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Profiling of *in vitro* activities of urea-based inhibitors against cysteine synthases from *Mycobacterium tuberculosis*

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ABSTRACT

CysK1 and CysK2 are two members of the cysteine/S-sulfocysteine synthase family in *Mycobacterium tuberculosis*, responsible for the *de novo* biosynthesis of L-cysteine, which is subsequently used as a building block for mycothiol. This metabolite is the first line defense of this pathogen against reactive oxygen and nitrogen species released by host macrophages after phagocytosis. In a previous medicinal chemistry campaign we had developed urea-based inhibitors of the cysteine synthase CysM with bactericidal activity against dormant *M. tuberculosis*. In this study we extended these efforts by examination of the *in vitro* activities of a library consisting of 71 urea compounds against CysK1 and CysK2. Binding was established by fluorescence spectroscopy and inhibition by enzyme assays. Several of the compounds inhibited these two cysteine synthases, with the most potent inhibitor displaying an IC_{50} value of 2.5 μM for CysK1 and 6.6 μM for CysK2, respectively. Four of the identified molecules targeting CysK1 and CysK2 were also among the top ten inhibitors of CysM, suggesting that potent compounds could be developed with activity against all three enzymes.

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Mycobacterium tuberculosis poses a major threat to human health, not at least due to the emergence of multidrug-resistant and totally drug-resistant strains (http://www.who.int/tb/publications/global_report/en/). Chemotherapy is further complicated by the ability of *M. tuberculosis* to persist in the lungs of infected individuals for decades by switching to a dormant or latent phase,[1] which also induces tolerance to current antibiotics.[2] Estimates by the WHO indicate that about one-third of the world's population is infected with persistent mycobacteria, providing a large reservoir for further spread of the disease. Reactivation of these dormant bacteria can occur either spontaneously or as a consequence of an immune-compromised state, e.g. HIV infection, resulting in active tuberculosis.[1,3] In the persistent phase, *M. tuberculosis* survives within granulomas in the lungs of infected individuals where the environment is characterized by hypoxia, nutrient starvation, and oxidative stress. Primarily nitrogen monoxide and derived reactive nitrogen (RNI) and reactive oxygen intermediates (ROI) are used by phagocytic cells to kill the internalized bacteria.[4] Mycothiol, the major low molecular weight thiol used by mycobacteria to maintain their redox homeostasis, contains a cysteine-derived sulfhydryl moiety.[5,6] Thus, the first line of defense of the pathogen against free radicals, and hence its long term survival within macrophages and granulomas, is directly linked to cysteine availability. Cysteine is also required for repair and/or *de novo* biosynthesis of mycobacterial iron-sulfur center containing proteins that are damaged by reactive ROS and RNI species generated by the host macrophages.[7]

Three independent cysteine biosynthesis pathways have been characterized in this pathogen (Fig. S1).[8–13] Each of the three metabolic pathways to *de novo* cysteine biosynthesis in *M. tuberculosis* relies on a specific cysteine synthase. All the three enzymes CysM, CysK1 and CysK2 contain PLP as cofactor and belong to the same enzyme family, with amino acid sequence identities in the range of 26–42%. CysK1 uses sulfide as sulfur donors, whereas CysM uses the thiocarboxylated protein CysO as sulfur source. CysK2 can utilize sulfide to produce cysteine, but shows a strong preference for thiosulfate as sulfur donor resulting in S-sulfocysteine as reaction product. Gene expression profiling consistently showed that CysM, CysO and CysK2 are upregulated under conditions that simulate dormancy.[14–17] *M. tuberculosis* mutants carrying transposon insertions in CysM and CysO are attenuated in macrophages and in a mouse TB-model, demonstrating the essential role of these proteins for pathogen survival.[18,19] In view of these findings and the

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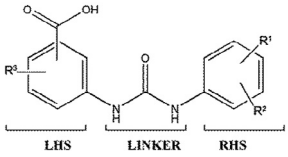
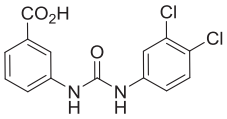
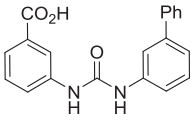
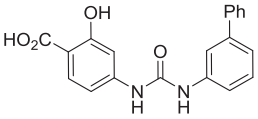
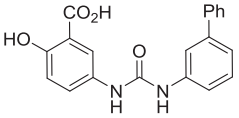
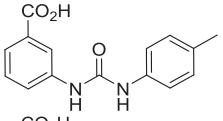
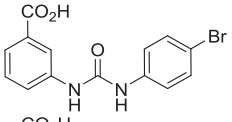
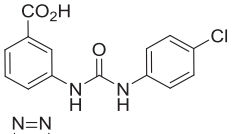
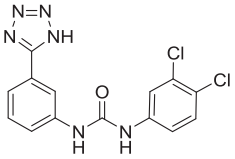
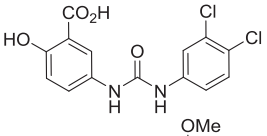
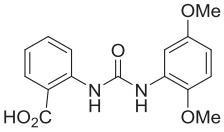
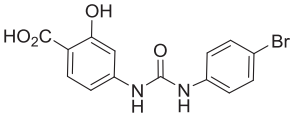
Table 1
Activities of urea compounds against mycobacterial cysteine synthases CysK1 and CysK2. Top: General structure of the urea compounds used in this study. LHS denotes left hand side and RHS right hand side, respectively.



Compound	Structure	CysK1		CysK2		CysM[a]	
		K_d (μM)	IC50 (μM)	K_d (μM)	IC50 (μM)	K_d (μM)	k_{obs} (min^{-1}) % activity[b]
1		241.1 ± 25.3	11.2 ± 4.5	48.7 ± 6.1	10.1 ± 2.4	0.32 ± 0.01	0.10 ± 0.01 0.3
2		96.8 ± 16.1	4.9 ± 0.7	14.9 ± 1.4	6.6 ± 1.9	1.7 ± 0.1	0.24 ± 0.06 0.6
3		n.d[c]	21.2 ± 9.6	n.d[c]	26.9 ± 11.0	4.5 ± 0.2	0.48 ± 0.18 1.2
4		no signal[d]	9.3 ± 4.6	102.0 ± 21.4	10.3 ± 4.1	2.2 ± 0.1	0.23 ± 0.05 0.6
5		no signal[d]	n/d	148.2 ± 15.0	n/d	8.0 ± 0.6	1.92 ± 1.08 4.9
6		no signal[d]	n/d	17.7 ± 1.9	27.6 ± 8.7	1.4 ± 0.4	0.48 ± 0.12 1.2
7		no signal[c]	n/d	16.9 ± 3.3	13.0 ± 8.4	3.4 ± 0.1	0.24 ± 0.06 0.6
8		no signal[d]	n/d	80.3 ± 9.8	n/d	no signal[d]	>20
9		75.7 ± 16.2	2.5 ± 0.5	no signal[d]	29.9 ± 9.4	no signal[d]	>20
24		59.9 ± 15.8	n/d	no signal[d]	n/d	no signal[d]	>20
31		n.d[c]	29.4 ± 12.2	no signal[d]	n/d	>300	>20

(*continued on next page*)

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