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FULL PAPER

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Discovery of 2-phenylquinazolines as potent *Mycobacterium avium* efflux pump inhibitors able to synergize with clarithromycin against clinical isolate

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Abstract

Nontuberculous mycobacteria (NTM), which include the *Mycobacterium avium* complex, are classified as difficult-to-treat pathogens due to their ability to quickly develop drug resistance against the most common antibiotics used to treat NTM infections. The overexpression of efflux pumps (EPs) was demonstrated to be a key mechanism of clarithromycin (CLA) resistance in NTM. Therefore, in this work, 24 compounds from an in-house library, characterized by chemical diversity, were tested as potential NTM EP inhibitors (EPIs) against *Mycobacterium smegmatis* mc^2 155 and *M. avium* clinical isolates. Based on the acquired results, 12 novel analogs of the best derivatives **1b** and **7b** were designed and synthesized to improve the NTM EP inhibition activity. Among the second set of compounds, **13b** emerged as the most potent NTM EPI. At a concentration of 4 µg/mL, it reduced the CLA minimum inhibitory concentration by 16-fold against the clinical isolate *M. avium* 2373 overexpressing EPs as primary mechanism of CLA resistance.

KEYWORDS
antimicrobial resistance, efflux pump inhibitors (EPI), *Mycobacterium avium*, nontuberculous mycobacteria (NTM), quinazoline

1 | INTRODUCTION

The genus *Mycobacterium* includes tuberculous (*Mycobacterium tuberculosis* complex) and nontuberculous mycobacteria (NTM, e.g., *Mycobacterium avium* complex [MAC], *Mycobacterium smegmatis*, *Mycobacterium abscessus*)[1] that are opportunistic pathogens responsible for a wide variety of human infections, especially in immunocompromised patients or individuals underlying lung diseases such as bronchiectasis, chronic obstructive pulmonary and cystic fibrosis.[2] MAC has also been associated with multifaceted infections of

household pets, livestock, and wild animals, attracting increased attention as potential zoonotic pathogens.[3] Human NTM infections are widespread in many countries including Asia, Europe, and most of the United States, but unfortunately, the absence of a systematic surveillance makes difficult to monitor the infection trends and have comprehensive epidemiologic data.[4] Moreover, treatment of infections caused by NTM poses a great clinical challenge, because it is generally long, expensive, and likely to fail. The treatment guidelines for MAC infections, proposed by the international respiratory medicine and infectious diseases societies (ATS, ERS, ESCMID, IDSA), recommend a combination of a macrolide-based regimen for patients with macrolide-susceptible MAC lung disease—such as clarithromycin (CLA) or azithromycin—ethambutol, and rifamycin, for at least 1 year, until culture conversion. During the first 2–3 months, injectable amikacin is suggested for patients with cavitary or advanced/severe bronchiectasis or in vitro macrolide-resistant MAC disease.[5] Worthy of note, MAC shows high intrinsic multidrug resistance (MDR) because of the decreased permeability of the mycobacterial cell wall and drug efflux mediated by efflux pumps (EPs). As commonly observed in the microbial world, overexpressing EPs is the first step in developing high-level resistance.[6] *M. avium* EPs, which are able to extrude macrolides and give rise to macrolide-resistant phenotypes, are mainly classified in two superfamilies: (i) ATP-binding cassette (e.g., MAV_3306 and MAV_1695) and (ii) Major Facilitator Superfamily (e.g., MAV_1406).[7,8] EPs cause a decrease in the intracellular antimicrobial concentration, with a subsequent increase of the minimum inhibitory concentration (MIC) values[6,7,9]; thus, the microorganisms acquire the ability of surviving in the presence of a sublethal drug concentration that may allow to point mutations on the macrolides target (i.e., the 23S rRNA) generating a high-level target-based macrolide resistance.[7] Due to the crucial role of EPs in the generation of MDR bacterial strains, their inhibition is considered as a valuable antimicrobial strategy based on the coadministration of the failing antibiotics with an efflux pump inhibitor (EPI), also called antimicrobial resistance breaker, which is able to restore the antibiotic efficacy.[10] The EPIs, devoid of any antibacterial activity, offer a great opportunity to fight antimicrobial resistance by renewing "old drugs" and preventing the evolutionary pressure on bacteria that evolve resistance only for compounds exerting bactericidal or bacteriostatic effects.[11] Therefore, a strategy to reduce the insurgence of resistance may be more rewarding than the modification of existing antibiotics or the identification of new antibacterials. In this direction, our research group has been involved for many years in the identification of novel EPIs, especially acting against NorA, the most studied EP of *Staphylococcus aureus*. Although there are limited similarities between the primary sequence of MAV_1406 (the most known *M. avium* MFS EP) and that of NorA, they both: (i) belong to the MF superfamily, (ii) are overexpressed after drug exposure/pressure and (iii) can extrude common substrates such as ethidium bromide (EtBr) and various antibiotics. As an indirect proof of similarity, we also demonstrated that some known NorA EPIs could inhibit NTM EPs and synergize with CLA against resistant *M. avium* strains.[12] However, there are few molecules characterized by a narrow chemical diversity that have been reported as NTM EPIs due to the lack of an in depth understanding around NTM EPs.[13,14] Indeed, there is a paucity of information regarding NTM EPs, particularly, in regard to three-dimensional structures. This lack of data precludes the possibility of employing a medicinal chemistry approach based on a structure-based drug design or the use of virtual screenings of compound libraries.

In this work, with the aim of expanding the array of compounds capable of inhibiting NTM EPs, we selected a set of 24 compounds from our in-house library, to be tested as NTM EPIs through a phenotypic approach. Of note, the selected molecules emerged from a previous scaffold hopping strategy aimed at increasing the chemical diversity of NorA EPIs.[15,16] The selected compounds are characterized by six different scaffolds (quinoline-4-carboxamide for compounds **1a** and **1b**, 1*H*-benzimidazole for compounds **2a** and **2b**, 4-hydroxyphthalazin-1(2*H*)-one for compounds **3a** and **3b**, pyridin-2-ol for compounds **4a** and **4b**, isoquinoline for compounds **5a** and **5b** and quinazoline for compounds **6a**, **6b**, **7a–g**, **8a–c**, **9a**, and **9b** (see Table 1). After a preliminary biological evaluation of this set of analogs against *M. smegmatis* mc^2155, some compounds bearing the carboxy-quinoline and the quinazoline scaffolds also exhibited a promising synergistic activity with CLA when tested in combination against the clinical isolate *M. avium* 2373 strain. Accordingly, a second set of new carboxy-quinoline (**1h–l**) and quinazoline (**10b**, **10c**, **11b**, **11c**, **12b**, **12c**, and **13b** [see Table 1]) analogs was designed, synthesized, and tested with the aim of finding novel and more potent NTM EPIs.

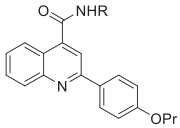
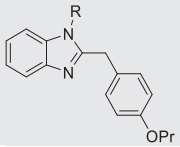
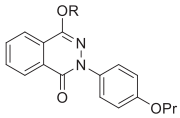
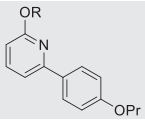
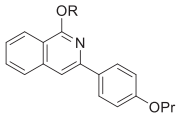
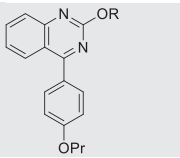
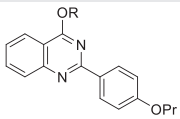
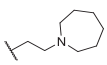
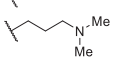
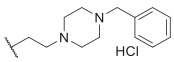
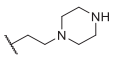
2 | RESULTS AND DISCUSSION

2.1 | Chemistry

Derivatives **1h–l** were synthesized according to the procedure reported in Scheme 1. Derivative **14**, synthesized according to the procedure reported by Cannalire et al.,[15] was reacted with appropriate amino-chains in the presence of TBTU, DIPEA and using dry DMSO as solvent to obtain the carboxamide analogs **1h–k**. Then, BOC removal from compound **1k** with TFA at 0°C in dry DCM yielded the piperidine derivative **1l**.

Compounds **10b**, **10c**, **11b**, **11c**, **12b**, **12c**, and **13b** were synthesized following the synthetic procedure reported in Scheme 2. The acyl chloride intermediates **15**,[18] **16**,[19] **17**,[20] and **18**,[21] were prepared and reacted with a solution of NH_3 7 M in MeOH or 33% NH_3 in H_2O to obtain the respective amide intermediates **19–22** in moderate to good yields. The nitro group reduction of derivatives **20** and **22** was performed using iron in an aqueous solution of acetic acid 0.6 M obtaining the corresponding amino derivatives **23** and **24** in good yields. The subsequent amide coupling of amino derivatives **19**, **21**, **23**, and **24** was carried out in the presence of 4-propoxybenzoyl chloride in dry THF and using Et_3N as a base, affording derivatives **25–28** which were then cyclized using

TABLE 1 MIC evaluation and synergism with CLA against *Mycobacterium smegmatis* mc^2 155 strain for compounds from in-house library **1a**, **1b**, **2a**, **2b**, **3a**, **3b**, **4a**, **4b**, **5a**, **5b**, **6a**, **6b**, **7a–g**, **8a–c**, **9a**, **9b**, and for the novel synthesized analogs **1h**, **1i**, **1j**, **1k**, **1l**, **10b**, **10c**, **11b**, **11c**, **12b**, **12c**, and **13b** in comparison with the reference compound **33**.

			M. smegmatis mc^2 155					
				Synergism				
Compd.		*R*	MIC (μg/mL)	MIC/fold	EPI conc (μg/mL)	CLA MIC (μg/mL)	N° fold CLA MIC reduction	Log D[17] (pH 7.40)
1a			64	1/4	16	4	2	3.04
1b			>512	1/4	128	0.5	16	3.31
				1/8	64	0.5	16	
				1/16	32	0.5	16	
				1/32	16	4	2	
2a			256	1/4	64	4	2	2.77
2b			>512	1/4	128	4	2	3.22
3a			256	1/4	64	0.5	16	2.52
				1/8	32	4	2	
3b			256	1/4	64	0.25	32	3.52
				1/8	32	4	2	
4a			64	1/4	16	4	2	2.90
4b			128	1/4	32	4	2	3.55
5a			32	1/4	8	4	2	3.89
5b			>512	1/4	128	≤0.125	≥64	4.32
				1/8	64	0.5	16	
				1/16	32	0.5	16	
				1/32	16	4	2	
6a			64	1/4	16	4	2	3.79
6b			64	1/4	16	4	2	4.33
7a			64	1/4	16	1	8	3.81
				1/8	8	4	2	
7b			>512	1/4	128	0.5	16	4.22
				1/8	64	0.5	16	
				1/16	32	4	2	
				1/32	16	4	2	
7c			32	1/4	8	4	2	4.19
7d			32	1/4	8	4	2	3.08
7e			>512	1/4	128	4	2	5.77
7f			32	1/4	8	4	2	2.47

(Continues)

TABLE 1 (Continued)

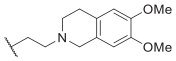
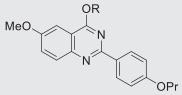
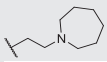
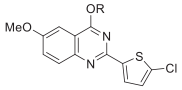
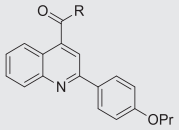
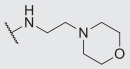
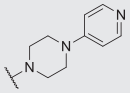
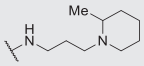
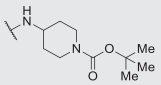
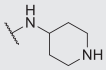
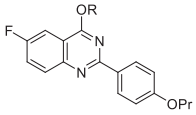
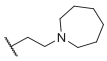
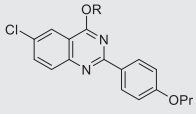
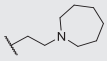
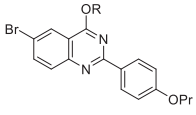
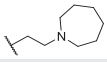
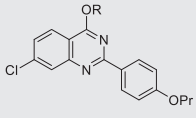
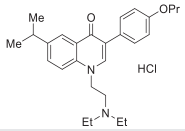
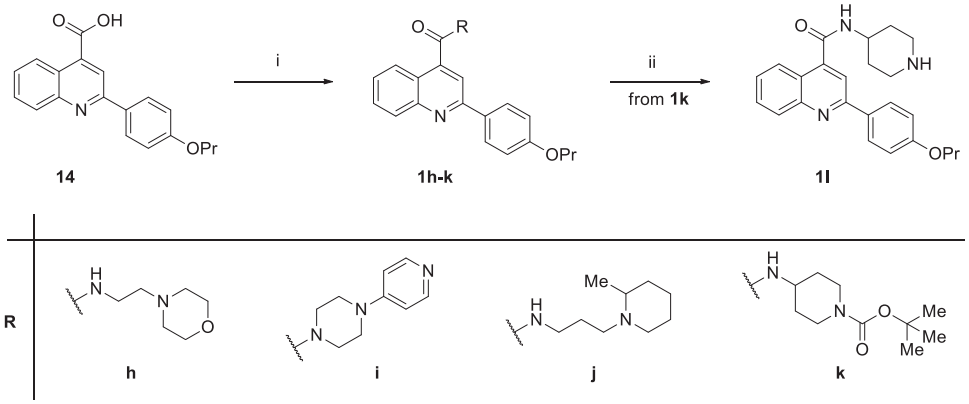
Compd.		R	*M. smegmatis* mc^2 155					Log D[17] (pH 7.40)
			MIC (μg/mL)	Synergism MIC/fold	Synergism EPI conc (μg/mL)	Synergism CLA MIC (μg/mL)	Synergism N° fold CLA MIC reduction	
7g			512	1/4	128	4	2	5.77
8a			32	1/4	8	2	4	3.60
				1/8	4	4	2	
8b			>512	1/4	128	4	2	4.07
8c			>512	1/4	128	4	2	4.03
9a			32	1/4	8	1	8	3.17
				1/8	4	4	2	
9b			>512	1/4	128	4	2	3.63
1h			>512	1/4	128	4	2	3.68
1i			32	1/4	8	2	4	3.87
				1/8	4	4	2	
1j			32	1/4	8	4	2	3.57
1k			>512	1/4	128	4	2	4.89
1l			32	1/4	8	4	2	1.07
10b			>512	1/4	128	1	8	4.44
				1/8	64	1	8	
				1/16	32	2	4	
				1/32	16	4	2	
10c			>512	1/4	128	2	4	4.41
				1/8	64	2	4	
				1/16	32	2	4	
				1/32	16	4	2	
11b			>512	1/4	128	4	2	4.84
11c			>512	1/4	128	2	4	4.81
				1/8	64	4	2	
12b			>512	1/4	128	4	2	5.04
12c			>512	1/4	128	4	2	5.01
13b			>512	1/4	128	0.25	32	4.82
				1/8	64	0.25	32	
				1/16	32	0.25	32	
				1/32	16	0.25	32	
				1/64	8	4	2	

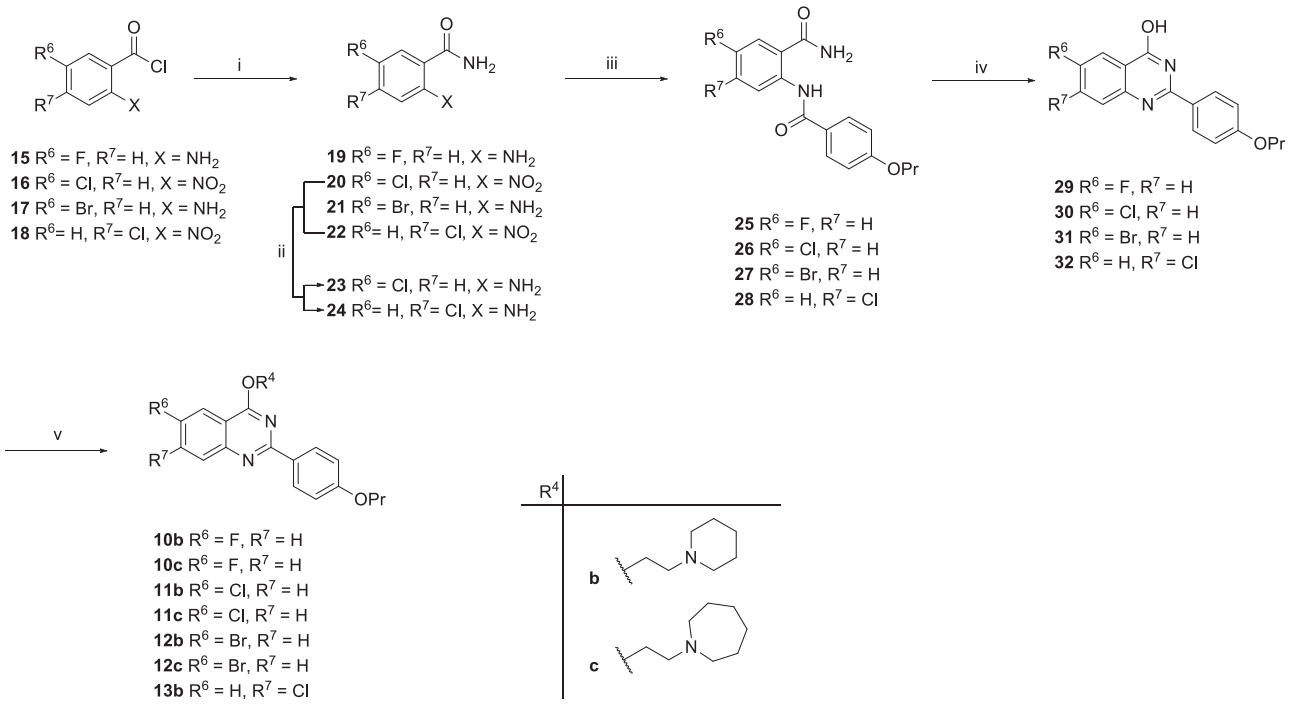
TABLE 1 (Continued)

Compd.	R	*M. smegmatis* mc^2 155 MIC (μg/mL)	Synergism MIC/fold	EPI conc (μg/mL)	CLA MIC (μg/mL)	N° fold CLA MIC reduction	Log D[17] (pH 7.40)
33 	-	32	1/4	8	2	4	3.63
CLA	-	-	-	-	8	-	1.63

Abbreviations: CLA, clarithromycin; EPI, efflux pump inhibitor; MIC, minimum inhibitory concentration.



SCHEME 1 Reagents and conditions: (i) appropriate amino-chains, TBTU, DIPEA, dry DMSO, room temperature (rt), 6–12 h, 30%–72%; (ii) TFA, dry DCM, 0°C, 4 h, 37%.



SCHEME 2 Reagents and conditions: (i) sol. NH_3 7 M in MeOH or NH_4OH 33%, THF or CH_3CN or benzene, rt, 2 h—overnight, 15%–82%; (ii) Fe, AcOH 0.6 M, rt, 72%–92%; (iii) Et_3N, dry THF, 4-propoxybenzoyl chloride, rt −60°C, 4–8 h, 84%–95%; (iv) *t*-BuOK, *t*-BuOH, rt, 3–4 h, 80%–92%; (v) K_2CO_3, appropriate chloroalkylamine, dry DMF, 80%–100°C, 2–5 h, 10%–30%.

t-BuOK in *t*-BuOH to give the quinazoline intermediates **29–32**. Alkylation of quinazoline scaffolds with appropriate alkylamino chains in dry DMF, and using K_2CO_3 as a base, gave regioselective *O*-alkylation affording derivatives **10b**, **10c**, **11b**, **11c**, **12b**, **12c**, and **13b**.

2.2 | Biological evaluation

The set of 24 compounds (**1a**, **1b**, **2a**, **2b**, **3a**, **3b**, **4a**, **4b**, **5a**, **5b**, **6a**, **6b**, **7a–g**, **8a–c**, **9a**, and **9b**),[15,16] selected from our in-house library and characterized by six different scaffolds, was first subjected to MIC evaluation against *M. smegmatis* mc^{2}155 (Table 1). This nonpathogenic NTM is commonly used in research laboratories due to its fast-growing ability and the expression of several EPs with significant homology with those of *M. avium* (a slow-growing NTM) [22] thus allowing reliable results in a reduced time frame. Most of the tested compounds exhibited MIC values ≥64 μg/mL, while six derivatives **5a**, **7c**, **7d**, **7f**, **8a**, and **9a** displayed MICs of 32 μg/mL. The determination of MIC values was essential to identify the concentration equal to ¼ MIC of each compound to be used as starting point in the synergistic assays with CLA against *M. smegmatis* mc^{2}155. Indeed, as for definition, EPI activity can be evaluated only at non-antibacterial concentrations, otherwise the final synergistic effect with CLA (or any other antibacterial agent) would be the result of a sum of activities. Therefore, all derivatives were tested in combination with CLA using ¼ MIC concentration and for compounds showing at least 4-fold CLA MIC reduction, scalar concentrations till $^1/_{32}$ MIC were also assayed. Among the tested derivatives, five compounds, **1b** (quinoline-4-carboxamide), **5b** (isoquinoline), and **7b**, **8a**, and **9a** (quinazoline) showed a synergistic activity with CLA (Table 1). C-6 -OMe quinazolines **8a** and **9a** were able to reduce the CLA MIC by 4- and 8-fold, respectively, exerting their synergistic activity with CLA at only ¼ MIC concentrations (8 μg/mL) (Table 1). On the other hand, the carboxy-quinoline derivative **1b** and the isoquinoline analog **5b**, when tested up to $^1/_{16}$ their MICs (32 μg/mL), were able to reduce the CLA MIC by 16-fold (from 8 to 0.5 μg/mL), while the slightly less active quinazoline analog **7b** retained this synergistic activity only at the higher concentration of 64 μg/mL ($^1/_8$ its MIC). Following an overall structure–activity relationship analysis, ethylpiperidine moiety is preferred over the diethylamino ethyl chain and, similarly to what observed for NorA inhibition,[15,16] phthalazinone, pyridine, and 4-aryl-quinazoline derivatives failed to produce any significant synergism with CLA against *M. smegmatis* mc^{2}155. On the other hand, the quinazoline analog **7b**, which showed synergistic activity with CLA against *M. smegmatis* mc^{2}155, was also reported as a NorA EPI derivative.[14] However, when the phenyl moiety of **7b** is shifted from C-2 to C-4 position (like in compound **6b**), the synergistic activity with CLA is lost, differently from that seen on *S. aureus*.[16] Then, focusing the attention on quinazoline derivatives, compounds **7b**, **8a**, and **9a** were able to synergize with CLA against *M. smegmatis* mc^{2}155; however, the presence of a -OMe group at C-6 position (**8a–c**) and a chlorothiophene at C-2 instead of a *p*-OPr-phenyl group (**9a**, **9b**) are detrimental for the activity in contrast with what observed for the NorA EPI activity, where these modifications afforded more potent analogs.[16] Indeed, it is well known that NTMs possess more lipophilic membrane than *S. aureus*, thus suggesting that more polar analogs such as compounds bearing the C-6 -OMe group instead of a hydrogen atom may be disadvantaged in crossing NTM membranes. A similar result was also observed by us within the series of 3-phenylquinolone analogs previously reported as NTM EPIs.[23,24] Even then, the introduction of an OMe group at the C-6 or C-7 position of the previously reported 3-phenylquinolone scaffold led to less active NTM EPIs than the respective unsubstituted analog, thus corroborating this hypothesis.

At this point, based on the activity against *M. smegmatis* mc^{2}155, the five best derivatives **1b**, **5b**, **7b**, **8a**, and **9a** were tested in combination with CLA against the *M. avium* clinical isolate MA2373 (Table 2). It should be noted that this strain is characterized by resistance to CLA but not by the common mutations on 23S rRNA, thus suggesting that the macrolide resistance was due to EP overexpression.[25] To confirm our hypothesis, we assessed the *M. avium* efflux activity of MA2373 by evaluating its ability to extrude EtBr. The clinical isolate MA878 (a CLA susceptible strain, CLA MIC = 1 μg/mL) was included for comparison. Figure 1 depicts the graph of the efflux activity recorded at three different time points (verapamil [VP] was used as pan-EPI to allow EtBr to accumulate in NTM cells). *T* = 0 min matches the conditions of EtBr accumulation (absence of glucose and VP at ½ MIC = 400 μg/mL), corresponding to 100% relative fluorescence. Three min after the removal of VP (early efflux measurement), an immediate decrease in fluorescence was detected in MA2373, confirming the presence of an overexpression of EPs that are responsible for the rapid EtBr extrusion. On the other hand, no early changes of fluorescence were observed in MA878, suggesting a basal expression of EPs. After 40 min (late efflux measurement), a remarkable decrease in fluorescence was observed in CLA-resistant *M. avium* MA2373 strain while only a lower effect was appreciable in CLA susceptible MA878. Late EtBr extrusion observed in *M. avium* MA878 could be ascribed to the basal activity of EP, always present in all microorganisms.

To further confirm that the overexpression of EPs is the main mechanism responsible for CLA resistance in MA2373 strain, we decided to test compound **33**,[23] previously reported by us as a potent *M. avium* EPI, in combination with CLA. Of note, **33** showed a comparable ability to synergize with CLA against both MA2373 and the previously used strain *M. avium* 104_{CLA3} (CLA resistant).[24] Indeed, in both cases, **33** at $^1/_8$ MIC (8 μg/mL) was able to reduce the CLA MIC by 4-fold (Table 2). Thus, once established that MA2373 is a suitable *M. avium* strain to evaluate the potential EPI activity of our compounds, we determined MIC values for the five best compounds **1b**, **5b**, **7b**, **8a**, and **9a**. Carboxy-quinoline derivative **1b** and quinazoline derivatives **7b** and **8a** retained their MIC values (512 and 32 μg/mL, respectively), like against *M. smegmatis* mc^{2}155, while derivative **9a** and the isoquinoline **5b** showed lower MICs (16 μg/mL). When tested at scalar concentrations (from $^1/_4$ to up $^1/_{64}$ their MIC value) for their synergistic activity with CLA against the MA2373

TABLE 2 MIC evaluation and synergism with CLA against *Mycobacterium avium* 2373 and 878 strains for compounds **1b**, **1i**, **5b**, **7b**, **8a**, **9a**, and **13b** in comparison with reference compound **33**.

	M. avium 2373					*M. avium* 878				
		Synergism					Synergism			
Compd.	MIC (μg/mL)	MIC/fold	EPI conc (μg/mL)	CLA MIC (μg/mL)	N° fold CLA MIC reduction	MIC (μg/mL)	MIC/fold	EPI conc (μg/mL)[a]	CLA MIC (μg/mL)	N° fold CLA MIC reduction
1b	512	1/4	128	≤0.5	≥1024	512	1/4	128	0.5	2
		1/8	64	1	512					
		1/16	32	2	256					
5b	16	1/4	4	>512	0	-[b]	-[b]	-[b]	-[b]	-[b]
7b	512	1/4	128	≤0.5	≥1024	512	1/4	128	1	0
		1/8	64	≤0.5	≥1024					
		1/16	32	≤0.5	≥1024					
		1/32	16	16	32					
8a	32	1/4	8	≤0.5	≥1024	-[b]	-[b]	-[b]	-[b]	-[b]
		1/8	4	32	16					
		1/16	2	>512	0					
9a	16	1/4	4	16	32	-[b]	-[b]	-[b]	-[b]	-[b]
		1/8	2	>512	0					
1i	32	1/4	8	>512	0	-[b]	-[b]	-[b]	-[b]	-[b]
13b	256	1/4	64	≤0.5	≥1024	512				
		1/8	32	≤0.5	≥1024					
		1/16	16	≤0.5	≥1024		1/4	128	0.25	4
		1/32	8	≤0.5	≥1024		1/8	64	1	0
		1/64	4	32	16					
33	64	1/4	16	16	≥64	-[b]	-[b]	-[b]	-[b]	-[b]
		1/8	8	256	4					
		1/16	4	512	2					
CLA				>512					1	

Abbreviations: CLA, clarithromycin; EPI, efflux pump inhibitor; MIC, minimum inhibitory concentration.

[a]Compounds **1b**, **7b**, and **13b** were tested at the higher concentration used against *M. avium* 2373 strain.

[b]Not tested.

clinical strain, all but one compound (**5b**) displayed an interesting activity. Indeed, carboxy-quinoline derivative **1b** was able of reducing the CLA MIC by 256-fold when tested at 32 μg/mL ($^1/_{16}$ MIC), while the quinazoline derivatives **7b** at $^1/_{32}$ MIC (16 μg/mL) reduced CLA MIC by 32-fold, **8a** at $^1/_8$ MIC (4 μg/mL) reduced CLA MIC by 16-fold, and **9a** at the same concentration ($^1/_4$ MIC) reduced CLA MIC by 32-fold (Table 2).

Considering the promising synergistic activity shown by the carboxyquinoline **1b** and quinazoline derivatives **7b**, **8a**, and **9a** with CLA against MA2373 strain, we decided to test their cytotoxic activity against human lung carcinoma epithelial-like A549 cell line to focus our development efforts on those compounds endowed with the best profile in terms of synergistic activity with CLA/cytotoxicity.

As shown in Figure 2, among the four compounds tested (**1b**, **7b**, **8a**, and **9a**), only derivatives **1b** and **7b** exhibited low cytotoxicity, even at higher concentrations of 16 and 32 μg/mL. On the other hand, C-6 methoxy quinazolines **8a** and **9a** displayed significant cytotoxic activity at those concentrations. Therefore, we focused our efforts on the design and synthesis of additional analogs bearing the two scaffolds of **1b** and **7b**. We decided to proceed focusing the modifications on the carboxy-quinoline nucleus modifying the less explored C-4 position amide group by replacing the ethylpiperidine chain of **1b** with five different alkyl amino chains, *N*-(2-morpholinoethyl) (**1h**), 4-(pyridin-4-yl) piperazine (**1i**), *N*-[3-(2-methylpiperidin-1-yl)propyl] (**1j**), *t*-butyl 4-piperidine-1-carboxylate (**1k**) and *N*-piperidin-4-yl (**1l**)) to enlarge the series of side chains on the carboxy quinoline scaffold. Regarding the design of additional

quinazoline analogs, we focused our modifications on C-6 and C-7 positions of the quinazoline core since the C-2 and C-4 positions have been, at least in part, already explored with derivatives **7a–g**, **9a**, and **9b**. Following the hypothesis that more lipophilic compounds could better penetrate inside the NTM membranes, we introduced halogen atoms (fluorine, chlorine, bromine) at C-6 position of the quinazoline scaffold (compounds **10b**, **10c**, **11b**, **11c**, and **12b**, **12c**, respectively) or a chlorine atom at C-7 position (compound **13b**).

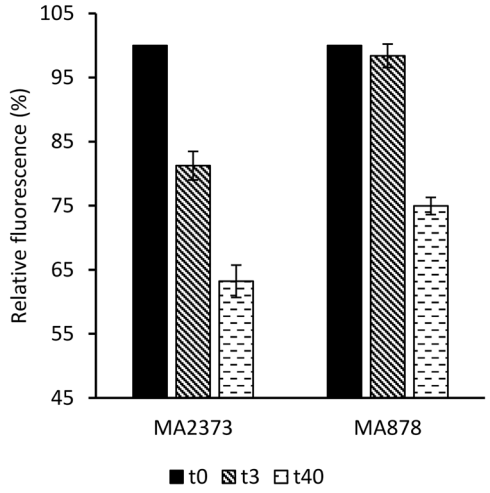


FIGURE 1 Relative fluorescence of ethidium bromide in *Mycobacterium avium* clinical isolates 2373 and 878 at $t = 0$ (no efflux), $t = 3$ min (early efflux measurement), and $t = 40$ (late efflux measurement).

The novel set of derivatives was then tested following the same procedure as described for the in-house library compounds. MIC evaluation and the subsequent synergy assay with CLA were first performed against *M. smegmatis* mc^{2}155 strain (Table 1) and then, only the best derivatives were tested against CLA-resistant MA2373 and CLA-sensitive *M. avium* 878 clinical strains (Table 2). The quinoline 4-carboxamide analogs exhibited different MIC values when tested against *M. smegmatis* mc^{2}155, strongly dependent on the nature of the alkyl amino chain substituent of the amide at C-4 position. However, when tested at ¼ MIC for their synergistic activity with CLA, none of them showed results with the only exception of piperazine-pyridinyl derivative (**1i**) able of reducing the CLA MIC only by 4-fold. Unfortunately, when tested against MA2373 clinical strain, it completely lost the synergistic activity with CLA. Considering the quinazoline series, all compounds exhibited high MIC values against *M. smegmatis* mc^2 155 (>512 μg/mL). The introduction, at C-6 position of the scaffold, of halogen atoms such as chlorine and bromine atoms (**11b**, **11c**, **12b**, and **12c**, respectively) proved to be detrimental for the NTM EPI activity against *M. smegmatis* mc^{2}155, while derivatives **10b** and **10c**, having a fluorine atom at C-6 position, showed the ability of synergizing with CLA reducing its MIC of 4-fold when tested at $^1/_{16}$ MIC (32 μg/mL). On the other hand, the C-7 chlorine quinazoline derivative **13b** exhibited an excellent synergistic activity with CLA, reducing its MIC by 32-fold when tested at $^1/_{32}$ MIC (16 μg/mL). Interestingly, when compound **13b** was tested against MA2373, it showed outstanding results being able at $^1/_{32}$ MIC (8 μg/mL) and $^1/_{64}$ MIC (4 μg/mL) to reduce CLA MIC by ≥1024- and 16-fold (from >512 to ≤0.5 and 32 μg/mL, respectively). Taken together, these results suggest that the synergistic effect of

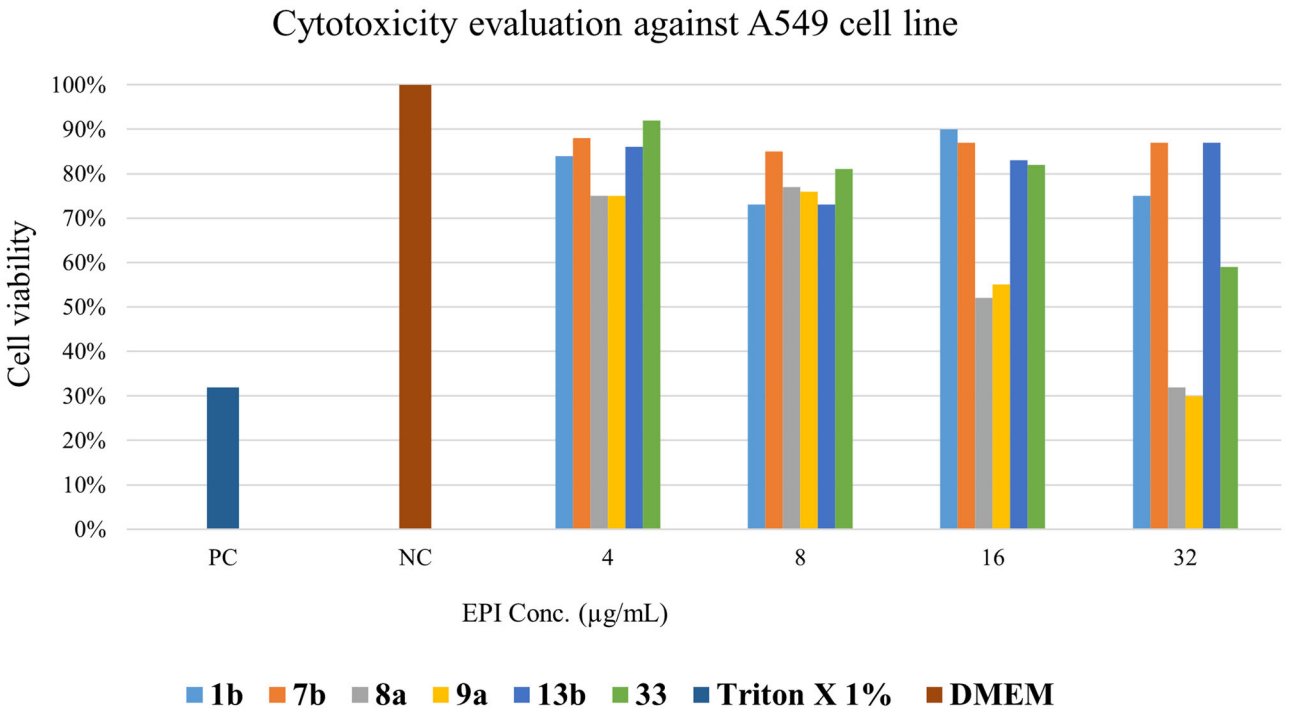


FIGURE 2 Cytotoxic activity of compounds **1b**, **7b**, **8a**, **9a**, and **13b** in comparison with that of reference **33** against A549 cell line. Triton X 1% (positive control—PC) and Dulbecco's modified Eagle's medium (DMEM) (negative control—NC) were included as controls. EPI, efflux pump inhibitors.

these compounds, in particular, of quinazoline analogs, is not guided by their lipophilicity. Indeed, quinazoline derivatives with a chlorine atom at C-6 (**10b**) or C-7 (**13b**) position showed a completely different synergism with CLA. Therefore, it can be assumed that the presence of a chlorine atom at C-7 position of the quinazoline core serves to increase the potential interactions between the compound and EP and/or to influence the overall electron distribution of the molecule as being an electron-withdrawing group.

To support the evidence that lipophilicity does not play a fundamental role in the EPI activity of the tested molecules, we extended the analysis of this property to all tested compounds against *M. smegmatis* mc^2155 strain by calculating LogD values in silico (Table 1). Although the role of lipophilicity is a well-known factor in the activity of antimicrobial drugs,[26] this cannot be translated to NTM EPIs. Indeed, most of the active and inactive tested compounds as NTM EPIs showed LogD values ranging from 3 to 5, confirming that lipophilicity does not guide EPI activity.

Additionally, as indirect evidence that the synergistic activity of the best compounds **1b**, **7b**, and **13b** was related to the inhibition of EPs, we evaluated them in combination with CLA against the MA878 strain, which has a weaker EP activity than MA2373 (see above for EtBr efflux assays in Figure 1). As expected, at the used concentrations, all three compounds did not exhibit any significant synergism with CLA against MA878 strain, thus suggesting that their synergistic activity is strongly dependent on the effect of EPs (Table 2).

Regarding compound **13b**, which showed the best synergistic activity with CLA against the MA2373 resistant strain, cytotoxicity experiments against the A549 cell line displayed that it possessed only a poor cytotoxic effect even at the highest tested concentration of 32 μg/mL (Figure 2). It is noteworthy that this concentration is eight times higher than the concentration at which the compound exhibited its synergistic effect with CLA against the clinical isolate MA2373 strain.

3 | CONCLUSION

Herein, we have reported efforts to identify new and potent NTM EPIs endowed with an acceptable safety profile. First, using our in-house library of 24 compounds, which were originally designed for a scaffold hopping approach in search of new *S. aureus* NorA EPIs and characterized by six different scaffolds, we endeavored to identify new NTM EPIs and gain insight into overlaps and/or differences in the structural requirements necessary to obtain *S. aureus* NorA and/or NTM EPIs. From this initial screening, five compounds characterized by three different scaffolds (carboxy-quinoline **1b**, isoquinoline **5b**, and quinazolines **7b**, **8a**, and **9a**) emerged as having synergistic activity with CLA against *M. smegmatis* mc^2155. Four of them, compounds **1b**, **7b**, **8a**, and **9a**, also confirmed their synergistic activity with CLA against MA2373 clinical isolate, characterized in this study for the presence of CLA efflux as the main mechanism of resistance. From the determination of the cytotoxic effect for compounds **1b**, **7b**, **8a**, and **9a** against human lung carcinoma epithelial-like A549 cell line, the safest derivatives were selected for further chemical development. Based on the obtained results, 12 novel analogs of the best derivatives **1b** and **7b**, were designed and synthesized to improve the NTM EPI activity. From the second set of compounds, **13b** emerged as the most potent NTM EPI, showing the ability to reduce the CLA MIC by ≥1024 when tested at 8 μg/mL and 16-fold when tested at 4 μg/mL against the MA2373 clinical isolate. Moreover, compound **13b** demonstrated a favorable safety profile as it was not cytotoxic to the A549 cell line even at the highest tested concentration of 32 μg/mL, which is eight times greater than the concentration that showed a synergistic effect with CLA against the resistant MA2373. Therefore, it represents the best NTM EPI identified to date. In addition to its potent activity and promising toxicity profile, it is noteworthy that compound **13b** exhibited exclusive synergistic effects against the MA2373 strain, which is resistant to CLA due to its capacity to extrude macrolides via EPs. Conversely, **13b** did not demonstrate synergism with CLA when tested against the sensitive MA878 strain. These results indirectly suggest that **13b** selectively synergizes with CLA, likely by inhibiting the EPs responsible for macrolide efflux. This observation is significant because it underscores that **13b** does not exhibit nonspecific synergistic activity with CLA, which would otherwise occur even in strains not resistant to macrolides.

The pursuit of new microbial EPIs remains in its nascent stage, and the approach has yet to be thoroughly validated in animal models. However, with the emergence of the concept of developing molecules capable of modulating microbial resistance, which could be synergistically combined with conventional antimicrobials, research in this field has garnered increased attention in recent years. In the realm of Gram-negative bacteria, significant strides have been made, both in identifying potent EPIs and elucidating the three-dimensional structures of EPs. Conversely, progress against Gram-positive bacteria, including NTMs, has been more modest. Consequently, substantial efforts are needed in this direction to establish the proof of concept that EPI molecules could play a pivotal role in future antimicrobial therapies.

In this context, the present work aims to improve the basic knowledge of the still theoretical feasibility of using EPIs in combination with macrolides to combat NTM infections. The discovery of the quinazoline derivative **13b**, which exhibits robust synergistic activity with CLA against a clinical isolate of *M. avium*, without toxicity to human cells, serves as a solid foundation for future investigations. Compound **13b** also holds promise for broader biological characterization, including validation of its efficacy in the context of intracellular NTM infections. Furthermore, once its favorable toxicity profile on other cell lines is confirmed, **13b** could serve as the first candidate for in vivo testing in animal models to assess its NTM EPI capacity. Finally, our results demonstrate that **13b** synergizes with CLA by inhibiting EPs in both *M. smegmatis* and *M. avium*, laying the groundwork for further experiments targeting other NTMs, including the notorious *M. abscessus*.

4 | EXPERIMENTAL

4.1 | Chemistry

4.1.1 | General

All starting materials, reagents, and solvents were purchased from common commercial suppliers and were used as such, unless otherwise indicated. Organic solutions were dried over anhydrous Na_2SO_4 and concentrated with a rotary evaporator at low pressure. All reactions were routinely checked by thin-layer chromatography on silica gel 60_{F254} (Merck) and visualized by using UV or iodine. Flash chromatography separations were carried out on Merck silica gel 60 (mesh 230–400). Yields were of purified products and were not optimized. ^{1}HNMR and ^{13}CNMR spectra (see the Supporting Information) were recorded at 400 and 101 MHz, respectively, using a Bruker Avance DRX-400 (Bruker Corporation). Chemical shifts are given in ppm (δ) relative to TMS. Spectra were acquired at 298 K. Data processing was performed with standard Bruker software TopSpin (Vers. 4.1.4) and the spectral data are consistent with the assigned structures. Detection mass was based on electrospray ionization (ESI) in positive polarity using an Agilent 1290 Infinity System equipped with an MS detector Agilent 6550UHD Accurate Mass Q-TOF.

Compounds **1h–l**, **10b** and **10c**, and **12b** were ≥95% pure as determined by LC/MS using an Agilent 1290 Infinity System machine equipped with DAD detector from 190 to 640 nm. The purity was revealed at 254 nm using a Phenomenex AERIS Widepore C4, 4.6, 100 mm (6.6 lm) with flow rate: 0.5 mL/min; acquisition time: 10 min; gradient: acetonitrile in water containing 0.1% of formic acid (0%–100% in 10 min); oven temperature, 30 C. Peak retention time is given in minutes.

Compounds **11b**, **11c**, **12c**, and **13c** were ≥95% pure as determined by HPLC analysis using a Jasco LC-4000 instrument equipped with a UV-Visible Diode Array Jasco MD-4015 and an XTerra MS C18 Column, 5 µm, 4.6 mm × 150 mm. The purity was revealed at 254 nm and the methods used have been specified for each compound. Chromatograms were analyzed using ChromNAV 2.0 Chromatography Data System software and the peak retention time is given in minutes.

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

4.1.2 | General procedure (A) for the synthesis of compounds **1h–k**

Under N_2 atmosphere to a solution of derivative **14** (1.0 eq) in dry DMSO (3 mL), DIPEA (4.0 eq), TBTU (1.2 eq), and proper amino chains (1.2 eq) were added, and the reaction was stirred for 6–12 h at room temperature (rt). The mixture was poured in ice/water and extracted with EtOAc (3 × 100 mL). The organic layer was washed with brine, dried over Na_2SO_4, and evaporated to dryness to give target compounds. Reaction crude was purified by flash column chromatography to give the title compounds.

N-(2-Morpholinoethyl)-2-(4-propoxyphenyl)quinoline-4-carboxamide (**1h**): General procedure (A): using (2-morpholin-4-ylethyl) amine, time = 12 h. Compound **1h** was obtained after purification by flash column chromatography eluting with $CHCl_3$/MeOH 97/3 as a white solid in 39% yield. ^{1}HNMR ($CDCl_3$, 400 MHz): δ_H 1.10 (3H, t, J = 7.5 Hz, $OCH_2CH_2\mathit{CH_3}$), 1.84–1.92 (2H, m, $OCH_2\mathit{CH_2}CH_3$), 2.50–2.57 (4H, m, morpholine CH_2 x2), 2.69 (2H, t, J = 5.8 Hz, $CONHCH_2\mathit{CH_2}N$), 3.69–3.74 (6H, m, $CONH\mathit{CH_2}CH_2N$ and morpholine CH_2 x2), 4.04 (2H, t, J = 6.5 Hz, $O\mathit{CH_2}CH_2CH_3$), 6.64–6.66 (1H, m, CON*H*), 7.07 (2H, d, J = 8.6 Hz, Ar–H), 7.56 (1H, t, J = 7.0 Hz, Ar–H), 7.77 (1H, t, J = 7.0 Hz, Ar–H), 7.92, (1H, s, Ar–H), 8.15 (2H, d, J = 6.7 Hz, Ar–H), 8.18–8.17 (2H, m, Ar–H). ^{13}CNMR ($CDCl_3$, 101 MHz): δ_C 10.61, 22.66, 35.80, 53.29, 57.26, 66.19, 69.75, 114.97, 116.65, 123.21, 125.08, 126.85, 128.98, 130.07, 131.31, 142.32, 148.90, 156.60, 160.83 (2C), 167.90 ppm. HPLC r_t: 4.81 min. HRMS (ESI) m/z $[M+H]^+$ calcd. for $C_{25}H_{29}N_3O_3$ 420.2282 found 420.22818.

[2-(4-Propoxyphenyl)quinolin-4-yl][4-(pyridin-4-yl)piperazin-1-yl]methanone (**1i**): General procedure (A): using 1-pyridin-4-ylpiperazine, time = 12 h. Compound **1i** was obtained after purification by flash column chromatography eluting with $CHCl_3$/MeOH 95/5 as a yellow solid in 72% yield. ^{1}HNMR ($CDCl_3$, 400 MHz): δ_H 1.08 (3H, t, J = 7.4 Hz, $OCH_2CH_2\mathit{CH_3}$), 1.82–1.91 (2H, m, $OCH_2\mathit{CH_2}CH_3$), 3.21–3.25 (2H, m, piperazine CH_2), 3.38–3.41 (2H, m, piperazine CH_2), 3.56–3.57 (2H, m, piperazine CH_2), 4.02 (3H, t, J = 6.6 Hz, $O\mathit{CH_2}CH_2CH_3$ and piperazine CH), 4.13–4.20 (1H, m, piperazine CH), 6.67 (2H, d, J = 6.8 Hz, Ar–H), 7.05 (2H, d, J = 6.9 Hz, Ar–H), 7.54 (1H, dt, J = 1.1 and 6.9 Hz, Ar–H), 7.74–7.80 (3H, m, Ar–H), 8.14 (2H, d, J = 6.8 Hz, Ar–H), 8.19 (1H, dd, J = 1.0 and 8.9 Hz, Ar–H), 8.32 (2H, d, J = 6.4 Hz, Ar–H). ^{13}CNMR ($CDCl_3$, 101 MHz): δ_C 10.60, 22.65, 41.23, 46.07, 46.22, 46.72, 69.78, 108.64, 115.03, 115.56, 122.71, 124.13, 127.21, 128.93, 130.40, 130.53, 131.13, 142.19, 148.39, 148.59, 155.15, 156.76, 160.96, 167.60 ppm. HPLC r_t: 4.31 min. HRMS (ESI) m/z $[M+H]^+$ calcd. for $C_{28}H_{28}N_4O_2$ 453.2185 found 453.22857.

N-[3-(2-Methylpiperidin-1-yl)propyl]-2-(4-propoxyphenyl)quinoline-4-carboxamide (**1j**): General procedure (A): using 3-(2-methylpiperidin-1-yl) propan-1-amine, time = 12 h. Compound **1j** was obtained after purification by flash column chromatography eluting with $CHCl_3$/MeOH 90/10 as a yellow solid in 30% yield. ^{1}HNMR ($CDCl_3$, 400 MHz): δ_H 1.06 (9H, m, $OCH_2CH_2\mathit{CH_3}$, piperidine CH_3, and CH x3), 1.38–1.40 (3H, m, piperidine CH and $NCH_2\mathit{CH_2}CH_2N$), 1.81–1.92 (4H, m, $NCH_2CH_2\mathit{CH_2}N$ and $OCH_2\mathit{CH_2}CH_3$), 2.14–2.16 (1H, m, piperidine CH), 2.44–2.47 (2H, m, $N\mathit{CH_2}CH_2CH_2N$), 2.88–2.91 (1H, m, piperidine CH), 2.99–3.01 (1H, m, piperidine CH), 3.54–3.57 (1H, m, piperidine CH), 3.58–3.60 (1H, m, piperidine CH), 3.73–3.74 (1H, m, piperidine CH), 4.00 (2H, t, J = 6.6 Hz, $O\mathit{CH_2}CH_2CH_3$), 7.03 (2H, d, J = 8.8 Hz, Ar–H), 7.53 (1H, t, J = 7.1 Hz, Ar–H), 7.72 (1H, t, J = 7.0 Hz, Ar–H), 7.95 (1H, s, Ar–H), 8.13–8.17 (3H, m, Ar–H), 8.25 (1H, d, J = 8.4 Hz, Ar–H), 8.69 (1H, s, CON*H*). ^{13}CNMR ($CDCl_3$, 101 MHz): δ_C 10.53, 17.55, 22.40, 22.58, 24.24, 24.96, 33.27, 40.10, 51.05, 52.75, 56.90, 69.64, 114.81, 116.27, 123.25, 125.28, 126.67, 128.82, 129.84, 129.90, 131.33, 143.00, 148.81, 156.44, 160.63, 167.68 ppm. HPLC r_t: 4.49 min. HRMS (ESI) m/z $[M+H]^+$ calcd. for $C_{24}H_{35}N_3O_2$ 446.2802 found 446.28028.

tert-Butyl 4-({[2-(4-propoxyphenyl)quinolin-4-yl]carbonyl}amino) piperidine-1-carboxylate (**1k**): General procedure (A): using *tert*-butyl 4-aminopiperidine-1-carboxylate, time = 6 h. Compound **1k** was obtained after purification by flash column chromatography eluting with Cy/EtOAc 60/40 as a white solid in 46% yield. ^{1}HNMR ($CDCl_3$, 400 MHz): δ_H 1.10 (3H, t, J = 7.4 Hz, $OCH_2CH_2CH_3$), 1.49–1.50 (11H, m, $OCH(CH_3)_3$ and piperidine CH_2), 1.84–1.91 (2H, m, $OCH_2CH_2CH_3$), 2.14–2.17 (2H, m, piperidine CH_2), 2.97 (2H, t, J = 11.2 Hz, piperidine CH_2), 4.02 (2H, t, J = 6.2 Hz, $OCH_2CH_2CH_3$), 4.17–4.30 (3H, m, piperidine CH_2 and CH), 6.17 (1H, s, CONH), 7.04 (2H, d, J = 7.6 Hz, Ar–H), 7.52 (1H, t, J = 7.5 Hz, Ar–H), 7.74 (1H, t, J = 8.0 Hz, Ar–H), 7.79 (1H, s, Ar–H), 8.06–8.11 (3H, m, Ar–H), 8.14–8.16 (1H, m, Ar–H). ^{13}CNMR ($CDCl_3$, 101 MHz): δ_C 10.53, 22.57, 28.44, 32.07, 42.80, 47.68, 47.78, 69.68, 79.84, 114.86, 115.94, 122.91, 124.73, 126.96, 128.84, 129.90, 130.15, 131.01, 142.64, 148.65, 154.69, 156.36, 160.81, 167.08 ppm. HPLC r_t: 6.49 min. HRMS (ESI) m/z $[M+H]^+$ calcd. for $C_{24}H_{28}ClN_3O_2$ 490.2701 found 490.27061.

N-[2-(Piperazin-1-yl)ethyl]-2-(4-propoxyphenyl)quinoline-4-carboxamide (**1l**): Under N_2 atmosphere, to a solution of compound **1k** (100 mg, 0.20 mmol) in dry DCM (4 mL), trifluoro acetic acid (0.61 mL) was added dropwise at 0°C. The reaction mixture was stirred at 0°C for 4 h. Then, it was poured into ice/water, pH was modified with $NaHCO_3$ up to 7–8, and extracted with DCM (3 × 100 mL). The organic layers were washed with brine, dried over Na_2SO_4 and evaporated to dryness to give a yellow solid. After purification by flash column chromatography eluting with $CHCl_3$/MeOH 97/3, compound **1l** was obtained as white solid in 37% yield. ^{1}HNMR (DMSO-$_{d6}$, 400 MHz): δ_H 1.02 (3H, t, J = 7.3 Hz, $OCH_2CH_2CH_3$), 1.40-1.51 (2H, m, piperazine CH_2), 1.76–1.81 (2H, m, $OCH_2CH_2CH_3$), 1.92 (2H, d, J = 11.8 Hz, piperazine CH_2), 2.63 (2H, t, J = 11.9 Hz, piperazine CH_2), 3.02–3.05 (2H, m, piperazine CH_2), 3.98–4.00 (1H, m, NH), 4.04 (2H, t, J = 6.5 Hz, $OCH_2CH_2CH_3$), 7.12 (2H, d, J = 8.6 Hz, Ar–H), 7.60 (1H, t, J = 7.2 Hz, Ar–H), 7.79 (1H, t, J = 7.0 Hz, Ar–H), 8.04 (1H, s, Ar–H), 8.09 (2H, t, J = 8.6 Hz, Ar–H), 8.28 (2H, d, J = 8.6 Hz, Ar–H), 8.77 (1H, d, J = 7.5 Hz, NH). ^{13}CNMR (DMSO-$_{d6}$, 101 MHz): δ_C 10.96, 22.60, 33.49, 45.71 (2 C), 48.07, 69.67, 115.27, 116.50, 123.67, 125.77, 127.12, 129.30, 129.84, 130.53, 131.08, 143.92, 148.42, 155.95, 160.83, 166.39 ppm. HPLC r_t: 3.97 min. HRMS (ESI) m/z $[M+H]^+$ calcd. for $C_{24}H_{27}N_3O_2$ 390.2176 found 390.21804.

4.1.3 | General procedure (B) for the synthesis of compounds **19–22**

To a solution of compounds **15–18** (1.0 eq) in THF or CH_3CN or benzene (4 mL/mmol), NH_4OH 33% (2.0 eq), or NH_3 7 M in MeOH (2.0 eq) was added dropwise at 0°C. The mixture was stirred at rt for 2 h to overnight. The mixture was filtered to eliminate the NH_4Cl formed, and the filtrate was evaporated under vacuum to obtain the crude that was treated with a saturated solution of $NaHCO_3$ to afford a precipitate that was filtered giving the target amide intermediates.

2-Amino-5-fluorobenzamide (**19**): General procedure (B): starting from derivative **15** and using NH_3 7 M in MeOH (time = 2 h), the title compound was obtained as a white solid in 32% yield. ^{1}HNMR (DMSO-d_6, 400 MHz): δ_H 6.46 (2H, s, NH_2), 6.68–6.71 (1H, m, Ar–H), 7.05 (1H, td, J = 2.9 and 6.1 Hz, Ar–H), 7.21 (1H, s, CONH), 7.40 (1H, dd, J = 2.7 and 7.6 Hz, Ar–H), 7.79 (1H, s, CONH).

5-Chloro-2-nitrobenzamide (**20**): General procedure (B): starting from derivative **16** and using NH_4OH 33%, (time = 12 h), the title compound was obtained as a white solid in 74% yield. ^{1}HNMR (DMSO-d_6, 400 MHz): δ_H 7.74–7.78 (2H, m, Ar–H), 7.81 (1H, s, CONH), 8.06 (1H, d, J = 8.6 Hz, Ar–H), 8.21 (1H, s, CONH).

2-Amino-5-bromobenzamide (**21**): General procedure (B): starting from derivative **17** and using NH_4OH 33%, (time = 12 h), the title compound was obtained as a yellow solid in 15% yield. ^{1}HNMR (DMSO-d_6, 400 MHz): δ_H 6.66 (1H, d, J = 8.8 Hz, Ar–H), 6.56 (2H, s, NH_2), 7.18 (1H, s, CONH), 7.26 (1H, dd, J = 1.9 and 8.6 Hz, Ar–H), 7.70 (1H, d, J = 1.8 Hz, Ar–H), 7.84 (1H, s, CONH).

4-Chloro-2-nitrobenzamide (**22**): General procedure (B): starting from derivative **18** and using NH_4OH 33%, (time = 2 h), the title compound was obtained as a yellow solid in 82% yield. ^{1}HNMR (acetone-d_6, 400 MHz): δ_H 7.08 (1H, s, CONH), 7.60 (1H, s, CONH), 7.71 (1H, d, J = 8.2 Hz, Ar–H), 7.79 (1H, d, J = 8.1 Hz, Ar–H), 8.01 (1H, s, Ar–H).

2-Amino-5-chlorobenzamide (**23**): To a suspension of compound **20** (400 mg, 1.99 mmol), in 0.6 M aqueous solution of acetic acid (20 mL), iron (777 mg, 13.9 mmol) was added, and the mixture was stirred at rt for 4 h. The reaction was filtered on Celite, and the filtrate was evaporated under vacuum to obtain a brown oil that was treated with a saturated solution $NaHCO_3$ and extracted with EtOAc (20 mL x3). Then, the organic phase was washed with brine and dried over with Na_2SO_4 that was filtered, and the solvent was concentrated under vacuum to obtain intermediate **23** as an orange solid (72% yield). ^{1}HNMR (DMSO-d_6, 400 MHz): δ_H 6.69 (1H, d, J = 1.9 Hz, Ar–H), 6.72 (2H, s, NH_2), 7.14–7.17 (2H, m, Ar–H and CONH), 7.59 (1H, d, J = 2.2 Hz, Ar–H), 7.84 (1H, s, CONH).

2-Amino-4-chlorobenzamide (**24**): Following the same procedure as for compound **23** and starting from compound **22** (200 mg, 0.99 mmol), the title compound was obtained as an orange solid (92% yield). ^{1}HNMR (acetone-d_6, 400 MHz): δ_H 6.49 (1H, dd, J = 2.1 and 8.4 Hz, Ar–H), 6.64 (2H, s, NH_2), 6.78 (1H, d, J = 1.9 Hz, Ar–H), 7.30–7.35 (2H, m, $CONH_2$), 7.56 (1H, d, J = 8.5 Hz, Ar–H).

4.1.4 | General procedure (C) for the synthesis of compounds **25–28**

Under N_2 atmosphere, to a solution of derivatives **19**, **21**, **23**, and **24** (1.0 eq) in dry THF (4 mL/mmol), Et_3N (3.0 or 5.0 eq) was added. After stirring for 10 min, a suspension of 4-propoxybenzoyl chloride (1.0 eq) in dry THF (3 mL/mmol) was dripped. The reaction mixture was stirred at rt or 60°C for 4–8 h. The reaction mixture was then poured in ice/water and the formed precipitate was filtered to obtain the target compounds.

N-(2-Carbamoyl-4-fluorophenyl)-4-propoxybenzamide (**25**): General procedure (C): starting from derivative **19**, (time = 5 h, *T* = 60°C) the title compound was obtained as a white solid in 90% yield. ^{1}HNMR (DMSO-d_6, 400 MHz): δ_H 0.95 (3H, t, *J* = 7.3 Hz, $OCH_2CH_2CH_3$), 1.69–1.74 (2H, m, $OCH_2CH_2CH_3$), 3.98 (2H, t, *J* = 6.4 Hz, $OCH_2CH_2CH_3$), 7.06 (2H, d, *J* = 8.6 Hz, Ar–H), 4.92 (1H, dt, *J* = 2.7 and 8.9 Hz, Ar–H), 7.68 (1H, dd, *J* = 2.5 and 9.5 Hz, Ar–H), 7.84 (2H, d, *J* = 8.5 Hz, Ar–H), 7.91 (1H, s, CONH), 8.47 (1H, s, CONH), 8.69–8.73 (1H, m, Ar–H), 12.68 (1H, s, CONH).

N-(2-Carbamoyl-4-chlorophenyl)-4-propoxybenzamide (**26**): General procedure (C): starting from derivative **23**, (time = 4 h, *T* = 60°C), the title compound was obtained as a white solid in 95% yield. ^{1}HNMR (DMSO-d_6, 400 MHz): δ_H 0.99 (3H, t, *J* = 7.3 Hz, $OCH_2CH_2CH_3$), 1.72–1.79 (2H, m, $OCH_2CH_2CH_3$), 4.02 (2H, t, *J* = 6.4 Hz, $OCH_2CH_2CH_3$), 7.12 (2H, d, *J* = 8.6 Hz, Ar–H), 7.63 (1H, dd, *J* = 2.1 and 9.0 Hz, Ar–H), 7.88 (2H, d, *J* = 8.6 Hz, Ar–H), 8.04–8.07 (2H, m, Ar–H and CONH), 8.53 (1H, s, CONH), 8.72 (1H, d, *J* = 9.0 Hz, Ar–H), 12.78 (1H, s, CONH).

N-(4-Bromo-2-carbamoylphenyl)-4-propoxybenzamide (**27**): General procedure (C): starting from derivative **21**, (time = 4 h, *T* = 60°C) the title compound was obtained as a yellow solid in 84% yield. ^{1}HNMR (DMSO-d_6, 400 MHz): δ_H 0.99 (3H, t, *J* = 7.4 Hz, $OCH_2CH_2CH_3$), 1.74–1.79 (2H, m, $OCH_2CH_2CH_3$), 4.03 (2H, t, *J* = 6.5 Hz, $OCH_2CH_2CH_3$), 7.11 (2H, d, *J* = 8.7 Hz, Ar–H), 7.74 (1H, dd, *J* = 1.9 and 7.0 Hz, Ar–H), 7.88 (2H, d, *J* = 8.7 Hz, Ar–H), 7.96 (1H, s, CONH), 8.09 (1H, d, *J* = 1.9 Hz, Ar–H), 8.52 (1H, s, CONH), 8.66 (1H, d, *J* = 9.0 Hz, Ar–H), 12.78 (1H, s, CONH).

N-(2-Carbamoyl-5-chlorophenyl)-4-propoxybenzamide (**28**): General procedure (C): starting from derivative **24**, (time= 8 h, *T* = 60°C) the title compound was obtained as a white solid in 71% yield. ^{1}HNMR (DMSO-d_6, 400 MHz): δ_H 0.95 (3H, t, *J* = 7.2 Hz, $OCH_2CH_2CH_3$), 1.71–1.74 (2H, m, $OCH_2CH_2CH_3$), 3.98 (2H, t, *J* = 6.1 Hz, $OCH_2CH_2CH_3$), 1.08 (2H, d, *J* = 8.3 Hz, Ar–H), 7.21 (1H, d, *J* = 8.2 Hz, Ar–H), 7.79–7.90 (4H, m, Ar–H), 8.46 (1H, s, CONH), 8.78 (1H, s, CONH), 13.00 (1H, s, CONH).

4.1.5 | General procedure (D) for the synthesis of compounds 29–32

Under N_2 atmosphere, to a suspension of derivatives **25–28** (1.0 eq) in *t*-BuOH (5 mL/mmol), *t*-BuOK (4.0 or 5.0 eq) was added. The reaction mixture was stirred at room temperature for 3–4 h. The reaction mixture was then poured in ice/water and the formed precipitate was filtered to obtain the target compounds.

6-Fluoro-2-(4-propoxyphenyl)quinazolin-4(1*H*)-one (**29**): General procedure (D): starting from derivative **25**, (time = 3 h) the title compound was obtained as a white solid in 80% yield. ^{1}HNMR (DMSO-d_6, 400 MHz): δ_H 1.01 (3H, t, *J* = 7.3 Hz, $OCH_2CH_2CH_3$), 1.75 (2H, m, $OCH_2CH_2CH_3$), 4.03 (2H, t, *J* = 6.5 Hz, $OCH_2CH_2CH_3$), 7.08 (2H, d, *J* = 8.6 Hz, Ar–H), 7.69–7.82 (3H, m, Ar–H), 8.16 (2H, d, *J* = 8.7 Hz, Ar–H), 12.55 (1H, s, NH).

6-Chloro-2-(4-propoxyphenyl)quinazolin-4(1*H*)-one (**30**): General procedure (D): starting from derivative **26**, (time = 4 h) the title compound was obtained as a white solid in 85% yield. ^{1}HNMR (DMSO-d_6, 400 MHz): δ_H 0.99 (3H, t, *J* = 7.4 Hz, $OCH_2CH_2CH_3$), 1.74–1.79 (2H, m, $OCH_2CH_2CH_3$), 4.02 (2H, t, *J* = 6.5 Hz, $OCH_2CH_2CH_3$), 7.03 (2H, d, *J* = 8.8 Hz, Ar–H), 7.71 (1H, d, *J* = 7.9 Hz, Ar–H), 7.82 (1H, dd, *J* = 2.3 and 8.7 Hz, Ar–H), 8.05 (1H, d, *J* = 2.3 Hz, Ar–H), 8.18 (2H, d, *J* = 8.8 Hz, Ar–H), 12.58 (1H, s, NH).

6-Bromo-2-(4-propoxyphenyl)quinazolin-4(1*H*)-one (**31**): General procedure (D): starting from derivative **27**, (time = 4 h) the title compound was obtained as a yellow solid in 90% yield. ^{1}HNMR (DMSO-d_6, 400 MHz): δ_H 0.99 (3H, t, *J* = 7.3 Hz, $OCH_2CH_2CH_3$), 1.72–1.81 (2H, m, $OCH_2CH_2CH_3$), 4.03 (2H, t, *J* = 6.5 Hz, $OCH_2CH_2CH_3$), 7.08 (2H, d, *J* = 8.7 Hz, Ar–H), 7.65 (1H, d, *J* = 8.7 Hz, Ar–H), 7.95 (1H, dd, *J* = 6.6 Hz, Ar–H), 8.16–8.20 (3H, m, Ar–H), 12.58 (1H, s, NH).

7-Chloro-2-(4-propoxyphenyl)quinazolin-4(1*H*)-one (**32**): General procedure (D): starting from derivative **28**, (time = 3 h) the title compound was obtained as a white solid in 92% yield. ^{1}HNMR (DMSO-d_6, 400 MHz): δ_H 0.96 (3H, t, *J* = 7.6 Hz, $OCH_2CH_2CH_3$), 1.68–1.77 (2H, m, $OCH_2CH_2CH_3$), 4.00 (2H, t, *J* = 6.4 Hz, $OCH_2CH_2CH_3$), 7.04 (2H, d, *J* = 8.5 Hz, Ar–H), 7.46 (1H, d, *J* = 8.47 Hz, Ar–H), 7.70 (1H, s, Ar–H), 8.07 (1H, d, *J* = 8.4 Hz, Ar–H), 8.14 (2H, d, *J* = 8.5 Hz, Ar–H), 12.44 (1H, s, NH).

4.1.6 | General procedure (E) for the synthesis of compounds 10b, 10c, 11b, 11c, 12b, 12c, and 13b

Under N_2 atmosphere, to a solution of derivatives **29–32** (1.0 eq) in dry DMF (10 mL/mmol), K_2CO_3 (4.0 eq), and appropriate chloroalkyl amine (2.0 or 4.0 eq) were added. The reaction mixture was stirred at 80–100°C for 2–5 h and then poured in ice/water. Reaction crudes were recovered after filtration of the precipitate formed or following an extraction with EtOAc (3 × 100 mL). Title compounds were obtained after purification by flash column chromatography.

6-Fluoro-4-[2-(piperidin-1-yl)ethoxy]-2-(4-propoxyphenyl) quinazoline (**10b**): General procedure (E): starting from derivative **29** and using 1-(2-chloroethyl)piperidine (time = 3 h, *T* = 80°C), compound **10b** was obtained after purification by flash column chromatography eluting with Cy/EtOAc 95/5 as a white solid in 10% yield. ^{1}HNMR ($CDCl_3$, 400 MHz): δ_H 1.09 (3H, t, *J* = 7.2 Hz, $OCH_2CH_2CH_3$), 1.56–1.58 (2H, m, piperidine CH_2), 1.85–1.88 (6H, m, $OCH_2CH_2CH_3$ and piperidine CH_2 x2), 2.89–2.91 (4H, m, piperidine NCH_2 x2), 3.22–3.24 (2H, m, OCH_2CH_2N), 4.03 (2H, t, *J* = 6.1 Hz, $OCH_2CH_2CH_3$), 5.00–5.04 (2H, m, OCH_2CH_2N), 7.03 (2H, d, *J* = 7.6 Hz, Ar–H), 7.56–7.60 (1H, m, Ar–H), 7.70–7.72 (1H, m, Ar–H), 7.96–7.99 (1H, m, Ar–H), 8.50 (2H, d, *J* = 7.6 Hz, Ar–H). ^{13}CNMR ($CDCl_3$, 101 MHz): δ_C 10.65, 22.69, 23.45, 24.98, 54.75, 57.01, 63.73, 69.70, 107.42 (*J* = 23.0 Hz), 114.44, 115.25 (*J* = 9.4 Hz), 123.50 (*J* = 25.3 Hz), 130.06, 130.38 (*J* = 8.1 Hz), 149.21, 158.81, 159.46, 161.28, 161.58, 165.78 ppm. HPLC r_t: 5.02 min. HRMS (ESI) *m/z* $[M+H]^+$ calcd. for $C_{24}H_{28}FN_3O_2$ 410.2239 found 410.22468.

4-[2-(Azepan-1-yl)ethoxy]-6-fluoro-2-(4-propoxyphenyl)quinazoline (**10c**): General procedure (E): starting from derivative **29** and using 1-(2-chloroethyl)azepane (time = 5 h, *T* = 80°C), compound **10c** was obtained

after purification by flash column chromatography eluting with Cy/EtOAc 98/2 as a white solid in 10% yield. ^{1}HNMR ($CDCl_3$, 400 MHz): δ_H 1.09 (3H, t, J = 7.42 Hz, $OCH_2CH_2CH_3$), 1.73–1.75 (4H, m, azepane CH_2 x2), 1.84–1.95 (6H, m, $OCH_2CH_2CH_3$ and azepane CH_2 x2), 3.23–3.25 (4H, m, azepane CH_2 x2), 3.46–3.48 (2H, m, OCH_2CH_2N), 4.03 (2H, t, J = 6.6 Hz, $OCH_2CH_2CH_3$), 5.12–5.14 (2H, m, OCH_2CH_2N), 7.03 (2H, d, J = 8.8 Hz, Ar–H), 7.59 (1H, dt, J = 2.7 and 8.9 Hz, Ar–H), 7.72, (1H, dd J = 2.8 and 8.3 Hz, Ar–H), 7.95–7.99 (1H, m, Ar–H), 8.49 (2H, d, J = 8.9 Hz, Ar–H). ^{13}CNMR ($CDCl_3$, 101 MHz): δ_C 10.64, 22.68, 27.06 (2C), 55.66, 55.74, 55.93, 69.70, 107.46 (J = 23.0 Hz), 114.44, 115.28 (J = 8.1 Hz), 123.52 (J = 24.7 Hz), 130.04, 130.37 (J = 5.0 Hz), 149.22, 158.81, 159.42, 161.28, 161.56 ppm. HPLC r_t: 5.19 min. HRMS (ESI) m/z $[M+H]^+$ calcd. for $C_{25}H_{30}FN_3O_2$ 424.2395 found 424.23997.

6-Chloro-4-[2-(piperidin-1-yl)ethoxy]-2-(4-propoxyphenyl) quinazoline (**11b**): General procedure (E): starting from derivative **30** and using 1-(2-chloroethyl)piperidine (time = 3 h, T = 80°C), compound **12b** was obtained after purification by flash column chromatography eluting with Cy/EtOAc 90/10 as a white solid in 13% yield. ^{1}HNMR ($CDCl_3$, 400 MHz): δ_H 1.09 (3H, t, J = 7.4 Hz, $OCH_2CH_2CH_3$), 1.56–1.60 (2H, m, piperidine CH_2), 1.85–1.90 (6H, m, $OCH_2CH_2CH_3$ and piperidine CH_2 x2), 2.90–2.91 (4H, m, piperidine NCH_2 x2), 3.23–3.24 (2H, m, OCH_2CH_2N), 4.03 (2H, t, J = 6.6 Hz, $OCH_2CH_2CH_3$), 5.05.06 (2H, m, OCH_2CH_2N), 7.02 (2H, d, J = 8.9 Hz, Ar–H), 7.75 (1H, dd, J = 2.3 and 8.9 Hz, Ar–H), 7.90 (1H, d, J = 8.9 Hz, Ar–H), 8.07 (1H, d, J = 2.1 Hz, Ar–H), 8.50 (2H, d, J = 8.9 Hz, Ar–H). ^{13}CNMR ($CDCl_3$, 101 MHz): δ_C 10.64, 22.67, 23.45, 24.95, 54.75, 56.99, 63.74, 69.70, 114.45, 115.50, 122.57, 129.56, 129.95, 130.22, 131.52, 134.51, 150.69, 160.14, 161.73, 165.27 ppm. HPLC, 1.0 mL/min, H_2O (0.1% DEA) 10%/CH_3CN 90% in 15 min, r_t: 8.51 min. HRMS (ESI) m/z $[M+H]^+$ calcd. for $C_{24}H_{28}ClN_3O_2$ 426.1948 found 426.1939.

4-[2-(Azepan-1-yl)ethoxy]-6-chloro-2-(4-propoxyphenyl)quinazoline (**11c**): General procedure (E): starting from derivative **30** and using 1-(2-chloroethyl)azepane (time = 2 h, T = 80°C), compound **12c** was obtained after purification by flash column chromatography eluting with EtOAc/Acetone 97/3 as a white solid in 15% yield. ^{1}HNMR ($CDCl_3$, 400 MHz): δ_H 1.09 (3H, t, J = 7.4 Hz, $OCH_2CH_2CH_3$), 1.63–1.65 (4H, m, azepane CH_2 x2), 1.81–1.90 (6H, m, $OCH_2CH_2CH_3$ and azepane CH_2 x2), 3.02–3.04 (4H, m, azepane NCH_2 x2), 3.25–3.27 (2H, m, OCH_2CH_2N), 4.03 (2H, t, J = 6.5 Hz, $OCH_2CH_2CH_3$), 4.90–5.00 (2H, m, OCH_2CH_2N), 7.02 (2H, d, J = 8.9 Hz, Ar–H), 7.72 (1H, dd, J = 2.3 and 8.9 Hz, Ar–H), 7.88 (1H, d, J = 8.9 Hz, Ar–H), 8.08 (1H, d, J = 1.8 Hz, Ar–H), 8.50 (2H, d, J = 8.9 Hz, Ar–H). ^{13}CNMR ($CDCl_3$, 101 MHz): δ_C 10.64, 22.67, 26.92, 27.07, 29.80, 55.75, 55.94, 64.38, 69.70, 114.43, 115.58, 122.65, 129.51, 130.04, 130.20, 131.47, 134.44, 150.65, 160.16, 161.69, 165.46 ppm. HPLC, 1.0 mL/min, H_2O (0.1% DEA) 10%/CH_3CN 90% in 15 min, r_t: 10.92 min. HRMS (ESI) m/z $[M+H]^+$ calcd. for $C_{25}H_{30}ClN_3O_2$ 440.2100 found 440.2096.

6-Bromo-4-[2-(piperidin-1-yl)ethoxy]-2-(4-propoxyphenyl) quinazoline (**12b**): General procedure (E): starting from derivative **31** and using 1-(2-chloroethyl)piperidine (time = 5 h, T = 80°C), compound **12b** was obtained after purification by flash column chromatography eluting with Cy/EtOAc 95/5 as a yellow solid in 22% yield. ^{1}HNMR ($CDCl_3$, 400 MHz): δ_H 1.09 (3H, t, J = 7.3 Hz, $OCH_2CH_2CH_3$), 1.58–1.59 (2H, m, piperidine CH_2), 1.87–1.88 (6H, m, $OCH_2CH_2CH_3$ and piperidine CH_2 x2), 2.91–2.93 (4H, m, piperidine NCH_2 x2), 3.24–3.26 (2H, m, OCH_2CH_2N), 4.00–4.03 (2H, m, $OCH_2CH_2CH_3$), 5.05–5.07 (2H, m, OCH_2CH_2N), 7.02 (2H, d, J = 8.5 Hz, Ar–H), 7.81–7.89 (2H, m, Ar–H), 8.23 (1H, s, Ar–H), 8.49 (2H, d, J = 8.5 Hz, Ar–H). ^{13}CNMR ($CDCl_3$, 101 MHz): δ_C 10.64, 22.67, 23.56, 25.11, 54.81, 57.06, 63.97, 69.70, 114.45, 116.02, 119.33, 125.90, 129.66, 129.98, 130.24, 137.06, 150.91, 160.23, 161.74, 165.17 ppm. HPLC r_t: 5.39 min. HRMS (ESI) m/z $[M+H]^+$ calcd. for $C_{24}H_{28}BrN_3O_2$ 470.1438 found 470.14435.

4-[2-(Azepan-1-yl)ethoxy]-6-bromo-2-(4-propoxyphenyl)quinazoline (**12c**): General procedure (E): starting from derivative **31** and using 1-(2-chloroethyl)azepane (time = 4 h, T = 100°C), compound **12c** was obtained after purification by flash column chromatography eluting with Cy/EtOAc 98/2 as a white solid in 30% yield. ^{1}HNMR ($CDCl_3$, 400 MHz): δ_H 1.09 (3H, t, J = 7.3 Hz, $OCH_2CH_2CH_3$), 1.67–1.69 (4H, m, azepane CH_2 x2), 1.83–1.95 (6H, m, $OCH_2CH_2CH_3$ and azepane CH_2 x2), 3.22–3.24 (4H, m, azepane CH_2 x2), 3.45–3.46 (2H, m, OCH_2CH_2N), 4.03 (2H, t, J = 6.6 Hz, $OCH_2CH_2CH_3$), 5.11–5.13 (2H, m, OCH_2CH_2N), 7.02 (2H, d, J = 8.8 Hz, Ar–H), 7.83–7.91 (2H, m, Ar–H), 8.23 (1H, d, J = 1.9 Hz, Ar–H), 8.49 (2H, d, J = 8.8 Hz, Ar–H). ^{13}CNMR ($CDCl_3$, 101 MHz): δ_C 9.50, 21.56, 26.03, 27.14, 54.85, 54.92, 64.37, 68.58, 113.30, 115.18, 118.00, 125.03, 128.45, 129.10, 129.17, 135.73, 149.74, 159.24, 160.53, 164.60 ppm. HPLC, 0.5 mL/min, H_2O (0.1% FA) 80%/CH_3CN 20% to CH_3CN 100% in 15 min, r_t: 6.58 min. HRMS (ESI) m/z $[M+H]^+$ calcd. for $C_{25}H_{30}BrN_3O_2$ 484.1594 found 484.15947.

7-Chloro-4-[2-(piperidin-1-yl)ethoxy]-2-(4-propoxyphenyl) quinazoline (**13b**): General procedure (E): starting from derivative **32** and using 1-(2-chloroethyl)piperidine (time = 2 h, T = 80°C), compound **13b** was obtained after purification by flash column chromatography eluting with Cy/EtOAc 80/20 as a white solid in 15% yield. ^{1}HNMR ($CDCl_3$, 400 MHz): δ_H 1.04 (3H, t, J = 7.3 Hz, $OCH_2CH_2CH_3$), 1.43–1.44 (2H, m, piperidine CH_2), 1.58–1.61 (4H, m, piperidine CH_2 x2), 1.80–1.85 (2H, m, $OCH_2CH_2CH_3$), 2.55–2.57 (4H, m, piperidine NCH_2 x2), 2.89 (2H, t, J = 6.0 Hz, OCH_2CH_2N), 3.98 (2H, t, J = 6.5 Hz, $OCH_2CH_2CH_3$), 4.79 (2H, t, J = 6.0 Hz, OCH_2CH_2N), 6.97 (2H, d, J = 8.5 Hz, Ar–H), 7.36 (1H, d, J = 8.6 Hz, Ar–H), 7.89 (1H, s, Ar–H), 8.00 (1H, d, J = 8.6 Hz, Ar–H), 8.46 (2H, d, J = 8.5 Hz, Ar–H). ^{13}CNMR ($CDCl_3$, 101 MHz): δ_C 10.52, 22.50, 24.08, 25.94, 54.98, 57.35, 64.75, 69.49, 113.35, 114.20, 124.92, 126.59, 126.67, 130.01, 130.15, 139.37, 152.69, 160.88, 161.50, 166.18 ppm. HPLC, 0.5 mL/min, H_2O (0.1% FA) 80%/CH_3CN 20% to CH_3CN 100% in 10 min, r_t: 5.90 min. HRMS (ESI) m/z $[M+H]^+$ calcd. for $C_{24}H_{28}ClN_3O_2$ 426.1943 found 426.194.

4.2 | Biological assays

4.2.1 | Bacterial strains

The reference strain *M. smegmatis* mc^{2}155 and *M. avium* (subsp. *hominissuis*) MA2373 and MA878 clinical strains, isolated in the

Laboratory of Clinical Mycobacteriology of the University Hospital of Pisa, Italy, were included in this study. Mycobacteria were grown at 37°C in Middlebrook 7H9 broth or Middlebrook 7H10 agar medium, both supplemented with 10% OADC.

4.2.2 | MIC determination

The resazurin microtiter assay described by Palomino et al.[27] was used to determine MIC of compounds and of CLA in the presence and absence of EPIs. Briefly, strains were grown in 7H9 broth containing 10% OADC to an optical density at 600 nm (OD_{600}) of 0.5 and diluted 1:20. Aliquots of 100 µL of the inoculum were added to each well of a 96-well plate containing 100 µL of 7H9 broth/OADC with two-fold serial dilution of compounds or CLA in absence or presence of each EPI. The plates were covered and incubated at 37°C. After 3 days incubation for *M. smegmatis* and 7 days for *M. avium*, 30 µL of 0.01% resazurin solution was added to each well and the plates were reincubated for 24 h. A change in color from blue to pink indicated the growth of bacteria, and the MIC was defined as the lowest concentration of compound or CLA that prevented this change in color.

4.2.3 | Fluorometric determination of drug efflux activity

M. avium strains were grown at 37°C with shaking in Middlebrook 7H9 broth supplemented with 0.2% glycerol, 0.05% tween 80%, and 10% ADC until an OD_{600} of 0.8. Bacteria were pelleted by centrifugation at 8000 rpm for 10 min, washed once, and resuspended in sterile phosphate-buffered saline (PBS) at 0.8 OD_{600}. Bacteria were loaded with EtBr at 3.125 µg/mL using 400 µg/mL of VP in the absence of glucose. The concentrations of EtBr and VP used in this study, which correspond to ½ MIC for reference *M. avium* ATCC 252910,[21] allow the highest EtBr accumulation without compromising the cellular viability. After 50 min of incubation at rt in the dark, the bacterial suspensions were pelleted, and cells were resuspended in sterile PBS at 0.8 OD_{600}. One hundred microliters of each suspension were added to wells in a white microtiter plate containing, in duplicate: (i) 100 µL of PBS with glucose (final concentration 0.4%) and without VP (conditions of efflux); (ii) 100 µL of VP at a final concentration of 400 µg/mL without glucose (conditions of accumulation). The plate was immediately read at 37°C excitation/emission 530/600 nm up to 40 min. Relative fluorescence at different efflux timepoints was normalized against the conditions of EtBr accumulation (no efflux).

4.2.4 | Cell cytotoxicity test

Cell culturing

Human lung carcinoma epithelial-like A549 cell line (ATCC-CCL 185) (LGC Standards) was cultured in tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM) (Euroclone) supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 10% heat-inactivated Fetal Bovine Serum (Euroclone). A549 cell line was cultured at 37°C in a 5% CO_2 atmosphere and split in new flasks by treatment with 1× Trypsin-EDTA solution (Euroclone) when cells reached the confluence.

3′-[1-phenyl-aminocarbonyl)-3,4-tetrazolium]bis(4-methoxy-6-nitro) benzenesulphonic acid (XTT) reduction assay on A549 cell line

In vitro cytotoxicity of the compounds was performed using the XTT reduction assay[28] as described by Del Gaudio et al.[29] with minor modifications. This assay is based on the reduction of sodium XTT by the activity of mitochondrial dehydrogenases in viable cells, into a water-soluble orange formazan product. XTT cellular reduction is improved by the use of *N*-methylphenazonium methyl sulfate (PMS) as an electron coupling agent. Formazan formation is measured spectrophotometrically at 450 nm, and the color intensity of the product can be correlated to the number of viable cells. Stock solutions of the compounds to be tested were prepared in DMEM culture media.

A549 cells were resuspended in complete DMEM, then 100 µL of the cell suspension was seeded in flat-bottom 96-well microtiter plates at a concentration of 104 cells/well and incubated for 24 h at 37°C in a 5% CO_2 atmosphere. Next day, culture media was removed and replaced with 100 µL of fresh complete DMEM containing the appropriate concentrations of the compounds to be tested (4, 8, 16, and 32 µg/mL). Culture media alone and 1% (v/v) Triton X-100 (Sigma) were also included as negative and positive controls, respectively. After 24 h of incubation at 37°C in a 5% CO_2 atmosphere, culture media was removed then 100 µL of pre-warmed PBS 1× (Euroclone) and 50 µL of freshly prepared XTT/PMS (Glentham Life Sciences) solution were added and the plate was incubated for 1 h at 37°C in a 5% CO_2 atmosphere protected from light. Finally, 100 µL of the solution was transferred into a new plate and absorbance at 450 nm was read using a microplate reader (MultiSkan GO microplate, Thermo Fisher Scientific). Two independent experiments with three replicates each were performed. Cytotoxicity was represented as the percentage of cell viability with respect to the negative control, the lower the viability value the higher the cytotoxic potential of the compound or material. If viability is reduced to <70% with respect to the negative control the test sample is considered as having a cytotoxic potential.

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CONFLICTS OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the Supporting Information of this article.

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