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Boosting Effect of 2-Phenylquinoline Efflux Inhibitors in Combination with Macrolides against *Mycobacterium smegmatis* and *Mycobacterium avium*

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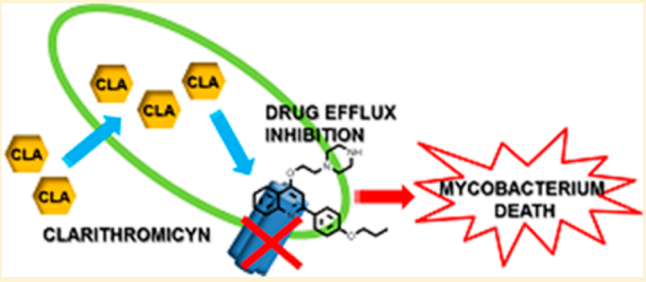
Supporting Information

ABSTRACT: The identification of efflux inhibitors to be used as adjuvants alongside existing drug regimens could have a tremendous value in the treatment of any mycobacterial infection. Here, we investigated the ability of four 2-(4′-propoxyphenyl)quinoline *Staphylococcus aureus* NorA efflux inhibitors (**1**−**4**) to reduce the efflux activity in *Mycobacterium smegmatis* and *Mycobacterium avium* strains. All four compounds were able to inhibit efflux pumps in both mycobacterial species; in particular, *O*-ethylpiperazinyl derivative **2** showed an efflux inhibitory activity comparable to that of verapamil, the most potent mycobacterial efflux inhibitor reported to date, and was able to significantly reduce the MIC values of macrolides against different *M. avium* strains. The contribution of the *M. avium* efflux pumps MAV_1406 and MAV_1695 to clarithromycin resistance was proved because they were found to be overexpressed in two *M. avium* 104 isogenic strains showing high-level clarithromycin resistance. These results indicated a correlation between increased expression of efflux pumps, increased efflux, macrolide resistance, and reduction of resistance by efflux pump inhibitors such as compound **2**. Additionally, compound **2** showed synergistic activity with clarithromycin, at a concentration below the cytotoxicity threshold, in an ex vivo experiment against *M. avium* 104-infected macrophages. In summary, the 2-(4′-propoxyphenyl)quinoline scaffold is suitable to obtain compounds endowed with good efflux pump inhibitory activity against both *S. aureus* and nontuberculous mycobacteria.



KEYWORDS: *nontuberculous mycobacteria, Mycobacterium avium complex, antimicrobial resistance, efflux pump, drug synergism*

INTRODUCTION

Although *Mycobacterium tuberculosis* infections have been commonly recognized as a worldwide threat,[1,2] other mycobacterial species also cause severe illness in humans. The increasing importance of nontuberculous mycobacteria (NTM) in clinical infections is now generally recognized worldwide.[3−6] NTM are ubiquitous, environmental, and biologically diverse microorganisms, some of which are associated with chronic and progressive pulmonary infections in susceptible individuals.[7,8] Treatment of infections caused by NTM poses a great clinical challenge because it is generally longer, costlier, more toxic, and significantly more likely to fail than tuberculosis therapy.[9] NTM intrinsic drug resistance and their predisposition to develop resistance during treatment[9] are the main causes for the lack of correlation between the in vitro susceptibility testing and the in vivo clinical response to the antimycobacterial drugs.[7,9] Nevertheless, introduction of the newer macrolides, clarithromycin (**CLA**) and azithromycin (**AZT**), constitutes a major therapeutic advance in the treatment of NTM infections. In fact, only for macrolides, a correlation between the in vitro activity and in vivo clinical response against *Mycobacterium avium* complex (MAC) infections has been demonstrated.[10,11]

M. avium spp. *hominissuis* and *Mycobacerium intracellulare* (grouped together in MAC) are the most frequent etiologic agents of pulmonary disease caused by NTM worldwide.[5,9,12,13] Their clinical relevance is arising due to severe opportunistic infections in human immunodeficiency virus (HIV) infected patients and other immunocompromised individuals (*M. avium*) as well as in immunocompetent individuals (*M. intracellulare*).[14−16] Furthermore, MAC is one of the most common isolated species in cystic fibrosis and chronic obstructive pulmonary disease patients[17,18] as well as in skin, soft-tissue infections, and lymphadenitis.[19] The recommended treatment for MAC infections consists of a macrolide, such as **AZT** or **CLA**, in combination with ethambutol and a rifamycin during at least one

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year until culture conversion.[7] Clinical outcomes for MAC infections depend significantly on the macrolide susceptibility of the infecting strain. Unfortunately, resistance to macrolides develops easily during the therapy.[20,21]

Recently, it has been reported that in Europe, North and South America, and Asia *M. avium* is significantly more prevalent, whereas in Australia and South Africa *M. intracellulare* prevails as the major cause of pulmonary disease caused by NTM.[13] *M. avium* spp. *hominissuis* (*M. avium* hereafter) is the most prevalent NTM in Europe, including Portugal.[6,13] To develop more efficient strategies for the treatment of *M. avium* infections, attention has been devoted to better comprehend its remarkable ability to acquire resistance and in particular the mechanisms involved in the macrolide resistance.

Efflux pump activity contributes to both intrinsic and acquired resistance to antibiotics of several bacterial species, including mycobacteria. In fact, this mechanism of resistance can also be induced upon drug exposure.[22,23] *M. avium* efflux activity has been related with macrolide-resistant phenotypes[24] and has been shown to be inhibited by phenothiazines and verapamil (**VP**).[24−26] Genomic analysis of *M. avium* showed the presence of several efflux pumps belonging to almost all of the known superfamilies such as the ATP-binding cassette (ABC), major facilitator (MF), resistance-cell-division-nodulation (RND), and small multidrug resistance (SMR). Recently, three efflux pumps, MAV_1406 (MF), MAV_1695, and MAV_3306 (ABC), induced by **AZT** exposure and related to the appearance of low-level resistance phenotypes, have been reported.[22] Thus, the identification of an efflux pump inhibitor (EPI) that could be used as an adjuvant for antimicrobial therapy will have a tremendous value in the treatment of any mycobacterial infection.[27]

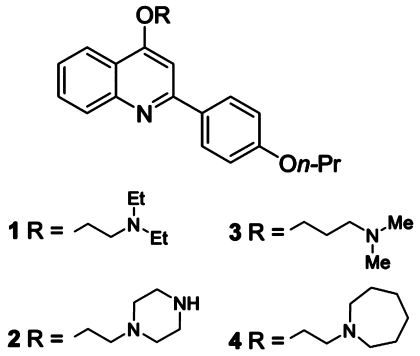

Figure 1. NorA EPIs evaluated against mycobacterial species.

Although limited similarities have been found by comparison of the primary sequence of MAV_1406 with that of NorA, the most studied efflux pump of *Staphylococcus aureus*,[28] some aspects are shared. They are (i) members of the MF superfamily, (ii) overexpressed after drug exposure, and (iii) involved in increased resistance to common efflux substrates such as ethidium bromide (EtBr) and quinolones.[29,30] These facts as well as literature data showing the correlation between the efflux activity and resistance to **CLA** in *M. avium*[22,24,25] led us to translate our previous experience on the identification of NorA inhibitors[31−37] toward the search for effective *M. avium* EPIs. In this work, we evaluate a small set of the most promising 2-(4′-propoxyphenyl)quinoline NorA EPIs (compounds **1**−**4**, Figure 1),[34,37] belonging to our large in-house collection,[31−37] for their efflux pump inhibition activity against both *M. smegmatis* and *M. avium* reference and clinical strains (Table 1). Structurally, they share a common 2-(4′-propoxyphenyl)-quinoline scaffold decorated with different *O*-alkylamino chains at the C-4 position (Figure 1). As a screening model we initially used *M. smegmatis* mc^{2}155 strain.[38] *M. smegmatis*, which expresses several efflux pumps with significant homology with those of *M. avium*, including MAV_1406, is a fast-growing mycobacterium that allows reliable results in a reduced time frame. Because all of the tested compounds showed different degrees of efflux pump inhibition activity against *M. smegmatis* mc^{2}155 strain,[38] they were also evaluated against *M. avium* 104 strain, a commonly used and well-characterized clinical strain.[39] Being the most promising EPI, compound **2** was further investigated against two *M. avium* 104 isogenic strains, obtained in our laboratory and presenting high-level **CLA** resistance (*M. avium* 104$_{CLA3}$ and *M. avium* 104$_{CLA4}$) as well as on a panel of four *M. avium* clinical strains.

RESULTS AND DISCUSSION

To set up the test, the 2-(4′-propoxyphenyl)quinolines **1**−**4** (Figure 1) were preliminarily assayed for their ability to inhibit EtBr efflux on *S. aureus* ATCC25923 (wild-type) and *S. aureus* ATCC25923$_{EtBr}$ (overexpressing NorA) by a real-time fluorometric assay.[29,30] At half of their MICs, all of the compounds increased the accumulation of EtBr, mainly in *S. aureus* ATCC25923$_{EtBr}$ (data not shown). All of these data highlighted

Table 1. Description of the Strains Used in This Study

strain	description	MICs, μg/mL		
		CLA	**ERY**	**AZT**
M. smegmatis mc^{2}155 (ATCC700084)	wild-type[a]	8	256	32
M. avium ATCC25291^T	wild-type[b]	8	128	64
M. avium 104	wild-type[c]	8	256	>512
M. avium 104$_{CLA3}$[d]	resistant to **CLA** due to overexpression of efflux pumps[e]	>512	>512	>512
M. avium 104$_{CLA4}$[d]	resistant to **CLA** overexpressing efflux pumps[e] and harboring the mutation A-2059G in 23S rRNA	>512	>512	>512
M. avium 386/08	wild-type	4	256	32
M. avium 47/07	resistant to **CLA** overexpressing efflux pumps[f] and harboring the mutation A-2058G in 23S rRNA[g]	>1024	>512	>512
M. avium HSB2	wild-type[f]	16	512	256
M. avium HSB3	wild-type[f]	8	512	256

[a]Reference 38. [b]Reference 40. [c]Reference 39. [d]*M. avium* 104 resistant to **CLA** obtained by serial passage of *M. avium* 104 in medium containing 2 μg/mL of **CLA**. [e]See Figure 3 for details. [f]See text for details. [g]Reference 24. **CLA** breakpoints: susceptible, ≤16 μg/mL; intermediate, 32 μg/mL; resistant ≥64 μg/mL.[41]

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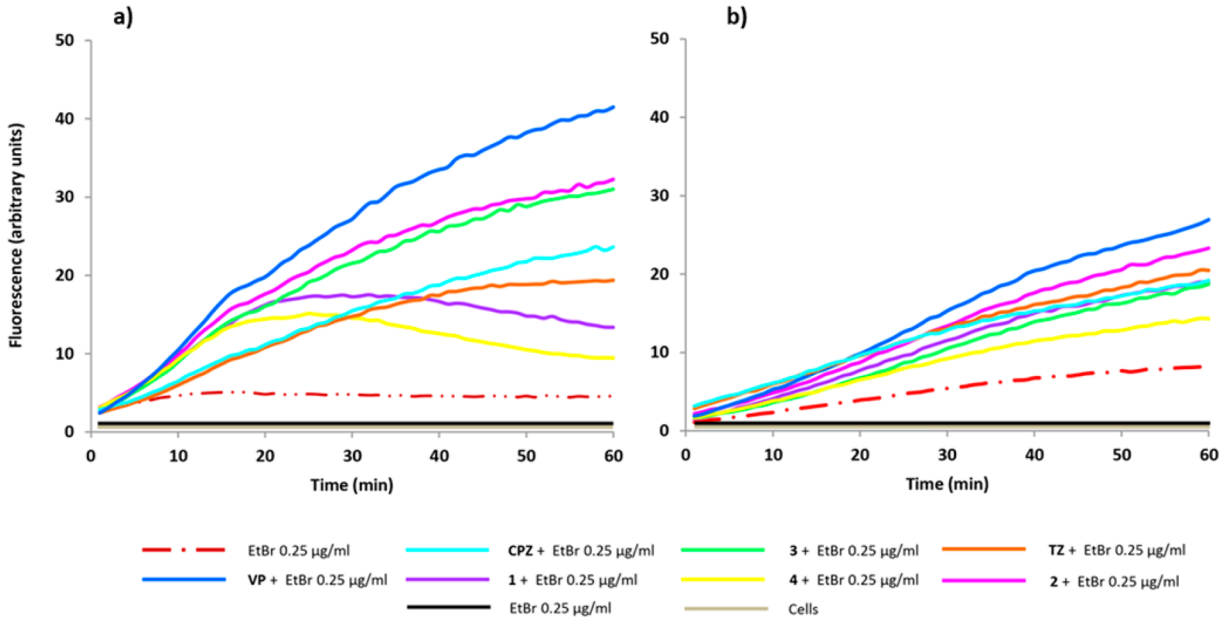

Figure 2. Effect of the 2-(4′propoxyphenyl)quinolines EPIs **1**−**4** on the EtBr accumulation by (a) *M. smegmatis* mc^{2}155 and (b) *M. avium* 104. Accumulation assays were performed in the presence of glucose with the tested compounds **1**−**4** and **VP**, **TZ**, and **CPZ** as controls at half of their MIC (for MIC values see Table S-1).

their strong efflux pump inhibitory activity against NorA, in agreement with what has been previously reported.[34,37] Then, we further proceeded with the screening of compounds **1**−**4** for their mycobacterial efflux pump inhibitory activity against *M. smegmatis* mc^{2}155 strain.

Effect of the 2-(4′-Propoxyphenyl)quinolines 1−4 against *M. smegmatis* mc^{2}155 Strain. *Effect of Compounds 1−4 on Accumulation and Efflux of EtBr.* The lowest concentration of EtBr that resulted in equilibrium between influx and efflux of EtBr by *M. smegmatis* mc^{2}155 was 0.25 μg/mL. This concentration of EtBr is deemed to be close to that able to saturate the intrinsic efflux systems. To evaluate whether 2-(4′-propoxyphenyl)quinoline compounds (**1**−**4**) possess inhibitory effects on *M. smegmatis* efflux systems, all compounds were tested at half of their MICs (Table S-1) in order to not compromise cell viability. Known mycobacterial EPIs such as **VP**, thioridazine (**TZ**), and chlorpromazine (**CPZ**) were included in the assays for comparative purpose.[23−26]

In Figure 2a, we can observe the effect of compounds **1**−**4** on the accumulation of EtBr by *M. smegmatis* exposed to 0.25 μg/mL of EtBr in the presence of glucose as energy source. All compounds were able to promote accumulation of EtBr (Figure 2a), with *O*-ethylpiperazinyl and *O*-propyldimethylamine derivatives (**2** and **3**, respectively) being the most effective.

The efflux inhibitory activity of each compound was expressed through the calculation of the relative final fluorescence (RFF) (Table 2). This value is a measure of the EtBr efflux inhibitory activity obtained by comparing the final fluorescence of the cells exposed to EtBr in the presence of the EPI (treated) normalized against the cells exposed only to EtBr (control).[42] The greater is the difference between the final fluorescence (min 60) of the treated cells and the control, the greater is the amount of EtBr accumulated inside the cells, which means a greater efflux pump inhibitory activity. The effect of the EPIs in the accumulation of EtBr was interpreted as follows: RFF values above zero indicated that cells (treated) accumulate more EtBr than those of the control (nontreated cells). Negative RFF values indicated that treated cells accumulated less EtBr than those of the control.

As can be observed in Table 2, all compounds, except **4**, showed high RFF values with compounds **2** and **3** endowed with the highest RFF values of 5.91 and 5.84, respectively ($P < 0.01$). The inhibitory activity of both compounds is comparable to that of **CPZ** and better than that of **TZ**. These results highlighted that the 2-phenylquinoline compounds **2** and **3** are the two most effective inhibitors of EtBr efflux by *M. smegmatis* pumps and showed their potential as mycobacterial EPIs.

*Effect of Compounds 2 and 3 against M. smegmatis mc^{2}155 Strain in the Presence of **CLA**.* Macrolides are an important component of NTM therapy, mainly of MAC therapy. For the screening of the synergistic activity of compounds **2** and **3** with macrolides against *M. smegmatis*, we chose **CLA** as the representative of the class. Compounds **2** and **3** were evaluated through synergism assays between **CLA** and increasing concentrations of both compounds, ranging from $^1/_{64}$ to $^1/_2$ of their respective MICs (see Table S-1). Compound **3** was able to reduce the MIC of **CLA** from 8 to 1 μg/mL (8-fold reduction) at half of its MIC, whereas the *O*-ethylpiperazinyl compound **2** was able to reduce the MIC of **CLA** from 8 μg/mL to <0.125 μg/mL (above 64-fold reduction) at half of its MIC and to 4 μg/mL (2-fold reduction) at one-fourth of the MIC (Table 3). The synergistic activity of each EPI with the tested macrolide or EtBr was determined through the calculation of the fractional inhibitory concentrations (FIC) that was interpreted as follows: FIC ≤ 0.25, synergism; 0.25 < FIC < 2, indifference; and FIC ≥ 2, antagonism. The FIC value for the combinations was classified as non-determinable (ND) when the MICs of the compounds were greater than the highest, less than to the lowest concentration tested.[43] The FIC value determined for the combination of compound **2** at half MIC plus **CLA** was ND. This result indicates a highly synergistic activity for compound **2** because the MIC values of **CLA** were reduced below 0.125 μg/mL.

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Table 2. Relative Final Fluorescence (RFF) Values Based on the Accumulation of EtBr against the Test Strains in the Presence of the Efflux Inhibitors[a]

	RFF ± SD							
compd	*M. smegmatis* mc²155	*M. avium* ATCC25291^T	*M. avium* 104	*M. avium* 104_{CLA3}	*M. avium* 104_{CLA4}	*M. avium* 386/08	*M. avium* 47/07	*M. avium* HSB2	*M. avium* HSB3
1	**1.65 ± 0.44****	–	0.76 ± 0.16*	–	–	–	–	–	–
2	**5.91 ± 0.35****	0.85 ± 0.24*	**1.23 ± 0.10****	**2.11 ± 0.60***	**1.05 ± 0.22****	**2.48 ± 0.41***	**1.00 ± 0.15****	**2.85 ± 0.75***	0.71 ± 0.26*
3	**5.84 ± 0.05****	–	0.77 ± 0.11**	–	–	–	–	–	–
4	0.98 ± 0.17**	–	0.29 ± 0.18	–	–	–	–	–	–
VP	**7.48 ± 1.01****	0.91 ± 0.07*	**1.77 ± 0.16****	**1.43 ± 0.16****	**1.58 ± 0.37****	**2.63 ± 0.21***	**1.51 ± 0.20****	**3.54 ± 1.10***	**1.37 ± 0.08*****
TZ	**2.49 ± 1.14***	0.33 ± 0.13*	0.80 ± 0.24*	**1.15 ± 0.14****	0.59 ± 0.01**	**1.84 ± 0.76***	0.83 ± 0.12**	1.08 ± 0.23	0.52 ± 0.11*
CPZ	**4.04 ± 0.27****	0.29 ± 0.14	0.77 ± 0.17*	0.33 ± 0.13*	0.60 ± 0.18*	**1.84 ± 0.01**	**1.18 ± 0.16****	0.56 ± 0.20	0.70 ± 0.33

[a]Values in boldface (>1) indicate enhanced accumulation of EtBr in the presence of EPIs. Each assay was performed in triplicate, and the results presented correspond to the average of three independent assays plus standard deviation (±SD). Results were considered significant when (*) $P < 0.05$ and highly significant when (**) $P < 0.01$ and (***) $P < 0.001$. (–) not tested.

Table 3. Synergistic Activity of Compounds 2 and 3 on MIC Values of CLA against *M. smegmatis* mc²155 Strain

	concn of EPI	CLA MIC, μg/mL (-fold reduction)/FIC
CLA (alone)		**8**
CLA + 2	1/2 MIC	<0.125 (↓>64)/ND
	1/4 MIC	4 (↓2)/0.5
	1/8 MIC	8/1
	1/16 MIC	8/1
	1/32 MIC	8/1
	1/64 MIC	8/1
CLA + 3	1/2 MIC	1 (↓8)/0.125
	1/4 MIC	8/1
	1/8 MIC	8/1
	1/16 MIC	8/1
	1/32 MIC	8/1
	1/64 MIC	8/1

Effect of the 2-(4′-Propoxyphenyl)quinolines 1−4 against *M. avium* Strains. *Effect of Compounds 1−4 on Accumulation and Efflux of EtBr by M. avium Strains.* Compounds **1**−**4** were then evaluated for their ability to inhibit EtBr efflux in *M. avium* 104, a clinical strain commonly used in mycobacterial research.[39] Similarly to that observed for *M. smegmatis*, compounds **1**−**3** were able to promote high levels of accumulation of EtBr, but at a lower extent than that observed for *M. smegmatis* (Figure 2b). Compounds **1** and **3** demonstrated similar activity to that observed for **TZ** and **CPZ**, with RFF values ranging from 0.76 to 0.8 (Table 2). Compound **2** demonstrated the most potent EtBr efflux inhibitory activity against *M. avium* 104 (RFF of 1.23, $P < 0.01$) comparable to that of **VP** (RFF of 1.77; $P < 0.01$), the most active reference compound. Due to the interesting EtBr efflux inhibitory activity of compound **2** against *M. avium* 104, we decided to further evaluate this compound against two *M. avium* 104 isogenic strains obtained in our laboratories, *M. avium* 104_{CLA3} and *M. avium* 104_{CLA4}, that show high-level resistance after prolonged exposure to **CLA**. Their high level of **CLA** resistance is due to the overexpression of two efflux pumps belonging to the MF and ABC transporters, namely, MAV_1406 and MAV_1695 (Figure 3), known to be associated with macrolide efflux.[22] On the basis of our previous experience we cannot exclude the contribution of other efflux pumps, belonging to these or to other classes, to the overall **CLA** resistance these strains.[23] In addition, *M. avium* 104_{CLA4} harbors a mutation in the macrolide target, the 23S rRNA (Table 1).

Whereas for *M. avium* 104 the steady-state accumulation level of EtBr took place at 0.25 μg/mL, for *M. avium* 104_{CLA3} and *M. avium* 104_{CLA4} the basal EtBr concentration was 0.50 μg/mL, in agreement with the efflux pumps overexpression. This means that both *M. avium* 104 isogenic resistant strains possess higher capability to handle increased concentrations of EtBr when compared to *M. avium* 104 (Figure 3b,c compared with 3a). Compound **2** has showed an EtBr efflux inhibitory activity comparable to that of **VP** and better than that of **TZ** and **CPZ** against both *M. avium* 104 and *M. avium* 104_{CLA4} strains (Figure 3a,c; Table 3). Interestingly, the highest EtBr efflux inhibitory activity of derivative **2** was observed against *M. avium* 104_{CLA3} strain with an RFF index of 2.11 (P value < 0.05), which is superior to that observed for **VP** (compare Figure 3b with 3a,c and Table 2). The potent efflux inhibitory activity of compound **2**

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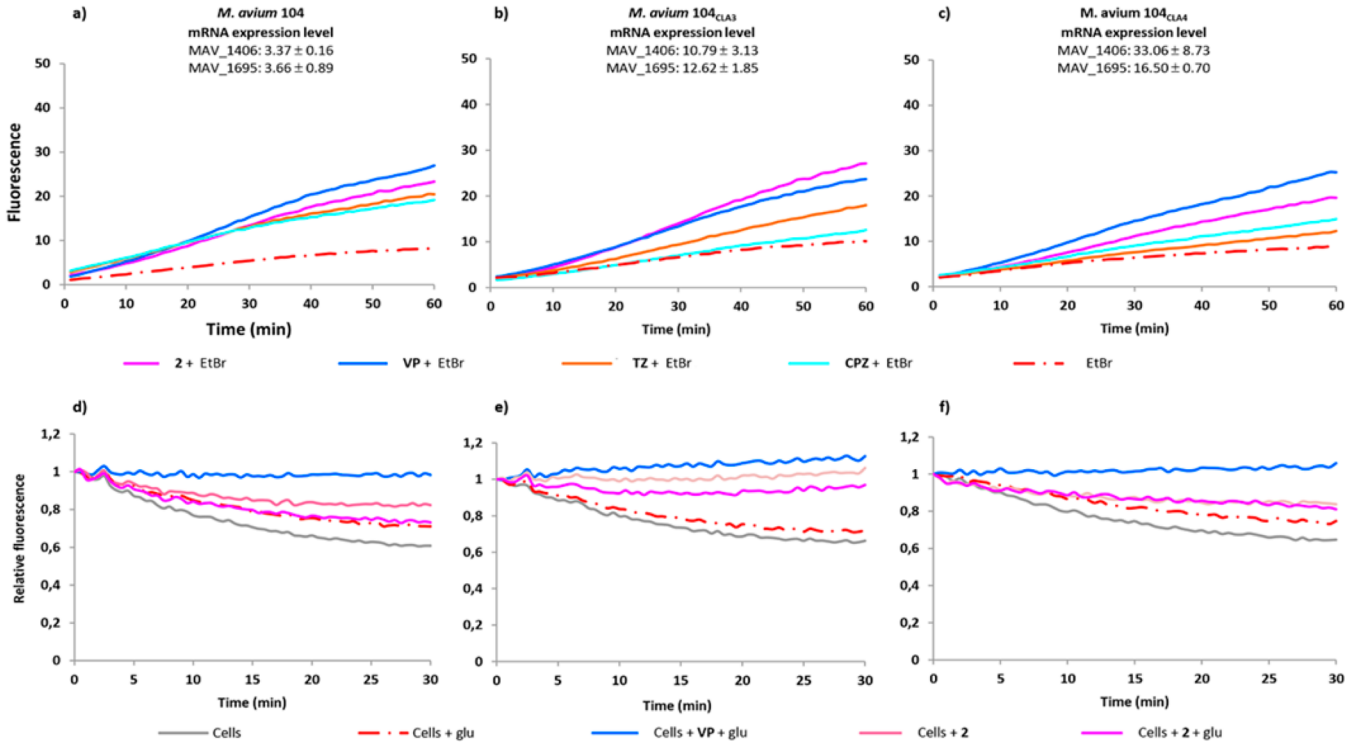

Figure 3. Accumulation assays of EtBr against (a) *M. avium* 104, (b) *M. avium* 104_{CLA3}, and (c) *M. avium* 104_{CLA4} strains in the presence of glucose with the tested compounds **2**, **VP**, **TZ**, and **CPZ**. Efflux assays of EtBr against (d) *M. avium* 104, (e) *M. avium* 104_{CLA3}, and (f) *M. avium* 104_{CLA4} strains for compounds **2** and **VP**. All of the compounds were tested at a concentration of half their MIC values (Table S-1). On the top of each graph is reported the mRNA expression level for the MAV_1406 and MAV_1695 efflux pumps.

was confirmed by the EtBr efflux assays (Figure 3d–f). In these assays, after **VP** has been removed from the cells (see Methods for details), compound **2** was able to inhibit efflux in a real-time manner in the three *M. avium* strains. In particular, the inhibition was higher against *M. avium* 104_{CLA3} than in *M. avium* 104 and *M. avium* 104_{CLA4} strains, following the same pattern observed in the accumulation assays (compare Figure 3a–c with 3d–f). Then, to assess the effect of compound **2** on other *M. avium* strains, we enlarged our study to a panel of four *M. avium* clinical strains with different phenotypes and genotypes as well as the *M. avium* ATCC25291^T strain, fully susceptible to **CLA** (Table 1). Data presented in Table 2 highlighted the capability of compound **2** to enhance EtBr accumulation in almost all *M. avium* strains tested; a lower effect in *M. avium* HSB3 and *M. avium* ATCC25291^T strains was observed. For the remaining strains the RFF values were comparable to those observed for **VP** and higher than those observed for **TZ** and **CPZ** (Table 2). Almost all of the tested compounds showed a weak intrinsic antimycobacterial activity with the only exception of compound **4**, which displayed interesting MIC values: the same (*M. smegmatis* mc^{2}155 and *M. avium* 104) or better (*M. avium* 104_{CLA3} and *M. avium* 104_{CLA4}) than those of **CLA** (Table S-1), coupled with a low level of EPI activity.

Effect of Compound 2 against M. avium Strains in the Presence of Macrolides and EtBr. Because compound **2** efficiently promotes accumulation and reduces efflux of EtBr on *M. avium* 104 and its isogenic strains resistant to **CLA**, we decided to expand our study to evaluate this compound for its synergistic activity in combination with different macrolides against the extended panel of clinical *M. avium* strains and, for comparative purpose, against *M. avium* ATCC25291^T strain. For these assays we have selected three macrolides: **CLA**, **AZT**, and erythromycin (**ERY**). The assays were performed at half and one-fourth of the MIC value of compound **2** (see Table S-1). The FIC values obtained for each combination are shown in Table 4. At half of its MIC value, compound **2** in combination with the three macrolides produced non-determinable FICs because the MIC values obtained were always out of the concentration range tested.

Compound **2**, at one-fourth of its MIC value, resulted in a significant synergistic activity with macrolides against some of the *M. avium* strains, except the resistant ones, with FIC values ranging from 0.0009 to 0.25. Against *M. avium* 104, compound **2**, at one-fourth of its MIC value, was also able to reduce the MIC of **CLA** by 8-fold, whereas 32- and 16-fold MIC reductions were observed in combination with **ERY** and **AZT**, respectively. The slight effect of compound **2** on the antimycobacterial activity of macrolides against *M. avium* 104_{CLA4} should be related to the presence, in this strain, of both the increased expression of efflux pumps and a mutation in the macrolide target, 23S rRNA. On the contrary, compound **2** was able to significantly reduce the resistance to **CLA** and **AZT** in *M. avium* 47/07, carrying a mutation in the 23S rRNA, and overexpress efflux pumps (mRNA expression levels MAV_1406, 39.25 ± 13.08; MAV_1695, 29.59 ± 9.46).[7−9] In the latter strain, for compound **2**, we cannot exclude the contribution of an additional mechanism involving cell membrane homeostasis, besides its EPI activity. For the remaining strains, several degrees of synergism were observed (Table 4).

With the non-antibiotic efflux substrate EtBr, all strains showed an increased susceptibility toward EtBr in the presence of compound **2** at half of its MIC with FICs values of 0.25 against *M. avium* ATCC25291^T, *M. avium* 104, and its isogenic strains and 0.125 against *M. avium* HSB3. FIC value was not

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Table 4. Synergistic Effect of Compound 2 on MIC Values of Macrolides and Ethidium Bromide for the *M. avium* Strains

	MICs, μg/mL (-fold reduction)/FIC							
	M. avium ATCC25291^T	*M. avium* 104	*M. avium* 104_{CLA3}	*M. avium* 104_{CLA4}	*M. avium* 386/08	*M. avium* 47/07	*M. avium* HSB2	*M. avium* HSB3
CLA	8	8	>512	>512	4	>1024	16	8
+ 2 at 1/2 MIC	**<0.0625 (↓>128)/ND**	**0.5 (↓16)/0.0625**	**32 (↓>16)/ND**	**512 (↓>2)/ND**	**<0.0312 (↓>128)/ND**	**<2 (↓>512)/ND**	**<0.0625 (↓>256)/ND**	**<0.0156 (↓>512)/ND**
+ 2 at 1/4 MIC	**<0.0625 (↓>128)/ND**	**1 (↓8)/0.125**	256 (↓>2)/ND	>512/ND	**0.25(↓8)/0.0625**	**1024 (↓>2)/ND**	**0.25 (↓64)/0.0156**	**2 (↓4)/0.0039**
ERY	128	256	>512	>512	256	>512	512	512
+ 2 at 1/2 MIC	**<1 (↓>128)/ND**	**<4 (>↓64)/ND**	**512 (↓>2)/ND**	**512 (↓>2)/ND**	**<0.125 (↓>2048)/ND**	**<2 (↓>256)/ND**	**<2 (↓>256)/ND**	**<1 (↓512)/ND**
+ 2 at 1/4 MIC	**32 (↓4)/0.25**	**8 (↓32)/0.0312**	>512/ND	>512/ND	**0.5(↓512)/0.0019**	>512/ND	**0.5 (↓1024)/0.0009**	**<1 (↓512)/ND**
AZT	64	>512	>512	>512	256	>512	256	256
+ 2 at 1/2 MIC	**<0.5 (↓>128)/ND**	**8 (↓64)/ND**	**512 (↓>2)/ND**	**512 (↓>2)/ND**	**<0.125 (↓>2048)/ND**	**<2 (↓>256)/ND**	**<2 (↓>128)/ND**	**<0.5 (↓512)/ND**
+ 2 at 1/4 MIC	64/1	**32 (↓16)/ND**	>512/ND	>512/ND	**4(↓64)/0.0156**	**512 (↓>2)/ND**	**1 (↓256)/0.0039**	**32 (↓8)/0.125**
EtBr	50	25	25	25	12.5	12.5	12.5	50
+ 2 at 1/2 MIC	**12.5 (↓>4)/0.25**	**6.25 (↓4)/0.25**	**6.25 (↓4)/0.25**	**6.25 (↓4)/0.25**	**<0.097 (↓>128)/ND**	**<0.097 (↓>128)/ND**	**<0.097 (↓>128)/ND**	**6.25 (↓8)/0.125**
+ 2 at 1/4 MIC	50/1	12.5 (↓2)/0.5	25/1	25/1	**3.125 (↓4)/0.25**	6.25(↓2)/0.5	6.25(↓2)/0.5	**12.5 (↓4)/0.25**

determinable against *M. avium* 386/08, 47/07, and HSB2 strains. On the contrary, compound **2** at one-fourth of its MIC demonstrated an indifferent effect for almost all of the strains with the exception of *M. avium* 386/08 and *M. avium* HSB3 (FIC values of 0.25).

Moreover, the results clearly indicated a correlation between increased expression of efflux pumps, increased efflux, macrolide resistance, and reduction of drug resistance by EPIs such as compound **2**. The expression of the efflux pumps was higher in *M. avium* 104_{CLA3} than in the parental strain *M. avium* 104; then, as expected, a greater reduction on the macrolide MIC values for *M. avium* 104_{CLA3} was observed. Due to their solubility problems, macrolides could not be tested at concentrations >512 μg/mL, and so we cannot establish the extent of their MIC reduction in the resistant strains.

Synergistic Activity of Compound 2 with CLA in *M. avium*-Infected Human Macrophages. Before evaluating the synergistic activity of compound **2** with **CLA** in *M. avium*-infected macrophages, we determined the in vitro cytotoxicity (CC_{50}) for both compounds alone against human monocyte-derived macrophages in order to know the nontoxic concentrations that can be used in the next step. Compound **2** showed a CC_{50} of 4.2 μg/mL, which is comparable to that of **TZ** (CC_{50} of 3.0 μg/mL),[44] whereas **CLA** was nontoxic up to a concentration of 20 μg/mL (Figure 4a,b). Then, we proceeded by evaluating the antimycobacterial activity in *M. avium* 104-infected macrophages of both compound **2** and **CLA** alone or in combination (Figure 4c). Because compound **2** was nontoxic for human macrophages at 2.5 μg/mL (Figure 4a), this concentration was selected to study its intracellular activity in *M. avium*-infected macrophages. Despite **CLA** was nontoxic up to a concentration of 20 μg/mL (Figure 4b), it was chosen to test such macrolide at 0.25 μg/mL, a concentration that is serum achievable, both alone and in the synergism assays, to allow a clear perception of the synergistic activity of compound **2** with a very low, non-antimicrobial, concentration of **CLA** against the intracellular *M. avium*. The results showed that compound **2** at 2.5 μg/mL concentration has only a weak intrinsic antimycobacterial activity because it was able to reduce the mycobacterial burden by only 11% after 3 days of treatment (Figure 4c). On the contrary, when compound **2** was tested in combination with **CLA** at 0.25 μg/mL, a significant antimicrobial potentiation of the macrolide activity was observed (7−10%).

In conclusion, this study evaluated the activity of four 2-(4′-propoxyphenyl)quinoline derivatives, previously reported as potent *S. aureus* NorA EPIs,[34,37] for both the inhibition of mycobacterial efflux pumps and the synergistic activity with macrolides against NTMs.[23−27] A stepwise screening process involving two mycobacterial species, *M. smegmatis* and *M. avium*, was employed. Among the tested compounds, *O*-ethylpiperazinyl derivative **2** emerged as the most potent EPI able to promote accumulation of EtBr against *M. smegmatis* mc^2155, *M. avium* 104, and its isogenic derivatives (*M. avium* 104_{CLA3} and *M. avium* 104_{CLA4}), as well as four *M. avium* clinical strains. Furthermore, a significant synergistic effect was observed upon combination of EPI **2** with different macrolides. Overall, our results showed that (i) compound **2** was able to strongly reduce the MIC values of macrolides against *M. avium* strains in vitro, (ii) the synergistic effect of **2** with macrolides is related to the inhibition of the efflux pumps, and (iii) the synergistic activity with **CLA** is retained in the ex vivo experiments at a concentration below the cytotoxicity threshold. The results described in this work

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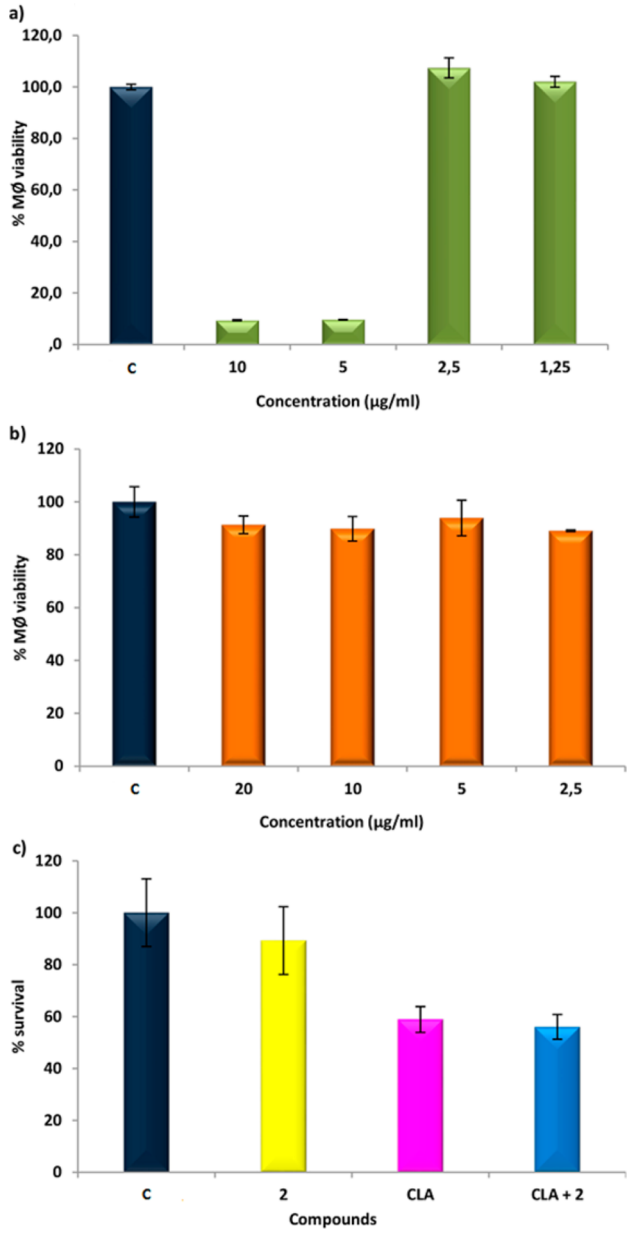

Figure 4. Cytotoxicity evaluation of (a) compound **2** and (b) **CLA** against human monocyte-derived macrophages; (c) evaluation of synergistic effect of compound **2** in combination with **CLA** against *M. avium*-infected macrophages, C, control.

confirmed that the 2-(4′-propoxyphenyl)quinoline scaffold is particularly suitable to confer efflux inhibitory activity also in mycobacteria, with a huge potential of further developments and improvements for a future clinical usage. In particular, derivative **2** emerged as a promising lead compound worthy of further optimization. The optimization strategy will aim at obtaining less toxic and more potent EPIs of NTMs to be used in combination with macrolides in particular against *Mycobacterium avium* complex, the causative agent of severe pulmonary infections in HIV co-infected, cystic fibrosis, and chronic obstructive pulmonary disease patients.[3−7,45]

METHODS

Reagents. Compounds *N*,*N*-diethyl-2-{[2-(4-propoxyphenyl)quinolin-4-yl]oxy}-ethanamine hydrochloride (**1**),[34] 4-[2-(1-piperazinyl)ethoxy]-2-(4′-propoxyphenyl)quinoline (**2**),[37] *N*,*N*-dimethyl-3-{[2-(4′-propoxyphenyl)-4-quinolinyl]-oxy}-1-propanamine hydrochloride (**3**),[37] and 4-[2-(1-azepanyl)ethoxy]-2-(4′-propoxyphenyl)quinoline (**4**)[37] (Figure 1), the syntheses of which were previously described, were selected for their interesting efflux inhibitory activity against *S. aureus* NorA efflux pump from an in-house library of NorA EPIs.

CPZ, **TZ**, **VP**, **ERY**, **CLA**, **AZT**, EtBr, phosphate-buffered saline (PBS), and glucose were purchased from Sigma-Aldrich (St. Louis, MO, USA). Middlebrook (MB) 7H9 and 7H11 and the oleic acid/albumin/dextrose/catalase (OADC) supplement were purchased from Difco (Madrid, Spain) and Becton and Dickinson (Sparks, MD, USA), respectively. The efflux inhibitors **CPZ**, **TZ**, and **VP** and the efflux substrate EtBr were prepared in sterile deionized water. **ERY**, **CLA**, **AZT**, and the 2-(4′-propoxyphenyl)quinoline compounds **1**−**4** were solubilized in DMSO. All solutions were prepared on the day of the experiment.

Bacterial Strains and MIC Determination. Mycobacterial strains used are described in Table 1 and comprised the reference strains *M. smegmatis* mc^2155[38] and *M. avium* $ATCC25291^T$,[40] *M. avium* 104 and its isogenic strains *M. avium* 104_{CLA3} and *M. avium* 104_{CLA4}, and four *M. avium* clinical strains. The determination of the MIC values of compounds **1**−**4**, **TZ**, **CPZ**, and **VP**, as well as of the antibiotics alone and in the presence of an EPI, was performed using the broth microdilution method. Briefly, strains were grown in MB7H9 plus 10% OADC at 37 °C until an OD_{600} of 0.8. The cultures were diluted in MB7H9 media, and the suspension was adjusted to be equal to that of the McFarland no. 0.5 standard. The final inoculum was prepared by diluting the adjusted bacterial suspension at 1:100. Aliquots of 0.1 mL were transferred in each well of the 96-well plate that contained 0.1 mL of each agent at concentrations prepared by 2-fold serial dilutions in MB7H9/OADC medium. The inoculated plates were sealed and incubated at 37 °C, and the results were registered after 3 and 7 days for *M. smegmatis* and *M. avium* strains, respectively. The MIC was defined as the lowest concentration of compound that inhibited visible growth.[46] The effect of each EPI on the activity of each antibiotic was represented by means of FIC calculated as FIC = MIC_{ATB} in combination/MIC_{ATB} alone. The FIC values were interpreted using the criteria established by Pillai et al.[47] The FIC value for the combinations was classified as non-determinable (ND) when the MICs of the compounds alone were greater than the highest, less than, or equal to the lowest concentration tested.[43]

Semiautomated Fluorometric Assays. The assessment of accumulation and extrusion of EtBr on a real-time basis by the mycobacterial strains was performed using a semiautomated fluorometric method, as previously described.[23,48]

Accumulation Assay. Strains were grown in 10 mL of MB7H9/OADC medium at 37 °C until an OD_{600} of 0.8. Cultures were centrifuged at 2940*g* for 3 min, the supernatant was discarded, the pellet was washed in PBS, and the OD_{600} was adjusted to 0.8 with PBS. To determine the concentration of EtBr at which there is equilibrium between influx and efflux of this substrate, the assays were performed in the presence of various concentrations of EtBr in the presence of glucose, to energize the cells, at a final concentration of 0.4%. The assays were prepared to a final volume of 100 µL containing 50 µL of the strain suspension (final OD_{600} of 0.4) plus 50 µL of EtBr solutions to final concentrations of 0.125, 0.25, 0.5, 1, 2, and

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3 μg/mL. The concentration for which there is equilibrium between the influx and efflux of EtBr corresponds to the one that does not exceed 10 arbitrary units of fluorescence in the presence of glucose as energy source. To assess the effect of compounds **1**−**4**, **TZ**, **CPZ**, and **VP** on the accumulation of EtBr, the assays were performed in a final volume of 100 μL containing 50 μL of the strain suspension (to a final OD_{600} of 0.4) and 50 μL of a solution containing the EtBr concentration previously selected and the concentration of the efflux inhibitor to be tested to a final concentration of half the MIC determined for each compound, in order to not compromise the cellular viability. In all assays was included a control containing the EtBr concentration to be tested without the efflux inhibitor with and without the cells, and cells only. The assays were conducted in a Rotor-Gene 3000 (Corbett Research, Sydney, Australia) at 37 °C, and the fluorescence was acquired at 530/585 nm at the end of every 60 s, during 60 min.[48] The activity of compounds **1**−**4**, **TZ**, **CPZ**, and **VP** on the accumulation of EtBr was determined according to the formula $RFF = (RF_{treated} - RF_{untreated})/RF_{untreated}$, where $RF_{treated}$ corresponds to the relative fluorescence at the last time point of EtBr accumulation curve (minute 60) in the presence of an inhibitor and $RF_{untreated}$ corresponds to the relative fluorescence at the last time point of the EtBr accumulation curve of the untreated control tube.[42] The RFF value is a measure of how effective the compound is on the inhibition of EtBr efflux (at a given concentration) by comparison of the final fluorescence at the last time point (60 min) of the treated cells with the cells treated only with EtBr. An index of activity >0 indicated that cells accumulate more EtBr under the condition used than those of the control (nontreated cells). In the case of negative RFF values, these indicated that treated cells accumulated less EtBr than those of the control condition. Values >1 in the presence of efflux inhibitors indicate enhanced accumulation of EtBr inside the cells.

Efflux Assay. Strain suspensions were adjusted to OD_{600} of 0.4 and exposed to conditions that promote maximum accumulation of EtBr: EtBr at the equilibrium concentration; no glucose; presence of the **VP** efflux inhibitor that caused maximum accumulation at half of the MIC; and incubation at 25 °C during 1 h. After the incubation, the EtBr-loaded cells were washed by centrifugation at 4860*g* during 5 min and resuspended in EtBr-free PBS, and the OD_{600} was adjusted to 0.8. The assays were prepared to a final volume of 100 μL containing 50 μL of the strain suspension (final OD_{600} of 0.4) plus 50 μL of compounds **1**−**4**, **TZ**, **CPZ**, and **VP** (at half of the MIC) with or without glucose, and control tubes with only cells with and without glucose. Fluorescence was measured in the Rotor-Gene 3000 at 37 °C, at the end of every 30 s, during 30 min. Efflux activity was quantified by comparing the fluorescence data obtained under conditions that promote efflux (presence of glucose and absence of efflux inhibitor) with the data from the control in which the mycobacteria are under conditions of no efflux (presence of an inhibitor and no energy source). Thus, the relative fluorescence corresponds to the ratio of fluorescence that remains per unit of time, relative to the EtBr-loaded cells.[48]

Efflux Pump Gene Expression Analysis. Total RNA was isolated using the RNeasy mini kit (QIAGEN, GmbH, Hilden, Germany) according to the manufacturer's instructions. Briefly, strains were grown until an OD_{600} of 0.8, and a 1 mL aliquot was removed and centrifuged at 16060*g* during 10 min. Then, 500 μL of supernatant was removed, and 1 mL of RNA*protect Bacteria reagent* (QIAGEN) was added. Cells were lysed with lysozyme at 3 mg/mL (Sigma) during 10 min and sonicated at 35 kHz (Gen-Probe, San Diego, CA, USA) during 15 min. The RNA was treated with RNase-free DNase I (QIAGEN) during 2 h and 15 min by on-column digestion at room temperature to reduce the presence of contaminating DNA and purified using the RNeasy kit (QIAGEN). All RNA samples were aliquoted and frozen at −20 °C until required. The relative expression level of the genes MAV_1406 and MAV_1695 were analyzed by RT-qPCR. The normalization of the data was done using the 16S rDNA for each experiment. The forward and reverse primers employed are described elsewhere.[22,49] The RT-qPCR procedure was performed in a Rotor-Gene 3000 thermocycler following the protocol recommended for the use with the QuantiTect SYBR Green RT-PCR Kit (QIAGEN). The determination of the relative mRNA expression level was performed using the comparative quantification cycle (*Cq*) method.[50] The relative amount of gene expression was assessed by comparison of the relative quantity of the respective mRNA in the presence of **CLA** with that of the nonexposed strain. A level of relative expression of 1 indicates that the expression level was identical to that of the unexposed strain. Genes showing expression levels ≥4 times, when compared with the unexposed strain, were considered to be overexpressed.[51]

Evaluation of Intracellular Activity of Compound 2. *Isolation of Human Monocyte-Derived Macrophages.* Blood was collected from healthy volunteers, and peripheral blood mononuclear cells were isolated by Ficoll−Paque Plus (GE Healthcare, Freiburg, Germany) density gradient centrifugation. Monocytes were differentiated into macrophages during 7 days in macrophage medium containing RPMI-1640 medium with 10% fetal calf serum (FCS), 1% GlutaMAX, 1 mM sodium pyruvate, 10 mM HEPES at pH 7.4, 100 IU/mL penicillin, 100 μg/mL streptomycin (Gibco, Life Technologies), and 20 ng/mL M-CSF (Immunotools, Friesoythe, Germany) and incubated at 37 °C in a 5% CO_2 atmosphere. Fresh medium was added at day 4 post isolation.

Cytotoxicity Evaluation. The effect of the compounds on human monocyte-derived macrophages was evaluated using AlamarBlue (Molecular Probes, Life Technologies) following the manufacturer's indications. Briefly, 3×10^5 cells were seeded in 96-well microplates, treated with the compounds, and then incubated at 37 °C in a 5% CO_2 atmosphere. After 3 days of treatment, cell viability was assessed. Briefly, 10% AlamarBlue was added to each well and incubated during 4 h at 37 °C and 5% CO_2. Fluorescence was measured at an excitation of 570 nm and emission of 595 nm in a Tecan's Infinite M200 plate spectrophotometer (Tecan Trading AG, Switzerland). For the subsequent intracellular assays, the compounds were used at concentrations that were shown to be nontoxic to the macrophages. The CC_{50} values were calculated by nonlinear regression (curve fit) using GraphPad Prism V5.01 software (La Jolla, CA, USA). The CC_{50} value corresponds to the highest concentration of compound at which 50% of the cells are viable relative to the control.[52]

Ex Vivo Synergy Assays. Human monocyte-derived macrophages were infected with *M. avium* 104 at a multiplicity of infection (MOI) 1:1 and were allowed to take up the bacteria for 3 h. Following the uptake, cells were washed three times with PBS to remove extracellular bacteria. Afterward, the selected compounds were added to the *M. avium*-infected macrophages at the desired concentrations. At 3 h and 1 and 3 days post infection, cells were lysed with 0.05% Igepal

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(Sigma-Aldrich). Serial dilutions of the lysate were placed on 7H11/10% OADC medium. Colony-forming units were counted upon 2 weeks of incubation at 37 °C. **CLA** was tested at 0.25 μg/mL and compound **2** at 2.5 μg/mL.

Statistics. Statistical analysis of the data was carried out using Student's *t* test. A *P* value <0.05 was considered statistically significant (two-tailed tested).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.5b00052.

Table S1: MICs of the compounds included in the study (PDF)

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Author Contributions

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Author Contributions

D.M. helped in the design of the experiments, performed the experiments with *M. smegmatis* and *M. avium*, and wrote the manuscript; R.C. helped both in the synthesis of the compounds and in the experiments with *Staphylococcus aureus* and wrote the manuscript; S.S.C. carried out the experiments with *Staphylococcus aureus* and revised the manuscript; G.M. synthesized the compounds and helped in their analysis; O.T. helped in compound analysis and manuscript revision; V.C. helped in the bibliographic search, the planning of the study, and manuscript revision; I.C. helped in the design of part of the experiments and revised the manuscript; M.V. designed all of the experiments and wrote the manuscript; S.S. conceived and designed the study and wrote the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

NTM, nontuberculous mycobacteria; **VP**, verapamil; **TZ**, thioridazine; **CPZ**, chlorpromazine; EtBr, ethidium bromide; **CLA**, clarithromycin; **AZT**, azithromycin; **ERY**, erythromycin; glu, glucose; RFF, relative final fluorescence; FIC, fractional inhibitory concentration

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