

# Rational Design and Synthesis of Thioridazine Analogues as Enhancers of the Anti-TB Therapy

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## Abstract

Tuberculosis, caused by *Mycobacterium tuberculosis*, is still one of the leading infectious diseases globally. Therefore, novel approaches are needed to face this disease. Efflux pumps are known to contribute to the emergence of *M. tuberculosis* drug resistance. Thioridazine has shown good anti-TB properties both *in vitro* and *in vivo*, likely due to its capacity to inhibit efflux mechanisms. Here we report the design and synthesis of a number of putative efflux inhibitors inspired by the structure of thioridazine. Compounds were evaluated for their *in vitro* and *ex vivo* activity against *M. tuberculosis* H37Rv. Compared to the parent molecule, some of the compounds synthesized showed higher efflux inhibitory capacity, less cytotoxicity, and a remarkable synergistic effect with anti-TB drugs both *in vitro* and in human macrophages, demonstrating their potential to be used as co-adjuvants for the treatment of tuberculosis.

## Introduction

Tuberculosis (TB) is a highly contagious airborne disease resulting from infection with *Mycobacterium tuberculosis*. The World Health Organization (WHO) currently estimates that one-third of the world's population is infected with *M. tuberculosis*, and in 2013, approximately 210 000 human lives were lost to TB.<sup>1</sup> The global health impact of *M. tuberculosis* is exacerbated by HIV co-infection, making TB one of the major AIDS-related opportunistic diseases.<sup>2</sup> This shocking failure to control TB is due to the combination of many factors, among which: the non-compliance of the patients to the required six months of multidrug therapy; the difficulty to provide it in a sustained and properly-dosed regimen, particularly in those parts of the world that are the least equipped to deal with the disease.<sup>3</sup> These issues have altogether led to the emergence of multidrug resistant (MDR) TB and extensively drug-resistant (XDR) TB. As

such, to address this global health crisis, the process of drug discovery must rely both on novel chemical anti-TB entities<sup>4,5</sup> and on an innovative approach that rationally prevents the raise of drug resistance.

After many years without delivering any new anti-TB drug, the discovery of bedaquiline for the treatment of MDR-TB, and a nourished pipeline of compounds already in clinical trials (<http://www.newtbdrugs.org/pipeline.php>) have renewed hope for the treatment of TB and especially MDR-TB. However, the lack of innovation in the overall therapeutic approach makes quite predictable that mutation-based resistant strains will inexorably emerge also for these novel drugs. The discovery of bedaquiline<sup>6</sup> and pretomanid<sup>7</sup> has showed that the whole cell phenotypic assay of large libraries of compounds, and the wise medicinal chemistry efforts toward the improvement of these molecules, are reliable methodological approaches to drive the research towards novel anti-TB agents.<sup>8</sup> Indeed, most of the efforts of some of the authors of this work has moved through this path.<sup>9-19</sup> While this approach has proved to be correct, it is nevertheless incomplete. It does not consider that, besides mutations, other mechanisms such as efflux play an important role in the development of resistance in *M. tuberculosis*.<sup>20-22</sup>

Efflux pumps are involved in several physiological processes such as detoxification of intracellular metabolites and cellular homeostasis.<sup>23</sup> Simultaneously, they contribute to the development of intrinsic and acquired drug resistance in many bacterial pathogens. Usually they confer low-to-intermediate level of resistance, however, the constant pressure of subinhibitory concentrations of the antibiotic promotes the selection of spontaneous mutants.<sup>20</sup> Therefore: *a*) efflux pumps are effectors of the innate drug-resistance machinery; *b*) they are crucial in conferring a low-level of drug resistance; *c*) they contribute to lower the concentration of the drug inside the mycobacterial cell, enabling the emergence of a high-level of resistance; *d*) it has

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3 been demonstrated that efflux inhibitors (EIs) are able to improve or restore the activity of old  
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5 drugs towards which the bacteria had become resistant.<sup>24</sup>  
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8 In spite of this knowledge, the extent to which an antimicrobial compound is a substrate of  
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10 efflux is seldom measured, and it is not considered an important parameter in the hit-to-lead  
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12 process for the optimization of novel anti-TB drugs. The recent discovery by Lee and colleagues  
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14 of spectinamides as anti-TB drug candidates,<sup>25</sup> also by virtue of their scarce tendency to be  
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16 extruded by efflux pumps, confirms the importance of taking into account efflux when the design  
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18 of new drugs is planned.<sup>26</sup> In addition to these findings, although the mechanism of adaptation of  
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20 *M. tuberculosis* to the hostile environment is still a matter of debate, Adams and colleagues have  
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22 recently reported that bacterial efflux pumps mediate multi-drug tolerance and the bacterial  
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24 survival in macrophages, leading to a long-lasting therapy.<sup>27,28</sup>  
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29 It is therefore possible to claim that inhibition of efflux would lead to a number of beneficial  
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31 effects: *a)* increasing the activity of the anti-TB drugs subject to efflux; *b)* keeping the  
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33 concentration of a given drug at the therapeutic dose, minimizing the possibility to select  
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35 mutants; *c)* drastically shorten the duration of treatment by reducing multi-drug tolerance. This  
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37 could be priceless in order to repurpose old drugs and preserve the efficacy of those novel  
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39 compounds, such as bedaquiline, that are either next to be introduced in the anti-TB arena or that  
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41 already are.  
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45 Thioridazine (TZ) is a neuroleptic with efflux inhibitory properties,<sup>29</sup> that has attracted the  
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47 attention of many researchers due to its ability to hit drug resistant strains.<sup>30</sup> In a clinical trial in  
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49 Argentina, TZ was used for the treatment of patients with XDR-TB in combination with anti-TB  
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51 drugs, with good results.<sup>31</sup> It has been demonstrated that TZ has a dual effect against *M.*  
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53 *tuberculosis*: it is an inhibitor of bacterial efflux pumps, and, also, it induces the acidification of  
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the phagosome, contributing to restriction of intracellular *M. tuberculosis* growth.<sup>30,32–34</sup> TZ is not the only inhibitor of efflux investigated for the treatment of TB. Recently chlorpromazine, another neuroleptic with efflux inhibitory properties, was tested along with its metabolites against *M. smegmatis* and exhibited synergistic activity when administered in combination with known anti-TB drugs.<sup>35,36</sup> Also the antiarrhythmic verapamil is an efflux pump inhibitor that has been shown to have an antimicrobial-potentiating effect in the treatment of *M. tuberculosis* both *in vitro* and *in vivo*.<sup>37</sup> It was shown that inhibition of the efflux pumps of *M. tuberculosis* by verapamil reduces the bacterial drug tolerance induced in the intracellular compartment inside the macrophages and in zebrafish granuloma-like lesions. Unfortunately, the use of TZ in the therapy of TB is severely limited by its neuroleptic properties and a number of severe toxicity issues. Inspired by these considerations, we herein report the design and synthesis of a number of TZ derivatives, and their biological evaluation as efflux inhibitors and synergistic effect with known anti-TB drugs both *in vitro* and on infected human monocyte-derived macrophages. Among the novel compounds, when compared to the parent molecule TZ, compound **10** showed higher efflux inhibitory ability, less toxicity, and a remarkable synergistic effect with first- and second-line drugs, especially rifampicin. As such, it may represent an interesting starting point for the development of more potent and less toxic efflux inhibitors to be used in combination with the current first- and second-line anti-TB drugs.

### Rational design

Since the lack of detailed information about the structure of the efflux systems, the new compounds were prepared following a Ligand-Based Drug Design approach. TZ was used as template, given its well-known and deeply investigated anti-TB properties when administered in combination with first-line drugs, as mentioned above.<sup>29,32–34,38,39</sup> The aim of this work was

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3 therefore to chemically modify TZ in order to obtain a compound with improved adjuvant  
4 properties while limiting the onset of side effects, especially at the CNS level. We reasoned that,  
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6 in first place, it was necessary to escape from the phenothiazine core because, according to the  
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8 SAR of antipsychotic drugs,<sup>40</sup> it is crucial in conferring the desired neuroleptic activity.  
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10 Moreover, although with a few exceptions,<sup>41</sup> a series of TZ analogues in which the  
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12 phenothiazine core was kept intact has already been reported as inhibitors of efflux in other  
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14 bacteria, but with scarce results.<sup>38,42</sup> In addition to this, efflux systems are known to be affine to  
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16 charged quaternary ammonium salts such as ethidium bromide (EtBr) and berberine, and to  
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18 molecules bearing basic amino groups (TZ, VP, reserpine), that likely occur to be protonated at  
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20 the acidic pH of the sites infected by *M. tuberculosis*. Given these premises, chemical  
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22 manipulation was carried out keeping intact the N-methylpiperidine moiety, whereas modifying  
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24 the nature of the heterocyclic attached to it and the length of the spacer.  
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32 Several heterocycles, embodying a variable number of rings, were attached by the nitrogen  
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34 atom to an N-methyl-2-piperidinyl appendage through an ethylene linker (compounds **9-15**, chart  
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36 1).  
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39 [Insert chart 1 here]  
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41 The carbazole (**9**) and the acridone (**12**), that are tricyclic flat structures, were chosen for their  
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43 close resemblance with the phenothiazine core. The indole (**10**), and the benzimidazole (**11**) are  
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45 among the most privileged scaffolds used in medicinal chemistry, and could afford extended  
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47 chemical manipulation of the series. The diphenylamines **13** and **14** were synthesized to check  
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49 whether the planar structure of the phenothiazine could be disrupted without loss of the  
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51 inhibitory activity. The pyrrolidine **15** was prepared to check whether a small ring could still be a  
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53 suitable substrate of efflux. Once the preliminary assays had corroborated the design of  
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compound **9**, other appendages were attached to the carbazole core in place of the N-methyl-2-piperidinyl one. To start, a less polar and flatter substrate such as the 2-ethylpyridine (**18**) was attached to the carbazole nitrogen; then, the distance between the nitrogen of carbazole and that of the appendage was shortened by one methylene unit in compound **19**.

The synthesis of compounds **5** and **6** was accomplished in the attempt to couple a moiety that is thought to be responsible for the EI activity to a potent anti-TB agent;<sup>11,16</sup> this would have led to the synthesis of the first rationally designed TB-GEI (Growth and Efflux Inhibitor), which could represent a superior therapeutic option for the treatment of TB. Indeed, the recent finding that bedaquiline, along with its antibacterial activity, is also a weak inhibitor of efflux mechanisms, accounts for this reasoning.<sup>43</sup> Finally, since the recent interest in the use of VP and its derivatives in the treatment of TB,<sup>44,45</sup> we have tried to merge the two structures of TZ and VP in one hybrid molecule (Figure 1), and therefore compounds **7** and **8** (chart 1) were synthesized and tested.

[Insert Figure 1 here]

## Chemistry

The synthesis of derivatives **5–15** was achieved through reaction of the suitable heterocyclic precursor and 2-(2-chloroethyl)-N-methylpiperidine hydrochloride, in dry DMF using NaH in excess, either at room temperature or heating for several hours, when necessary (Scheme 1). When not commercially available, the synthesis of the heterocyclic precursors was achieved in good yields according to a well-established procedure, as in the case of intermediates **1** and **2**.<sup>13,16</sup> Compound **3**<sup>46</sup> was prepared reacting 3,4-dimethoxyaniline with triethylamine and 2-iodopropane in refluxing methanol, differently from what already reported. For the synthesis of compound **18**, first 2-(hydroxymethyl)-pyridine was treated with *p*-toluenesulfonyl chloride and

triethylamine in dichloromethane to give the tosyl derivative **16**, and then the ameliorated leaving group is displaced by carbazole according to the conditions reported by Cheng (Scheme 2).<sup>47</sup> In the case of compound **19**, carbazole was first treated with dichloroethane to give N-(chloroethyl)carbazole **17**, that was reacted with piperidine in DMF at room temperature using K<sub>2</sub>CO<sub>3</sub> as the base to give **19** (Scheme 2).<sup>48</sup> For the synthesis of compound **8**, 3,4-dimethoxyphenyl acetonitrile was first treated with sodium hydride and 2-bromopropane to give intermediate **4**;<sup>49</sup> several attempts to couple this derivative with 2-(2-chloroethyl)-N-methylpiperidine hydrochloride with the use of various bases, performed according to different literature protocols,<sup>49-51</sup> failed to yield the desired product. The synthesis was finally accomplished by heating the two reagents in DMF in a microwave oven, using an excess of sodium amide as the base. Interestingly, the same synthetic conditions did not give any product with conventional heating also after many days at 120 °C. Compound **8** was tested preliminarily as a racemic mixture.

[Insert Scheme 1 here]

[Insert Scheme 2 here]

## Results and Discussion.

A total of 13 compounds were synthesized in this study (**5-15**, **18**, **19**, chart 1), that underwent biological assays. In order to set up a faster and cheaper model for screening the activity of the compounds, they were first evaluated for their antimycobacterial and efflux inhibitory activity against *M. smegmatis* mc<sup>2</sup>155 reference strain. For those compounds showing inhibitory activity higher than TZ, cytotoxicity on human-monocyte derived macrophages was assessed. Compounds showing less cytotoxicity and higher efflux inhibitory activity than that of TZ, were further evaluated on *M. tuberculosis* H37rv, as described herein.



### Antimycobacterial and efflux inhibitory activity against *M. smegmatis* mc<sup>2</sup>155

The compounds were first tested for their capability to inhibit the growth of *M. smegmatis* strain mc<sup>2</sup>155 (Table 1). The compounds demonstrated extremely poor or absent antimycobacterial activity against *M. smegmatis*, especially if compared with TZ, which was used as positive control. This was considered a good result, as high bactericidal/bacteriostatic activity could bias the following experiments of synergy with first- and second-line anti-TB agents. Due to the reduced solubility and the low antimycobacterial activity, it was not possible to determine the exact MIC values for some compounds (*i.e.* **5-8**, **11**, **13**, **15**, **18**, **19**). As such, when bacterial growth is still noticed at the maximum concentration tested, the MIC was indicated by “>MIC”. For instance, >256 µg/mL means that the MIC of this compound is higher than 256 µg/mL, as it was technically impossible to determine it. After determining their MICs, the compounds were tested for their capability to inhibit efflux of EtBr from *M. smegmatis* mc<sup>2</sup>155 cells by real-time fluorometry, using TZ and VP as controls (Table 1, relative final fluorescence (RFF) values). According to a widely used protocol for this kind of assay,<sup>52</sup> in order not to compromise the cellular viability, all of the compounds were tested at ½ of their MIC. For those compounds for which the exact MIC could not be determined (above reported), the last concentration value that could be technically determined was considered ½ of the MIC. Despite the small set of compounds prepared for this preliminary study, a few SAR considerations can be made. In general, the majority of the compounds bearing the N-methyl-2-piperidinyl appendage had inhibitory effect on the efflux of EtBr. We were pleased to notice that compound **7**, designed as a hybrid molecule resembling both VP and TZ, had a very good inhibitory activity of EtBr efflux. However, the same does not apply to the other hybrid compound **8**. It might be then speculated that the N-methyl-2-piperidinyl appendage must be attached, through an ethylene

linker, to another nitrogen atom of the heterocyclic, yielding a more polar structure. When the N-methyl-2-piperidinyl appendage is attached through an ethylene linker either to tricyclic (compounds **9**, **12**), bicyclic (compounds **10**, **11**) or more flexible ring structures (compounds **13**, **14**), a good inhibitory activity, sometimes higher than that of the parent compound TZ, is noticed. However, bulkier heterocyclic (compounds **5** and **6**) or small rings (compound **15**), have a detrimental impact on the efflux inhibitory properties. Surprisingly, the benzimidazole derivative **11** showed an exceptionally high activity, resulting by far more potent than VP. The distance between the nitrogen of the appendage and that of the heterocyclic seems to have pharmacological significance, since the activity of compound **19** is considerably low. Finally, the presence of a tertiary aliphatic amino moiety seems to be determinant, being compound **18** the least active of the series.

#### Cytotoxicity evaluation of compounds **7**, **9**, **10**, **11** and **12**

Compounds **7**, **9**, **10**, **11** and **12**, that resulted more active than TZ in inhibiting the efflux of EtBr out of *M. smegmatis* cells, were evaluated against human-monocyte derived macrophages to assess their *ex vivo* cytotoxicity toward eukaryotic cells (Table 1). We were pleased to notice that, with the exception of compound **12** ( $IC_{50} = 0.762 \mu\text{g/mL}$ ) all of the tested compounds were found to be less cytotoxic than TZ in this assay. Compounds **9** and **10**, ( $IC_{50} = 8.8 \mu\text{g/mL}$  and  $10.5 \mu\text{g/mL}$ , respectively) are two-fold less cytotoxic than TZ ( $IC_{50} = 5.6 \mu\text{g/mL}$ ), whereas compounds **7** and **11** ( $IC_{50} = 84.8 \mu\text{g/mL}$  and  $170.8 \mu\text{g/mL}$ ) were found to be safe towards human macrophages. Overall, these preliminary data clearly suggest that the replacement of the phenothiazine core with other ring systems allows maintaining the efflux inhibitory activity with the possibility to decrease the cytotoxicity.

[Insert Table 1 here]

[Insert Figure 2 here]

Compounds **7**, **9**, **10** and **11**, that resulted more active than TZ in inhibiting the efflux of EtBr from *M. smegmatis*, and less cytotoxic against human-monocyte derived macrophages, were selected for additional biological studies against *M. tuberculosis* H37Rv strain. Compound **12**, although presenting higher RFF values than that of TZ, was excluded based on its higher cytotoxicity.

#### **Evaluation of compounds 7, 9, 10 and 11 antimycobacterial and efflux inhibitory activity against *M. tuberculosis* H37Rv**

Compounds **7**, **9**, **10** and **11** were tested against the pan-susceptible reference strain H37Rv, following the same rationale used for *M. smegmatis*, i.e. the determination of antimycobacterial activity and the evaluation of their effect on EtBr efflux (Table 2). As observed for *M. smegmatis*, the selected derivatives showed no antimycobacterial activity against *M. tuberculosis*, therefore excluding the possibility of bias during the following assays. With regard to their activity as inhibitors of EtBr efflux, the compounds were tested with the same procedure used as in the case of *M. smegmatis*. Compound **9** showed similar activity to that of TZ, whereas compounds **7**, **10** and **11** were found to be 2-4 fold more potent than TZ, although less active than VP (Table 2, Figure 2). Although quite active, the benzimidazole derivative **11** failed to reproduce the extraordinary efflux inhibitory effect observed against *M. smegmatis*. This is not surprising and can be somehow explained on the basis of the faster metabolism of *M. smegmatis* compared to that of *M. tuberculosis*, that accounts for the higher susceptibility to the efflux inhibitory effect. Our own experience shows that RFF values are always scaled down in *M. tuberculosis* in comparison to those observed for the same compounds in *M. smegmatis*, although maintaining the inhibitory relation.<sup>53,54</sup> Regardless, we can claim that, in the conditions reported,

all of the tested compounds have maintained their superior capability of inhibiting efflux compare to TZ against *M. tuberculosis*.

[Insert Table 2 here]

**Evaluation of synergistic effect of the selected TZ analogues in combination with first- and second-line anti-TB drugs and determination of the fractional inhibitory concentration (FIC).**

Once that the inhibition of efflux was confirmed, the study went ahead evaluating compounds **7**, **9**, **10** and **11** for their effect in combination with the current first-line (isoniazid, INH; rifampicin, RIF) and second-line (amikacin, AMK; ofloxacin, OFX) drugs used for the treatment of TB. Their effect was also tested in combination with EtBr, to correlate its efflux with the efflux of the anti-TB drugs.<sup>55</sup> The MICs of INH, RIF, AMK and OFX were calculated in the absence and in the presence of scalar sub-inhibitory concentrations of each compound against *M. tuberculosis* H37Rv, that is known to have intrinsic and readable efflux activity of these anti-TB drugs<sup>22,54</sup> (Table 3).

[Insert Table 3 here]

Initially, compounds **7**, **9**, **10** and **11** were tested at  $\frac{1}{2}$  of their MIC (see Table 3 for details), and then, according to the protocol widely used for this sort of investigations,<sup>52</sup> they were tested by two-dimensional broth microdilution checkerboard assay, to evaluate the extent of the synergism. Also in this case, as already explained in the case of the efflux inhibition assays, for those compounds for which the exact MIC could not be determined,  $\frac{1}{2}$  of the MIC was considered the concentration that was equal to the highest concentration where it was obtained growth. When tested at these concentrations, all of the newly synthesized compounds and the controls demonstrated very high synergistic effect with both the antibiotics and EtBr. In this

case, the MIC values of INH, RIF, AMK, OFX and EtBr could be reduced more than 64-fold (Table 3). Compound **10**, for which the inhibitory effect on efflux was already assessed, had in general a very high synergistic effect with the tested drugs and, in particular, with RIF. In fact, compound **10** was able to improve the activity of RIF by 4-fold at concentrations as low as 8  $\mu\text{g/mL}$  (approx. 1/64 of its MIC), demonstrating a potent synergistic effect. Moreover, at a concentration of 64  $\mu\text{g/mL}$  (approx. 1/8 of its MIC), compound **10** was also able to improve the activity of AMK and OFX by more than 128-fold, and EtBr by 8-fold (Table 3). Importantly, we have noticed that the synergistic activity demonstrated by the compounds in combination with EtBr correlates well with the results obtained with the inhibition of EtBr efflux by RFF.

One may argue that TZ, considering the absolute concentrations at which the synergistic activity is measured, is slightly more active than compound **10**. However, seen under a more widespread perspective, compound **10** is less toxic, more active with regard the inhibition of efflux, and unlikely to possess CNS-related properties. This can be considered a good achievement, in particular considering that the aim of this paper was the selection of compounds devoid of antimycobacterial activity, with reduced toxicity to the human macrophage and with higher efflux inhibitory capability, so as to promote the antimycobacterial activity of the anti-TB drugs and be considered as a future adjuvant in the fighting against TB.

Surprisingly, despite the high activity in inhibiting the efflux, compound **11** was found to be not active in the combination assay. Compounds **7** and **9** were found to be slightly less active than **10** in the combination assay; however, in the case of compound **7**, it must be noticed that it is able to improve the activity of RIF by 4-fold also at very low concentrations. It is intriguing to notice that compounds **7** and **9**, as compound **10**, demonstrated to have a significant synergistic effect in particular with RIF. The reason of this preferred synergism is worth of further

investigation but, at this moment, it can only be speculated that **10**, as well as the other derivatives belonging to this series, although to a lesser extent, have high affinity for interfering with efflux systems that extrude RIF. However, it cannot be ruled out that the two derivatives interfere with other mechanism of resistance used by the cell in the presence of RIF. Finally, it is worth of consideration the fact that compounds **7**, **9** and **10** were more active in the combination assay than VP, another drug often investigated for its potential role as adjuvant in the TB treatment.

The synergistic activity between the compounds was also represented as the fractional inhibitory concentration (FIC), that is a value expressing the synergistic activity of the compounds in combination with the antibiotics and EtBr (Figure 3). FIC was determined for each anti-TB drug by dividing the MIC of each drug when used in combination, by the MIC of each drug used alone. Since a drug cannot interact with itself, the effect of a self-drug combination will always be additive, with an FIC index of 1. As such, when the FIC value is lower than 0.25, a synergistic effect is noticed between the modulator and the antimicrobial. When above 2, there is antagonism between the compounds tested, whereas within these two FIC values, indifference is noticed.<sup>24</sup>

[Insert figure 3 here]

Isobolograms were constructed, by plotting changes in the MIC of antibiotics as a function of the tested compounds concentration (Figure 3 and S1 – example for compound **10**). As it can be seen, for the majority of the combinations of compound **10** with the antibiotics synergy was observed, with FIC values ranging from 1 to not possible to determine (ND) due to the very high synergistic effect, depending on the antibiotic combination (see legend of Figure 3 supporting information for details).

### Evaluation of the intracellular anti-TB activity of the selected TZ analogues.

Finally, we evaluated the intracellular antimycobacterial activity of compounds **7**, **9**, **10** and **11** alone and in combination with INH or RIF against *M. tuberculosis*-infected human monocyte-derived macrophages (Figure 4). This assay is a fair reproduction of what actual happens during the TB infection, and therefore it can help in obtaining a clearer idea of the potential of our compounds. INH and RIF were used at  $\frac{1}{2}$  of its MIC (0.05 and 0.5  $\mu\text{g/mL}$ , respectively), therefore having no direct inhibitory effect on the bacteria at these concentrations. The compounds **7**, **9**, **10** and **11** were tested at nontoxic concentrations (16  $\mu\text{g/mL}$ , 0.078  $\mu\text{g/mL}$ ; 1.25  $\mu\text{g/mL}$ , and 40  $\mu\text{g/mL}$ , respectively) determined against human-monocyte derived macrophages (Figure S2). Above these concentrations, macrophage viability is reduced below 90%, nullifying the assay. At these concentrations, the compounds tested showed to be devoid of any antimycobacterial activity against intracellular *M. tuberculosis*. Consistent with the findings above reported, we were pleased to notice that the co-administration of the compounds with INH or RIF (at a non-antibiotic inhibitory concentration) led to a strong enhancement of the killing activity of the human macrophages against the H37Rv strain. This is comparable with the results previously obtained with TZ and other efflux inhibitors that has been shown to have a dual bacterial-host boosting effect against *M. tuberculosis*.<sup>56</sup>

### Conclusion

The contribution of efflux systems to *M. tuberculosis* drug resistance and tolerance has been increasingly recognized, although only a few medicinal chemistry efforts have been made towards the synthesis of *M. tuberculosis* efflux inhibitors. Despite the amount of literature establishing TZ as a valuable agent against TB, the numerous toxicity issues have always hampered its safe administration as an adjuvant of TB therapy. To our knowledge, this is the first

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3 medicinal chemistry campaign aimed at modifying TZ with regard to its potential as adjuvant of  
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5 TB therapy, with specific assays such as the determination of the efflux inhibition and the  
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7 synergistic activity in combination with known anti-TB drugs both *in vitro* and *ex vivo*.  
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9 Compared to TZ, the representative derivatives tested were found to be less toxic toward human  
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11 macrophages, ranging from two-fold (compound **9** and **10**) to more than 50-fold (compound **11**).  
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13 Compound **7**, intended as a hybrid of TZ and VP, showed a higher inhibitory activity than that of  
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15 TZ itself and much less toxicity, therefore confirming the rationale of the design. More  
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17 importantly, we have escaped from the phenothiazine scaffold, thus assuming a reduction of the  
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19 onset of side effects at the CNS level. The newly synthesized compounds showed high  
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21 synergistic activity when tested in combination with some of the most important first-line (INH,  
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23 RIF) and second-line (AMK, OFX) drugs used for the treatment of TB. In particular compound  
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25 **10** was able to improve the activity of RIF at concentration as low as approximately 1/64 of its  
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27 MIC. Additionally, these compounds were able to improve the activity of INH and RIF against  
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29 intracellular *M. tuberculosis*. Considering the data on inhibition of EtBr efflux, coupled to the  
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31 low antimycobacterial activity, it might be claimed that the synergetic activity is due to their  
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33 ability to hamper the intrinsic mycobacterial drug efflux.  
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41 Finally, we confirmed *M. smegmatis* mc<sup>2</sup>155 as a suitable surrogate of *M. tuberculosis* for the  
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43 preliminary assessment the efflux inhibitory activity, at a lower cost and time consuming rate.  
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45 Altogether, these findings provide a solid base to further investigate compound **10** as a booster of  
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47 the antimycobacterial chemotherapy when associated with first- and second-line drugs, by virtue  
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49 of its capability to block the intrinsic efflux activity of mycobacteria. This might represent a  
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51 lateral approach toward the cure of TB. Studies expanding this versatile series and its mechanism  
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of action and activity toward *M. tuberculosis* drug resistant strains, especially resistant to isoniazid and rifampicin (MDR-TB), are currently underway in our research groups.

## EXPERIMENTAL PART

### Chemistry

**General information.** All the reagents were purchased from Sigma-Aldrich, Alfa-Aesar and Enamine at reagent purity and, unless otherwise noted, were used without any further purification. Dry solvents used in the reactions were obtained by distillation of technical grade materials over appropriate dehydrating agents. MCRs were performed using CEM Microwave Synthesizer-Discover model. Reactions were monitored by thin layer chromatography on silica gel-coated aluminium foils (silica gel on Al foils, SUPELCO Analytical, Sigma-Aldrich) at both 254 and 365 nm wavelengths. When indicated, intermediates and final products were purified through silica gel flash chromatography (silica gel, 0.040-0.063 mm), using appropriate solvent mixtures.

$^1\text{H}$ -NMR and  $^{13}\text{C}$ -NMR spectra were recorded on a BRUKER AVANCE spectrometer at 300, 400 and 100 MHz respectively, with TMS as internal standard.  $^1\text{H}$ -NMR spectra are reported in this order: multiplicity and number of protons. Standard abbreviation indicating the multiplicity was used as follows: s = singlet, d = doublet, dd = doublet of doublets, t = triplet, q = quadruplet, m = multiplet and br = broad signal. HPLC/MS experiments were performed with HPLC: Agilent 1100 series, equipped with a Waters Symmetry C18, 3.5  $\mu\text{m}$ , 4.6 mm x 75 mm column and MS: Applied Biosystem/MDS SCIEX, with API 150EX ion source. HRMS experiments were performed with LTQ ORBITRAP XL THERMO.

All compounds were tested as 95% purity samples or higher (by HPLC/MS).

The synthesis and chemical characterizations of intermediates 1-4, 16, 17 is reported in the Supporting Information.

**General procedure for the synthesis of compounds 5, 6, 9-15, 18.** To a suspension of sodium hydride (60% suspension in mineral oil, 3 eq) in DMF (5 mL/mmol) was added the suitable heterocyclic compound (1 eq) at 0 °C. Reaction mixture was stirred for 15 minutes at the same temperature and then 2-(2-chloroethyl)-piperidine hydrochloride (1.3 eq) was added portion wise. The mixture was allowed to react at room temperature until consumption of the starting material. In some cases (**5**, **6**, **13**) reflux heating was necessary. After quenching with water, the mixture was extracted with ethyl acetate (3 × 10 mL), and the organic layers were washed with water and brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated under reduced pressure. The crude material was purified through flash chromatography. Reaction times and conditions, yields, purification methods and other analytical data are reported in the supporting information.

**N-isopropyl-3,4-dimethoxy-N-(2-(1-methylpiperidin-2-yl)ethyl)aniline (7).** To a solution of **3** (60 mg; 0.38 mmol) in dry toluene (2 mL) was added sodium amide (120 mg; 3.07 mmol) and 2-(2-chloroethyl)piperidine hydrochloride (244 mg; 1.23 mmol). The reaction mixture was stirred and heated in a microwave oven at 140 °C for 30 minutes. After quenching with brine (25 mL), the mixture was extracted with ethyl acetate (3 × 10 mL), and the combined organic layers were dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and evaporated under reduced pressure. The crude material was then purified through flash chromatography eluting with dichloromethane/methanol 9:1. Yield: 16%. <sup>1</sup>H-NMR (400 MHz-CDCl<sub>3</sub>): □ 1.10-1.15 (m, 6 H); 1.20-1.87 (m; 8H), 2.30-2.35 (bs; 5H); 2.95-3.10 (m; 2H), 3.15-3.25 (m, 1H); 3.65-3.75 (m, 1H); 3.84 (s, 3H); 3.87, (s, 3H); 6.41 (d, J = 9 Hz, 1H); 6.49 (s, 1H); 6.79 (d, J = 9 Hz, 1H). <sup>13</sup>C-NMR (100.6 MHz-CDCl<sub>3</sub>): □ 19.4, 20.2, 23.3, 28.3, 41.3, 53.8, 55.9, 56.3, 105.6, 111.9, 142.6, 143.7, 149.5. HRMS (ESI) calculated for C<sub>19</sub>H<sub>32</sub>N<sub>2</sub>O<sub>2</sub> [M + H]<sup>+</sup> 321,2464, found 321.25365.

**2-(3,4-Dimethoxyphenyl)-2-isopropyl-4-(1-methylpiperidin-2-yl)butanenitrile (8).** To a solution of **4** (70 mg; 0.31 mmol) in dry toluene (2 mL) was added sodium amide (124 mg; 3.19 mmol) and 2-(2-chloroethyl)piperidine hydrochloride (252 mg; 1.25 mmol). The reaction mixture was stirred and heated in a microwave oven at 140 °C for 30 minutes. After quenching with brine (25 mL), the mixture was extracted with ethyl acetate (3 × 10 mL), and the combined organic layers were dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. The crude material was then purified through flash column chromatography eluting with dichloromethane/methanol 9:1. Yield: 50%. <sup>1</sup>H-NMR (300 MHz-CDCl<sub>3</sub>): □ 0.8 (dd, J<sub>1</sub> = 4 Hz, J<sub>2</sub> = 7 Hz, 3 H); 1.23-1.80 (m; 11H), 1.85-2.40 (m; 8H); 2.80-2.95 (m; 1H), 3.88 (s, 3H); 3.90 (s, 3H); 6.80-7.00 (m, 3H). <sup>13</sup>C-NMR (100.6 MHz-CDCl<sub>3</sub>): □ 18.7, 19.0, 24.0, 28.2, 29.9, 32.1, 32.5, 37.9, 53.4, 55.9, 56.0, 63.0, 109.1, 109.4, 111.0, 118.8, 121.1, 130.5, 148.3, 149.0. HRMS (ESI) calculated for C<sub>21</sub>H<sub>32</sub>N<sub>2</sub>O<sub>2</sub> [M + H]<sup>+</sup> 345.2464, found 345.25365.

**9-(2-(Piperidin-1-yl)ethyl)-9H-carbazole (19).** To a solution of piperidine (33 µl; 0.33 mmol) in DMF (15 mL/mmol) was added potassium carbonate (138 mg, 1 mmol), this mixture was stirred at room temperature for 20 minutes and then the intermediate **17** (10 mg, 0.43 mmol) was added. Reaction mixture was stirred to 60 °C overnight. After cooling, brine (25 mL) was added and the mixture was extracted with ethyl acetate (3 × 10 mL). The combined organic layers were washed with water and brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated under reduced pressure. The crude material was purified through flash chromatography eluting with petroleum ether/ethyl acetate 95/5, yielding the title compound as a white solid. Yield: 19%. <sup>1</sup>H-NMR (400 MHz-CDCl<sub>3</sub>): □ 1.24-1.60 (m, 6H); 3.07 (bs, 2H); 3.39 (bs, 2H); 4.49 (t, J = 8 Hz, 2H); 4.62 (t, J = 8 Hz, 2H); 7.23-7.28 (m, 2H); 7.46-7.79 (m, 4H), 8.12 (d, J = 8 Hz, 2H). <sup>13</sup>C-NMR (100.6

MHz-CDCl<sub>3</sub>):  $\delta$  24.2, 25.4, 42.1, 44.7, 62.8, 108.7, 119.1, 120.3, 123.0, 125.7, 140.5, 155.0.

HRMS (ESI) calculated for C<sub>19</sub>H<sub>22</sub>N<sub>2</sub> [M + H]<sup>+</sup> 279.1783, found 279.39258

## Biology

### Evaluation of the novel synthesized compounds against mycobacteria

#### Reagents and mycobacterial strains

Thioridazine (TZ), verapamil (VP), isoniazid (INH), rifampicin (RIF), amikacin (AMK), ofloxacin (OFX), EtBr, phosphate-buffered saline (PBS), and glucose were purchased from Sigma-Aldrich (St. Louis, MO, USA). All solutions were prepared in deionized water, except rifampicin and the TZ analogues **5-15**, **18** and **19**, which were prepared in DMSO. All solutions were prepared on the day of the experiment. Middlebrook 7H9 (MB7H9) and the OADC supplement (oleic acid/albumin/dextrose/catalase) were purchased from Difco (Madrid, Spain) and Becton and Dickinson (Sparks, MD, USA) respectively. The mycobacterial reference strains *M. smegmatis* mc<sup>2</sup>155 ATCC700084 and *M. tuberculosis* H37Rv ATCC27294<sup>T</sup> were used to evaluate the biologic activity of TZ analogues.

The determination of the minimum inhibitory concentration (MIC), the evaluation of the synergistic effect of TZ analogues **7**, **9**, **10** and **11** with selected antibiotics and EtBr by broth microdilution checkerboard assay, the evaluation of efflux inhibitory activity of the compounds by real-time fluorometry and the cytotoxicity evaluation are reported in detail in the Supporting Information.

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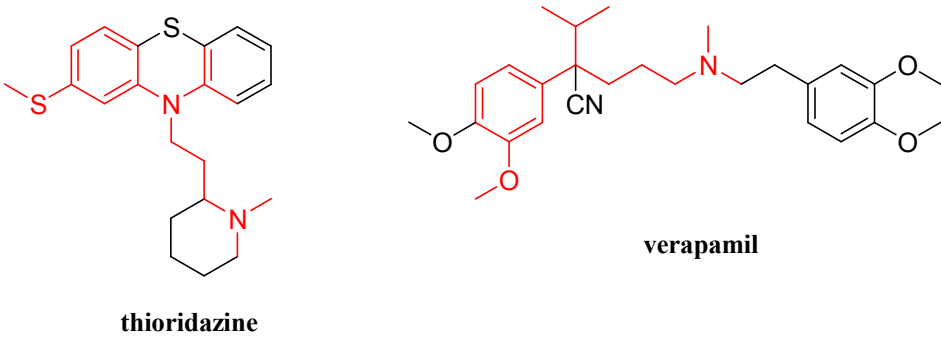
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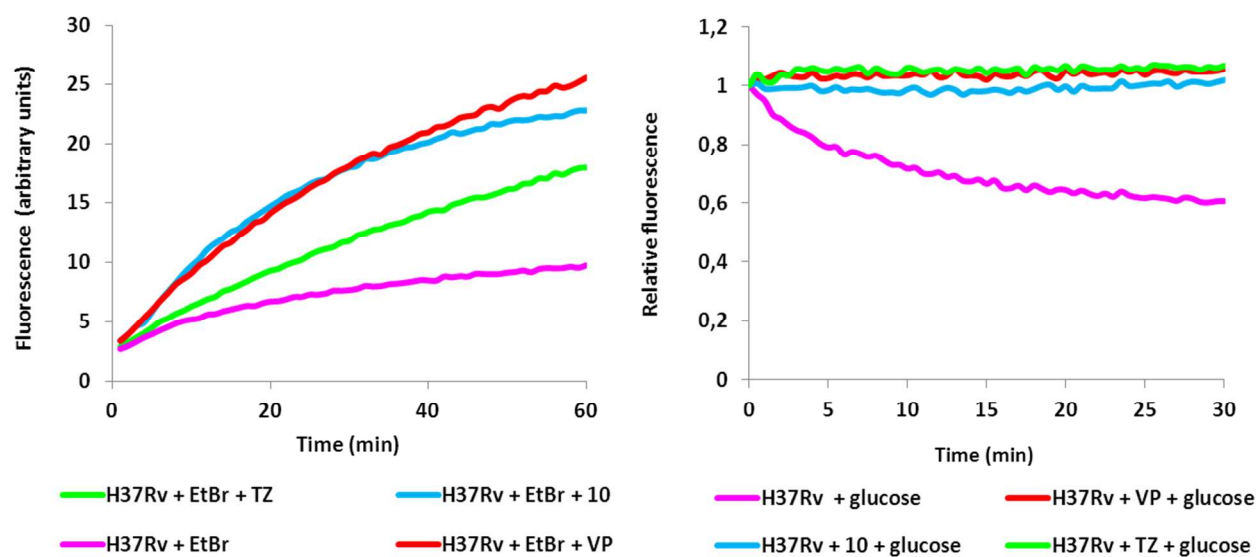
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**Figure 1.** Structure of thioridazine and verapamil. In red the backbone that the two molecules share is shown.

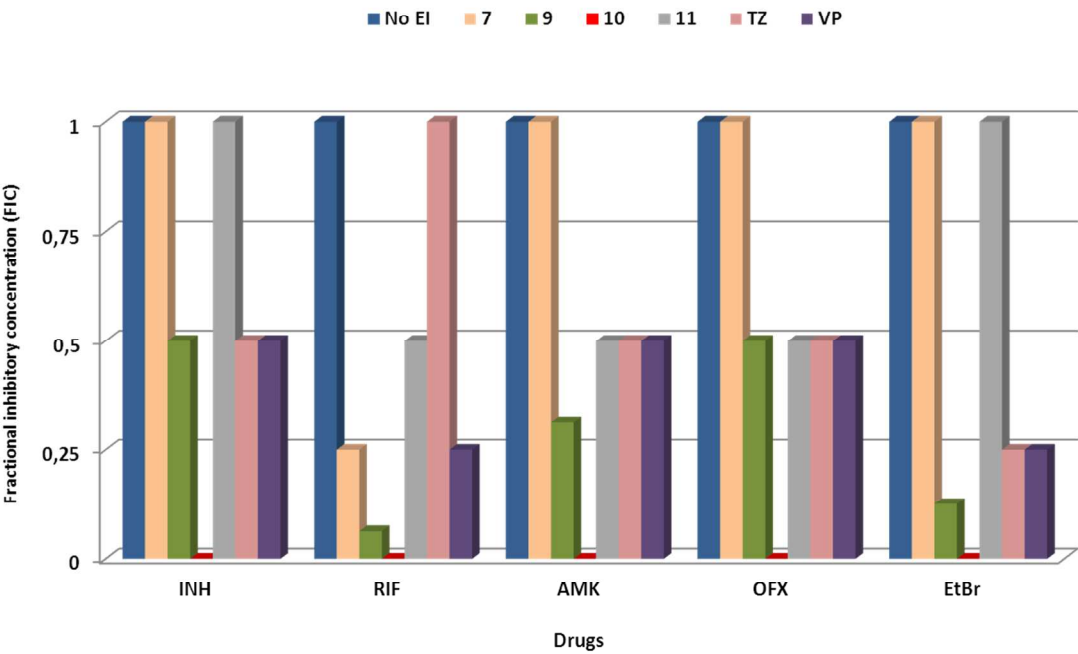


**Figure 2.** Effect of thioridazine (TZ), verapamil (VP) and compound 10 on the accumulation (left) and efflux (right) of ethidium bromide (EtBr) at 0.5  $\mu\text{g/mL}$  by *M. tuberculosis* H37Rv. **Left panel:** *M. tuberculosis* H37Rv was loaded with 0.5  $\mu\text{g/mL}$  of EtBr with and without compounds. Verapamil (VP) and thioridazine (TZ) were tested at  $\frac{1}{2}$  of their MIC whereas compound **10** was tested at 256  $\mu\text{g/mL}$ , (see text for details). The increase in the fluorescence is directly proportional to the amount of EtBr that is retained within the cells. **Right panel:** *M. tuberculosis* H37Rv was loaded with 0.5  $\mu\text{g/mL}$  of EtBr in the presence of VP at  $\frac{1}{2}$  of their MIC. Efflux of EtBr took place at 37  $^{\circ}\text{C}$  in the presence of glucose and was inhibited in the presence of the compounds at the same concentrations used on the accumulation assays (compare pink curve – no inhibitor – with the remaining curves – with inhibitor).

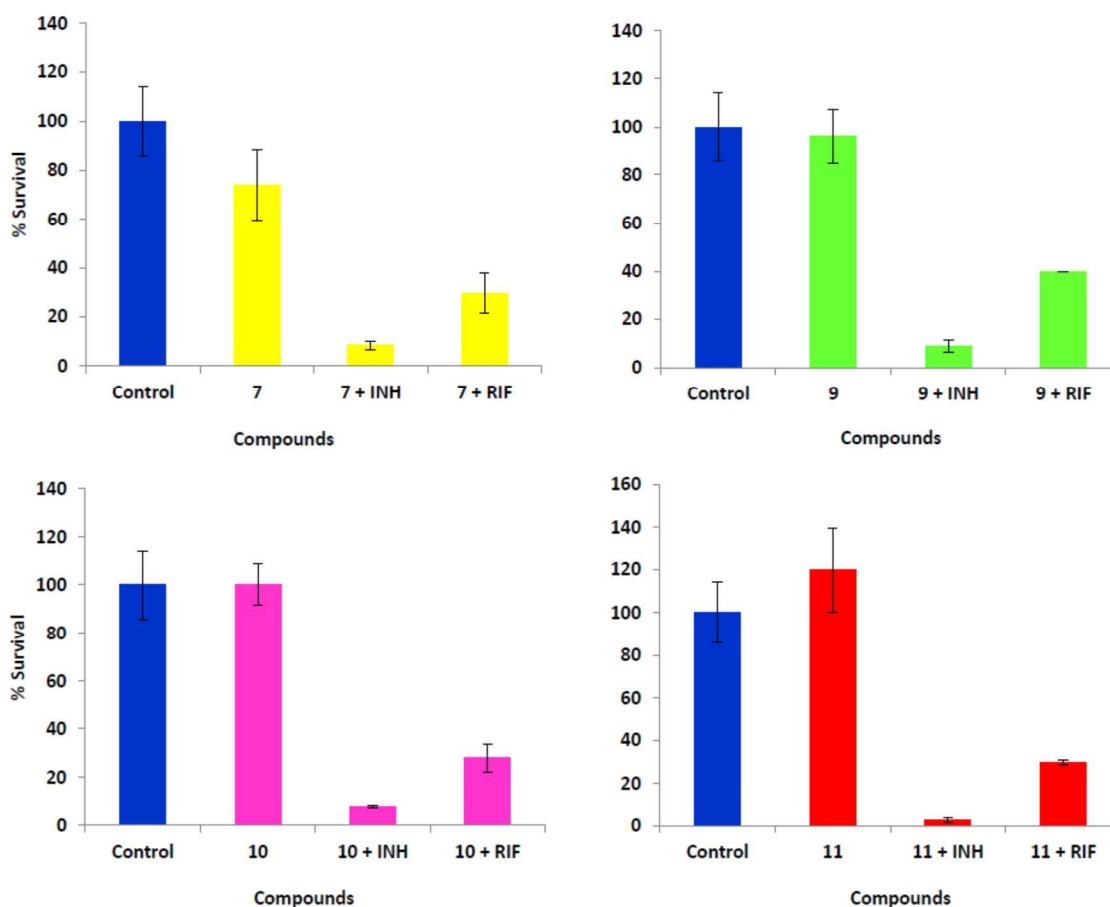




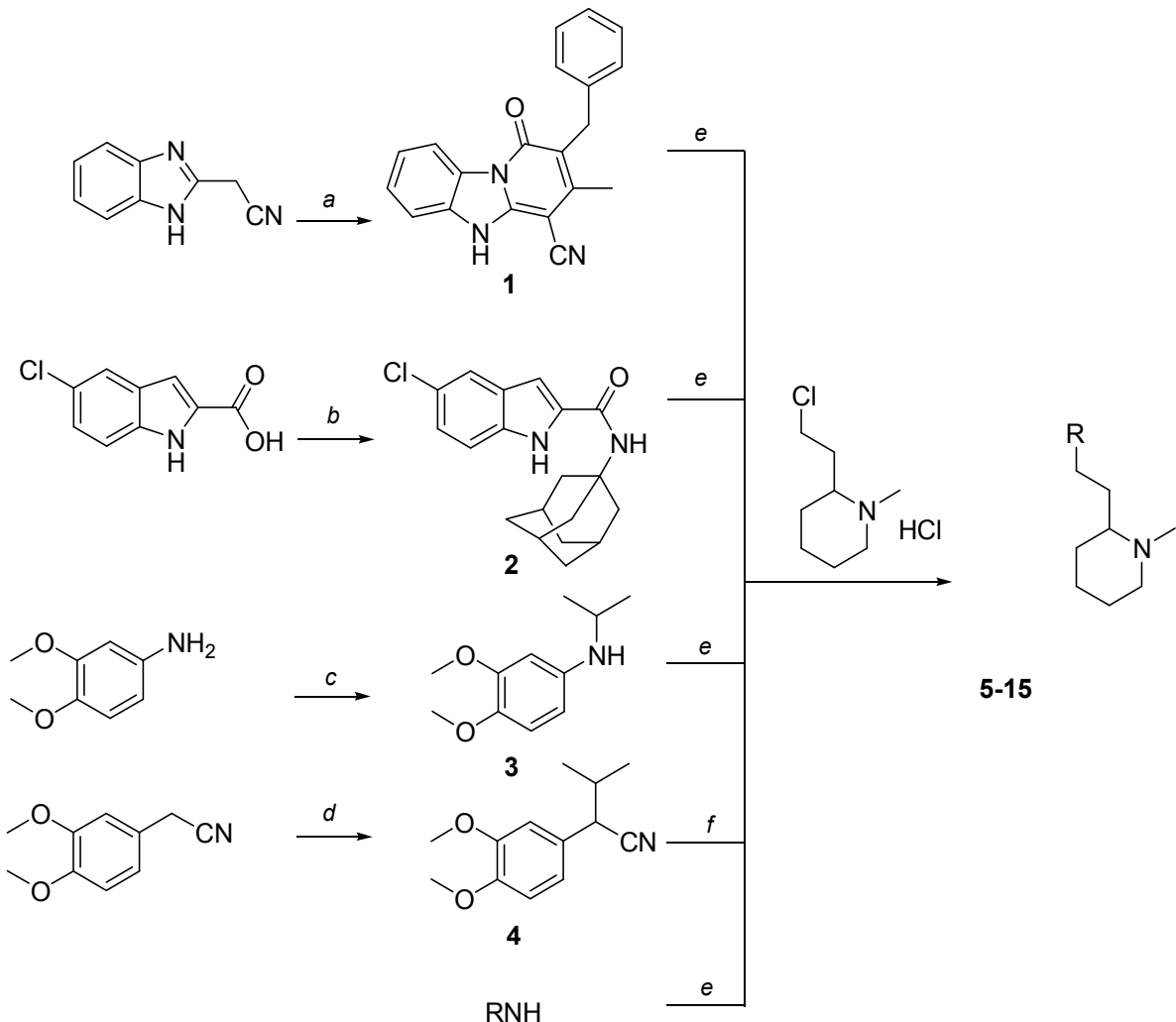
**Figure 3.** Fractional inhibitory concentration (FIC) determination for each combination against *M. tuberculosis* H37Rv. The effect of each compound on the activity of each antibiotic was represented by means of fractional inhibitory concentration (FIC) calculated as follows:  $FIC = MIC_{\text{antibacterial in combination}} / MIC_{\text{antibacterial alone}}$ . The FIC values were interpreted based on the criteria established as follows:  $FIC \leq 0.25$ , synergism;  $0.25 > FIC < 2$ , indifference and  $FIC \geq 2$ , antagonism.<sup>24</sup> The FIC value for the combinations was classified as ND (non-determinable) when the MICs of the compounds were greater than the highest, less than, or equal to the lowest concentration tested (Moody, 1992).<sup>57</sup> As such, FIC values were calculated at the following concentrations of the compounds: **7**, **10** and **11**, 128  $\mu\text{g/mL}$ ; **9**, 32  $\mu\text{g/mL}$ ; VP, 64  $\mu\text{g/mL}$ , and TZ, 3.75  $\mu\text{g/mL}$  (see Table 3 for further information).



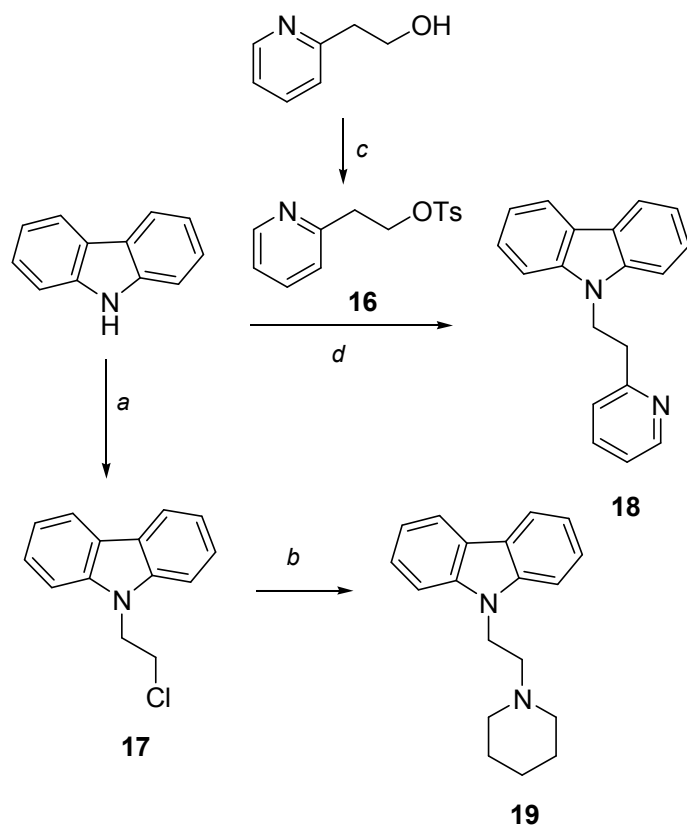
**Figure 4.** Determination of the intracellular activity of compounds **7**, **9**, **10** and **11** against *M. tuberculosis*-infected human macrophages. For the evaluation of the intracellular synergistic activity against *M. tuberculosis*-infected human monocyte-derived macrophages, INH and RIF were used at  $\frac{1}{2}$  of its MIC (0.05 and 0.5  $\mu\text{g/mL}$ , respectively). Macrophage viability was determined after 3 days of treatment with the compounds and for the subsequent intracellular assays, these were used at concentrations that were shown to be non-toxic for the macrophages: compound **7**, 16  $\mu\text{g/mL}$ , **9**, 0.078  $\mu\text{g/mL}$ ; **10**, 1.25  $\mu\text{g/mL}$ , and **11**, 40  $\mu\text{g/mL}$ . Above these concentrations macrophage viability is reduced below 90%. The results are presented as a mean of the percentage of the survival  $\pm$  SD. (Figure S2)



**Scheme 1.**<sup>a,b</sup> Preparation of the thioridazine analogues.



<sup>a</sup>Reagents and conditions: a) Ammonium acetate, toluene,  $\alpha$ -benzyl-ethylacetoacetate, 150 °C, 1.5 h, 40%; b) 1-aminoadamantane, EDC·HCl, HOBT, triethylamine, DMF, rt, 24h, 49%; c) Isopropyl iodide, triethylamine, MeOH, reflux, 18h, 50%; d) Isopropyl bromide, NaH, DMF, rt, 18h, 47%; e) RNH, NaH, DMF, reflux, 18-72h, 9-70%; f) NaNH<sub>2</sub>, Toluene,  $\mu$ W, 110 °C, 30 min, 50%. <sup>b</sup>For complete structures see chart 1.

**Scheme 2.**<sup>a</sup> Preparation of the carbazole derivatives.

<sup>a</sup>Reagents and conditions: *a*) Dichloroethane, KOH, K<sub>2</sub>CO<sub>3</sub>, TBAF, 60 °C, 18h, 40%; *b*) piperidine, K<sub>2</sub>CO<sub>3</sub>, DMF, 60°C, 24h, 44%; *c*) *p*-Toluensulfonyl chloride, triethylamine, DCM, rt, 18h, 75%; *d*) NaH, DMF, rt, 24h, 4%.

comp	R	comp	R	comp	R
5		9		13	
6		10		14	
7		11		15	
8 <sup>b</sup>		12			

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**Table 1.** Screening of the antimycobacterial and efflux inhibitory activity of the compounds against *M. smegmatis* mc2 155 and their toxicity assessed by the index of cytotoxicity (IC) towards human monocyte derived macrophages.

comp	<i>M. smegmatis</i> mc <sup>2</sup> 155		IC <sub>50</sub> <sup>c</sup> µg/mL(µM)
	MIC (µg/mL) <sup>a</sup>	RFF ± SD <sup>b</sup>	
<b>5</b>	>64 <sup>d</sup>	0.66 ± 0.13*	nd <sup>e</sup>
<b>6</b>	>256 <sup>d</sup>	0.17 ± 0.14	nd
<b>7</b>	>256 <sup>d</sup>	<b>1.17 ± 0.46*</b>	84.8 (264.7)
<b>8</b>	>256 <sup>d</sup>	0.72 ± 0.19*	nd
<b>9</b>	<b>256</b>	<b>0.95 ± 0.15*</b>	8.8 (30)
<b>10</b>	<b>64</b>	<b>0.81 ± 0.27*</b>	10.5 (32.51)
<b>11</b>	>256 <sup>d</sup>	<b>15.34 ± 0.82***</b>	170.8 (701.8)
<b>12</b>	64	<b>0.94 ± 0.079**</b>	0.762 (2.37)
<b>13</b>	>64 <sup>d</sup>	0.61 ± 0.16*	nd
<b>14</b>	256	0.76 ± 0.04**	nd
<b>15</b>	>256 <sup>d</sup>	0.39 ± 0.16*	nd
<b>18</b>	>64	-0.17 ± 0.16	nd
<b>19</b>	>256 <sup>d</sup>	0.08 ± 0.18	nd
<b>TZ</b>	<b>30</b>	<b>0.82 ± 0.1*</b>	5.6 (13.78)
<b>VP</b>	<b>800</b>	<b>2.21 ± 0.14**</b>	50.5 (116.6)

<sup>a</sup> Determined by microdilution; <sup>b</sup> Relative final fluorescence based on accumulation of EtBr at 0.25 µg/mL; the results are presented as the average of three independent assays plus standard deviation (± SD). The results were considered significant when \*P<0.05 and highly significant when \*\*P<0.01 and \*\*\*P<0.001; <sup>c</sup> Index of Cytotoxicity (IC) determined in human monocyte derived macrophages; <sup>d</sup> Due to the reduced solubility of the compounds it was not possible to test at higher concentrations; <sup>e</sup> not determined.

**Table 2.** Evaluation of the antimycobacterial and efflux inhibitory activity of the selected compounds against *M. tuberculosis* H37Rv.

comp	<i>M. tuberculosis</i> H37Rv	
	MIC (µg/mL) <sup>a</sup>	RFF ± SD <sup>b</sup>
7	>256 <sup>c</sup>	0.84 ± 0.02*
9	128	0.33 ± 0.11
10	>256 <sup>c</sup>	1.22 ± 0.09*
11	>256 <sup>c</sup>	1.00 ± 0.19
TZ	15	0.27 ± 0.02
VP	512	1.94 ± 0.005**

<sup>a</sup> Determined by microdilution; <sup>b</sup> Relative final fluorescence (RFF) based on accumulation of EtBr at 0.5 µg/mL; the results are presented as the average of three independent assays plus standard deviation (± SD). The results were considered significant when \*P<0.05 and highly significant when \*\*P<0.01; <sup>c</sup> Due to the reduced solubility of the compounds it was not possible to test at higher concentrations.

**Table 3.** Evaluation of the synergistic effect and determination of the modulation factor (MF) of the selected compounds with first- and second- line drugs against *M. tuberculosis* H37Rv.

comp	test conc <sup>a</sup>	MIC (μg/mL) against <i>M. tuberculosis</i> H37Rv (MF) <sup>g</sup>				
		INH <sup>b</sup>	RIF <sup>c</sup>	AMK <sup>d</sup>	OFX <sup>e</sup>	EtBr <sup>f</sup>
<b>no comp</b>		0.1	1	2	2	12.5
<b>7</b>	256	0.1	<0.0156(>↓64)	2	1	12.5
	128	0.1	0.25(↓4)	2	2	12.5
	64	0.1	0.25(↓4)	2	2	12.5
	32	0.1	0.25(↓4)	2	2	12.5
	16	0.1	0.25(↓4)	2	2	12.5
	8	0.1	0.25(↓4)	2	2	12.5
	4	0.1	0.25(↓4)	2	2	12.5
<b>9</b>	64	<0.00078 (↓>128)	<0.0156(>↓64)	<0.0156 (>↓128)	<0.0156(>↓128)	<0.195 (>↓64)
	32	0.05	0.0625 (↓16)	0.625 (↓4)	1	1.56 (↓4)
	16	0.1	0.5	1	1	6.25
	8	0.1	0.5	1	1	12.5
	4	0.1	1	1	1	12.5
	2	0.1	1	1	1	12.5
	1	0.1	1	1	1	12.5
<b>10</b>	256	<0.00078 (↓>128)	<0.0156(>↓64)	<0.0156 (>↓128)	<0.0156(>↓128)	<0.195 (>↓64)
	128	<0.0015 (>↓64)	<0.0156(>↓64)	<0.0156 (>↓128)	<0.0156(>↓128)	<0.195 (>↓64)
	64	0.05	<0.0156(>↓64)	>0.0156 (>↓128)	<0.0156(>↓128)	1.56 (↓8)
	32	0.1	0.0312(↓32)	1	1	6.25
	16	0.1	0.0625(↓16)	1	1	12.5
	8	0.1	0.25(↓4)	1	1	12.5
	4	0.1	0.5	1	1	12.5
<b>11</b>	256	<0.00078 (↓>128)	<0.0156(>↓64)	<0.0156 (>↓128)	<0.0156(>↓128)	<0.195 (>↓64)
	128	0.1	0.5	1	1	12.5
	64	0.1	0.5	1	1	12.5
	32	0.1	0.5	1	1	12.5
	16	0.1	0.5	1	1	12.5
	8	0.1	0.5	1	1	12.5
	4	0.1	0.5	1	1	12.5
<b>TZ</b>	7.5	<0.00078 (↓>128)	<0.0156(>↓64)	<0.0156 (>↓128)	<0.0156(>↓128)	<0.195 (>↓64)
	3.75	0.05	1	1	1	3.125 (↓4)
	1.88	0.1	1	1	1	6.25



	0.94	0.1	1	1	1	6.25
	0.47	0.1	1	1	1	6.25
	0.23	0.1	1	1	1	6.25
	0.12	0.1	1	1	1	6.25
	128	<0.00078 (↓>128)	<0.0156(>↓64)	<0.0156 (>↓128)	<0.0156(>↓128)	<0.195 (>↓64)
	64	0.05	0.25 (↓4)	1	1	3.125 (↓4)
	32	0.1	0.5	1	1	6.25
VP	16	0.1	0.5	1	1	6.25
	8	0.1	0.5	1	1	12.5
	4	0.1	1	1	1	12.5
	2	0.1	1	1	1	12.5

<sup>a</sup> Concentration at which the compound was tested (in µg/mL); <sup>b</sup> Isoniazid; <sup>c</sup> Rifampin; <sup>d</sup> Amikacin; <sup>e</sup> Ofloxacin; <sup>f</sup> Ethidium bromide; TZ, thioridazine; VP, verapamil. <sup>g</sup> modulation factor. The modulation factor (MF) was calculated with the following formula:  $MF = \frac{MIC_{antibiotic}}{MIC_{combination}}$  The modulation factor reflects a reduction of the MIC values of a given antibiotic in the presence of an inhibitor and was considered significant when  $MF \geq 4$  (≥four-fold reduction). All experiments were repeated at least three times.

**Supporting Information Available:** <sup>1</sup>H NMR, <sup>13</sup>C NMR description of compounds **5**, **6**, **9-15**, **18**, the synthesis of compounds **1-4**, **16**, **17** and the biological methods used for the determination of the MIC, the RFF and the synergistic activity are reported in the supporting information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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#### Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. # These authors contributed equally.

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## Abbreviations

AMK, amikacin; DMF, *N,N*-dimethyl formamide; DOTS, directly observed therapy short-course; EI, efflux inhibitor; INH, isoniazid; MIC, minimum inhibitory concentration; MDR-TB, multidrug-resistant tuberculosis; *Mtb*, *Mycobacterium tuberculosis*; RIF, rifampicin; TB, tuberculosis; TEA, triethylamine ; XDR-TB, extensively drug-resistant tuberculosis.

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