Published in final edited form as:

J Fluor Chem. 2017 April ; 196: 44–56. doi:10.1016/j.jfluchem.2016.07.020.

Strategic incorporation of fluorine in the drug discovery of new-generation antitubercular agents targeting bacterial cell division protein FtsZ*

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Abstract

This article presents an account of our research on the discovery and development of new-generation fluorine-containing antibacterial agents against drug-resistant tuberculosis, targeting FtsZ. FtsZ is an essential protein for bacterial cell division and a highly promising therapeutic target for antibacterial drug discovery. Through design, synthesis and semi-HTP screening of libraries of novel benzimidazoles, followed by SAR studies, we identified highly potent lead compounds. However, these lead compounds were found to lack sufficient metabolic and plasma stabilities. Accordingly, we have performed extensive study on the strategic incorporation of fluorine into lead compounds to improve pharmacological properties. This study has led to the development of highly efficacious fluorine-containing benzimidazoles as potential drug candidates. We have also performed computational docking analysis of these novel FtsZ inhibitors to identify their putative binding site. Based on the structural data and docking analysis, a plausible mode-of-action for this novel class of FtsZ inhibitors is proposed.

Keywords

Mycobacterium tuberculosis; Antibacterial; Fluorine-containing benzimidazoles; FtsZ; Structure-activity relationship; Metabolic and plasma stability; Docking; Molecular modeling

1. Introduction

Tuberculosis (TB) was responsible for 1.5 million deaths in 2014, which was second only to HIV/AIDS related fatalities among all infectious diseases [1,2]. TB is caused by *Mycobacterium tuberculosis* (*Mtb*) and is highly contagious when airborne [3]. It has been

*This article is dedicated to Professor Steven H. Strauss on the occasion of his 2016 ACS Award for Creative Work in Fluorine Chemistry.

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estimated that 90% of humans who are exposed to or infected with the pathogen have latent-TB, which has a 10% chance of progressing to an active-TB diseased state during their lifetime [4]. Recent statistics from the World Health Organization (WHO) indicate an estimated 9.6 million new TB cases globally in 2014 [1,2]. Among these cases, 12% were HIV-positive individuals with 0.4 million deaths, which demonstrates the ability of the bacteria to target immunocompromised patients [1,2]. In 2014, 480,000 cases of MDR-TB were estimated, but only 123,000 cases were actually detected and reported [1,2]. MDR-TB, which is resistant to at least one of the three second-line antibiotics and fluoroquinolones, is classified as extensively drug resistant-TB (XDR-TB). About 9.7% of MDR-TB cases have been classified as XDR-TB by WHO in 2015 and 105 countries have reported at least one case of XDR-TB [1,2].

1.1. FtsZ as the target for the drug discovery of new-generation anti-TB agents

The globally spread resistance to existing therapeutics has become a major clinical challenge for effective treatment of the disease. Thus, in order to address the drug resistance issue, there is an urgent need for the identification of novel therapeutic targets. Therefore, FtsZ, an essential bacterial cytokinesis protein, has been recognized as a highly promising therapeutic target [5–9]. FtsZ polymerizes bi-directionally in the presence of GTP, at the center of the cell to form “Z-ring” [10–14]. Several other cell division proteins are recruited to undergo Z-ring contraction, resulting in septum formation and cell division [10–14]. Consequently, the inhibition of critical FtsZ assembly would block the cell division, which should lead to bacterial growth inhibition and cell death [5,7,8,15–17]. Crystal structures of the head-to-head lateral FtsZ dimer of *M. tuberculosis* (pdb: 1RLU) [12], as well as the head-to-tail longitudinal FtsZ dimer of *M. jannaschii* (pdb: 1W5A) [18] are shown in Fig. 1. The key function of FtsZ in the Z-ring formation and bacterial cell division is illustrated in Fig. 2 [5,19].

As described above, FtsZ emerged as a promising new target for antibacterial drug discovery. Thus, in the last decade, FtsZ inhibitors have been actively investigated for pathogen specific as well as broad-spectrum antibacterial drug discovery against a variety of pathogens [5,8,19–22]. As a result, several types of compounds have been identified as promising leads for antibacterial drug development, *e.g.*, OTBA [23], 2-alkoxycarbonylamino-pyridines [24], 2-carbamoylpteridine [25], taxanes [8,15,26], benzimidazoles [5,27–32], GTP analogs [33,34], benzo[c]phenanthridines [35,36], isoquinolines [35,37,38], PC190723 [39,40], Zantrins [6] and chrysopaentins [41]. However, against *Mtb*-FtsZ, only taxanes [15] and benzimidazoles [27] have been studied as promising leads after the pioneering work by the Southern Research Institute group [24,25,37,42].

1.2. Strategic incorporation of fluorine into drug leads to improve their potency and pharmacological properties

The importance of fluorine in medicinal chemistry is evident based on the fact that a large number of fluorinated compounds have been approved by the FDA for medical use [43–47]. In fact, fluorine is recognized as the second “favorite heteroatom” after nitrogen in the current drug design [48]. It has been demonstrated that the replacement of a C-H or C-O

bond with a C-F bond in biologically active compounds often introduces beneficial pharmacological properties such as higher metabolic stability, increased binding to target molecules, and enhanced membrane permeability [49,50]. Small atomic radius, high electronegativity, nuclear spin of $\frac{1}{2}$, and low polarizability of the C-F bond are among the special properties that make fluorine and organofluorine groups very attractive in chemical biology and medicinal chemistry. Accordingly, it is currently a common practice in drug discovery to explore fluoro-analogs of lead compounds under development. This account describes and discusses our case study on the strategic incorporation of fluorine into new-generation anti-TB agents based on the benzimidazole scaffold, targeting *Mtb*-FtsZ.

2. Effects of the fluorine and organofluorine substitutions on the potency and metabolic stability of benzimidazole-based FtsZ inhibitors as novel anti-TB agents

We started our drug discovery project for new-generation anti-TB agents, targeting the key cell division protein of *M. tuberculosis*, *Mtb*-FtsZ, from the design, moderately high throughput (HTP) synthesis of libraries of 2,5,6- and 2,5,7-trisubstituted benzimidazoles, and their whole cell screening. Moderately HTP screening of those compound libraries by the microplate Alamar blue assay [27,51] against *M. tuberculosis* H37Rv at 5 $\mu\text{g/mL}$ concentration identified a good number of hit compounds [27]. Furthermore, selected early lead compounds from these hits exhibited promising MIC values in the range of 0.39–6.1 $\mu\text{g/mL}$ against drug sensitive (H37Rv) as well as drug resistant clinical strains of *M. tuberculosis* (Fig. 3) [27]. It has been shown that these lead compounds inhibited FtsZ assembly in a dose dependent manner, and enhanced the GTPase activity of *Mtb*-FtsZ [27].

Moreover, benzimidazoles, SBZ0101 (**1**) and SBZ0121 (**2**) (Fig. 3), were found to be bactericidal through kill characteristics analysis [31]. It has also been shown that **1** and **2** are active against non-replicating *Mtb* grown under low oxygen conditions [31], which is unexpected and very exciting since it indicates that these compounds have potential to be effective against latent TB infection.

SBZ0101 (**1**) was evaluated in two different animal models of acute TB infection to investigate if **1** can reduce the bacterial load during an acute infection and inhibit dissemination from the site of infection to secondary sites [31]. In the first acute infection animal study which uses immune incompetent GKO mice (C57BL/6-Ifngtm1ts, Jackson Laboratories), **1** reduced the bacterial load of *M. tuberculosis* H37Rv by 0.71–0.17 $\log_{10}$ CFU in the lungs and 0.41–0.36 $\log_{10}$ CFU in spleen [31]. In the second dissemination model of infection using immune competent C57BL/6 mice, **1** reduced the bacterial load of *M. tuberculosis* Erdman in the spleen by $\log_{10}$ 1.6–0.49 CFU [31]. Thus, these animal studies show that **1** has some encouraging efficacy as an early lead compound against an acute infection and can prevent dissemination to secondary sites.

Since these two early lead compounds have shown promising antibacterial activities *in vitro* and *in vivo*, a host of preclinical evaluations on the pharmacological properties of **1** were performed. In these evaluations, **1** exhibited very good permeability (Caco2/TC7), safe for

hERG affinity, not an inhibitor of cytochrome P450 3A4, and very clean kinase profile (only one kinase in selected 50 kinases panel). Also, **1** did not show cytotoxicity ($IC_{50} > 400 \mu M$) in Vero cells [31]. However, **1** was found to have an issue with plasma stability in mice, which was identified as the most urgent need for improvement. Although **1** was stable in human blood plasma (only 5% clearance), it was moderately unstable in murine blood plasma (56% clearance) at 37 °C for 4 h. SBZ0121 (**2**) was even worse, *i.e.*, 9% and 98.5% clearance in human and murine plasma, respectively. The stability of **1** in murine plasma was found to be improved to 10% clearance in the presence of a hydrolase inhibitor, phenylsulfenylmethyl fluoride (PMSF). Thus, it was clear that the carbamate moiety of **1** was the cause of the instability. Metabolic stability (against liver microsomes) of **1** and **2** were problematic as well, especially in rat and mouse (92–100% clearance) although the stability in human microsomes was better (78 and 83% clearance, respectively) (Table 1, entries 1 and 2).

Accordingly, we synthesized and examined the 2,2,2-trifluoroethyl ester derivatives of **1** and **2**, *i.e.*, SBZ0105 (**3**) and SBZ0125 (**4**), which showed considerable increase in potency (*e.g.*, **4**: MIC 0.31 $\mu g/mL$) and substantial improvements in stabilities in both human and rat (Table 1, entries 3 and 4) [28]. It is noteworthy that **4** exhibited only 1% clearance in human plasma, 20% clearance in human microsomes and 10% clearance in rat microsomes. However, the introduction of a trifluoroethyl ester did not improve the stabilities in murine plasma and microsomes.

In the meantime, we performed extensive SAR study and optimization of these early leads, **1** and **2**, through systematic structural modifications at the 5 and 6 positions, keeping the cyclohexyl group at the 2 position intact [28]. This SAR study led to the identification of a highly potent lead compound, SBZ0131 (**5**) (MIC 0.06 $\mu g/mL$), bearing a 6-*N,N*-dimethylamino group (Table 1, entry 5) [28]. It was found that the introduction of an *N,N*-dimethylamino group in place of an *N,N*-diethylamino or pyrrolidinyl group at the 6 position increased the potency substantially. However, as easily anticipated, the plasma (murine: 88% clearance) and metabolic stabilities (95–99% clearance in human, rat and murine microsomes: entry 5) of this highly potent compound were poor, except for the stability in human blood plasma (6% clearance). Thus, **5** cannot be a good compound to examine *in vivo* although it is an excellent compound for *in vitro* and cell-based studies.

Accordingly, we turned our attention to the corresponding 5-benzamidobenzimidazoles, which should be more stable than benzimidazoles bearing a 5-carbamate group [28]. However, the most basic 5-benzamido-2-cyclohexylbenzimidazole **6** was found to be as labile as **5** in human and murine microsomes, although there was a substantial improvement (95–48% clearance) in rat microsomes (Table 1, entry 6). Therefore, we explored substituent effects on the metabolic lability of 5-benzamido-2-cyclohexyl-benzimidazoles. We introduced F, CF₃, MeO, CHF₂O, and CF₃O groups to the 4-position of the 5-benzamido moiety of **6** to examine their effects (Fig. 4) [28]. As Table 1 (entries 7–11) shows, the 4-F group only moderately improved the stability in human microsomes, whereas the 4-CF₃ group dramatically improved the stability in the microsomes of three species (entries 7 and 8). The 4-MeO group also exhibited slightly weaker but similar improvement in metabolic stability as compared to the 4-CF₃ group, and increased potency (**9**: MIC 0.31 $\mu g/mL$) (entry

9). These results strongly suggest that the 4 position of the 5-benzamido moiety is the major site for hydroxylation by cytochrome P450 oxidases (CYPs).

Next, we introduced 4-CHF₂O and 4-CF₃O groups, which are increasingly popular fluorine-containing substituents in medicinal chemistry, to examine their effects [28]. As Table 1 shows, the introduction of the 4-CHF₂O group (entry 10) did not improve the metabolic stability as well as MIC as compared to the 4-MeO group, suggesting a possible lability of its C-H bond against oxidation. In contrast, the introduction of 4-CF₃O group (entry 11) exhibited clear improvement in metabolic stability (39%, 14% and 45% clearance in human, rat, and murine microsomes, respectively) and MIC (0.16 µg/mL). The stability in blood plasma was also improved (0% and 24% clearance in human and murine plasma, respectively). Because of its improved plasma and metabolic stability, SBZ01325 (**11**), was selected for *in vivo* efficacy study on the acute TB model in mice [30].

Since it was found that the introduction of 4-CF₃O group improved both potency and metabolic/plasma stability, we synthesized analogs of SBZ01325 (**11**) bearing a benzoxazole skeleton (SBO01325, entry 12), a 6-methoxy group (entry 13), a 2-(1-ethylpropyl) group (entry 14) and a 2-benzyl group (entry 15) as shown in Table 1 [28]. Among these analogs, only SBZ02325 (**14**) maintained high potency (MIC 0.31 µg/mL) with good stability in rat microsomes (12% clearance) (Table 1, entry 14). We also synthesized the 2-(4,4-difluorocyclohexyl) analog of **11** (SB01325-2F₂: **16**, Fig. 4) and examined its potency and metabolic stability (entry 16) [52]. To our surprise, the introduction of a *gem*-difluoromethylene group at the 4 position of the 2-cyclohexyl moiety completely shut down the metabolism by CYPs. The result clearly indicates that the cyclohexyl group at the 2 position is a site for CYP-metabolism and the 4,4-*gem*-difluorocyclohexyl group plays a key role in extremely high metabolic stabilization. At the same time, however, the introduction of the *gem*-difluorocyclohexyl group substantially reduced its potency, and thus this unique modification was not pursued further. It should be noted that the 4,4-*gem*-difluorocyclohexyl group alone is not responsible for the remarkable enhancement of metabolic stability, *i.e.*, the corresponding 4,4-*gem*-difluorocyclohexyl analog of **11**, bearing a *n*-butyl carbamate group at the 5 position (SBZ0131-2F₂: **17**, Fig. 4) [52] did not show exceptional metabolic stability, although it was considerably better than the parent SBZ0131 (**5**) (entry 17). In addition, we examined the effects of a 4-CF₃S group on the potency and metabolic stability, as compared to that of a 4-CF₃O group by synthesizing the CF₃S analog of **11** (SBZ01326: **18**) [52]. As Table 1 shows, the potency was only slightly affected by the CF₃S group, but metabolic stability in human microsomes was dramatically reduced (95% clearance), keeping those in rat and murine microsomes at good to moderate level (22% and 45% clearance, respectively) (entry 18). The result may indicate that the oxidation of sulfur by CYP3A4 takes place in human microsomes.

SBZ01325 (**11**) exhibited substantially improved *in vivo* efficacy as compared to that of **1**, but it was still far weaker than the current front line TB drug, isoniazid (INH), in an acute infection model in mice, which is mainly attributable to its insufficient metabolic stability [31]. Accordingly, we continued our SAR study and optimization of the metabolic stability of SBZ01325 series of benzimidazoles. We focused our attention to the potential blockage or mitigation of metabolism (hydroxylation/oxidation by CYPs and hydrolysis of amide by

hydrolases) through substitution of the 2 position of the 5-benzamido moiety. Thus, we investigated the effect of 2-fluoro group on the metabolic stability and potency.

We synthesized 2,4-difluoro analog (**19**) [28], 4-bromo-2,6-difluoro analog (**20**) [52], and 2,6-difluoro-4-methoxy analog (**21**) [52] of SBZ0132 (**6**) and examined their potency and metabolic stability. As Table 1 shows (entries 19, 21 and 22), the introduction of a 2,6-difluorobenzamido group considerably reduced the potency and the blocking of two *ortho* positions of the benzamido group did not improve metabolic stability. A 4-methoxy group was more effective than 4-bromo group to increase metabolic stability. The introduction of a 6-methoxy group in place of an *N,N*-dimethylamino group substantially reduced potency although metabolic stability is in the same range (Table 1, entry 20) [53].

Since the introduction of a fluorine substituent at the 2 position of 5-benzamido moiety was found to be effective in improving metabolic stability without affecting potency, we synthesized the 2-fluoro analog (SBZ013251: **23**) of SBZ01325 (**11**), as well as 2-fluoro analog (SBZ013221: **24**), and 2,3-difluoro analog (SBZ0132211: **25**) of SBZ01322 (**8**) and examined their potency and metabolic stability (Fig. 5) [32,52]. These three analogs exhibited impressive metabolic stability in human (5–13% clearance), rat (0–17% clearance) and murine (4–17% clearance) microsomes and maintained high potency (MIC 0.16–0.39 µg/kg) (Table 1, entries 23–25). SBZ013251 (**23**) and SBZ0132211 (**25**) also showed excellent stability in human (2 and 1% clearance) and murine (12 and 11% clearance) plasma. Accordingly, these three analogs were evaluated for their *in vivo* efficacy in an acute TB infection model in mice [32].

Treatment with SBZ013251 (**23**) reduced the *M. tuberculosis* Erdman bacterial load in the lungs by log₁₀ 6.1/6.3 CFU *via* oral (PO)/Intraperitoneal (IP) administration, respectively. SBZ013221 (**24**) also reduced the bacterial load in the lungs by 5.6/5.8 Log₁₀ CFU *via* PO/IP administration, respectively. Treatment with both **23** and **24** totally cleaned the bacteria in the spleen when the compounds were delivered either PO (>log₁₀ 4.96 CFU reduction) or IP (>log₁₀ 3.89 CFU reduction) [32]. These results are truly remarkable as novel drug lead compounds, which exhibited comparable efficacy to the gold standard anti-TB drug, INH, used as a positive control. In contrast, treatment with **25** resulted in modest 1.7/2.5 log₁₀ CFU reduction *via* PO, as well as 2.1/3.4 log₁₀ CFU reduction *via* IP, in bacterial load in the lungs and spleen, respectively [32]. Since **25** showed only slightly weaker potency and lower metabolic stability in mice than **23** (Table 1, entry 25), the substantial difference in their *in vivo* efficacies indicates a limited correlation between the MIC/metabolic stability *in vitro* and the activity *in vivo*. However, the kill characteristics of **25**, compared to those of **23** and **24**, clearly indicate the difference in potency, predicting poorer outcome in efficacy *in vivo* (see Fig. 9, Section 3.2.2.) [32]. These results demonstrate the importance of the determination of kill characteristics for antibacterial drug discovery. More detailed description and discussion on the biological evaluations are presented in Section 3.2.

3. Biological evaluations of fluorine-containing benzimidazoles as anti-TB agents, targeting *Mtb*-FtsZ

3.1. *In vitro* potency and *in vivo* efficacy of SBZ01325 (**11**)

3.1.1. *In vitro* potency of **11** against *M. tuberculosis* H37Rv and clinical strains

—The MIC value for SBZ01325 (**11**) against a common laboratory strain of *M. tuberculosis* H37Rv (drug-sensitive) as well as different clinical strains of *M. tuberculosis*, *i.e.*, TN587 (INH-resistant, Kat G S315T mutation) [54], NHN382 (INH-resistant, Kat G-Del) [54], W210 (Kat G wild type) and NHN20 (INH-resistant) is 0.16 µg/mL based on the microplate Alamar Blue assay (MABA) [27,51]. The results clearly indicate that **11** is equally potent against drug-sensitive and drug-resistant strains of *M. tuberculosis* [30]. The antibacterial activity was found to be concentration dependent with a typical sigmoidal curve. Also, there was no difference in kill characteristics among the different *M. tuberculosis* strains and clinical isolates [31]. These results strongly suggest that *Mtb*-FtsZ is a novel target and the inhibitors of this target can completely circumvent the resistance to existing anti-TB drugs. Also, **11** is not cytotoxic (IC₅₀ > 100 µM) to Vero cells [30].

3.1.2. Frequency of resistance (FOR) assessment—In order to examine potential drug resistance caused by genetic mutations upon exposure of bacteria to drugs in the case of benzimidazole-based *Mtb*-FtsZ inhibitors, *M. tuberculosis* H37Rv (26,000 cells) was plated on 1.6 µg/mL of **11**, which is 10 times the experimentally determined MIC value (0.16 µg/mL) as mentioned above. This is a standard method to determine the “frequency of resistance”, *i.e.*, probability of the occurrence of a mutation in the bacterial population that provides a selective advantage upon exposure to a drug, conferring resistance. No mutants were observed, and no single resistant colony was obtained, either. Thus, the FOR should be extremely low [30].

3.1.3. *Mtb*-FtsZ-polymerization inhibitory activity—As mentioned above, the early lead compound **1** was found to inhibit the polymerization of *Mtb*-FtsZ in dose-dependent manner, which clearly indicated that *Mtb*-FtsZ was the molecular target of **1** and constituted the most important mode of action (MOA) for this novel class of anti-TB agents. To confirm that a more potent lead **11** shares the same MOA, transmission electron microscopy (TEM) images of *Mtb*-FtsZ treated with **11** were taken to examine the ability of **11** to inhibit the polymerization and aggregation of *Mtb*-FtsZ [30]. As Fig. 6 shows, the treatment of *Mtb*-FtsZ (5 µM) with **11** at 40 µM following addition of GTP (25 µM) produced fewer, shorter and thinner FtsZ polymers (Fig. 6B) as compared to control, *i.e.*, untreated protein (Fig. 6A). The inhibitory effect at 80 µM was even more remarkable, wherein only a few dispersed FtsZ polymers and aggregates of FtsZ nucleates were observed (Fig. 6C). These studies confirm that *Mtb*-FtsZ is the molecular target of **11**, as anticipated for this class of novel benzimidazoles [30].

3.1.4. Killing characteristics of **11 against *M. tuberculosis* H37Rv**—To examine the killing characteristics of **11**, the bacterial growth of *M. tuberculosis* H37Rv and the bactericidal effect of **11** were monitored by OD₆₀₀ nm over 7 days in the presence of different concentrations of **11** (Fig. 7) [30]. As Fig. 7A shows, **11** is a concentration-

dependent anti-TB agent. It is noteworthy that bacterial growth was affected by sub-MIC concentrations (0.16–0.02 µg/mL) of **11** (Fig. 4A). As Fig. 7B clearly indicates, **11** is bactericidal even at a sub-MIC concentration (0.08 µg/mL).

3.1.5. *In vivo* efficacy of 11 in an acute TB infection model in mice—The *in vivo* efficacy of **11** was evaluated in an acute murine model of TB infection with C57BL/6-Ifngtm1ts (GKO) mice (Jackson Laboratories) using 50 mg/kg dose twice a day (b.i.d) *via* IP administration (Fig. 8) [30]. INH was used as a control *via* IP using 20 mg/kg does once a day (qd). In this acute model, all mice treated with **11** reduced the bacterial load by 1.7 log₁₀ CFU (p value, 0.0001) in the lungs and that by 2.7 log₁₀ CFU (p value 0.0002) in the spleen with one mouse having no detectable bacteria at the lowest level of detection [30]. It should be noted that **11** is the first lead compound in this class exhibited significant *in vivo* efficacy in both the lungs and spleen, as a result of the strategic incorporation of an organofluorine group.

3.2. *In vitro* potency and *in vivo* efficacy of SBZ013251 (23), SBZ013221 (24) and SBZ0132211 (25)

3.2.1. *In vitro* potency of 23, 24 and 25 against *M. tuberculosis* H37Rv and clinical strains—As Table 2 shows, advanced lead compounds, **23**, **24** and **25**, are almost equally potent (**24** is the most potent with MIC 0.18 µg/mL) against *M. tuberculosis* H37Rv and clinical strains with different level of drug resistance, in a similar manner to **11** and other lead compounds in this series [32]. Also, the cytotoxicities of these three lead compounds are negligible (IC₅₀ > 200 µM) in Vero cells [32]. The growth inhibition of *M. tuberculosis* H37Rv by these three lead compounds was sigmoidal and concentration-dependent, which are indicative of *in vivo* efficacy, as exemplified in the case of **11**, described above.

3.2.2. Killing characteristics of 23, 24 and 25 against *M. tuberculosis* H37Rv—The killing characteristics of **23**, **24** and **25** against *M. tuberculosis* H37Rv were determined by monitoring the time course of bacterial growth and their bactericidal effect in the presence of these *Mtb*-FtsZ inhibitors over 7 days in the same manner as that described for **11** (see Section 3.1.4) [32]. Results are summarized in Fig. 9. It is clear that all three lead compounds are dose-dependent anti-TB agents. SBZ013251 (**23**) reduced the number of viable bacteria by 2.8 log₁₀ CFU at 1× MIC by day 2 and by 2.9 log₁₀ CFU at day 6 (Fig. 9d). SBZ013221 (**24**) reduced the number of bacteria by 2.5 log₁₀ CFU at day 2 at 1× MIC and continued to reduce the number through day 6 by almost 4 log₁₀ CFU (Fig. 9e). SBZ0132211 (**25**) exhibited weaker bactericidal activity reducing the bacterial load only by 0.8–1.7 log₁₀ CFU in the first 2 days when dosed at the MIC level (Fig. 9f). Furthermore, **25** required 3–6× MIC concentration to achieve the same level of bacterial viability, *i.e.*, 3.6–4.4 log₁₀ CFU reduction in bacterial load, as that with **23** or **24** at 1× MIC (Fig. 9f). Accordingly, it was anticipated that there would be substantial difference in the *in vivo* efficacy between **25** and **23** or **24** in an acute TB infection model in mice.

3.2.3. *In vivo* efficacy of 23, 24 and 25 in a TB murine model of infection—The *in vivo* efficacy of **23**, **24** and **25** were evaluated in an acute infection model of *M.*

tuberculosis Erdman in C57BL/6-Ifngtm1ts (GKO) mice (Jackson Laboratories), in a manner similar to the case of **11** described in the Section 3.1.5 [32]. Results are summarized in Table 3 and data plots are shown in Fig. 10. Each test compound was delivered at 50 mg/kg dose *via* IP and PO twice a day (b.i.d), starting on day 5 post infection till the endpoint of day 15 [32]. SBZ013251 (**23**) reduced the bacterial load in the lungs by log₁₀ 6.1 CFU *via* PO and log₁₀ 6.3 CFU *via* IP (Table 3, entries 3 and 4). In one of the 5 animals treated with **23** *via* PO and one of the 4 animals treated with **23** *via* IP, the lungs appeared clear. In a similar manner, **24** reduced the bacterial load in the lungs by 5.6 Log₁₀ CFU *via* PO and 5.8 log₁₀ CFU *via* IP, respectively (entries 5 and 6). In one of the 5 animals treated with **24** *via* PO, the lungs appeared clear, but all of the animals treated with **24** *via* IP had some counts in the lungs (Fig. 10A and B). It is worthy of note that **23** and **24** demonstrated efficacy comparative to the front-line and gold standard anti-TB drug, INH, which was delivered *via* IP at 20 mg/kg dose once a day (q.d.) as a positive control (entries 9 and 10) [32]. Further, there were no detectable bacteria in the spleen of mice groups treated with **23** and **24** either *via* PO or IP. treated groups when delivered either IP or PO (Fig. 10A and B). In contrast, treatment with **25** resulted in a statistically significant but modest reductions in bacterial load in the lungs and spleen, *i.e.*, 1.7 and 2.5 log₁₀ CFU (*via* PO); 2.1 and 3.4 log₁₀ CFU (*via* IP), respectively (entries 7 and 8; Fig. 10C) [32].

4. Computational docking study on the putative binding site of the advanced lead SBZ013251 (**23**) in *Mtb*-FtsZ

4.1. Docking study on the putative binding site of **23** in *Mtb*-FtsZ

4.1.1. *Mtb*-FtsZ head-to-tail longitudinal model structure—In order to understand the molecular basis of the MOA of the highly efficacious novel anti-TB drug lead compounds, targeting *Mtb*-FtsZ described above, we performed molecular modeling study using computational docking methods. In this study, we first constructed a key *Mtb*-FtsZ longitudinal dimer model based on the protein X-ray crystallography data of the head-to-head lateral dimers of *Mtb*-FtsZ, as well as the head-to-tail longitudinal dimer of *Methanococcus jannaschii* FtsZ (*Mj*-FtsZ). Curved head-to-tail trimer of GDP-bound *Mtb*-FtsZ crystal structure was also used in this study.

The protein crystal structures of head-to-head lateral dimers of *Mtb*-FtsZ with GTPγS (1RLU), GDP (1RQ7) and citrate (1RQ2) are available in the RCSB Protein Data Bank (PDB) [12]. However, no crystal structure of head-to-tail dimer of *Mtb*-FtsZ has been reported to date. Nevertheless, the crystal structure of head-to-tail dimer of *Mj*-FtsZ is available (1W5A) [18]. Our preliminary global docking study to identify potential binding sites of benzimidazole-based FtsZ inhibitors in the 1RLU and 1RQ7 structures, using AutoDock [55] and Dock [56] programs, indicated that a binding site near the nucleotide binding site was the most promising. Since it has been shown that the novel benzimidazole-based FtsZ inhibitors accelerate the GTPase activity of *Mtb*-FtsZ and destabilize FtsZ polymers, it was quite reasonable that the binding site near the GTP binding site was identified through a global docking study. As the GTP binding site in *Mtb*-FtsZ is located at the interface of the head-to-tail longitudinal dimer, there was a clear need to construct a head-to-tail dimer model through protein alignment of the crystal structures of GTPγS-

bound *Mtb*-FtsZ (1RLU) [12] and GTP-bound *Mj*-FtsZ (1W5A) [18]. As Fig. 11A shows, the alignment of *Mtb*-FtsZ and *Mj*-FtsZ, using the MOE program (ver. 2015.10) is excellent (rmsd ~1.4 Å). This is not surprising because FtsZ is a highly conserved protein essential for cell division among a variety of bacterial species. In a similar manner, a GDP-bound FtsZ head-to-tail dimer model was constructed by protein alignment of GDP-bound *Mtb*-FtsZ (1RQ7) and GTP-bound *Mj*-FtsZ (1W5A). We also used the crystal structure of a GDP-bound curved *Mtb*-FtsZ trimer (4KWE) [57] (Fig. 11B) for docking in connection to the proposed mode of action for these FtsZ inhibitors, blocking the polymerization of FtsZ monomers and destabilizing FtsZ polymers, as discussed below.

4.1.2. *Mtb*-FtsZ dimer model structure bound to FtsZ-inhibitor SBZ013251 (23) and GTP γ S—SBZ013251 (**23**) was docked to the *Mtb*-FtsZ head-to-tail dimer model (Fig. 11A) and the best docking energy was scored using the MOE program [58]. Fig. 12 shows the docking site and docking pose of **23**, as well as its relative position to GTP γ S. The docking energy score is -8.49 kcal/mol and the distance to GTP γ S is ~5 Å. Fig. 13 illustrates the 2D interaction diagram of **23** with the amino acid residues in the binding site. As Figs. 12 and 13 indicate, FtsZ-inhibitor **23** binds to the hydrophobic interface of two FtsZ subunits, very close to the T7 loop, which is known to be critical to FtsZ's GTPase activity. Also, its closeness to the phosphate moieties of GTP γ S strongly imply plausible mechanisms to accelerate GTP hydrolysis through more detailed analysis.

4.1.3. Plausible mode of action for the benzimidazole-based *Mtb*-FtsZ inhibitors—Li et al. reported the structure of novel curved head-to-tail trimer of *Mtb*-FtsZ and proposed that FtsZ protofilaments use a “hinge-opening” mechanism for the generation of constrictive force for Z-ring contraction in bacterial cell division [57]. The proposed mechanism provided a strong basis for us to deduce a highly plausible MOA of the benzimidazole-based FtsZ inhibitors. Fig. 14 illustrates our proposed mechanism or MOA of FtsZ inhibitors that block the polymerization of FtsZ monomers and protofilaments, as well as destabilize FtsZ polymers. Under a normal bacterial cell division process, GTP binds to FtsZ monomers to promote the formation of FtsZ protofilaments and straight FtsZ polymers. When the length of a FtsZ polymer reaches certain length, GTP is hydrolyzed to GDP to form a curved FtsZ polymer for Z-ring formation and constriction. However, in the presence of the benzimidazole-based FtsZ inhibitors, which binds to FtsZ close to the GTP binding site and accelerate the GTP hydrolysis, FtsZ monomers and nucleates cannot form normal straight FtsZ protofilaments and polymers. The fact that this type of FtsZ inhibitors can degrade preformed FtsZ polymers strongly indicates the ability of the FtsZ inhibitors to interact with GTP-bound FtsZ polymers, enhancing the GTPase activity of FtsZ to hydrolyze GTP to GDP and further degrading FtsZ polymers.

To further probe this proposed MOA, FtsZ inhibitor **23** was docked to the “hinge-opened” GDP-bound dimer unit of the curved *Mtb*-FtsZ trimer. There were two possible binding sites, *i.e.*, one site very close to GDP (Fig. 15A) and the other close to the T7 loop (Fig. 15B) with docking scores -7.18 kcal/mol and -7.09 kcal/mol, respectively. Accordingly, the interactions of **23** with the “hinge-opened” FtsZ dimer unit appear to be considerably weaker than those with the head-to-head longitudinal FtsZ dimer. Nevertheless, **23** may still

contribute to the further destruction of FtsZ protofilaments. Also, we have found that **23** binds to the GDP-bound FtsZ head-to-tail dimer model strongly with energy score -8.89 kcal/mol (Fig. 15C). This might suggest that this type of FtsZ inhibitors trigger the degradation of FtsZ architecture *via* the “hinge-opening” or another destabilization mechanism, which does not need to involve GTP hydrolysis.

5. Conclusions

Emergence of severe drug resistance to currently available anti-TB drugs is imposing a serious threat to human health worldwide. Most of anti-TB drugs target cell wall biosynthesis, nucleic acid synthesis and protein synthesis. In the last decade, FtsZ, an essential protein for bacterial cell division and growth has emerged as a highly promising new molecular target for the discovery of novel antibacterial agents. FtsZ is a highly conserved and ubiquitous bacterial protein that plays a critical role in bacterial cytokinesis. Thus, the inhibition of FtsZ assembly causes the disruption of septum formation and bacterial cell division, leading to cell death. Since FtsZ is a totally new target for anti-TB agents, the FtsZ-inhibitors should not have any resistance to the drug-resistant strains for clinically used anti-TB drugs at present. We found that novel benzimidazoles inhibited the nucleation/aggregation/polymerization of *Mtb*-FtsZ effectively in a dose-dependent manner through enhancement of its GTPase activity through design and synthesis of compound libraries, as well as semi-HTP screening. For the optimization of lead anti-TB benzimidazoles, we have extensively investigated the strategic incorporation of fluorine and organofluorine groups into these lead compounds in order to increase the potency against drug-sensitive and drug-resistant *M. tuberculosis* strains, as well as enhance metabolic and plasma stabilities. As described above, we have successfully developed highly efficacious fluorine-containing benzimidazoles as potential new-generation anti-TB agents through these concentrated efforts.

Two novel fluorine-containing FtsZ-inhibitors, SBZ013251 (**23**) and SBZ13221 (**24**), exhibited impressive *in vivo* efficacy, equivalent to that of the gold standard anti-TB drug, isoniazid (INH), against *M. tuberculosis* Erdman in an acute murine model of TB infection, as well as the same high potency against drug-sensitive and drug-resistant *M. tuberculosis* strains. The latter results confirm that FtsZ-inhibitors are indeed effective against *M. tuberculosis* strains resistant to the currently used anti-TB drugs. As the CLogP values in Table 1 indicate, these highly potent advanced lead compounds are hydrophobic, which require formulations using excipients for solubilization. Thus, the optimization efforts are continuing to lower the CLogP values, including further modifications and prodrug approaches as well as nano-formulations.

We have also performed computational docking study to identify putative binding site of SBZ013251 (**23**) bearing one fluorine and one trifluoromethoxy group, using the MOE program, which has led to a plausible unique mode of action for this class of novel FtsZ-inhibitors, which block the polymerization of FtsZ monomers and protofilaments, as well as destabilize and degrade FtsZ polymers.

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Fig. 1. Crystal structures of FtsZ. (A) head-to-head lateral FtsZ dimer of *M. tuberculosis* (pdb: 1RLU) [12]; (B) head-to-tail longitudinal FtsZ dimer of *M. jannaschii* (pdb: 1W5A) [18].

Fig. 2. (A) FtsZ monomers polymerize in the presence of GTP, forming protofilaments. (B) Protofilaments line up at the center of the cell to form the highly dynamic Z-ring, which is anchored to the bacterial cell membrane by FtsA and ZipA. FtsZ monomers readily exchange with FtsZ molecules incorporated into the Z-ring. (C) Contraction of the Z-ring leads to invagination of the cell membrane. (D) Septation is completed and the Z-ring dissipates. Adapted from Reference [19].

Fig. 3.
Early lead compounds from hit benzimidazoles [28].

Fig. 4.
Fluorine-containing benzimidazoles.

Fig. 5.
Highly potent fluorine-containing benzimidazoles.

Fig. 6.

TEM images of *Mtb*-FtsZ. FtsZ (5 μ M) was polymerized by GTP (25 μ M) in the absence (A) and presence of **11** at 40 μ M (B) and 80 μ M (C). Images are at 49,000 $\times$ magnification (scale bar 500 nm). Adapted from Reference [30].

Fig. 7.

Killing characteristics of **11** against whole bacteria. The time dose curves were generated from OD600 nm (a) and from CFU enumeration (b) data. Different concentrations of the compound were tested in triplicate and the mean and standard deviation of the OD600 nm values or the CFU counts from Day 0, 2, 4, and 6 were plotted against time using GraphPad Prism Version 5.0d. Adapted from Reference [30].

Fig. 8.

Efficacy of **11** in an acute murine model of TB infection. Scatter plot of the CFU counts from the lung and spleens of infected mice after drug therapy with **11** delivered IP at 50 mg/kg b.i.d. The colony counts were converted to logarithms. The lower level of detection was 1 $\log_{10}$ CFU. Outliers were identified by the Grubbs' Test using an online calculator (GraphPad Software). A scatter plot of the CFU data from the lung and spleen of individual mice from the treatment and control groups were plotted with the mean and SE from each group using GraphPad Prism Version 5.0d. Adapted from Reference [30].

Fig. 9. Killing characteristics of **23**, **24** and **25**. Separate curves were generated for each compound. The time dose curves were generated from OD_{600nm} (a–c) and from CFU enumeration (d–f) data. Different concentrations of the compound were tested in triplicate and the mean and standard deviation of the OD_{600nm} values or the CFU counts from Day 0, 2, 4, and 6 were plotted against time using GraphPad Prism Version 5.0d (GraphPad Software).

Fig. 10.

In vivo efficacy data for **23**, **24** and **25** in an acute murine model of TB infection.

Fig. 11.
(A) Protein alignment of GTP γ S-bound *Mtb*-FtsZ (1RLU, purple) and GTP-bound *Mj*-FtsZ (1W5A, blue); (B) Crystal structure of curved head-to-tail trimer of GDP-bound *Mtb*-FtsZ (4KWE). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 12.
Docked pose of SBZ013251 (**23**) with GTP γ S-bound *Mtb*-FtsZ head-to-tail dimer model.

Fig. 13.
2D interaction diagram of **23** with GTP γ S-bound *Mtb*-FtsZ head-to-tail dimer model.

Fig. 14. Proposed mode of action for the benzimidazole-based *Mtb*-FtsZ inhibitors. Adapted from Reference [57] with permission and modified.

Fig. 15.

Docked poses of SBZ013251 (**23**) to (A) "hinge-opened" dimer unit of the curved GDP-bound *Mtb*-FtsZ trimer, close to the GDP binding site; (B) "hinge-opened" dimer unit of the curved GDP-bound *Mtb*-FtsZ trimer, close to the T7 loop; (C) the binding pocket between the T7 loop and GDP binding site in the head-to-tail longitudinal GDP-bound *Mtb*-FtsZ dimer model.

Table 1

Fluorine-containing benzimidazoles, their potency and metabolic stability.^a

Comp. No./Entry	R ¹	R ²	R ³	Code	MIC (μg/mL)	H37Rv	CLogP ^b	% metabolic clearance human/rat/mouse ^c
1	Cy	OBu- <i>n</i>	Et ₂ N	SBZ0101	2.0		7.15	78/92/98
2	Cy	OBu- <i>n</i>	(CH ₂ CH ₂) ₂ N	SBZ0121	1.0		6.20	83/100/97
3	Cy	OCH ₂ CF ₃	Et ₂ N	SBZ0105	1.56		6.36	71/56/100
4	Cy	OCH ₂ CF ₃	(CH ₂ CH ₂) ₂ N	SBZ0125	0.31		5.41	20/10/96
5	Cy	OBu- <i>n</i>	Me ₂ N	SBZ0131	0.06		6.09	97/95/99
6	Cy	Ph	Me ₂ N	SBZ0132	1.56		5.66	97/46/97
7	Cy	4-F-Ph	Me ₂ N	SBZ01321	0.63		5.87	78/48/96
8	Cy	4-CF ₃ -Ph	Me ₂ N	SBZ01322	0.44		6.67	25/10/44
9	Cy	4-MeO-Ph	Me ₂ N	SBZ01323	0.31		5.78	39/26/49
10	Cy	4-CHF ₂ -O-Ph	Me ₂ N	SBZ01324	0.31		6.23	55/40/82
11	Cy	4-CF ₃ -O-Ph	Me ₂ N	SBZ01325	0.16		6.89	39/14/45
12 [*]	Cy	4-CF ₃ -O-Ph	Me ₂ N	SBO01325 [*]	6.25		6.74	56/22/71
13	Cy	4-CF ₃ -O-Ph	MeO	SBZ01A25	1.44		6.0	46/64/95
14	Et ₂ CH	4-CF ₃ -O-Ph	Me ₂ N	SBZ02325	0.31		6.76	68/12/45
15	PhCH ₂	4-CF ₃ -O-Ph	Me ₂ N	SBZ03325	1.25		6.34	27/81/49
16	4,4-F ₂ -Cy	4-CF ₃ -O-Ph	Me ₂ N	SBZ01325-2F ₂	1.25		6.27	9/0/0
17	4,4-F ₂ -Cy	OBu- <i>n</i>	Me ₂ N	SBZ0131-2F ₂	0.63		5.48	80/55/94
18	Cy	4-CF ₃ -S-Ph	Me ₂ N	SBZ01326	0.67		7.39	95/22/45
19	Cy	2,4-F ₂ -Ph	Me ₂ N	SBZ013201	0.31		5.63	55/40/64
20	Cy	2,4-F ₂ -Ph	MeO	SBZ01A201	1.32		4.74	52/61/63
21	Cy	2,6-F ₂ -4-Br-Ph	Me ₂ N	SBZ0132021	1.56		6.09	52/61/63

Comp. No./Entry	R ¹	R ²	R ³	Code	MIC (µg/mL) H37Rv	CLogP ^b	% metabolic clearance human/rat/mouse ^c
22	Cy	2,6-F ₂ -4-MeO-Ph	Me ₂ N	SBZ0132022	2.5	5.32	23/20/46
23	Cy	2-F-4-CF ₃ -O-Ph	Me ₂ N	SBZ013251	0.16/0.31	6.68	13/17/4
24	Cy	2-F-4-CF ₃ -Ph	Me ₂ N	SBZ013221	0.16	6.42	12/14/13
25	Cy	2,3-F ₂ -4-CF ₃ -Ph	Me ₂ N	SBZ0132211	0.39	6.50	5/0/17

^aSome non-fluorine-containing benzimidazoles are listed as well for comparison purpose.

^bCalculated by the ChemDraw program.

^cMetabolic stability was determined by incubating for 4 h at 37 °C and then analyzing the samples by LC–MS/MS.

* 2-cyclohexyl-5-dimethylamino-6-(4-trifluoromethoxy)benzamido-1,3-benzoxazole.

Table 2

In vitro anti-TB activity of **23**, **24** and **25** against *M. tuberculosis* H37Rv and clinical strains^a (MIC µg/mL).

Benzimidazoles	H37RV	NHN382	TN587	W210	NHN20
SBZ013251 (23)	0.31 ± 0.22	0.31 ± 0	0.31 ± 0	0.47 ± 0.22	0.24 ± 0.11
SBZ013221 (24)	0.16 ± 0.1	0.16 ± 0	0.24 ± 0.11	0.31 ± 0	0.16 ± 0
SBZ0132211 (25)	0.39 ± 0.16	0.37 ± 0.24	0.31 ± 0	0.47 ± 0.22	0.39 ± 0.33

^aFor the resistance characteristics of these clinical strains, see Section 3.1.1.

Table 3
in vivo efficacy of **23**, **24** and **25** in an acute murine model of TB infection *via* IP or PO b.i.d.

Entry	Benzimidazole	Route	log ₁₀ CFU Lungs	log ₁₀ CFU Spleen
1	Control	IP	6.69 ± 0.05 n = 2	N/A
2		PO	6.31 ± 0.04 n = 5	N/A
3	SBZ013251 (23)	IP	0.39 ± 0.63 n = 4	-4.96
4		PO	0.22 ± 0.34 n = 5	-3.89
5	SBZ013221 (24)	IP	0.91 ± 0.64 n = 4	-4.96
6		PO	0.66 ± 0.43 n = 5	-3.89
7	SBZ0132211 (25)	IP	4.59 ± 0.71 n = 3	-3.36
8		PO	4.58 ± 0.21 n = 5	-2.49
9	INH	IP	0.12 ± 0.16 n = 5	-4.96
10		PO	0 ± 0 n = 5	-3.89

n = number of animals survived. = difference in log₁₀ CFU between the means from the test and control groups; p values < 0.001 unless otherwise noted.

* p value < 0.01.