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

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Mycobacterial carbonic anhydrases, such as the essential protein Rv3588c, are attractive drug targets since they constitute a different class of carbonic anhydrases than those found in humans. A natural product library has been screened for compounds that inhibit mycobacterial carbonic anhydrases but lack the much-exploited sulfonamide/sulfamate group. The identified inhibitors of mycobacterial Rv3588c are likely to affect the enzyme at a site different from the catalytic centre – a concept which may be exploited for novel specific anti-mycobacterials.

Tuberculosis is a chronic infection caused by the tubercle bacillus *Mycobacterium tuberculosis*, typically affecting the lungs, but also other parts of the body. According to WHO estimates, about a third of the world's population has been infected with the bacillus and the disease accounted for more than one million deaths in 2012.[1] While the disease had been decreasing steadily over past decades in developed countries, this pattern was reversed with the arrival of HIV and the increased mobility of people around the world.

Treatment for tuberculosis requires the use of multiple drugs for at least six months. Active tuberculosis is treated, usually successfully, with isoniazid in combination therapy with other antibiotics, including rifampicin, ethambutol, pyrazinamide, and streptomycin. However, there has been a rapid development of multi-drug resistant tuberculosis strains, accounting for more than 20% of new cases in a number of countries.[2-5]

Carbonic anhydrases are metal-dependent enzymes, catalysing the reversible hydration of CO_2 to form HCO_3^- and H^+, thus being a pivotal enzyme class for carbon fixation and pH regulation in living organisms. Enzymes with carbonic anhydrase activity are separated into five different classes (α-ζ).[6, 7] With exception of the ζ carbonic anhydrases, which uses cadmium as an alternative metal co-factor,[8] all other carbonic anhydrases are described as zinc-dependent. The seven human carbonic anhydrases belong to class α; β-carbonic anhydrases are found in eubacteria, archaea, algae and plants.[9] Importantly, the identified carbonic anhydrases from *M. tuberculosis* belong to class β. The *M. tuberculosis* genome (http://genome.tbdb.org) encodes four proteins with similarity to carbonic anhydrases : Rv3588c is essential for *M. tuberculosis* growth *in vivo*[10] and therefore a potential drug target. The physiological function of Rv1284 is not clear, but it is deemed to be essential based on Himar1-based transposon mutagenesis in strain H37Rv.[11] Additionally, its transcription levels are up-regulated under starvation conditions which are used to model persistent bacteria.[12] Very little is known so far about Rv3525c; based on homology with *Escherichia coli* acetyltransferase, however, transferase activity is predicted for this protein. Rv3273, a transmembrane protein, has not been found to be essential for survival of the pathogen.[10,11]

The common feature of enzymes belonging to the α- and β-families of carbonic anhydrases, respectively, is the existence of a zinc ion in the active site, but there are also considerable structural differences. Most notably, the β-carbonic anhydrases are oligomeric enzymes in solution; the fundamental structural unit is a dimer, but tetramers (Rv1284, Rv3588c)[13] and octamers (*Pisum sativum* β-carbonic anhydrase)[14] have also been observed. At present, there are no experimental structures of mycobacterial carbonic anhydrases in complex with small molecule inhibitors other than SCN^-, which is iso-electronic to the substrate of these enzymes, CO_2. Assessment of the active sites of Rv1284 and Rv3588c shows that access to the catalytic centres is restricted by the dimer interface. Furthermore, the active sites of β-carbonic anhydrases are generally much smaller (~7 $Å^3$ in Rv1284) than those of human α-carbonic anhydrases (~100 $Å^3$ in human CAII). From this analysis, one can derive the structural paradigm that binding of classical carbonic anhydrase inhibitors with the shape/extent of current clinical inhibitors in the active site is not possible for steric reasons.[9,15]

Phenol-based natural products have previously been shown to affect β-carbonic anhydrases from pathogenic organisms, as well as human α-carbonic anhydrases I and II.[15] Many of these natural products selectively inhibited the mycobacterial and fungal β-carbonic anhydrases, providing the first evidence that chemotypes lacking the classical sulfonamide/sulfamate motif (frequently employed with carbonic anhydrase inhibitors) display β- over α-carbonic anhydrase enzyme selectivity.

Here, we established a work flow to screen a subset of a unique natural product-based library to identify potential non-classical inhibitors of mycobacterial carbonic anhydrases. This in-house compound library currently consists of 352 distinct structures, the majority of which have been obtained from Australian natural sources, such as endophytic fungi,[16] macrofungi,[17] plants,[18] and marine invertebrates[19,20]. Approximately 15% of this library contains semi-synthetic natural product analogues[17,21] while a small percentage (~5%) are known commercial drugs or synthetic compounds inspired by natural products.

The work flow detailed in this study is based on the initial screening of a natural product discovery library by differential scanning fluorimetry (DSF), which provides an inexpensive and accessible first-line screening method.[22] In particular, this method

has recently been applied in similar studies, including carbonic anhydrases.[23] Using purified recombinant Rv1284 and Rv3588c as well as their N-terminally His_6-tagged fusion constructs (see Supplementary Materials and Methods for details), we tested their behaviour in DSF assays. Appropriate conditions could be identified for (His_6-)Rv1284, but the data for (His_6-)Rv3588c showed consistent high binding of the amphiphilic dye even at low temperatures, thus rendering (His_6-)Rv3588c not amenable to this screening technique. We thus subjected a panel of 90 compounds obtained from the in-house natural product library to protein-ligand binding assays by DSF using His_6-Rv1284, and identified 22 compounds with a $|\Delta T_m|$ larger than 0.8 K. Notably, the majority of compounds elicited a negative shift of the melting temperature, indicating destabilisation of the protein in the presence of the compound. This result would be in agreement with disassembly of the preferred oligomeric state of the protein. His_6-Rv3588c has previously been reported to adopt dimeric and tetrameric states in a pH-dependent manner.[13] In our hands, the quaternary solution structure of the His_6-fusion proteins as assessed by size exclusion chromatography reveals several oligomeric species. In contrast, the untagged proteins show a consistent mixture mainly consisting of monomer and dimer species (Figure S2). The effect of natural products (identified by DSF as potential ligands) on the quaternary structure of Rv3588c was then investigated by size exclusion chromatography at acidic and basic pH. As evident from Figure S2, none of the identified compounds caused significant changes in the quaternary solution structure of Rv3588c.

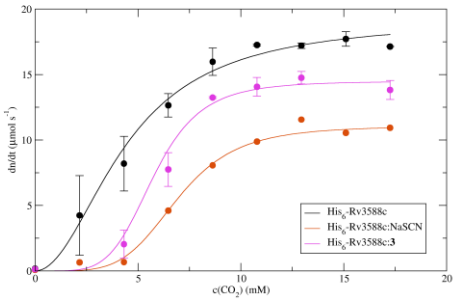

Figure 1. Enzyme kinetics of His_6-Rv3588c (c = 2.5 µM) in the absence (black) and presence (orange) of 25 µM NaSCN or 25 µM (+)-mispyric acid (**3**). Michaelis-Menten profile with fit to the Hill equation (Non-inhibited enzyme: n = 2.0 [0.34], k = 4.4 [0.39] mM, A = 19.2 [1.1], R^2 = 1.01; NaSCN: n = 4.8 [0.79], k = 6.9 0.23] mM, A = 11.1 0.38], R^2 = 0.986; **3**: n = 5.0 [0.89], k = 5.8 [0.17] mM, A = 14.5 [0.40], R^2= 0.936; numbers in brackets denote standard errors).

Despite a number of previous studies on enzyme activity and inhibition of Rv1284 and Rv3588c,[15,24,25] no Michaelis-Menten profiles or mechanistic studies for these two enzymes have been reported. We thus assessed the CO_2 hydration activity of the two mycobacterial carbonic anhydrases using stopped flow assays monitored by the absorbance change of the pH indicator *m*-cresol purple.[26] Since the pH optimum for both enzymes had previously been reported,[13] we chose to conduct all enzymatic assays at pH 8.5. The Michaelis-Menten profiles, plotting v_{max} vs. $c(CO_2)$, of Rv1284, Rv3588c and His_6-Rv3588c clearly revealed a cooperative behaviour that cannot be described with the Michaelis-Menten equation, but rather the Hill equation (see Figure 1). For Rv3588c, the apparent K_m values of both fusion and untagged proteins are very similar (3-4 mM), as are their turn-over numbers k_{cat}/K_m (see Table S3). This indicates that the enzymatic activity of Rv3588c is not significantly affected by the presence of an N-terminal His_6-fusion peptide. The catalytic efficiencies of all enzymes tested in this study are in agreement with data reported earlier.[27] It has previously been observed that His_6-Rv1284 does not display significant enzymatic activity in the CO_2 hydration assay,[13] and experiments in this present study confirm this finding.

In order to assess the enzyme inhibition ability of the identified compounds, they were subjected to an enzymatic CO_2 hydration assay using stopped flow kinetic measurements. For these enzymatic assays, we focused on His_6-Rv3588c, since this enzyme is essential for *M. tuberculosis* growth *in vivo*,[10] and the presence of the His_6-tag does not appear to significantly affect enzymatic activity.

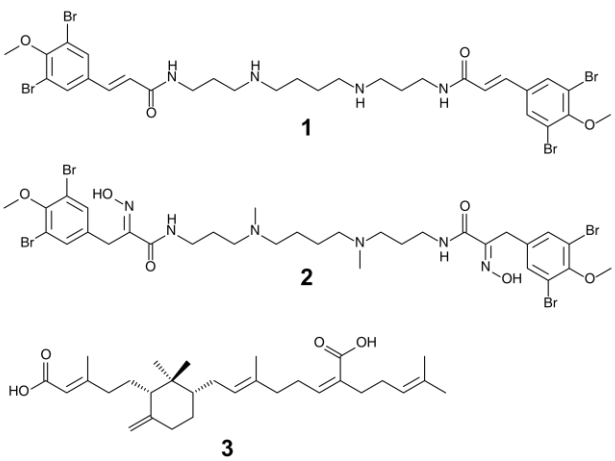

Figure 2. Chemical structures of the identified novel inhibitors of Rv3588c that include ianthelliformisamine C (**1**), spermatinamine (**2**), (+)-mispyric acid (**3**).

As a positive control, inhibition by 25 µM thiocyanate was investigated by means of a Michaelis-Menten profile (see Figure 1). The presence of SCN^- leads to a reduced v_{max} and an increase in the apparent K_m, as well as a higher Hill coefficient (n = 4.8). Therefore, Michaelis-Menten profiles for the 22 identified compounds were acquired. Out of these, 12 compounds were moderate effectors of enzyme activity, but three compounds (**1**-**3**, Figure 2 and Supplementary Table S4) clearly displayed substantial inhibitory effects [K_i = 16 µM (**1**), 23 µM

(**2**), and 10 μM (**3**)]. Since the presence of **1**-**3** result in a reduced v_{max}, in addition to an increased apparent K_m, these compounds clearly can not be classified as competitive inhibitors, in agreement with our hypothesis that they interact with the enzyme in a location distant from the active site.

To determine if these inhibitors of Rv3588c carbonic anhydrase activity were effective against the whole organism, we tested the compounds against *M. tuberculosis* H37Ra, which is commonly used as a surrogate for virulent *M. tuberculosis* strains in compound screening.[28] The minimum concentrations that inhibit the growth of 90% of bacteria (MIC90) determined for **1** and **2** were 12.5 and 6.3 μM, respectively. Compound **3** was inactive against mycobacteria (MIC90 > 50 μM), which may be due to poor cell permeability since **3** is a highly lipophilic compound with a calculated logP of ~8.

The natural products **1**-**3** have all been previously published, and various biological activities have been reported.[17,29-32] Ianthelliformisamine C (**1**) was first isolated from the marine sponge *Suberea ianthelliformis* and displayed moderate inhibitory activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus* with IC_{50} values of 8.9 and 4.1 μM, respectively.[30] Spermatinamine (**2**) was originally isolated from the sponge *Pseudoceratina* sp. and was the first natural product inhibitor of isoprenylcysteine carboxyl methyltransferase.[31] This enzyme catalyses the carboxyl methylation of oncogenic proteins in the final step of a series of post-translational modifications and has been proposed as an attractive and novel anticancer target.[31] (+)-Mispyric acid (**3**) was first identified from the stem bark of the rainforest plant, *Mishocarpus pyriformis*, and was shown to inhibit DNA polymerase β with an IC_{50} of 20 μM.[32]

The present study is an important step in the investigation of non-classical carbonic anhydrase ligands as specific inhibitors for members of the β-class of these enzymes. With compounds **1**-**3**, we have obtained chemical scaffolds as novel effectors of β-carbonic anhydrases that explore a different chemical space than the much-exploited sulfonamide or sulfamate functionalities. All these compounds share an extended shape that restricts access to the active site of β-carbonic anhydrases geometrically. Since none of the compounds investigated in this study showed any effect on the quaternary structure of the Rv3588c in solution, their inhibitory effect is most likely due to conformational changes in the protein elicited upon binding, but in a site distant from the active site. Additionally, compounds **1**-**3** possess significant potential for medicinal chemistry and library generation. Given that β-carbonic anhydrases are a shared protein family in mycobacteria, but also fungi and yeast, we believe that **1**-**3** are attractive starting points for developing novel anti-infective therapeutics against these pathogens. Furthermore, targeting carbonic anhydrases at sites different from the catalytic centre, decreases the likelihood of inhibition of host carbonic anhydrases.

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Notes and references

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