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Dichloro-naphthoquinone as a non-classical inhibitor of the mycobacterial carbonic anhydrase Rv3588c

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The soluble mycobacterial carbonic anhydrases Rv3588c and Rv1284 belong to a different class of carbonic anhydrases than those found in humans, making them attractive drug targets by using the inherent differences in the folds of the different classes. By screening a natural product library, we identified naphthoquinone derivatives as a novel non-classical inhibitor scaffold of mycobacterial carbonic anhydrases that lack the sulfonamide/sulfamate group and thus did not affect human carbonic anhydrase II.

Tuberculosis (TB) is an infectious diseases of global burden, affecting more than a third of the world's population with 9.6 million cases and 1.5 million deaths in 2014.[1] The infection, caused by the tubercle bacillus *Mycobacterium tuberculosis*, is treated with an extended course of combination therapy (i.e. multiple drugs taken at the same time). Many of the drugs commonly used today have adverse side effects, including nausea, vomiting, loss of appetite, fever, and jaundice. Current first-line drugs used to treat TB include isoniazid, rifampin, ethambutol, and pyrazinamide. However, resistance to these first-line drugs is increasing, with 480,000 cases of multi-drug resistant (MDR) TB reported worldwide in 2015.[2] Second-line drugs, such as kanamycin and fluoroquinolones, are used to treat patients with resistant TB, but this requires a complex treatment regimen and can cause severe side effects.[3] Extensively-drug resistant (XDR) TB strains, first detected in 2006, are resistant to first- and second-line anti-tubercular antibiotics, have occurred in 117 countries and represent approximately 10% of MDR-TB cases.[2] Thus, there is an urgent need to develop new, effective anti-TB drugs with efficacy against drug resistant mycobacterial strains, that account for up to 20% of new TB cases in some countries.[4,5]

Carbonic anhydrases (CA) are divided into five classes, α-ζ. Human carbonic anhydrases all belong to class α, while β-CAs are found in plants, algae, and bacteria. Two soluble β-CAs (Rv1284 and Rv3588c) found in *M. tuberculosis* have been determined as essential for the survival of the bacterium.[6,7] Their absence from the human host as well as significant structural differences compared to the α-CA enzymes in mammals make β-CAs promising targets for new therapeutic drugs.[8] Carbonic anhydrases are metal-dependent enzymes with major importance for the pH homeostasis of organisms since they catalyse the reversible hydration of CO_2 to form HCO_3^- and H^+.

Sulfonamides and their derivatives are well-established inhibitors of mammalian carbonic anhydrase enzymes.[9] In these "classical" carbonic anhydrase inhibitors, which include acetazolamide, topiramate and many others, the sulfonamide moiety ($-SO_2-NH_2$) provides anchorage of the drug to the active site zinc ion. The conjugated aliphatic, aromatic or heterocyclic groups engage in interactions with amino acid residues lining the cone-shaped active site cavity. Only few compounds that lack the sulfonamide moiety have been identified as inhibitors of carbonic anhydrase enzymatic activity.

In a previous investigation of a library of phenol-based natural products against a panel of β-carbonic anhydrases from pathogenic organisms two compounds were identified that displayed sub-micromolar inhibition of CA enzymatic activity.[10] Moreover, both compounds showed a preference of more than two orders of magnitude higher for fungal and mycobacterial carbonic anhydrases (Rv1284 and Rv3588c) compared to human members of this enzyme family. Importantly, the exceptionally small volume of the catalytic sites of Rv1284 and Rv3588c do not seem to allow binding of molecules with an extent larger than substrate analogues to the dimeric enzymes.[11,12] Further support of this notion was provided by a subsequent study revealing the redox regulation of the active site of Rv1284.[13] A range of chemical probes including the

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natural product polycarpine demonstrated redox effects on one of the active site cysteine residues of Rv1284 from an

These findings suggest that larger molecules may target sites other than the active sites to modulate the enzyme activity of mycobacterial carbonic anhydrases. Accordingly, one can hypothesise that it is possible to inhibit these enzymes with particular effectors in a non-competitive fashion using specific structural features absent from mammalian carbonic anhydrases.

Here, we tested a set of compounds (N = 100) recently added to Davis Open Access Compound Library (see below) as potential non-classical inhibitors of the mycobacterial carbonic anhydrases Rv3588c and Rv1284 (see ESI materials and methods for details) using our established platform consisting of a thermal denaturation assay as a first step to identify potential small-molecule ligands, followed by validation of hit compounds in stopped-flow enzyme assays.[8] From the ligand binding assay, we identified compounds **1**-**3** based on their substantial temperature shifts (see Table 1). Thermal denaturation of 40 μM Rv1284 in the absence and presence of compounds was monitored using SYPRO Orange (6.5×); experiments were performed with a Roche LightCycler 480 (Roche, Basel, Switzerland) as reported previously.[13] Data were analysed using the software DMAN[14] and ΔT_m values were calculated as difference between ligand and DMSO control experiments. Among the remaining 97 compounds, 20 showed a temperature shift of at least 1 K, and 76 compounds between 0 and 1 K.

Based on the identified naphthoquinone scaffold of the synthetic compounds **1**-**3**, molecules with structural similarity were identified from the larger Davis Open Access Compound Library (N = 472), which led to the addition of compounds **4**-**8** for further testing. The majority (53%) of compounds in this library are natural products that have been obtained from Australian natural sources, such as endophytic fungi,[15] plants,[16] macrofungi,[17] and marine invertebrates.[18] Approximately 28% of this library constitute semi-synthetic natural product analogues,[19] while a smaller percentage (19%) are known commercial drugs or synthetic compounds inspired by natural products.

In order to determine the importance of the oxygen atoms in the initial hits, **1**-**3**, compound **9** (2,3-dichloronaphthalene) was sourced commercially. To further probe the positions of the chloro-substituents in the initial hit **1**, a small library (N = 6) of N-alkylated derivatives was synthesised (**10**-**15**) with starting material **1** in yields ranging from 27% to 88% using a previously reported procedure (for details see ESI materials and methods).[20]

apparent surface-bound location that was confirmed by site-directed mutagenesis.

The effectiveness of compounds **1**-**15** as inhibitors of mycobacterial carbonic anhydrase Rv3588c was evaluated in assays of enzymatic CO_2 hydration catalysed by each enzyme (see ESI materials and methods for details). As is evident from the results listed in Table 1, 2,3-dichloronaphthoquinone (**1**) elicited the most substantial reduction (by ~80%) of the enzymatic activity of Rv3588c. Given the selective activity of 2,3-dichloronaphthoquinone (**1**) among the compounds tested in this study, the inhibitory effect of this compound on Rv3588c appears to be due to a specific interaction with the protein, rather than an unspecific redox reaction that would result in an oxidised state of the enzyme. The structure-activity relationships obtained from comparison of naphthoquinone **1** with naphthalene derivatives **2**-**4** and **6**-**15** reveal that (i) halogen or single hetero-atom substituents in positions 2 and 3 maximise the inhibitory activity; (ii) extended substituents in positions 2 and/or 3 diminish inhibitory activity. Comparison of compounds **1** and **9** highlights that oxygen substituents in positions 1 and 4 are required. Furthermore, the absence of inhibitory effects observed with quinone **5** highlight that the underlying naphthalene scaffold is a requirement.

In order to evaluate the specificity of the inhibitory effects of 2,3-dichloronaphthoquinone (**1**) for either mycobacterial CA, we next tested the relative activity of Rv1284 in the presence of compounds **1**-**15** (see Table 1). Clearly, the naphthoquinone derivatives were less effective in inhibiting Rv1284, with 2-methoxy-naphthoquinone (**4**) showing the largest reduction of Rv1284 enzyme activity (by ~40%).

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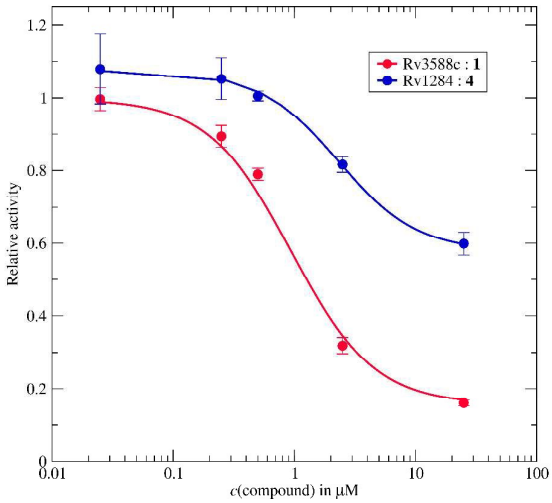
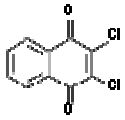
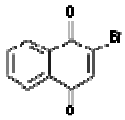
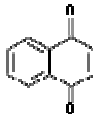


Figure 1: Dose-response data acquired for 2,3-dichloronaphthoquinone (**1**) and N-terminally His-tagged Rv3588c (red; IC_{50}: 1.2 µM, max. inhibition: 84%), and 2-methoxy-naphthoquinone (**4**) and Rv1284 (blue; IC_{50}: 2.3 µM, max. inhibition: 40%) at a final protein concentration of 2.5 µM. Each data point represents the mean of at least three independent measurements; error bars indicate the standard error. The solid lines show the fits of a logistic IC_{50} equation using the software SDAR.[21] Figure prepared with Grace-5.1.25 (http://plasma-gate.weizmann.ac.il/Grace/) and Inkscape

Compound		DSF assay		Enzyme assay‡					
		Rv1284		Rv3588c§		Rv1284		human CAII	
		ΔT_m (K)	*N*	Relative activity (%)	*N*	Relative activity (%)	*N*	Relative activity (%)	*N*
1		-10.2	3	16.1 (4.6)	6	81.5 (4.9)	3	96.8 (0.8)	2
2		-10.1	3	68.1 (9.0)	3	72.2 (14.0)	3	108.9 (9.3)	2
3		-8.8	3	94.8 (9.9)	3	76.6 (11.5)	3		

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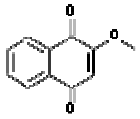
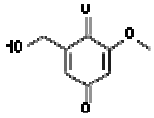
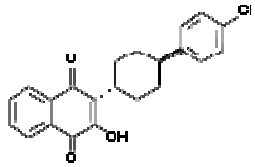
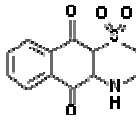
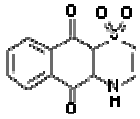
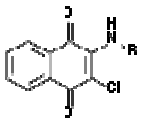
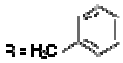
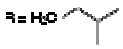
4		-0.5	3	84.5 (4.7)	2	62.3 (6.7)	3	104.4 (13.1)	2
5		+0.1	3	103.7 (25.0)	2	101 (10.8)	2		
6		-0.5	3	80.7 (5.2)	2	83.1 (2.5)	3		
7		-0.4	3	80.6 (4.3)	2	79.5 (5.1)	3		
8		-1.1	3	78.3 (0.9)	2	74.6 (8.4)	2		
9		+1.3	3	82.8 (8.7)	3	80.3 (4.3)	3		
									
10		-1.5	3	98.8 (2.4)	3	67.2 (11.5)	3		
11		-2.5	3	A99.1 (2.5)	3	96.5 (1.2)	2		
12		-0.7	3	93.2 (1.7)	3	110 (19.7)	2		
13		-1.5	3	103 (4.3)	3	103 (3.9)	2		
14		-0.9	3	110 (6.7)	3	79.4 (8.9)	2		
15		-0.6	3	93.8 (3.2)	3	68.5 (8.7)	3		

Table 1: Identification of hits in the natural product library and assessment of relative inhibitory activity of compounds in the CO_2 hydration assay catalysed by mycobacterial carbonic anhydrases.

In the context of discovering novel anti-mycobacterial lead molecules, any candidate molecules should be specific for mycobacterial CAs. Therefore, the possible modulation of the catalytic activity of human carbonic anhydrase II by the three most active compounds in this study (**1**, **2**, **4**) were evaluated. None of the three compounds displayed any significant effects on human CA II (see Table 1).

With compounds **1** and **4** emerging as the most active inhibitors against Rv3588c and Rv1284, respectively, we finally compared the efficacy of both compounds in dose-response experiments against their preferred target (Figure 1). From these results, the IC_{50} value of 2,3-

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dichloronaphthoquinone (**1**) with respect to Rv3588c was determined to be 1.2 μM with a maximum inhibition of 84%. In contrast, 2-methoxy-naphthoquinone (**4**) inhibited Rv1284 with an IC_{50} value of 2.3 μM but at a much lower maximum inhibition (40%). Notably, the synthetic compound **1** has previously been reported to exhibit antiproliferative and antifungal activities.[22] The natural product **4** was first isolated from the plant *Impatiens balsamina* L. Several studies have revealed that **4** possesses antifungal, antipruritic, and cytotoxicity effects.[23-25]

Conclusions

With the vast majority of currently known inhibitors of carbonic anhydrases relying on the sulfonamide moiety as an anchoring group to the active site metal ion, the naphthoquinone skeleton constitutes a non-classical inhibitor scaffold with respect to CA enzymes. As highlighted in previous studies,[8,13] the crystal structures of the β-CAs Rv1284 and Rv3588c[11] reveal that there are substantial steric constraints at the entry and exit sites of the catalytic centres and non-linear molecules with more than 3 or 4 atoms are virtually not able to enter the active sites of these proteins. Such differences in the three-dimensional structures and topologies between carbonic anhydrases of different classes may offer an opportunity to design inhibitor molecules specifically targeting non-mammalian members of this enzyme family. In this study, we identified the natural product 2,3-dichloronaphthoquinone (**1**) as a non-classical inhibitor of Rv3588c. To our knowledge, this is the most active non-classical mycobacterial CA inhibitor to date, with an IC_{50} value of 1.2 μM. Due to the steric constraints inherent in the fold of mycobacterial CAs, we suggest that this effector binds to a cleft on the surface of the protein, similar to the phenomenon observed earlier with Rv1284.[13] Among the mycobacterial CAs, the compound shows specificity for Rv3588c and, in agreement with the absence of features of classical CA inhibitors, shows no significant inhibitory activity of human CA II. Future studies should focus on structure-activity relationships of non-carbonyl derivatives of **1**.

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Conflict of Interest

The authors declare no competing interests.

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‡ Measured in the presence of 25 μM inhibitor and compared to protein in the absence of inhibitor; values in brackets indicate the standard error.
§ N-terminally His-tagged protein.

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Dichloro-naphthoquinone as a non-classical inhibitor of the mycobacterial carbonic anhydrase Rv3588c

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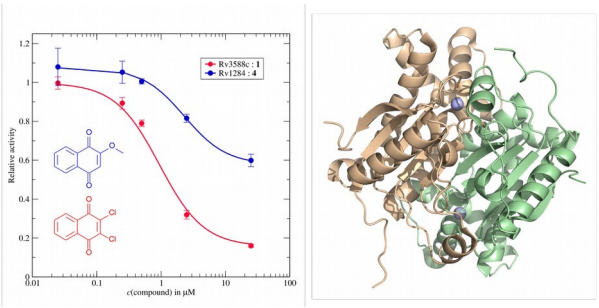

This study reports the most active non-sulfonamide mycobacterial CA inhibitor to date.