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β-CA-specific inhibitor dithiocarbamate Fc14–584B: a novel antimycobacterial agent with potential to treat drug-resistant tuberculosis

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ABSTRACT

Inhibition of novel biological pathways in Mycobacterium tuberculosis (Mtб) creates the potential for alternative approaches for treating drug-resistant tuberculosis. In vitro studies have shown that dithiocarbamate-derived β-carbonic anhydrase (β-CA) inhibitors Fc14–594A and Fc14–584B effectively inhibit the activity of Mtб β-CA enzymes. We screened the dithiocarbamates for toxicity, and studied the in vivo inhibitory effect of the least toxic inhibitor on M. marinum in a zebrafish model. In our toxicity screening, Fc14–584B emerged as the least toxic and showed minimal toxicity in 5-day-old larvae at 300 μM concentration. In vitro inhibition of M. marinum showed that both compounds inhibited growth at a concentration of 75 μM. In vivo inhibition studies using 300 μM Fc14–584B showed significant (p > .05) impairment of bacterial growth in zebrafish larvae at 6 days post infection. Our studies highlight the therapeutic potential of Fc14–584B as a β-CA inhibitor against Mtб, and that dithiocarbamate compounds may be developed into potent anti-tuberculosis drugs.

INTRODUCTION

Tuberculosis (TB) caused by Mtб is highly contagious and easily spreads through airborne droplets. The latest estimates show that 2 billion people worldwide are currently infected with the latent form of TB. In 2015, 10.4 million people developed active TB, and 1.8 million people died of the disease. Anti-TB drugs were introduced 40 years ago, but these have become less effective due to the development of drug resistance. There is an urgent need for safe and potent new drugs for the treatment of multi-drug resistant (MDR)-TB. In addition, it would be highly desirable for these new drugs to be effective against the latent form of TB.

Using sequenced mycobacterial genomes and proteome analyses, it is possible to identify pathways that are essential for the life cycle of Mtб. Carbonic anhydrase (CA) enzymes of pathogenic microorganisms are possible novel drug targets. CA enzymes catalyze the reversible hydration of carbon dioxide (CO2) to bicarbonate (HCO3–) and protons (H+), and are essential for many physiological processes, such as fatty acid biosynthesis, regulation of pH homeostasis, and survival of cells under hypoxia. Several studies have shown that the enzymatic activity of α- and β-CAs can be successfully inhibited both in vitro and in vivo using various inhibitors, including sulfonamides and phenolic acids. In the past, research has shown that ethoxzolamide, a sulfonamide CA inhibitor, attenuates virulence of Mtб by inhibiting the expression of virulence factors that are crucial for pathogenesis. In addition, recent research showed that CA inhibitor ethoxzolamide significantly reduced extracellular DNA (eDNA) export as bicarbonate positively influences eDNA export in a pH-dependent manner in M. avium, M. abscessus, and M. chelonae. The eDNA is an integral part of biofilm matrix of many pathogens, including Mtб, and bacteria within biofilm are more tolerant to antibiotics than microorganisms grown planktonically. These studies suggest that β-CAs are involved in expression of virulence factors and the export of eDNA in mycobacterial species, and that inhibition of mycobacterial CAs using chemical inhibitors could attenuate the virulence and reduce biofilm formation.

Studies have shown that the β-CAs are essential for growth and survival of Mtб in the host organism. Mtб is capable of survival and growth in adverse host environments, and has three β-CAs. Importantly, humans lack β-CAs, suggesting that the drugs targeted against the β-CAs of Mtб would be less harmful with fewer side effects. Thus, the β-CAs of Mtб could serve as excellent targets for drug development.

Supuran’s group has previously identified a novel class of antimycobacterial agents that target the β-CAs of Mtб. These dithiocarbamates (DTCs) inhibit both Mtб CA1 and CA3 in vitro by binding to the active site of the enzymes. However, to date, none of these agents have been screened for toxicity and safety in animals and no in vivo inhibition studies have been conducted using model organisms.

M. marinum is a close relative of Mtб and a natural pathogen of zebrafish (Danio rerio). The zebrafish model has been successfully used for modeling different aspects of human tuberculosis during the last 15 years. In the adult zebrafish model, the role of adaptive immune responses and the wide spectrum of disease
outcomes including latent and reactivated infection have been assessed.\textsuperscript{17,18} The transparent zebrafish larvae, on the other hand, is well-established as the perfect model for dissecting the early stages of active mycobacterial infection\textsuperscript{19,20} and as a platform for rapid discovery and toxicity testing of new antibiotics.\textsuperscript{21}

In this study, we evaluated the safety and toxicity of the two DTCs, Fc14–594A and Fc14–584B, and studied the inhibitory properties of these drugs \textit{in vitro} and \textit{in vivo} using \textit{M. marinum} and zebrafish as model organisms. The structures of the compounds that were used in the present study are shown in Figure 1.

\section*{Materials and methods}

\subsection*{Inhibitors}

The two DTCs Fc14–594A and Fc14–584B (Figure 1) used in the study were prepared from the corresponding amine by reacting with carbon disulfide in the presence of a base as reported earlier.\textsuperscript{16} \textit{In vitro}, the DTCs were investigated as specific inhibitors of two \textit{Mtb} \( \beta \)-CA enzymes (\textit{Mtb} CA1 and \textit{Mtb} CA3).\textsuperscript{13} The DTC compounds were dissolved in deionized and distilled water (ddH\textsubscript{2}O) to prepare 100 mM stock solutions. Series of dilutions of each compound were carried out in ddH\textsubscript{2}O before toxicological experiments.

\subsection*{Maintenance of zebrafish and ethical statement}

Wild type zebrafish of the AB strains were maintained at 28.5 \textdegree{}C in an incubator, as described previously.\textsuperscript{17} The 1–2 h post fertilization (hpf) embryos were collected from breeder tanks using a sieve and rinsed with embryonic medium [5.0 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl\textsubscript{2}, 0.33 mM MgSO\textsubscript{4}, and 0.1% w/v Methylene Blue (Sigma-Aldrich, Germany)]. All zebrafish experiments were done at room temperature. After the fixation, the larvae were transferred to PBS and fixed in 4% paraformaldehyde (PFA) in PBS for 3 h at room temperature. After the fixation, the larvae were transferred to 70% ethanol and stored at 4 \textdegree{}C before being embedded in paraffin. The paraffin embedded samples were sectioned into 5 \textmu{}M slices for the histochemical staining. The fixed sections containing samples were deparaffinized in xylene, rehydrated in an alcohol series, and histologically stained with Mayer’s Hematoxylin and Eosin Y (both from Sigma-Aldrich). After dehydration, the slides were mounted with EntellanNeu™ (Merck; Darmstadt, Germany). The slides containing the tissues were examined for the presence of pathological changes according to OECD guidelines \textsuperscript{18} and photographed using a Nikon Microphot microscope (Nikon Microphot-FXA, Japan). All the procedures were carried out at room temperature.

\subsection*{Isolation of total RNA and reverse transcription}

Three strains of \textit{M. marinum} (ATCC 927, ATCC BAA-535/M, and E11) were cultured, as described in the Materials and Methods section. \textit{M. marinum} infections of zebrafish larvae, but without Hygromycin B. The RNA extraction was performed from bacterial pellets of 30 mg using RNeasy® Mini kit (Qiagen, Hilden, Germany), following the manufacturer’s instructions. Purity and concentration of total RNAs from bacterial samples were determined using a NanoDrop Spectrophotometer (ThermoScientific, Waltham, MA) at 260 and 280 nm. A reverse transcriptase-reaction was performed for 50 ng of total RNA in a volume of 50 \mu{}l using a First Strand cDNA Synthesis kit (High-Capacity cDNA Reverse Transcription Kits,
Phylogenetic and sequence analyses

A selection of insect, parasite and mycobacterium β-CA amino acid sequences were retrieved from UniProt. An analysis of the *M. marinum* genome was made using the exonerate program to identify any β-CA sequences therein. A similar analysis was performed using the genome of *T. spiralis*. The two *M. marinum* sequences and one *T. spiralis* sequence produced from these predictions were included with the other UniProt sequences for phylogenetic analysis. A maximum likelihood phylogenetic analysis of the final 9 β-CAs was performed using PhyML. For this analysis, the LG amino acid substitution model was used during a run of 1000 bootstraps. The alpha, transition/transversion, and proportion of invariable sites parameters were all set to empirical, with all other parameters as default. The results were visualized using the FigTree program (http://tree.bio.ed.ac.uk/software/figtree/).

Expression analysis of β-CAs from *M. marinum*

Primers for polymerase chain reaction (PCR) for three β-CAs of *M. marinum* (β-CA1 F 5'-atggccacacagctgggatc-3', R 5'-ggtctgcttggctcccgcgatag-3'; β-CA2, F1 5'-gtgacggttaccgacgactacc-3', R1 5'-ccggatctgcttgatgtgctg-3'; and β-CA3, F2 5'-atccctgatggcttgacgca-3', R2 5'-ccctggctctgatcctgtc-3') were manually designed for full length of transcript. The PCR reactions were performed with an initial denaturation step at 95 °C for 3 min followed by 35 cycles, 55 °C annealing temperature and 72 °C for 10 s elongation step. Following the PCR, the samples were analyzed on a 0.7% agarose gel using a standard DNA markers (100 bp and 1 kb) (Promega). The ethidium bromide gels were observed under UV-light (GelDoc) and photographed.

Quantitative real-time PCR

Primers for Quantitative Real-Time PCR (qRT-PCR) were designed based on cDNA sequences taken from NCBI/UniProt using the program Primer Express® Software v2.0 (Applied Biosystems) (β-CA1 F 5'-gacctggtcttcacctgctc-3', R 5'-gtacctcgttctccagctc-3'; β-CA2 F 5'-ccacgacctgccttggtggc-3', R 5'-ccgagcttgctctgcttgctc-3'; β-CA3 5'-cgaagaacctggccgagatg-3', 5'-cttctggctccgctcgatg-3'). The qRT-PCR was performed using the SYBR Green PCR Master Mix Kit in an ABI PRISM 7000 Detection System™ according to the manufacturer's instructions (Applied Biosystems). The PCR conditions consisted of an initial denaturation step at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s (denaturation) and 60 °C for 1 min (elongation). The data were analyzed using the ABI PRISM 7000 SDS™ software (Applied Biosystems). Every PCR was performed in a total reaction volume of 15 μl containing 2 μl of first strand cDNA (20 ng cDNA), 1 x Power SYBR green PCR Master Mix™ (Applied Biosystems, Foster City, CA, USA), and 0.5 μM of each primer. The final results are given as relative expression values, calculated according to the Pfaffl's Equation.

Determination of minimal inhibitory concentration in *in vitro* cultures of *M. marinum*

For the determination of minimal inhibitory concentration (MIC), the protocol used here was modified from Hall et al. Briefly, wild type *M. marinum* (ATCC 927) was grown on Middlebrook 7H10 agar plates (BD) for 6 days at +29 °C. Bacterial mass was scraped from the plate and transferred into PBS pH 7.4 containing 0.2% Tween 80 (SIGMA) to obtain an OD600 of 0.08–0.100. 200 μl of this bacterial suspension was mixed with 11 ml of Middlebrook 7H9 Broth OADC (BD) (no tween, no glycerol) by vortexing. The bacterial concentration was determined by plating on 7H10 agar (BD) and purity by plating on LB agar (SIGMA). Plates were incubated for 6 days at +29 °C. The bacterial concentration was between 1.4 x 10^5 and 4.7 x 10^5 cfu/ml. 50 μl of this bacterial suspension was pipetted per well onto sterile, clear 96-well tissue culture treated plates (Corning Costar from SIGMA). The filter sterilized inhibitors dissolved in Middlebrook 7H9 Broth OADC (no tween, no glycerol) were added on top of bacteria in a volume of 50 μl. A concentration range of 0.3 pM–300 nM using a 10-fold dilution series was tested in two separate experiments on 2–6 replicate wells. A concentration range of 18.75-300 nM using a 2-fold dilution series was in two separate experiments on six replicate wells. The lids were sealed onto the plates with parafilm and the cultures were incubated at +28.5 °C for 5 days. The result was determined by assessing the turbidity of the cultures both by visual inspection and by an OD600 measurement using Perkin Elmer Envision multi-reader scan measurement. Five horizontal and five vertical points 0.72 mm apart were measured from each well. The sum of the readings was calculated for each sample. The background signal from wells containing medium only was subtracted from all values.

The nature of inhibition of the tested agents was also examined. Bacteria were grown as for MIC determination by making serial inhibitor dilutions. After initial incubation, fresh 7H9 medium was added to dilute the inhibitors at a ratio of 1:2 and 1:4. Cultures were analyzed after six days of incubation as in MIC determination by comparing to undiluted duplicate culture wells.

*M. marinum* transformation

A fluorescent *M. marinum* (wasabi) strain was generated using a modified protocol for *M. tuberculosis* electroporation. *M. marinum* ATCC 927 was grown in Middlebrook 7H9 Broth OADC (BD) with 0.2% Tween 80 (SIGMA) starting from an OD600 of 0.07–0.100 to an OD600 of 0.700–0.800. 20 ml of culture was harvested for bacteria and washed three times in 10% glycerol. 0.1–1 μg of purified pTEC15 plasmid DNA was mixed with 500 μl *M. marinum* in 10% glycerol and incubated for 5 min. Cells were transformed in 2 mm cuvettes with a single pulse of 2.5 kV, 25 μF (1000 Ω resistance) using Gene pulser II Electroporation System (Bio-Rad). Transformed cells were resuspended in 4 ml 7H9 medium. After overnight incubation at 29 °C with gentle shaking, transformants were selected on 7H10 agar plates with 75 μg/ml hygromycin B. Correct transformants were verified by fluorescence microscopy. pTEC15 was a gift from Lalita Ramakrishnan (Addgene plasmid #30174).

*M. marinum* infections of zebrafish embryos

*M. marinum* ATCC 927 containing pTEC15 plasmid for constitutive green fluorescence was grown for 4 days in Middlebrook 7H9 Broth OADC (BD) with 0.2% Tween 80 (SIGMA) and 75 μg/ml of Hygromycin B (VWR) starting from an OD600 of 0.07–0.100 to an OD600 of 0.760–0.890. The bacteria were pelleted and dissolved in the appropriate volume of PBS containing 0.6 mg/ml of phenol red as a tracer (SIGMA). Wild-type (AB) zebrafish embryos were manually dechorionated at 22-24 hpf and put into E3-water containing 0.00045% phenylthiourea (PTU)(SIGMA) to inhibit...
pigmentation. The embryos were anesthetized with 0.02% Tricaine (SIGMA). Using aluminosilicate capillary needles and a Pneumatic PicoPump PV820 (World Precision Instruments) 1 nl of bacterial suspension was injected into the caudal vein of the zebrafish 27-31 hpf and the bacterial concentration was verified by plating. The infected fish were kept at 28.5°C in 1 ml of PTU-E3 medium with or without the inhibitor on 24-well plates.

**Determination of bacterial load from infected zebrafish larvae**

On day 5 post infection the fish were euthanized with an overdose of Tricaine (SIGMA), transferred onto a black 96-well Proxplate (PerkinElmer), and embedded on their side in the middle of the well in 50 μl of 1% low melt agarose (SIGMA). Eight non-infected fish were embedded for measuring background values. Prior to green fluorescence scan measurement with Perkin Elmer Envision multireader, 50 μl of PBS was added on top of each sample. The scan measurement was carried out on 5 horizontal and 5 vertical dots 0.5 mm apart from 6.5 mm height with 100% excitation at 493 nm, 509 nm emission and 500 flashes per point. The average signal (relative fluorescence units, RFU) from each well was calculated. The average background signal was subtracted from the measured samples.

**Statistical analysis**

The GraphPad Prism software (5.02) was used to perform statistical analysis. Due to small sample numbers, we used a non-parametric two-tailed Mann–Whitney test the determination of statistical significance of differences between the drug treated and non-treated group. For statistical analysis of the toxicity parameters, a two-tailed Fisher’s test was used, p values below .05 were considered significant.

**Results**

**Expression analysis shows transcription of all three β-CA genes in M. marinum**

Bioinformatic analysis of the *M. marinum* genome showed the presence of three β-CA genes. We experimentally validated the expression of the β-CA genes in log-phase cultures of *M. marinum* strain ATCC 927 using RT-qPCR. The PCR bands were 500 bp for β-CA 1, 490 bp for β-CA 2, and 600 pb for β-CA 3 (Figure 2(A)) as expected. In qRT-PCR comparing β-CAs expression in three different strains of *M. marinum* (M, ATCC 927 and E11), we found the expression to be highest in ATCC 927 (Figure 2B–D). (Our molecular analysis thus confirmed the presence of the β-CA genes in *M. marinum.*)

**M. marinum β-CA sequences are similar to the β-CA sequences of *M. tuberculosis***

*Mtb* contains three β-CA sequences; two of the enzymes (β-CA 1 and β-CA 2) can be specifically inhibited using DTCs *in vitro*. To investigate if the β-CA sequences of *M. marinum* are closely related to *Mtb* β-CAs, we first performed multiple sequence alignment (MSA) studies. The MSA showed very high conservation of nucleotides between the sequences (Mtb ca1 vs. *M. marinum* ca2 = 83.95, Mtb ca2 vs. *M. marinum* ca1 = 83.17 and Mtb ca3 vs. *M. marinum* ca3 = 68.86) (The names and numbering of the β-CAs were based on the UniProt entry) (Supplementary Figure 1 online). The subcellular localization information accessed from tuberculist (http://tuberculist.epfl.ch) database suggested that β-CA 1 and 2 are cytoplasmic, and β-CA 3 is a membrane-associated protein and these predicted localizations match those of the *Mtb* β-CAs. The phylogenetic analysis showed that each of the three *Mtb* β-CA sequences are most closely related to a corresponding *M. marinum* β-CA sequence (Figure 3).

**DTC Fc14–584B is safer compared to Fc14–594A in zebrafish embryos/larvae**

The toxic effects of DTCs Fc14–584B and Fc14–594A on developing zebrafish embryos were dose dependent (Figures 4 and 5). Fc14–594A had an LC50 value of 18.5 μM (Figure 5(A)). The DTC Fc14–584B was less toxic, and had an LC50 value of 498.1 μM (Figure 5(B)).

To assess the toxicity of the two inhibitors, Fc14–594A and Fc14–584B, in a preliminary experiment, we examined 1–5 dpf zebrafish exposed to different concentrations of the drugs, and compared the observable developmental parameters with those of non-treated fish. Figure 4 shows representative pictures of larvae subjected to different concentrations of inhibitors. As Fc14–584B was clearly better tolerated at higher concentrations, we carried out a more detailed analysis with this drug at 300 and 500 μM concentrations (Figure 6). A 300 μM concentration did not affect survival, hatching, movement, edema, or yolk sack utilization during the first 5 dpf. Some larvae subjected to 300 μM of Fc14–584B showed mild abnormalities in body shape (curving of the back).
and heartbeat (difference to controls not statistically significant). Phenotypic studies suggested that \(\text{Fc14} \rightarrow 584\text{B}\), is safer than \(\text{Fc14} \rightarrow 594\text{A}\) and has limited adverse effects on the embryos at 300 \(\mu\text{M}\) concentration. Thus, \(\text{Fc14} \rightarrow 584\text{B}\) was selected for further in vivo testing.

**The DTCs \(\text{Fc14} \rightarrow 584\text{B}\) and \(\text{Fc14} \rightarrow 594\text{A}\) did not induce any histological defects in the zebrafish larvae**

To see any damage to the tissues of the zebrafish embryos, we studied the histological structures of 5 dpf larvae treated with different concentrations of \(\text{Fc14} \rightarrow 594\text{A}\) and \(\text{Fc14} \rightarrow 584\text{B}\) and compared the findings with the control group of 5 dpf zebrafish larvae. The semi thin (5 \(\mu\text{m}\)) sagittal sections of the larvae stained with hematoxylin and eosin did not show any apparent morphological changes compared to the control group larvae. The histochemical studies of the drug treated larvae suggested that these drugs do not cause any histological damage to the internal tissue at the \(\text{LC}_{50}\) dose or lower.

**DTCs \(\text{Fc14} \rightarrow 584\text{B}\) and \(\text{Fc14} \rightarrow 594\text{A}\) inhibit the growth of \(M.\ marinum\) in vitro**

We then sought to determine, whether the selected DTCs inhibit the growth of \(M.\ marinum\) in vitro. We carried out standard MIC-tests using liquid cultures of \(M.\ marinum\) on a 96-well plate. In addition to a visual inspection, the optical density of the cultures was measured after 6 days. In a preliminary experiment, we titrated a concentration range from 3 nM to 300 \(\mu\text{M}\) with a 10-fold dilution series and found no growth at 300 \(\mu\text{M}\) concentration and reduced growth at 30 \(\mu\text{M}\) (data not shown). Based on these results we carried out two rounds of MIC tests using concentrations between 18.75 \(\mu\text{M}\) and 300 \(\mu\text{M}\) with a 2-fold dilution series. A dose response in growth inhibition was seen for both compounds (Figure 7(A–D)). The MIC of both compounds was 75 \(\mu\text{M}\) (Figure 7(A–D)). In terms of growth resumption, none of the inhibited mycobacterial cultures showed any signs of revival with inhibitor concentrations below MIC after inhibitor dilution by 1:4. This suggests that the tested agents acted as bactericidal rather than bacteriostatic inhibitors (data not shown).

**DTC \(\text{Fc14} \rightarrow 584\text{B}\) inhibits the growth of \(M.\ marinum\) in vivo in zebrafish larvae**

Based on our results from toxicity and MIC testing, we continued with compound \(\text{Fc14} \rightarrow 584\text{B}\) to in vivo testing. We infected fish 1-dpf with green fluorescent \(M.\ marinum\) (average infection dose 471 ± 143 bacteria) (Figure 8(A)). \(\text{Fc14} \rightarrow 584\text{B}\) was added to the embryonic medium at a concentration of 300 \(\mu\text{M}\). As zebrafish larvae can be kept transparent, the bacterial load at 6 days post infection (dpi) could be measured by fluorescence. The fluorescent
Figure 4. Effects of dithiocarbamates Fc14–594A and Fc14–584B on developing embryos. Developmental images of 1–5 dpf embryos exposed to different concentrations of Fc14–594A and Fc14–584B β-CA inhibitor compounds. (A) Row shows the images of control group embryos (not treated with inhibitors) with normal embryonic development. (B) Row shows the images of zebrafish embryos exposed to Fc14–594A. The embryos exposed to 20 μM concentration of Fc14–594A showed short and curved body structure with mild edema (arrows), curved tail (bullet), and unutilized yolk sac (arrow head) and the embryos exposed to 30 μM Fc14–594A did not survive beyond 3 dpf. (C) Row shows the images of embryos exposed to Fc14–584B. The embryos exposed to concentrations up to 300 μM of Fc14–584B generally had a normal embryonic development with no significant phenotypic defects. The embryos exposed to 600 μM of Fc14–584B did not survive beyond 3 dpf.

Figure 5. LC_{50} determination of the two compounds. The LC_{50} dose for the drugs, Fc14–584B and Fc14–594A was determined based on cumulative mortality of 5 days after the exposure of embryos to the different concentration of the drugs. The LC_{50} were determined after three independent experiments with similar experimental conditions (n = 30).
Figure 6. Effect of dithiocarbamate Fc14–584B on phenotypic parameters of the developing zebrafish embryos. The effect of 300 and 500 μM concentrations of Fc14–584B on survival, movement, yolk sack, hatching, heartbeat, body shape, and edema of the zebrafish embryos was recorded 1–5 dpf. For each concentration, \( n = 30 \). * \( p < .05 \) by two-tailed Fisher’s test.

Figure 7. Dithiocarbamates Fc14–584B and Fc14–594A inhibit the growth of *M. marinum*. MIC was determined in liquid cultures by visual inspection and turbidity measurement in two separate experiments (AB and CD). In both experiments \( n = 6 \). * \( p \) values in the pictures are two-tailed t-test values of comparisons to 0 μM.
signal correlates with the bacterial load measured from the same samples with an M. marinum specific qPCR method. In the three separate experiments, the treated groups significantly lower bacterial numbers compared to the controls (range from 2.9 to 8.9-fold difference \( p < .05 \)) in median bacterial loads, a representative experiment shown in Figure 8(C). These results provide evidence of the in vivo efficacy of DTC Fc14–584B as an antitubercular drug.

**Discussion**

Tuberculosis is currently one of the deadliest infectious, causing almost 2 million yearly deaths worldwide. The TB epidemic is a global problem that is aggravated by the recent emergence of multidrug-resistant strains of M. tuberculosis that are untreatable with common antibiotic regimens. This alarming situation emphasizes the need to develop novel antibiotics against previously unexploited targets.

Recent studies have shown that inhibitors of CAs, such as sulfonamides, coumarines, and sulfamides, are very effective in inhibiting the activity of CA enzymes of Mtbb at subnanomolar concentrations in vitro, thus validating the CA enzymes as potential antituberculosis targets. DTCs, Fc14–594 A and Fc14–584B, belonging to a structurally distinct class of CA inhibitors have been developed and identified in vitro as potential drug candidates against \( \beta \)-CA enzymes. Despite the promise of \( \beta \)-CA enzymes as targets for developing anti-TB agents, so far, no in vivo studies have explored the potential of targeting the \( \beta \)-CA enzyme. The aim of our study was to evaluate the safety and toxicity of the two novel DTCs (Fc14–594 A and Fc14–584B), and subsequently use the less toxic inhibitor for in vivo studies using M. marinum and zebrafish larvae as model organisms. Zebrafish have been widely used for acute and chronic toxicity testing and zebrafish larvae as model organisms. Zebrafish

In conclusion, we have identified DTC Fc14–584B as a potent anti-mycobacterial drug candidate that targets mycobacterial \( \beta \)-CA enzymes. Fc14–584B specifically inhibits purified \( \beta \)-CAs of Mtbb at nanomolar and subnanomolar concentrations. In a bacterial culture, the compound Fc14–584B effectively inhibits the growth of M. marinum. We have also demonstrated that the compound is safe for use in zebrafish and causes no significant phenotypic or histological abnormalities in 5 dpf zebrafish larvae. Importantly, we have demonstrated that Fc14–584B significantly impairs the growth of M. marinum in vivo in the zebrafish larval model. To our knowledge, this is the first report on invasive M. marinum and its susceptibility to inhibitors of \( \beta \)-CA in vivo in a vertebrate model. Currently, the DTC Fc14–584B is in process towards preclinical...
characterization, using adult tuberculosis zebrafish model, for the treatment of latent and active tuberculosis disease.

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Disclosure statement

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