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2-Carboxyquinoxalines kill *Mycobacterium tuberculosis* through non-covalent inhibition of DprE1

João Neres, Ruben C Hartkoorn, Laurent R. Chiarelli, Ramakrishna Gadupudi, Maria Rosalia Pasca, Giorgia Mori, Davide Farina, Svetlana Savina, Vadim Makarov, Gaëlle S. Kolly, Elisabetta Molteni, Claudia Binda, Neeraj Dhar, Stefania Ferrari, Priscille Brodin, Vincent Delorme, Valerie Landry, Ana Luisa de Jesus Lopes Ribeiro, Alberto Venturelli, Puneet Saxena, Florence Pojer, Antonio Carta, Rosaria Luciani, Alessio Porta, Giuseppe Zanoni, Edda De Rossi, Maria Paola Costi, Giovanna Riccardi, and Stewart T. Cole

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Title: 2-Carboxyquinoxalines kill *Mycobacterium tuberculosis* through non-covalent inhibition of DprE1

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28 33 **Abstract:** Phenotypic screening of a quinoxaline library against replicating *Mycobacterium*
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30 34 *tuberculosis* led to the identification of lead compound Ty38c (3-((4-methoxybenzyl)amino)-6-
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32 (trifluoromethyl)quinoxaline-2-carboxylic acid). With an MIC₉₉ and MBC of 3.1 μM, Ty38c is
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34 bactericidal and active against intracellular bacteria. To investigate its mechanism of action we
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36 37 isolated mutants resistant to Ty38c and sequenced their genomes. Mutations were found in
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39 38 *rv3405c*, coding for the transcriptional repressor of the divergently expressed *rv3406* gene.
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41 39 Biochemical studies clearly showed that Rv3406 decarboxylates Ty38c into its inactive keto
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43 40 metabolite. The actual target was then identified by isolating Ty38c-resistant mutants of an *M.*
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45 41 *tuberculosis* strain lacking *rv3406*. Here, mutations were found in *dprE1*, encoding the
46
47 42 decaprenylphosphoryl-D-ribose oxidase DprE1, essential for biogenesis of the mycobacterial cell
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49 43 wall. Genetics, biochemical validation, and X-ray crystallography revealed Ty38c to be a non-
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51 44 covalent, non-competitive DprE1 inhibitor. Structure-activity relationship studies generated a
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53 45 family of DprE1 inhibitors with a range of IC₅₀s and bactericidal activity. Co-crystal structures
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4 46 of DprE1 in complex with eight different quinoxaline analogs provided a high-resolution
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6 47 interaction map of the active site of this extremely vulnerable target in *M. tuberculosis*.
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11 INTRODUCTION

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14 50 More than 130 years after Koch's discovery of *Mycobacterium tuberculosis* as its etiological
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16 51 agent, tuberculosis (TB) still affects humankind and was responsible for 1.3 million deaths in
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18 52 2012^{1, 2}. This disease reemerged in recent decades as an increasingly important public health
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21 53 problem due to the appearance of multidrug resistant (MDR-TB) and extensively drug resistant
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23 54 (XDR-TB) strains with high mortality rates, the synergy with the HIV/AIDS pandemic and
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25 55 increased poverty^{1, 3, 4}. After decades of relative inactivity in TB drug discovery, a promising
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28 56 pipeline of TB drug candidates in different stages of development has emerged recently⁵. In
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30 57 2012, bedaquiline, the first new TB drug approved since the 1960s, brought new hope for many
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32 58 patients with MDR-TB⁶. Several molecules are now in preclinical studies, phase II and III
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35 59 clinical trials, but the pipeline still needs more novel scaffolds to provide backup drugs given the
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37 60 high attrition rate observed during clinical development^{5, 7}. Phenotypic screens have emerged as
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40 61 an efficient means of identifying active compounds for TB drug discovery, especially as almost
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42 62 all hits from target-based screens, which provided potent enzyme inhibitors failed to display
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44 63 useful bactericidal activity against *M. tuberculosis*⁸.

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47 64 Here, we report the discovery of a family of quinoxalines with antitubercular activity,
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49 65 following a phenotypic screen of a chemical library against replicating *M. tuberculosis*. The lead
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51 66 compound Ty38c (3-((4-methoxybenzyl)amino)-6-(trifluoromethyl)quinoxaline-2-carboxylic
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53 67 acid) is active against extracellular and intracellular *M. tuberculosis*. We have elucidated both a
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55 68 mechanism of resistance to Ty38c and its mechanism of action, and validated the findings using
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3 69 biochemical assays and X-ray crystallography. Furthermore, the synthesis and structure activity
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5 70 relationship studies of analogs of Ty38c provide valuable information regarding this novel
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8 71 DprE1 inhibitor scaffold.
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13 73 **RESULTS AND DISCUSSION**

16 74 **A phenotypic screen identifies a tuberculocidal quinoxaline scaffold**

17 75 Single point screening of a library containing 266 quinoxaline analogs against *M. tuberculosis*
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19 76 H37Rv using the resazurin reduction assay revealed five compounds with MIC₉₉ <15 μM. In
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21 77 order to focus on non-nitroaromatic scaffolds, with potentially novel mechanisms of action, we
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23 78 selected a 2-carboxyquinoxaline cluster with 3 compounds: Ty38c, Ty21c (the ethyl ester of
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25 79 Ty38c) and Ty36c (Table 1). Ty38c and Ty21c killed intracellular H37Rv in a macrophage
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27 80 model with IC₅₀s of 2.5 and 6.1 μM, respectively. Both compounds were inactive against the
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29 81 non-replicating ss18b *M. tuberculosis* strain (MIC₉₉ >100 μM), suggesting that they inhibit a
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31 82 function essential for growth. Ty38c and Ty21c presented selectivity indexes (TD₅₀/MIC₉₉) of 12
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33 83 and 15, respectively, based on their HepG2 cytotoxicity (Table 1). Ty38c was confirmed to be
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35 84 bactericidal with an MBC equal to its MIC₉₉ of 3.1 μM (1.2 μg/ml). Cidality was also directly
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37 85 visualized using microfluidics-based time-lapse microscopy of *M. tuberculosis* expressing GFP⁹.
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39 86 Exposure of H37Rv to 5 μM Ty38c caused a dramatic reduction in the growth rate of individual
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41 87 bacteria (Fig. 1, Movie Supplementary Movie 1), although some cells continued to divide
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43 88 without elongation. Cell lysis occurred after a certain lag (25-30 h), and most of the cells lysed
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45 89 over the 7-day exposure period.
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91 **Rv3406 is responsible for primary resistance of *M. tuberculosis* to Ty38c**

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4 92 To identify the molecular target(s) of Ty38c, we isolated spontaneous resistant mutants of *M.*
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6 93 *tuberculosis* H37Rv; these arose at a frequency of 1×10^{-6} on solid medium containing 20 μM
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8 94 Ty38c, and their Ty38c-resistance profile was confirmed in liquid culture (Supplementary Table
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10 95 2). Whole-genome sequencing and bioinformatics analysis of four independent mutants (TRC1 -
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12 96 TRC4) revealed that each mutant carried a different non-synonymous single nucleotide
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14 97 polymorphism (SNP) or a single base deletion in the *rv3405c* gene (Supplementary Table 2).
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16 98 This gene codes for a transcriptional regulator of the TetR family. Sanger sequencing of *rv3405c*
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18 99 confirmed the mutations in these four mutants and in six additional independently isolated
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20 100 Ty38c-resistant mutants (TRC5 – TRC10; Supplementary Table 2).
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25 101 The *rv3405c* gene is expressed divergently from the neighboring *rv3406* gene encoding
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27 102 an iron and α -ketoglutarate (α -KG) dependent sulfate ester dioxygenase, which oxidizes
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29 103 medium-chain alkyl-sulfate esters like 2-ethylhexyl sulfate (2-EHS, Fig. 2A)¹⁰. Interestingly, a
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31 104 unique palindrome (TGTAGTCAtcTGACTACA) is found between *rv3405c* and *rv3406* that
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33 105 could represent the DNA binding sequence of *rv3405c*. Quantitative Real-Time PCR (qRT-PCR)
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35 106 experiments confirmed that the Ty38c-resistant mutants (TRC5 and TRC6), have significantly
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37 107 increased transcription of *rv3406* (30- and 47.2-fold respectively), as well as *rv3405c* itself
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39 108 (15.7-fold increase) (Supplementary Table 3). This therefore suggests that Rv3405c is a
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41 109 transcriptional repressor controlling both *rv3405c* and *rv3406* and that mutations in Rv3405c
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43 110 lead to their overexpression, in agreement with a recent report in *M. bovis* BCG¹¹.
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49 111 To confirm that overexpression of Rv3406 causes Ty38c resistance, *rv3405c* or *rv3406*
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51 112 were cloned into a pSODIT-2 expression vector, the resultant plasmids were transformed into
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53 113 H37Rv and the susceptibility of selected transformants to Ty38c determined (Supplementary
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55 114 Table 4). Compared to H37Rv:pSODIT (MIC₉₉ 2.5 $\mu\text{g/ml}$), the data clearly showed that
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3 115 overexpression of *rv3406* in H37Rv:pSODIT/*rv3406* conferred an >8-fold increase in resistance
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5 116 to Ty38c (MIC₉₉ >20 μg/ml), whereas H37Rv:pSODIT/*rv3405c* showed the same susceptibility
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8 117 to Ty38c as H37Rv:pSODIT. Importantly, transformation of pSODIT/*rv3405c* into the Ty38c-
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10 118 resistant mutant TRC5 complemented the resistance phenotype of TRC5 (MIC₉₉ 2.5-5 μg/ml)
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12 119 compared to the vector control (MIC₉₉ >20 μg/ml). This confirmed that mutations in Rv3405c
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14 120 prevent transcriptional repression of *rv3406*, and that Rv3406 overexpression leads to Ty38c
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16 121 resistance.
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22 123 **Rv3406 inactivates Ty38c**

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25 124 To understand the putative resistance mechanism caused by Rv3406, we expressed and purified
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27 125 this protein (Supplementary Fig. 1). The activity of the recombinant Rv3406 enzyme was
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29 126 followed by the rate of oxygen consumption using α-KG and 2-EHS as substrates (Table 2).
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31 127 First, we tested whether Ty38c (up to 250 μM) could inhibit Rv3406 in standard assay
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33 128 conditions, but this was not the case. We then determined whether Ty38c could replace either
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35 129 αKG or 2-EHS as a substrate and be metabolized by the enzyme. Rv3406 was enzymatically
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37 130 active in the presence of Ty38c and 2-EHS (in the absence of α-KG), but not when incubated
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39 131 with Ty38c and α-KG in the absence of EHS, showing that Rv3406 can use Ty38c instead of α-
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41 132 KG as a substrate. Subsequent kinetic analysis showed that Ty38c presents a 14-fold lower k_{cat}
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43 133 value and a 17-fold higher K_m value as compared to α-KG (Table 2), indicating that Rv3406
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45 134 metabolizes Ty38c significantly less efficiently than α-KG in the assay conditions. We isolated
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47 135 and purified the reaction products from the reaction mixture, and through NMR and mass
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49 136 spectrometry, the main metabolite was identified as a keto derivative of Ty38c (QN113),
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51 137 resulting from oxidative decarboxylation by Rv3406 (Fig. 2B). In parallel, we independently
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3 138 synthesized and characterized QN113 and thus confirmed the metabolite's identity (see
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6 139 Supporting Information). QN113 was inactive against wild-type H37Rv and the TRC5 mutant
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8 140 (MIC₉₉ > 40 µg/ml). Together, the data clearly demonstrate that resistance results from
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10 141 decarboxylative inactivation of Ty38c by Rv3406 *in vitro* and most likely in *M. tuberculosis* as
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12 142 well, following derepression of the *rv3406* gene.

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15 143 To obtain structural information regarding the mode of binding of Ty38c to Rv3406 and
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17 144 potentially use a structure-based approach to design improved Ty38c analogs that avoid
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19 145 decarboxylation, we attempted to obtain crystal structures of the complex. However, only
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21 146 crystals of native Rv3406 protein with bound Fe²⁺ were obtained, which diffracted to 2.0 Å
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23 147 (Supplementary Fig. 2 and Supplementary Table 7).
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28 29 30 149 **DprE1 is the target of Ty38c**

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32 150 To find the actual target of Ty38c, responsible for its anti-mycobacterial activity, we constructed
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34 151 H37RvΔ*rv3406*, an H37Rv strain lacking Rv3406, by recombineering¹², and confirmed the
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36 152 correct replacement of *rv3406* with a hygromycin cassette by PCR. The susceptibility of
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38 153 H37RvΔ*rv3406* to Ty38c was identical to that of wild type H37Rv. We then isolated
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40 154 spontaneous Ty38c-resistant mutants in H37RvΔ*rv3406*, which arose at a much lower frequency
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42 155 (1x10⁻⁸) than was seen with H37Rv. Two mutants (TRC11 and TRC12) showed 4-fold resistance
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44 156 to Ty38c, but were as susceptible to moxifloxacin as wild-type H37Rv. Whole genome
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46 157 sequencing revealed two different non-synonymous SNPs in the *dprE1* gene (g49t and t1103c,
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48 158 translating to G17C and L368P, respectively). No cross-resistance was observed between either
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50 159 of these *dprE1* mutants and the prototypic DprE1 inhibitor BTZ043, but mutations that result in
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52 160 resistance to benzothiazinones (NTB1 mutant, C387S¹³) conferred cross-resistance to Ty38c (4-
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3 161 fold increase in MIC₉₉). These data suggested that Gly17, Leu368 and Cys387 are involved in
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5 162 Ty38c binding.
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8 163 Additional genetic validation of DprE1 as the target of Ty38c was performed using a
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10 164 conditional expression system in *M. tuberculosis*, in which *dprE1* is overexpressed following
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12 165 addition of pristinamycin. Over-expression of *dprE1* caused a >16-fold increase in resistance to
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14 166 both BTZ043 and Ty38c, whilst not affecting susceptibility to the control drug (Supplementary
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16
17 167 Table 5).
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20 168 Biochemical assays using recombinant *M. tuberculosis* DprE1, and the G17C and L368P
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22 169 mutant enzymes were used to assess enzyme inhibition. As previously reported for *M. smegmatis*
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24 170 DprE1¹⁴, the *M. tuberculosis* enzyme presents non-Michaelis-Menten behavior, with a
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26 171 sigmoidal-shaped initial velocity *versus* substrate concentration curve, so the data were fitted to
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28 172 the Hill equation¹⁵. The G17C and L368P mutants presented $K_{0.5}$ values similar to the wild type
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30 173 enzyme, but were 34- and 6.6-fold less efficient, respectively, based on the determined k_{cat} values
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32 174 (Table 3). Ty38c effectively inhibited wild-type DprE1 with an IC₅₀ of 41 nM and behaved as a
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34 175 non-competitive inhibitor, with a K_i of 25.9 nM. The G17C and L368P mutants were
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36 176 significantly less susceptible to Ty38c, with IC₅₀ values of 0.15 and 1.3 μ M, respectively (Table
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38 177 3), in agreement with the MIC₉₉ determined against the corresponding Ty38c-resistant
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40 178 H37Rv Δ rv3406 *dprE1* mutants.
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48 180 **SAR studies on Ty38c reveal key features required for DprE1 inhibition**

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50 181 Given the potency of Ty38c and the novelty of this scaffold as an antitubercular agent, we
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52 182 pursued Structure-Activity Relationship (SAR) studies to improve it and understand the
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54 183 substituent requirements needed to achieve DprE1 inhibition and activity against *M.*
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3 184 *tuberculosis*, whilst trying to avoid inactivation by Rv3406. SAR studies focused on the 2-
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6 185 carboxylate, the 6-trifluoromethyl and the 3-benzylamine moieties (synthesis described in the
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8 186 Supporting Information). All Ty38c analogs were tested in biochemical assays as substrates for
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11 187 Rv3406 or inhibitors of DprE1 (Tables 4, 5 and Supplementary Table 6), and for their activity
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13 188 against *M. tuberculosis* H37Rv, its *rv3405c* (TRC4) and Δ *rv3406* DprE1 mutants (G17C and
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15 189 L368P), and against intracellular H37Rv in the macrophage model of infection.

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18 190 Compounds designed to resist the activity of Rv3406, where the 2-carboxylate group was
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20 191 replaced by a methyl group (QN106, QN107, QN108 and QN109) or by a carboxamide (QN102,
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22 192 QN104, QN103 and QN105), were not active against *M. tuberculosis* and showed only residual
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24 193 inhibition of DprE1 (Table 4). As mentioned above, the keto analogue QN113 was inactive
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27 194 against DprE1. Interestingly, some of the 2-carboxyl ethyl esters (Tables 4 and Supplementary
28
29 195 Table 6), namely Ty21c, QN101, QN144 and QN141, presented reasonable MIC₉₉ against *M.*
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31 196 *tuberculosis* H37Rv (3.1-12.5 μ M), but none was significantly active against DprE1 (IC₅₀ \geq 50
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34 197 μ M). These results indicate that the esters' activity against the bacterium is likely due to
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36 198 hydrolysis to the free acid, during the long incubation period in culture medium, or to the action
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39 199 of mycobacterial esterases.

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41 200 Absence of the 6-trifluoromethyl moiety in quinoxalines QN111, QN110 and QN112
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43 201 (Table 4), led to near complete loss of whole cell activity (MIC₉₉ \geq 50 μ M) and DprE1 inhibition
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45 202 (IC₅₀ between 16 and 33 μ M) but did not prevent decarboxylation by Rv3406, with turnover
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47 203 rates between 0.35 and 0.62 min⁻¹.

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50 204 In the 2-carboxy-6-trifluoromethyl-quinoxaline series (Table 6) there was significant
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52 205 modulation of the various parameters tested depending on the modifications introduced in
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55 206 position 3 of the quinoxaline ring. A benzyl group (present in Ty38c) is preferred to a phenyl in
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3 207 this position (QN131 versus Ty38c), likely due to the flexibility introduced by the methylene
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5 208 spacer, which may improve interaction with the active site of DprE1. QN130, a synthetic
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7 209 intermediate lacking the benzyl moiety was not active. The remaining eighteen Ty38c analogs
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9 210 had benzyl groups with varied substitution patterns (Table 5). The best compounds in this series,
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11 211 with an MIC₉₉ of 3.1 μM, were Ty38c, QN114 and QN124, which had single substitutions in the
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13 212 *para* position of the benzene ring (OMe, OEt and Cl, respectively), and were also among the
14
15 213 most potent DprE1 inhibitors (IC_{50s} of 0.041 - 0.088 μM). Minor modifications on the benzyl
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17 214 group were not favorable, leading to a 2- to 16-fold increase in MIC₉₉ (e.g.: replacing the
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19 215 methoxy group by methyl, fluoro, trifluoromethyl or nitrile (Ty38c *versus* QN119, Ty36c,
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21 216 QN127 or QN129, respectively) . However, these compounds retained DprE1 inhibition (IC_{50s} of
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23 217 0.072 - 0.12 μM), implying that they might not reach the target as efficiently in the bacterium.
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25 218 Compounds with a single substituent in the *meta* position, or with double substitutions in the
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27 219 *meta* and *para* positions showed a significant increase in MIC₉₉ accompanied by a drop in
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29 220 potency against DprE1 (Table 5).

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36 221 A modest positive correlation ($R^2 = 0.21$) was found between MIC₉₉ values and DprE1
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38 222 inhibition for the Ty38c analogs modified in the 3-benzyl moiety (Table 5 and Supplementary
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40 223 Fig. 3A). All compounds in this series were decarboxylated by Rv3406, with rates varying
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42 224 between 0.08 min⁻¹ for QN122 to 1.19 min⁻¹ for Ty36c. These data shows that modifications in
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44 225 the substituent in position 3 of the quinoxalines can effectively modulate the Rv3406-substrate
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46 226 ability. Importantly, there was no correlation between MIC₉₉ values and Rv3406 activity
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48 227 (Supplementary Fig. 3B) and Ty38c, the compound with the lowest MIC₉₉, was also the best
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50 228 substrate for Rv3406. Therefore, despite playing a role in the development of resistance to the 2-
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52 229 carboxyquinoxalines *in vitro*, Rv3406 does not seem to significantly affect their antitubercular
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3 230 activity against exponentially growing wild type *M. tuberculosis* probably due to its repression
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6 231 mediated by Rv3405c.
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8 232 The SAR data presented above shows that the 6-trifluoromethyl-2-carboxyquinoxalines
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10 233 with a *para*-substituted benzyl group in position 3 are the best compounds at inhibiting DprE1,
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12 234 and at killing *M. tuberculosis in vitro*. All compounds displayed 2-fold higher MIC₉₉ against a
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14 235 selected *rv3405c* mutant compared to H37Rv and, in most cases, the MIC₉₉ increased 4-fold
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16 236 against the G17C and L368P DprE1 mutants (TRC11 and TRC12, respectively). Good MIC₉₉ *in*
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18 237 *vitro* did not always translate into activity against intracellular bacteria, as only Ty38c and Ty21c
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20 238 were bactericidal intracellularly, with IC₅₀ values in the same range as their MIC₉₉. This fact
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22 239 underlines the importance of evaluating compounds not only against *M. tuberculosis* in culture
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24 240 but also in infected host cells, to have a complete view of their potential efficacy *in vivo*. All the
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26 241 modified benzyl analogs of Ty38c showed IC₅₀s between 41 and 220 nM, hence this moiety has
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28 242 the potential to accommodate substantial modifications in order to improve stability, cytotoxicity
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30 243 or even to prevent inactivation by Rv3406.
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39 245 **Ty38c and analogs interact with key residues in the active site of DprE1**

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41 246 To understand the SAR data at a structural level, we co-crystallized *M. tuberculosis* DprE1 with
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43 247 several Ty38c analogs. Complexes with eight quinoxalines (Ty38c, Ty21c, QN114, Ty36c,
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45 248 QN118, QN124, QN127 and QN129) were obtained, diffracting to 1.8 - 2.5 Å, with crystals in
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47 249 the space group previously found for the PBTZ169 complex¹⁶ (Supplementary Table 7). Co-
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49 250 crystallization attempts with analogs lacking the 2-carboxylate or the 6-CF₃ groups generally led
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51 251 to good quality crystals that displayed no electron density for the compounds in the active site of
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53 252 DprE1.
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3 253 The overall structure of DprE1 in the quinoxaline complexes was identical to that of
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5 254 previously reported structures^{14, 17, 18}. The two loops that were disordered in most available
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8 255 DprE1 structures are resolved to different extents in the new complexes. While the 267-298 loop
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10 256 is fully resolved in monomer A of almost all structures, electron density was observed for the
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12 257 whole 315-329 loop in only two cases (Ty36c and QN129 complexes), providing important clues
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15 258 regarding the flexibility and potential interactions of these residues with the 2-
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17 259 carboxyquinoxalines. This loop could also be involved in interactions with the cell membrane or
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20 260 with other protein partners involved in the DPA biosynthetic pathway^{14, 19}. Residue Tyr314,
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22 261 which when mutated to a histidine leads to resistance to TCA1¹⁸ and other reported non-
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24 262 covalent DprE1 inhibitors (Supplementary Fig. 7), is located at the edge of this loop and close to
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27 263 the active site, but does not interact with the quinoxalines presented here.

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29 264 In all complexes, the common 2-carboxy-6-trifluoromethylquinoxaline core is invariably
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31 265 observed in the same position, next to the FAD flavin ring, with an angle of 22° between the
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33 266 planes defined by the two ring systems (Fig. 3). The trifluoromethyl group is located in a small
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35 267 hydrophobic pocket formed by His132, Gly133, Lys367, Lys134, Ser228 and Phe369, as
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37 268 previously observed for the same group in BTZ043 or PBTZ169^{14, 16}. Key hydrogen bonds are
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39 269 formed between the side-chain of Lys418, an essential catalytic residue¹⁴, and the carboxylate
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41 270 group and nitrogen 1 of the quinoxaline ring. The hydroxyl group of Tyr60 also forms a
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43 271 hydrogen bond with the carboxylate. In the Ty36c and QN129 complexes, the 315-329 loop
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45 272 seems to be stabilized by an extra electrostatic interaction between the side-chain of Arg325 and
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47 273 the quinoxaline's carboxylate. However, this loop presents high B-factors, and adopts alternative
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49 274 conformations in other DprE1-quinoxaline complexes (Supplementary Fig. 4).
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4 275 Major differences were observed in the mode of binding of the benzyl moiety of Ty38c
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6 276 and its analogs (Fig. 3A-D). In monomer B, these inhibitors were always present in a bent
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8 277 conformation, the planes defined by the quinoxaline and benzene rings being approximately
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10 278 perpendicular in the various structures (Fig. 3C and 3D). The benzene ring is placed near the side
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12 279 chain of Leu363 and induces a conformational change of the side-chain of Trp230 compared to
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15 280 previously published structures. In monomer A, the situation varied between inhibitors: QN124,
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17 281 QN127 and QN129 adopt the bent conformation (identical to that observed in monomer B),
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20 282 Ty38c, Ty36c and QN114 are present in a planar conformation (benzyl and quinoxaline rings
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22 283 approximately co-planar), and QN101 is apparently in two populations of bent and co-planar
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24 284 conformations (Fig. 3A and 3B). In the planar conformation, the benzyl group is surrounded by
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27 285 the side-chains of Leu317, Asn324 and Arg325.

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29 286 Interestingly, when the compound was present in the bent conformation in monomer A,
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31 287 extra electron density was observed in its vicinity (Fig. 3E), and here we modeled a tri-
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33 288 propylene-glycol (tri-PG) molecule, as poly-propylene glycol is present in the crystallization
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35 289 conditions. Following refinement, the modeled tri-PG molecule presented average B-factors of
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37 290 $59 - 75 \text{ \AA}^2$, compared to $40 - 45 \text{ \AA}^2$ for the inhibitors in the same monomer. The tri-PG was
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39 291 located between the quinoxaline ring and residues Pro316, Leu363, Trp230, Ala244, Ser246 and
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41 292 Ser228, in approximately the same location where extra electron density was found in the DprE1
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43 293 complex with PBTZ169¹⁶, and this pocket may correspond to the binding site of the DPR
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45 294 substrate of DprE1.

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50 295 In addition, a co-crystal structure of Ty21c (ethyl ester of Ty38c) was obtained, in which
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52 296 electron density was clearly observed in the active site of monomer A (Supplementary Fig. 5B).
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55 297 The structure was refined after fitting Ty38c here, bound in the same manner as in the DprE1-

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3 298 Ty38c complex (Supplementary Figs. 5A and 5B). No electron density to account for the ethyl
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6 299 moiety of the ester was observed, which could have undergone hydrolysis according to our
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8 300 hypothesis discussed above.

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10 Gly17 and Leu368, mutated to Cys and Pro, respectively in DprE1 in Ty38c-resistant
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12 301 mutants do not interact directly with the quinoxaline inhibitors (Figs. 3A, 3C, Supplementary
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14 302 Figs. 6A and 6B). Gly17 is located near Tyr60 (Supplementary Fig. 6A), therefore a mutation to
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16 303 a cysteine might induce conformational adjustments affecting the interaction of this residue with
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18 304 the carboxylate of the inhibitors. A Leu368Pro mutation could affect adjacent residues, including
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20 305 Lys367, part of the trifluoromethyl binding pocket (Supplementary Fig. 6B), eventually
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22 306 interfering with binding of Ty38c and its analogs.
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27 308 Overall, despite substantial structural differences when compared to the
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29 309 benzothiazinones, the quinoxalines occupy approximately the same space in the active site as
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31 310 BTZ043 or PBTZ169, and the non-covalent inhibitor TCA1 (Fig. 3F). The main difference
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33 311 between PBTZ169 and the quinoxaline family of inhibitors resides in the absence of the covalent
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35 312 bond with Cys387 for the quinoxalines, which seems to be compensated, in part, by strong
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37 313 electrostatic interactions between Lys418 and Arg325, and the carboxylate group.
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43 315 **CONCLUSIONS**

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46 316 A change in the TB drug discovery paradigm in recent years led to a move away from target-
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48 317 based screens to whole-cell screens²⁰. Surprisingly, despite the use of distinct chemical libraries
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50 318 with broad chemical diversity, many of the hit compounds were found to inhibit a very limited
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52 319 number of targets in mycobacteria. Examples of these “promiscuous targets”, inhibited by a
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3 320 range of structurally unrelated molecules, are the trehalose monomycolate transporter MmpL3
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6 321 (targeted by compounds SQ109, AU1235, BM212 and C215 among others) and DprE1⁵.
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8 322 DprE1 is a highly vulnerable and fully validated TB drug target, essential for the
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10 323 decaprenylphosphoarabinose (DPA) pathway, crucial for cell wall biosynthesis and
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12 324 mycobacterial growth²¹⁻²³. BTZ043 and PBTZ169, among the most potent TB drug candidates
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14 325 discovered so far (MIC₉₉ of 1 and 0.3 ng/ml, respectively), are suicide, covalent inhibitors of
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16 326 DprE1^{14b, 18}; PBTZ169 is expected to enter clinical trials in the near future¹⁶. An impressive
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18 327 range of eight compound scaffolds with antitubercular activity has been recently shown to target
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20 328 DprE1 as covalent or non-covalent inhibitors (Supplementary Fig. 7).
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24 329 Here, we report a new family of DprE1 inhibitors, the 2-carboxy-6-
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26 330 trifluoromethylquinoxalines, discovered in a whole-cell screen, and disclose initial SAR data.
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28 331 The best compound Ty38c exhibits strong activity against replicating *M. tuberculosis in vitro* as
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30 332 well as in macrophages. Target finding for Ty38c was not straightforward. Spontaneous resistant
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32 333 mutants of *M. tuberculosis* to Ty38c presented mutations in Rv3405c, which controls expression
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34 334 of *rv3406*¹¹. Rv3406 is an α -ketoglutarate-dependent sulfate ester dioxygenase of broad
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36 335 substrate specificity¹⁰, which decarboxylates Ty38c to an inactive keto metabolite. The
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38 336 decarboxylating activity of Rv3406 on aromatic carboxylates reported here could prove
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40 337 important for other potential antitubercular drugs with carboxyl groups, which might undergo
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42 338 similar inactivation.
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48 339 To find the actual target of Ty38c, we constructed a H37Rv Δ *rv3406* knock-out mutant
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50 340 and used this to generate new Ty38c-resistant mutants, which contained *dprE1* point mutations.
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52 341 DprE1 was then confirmed as the target of Ty38c by genetic and biochemical means. The most
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3 342 potent Ty38c analogs displayed IC_{50} s <100 nM, and Ty38c was found to be a non-competitive
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5 343 inhibitor of DprE1, with a K_i of 25.9 nM.
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8 344 SAR studies on the Ty38c scaffold, supported by crystallographic data, have shown that
9
10 345 the carboxylate group forms crucial electrostatic and hydrogen bond interactions with Lys418,
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12 346 Tyr60 and possibly Arg325. The 6-trifluoromethyl group of Ty38c seems to be optimal for
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15 347 binding. Position 3 in the quinoxaline ring was found to be more amenable to modifications.
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17 348 Interestingly, the 3-benzyl group in these compounds mimics the cyclohexylmethylpiperazine
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19 349 moiety of PBTZ169 (Fig. 5D), which was found to accommodate various modifications and fully
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21 350 retain MIC against *M. tuberculosis*. These moieties in Ty38c and PBTZ169 have a hydrophobic
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23 351 character and project towards the exposed surface of the protein and so they might also interact
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25 352 with protein partners of DprE1, or with the cell membrane. Importantly, the apparent lack of
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27 353 specific interactions of the benzyl group in Ty38c offers the opportunity to introduce structural
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29 354 changes in order to improve potency and ADME/T properties.
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34 355 A major strength of the present work is the richness of the high-resolution structural data
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36 356 presented for DprE1 in complex with a variety of 2-carboxyquinoxaline derivatives. Combined
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38 357 with previous structural information^{14, 16-18}, a detailed model of the various interactions possible
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40 358 between small molecule inhibitors and the active site of the enzyme can now be generated. This
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42 359 interaction map is extended by two new positions in DprE1 that modulate quinoxaline binding,
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44 360 Gly17 and Leu368. The apparent promiscuity of DprE1 in binding a large range of chemical
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46 361 scaffolds might be attributable to the space available close to the FAD flavin ring, which is then
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48 362 able to form stacking interactions with various heterocyclic ring systems, thereby occluding part
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50 363 of the expected substrate binding site. In addition, the flexibility of the 315-329 loop, located
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52 364 immediately above the active site cavity, may also favor access of compounds to the active site
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3 365 and thus account for the large range of scaffolds. Furthermore, the association of DprE1 with the
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5 366 cell membrane should facilitate access to more hydrophobic inhibitors ²⁴. The wealth of new
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8 367 structural insight obtained in the present study will underpin structure-based drug design of
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11 368 DprE1 inhibitors.

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18 371 **METHODS**

20 372 **Synthesis of Ty38c and analogs**

22 373 Ty38c and its derivatives were synthesized through the adaptation of previously published
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25 374 procedures ²⁵. Synthetic routes, experimental details and compound characterization data are
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28 375 provided in the Supporting Information.

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32 377 **Library-screening**

34 378 A library of 266 quinoxaline analogs was screened at the concentration of 20 μ M for anti-
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37 379 tuberculosis activity on log-phase *M. tuberculosis* H37Rv using the resazurin reduction assay
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39 380 (REMA) ²⁶. Compounds that showed >80% inhibition of H37Rv growth were subsequently
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41 381 analyzed for their minimal inhibitory concentrations (MIC₉₉) against log phase H37Rv, non-
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44 382 replicating ss18b ^{27,28}; intracellular activity against H37Rv ²⁹ and cytotoxicity against the human
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46 383 hepatocellular carcinoma cell line, HepG2. The minimum bactericidal activity (MBC) of the lead
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48 384 compound, Ty38c, was then determined using a colony forming unit (cfu) inhibition assay.

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53 386 **Rv3406 enzymatic assays and steady state kinetics**

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3 387 The enzymatic activity of Rv3406 was determined by measuring the rate of oxygen consumption
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6 388 with a Hansatech Oxygraph oxygen electrode, using α -ketoglutarate (α KG) and 2-
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8 389 ethylhexylsulfate (2-EHS) as substrates, at 25°C with atmospheric oxygen. The standard reaction
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11 390 mixture contained 20 mM imidazole pH 7.0, 0.05 mM 2-EHS, 0.5 mM α KG, 0.1 mM FeSO₄, 0.2
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13 391 mM sodium ascorbate, in a final volume of 1 ml, and the reaction was started by adding enzyme
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16 392 solution (5 μ M).

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18 393 Steady-state kinetics parameters were determined as follows: for α -KG and Ty38c at fixed
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20 394 concentration of 0.05 mM 2-EHS; for 2-EHS at 0.5 mM α -KG. In all cases the reaction was
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23 395 started by addition of the enzyme and activity assayed at >8 different substrate concentrations.
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25 396 All experiments were performed in duplicate, and the kinetic constants, K_m and k_{cat} , were
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28 397 determined fitting the data to the Michaelis-Menten equation using Origin 8 software. Rv3406
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30 398 activity towards Ty38c analogs was determined in triplicate, using 0.2 mM of each compound.
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32 399 The metabolite resulting from the inactivation of Ty38c by Rv3406 was isolated following
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35 400 incubation with the enzyme at 37°C for 5 hours (details in Supporting Information).
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402 **DprE1 inhibition assays**

403 DprE1 assays were performed on the *M. tuberculosis* enzyme, in black 96-well half-area plates
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404 (Corning 3686), in a final volume of 30 μ l. DprE1 (300 nM), FAD (1 μ M), Horseradish
405 peroxidase (2 μ M, Sigma-Aldrich P-6782) and Amplex Red (50 μ M, Life Technologies A-
406 22177) in 50 mM glycylglycine pH 8.0, 200 mM potassium glutamate and 0.002% Brij-35 was
407 incubated at 30°C with the test compound (DMSO stock, final conc.: 1%) for 10 min, followed
408 by the addition of farnesyl-phosphoryl- β -D-ribofuranose (FPR) to 300 μ M. The conversion of
409 Amplex Red to resorufin was followed by fluorescence measurement (excitation/emission:

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3 410 560/590 nm) on a Tecan M200, in kinetic mode, at 30°C. A negative control with no inhibitor
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6 411 was used, and the background rate (no added FPR) subtracted from measured rates. Fluorescence
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8 412 units were converted to resorufin concentration using a calibration curve in assay buffer. Kinetic
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10 413 constants were calculated using Prism (GraphPad Software). Steady-state kinetic constants were
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12 414 determined by fitting data to the Hill equation (Equation 1)¹⁵. IC₅₀ values were determined by
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14 415 fitting log[I] and normalized response to Equation 2, and the K_i for Ty38c was determined using
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16 416 an adapted equation for non-competitive inhibition of enzymes with sigmoidal behavior
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20 417 (Equation 3).

$$V = \frac{V_{max} \times [S]^h}{(K_{0.5})^h + [S]^h} \quad \text{Equation 1}$$

$$V = \frac{100}{\{1 + 10^{(\log(IC_{50} - [I]) \times h)}\}} \quad \text{Equation 2}$$

$$V = \frac{\frac{V_{max}}{\left(1 + \frac{I}{K_i}\right)} \times [S]^h}{(K_{0.5})^h + [S]^h} \quad \text{Equation 3}$$

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32 421 **DprE1 crystallization and structure determination.** Crystals of *M. tuberculosis* DprE1 in
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34 422 complex with Ty38c, Ty36c, Ty21c, QN127, QN124, QN129, QN118 and QN114 were obtained
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36 423 by the hanging-drop vapor diffusion method at 18°C. One or 0.5 μL of DprE1 (7 mg/ml) in 20
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38 424 mM Tris pH 8.0, 100 mM NaCl and 0.7 mM of the carboxyquinoxaline (DMSO stock, final
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40 425 conc.: 7%), was mixed with 1 μl of the reservoir solution containing 100 mM imidazole pH 6.9-
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42 426 7.5 and 34-39% polypropyleneglycol 400. Yellow crystals grew in approximately 1-3 days and
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44 427 were transferred to a cryo-protectant (reservoir solution with 25% glycerol) prior to, or frozen
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46 428 directly by flash-cooling in liquid nitrogen. X-ray data were collected at the X06DA beamline of
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48 429 the Swiss Light Source synchrotron (Villigen). Data processing and scaling are described in the
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50 430 Supporting Information.
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3 431 **Accession Codes:** Coordinates and structure factors for the crystal structures here reported have
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6 432 been deposited in the Protein Database with access code 4CVY for Rv3406, and the following
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8 433 codes for the DprE1 complexes: 4P8K (Ty38c), 4P8L (Ty36c), 4P8M (QN114), 4P8N (QN118),
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10 434 4P8P (QN124), 4P8C (QN127), 4P8T (QN129) and 4P8Y (Ty21c).
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27 530 **Acknowledgments:**

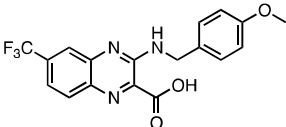
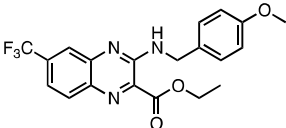
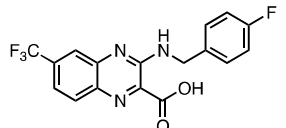
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49 539 **ASSOCIATED CONTENT**

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52 540 **Supporting Information:** Supplementary movies, tables, figures, methods, and references. This
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54 541 material is available free of charge via the Internet at <http://pubs.acs.org>.
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544 **Table 1.** Biological profile of the five confirmed hits in the quinoxaline library screening.

Structure	H37Rv MIC ₉₉ (μM)	Intracellular activity IC ₅₀ (μM)	HepG2 cytotoxicity TD50 (μM)
Ty38c 	3.1	2.5	37
Ty21c 	3.1	6.1	48
Ty36c 	12.5	>20	25

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547 **Table 2.** Kinetic analysis of the enzymatic activity of *M. tuberculosis* Rv3406.

Substrate	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{min}^{-1} \text{mM}^{-1}$)
α -KG ^a	0.0094 ± 0.0012	21.8 ± 1.0	2319 ± 106
Ty38c ^a	0.16 ± 0.01	1.54 ± 0.08	9.6 ± 0.3
2-EHS ^b	0.0088 ± 0.0005	19.2 ± 0.9	2186 ± 97

548 ^a assays were performed at a concentration of 0.05 mM 2-EHS. ^b The kinetic549 analysis for 2-EHS was performed at a concentration of 0.5 mM α -KG.

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4 552 **Table 3.** Enzymatic characterization and Ty38c inhibition of *M. tuberculosis* DprE1 wild type,
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6 553 G17C and L368P mutants.

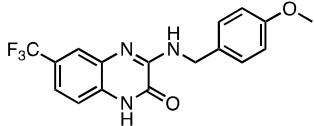
	Wild type	G17C	L368P
$K_{0.5}^a$ (mM)	242 ± 8	311 ± 20	308 ± 13
h^a	2.6 ± 0.2	2.6 ± 0.4	3.3 ± 0.4
k_{cat} (min ⁻¹) ^b	4.1 ± 0.6	0.12 ± 0.01	0.62 ± 0.02
IC ₅₀ Ty38c (μM)	0.041	0.15	1.3
K_i (μM)	0.0259	n.d. ^c	n.d.

19 554 ^a Data were fitted to the Hill equation for enzymes with sigmoidal behavior; ^b The enzyme
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21 555 concentration in the assay was 0.3, 1.5 and 1.2 μM for the wild type, G17C and L368P
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23 556 mutants, respectively; ^c n.d.: not determined.

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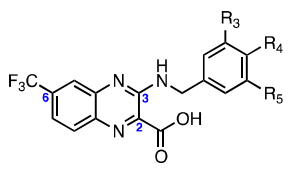
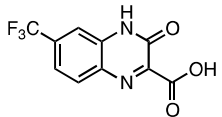
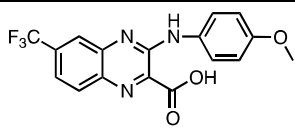
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559 **Table 4.** Biological and biochemical characterization of Ty38c analogs with modifications in
 560 positions 2 and 6 of the quinoxaline ring.

Compound	Structure					MIC ₉₉ (μM)			Rv3406 rate ^a (min ⁻¹)	DprE1 inhibition IC ₅₀ (μM) or % inhibition at 50 μM
	R ₁	R ₂	R ₃	R ₄	R ₅	H37Rv	H37Rv rv3405c mutant TRC4	H37Rv Δrv3406 dprE1 L368P		
Ty38c	CF ₃	COOH	H	OMe	H	3.1	6.3	12.5	1.07	0.041
Ty21c	CF ₃	COOEt	H	OMe	H	3.1	6.3	12.5-25	0.09	(54%)
QN101	CF ₃	COOEt	H	OEt	H	6.3-12.5	12.5	>100	0.07	n.i. ^b
QN102	CF ₃	CONH ₂	H	OMe	H	>100	>100	>100	0.04	n.i.
QN103	CF ₃	CONH ₂	H	CF ₃	H	>100	>100	>100	0.03	n.i.
QN104	CF ₃	CONHMe	H	OMe	H	>100	>100	>100	0.02	n.i.
QN105	CF ₃	CONHMe	H	CF ₃	H	>100	>100	>100	0.02	n.i.
QN106	CF ₃	Me	H	OMe	H	100	100	100	0	22
QN107	CF ₃	Me	OMe	OMe	H	>100	>100	>100	0	20
QN108	CF ₃	Me	OMe	OMe	OMe	>100	>100	>100	0	(49%)
QN109	CF ₃	Me	H	CF ₃	H	100	100	100	0	n.i.
QN110	H	COOH	H	OMe	H	100	>100	100	0.62	23
QN111	H	COOH	OMe	OMe	H	>100	>100	>100	0.35	33
QN112	H	COOH	H	F	H	50-100	100	50-100	0.56	16
QN113						>100	>100	>100	0	n.i.

561 ^a Rv3406 rate determined at 200 μM of the test compound, and calculated by dividing the measured enzyme velocity
 562 by the enzyme concentration; ^b n.i.: no inhibition.

566 **Table 5.** Biological and biochemical characterization of Ty38c analogs on the 3-benzyl moiety.

Compound	Structure			MIC ₉₉ (μM)			Rv3406 rate ^a (min ⁻¹)	DprE1 inhibition IC ₅₀ (μM) or % inhibition at 50 μM
	R ₃	R ₄	R ₅	H37Rv	H37Rv <i>rv3405c</i> mutant TRC4	H37Rv <i>Δrv3406</i> <i>dprE1</i> L368P		
								
Ty38c	H	OMe	H	3.1	6.3	12.5	1.07	0.041
QN114	H	OEt	H	3.1	6.3	12.5	0.26	0.088
QN115	OMe	OMe	H	50	100	100	0.67	0.16
QN116	OMe	OMe	OMe	100	>100	100	0.33	0.20
QN117	OMe	H	H	25	50	50	0.28	0.083
QN118	F	OMe	H	6.3-12.5	12.5	25	0.22	0.080
QN119	H	Me	H	12.5	12.5-25	25-50	0.38	0.10
QN120	Me	H	H	25	25-50	50	0.17	0.22
QN121	Me	F	H	6.3	12.5	25	0.25	0.13
Ty36c	H	F	H	12.5	25	25	1.19	0.072
QN122	F	H	H	25-50	50	50	0.08	0.16
QN123	F	F	H	25	25-50	50-100	0.27	0.15
QN124	H	Cl	H	3.1	12.5	25	0.21	0.050
QN125	Cl	H	H	25	50-100	50-100	0.25	0.13
QN126	Cl	Cl	H	12.5-25	12.5	25-50	0.27	0.11
QN127	H	CF ₃	H	6.3	12.5	25	0.2	0.12
QN128	CF ₃	H	H	12.5	25	50	0.17	0.16
QN129	H	CN	H	50	50	100	0.26	0.067
QN130				>100	>100	>100	0.05	(40%)
QN131				12.5-25	50	50	0.84	0.22

567 ^a Rv3406 rate determined at 200 μM of the test compound, and calculated by dividing the measured enzyme velocity
 568 by the enzyme concentration.

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6 572 **FIGURE LEGENDS:**
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9 573 **Fig. 1.** Single-cell analysis of bactericidal activity of Ty38c. *M. tuberculosis* expressing GFP was
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11 574 grown in a microfluidics device and exposed to 5 μ M Ty38c between days 5-12. Imaging
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13 575 was carried out at 1-hour intervals during 18 days on fluorescence (FITC) and phase
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16 576 channels. Representative time-series snapshots of an imaged XY-point in the microfluidic
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18 577 device are shown. The medium conditions are indicated on top left (7H9 - no drug;
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20 578 Ty38c). Days are indicated on top right. The scale bar shown at bottom left represents 5
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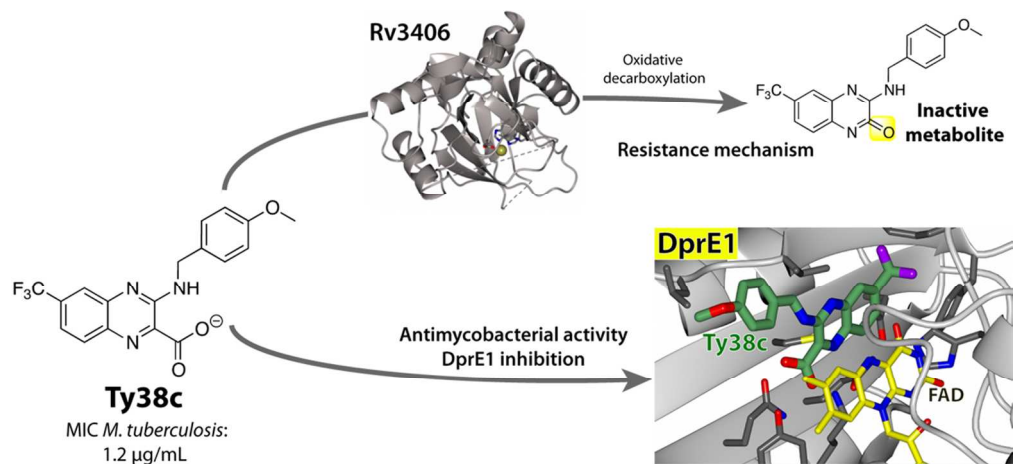
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29 581 **Fig. 2.** Reactions catalyzed by Rv3406. (A) Oxidation of α -KG by Rv3406 in the presence of 2-
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31 582 EHS leads to the formation of succinate and 2-ethylhexanal. (B) Oxidation of Ty38c and
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33 583 its analogs by Rv3406 in the same conditions leads to the decarboxylation of the
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36 584 compound, affording the respective keto derivative.
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41 586 **Fig. 3.** Crystal structures of DprE1 in complex with Ty38c and analogs. (A) Active site with
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43 587 Ty36c bound in a planar conformation (monomer A in the asymmetric unit). (B)
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45 588 Superposed structures of Ty38c and six analogs, in the conformation observed in
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48 589 monomer A. The inhibitor QN118 presented two alternative conformations in this
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50 590 monomer. (C) Active site with Ty36c bound in a bent conformation (monomer B), with
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52 591 the disordered loop (316-331) represented by a dashed line. Gly17 and Leu368
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55 592 (represented as spheres), when mutated to Cys and Pro, respectively, confer resistance.
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57 593 The residues forming the pocket where the trifluoromethyl group is bound are shown as a
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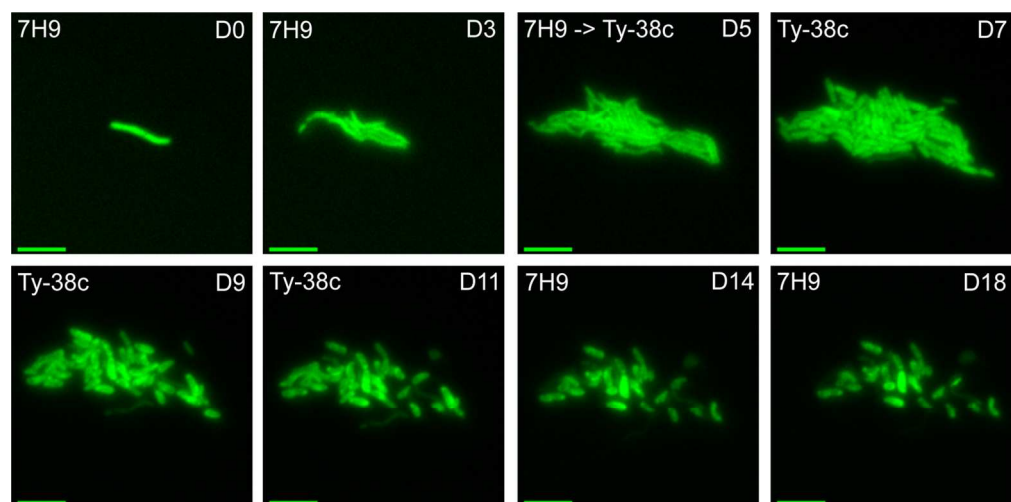
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3 594 semi-transparent surface. **(D)** Superposed structures of Ty38c and six analogs, in the
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5 595 conformations observed in monomer B. **(E)** $2F_o-F_c$ electron density map contoured at 1.0
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8 596 RMSD ($0.2291 \text{ e}/\text{\AA}^3$) for QN124 and tri-propylene-glycol (tri-PG), showing the
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10 597 surrounding residues in the DprE1-QN124 complex. **(F)** Superposition of the DprE1
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12 598 active site-bound conformations of PBTZ169 (PDB 4NCR (*10*)), TCA1 (PDB 4KW5
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15 599 (*19*)) and Ty38c.
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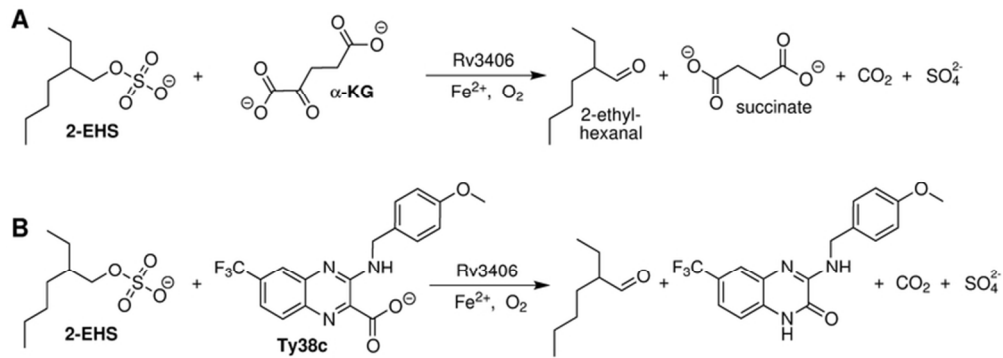
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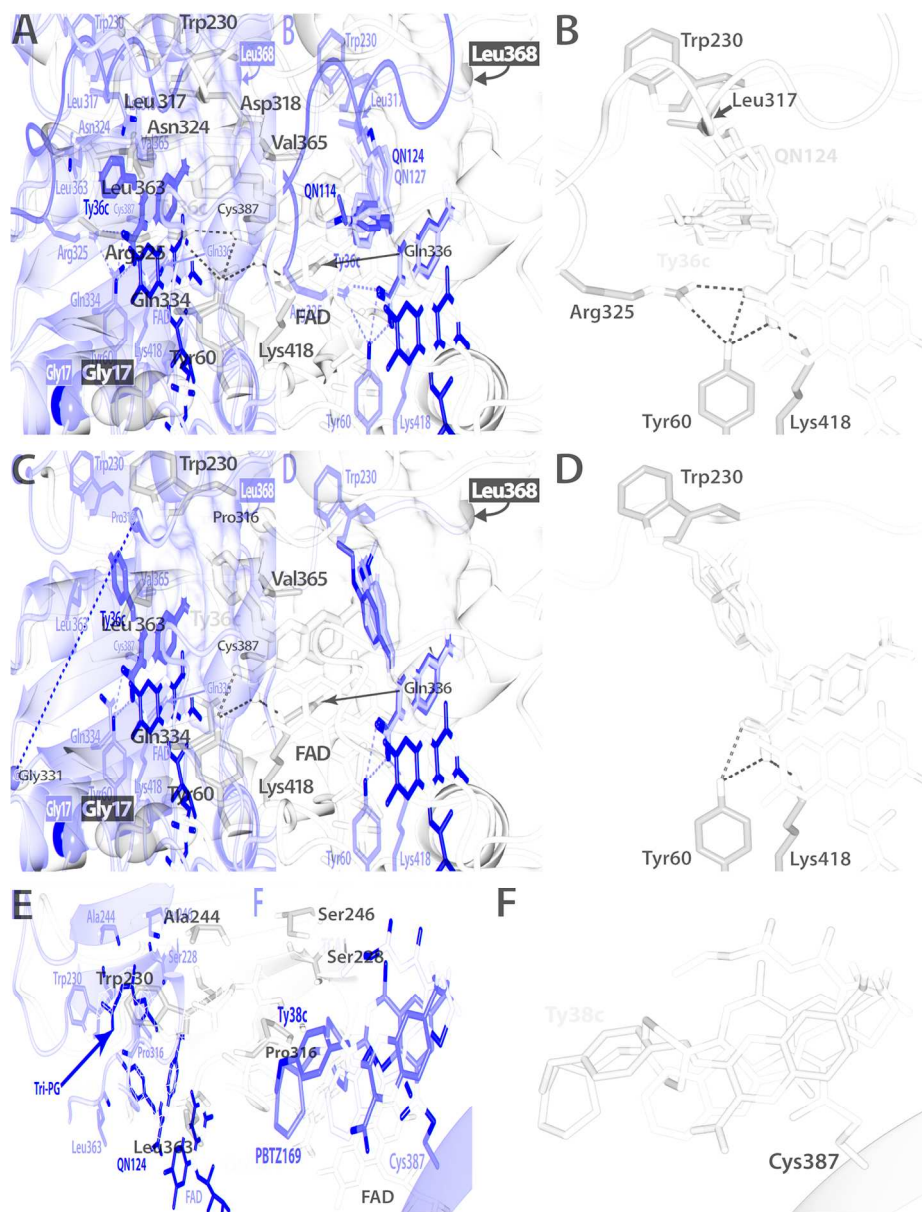
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