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FULL PAPER

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Quinoxaline-based efflux pump inhibitors restore drug susceptibility in drug-resistant nontuberculous mycobacteria

Paola Corona[1] | Roberta Ibba[1,2] | Sandra Piras[1] | Paola Molicotti[3] | Alessandra Bua[3] | Antonio Carta[1]

[1]Department of Medical, Surgical and Experimental Sciences, University of Sassari, Sassari, Italy

[2]Department of Biotechnology, Chemistry, and Pharmacy, DoE Department of Excellence 2018–2022, University of Siena, Siena, Italy

[3]Department of Biological and Medicinal Sciences, University of Sassari, Sassari, Italy

Correspondence

Roberta Ibba, Department of Medical, Surgical and Experimental Sciences, University of Sassari, Via Muroni, 23A, 07100 Sassari, Italy.
Email: ribba@uniss.it

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Abstract

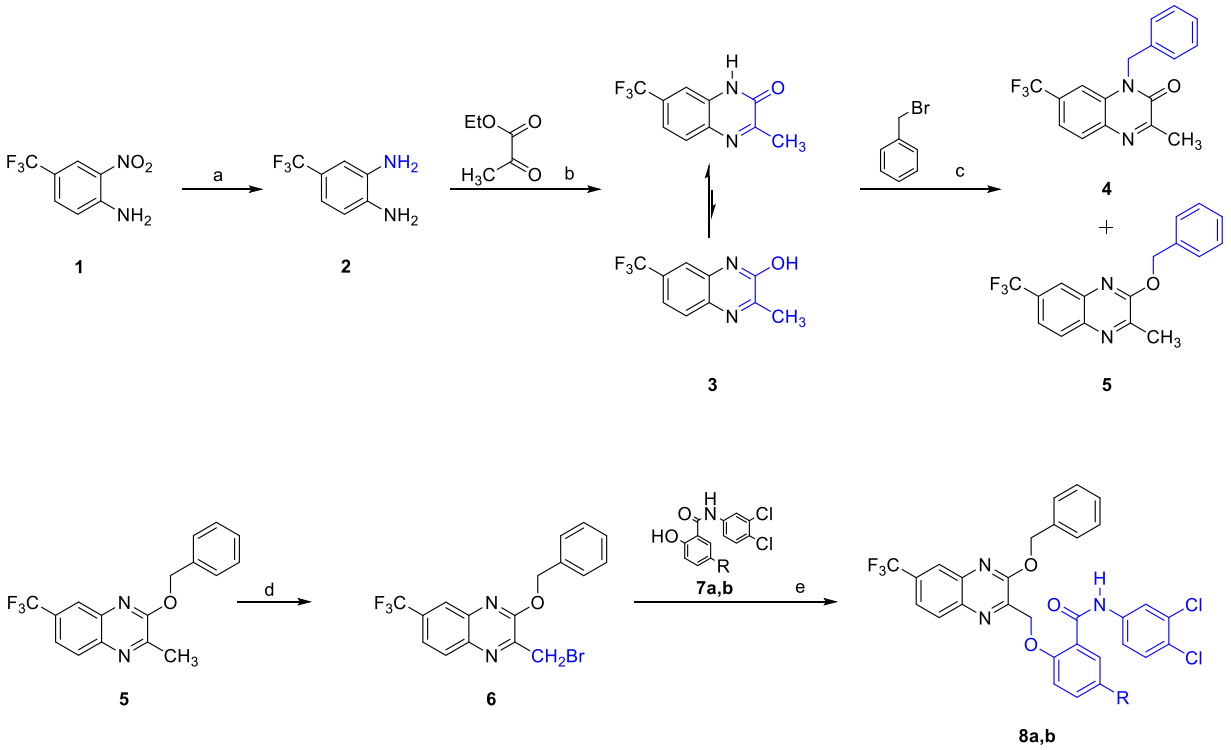
Nontuberculous mycobacteria (NTM) comprise several ubiquitous, environmentally localized bacteria that may be responsible for serious human diseases. NTM-associated pulmonary infections largely affect individuals with underlying respiratory disease or chronic disease and immunosuppressed patients. *Mycobacterium simiae* and *M. abscessus* are two NTMs responsible for lung disease in immunocompetent and immunocompromised individuals. In this study, two NTM strains were isolated from two patients admitted to an Italian hospital and were identified as *M. simiae* and *M. abscessus*. The two NTMs were tested for drug susceptibility against different antibiotics. To restore drug susceptibility, a new series of 2-aryl-3-phenoxymethyl-quinoxaline derivatives (QXs) was designed, synthesized, and investigated as efflux pump inhibitors (EPIs) against two clinical isolates of the above-cited NTMs, evaluating how EPIs can influence the drug minimal inhibitory concentration values and, therefore, the activity. The different\ resistance levels tracked in the clinical strains were reduced by EPIs, and in several cases, the susceptibility was completely restored. QXs also resulted as potential chemical probes to be used in drug susceptibility tests to identify the resistance origin when detected.

KEYWORDS

efflux pump inhibitors, medicinal chemistry, multidrug resistance, nontuberculous mycobacteria, quinoxaline derivatives

1 | INTRODUCTION

Besides *Mycobacterium tuberculosis* and *M. leprae*, several mycobacterial species can be found as ubiquitous living organisms across the world. Nontuberculous mycobacteria (NTM) include several species found in the environment, particularly in water and soil. Almost 200 species are currently recognized, but only a few are pathogenic to humans. They are defined as opportunistic pathogens as they infect mainly pathological or physiological immunocompromised individuals. They can be classified by the growth rates into slow-growing NTMs and rapid-growing NTMs.[1] NTM-caused infection can involve different sites in human bodies, although lung, skin, and mucosal infections are the most commonly diagnosed.[2,3] Pulmonary infections associated with NTM mostly affect individuals with underlying respiratory diseases such as cystic fibrosis or chronic diseases and immunosuppressed patients.[4,5] *M. simiae* is a slow-growing, photochromogenic, environmental NTM whose reservoir is mainly water.[6,7] It was initially considered



SCHEME 1 Synthetic route designed and performed to obtain derivatives **8a** and **8b** (**a**: R = H; **b**: R = Cl. (a) H_2/Pd, EtOH, rt; (b) EtOH, HCl 2N, rt; (c) DMF, K_2CO_3, rt; (d) Br_2, CH_3COOH, CH_3COONa, 80°C; (e) $CHCl_3$, H_2O, NaOH, BTAC, reflux, yield: 78% (**8a**) and 63% (**8b**).

nonpathogenic and only rarely associated with disease in humans. More recently it has been associated with pulmonary disease in both immunocompromised and immunocompetent patients. No treatment regimen was found to be effective against *M. simiae* caused pulmonary infections, not even multiple antimycobacterial associations, especially in subjects whose therapeutic treatment is difficult as this microorganism has intrinsic and acquired resistance to first-line drugs but also macrolides.[8] *Mycobacterium abscessus* is a fast-growing, ubiquitous mycobacterium in soil and water responsible for a wide variety of human infections; skin and respiratory tract are particularly affected. Infections caused by this NTM are difficult to treat due to the natural and acquired resistance to drugs and disinfectants. It is responsible for pneumonia mainly in hosts with underlying structural lung diseases, such as cystic fibrosis, bronchiectasis, and previous tuberculosis.[9] According to the 2007 American Thoracic Society/Infectious Disease Society of America guidelines, treatment regimens remain limited with current antimicrobial agents and, therefore, abscessus lung disease is considered a chronic incurable disease.[10] *M. simiae* and *M. abscessus* infections have been revealed in patients in different states worldwide and drug susceptibility test to commonly used drugs has been performed on isolates, showing drug resistance for different tested drugs.[6,10,11] Intrinsic resistance can be due to a combination of different factors, such as the altered permeability of the cell envelope, low-affinity antibiotic target, drug efflux systems, and antibiotic neutralizing enzymes. Although acquired drug resistance is mainly caused by antibiotic target mutation or overexpression of efflux pumps and neutralizing enzymes, proved by upregulations or mutations detected in the bacterial genome.[12] The Mycobacterial membrane protein Large (MmpL) transporters belong to RND, Resistance-Nodulation Cell Division, an important family of multidrug resistance pumps. MmpLs are highly conserved between *M. tuberculosis* and NTMs. They are transporters that participate in the transport of the substrates through the periplasm to the extracellular environment and are involved in drug resistance mechanisms.[13] A high abundance of MmpLs in NTMs has been proved to be associated with drug resistance.[13–16] It was also found that efflux inhibitors such as verapamil,[17] reserpine, and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP)[14] increased drug susceptibility.[18] Efflux pump inhibitors can also be used as chemical probes. As target mutation and efflux pump overexpression are the most detected causes connected with drug resistance, effective efflux pump inhibitors (EPIs) can also be used in drug susceptibility tests to elucidate the eventual drug resistance origin. Clearly, there is an urgent need for the development of effective drugs or regimens against drug-resistant NTMs and fast enlightening of drug resistance origin. In the last decade, our research group has focused its attention on the synthesis and biological activity of compounds with antimycobacterial activity.[19–21] Furthermore, we synthesized effective EPIs active in enhancing chemotherapeutics in cancer cell lines,[22,23] *M. tuberculosis*,[24] and several bacterial, and fungal strains.[25] These latter compounds bear a quinoxaline (QX) scaffold, differently substituted in positions 2 and 3. A simple phenoxymethyl functionalization in position 3 and a variously

functionalized aryl moiety in position 2. The high and interesting inhibition of different EP was paired with very low solubility, therefore, the QX compounds from the first generation were used as hit compounds to obtain more soluble but still active EP inhibitors. To increase solubility, methoxy groups from parental series were replaced by chlorine atoms and EPI activity was studied against several pathogen strains and cancer cell lines. We present here a new series of 2-aryl-3-phenoxymethyl-quinoxaline derivatives (QXs) that has been proved active as EPIs against two NTM clinical isolates.

2 | RESULTS AND DISCUSSION

2.1 | Chemistry

The designed compounds (**8a**, **8b**, **10a**, **10b**) were obtained via the synthetic routes depicted in Schemes 1 and 2, while in Scheme 3 the synthetic process used to gain intermediates **7a**,**b** is reported. The key intermediate **5** was obtained starting with proper 2-nitroaniline (**1**) whose nitro-group was reduced to get the desired *o*-dianiline (**2**) which was condensed with ethyl 2-oxopropanoate to obtain a mixture of proper quinoxaline-2(1*H*)-one and quinoxaline-2-ol (**3**) in keto-enol tautomerization equilibrium. The following substitution of mixture **3** with benzyl bromide produced the undesired *N*-substituted product (**4**) and the desired key intermediate **5** bearing the *O*-benzyl moiety in a ratio of 7:3. This validated synthetic strategy to obtain the proper intermediates[22,23,26] was then implemented in two final steps to get the desired compounds. The subsequent bromination and halogen substitution yielded designed compounds **8a** and **8b**.

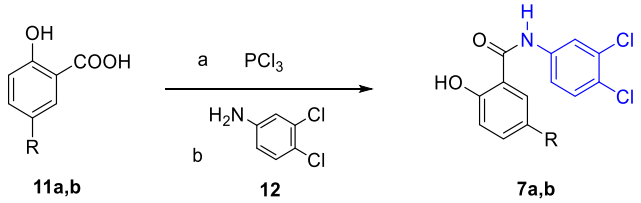
Instead, derivatives **10a** and **10b** were obtained by a faster synthetic route, as depicted in Scheme 2. Proper *o*-dianiline (**2**) was condensed with 1,4-dibromobutane-2,3-dione to get the second key intermediate 2,3-bis(bromomethyl)-6-(trifluoromethyl)quinoxaline (**9**). Final compounds were obtained by halogen substitution with the right salicylic amide derivative (**7a**,**b**).

Intermediates **7a**,**b** were prepared by activating the proper salicylic acid derivative (**11a**,**b**) to a more reactive chloride derivative, and the following coupling with the desired 3,4-dichloroaniline (**12**) produced the designed salicylic amide intermediates, as shown in Scheme 3.

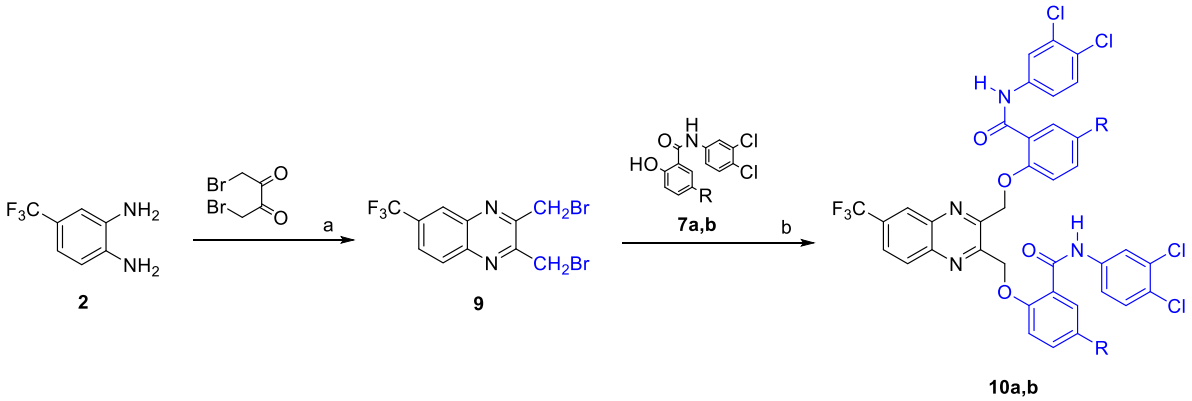
2.2 | Biology

2.2.1 | Enhancing the effect of efflux pump inhibitors on antimicrobial activity

The enhancement of antimycobacterial-drugs activity exerted by the synthesized efflux pump inhibitors (**8a**,**b** and **10a**,**b**) was evaluated by REMA assays. They were performed in parallel by administering the sole drug or the association of each drug with each synthesized EPI. Minimum inhibitory concentrations (MICs) were measured for antimycobacterial drugs alone and when coadministered with EPIs. Table 1 shows the drug susceptibility of both NTM clinical isolated strains to six drugs commonly used in therapy: azithromycin, amikacin, ciprofloxacin, levofloxacin, moxifloxacin, and linezolid. According to the recorded MIC values, *M. abscessus* turned out as resistant to all the tested compounds except for linezolid, also *M. simiae* resulted being a multidrug-resistant (MDR) strain being susceptible to the sole azithromycin. The MICs for the four QXs were higher than 256 μg/ml, proving no direct antimycobacterial activity. Enhancement of antimycobacterial activity acted by QX



SCHEME 3 Synthetic route designed and performed to synthesize intermediates **7a** and **7b** (**a**: R = H; **b**: R = Cl). (i) Chlorobenzene, reflux; (ii) chlorobenzene, reflux. Final yield: 85% (**7a**) and 93% (**7b**).



SCHEME 2 Synthetic route designed and performed to obtain derivatives **10a** and **10b** (**a**: R = H; **b**: R = Cl). (a) EtOH, reflux; (b) $CHCl_3$, H_2O, NaOH, BTAC, reflux, yield: 45% (**10a**) and 95% (**10b**).

TABLE 1 Drug susceptibility test results obtained by the REMA test.

NTM	MIC (μg/ml)					
	Azithromycin	Amikacin	Ciprofloxacin	Levofloxacin	Moxifloxacin	Linezolid
Mycobacterium abscessus	16	16	16	16	4	2
Mycobacterium simiae	1	16	32	32	4	>16

Abbreviations: MIC, minimum inhibitory concentration; NTM, nontuberculous mycobacteria; REMA, resazurin microtiter assay.

TABLE 2 MIC values measured by REMA test for the clinical isolate of *Mycobacterium abscessus* strain treated with azithromycin (Azk) and amikacin (Amk) alone and in association with EPIs **8a**,**b** and **10a**,**b**.

M. abscessus	EPI	MIC (μg/ml)		*n*-Fold reduction	EPI conc.	MIC (μg/ml)		*n*-Fold reduction	EPI conc.
	MIC (μg/ml)	Azm	Azm + EPI		(μg/ml)	Amk	Amk + EPI		(μg/ml)
8a	>256	16	2	8	16	16	8	2	64
8b	>256	16	2	8	16	16	16	-	128
10a	>256	16	2	8	16	16	16	-	128
10b	>256	16	8	2	64	16	8	2	64

Note: *n*-Fold reduction of MICs is reported.

Abbreviations: EPI, efflux pump inhibitor; MIC, minimum inhibitory concentration; REMA, resazurin microtiter assay.

derivatives acting as efflux pump inhibitors was reported in Tables 2 and 3, in which the ineffective coadministration results were not reported. The full results are reported in the Supporting Information: Tables SI1 and SI2.

Replication of *M. abscessus* MDR strain was invalidated when azithromycin was coadministered with EPIs **8a**, **8b**, and **10a** with an eightfold reduction of the drug MIC values, as shown in Table 2. Derivative **10b** reduced it only by two-old and it can be deemed as not considerable. While the antimycobacterial effect of amikacin was modulated by our QXs but drug susceptibility was not fully recovered, proving that the mechanism of amikacin resistance is not chargeable to the sole efflux pumps activity. Along the same line, ciprofloxacin, levofloxacin, moxifloxacin, and linezolid MIC values were not improved by the coadministration with our EPIs, proving that the drug resistance in this mycobacteria strain is not due to efflux pump overexpression.

Multidrug resistance of clinical *M. simiae* was reverted by the association of antimycobacterial drugs with our EPIs, as reported in Table 3. In the presence of all the tested compounds, the MICs of amikacin were reduced and compound **8a** restored drug susceptibility with a fourfold enhancement. Levofloxacin activity was improved four-fold by all the QX derivatives while ciprofloxacin resistance was affected by the sole compound **10a** with a MIC reduction of four times. Azithromycin, moxifloxacin, and linezolid resistance were not reverted by the association with our EPIs.

Compound **8a** showed the widest activity improving the MIC values of all the reported antimycobacterial drugs. From a structure–activity relationship point of view, the slight structural differences among the four compounds resulted in a comparable activity of the QX derivatives when tested as efflux pump inhibitors in these NTM strains. We can highlight that the addition of a second, more hindered side-chain provided in compounds **10a** and **10b** did not result in a significant improvement of the activity. It also proved that the binding site is huge enough to accommodate huge molecules such as **10a** and **10b**, but it is occupied also by slightly smaller molecules such as **8a**,**b**. These results proved quite clearly that EPIs can be used in coadministration to revert drug resistance in MDR NTM, as well as chemical probes, during in vitro assays, to identify the resistance origin.

3 | CONCLUSION

Starting from the previously acquired knowledge of the syntheses and biological activities of EPIs active against several bacteria, fungi, and human cancer cells, four new 6-trifluoromethylquinoxaline derivatives (QXs) have been synthesized, and tested, in association with azithromycin, amikacin, ciprofloxacin, levofloxacin, moxifloxacin, and linezolid against two NTM clinical isolated strains, *M. abscessus* and *M. simiae*. The former one was found resistant to all the tested compounds except for linezolid, also *M. simiae* strain was proved to be MDR turning out as susceptible to the sole azithromycin. Based on our experiments compound **8a** showed the widest activity in improving the MIC values of all drugs versus both NTM. The introduction of an additional Cl atom in the side chain (compound **8b**) does not improve the activity of this series of derivatives but, on the contrary, it worsens the MIC values against both NTMs. Also, the addition of a second side-chain (compounds **10a**,**b**) did not result in an improvement in the activity. In conclusion, we can state that compound **8a** can be considered the hit compound for further biological studies. The most active EPI of this series of QXs could also be used as chemical probes during drug sensitivity tests in vitro, they would be a useful tool to be employed before selecting the best regimen for NTM infection treatments.

TABLE 3 MIC values measured by REMA test for clinical isolate of *Mycobacterium simiae* strain treated with amikacin (Amk), ciprofloxacin (Cipro), and levofloxacin (Levo) alone and in association with EPIs **8a**,**b** and **10a**,**b**.

M. simiae	EPI MIC (μg/ml)	MIC (μg/ml) Amk	Amk + EPI	*n*-Fold reduction	EPI conc. (μg/ml)	MIC (μg/ml) Cipro	Cipro + EPI	*n*-Fold reduction	EPI conc. (μg/ml)	MIC (μg/ml) Levo	Levo + EPI	*n*-Fold reduction	EPI conc. (μg/ml)
8a	>256	16	2	4	16	32	16	2	64	32	8	4	32
8b	>256	16	8	2	64	32	>16	-	128	32	8	4	32
10a	>256	16	8	2	64	32	8	4	32	32	8	4	32
10b	>256	16	8	2	64	32	>16	-	128	32	8	4	32

Note: *n*-Fold reduction of MICs is reported.

Abbreviations: EPI, efflux pump inhibitor; MIC, minimum inhibitory concentration; REMA, resazurin microtiter assay.

4 | EXPERIMENTAL

4.1 | Chemistry

4.1.1 | General remarks

Melting points (m.p.) were measured with a Köfler hot stage Microscopes Polytherm from Wagner & Munz or Electrothermal Mel-Temp™ Digital Melting Point Apparatus from Thermo Fisher Scientific and are uncorrected. Nuclear magnetic resonance (^{1}H-NMR and ^{13}C-NMR) spectra were determined in deuterated dimethyl sulfoxide (DMSO-d_6) or $CHCl_3$/DMSO-d_6 (ratio 9:1) and were recorded with a Bruker Avance III 400 NanoBay (400 MHz) or an XL-200 (200 MHz). Chemical shifts (δ) are reported in parts per million (ppm) downfield from tetramethylsilane used as an internal standard. Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; m, multiplet; br s, broad singlet; dd, double doublet. Mass spectra (MS) were performed on the combined Liquid Chromatograph-Agilent 1100 series Mass Selective Detector. Analytical thin-layer chromatography (TLC) was performed on Merck silica gel F-254 plates. Pure compounds showed a single spot in TLC. For flash chromatography, Merck silica gel 60 was used with a particle size of 0.040–0.063 mm (230–400 mesh ASTM). Elemental analyses were measured on a Perkin-Elmer 2400 instrument and the results were within ±0.4% of theoretical values. Full NMR spectra of active compounds **8a,b** and **10a,b** are depicted in the Supporting Information: Figures SI1–SI8.

The InChI codes of the investigated compounds, together with some biological activity data, are provided as Supporting Information.

4.1.2 | General synthesis of the starting materials and known intermediates

2-Nitro-4-(trifluoromethyl)aniline (**1**), ethyl 2-oxopropanoate, (bromomethyl)benzene, 1,4-dibromobutane-2,3-dione, 2-hydroxybenzoic acid (**11a**), 4-chloro-2-hydroxybenzoic acid (**11b**), 3,4-dichloroaniline (**12**) and inorganic reagents were commercially available and were purchased by Sigma Aldrich. Intermediates **1–6**, and **9** were prepared following the procedure we previously described.[22,23,26] The known *N*-(3,4-dichlorophenyl)-2-hydroxybenzamide (**7a**) and 5-chloro-*N*-(3,4-dichlorophenyl)-2-hydroxybenzamide (**7b**) intermediates were prepared as reported by Waisser et al.[27] and are here fully characterized.

4.1.3 | General procedure for the synthesis of intermediates **7a** and **7b**

A solution of 2.2 mmol of 2-hydroxybenzoic acid (**11a**) or 4-chloro-2-hydroxybenzoic acid (**11b**) in chlorobenzene (6.6 ml) and an additional 0.1 ml (1.15 mmol) of PCl_3 was prepared. The solution was stirred at reflux temperature for 45 min when 2.2 mmol of 3,4-dichloroaniline (**12**) was added. The mixture was left at reflux temperature until the

reaction was completed, 7 h (**7a**) or 5 h (**7b**). Solvent was removed under reduced pressure and the solid product obtained was recrystallized by ethanol.

*N-(3,4-Dichlorophenyl)-2-hydroxybenzamide (**7a**)*

Compound **7a** was obtained as white solid, m.p.: 210–212°C. Yield, 85%. TLC (petroleum ether/ethyl acetate 7:3): R_f 0.57. ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 11.80 (1H, br s, NH), 10.42 (1H, s, OH), 8.07 (1H, d, J = 1.6 Hz, H-2'), 8.01 (1H, d, J = 6.2 Hz, H-6), 7.62 (1H, dd, J = 1.6 Hz and J = 6 Hz, H-6'), 7.50–7.40 (2H, m, H-4, 5'), and 7.10–6.90 (2H, m, H-3, 5). LC/MS: m/z 283 $[M+H]^+$. Elem. Anal. calcd. (%) for $C_{13}H_9Cl_2NO_2$: C, 55.35; H, 3.22; N, 4.96. Found: C, 55.42; H, 3.36; N, 5.03.

*5-Chloro-N-(3,4-dichlorophenyl)-2-hydroxybenzamide (**7b**)*

Compound **7b** was obtained as white solid, m.p.: 252–254°C. Yield, 93%. TLC (petroleum ether/ethyl acetate 8:2): R_f 0.5. ^{1}H-NMR ($CHCl_3$/DMSO-d_6, 200 MHz) δ: 11.90 (1H, br s, NH), 10.39 (1H, s, OH), 8.10 (1H, d, J = 2.8 Hz, H-6), 8.02 (1H, d, J = 2.4 Hz, H-2'), 7.62 (1H, dd, J = 2.4 Hz and J = 8.8 Hz, H-6'), 7.42 (1H, d, J = 8.8 Hz, H-5'), and 6.96 (1H, d, J = 8.8 Hz, H-3). LC/MS: m/z 317 $[M+H]^+$. Elem. Anal. calcd. (%) for $C_{13}H_8Cl_3NO_2$: C, 49.32; H, 2.55; N, 4.42. Found: C, 49.41; H, 2.67; N, 4.56.

4.1.4 | General procedure for the synthesis of derivatives **8a** and **8b**

To a solution of equimolar amounts of 3-(benzyloxy)-2-(bromomethyl)-6-(trifluoromethyl)quinoxaline (0.75 mmol) (**6**) and the suitable intermediate **7a** or **7b** in chloroform (10 ml), a second solution of 0.75 mmol of benzyltriethylammonium chloride (BTAC) and 1.12 mmol of NaOH in 10 ml of water was added dropwise. The reaction mixture was stirred at 90°C until reaction completion, 27 h (**8a**) or 168 h (**8b**). The mixture was cooled down to room temperature and then the two organic/aqueous layers were separated. The aqueous solution was extracted three times with chloroform (3 × 20 ml). The organic phases were collected and dried with anhydrous Na_2SO_4, filtered, and then evaporated under reduced pressure. The crude solids obtained were purified by chromatography on silica gel with a mixture of petroleum ether/ethyl acetate as eluent, in a proper ratio for **8a** (7:3) and **8b** (8:2).

*2-{[3-(Benzyloxy)-6-(trifluoromethyl)quinoxalin-2-yl]methoxy}-N-(3,4-dichlorophenyl)benzamide (**8a**)*

Compound **8a** was obtained as white solid, m.p.: 188–191°C. Yield, 78%. TLC (petroleum ether/ethyl acetate 7:3): R_f 0.68. ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 10.73 (1H, br s, NH), 8.22 (1H, s, Ar-H), 7.97–7.84 (4H, m, 4 Ar-H), 7.67–7.54 (4H, m, 4 Ar-H), 7.49–7.34 (5H, m, 5 Ar-H), 7.16 (1H, t, Ar-H), 5.73 (2H, s, CH_2), and 5.62 (2H, s, CH_2). ^{13}C-NMR (APT, DMSO-d_6, 100 MHz) δ: 164.06 (C), 155.72 (C), 155.62 (C), 155.53 (C), 148.38 (C), 148.32 (C), 139.30 (C), 138.96 (C), 138.81 (C), 135.79 (C), 133.03 (CH), 131.56 (C), 130.94 (CH), 130.54 (CH), 130.11 (CH), 129.69 (CH), 128.46 (CH ×x 2), 128.43 (CH), 127.97 (CH ×x 2), 125.12 (C), 124.19 (CH), 123.67 (C), 121.69 (CH), 121.24 (CH), 120.04 (CH), 114.24 (CH), 68.32 (CH_2), and 67.31 (CH_2). LC/MS: m/z 598 $[M+H]^+$. Elem. Anal. calcd. (%) for $C_{30}H_{20}Cl_2F_3N_3O_3$: C, 60.22; H, 3.37; N, 7.02. Found: C, 60.33; H, 3.45; N, 7.13.

*2-{[3-(Benzyloxy)-6-(trifluoromethyl)quinoxalin-2-yl]methoxy}-5-chloro-N-(3,4-dichlorophenyl)benzamide (**8b**)*

Compound **8b** was obtained as white solid, m.p.: 160–163°C. Yield, 63%. TLC (petroleum ether/ethyl acetate 8:2): R_f 0.45. ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 10.71 (1H, s, NH), 8.21 (1H, s, Ar-H), 7.96–7.88 (3H, m, 3 Ar-H), 7.77 (1H, s, Ar-H), 7.57–7.56 (5H, m, 5 Ar-H), 7.46–7.38 (4H, m, 4 Ar-H), 5.71 (2H, s, CH_2), and 5.60 (2H, s, CH_2). ^{13}C-NMR (DMSO-d_6, 100 MHz) δ: 162.76 (C), 155.51 (C), 154.55 (C), 148.06 (C), 138.96 (C), 138.53 (C), 135.75 (C), 132.26 (CH), 130.96 (C), 130.57 (CH), 130.16 (C), 129.71 (CH), 129.62 (CH), 128.41 (CH ×x 2), 128.07 (CH), 127.96 (CH ×x 2), 125.60 (C), 125.49 (C), 125.39 (C), 125.12 (C), 124.21 (CH), 122.66 (CH), 122.41 (C), 121.27 (CH), 120.06 (CH), 116.56 (CH), 68.33 (CH_2), and 67.79 (CH_2). LC/MS: m/z 632 $[M+H]^+$. Elem. Anal. calcd. (%) for $C_{30}H_{19}Cl_3F_3N_3O_3$: C, 56.94; H, 3.03; N, 6.64. Found: C, 57.07; H, 3.16; N, 6.73.

4.1.5 | General procedure for the synthesis of derivatives **10a** and **10b**

A solution of 2,3-bis(bromomethyl)-6-(trifluoromethyl)quinoxaline) (1.0 mmol) (**9**) and the suitable intermediate **7a** or **7b** (4.0 mmol) in chloroform (15 ml), was added with a second solution of 2.0 mmol of BTAC and 3.0 mmol of NaOH in 15 ml of water. The reaction mixture was stirred at 90°C until reaction completion, 120 h (**10a**) or 168 h (**10b**). The mixture was cooled down to room temperature and the crude solid was obtained. The precipitate was collected by filtration in vacuo and washed with water. The two organic/aqueous layers were separated. The aqueous solution was extracted in chloroform (3 × 20 ml). The chloroform solutions were combined and washed with brine and dried with anhydrous Na_2SO_4. By evaporation under reduced pressure of the organic solution, an additional solid product was obtained. The crudes were purified by flash chromatography on silica gel with a mixture of petroleum ether/ethyl acetate as eluent, in a ratio of 7:3, respectively.

*2,2'-[{[6-(Trifluoromethyl)quinoxaline-2,3-diyl]bis(methylene)}bis-(oxy)]bis[N-(3,4-dichlorophenyl)benzamide] (**10a**)*

Compound **10a** was obtained as brown solid, m.p.: 173–176°C. Yield, 45%. TLC (petroleum ether/ethyl acetate 7:3): R_f 0.18. ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 10.82 (1H, s, NH), 10.74 (1H, s, NH), 8.13–8.08 (3H, m, 3 Ar-H), 7.90 (2H, d, J = 12 Hz, 2 Ar-H), 7.83 (1H, d, J = 8 Hz, Ar-H), 7.76 (1H, d, J = 8 Hz, Ar-H), 7.61–7.43 (8H, m, 8 Ar-H), 7.18–7.13 (2H, m, 2 Ar-H), and 5.87 (4H, s, 2 CH_2). ^{13}C-NMR (DMSO-d_6, 100 MHz) δ: 164.50 (C), 164.17 (C), 155.36 (C ×x 2), 155.17 (C × 2), 152.77 (C), 152.36 (C), 141.47 (C), 139.20 (C), 138.79 (C), 138.66 (C), 132.76 (CH), 132.53 (CH), 130.97 (C), 130.50 (CH),

130.39 (CH × 2), 130.34 (CH), 130.12 (CH), 129.23 (CH), 125.49 (C), 125.16 (C), 124.46 (C), 123.75 (C), 122.10 (C), 121.75 (CH), 121.60 (CH), 121.03 (CH ×x 2), 120.64 (CH), 120.52 (CH), 119.81 (CH), 113.80 (CH), 113.71 (CH), 68.77 (CH_2), and 68.49 (CH_2). LC/MS: *m/z* 785 $[M+H]^+$. Elem. Anal. calcd. (%) for $C_{37}H_{23}Cl_4F_3N_4O_4$: C, 56.51; H, 2.95; N, 7.12. Found: C, 56.60; H, 3.00; N, 7.20.

*6,6'-[{[6-(Trifluoromethyl)quinoxaline-2,3-diyl]bis(methylene)}bis-(oxy)]bis[3-chloro-*N*-(3,4-dichlorophenyl)benzamide] (**10b**)*

Compound **10b** was obtained as white solid, m.p.: 232−−235°C. Yield, 95%. TLC (petroleum ether/diethyl ether 7:3): R_f 0.23. ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 10.82 (1H, s, NH), 10.74 (1H, s, NH), 8.15–8.06 (3H, m, 3 Ar-H), 7.91–7.88 (2H, m, 2 Ar-H), 7.83 (1H, d, J = 8 Hz, Ar-H), 7.76 (1H, d, J = 8 Hz, Ar-H), 7.61–7.43 (6H, m, 6 Ar-H), 7.18–7.13 (2H, m, 2 Ar-H), and 5.85 (4H, s, 2 CH_2). ^{13}C-NMR (DMSO-d_6, 100 MHz) δ: 163.63 (C), 163.33 (C), 154.49 (C × 2), 154.41 (C × 2), 152.91 (C), 152.53 (C), 141.94 (C), 139.68 (C), 138.97 (C), 138.86 (C), 132.54 (CH), 132.29 (CH), 131.49 (C), 131.45 (C), 131.10 (CH), 131.03 (CH × 2), 130.87 (CH), 130.64 (C), 129.99 (CH), 129.73 (CH), 128.92 (CH), 126.79 (C), 126.25 (C), 126.04 (C), 125.93 (C), 125.90 (C), 122.24 (CH), 121.55 (CH), 120.03 (CH), 120.34 (CH), 116.40 (CH), 116.28 (CH), 69.68 (CH_2), and 68.36 (CH_2). LC/MS: *m/z* 853 $[M+H]^+$. Elem. Anal. calcd. (%) for $C_{37}H_{21}Cl_6F_3N_4O_4$: C, 51.96; H, 2.47; N, 6.55. Found: C, 52.03; H, 2.58; N, 6.64.

4.2 | Biological assays

4.2.1 | Antimycobacterial drug susceptibility and antimycobacterial enhancement assay

In this study, two NTM strains, isolated from two patients admitted to a respiratory ward, were identified as *M. simiae* and *M. abscessus* by MALDI TOF (Biomerieux). The drug susceptibility and the antimycobacterial enhancement activity were determined by REMA against the two NTM strains. Stock cultures were prepared from isolated colonies selected on Middlebrook 7H11 agar plates and diluted in Middlebrook 7H9. Strains were grown in Middlebrook 7H11 medium supplemented with OADC. The bacterial suspensions (at the standard turbidity of 1 McFarland) were diluted 1:10, 1:100, and 1:10,000 with Middlebrook 7H9 and inoculated in duplicate Middlebrook 7H11 plates containing serial concentrations of the tested drugs (azithromycin, amikacin, ciprofloxacin, levofloxacin, moxifloxacin, and linezolid) alone or in association with our four EPIs (**8a**, **8b**, **10a**, and **10b**). All the tested compounds were previously dissolved in DMSO at 100 mM as stock, then serially diluted in cell media. The final bacterial inoculum was approximately 1–5 × 10^5 CFU/ml. Assays were performed in sterile 96-well microtiter plates with round bottom wells, sealed in a plastic bag. Plates were incubated at 37°C and read after 72 h. After the visual reading, to each well was added 30 µl of 0.02% resazurin, and plates were incubated overnight. MIC was defined by a change in color. Bacterial growth causes reagent reduction: a change in color from blue (oxidized state) to pink (reduced) indicated bacterial growth, and MIC was measured as the lowest drug concentration that prevented the color change. A lower drug MIC value when the drug was coadministered with EPI indicated the antimycobacterial activity enhancement by efflux pump inhibition.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ORCID

Roberta Ibba http://orcid.org/0000-0003-3332-3845

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