Antimycobacterial Compounds by a Lux-Based phoP2 Promoter-Reporter Platform

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Abstract: The emergence of multidrug-resistant strains and hyper-virulent strains of Mycobacterium tuberculosis are big therapeutic challenges for tuberculosis (TB) control. Repurposing bioactive small-molecule compounds has recently become a new therapeutic approach against TB. This study aimed to identify novel anti-TB agents from a library of small-molecule compounds via a rapid screening system. A total of 320 small-molecule compounds were used to screen for their ability to suppress the expression of a key virulence gene, phop, of the M. tuberculosis complex using luminescence (lux)-based promoter-reporter platforms. The minimum inhibitory and bactericidal concentrations on drug-resistant M. tuberculosis and cytotoxicity to human macrophages were determined. RNA sequencing (RNA-seq) was conducted to determine the drug mechanisms of the selected compounds as novel antibiotics or anti-virulent agents against the M. tuberculosis complex. The results showed that six compounds displayed bactericidal activity against M. bovis and BCG, of which Ebselen demonstrated the lowest cytotoxicity to macrophages and was considered as a potential antibiotic for TB. Another ten compounds did not inhibit the in vitro growth of the M. tuberculosis complex and six of them downregulated the expression of phoP/R significantly. Of these, ST-193 and ST-193 (hydrochloride) showed low cytotoxicity and were suggested to be potential anti-virulence agents for M. tuberculosis.

Keywords: lux-based promoter-reporter platforms; small-molecule compounds; Mycobacterium tuberculosis complex; anti-virulence agents; antibiotics

1. Introduction

Tuberculosis (TB) remains a significant global health problem today. According to the World Health Organization Global TB report in 2021, TB ranked 13th among the leading causes of death worldwide [1]. Approximately 10 million people developed TB, which claimed over 1.5 million lives in 2020 [1]. Approximately 10 million people developed TB, which claimed over 1.4 million lives in 2019 [1]. TB infections associated with multidrug-resistant (MDR), extensively drug-resistant (XDR), and totally drug-resistant (TDR) Mycobacterium tuberculosis strains have been increasing in recent years [1–4]. This situation poses a significant threat to global TB control, particularly in resource-poor countries with a high prevalence of AIDS. However, according to the global new TB drug development pipeline built by the Stop TB Partnership Working Group on New Drugs...
in 2021, only a few anti-TB drugs were in phase III clinical trials [5], and some of them could not penetrate through complex lung lesions and the MTB cell wall, thereby failing to eliminate *M. tuberculosis* [6,7]. Hence, there is an urgent need to develop new anti-TB treatments against *M. tuberculosis* strains with various patterns of drug resistance.

Drug repurposing is a novel strategy to treat TB, which uncovers known compounds that have the unexpected potential to treat TB [8]. Repurposed drugs such as antibiotics, antifungal, antiviral, and anticancer drugs were potential anti-TB drugs; however, their effectiveness and mechanisms have not been clearly understood [9]. Of them, repurposed antiviral drugs were the least frequently studied. Only a few antiviral drugs, including Isoprinosine, were identified [9,10]. Nevertheless, anti-virulence therapy is an innovative therapeutic strategy to treat multidrug-resistant organisms. It focuses on disarming bacterial virulence factors that facilitate disease development instead of killing the bacteria [11] and often penetrates through the host cell members to eliminate MTB. A previous study discovered a small-molecule compound, 2-phospho-L-ascorbic acid (2P-AC), could reduce mycobacterial survival in macrophage infections [12]. It underlined the potential for the development of anti-virulence agents against *M. tuberculosis*.

Several thousands of small-molecule compounds are approved and passed phase I clinical drug trials, meaning that they have completed extensive preclinical and clinical studies and have well-characterized bioactivities, safety, and bioavailability properties. These compounds could be potentially repurposed to inhibit *M. tuberculosis* virulence. As such, a rapid and high-throughput platform that can screen effective anti-virulence agents for TB is warranted. *PhoP*, which is a key virulence gene of TB, is a global transcriptional regulator of lipid metabolism and hypoxic response and controls the expression of ~2% of the genes in *M. tuberculosis* [13]. It was shown that disruption of *phop* in *M. tuberculosis* caused impaired multiplication within macrophages, suggesting that this gene possibly plays an essential role in the intracellular growth of *M. tuberculosis* [14]. In addition, our previous study identified a common mutation in the promoter region of *phop*, which could confer aggressive intramacrophage growth of hypervirulent *M. tuberculosis* strains [15]. Therefore, we hypothesized that suppression of *phop* expression might be able to impair intramacrophage survival of *M. tuberculosis*, facilitating the host’s immune system to eradicate the bacteria.

The present study aimed to screen small-molecule compounds that were likely to suppress the expression of *phop* in *Mycobacterium bovis* BCG using a *lux*-based promoter-reporter platform. Compared with *M. tuberculosis*, *M. bovis* BCG has a lower biosafety level (risk group 2 and biosafety level 2 practices), despite its genome being >99.95% identical to that of *M. tuberculosis* reference strain H37Rv [16]. This allowed the screening process to be conducted in the BSL-2 laboratory. A library of 320 small-molecule compounds with antiviral activities was screened for their anti-TB potency. The compounds that could reduce the *lux* signal were selected for further validation on (i) their abilities to inhibit in vitro growth of the *M. tuberculosis* complex, (ii) cytotoxicity to THP-1 macrophage, and (iii) dysregulated expression of *phop* and its associated gene networks. Our findings eventually suggested one potential antibiotic and two anti-virulence agents for anti-TB therapy.

2. Results

2.1. Construction and Validation of the Lux-Based Promoter-Reporter Platform

The mycobacterial reporter plasmid -pMV306G13+Lux and the *lux*-based *phop* promoter-reporter plasmid (pMV306PhoP+Lux) were constructed as shown in Figure 1A,B. To validate the platform, ethoxzolamide (ETZ), which could downregulate *phop* expression, was used to demonstrate if the *lux* signal could represent *phop* expression in *M. bovis* BCG. We firstly showed that there was no significant difference (*p > 0.05*) in OD600 between *M. bovis* BCG with and without ETZ treatment (Figure 1(Ci)). This indicated that ETZ did not affect bacterial growth. As we expected, the *lux* signal in *M. bovis* BCG after 24 h of ETZ treatment declined prominently (*p < 0.001* vs. the control group without treatment at 24 h, Figure 1(Cii)). Our results also demonstrated a statistical effect size of 0.886, indicating...
a high quality of screening assay. Meanwhile, RT-qPCR revealed that the expression of the luxA and phoP genes was significantly suppressed \( (p < 0.001) \) after ETZ treatment (Figure 1D). Taken together, the result highlighted that phop expression in M. bovis BCG after drug treatment could be illustrated by referring to its lux signal.

2.2. Screening of 320 Antiviral Compounds and 3 Anti-TB Drugs

M. bovis BCG transformed with pMV306Adaptor+Lux (control set) and pMV306PhoP+Lux (phoP set) were, respectively, treated with a total of 320 antiviral compounds and 3 anti-TB drugs. For the control set, the lux signals were consistently below 50 CPS (no difference existed compared with the blank wells, \( p > 0.05 \)) after all compound treatments, except for triciribine (CPS = 155) (Figure 2A). For the PhoP set, the drug-free controls (Figure 2B, green dots) showed an average lux signal at around 300 CPS, which is regarded as the basal lux intensity induced by the phop promoter in the absence of compound challenges. When M. bovis BCG was treated with the three anti-TB drugs (Figure 2B, red dots), no significant change of lux signals was observed. This indicated that these three common anti-TB drugs could not repress phoP. Notably, 16 antiviral small compounds (CPS \( \approx 0 \), blue dots in Figure 2B) presented a significant suppression effect on lux signals after a 4 h treatment when compared with the drug-free control (Figure 2C). These 16 compounds, which are listed in Supplementary Table S4, were selected for further experimental validation.

Figure 1. Cont.
Figure 1. Construction and validation of the lux-based promoter-reporter platform. (A) pMV306G13+Lux. (B) pMV306PhoP+Lux. (C) Mean (± SD, n = 3) lux signal (in log lux) and OD$_{600}$ in *M. bovis* BCG with and without (control) ETZ treatment at time 0 h and 24 h. (D) LuxA and phoP expression of *M. bovis* BCG with and without (control) ETZ treatment. Data are presented as means ± SD (n = 3) and analyzed using an unpaired t-test, ***p < 0.001 vs. BCG without ETZ treatment.
Figure 2. Screening of 320 antiviral compounds and 3 anti-TB drugs. (A) \(\text{OD}_{600}\) and LUX of all samples with/without 4 h compound treatment in negative control platform of *M. bovis* BCG. (B) \(\text{OD}_{600}\) and LUX of all samples in the *phoP* promoter-reporter platform. Green dots represent BCG samples (including pMV306Adaptor+Lux) without any compound treatment. Red dots represent samples treated with the three anti-TB drugs. Blue dots represent samples treated with the 16 selected compounds, while black dots represent samples treated with other compounds. (C) Comparison of lux signals between the compound treatment and control samples. **\(p < 0.01\).
2.3. MICs and MBCs of Six Compounds against BCG/M. Tuberculosis

After 4 h of treatment with the above 16 compounds, M. bovis BCG were washed and subcultivated in drug-free media, followed by incubation for 14 days. No viable M. bovis BCG was observed after treatment with six compounds, namely Trifluoperazine (dihydrochloride), Elvitegravir, NH125, Ebselen, Letrazuril, and Shikonin, whereas confluent growth was observed after treatment with the remaining 10 compounds. Subsequently, the MICs and MBCs of the six compounds against M. bovis BCG, M. tuberculosis H37Rv, and the two drug-resistant clinical isolates of M. tuberculosis, HKU14621 (MDR-TB) and WC274 (XDR-TB), were determined. Except for Elvitegravir and Letrazuril, these compounds demonstrated bactericidal effects on M. bovis BCG as well as all M. tuberculosis strains at a concentration of 100 µM or below (Table 1). Notably, the MICs and MBCs against the MDR-TB and XDR-TB strains were almost identical to those against M. tuberculosis H37Rv, suggesting that these compounds can kill MDR- and XDR-M. tuberculosis clinical isolates as effective as the pan-susceptible reference strain (Table 1).

Table 1. The MICs and MBCs of six compounds against BCG and MTB (n = 3).

| Compounds                  | BCG MIC (µM) | BCG MBC (µM) | H37Rv MIC (µM) | H37Rv MBC (µM) | MDR-MTB MIC (µM) | MDR-MTB MBC (µM) | XDR-MTB MIC (µM) | XDR-MTB MBC (µM) |
|----------------------------|--------------|--------------|----------------|----------------|------------------|------------------|------------------|------------------|
| Ebselen                    | 50           | 100          | 100            | 200            | 50               | 100              | 50               | 50               |
| Elvitegravir               | 100          | 200          | >400           | >400           | >400             | >400             | >400             | >400             |
| Letrazuril                 | 25           | 100          | 200            | >400           | 200              | 400              | 200              | >400             |
| NH125                      | 25           | 50           | 50             | 100            | 25               | 50               | 25               | 50               |
| Shikonin                   | 25           | 50           | 50             | 100            | 25               | 100              | 25               | 100              |
| Trifluoperazine (dihydrochloride) | 12.5     | 25           | 25             | 50             | 25               | 50               | 25               | 50               |

2.4. RNA-Seq Transcriptome Analysis

To unveil the genetic mechanisms of drug actions of the 16 selected compounds on the M. tuberculosis complex, the transcriptomes of M. bovis BCG after compound treatment were profiled using RNA-seq. Based on the genome-wide differential expression patterns, the datasets could be clustered into two groups by a principal component analysis (PCA) and hierarchical clustering (Figure 3A, B). The clustering separated the compounds with bactericidal activity from those without an inhibitory effect. The compound group in which M. bovis BCG “survived” after the treatment was labelled as the survival (S) group, whereas the compounds that led to the “death” of M. bovis BCG were labelled as the dead (D) group. Interestingly, four compounds that belonged to the S group, namely Pirodavir, AEBSF (hydrochloride), Bicyclol, and ABX464, exhibited similar transcriptome profiles and merged into the same cluster as the D group (Figure 3B). Hence, we labelled these four compounds as S2 while the other six compounds were named S1.

2.5. Expression of phoP-Associated Pathways upon Compound Treatment

PhoP was differentially expressed between S1 and the other groups (two-way ANOVA, p < 0.05 vs. S2 and Control and p < 0.001 vs. D), while phoR was significantly downregulated in S1 when compared with D (two-way ANOVA, p < 0.05) (Figure 3C). This indicated that the compounds in S1 could effectively downregulate phoP expression in M. bovis BCG. Next, the expression of phoP and its downstream gene network was investigated specifically. Interestingly, in addition to phoP, the related genes in the associated pathways were consistently downregulated when M. bovis BCG was treated with two compounds, ST-193 (B4) and ST-193 (hydrochloride) (B6) (Figure 3D). This implicated that these two compounds might dysregulate the entire phoP-associated pathways.
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Trifluoperazine (dihydrochloride) 12.5 25 25 50 25 50 25 ... oride) (B6) (Figure 3D). This implicated that these two compounds might dysregulate the entire phoP-associated pathways.

Figure 3. Cont.

Figure 3. RNA-Seq transcriptome analysis and representative genes involved in the phoP-pathway. (A) Principal component analysis (PCA) of BCGs after treatment with 16 compounds in 3 groups based on the gene expression detected in all samples. (B) Heatmap showing a clustering analysis of gene expression levels for the three groups of BCGs (p < 0.05, FC > 2 or < 0.5). (C) Expression of phoP and phoR in S1, S2, D, and the control groups. Data are presented as means ± SD (n = 6 for S1 and D, n = 4 for S2, and n = 1 for the control group) and were analyzed using a two-way ANOVA, * p < 0.05, *** p < 0.001, while those without a label have no significances. (D) The expression of genes regulated by phoP/R in BCGs after treatment with the 16 compounds.
2.6. Molecular Regulation Associated with the Anti-Virulence Process

To identify differentially expressed genes (DEGs) between the S and D groups, genes with at least a 2-fold difference and an adjusted \( p < 0.05 \) were selected. Altogether, 52 DEGs (27 upregulated and 25-downregulated) were identified in groups D vs. S (Figure 4A, Supplementary Table S6A). Moreover, for the DEGs between the D and S2 groups, only one gene, \( \text{phoY1} \), was identified (Figure 4B, Supplementary Table S5B). Next, a gene ontology (GO) enrichment analysis was performed to reveal the important biological processes and molecular functions dysregulated between the S and D groups. We demonstrated that genes involved in multidrug resistance mechanisms (efflux pump), DNA repair system, PPE family protein, and polyketide synthesis were downregulated in the dead group, while genes related to the DNA repair process and cell wall biogenesis were downregulated in the survival (S) group (Figure 4C, Supplementary Table S6A). The results highlighted the importance of these processes during anti-virulence.

![Figure 4](image_url)

**Figure 4.** Molecular regulation associated with the anti-virulence process. (A) Volcano plot of the DEGs between D and S. (B) Volcano plot of the DEGs between D and S2. (C) Molecular functions and biological processes enriched in the DEGs for the survival groups.

2.7. Prediction of Drug Targets in the Dead Group

As described above, six compounds exhibited bactericidal activity against \( M. \text{bovis} \) BCG. The DEG profiles were investigated to identify the potential drug targets of these compounds. Some common downregulated genes (logFC \( < -2 \) and FDR \( < 0.1 \) when compared with the DMSO control sample), including \( \text{cysA2}, \text{frdC}, \) and \( \text{glpD2}_2 \), were identified when \( M. \text{bovis} \) BCG was treated with Ebselen (A3), NH125 (A9), and Shikonin (B2) (Supplementary Table S6B). It should be noted that the sequences of \( \text{cysA2}, \text{frdC}, \) and \( \text{glpD2}_2 \) in \( M. \text{bovis} \) BCG were almost 100% identical to those in \( M. \text{tuberculosis} \) H37Rv. It is consistent with our observation in phenotypic drug susceptibility tests that Ebselen,
NH125, and Shikonin shared very similar MICs and MBCs against the *M. bovis* BCG and *M. tuberculosis* strains. Therefore, cysA2, frdC, and glpD2 were considered as potential drug targets of these small compounds.

### 2.8. THP-1 Cytotoxicity of the Small-Molecule Compounds

The cytotoxicity of the 16 selected small compounds was determined using a THP-1 macrophage. Overall, five compounds were considered as non-cytotoxic to human macrophages given that their CC₅₀ were greater than 200 µM, the highest achievable concentration in this study (Figure 5, Supplementary Table S7). Of these five compounds, ST-193 and ST-193 (hydrochloride) were shown to dysregulate the expression of most genes in the *phop*-associated pathways without inhibiting the in vitro growth of *M. bovis* BCG, suggesting that they were potential anti-virulence agents in anti-TB therapy. Moreover, among the compounds that had a bactericidal effect on *M. tuberculosis*, only Ebselen had the CC₅₀ (>200 µM) greater than its MIC (50 µM) and MBC (100 µM) against the *M. tuberculosis* complex. It was, therefore, considered as a potential antibiotic for *M. tuberculosis*.

**Figure 5.** LDH results in 16 compounds in THP-1 cells. (A) Cell viability after being treated with drugs in the S1 group. (B) Cell viability after being treated with drugs in the S2 group. (C) Cell viability after being treated with drugs in the D group. Data are presented as means ± SD (n = 3).
3. Discussion

Repurposing bioactive small-molecule compounds has been suggested as a new therapeutic approach against M. tuberculosis infection. Unlike previous studies, which focused on one or several compounds [12], we examined the anti-TB potency of a library of 320 small-molecule compounds with antiviral activity. As antiviral agents, these compounds are expected to effectively penetrate through the host cell members, which is an important feature of the drugs used to treat intracellular pathogens, such as the M. tuberculosis complex. In addition to identifying new candidates of antibiotic from these compounds, this study aimed to discover anti-virulence agents for M. tuberculosis that impaired the bacterial virulence factors without killing the organisms [11]. Notably, the development of anti-virulence drugs requires an in-depth understanding of the roles that diverse virulence factors have in disease processes. Previous studies from our team and other research groups suggested that phop is essential for the intracellular growth of the M. tuberculosis complex inside macrophages [14,15]. Disruption of phop could impair intramacrophage and facilitate the host’s immune system to eradicate the bacteria.

Instead of individual measurement of phop expression using RT-qPCR, we developed a lux-based promoter-reporter screening platform to select potential agents that can inhibit phoP promoter activity. This platform enabled the real-time measurement of phop expression in viable M. bovis BCG culture in response to the compound challenge, in terms of lux signal, in a batch of 96 samples. In the screening platform validation, we successfully demonstrated that ETZ could reduce the RNA quantity of both phop and lux signals, indicating the luminescence generated by the phoP promoter-reporter plasmid, pMV306PhoP+Lux, corresponded to the phop expression in M. BCG bovis.

By using our screening platform, 16 out of 320 (5.0%) small-molecule compounds were identified as potential candidates against M. tuberculosis infection. Surprisingly, six of them: trifluoperazine (dihydrochloride), elvitegravir, NH125, ebselen, letrozuril, and shikonin did not show a decrease in phop gene expression and directly eliminated M. bovis BCG. The decrease in their lux signals was possibly caused by bacterial death. In the molecular regulation analysis, we highlighted that dysregulation of the multidrug resistance mechanisms, DNA repair system, PPE family protein, and polyketide synthesis would determine M. tuberculosis death. Among the six compounds, only Ebselen exhibited a CC50 greater than its MIC and MBC against the M. tuberculosis complex, including the H37Rv, MDR-MTB and XDR-MTB strains. Our RNA-seq analysis identified that Ebselen displayed bactericidal activity in M. bovis BCG through cysA2 and glpD2 downregulation, which are the major regulators of sulfur and glycerol metabolism, respectively. CysA2 is an essential regulator in the sulfur assimilation pathway [17], and its dysregulation could impair M. tuberculosis survival in macrophages [18]. In parallel, a previous study demonstrated that the inhibition of glycerol metabolism by 2-aminooquinazolinones via glpD2 downregulation could kill M. tuberculosis in vitro [19]. Taken together, dysfunctions of sulfur and glycerol metabolism are possible drug mechanisms for Ebselen. It might also be effective on M. tuberculosis, as its sequences of cysA2 and glpD2 were almost 100% identical to those in M. bovis BCG. Hence, Ebselen could be considered as a potential antibiotic for the M. tuberculosis complex. In addition, it is interesting to highlight that phoY1, which controls phosphate sensing in M. tuberculosis, thereby affecting its susceptibility to antibiotics [20], was the only downregulated DEG between the dead group and the survival groups. This might indicate the importance of phoY1 on M. tuberculosis survival.

Among the remaining 10 survival candidates, 6 of them, Saquinavir, Clemizole (hydrochloride), Bay 41-4109 (racemate), ST-193, ST-193 (hydrochloride) and Saquinavir (Mesylate), exhibited a distinct transcriptome profile and a lower gene expression of phop. In the molecular regulation analysis, genes related to the DNA repair process and cell wall biogenesis in this group were significantly downregulated. It was found that the activity of the PhoP-related system interacted with genes involved in the fadD family, which regulates the fatty acid β-oxidative pathway [21]. Our results revealed that genes in the fadD family (fadD29 and fadD22) are of lower expression in M. bovis BCG after being treated.
with these six compounds. Disruption of fatty acid biosynthesis could inhibit bacterial differentiation and growth [22]. These six compounds are potential anti-virulence agents because they inactivated M. bovis BCG through DNA damage, cell wall destruction, and differentiation inhibition, thereby facilitating the host’s immune system to eradicate the bacteria. Interestingly, ST-193 and ST-193 (hydrochloride), with CC50 greater than 200 µM, dysregulated most genes in the phoP-associated network in M. bovis BCG, so they are the most desirable anti-virulence agents. In brief, many studies revealed that ST-193 effectively treats guinea pig models of Lassa virus infections [23–26]. ST-193 was found to suppress the conformational rearrangement of the arenavirus envelope glycoprotein, which is necessary for membrane fusion [27]. However, the drugs possibly affect more targets that we had not uncovered; therefore, the mechanism and effectiveness of ST-193 and ST-193 (hydrochloride) against M. tuberculosis warrant further investigation.

For proof-of-concept, only 320 small compounds were screened in this study. Despite this small-scale screening, three (0.94%) compounds were identified as potential antibiotic and anti-virulence agents against M. tuberculosis. In the future, the discovery of more new candidates of anti-TB drugs is anticipated if more drug repurposing compounds are screened using the promoter-reporter platforms coupled with automatic high-throughput screening instruments. Current TB treatment relies on a synergistic combination of drugs administered for the desired time to ensure definitive non-relapsing cures and to avoid drug-resistant mutants [28]. In 2021, the World Health Organization (WHO) established a “Position statement on innovative clinical trial design for development of new TB treatments” to outline clinical trial designs [29]. Anti-virulence agents facilitate the development of new TB therapies and provide us an avenue to establish our innovative TB treatment in clinical trials. However, it is necessary to see if the anti-virulence agents have synergistic or antagonistic interactions with existing anti-TB drugs. Fractional inhibitory concentrations of the compounds and the current anti-TB drugs should be determined by checkerboard assay in future studies.

In conclusion, our study successfully identified Ebselen as the most desirable antibiotic, while ST-193 and ST-193 (hydrochloride) were proposed to be potential candidates of anti-virulence for the M. tuberculosis complex.

4. Materials and Methods

4.1. Bacterial Strains

The Mycobacterium bovis vaccine strain, bacille Calmette-Guérin (BCG)-1 (Russia) (M. bovis BCG), which was used for the screening of small compounds, was collected from Queen Mary Hospital, Hong Kong. The organism was revived using Middlebrook 7H9 broth and 7H10 agar including OADC (10%), glycerol (0.2% in 7H9 broth and 0.5% in 7H10 agar), sodium pyruvate (4.4 mg/mL), and pancreatic digest of casein (1mg/mL) at 37 °C for 14 days.

M. tuberculosis H37Rv and two drug-resistant clinical isolates of M. tuberculosis, namely HKU1462 (an MDR-TB strain co-resistant to isoniazid and rifampicin) and WC274 (an XDR-TB strain resistant to isoniazid, rifampicin, pyrazinamide, ethambutol, streptomycin, fluoroquinolones, and injectable aminoglycoside), collected from the same hospital, were used to determine the inhibitory and bactericidal activities against M. tuberculosis. The resuscitation of the archived clinical isolates was performed using Lowenstein–Jensen (LJ) medium. The detailed drug susceptibility pattern of the clinical isolates is shown in Supplementary Table S1.

4.2. Construction of Lux-Based Promoter-Reporter Plasmids and a Negative Control

The Mycobacterial reporter plasmid pMV306G13+Lux was obtained from Brian Robertson and Siouxsie Wiles (Addgene plasmid #26160, Figure 1A) [30], including a kanamycin-resistant gene, luxCDABE, and a promoter G13 located between the NotI and NcoI restriction enzyme sites. The promoters P_{hsp}60, deriving from pSMT3, were inserted into pMV306 vectors to form the integrating vectors -pMV306hsp. Subsequently, the lux operon (luxCDABE) from pMU1*
was cloned into pMV306hsp, generating plasmids of pMV306hsp+Lux. Finally, G13 of *M. marinum* replaced *P*._hsp60 in plasmid pMV306hsp+Lux to form lux-based promoter-reporter plasmids pMV306G13+LuxAB+LuxCDE (pMV306G13+Lux) [30].

To construct a lux-based phoP promoter-reporter, pMV306G13+Lux and *phoP* promoters were digested with NcoI and NotI (New England Biolabs, Ipswich, MA, USA), followed by DNA purification (QIAGEN, Hilden, Germany) and ligation. The purified plasmids (100 ng per reaction) and the target promoters were incubated with T4 DNA ligase (New England BioLabs, USA) overnight at room temperature. The G13 promoter in pMV306G13+Lux was eventually replaced by the *phop* promoter (Figure 1B, Supplementary Table S2A) to generate pMV306PhoP+Lux.

The negative control pMV306Adaptor+Lux was similarly created by replacing the G13 promoter with an adaptor (5′-GGCCGCTTAGCTTTC-3′, a random sequence without the promoter activity). All plasmids used in this study are listed in Supplementary Table S2B.

### 4.3. Transformation of Lux-Based Reporter Plasmids into *M. bovis* BCG

Lux-based promoter-reporter plasmids (pMV306PhoP+Lux) and negative control (pMV306Adaptor+Lux) were transformed into *M. bovis* BCG by electroporation. The sequences of the plasmid were confirmed by PCR-sequencing. The lux signal of transformed *M. bovis* BCG was validated by an IVIS Lumina imaging system (Perkin-Elmer, Shanghai, China).

### 4.4. Validating the Correlation of the Lux Signal of the Promoter-Reporter Screening Platform with *phop* Gene Expression in *M. bovis* BCG

Ethoxzolamide (ETZ) was shown to inhibit the PhoPR regulon in *Mycobacterium tuberculosis* via binding to *phop* promoter regions [31]. In this study, *M. bovis* BCG transformed with pMV306PhoP+Lux were treated with 200 µg/mL ETZ (experimental group) and DMSO (control group) at 37 °C for 24 h. The optical density via absorbance at 600 nm (OD$_{600}$) and lux signals from all samples were measured by a Benchmark Plus Microplate Spectrophotometer (BIO-RAD) and a VICTOR3 Multilabel Plate Reader (Perkin-Elmer), respectively. The OD$_{600}$ of the culture medium was used to eliminate the background noise. The statistical effect size (Z-factor) was calculated as follows: $Z = 1 - [(3SD of sample + 3SD of control)/(|mean of sample − mean of control|)]$, where sample is the lux signals after treatment with ETZ, while control is the lux signals without drug treatment. A Z-factor between 0.5 and 1.0 indicates an excellent assay [32].

For the luxA and *phop* expression analysis, total RNA was extracted from the experimental and control group for quantitative reverse transcription PCR (RT-qPCR). The fold-changes in gene expression of luxA and *phop* were calculated based on a housekeeping gene recX [33]. The primers used for RT-qPCR are listed in Supplementary Table S2C.

### 4.5. Screening Experiments

*M. bovis* BCG transformed with pMV306PhoP+Lux and pMV306Adaptor+Lux (negative control) were treated with 320 antiviral small compounds (MedChemExpress, USA) and three first-line anti-TB drugs (ethambutol, isoniazid, and rifampicin) (Supplementary Table S3). The assay was carried out in a 96-well plate. Each compound (2 µL, 10 mM) was dispensed in 100 µL of *M. bovis* BCG suspension (OD$_{600}$ ≈ 0.2) in triplicate and eventually had a concentration of 200 µM. The concentration of the antiviral compounds used to treat MTB was indicated by a previous study that applied 140 to 351 µM of anti-virulence drugs (L-ascorbic acid and 2P-AC) to treat MTB [8]. To eliminate the bactericidal effects of the three anti-TB drugs, their final concentrations were adjusted to two-fold lower than their MICs to *M. bovis* BCG, which were 3.8 µg/mL (ethambutol), 0.08 µg/mL (isoniazid), and 0.4 µg/mL (rifampicin) [34]. These compounds were dissolved in DMSO or water. Meanwhile, 100 µL of BCG suspensions without treatment were involved as drug-free control. OD$_{600}$ and lux were measured after 4 h of treatment. Those showing lux inhibition (CPS-photon count per second ≈ 0) were selected. The selected *M. bovis* BCG suspensions
were washed with 7H9 medium twice to remove residual compounds and then were inoculated on drug-free 7H10 medium, followed by incubation at 37 °C for 14 days to determine their viabilities.

4.6. Minimal Inhibitory Concentrations (MICs) and Minimal Bactericidal Concentrations (MBCs) against M. tuberculosis Complex

Compounds showing bactericidal effects on M. bovis BCG after 14 days of incubation were selected for further investigation. The MICs and MBCs of them against M. bovis BCG, M. tuberculosis H37Rv, HKU14621 (MDR clinical isolate), and WC276 (XDR clinical isolate) were determined using the microbroth dilution method according to the Clinical and Laboratory Standards Institute (CLSI) [35]. The selected compounds were diluted from 400 µM to 1.5625 µM using the “two-fold serial dilution” method. The MIC and MBC experiments were conducted in triplicate.

4.7. RNA-Seq Transcriptome Analysis

For RNA-seq analysis, untransformed M. bovis BCG (i.e., with no plasmid) was treated for 4 h with the selected compounds at a concentration of 200 µM or two-fold lower than their respective MICs if they exhibited inhibitory or bactericidal activity in previous MIC and MBC experiments. Total RNA was extracted with ribosomal RNA depletion after a 4 h treatment, followed by a strand-specific library (the bacterial cDNA library) construction with the quality control process. Subsequently, RNA-Seq was performed using the HiSeq X Ten system. Raw reads from RNA-seq were checked by FastQC v0.11.9 and trimmed by TrimGalore v0.6.7. The filtered clean reads were mapped to the reference genome (Mycobacterium bovis BCG strain Russia 368, complete genome) by HISAT2 (version 2.2.1). StringTie and ASprofile were used for transcript assembly and quantification, respectively. The data quality is shown in Supplementary Table S4A. Cufflinks software was then used to quantitate the transcripts and gene expression levels using mapped reads’ positional information on the gene. The gene expression levels were calculated in the form of fragments per kilobase million (FPKM) by GenomicFeatures (R package) and are listed in Supplementary Table S4B. Genes with undetected expression levels in all the samples were excluded in the following analysis. The calculated FPKM was used to perform a principal component analysis (PCA) by the R function pcromp. A three-dimensional figure with the top three principal components (PC1, PC2, and PC3) was generated by R. The differential mRNA expression was analysed by DESeq2 using Trinity v2.8.4 with default parameters [36]. Pairwise comparisons and a clustering analysis were performed using the Trinity v2.8.4 package (Biostars, New Taipei, Taiwan). Genes with at least a 2-fold change with adjusted $p < 0.05$ were identified as differentially expressed genes (DEGs). A gene ontology (GO) enrichment analysis was performed by the GENEONCOLOGY and PATRIC tools [37–40], and the chord plot was created by GO plot 1.0.2. Meanwhile, the mechanisms of the drugs that could kill M. tuberculosis were predicted and illustrated via the Kyoto Encyclopedia of Genes (KEGG) pathway database. In brief, significantly downregulated genes in M. bovis BCG treated with antibiotics ($\log_{10}PC < -2$ and FDR < 0.01 compared with the DMSO control sample) that probably are critical for M. tuberculosis metabolism were selected and predicted as potential drugs of M. tuberculosis elimination. No biological replicates were conducted for the RNA-Seq experiment.

4.8. Cell Viability Assay—LDH Assay

The THP-1 monocyte cell line (ATCC TIB-202) was cultured in triplicate at a density of $5 \times 10^4$ cells per well of a 96-well plate containing RPMI1640 medium (GIBCO, Waltham, MA, USA) supplemented with 5% (v/v) fetal bovine serum (GIBCO, USA) at 37 °C with 5% CO$_2$. The cells were then treated with 10 µL of sterile water and 10 µL of the compound (concentrations ranging from 0.2 µM to 200 µM) to determine the spontaneous LDH activity (control group one) and compound-treated LDH activity, respectively, using a CyQUANT™
LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Additionally, cells of control group two were treated with nothing to measure LDH activity.

4.9. Statistical Analysis

Data were expressed as means ± SD, and p < 0.05 was significant as measured by two-sample t-tests for the promoter-reporter validation and antiviral compound screening using GraphPad Prism (GraphPad Inc., San Diego, CA, USA). A two-way ANOVA was performed in the \textit{phoP} and \textit{phoR} expression investigation.

5. Conclusions

In conclusion, our study successfully identified Ebselen as the most desirable antibiotic, while ST-193 and ST-193 (hydrochloride) were proposed to be potential anti-virulence agents for the \textit{M. tuberculosis} complex.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antibiotics11030369/s1, Table S1: Mutations in HKU14621 and WC274; Table S2: Plasmid construction and validation; Table S3: List of anti-viral compound library and anti-TB drugs; Table S4: RNA-Seq transcriptome analysis; Table S5: List of DEGs identified between D and S1 samples and List of DEGs identified between D and S2 samples; Table S6: GO analysis of the DEGs, molecular functions and biological processes enriched in DEGs for the survived groups and five compounds significantly downregulated genes expression (logFC < −2, FDR < 0.1) in BCG Russia compared with DMSO control sample (no drug treatment), predicting as potential drug targets of MTB; Table S7: Cell viability and CC50 of 16 compounds treating THP-1 cells.

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