Substituted N-Phenyl-5-(2-(phenylamino)thiazol-4-yl)isoxazole-3-carboxamides Are Valuable Antitubercular Candidates that Evade Innate Efflux Machinery

Journal of Medicinal Chemistry

Article

pubs.acs.org/jmc

Substituted *N*-Phenyl-5-(2-(phenylamino)thiazol-4-yl)isoxazole-3-carboxamides Are Valuable Antitubercular Candidates that Evade Innate Efflux Machinery

Elisa Azzali,[†,‡,▽] Diana Machado,[§] Amit Kaushik,[∥,⊥] Federica Vacondio,[‡] Sara Flisi,[#] Clotilde Silvia Cabassi,[#] Gyanu Lamichhane,[∥,⊥] Miguel Viveiros,[§] Gabriele Costantino,[†,#,▽] and Marco Pieroni*[,†,‡]

[†]P4T group, and [‡]Department of Food and Drug, University of Parma, Parco Area delle Scienze 27/A, 43124 Parma, Italy

[§]Global Health and Tropical Medicine, GHTM, Instituto de Higiene e Medicina Tropical, IHMT, Universidade Nova de Lisboa, UNL, Rua da Junqueira, 100, 1349-008 Lisbon, Portugal

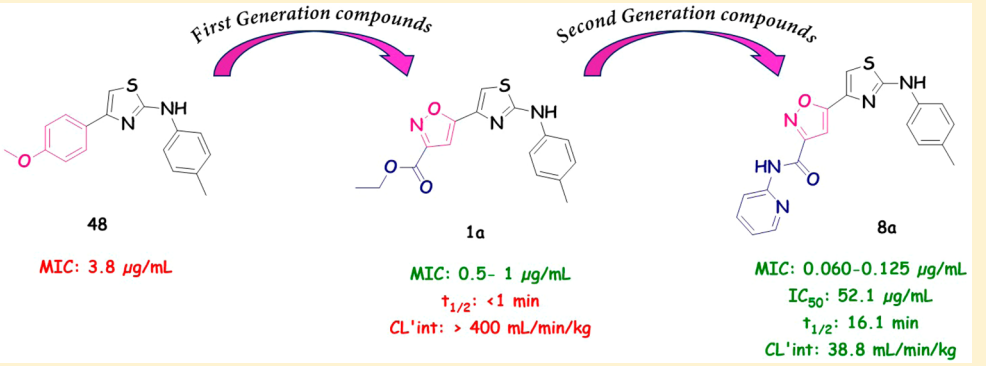
[∥]Center for Tuberculosis Research, Department of Medicine, Johns Hopkins University, 1503 East Jefferson Street, Baltimore, Maryland 21231-1002, United States

[⊥]Taskforce to Study Resistance Emergence & Antimicrobial development Technology, Department of Medicine, Johns Hopkins University, Baltimore, Maryland 21287, United States

[#]Department of Veterinary Science, University of Parma, via del Taglio 10, 43126 Parma, Italy

[▽]Centro Interdipartimentale Misure (CIM) 'G. Casnati', University of Parma, Parco Area delle Scienze 23/A, 43124 Parma, Italy

Supporting Information



ABSTRACT: Tuberculosis remains one of the deadliest infectious diseases in the world, and the increased number of multidrug-resistant and extremely drug-resistant strains is a significant reason for concern. This makes the discovery of novel antitubercular agents a cogent priority. We have previously addressed this need by reporting a series of substituted 2-aminothiazoles capable to inhibit the growth of actively replicating, nonreplicating persistent, and resistant *Mycobacterium tuberculosis* strains. Clues from the structure−activity relationships lining up the antitubercular activity were exploited for the rational design of improved analogues. Two compounds, namely *N*-phenyl-5-(2-(*p*-tolylamino)thiazol-4-yl)isoxazole-3-carboxamide **7a** and *N*-(pyridin-2-yl)-5-(2-(*p*-tolylamino)thiazol-4-yl)isoxazole-3-carboxamide **8a**, were found to show high inhibitory activity toward susceptible *M. tuberculosis* strains, with an MIC_{90} of 0.125−0.25 μg/mL (0.33−0.66 μM) and 0.06−0.125 μg/mL (0.16−0.32 μM), respectively. Moreover, they maintained good activity also toward resistant strains, and they were selective over other bacterial species and eukaryotic cells, metabolically stable, and apparently not susceptible to the action of efflux pumps.

INTRODUCTION

Tuberculosis (TB), a highly contagious respiratory disease that results from infection with *Mycobacterium tuberculosis* (Mtb), was recently declared as the number one infectious disease as it killed more people than HIV/AIDS or malaria.[1] According to the World Health Organization (WHO), in 2015, there were an estimated 10.4 million new TB cases and 1.4 million people died from this illness.[2,3] Eleven percent of all the new cases occurred in HIV infected patients, among which TB is the leading cause of death, especially in Africa. While it is clear from these statistics that existing treatment for TB is inadequate, the growing number of drug-resistant Mtb strains is an additional concern that needs to be addressed.[4] Resistant Mtb strains are generally grouped into three distinct phenotypes: (a) multidrug resistant TB (MDR-TB), resistant to at least isoniazid (INH)

Received: June 1, 2017
Published: July 27, 2017

DOI: 10.1021/acs.jmedchem.7b00793
J. Med. Chem. XXXX, XXX, XXX−XXX

and rifampicin (RIF), (b) extensively drug-resistant TB (XDR-TB), resistant to INH, RIF, fluoroquinolones and, at least, one of the three injectable second-line drugs, and (c) totally drug-resistant TB (TDR-TB), a nonconsensual designation for strains that are virtually untreatable since have lost susceptibility to all of the molecules belonging to the anti-TB arsenal.[5−8] The WHO has estimated that about 3.7% of the new cases, and 20% of those previously treated, are sustained by MDR-TB strains and about 9% of the patients suffering from MDR-TB are eventually infected with XDR-TB. India, China, and the Russian Federation accounted for 45% of the combined 580000 drug-resistant cases recorded in 2015. Since the tight trade ties among these countries, United States, and the European Union, the spread of the disease also in the developed countries is a highly realistic matter of concern.[2]

The existing TB treatment requires daily intake of multiple drugs for, at least, six months.[9] The long-term use of these drugs is often associated with the onset of several and severe side effects that lead to patient noncompliance. In turn, this leads to the appearance of resistant strains, which require the use of less effective and more toxic drugs for a longer period and drastically increase the financial burden for healthcare, jamming the treatment of TB in a vicious circle of difficult resolution.

Since the introduction of RIF in 1967,[10] bedaquiline[11,12] is the only new chemical entity (NCE) developed for the treatment of TB that has reached the market, although its use is restricted to the treatment of MDR- and XDR-TB. Unfortunately, after its introduction in the clinical practice, an unexpected number of abnormal deaths has been reported, probably due to the serious side effects associated with significant cardiac arrhythmia.[13−15] Delamanid, a nitroimidazole inhibiting mycolic acid biosynthesis, is another novel drug that has received conditional approval for MDR-TB by the European Medicines Agency in 2014.[16] Also in this case, severe side effects such as cardiac arrhythmia and general central nervous system (CNS) toxicity, especially when used in combination with INH or fluoroquinolones,[17] have cooled down the initial enthusiasm caused by the introduction of this novel anti-TB agent in therapy. In addition, mutations in the Mtb genome causing resistance to bedaquiline and delamanid have been recently documented.[18] Therefore, efforts to develop novel anti-TB therapeutic options that are safe and effective against drug-resistant Mtb are still needed.

After decades of oblivion, the emerging TB drug pipeline is nowadays nourished with a number of novel molecules that were developed following different drug discovery approaches.[19,20] Some of these compounds were prepared starting from known inhibitors of old TB targets such as RNA polymerase, as in the case of rifapentine from RIF.[21] On a similar vein, second-line anti-TB therapeutics such as fluoroquinolones,[22,23] although conceived to treat infections brought by Gram-positive and Gram-negative microorganisms, have recently entered the clinical trials to evaluate their use as first-line agents.[24,25] However, it is debatable whether these approaches based on "drug-repositioning" might lead to long-term results, as the occurrence of cross-resistance is rather predictable. Target-based approaches have produced encouraging results, especially when directed toward the so-called promiscuous targets, proteins whose functionality can be inhibited by more than one chemical entity.[26] Two representative Mtb promiscuous targets, that have been intensively studied in the last years, are decaprenylphosphoryl-β-D-ribose 2′-oxidase (DprE1),[27] and one of the mycobacterial membrane transport proteins, large (MmpL3),[28] both inhibited by several different chemical classes of compounds.[29−37] Although the target-based approach is a valuable method for drug identification, it does not take under consideration the thick Mtb cell wall and the role of efflux machinery, leading sometimes to disappointing discrepancies between the biochemical and the whole-cell phenotype assays. For these reasons, phenotypic high-throughput screening (HTS), followed by ligand-based optimization, remains the most profitable strategy to identify novel antibacterial agents in general and novel anti-TB leads in particular.

Recently, the whole-cell phenotypic screening of an in-house chemical library led us to identify a number of molecules embodying a 2-aminothiazole scaffold endowed with an interesting anti-TB activity.[38] Although the anti-TB activity of aminothiazoles and benzothiazoles was already reported elsewhere,[39,40] no attempt was made to establish a reliable structure−activity relationship (SAR) for this chemical class. This prompted us to synthesize several structurally related derivatives in which the 2-aminothiazole core was kept intact whereas different substituents were introduced at the positions C-4, C-5, and at the 2-amino group.

Some of the 2-aminothiazoles synthesized (Figure 1), **47** and **48**,[38] exhibited good inhibitory activity toward both the actively

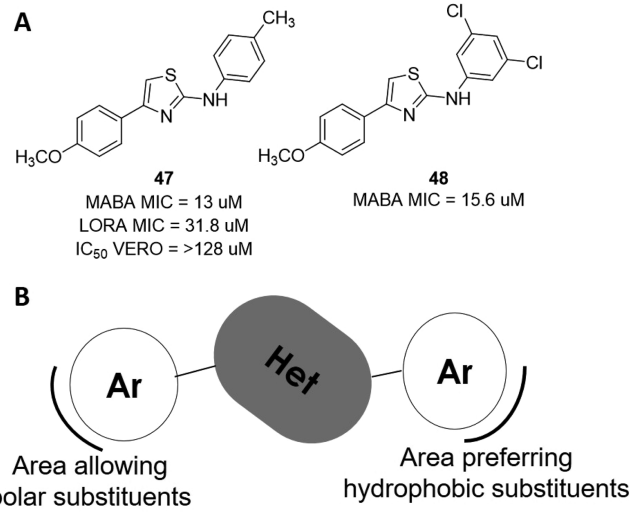


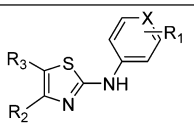
Figure 1. (A) Structure of compounds **47** and **48**, along with some selected biological data. (B) Sketched pharmacophore for the anti-TB 2-aminothiazoles.

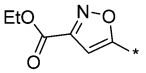
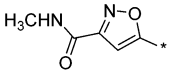
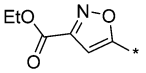
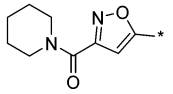
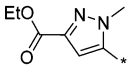
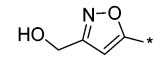
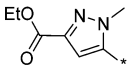
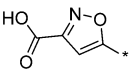
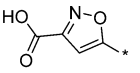
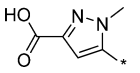
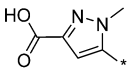
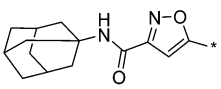
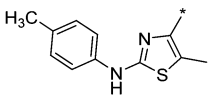
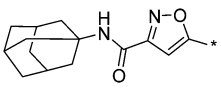
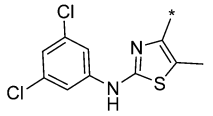
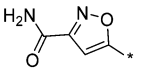
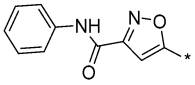
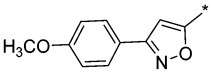
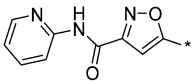
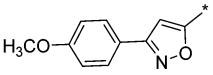
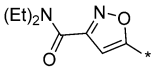
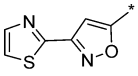
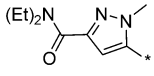
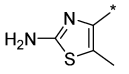
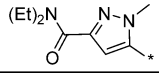
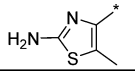
replicating Mtb strains and the nonreplicating persistent (NRP) mycobacteria in a low oxygen recovery assay (LORA). More importantly, when evaluated against a panel of single-drug resistant (SDR) Mtb strains, representative compounds maintained the same inhibitory activity as toward the wild-type, indicating that these substituted 2-aminothiazoles were acting with a mechanism of action different from those of the currently marketed drugs,[38] therefore encouraging further investigation around this nucleus.

By leveraging the SAR data collected and the hypothetical pharmacophore designed, we herein report the design and synthesis of a novel series of anti-TB derivatives based on the 2-aminothiazole scaffold, with two compounds, **7a** and **8a**, showing a remarkable anti-TB activity in the submicromolar range. To expand the set of information already reported, an in-

DOI: 10.1021/acs.jmedchem.7b00793
J. Med. Chem. XXXX, XXX, XXX−XXX

Chart 1. Structure of the Synthesized Final Compounds[a]



Comp	R_1	R_2	R_3	X	Comp	R_1	R_2	R_3	X
1a	4-CH_3		H	C	**11a**	4-CH_3		H	C
1b	3,5-Cl		H	C	**12a**	4-CH_3		H	C
2a	4-CH_3		H	C	**13a**	4-CH_3		H	C
2b	3,5-Cl		H	C	**14**	4-CH_3	$C(O)CH_3$	CH_3	C
3a	4-CH_3		H	C	**15**	3,5-Cl	$C(O)CH_3$	CH_3	C
3b	3,5-Cl		H	C	**16a**	4-CH_3		H	C
4a	4-CH_3		H	C	**16b**	3,5-Cl		H	C
4b	3,5-Cl		H	C	**16c**	H		H	N
5a	4-CH_3		H	C	**17a**	4-CH_3		H	C
5b	3,5-Cl		H	C	**18b**	3,5-Cl		H	C
6a	4-CH_3		H	C	**19b**	3,5-Cl		H	C
7a	4-CH_3		H	C	**20a**	4-CH_3		H	C
8a	4-CH_3		H	C	**20b**	3,5-Cl		H	C
9a	4-CH_3		H	C	**21a**	4-CH_3		H	C
10a	4-CH_3		H	C	**22a**	4-CH_3		H	C
10b	3,5-Cl		H	C	**22b**	3,5-Cl		H	C

[a]Indicates the point of attachment.

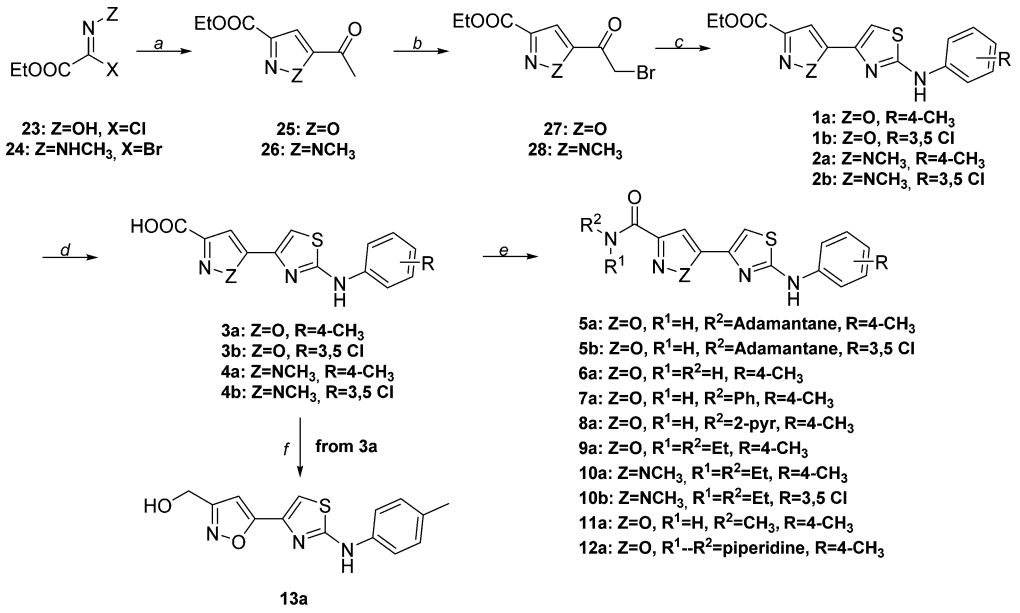
depth study that included several other biological assays was carried out for these leads; in particular, their cytotoxicity toward human monocyte-derived macrophage (HMDM), their selectivity over other bacterial species and fungi, their metabolic profiles in human liver microsomes (HLM), and their capability to interact with Mtb efflux systems, were measured. More importantly, the activity toward a set of resistant Mtb phenotypes (MDR- and XDR-TB) was as well evaluated.

RESULTS AND DISCUSSION

Rational Design. Compounds **47**[38] and **48**,[38] previously reported by our group,[38] are representative examples of a series of substituted 2-aminothiazoles endowed with lead-like anti-TB characteristics. The main structural feature of these molecules (Figure 1) is the presence of two aromatic rings, suitably substituted, connected to each other through a five-membered heterocycle such as the 2-aminothiazole. The anti-TB efficacy

DOI: 10.1021/acs.jmedchem.7b00793
J. Med. Chem. XXXX, XXX, XXX−XXX

Scheme 1. Preparation of the Compounds 1−13[a,b]



[a]Reagents and conditions: (a) 3-butyn-2-one, triethylamine, benzene, 60 °C, 1 h (55−68%); (b) Br_2, chloroform, AcOH, 50 °C, 1 h (56−91%); (c) 1-(*p*-tolyl)thiourea or 1-(3,5-dichlorophenyl)thiourea, EtOH, reflux, 2 h (40−95%); (d) LiOH, THF/MeOH/H_2O, rt, 2 h (77−98%); (e) R-NH_2, TBTU, EDC-HCl, TEA, DMF, rt, 2 h (25−85%) or NH_4OH, rt, overnight, 82%; (f) **3a**, $NaBH_4$, MeOH, rt, 30 min, 93%. [b]For details, see Chart 1.

of this structural pattern was corroborated by the activity of a series of diarylimidazoles, reported by our group, in which the two aromatic rings were connected through a different five-membered ring such as the imidazole.[41] After several rounds of modifications, we were able to establish that the phenyl ring attached to the 2-amino group tolerated the presence of lipophilic functional groups (i.e., halogens, small alkyls), whereas in the phenyl ring attached at the C-4, the presence of polar substituents (i.e., the methoxy moiety) conferred good activity along with reduced cytotoxicity.[38] With the aim of further validating the proposed pharmacophore, we planned to synthesize a novel series of 2-aminothiazoles in which the aromatic ring at the position C-4 of the 2-aminothiazole was replaced by polar heterocyclic structures, chosen depending on the different physicochemical properties and the synthetic accessibility. Therefore, a pyridine, an ethyl isoxazole-3-carboxylate, an ethyl 1-methylpyrazole-3-carboxylate, an 1,2,3-triazole, and a 2-amine-5-methylthiazole were selected as C-4 appendages.

Pyridine was prepared as it is a known bioisostere of benzene, and it is the simplest six-membered aromatic ring containing a heteroatom. 4-(2-Pyridyl)-2-aminothiazoles have already been disclosed as valuable anti-TB chemotypes[42] and partially developed with respect to their SAR.[40,43] To add further information, in the small set of compounds prepared, the pyridine nitrogen is placed at the *para*-position to conform to the position of the polar methoxy moiety in compounds **47** and **48**.

The ethyl isoxazole-3-carboxylate moiety was chosen in first instance as it has already proved to be a valuable moiety in order to promote good anti-TB activity when properly substituted.[44−48] Moreover, while this work was being prepared, GlaxoSmithKline disclosed a number of anti-TB chemotypes suitable for further investigation, among which was a singleton structurally similar to our derivatives **1a** and **1b**.[49] Finally, the synthesis of the isoxazole nucleus can be easily achieved through an efficient 1,3-dipolar cycloaddition protocol, and the ethyl carboxylate obtained can be further functionalized.

In a similar way, also other heterocycles such as the ethyl 1-methylpyrazole-3-carboxylate and the 1,2,3-triazole were selected because of their presence in a number of molecules recently reported to have good anti-TB activity. A series of triazole derivatives was synthesized to target InhA, showing inhibition of Mtb growth in the low micromolar range.[50] Also in the case of the pyrazole, numerous derivatives embodying this structural motif were found to display a broad spectrum of pharmaceutical activities, among which the inhibition of Mtb growth has been recently reviewed.[51] In addition to that, the synthesis of these derivatives relies on the same synthetic protocol employed for the preparation of isoxazoles and starts from the same chemicals.

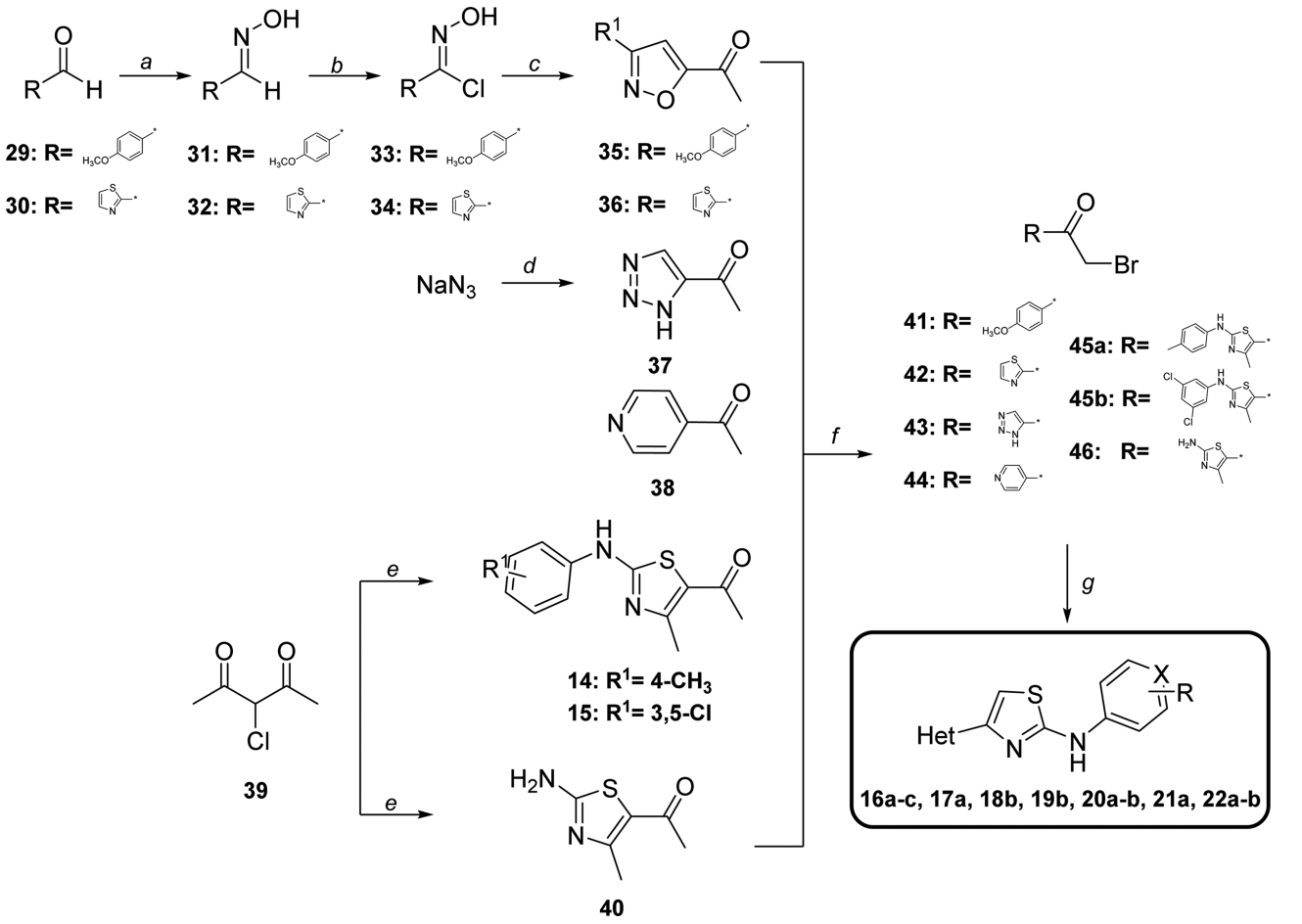
Finally, a substituted 5-methyl-2-aminothiazole was attached to position C-4 to obtain a series of bithiazoles. Although the biological properties of numerous 2-aminothiazoles are well established, little is known about the biological activity of bithiazoles in general and as anti-TB agents in particular. Among other biological properties,[52] bithiazoles are reported to be inhibitors of DNA-gyrase,[53] a well validated target for the treatment of TB. Indeed, along with the quinolones mentioned above, many other molecules targeting DNA-gyrase, belonging to different chemical series (aminopyrazinamides, thiazolopyridine ureas, thiazole-aminopiperidine hybrid analogues), have been recently reported as promising anti-TB chemotypes.

At the 2-amino group of the 2-aminothiazole, either the *p*-tolyl or the 3,5-dichlorophenyl appendages were maintained because they have previously been demonstrated to promote good anti-TB characteristics (see compounds **47** and **48**).[38]

Chemistry. All of the target compounds evaluated (**1a,b**, **2a,b**, **3a,b**, **4a,b**, **5a,b**, **6−9a**, **10a,b**, **11−13a**, **14**, **15**, **16a−c**, **17a**, **18b**, **19b**, **20a,b**, **21a**, and **22a,b**), reported in Chart 1, are characterized by the presence of the 2-aminothiazole moiety,

DOI: 10.1021/acs.jmedchem.7b00793
J. Med. Chem. XXXX, XXX, XXX−XXX

Scheme 2. Preparation of the Compounds 16−22[a,b]



[a]Reagents and conditions: (a) $NH_2OH{\cdot}HCl$, 1N NaOH, EtOH, H_2O, rt; 30 min, (91−99%); (b) NCS, DMF, rt, 1 h, 100% or NCS, pyridine, DCM, 40 °C, 3 h, 99%; (c) 3-butyn-2-one, triethylamine, benzene, 60 °C, 1 h (67−68%); (d) 3-butyn-2-one, DMF, 60 °C, 1 h, 30%; (e) proper thiourea, EtOH, reflux, (40−73%); (f) Br_2, chloroform, AcOH, 50 °C, 1 h, (35−91%) or Br_2, 1,4-dioxane/diethyl ether, rt, 1 h, 50% or Br_2, HBr solution, 1,4-dioxane, 50 °C, 1 h, (15−70%) see experimental section for details; (g) proper arylthiourea, EtOH, reflux, 2 h, (24−84%). [b]For details, see Table 1.

synthesized according to the established Hantzsch protocol, refluxing the appropriate α-bromoketone with the proper thiourea in absolute ethanol (Schemes 1 and 2).[54] When not commercially available, thioureas are prepared by refluxing the corresponding anilines and ammonium thiocyanate in a solution of 1N hydrochloric acid. Depending on the nature of the heterocycle, the desired α-bromoketone intermediates were obtained with different procedures. The other synthetic pillar for the compounds reported in this study is the 1,3-dipolar cycloaddition, used to prepare the isoxazole, triazole, and 1-methylpyrazole intermediates. The isoxazole (Scheme 1) was prepared by reacting the nitrile oxide generated in situ by the properly substituted chloroxime and 3-butyn-2-one. When not commercially available, the chloro-oximes were prepared by condensation of the proper aldehyde with hydroxylamine hydrochloride, followed by the treatment of the resulting aldoxime with *N*-chlorosuccinimide, to give intermediates **23** and **24** (Scheme 2). In a similar way, the synthesis of the pyrazole ring was carried out according to the procedure of Oh et al. by reacting a nitrile-imine dipole, generated by base-promoted dehydrohalogenation of hydrazonoyl halide and the 3-butyn-2-one.[55] The hydrazonoyl halide **24** was prepared by condensation of methylhydrazine and ethyl glyoxylate, followed by the bromination of the resulting hydrazine with *N*-bromosuccinimide in a mixture of ethyl acetate and dichloromethane (Scheme 1). Finally, the triazole ring **37** was prepared by "click" reaction of 3-butyn-2-one with sodium azide stirred in DMF at 60 °C. All of the above-reported ketones (**14**, **15**, **25**, **26**, **35**−**38**, **40**) were then brominated with bromine and glacial acetic acid in chloroform at 50 °C, affording the α-bromoketones (**27**, **28**, **41**−**46**) in good yields, although sometimes with traces of the corresponding α-dibromoketones as collateral products. The synthesis of the bithiazoles started with the reaction of 3-chloropentane-2,4-dione and the appropriate thiourea in absolute ethanol at reflux. The reaction afforded in high yields the ketones **14** and **15** that were brominated according to a procedure slightly different from that reported, that is by using bromine and HBr solution in 1,4-dioxane at 50 °C. The ethyl ester moiety was hydrolyzed to carboxylic acid with LiOH in a solution of THF/MeOH/H_2O at room temperature.[56] For the synthesis of amides, the suitable carboxylic acids were first activated with 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC) and 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) and then coupled with the appropriate amine at room temperature using trimethylamine as the base to yield the corresponding amides **5** and **7**−**12** in good overall yields.[57] Amide **6a** was obtained by stirring the corresponding ethyl

DOI: 10.1021/acs.jmedchem.7b00793
J. Med. Chem. XXXX, XXX, XXX−XXX

Table 1. Preliminary Activity of the Compounds Synthesized against Wild-Type *M. tuberculosis* H37Rv

compd	MIC_{90}[a] (μg/mL)	compd	MIC_{90} (μg/mL)	compd	MIC_{90} (μg/mL)	compd	MIC_{90} (μg/mL)
1a	<4[b]	**5a**	>64	**11a**	>64	**17a**	4−8
1b	4−8	**5b**	>64	**12a**	16−32	**18b**	>64
2a	16−32	**6a**	>64	**13a**	16−32	**19b**	>64
2b	>64	**7a**	<4[b]	**14**	>64	**20a**	4−8
3a	>64	**8a**	<4[b]	**15**	>64	**20b**	>64
3b	>64	**9a**	16−32	**16a**	>64	**21a**	>64
4a	>64	**10a**	>64	**16b**	>64	**22a**	16−32
4b	>64	**10b**	>64	**16c**	>64	**22b**	8−16
INH[c]	0.03−0.06						

[a]Minimun inhibitory concentration, determined by microdilution. [b]Compounds retested to evaluate the actual activity (see Table 2). [c]Isoniazid.

ester derivative **3a** and NH_4OH overnight at room temperature whereas, starting from the same parent compound, alcohol **13a** was obtained after reduction with sodium borohydride.

Evaluation of the Antimycobacterial Activity. A total of 32 compounds (Chart 1, **1a,b**, **2a,b**, **3a,b**, **4a,b**, **5a,b**, **6**−**9a**, **10a,b**, **11**−**13a**, **14**, **15**, **16a**−**c**, **17a**, **18b**, **19b**, **20a,b**, **21a**, **22a,b**) were synthesized and tested for their ability to inhibit the growth of actively replicating Mtb strain H37Rv in a microdilution assay (see biological methods). The whole set of compounds prepared can be ideally divided into two groups. In the "first-generation", the aim was to identify those heterocycles conferring good activity; feedback from this preliminary evaluation drove the synthesis of improved "second-generation" analogues. Finally, the most promising compounds were evaluated for their stability in human liver microsomes (HLM), toxicity against human monocyte derived macrophage (HMDM), interaction with efflux pumps, and selectivity over other microorganisms, including MDR and XDR-TB strains. While 21 compounds failed to inhibit the growth of Mtb at concentrations up to 64 μg/mL, the minimum inhibitory concentration (MIC) of 12 compounds were within a therapeutically interesting range, with some of them exhibiting MIC_{90} lower than 1 μg/mL.

First-Generation Compounds. The first round of synthetic efforts led to 10 derivatives (**1a,b**, **2a,b**, **16a**−**c**, **17a**, **18b**, **19b**, **22a,b**) that were evaluated toward actively replicating Mtb. The 4-(pyridin-4-yl)-2-aminothiazoles **16a**−**c** and the 4-(triazol-5-yl)-2aminothiazole **19b** were found to be devoid of any anti-TB activity (Table 1, MIC_{90} > 64 μg/mL). It is well-known that a decrease in the ClogP, especially in the case of anti-TB agents, is often accompanied by the loss of activity because of poor cell membrane penetration. However, because of the remarkable activity of some of the derivatives described in this work, for these compounds the lack of activity is probably due to weak interactions with the molecular target. The substituted 4-(isoxazol-5-yl)-2-aminothiazole derivatives **1a** and **1b** were found to show good activity (Table 1, MIC_{90} < 4 μg/mL and 4−8 μg/mL, respectively). Substitution of the oxygen atom with an *N*-methyl moiety led to a sharp reduction of the anti-TB potency, as in the case of compounds **2a** and **2b** (MIC_{90} = 16−32 μg/mL and >64 μg/mL, respectively). Regarding the bithiazole derivatives, the symmetric compounds **17a** and **18b** showed conflicting results, in that the first had an encouraging MIC_{90} of 4−8 μg/mL, whereas the latter, a strictly close analogue, was found to be completely inactive (MIC_{90} > 64 μg/mL). A similar trend is maintained by compounds **22a** and **22b** (MIC_{90} = 8−16 μg/mL and 16−32 μg/mL, respectively), in which the symmetry of the molecule is lost due to removal of the phenyl ring attached at the 2-amino group. To some extent, and based on the biological feedback from compounds **1a**−**b** and **2a**−**b**, it can be speculated that the *p*-tolyl moiety performs better than the 3,5-dichlorophenyl one in order to provide the best anti-TB activity. However, it is highly arguable whether the different substitution pattern might be accountable for the large difference in the potency shown by compounds **17a** and **18b**. Because of these inconsistencies, bithiazoles were put aside, and our attention was focused on the synthesis of "second-generation" 4-(isoxazol-5-yl)-2-amino-thiazole derivatives and a few 4-(1-methylpyrazol-5-yl)-2-aminothiazoles.

Second-Generation Derivatives. Although derivatives **1a** and **1b** showed good activity, the ester moiety represented an evident weakness, as it can be easily hydrolyzed in the biological systems to give the corresponding acid. Indeed, experimental evidence were obtained by assessing the metabolic stability of compounds **1a** and **1b** in human liver microsomes (HLM). As expected, compounds **1a** and **1b** had only poor metabolic stability (**1a**, $t_{1/2}$ < 1 min, CL'_{int} > 400 mL/min/kg; **1b** $t_{1/2}$ = 3.3 ± 0.3 min, CL'_{int} = 188.8 mL/min/kg) and the HPLC−MS analysis clearly revealed that the ester moiety was rapidly cleaved to give the corresponding acids. Because sometimes biotransformations are beneficial to improve the activity of molecules, and are employed as a strategy for the activation of the so-called prodrugs, we synthesized and tested the corresponding acids derived from **1a** and **1b**. Unfortunately, these derivatives were found to be devoid of any anti-TB activity (Table 1, **3a**, **3b** MIC_{90} > 64 μg/mL), likely due to permeability issues. The same results applied to the pyrazol-5-yl-2-aminothiazoles counterparts (Table 1, **4a**, **4b** MIC_{90} > 64 μg/mL). As such, we reasoned that improving the stability of the compounds was a priority matter in order to make meaningful any further investigation. Although inactive, derivative **3a** was tested with regard to its metabolic stability in order to ascertain whether the ester moiety was the only metabolic soft spot in the molecule.[46] We were pleased to notice a drastic improvement in the metabolic stability of this compound (**3a**, $t_{1/2}$ = 14.3 ± 1.1 min, CL'_{int} = 43.7 mL/min/kg), proving that the ester functionality was the only issue hampering the use of these molecules in the biological systems. Considering these findings, we reasoned that preparing acid isosteres, with improved stability to hydrolysis, could couple good activity and reasonable stability. The first round of modification was focused on the synthesis of various carboxamides that are generally more resistant than esters to hydrolysis. The amide nitrogen was either left unsubstituted, mono-, or disubstituted with moieties different for size and physicochemical characteristics in order to evaluate their contribution to activity and stability. A primary amide led to

DOI: 10.1021/acs.jmedchem.7b00793
J. Med. Chem. XXXX, XXX, XXX−XXX

Table 2. Activity, Activity toward Nonreplicating Strains, Cytotoxicity, Metabolic Stability, and Evaluation of the Selectivity of Compounds 1a, 7a, and 8a

					antimicrobial activity (μg/mL)				
compd	MIC_{90} μg/mL[a] (μM)	NRP-TB[b] (μg/mL)	IC_{50}[c] μg/mL (μM)	$T_{1/2}$ (min) HLM[d] CL'_{int}[e]	*P. aer.*[f]	*E. coli*[g]	*E. fae.*[h]	*S. aur.*[i]	*C. alb.*[j]
1a	0.5−1.0 (1.5−3.0)	nd	nd	3.3 ± 0.3 188.8	nd	nd	nd	nd	nd
7a	0.125−0.25 (0.33−0.66)	1.0	70 (186)	35.1 ± 1.4 17.8	100	100	100	>100	>100
8a	0.06−0.125 (0.16−0.32)	1.0	53 (140)	16.1 ± 0.2 38.8	100	100	100	100	100
INH	0.03−0.06 (0.22−0.44)	nd	nd	nd	nd	nd	nd	nd	nd

[a]Minimum inhibitory concentration, determined by microdilution. [b]Nonreplicating H37Rv strain, MIC determined by microdilution. [c]Index of cytotoxicity (IC) determined in human monocyte-derived macrophages. [d]Measured in human liver microsomes and expressed in minutes. [e]Intrinsic clearance was calculated as (0.693/in vitro $t_{1/2}$) × (mL incubation/mg microsomes) × (45 mg microsomes/g liver) × (20 g liver/kg body weight). [f]*Pseudomonas aeruginosa* ATCC 27853. [g]*Escherichia coli* ATCC 25922. [h]*Enterococcus faecium* ATCC 35667. [i]*Staphylococcus aureus* ATCC25923. [j]*Candida albicans* ATCC 11006; nd: not determined.

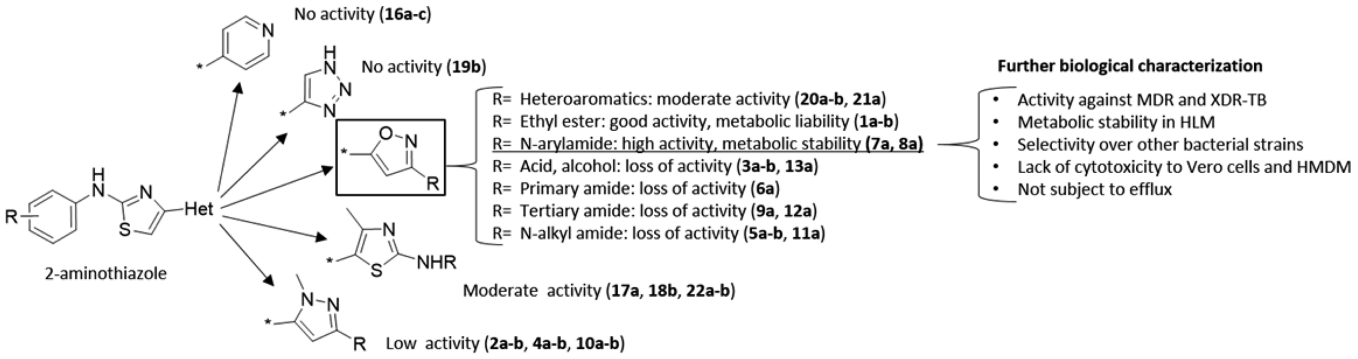


Figure 2. Visual representation of SAR emerged from this study and biological characterization of the lead compounds **7a** and **8a**.

a complete loss of activity (Table 1, **6a**, MIC_{90} > 64 μg/mL) as in the case of the acid. Secondary amide **11a**, that is a close analogue of ester **1a**, showed the same unpleasant result (MIC_{90} > 64 μg/mL). A diethyl substitution led to compound **9a**, that showed some hint of activity (MIC_{90} = 16−32 μg/mL), although still unsatisfactory compared to that of the corresponding ethyl ester. It can be concluded that small-sized aliphatic substituents at the amide nitrogen are not suitable to convey the desired potency. To see the effect of bigger aliphatic groups, the 1-adamantyl and the 1-piperidinyl moieties were selected as their lipophilic structures can enhance the penetration through the Mtb cell wall. In addition, a number of preclinical and clinical anti-TB candidates such as *N*-adamantan-2-yl-*N*′-((*E*)-3,7-dimethyl-octa-2,6-dienyl)-ethane-1,2-diamine dihydrochloride (SQ-109),[58] [1-(2-adamantyl)-3-(2,3,4-trifluorophenyl)urea] (AU-1235),[36] and some indolecarboxamides show the 1-adamantyl functional group in their structure. However, compounds **5a**, **5b**, and **12a** were found to be inactive up to a concentration of 64 μg/mL. Therefore, it can be proposed that aliphatic groups, either bulky or small-sized, resulting in secondary or tertiary amides, are not suitable to confer the desired anti-TB activity. On the other hand, those compounds bearing an aromatic or heteroaromatic ring attached at the amide nitrogen were found to be active toward the replicating Mtb phenotype in the sub μg/mL range (**7a**, MIC_{90} = 0.125−0.250 μg/mL, 0.33−0.66 μM; **8a**, MIC_{90} = 0.06−0.125 μg/mL, 0.16−0.32 μM). As reported in Table 2, considering the different molecular weights, compounds **7a** and **8a** are therefore slightly more active than isoniazid, that is one of the most potent anti-TB drug ever discovered. To evaluate whether abolishing the amide linker was detrimental for the activity, compounds **20a** and **20b**, in which an aromatic ring was directly attached at the C-3 position of the isoxazole, were synthesized and tested. In this case, the 4-methoxyphenyl moiety was selected because inspired by the structure of compounds **47** and **48**.[38] Although it can be clearly seen that the amide moiety is crucial to confer high potency, this modification led to contradictory results, (**20a**, MIC_{90} = 4−8 μg/mL; **20b**, MIC_{90} > 64 μg/mL), making difficult the assessment of their collocation within the SAR. Compound **21a** was synthesized as the 2-thiazole is considered a bioisostere of the ester moiety, but unfortunately it did not show any activity up to the highest concentration tested (MIC_{90} > 64 μg/mL), further establishing the amide as favorable functional group for the anti-TB activity. Finally, alcohol **13a**, although being more active than the acid counterpart, showed to be several-fold less active than the corresponding ester **1a** (MIC_{90} = 16−32 μg/mL). All of these findings are summarized in Figure 2, where a visual representation of the SAR is reported.

Further Biological Characterization. Because our first concern was to improve the metabolic stability of the hit compounds, **7a** and **8a** were evaluated for their $t_{1/2}$ (min) and Cl'_{int} (mL/min/kg) in HLM. We were pleased to notice that, compared to the esters, both the hit derivatives showed a several-fold improvement in the metabolic stability (**7a**, $t_{1/2}$ = 35.1 ± 1.4 min, CL'_{int} = 17.8 mL/min/kg; **8a**, $t_{1/2}$ = 16.1 ± 0.2 min, CL'_{int} = 38.8 mL/min/kg), with half-lives and clearance values in the range of those of many marketed drugs. In addition, compounds **7a** and **8a** were also found to be also more stable than the acid counterpart **3a** ($t_{1/2}$ = 14.3 ± 1.1 min, CL'_{int} = 43.7 mL/min/kg). Then we wanted to check whether the strong activity was specific for Mtb or if it was due to general toxicity. Thus, compounds **7a** and **8a** were evaluated in both eukaryotic and other prokaryotic cells. Although we have already reported that 2-aminothiazoles were found to be devoid of cytotoxicity toward VERO cells, we have further confirmed

DOI: 10.1021/acs.jmedchem.7b00793
J. Med. Chem. XXXX, XXX, XXX−XXX

Table 3. Additional Evaluation of Compounds 7a and 8a against Resistant Phenotypes and with Regard to Their Tendency to Be Effluxed

		MIC$_{90}$ (μg/mL)[b]					
compd	inhibition of EtBr efflux RFF[a]	no EI	+VP[c]	+TZ[d]	MTB1-MDR[e]	MTB2-MDR[f]	MTB3-XDR[g]
7a	0.22	2.0	2.0	2.0	2.0	1.0	2.0
8a	0.17	2.0	2.0	1.0	2.0	1.0	2.0

[a]Relative final fluorescence, based on accumulation of EtBr at 0.25 μg/mL for Mtb H37Rv strain in the absence of glucose and the compounds at concentrations of 2.6 μM. Verapamil and thioridazine were used as internal control at 18.5 μM: RFF verapamil = 1.45, RFF thioridazine = 1.15. [b]Minimum inhibitory concentration, determined by microdilution. [c]Verapamil, added at the concentration of 64 μg/mL. [d]Thioridazine, added at the concentration of 3.75 μg/mL. [e]MTB1 strain, resistant to INH and RIF. [f]MTB2 strain, resistant to INH and RIF. [g]MTB3 strain, resistant to INH, RIF, OFX, AMK, and CAP.

the safety of this class in HMDM, that are the cells where Mtb mostly resides during the infection, and where anti-TB drugs exert their action.[59] Also in this case, we were pleased to notice that both the compounds resulted in being toxic at concentrations up to >500-fold the MIC (**7a**, IC$_{50}$ = 70 μg/mL; **7b**, IC$_{50}$ = 53 μg/mL). Compounds **7a** and **8a** were also tested against a panel of bacteria other than mycobacteria (Gram-positive, Gram-negative, and fungi) to assess their selectivity of action, resulting highly specific for Mtb, as they did not inhibit the growth of *Pseudomonas aeruginosa*, *Escherichia coli*, *Enterococcus faecium*, *Staphylococcus aureus*, and *Candida albicans* (see Table 2 for MICs). To summarize, the activity of compounds **7a** and **8a** toward Mtb is highly specific, as they neither are toxic toward the human cells where the Mtb harbors nor toward the most common bacterial strains, preventing the occurrence of antimicrobial resistance.

Derivatives **7a** and **8a** were also tested against a panel of Mtb resistant strains. Some of the 2-aminothiazoles previously reported were reported to maintain their activity against a panel of SDR-TB strains.[38] In this work, we expanded the set of information by testing the novel 2-aminothiazoles synthesized against two MDR-TB and one XDR-TB strains. We were very pleased to notice that also in this case compounds **7a** and **8a** maintained, toward the resistant strains, the same activity as toward the wild-type strain (Table 3).

On a similar vein, compounds were tested against a model of persistent Mtb strain. Molecules bearing a 2-aminothiazole scaffold have already demonstrated to possess a moderate activity against nonreplicating Mtb strains in a carbon starvation model,[60] a behavior confirmed also in a low oxygen recovery assay (LORA) as previously reported by us.[38] Since the introduction of an *N*-arylisoxazole-3-carboxamide moiety, we have investigated whether the activity toward nonreplicating strains was maintained. Compounds **7a** and **8a**, tested using the Wayne model, were found to maintain good activity, although slightly lesser than toward the actively replicating phenotypes, also toward nonreplicating bacteria (Table 2), further confirming the versatility of this scaffold.

Finally, compounds were also evaluated for their susceptibility to be substrates of the efflux systems. After the exposure to an antibiotic, the activity of bacterial efflux pumps increases and this increased activity results in the reduction of the intracellular levels of the antibiotic, which enable the survival of a low-level resistant subpopulation. During this period, mutants with alterations in the genes that favor drug resistance can be selected, therefore ensuring the establishment of an antibiotic resistant population presenting clinically significant, high-level resistance. In spite of this knowledge, the effects of an antimicrobial compound toward efflux is seldom measured, and it is not considered an important parameter in the hit-to-lead process for the optimization of novel anti-TB drugs.[61,62] The recent discovery by Lee and colleagues of spectinamides as anti-TB drug candidates, also by virtue of their scarce tendency to be extruded by efflux pumps, confirms the importance of taking into account efflux when the design of new drugs is planned.[63] Taking into account all of these considerations, we tested some of our newly synthesized derivatives to investigate their effect toward efflux. In particular, from one side we wanted to investigate whether these compounds could be considered GEIs (growth and efflux inhibitors), a concept already reported by some of us,[59] aimed at discovering dual action anti-TB chemotypes. On the other side, we wanted to preliminarily evaluate the tendency of derivatives **7a** and **8a** to be suitable substrates of the mycobacterial efflux machinery so as to predict the likeliness of resistance development by Mtb. First, compounds **7a** and **8a** were evaluated for its activity as efflux inhibitors by EtBr real-time fluorometry and showed to be considerably less active (RFF = 0.22 and 0.17, respectively) than VP and TZ (Table 3), two of the most potent and characterized mycobacterial efflux pump inhibitors reported so far. These results demonstrate that none of these compounds is capable of inhibiting EtBr efflux by Mtb cells at sublethal concentrations. Then we have determined the MICs of compounds **7a** and **8a** alone and in the presence of VP and TZ at concentrations of 64 and 3.75 μg/mL, respectively. Indeed, at these concentrations, VP and TZ have already demonstrated exhibition of strong synergistic effect with many first- and second-line anti-TB drugs that are substrates of efflux systems, considerably lowering their absolute MICs also toward wild-type strains. The results showed no significant changes in the MIC values of both compounds (Table 3), indicating that they have little probability to be extruded by the Mtb efflux pumps.

Although aware that 2-aminothiazoles may cause a false positive interacting with every protein,[64] and taking into consideration the call made by some editors of the ACS journals with regard to the early identification of PAINS,[65] we would like to point out that the data in our hand rule out the possibility of an unselective and unspecific mechanism of action. Indeed, the compounds in this study were tested toward several cellular lines (Mtb H37Rv, MDR-TB, XDR-TB, HMDM, *P. aeruginosa*, *E. coli*, *E. faecium*, *S. aureus*, and *C. albicans*), according different methods, and also in the presence of liver subcellular fractions (microsomes), yielding a wide range of activities and allowing an analysis of their SAR. Moreover, some of these derivatives were not recognized as PAINS using the software "False Positive Remover" (http://www.cbligand.org/PAINS/) nor as aggregator according to the software "Aggregator Advisor" (http://advisor.bkslab.org/).

DOI: 10.1021/acs.jmedchem.7b00793
J. Med. Chem. XXXX, XXX, XXX−XXX

Often, the description of the complete mechanism of action for the majority of antibacterials has been unravelled long after their introduction in the market, and this applies also to many currently used anti-TB compounds. This happens because their antimycobacterial properties and good tolerability for the patient overcome the need for an exhaustive search for the description of their multitarget activity. In the case of the molecules reported in our manuscript, we can assert that the molecular target is very likely different from that of the first- and second-line anti-TB drugs, as they are active against MDR and XDR-TB strains. However, the actual enzyme(s) toward which compounds **7a** and **8a** exerts their inhibitory action is/are still unknown. The isoxazole-2-aminothiazole scaffold, that is assumed to be the pharmacophore for these derivatives, is quite an unexplored moiety in the field of medicinal chemistry. In the literature, only a few examples of enzymes targeted by structurally related molecules can be retrieved. Analogues were reported to inhibit the activity of the antigen-induced slow reacting substance of anaphylaxis (SRS-A-like material) and proved to be efficient in preventing anaphylaxis in guinea pigs.[66] In another work, similar compounds were tested as correctors by targeting the cystic fibrosis transmembrane conductance regulator (CFTR) that is a chloride channel present in the membrane of epithelial cells.[67] In another study, analogues were also found to be active against histone deacetylase 8 (HDAC8), an anticancer target.[68] Although HDAC inhibitors are known to possess a plethora of biological activities, spanning from the anticancer to the antiparasitic,[69,70] and although the presence of potential orthologue(s) in Mtb cannot be excluded, to hypothesize that the molecules herein reported might be HDAC inhibitors is highly speculative and, as such, of no use. If the analysis is limited to the 2-aminothiazole scaffold alone, a couple of works have attempted to identify the suitable molecular target. In both the works,[71,72] the synthesis of 2-aminothiazoles took the cue from the structure of thiolactomycin (TLM), that is, an inhibitor of β-ketoacyl synthase (KAS). As such, the authors have reasoned that also the 2-aminothiazoles should possess inhibitory activity toward this enzyme. However, because of the general lack of cellular activity, the drastic difference in the structures of TLM and 2-aminothiazoles, and, in particular, because of the remarkable discrepancies between biochemical and cellular results, it is reasonable to doubt that KAS might be the target, or the only one, also for our derivatives. Recently, Chiarelli et al. have reported a phenotypic based target screening approach in which a set of substituted 2-aminothiazoles were found to be inhibitors of the CTP synthetase PyrG,[73] a validated bactericidal target in Mtb. Also in this case, the substantial structural differences with the derivatives reported by Chiarelli et al. and our molecules make it highly unlikely that they share the same mechanism of action.

CONCLUSION

The final aim of this work was to identify a novel valuable anti-TB chemotype to be submitted for advanced in vivo studies. To this end, we have synthesized a total of 32 compounds focused on the 2-aminothiazole core, with various heterocycles rationally attached at the C-4 position. On the basis of the activity against wild-type Mtb, the most promising derivatives were selected and evaluated especially with regard to those characteristics that are highly desirable in an anti-TB candidate. Compared to the phenyl ring, heterocycles such as the isoxazole and the thiazole ring exhibited remarkable anti-TB potency. In particular, when the isoxazole-3-carboxylate moiety is suitably functionalized, as in the case of compounds **7a** and **8a**, anti-TB activity in the sub μg/mL range could be obtained. It is worthwhile to highlight that among the antibiotics that are listed in WHO's recommendation for treatment of all forms of TB, the activity of our compound **8a** would rank among the best. Along with the improved inhibitory activity, these modifications also led to a noticeable improvement of the metabolic stability. To further promote the advancement of these compounds, additional biological assays were carried out. Both **7a** and **8a** maintained remarkable activity against a panel of MDR-TB and XDR-TB strains, indicating that the mechanism of action by which they exert their inhibitory activity is different from that of the currently used anti-TB agents. Also, the mechanism of action is highly specific for Mtb, as compounds **7a** and **8a** failed to show any inhibitory activity either against eukaryotic cells or against a panel of Gram-positive and Gram-negative microorganisms. Finally, although the extent to which an antimicrobial compound is a substrate of efflux is seldom measured, we consider it an important parameter in the hit-to-lead process and we have demonstrated that both of these compounds are likely not subject to the effect of mycobacterial efflux pumps. Along with the specific mechanism of action, this increases the bar for the development of resistance to these molecules. In addition to these promising biological characteristics, it must be considered that these 2-aminothiazoles have physicochemical properties that indicate great potential for absorption and permeation when used as orally available compounds, as they do not show any violation of the four physicochemical characteristics defined by the Lipinski Rule of Five.[74] Indeed, their molecular weight is lower than 500 g/mol, the total number of oxygen and nitrogen atoms is lesser than 10, the number of hydrogen bond donor is lesser than 5, and, also, the ClogP is lower than 5 (calculated at http://www.molinspiration.com/), a considerable improvement over that of bedaquiline (ClogP = 7.25) and some indole-2-carboxyamides in clinical studies.[75] Considering all of this information, the ease of synthesis and the promising physicochemical characteristics, compounds **7a** and **8a** can be considered mature leads for further characterization, especially with regard to their in vivo activity and the determination of their mechanism of action. This investigation is currently ongoing in our laboratories.

EXPERIMENTAL SECTION

Chemistry. *General Information.* All the reagents were purchased from Sigma-Aldrich and Alfa-Aesar at reagent purity and, unless otherwise noted, were used without any further purification. Dry solvents used in the reactions were obtained by distillation of technical grade materials over appropriate dehydrating agents. Reactions were monitored by thin layer chromatography on silica gel-coated aluminum foils (silica gel on Al foils, Supelco Analytical, Sigma-Aldrich) at both 254 and 365 nm wavelengths. Where indicated, intermediates and final products were purified through silica gel flash chromatography (silica gel, 0.040−0.063 mm), using appropriate solvent mixtures.

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance spectrometer at 400 and 100 MHz, respectively, with TMS as internal standard. ^{1}H NMR spectra are reported in this order: multiplicity and number of protons. Standard abbreviation indicating the multiplicity was used as follows: s = singlet, d = doublet, dd = doublet of doublets, t = triplet, q = quadruplet, m = multiplet and br = broad signal. HPLC/MS experiments were performed with HPLC, Agilent 1100 series, equipped with a Waters Symmetry C18, 3.5 μm, 4.6 mm × 75 mm column; and MS, Applied Biosystem/MDS SCIEX, with API

DOI: 10.1021/acs.jmedchem.7b00793
J. Med. Chem. XXXX, XXX, XXX−XXX

150EX ion source. HRMS experiments were performed with LTQ Orbitrap XL Thermo.

All compounds were tested as 95−100% purity samples (by HPLC/MS).

General Procedure for Hantzsch Synthesis. The suitable α-bromoketone (1 equiv) and the proper thiourea (1 equiv) were solubilized in anhydrous ethanol (20 mL/mmol) and reacted at 70 °C until consumption of the starting materials as indicated by TLC. After cooling, the solvent was evaporated and the crude material was purified by flash column chromatography or in a few cases, by precipitation. Purification conditions, yields, and analytical data are reported in the Supporting Information. TLC for control (7:3 petroleum ether/ethyl acetate).

α-Bromination of the Ketones: Method A. Acetic acid (0.1 mL/mmol) was added to a solution of the ketone (1 equiv) in chloroform (1.5 mL/mmol), and reaction mixture was heated at 50 °C. Then a solution of Br_2 (1.05 equiv) in chloroform (0.4 mL/mmol) was added dropwise, and the mixture was stirred at the same temperature for 1 h. After consumption of the starting material according to TLC (9:1 petroleum ether/ethyl acetate), $NaHCO_3$ (aq sat solution, 5 mL/mmol) was added and the mixture was extracted with dichloromethane (3 times). The combined organic layers were washed with brine, dried over Na_2SO_4, and after removal of the solvent in vacuo, the crude material was purified by flash column chromatography. Purification conditions, yields and analytical data are reported in the Supporting Information.

α-Bromination of the Ketones: Method B. A solution of Br_2 (0.8 equiv) in 1,4-dioxane (4 mL/mmol) was added to a solution of the appropriate ketone (1 equiv) solubilized in HBr (48% solution, 4 mL/mmol) preheated to 50 °C. After consumption of the starting material according to TLC (9:1 petroleum ether/ethyl acetate), saturated $NaHCO_3$ aq solution (5 mL/mmol) was added, and the mixture was extracted with chloroform (3 times). The combined organic layers were washed with brine, dried over Na_2SO_4, and after removal of the solvent in vacuo, the crude material was purified by flash column chromatography. Purification conditions, yields, and analytical data are reported in the Supporting Information.

General Procedure for the Hydrolysis of the Esters. The appropriate ester (1 equiv) and $LiOH{\cdot}H_2O$ (4 equiv) were dissolved in a solution of THF/MeOH/H_2O (3:1:1, 1 mL/mmol) and stirred at room temperature until consumption of the starting material as indicated by TLC (7:3 petroleum ether/ethyl acetate, then 9:1 dichloromethane/methanol). The reaction mixture was then evaporated under reduced pressure, and the crude obtained was taken up with H_2O, acidified with 2 N HCl and extracted with ethyl acetate (3 × 10 mL). After evaporation of the solvent, the product is used for the next reaction step without further purification.

*5-(2-(p-Tolylamino)thiazol-4-yl)isoxazole-3-carboxamide (**6a**).* A suspension of compound **1a** (50 mg, 0.15 mmol) in NH_4OH (2 mL) was stirred overnight at room temperature, then H_2O (8 mL) was added and the mixture extracted with ethyl acetate (3 × 8 mL). The organic layers were treated with water, washed with brine, and dried over Na_2SO_4. After filtration, the solvent was removed in vacuo and the crude material was purified by flash column chromatography eluting with petroleum ether/ethyl acetate 7:3 to give the title compound as a white powder in 82% yield. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.27 (s, 3H), 7.02 (s, 1H), 7.16 (d, J = 8 Hz, 2H), 7.55 (d, J = 8 Hz, 2H), 7.62 (s, 1H), 7.90 (s, 1H), 8.20 (s, 1H), 10.30 (s, 1H). ^{13}C NMR (100.6 MHz, DMSO-d_6): δ = 20.86, 100.49, 109.72, 117.88, 129.95, 131.22, 138.43, 138.78, 159.83, 160.49, 164.95, 166.73. HRMS (ESI) calculated for $C_{16}H_{15}N_3O_3S$ $[M + H]^+$ 301.0681, found 301.0660.

General Procedure for the Synthesis of Amides. *O*-(Benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU, 1 equiv) and *N*-(3-(dimethylamino)propyl)-*N*′-ethylcarbodiimide hydrochloride (EDC·HCl, 1 equiv) were added to a solution of the proper carboxylic acid (1 equiv) in dry DMF (4 mL/mmol). The reaction mixture was stirred at room temperature under N_2 for 15 min, then triethylamine (1.5 equiv) and the suitable amine (1 equiv) were added to the mixture that was stirred at the same temperature until the complete consumption of the starting material, as indicated by TLC (usually 7:3 petroleum ether/ethyl acetate). Water (10 mL) was added, and the mixture extracted with ethyl acetate (3 × 10 mL). The organic layers were treated with water, washed with brine, and dried over Na_2SO_4. After filtration, the solvent was removed in vacuo and the crude material was purified by flash column chromatography to give the title compounds. Purification conditions, yields, and analytical data are reported in the Supporting Information.

*(5-(2-(p-Tolylamino)thiazol-4-yl)isoxazol-3-yl)methanol (**13a**).* Sodium borohydride (14 mg; 0.36 mmol) was added to solution of **1a** (20 mg; 0.06 mmol) in anhydrous methanol at 0 °C (1 mL), and the reaction mixture was stirred at room temperature for 30 min. After the complete consumption of the starting material, as indicated by TLC (8:2 petroleum ether/ethyl acetate), the mixture was cooled to 0 °C and NH_4Cl (aq satd soln, 5 mL) was added. The aqueous layers were extracted with ethyl acetate (3 × 8 mL), and the combined organic layers were washed with brine and dried over Na_2SO_4. After filtration, the solvent was removed in vacuo and the crude material was purified by silica gel flash chromatography column eluting with dichloromethane/methanol from 98:2 to 95:5 to give compound **13a** as a white powder in 93% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.38 (s, 3H), 4.84 (s, 2H), 6.66 (s, 1H), 7.14 (s, 1H), 7.23−7.27 (m, 2H), 7.29−7.31(m, 2H). ^{13}C NMR (100.6 MHz, DMSO-d_6): δ = 20.86, 55.45, 100.49, 108.58, 117.78, 129.92, 131.12, 138.87, 139.10, 164.78, 165.30, 165.49. HRMS (ESI) calculated for $C_{16}H_{15}N_3O_3S$ $[M + H]^+$ 288.0728, found 288.0699

General Procedure for the 1,3-Dipolar Cycloaddition. Triethylamine (1 equiv) was added dropwise to a solution of the suitable halogenated oxime (1 equiv) and 3-butyn-2-one (2 equiv) in benzene (1.8 mL/mmol) at 0 °C. The reaction mixture was stirred either at room temperature or at 60 °C for 1 h. After this period, 1 N HCl (5 mL/mmol) was added and the mixture extracted with ethyl acetate (3 × 10 mL). The organic layers were washed with water and brine and dried over Na_2SO_4. After filtration, the solvent was removed in vacuo and the crude material was purified by flash column chromatography.

With this procedure, starting from intermediates **23**, **24**, **33**, and **34**, the isoxazole derivatives **25**, **35**, and **36**, and the pyrazole derivative **26**, were synthesized. Detailed purification conditions, yields, and analytical data are reported in the Supporting Information.

*Synthesis of (Z)-Ethyl 2-Bromo-2-(2-methylhydrazono)acetate (**24**).* A solution of methylhydrazine (228 μL; 4.34 mmol) in methanol (0.33 mL/mmol) was heated at 40 °C, then ethyl glyoxalate (50% solution in toluene, 400 μL; 4.03 mmol) was added while maintaining the temperature below 50 °C. The mixture was allowed to stir at 50 °C until consumption of the starting material as revealed by TLC (8:2 petroleum ether/ethyl acetate, stained with $KMnO_4$). Solvents and excess methylhydrazine were distilled off, and the crude material was purified by flash column chromatography eluting petroleum ether/ethyl acetate from 75:25 to 50:50. Yield 50%. The product obtained (50 mg, 0.38 mmol) was solubilized in dichloromethane (1.38 mL) and added to a slurry of *N*-bromosuccinimide (68 mg; 0.38 mmol) in ethyl acetate (1.38 mL) maintained at 0 °C. The reaction mixture was stirred for 1 h at 5 °C, then the solvents were evaporated under reduced pressure and the crude material used for the next reaction step without purification, as the product is instable and easily decomposes when left at the air.

General Procedure for the Synthesis of the Oximes. NaOH 10% (0.5 mL/mmol) was added to a solution of hydroxylamine hydrochloride (1.2 equiv) in water (0.7 mL/mmol). Then a solution of the suitable aldehyde (1 equiv) in ethanol (3 mL/mmol) was added dropwise. The reaction mixture was stirred at room temperature for 30 min, then the solvents were evaporated under reduced pressure and the residue solubilized in water and extracted with ethyl acetate. The combined organic layers were washed with brine and dried over Na_2SO_4. After filtration, the solvent was removed in vacuo and the crude material was used for the next reaction step without further purification.

*(E)-4-Methoxybenzaldehyde oxime (**31**).* Yield 99%. ^{1}H NMR (400 MHz-$CDCl_3$): δ = 3.86 (s, 3H), 6.92 (d, J = 8 Hz, 2H) 7.53 (d, J = 8 Hz, 2H), 8.11 (s, 1H).

DOI: 10.1021/acs.jmedchem.7b00793
J. Med. Chem. XXXX, XXX, XXX−XXX

*(E)-Thiazole-4-carbaldehyde oxime (**32**).* Yield 91%. ^{1}H NMR (400 MHz-DMSO-d_6): δ = 7.95 (s, 1H), 8.23 (s, 1H), 9.15 (s, 1H), 11.3 (s, 1H).

*(Z)-N-Hydroxy-4-methoxybenzimidoyl Chloride (**33**).* *N*-Chlorosuccinimide (103 mg; 0.77 mmol) was added to a stirred solution of compound **31** (100 mg, 0.64 mmol) in DMF (2 mL), and the reaction mixture was reacted at room temperature for 1 h. After the complete consumption of the starting material according to TLC (95:5 dichloromethane/methanol), water (5 mL) was added, and the mixture was extracted with ethyl acetate (3 × 10 mL). The combined organic layers were washed with brine and dried over Na_2SO_4. After filtration, the solvent was removed in vacuo and the crude material was used for the next reaction step without further purification.

*(Z)-N-Hydroxythiazole-4-carbimidoyl Chloride (**34**).* Following a similar procedure, but using (*E*)-thiazole-4-carbaldehyde oxime **32** as the starting material, and a mixture of pyridine (6 μL, 0.078 mmol) and dichloromethane (1.5 mL) as the solvent, compound **34** was prepared and used for the next reaction step without further purification.

*1-(1H-1,2,3-Triazol-5-yl)ethanone (**37**).* A solution of 3-butyn-2-one (114 μL, 1.46 mmol) in DMF (0.4 mL/mmol) was added over a period of 30 min to a suspension of NaN_3 (143 mg, 2.20 mmol) in DMF (1 mL/mmol) preheated at 60 °C. The reaction mixture was stirred at the same temperature for 1 h, and then water (10 mL) was added and the slurry obtained was washed with dichloromethane (2 × 10 mL). The solvent was separated, and the aqueous layers were acidified with 3 N HCl and extracted with ethyl acetate (3 × 10 mL). The combined organic layers were washed with brine and dried over Na_2SO_4. After filtration, the solvent was removed in vacuo and the crude material was purified by flash column chromatography eluting petroleum ether/ethyl acetate from 9:1 to 7:3. Yield 30%. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.56 (s, 3H), 8.53 (s, 1H).

*2-Bromo-1-(pyridin-4-yl)ethanone (**44**).* A solution of Br_2 (294 μL; 5.76 mmol) in diethyl ether (2.8 mL) was added dropwise over a period of 30 min to a solution of 4-acetylpyridine (456 μL; 4.10 mmol) in 1,4-dioxane/diethyl ether 1/1 (4.8 mL) cooled to 0 °C. The reaction mixture was stirred overnight, then saturated $NaHCO_3$ aq solution (20 mL) was added and the mixture extracted with ethyl acetate (3 × 10 mL). The combined organic layers were treated with water (3 × 10 mL), washed with brine, and dried over Na_2SO_4. After filtration, the solvent was removed in vacuo and the crude material was purified by flash column chromatography eluting petroleum ether/ethyl acetate 7:3. Yield 50%. ^{1}H NMR (300 MHz $CDCl_3$): δ = 3.10 (s, 2H), 8.53 (d, J = 6 Hz, 2H), 8.62 (d, J = 6 Hz, 2H). TLC for control (8:2 petroleum ether/ethyl acetate).

Biology. *Inhibition of M. tuberculosis H37Rv.* Minimum inhibitory concentration (MIC_{90}) for Mtb was determined using standard broth macrodilution in 15 mL sterile conical tubes containing 2.5 mL of 7H9 broth. Standard broth microdilution method (using 96-well plates) was used for other organisms as per Clinical and Laboratory Standard Institute (CLSI) recommendations.[76] Middlebrook 7H9 broth was used for Mtb growth as per CLSI guidelines (Desmond, 2011). In summary, 10^5 bacilli grown to exponential phase in liquid medium were inoculated into each well containing drug at 2-fold dilutions ranging from 64 to 0.03 μg/mL. Growth medium alone and without drug but inoculated with 10^5 bacilli were included as negative and positive controls, respectively. Appropriate drugs (INH and RIF) were included as positive control for growth inhibition. Growth was evaluated by visual inspection for the presence of bacterial pellet following incubation for 14 days at 37 °C. The first well in which bacterial pellet is absent and therefore growth is not observable is considered the MIC_{90} as per the standard CLSI guidelines. MIC_{90} is expressed as a range spanning two concentrations: the higher concentration represents the lowest concentration at which bacterial growth could not be observed.

Inhibition of Resistant Mtb Strains. Mtb drug-resistant clinical isolates (Mtb1, Mtb2, and Mtb3) were obtained from patients diagnosed with active drug-resistant TB in Lisbon in 2008 and 2009.[77] MICs of compounds **7a** and **8a** was conducted by the 96-well broth microdilution method using a tetrazolium microplate-based assay with slight modifications.[78] Mtb strains were grown in MB7H9 plus 10% OADC supplement at 37 °C until an OD600 nm of 0.8. The inoculum was prepared by diluting the bacterial cultures in MB7H9/OADC to a final density of approximately 10^5 cells/mL.[79] Briefly, aliquots of 0.1 mL of inoculum were transferred to each well of the plate that contained 0.1 mL of each compound at concentrations prepared from 2-fold serial dilutions in MB7H9/OADC medium. Growth controls and a sterility control were included in each assay. The inoculated plates were sealed in plastic bags and incubated at 37 °C during 7 days. After 7 days of incubation, MTT was added to each well to a final concentration of 2.5% and the plates incubated overnight. The bacterial viability was registered for each well based on the MTT color change, and the MIC was defined as the lowest concentration of compound that totally inhibited bacterial growth (no color change). The assays were performed in triplicate. MICs of the compounds in the presence of the inhibitors verapamil or thioridazine were performed as described above with the exception that each inhibitor was added to each drug-containing well at 1/4 MIC (VP, 64 μg/mL; and TZ, 3.75 μg/mL). The results were interpreted as described above.

Inhibition of Nonreplicating Mtb Strains. Mtb H37Rv ATCC27924 cultures were adapted to hypoxic conditions as described earlier[80] with modifications. Briefly, Mtb cells were grown in tubes containing 8 mL of MB7H9. The tubes were tightly capped, sealed with parafilm, and incubated standing at 37 °C during 8 weeks. Methylene blue was added as a redox indicator (final concentration of 1.5 μg/mL) to all tubes to monitor oxygen depletion. MIC determination of the compounds **7a** and **8a** against the NRP Mtb cells was determined in 96-well microtiter plates in an anaerobic jar by exposing the hypoxic cells to varying concentrations of compounds for 5 days at 37 °C. An anaerobic indicator strip was placed inside the jar to visually confirm the removal of oxygen during the incubation. After this period the plates were transferred to normal atmosphere for recovery during 2 days at 37 °C. Then, MTT was added to each well to a final concentration of 2.5% and the plates incubated overnight at room temperature. The bacterial viability was registered for each well based on the MTT color change, and the MIC was defined as the lowest concentration of compound that totally inhibited bacterial growth (no color change). The assays were performed in triplicate.

Cytotoxicity Assays toward Human Monocyte-Derived Macrophage (HMDM). Cellular toxicity was assayed against human monocyte-derived macrophages. Blood was collected from healthy volunteers and peripheral blood mononuclear cells isolated by Ficoll-Paque Plus (GE Healthcare, Freiburg, Germany) density gradient centrifugation. Monocytes were differentiated into macrophages during 7 days in macrophage medium containing RPMI-1640 medium with 10% fetal calf serum (FCS), 1% GlutaMAX, 1 mM sodium pyruvate, 10 mM HEPES at pH 7.4, 100 IU/mL penicillin and 100 μg/mL streptomycin (Gibco, Life Technologies), and 20 ng/mL M-CSF (Immunotools, Friesoythe, Germany) and incubated at 37 °C with 5% CO_2. Fresh medium was added at day 4 post isolation. The effect of the compounds **7a** and **8a** was evaluated using AlamarBlue (Molecular Probes, Life Technologies) according to the manufacturer's instructions. Briefly, 5 × 10^4 cells were seeded in 96-well microplates, treated with the compounds, and then incubated at 37 °C in a 5% CO_2 atmosphere. After 3 days of exposure, cell viability was assessed. Briefly, 10% AlamarBlue was added to each well and incubated at 37 °C and 5% CO_2. Fluorescence was measured with a 540/35 excitation filter and a 590/20 emission filter in a Synergy HT multimode microplate reader (BioTek Instruments, Inc., Vermont, USA). The IC_{50} value corresponds to the highest concentration of compound at which 50% of the cells are viable relative to the control.[81]

*Antimicrobial Activity of Compounds **7a** and **8a**.* Bacterial and fungal reference strains (*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC25923, *E. faecium* ATCC 35667, and *Candida albicans* ATCC 11006) were purchased from Mast Diagnostic (Germany). The antimicrobial activity of **7a** and **8a** was evaluated as reported elsewhere[82,83] following the CLSI guidelines (2008). Powder compounds were dissolved in sterile water with 5% DMSO. DMSO concentration were increased to 55% to dissolve macroscopic

DOI: 10.1021/acs.jmedchem.7b00793
J. Med. Chem. XXXX, XXX, XXX−XXX

aggregation of the compounds, and solutions were sonicated for about 30 min before use. The final concentration of **7a** and **8a** stock solution was 500 μg/mL. The bacterial suspension was standardized following the Clinical and Laboratory Standards Institute guidelines. Briefly, the log-growing phase was reached by incubating each strain in Mueller Hinton broth (MH) (Difco, USA) at 37 °C in a shaker at 225 rpm for 3−4 h. After being pelleted at 1000*g* for 20 min, the bacterial suspension was adjusted spectrophotometrically at 600 nm to an optical density value in the range 0.08−0.13, containing approximately 10^8 colony forming unit (CFU)/mL in phosphate buffer (PB) 10 mM pH 7. Then 10 μL of bacterial suspension containing 10^6 CFU/mL were inoculated into each well to obtain a final concentration of approximately 5×10^5 CFU/mL. For *C. albicans* susceptibility, tests were performed by broth microdilution methodology in RPMI according to the CLSI M27-A3 guidelines (2008). Briefly, *C. albicans* culture was adjusted by adding PB to a 0.5 McFarland standard which corresponds to a concentration of $(1-5) \times 10^6$ CFU/mL. Fungal suspension was further diluted in broth to obtain a final concentration of about 10^3 CFU/mL and used within 30 min after its preparation for microdilution assay. Compounds were serially diluted into 96 U bottomed microtiter plate wells from 200 μg/mL to 0.4 μg/mL concentration in a volume of 50 μL. In each well 50 μL of bacterial/fungal suspension was added (final volume 100 μL/well). Therefore, the final concentrations were ranging from 100 to 0.2 μg/mL. For every bacterial/fungal strain, a growth and sterility control were set. Plates were incubated at 37 °C for 24 h (48 h for fungal strains) in aerobic atmosphere. The minimum inhibitory concentration (MIC) is defined as the lowest concentration of antimicrobial at which there is no visible growth of the organism. Because of the turbidity of the suspension at higher concentrations, the test was also evaluated through viability staining with resazurin. In this case, MIC was considered as the lower concentration of the compounds with no color change from blue to pink (no cells metabolic activity).

Evaluation of Efflux Inhibitory Activity of Compounds by Real-Time Fluorometry. The EtBr accumulation by the mycobacterial strains was assessed on a real-time basis using a fluorometric method, as previously described.[61,84] Mtb H37Rv was grown in MB7H9 supplemented with 10% OADC and 0.05% Tween 80 until $OD_{600\ nm}$ of 0.8. For the accumulation of EtBr, the cells were collected by centrifugation at 2940*g* for 3 min, the pellet washed in PBS, and the $OD_{600\ nm}$ of the suspension adjusted to 0.8 with PBS. To assess the effect of compounds **7a** and **8a** on EtBr accumulation, the assays were performed in a final volume of 0.1 mL containing 0.05 mL of the cellular suspension (final $OD_{600\ nm}$ of 0.4) and 0.05 mL of a solution of EtBr to a final concentration of 0.25 μg/mL, and the compound to be tested to a final concentration of half MIC, in order to not compromise the cell viability. Verapamil and thioridazine were included as controls and used at 18.5 μM. The assays were conducted in a Rotor-Gene 3000 (Corbett Research, Sydney, Australia) at 37 °C, and the fluorescence acquired at 530/585 nm at the end of every 60 s during 60 min. The activity of the compounds was evaluated by the relative final fluorescence (RFF) index as previously described.[85]

Stability Studies in Human Liver Microsomes. Stability of selected compounds in the presence of pooled HLM (X200 pooled, Xenotech LLC, USA) was assessed by incubation of a 1 μM concentration for 60 min in the presence of HLM (1 mg protein mL^{-1}), at 37 °C, in the presence of a NADPH-regenerating system (2 mM $NADP^+$, 10 mM glucose-6-phosphate, 0.4 U mL^{-1} glucose-6-phosphate dehydrogenase, 5 mM $MgCl_2$) in 100 mM PBS buffer solution pH 7.4. The reaction mixtures were preheated (37 °C) for 5 min before adding the parent compound. At fixed time points (t = 0, 15, 30, 60 min), aliquots of samples were withdrawn, deproteinized with two volumes of acetonitrile, centrifuged (9000*g*, 4 °C, 10 min), and the supernatant analyzed by injection in HPLC-MS/MS system. The chromatographic separation was performed employing a gradient elution starting from 70% water + 0.1% formic acid (solvent A):30% methanol (solvent B) to 90%B:10%A in 10 min; 90%B:10%A was kept for further 5 min, then back to 70%A:30%B, and further 5 min of reconditioning time. HPLC-MS/MS analysis employed a Thermo Quantum Access Max TSQ triple quadrupole mass spectrometer (Thermo, USA) equipped with an heated-electrospray ionization (H-ESI) interface and coupled to an Accela UHPLC system (Thermo, USA) constituted of a quaternary pump, a degasser, and a thermostated autosampler. Compounds were analyzed in positive ion mode using both total ion monitoring mode, over a mass range from 50 to 500 amu, and single ion monitoring mode. Data were acquired and analyzed employing Thermo Excalibur 1.4 software (Thermo, USA).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.7b00793.

^{1}H NMR spectra of the intermediates and the ^{1}H NMR, ^{13}C NMR, and HRMS and the of the title compounds (PDF)

Molecular formula strings (CSV)

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +39 0521 905054. E-mail: marco.pieroni@unipr.it.

ORCID

Elisa Azzali: 0000-0001-8344-1634

Marco Pieroni: 0000-0001-9190-3712

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Maria Giovanna Massanova is kindly acknowledged for the help in the synthesis of derivatives. The Centro Interdipartimentale Misure "G. Casnati" is kindly acknowledged for the contribution in the analytical determination of the molecules synthesized. D.M. was supported by grant SFRH/BPD/100688/2014 from Fundação para Ciência e a Tecnologia (FCT), Portugal. D.M. and M.V. are thankful to FCT for funding to the Global Health and Tropical Medicine (GHTM) Research Center (grant UID/Multi/04413/2013).

■ ABBREVIATIONS USED

AMK, amikacin; CAP, capreomycin; DMF, *N*,*N*-dimethylformamide; DOTS, directly observed therapy short-course; EDC, 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide; EDG, electron-donor group; EWG, electron-withdrawing group; EI, efflux inhibitor; INH, isoniazid; LORA, low oxygen recovery assay; MIC, minimum inhibitory concentration; MDR-TB, multidrug-resistant tuberculosis; MOX, moxifloxacin; Mtb, *Mycobacterium tuberculosis*; NRP-TB, nonreplicating persistent tuberculosis; OFX, ofloxacin; RIF, rifampin; SAR, structure−activity relationships; SM, streptomycin; TB, tuberculosis; TBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TEA, triethylamine; THF, tetrahydrofuran; XDR-TB, extensively drug-resistant tuberculosis

■ REFERENCES

(1) *The Top 10 Causes of Death*; World Health Organization: Geneva, 2017; http://www.who.int/mediacentre/factsheets/fs310/en/ (accessed Feb 16, 2017).

DOI: 10.1021/acs.jmedchem.7b00793
J. Med. Chem. XXXX, XXX, XXX−XXX

(2) *Global Tuberculosis Report 2016*; World Health Organization: Geneva, 2017; http://www.who.int/tb/publications/global_report/en/ (accessed Feb 16, 2017).
(3) Dheda, K.; Barry, C. E.; Maartens, G. Tuberculosis. *Lancet* **2016**, *387* (10024), 1211−1226.
(4) *Surveillance of Drug Resistance in Tuberculosis*; World Health Organization: Geneva, 2017; http://www.who.int/tb/publications/mdr_surveillance/en/ (accessed Feb 16, 2017).
(5) Duncan, K. Progress in TB Drug Development and What Is Still Needed. *Tuberculosis* **2003**, *83* (1−3), 201−207.
(6) Caminero, J. A.; Sotgiu, G.; Zumla, A.; Migliori, G. B. Best Drug Treatment for Multidrug-Resistant and Extensively Drug-Resistant Tuberculosis. *Lancet Infect. Dis.* **2010**, *10* (9), 621−629.
(7) *Drug-Resistant TB*; Centers for Disease Control and Prevention: Atlanta, GA, 2017; https://www.cdc.gov/tb/topic/drtb/ (accessed Feb 16, 2017).
(8) Migliori, G. B.; De Iaco, G.; Besozzi, G.; Centis, R.; Cirillo, D. M. First Tuberculosis Cases in Italy Resistant to All Tested Drugs. *Euro Surveill.* **2007**, *12* (5), E070517.1.
(9) Pasipanodya, J. G.; Gumbo, T. A Meta-Analysis of Self-Administered vs Directly Observed Therapy Effect on Microbiologic Failure, Relapse, and Acquired Drug Resistance in Tuberculosis Patients. *Clin. Infect. Dis.* **2013**, *57* (1), 21−31.
(10) Sensi, P. History of the Development of Rifampin. *Clin. Infect. Dis.* **1983**, *5* (Suppl 3), S402−S406.
(11) Andries, K.; Verhasselt, P.; Guillemont, J.; Göhlmann, H. W. H.; Neefs, J.-M.; Winkler, H.; Gestel, J. V.; Timmerman, P.; Zhu, M.; Lee, E.; Williams, P.; Chaffoy, D.; de Huitric, E.; Hoffner, S.; Cambau, E.; Truffot-Pernot, C.; Lounis, N.; Jarlier, V. A Diarylquinoline Drug Active on the ATP Synthase of Mycobacterium Tuberculosis. *Science* **2005**, *307* (5707), 223−227.
(12) Mahajan, R. Bedaquiline: First FDA-Approved Tuberculosis Drug in 40 Years. *Int. J. Appl. Basic Med. Res.* **2013**, *3* (1), 1−2.
(13) Kakkar, A. K.; Dahiya, N. Bedaquiline for the Treatment of Resistant Tuberculosis: Promises and Pitfalls. *Tuberculosis* **2014**, *94* (4), 357−362.
(14) Fox, G. J.; Menzies, D. A Review of the Evidence for Using Bedaquiline (TMC207) to Treat Multi-Drug Resistant Tuberculosis. *Infect. Dis. Ther.* **2013**, *2* (2), 123−144.
(15) Guglielmetti, L.; Jaspard, M.; Le Dû, D.; Lachâtre, M.; Marigot-Outtandy, D.; Bernard, C.; Veziris, N.; Robert, J.; Yazdanpanah, Y.; Caumes, E.; Fréchet-Jachym, M. French MDR-TB Management Group. Long-Term Outcome and Safety of Prolonged Bedaquiline Treatment for Multidrug-Resistant Tuberculosis. *Eur. Respir. J.* **2017**, *49*, 1601799.
(16) Sotgiu, G.; Pontali, E.; Centis, R.; D'Ambrosio, L.; Migliori, G. B. Delamanid (OPC-67683) for Treatment of Multi-Drug-Resistant Tuberculosis. *Expert Rev. Anti-Infect. Ther.* **2015**, *13* (3), 305−315.
(17) Harausz, E.; Cox, H.; Rich, M.; Mitnick, C. D.; Zimetbaum, P.; Furin, J. QTc Prolongation and Treatment of Multidrug-Resistant Tuberculosis. *Int. J. Tuberc. Lung Dis. Off. J. Int. Union Tuberc. Lung Dis.* **2015**, *19* (4), 385−391.
(18) Bloemberg, G. V.; Keller, P. M.; Stucki, D.; Trauner, A.; Borrell, S.; Latshang, T.; Coscolla, M.; Rothe, T.; Hömke, R.; Ritter, C.; Feldmann, J.; Schulthess, B.; Gagneux, S.; Böttger, E. C. Acquired Resistance to Bedaquiline and Delamanid in Therapy for Tuberculosis. *N. Engl. J. Med.* **2015**, *373* (20), 1986−1988.
(19) *Clinical Pipeline*; Working Group for New TB Drugs: New York, 2016; http://www.newtbdrugs.org/pipeline/clinical (accessed Feb 16, 2017).
(20) Stehr, M.; Elamin, A. A.; Singh, M. Filling the Pipeline - New Drugs for an Old Disease. *Curr. Top. Med. Chem.* **2014**, *14* (1), 110−129.
(21) Sterling, T. R.; Villarino, M. E.; Borisov, A. S.; Shang, N.; Gordin, F.; Bliven-Sizemore, E.; Hackman, J.; Hamilton, C. D.; Menzies, D.; Kerrigan, A.; Weis, S. E.; Weiner, M.; Wing, D.; Conde, M. B.; Bozeman, L.; Horsburgh, C. R.; Chaisson, R. E. TB Trials Consortium PREVENT TB Study Team. Three Months of Rifapentine and Isoniazid for Latent Tuberculosis Infection. *N. Engl. J. Med.* **2011**, *365* (23), 2155−2166.
(22) Pieroni, M.; Sabatini, S.; Massari, S.; Kaatz, G. W.; Cecchetti, V.; Tabarrini, O. Searching for Innovative Quinolone-like Scaffolds: Synthesis and Biological Evaluation of 2,1-Benzothiazine 2,2-Dioxide Derivatives. *MedChemComm* **2012**, *3* (9), 1092−1097.
(23) Tabarrini, O.; Sabatini, S.; Massari, S.; Pieroni, M.; Franzblau, S. G.; Cecchetti, V. 6-Hydrogen-8-Methylquinolones Active Against Replicating and Non-Replicating *Mycobacterium Tuberculosis*: Anti-mycobacterial 6-Desfluoroquinolones. *Chem. Biol. Drug Des.* **2012**, *80* (5), 781−786.
(24) Ruan, Q.; Liu, Q.; Sun, F.; Shao, L.; Jin, J.; Yu, S.; Ai, J.; Zhang, B.; Zhang, W. Moxifloxacin and Gatifloxacin for Initial Therapy of Tuberculosis: A Meta-Analysis of Randomized Clinical Trials. *Emerging Microbes Infect.* **2016**, *5*, e12.
(25) Wallis, R. S.; Maeurer, M.; Mwaba, P.; Chakaya, J.; Rustomjee, R.; Migliori, G. B.; Marais, B.; Schito, M.; Churchyard, G.; Swaminathan, S.; Hoelscher, M.; Zumla, A. Tuberculosis—advances in Development of New Drugs, Treatment Regimens, Host-Directed Therapies, and Biomarkers. *Lancet Infect. Dis.* **2016**, *16* (4), e34−e46.
(26) Chiarelli, L. R.; Mori, G.; Esposito, M.; Orena, B. S.; Pasca, M. R. New and Old Hot Drug Targets in Tuberculosis. *Curr. Med. Chem.* **2016**, *23* (33), 3813−3846.
(27) Manina, G.; Pasca, M. R.; Buroni, S.; De Rossi, E.; Riccardi, G. Decaprenylphosphoryl-β-D-Ribose 2'-Epimerase from Mycobacterium Tuberculosis Is a Magic Drug Target. *Curr. Med. Chem.* **2010**, *17* (27), 3099−3108.
(28) Domenech, P.; Reed, M. B.; Barry, C. E., 3rd Contribution of the Mycobacterium Tuberculosis MmpL Protein Family to Virulence and Drug Resistance. *Infect. Immun.* **2005**, *73* (6), 3492−3501.
(29) Makarov, V.; Lechartier, B.; Zhang, M.; Neres, J.; van der Sar, A. M.; Raadsen, S. A.; Hartkoorn, R. C.; Ryabova, O. B.; Vocat, A.; Decosterd, L. A.; Widmer, N.; Buclin, T.; Bitter, W.; Andries, K.; Pojer, F.; Dyson, P. J.; Cole, S. T. Towards a New Combination Therapy for Tuberculosis with next Generation Benzothiazinones. *EMBO Mol. Med.* **2014**, *6* (3), 372−383.
(30) Peng, C.-T.; Gao, C.; Wang, N.-Y.; You, X.-Y.; Zhang, L.-D.; Zhu, Y.-X.; Xv, Y.; Zuo, W.-Q.; Ran, K.; Deng, H.-X.; Lei, Q.; Xiao, K.-J.; Yu, L.-T. Synthesis and Antitubercular Evaluation of 4-Carbonyl Piperazine Substituted 1,3-Benzothiazin-4-One Derivatives. *Bioorg. Med. Chem. Lett.* **2015**, *25* (7), 1373−1376.
(31) Tiwari, R.; Miller, P. A.; Chiarelli, L. R.; Mori, G.; Šarkan, M.; Centárová, I.; Cho, S.; Mikušová, K.; Franzblau, S. G.; Oliver, A. G.; Miller, M. J. Design, Syntheses, and Anti-TB Activity of 1,3-Benzothiazinone Azide and Click Chemistry Products Inspired by BTZ043. *ACS Med. Chem. Lett.* **2016**, *7* (3), 266−270.
(32) La Rosa, V.; Poce, G.; Canseco, J. O.; Buroni, S.; Pasca, M. R.; Biava, M.; Raju, R. M.; Porretta, G. C.; Alfonso, S.; Battilocchio, C.; Javid, B.; Sorrentino, F.; Ioerger, T. R.; Sacchettini, J. C.; Manetti, F.; Botta, M.; De Logu, A.; Rubin, E. J.; De Rossi, E. MmpL3 Is the Cellular Target of the Antitubercular Pyrrole Derivative BM212. *Antimicrob. Agents Chemother.* **2012**, *56* (1), 324−331.
(33) Onajole, O. K.; Pieroni, M.; Tipparaju, S. K.; Lun, S.; Stec, J.; Chen, G.; Gunosewoyo, H.; Guo, H.; Ammerman, N. C.; Bishai, W. R.; Kozikowski, A. P. Preliminary Structure-Activity Relationships and Biological Evaluation of Novel Antitubercular Indolecarboxamide Derivatives against Drug-Susceptible and Drug-Resistant Mycobacterium Tuberculosis Strains. *J. Med. Chem.* **2013**, *56* (10), 4093−4103.
(34) Lun, S.; Guo, H.; Onajole, O. K.; Pieroni, M.; Gunosewoyo, H.; Chen, G.; Tipparaju, S. K.; Ammerman, N. C.; Kozikowski, A. P.; Bishai, W. R. Indoleamides Are Active against Drug-Resistant Mycobacterium Tuberculosis. *Nat. Commun.* **2013**, *4*, 2907.
(35) Remuiñán, M. J.; Pérez-Herrán, E.; Rullás, J.; Alemparte, C.; Martínez-Hoyos, M.; Dow, D. J.; Afari, J.; Mehta, N.; Esquivias, J.; Jiménez, E.; Ortega-Muro, F.; Fraile-Gabaldón, M. T.; Spivey, V. L.; Loman, N. J.; Pallen, M. J.; Constantinidou, C.; Minick, D. J.; Cacho, M.; Rebollo-López, M. J.; González, C.; Sousa, V.; Angulo-Barturen, I.; Mendoza-Losana, A.; Barros, D.; Besra, G. S.; Ballell, L.; Cammack, N. Tetrahydropyrazolo[1,5-a]Pyrimidine-3-Carboxamide and N-Benzyl-

DOI: 10.1021/acs.jmedchem.7b00793
J. Med. Chem. XXXX, XXX, XXX−XXX

6',7'-Dihydrospiro[Piperidine-4,4'-Thieno[3,2-c]Pyran] Analogues with Bactericidal Efficacy against Mycobacterium Tuberculosis Targeting MmpL3. *PLoS One* **2013**, *8* (4), e60933.
(36) Grzegorzewicz, A. E.; Pham, H.; Gundi, V. A. K. B.; Scherman, M. S.; North, E. J.; Hess, T.; Jones, V.; Gruppo, V.; Born, S. E. M.; Korduláková, J.; Chavadi, S. S.; Morisseau, C.; Lenaerts, A. J.; Lee, R. E.; McNeil, M. R.; Jackson, M. Inhibition of Mycolic Acid Transport across the Mycobacterium Tuberculosis Plasma Membrane. *Nat. Chem. Biol.* **2012**, *8* (4), 334−341.
(37) Tantry, S. J.; Degiacomi, G.; Sharma, S.; Jena, L. K.; Narayan, A.; Guptha, S.; Shanbhag, G.; Menasinakai, S.; Mallya, M.; Awasthy, D.; Balakrishnan, G.; Kaur, P.; Bhattacharjee, D.; Narayan, C.; Reddy, J.; Naveen Kumar, C. N.; Shandil, R.; Boldrin, F.; Ventura, M.; Manganelli, R.; Hartkoorn, R. C.; Cole, S. T.; Panda, M.; Markad, S. D.; Ramachandran, V.; Ghorpade, S. R.; Dinesh, N. Whole Cell Screen Based Identification of Spiropiperidines with Potent Antitubercular Properties. *Bioorg. Med. Chem. Lett.* **2015**, *25* (16), 3234−3245.
(38) Pieroni, M.; Wan, B.; Cho, S.; Franzblau, S. G.; Costantino, G. Design, Synthesis and Investigation on the Structure−activity Relationships of N-Substituted 2-Aminothiazole Derivatives as Antitubercular Agents. *Eur. J. Med. Chem.* **2014**, *72*, 26−34.
(39) Roy, K. K.; Singh, S.; Sharma, S. K.; Srivastava, R.; Chaturvedi, V.; Saxena, A. K. Synthesis and Biological Evaluation of Substituted 4-Arylthiazol-2-Amino Derivatives as Potent Growth Inhibitors of Replicating Mycobacterium Tuberculosis H37RV. *Bioorg. Med. Chem. Lett.* **2011**, *21* (18), 5589−5593.
(40) Mjambili, F.; Njoroge, M.; Naran, K.; De Kock, C.; Smith, P. J.; Mizrahi, V.; Warner, D.; Chibale, K. Synthesis and Biological Evaluation of 2-Aminothiazole Derivatives as Antimycobacterial and Antiplasmodial Agents. *Bioorg. Med. Chem. Lett.* **2014**, *24* (2), 560−564.
(41) Pieroni, M.; Wan, B.; Zuliani, V.; Franzblau, S. G.; Costantino, G.; Rivara, M. Discovery of Antitubercular 2,4-Diphenyl-1H-Imidazoles from Chemical Library Repositioning and Rational Design. *Eur. J. Med. Chem.* **2015**, *100*, 44−49.
(42) Ananthan, S.; Faaleolea, E. R.; Goldman, R. C.; Hobrath, J. V.; Kwong, C. D.; Laughon, B. E.; Maddry, J. A.; Mehta, A.; Rasmussen, L.; Reynolds, R. C.; Secrist, J. A.; Shindo, N.; Showe, D. N.; Sosa, M. I.; Suling, W. J.; White, E. L. High-Throughput Screening for Inhibitors of Mycobacterium Tuberculosis H37Rv. *Tuberculosis* **2009**, *89* (5), 334−353.
(43) Meissner, A.; Boshoff, H. I.; Vasan, M.; Duckworth, B. P.; Barry, C. E.; Aldrich, C. C. Structure-Activity Relationships of 2-Amino-thiazoles Effective against Mycobacterium Tuberculosis. *Bioorg. Med. Chem.* **2013**, *21* (21), 6385−6397.
(44) Lilienkampf, A.; Pieroni, M.; Franzblau, S. G.; Bishai, W. R.; Kozikowski, A. P. Derivatives of 3-Isoxazolecarboxylic Acid Esters - A Potent and Selective Compound Class against Replicating and Nonreplicating Mycobacterium Tuberculosis. *Curr. Top. Med. Chem.* **2012**, *12* (7), 729−734.
(45) Lilienkampf, A.; Pieroni, M.; Wan, B.; Wang, Y.; Franzblau, S. G.; Kozikowski, A. P. Rational Design of 5-Phenyl-3-Isoxazolecarboxylic Acid Ethyl Esters as Growth Inhibitors of *Mycobacterium Tuberculosis*. A Potent and Selective Series for Further Drug Development. *J. Med. Chem.* **2010**, *53* (2), 678−688.
(46) Mao, J.; Yuan, H.; Wang, Y.; Wan, B.; Pieroni, M.; Huang, Q.; van Breemen, R. B.; Kozikowski, A. P.; Franzblau, S. G. From Serendipity to Rational Antituberculosis Drug Discovery of Mefloquine-Isoxazole Carboxylic Acid Esters. *J. Med. Chem.* **2009**, *52* (22), 6966−6978.
(47) Pieroni, M.; Lilienkampf, A.; Wan, B.; Wang, Y.; Franzblau, S. G.; Kozikowski, A. P. Synthesis, Biological Evaluation, and Structure−Activity Relationships for 5-[(*E*)-2-Arylethenyl]-3-Isoxazolecarboxylic Acid Alkyl Ester Derivatives as Valuable Antitubercular Chemotypes. *J. Med. Chem.* **2009**, *52* (20), 6287−6296.
(48) Pieroni, M.; Lilienkampf, A.; Wang, Y.; Wan, B.; Cho, S.; Franzblau, S. G.; Kozikowski, A. P. NOC Chemistry for Tuberculosis-Further Investigations on the Structure-Activity Relationships of Antitubercular Isoxazole-3-Carboxylic Acid Ester Derivatives. *ChemMedChem* **2010**, *5* (10), 1667−1672.
(49) Ballell, L.; Bates, R. H.; Young, R. J.; Alvarez-Gomez, D.; Alvarez-Ruiz, E.; Barroso, V.; Blanco, D.; Crespo, B.; Escribano, J.; González, R.; Lozano, S.; Huss, S.; Santos-Villarejo, A.; Martín-Plaza, J. J.; Mendoza, A.; Rebollo-Lopez, M. J.; Remuiñan-Blanco, M.; Lavandera, J. L.; Pérez-Herran, E.; Gamo-Benito, F. J.; García-Bustos, J. F.; Barros, D.; Castro, J. P.; Cammack, N. Fueling Open-Source Drug Discovery: 177 Small-Molecule Leads against Tuberculosis. *ChemMedChem* **2013**, *8* (2), 313−321.
(50) Menendez, C.; Gau, S.; Lherbet, C.; Rodriguez, F.; Inard, C.; Pasca, M. R.; Baltas, M. Synthesis and Biological Activities of Triazole Derivatives as Inhibitors of InhA and Antituberculosis Agents. *Eur. J. Med. Chem.* **2011**, *46* (11), 5524−5531.
(51) Keri, R. S.; Chand, K.; Ramakrishnappa, T.; Nagaraja, B. M. Recent Progress on Pyrazole Scaffold-Based Antimycobacterial Agents. *Arch. Pharm. (Weinheim, Ger.)* **2015**, *348* (5), 299−314.
(52) Tassini, S.; Sun, L.; Lanko, K.; Crespan, E.; Langron, E.; Falchi, F.; Kissova, M.; Armijos-Rivera, J. I.; Delang, L.; Mirabelli, C.; Neyts, J.; Pieroni, M.; Cavalli, A.; Costantino, G.; Maga, G.; Vergani, P.; Leyssen, P.; Radi, M. Discovery of Multitarget Agents Active as Broad-Spectrum Antivirals and Correctors of Cystic Fibrosis Transmembrane Conductance Regulator for Associated Pulmonary Diseases. *J. Med. Chem.* **2017**, *60* (5), 1400−1416.
(53) Brvar, M.; Perdih, A.; Renko, M.; Anderluh, G.; Turk, D.; Solmajer, T. Structure-Based Discovery of Substituted 4,5′-Bithiazoles as Novel DNA Gyrase Inhibitors. *J. Med. Chem.* **2012**, *55* (14), 6413−6426.
(54) Hantzsch, A. Condensationsprodukte Aus Aldehydammoniak Und Ketonartigen Verbindungen. *Ber. Dtsch. Chem. Ges.* **1881**, *14* (2), 1637−1638.
(55) Oh, L. M.; Wang, H.; Shilcrat, S. C.; Herrmann, R. E.; Patience, D. B.; Spoors, P. G.; Sisko, J. Development of a Scalable Synthesis of GSK183390A, a PPAR α/γ Agonist. *Org. Process Res. Dev.* **2007**, *11* (6), 1032−1042.
(56) Pieroni, M.; Annunziato, G.; Azzali, E.; Dessanti, P.; Mercurio, C.; Meroni, G.; Trifiró, P.; Vianello, P.; Villa, M.; Beato, C.; Varasi, M.; Costantino, G. Further Insights into the SAR of α-Substituted Cyclopropylamine Derivatives as Inhibitors of Histone Demethylase KDM1A. *Eur. J. Med. Chem.* **2015**, *92*, 377−386.
(57) Pieroni, M.; Azzali, E.; Basilico, N.; Parapini, S.; Zolkiewski, M.; Beato, C.; Annunziato, G.; Bruno, A.; Vacondio, F.; Costantino, G. Accepting the Invitation to Open Innovation in Malaria Drug Discovery: Synthesis, Biological Evaluation, and Investigation on the Structure-Activity Relationships of Benzo[b]Thiophene-2-Carboxamides as Antimalarial Agents. *J. Med. Chem.* **2017**, *60*, 1959−1970.
(58) Tahlan, K.; Wilson, R.; Kastrinsky, D. B.; Arora, K.; Nair, V.; Fischer, E.; Barnes, S. W.; Walker, J. R.; Alland, D.; Barry, C. E., 3rd; Boshoff, H. I. SQ109 Targets MmpL3, a Membrane Transporter of Trehalose Monomycolate Involved in Mycolic Acid Donation to the Cell Wall Core of Mycobacterium Tuberculosis. *Antimicrob. Agents Chemother.* **2012**, *56* (4), 1797−1809.
(59) Pieroni, M.; Machado, D.; Azzali, E.; Santos Costa, S.; Couto, I.; Costantino, G.; Viveiros, M. Rational Design and Synthesis of Thioridazine Analogues as Enhancers of the Antituberculosis Therapy. *J. Med. Chem.* **2015**, *58* (15), 5842−5853.
(60) Grant, S. S.; Kawate, T.; Nag, P. P.; Silvis, M. R.; Gordon, K.; Stanley, S. A.; Kazyanskaya, E.; Nietupski, R.; Golas, A.; Fitzgerald, M.; Cho, S.; Franzblau, S. G.; Hung, D. T. Identification of Novel Inhibitors of Nonreplicating Mycobacterium Tuberculosis Using a Carbon Starvation Model. *ACS Chem. Biol.* **2013**, *8* (10), 2224−2234.
(61) Machado, D.; Couto, I.; Perdigão, J.; Rodrigues, L.; Portugal, I.; Baptista, P.; Veigas, B.; Amaral, L.; Viveiros, M. Contribution of Efflux to the Emergence of Isoniazid and Multidrug Resistance in Mycobacterium Tuberculosis. *PLoS One* **2012**, *7* (4), e34538.
(62) Coelho, T.; Machado, D.; Couto, I.; Maschmann, R.; Ramos, D.; von Groll, A.; Rossetti, M. L.; Silva, P. A.; Viveiros, M. Enhancement of Antibiotic Activity by Efflux Inhibitors against

DOI: 10.1021/acs.jmedchem.7b00793
J. Med. Chem. XXXX, XXX, XXX−XXX

Multidrug Resistant Mycobacterium Tuberculosis Clinical Isolates from Brazil. *Front. Microbiol.* **2015**, *6*, 330.
(63) Lee, R. E.; Hurdle, J. G.; Liu, J.; Bruhn, D. F.; Matt, T.; Scherman, M. S.; Vaddady, P. K.; Zheng, Z.; Qi, J.; Akbergenov, R.; Das, S.; Madhura, D. B.; Rathi, C.; Trivedi, A.; Villellas, C.; Lee, R. B.; Rakesh; Waidyarachchi, S. L.; Sun, D.; McNeil, M. R.; Ainsa, J. A.; Boshoff, H. I.; Gonzalez-Juarrero, M.; Meibohm, B.; Böttger, E. C.; Lenaerts, A. J. Spectinamides: A New Class of Semisynthetic Antituberculosis Agents That Overcome Native Drug Efflux. *Nat. Med.* **2014**, *20* (2), 152−158.
(64) Devine, S. M.; Mulcair, M. D.; Debono, C. O.; Leung, E. W. W.; Nissink, J. W. M.; Lim, S. S.; Chandrashekaran, I. R.; Vazirani, M.; Mohanty, B.; Simpson, J. S.; Baell, J. B.; Scammells, P. J.; Norton, R. S.; Scanlon, M. J. Promiscuous 2-Aminothiazoles (PrATs): A Frequent Hitting Scaffold. *J. Med. Chem.* **2015**, *58* (3), 1205−1214.
(65) Aldrich, C.; Bertozzi, C.; Georg, G. I.; Kiessling, L.; Lindsley, C.; Liotta, D.; Merz, K. M.; Schepartz, A.; Wang, S. The Ecstasy and Agony of Assay Interference Compounds. *ACS Chem. Biol.* **2017**, *12* (3), 575−578.
(66) Chiarino, D.; Grancini, G.; Frigeni, V.; Biasini, I.; Carenzi, A. N-(4-Isoxazolylthiazol-2-Yl)Oxamic Acid Derivatives as Potent Orally Active Antianaphylactic Agents. *J. Med. Chem.* **1991**, *34* (2), 600−605.
(67) Pesce, E.; Bellotti, M.; Liessi, N.; Guariento, S.; Damonte, G.; Cichero, E.; Galatini, A.; Salis, A.; Gianotti, A.; Pedemonte, N.; Zegarra-Moran, O.; Fossa, P.; Galietta, L. J. V.; Millo, E. Synthesis and Structure−activity Relationship of Aminoarylthiazole Derivatives as Correctors of the Chloride Transport Defect in Cystic Fibrosis. *Eur. J. Med. Chem.* **2015**, *99*, 14−35.
(68) Sundarapandian, T.; Shalini, J.; Sugunadevi, S.; Woo, L. K. Docking-Enabled Pharmacophore Model for Histone Deacetylase 8 Inhibitors and Its Application in Anti-Cancer Drug Discovery. *J. Mol. Graphics Modell.* **2010**, *29* (3), 382−395.
(69) Andrews, K. T.; Haque, A.; Jones, M. K. HDAC Inhibitors in Parasitic Diseases. *Immunol. Cell Biol.* **2012**, *90* (1), 66−77.
(70) Falkenberg, K. J.; Johnstone, R. W. Histone Deacetylases and Their Inhibitors in Cancer, Neurological Diseases and Immune Disorders. *Nat. Rev. Drug Discovery* **2014**, *13* (9), 673−691.
(71) Makam, P.; Kannan, T. 2-Aminothiazole Derivatives as Antimycobacterial Agents: Synthesis, Characterization, in Vitro and in Silico Studies. *Eur. J. Med. Chem.* **2014**, *87*, 643−656.
(72) Al-Balas, Q.; Anthony, N. G.; Al-Jaidi, B.; Alnimr, A.; Abbott, G.; Brown, A. K.; Taylor, R. C.; Besra, G. S.; McHugh, T. D.; Gillespie, S. H.; Johnston, B. F.; Mackay, S. P.; Coxon, G. D. Identification of 2-Aminothiazole-4-Carboxylate Derivatives Active against Mycobacterium Tuberculosis H37Rv and the β-Ketoacyl-ACP Synthase MtFabH. *PLoS One* **2009**, *4* (5), e5617.
(73) Esposito, M.; Szadocka, S.; Degiacomi, G.; Orena, B. S.; Mori, G.; Piano, V.; Boldrin, F.; Zemanová, J.; Huszár, S.; Barros, D.; Ekins, S.; Lelièvre, J.; Manganelli, R.; Mattevi, A.; Pasca, M. R.; Riccardi, G.; Ballell, L.; Mikušová, K.; Chiarelli, L. R. A Phenotypic Based Target Screening Approach Delivers New Antitubercular CTP Synthetase Inhibitors. *ACS Infect. Dis.* **2017**, *3*, 428−437.
(74) Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J. Experimental and Computational Approaches to Estimate Solubility and Permeability in Drug Discovery and Development Settings. *Adv. Drug Delivery Rev.* **2001**, *46* (1−3), 3−26.
(75) Kondreddi, R. R.; Jiricek, J.; Rao, S. P. S.; Lakshminarayana, S. B.; Camacho, L. R.; Rao, R.; Herve, M.; Bifani, P.; Ma, N. L.; Kuhen, K.; Goh, A.; Chatterjee, A. K.; Dick, T.; Diagana, T. T.; Manjunatha, U. H.; Smith, P. W. Design, Synthesis, and Biological Evaluation of Indole-2-Carboxamides: A Promising Class of Antituberculosis Agents. *J. Med. Chem.* **2013**, *56* (21), 8849−8859.
(76) Gavan, T. L.; Town, M. A. A Microdilution Method for Antibiotic Susceptibility Testing: An Evaluation. *Am. J. Clin. Pathol.* **1970**, *53* (6), 880−885.
(77) Perdigão, J.; Macedo, R.; Silva, C.; Machado, D.; Couto, I.; Viveiros, M.; Jordao, L.; Portugal, I. From Multidrug-Resistant to Extensively Drug-Resistant Tuberculosis in Lisbon, Portugal: The Stepwise Mode of Resistance Acquisition. *J. Antimicrob. Chemother.* **2013**, *68* (1), 27−33.
(78) Caviedes, L.; Delgado, J.; Gilman, R. H. Tetrazolium Microplate Assay as a Rapid and Inexpensive Colorimetric Method for Determination of Antibiotic Susceptibility of Mycobacterium Tuberculosis. *J. Clin. Microbiol.* **2002**, *40* (5), 1873−1874.
(79) Eliopoulos, G. M.; Wennersten, C. B.; Gold, H. S.; Moellering, R. C., Jr. In Vitro Activities in New Oxazolidinone Antimicrobial Agents against Enterococci. *Antimicrob. Agents Chemother.* **1996**, *40* (7), 1745−1747.
(80) Wayne, L. G.; Hayes, L. G. An in Vitro Model for Sequential Study of Shiftdown of Mycobacterium Tuberculosis through Two Stages of Nonreplicating Persistence. *Infect. Immun.* **1996**, *64* (6), 2062−2069.
(81) Viveiros, M.; Martins, M.; Rodrigues, L.; Machado, D.; Couto, I.; Ainsa, J.; Amaral, L. Inhibitors of Mycobacterial Efflux Pumps as Potential Boosters for Anti-Tubercular Drugs. *Expert Rev. Anti-Infect. Ther.* **2012**, *10*, 983−998.
(82) Cabassi, C. S.; Taddei, S.; Cavirani, S.; Baroni, M. C.; Sansoni, P.; Romani, A. A. Broad-Spectrum Activity of a Novel Antibiotic Peptide against Multidrug-Resistant Veterinary Isolates. *Vet. J.* **2013**, *198* (2), 534−537.
(83) Romani, A. A.; Baroni, M. C.; Taddei, S.; Ghidini, F.; Sansoni, P.; Cavirani, S.; Cabassi, C. S. In Vitro Activity of Novel in Silico-Developed Antimicrobial Peptides against a Panel of Bacterial Pathogens. *J. Pept. Sci.* **2013**, *19* (9), 554−565.
(84) Rodrigues, L.; Wagner, D.; Viveiros, M.; Sampaio, D.; Couto, I.; Vavra, M.; Kern, W. V.; Amaral, L. Thioridazine and Chlorpromazine Inhibition of Ethidium Bromide Efflux in Mycobacterium Avium and Mycobacterium Smegmatis. *J. Antimicrob. Chemother.* **2008**, *61* (5), 1076−1082.
(85) Machado, L.; Spengler, G.; Evaristo, M.; Handzlik, J.; Molnár, J.; Viveiros, M.; Kiec-Kononowicz, K.; Amaral, L. Biological Activity of Twenty-Three Hydantoin Derivatives on Intrinsic Efflux Pump System of Salmonella Enterica Serovar Enteritidis NCTC 13349. *In Vivo* **2011**, *25* (5), 769−772.

DOI: 10.1021/acs.jmedchem.7b00793
J. Med. Chem. XXXX, XXX, XXX−XXX