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Recent advances in the design of inhibitors of mycobacterial transcriptional regulators to boost thioamides anti-tubercular activity and circumvent acquired-resistance

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Abstract

With more than 1.6 million deaths per year, tuberculosis (TB) is today the major cause of mortality resulting from a bacterial infection. The principal obstacles to the global control of this infectious disease include the difficulties to detect and cure a sufficient number of cases to interrupt transmission. In addition, multidrug-resistant-TB (MDR-TB) has emerged as a major concern worldwide. Therapeutic solutions to stem this scourge are based on a 2 to 4 year treatment regimen of less effective second-line drug cocktails, often associated with serious side effects, which reduces patients compliance and thus leads to high rates of recurrence and mortality. In parallel to the search for new targets and new class of antibiotics, the development of strategies to improve the efficacy of drugs already used in the clinic have been proposed. One of these strategies takes advantage of the fact that many first-line and second-line anti-tubercular drugs are pro-drugs. Indeed, they need to be bioactivated by drug-specific mycobacterial enzymes in order to develop their anti-bacterial activity. Thioamides (ethionamide or prothionamide) are among the most frequently used drugs for the treatment of drug-resistant tuberculosis. Bioactivation of ethionamide occurs through the catalysis of a flavin-dependent Baeyer-Villiger monooxygenase called EthA. The resulting NAD-adduct inhibits InhA, an enoyl-ACP reductase of the type II fatty acid synthase (FAS-II) system, involved in cell-wall synthesis. The transcriptional repressor EthR, in turn, tightly controls the expression of *ethA*. A new therapeutic concept emerged from this observation and the development of EthR inhibitors was proposed as a solution to improve ethioanmide activity. In this chapter, we outline the most recent efforts of different research groups, using modern drug discovery strategies that led to the design, discovery and optimization of the first compounds able to inhibit EthR and boost the bioactivation of ETH *in vitro* and *in vivo*. We finally discuss the limitations of this approach and the breakthroughs in the identification of new chemical series and alternative bioactivation pathways that paves the way for the clinical development of a combination allowing thioamides to return fully active against all sensitive or resistant clinical isolates.

Key words

Transcriptional repressors EthR and EthR₂, Thioamides boosting strategies, EthR and EthR₂ inhibitors, modern medicinal chemistry strategies to improve antitubercular activity of prodrugs.

Introduction

According to the WHO most recent statistics, tuberculosis (TB) is today the first leading cause of death by an infectious agent and is one of the top 10 causes of death worldwide.¹ In 2017, *Mycobacterium tuberculosis* infections led to an estimated 1.3 million deaths among HIV-negative people and 300,000 additional deaths among HIV-positive people. Globally, 10 million people developed the disease in 2017. Around 87% of TB cases were diagnosed in the 30 countries listed by WHO as high TB burden. Drug-sensitive TB treatment, established in the 1980s, typically lasts 6 months, and consists of a combination of four drugs during 2 months, followed by a bi-therapy with isoniazid and rifampicin for the last 4 months of treatment. Most anti-TB drugs have been discovered during the 50s and the 60s and their chronic use often causes important side effects leading to insufficient patient compliance, thereby opening the way to the spread of multiple drug-resistant TB strains (MDR).² MDR-TB must then be treated with second line drugs, with a narrow therapeutic index, resulting in even poorer compliance and the inexorable emergence of extensively drug-resistant TB (XDR). Even though a more systematic use of diagnostics and the deployment of treatments have saved 53 million lives between 2000 and 2016, these regimens have remained unchanged since their implementation in the 70s³ and only recently, two new drugs, bedaquiline and delamanid, have been approved for the treatment of MDR-TB.^{4,5} Hopefully, during the TB Innovation Summit, held in New York in September 2018, there has been a growing awareness from policy-makers that finally share the objective of tackling the sensitive and drug-resistant TB threat.

Many anti-TB drug development efforts have been undertaken in the last two decades since the full sequencing of *Mycobacterium tuberculosis* (Mtb) genome and the identification of new drug targets.⁶ Unfortunately, target based screenings did not afford the expected outcome. This is partly due to our inability to predict and improve the behavior of the identified hits in terms of membrane permeability, penetration of the thick waxy mycobacterial cell wall and active drug efflux from the bacilli.^{7,8} In order to identify compounds with suitable membrane permeability and not prone to efflux, anti-TB drug development efforts have also been focused on phenotypic-based screenings. However, identifying the mode of action of hits or discarding frequent hitters has been a recurring issue.

On top of acting on a new target, in order to be efficient against drug-sensitive as well as MDR and XDR Mtb strains, the ideal new chemical entity would also require to shorten treatment duration.⁹ Since the origin of the TB polytherapy, only pyrazinamide (PZA, discovered in 1952) was able to shorten treatment from 9-12 to 6 months.¹⁰ This unique property of PZA is due to its remarkable activity against “persisters”, a subpopulation of bacilli progressively less sensitive to drugs during treatment. The critical and irreplaceable role of PZA has been illustrated in many clinical trials where its removal led to an inferior therapeutic outcome, especially in terms of disease relapse.¹¹ However, dramatically, recent years have seen an increased worldwide burden of PZA resistance. PZA resistance is now ubiquitous, with an estimated one in six incident TB cases and more than half of all MDR-TB cases being resistant to this antibiotic globally. With a burden of 1.4 million new PZA resistant TB cases annually, the situation is now so critical that some authors caution the scientific and medical community against relying on PZA in current and future TB drug regimens.¹²

Pro-drugs in TB treatment

The common point of most of the anti-tuberculosis compounds used in first and second-line such as isoniazid, ethionamide, prothionamide, delamanid and PZA, is that they are prodrugs. Comparable to Trojan horses, these small molecules display physico-chemical properties that facilitate their penetration through the peculiar mycobacterial cell wall. Inside, they are bioactivated by specific mycobacterial enzymes into molecules that are toxic for the mycobacterium. Some of them are then

trapped in the bacterial cytoplasm of the bacilli.¹³ Unsurprisingly, one drawback of these compounds is that the most frequent resistance mechanism occurs by mutation in the bioactivation pathways. As an example, 94% of the clinical strains resistant to isoniazid (INH) carry mutations in the INH-activating catalase/peroxidase KatG.¹⁴ Likewise, it was reported that 87% of PZA-resistant clinical isolates are mutated in the PZA-activating enzyme PncA.¹⁵ Clinical isolates showing mutations in Ddn, the key enzyme involved in bioactivation of delamanid, have also been reported.¹⁶ These crucial observations strongly suggest that the anti-mycobacterial potency of these prodrugs is, at least *in vitro*, limited by the rate of their bioactivation.

Thioamide prodrugs as good candidates for boosting strategies.

Ethionamide (ETH) and prothionamide were developed in the late 50s as analogues of isonicotinic acid.¹⁷ They both show potent activity against Mtb *in vitro* and *in vivo* and they are key components of existing treatment regimens for MDR-TB in adults and children.¹⁸ Consequently, as the number of MDR and XDR cases is continuously increasing worldwide, the importance of ETH is steadily increasing. However, ETH has a narrow therapeutic index, as the dose required to inhibit Mtb growth generally causes serious adverse effects, such as gastrointestinal disorders, hepatitis, and various mental disturbances.¹⁸ In fact, ETH activity is linked to its level of bioactivation, which occurs in the bacteria through multiple oxidation steps catalyzed by the flavin-dependent mycobacterial Baeyer-Villiger monooxygenase EthA. However, ETH is also prone to metabolism in the liver through flavin-dependent monooxygenases, which may contribute to its toxicity.¹⁹ In Mtb, the bioactivation triggers the production of ethionamide S-oxide, then reactive radical intermediates that are trapped within the bacterial cell as a NAD-adduct.^{20–23} This metabolite effectively inhibits InhA, an essential enzyme involved in the FAS-II pathway, responsible for the biosynthesis of mycolic acids (Figure 1).²⁴

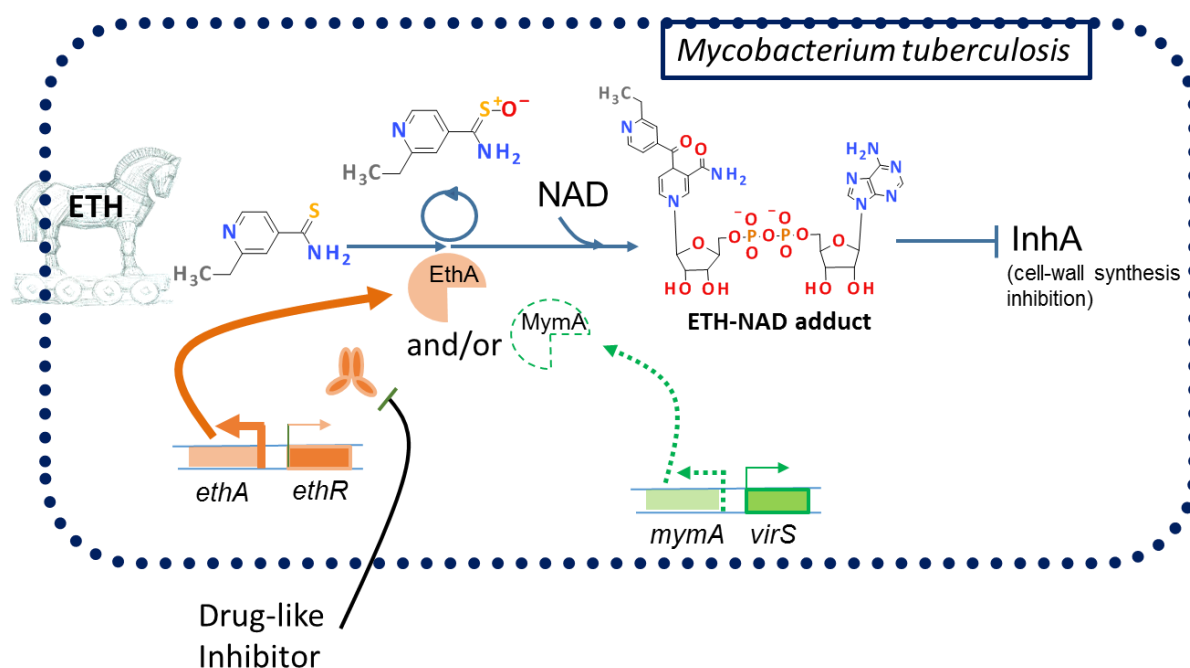


Figure 1. Baeyer-Villiger monooxygenases EthA and to a lesser extent, MymA, are required for bioactivation of ethionamide (ETH). Development of EthR inhibitors is proposed as a therapeutic strategy to boost the expression of *ethA* and thus the bioactivation of ETH inside the mycobacteria.

EthR as a validated target to boost the bioactivation of ethionamide

The gene *ethA* is arranged with *ethR* in a divergent operon with a shared intergenic promoter region. EthR is a member of the well-characterized TetR family of transcriptional regulators.^{20,25,26} It tightly

controls the expression level of *ethA*. Therefore, chromosomal inactivation of *ethR* was shown to be associated with ETH hypersensitivity.²⁰ Likewise, the reduction of the production of EthA by overproduction of EthR²⁰ or by mutation in the promoter of *ethA*²⁷ impaired ETH activation. EthA was the first key enzyme responsible for ETH bioactivation identified within the bacteria. More recently, Grant et al. reported the existence of a second Baeyer-Villiger monooxygenase, called MymA, that is, to a lesser extent, also implicated in the bioactivation of the prodrug into active metabolites (Figure 1).²⁸ Moreover, Ang et al. confirmed that deletion of the *ethA/R* locus did not confer high level of resistance to ETH in Mtb which is in line with the existence of an alternative bioactivation pathway for ETH.²⁹ Ethionamide resistance in Mtb occurs mainly by *ethA* mutations, deletion or mutation in *msha* coding for glycosyltransferase involved in mycothiol biosynthesis, mutations in *ndh* which codes for a type II NADH dehydrogenase, or mutations in *inhA* and/or its promoter region (c-15t).^{30–34} Rare mutations in *ethR* relative to thioamides resistance have also been described.^{32,35,36} Mutation in the intergenic region of *ethA-ethR* (t-11c), in combination with an *inhA* promoter mutation was described as leading to a high level of resistance.²⁷ The binding of c-di-GMP to EthR in its dimeric conformation has also been shown to limit *ethA* expression and to enhance resistance to ETH.³⁷ Finally, EthR-DNA binding has been shown to be controlled by PknF-mediated phosphorylation.³⁸

Based on the knowledge that EthA is a central element in ETH bioactivation and antibacterial activity, the search for EthR inhibitors was considered as a promising strategy in order to amplify the sensitivity of Mtb to the prodrug, by increasing expression levels of *ethA* (Figure 1).³⁹

Biological assays used for hits identification

Numerous strategies were developed to identify hits using structure-based or fragment-based drug design approaches, *in silico* virtual screenings, and low or high-throughput target-based whole-cell screenings.

Three main assays were developed to identify and optimize EthR inhibitors. A binding assay, known as thermal shift assay (TSA)⁴⁰, was used to monitor the thermal unfolding of the protein EthR, using a fluorescent dye. A ligand of EthR, while binding to the protein, will increase its thermal stability. Thus, the difference between the melting temperatures of the holo and apo forms of EthR, expressed as ΔT_m , was shown to correlate with the affinity of the ligand for the protein.⁴¹ A functional assay based on surface plasmon resonance (SPR) was optimized to measure the binding of EthR to its DNA operator in the presence of increasing doses of inhibitor. IC₅₀ represents the concentration of the compound that inhibits 50% of this interaction.⁴² Finally, a phenotypic cell-based assay was used to monitor the potency of EthR inhibitors, expressed as EC₅₀, to impair, in combination with subactive doses of ethionamide (MIC₉₉/10), the growth of H37Rv-GFP strains with or without the presence of macrophages.⁴³

Target-based screenings

The key event that initiated target-based screenings was the independent publications of two crystal structures of the transcriptional regulator EthR in 2004 (Figure 2).^{39,44} The first structure revealed that the repressor is a homodimer organized in two functional domains: a DNA-binding domain comprising three α -helices connected to an α -helical core domain involved in dimerization of the repressor and a ligand binding domain. In the later, a long highly hydrophobic ester, hexadecyloctanoate (HexOc), was found embedded. The cavity is a long and linear tunnel, mainly composed of hydrophobic residues, at the exception of 2 asparagines (N176 and N179), with one open end, at the C-terminal domain. A second entrance may be accessible on the opposite site, through the rotation of the side chain of F114. Except this change, the ligand binding is quite rigid. By analogy with other members of the TetR family of repressors, it was supposed that HexOc induces

conformational changes, which move the DNA-binding domains apart by approximately 18 Å. In this liganded conformation, EthR loses its capacity to bind to its operator. The second crystal structure showed the same overall architecture that is structurally incompatible with DNA binding. In this study, two molecules of 1,4-dioxane were found in the core domain of each monomer. This observation led to the conclusion that occupation of the upper part of the ligand-binding domain with small hydrophobic ligands may be sufficient to induce conformational changes that impair the DNA-binding function of EthR.⁴⁵ This hypothesis was later confirmed with an EthR variant where G106, was replaced by a tryptophane (Figure 2). It was shown experimentally, by SPR, that this variant was unable to bind DNA. X-ray crystallography revealed an apo conformation of this variant equivalent to the holo conformation of the wild-type structure of EthR.⁴⁶

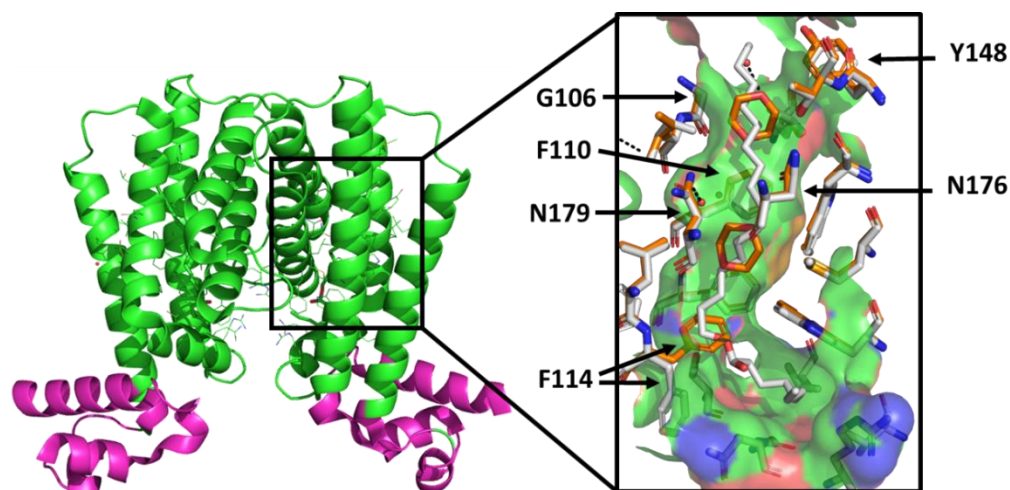


Figure 2. X-ray structure of EthR liganded by HexOc (white) superimposed with the X-ray structure containing two molecules of 1,4-dioxane (orange). Key residues are labelled.^{39,44}

Based on this information, a 3D-pharmacophore of a putative EthR inhibitor was defined and used by Willand *et al.* to select compounds from an in-house chemical library.⁴² These compounds were screened for their capacity to interfere with the DNA-binding and -recognition functions of EthR using SPR.^{42,47} This screening retrieved one main family of compounds sharing a 1,2,4-oxadiazole scaffold. The hit compound BDM14500 ($IC_{50} = 38 \mu M$) was successfully co-crystallized with EthR. As expected, the repressor was in a conformation incompatible with DNA binding. After several rounds of optimization, the cyanoacetyl analogue BDM31343 was identified as a more potent inhibitor of EthR ($\Delta T_m = 3.2^\circ C$, $IC_{50} = 3.3 \mu M$)⁴² and booster of ethionamide activity ($EC_{50} = 1.5 \mu M$)⁴¹. Moreover, among the various synthesized analogues, this compound showed the most favorable pharmacokinetic profile for *in vivo* experiments.⁴² Further structure–activity and structure–property relationships exploration in the 1,2,4-oxadiazole series led to compounds with improved potency, such as the lead compound BDM41906 ($\Delta T_m = 9.3^\circ C$, $IC_{50} = 0.4 \mu M$, $EC_{50} = 0.06 \mu M$), which showed optimal microsomal stability and mice exposure after oral administration (Figure 3).^{47–49}

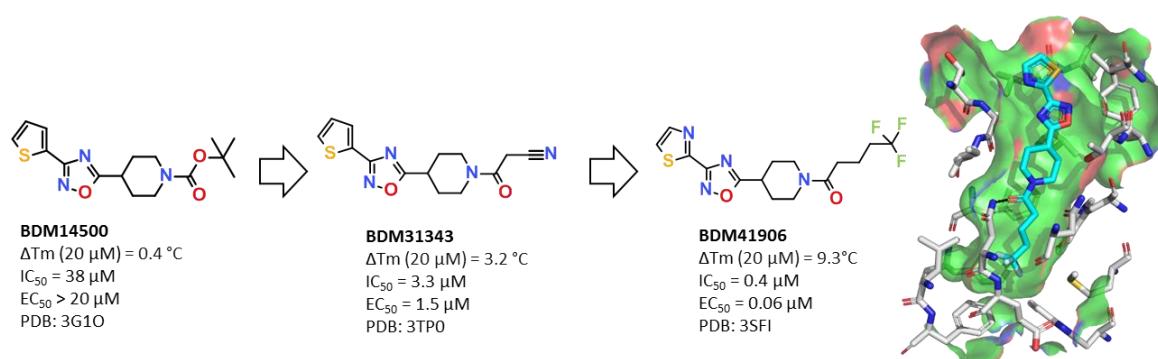


Figure 3. Chemical structures of EthR inhibitors identified and optimized through a structure-based approach.^{41,42,49}

Target-guided synthesis

In the last two decades, a large number of target-guided synthesis (TGS) approaches have been used in which a protein is actively engaged in the catalytic formation of irreversible chemical bounds from complementary molecules. The most representative example is *in situ* click-chemistry, where the spatially close binding of an azide and an alkyne into a specific pocket of a protein, allows the 1,3-dipolar Huisgen cycloaddition to yield a triazole derivative that shows higher affinity.⁵⁰ EthR was successfully used by Willand et al. as a catalytic template to allow the reaction of an azido derivative with a large set of small alkynes. Compound BDM14801, the acetylazido analogue of BDM31343 in the 1,2,4-oxadiazole series, was synthesized and displayed a slightly lower potency to disrupt EthR/DNA interaction than compound BDM31343 (IC_{50} = 7.4 μM vs 3.3 μM).⁵¹ Co-crystallization with EthR followed by X-ray diffraction showed the azido group pointing toward the bottom of the pocket. Therefore, an *in situ* click-chemistry experiment was performed with EthR and BDM14801 in mixture with six different sets of 10 alkynes. The predominant *in situ* synthesis of the clicked compound BDM14950 (Figure 4) was monitored by mass-spectrometry (SIM-LC/MS). This triazole derivative was resynthesized and displayed a 10-fold higher potency than the starting azido derivative (IC_{50} = 0.58 μM vs 7.4 μM), however it was less active on bacteria in combination with ethionamide probably due to a lack of permeability (EC_{50} = 20 μM vs 1.2 μM). Nevertheless, the co-crystal structure of BDM14950 with EthR revealed that F114 closing the ligand binding domain had to flip to allow occupancy of a previously hidden hydrophobic pocket, as observed with the HexOc ligand identified in the first cocrystal structure obtained with EthR.³⁹ Although this EthR-guided synthesis did not deliver ethionamide boosters with improved potency, it led to a better understanding of the flexibility of the regulation domain and the existence of additional interaction pockets. On top of that, this work confirmed that starting from small molecules allows the optimized compounds to reach more efficiently their target within the bacteria. This encouraged us to initiate a fragment-based approach.

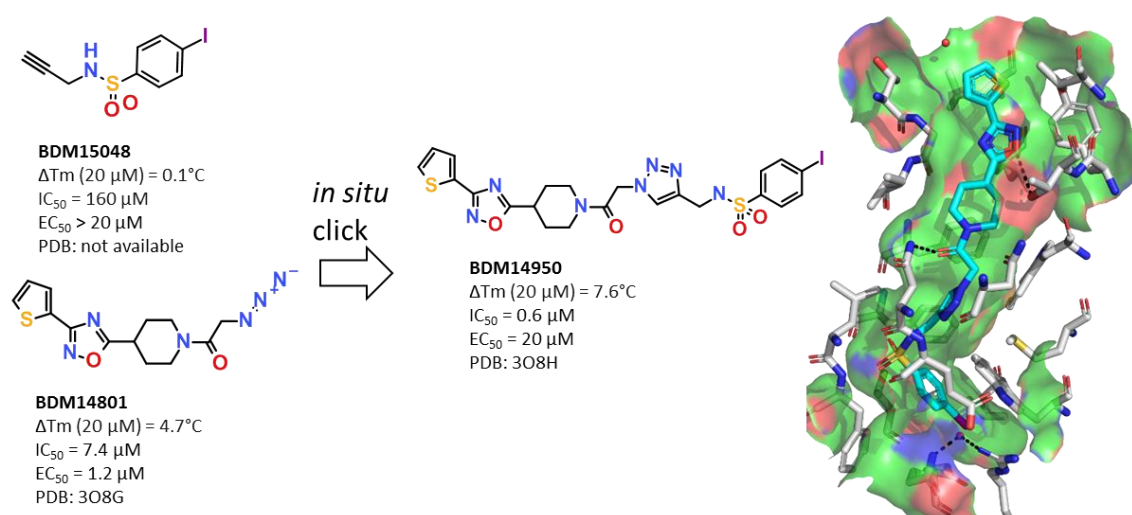


Figure 4. Chemical structure and protein interactions of an EthR inhibitor identified through *in situ* click-chemistry.⁵¹

Fragment-based approach

Fragment-based drug design (FBDD) approaches have become popular in medicinal chemistry to discover lead compounds and develop clinical candidates in many therapeutic areas and particularly in infectious diseases.^{52–54} Because fragments display small size and low molecular weight, they tend to present better physicochemical properties, especially solubility, than lead-like or drug-like compounds, which makes them ideal tools to counteract the low permeability of the waxy and thick Mtb cell envelope.⁵⁵ This fundamental advantage compensates for the low affinity and potency of the hits, which identification requires the use of sensitive biophysical techniques to detect their binding. Fragment-based approaches have been successfully applied to identify EthR inhibitors and improve their potency. Following the *in situ* click-chemistry approach described above, Villemagne *et al.* selected the small alkyne BDM15048 as a starting scaffold for fragment-based optimization. Although this small molecule (14 heavy atoms) displayed a weak affinity for EthR and potency to disrupt EthR/DNA interaction (ΔT_m = 0.1°C, IC_{50} = 160 μM), its co-crystallization with EthR revealed that the fragment occupies simultaneously two different subdomains of the ligand binding domain. At the portal, one molecule of BDM15048 is engaged in an H-bond with Y148 and deeper in the pocket the second one is H-bonded to N179. This key structural information opened the way to the development of growing, merging and linking strategies to optimize the hit (Figure 5).⁵⁶ In the growing strategy, *in silico* screening of a focused library of 976 virtual phenylsulfonamide analogues of BDM15048 led to the identification of BDM41329 which showed improved affinity and potency (ΔT_m = 6.1°C, IC_{50} = 4.9 μM) and was able to achieve a ten-fold boost of ETH activity on Mtb with an EC_{50} of 5.7 μM . Further improvement, especially the replacement of the isopentyl tail by a trifluoropropyl chain and the modification of the sulfonamide by an amide linker, led to the lead compound BDM43266 (MW= 314 g.mol⁻¹, ΔT_m = 11.2°C, IC_{50} = 0.40 μM , EC_{50} = 0.08 μM). The noteworthy improvement in activity was explained by its strong interaction with EthR thanks to the formation of two hydrogen bonds (instead of one for BDM15048 and BDM41329) with the two asparagines (N176 and N179) located in the middle of the ligand binding domain.

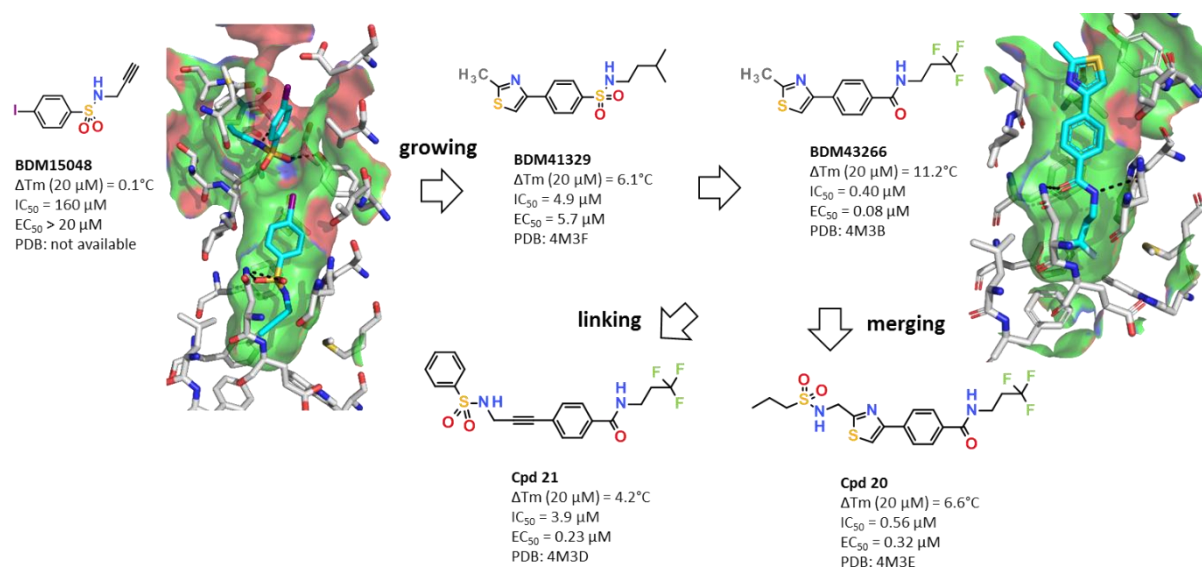


Figure 5. Chemical structures of EthR inhibitors identified and optimized through fragment-based approaches.⁵⁶

A structure-guided fragment-based approach has also allowed Surade *et al.* to obtain new inhibitors of EthR.⁵⁷ The screening of a 1250 fragment library using a thermal-shift fluorescent assay led to the identification of 86 hits. The use of the functional SPR assay to measure the inhibition of the interaction between EthR and the *ethA* promoter, in the presence of hits at 500 μM , allowed the selection of 4 compounds for X-ray crystallography experiments. Three hits contained an arylsulfone scaffold which was hydrogen-bonded to N179 and two of them formed an additional hydrogen bond with N176. A fourth hit, compound 1 (ΔT_m = 5.5°C, IC_{50} = 280 μM), was also bound to N179 while a second molecule occupied simultaneously the buried hydrophobic domain opened by the rotation of F114 as previously described (Figure 6). Replacement of the cyclopentyl group by a phenyl ring followed by the linking of the two fragments with a disulfide bond led to the improved analogue 9 (IC_{50} = 1.0 μM). Although more potent *in vitro* in the functional test, this compound showed only an 8-fold ethionamide boosting efficacy at a concentration of 1 μM . This unexpected weak activity was attributed by the authors to the relatively permeability of the more complex structures. Indeed, a growing strategy starting from compound 1 afforded analogues with better *in vitro* and *ex vivo* potencies such as compound 14 (EC_{50} = 0.04 μM), which had also intrinsic bactericidal activity at 1 μM (Figure 6).^{58,59}

Native electrospray ionization-mass spectroscopy (ESI-MS) was recently developed to study the interactions and stoichiometry of EthR/DNA (62 bp) complexes.⁶⁰ This technique revealed hexamerization or tetramerization of EthR on DNA in solution instead of octamerization as it was previously shown by SPR.⁶¹ ESI-MS was then used to screen fragments at a small scale.⁶² The assay was performed with the full-length protein but also with a 31-bp shortened DNA sequence to increase the sensitivity for the detection of tight binders. This assay was validated by testing the published 1,2,4-oxadiazole inhibitor BDM31381⁴². From a fragment-library of 80 molecules, two new hits able to disrupt the EthR-DNA interaction *in vitro* in the micromolar range (IC_{50} = 150 and 190 μM in the native MS assay, IC_{50} = 460 and 610 μM in the SPR experiment) were identified. Their binding to the hydrophobic channel of the transcriptional regulator was shown by co-crystallization and X-ray diffraction (Figure 6).

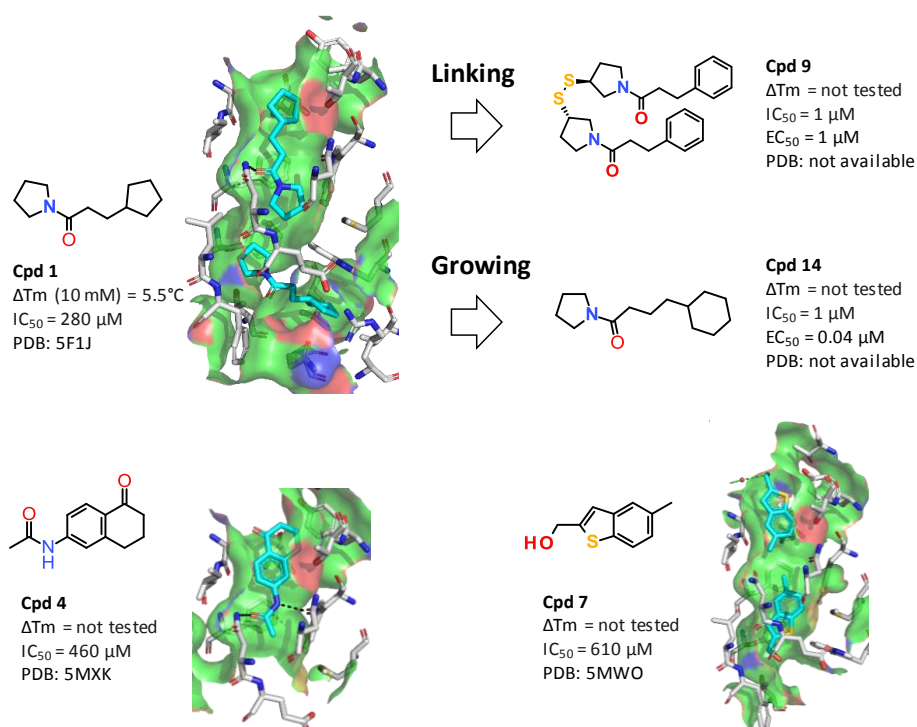


Figure 6. Chemical structures of EthR inhibitors identified through fragment-based approaches.^{57–59,62}

Phenotypic-based screening

A high-throughput target-based phenotypic assay was developed by Flipo et al.⁶³ to identify and optimize potent EthR inhibitors in a mycobacterial environment, in order to reveal compounds able to cross the mycobacterial cell wall and to inhibit the binding of EthR to DNA. This phenotypic assay, based on a fluorescent methylumbelliferone reporter system in *M. smegmatis*, was used for the screening of a 14,640 compounds library. 76 compounds inducing a positive response, together with 244 direct analogues, were selected for dose-response experiments. Among the 320 compounds, 22 showed IC_{50} s below 10 μM , and 3 from the N-phenylphenoxyacetamide family were able to thermally stabilize EthR with ΔT_m greater than 4°C. Compound BDM5683 showed the best profile with an IC_{50} of 2.9 μM and a ΔT_m of 6.4°C (Figure 7). Rapid optimization was carried out through the parallel synthesis of a focused library based on the N-phenylphenoxyacetamide motif. The 960 resulting molecules were screened on EthR using TSA. Seven compounds displaying a shift of the melting temperature of EthR greater than 7°C were tested on Mtb infected macrophages in combination with ethionamide ($MIC_{99/10}$). Among them, BDM31827 displayed the highest potency (EC_{50} = 0.21 μM) and showed no self antimycobacterial activity. This work is a good example of the use of a screening cascade, that relies on a target-based phenotypic assay in a whole bacteria and on a ligand binding assay, for the rapid identification of potent EthR inhibitors with *ex vivo* activities.

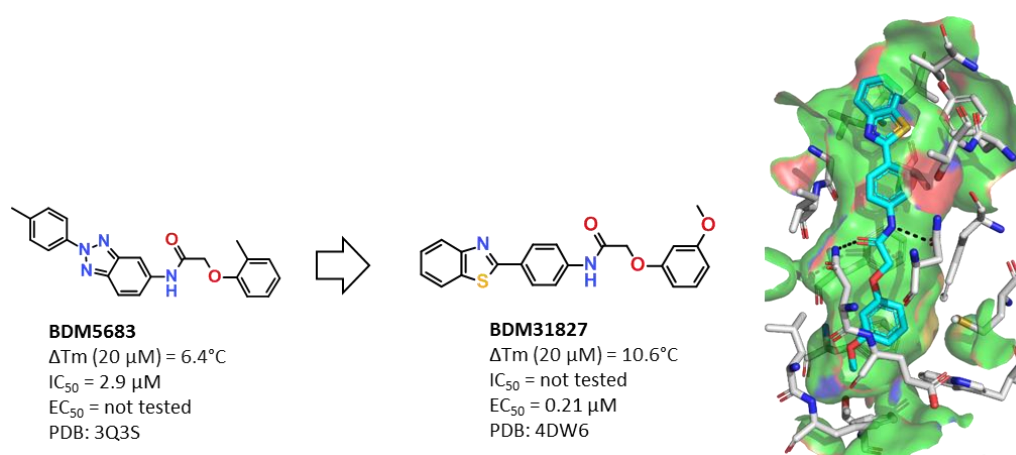


Figure 7. Chemical structures of EthR inhibitors identified through a high-throughput phenotypic screening.⁶³

In silico screening

Tatum *et al.*⁶⁴ designed a large *in silico* structure-based screening, using a filtered set of 409,201 compounds from the ZINC database, to ensure a high level of diversity. This led to the identification of a number of novel EthR inhibitor scaffolds. Testing of 85 hits in subsequent thermal shift assay provided 19 distinct chemical structures causing a significant increase in protein stabilization with ΔT_m in the range of 0.4 to 9.2°C. Only one compound was shown to destabilize the protein with a negative ΔT_m . From that set, two compounds (25 and 48, Figure 8) demonstrated ethionamide boosting activities on Mtb in the micromolar range (EC_{50} = 0.76 μM and 34 μM respectively). Predicted binding poses were confirmed with crystal structures of liganded EthR.

In another vein, Mugumbate *et al.* used a combination of ligand-based and structure-based chemogenomic approaches to unravel the molecular targets of phenotypic hits identified through a screening conducted by Ballell *et al.* at GlaxoSmithKline.⁶⁵ From that study, they identified 35 putative EthR inhibitors, and eight exhibited IC_{50} in the range of 3.9 to 50 μM in SPR assay. New crystal structures were obtained and showed direct interactions between the molecules and key residues in the hydrophobic ligand binding domain of EthR. Moreover, some of these compounds displayed also a concomitant ability to inhibit InhA. The most potent EthR inhibitor identified through this approach is compound GSK2032710a displaying an IC_{50} of 3.9 μM (Figure 8).

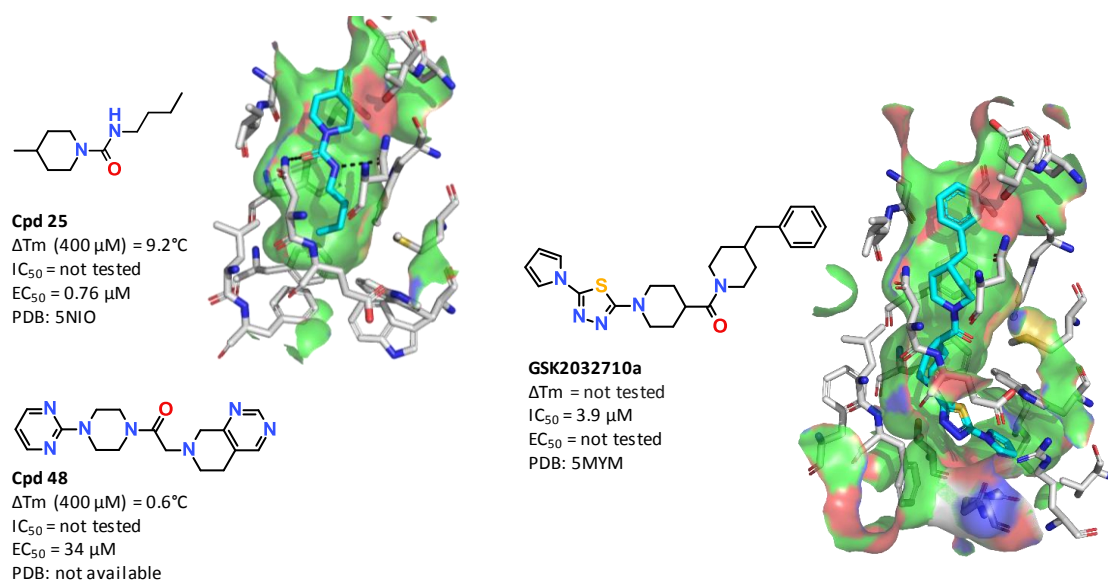


Figure 8. Chemical structures of EthR inhibitors identified through *in silico* screening.^{64,66}

X-ray structures analysis

In the different approaches developed to identify drug-like ligands of EthR, a large quantity of structural information on ligand/protein complexes have been generated. The search for new chemical structures of EthR inhibitors has been fueled by a tremendous amount of crystallographic structural data. In most of the approaches described hereinabove, co-crystal structures and *in silico* modeling were the key tools to drive the optimization process. The starting point of all this work is the publication of the three first crystal structures of the protein in complex with non-drug-like molecules such as 1,4-dioxane (PDB: 1T56)⁴⁴ or HexOc (PDB: 1U9N and 1U9O)³⁹. Since then, more than 80 structures of the holoprotein have been solved and are available in the protein data bank. Tanina *et al.* studied this source of information in order to shed light on hot spot residues.⁶⁷ The majority of the inhibitors were H-bonded to N179, and most of them interacted with aromatic residues lining the cavity, and in particular with F110 (Figure 2). More importantly, in all crystal structures of EthR, the dimeric functional protein adopts a conformation incompatible with binding to two consecutive major grooves of B-form DNA, with the HTH-domains of each monomer separated by an average distance of 43.1 Å. The large number of X-ray structures obtained of the liganded repressor contrasts with our inability to obtain crystals with the apoprotein. It is likely the apoprotein is less structured than the liganded one, and that a large loss of conformational entropy occurs upon ligand binding, which is compensated by a rather large contribution of hydrophobic interactions in the binding affinity of ligands in all the disclosed series.

In vivo proof of concept

From these different chemical families of EthR inhibitors and ethionamide boosters, identified through the wide variety of modern techniques used in medicinal chemistry, only three displayed relevant *in vivo* activities in an acute animal model of Mtb infection. After several rounds of optimization, the member of the 1,2,4-oxadiazole family, BDM31343, was selected for complementary ADME experiments that clearly demonstrated that it had a favorable pharmacokinetic profile (AUC = 82 µg.mL⁻¹.h after IP administration at 100 mg/kg). Given its wide distribution in animal tissues⁶⁸, BDM31343 was therefore the best candidate to validate the strategy of boosting the antituberculous activity of ETH *in vivo*. The combination of ETH (*per os* at 3, 9, 18 and 27 mg/kg) with BDM31343 (ip administration, BID at 50 mg/kg) led, in all groups, to a decrease of the bacterial load greater than the one obtained with the antibiotic administered alone.⁴² After a second optimization phase, the new lead compound BDM41906 showed significantly improved properties in terms of *in vitro* potency (EC₅₀ = 60 nM), solubility (410 µg.mL⁻¹), microsomal stability (t_{1/2} = 81 min, Cl_{int} = 15 µL.min⁻¹.mg⁻¹), and pharmacokinetics (AUC = 98.6 µg.mL⁻¹.h after *per os* administration of 20 mg/kg).⁴⁹ Its capacity to reduce the mycobacterial load synergistically with ETH in female BalbC mice intravenously infected with Mtb bacilli was then evaluated. BDM41906 was administered orally at 20 mg/kg, 6 days a week in combination with ETH, during four weeks. This study revealed that BDM41906 in combination with ETH 12.5 mg/kg was as efficient as ETH 50 mg/kg. Thus, the boosting effect on ETH efficacy observed with BDM41906 (4-fold) was better than the one previously shown with BDM31343 (3-fold), at a lower dose.^{41,49,69} In a second experiment, ETH and BDM41906 were co-encapsulated in biodegradable polymeric cyclodextrin nanoparticles (pCD NPs) and administrated by endotracheal aerosol administration using a Microsprayer®. This led to a significant decrease (p < 0.01) of the pulmonary bacterial load compared to ETH alone. The treatment of infected mice with only 6 doses of pCD NPs containing the combination, led to a 3 log decrease in lungs CFU in comparison to untreated animals.⁷⁰ In a similar experiment, BDM43266 was co-loaded with ETH in pCD.⁷¹ Administration via endotracheal route of the pCD nanoparticles co-loaded with ETH and

BDM43266 in Mtb challenged mice led to a 2 log lower ($p < 0.05$) pulmonary bacterial load of two logs, compared to the non-treated group (to be published).

Limitations to the development of EthR inhibitors

ETH is recommended as a second line drug by WHO in the DOTS-plus program, which means that it is exclusively prescribed to patients with MDR-TB. It has been documented that some MDR strains show cross-resistance to INH and ETH. Thus, the efficacy of the ETH-BDM41906 combination was evaluated on a series of MDR Mtb clinical strains isolated at the Lille-University hospital and at the Department of Health in Belgium (WIV-ISP) from patients epidemiologically unrelated, natives from EU, Africa or Asia. The MIC of ETH on five MDR strains sensitive to ETH was measured with and without BDM41906. All strains showed a marked decrease of the ETH MIC in the presence of the booster. Then, a series of 19 MDR strains that are also resistant to ETH were tested. For 13 strains, the adjunction of BDM41906 in the culture restored the “clinical sensitivity” to ETH with a reduction of the MIC inferior to $5 \mu\text{g.mL}^{-1}$. The 6 remaining strains did not recover, or partially recovered, sensitivity to ETH in the presence of BDM41906. Genetic characterization of these strains revealed that the 5 of them were indeed mutated in *ethA*. These observations are in agreement with the concept, as the adjunction of booster to a strain mutated in *EthA*, does not translate in a modification of the MIC of ETH.⁷²

Same conclusion was drawn by Grau et al., as the combination of 2-phenylethylbutyrate with *EthA* - activated prodrugs (ethionamide, isoxyl or thiacetazone) increased the growth inhibitory effect of these antibiotics on five drug-susceptible and two drug-resistant clinical isolates of Mtb. In contrast, five strains found resistant to the combination harbored mutations in the *ethA* locus.^{73,74}

Identification of new bioactivation pathways to revert resistance

In the quest to explore further the chemical space of EthR inhibitors, the key replacement of the 1,2,4-oxadiazole scaffold by a spiroisoxazoline motif, led to the discovery of SMART-420.⁷²

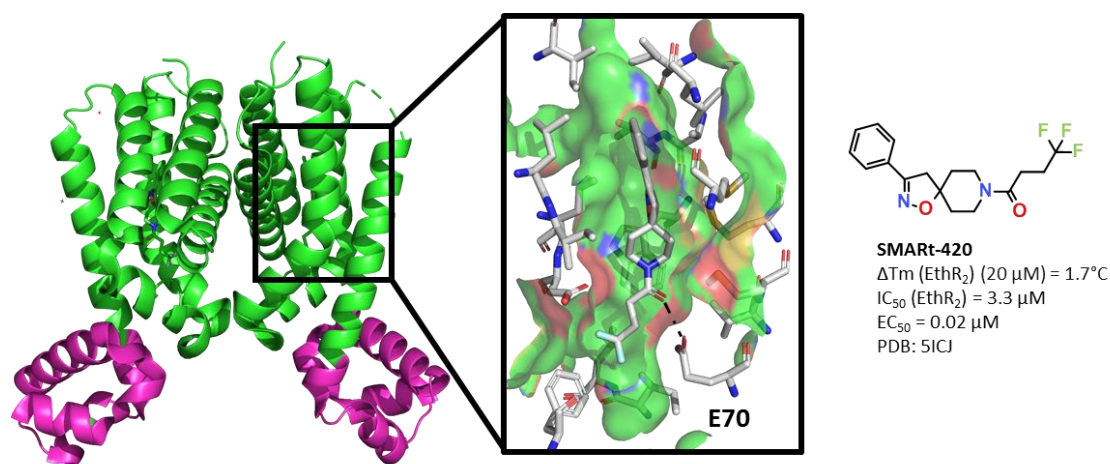


Figure 9. X-ray structure of EthR₂ liganded with SMART-420.⁷²

This compound lost its ability to bind to EthR, however it remained very potent to boost ETH in the infected macrophage assay with EC_{50} in the low nanomolar range (EC_{50} = 20 nM). The analysis of the transcriptome of *M. bovis* BCG treated with SMART-420 revealed a strong induction of the expression of two cryptic genes coding a transcriptional regulator and an oxidoreductase (named respectively EthR₂ and EthA₂). The direct binding of SMART-420 to EthR₂ was shown by TSA (ΔT_m = 1.7°C at 20 μM and 4.0 at 100 μM) and co-crystallization.⁷⁵ EthR₂, like EthR, crystallizes in a homodimeric conformation, with SMART-420 liganded within the hydrophobic binding domain of each monomer,

stabilized by H-bonds with E70 (Figure 9). The dose-dependent inhibition of EthR₂/DNA interaction was assessed by SPR (IC₅₀ = 3.3 μM) and a reporter gene assay developed in mammalian cells. The combination of ETH and SMART-420 allowed to boost the thioamide prodrug in drug-sensitive and drug-resistant strains even those carrying *ethA* mutations. Reversion of resistance was also observed in a mice model infected with an ETH-resistant Mtb bacilli mutated in *ethA*. Treatment of mice with a combination of ETH and SMART-420 (both at 50 mg/kg) allowed a 4.6 log reduction of the bacterial load in the lungs of animals. Finally, in a recent study, Prieri *et al.* demonstrated that the two bioactivation pathways triggered in Mtb by the two boosters BDM41906 and SMART420 share the same substrate profile. Indeed, by exploring the structure-bioactivation relationships in a series of thioamide analogues of ETH substituted in position 2 of the pyridine ring, we observed a strong correlation in their MICs when combined with either booster.⁷⁶ Knowing that InhA is also the final target of ETH when combined with either booster,⁷² the bioactivated intermediates may be the same and this characterization and comparison is in progress. Furthermore, a fragment-based screening has revealed new chemical scaffolds of EthR₂ inhibitors, which opens the way to the optimization of new inhibitors.⁷⁷

Conclusions

The boosting of ETH activity through the inhibition of Mtb transcriptional regulator has been validated as promising new anti-TB strategy. This approach, relying on the detailed understanding of the molecular processes that trigger the bioactivation of ethionamide, has not only allowed the identification of boosters of ETH activity, but has also led to compounds capable of derepressing a cryptic bioactivation pathway, whose awakening can erase acquired resistance to the drug. This discovery opens the way for the preclinical development of an optimized analogue in this series to treat patients infected with ETH resistance strains, especially those harbouring *ethA* mutation.⁷⁸ This work also paves the way for the identification of boosters of the MymA pathway, but also of other prodrugs bioactivation pathways such as isoniazid or nitroimidazoles like pretomanid or delamanid. This strategy, if successful, will contribute to the repelling of the advent of a dreaded post-antibiotics era.

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