Supporting Information

Non-classical β-carbonic anhydrase inhibitors - Towards novel anti-mycobacterials

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Supplementary Materials and Methods
Subcloning of Rv1284 and Rv3588c. Preparation of recombinant proteins. Differential scanning fluorimetry. Enzyme activity assays. Determination of quaternary structure in solution. Bacterial growth assay.

Figure S1
Purity of recombinant proteins used in this study.
Denaturing-reducing SDS-PAGE (12%) using single gels[1] of fractions obtained in the final chromatographic separation. The gels were stained with Coomassie following standard procedures. The gels show no signs of contaminating proteins.

Figure S2
Size exclusion chromatography and quaternary structure of Rv3588c in solution, in absence and presence of compounds **1-3**.

Table S3
Enzyme parameters of carbonic anhydrase proteins tested in this study.

Table S4
Compounds from a subset (n = 90) of the in-house natural product library identified in differential scanning fluorimetry against His_6-Rv1284 with $|\Delta T_m| > 0.8$ K and their inhibitory activity in the CO_2 hydration reaction catalysed by Rv3588c.

Supplementary Materials and Methods

Subcloning of Rv1284 and Rv3588c. The cDNA constructs of His_6-tagged Rv1284 and Rv3588c in pCR-T7[2] were used as template in a PCR reaction with *Pfu*Ultra II Fusion HS DNA Polymerase (Agilent, Mulgrave, Victoria, Australia) to obtain the genes of untagged wild-typ proteins. For Rv1284, the oligonucleotides used were 5'-G GAA TTC CAT ATG ACG GTT ACC GAC GAC TAC CTG G-3' (coding) and 5'-CGC GGA TCC TTA GGG CGT GAC CTC GTT GAG TTT G-3' (non-coding); for Rv3588c, the oligonucleotides were 5'-G GAA TTC CAT ATG CCC AAC ACC AAT CCG GTA GC-3' (coding) and 5'-CGC GGA TCC TTA GAC CTC CTC GCC GAT GTT GC-3' (non-coding). Purified PCR products were ligated into pRSET_6c[3] via the *Nde*I and *BamH*I restriction enzyme sites. Chemically competent *Escherichia coli* XL1Blue cells generated in house were used for transformation. Single colonies were picked, propagated and cDNA was purified using the NucleoBond Xtra kit (MN; Scientifix Life, Brisbane, Queensland, Australia). All constructs were validated by DNA sequencing using BigDye chemistry.

Preparation of recombinant proteins. The bacterial expression plasmids were transformed into *Escherichia coli* BL21-AI cells, and a liquid overnight culture in 1 L LB^+ medium with 50 µg mL^{-1} ampicillin was grown at 37°C. A total of 8 L of LB^+ medium (50 µg mL^{-1} ampicillin) were inoculated with the overnight culture. The cells were grown at 37°C for 4 hrs. For induction, 0.2% arabinose was added, and in case of the untagged proteins, IPTG at a final concentration of 0.5 mM was also added. Incubation continued for another 4 hrs at 37°C. After harvest, the cells were resuspended (100 mM NaCl, 1 mM EDTA, 20 mM TRIS (pH 8), 0.1% Triton X-100, 1 mM PMSF, 5 mM benzamidinium chloride), and lysed by multiple freeze-thaw cycles and subsequent sonication. The resulting suspension was cleared by ultracentrifugation (100000×*g*, 45 min, 4ºC).
For the His-tagged proteins, the supernatant from the ultracentrifugation step was diluted 3-fold with equilibration buffer (100 mM NaCl, 20 mM TRIS, pH 8.0) and subjected to immobilised metal ion affinity chromatography using Ni^{2+}-NTA resin (QIAGEN). The fractions obtained from step elution with 20 mM, 50 mM, 100 mM, 200 mM and 500 mM imidazole in 100 mM NaCl, 20 mM TRIS (pH 8.0) were pooled appropriately, dialysed against 20 mM TRIS (pH 8.0) and purified in a second step by anion exchange chromatography with Q-Sepharose (GE Health). Protein was eluted with a gradient 0-1 M NaCl, 20 mM TRIS (pH 8.0), appropriate fractions were pooled and concentrated by ultrafiltration. The buffer was exchanged to 5% glycerol, 150 mM NaCl, 20 mM TRIS (pH 8.0) by multiple wash steps in the final ultrafiltration cycles. The untagged proteins were purified by anion exchange chromatography with QA52 (0-1 M NaCl, 20 mM TRIS, pH 8.0) and subsequent cation exchange chromatography using SP-Sepharose (20 mM NaAc, pH 4.5) where the desired proteins appeared in the flow-through and wash fractions. All purification steps were monitored by SDS-PAGE; representative results from the final purification steps are shown in Figure S1.

Differential scanning fluorimetry
The optimal ratio of protein and fluorescence dye was optimised by testing a 4×5 matrix of conditions varying the protein concentration from 2.5 µM to 40 µM, and SYPRO Orange (Invitrogen; Life Technologies, Mulgrave, Victoria, Australia) concentration between 5× and 20×, using a sample volume of 20 µl with a buffer composed of 100 mM NaCl, 20 mM HEPES (pH 7.5). For Rv1284, the best conditions were determined to contain 40 µM protein and 6.5× SYPRO Orange. At least three technical replicas were then tested for for each ligand using the optimised protein-dye ratio. Ligands were added at a final concentration of 250 µM in 20 µl sample aliquots, with a final DMSO concentration of 5%. Experiments were conducted on a Roche LightCycler 480,

and analysed using the software DMAN.[4] ΔT_m values were calculated as difference between ligand and DMSO control experiments.

Enzyme activity assays

Kinetic analysis of CO_2 hydration activity of the proteins in this study was done using a stopped flow assay following the methodology previously reported by Khalifah.[5] The change of absorbance of *m*-cresol purple was monitored at a wavelength of 572 nm using a Bio-Logic SFM-100 MOS LED stopped flow instrument. All solutions were made with freshly filtered and degassed deionised water; CO_2 was obtained from BOC, Australia, and other chemicals from Sigma-Aldrich, Australia. Inhibitor stock solutions were prepared in DMSO or methanol at 60 mM concentration. For an individual experiment, the sample buffer contained 100 mM Na_2SO_4, 25 mM TAPS (pH 8.5), 50 µM *m*-cresol purple, 5 µM protein and 25 µM inhibitor. The substrate buffer consisted of CO_2-saturated water, diluted with degassed water to achieve final CO_2 concentrations between 2 and 17 mM. The reactions are followed for the first 10 sec, and at least five traces are analysed for each individual experiment to determine the initial rates of the reaction. Assuming non-competitive inhibition, the inhibition constant K_i can be estimated from v_{max} and K_m in the absence and v_{max}', K_m' in the presence of inhibitor according to: $K_i = v_{max}' \cdot c(\text{inhibitor}) / v_{max}$.[6]

Determination of quaternary structure in solution

Effects of ligand binding on the quaternary structure of proteins was assessed by size exclusion chromatography coupled to a light scattering and refractive index detector system (SEC-MALS), comprising of a BioRad DuoFlow HPLC with Wyatt miniDawn TREOS multi-angle light scattering and Shimadzu refractive index detector. Monomer and dimer contents were determined by integration of the chromatograms using the software SDAR.[7]

Bacterial growth assay

Compounds were serially diluted in 10 µL of purified H_2O in triplicate in 96 well microtiter plates. *M. tuberculosis* H37Ra was grown in complete Middlebrook 7H9 media (Bacto, Australia) containing albumin, dextrose and catalase (ADC), 20% Tween 80 and 50% glycerol (Sigma-Aldrich, Australia). A bacterial suspension (90 µL) at OD_{600nm} of 0.001 was added to the wells and incubated for 7 days. Resazurin (10 µL; 0.05% w/v; Sigma-Aldrich, Australia) was then added, incubated for 24 h at 37°C, and fluorescence measured at 590 nm using a FLUOstar Omega microplate reader (BMG Labtech, Germany). After subtraction of background fluorescence from all wells, the percentage mycobacterial survival was determined by comparing the fluorescence of wells containing compounds compared to control wells not treated with compound.

Figure S1
Purity of recombinant proteins used in this study as assessed by 12% SDS-PAGE.

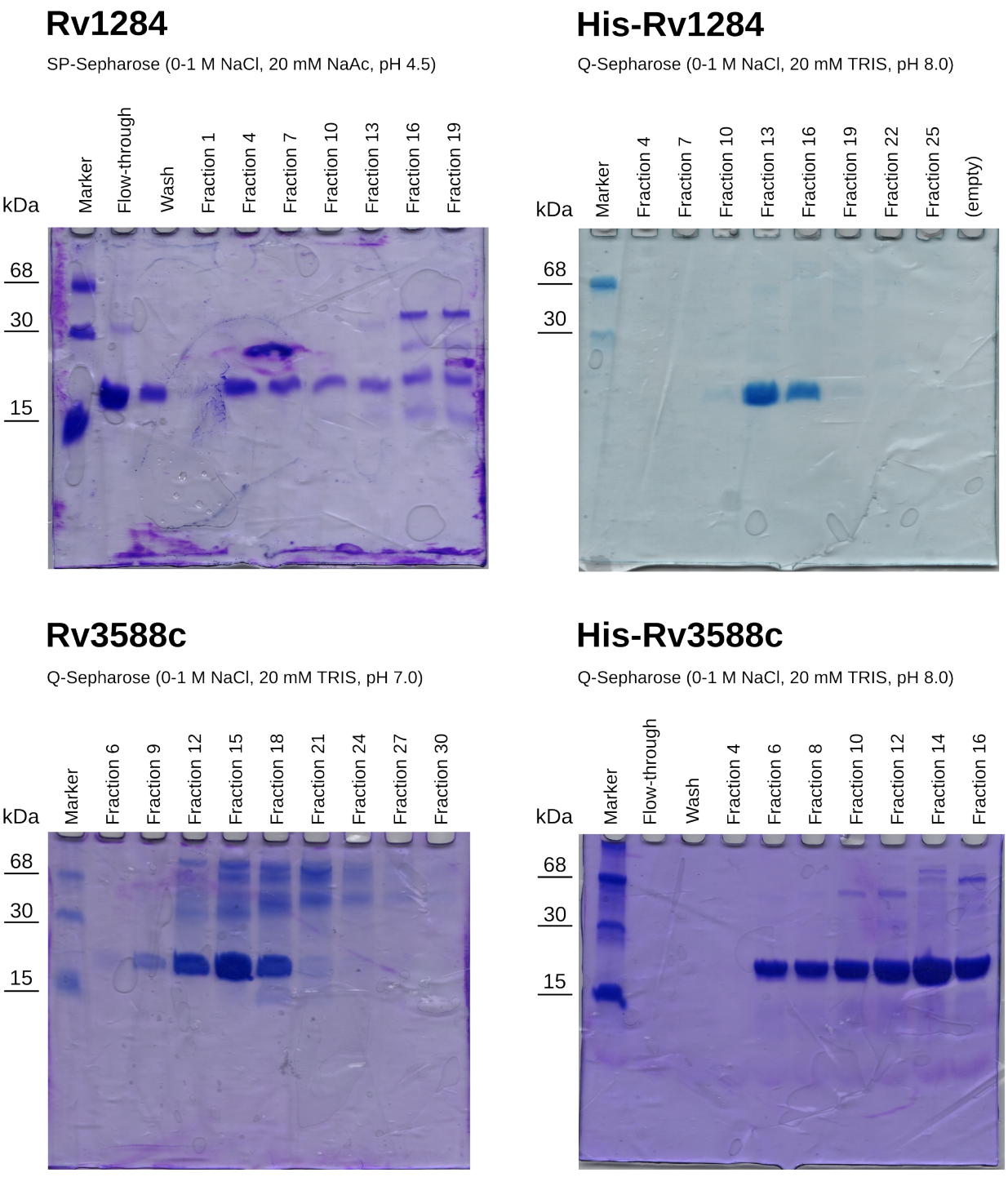
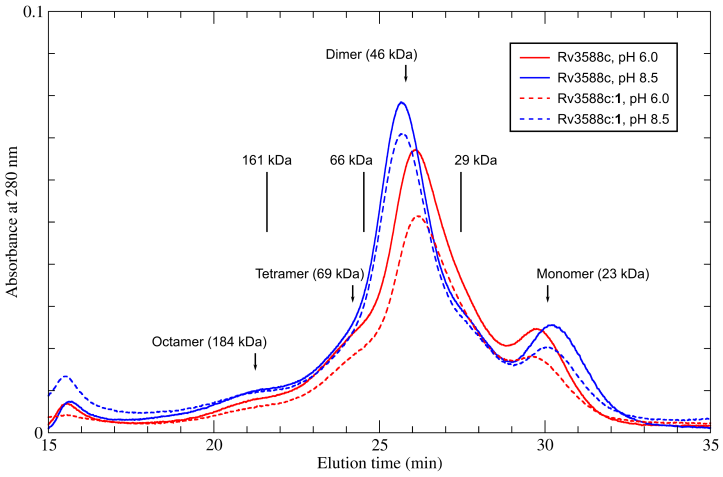
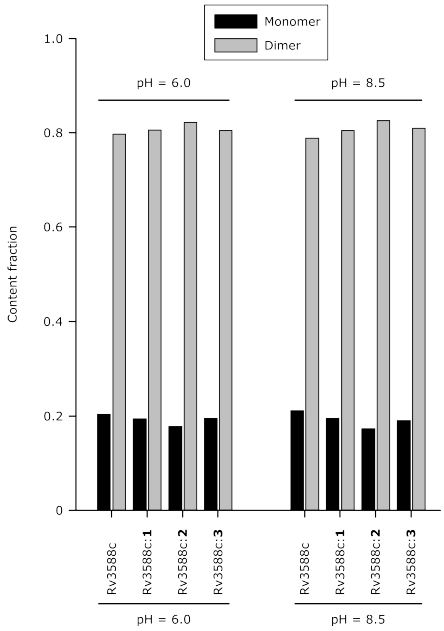


Figure S2
Left: Size exclusion chromatograms of untagged Rv3588c in the absence and presence of compound **1** at acidic and basic pH. Right: Monomer and dimer contents of untagged Rv3588c in the absence and presence of compounds **1-3** (see Supplementary Table S4). The contents have been calculated based on size exclusion chromatography at pH 6.0 and 8.5.



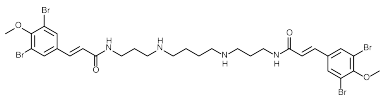
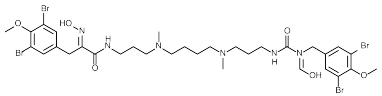
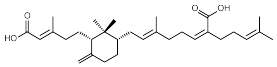


Supplementary Table S3
Enzyme parameters of carbonic anhydrase proteins tested in this study.

	Rv3588c	**His_6-Rv3588c**	**Rv1284**	**His_6-Rv1284**
K_m	3.5 mM	4.4 mM	7.9 mM	not active
k_{cat}/K_m	2.7×10^7 s^{-1} M^{-1}	1.5×10^7 s^{-1} M^{-1}	1.4×10^5 s^{-1} M^{-1}	
Hill coefficient[a]	2.3 [0.86]	2.0 [0.34]	2.5 [3.0]	

[a]Numbers in brackets denote the standard error.

Supplementary Table S4
Compounds from a subset (n = 90) of the Davis natural product library identified with inhibitory activity in the CO_2 hydration reaction catalysed by Rv3588c.

Compound				His_6-Rv1284	Rv3588c / His_6-Rv3588c			MIC90[b]
ID	**Trivial/IUPAC Name**	**Structure**	**Reference**	**DSF** ΔT_m **(K)**	**MM profile**	K_i **μM**	**Hill**[a]	
NaSCN	sodium thiocyanate				v_{max} decrease, K_m increase	8	4.8 [0.79]	
RAD168	ianthelliformisamine C (**1**)		8	-1.0	v_{max} decrease, K_m increase	16	4.0 [2.3]	12.5 μM
RAD169	spermatinamine (**2**)		9	-1.2	K_m increase	23	5.9 [2.6]	6.3 μM
RAD050*	(+)-mispyric acid (**3**)		10	-5.4	v_{max} decrease, K_m increase	10	4.0 [0.89]	> 50 μM

[a]Numbers in brackets denote the standard error.
[b]Minimum inhibitory concentration required to inhibit the growth of 90% of *M. tuberculosis* H37Rv.

References

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