

Accepted Manuscript

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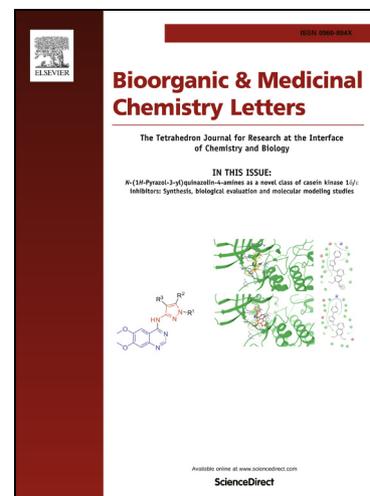
PII: S0960-894X(17)30773-4
DOI: <http://dx.doi.org/10.1016/j.bmcl.2017.07.067>
Reference: BMCL 25180

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 26 May 2017
Revised Date: 23 July 2017
Accepted Date: 25 July 2017

Please cite this article as: Clark, M.P., Wang, T., Perola, E., Deininger, D.D., Zuccola, H.J., Jones, S.M., Gao, H., VanderVen, B.C., Russell, D.G., Shoen, C.M., Cynamon, M.H., Thomson, J.A., Locher, C.P., 2-*N*-Arylthiazole Inhibitors of *Mycobacterium tuberculosis*, *Bioorganic & Medicinal Chemistry Letters* (2017), doi: <http://dx.doi.org/10.1016/j.bmcl.2017.07.067>

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2-N-Arylthiazole Inhibitors of *Mycobacterium tuberculosis*.

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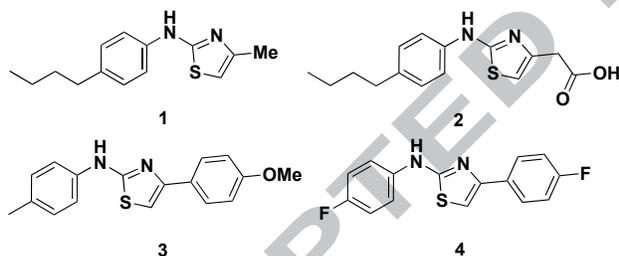
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Abstract: To develop agents for the treatment of infections caused by *Mycobacterium tuberculosis*, a novel phenotypic screen was undertaken that identified a series of 2-N-aryl thiazole-based inhibitors of intracellular *Mycobacterium tuberculosis*. Analogs were optimized to improve potency against an attenuated BSL2 H37Ra laboratory strain cultivated in human macrophage cells *in vitro*. The insertion of a carboxylic acid functionality resulted in compounds that retained potency and greatly improved microsomal stability. However, the strong potency trends we observed in the attenuated H37Ra strain were inconsistent with the potency observed for virulent strains *in vitro* and *in vivo*.

Tuberculosis (TB) is a chronic infectious disease caused by *Mycobacterium tuberculosis* (Mtb). It has been estimated that as much as one third of the world's population is infected with latent Mtb.^{1,2} Approximately one in ten latent infections progresses into active tuberculosis disease.^{1,2} Mtb is classified as a Gram positive bacteria that has a cell wall, but lacks a phospholipid outer membrane. Its unique biology of surviving in an intracellular state within the human macrophage combined with its ability to reduce its metabolic rate and enter dormancy in response to drug treatment makes treating with standard antibiotics very difficult. Without an effective vaccine, the current standard of care relies on a long regimen of four anti-TB drugs selected from isoniazid, rifampicin, pyrazinamide, ethambutol and streptomycin for two months followed by two of these drugs for an additional four months. This burdensome regimen is made more difficult by lack of patient compliance and oversight. The failure of patient compliance has contributed to the rapid increase in multidrug-resistant (MDR-TB) and extensively drug-resistant (XDR-TB) strains that have become resistant to many of the known TB therapies.³ XDR-TB has been reported by 84 countries. An important characteristic of this infection is the formation of granulomatous lesions, consisting of clusters of infected macrophages, T lymphocytes, B lymphocytes and fibroblasts. It has been suggested that Mtb utilizes these granulomas to avoid destruction by the immune system of the host.⁴ Co-infection with HIV as a major driver for the disease along with the rapid spread of deadly MDR and XDR strains of Mtb reinforces the need for new anti-TB therapies.

Our objective was to utilize a unique phenotypic approach to identify screening hits with an advantage over more traditional Mtb screening approaches, specifically to identify small molecules that kill *M. tuberculosis* inside its host cell. An initial screening set of 340,000 compounds was screened in murine J774 macrophages infected with Mtb engineered to fluoresce (mCherry)^{5,6} and multiple series of inhibitors were identified. By virtue of this screening approach, identified hits offered the potential advantage of not only possessing anti-mycobacterial activity, but also the ability to enter the macrophage and penetrate the Mtb bacterial membrane. Compound **1** was identified as a compound of high interest due to its strong potency ($IC_{50} < 40$ nM) in the Mtb-infected macrophage assay. Additionally, the low molecular weight (MW = 246) and structural simplicity were appealing as a starting point for medicinal chemistry lead optimization. Interestingly, the original screening hit was thought to be thiazole carboxylic acid **2**. However, hit validation analysis of the sample used for the phenotypic screen showed that thiazole **2** had undergone a decarboxylation over time in storage to give 4-methylthiazole **1**. Both compounds were re-synthesized and shown to be potent with a slight edge in potency for the methyl thiazole analog **1**. Interestingly, other examples of *N*-aryl-thiazole inhibitors of Mtb have been published recently,^{7,8} highlighted by compounds **3** and **4**, which reinforces the utility of these thiazole-based inhibitors.



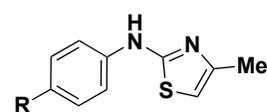
Initial SAR exploration focused on the 4-methylthiazoles derived from **1** (Table 1). An early goal for this series was to reduce lipophilicity (cLogP for **1** = 5.7) and improve upon the poor microsomal stability of **1**. Metabolite ID studies (data not shown) with compound **1** identified the butyl side chain as the primary site for metabolism.

Compounds were designed, synthesized and then tested in H37Ra Mtb-infected macrophages as our primary assay.⁹

Table 1 shows our exploratory efforts to replace this side chain while retaining potency and reducing oxidative metabolism. Initial analogs focused on various alkyl side chains. Strong potency was retained with *sec*-butyl, isopropyl and *tert*-butyl side chains (compounds **5**, **6** and **9**). A reduction in potency was observed with side chains smaller than isopropyl, exemplified by the ethyl and methyl substitutions (compounds **7** and **8**). Extending the butyl side chain to > 4 carbons retained potency (**10**, **11**) but offered no additional advantages. Limited exploration of the

meta position of the phenyl ring was explored. Moving the *n*-butyl side chain to the meta-position dropped potency more than 70-fold (macrophage IC₅₀ = 360 nM) relative to compound **1**. A more moderate 7-fold drop in potency was observed with the ethyl analog (macrophage IC₅₀ = 840 nM) relative to compound **7**. Moving the ethyl functionality position resulted in complete loss of potency (macrophage IC₅₀ > 20 μM). For this reason, we focused our efforts on the para-position. Introduction of polar functionalities designed to reduce the lipophilicity of the alkyl side chain showed good tolerance for ethers and alcohols. Ethers (**13**, **14**, **19**) showed consistently good potency, while alcohol **12** retained potency and the 2-hydroxybutyl analog (**16**) only lost 10-fold in potency. The terminal alcohol (compound **15**) showed a 100-fold reduction in potency. Side chains containing a basic amine (**17**, **18**) were not tolerated. Despite success in identifying additional polar side chains that conferred good potency, stability as measured in rat liver microsomes was generally poor. The one exception to this trend is compound **15**, which is the least lipophilic (cLogP = 2.0) relative to the remaining compounds with RLM data (cLogP range = 3.1 to 5.6).

Table 1. 4-methylthiazole analogs.



Compd	R	Macrophage ^a IC ₅₀ (μM)	RLM ^b stability (% unchanged at 30 min)	Compd	R	Macrophage ^a IC ₅₀ (μM)	RLM ^b stability (% unchanged at 30 min)
1		<0.005	1	12		0.060	12
5		<0.040	0	13		0.150	4
6		0.010	0	14		<0.040	1
7		0.120	0	15		3.19	73
8	Me	> 20	ND	16		0.400	1
9		<0.040	0	17		>20	0
10		0.040	3	18		>20	ND
11		0.050	ND	19		0.070	10

^aA reporter strain of Mtb expressing firefly luciferase was used to infect THP-1 cells and measure bacterial growth at 50% inhibitory concentration at five days post-infection; ^brat liver microsome stability measured as % unchanged at 30 minutes (1 μ M); ND = not determined

We next turned our attention to the thiazole carboxylic acids related to **2** (Table 2). Our hypothesis was the methyl group at the 4-position of the electron rich thiazole ring might also be an easy target for CYP-mediated metabolism and the acetic acid functionality could serve to stabilize the methyl thiazole ring system. The SAR shown in Table 2 demonstrates the high tolerance for variable substitutions at the thiazole 4-position (R^3) of the thiazole ring in this sub-series. The acetic acid analogs at the 4-position of the thiazole ring (**2**, **21**, **22**) showed similar potency in the macrophage assay. Replacing the carboxylic acid functionality with a primary carboxamide (**20**) resulted in a 5-fold decrease in potency while attaching the carboxylate directly to the 4-position of the thiazole (**23**) resulted in total loss of potency. As shown in Table 2, analogs containing the carboxylic acid functionality generally showed lower clearance and good oral exposure in mice, highlighted by acid analogs **2**, **21**, **22** and **24-27**. Attempts to incorporate an ether functionality into the phenyl side chain generally resulted in a loss of potency (data not shown). The best alkyl ether side chain was compound **28**, which showed good exposure in mouse, but the potency was less than desirable.

Table 2. *N*-aryl-2-aminothiazole carboxylate analogs with mouse pharmacokinetic parameters

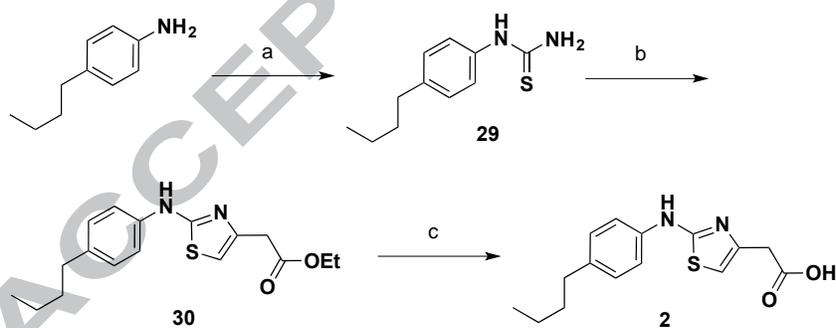
	R_1		R_3	M acr op ha ge ^a IC 50 (μ M)	Mouse PK ^b iv Cl ^c , $T_{1/2}$ (h) AUC ^d , Cmax ^e	
2	<i>n</i> -Bu	H		0.15	5, 3.1	4.9, 4.8
20	<i>n</i> -Bu	H		0.86	--	--
21	<i>n</i> -Bu	H		0.14	4, 0.9	4.8, 4.5

22	<i>n</i> -Bu	H		0.14	18, 5	1.4, 1.8
23	<i>n</i> -Bu	H		> 20	--	--
24	<i>t</i> -Bu,	H		0.07	4, 1.1	24.1, 4.0
25	<i>n</i> -Bu	Me		0.10	7, 1.1	4.8, 4.1
26	<i>n</i> -Bu	Cl		0.07	5, 1.1	6, 2.9
27	(+/-)-2-Bu	H		0.07	3, 2.8	14.4, 6.6
28		H		0.27	1.5, 1.6	15, 4.0

^aA reporter strain of Mtb expressing firefly luciferase was used to infect THP-1 cells and measure bacterial growth at 50% inhibitory concentration at five days post-infection; ^bmouse PO at 3 mg/kg, mouse IV at 1 mg/kg; ^cCl measured (mL/min/kg); ^dAUC measured ($\mu\text{g}\cdot\text{h}/\text{mL}$); ^enormalized to actual dose ($\mu\text{g}/\text{mL}$).

The thiazole analogs were prepared as shown in Scheme 1. All compounds were prepared in a similar fashion.

Starting with 4-butylaniline, conversion to the primary thiourea, **29**, is followed by alkylation and subsequent cyclocondensation with ethyl 4-chloro-3-oxobutanoate to afford the thiazole intermediate, **30**. For compound **2**, the final step was ester hydrolysis under basic conditions.



Scheme 1. Synthesis of thiazole acid **2**. Reagents and conditions: (a) NH_4SCN , HCl , $105\text{ }^\circ\text{C}$; (b) ethyl 4-chloro-3-oxobutanoate, EtOH , $90\text{ }^\circ\text{C}$; (c) LiOH , MeOH .

Given the strong potency in the Mtb macrophage assay coupled with good plasma exposure in mice, we advanced compounds **2**, **24**, **26** and **27** as well as the methyl thiazole analog, **1**, to be evaluated in a murine model of Mtb infection. Intranasal infections of BALB/c mice with 10^6 cfu of the Mtb Erdman (BSL3) isolate (six mice per group)

were performed as previously described.¹⁰ An early control (EC) group was euthanized at the initiation of therapy to determine the infection load and a late control (LC) group was used to determine the infection load at the end of an experiment and to confirm virulence. Mice were treated daily (5 days/week) for four weeks (100 mg/kg) and colony forming units (cfu) were determined from Mtb recovered from lung homogenates. Rifampin (10 mg/kg) was used as a positive control antibiotic.

Unfortunately, none of the five analogs showed a statistically significant reduction of Mtb cfu in lung homogenates from infected mice. The lack of efficacy in the mouse model led us to further explore the lack of *in-vitro* to *in-vivo* translation. Comparison between BSL2 broth and macrophage assays shows a strong correlation in activity (Table 3). However, upon testing these analogs in multiple BSL3 Mtb strains, we were surprised to see a substantial reduction in potency. First, a significant drop in potency was observed for compounds **24**, **26** and **27** in the BSL3 H37Rv-7H9 assay with an average reduction of 120-fold. In this case, the H37Rv-7H9 assay utilizes the same medium as the macrophage assay with glucose and glycerol as the carbon source. An additional drop in activity was observed when these analogs were tested in the H37Rv-7H12, Erdman 7H12 and H37Rv low oxygen recovery (LORA) BSL3 assays. The low oxygen recovery assay duplicates the H37Rv-7H12 assay but under anaerobic conditions.¹¹ All three of these assays utilize a different medium with palmitic acid as the source of carbon, suggesting that the source of carbon as well as the strain (BSL2 vs BSL3) both play a significant role in the lack of *in vitro* translation. The inability of these compounds to show efficacy in the mouse model was consistent with the limited potency observed against the virulent BSL3 strains.

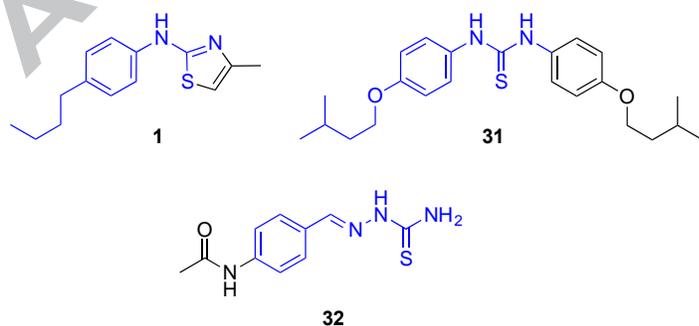
Table 3. Potency of thiazole analogs in Mtb BSL2 and BSL3 strains

Compd	H37Ra broth ^a BSL-2 MIC (μM)	H37Ra macrophage ^b BSL2 IC ₅₀ (μM)	H37Rv 7H9 ^b BSL-3 IC ₅₀ (μM)	Erdman 7H9 ^c BSL-3 MIC (μM)	H37Rv 7H12 ^b BSL-3 IC ₅₀ (μM)	H37Rv LORA ^b BSL-3 IC ₅₀ (μM)	Erdman 7H12 ^b BSL-3 IC ₅₀ (μM)
1	0.04	0.04	ND	7	47	12	>100
2	0.16	0.15	ND	7.8	91	17.8	56
24	0.16	0.07	9.4	6.3	>100	>100	63
26	0.39	0.07	6.3	12.5	>100	5.6	54

27	0.16	0.07	9.4	6.3	>100	96	>100
rifampin	0.08	0.03	--	--	--	--	--

^a Concentration (μM) of test compound resulting in 70% inhibition of growth compared to control wells was reported as MIC in the BSL2 H37Ra broth assay; ^b Reporter strain of Mtb expressing firefly luciferase was used to infect THP-1 cells and measure bacterial growth at 50% inhibitory concentration at five days post-infection; ^c Concentration (μM) of test compound resulting in 90% inhibition of growth compared to control wells was reported as MIC; ND = not determined

While it has been established that H37Ra is a useful surrogate for identifying and optimizing anti-Mtb activity, it has also been shown that false positives can sometimes be identified.¹⁰ Franzblau and Collins observed a good correlation between the activities against H37Ra and H37Rv strains across a panel of 30 known anti-Mtb agents.¹² One of the few agents in their study that did not show translation from H37Ra to H37Rv was thiacetazone **31** (TAC). Given that the SAR of the *N*-aryl-2-aminothiazole series suggests the 2-aminothiazole as the core scaffold responsible for the anti-Mtb activity, we hypothesized that this series of inhibitors might undergo sulfur oxidation to induce formation of a bioactive metabolite, similar to historic Mtb treatments, such as isoxyl (ISO), **31**, and thiacetazone (TAC), **32**. Both of these compounds are known to inhibit mycolic acid biosynthesis after bioactivation.^{13,14} While our aminothiazoles are not free thiocarbamides, as in **31** and **32**, the thiazole functionality does contain an embedded thiourea. In 2003, it was shown that the anti-Mtb effect of isoxyl could be partially reversed by the addition of oleic acid.¹⁵ We carried out similar oleic acid reversal experiments with both isoxyl and our *N*-aryl-2-aminothiazole inhibitors (compounds **1**, **2** and **27**) and confirmed partial loss of activity (4 fold for compounds **2** and **27** and 8 fold for compound **1**). In this experiment, oleic acid was able to diminish compound inhibition at concentrations typically around 25 μM . It is possible that the monooxygenase (EthA) that is responsible for bioactivation of ISO and TAC may also be an agent for activation of our 2-aminothiazole Mtb inhibitors.



Starting from a phenotypic screening effort using an intracellular (macrophage) H37Ra Mtb assay, a class of potent 2-*N*-arylaminothiazoles was identified. It was found that the 4-methylthiazole ring system could be stabilized against oxidative metabolism by the introduction of an acetic acid functionality with minimal loss in potency. The strong anti-mycobacterial activity observed in our H37Ra broth and macrophage assays did not consistently correlate with activity against the more virulent BSL3 strains (e.g., H37Rv strain) *in vitro* and did not afford protection in a mouse model when evaluated *in vivo*. Our data showed that the diminished potency of these thiazoles against virulent BSL3 strains was dependent on the strain used in our *in vitro* assays and also the carbon source. Preliminary experiments were conducted that showed a possible overlap of mechanism of bioactivation with historical anti-Mtb treatments such as isoxyl and thiacetazone.

Acknowledgements.

We thank Yuzhou Xu and Jie Wang of Shanghai ChemPartner for biological assay support, Xun Tao and Min Guo of Shanghai ChemPartner for PK assay support, and Scott Franzblau and Baojie Wang of the University of Illinois Chicago for evaluations of MIC compounds *in vitro*.

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