for $C_{24}H_{24}N_4O_3H^+$: 417.1927, Found: 417.1924 (Δ = -0.7 ppm).

2-Furyl-5-(1-naphthylamido)-6-piperidinyl-1*H*-benzo[d]-imidazole (5h)

White solid; 62 % yield; mp 202-204 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.58 (broad s, 6 H), 2.86 (broad s, 4 H), 6.10 (m, 1 H), 6.55 (m, 2 H7.49 (m, 2 H), 7.59 (m, 2 H), 7.86 (dd, 1 H, *J* = 0.8 Hz, 6.96 Hz), 7.92 (m, 1 H), 8.04 (d, 1 H, *J* = 8.28 Hz), 8.51 (d, 1 H, *J* = 9.4 Hz), 9.32 (s, 1 H), 9.82 (s, 1 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 23.78, 26.90, 54.63, 102.3, 109.4, 111.4, 124.7, 124.9, 125.2, 126.7, 127.3, 128.2, 129.9, 130.2, 130.7, 133.8, 135.3, 139.7, 143.0, 144.4, 145.2, 167.9; HRMS (ESI) *m/z* calcd for $C_{27}H_{24}N_4O_2H^+$: 437.1971, Found: 437.1964 (Δ = 1.6 ppm).

5-Benzenesulfonamido-2-cyclohexyl-6-(*N*',*N*'-dimethyl-*N*-ethylethylenediamino)-1*H*-benzo[d]imidazole (5i)

White solid; 19 % yield; mp 187-188 °C; ^{1}H NMR (400 MHz, METHANOL-d_4) δ 0.60 (t, 3 H, *J* = 7.2 Hz), 1.21 - 1.51 (m, 7 H), 1.56 - 1.70 (m, 2 H), 1.73 - 1.82 (m, 2 H), 1.82 - 1.93 (m, 2 H), 2.00 - 2.10 (m, 2 H), 2.32 - 2.43 (m, 8 H), 2.74 (q, 2 H, *J* = 7.2 Hz), 2.80 - 2.94 (m, 3 H), 7.29 (s, 1 H), 7.37 - 7.45 (m, 2 H), 7.46 - 7.52 (m, 1 H), 7.71 (s, 1 H), 7.73 - 7.78 (m, 2 H);^{13}C NMR (100 MHz, METHANOL-d_4) δ 11.9, 25.6, 25.8, 31.4, 38.4, 43.6, 49.3, 53.7, 56.3, 100.0, 126.7, 128.5, 131.5, 132.1, 138.1, 140.9, 160.2; HRMS (ESI) *m/z* calcd for $C_{25}H_{35}N_5O_2SH^+$: 470.2590, Found: 470.2590 (Δ = 0.0 ppm).

5-Benzenesulfonamido-2-cyclohexyl-6-(*N*',*N*',*N*-trimethyl-ethylenediamino)-1*H*-benzo[d]imidazole (5j)

White solid; 47 % yield; mp >200 °C; ^{1}H NMR (400 MHz, CD_3OD) δ 1.19 - 1.53 (m, 7 H), 1.57 - 1.69 (m, 2 H), 1.77 (m, 1 H), 1.86 (m, 2 H), 2.04 (m, 2 H 2.54 (s, 3 H), 2.90 (m, 7 H), 7.40 (d, 2 H, *J* = 12.00 Hz), 7.52 (t, 2 H, *J* = 16.00 Hz), 7.61 (t, 1 H, *J* = 16.00 Hz), 7.86 (d, 2 H, *J* = 4.00 Hz);^{13}C NMR (100 MHz, METHANOL-d_4) δ 25.4, 25.6, 31.2, 38.1, 42.6, 43.3, 50.6, 55.0, 107.5, 107.8, 126.9, 128.9, 132.9, 139.9, 141.1, 160.4; HRMS (ESI) *m/z* calcd for $C_{24}H_{33}N_5O_2SH^+$: 456.2430, Found: 456.2433 (Δ = -0.7 ppm).

2-Cyclohexyl-5-cyclopropylamido-6-pyrrolidinyl-1*H*-benzo-[d]imidazole (5k)

Light brown solid; 44 % yield; mp 165-166 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.77 - 0.93 (m, 4 H), 1.00 - 1.40 (m, 10 H), 1.46 - 1.73 (m, 5 H), 1.76 - 1.79 (m, 1 H), 1.87 - 2.07 (m, 6 H), 2.68 - 2.84 (m, 1 H), 2.99 (br. s., 4 H), 7.46 (s, 1 H), 8.44 (s, 1 H), 8.93 (s, 1 H);^{13}C NMR (100 MHz, $CDCl_3$) δ 8.0, 14.3, 16.4, 22.8, 24.6, 26.0, 26.3, 29.9, 31.9, 38.6, 53.8, 103.0, 109.5, 129.5, 136.0, 159.6, 172.0; HRMS (ESI) *m/z* calcd for $C_{21}H_{28}N_4OH^+$: 353.2336, Found: 353,2341 (Δ = -1.4 ppm).

6-*N,N*-Diethylamino-2-phenyl-5-*4-methylbenzamido)-1*H*-benzo[d]imidazole (5l)

White solid; 44 % yield; mp >200 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.96 (t, 6 H, *J* = 7.2 Hz), 2.44 (s, 3 H), 3.02 (q, 4 H, *J* = 7 Hz), 7.09 (t, 2 H, *J* = 7.6 Hz), 7.24 (m, 3 H), 7.64 (s, 1 H), 7.84 (d, 2 H, *J* = 8.4 Hz), 7.94 (d, 2 H, *J* = 7.6 Hz), 9.14 (s, 1 H), 10.36 (s, 1 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 12.98, 21.48, 50.53, 101.6, 113.3, 126.5, 127.0, 128.5, 129.3, 129.6, 129.9, 132.3, 132.5, 133.1, 135.9, 142.1, 152.5, 165.6; HRMS (ESI) *m/z* calcd for $C_{25}H_{26}N_4OH^+$: 399.2185, Found: 399.2184 (Δ = - 0.3 ppm).

2-(4-Bromophenyl)-5-ethoxycarbonylamino-7-(2-methoxy-benzamido)-1*H*-benzo[d]midazole (10a)

Off-white solid; 52 % yield; mp >200 °C; ^{1}H NMR (400MHz, acetone-d_6) δ 1.28 (t, 3 H, *J* = 7.08 Hz), 4.01 (s, 3 H), 4.18 (m, 2 H), 7.08 (m, 1 H), 7.16-7.22 (m, 2 H), 7.31 (d, 1 H, *J* = 8.32 Hz), 7.55 (m, 1 H), 7.71 (d, 1 H, *J* = 8.32 Hz), 7.85 (s, 1 H), 7.90 (m, 1 H), 8.19 (d, 1 H, *J* = 8.28 Hz), 8.21 (m, 1 H), 8.35 (s, 1 H), 8.73 (s, 1 H); ^{13}C NMR (100 MHz, acetone-d_6) δ 14.11, 55.88, 56.15, 60.16, 95.77, 103.6, 112.2, 120.8, 121.1, 121.5, 121.6, 123.0, 127.5, 129.7, 131.2, 131.9, 132.0, 133.4, 134.1, 157.8; HRMS (ESI) *m/z* calcd for $C_{24}H_{21}BrN_4O_4H^+$: 509.0818, Found: 509.0814 (Δ = 0.8 ppm).

2-(4-Bromophenyl)-5-ethoxycarbonylamino-7-cyclopentyl-amido-1*H*-benzo[d]midazole (10b)

Pale brown solid; 41 % yield; mp >200 °C; ^{1}H NMR (400 MHz, acetone-d_6) δ 1.28 (t, 3 H, *J* = 7.08 Hz), 1.64 (m, 2 H), 1.77 (m, 2 H), 1.96 (m, 4 H), 3.08 (m, 1 H), 4.18 (m, 2 H), 7.70 (d, 1 H, *J* = 8.8 Hz), 7.73 (s, 1 H), 8.10 (d, 1 H, *J* = 8.8 Hz), 8.69 (s, 1 H); ^{13}C NMR (100 MHz, acetone-d_6) δ 15.06, 26.80, 31.30, 47.00, 61.12, 96.69, 105.0, 124.1, 128.9, 130.5, 132.9, 136.7, 149.6, 154.7, 175.6; HRMS (ESI) *m/z* calcd for $C_{22}H_{23}BrN_4O_3H^+$: 471.1024, Found: 471.1021 (Δ = 0.6 ppm).

2-(4-Bromophenyl)-5-ethoxycarbonylamino-7-benzamido-1H-benzo[d]midazole (10c)

Off-white solid; 50 % yield; mp >200 °C; ^{1}H NMR (400 MHz, acetone-d_6) δ 1.28 (t, 3 H, *J* = 7.1 Hz), 4.18 (m, 2 H), 7.63-7.72 (m, 4 H), 7.86 (s, 1 H), 8.12 (d, 1 H, *J* = 8.8 Hz), 8.80 (s, 1 H), 9.62 (s, 1 H); ^{13}C NMR (100 MHz, acetone-d_6) δ 15.04, 61.18, 124.2, 128.3, 129.0, 129.2, 129.6, 130.3, 132.7, 132.8, 132.9, 135.8, 154.87; HRMS (ESI) *m/z* calcd for $C_{23}H_{19}BrN_4O_3H^+$: 479.0723, Found: 479.0719 (Δ = 0.8 ppm).

7-(Benzyoxycarbonylamino)-2-phenyl-5-(*tert*-butoxycarbonyl-amino)-1*H*-benzo[d]imidazole (10d)

White solid, 55 % yield; mp >200 °C ^{1}H NMR (400 MHz, acetone-d_6) δ 1.50 (s, 9 H), 5.26 (s, 2 H), 7.34-7.40 (m, 3 H), 7.49-7.51 (m, 5 H), 7.72 (s, 1 H), 8.15 (d, 2 H, *J* = 7.0 Hz), 8.48 (s, 1 H); ^{13}C NMR (100 MHz, acetone-d_6) δ 28.68, 67.33, 79.78, 127.4, 128.9, 129.0, 129.4, 129.8, 130.4, 131.3, 137.9, 154.0; HRMS (ESI) *m/z* calcd for $C_{26}H_{26}N_4O_4H^+$: 459.2027, Found: 459.2032 (Δ = - 1.1 ppm).

7-(Cyclohexylamido)-5-ethoxycarbonylamino-2-(4-methoxy-carbonylphenyl)-1H-benzo[d]midazole (10e)

White solid; 53 % yield; mp >200 °C; ^{1}H NMR (400 MHz, acetone-d_6) δ 1.24-1.28 (m, 6 H), 1.55-1.66 (m, 3 H), 1.76 (m, 2 H), 1.99 (m, 2 H), 2.58 (m, 1 H), 3.90 (s, 3 H), 4.16 (q, 2 H, *J* = 7.2 Hz), 7.81 (s, 1 H), 8.10 (m, 3 H), 8.25 (m, 2 H), 8.70 (s, 1 H), 9.17 (s, 1 H); ^{13}C NMR (100 MHz, acetone-d_6) δ 15.03, 26.61, 46.53, 52.56, 61.15, 97.71, 105.4, 127.0, 131.6, 135.1, 136.9, 149.5, 154.8, 166.8, 175.4; HRMS (ESI) *m/z* calcd for $C_{25}H_{28}N_4O_5H^+$: 465.2129, Found: 465.2138 (Δ = - 1.9 ppm).

5-Ethoxycarbonylamino-7-(4-methoxybenzamido)-2-(4-methoxycarbonylphenyl)-1*H*-benzo[d]midazole (10f)

Off-white solid; 52 % yield; m.p. >200 °C; ^{1}H NMR (400MHz, d6-acetone) δ 1.28 (t, 3 H, *J* = 9.6 Hz), 3.92 (s, 6 H), 4.22 (q, 2 H, *J* = 9.3 Hz), 7.14 (d, 2 H, *J* = 11.8), 7.88 (s, 1 H), 7.68 (s, 1 H), 8.07-8.33 (m, 6 H), 8.54 (s, 1 H), 8.82 (s, 1 H), 9.33 (s, 1 H), 12.16 (s, 1 H); ^{13}C NMR (100 MHz, d6-acetone) δ 14.09, 51.61, 55.06, 60.21, 96.00, 103.9, 113.9, 126.2, 129.1, 129.9, 134.3, 135.2, 136.3, 148.8, 154.0, 162.8, 165.8 ; HRMS (ESI) *m/z* calcd for $C_{26}H_{24}N_4O_6H^+$: 489.1774, Found: 489.1774 (Δ = - 0.0 ppm).

7-Benzamido-5-ethoxycarbonylamino-2-(4-methoxycarbonyl-phenyl)-1*H*-benzo[d]midazole (10g)

Brown solid; 51 % yield; m.p. > 200 °C; ^{1}H NMR (400 MHz, acetone-d_6) δ 1.28 (t, 3 H, *J* = 7.2 Hz) 3.91 (s, 3 H), 4.20 (q, 2 H, *J* = 7.2 Hz), 7.61 (m, 3 H), 7.87 (s, 1 H), 8.10 (m, 4 H),

8.28 (d, 2 H, *J* = 6.3 Hz), 7.79 (s, 1 H); ^{13}C NMR (100 MHz, acetone-d_6) δ 15.09, 52.55, 61.20, 97.20, 105.0, 127.2, 128.1, 129.7, 130.7, 131.7, 132.8, 135.2, 136.0, 154.7, 166.8; HRMS (ESI) *m/z* calcd for $C_{25}H_{22}N_4O_5H^+$: 459.1660, Found: 459.1668 (Δ = - 1.7 ppm).

5-Ethoxycarbonylamino-7-(4-fluorobenzamido)-2-(1-furyl)-1*H*-benzo[d]imidazole (10h)

Light brown solid; 51 % yield; mp turned black at 140 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.25 (t, 3 H, *J* = 7.2 Hz) 4.17 (q, 2 H, *J* = 7.2 Hz) 6.42 (br. s., 1 H) 7.01 (br. s., 1 H) 7.08 (t, 2 H, *J* = 8.3 Hz) 7.30 - 7.51 (m, 2 H) 7.69 (br. s., 3 H) 7.96 (m, 3 H) 9.12 (br. s., 1 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.68, 61.51, 110.84, 112.35, 115.74, 115.95, 130.03, 130.12, 130.64, 134.42, 143.19, 144.05, 144.97, 154.81, 163.93, 165.41, 166.44; HRMS (ESI) *m/z* calcd for $C_{21}H_{17}FN_4O_4H^+$: 409.1316, Found: 409.1312 (Δ = 1.0 ppm).

7-Benzamido-5-ethoxycarbonylamino-2-(1-furyl)-1*H*-benzo-[d]imidazole (10i)

Light brown solid; 52 % yield; mp turned black at 140 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.26 (t, 3 H, *J* = 7.2 Hz), 4.18 (q, 2 H, *J* = 7.2 Hz), 6.43 (br. s, 1 H), 7.04 (br. s, 1 H), 7.31 - 7.68 (m, 5 H), 7.97 (m, 3 H), 9.14 (br. s., 1 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.7, 61.5, 112.4, 127.7, 128.8, 132.2, 134.5, 143.1, 144.1, 154.8, 166.5; HRMS (ESI) *m/z* calcd for $C_{21}H_{18}N_4O_4H^+$: 391.1401, Found: 391.1406 (Δ = -1.3 ppm).

Antibacterial activity. *F. tularensis* LVS provided by Dr. J. Petersen (Centers for Disease Control, Fort Collins, CO) was grown to an OD_{600} of ~0.6, frozen at -80°C in 10 % glycerol and used as a standard bacterial stock for these studies. For each evaluation bacteria were prepared fresh by growth from the standard stocks on Cystine Heart Agar supplemented with 2% hemoglobin (BD, Franklin Lakes, NJ) grown at 37 °C for 48-72 h. Bacteria recovered from the Cystine Heart Agar-Hemoglobin (CHAB) plates were used to inoculate 50 mL Mueller-Hinton broth (BD) supplemented with 0.025% ferric pyrophosphate (Sigma-Aldrich), 2% IsoVitaleX (BD), 0.1% glucose (Sigma-Aldrich). Broth cultures were then incubated for 18 h at 37°C passed 1:20 and incubated for an additional 8 h at 37 °C. Bacteria were then diluted to a concentration of $1x10^7$ colony forming units (CFU)/mL in modified Mueller-Hinton (MMH) broth and 50 µL added to each well for each test plate. Trisubstituted benzimidazole compound library was screened in a percent inhibition high-throughput fashion against *F. tularensis* LVS in a 96-well plate format. All compounds were diluted in MMH broth to concentrations of 10, 2 and 0.4 µg/mL in a 50µL volume/well. For MIC determination, compounds were added to the 96-well plate starting at 512 µg/mL in the first column and serially diluted 1:2 to column 12 for a final concentration of 0.25 in MMH broth. MIC plates were incubated at 37°C for 18 h at which time 10 µL of Alamar Blue (Invitrogen, Carlsbad, California) was added to each well and plates were incubated for an additional 4 h. Reduction of Alamar Blue was determined by absorbance readings at wavelengths of 570 and 600 nm using a microplate reader (Biotek, Winooski, VT). Percent growth reduction was calculated and MIC_{90} values were determined by plotting the percentage inhibition calculated from spectrophotometric readings over the drug concentration series. Bacterial growth and MIC was confirmed by optical density. Non-linear regression analysis was performed on % growth inhibition curves to determine MIC_{90} values.

***Francisella ex vivo* model of efficacy.** RAW 264.7 mouse macrophages (American Type Tissue Collection (ATCC), Manassas, VA) were used to assess intracellular efficacy of our compounds. RAW cells were cultured in complete growth medium (ATCC) supplemented with 10% fetal bovine serum (Atlas Biologicals, Fort Collins, CO. Bacteria were added to $2.5x10^5$ RAW cells per well in a 24-well tissue culture plate at a multiplicity of infection of 50 CFU per cell in a 0.5 mL media. The plates were then centrifuged at 2,400 x g for 2 min and placed at 37 °C/5% CO_2 to incubate for 1 h. Supernatant was then removed; plate washed once with 2 ml PBS and 1 mL of 0.05 mg/mL gentamicin (Sigma-Aldrich) in complete media added. Plates were then incubated for 1 h and washed 2 times with 2 mL PBS.

Selected compounds were then added to each well at 50, 10, and 2 µg/mL in complete media in triplicate. Untreated, 1 µg/mL doxycycline (Sigma-Aldrich), and 50 µg/mL thiabendazole (Sigma-Aldrich) were included as controls. Plates were incubated for 18 h and cells observed for signs of infection. Cells were washed 3 times with 2 mL PBS and 1 mL sterile ddH_2O added to each well. Each well was thoroughly mixed/scraped to insure complete cell lysis. Lysates were then serially diluted 1:10 and inoculum plated from the 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilutions onto CHAB agar plates. Plates were incubated for 48 h at 37 °C. Colonies from each plate was counted and CFU/ml lysate was calculated. MIC_{90} was calculated using linear regression analysis of compound dilution series to determine specific compound concentration that exhibits 90% growth reduction of bacteria.

Cytotoxicity Assay. The cytotoxicity of the compounds was tested against Vero cells. Epithelial cells from the kidneys of the African Green Monkey were used to start the Vero cell line. Vero cells were grown in L15 media without CO_2. Serial 2-fold dilutions of the drugs were prepared (200 µg/mL to 50 µg/mL) in triplicate using 96-well microtiter plates. The cells were added to the plates to a final concentration of 1.25×10^5/well in media containing Resazurin for a final concentration 25 µg/mL. The plates were incubated for 3 days at 37 °C. The LD was the lowest drug concentration that inhibited cell growth and the resazurin was not reduced.

5. Acknowledgements

This research was supported by grants from the National Institutes of Health (Grant AI078251 to I.O.) and (U01082164 to R.A. S.), as well as the NYSTAR (to I.O.).

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