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Small-molecule SMARt751 hypersensitizes *M. tuberculosis* resistant to ethionamide by upregulating the *mymA* operon

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Abstract: The sensitivity of *Mycobacterium tuberculosis* to prodrugs is dependent on the efficacy of the bioactivation process transforming them into the active antibacterial moieties. For the prodrug ethionamide (ETH), boosting its bioactivation has revealed a high potential to increase the sensitivity of the bacteria as well as to revert resistance by rerouting bioactivation pathways. Medicinal chemistry coupled to a highly focussed phenotypic assay selected an N-acylated 4-phenylpiperidine that was observed to interact with VirS and stimulate the expression of the *mymA* operon, recently described for its ability to bio-activate ETH. SMARt751, the front runner in this chemical series, is highly potent, shows prolonged ETH boosting effect in mice, and reverts resistance of *ethA*-mutated clinical strains. A model extrapolating animal pharmacokinetic and PD parameters to human predicts that as little as 25 mg of SMARt751 daily would allow a 4-fold reduction of ETH doses while keeping the same efficacy and reducing its side effects.

Introduction

The DOTS (Directly Observed Treatment Short Course) strategy launched by WHO in 1995 had, and continues to have, a substantial impact on the tuberculosis (TB) pandemic. Nonetheless, in its latest report, WHO estimates that in 2019, 10 million individuals developed the disease and 1.4 million died, including 208,000 within the HIV-positive population (*1*). Aside from this, the increasing number of bacteria resistant to the core antibiotics of the recommended anti-TB polytherapy is now putting the whole strategy at risk and is one of the major obstacles to the objective of "a world free from TB" envisioned by WHO in 2035 (*2*). Worldwide in 2019, close to half a million people developed rifampicin-resistant TB, of which 78% had multidrug-resistant TB (MDR-TB) (*1*). The efficacy of the treatment is around 60% for MDR-TB and can drop to 26% for extensively drug-resistant TB (XDR-TB) (e.g. MDR-TB with resistance to fluoroquinolones and one injectable aminoglycoside) (*3*). In this context, the *End TB Strategy* proposed by WHO includes the development of new universal treatments based on pan-active drugs, and the treatment of latent TB individuals, at least those at high risk of reactivation. The recent discovery of antibiotics such as bedaquiline, delamanid and pretomanid has now initiated the possibility to assemble completely new anti-tuberculosis regimens. Nevertheless, more candidates are needed to ensure that new efficacious and safe combinations can be developed.

Many anti-tuberculosis antibiotics are prodrugs that need to be transformed in active compounds by specific enzymes of the mycobacterium. This is the case for isoniazid, pyrazinamide, *p*-aminosalicylic acid, ethionamide (ETH), prothionamide, delamanid, pretomanid (reviewed in (*4*)), clofazimine (*5*) and TBA-354 (*6*).

We have shown that small molecules specifically developed to inhibit the transcriptional repressor EthR (Rv3855) boost the production of EthA (Rv3854c), which increases dramatically the bioactivation and thus the antibacterial activity of ETH (*7*-*17*).

During the last decade, analyses of clinical isolates resistant to ETH revealed the major implication of mutations in *ethA* (reviewed in (*18*)). Recently, by doing key modifications in the structure of BDM41906, one of our potent inhibitors of EthR (Fig. 1a), we identified compounds activating an alternative bioactivation pathway of ETH. SMARt420, the lead compound of this new chemical series, was shown to stimulate the expression of the putative oxidoreductase Rv0077c (named EthA2) by inhibiting the transcriptional repressor Rv0078 (named EthR2) (Fig. 1a). Consequently, SMARt420 reverts resistance to ETH for bacteria mutated in *ethA* (*19*).

Searching for dual inhibitor of EthR and EthR2, we designed ligands using the crystal structure of both proteins, followed by the selection of active compounds through phenotypic and functional assays. Unexpectedly, this approach identified small molecules controlling the expression of the VirS-MymA regulon, which has been recently described as able to bio-activate ETH (*20*). This new family of small molecules revealed to be highly effective ETH-boosters *in vitro* and *in vivo*, fully active against a broad panel of resistant clinical isolates, and showed encouraging safety margins in early preclinical screening studies with low risk of drug-drug-interactions.

Results

Rational design and optimization identify highly potent ETH-boosters *in vitro*.

Whereas previous ETH-boosters SMARt420 and BDM41906 shared common features (Fig. 1a), it was noteworthy that the former displayed only some residual binding to EthR and the latter showed no affinity for EthR2 (*19*). With the objective of targeting both regulators with one molecule, and based on the structure-activity relationships collected from the two different chemical series, we hypothesized that an N-trifluorobutyrylpiperidine motif substituted on the position 4 of the piperidine by a phenyl ring could accommodate the ligand-binding domain of both EthR and EthR2. Compound SMARt647 was designed and synthesized accordingly to these criteria (Fig. 1b and Scheme S1).

Affinities of SMARt647 for EthR and EthR2 were then compared by determining the denaturation temperature of these two proteins in complex with the small molecule. SMARt647 increased the thermostability of EthR by 3.5 °C and of EthR2 by 0.5 °C (Table). To measure the potency of SMARt647 to inhibit the binding of EthR and EthR2 with their cognate operator DNA, we used our previously described synthetic mammalian gene circuits translating Protein-DNA interactions in *SEAP* (SEcreted Alkaline Phosphatase) readout (*19*). In this functional test, whereas active in a dose-dependent manner, SMARt647 revealed to be less potent than BDM41906 and SMARt420 to inhibit the DNA binding of EthR and EthR2, respectively (Table 1 and Fig. S1).

Contrasting with these results, SMARt647 (EC_{50} = 11 nM) showed to be much more potent than SMARt420 (EC_{50} = 1.5 µM) to boost (by 10-fold) the activity of ETH against *M. tuberculosis in vitro* (Table 1).

With the objective to perform *in vivo* efficacy studies, we measured the *in vitro* microsomal stability of SMARt647 together with systemic exposure in mice. The microsomal study indicated that SMARt647 was rapidly metabolized to a less potent phenolic derivative (Clint (Intrinsic clearance) = 23.6 ml/min/g liver). Formulated in hydroxypropyl-beta-cyclodextrine (Kleptose HP; Roquette) and given orally at the dosage of 20 mg per kg of body

weight to C57BL/6 female mice, SMARt647 expectedly showed a relatively poor exposure (C_{max} = 40 ng/ml, AUC = 25.7 ng.h/ml) (Fig. S2). Liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis performed on blood samples from treated mice revealed later that the phase-1 phenolic metabolite was glucuronidated *in vivo*. Introduction of a fluorine atom at the metabolically unstable para-position of the phenyl ring of SMARt647 led to SMARt751 (Fig. 1b and Scheme S1), which showed highly improved metabolic stability on mouse microsomes (CLint = 0.74 ml/min/g liver [Fig. S3]).

The *in vitro* potency of SMARt751 was equivalent to the one of SMARt647, with a concentration of 10 nM being sufficient to boost 10-fold the activity of ETH against *M. tuberculosis* H37Rv. Similarly, 33 nM was enough to revert ETH-resistance for the *M. tuberculosis* strain E1, which is a spontaneous mutant of the Beijing strain W4 resistant to ETH due to a mutation in EthA (Rv3854-Gly343Ala).

The higher intrinsic metabolic stability of SMARt751 resulted in improved PK parameters in mice (Fig. S3). SMARt751 was rapidly absorbed following oral administration at the two doses of 0.1 and 1 mg per kg of body weight tested in C57BL/6 female mice. The maximum blood concentrations obtained at these two doses were 5.9 ng/ml (19.4 nM) and 84 ng/ml (278 nM), respectively. Importantly, administration of 1 mg per kg was sufficient to maintain a blood concentration above its *in vitro*-EC_{50} (10 nM) during more than 8 hours.

In summary, SMARt751 appeared to be a highly potent booster of ETH *in vitro* with physicochemical properties and metabolic stability permitting its evaluation in a tuberculosis mouse model.

A very low dose of SMARt751 boosts ETH in mice.

The *in vivo* efficacy of SMARt751 to boost ETH was first evaluated using a fast-acute mouse model of infection (*21*). This model, involving 8 days of treatment, allowed us to quickly test a large panel of combinations of SMARt751 and ETH. An ETH dose response curve was performed in absence or presence of different doses of SMARt751. The efficacy of the various combinations was compared using their respective ED99, that is, the dose eliminating at least 99% of the bacteria in the lungs (2 log colony forming units [CFU] reduction).

The ED99 and the 95 % Confidence Interval (CI95%) were calculated for each experimental condition. Doses of SMARt751 as low as 0.1 mg/kg were sufficient to obtain the maximum boosting effect. The ED99 of ETH shifted from 23 mg per kg (CI95% : 18-31 mg/kg) when used alone, to < 3 mg per kg when used in combination with 0.1 mg per kg of SMARt751 (Fig. 2). This concentration of 0.1 mg/kg was shown to be as effective as the highest dose used in this assay (10 mg/kg). In contrast, SMARt751 given alone showed no effect on the bacterial pulmonary load in mice treated with doses ranging from 0.001 to 1 mg per kg of body weight (Fig. S4). Although expected fluctuations from one mouse experiment to another were observed, ETH ED99 shifts were systematically obtained in 3 additional experiments using either 0.1 mg/kg or 10 mg/kg of SMARt751 (see Report S1).-

SMARt751 shows long-lasting boosting effect *in vitro* and *in vivo*.

To evaluated whether a continuous contact of the bacteria with SMARt751 is required to boost ETH, a culture of fluorescent *M. tuberculosis* was treated for 24 hours with 1 µM SMARt751, extensively washed, and finally submitted to phenotypic assay to measure how this pre-treatment affected its sensitivity to ETH. Figure 3a shows that pre-exposing *M. tuberculosis*

to SMARt751 during 24h, eliminating the compound and treating subsequently the culture with ETH for 5 days, was as efficient as exposing the bacteria to both ETH and SMARt751 during the same period of time.

Further, this pre-boosting effect was tested in our fast-acute tuberculosis mice model, in which TB-infected mice were treated over 8 days with ETH (10 mg per kg) on a daily basis, combined with doses of SMARt751 (1 mg per kg) spaced by 1, 2, or 4 days. Notably, administration of SMARt751 every 2 or 4 days with ETH daily was as efficient as a daily co-administration (Fig. 3b).

Based on the efficacy results and whole blood concentration of SMARt751 in TB-infected mice, we can conclude that maintaining a constant systemic exposure of the compound is not absolutely necessary to drive the boosting effect. This observation opens interesting and diverse therapeutic perspectives, such as the possibility, if needed, to treat patients less often with SMARt751 than with ETH.

SMARt751 efficiently boosts ETH in mice with chronic TB.

The potency of SMARt751 was then measured in a chronic mouse model of infection. C57BL/6 mice were infected with ~100 CFU of *M. tuberculosis H37Rv* and left untreated for 6 weeks, allowing the bacilli to reach a steady-state level of infection of $\sim 10^6$ bacteria per lung. Combinations of ETH and SMARt751 were then given orally for 8 weeks (daily, 7 times per week). In this situation, 0.3 mg per kg of SMARt751 was enough to shift the ED99 dose of ETH (Dose that eliminates 99% of the bacterial load) from 24 mg/kg (CI95%: 19-31 mg per kg) to 9.2 (CI95%: 7-12 mg/kg) (Fig. 4). Whereas fluctuations between replicated experiments were observed, comparable ETH ED99 shifts were obtained in 2 additional experiments using 10 mg/kg of SMARt751 (see Report S3).

Notably, the maximum effect of the dose-response (low plateau) observed with ETH alone was not different from the one obtained in the presence of SMARt751, illustrating that this compound has indeed no intrinsic antibacterial activity at doses shown in Fig.4, but instead boosts ETH up to its maximum potency.

To quantify the real amplitude of the boosting effect of SMARt751 at the various doses of ETH tested in mice, it was important to report the efficacy of ETH, not as a function of the dose, but rather as a function of the plasma exposure. Mice experiments showed that pre- or co-administration of SMARt751 had no influence on the pharmacokinetic (PK) profile of ETH (Fig. 5.A. and Report S4), confirming that the observed increase of ETH potency *in vivo* is indeed mediated by the boosting effect of SMARt751 and not by pharmacokinetic drug-drug interaction effects. Then, we determined whether the exposure of ETH was linear for doses ranging from 3.4 mg per kg to 90 mg per kg. Oral administration of ETH to mice revealed marked non-linear pharmacokinetics (Fig. 5.B.), in contrast to what has been described in human (*22*, *23*). Considering the relationship between dose and exposure (AUC = Dose x F /CL, where F is the oral bioavailability and CL is the blood clearance), the boosting effect of SMARt751 in mice was derived from the ratio $AUC_{ED99}/AUC^{B}_{ED99}$, where AUC_{ED99} and AUC^{B}_{ED99} are the exposure of ETH required to obtain 99% reduction of the bacterial load in the lungs, in the absence (AUC_{ED99}), or in the presence (AUC^{B}_{ED99}) of SMARt751, respectively. Based on our exposure data, ETH was boosted approximately 19-fold in the fast-acute mouse model (640 ng.h/ml, against 34 ng.h/ml) and 6-fold in the chronic model (640 ng.h/ml, against 105 ng.h/ml).

SMARt751 reverts resistance to ETH in mice.

The combination of ETH-SMARt751 in mice infected with bacteria resistant to ETH was also examined. In this assay, C57BL/6 mice were infected by intratracheal instillation with ~10^5 CFU of the *ethA*-mutated *M. tuberculosis* strain E1. Seven days after infection, the mice were treated with ETH, alone or in combination with SMARt751 at 6 mg per kg of body weight. Daily administration by gavage of up to 50 mg per kg of ETH alone for 21 days was ineffective in reducing the bacterial load in the lungs, confirming the strong resistance of the strain E1 to ETH.

In contrast, treatment with a combination of ETH (25 mg per kg of body weight) and SMARt751 (6 mg per kg of body weight) resulted in a substantial ~6.3 log reduction in CFU for this group, showing the strong bactericidal activity of the combination against this ETH resistant strain (Fig. 6 and Report S5). Notably, the efficacy of the combination ETH-SMARt751 was better than the efficacy observed with 25 mg per kg of body weight of isoniazid. No detectable effect was observed with SMARt751 administered alone, confirming that the anti-TB activity of the combination against these bacteria mutated in *ethA* is specifically due to the restoration of the ETH activity.

SMARt751 reverts resistance on clinical isolates.

Mechanisms of resistance to ETH are diverse, possibly including mutations in *ethA, ethR, inhA, mshA* (*24*). The potency of SMARt751 to revert ETH resistance was assessed on a panel of 37 ETH-resistant (ETH^r) *M. tuberculosis* clinical strains (MIC [Minimal inhibitory concentration] ≥ 4 µg/ml) selected for their diversity (7 lineages) and their profile of drug resistance (all clones are isoniazid resistant including 32 MDR, out of which 10 are pre-XDR and 3 are XDR). Notably, ten isolates are mutated in *ethA* and 12 in the promoter of *inhA* (Table S1).

Standardized respirometry experiments in MGIT-960 showed that for 33 out of the 37 ETH resistant isolates, 150 nM of SMARt751 was sufficient to revert their ETH MIC below or equal to 0.8 µg/ml. Fourteen strains were randomly chosen in this list of 33. For all of them, 10 nM of SMARt751 was sufficient to reduce the ETH MIC below 0.8 µg/ml (Table S1). One of the 37 clinical isolate highly resistant to ETH (≥256 µg/ml) required 300 nM of SMARt751 to shift its ETH MIC to 0.8 µg/ml. The three remaining isolates of the list of 37 recovered an ETH MIC of 2 µg/ml in the presence of SMARt751. The genome of these 3 clinical isolates were fully sequenced. Whereas the 3 sequences confirmed mutation in the *inhA* promoter and revealed nucleotide polymorphisms classically observed between clinical strains, no single nucleotide polymorphisms (SNPs) in genes described to be specifically involved in ETH resistance nor in the *mymA* regulon were identified. In summary, SMARt751 reverts resistance to ETH on all clinical isolates tested in this study, independently of their lineage or background of resistance to other antibiotics, including isoniazid.

Pharmacological data from preclinical species allow to estimate human PK parameters.

Physicochemical properties, *in vitro* ADME, and *in vivo* drug metabolism and PK profiles in preclinical species (mouse, rat, dog) were evaluated to support progression of SMARt751 and dose prediction modelling in human (Table S2). SMARt751 displayed satisfactory solubility (53–103 µg/ml) in physiologically relevant media. Permeability values across Caco-2 cell monolayers were high in both directions with no notable efflux. Therefore, neither solubility nor permeability is expected to limit oral absorption of SMARt751. The CLint

of SMARt751 was below the lower limits of quantification (LLOQ) of the method for both human microsomes and hepatocytes. Moderate plasma protein binding (PPB) and low blood-to-plasma partitioning ratios (B/P) were observed in human and preclinical species.

PK of SMARt751 after intravenous and oral administration at various doses were evaluated in rodents and dogs. The compound exhibited moderate to high CL, well predicted considering CLint in hepatocytes, and moderate to high volume of distribution (Table S3). Oral bioavailability was low in rats and moderate in mice and dogs, in agreement with expected first-pass effect.

Human PK parameters were estimated using a physiologically based PK model. This model accurately predicted (with only a 2-fold error) the experimentally observed PK profiles in preclinical species based on the corresponding *in vitro* ADME parameters. The prediction for human estimates a moderate CL (9 ml/min/kg), a high volume of distribution (7.8 l/kg), and a moderate oral bioavailability (50%).

Exposure at the target site of action over a desired period of time is fundamental to elicit the desired effect of any treatment. In the present boosting strategy, the desired effect required the presence of both ETH and SMARt751, although *in vitro* and *in vivo* data show a long-lasting boosting effect for SMARt751, suggesting that the active concentrations of the two drugs are not necessarily required to be reached in a synchronized manner. Ethionamide shows high values of permeability, solubility, and distribution, which allows it to cross the blood-brain barrier and to achieve concentrations in the brain and cerebral-spinal fluid equivalent to plasma. Similarly, SMARt751 displays high passive permeability and bioavailability. SMARt751 is not an *in vitro* substrate of the permeability glycoprotein (P-gp) given that the efflux ratio measured was similar in the absence and presence of P-gp inhibitors. These observations are consistent with *in vivo* data in preclinical species. In particular, the good *in vitro* permeability of SMARt751 translates to a high brain *in vivo* partition coefficient (Kp) in mice, with an estimated brain:blood ratio of 1.7-1.8 (Fig. S5). These values remained unchanged after pre-treatment with P-gp inhibitor elacridar (*25*). Altogether, these data support a perfusion-limited physiologically based PK model for the SMARt751 distribution, inferring that it is unlikely that organs exposed to ETH are not similarly exposed to SMARt751.

Modelling of the efficacy of the ETH-SMARt751 combination in human predicts high therapeutic benefits.

The efficacy measurements from the acute and chronic TB-mice assays presented above, together with the PK of ETH and ADME properties of SMARt751, have been used to estimate a set of pharmacodynamic (PD) parameters in mice (*26*). The translation/scaling of these parameters from mice to human assumes that first, the growth rates and maximum killing rates are the same in both species, and second, the unbound drug concentration in plasma is the driver of both ETH and SMARt751 efficacy. Considering the scaled PD parameters and the PK parameters distributions of a human adult cohort (*22*), the Early Bactericidal Activity in the 14 first days of treatment ($EBA_{0\text{-}14}$) performed during Phase-2 was simulated to see the effect of SMARt751 on 3 different ETH daily doses (250 mg, 500 mg, and 750 mg) (Fig. 7). The simulations indicated that as little as 25 mg of SMARt751 daily (0.35 ± 0.1 mg per kg of body weight) would drive a 4-fold reduction of any of the ETH doses, while keeping the same respective $EBA_{0\text{-}14}$ performance. (See Report S6 for details).

Drug-Drug interactions and safety of SMARt751

Taking into account that SMARt751 will have to be administered not only with ETH but also in combination with other antitubercular compounds, drug-drug interactions liabilities have been evaluated. The potential of SMARt751 to directly inhibit cytochrome P450 (CYP) isoforms 1A2, 2C9, 2C19, 2D6, and 3A4 was evaluated in human liver microsomes with specific probe substrates. No inhibition was observed at concentrations up to 25 µM for all isoforms tested, with the exception of 2C19 (IC_{50} = 2.1 µM). Complementary studies on CYP3A4 and CYP2C19 ruled out any time- or metabolism-dependent inhibition of SMARt751. CYP induction potential of SMARt751 was carried out in cultured human hepatocytes. The increase of expression of CYP1A2, CYP2D6, and CYP3A4 mRNA was lower than 2-fold for all the concentrations tested (up to 10 µM) (Report S7). In conclusion, considering the expected blood concentrations of SMARt751 in human (C_{max}=23.5 ng/ml; 0.08 µM), our results based on static models suggest a low risk of SMARt751 perpetrating drug interactions or perturbing the PK of co-administered drugs via these mechanisms.

In vitro safety pharmacology studies identified hERG inhibition with an IC_{50} of 27 µM, although in a subsequent study using the *ex vivo* Rabbit Ventricular Wedge (RVW) model at 10, 30 and 100 µM, QT shortening (up to 38%) was observed from 10 µM and a decrease in Tp-e interval at the high dose of 100 µM. Based on these results, the risk of arrhythmia in humans with SMARt751 is considered low at the expected therapeutic dosage (*27*).

The potential off-target pharmacological activity of SMARt751 was also assessed in an enhanced cross-screen profiling panel of assays representing 50 human pharmacological targets covering ion channels, transporters, enzymes, and phenotypic assays. No activity of SMARt751 with an XC_{50} of less than 1 µM was detected in any of these assays. Whereas evaluation identified activity on 2 specific assays (GABA-A antagonism and PXR), follow-up studies concluded a low risk of secondary effect at expected therapeutic dosages for both hits, as described in Report S7.

Finally, preliminary toxicity studies of SMARt751 were carried out in mice using an oral repeat-dose protocol. No acute toxicity nor noticeable findings were observed after 4 days at 20 mg per kg per day, which corresponds to 60 times the observed therapeutic dose in mice. The findings observed at higher doses in the liver at 60 and 200 mg per kg per day are described in the supplementary material. Altogether, and based on our clinical dose projections in human, the preliminary safety profile supports continued progression to further preclinical studies.

SMARt751 boosts ETH activation by controlling the *mymA* operon.

Selection of spontaneous resistant mutants is an effective way to gain insight into the mode of action of small molecules and may help to develop molecular tools to monitor resistance during clinical trials and later clinical use. The minimal ETH concentration that allowed for the selection of resistant mutants in the absence of SMARt751 was 10 µg/ml (around 5-fold the MIC). Under this ETH concentration, spontaneous mutants appeared at a frequency of $4x10^{-8}$. Strikingly, no mutant could be obtained by plating 10^8 bacteria on solid medium containing as low as 2 µg/ml of ETH in combination with 0.15 µM of SMARt751. Combinations of ETH 0.5 µg/ml and SMARt751 at 0.64 µM, 0.32 µM, and 0.15 µM allowed for the selection of mutants at a frequency of $6x10^{-8}$, $4x10^{-7}$, and $3x10^{-6}$, respectively.

Nine mutants were selected for whole-genome sequencing. A relatively complex pattern of mutations was observed in these clones. Strikingly, all mutants harboured mutations in at least one gene of the regulon *virS-mymA* (Table S4). Mutations in *ethA* were also observed in 8 of the

9 clones. More precisely, in addition to their mutation in *ethA*, four of the 8 clones had non-synonymous SNPs in *Rv3083* (*mymA*), one had a SNP in *Rv3082c* (*virS*) and three had a 3.3 kb deletion covering the end of *rv3080* (*pknK*), *rv3081*, *virS* and the beginning of *mymA*. MymA is a close homologue of the Baeyer-Villiger monooxygenase EthA and has recently been shown to bioactivate thioamides (*20*). Loss of MymA function was also shown to confer some resistance to ETH, additively to the resistance associated to EthA loss of function. To rule out the involvement of other mutations identified in the nine spontaneous mutants, we tested the phenotype of *mymA*, *virS* and *ethA* mutants independently obtained and kindly provided by Dr Hung (*20*). The first clone (Rv3083::TN), which contains a transposon in *mymA*, confirmed the inability of SMARt751 to boost ETH in this context (Table S4). An equivalent phenotype was observed for clone 3-RM4, which contains a frame shift in the C-terminus portion of VirS (Table S4). Finally, the third clone, containing a transposon in EthA (Rv3854c::TN) was shown to be resistant to ETH, but sensitive to the SMARt751-ETH combination (Table S4). In summary, these data show that resistance to ETH-SMARt751 can arise either from simultaneous mutations in *ethA* and in the *mymA* regulon, or more rarely by single mutations in this regulon. In particular, the clone mutated in *virS* suggests that SMARt751 can control the expression of *mymA* through interaction with VirS, which has been described as the transcriptional regulator controlling the expression of the *mymA* operon (*28*).

To gain knowledge on the mechanism of action of SMARt751, we analysed the transcriptomic profile of *M. tuberculosis* exposed to 10 µM of this compound during 24 hours. Bacteria treated with SMARt751 showed 12 genes more than 3-fold up-regulated (Fig. 8a). The most overexpressed mRNAs correspond to the *mymA* operon (*rv3083 to rv3089*). These data, together with the identification of *mymA* mutants resistant to the combination SMARt751-ETH, suggest that this operon is a central player in the reversion of ETH resistance induced by SMARt751. Interestingly, no modification of the transcription level of *ethA* and *ethR* was detected, which is in agreement with the modest thermostabilisation of EthR by SMARt751 that was observed by thermal shift assay (3.5°C instead of 8.5°C by BDM41906). Notably, *rv0077c*, the gene corresponding to the oxidoreductase EthA2, was also overexpressed in the presence of SMARt751, highlighting the complexity of the mechanisms possibly involved in ETH activation (*19*) (Table S5).

To refine this picture, we quantified the modifications of the global proteome of *M. tuberculosis* upon treatment with SMARt751. Total protein extracts of two independent cultures of *M. tuberculosis* treated with 1 µM of SMARt751 or with DMSO during 72h were quantified by isobaric labelling-based mass spectroscopy. Approximately 2200 proteins were identified. Treatment of the bacteria with SMARt751 led to the modification of the production of about 19 proteins.

Figure 8a lists the proteins for which the production is increased or decreased more than 2-fold. Strikingly, all the proteins of the MymA-VirS regulon were overproduced upon treatment of the bacteria with SMARt751, in agreement with the effect of this compound observed in transcriptomic experiments. In contrast and surprisingly, the production of EthA and EthR was shown to be diminished in these conditions, whereas no obvious regulation upon treatment with SMARt751 had been observed for these genes at the transcription level.

As described above, *virS* mutants emerged by selecting clones spontaneously resistant to the ETH – SMARt751 combination. VirS (Rv3082c) has been shown to be a major regulator of the *mymA* operon. To study the possible interaction between SMARt751 and VirS, we first

adapted the synthetic mammalian reporter system already presented above. In this assay, binding of VirS to a chimeric DNA that includes the intergenic region of the *virS-mymA* operon fused to the *hCMVmin* promoter is expected to lead to the expression of downstream *SEAP* reporter gene. In the absence of SMARt751, we observed a strong production of SEAP, confirming the binding of VirS to the *mymA* promoter in this context. Addition of SMARt751 led to a strong inhibition of SEAP production, showing that SMARt751 modulates the binding function of VirS on its DNA operator (Fig. S6.). Alternatively, the thermostability of purified VirS was measured in the absence and in the presence of SMARt751. Thermal-shift assay showed a strong dose-dependent thermo-stabilizing effect of SMARt751 on VirS of up to 8°C (Fig. 8b). A full stabilisation of the protein was reached in the presence of approximately 2 molar-equivalent of SMARt751, revealing a high-affinity couple (Fig. 8c).

Discussion

Isoniazid, targeting the mycobacterial enoyl-reductase InhA, is one of the most potent TB drugs. Unfortunately, the progressive accumulation in the clinical field of isoniazid-resistant *M. tuberculosis* isolates seriously compromises the future of this antibiotic. Resistance to isoniazid is predominantly due to mutations in KatG, the catalase-peroxidase involved in the bio-activation of this pro-drug. As inhibiting mycobacterial enoyl reductase InhA is one of the most effective means of killing *M. tuberculosis*, important efforts have been developed to replace isoniazid by direct InhA inhibitors, but to date no orally active candidate has reached clinical phase II. An alternative to this approach would be the use of ETH in replacement of isoniazid. ETH is a prodrug also targeting InhA. In contrast to isoniazid, the bio-activation of ETH in *M. tuberculosis* is independent of KatG, making this antibiotic fully active against isoniazid-resistant clinical isolates mutated in the *katG* gene. In combination with moxifloxacine, ETH has been pointed as an essential asset for the successful treatment of MDR-TB (*29*). In its later consolidated guidelines, WHO recommended the inclusion of ETH in an all-oral shorter regimen for the treatment of MDR-TB where the injectable aminoglycoside agent is replaced by bedaquiline (*30*). Nevertheless, ETH has a less favourable therapeutic index than isoniazid, as the dose required to inhibit *M. tuberculosis* growth sometimes causes adverse effects such as gastrointestinal disorders, hepatitis, and more rarely various mental disturbances (*31*, *32*).

For a long time, it was assumed that the bioactivation of ETH was only achieved by the Baeyer-Villiger monooxygenase EthA (*33-35*), leading to the formation of a stable ETH-NAD adduct inhibiting InhA (*36, 37*). As the production of EthA is regulated by the TetR-type transcriptional repressor EthR (*38*), we and others have successfully developed small molecule inhibitors of EthR to stimulate the transcription of *ethA* (*7-9, 12, 16*), improve the bio-activation of ETH, and consequently boost its antibiotic activity (*13*). Recently, two additional regulons have been described to be able to participate to the bioactivation of ETH, the regulon *Rv0077-Rv0078* (*ethA2-ethR2*), and the regulon *virS-mymA* coding Rv3083 to Rv3089. Our group has shown that the small molecule SMARt420 inhibits the transcriptional repressor Rv0078, stimulates the expression of the oxidoreductase Rv0077c and restores the bioactivation of ETH in the clinical strains mutated in EthA. Here, we identified and developed a new series of compounds that stimulate the expression of the *mymA* operon. Notably, SMARt751, the best representative of this new generation of boosters, showed high potency to boost the activity of ETH against a large panel of MDR and XDR clinical isolates, including bacteria mutated in *ethA* or in the promotor of *inhA*. Transcriptomics and quantitative proteomics showed that SMARt751

stimulated expression of all the genes and production of all the respective proteins of the *mymA* operon. Moreover, SMARt751 also causes upregulation of the *virS* gene, which leads to a marked overproduction of the VirS protein. The direct interaction between VirS and SMARt751 *in vitro* was shown by thermal-shift assay (Fig. 8b, c). By using a mammalian reporter assay eliminating risks of interactions of VirS with other mycobacterial proteins, we then showed that the binding of VirS to the intergenic region between *virS* and *mymA* was impaired in the presence of SMARt751.

These data suggest that the interaction of SMARt751 with VirS modulates its regulator function by impairing its DNA binding capacity. At the same time, treatment of bacteria with SMARt751 resulted in the overexpression of the *mymA* operon. In the light of previous reports showing that VirS acts as a transcriptional activator of *mymA* (*28*), the precise mechanism by which SMARt751 stimulates the expression of both *mymA* and *virS* remains to be elucidated.

Selection of mutants resistant to the ETH+SMARt751 combination pointed not only to MymA, and VirS, but also to Rv3080 (PknK), which has been shown to modulate the expression of the *mymA* operon through phosphorylation of VirS (*39*). Interestingly, it has been reported that functional loss of *mymA* resulted in reduced ability to persist specifically in the spleen of infected guinea pigs(*40*) This could suggest that appearance of mutations inactivating the effect of SMARt751 would be counter-selected in the clinical field because of their impact on bacterial virulence. As observed in transcriptomic data, SMARt751 also stimulated the expression of Rv0077c (EthA2), meaning that this enzyme could participate in the boosting of ETH+SMARt751 and to the reversion of resistance.

Altogether SMARt751 harbours many major assets. The mechanism by which it boosts the activity of ETH allows to circumvent the most frequent mechanism of ETH-resistance found in clinical strains, including mutations in *ethA*, in *ethR* promoter, as well as in *inhA* promoter. Some attention will however be paid onto strains of the particular *M. tuberculosis* sub-lineage 4.8 that harbour a large deletion covering the VirS-MymA regulon (*41*). Treating chronically infected mice with an oral dose of 1 mg per kg of SMARt751 allowed a 6-fold reduction in the effective plasma concentration of ETH. Furthermore, the observed efficacy of the ETH-SMARt751 combination against ETH-resistant TB in mice highlights the therapeutic potential of this combination to revert ETH resistance in the clinical field. The long-lasting boosting effect of SMARt751 observed *in vitro* and *in vivo* may uncover the high potential of targeting transcriptional mechanisms and opens interesting avenues for the administration of such boosters in humans. SMARt751 has shown no effect on the viability of the human cell line (HepG2), nor *in vitro* genetic toxicity and a low risk for arrhythmia. Effects on the liver in mice were recorded at doses higher than 20 mg per kg, suggesting reasonable safety margins with respect to expected PK parameters in man. Despite its high lipophilicity, SMARt751 displayed favourable PK properties following oral administration in mice and dogs. High oral *in vivo* doses (up to 200 mg/kg) were used in rat to counteract the expected first pass effect and achieve C_{max} and AUC values that would support evaluation of the safety window. No inhibition nor induction of the major CYP450 isoenzymes was observed in the preliminary study carried out in human microsomes and hepatocytes in the range of tested concentrations of SMARt751, suggesting low risk of drug-drug interactions via these mechanisms, which is an important parameter in the context of the TB poly-therapy and sometimes anti-retroviral co-therapy. Regarding scalability aspects, SMARt751 is a crystalline and low molecular weight molecule that can be prepared using a short and straightforward synthetic route. Finally, SMARt751 also shows good

cerebrospinal fluid penetration. As ETH is recommended in the treatment of drug-susceptible tuberculous meningitis (*42*), the ETH-SMARt751 combination may open interesting clinical perspectives to reduce side effects in the treatment of this deadliest form of tuberculosis.

Translating PK and PD data from *in vitro* and animal models to human efficacious dose prediction remains a challenge, obviously more complex when considering combination of compounds. The new physiologically based PK (PBPK) model used here integrates the efficacy measurements of the combination ETH-SMARt751 in mice, the biotransformation and PK of both compounds, and translates these parameters to human. The model predicts that as little as 25 mg per day (around 0.35 mg per kg) of SMARt751 would be sufficient to cut the dose of ETH in human by 4-fold. Based on clinical data, this would massively improve ETH tolerability and allow consideration of this combination as an ideal mycolic acid biosynthesis inhibitor, not only in second line treatment but also as a possible substitute for isoniazid within a new first line regimen.

Materials and Methods

Study design

The objective of the work was to develop, up to preclinical stage, a small molecule that increases the sensitivity of *M. tuberculosis* to ETH as well as to revert resistance by rerouting bioactivation pathways of this antibiotic. The structure of the rationally-designed molecules synthesized and recrystalized in this study were controlled by NMR. Thermostabilisation of the putative targets Rv3855 (EthR), Rv0078 (EthR2), VirS by our synthetic compounds was measured by TSA. The ability of our small molecules to interfere with the binding of these 3 transcriptional regulators on their cognate DNA operator was measured using a mammalian reporter assay. The ETH-boosting effect of the small molecules was quantified both on axenic cultures of ETH-sensitive and ETH-resistant *M. tuberculosis-GFP*, and on infected macrophages. Chemical modifications performed to improve the metabolic stability led to SMARt751. Physicochemical properties, *in vitro* ADME, *in vivo* drug metabolism and PK profile of SMARt751 were studied in 3 preclinical species (mouse, rat, dog). Cytochrome P450 interactions and *in vitro* ADME were also studied in *in vitro* human assays. Preliminary assessment of the safety of SMARt751 was investigated in safety pharmacology studies (hERG Inhibition, off-target activity on 50 human targets, RVW, rat EEG and behavioural endpoints on *in vivo* studies), genetic toxicology (screening AMES and MLA) and an *in vivo* general toxicity study in mice. Animal experiments were performed in a fast-acute, an acute, and a chronic mice models of tuberculosis. Selection of spontaneous resistant bacteria, transcriptomic and proteomic experiments confirmed the central role of the VirS-MymA operon in the boosting effect of SMARt751. Mice were purchased from the vendor and randomly assigned to treatment groups. Antibiotics were prepared and administered in a nonblinded fashion, both in vitro and in vivo. **Fig. 2** shows a dose response experiment (one mouse per dose level) that is representative of 4 independent experiments detailed in Report S1. **Fig.3.A.** Each point on the curves are the average of 6 replicates.**Fig. 3.B.** This experiment included minimum 2 mice per point detailled in Report S2. **Fig. 4.** shows a dose-response experiment including 1 to 2 mice per dose that is representative of 3 independent experiments detailed in Report S3. **Fig. 5.** These experiments included 3 mice per group. **Fig. 6**. This experiment included 4 to 5 mice per group. **Fig. 8.** Experiments A. and B. included respectively 3 and 4 replicates per conditions.

M. tuberculosis ethA^{-} strain E1 and *M. tuberculosis* H37Rv-GFP

These strains are described in Supplementary Materials and Methods (*43*).

Thermal Shift Assay on Rv0078, Rv3855, VirS

The recombinant *M. tuberculosis* proteins EthR2 (Rv0078) and EthR (Rv3855) were purified as described previously (*13, 19*). Recombinant VirS was expressed and purified based on the protocol described by Singh et al. (*44*).The fluorescent dye SYPRO® Orange (SigmaAldrich) was used to monitor protein unfolding. See Supplementary Materials and Methods for additional details.

Mammalian SEAP reporter assays

Construction of EthR and EthR2 chimeric mammalian transcriptional regulators as well as the corresponding *ethR* and *ethR2* DNA operator fused to the human cytomegalovirus derived promotor ($P_{hCMVmin}$) have been described previously (*19*). A similar strategy was used to adapt

this assay to report on the binding of VirS onto its DNA operator. The method is described in the legend of the Fig. S6.

Extracellular potency assay on *M. tuberculosis*

Ethionamide (ETH, Sigma, E6005-5G) was diluted in DMSO at 0.1 mg/ml, and aliquots were stored frozen at −20 °C. Test-compounds (booster candidates) were resuspended in DMSO at a concentration of 10 µM. ETH and test-compounds were transferred to a 384-well low-volume polypropylene plate (Corning, no. 3672) and used to prepare assay plates. On the day of the experiment, 50 µl of a culture of either *M. tuberculosis*-GFP H37Rv or *M. tuberculosis* E1-GFP (*ethA*$^{-}$) (OD_{600nm} = 0.02) was transferred to each assay plate and incubated at 37ºC for 5 days. The fluorescence intensity was measured at Ex/Em = 485/535 nm using a *Victor Multilabel Plate Reader* (PerkinElmer). See Supplementary Materials and Methods for additional details.

Long-lasting effect of SMARt751 *in vitro*

H37Rv-GFP, was diluted to an $OD_{600\ nm}$ of 0.1 and treated with 0.3 µM SMARt751 24 hours before adding to assay plates containing DRC of ETH or DRC of ETH+0.3 µM SMARt751. H37Rv-GFP was treated with DMSO and added to a DRC of ETH as control. Pre-boosted bacteria were centrifuged, washed twice with PBS, diluted in fresh culture medium to an $OD_{600\ nm}$, and transferred (50 µl) to assay plate, which were incubated at 37ºC for 5 days. The fluorescence intensity was measured at Ex/Em = 485/535 nm using a *Victor Multilabel Plate Reader* (PerkinElmer). Microplates containing compounds dilutions were prepared as described in the paragraph entitled “Extracellular potency assay on *M. tuberculosis*”.

Long-lasting effect *in vivo*

This experiment was carried out similarly to the one described in the “*in vivo* mice fast-acute assay” except that ETH was administered on a “everyday schedule”, while SMARt751 was given either every day, every 2 days (on day 1, 3, 5, and 7 post infection), or every 4 days (on day 1 and 5 post infection).

***in vivo* mice chronic assay**

The effect of SMARt751 on the efficacy of ETH against *M. tuberculosis* was evaluated in a chronic murine model of intratracheal infection as previously described (*19*). See Report S3 for details and statistical data.

***in vivo* mice fast-acute assay**

Experiments were carried out as previously described (*21*). See Report S1 for a description of experimental conditions, groups and statistical data.

Efficacy study in mice infected with the ETH-resistant *M. tuberculosis* strain E1.

One week after infection (with ~10^5 mycobacteria), either with strain H37Rv (control) or with strain E1, the mice (C57BL/6) were treated 7 days per week for 3 weeks with the doses of ETH and SMARt751 indicated in Report S5.

Solubility of Solid Compounds in Simulated Gastric Fluid (SGF), Fasted Simulated Intestinal Fluid (FaSSIF) and Fed Simulated Intestinal Fluid (FeSSIF).

This experiment determines the solubility of solid compounds in simulated gastric fluid (SGF) at pH = 1.2, fasted simulated intestinal fluid (FaSSIF) at pH 6.5, and fed simulated intestinal fluid (FeSSIF) at pH = 6.5. See Supplementary Materials and Methods for additional details.

***In Vitro* Investigation of the Passive Membrane Permeability in human Caco-2 cells at pH7.4**

This assay was designed to determine passive cellular permeability (Papp) by using Caco-2 cell line (CacoReady cells). See Supplementary Materials and Methods for a description of the experiment.

Intrinsic clearance, plasma protein binding, and blood to plasma ratio.

These experiments are described in Supplementary Materials and Methods.

***In vitro* CYP inhibition in human liver microsomes**

The objective of this study was to evaluate the direct inhibition potential of SMARt751 on CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 in human liver microsomes under established incubation conditions using probe substrates and controls for each CYP enzyme and LC-MS/MS based assays. The study was conducted in human liver microsomes. See Supplementary Materials and Methods for a full description of the experiment.

Cytochrome P450 Induction Potential of the Test Compound SMARt751

Cryopreserved human hepatocytes were supplied by Triangle Research Laboratories and seeded according to the supplier's protocol in supplemented Williams E medium with serum. The hepatocytes were seeded on a 96-well collagen coated plate at a density of 1×10^6 hepatocytes/ml (100 µl per well). The plate was incubated at 37°C for 4 hours before the seeding medium was replaced with 100 µl of supplemented Williams E medium without serum. The cells were cultured further for 24 hours before addition of the test compound in serum-free culture media (final test compound concentrations 10, 4, 1, 0.4, 0.1, 0.04 µM). Positive control inducers (omeprazole for CYP1A2, phenobarbital for CYP2B6 and rifampicin for CYP3A4) were incubated alongside the test compounds. Negative control wells were also included where the test compound or positive control inducer was replaced by vehicle alone (0.1 % DMSO in Williams E media for positive controls and test compound). The experiment was performed in triplicate. Upon completion of the 72-hour dosing period, the hepatocytes were washed twice with Williams E media. The hepatocytes were then lysed and the lysate was stored at -80°C until the qRT-PCR assay was performed. Total RNA was extracted from lysate, RNA quality and quantity were assessed and reverse transcription was performed using the high capacity RNA-to-cDNA kit (Applied Biosystems) and Veriti 96 well Thermal Cycler (Applied Biosystems). Quantitative PCR analysis was performed on cDNA, using Applied Biosystems designed TaqMan gene expression assays for the target genes CYP1A2, CYP2B6, CYP3A4 and endogenous control (PPIA). Samples were analysed using an ABI 7900 HT real time PCR system. Relative fold mRNA expression level of the target genes was determined based on the threshold cycle (CT) data of target gene relative to endogenous control for each reaction (Δ CT), normalised to the negative control (ΔΔ CT). Fold induction was calculated using the 2-ΔΔCt method. To determine whether the level of relative cytochrome P450 isoform mRNA expression was statistically different in the test compound samples compared to the appropriate negative

controls, a one-way ANOVA with two-tailed Dunnetts post-test was performed. A p-value of less than 0.05 was considered as the threshold for significance.

In vivo PK studies

The PK and oral bioavailability of SMARt751 were investigated in mouse, rat and dog following single intravenous (i.v.), and oral administration. To support efficacy studies, oral PK studies were also performed in female mice C57BL/6, same strain used for efficacy model. In order to investigate in vivo CL and volume of distribution (Vss), intravenous PK studies were conducted in male CD-1 mice, male SD rats and male beagle dogs. 3 animals per group were used for each study performed. A dose of 1 mg/kg was administered intravenously, in a bolus form to mice and infused over a 30 min period to rats and dogs. For all the intravenous studies compound was dissolved in 5% DMSO/20% Encapsin™ in saline. A dose of 5 mg/kg was orally administered by gavage to the rodent species and a dose of 3 mg/kg was orally dosed to the dogs. In all the studies, SMARt751 was formulated as a suspension of 1% methylcellulose to investigate oral PK and to estimate oral bioavailability. Peripheral blood samples were obtained at 5, 15, 30 min, 1, 2, 4, 6 ,8 and 24 h after intravenous administration in mice, 0.25, 0.5 (before end of infusion), 0.58, 0.75, 1, 1.5, 2, 3, 5, 7 and 24 h after intravenous dosing in rats and dogs and 0.25, 0.5, 0.75, 1, 2, 4, 6, 8 and 24 h after oral administration for all the species. Blood was 1/2 diluted with milliQ water and immediately frozen on dry ice until analysis. Quantification was performed by means of LC-MS/MS (API4000), with a lower limit of quantification of 1 ng/ml. PK parameters, namely CL, volume of distribution at steady state (Vss) and bioavailability (F%), were estimated using Phoenix 64 (Pharsight, Certara).

Human dose projection for Phase 2 EBA $_{0\text{-}14}$

The human dose projections of SMARt751 and its estimated effect on the ETH dose reduction presented in this paper are based on the semi-mechanistic mathematical PD model for TB established in Muliaditan *et al.* (*26*). A full description of the model and computations is available in Report S6.

Chromosomic DNA sequencing

Preparation of chromosomic DNA. H37Rv spontaneous mutants to ETH-SMARt751 combinations were grown at 37ºC until a saturated culture was obtained. 1 ml of saturated culture of each mutant was heat-inactivated 30 min at 95°C. Total genomic DNA was extracted using MasterPure DNA Purification kit (EpicentreBio, Cat nº MCD85201), according to manufacturer's instructions. Briefly, bacterial pellets were collected by centrifugation Supernatant were discarded and 25 µl of water was added. Pellets were vortexed briefly for 10 sec. Then, 300 µl of Lysis Solution containing Proteinase K was added and samples were mixed thoroughly. Samples were incubated at 65°C for 15 min with a brief vortex-mix every 5 min. Samples were cooled to 37ºC and 1 µl of a 5 µg/µl RNaseA solution was added to each sample, which were mixed thoroughly and incubated at 37°C for 30 min. Samples were cooled on ice for 5 min. DNA was precipitated by adding 175 µl of MPC Protein Precipitation Reagent (Epicentre) to 300 µl of lysed sample followed by vortexing for 10 seconds. Debris were pelleted by centrifugation at 4°C for 10 min at ≥10,000 x g. Supernatants were transferred to clean microcentrifuge tubes to which 500 µl of isopropanol was added. The tubes were inverted several times to mix well the two solutions. The DNA was collected by centrifugation at 4°C for 10 min at ≥10,000 x g and by carefully discarding the isopropanol. The DNA pellet was rinsed twice with 70% ethanol, resuspended in 35 µl of TE Buffer. DNA was quantified by Qubit dsDNA HS

Assay kit (ThermoFisher Scientific) and send to Fisabio (Valencia, Spain) for library preparation and whole genome sequencing.

Transcriptomic analysis

M. tuberculosis H37Rv was grown at 37 °C in 60 ml 7H9 supplemented with 2% (w/v) glucose and 0.025% (v/v) tyloxapol (Sigma) to an OD_{600nm} of 0.65. Ten 10 ml of culture was treated with each SMARt751 or DMSO to a final concentration of 10 µM or equivalent amount of DMSO. This procedure was done in triplicate. Incubation was continued for 24 hours at 37 °C. Mycobacteria were harvested by 10 min centrifugation at 5,000 × *g* at 4 °C, resuspended in 1 ml of RNApro™ (FastRNA Pro Blue Kit, MP biomedicals) and homogenized in impact-resistant 2 ml tubes containing 0.1 mm silica spheres (Lysing Matrix B, MP biomedicals) using a FastPrep FP120 cell disrupter (Thermo Fisher Scientific) at 6.0 Hz for 40 sec. The ribolysed cells were centrifuged at 12,000 × *g* to remove cellular debris and RNA was purified following the manufacturer's instructions. Ribosomal RNA(rRNA) depletion was performed using QIAseq FastSelect –5S/16S/23S Kits; Qiagen). Libraries for Illumina sequencing were prepared with the TruSeq RNA sample preparation kit version 2.0 rev. A (Illumina Inc.). All cDNA libraries were uniquely indexed. cDNA libraries were sequenced using an Illumina NextSeq 500 system (Illumina Inc.) in high-output mode. All samples were multiplexed on one lane of the flow cell and sequenced in single-read sequencing mode with reads lengths of 150 bp. Raw RNA-seq reads were processed with Illumina quality control tools using default settings. Sequences shorter than 50 bp and/or that contained any 'Ns' and/or with a mean quality score lower than 30 were removed using PRINSEQ (http://prinseq.sourceforge.net/index.html). Next, rRNA-specific reads were filtered out by mapping all the reads on *M. tuberculosis* rRNAs sequences using Bowtie2 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml). Analysis of the RNA sequencing data was conducted using the SPARTA open source software package with default parameters (https://sparta.readthedocs.io/en/latest/).

Proteomics

Protein extraction: M. tuberculosis H37Rv was cultured in 7H9 medium without glycerol and supplemented with 2% (w/v) glucose and 0.025% (v/v) tyloxapol at 37 °C for about 7 days until an $OD_{600\ nm}$ of 0.8 was reached. The culture was diluted to an $OD_{600\ nm}$ of 0.2, split in 3 subcultures. Two of them were further incubated for 72 h in the presence of 1 µM SMARt751 and the third one incubated with DMSO. Protein extracts were prepared by centrifuging the culture and washing the pellet with PBS before resuspending in 1 ml lysis buffer (50 mM Tris-HCl, pH 7.4, 0.8 (v/v)% NP40, 1.5 mM $MgCl_2$, 5% glycerol, 150 mM NaCl, 25 mM NaF, 1 mM Na_3VO_4, 1 mM dithiothreitol (DTT) and one Complete EDTA-free protease inhibitor tablet (Roche)), which was then disrupted using a TissueLyser II (Qiagen) for three cycles at full amplitude for 5 min in refrigerated supports. Bacterial lysates were centrifuged at 14,000 × *g* for 30 min and the supernatant filtered using Millex LG (PTFE) 0.2 µm/13 mm diameter. Filtered supernatants were further ultracentrifuged at 4 °C for 60 min (140,000 × *g*). Samples were prepared in quadruplicate.

Chemoproteomics methods: SDS sample buffer (2% SDS, 10% glycerol, 0.005% bromophenol blue, 100mM Tris HCl, 125mM Tris Base) was added to 37.5 µl of the *M. tuberculosis* protein extract. The contained proteins were digested according to a modified single pot solid-phase sample preparation (SP3) protocol (*45, 46*) described in Method S1.

Genetic and general toxicology, safety pharmacology.

Ames(*47*) and Mouse Lymphoma (MLA) mutagenicity assays (*48*), the Human Ether-a-go-go-related Gene (hERG) assay (*47*), QT interval evaluation (*49*) and the arterially perfused rabbit left ventricular wedge assay (RVW) (*49*) were performed as described previously.

Rat EEG was used to evaluate the activity SMARt751 after a single administration at 100 mg per kg of body weight and 300 mg per kg of body weight and after the repeated administration, over 7 days, at 300 mg per kg of body weight, on the electroencephalographic (EEG) parameters by EEG trace monitoring in male Crl:WI(Han) rats (*50*).

Mouse 4-day toxicology of SMARt751 was evaluated on male Crl:CD1(ICR) mice (6/group) at doses of 0 (vehicle), 20, 60 or 200 mg per kg of body weight/day once daily for 4 days by oral gavage at a dose volume of 10 ml per kg of body weight. Six additional males were added to the 60 mg per kg of body weight/day dose group and were dosed for four days to assess brain concentration compared to blood concentration. The following endpoints/parameters were evaluated: clinical observations, body weights, limited clinical chemistry results, organ weights (liver including gall bladder, brain, heart, kidneys and testes) and macroscopic and microscopic observations (heart, kidneys, mesenteric lymph node, skeletal muscle, testes, liver and gall bladder). Toxicokinetic (composite) evaluation was performed on samples collected from SMARt751 dosed animals on Days 1 and 4. Brain tissue and blood samples from animals given 60 mg per kg of body weight/day were analysed for SMARt751 concentration on Day 4 (3 males/time point at 0.5, 3 and 24 hours after dosing).

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Figure captions

Fig. 1. Structures. (**A**) BDM41906 and SMARt420. (**B**) SMARt647 and SMARt751

Fig. 2. Bacterial load in the lung of mice after 8 days of treatment in a fast-acute infection model. Mice were infected with 10^5 CFU of *M. tuberculosis* H37Rv by intratracheal instillation. Treatment was started the next day, given once daily during 8 days, and lung CFUs were counted on day 9, 24 hours after the last administration, with the objective of comparing the efficacy of ETH with combinations of ETH and SMARt751 (S751). Each point represents data from an individual mouse that received ETH or ETH + S751. To serve as a reference, a full dose-response curve of ETH was established in the range of concentration between 1 and 100 mg per kg body weight (black circles). ETH dosages were given in the presence of different concentrations of S751 ranging from 0.1 mg per kg to 10 mg per kg (coloured figures). The pulmonary bacterial load was assessed by numeration of CFU on 7H11 plates. LogDose vs logCFU were fitted to a 4-parameter sigmoidal curve where top (mean CFU for untreated mice) and bottom (shared for all groups, as determined by previous experiments) were constrained. Dotted lines show CI95% for each curve. The estimated ED99 of ETH administered in combination with S751 were systematically out of the CI95% of ETH ED99 in monotherapy (see Report S1).

Fig. 3. (**A**) Dose-response growth curve (DRC) of *M. tuberculosis* H37Rv-GFP treated with 1 µM of SMARt751 (S751) during 24h, washed, and subsequently treated with various doses of ETH (red curve). DRC of bacteria treated with ETH alone (black curve). DRC of bacteria treated with ETH in combination with 1 µM of S751 (blue curve). Each point on the curves are the average of 2 independent experiments, each with replicates (n_{total}=6). (**B**) Eight-day treatment efficacy of TB-infected mice that have received daily administration of ETH 10 mg per kg of body weight alone (in blue) or in combination with SMARt751 (S751) (1 mg per kg of body weight; in red) given daily, every 2 days, every 4 days, or every 8 days. Control-mice received moxifloxacin daily at 30 mg per kg of body weight. ** $P \leq 0.01$; ****$P \leq 0.0001$. For a full description of the results, see Report S2.

Fig. 4. Dose-response curves in chronically infected mice comparing ETH alone (black circles), and ETH in combination with SMARt751 (S751) at doses of 0.3 mg per kg (in yellow) or 1 mg per kg (in red). After 2 months of oral treatment once daily, the pulmonary bacterial load was assessed by numeration of CFU on Middlebrook 7H11 agar plates. Two mice were used per time point and combination. The intersection points between the dashed line and the curves allows to identify the dose of ETH that eliminate 99% of the bacterial load (ED99) in the absence (24 mg per kg for the black curve) or in presence (9.2 mg per kg for the red curve) of S751. LogDose vs logCFU were fitted to a 4-parameter sigmoidal curve where top (mean CFU for untreated mice) and bottom (shared for all groups, as determined by previous experiments) were constrained. Dotted lines show CI95% for each curve. For a full description of the results, see Report S3.

Fig. 5. (**A**) Whole blood concentration-time curve following a single oral administration of ETH (3.5 mg per kg of body weight) with (red) or without (blue) co-administration of SMARt751 (S751) (10 mg per kg) (**B**) Whole blood concentration-time curves following a single oral

administration of 3.4 (red), 15 (blue) or 90 (black) mg per kg of body weight of ETH to mice. Estimated AUC from the 3 curves revealed the non-linearity of the pharmacokinetic of ETH. The dashed line is the limit of quantification of ETH.

Fig. 6. Average log CFU per lung obtained through spread-plating of lung homogenates on Middlebrook 7H11 agar plates. Mice (n = 5) infected with the ETH-resistant strain E1 were treated with ETH monotherapy (blue) or with combinations of ETH and SMARt751 (S751) (magenta). Isoniazid (INH) was used at 25 mg per kg of body weight as positive control. Treatment concentrations, in mg per kg of body weight, are indicated within brackets. Data shown are average +/- SD. For a full description of the results and statistics, see Report S5. (****$P \leq 0.001$)

Fig. 7. Daily reduction of the bacterial load. $EBA_{0\text{-}14}$ simulation of ETH (alone) and in combination with 25 mg of SMARt751 for different doses of ETH. Simulations based on a PK/PD mathematical model translating PD parameters from mice models to human. The PK parameters of ETH in human are taken from Zhu et al. (22). (QD: once a day)

Fig. 8. Target engagement. (**A**) Transcriptomic and proteomic profile of *M. tuberculosis* H37Rv treated with SMARt751. (**B**) Thermal denaturation of VirS (10 µM) monitored using SYPRO Orange in the presence of increasing SMARt751 (S751) concentrations. Melting temperature (Tm) of VirS is obtained using the first-derivative of the fluorescence as a function of temperature (-dF/dT). (**C**) The plot represents the Tm of VirS as a function of the SMARt751/protein molar ratio.

Table

Table 1. **Δ**Tm-EthR and ΔTm-EthR2 values are the differences (in °C) between the thermal denaturation turning point of the corresponding proteins alone and the one of the proteins in combination with the listed compounds. BV-EthR and BV-EthR2 IC_{50} values are the concentration of compounds which displaces 50% of the binding of the corresponding protein to its DNA operator measured using a mammalian reporter gene circuit (SEAP test; see Fig. S1). ETH-Boost EC_{50} values are the concentration of compound required to make ETH ten times more potent, either on *M. tuberculosis* H37Rv or on *M. tuberculosis* strain E1 ($ethA^-$) resistant to ETH.

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N.W., B.D., A.B., P.B., M.F. are listed as inventors of Smart751 on patent FR3000065A1 (WO2014096378) entitled "Bicyclic compounds having an activity potentiating the activity of an antibiotic active against mycobacteria-composition and pharmaceutical product comprising such compounds". A.B. is a consultant for Bioversys. B.D. is paid as the Scientific Director of Institut Pasteur de Lille.

(IF YOU DECLARE NO CONFLICT OF INTEREST **YOU MUST** ADD HERE YOUR INITIALS). **X.X., Y.Y., Z.Z.**, … , declare no conflict of interest.

Supplementary materials

Supplementary methods