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Research paper

Optimization of pyridylpiperazine-based inhibitors of the *Escherichia coli* AcrAB-TolC efflux pump

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ABSTRACT

Multidrug-resistant *Escherichia coli* is a continuously growing worldwide public health problem, in which the well-known AcrAB-TolC tripartite RND efflux pump is a critical driver. We have previously described pyridylpiperazines as a novel class of allosteric inhibitors of *E. coli* AcrB which bind to a unique site in the protein transmembrane domain, allowing for the potentiation of antibiotic activity. Here, we show a rational optimization of pyridylpiperazines by modifying three specific derivatization points of the pyridine core to improve the potency and the pharmacokinetic properties of this chemical series. In particular, this work found that the introduction of a primary amine to the pyridine through ester (**29**, BDM91270) or oxadiazole (**44**, BDM91514) based linkers allowed for analogues with improved antibiotic boosting potency through AcrB inhibition. *In vitro* studies, using genetically engineered mutants, showed that this improvement in potency is mediated through novel interactions with distal acidic residues of the AcrB binding pocket. Of the two leads, compound **44** was found to have favorable physico-chemical properties and suitable plasma and microsomal stability. Together, this work expands the current structure-activity relationship data on pyridylpiperazine efflux pump inhibitors, and provides a promising step towards future *in vivo* proof of concept of pyridylpiperazines as antibiotic potentiators.

1. Introduction

Multidrug-resistant bacteria (MDR) are now recognized by the World Health Organization (WHO) as a public health emergency that seems to have no end [1]. Over the last 80 years, there has been a race between the production of antibiotics and the continual evolution of resistance that is mainly driven by the wide employment of antibiotics, whose overuse exerts an intense selective pressure upon bacteria favoring the appearance of resistant strains. According to a 2014 report by economist Jim O'Neills, an overlook to the current situation could lead to a 10 million deaths per year global toll with a cost exceeding 100 000 billion USD [2]. As of 2019, each year 4.95 million individuals die of infectious diseases, from which approximately 12% are associated to bacteria

resistant to multiple antibiotics [1]. Thus, the WHO established carbapenem-resistant and extended-spectrum beta-lactamases (ESBL)--producing Enterobacterales as a critical priority for the development of new therapeutic solutions [3]. The mechanisms by which Gram-negative bacteria escape the action of antibiotics are diverse but one that dominates is their ability to intrinsically prevent antibiotic accumulation within the cells via efflux pump transporters belonging to the RND superfamily, such as the AcrAB-TolC tripartite system. In addition to being associated with antibiotic resistance [4,5], these bacterial efflux pumps are also involved in crucial physiological processes like intercellular communication (quorum sensing) [6,7], pathogenicity through protection against host defense [8,9], virulence factors export [10,11], and biofilm formation [12]. Together, efflux pumps have become of great interest to be considered as a valuable antimicrobial target [13,14].

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Abbreviations

Ac	acetate	LLE	ligand-lipophilicity efficiency
AgOTf	silver triflate	MeCN	acetonitrile
AMR	antimicrobial resistance	MIC	minimal inhibitory concentration
B ₂ pin ₂	bis(pinacolato)diboron	MsCl	mesyl chloride
BINAP	2,2'-bis(diphenylphosphino)-1,1'-binaphthyl	<i>n</i> BuLi	<i>n</i> -butyllithium
Boc	<i>tert</i> -butoxy carbonyl	ND	not determined
cLogP	calculated LogP	NMP	naphthylmethyl-piperazine
COMU	(1-Cyano-2-ethoxy-2-oxoethylideneaminoxy) dimethylamino-morpholino-carbenium hexafluorophosphate	PAβN	Phenylalanine-Arginine-β-Naphthylamide
DCM	dichloromethane	PBS	phosphate buffer saline;
DME	dimethoxyethane	RND	resistance-nodulation-cell division
DMF	<i>N,N</i> -dimethylformamide	r. t.	room temperature
DMSO	dimethylsulfoxide	SAR	structure-activity relationship
EPI	efflux pump inhibitor	S _N Ar	nucleophilic aromatic substitution
eq	equivalent	t _{1/2}	half-life
ESBL	extended-spectrum beta-lactamase	T3P	propanephosphonic acid anhydride
Et	ethyl	TBAF	tetrabutylammonium fluoride
HBTU	<i>N,N,N',N'</i> -tetramethyl- <i>O</i> -(1 <i>H</i> -benzotriazol-1-yl)uronium hexafluorophosphate	<i>t</i> Bu	<i>tert</i> -butyl
HOBt	hydroxy benzotriazole	TEA	triethylamine
<i>i</i> Pr	isopropyl	TFA	trifluoroacetic acid
		THF	tetrahydrofuran
		Troc	2,2,2-trichloroethoxycarbonyl
		USD	United States dollar
		WHO	World Health Organization
		WT	wild-type

The major clinically relevant efflux systems in Gram-negative bacteria belong to the Resistance-Nodulation-Cell Division (RND) superfamily. Enterobacterales such as *Klebsiella pneumoniae* and *Escherichia coli* encode a number of tripartite RND-pumps in their genomes but express only one constitutively, namely the AcrAB-TolC efflux pump, which is greatly involved in multidrug resistance due to its broad substrate specificity [15,16].

Several AcrAB-TolC efflux pump inhibitors (EPIs) have been described in the literature such as the peptidomimetic PABN, naphthylmethyl-piperazine (NMP) and their analogues, as well as the pyranopyridines of the MBX series [17–20]. We recently reported the discovery of a novel class of AcrB inhibitors, known as pyridylpiperazines, that were identified through fragment-based screening [21]. The original pyridylpiperazine hit **1** (BDM73185) (Fig. 1) was confirmed to

bind an allosteric pocket in the transmembrane domain of AcrB, highlighting a new binding site for RND efflux pumps. Compound **1** has no intrinsic antibacterial activity (MIC₉₀ > 500 μM) but is able to boost several AcrAB-TolC antibiotic substrates such as chloramphenicol, pyridomycin, ciprofloxacin, tetracycline and erythromycin [21].

Early optimization of compound **1** led to the identification of compound **2** (BDM88832) (Fig. 1), where the trifluoromethyl group has been replaced by an iodine atom, conferring a 5-fold increase in potency. Co-crystallization of compound **2** with AcrB R971A at 2.8 Å resolution allowed us to decipher the binding mode of this new chemical series (Fig. 2A) [21].

Based on the X-ray structure, we outlined new strategies to optimize the potency of EPIs starting from BDM88832, their physico-chemical properties as well as their stability in physiological fluids. The 3D-structural analysis of BDM88832 bound to AcrB allowed us to define three derivatization points around the pyridine core (Fig. 2B). First, substituents introduced in position 2 of the pyridine (orange circle) had a basic moiety to maintain the interaction with Asp408. Chlorine atom in position 3 (purple circle) was then replaced with small substituents to target the interaction with the backbone of Lys940 via a hydrogen or halogen bond [22,23]. Lastly, we introduced more diverse substituents in position 5 of the pyridine ring (blue circle) as they occupy the entrance of the binding pocket which is wider and open to the solvent. This led to the selection of two compounds which were then tested in combination with antibiotics to confirm the boosting capacity of these efflux pump inhibitors.

2. Chemistry

2.1. Modifications in position 2 of the pyridine ring

First, the piperazine ring was replaced by substituted piperazines or analogues that could retain the interaction with Asp408 through a hydrogen bond or a salt bridge, such as oxopiperazine (compound **3**), methylpiperazines (compounds **4**, **5** and **6**), homopiperazine (compound **7**), aminopiperidine (compound **8**), ethylenediamine (compound **9**) and tetrahydropyridine (compound **10**). To introduce modifications in position 2 of the pyridine ring, we used the 2,3-dichloro-5-

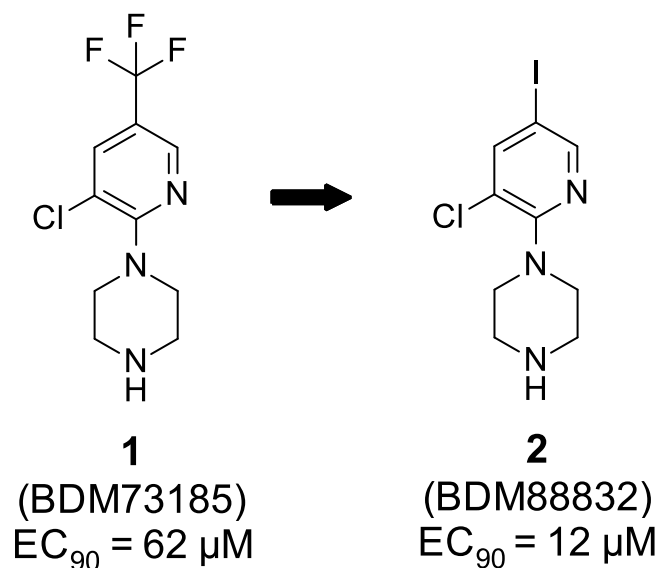


Fig. 1. Structure of hit **1** and compound **2**.

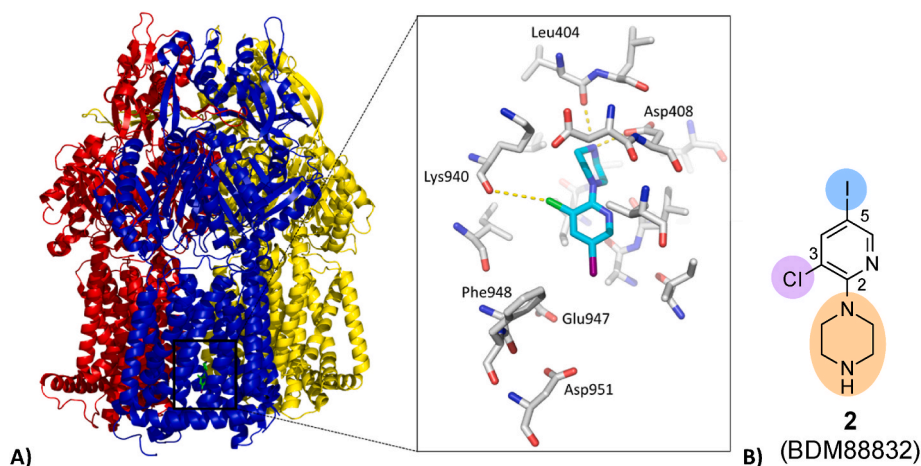


Fig. 2. A) Views of compound **2** (in teal) in *E. coli* AcrB R971A (PDB: 7OUL). Each AcrB protomer is represented in yellow, blue or red on the full view, whilst AcrB residues are represented in grey on the zoom; B) Derivatization points around compound **2**.

(trifluoromethyl)pyridine as a starting building block. Compounds **3**, **6–9** were obtained by nucleophilic aromatic substitution (S_NAr) with the corresponding cyclic or aliphatic amine. *R* and *S* enantiomers **4** and **5** were also synthesized by S_NAr with the corresponding enantiopure *tert*-butyl 3-methylpiperazine-1-carboxylate followed by deprotection in acidic conditions. Finally, a Suzuki coupling [24] with *tert*-butyl 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-3,6-dihydro-2H-pyridine-1-carboxylate followed by the cleavage of the Boc protecting group afforded compound **10** (Scheme 1).

2.2. Modifications in position 3 of the pyridine ring

The chlorine atom in position 3 was first removed (compound **11**), then replaced with a bromine atom (compound **12**), an electron-withdrawing group (trifluoromethyl, compound **14**) and a hydrogen bond donor group (hydroxymethyl, compound **13**) to potentially interact with the Lys940 backbone.

Compound **2** was synthesized in three steps from 5-bromo-2,3-dichloro-pyridine as previously described in Plé, Tam et al. [21]. 1-[5-(trifluoromethyl)-2-pyridyl]piperazine (compound **11**) was

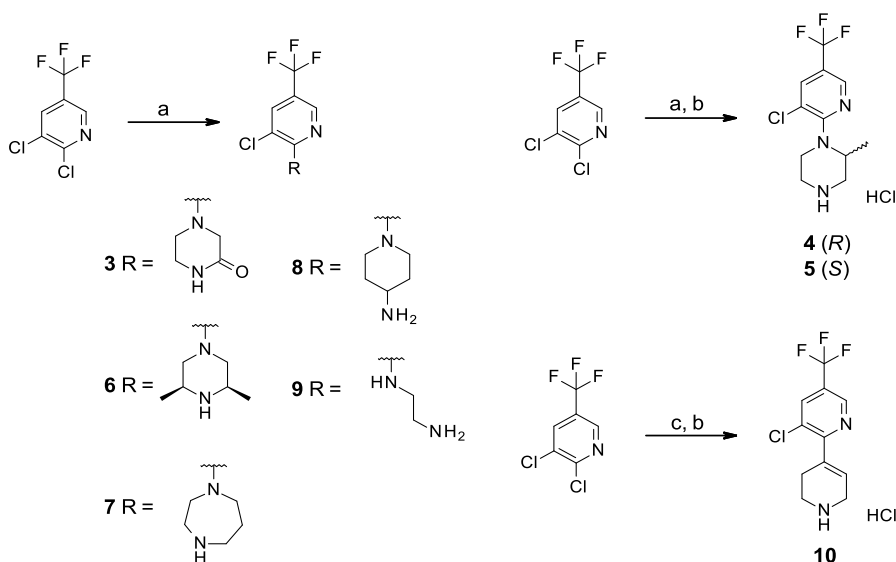
commercially available. Compounds **12** and **14** were obtained starting respectively from 3-bromo-2-chloro-5-(trifluoromethyl)pyridine and 2-chloro-5-iodo-3-(trifluoromethyl)pyridine. The Boc-piperazine was introduced by S_NAr and the Boc protecting group was then cleaved using acidic conditions (Scheme 2).

The synthesis of compound **13** started with 3-bromo-2-chloro-5-(trifluoromethyl)pyridine. A formylation using *n*-BuLi and DMF allowed to obtain the corresponding aldehyde. This intermediate was then reduced with $NaBH_4$ to give the corresponding alcohol. Next, an S_NAr was performed to introduce the Boc-piperazine, which was then deprotected in acidic conditions to yield compound **13** (Scheme 2).

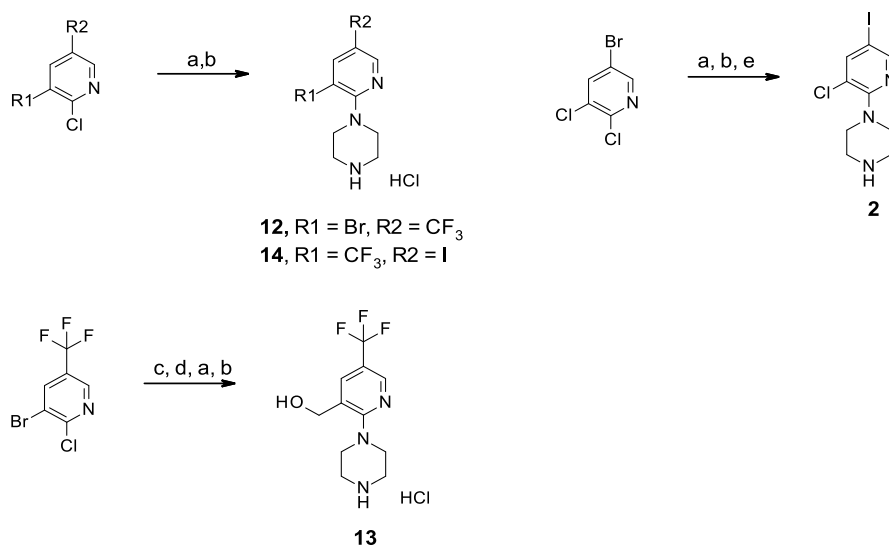
2.3. Modifications in position 5 of the pyridine ring

2.3.1. Introduction of small polar substituents

The first modification considered in position 5 of the pyridine ring was the introduction of esters (compounds **15** and **16**), amides (compounds **18** and **20**), sulfonamide (compound **21**) and alcohol (compound **22**) to determine if the iodine atom could be replaced by small polar substituents.



Scheme 1. Synthetic pathway for compounds **3–10**. Reagents and conditions: (a) Corresponding amine (1.5–3 eq.), TEA (1.3 eq.), MeCN, 50–70 °C, 3–48 h; (b) HCl 4 M (10 eq.), 1,4-dioxane, r.t., 5–16 h; (c) *tert*-butyl 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-3,6-dihydro-2H-pyridine-1-carboxylate (1 eq.), K_2CO_3 (1.5 eq.), Pd(PPh_3) $_2Cl_2$ (0.1 eq.), DME/EtOH/ H_2O 2:1:2, 90 °C, 4 h.



Scheme 2. Synthetic pathway for compounds **2** and **12–14**. Reagents and conditions: a) Boc-piperazine (1.5–4.5 eq.), TEA (1.3 eq.), MeCN, 80 °C, 20–96 h; b) HCl 4 M (10 eq.), 1,4-dioxane, r.t., 2h30-72 h; c) *n*-BuLi 1.6 M (1.25 eq.), DMF (2.5 eq.), Et₂O, –78 °C–0 °C, 3 h; d) NaBH₄ (1.1 eq.), MeOH, 0 °C, 1 h; e) CuI (7 mol%), (1*R*,2*R*)-*N*1,*N*2-dimethylcyclohexane-1,2-diamine (0.1 eq.), NaI (2 eq.), 1,4-dioxane, 110 °C, 72 h.

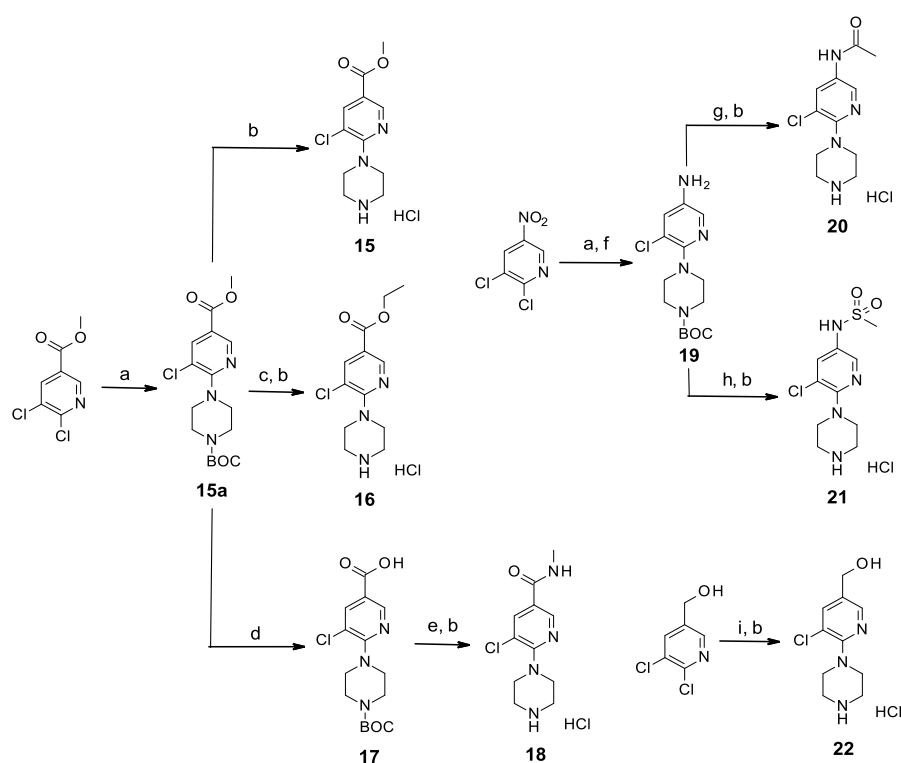
Compounds **15**, **16** and **18** were obtained starting from methyl 5,6-dichloropyridine-3-carboxylate. S_NAr was first performed with Boc-piperazine to afford **15a**. Direct deprotection with hydrochloric acid yielded compound **15**. β-elimination with sodium ethoxide in ethanol starting from **15a** yielded the corresponding carboxylic acid, which was further alkylated with iodoethane. Boc cleavage afforded compound **16** (Scheme 3).

Finally, saponification of the ethyl ester moiety of **15a** allowed the formation of intermediate **17**. Subsequent coupling with methylamine using isopropyl chloroformate as activating agent [25], followed by deprotection of the piperazine, allowed the obtention of compound **18**

as a hydrochloride salt.

Compounds **20** and **21** were synthesized starting from 2,3-dichloro-5-nitro-pyridine. Following previous synthesis, Boc-piperazine was first introduced by S_NAr. Then, the nitro group was reduced using bis(pinacolato)diboron (B₂pin₂) in isopropanol following a protocol described by Lu et al. to give intermediate **19** [26]. Acylation of the newly formed aniline with acetic anhydride followed by Boc-cleavage with hydrochloric acid yielded compound **20**, while sulfonylation with methanesulfonyl chloride (MsCl) followed by deprotection gave compound **21**.

Lastly, compound **22** was obtained via Buchwald coupling between



Scheme 3. Synthetic pathway for compounds **15–22**. Reagents and conditions: a) Boc-piperazine (1.5 eq.), TEA (1.3 eq.), MeCN, 50–80 °C, 1–16 h; b) HCl 4 M (10–30 eq.), 1,4-dioxane, r.t., 3–72 h; c) NaOEt 21% in EtOH (5 eq.), 70 °C, 20 h, followed by C₂H₅I (55 eq.), K₂CO₃ (2 eq.), DMF, r.t. to 100 °C, 16 h; d) NaOH (8 eq.), water, methanol, 65 °C, 1 h; e) Isopropyl chloroformate (1.5 eq.), TEA (1.2 eq.), THF, 0 °C to r.t., 16 h, followed by methylamine (1.5 eq.), TEA (2 eq.), THF, 0 °C to r.t., 2h30; f) B₂pin₂ (3.1 eq.), tBuOK (1.2 eq.), iPrOH, 110 °C, 4 h; g) Acetic anhydride (43 eq.), r.t., 72 h; h) MsCl (1.5 eq.), pyridine, r.t., 2 h; i) Boc-piperazine (1.5 eq.), Pd(OAc)₂ (2 mol%), BINAP (3 mol%), tBuONa (1.4 eq.), toluene, 110 °C, 8 h.

Boc-piperazine and (5,6-dichloro-3-pyridyl)methanol using palladium acetate and BINAP [27], followed by the cleavage of the Boc-group with hydrochloric acid.

2.3.2. Introduction of substituents on the ester and amide linkers

The introduction of diverse substituents on the ester and amide functions was then explored, including hydrophobic groups (phenyl: compound **23**, benzyl: compound **24**, phenethyl: compound **25**, phenylpropyl: compound **26**) as well as more polar substituents bearing amino (compounds **27–29** and **31–33**) or hydroxy groups (compound **30**) (Scheme 4).

First, esters **25** and **27** were formed after activation of the carboxylic acid **17** with isopropyl chloroformate but this led to the desired products with modest yields (37% and 33% respectively) [25]. The protocol described by MacMillan et al. using COMU as a coupling agent was then used for the synthesis of compounds **23**, **24**, **26**, **28**, **29**, **31**, **32** and **33** [28], allowing an improvement of the yield (average yield: 51%). Triethylamine was used for coupling reactions involving primary alcohols or anilines (**24**, **26**, **31**, **32**, **33**), while potassium carbonate was used for the coupling reactions with phenols (**23**, **28** and **29**). However, these coupling conditions were not optimal due to the formation of an ethyl ester as a by-product probably formed by the release of ethanol from COMU, explaining the moderate yields obtained.

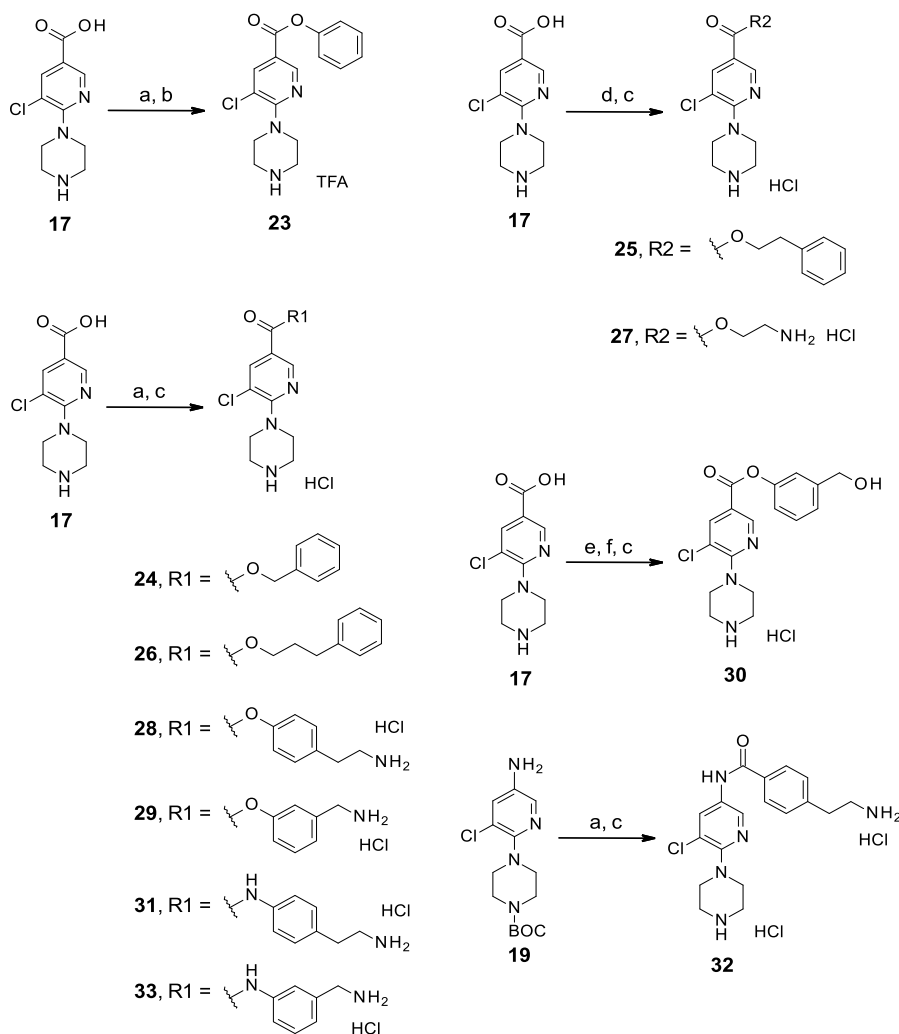
Lastly, the synthesis of the ester derivative bearing an alcohol (compound **30**) was performed with 3-[[*tert*-butyl(dimethyl)silyl]oxymethyl]phenol using HOBt/HBTU as coupling agents to improve the

yield of the esterification step (63% yield on the coupling step).

After the coupling step, all compounds were deprotected using HCl 4 M in dioxane, except compound **23** whose Boc protecting group was cleaved by TFA in dichloromethane due the formation of an unidentified product when using hydrochloric acid.

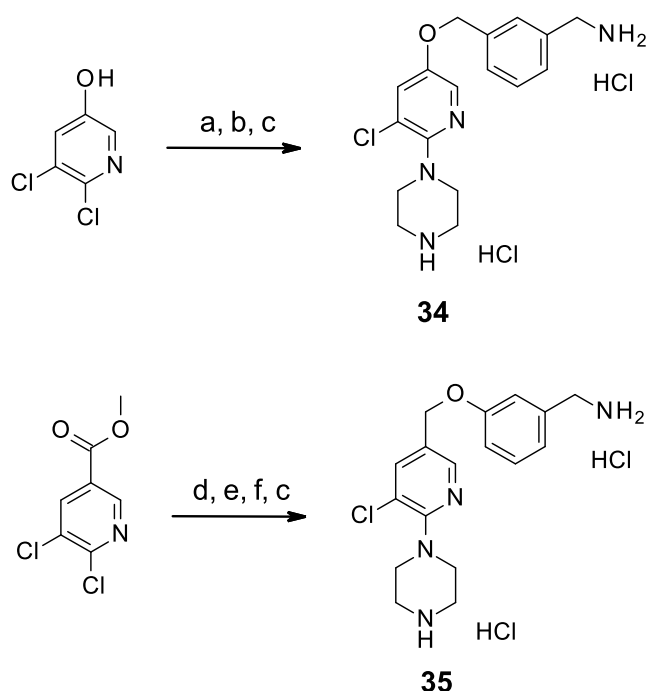
2.3.3. Introduction of ether linkers

Analogues of compound **29** bearing an ether linker instead of an ester bound (compounds **34** and **35**) were then synthesized (Scheme 5). On one hand, a nucleophilic substitution was performed with 5,6-dichloropyridin-3-ol and the corresponding benzylbromide derivative in presence of potassium carbonate. The Boc-piperazine was then introduced by Buchwald coupling using palladium acetate, BINAP and sodium *tert*-butoxide [27]. Finally, deprotection in HCl 4 M allowed the formation of compound **34**. On the other hand, an S_NAr allowed the introduction of the Boc-piperazine on methyl 5,6-dichloropyridine-3-carboxylate. This was followed by the reduction of the ester moiety with lithium borohydride. This synthetic pathway allowed an improved yield (61% on two steps) compared to the Buchwald coupling performed on the commercially available (5,6-dichloro-3-pyridyl)methanol (31% yield) to obtain compound **22** (Scheme 3). The obtained alcohol was then activated with mesyl chloride (MsCl) to undergo nucleophilic substitution with *tert*-butyl *N*-[(3-hydroxyphenyl)methyl]carbamate. Deprotection with HCl 4 M in dioxane finally yielded compound **35**.



Scheme 4. Synthetic pathway for compounds **23–33**.

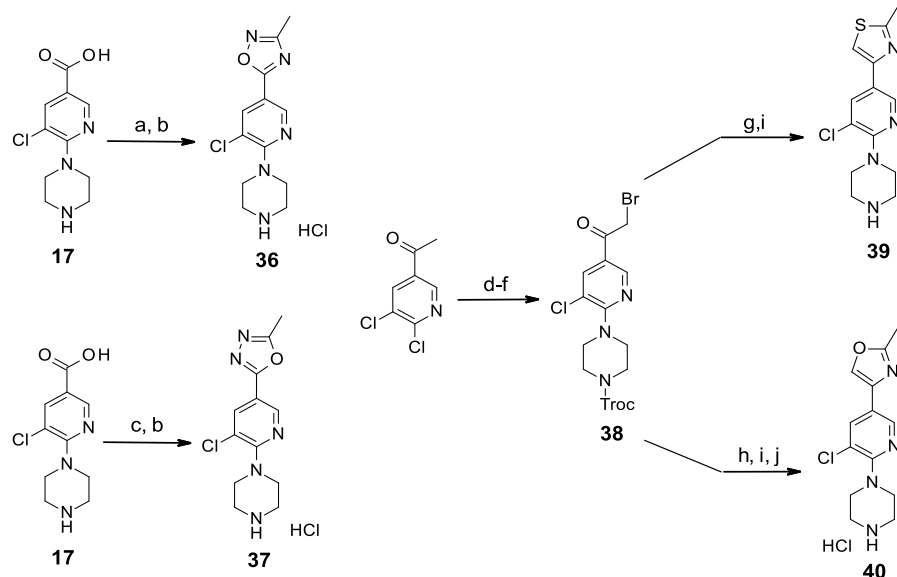
Reagents and conditions: a) Carboxylic acid (1 eq.), corresponding alcohol, phenol or aniline (1.5 eq.), TEA or K_2CO_3 (2–3 eq.), COMU (1.5–3 eq.), EtOAc, r. t. $-50^\circ C$, 1–48 h; b) TFA (40 eq.), DCM, r.t., 1h30; c) HCl 4 M (10–30 eq.), 1,4-dioxane, r.t., 4–36 h; d) Isopropyl chloroformate (1.5 eq.), TEA (1.2 eq.), THF, $0^\circ C$ to r.t., 16 h, then corresponding alcohol (1.2 eq.), $tBuOK$ (1.6 eq.), THF, $0^\circ C$ to r.t., 4 h-4h30; e) 3-[[*tert*-butyl(dimethyl)silyl]oxymethyl]phenol (1.5 eq.), HBTU (1.3 eq.), HOBt (0.3 eq.), K_2CO_3 (3 eq.), DCM, r.t., 16 h; f) TBAF (1.1 eq.), THF, r.t., 2h15.



Scheme 5. Synthetic pathway for compounds 34–35. Reagents and conditions: a) Tert-butyl N-[(3-bromomethyl)phenyl]methylcarbamate (2 eq.), K_2CO_3 (2.5 eq.), DMF, 80 °C, 1 h; b) Boc-piperazine (1.8 eq.), Pd(OAc) $_2$ (4 mol%), BINAP (4 mol%), tBuONa (1.7 eq.), toluene, 110 °C, 16 h; c) HCl 4 M (20 eq.), 1,4-dioxane, r.t., 20 h; d) Boc-piperazine (1.5 eq.), TEA (1.3 eq.), MeCN, 80 °C, 16 h; e) $LiBH_4$ (4 eq.), THF, 0 °C to r.t., 72 h; f) MsCl (2 eq.), DCM, 0 °C to r.t., 2 h, then tert-butyl N-[(3-hydroxyphenyl)methyl]carbamate (1.2 eq.), Cs_2CO_3 (1.3 eq.), DMF, 70 °C, 4 h.

2.3.4. Introduction of five-membered aromatic heterocycles

To further investigate the replacement of the ester linker, bioisosteres were considered, in particular five-membered heterocycles such as thiazole (compound 39), oxazole (compound 40), and oxadiazoles (compounds 36, 37, 42). All heterocycles were first synthesized with a methyl substituent (Scheme 6), and then analogues of compound 42 bearing an 1,2,4-oxadiazole substituted with amino chains were synthesized (Scheme 7).



Scheme 6. Synthetic pathway for compounds 36–40. Reagents and conditions: a) *N*'-hydroxyacetamide (1 eq.), T3P (2.5 eq.), TEA (5 eq.), EtOAc, 80 °C, 16 h; b) HCl 4 M (10 eq.), 1,4-dioxane, r.t., 16–40 h; c) Acetohydrazide (1.4 eq.), T3P (3.5 eq.), TEA (5 eq.), EtOAc, 80 °C, 48 h; d) Troc-piperazine (1.5 eq.), TEA (3 eq.), MeCN, 80 °C, 6 h; e) Phenyltrimethylammonium tribromide (1 eq.), THF/MeOH 4:1, 50 °C, 5 h; f) HCl 0.1 M (4 eq.), THF, r.t., 3h30; g) Thioacetamide (1.3 eq.), absolute EtOH, 78 °C, 3 h; h) Acetamide (1.4 eq.), AgOTf (2.1 eq.), EtOAc, 70 °C, 20 h, protected from light; i) Zinc dust (10–20 eq.), acetic acid (10–20 eq.), THF, r.t., 20–36 h; j) HCl 4 M (10 eq.), dioxane, r.t., 10 min.

Firstly, carboxylic acid 17 reacted with *N*'-hydroxyacetamide and T3P to form a 1,2,4-oxadiazole [29], which was subsequently deprotected with HCl 4 M in dioxane to give compound 36. Secondly, the same intermediate 17 reacted with acetohydrazide and T3P to obtain the corresponding 1,3,4-oxadiazole [29], which was also deprotected with hydrochloric acid to yield compound 37.

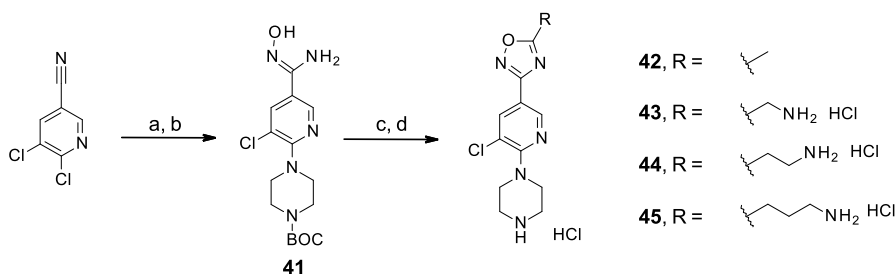
The thiazole and oxazole derivatives 39 and 40 were both obtained by Hantzsch-type cyclisation starting from 1-(5,6-dichloro-3-pyridyl) ethenone. An S_NAr reaction allowed the introduction of the Troc-protected piperazine, followed by a bromination step using phenyltrimethylammonium tribromide to yield intermediate 38 [30]. The obtained alpha bromoketone 38 was then reacted with thioacetamide in absolute ethanol to form the thiazole ring [31], or with acetamide and silver triflate to give the oxazole ring [32]. Both intermediates were finally deprotected in reductive conditions using zinc dust and acetic acid [33]. To facilitate its purification, HCl 4 M in dioxane was added to compound 40 to yield a hydrochloric salt.

1,2,4-oxadiazoles 42–45 were obtained starting from the same amidoxime intermediate 41 synthesized in two steps by S_NAr to introduce the Boc-piperazine on the 5,6-dichloropyridine-3-carbonitrile, followed by reaction with hydroxylamine hydrochloride to form the amidoxime. The 1,2,4-oxadiazole ring was then formed by coupling with the corresponding carboxylic acid using T3P [29]. A deprotection step with hydrochloric acid yielded the final compounds.

3. Structure-activity relationships

All synthesized compounds were tested in dose response on *E. coli* BW25113 alone and in combination with a sub-inhibitory dose (8 μ g/mL) of pyridomycin, the natural antibiotic effluxed by the AcrAB-TolC pump used in our assay as model substrate (MIC = 12.5–25 μ g/mL on *E. coli* BW25113, and MIC = 0.78 μ g/mL on *E. coli* BW25113 Δ acrB). The aim of this biological evaluation was to identify compounds without intrinsic antibacterial activity but able to potentiate pyridomycin activity by inhibiting AcrB.

When tested alone, all compounds were confirmed to have no intrinsic antibiotic activity on *E. coli* (MIC greater than 250 μ M), with the exception of compound 26 (MIC = 250 μ M) and compound 36 (MIC >125 μ M, maximum tested concentration due to solubility issues).



Scheme 7. Synthetic pathway for compounds **41–47**. Reagents and conditions: a) Boc-piperazine (1.5 eq.), TEA (1.3 eq.), MeCN, 50 °C, 1 h; b) $\text{NH}_2\text{OH}\cdot\text{HCl}$ (1.5 eq.), TEA (1.6 eq.), absolute ethanol, reflux, 1 h; c) Corresponding carboxylic acid (1.1 eq.), T3P (3.5 eq.), TEA (5 eq.), EtOAc, 80 °C, 16–24 h; d) HCl 4 M (10–20 eq.), 1,4-dioxane, r.t., 16h.

3.1. Replacement of the piperazine at position 2

Initial structure-activity relationships and co-crystallography showed that the basic nitrogen atom was essential for activity as it can form a salt bridge with Asp408 (Fig. 2). As the binding pocket around the piperazine appears to be very narrow in the crystal structure, only small substituents were introduced on this heterocycle or it was replaced by close analogues (Table 1). Firstly, small substituents such as methyl or carbonyl groups have been introduced on the piperazine ring. When the basicity of the aliphatic nitrogen atom was reduced (compound **3**) or the alpha positions of the basic aliphatic nitrogen were hindered (compound **6**), the compounds were unable to boost antibiotic activity by inhibiting AcrB ($\text{EC}_{90} > 500 \mu\text{M}$). Similar results were obtained when substituting the alpha position of the aryl nitrogen of the piperazine (compounds **4**, **5**). Replacing the piperazine with a homopiperazine (compound **7**) decreased drastically the potency ($\text{EC}_{90} > 500 \mu\text{M}$) which indicated that the increase in ring size was not tolerated. Compound **8** with an extracyclic nitrogen atom was 8 times less potent than the hit compound **1** ($\text{EC}_{90} = 500 \mu\text{M}$). Opening the piperazine ring (compound **9**) or changing the ring geometry by introducing a double bond (compound **10**) also proved to be detrimental for the potency. Overall, the unsubstituted piperazine ring could not be replaced advantageously, as its basicity alongside its geometry appeared to allow for optimal interaction with Asp408.

3.2. Investigation of the substituent in position 3

Next, the substituent in position 3 of the pyridine core was investigated (Table 2). As expected, the removal of the chlorine atom abolished EPI activity (compound **11**, $\text{EC}_{90} > 500 \mu\text{M}$). The replacement of the chlorine atom by a bromine atom allowed a small improvement in potency (compound **12**, $\text{EC}_{90} = 16 \mu\text{M}$), while the introduction of other electron-withdrawing groups like a hydroxymethyl group (compound **13**) or a trifluoromethyl group (compound **14**), resulted in more than an 8-fold decrease in potency (compounds **13** vs **1** and **2** vs **14**).

In summary, the presence of a halogen atom in position 3 of the pyridine core was favorable to maintain the potency of this chemical series. This is consistent with the co-crystallography structure where the formation of a halogen bond can be observed (Fig. 2A).

3.3. Investigation of the substituent in position 5

3.3.1. Introduction of small polar moieties

Previous SARs have evaluated the possibility of replacing the trifluoromethyl group of hit **1** with a halogen atom or electron-donating moieties like methyl or methoxy groups [21]. In this work, we investigated the possibility to introduce novel electron-donating (amide and sulfonamide) as well as electron-withdrawing (ester and alcohol) groups that could be easily chemically modified (Table 3).

First, methyl and ethyl esters (compounds **15** and **16**) were introduced in position 5 of the pyridine ring. The presence of the ester

function was well tolerated, as it led to a 3- to 5-fold improvement in potency compared to the hit **1** (compound **15**, $\text{EC}_{90} = 20 \mu\text{M}$, compound **16**, $\text{EC}_{90} = 12 \mu\text{M}$). Surprisingly, the amide analogue of ester **15** (compound **18**) was found to be completely inactive ($\text{EC}_{90} > 250 \mu\text{M}$). Then, the introduction of amide or sulfonamide functions linked to the aromatic ring via the nitrogen atom were also investigated (compounds **20** and **21**). Amide **20** and sulfonamide **21** were respectively poorly active ($\text{EC}_{90} = 187 \mu\text{M}$) or inactive ($\text{EC}_{90} > 250 \mu\text{M}$). Finally, reduction of the ester to give the corresponding alcohol was strongly detrimental for the activity (compound **22**, $\text{EC}_{90} > 250 \mu\text{M}$).

Only esters **15** and **16** gave interesting results, replacement of the ester bond with amides, a sulfonamide or alcohol was not tolerated. Based on these data, it was decided to further explore the SARs by synthesizing different esters bearing a phenyl group (Table 4).

3.3.2. Introduction of a phenyl ring on the ester function

Replacing the methyl ester (compound **15**) by a phenyl ester (compound **23**) maintained EPI potency ($\text{EC}_{90} = 23 \mu\text{M}$, Table 4). Introducing one or two methylene spacers between the oxygen atom and the aromatic ring decreased the potency by 3- to 6-fold (compounds **24** and **25**, $\text{EC}_{90} = 156$ and $62 \mu\text{M}$ respectively) while adding three methylene groups drastically reduced the potency (**26**, $\text{EC}_{90} = 250 \mu\text{M}$), showing that lengthening the alkyl chain had a strong detrimental impact on activity.

3.3.3. Introduction of a basic moiety to interact with acidic residues

In the co-crystalline structure obtained with compound **2**, two acidic residues, namely Glu947 and Asp951, are located at a distance of 6.3 and 8.6 Å respectively of the carbon atom in position 5 of the pyridine core (Fig. 2). It was thus hypothesized that addition of a positively charged group could establish a new ionic interaction with AcrB to improve affinity. The introduction of a basic moiety in position 5 of the pyridylpiperazine was therefore considered (Table 5).

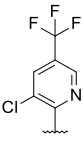








Addition of an ethylamine chain directly connected to the ester linker was detrimental to the activity (compound **27**, $\text{EC}_{90} > 250 \mu\text{M}$). Conversely, adding an aminoethyl or aminomethyl group on the phenyl ring led to a marked improvement in potency (compounds **28** and **29**, $\text{EC}_{90} = 3$ and $0.6 \mu\text{M}$ respectively) which could be the result of a new contact with Glu947 or/and Asp951. Replacing the amino group with another hydrogen-bond donor group, such as a hydroxyl, resulted in compound **30** which is 40-times less potent than compound **29** ($\text{EC}_{90} = 26 \mu\text{M}$), confirming the importance of a basic moiety to potentially interact with the acidic residues of the binding pocket.

Amides or ethers were also considered as linkers. As previously observed, amides were less potent than the corresponding ester derivatives (compounds **28** vs **31** and **29** vs **33**). Likewise, ether linkers led to 16- to 38-fold less active compounds (compounds **34**, **35**).

3.3.4. 5-Membered aromatic heterocycles as ester bioisosteres

As esters are known to be metabolically unstable, especially in plasma [34], the replacement of the ester linker with several bioisosteres

Table 1
Biological activities of compounds 1, 3–10.

Compound	Structure	EC ₉₀ ^a
1 (BDM73185)		62 μM
3		>500 μM
4		>500 μM
5		>500 μM
6		>500 μM
7		>500 μM
8		500 μM
9		>500 μM
10		>250 μM

^a EC₉₀ represents the 90% maximal effective concentration of tested compounds that prevents the growth of *E. coli* BW25113 in the presence of 8 μg/mL pyridomycin as measured by resazurin reduction. Data are the result of at least two biological replicates and are presented as mean values.

was also investigated. 5-membered aromatic heterocycles, such as 1,2,4- and 1,3,4-oxadiazoles, a thiazole and an oxazole [34–38], were therefore introduced (Table 6).

Firstly, compound 36 did not display any activity, which could be attributed to a low solubility in DMSO. Introducing a 1,3,4-oxadiazole, a thiazole or an oxazole led to weakly active or inactive compounds (compounds 37, 39, 40, EC₉₀ > 125 μM) whereas a 1,2,4-oxadiazole ring with a methyl substituent at position 5 was better tolerated (compound 42, EC₉₀ = 78 μM).

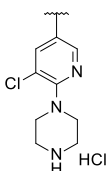






Based on these data, we considered the introduction of an amino group on the oxadiazole via different alkyl chain length to interact with Glu947 and/or Asp951. It appears that the aminoethyl derivative

Table 2
Biological activities of compounds 1, 2 and 11–14.

Compound	R1	R2	EC ₉₀ ^a
1 (BDM73185)	-Cl	-CF ₃	62 μM
11	-H	-CF ₃	>500 μM
12	-Br	-CF ₃	16 μM
13	-CH ₂ -OH	-CF ₃	>500 μM
2 (BDM88832)	-Cl	-I	12 μM
14	-CF ₃	-I	94 μM

^a EC₉₀ represents the 90% maximal effective concentration of tested compounds that prevents the growth of *E. coli* BW25113 in the presence of 8 μg/mL pyridomycin as measured by resazurin reduction. Data are the result of at least two biological replicates and are presented as mean values.

Table 3
Biological activities of compounds 1, 15, 16, 18 and 20–22.

Compound	Structure	EC ₉₀ ^a
1 (BDM73185)		62 μM
15		20 μM
16		12 μM
18		>250 μM
20		187 μM
21		>250 μM
22		>250 μM

^a EC₉₀ represents the 90% maximal effective concentration of tested compounds that prevents the growth of *E. coli* BW25113 in the presence of 8 μg/mL pyridomycin as measured by resazurin reduction. Data are the result of at least two biological replicates and are presented as mean values.

(compound 44, EC₉₀ = 8 μM) was the most potent compared to aminomethyl or aminopropyl derivatives (compound 43, EC₉₀ = 81 μM and compound 45, EC₉₀ = 21 μM respectively).

4. *E. coli* Ala446Pro and Ser450Pro AcrB mutants

Previous research demonstrated that single point mutations near the pyridylpiperazine binding pocket of AcrB (namely Ser450Pro or Ala446Pro) had no impact on AcrB function, but were sufficient to prevent pyridylpiperazines binding to AcrB, conferring resistance to this chemical series in *E. coli*. To confirm that the analogues developed here remained on target and act by directly inhibiting AcrB, the most promising analogues, 29 and 44, were tested in combination with a subactive dose of pyridomycin on the *E. coli* AcrB Ala446Pro and *E. coli* AcrB Ser450Pro strains (Table 7). As expected, both compounds are no longer able to boost pyridomycin in the AcrB mutated strains, implying that 29 and 44 act by inhibiting AcrB.

Table 4
Biological activities of compounds **1**, **15** and **23–26**.

Compound	Structure	EC ₉₀ ^a
1 (BDM73185)		62 μM
15		20 μM
23		23 μM
24		156 μM
25		62 μM
26		250 μM

^a EC₉₀ represents the 90% maximal effective concentration of tested compounds that prevents the growth of *E. coli* BW25113 in the presence of 8 μg/mL pyridomycin as measured by resazurin reduction. Data are the result of at least two biological replicates and are presented as mean values.

5. *E. coli* Glu947 and Asp951 AcrB mutants

In order to better understand the binding mode of pyridylpiperazine compounds and to validate the putative interactions with AcrB acidic residues Glu947 or Asp951, *E. coli* AcrB mutants were engineered where these amino-acids were substituted into their corresponding amide residue (Gln or Asn). These substitutions were chosen because they result in the loss of charge of the amino acid residue, without impacting its size or its ability to form hydrogen bonds with the EPI. Importantly, these mutations did not impact AcrB activity, as the pyridomycin susceptibility of the mutant strains was not altered compared to the wild-type.

Eight compounds were tested in combination with pyridomycin on the two mutants (Table 8). We observed that all compounds bearing a primary amine showed at least an 8-fold increase in EC₉₀ on one or both mutants (Glu947Gln or Asp951Asn), with the exception of compound **43**. The most potent compound on the wild-type strain (**29**, EC₉₀ = 0.6 μM) showed to be less potent on the Asp951Asn mutant (EC₉₀ = 62 μM) and on the Glu947Gln mutant (EC₉₀ > 62 μM) and in the same range of activity than **23** on the wild-type strain. On the contrary, the alcohol derivative, compound **30**, did not display significant increased EC₉₀. These data confirm the involvement of the two acid residues in the interaction with the basic inhibitors through a possible ionic bond. It cannot be excluded that these two acidic residues may also facilitate the entry of compounds into the binding pocket by interaction via the piperazine in position 2.

6. Boosting of a panel of antibiotics by compounds **29** and **44**

The ability of the most promising compounds **29** and **44** to inhibit the efflux of AcrAB-TolC substrates other than pyridomycin was further evaluated by measuring the MICs of a panel of antibiotics with and without 100 μM of each EPI. The panel included chloramphenicol, two β-lactam antibiotics (oxacillin and piperacillin), fusidic acid, novobiocin, linezolid, erythromycin and ciprofloxacin (Table 9).

Both compounds were able to boost all antibiotics tested. More particularly, the MICs of piperacillin, novobiocin, linezolid and erythromycin were decreased by at least 8-fold in combination with both EPIs. As expected, compound **29** was more effective than compound **44**.

Table 5
Biological activities of compounds **1**, **15**, **23**, **27–35**.

Compound	Structure	EC ₉₀ ^a
1 (BDM73185)		62 μM
15		20 μM
23		23 μM
27		>250 μM
28		3 μM
29 (BDM91270)		0.6 μM
30		26 μM
31		187 μM
32		47 μM
33		156 μM
34		10 μM
35		23 μM

^a EC₉₀ represents the 90% maximal effective concentration of tested compounds that prevents the growth of *E. coli* BW25113 in the presence of 8 μg/mL pyridomycin as measured by resazurin reduction. Data are the result of at least two biological replicates and are presented as mean values.

7. Physico-chemical and *in vitro* pharmacokinetic properties

The physico-chemical properties and the plasma and microsomal stability of the reference compound **2** and the two most promising compounds **29** and **44** were then measured (Table 10). The three compounds showed good aqueous solubility and low lipophilicity. An improvement in ligand-lipophilicity efficiency (LLE) was achieved with compounds **29** and **44** compared to the starting iodo derivative **2** and these two compounds show similar LLE values despite their difference in potency.

Regarding pharmacokinetic properties, compound **2** was rapidly degraded in murine microsomes ($t_{1/2}$ = 9 min) although it was stable in murine plasma ($t_{1/2}$ > 6 h). The most potent compound **29** was found to be completely unstable in plasma ($t_{1/2}$ = 0 min) due to its rapid hydrolysis into the corresponding carboxylic acid. In contrast, compound **44** proved to be stable in both plasma and microsomes ($t_{1/2}$ > 6 h and $t_{1/2}$ > 40 min, respectively). This compound is thus the best compromise between activity and stability for further *in vivo* experiments.

Table 6
Biological activities of compounds 15, 36, 37, 39, 40, 42–45.

Compound	Structure	EC ₉₀ ^a
15		20 μM
36		>125 μM ^b
37		187 μM
42		78 μM
39		187 μM
40		>250 μM
43		81 μM
44 (BDM91514)		8 μM
45		21 μM

^a EC₉₀ represents the 90% maximal effective concentration of tested compounds that prevents the growth of *E. coli* BW25113 in the presence of 8 μg/mL pyridomycin as measured by resazurin reduction. Data are the result of at least two biological replicates and are presented as mean values.

^b Maximal concentration tested due to insolubility of compound 36 in DMSO.

8. Conclusion

We have previously described pyridylpiperazine compounds binding to an allosteric pocket within the transmembrane domain of AcrB, highlighting a new chemical series with an original binding site for RND efflux pump inhibitors.

In this work, we aimed to optimize the EPI potency, the physico-

Table 7
Biological activities of compounds 29 and 44 on *E. coli* AcrB WT, *E. coli* AcrB-Ala446Pro and *E. coli* AcrB-Ser450Pro.

	EC ₉₀ <i>E. coli</i> WT ^a	EC ₉₀ <i>E. coli</i> AcrB Ala446Pro ^a	EC ₉₀ <i>E. coli</i> AcrB Ser450Pro ^a
29 (BDM91270)	0.6 μM	>62 μM	>62 μM
44 (BDM91514)	8 μM	>250 μM	>250 μM

^a EC₉₀ represents the 90% maximal effective concentration of tested compounds that prevents the growth of *E. coli* BW25113 WT, AcrB-Ala446Pro or AcrB-Ser450Pro in the presence of 8 μg/mL pyridomycin as measured by resazurin reduction. All data are the result of at least two biological replicates and are presented as mean values.

chemical properties and stability in physiological fluids of the pyridylpiperazine series. As a result, we designed, synthesized and tested 40 compounds on *E. coli* in combination with a sub-inhibitory dose of pyridomycin to enrich the SARs. Experimental data established the requirement of a piperazine ring in position 2 of the pyridine to interact with Asp408. The importance of a chlorine or bromine atom in position 3, which forms a halogen bond with Lys940, was also confirmed. Finally, through rational design, we uncovered substituents in position 5 which greatly improved the potency, notably by introducing phenyl esters substituted with basic amines (compounds 28 and 29) that could interact with Glu947 and/or Asp951. Compound 29 was identified as a very remarkable candidate, being 20-fold more potent than the starting iodo derivative 2 (EC₉₀ = 0.6 μM and 12 μM respectively). However, this compound was highly unstable in plasma. The replacement of the cleavable ester bond with a bioisostere such as a 1,2,4-oxadiazole ring (compound 44) allowed to improve the plasma stability. Moreover, compound 44 displayed a favorable microsomal stability.

We then showed that a single point mutation in AcrB (Ser450Pro or Ala446Pro) prevents compounds 29 and 44 from boosting pyridomycin activity, demonstrating that this chemical series acts by directly inhibiting AcrB. To verify the hypothesized interaction of the primary amine bearing EPIs with one or both of the acidic residues Glu947 and Asp951, two *E. coli* mutant strains (AcrB-Glu947Gln and AcrB-Asp951Asn) were generated. At least 6 compounds showed significantly higher EC₉₀ on those strains compared to the WT strain, confirming the role of these two residues either to form an ionic bond with the inhibitors or enhance their entry into the binding pocket.

Out of the 40 inhibitors synthesized, the most promising compounds, 29 (BDM91270) and 44 (BDM91514), were tested on a panel of eight antibiotics, where they proved to boost all AcrAB-TolC substrates tested, confirming their role as EPIs.

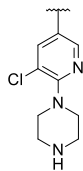
To conclude, we identified that compound 44 (BDM91514) has the best physicochemical and *in vitro* biological features to further progress it into evaluations as an antibiotic potentiator. This EPI shows no intrinsic antibacterial activity, is potent (EC₉₀ = 8 μM) and is able to boost several AcrAB-TolC antibiotic substrates. Moreover, by using *E. coli* *acrB* strategically designed point mutations, we confirmed that the interaction with AcrB Glu947 and/or Asp951 is of paramount importance for compound 44 binding mode. Together, our rational designed compound 44 represents a current excellent candidate to perform *in vivo* experiments, paving the way towards further EPI scaffolds that can help to combat MDR through efflux pump inhibition.

9. Experimental section

9.1. Chemistry

Solvents for synthesis, analysis and purification were purchased as analytical grade from commercial suppliers and used directly without further purification. Chemical reagents were purchased from Fisher scientific, Merck, Fluorochem, Enamine or TCI as reagent grade and used without further purification. Compound 11, 1-[5-(Trifluoromethyl)pyrid-2-yl]piperazine, was purchased from Fluorochem.

UPLC-MS Waters system was equipped with a UPLC I SMP MGR-FTN sample manager, an ACQUITY UPLC I-Class eK photodiode array detector (210–400 nm) and an ACQUITY QDa (Performance) detector

Table 8Biological activities of compounds **28–30**, **34**, **35**, **43–45** on *E. coli* AcrB WT, *E. coli* AcrB-Glu947Gln and *E. coli* AcrB-Asp951Asn.

Compound	Structure	EC ₉₀ <i>E. coli</i> WT ^a	EC ₉₀ <i>E. coli</i> Glu947Gln ^b (fold difference)	EC ₉₀ <i>E. coli</i> Asp951Asn ^b (fold difference)
28		3 μM	8 μM (x3)	23 μM (x8)
29 (BDM91270)		0.6 μM	>62 μM (>x103)	62 μM (x103)
30		26 μM	62 μM (x2.4)	62 μM (x2.4)
34		10 μM	>250 μM (>x25)	250 μM (x25)
35		23 μM	>250 μM (>x11)	>250 μM (>x11)
43		81 μM	250 μM (x3)	250 μM (x3)
44 (BDM91514)		8 μM	>250 μM (>x31)	>250 μM (>x31)
45		21 μM	>250 μM (>x12)	>250 μM (>x12)

^a EC₉₀ represents the 90% maximal effective concentration of tested compounds that prevents the growth of *E. coli* BW25113 in the presence of 8 μg/mL pyridomycin as measured by resazurin reduction.

^b EC₉₀ represents the 90% maximal effective concentration of tested compounds that prevents the growth of *E. coli* BW25113 AcrB-Glu947Gln or AcrB-Asp951Asn in the presence of 3 μg/mL pyridomycin as measured by resazurin reduction. All data are the result of at least two biological replicates and are presented as mean values.

Table 9Minimal Inhibitory concentration (MIC₉₅) of a panel of antibiotics on *E. coli* WT in the presence or absence of 100 μM of compound **29** or compound **44**.

	MIC ^a (μg/mL) without EPI	MIC ^a (μg/mL) with 29	Fold change ^b	MIC ^a (μg/mL) with 44	Fold change ^b
Chloramphenicol	8	2	4	2	4
Oxacillin	>128	5	> 26	40	3
Piperacillin	4 ^c	<0.5	> 8	<0.5	> 8
Fusidic acid	>128	5	> 26	32	4
Novobiocin	80	1.2	64	10	8
Linezolid	>128	8	> 16	16	8
Erythromycin	128 ^c	4	32	16	8
Ciprofloxacin	0.01	<0.005	>2	<0.005	>2

^a MIC determined on *E. coli* BW25113 by using the resazurin reduction assay. All data are the result of four biological experiments with two technical repeats each and are presented as mean values.

^b Fold change superior to 8 written in bold characters.

^c Values reported are shifted as the assay was performed overnight (up to 18 h) as compared to the 4 h incubation period in our previous work [21].

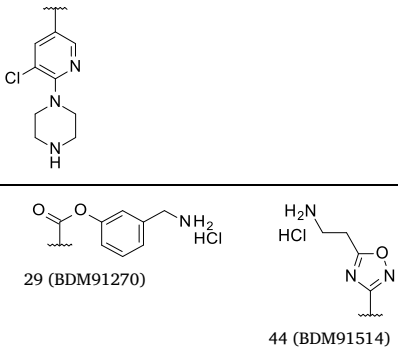
(scan 30–1250). Acquity BEH C18 column (50 mm × 2.1 mm, 1.7 μm, Waters) was used. The injection volume was 0.5 μL. A mixture of water and acetonitrile was used as mobile phase in gradient-elution. The pH of the mobile phase was adjusted with HCOOH and NH₄OH to form a buffer solution at pH 3.8. The analysis time was 5 min (at a flow rate of 600 μL/min), 10 min (at a flow rate at 600 μL/min) or 30 min (at a flow rate at 600 μL/min). Purity (%) was determined using UV detection (215 nm), and all isolated compounds showed purity greater than 95%.

HRMS analysis was performed on a LC-MS system equipped with a LCT Premier XE mass spectrometer (Waters), using a XBridge C18 column (50 mm × 4.6 mm, 3.5 μm, Waters). A gradient starting from 98% H₂O 5 mM Ammonium Formate pH 3.8 and reaching 100% CH₃CN 5 mM Ammonium Formate pH 3.8 within 3 min at a flow rate of 1 mL/min was used.

NMR spectra were recorded on a Bruker DRX-300 spectrometer. The results were calibrated to signals from the solvent as an internal reference [e.g. 2.50 (residual DMSO-*d*₆) and 39.52 (DMSO-*d*₆) ppm for ¹H and ¹³C NMR spectra respectively]. Chemical shifts (δ) are in parts per million (ppm) downfield from tetramethylsilane (TMS). The assignments were made using one-dimensional (1D) ¹H and ¹³C spectra and two-dimensional (2D) HSQC-DEPT, COSY and HMBC spectra. NMR

Table 10

Potency, physico-chemical properties, plasmatic and microsomal stability of compounds **2**, **29** and **44**.



Compound	2 (BDM88832)	29 (BDM91270)	44 (BDM91514)
EC ₉₀ ^a	12 μM	0.6 μM	8 μM
Solubility (PBS) ^b	>200 μM	180 μM	>200 μM
cLogP ^c	1.58	1.58	0.40
LLE ^d	3.34	4.57	4.65
Plasmatic stability ^e	t _{1/2} > 6h	t _{1/2} = 0 min	t _{1/2} > 6h
Microsomal stability ^f	t _{1/2} = 9 min CL _{int} = 241 μL/min/mg	ND	t _{1/2} > 40 min CL _{int} < 2 μL/min/mg

^a EC₉₀ represents the 90% maximal effective concentration of tested compounds that prevents the growth of *E. coli* BW25113 in the presence of 8 μg/mL pyridomycin as measured by resazurin reduction.

^b Solubility measured in PBS pH 7.4 starting from a 10 mM solution in DMSO.

^c calculated with DataWarrior.

^d LLE = pEC₉₀ - clogP.

^e Stability in mouse plasma.

^f Stability in mouse microsomes. Intrinsic clearance (CL_{int}) is expressed as μL/min/mg proteins. ND not determined.

coupling constants (*J*) are reported in Hertz (Hz), and splitting patterns are indicated as follows: s for singlet, brs for broad singlet, d for doublet, t for triplet, q for quartet, p for pentet, dd for doublet of doublet, ddd for doublet of doublet of doublet, qd for quartet of doublet, m for multiplet, δ for chemical shift, *J* for coupling constant.

Flash chromatography purifications were done on prepacked columns Reveleris® flash cartridges (40 μm, Büchi® FlashPure or 15–40 μm, Macherey-Nagel® Chromabond) under pressure with an Interchim Puriflash® 430 instrument. Products were detected by UV absorption at 254 nm and by ELSD.

Reverse flash chromatography was performed using a CombiFlash® C₁₈ Rf200 with C₁₈ silica gel cartridges. UV detection (215 and 254 nm) was used to collect the desired product.

Preparative HPLC was performed using a Buchi Pure C-830 system with an OmniSphere 10 μm column C₁₈ Dynamax (250 mm × 41.4 mm) from Agilent Technologies. A gradient starting from 20% MeCN/80% H₂O/0.1% formic acid and reaching 100% MeCN/0.1% formic acid at a flow rate of 80 mL/min was used. UV detection (215 and 254 nm) was used to collect the desired product.

9.1.1. General methods

9.1.1.1. Procedure A: introduction of an amine substituent in position R1 of the pyridine by S_NAr. In a tube were introduced the corresponding chloro-pyridine derivative (0.7–4.1 mmol, 1 eq.), the cyclic or linear amine (1.5–4.5 eq.) and triethylamine (1.3 eq.) in 1–8 mL of acetonitrile. The resulting mixture was stirred at 50–80 °C during 1–96 h. After evaporation of the solvent, the crude was purified by flash chromatography to give the desired compound.

9.1.1.2. Procedure B: Boc-cleavage with HCl. In a round-bottomed flask

containing the corresponding Boc-protected compound (0.132–0.0395 mmol, 1 eq.) in 0.5–1.0 mL of 1,4-dioxane, HCl 4 M in 1,4-dioxane (10 eq.) was added. The mixture was stirred at room temperature during 5 hours–3 days. The solvent was then evaporated under reduced pressure, petroleum ether was added and the mixture was filtered to give the desired compounds.

9.1.1.3. Procedure C: coupling with isopropyl chloroformate. A 1 M solution of isopropyl chloroformate in toluene (1.5 eq) was added in 2.5 mL of anhydrous THF under argon. Carboxylic acid **17** (2.45 mmol, 1 eq.) and TEA (1.2 eq.) were dissolved in 2.5 mL of anhydrous THF and added dropwise to the isopropyl chloroformate solution at 0 °C. The reaction was allowed to warm up to r.t. and stirred overnight. A solution of saturated NaHCO₃ was added and the product was extracted twice with EtOAc. The organic layer was washed once with a saturated solution of NaHCO₃, once with brine, dried over MgSO₄ and evaporated under reduced pressure.

A solution of the previous compound (0.5 mmol, 1 eq) dissolved in 1 mL anhydrous THF was added dropwise at 0 °C to a solution of the corresponding alcohol or amine (1.2–1.5 eq) and TEA or *t*BuOK (1.6–2.0 eq) dissolved in 1 mL anhydrous THF at 0 °C. The mixture was stirred at r.t. for 2–4h. The reaction was diluted with EtOAc and then washed twice with a 1 N HCl aqueous solution and twice with a saturated solution of NaHCO₃. The organic layers were combined, washed with a saturated solution of NaCl, dried over MgSO₄ and evaporated under vacuum. The intermediate was purified by flash chromatography.

9.1.1.4. Procedure D: coupling with COMU. The corresponding carboxylic acid (1–1.5 eq.) was stirred at r.t. with TEA or K₂CO₃ (2–3 eq) and COMU (1.5–3 eq) for 10 min in EtOAc (1.5 mL). Then, the corresponding alcohol or amine was added (1–1.5 eq) and the reaction was allowed to stir at room temperature. If the product precipitated, it was isolated by filtration. Otherwise the reaction was washed once with an aqueous solution of HCl 1 N, twice with a saturated solution of NaHCO₃ and the product was extracted with EtOAc. The combined organic layers were washed with brine, dried over MgSO₄ and evaporated under vacuum. The intermediate was purified by reverse or normal phase chromatography.

9.1.1.5. Procedure E: Synthesis of 1,2,4-oxadiazole derivatives starting from *tert*-butyl 4-[3-chloro-5-[(*Z*)-*N*'-hydroxycarbamimidoyl]-2-pyridyl]piperazine-1-carboxylate (**41**). To a mixture of intermediate **41** (1 eq.), the corresponding carboxylic acid (1.1 eq) and TEA (5 eq) in 2 mL of EtOAc, was added dropwise T3P 50% in EtOAc (3.5 eq). The reaction was heated at 80 °C for 16–24h. The reaction was diluted with EtOAc, washed twice with water and twice with a saturated solution of NaHCO₃. The organic layer was then washed with brine, dried over MgSO₄ and evaporated under reduced pressure.

9.1.2. Compounds description

9.1.2.1. 4-[3-chloro-5-(trifluoromethyl)-2-pyridyl]piperazin-2-one (3). Using **procedure A**, starting with 2,3-dichloro-5-(trifluoromethyl)pyridine (0.7 mmol) and piperazin-2-one (1.5 eq.), in 1 mL of MeCN at 70 °C for 24h. The crude was purified with a gradient dichloromethane/methanol 100:0 to 90:10 to give **3** as a white powder. Yield: 76%; LC-MS (ESI) m/z [M+H]⁺ = 280; ¹H NMR (300 MHz, DMSO-*d*₆), δ (ppm): 3.28–3.31 (m, 2H), 3.72 (t, *J* = 5.1 Hz, 2H), 4.0 (s, 2H), 8.04 (s, 1H), 8.23–8.24 (m, 1H), 8.57–8.58 (m, 1H); ¹³C NMR (75 MHz, DMSO-*d*₆), δ (ppm): 39.0, 45.2, 51.5, 118.9 (q, ²J_{CF} = 32 Hz), 119.6, 123.4 (q, ¹J_{CF} = 271 Hz), 136.5 (q, ³J_{CF} = 3 Hz), 143.1 (q, ³J_{CF} = 4 Hz), 158.3, 166.7; HRMS (TOF, ES+) m/z [M+H]⁺: calcd. for C₁₀H₁₀ClF₃N₃O₃ 280.0464, found 280.0457.

9.1.2.2. tert-butyl (3R)-4-[3-chloro-5-(trifluoromethyl)-2-pyridyl]-3-methyl-piperazine-1-carboxylate (4a). Using **procedure A**, starting with 2,3-dichloro-5-(trifluoromethyl)pyridine (0.7 mmol) and *tert*-butyl (3R)-3-methylpiperazine-1-carboxylate (2.25 eq.) in 1 mL of MeCN at 50 °C for 48 h. The crude was purified with a gradient cyclohexane/EtOAc 100:0 to 95:5 to give **4a** as a colorless oil. Yield: 20%; LC-MS (ESI) m/z $[M+H]^+$ = 380; 1H NMR (300 MHz, CD_2Cl_2), δ (ppm): 1.23 (d, J = 6.6 Hz, 3H), 1.50 (s, 9H), 3.12–3.19 (m, 1H), 3.25–3.30 (d, J = 13.6 Hz, 1H), 3.36–3.46 (m, 1H), 3.67 (d, J = 13.4 Hz, 1H), 3.80 (d, J = 13.4 Hz, 1H), 3.95–4.08 (m, 1H), 4.32–4.34 (m, 1H), 7.82–7.83 (m, 1H), 8.43–8.44 (m, 1H).

9.1.2.3. (2R)-1-[3-chloro-5-(trifluoromethyl)-2-pyridyl]-2-methyl-piperazine; hydrochloride (4). Using **procedure B**, starting with compound **4a** (0.132 mmol) and 10 eq. HCl 4 M for 5 h to give **4** as a white powder. Yield: 99%; LC-MS (ESI) m/z $[M+H]^+$ = 280; 1H NMR (300 MHz, CD_2Cl_2), δ (ppm): 1.48 (d, J = 5.7 Hz, 3H), 3.21–3.49 (m, 4H), 3.64–3.87 (m, 2H), 4.39–4.46 (m, 1H), 7.86 (d, J = 1.7 Hz, 1H), 8.46 (d, J = 0.9 Hz, 1H), 9.88–10.16 (m, 2H); ^{13}C NMR (75 MHz, CD_2Cl_2), δ (ppm): 16.0, 41.6, 43.2, 47.4, 49.4, 121.8 (q, $^2J_{CF}$ = 32 Hz), 122.9, 123.7 (q, $^1J_{CF}$ = 271 Hz), 136.7 (q, $^3J_{CF}$ = 3 Hz), 143.5 (q, $^3J_{CF}$ = 4 Hz), 159.6; HRMS (TOF, ES+) m/z $[M+H]^+$: calcd. for $C_{11}H_{14}ClF_3N_3$ 280.0828, found 280.0814.

9.1.2.4. tert-butyl (3S)-4-[3-chloro-5-(trifluoromethyl)-2-pyridyl]-3-methyl-piperazine-1-carboxylate (5a). Using **procedure A**, starting with 2,3-dichloro-5-(trifluoromethyl)pyridine (0.7 mmol) and *tert*-butyl (3S)-3-methylpiperazine-1-carboxylate (2.25 eq.) in 1 mL of MeCN at 50 °C for 48 h. The crude was purified with a gradient cyclohexane/EtOAc 100:0 to 95:5 to give **5a** as a colorless oil. Yield: 22%; LC-MS (ESI) m/z $[M+H]^+$ = 380; 1H NMR (300 MHz, CD_2Cl_2), δ (ppm): 1.23 (d, J = 6.6 Hz, 3H), 1.50 (s, 9H), 3.15–3.20 (m, 1H), 3.25–3.30 (d, J = 13.3 Hz, 1H), 3.37–3.46 (m, 1H), 3.67 (d, J = 13.1 Hz, 1H), 3.81 (d, J = 13.1 Hz, 1H), 3.98–4.07 (m, 1H), 4.32–4.34 (m, 1H), 7.82–7.83 (m, 1H), 8.43–8.44 (m, 1H).

9.1.2.5. (2S)-1-[3-chloro-5-(trifluoromethyl)-2-pyridyl]-2-methyl-piperazine; hydrochloride (5). Using **procedure B**, starting with compound **5a** (0.040 mmol) and 12 eq. HCl 4 M for 7 h to give **4** as a white powder. Yield: 96%; LC-MS (ESI) m/z $[M+H]^+$ = 280; 1H NMR (300 MHz, CD_2Cl_2), δ (ppm): 1.48 (d, J = 5.3 Hz, 3H), 3.2–3.43 (m, 4H), 3.58–3.84 (m, 2H), 4.37–4.48 (m, 1H), 7.87 (d, J = 1.8 Hz, 1H), 8.46 (d, J = 1.2 Hz, 1H), 9.91–10.31 (m, 2H); ^{13}C NMR (75 MHz, CD_2Cl_2), δ (ppm): 16.0, 41.6, 43.2, 47.4, 49.4, 121.8 (q, $^2J_{CF}$ = 33 Hz), 122.9, 123.5 (q, $^1J_{CF}$ = 271 Hz), 136.8 (q, $^3J_{CF}$ = 3 Hz), 143.5 (q, $^3J_{CF}$ = 4 Hz), 159.6; HRMS (TOF, ES+) m/z $[M+H]^+$: calcd. for $C_{11}H_{14}ClF_3N_3$ 280.0828, found 280.0821.

9.1.2.6. (3R,5S)-1-[3-chloro-5-(trifluoromethyl)-2-pyridyl]-3,5-dimethyl-piperazine (6). Using **procedure A**, starting with 2,3-dichloro-5-(trifluoromethyl)pyridine (0.7 mmol) and (2R,6S)-2,6-dimethylpiperazine (1.5 eq.) in 1 mL of MeCN at 50 °C for 3 h. The crude was purified with a gradient dichloromethane/methanol 100:0 to 90:10 to give **6** as a colorless oil. Yield: 93%; LC-MS (ESI) m/z $[M+H]^+$ = 294; 1H NMR (300 MHz, CD_2Cl_2), δ (ppm): 1.15 (d, J = 6.4 Hz, 6H), 2.61 (dd, J = 10.7, 12.6 Hz, 2H), 3.02–3.15 (m, 3H), 3.98 (d, J = 12.6 Hz, 2H), 7.77 (dd, J = 0.5, 2.2 Hz, 1H), 8.37 (dd, J = 1.0, 2.2 Hz, 1H); ^{13}C NMR (75 MHz, CD_2Cl_2), δ (ppm): 19.2, 51.2, 55.0, 119.7 (q, $^2J_{CF}$ = 32 Hz), 120.8, 123.8 (q, $^1J_{CF}$ = 271 Hz), 136.4 (q, $^3J_{CF}$ = 3 Hz), 143.3 (q, $^3J_{CF}$ = 4 Hz), 160.0; HRMS (TOF, ES+) m/z $[M+H]^+$: calcd. for $C_{12}H_{16}ClF_3N_3$ 294.0985, found 294.0978.

9.1.2.7. 1-[3-chloro-5-(trifluoromethyl)-2-pyridyl]-1,4-diazepane (7). Using **procedure A**, starting with 2,3-dichloro-5-(trifluoromethyl)pyridine (0.7 mmol) and 1,4-diazepane (3 eq.) in 1 mL of MeCN at 50 °C

for 16 h. The crude was purified with a gradient dichloromethane/methanol 100:0 to 90:10 to give **7** as a yellow oil. Yield: 77%; LC-MS (ESI) m/z $[M+H]^+$ = 280; 1H NMR (300 MHz, CD_2Cl_2), δ (ppm): 1.86–1.95 (m, 2H), 2.14 (br s, 1H), 2.85–2.90 (m, 2H), 3.03–3.08 (m, 2H), 3.78–3.87 (m, 4H), 7.68–7.70 (m, 1H), 8.27–8.29 (m, 1H); ^{13}C NMR (75 MHz, CD_2Cl_2), δ (ppm): 31.1, 48.4, 49.8, 50.5, 54.1, 116.5, 117.1 (q, $^2J_{CF}$ = 33 Hz), 124.2 (q, $^1J_{CF}$ = 271 Hz), 136.9 (q, $^3J_{CF}$ = 3 Hz), 143.0 (q, $^3J_{CF}$ = 4 Hz), 158.7; HRMS (TOF, ES+) m/z $[M+H]^+$: calcd. for $C_{11}H_{14}ClF_3N_3$ 280.0828, found 280.0829.

9.1.2.8. 1-[3-chloro-5-(trifluoromethyl)-2-pyridyl]piperidin-4-amine (8). Using **procedure A**, starting with 2,3-dichloro-5-(trifluoromethyl)pyridine (0.7 mmol) and 4-aminopiperidine (1.5 eq.) in 1 mL of MeCN at 50 °C for 5 h. The crude was purified with a gradient dichloromethane/methanol +2% TEA 100:0 to 90:10 to give **8** as a white powder. Yield: 78%; LC-MS (ESI) m/z $[M+H]^+$ = 280; 1H NMR (300 MHz, DMSO- d_6), δ (ppm): 1.45–1.58 (m, 2H), 1.90–1.95 (m, 2H), 2.93–3.09 (m, 3H), 3.93–3.98 (m, 2H), 5.24 (br s, 2H), 8.16–8.17 (m, 1H), 8.53–8.54 (m, 1H);

^{13}C NMR (75 MHz, DMSO- d_6), δ (ppm): 32.3, 46.9, 47.6, 118.1 (q, $^2J_{CF}$ = 32 Hz), 119.8, 123.5 (q, $^1J_{CF}$ = 271 Hz), 136.2 (q, $^3J_{CF}$ = 3 Hz), 143.1 (q, $^3J_{CF}$ = 4 Hz), 160.5; HRMS (TOF, ES+) m/z $[M+H]^+$: calcd. for $C_{11}H_{14}ClF_3N_3$ 280.0828, found 280.0812.

9.1.2.9. N'-[3-chloro-5-(trifluoromethyl)-2-pyridyl]ethane-1,2-diamine (9). Using **procedure A**, starting with 2,3-dichloro-5-(trifluoromethyl)pyridine (0.7 mmol) and ethane-1,2-diamine (3 eq.) in 1 mL of MeCN at 50 °C for 16 h. The crude was purified with a gradient dichloromethane/methanol 100:0 to 80:20 to give **9** as a white powder. Yield: 72%; LC-MS (ESI) m/z $[M+H]^+$ = 240; 1H NMR (300 MHz, DMSO- d_6), δ (ppm): 2.41 (brs, 2H), 2.73 (t, J = 6.3 Hz, 2H), 3.42 (q, J = 6.1 Hz, 2H), 7.21 (brs, 1H), 7.93–7.94 (m, 1H), 8.30–8.32 (m, 1H); ^{13}C NMR (75 MHz, DMSO- d_6), δ (ppm): 40.5, 43.9, 113.2 (q, $^2J_{CF}$ = 32 Hz), 114.1, 124.1 (q, $^1J_{CF}$ = 271 Hz), 132.7 (q, $^3J_{CF}$ = 3 Hz), 143.7 (q, $^3J_{CF}$ = 4 Hz), 156.1; HRMS (TOF, ES+) m/z $[M+H]^+$: calcd. for $C_8H_{10}ClF_3N_3$ 240.0515, found 240.0520.

9.1.2.10. tert-butyl 4-[3-chloro-5-(trifluoromethyl)-2-pyridyl]-3,6-dihydro-2H-pyridine-1-carboxylate (10a). To a mixture of 2,3-dichloro-5-(trifluoromethyl)pyridine (0.7 mmol, 1 eq.), *tert*-butyl 4-(4,4,5,5-tetra-methyl-1,3,2-dioxaborolan-2-yl)-3,6-dihydro-2H-pyridine-1-carboxylate (1 eq.), and potassium carbonate (1.5 eq.) in 10 mL of a 2:1:2 dimethoxyethane/ethanol/water mixture at room temperature was added Pd(PPh $_3$) $_2Cl_2$ (0.1 eq.). The resulting reaction mixture was degassed and heated at 90 °C for 4 h. The reaction mixture was then cooled to room temperature, diluted with water and extracted twice with DCM. The combined organic layers were next dried over MgSO $_4$ and concentrated under reduced pressure. The crude was purified by flash chromatography (cyclohexane/EtOAc 100:0 to 90:10) to give compound **10a** as a yellow powder. Yield: quantitative; LC-MS (ESI) m/z $[M+H]^+$ = 363; 1H NMR (300 MHz, CD_2Cl_2), δ (ppm): 1.52 (s, 9H), 2.59–2.66 (m, 2H), 3.67 (t, J = 5.6 Hz, 2H), 4.15 (q, J = 3.0 Hz, 2H), 5.36 (t, J = 1.1 Hz, 1H), 8.00–8.01 (m, 1H), 8.76–8.77 (m, 1H).

9.1.2.11. 3-chloro-2-(1,2,3,6-tetrahydropyridin-4-yl)-5-(trifluoromethyl)pyridine; hydrochloride (10). Using **procedure B**, starting with compound **10a** (0.69 mmol) and 8 eq. HCl 4 M for 16 h to give **10** as a white powder. Yield: 49%; LC-MS (ESI) m/z $[M+H]^+$ = 263; 1H NMR (300 MHz, DMSO- d_6), δ (ppm): 2.70–2.78 (m, 2H), 3.24–3.33 (m, 2H), 3.76–3.84 (m, 2H), 6.35–6.40 (m, 1H), 8.50–8.51 (m, 1H), 8.94–8.95 (m, 1H), 9.64 (brs, 2H); ^{13}C NMR (75 MHz, DMSO- d_6), δ (ppm): 23.6, 39.8, 40.8, 122.8 (q, $^1J_{CF}$ = 272 Hz), 124.9 (q, $^2J_{CF}$ = 32 Hz), 125.5, 129.0, 132.7, 135.9 (q, $^3J_{CF}$ = 3.5 Hz), 144.0 (q, $^3J_{CF}$ = 4 Hz), 158.5; HRMS (TOF, ES+) m/z $[M+H]^+$: calcd. for $C_{11}H_{11}ClF_3N_2$ 263.0563, found 263.0562.

9.1.2.12. 1-[3-bromo-5-(trifluoromethyl)-2-pyridyl]piperazine; hydrochloride (12). Using **procedure A**, starting with 3-bromo-2-chloro-5-(trifluoromethyl)pyridine (0.7 mmol) and *tert*-butyl piperazine-1-carboxylate (3 eq.) in 1 mL of MeCN at 80 °C for 48 h. The crude was purified with a gradient cyclohexane/EtOAc 100:0 to 80:20. The resulting intermediate was directly deprotected. Using **procedure B**, starting with *tert*-butyl 4-[3-bromo-5-(trifluoromethyl)-2-pyridyl]piperazine-1-carboxylate (0.49 mmol) and 10 eq. HCl 4 M for 72 h to give **12** as a white powder. Yield: 63%; LC-MS (ESI) *m/z* [M+H]⁺ = 310; ¹H NMR (300 MHz, DMSO-*d*₆), δ (ppm): 3.16–3.24 (m, 4H), 3.63–3.67 (m, 4H), 8.40 (dd, *J* = 0.5, 2.2 Hz, 1H), 8.64–8.65 (m, 1H), 9.66 (brs, 2H); ¹³C NMR (75 MHz, DMSO-*d*₆), δ (ppm): 42.3, 45.6, 110.5, 120.0 (q, ²*J*_{CF} = 33 Hz), 123.0 (q, ¹*J*_{CF} = 273 Hz), 139.9 (q, ³*J*_{CF} = 2.4 Hz), 143.7 (q, ³*J*_{CF} = 4.1 Hz), 160.4; HRMS (TOF, ES⁺) *m/z* [M+H]⁺: calcd. for C₁₀H₁₂BrF₃N₃ 310.0167, found 310.0152.

9.1.2.13. 2-chloro-5-(trifluoromethyl)pyridine-3-carbaldehyde (13a). To a 1.6 M solution of *n*-BuLi (1.25 eq.) in 40 mL anhydrous Et₂O cooled to –78 °C was added a solution of 3-bromo-2-chloro-5-(trifluoromethyl)pyridine (19.2 mmol, 1 eq.) in 15 mL of Et₂O under argon. The resulting mixture was allowed to react at this temperature for 1 h under argon. Anhydrous DMF (2.5 eq.) was then added dropwise. The mixture was stirred at –78 °C for 1 h under argon and then allowed to warm up to 0 °C and finally left to react for an additional hour. Aqueous HCl 1 M (50 mL) was added to the solution at 0 °C and then stirred for 20 min. The resulting mixture was extracted with Et₂O (3 × 40 mL), the combined organic layers were dried over MgSO₄, and concentrated under reduced pressure. The residue was purified by flash chromatography (cyclohexane/EtOAc 100:0 to 95:5) to give **13a** as a yellow oil. Yield: 19%; LC-MS (ESI) *m/z*: not ionizable; ¹H NMR (300 MHz, CD₂Cl₂), δ (ppm): 8.48–8.49 (m, 1H), 8.89–8.90 (m, 1H), 10.42 (s, 1H).

9.1.2.14. tert-butyl 4-[3-(hydroxymethyl)-5-(trifluoromethyl)-2-pyridyl]piperazine-1-carboxylate (13b). Sodium borohydride (1.1 eq.) was added to a solution of 2-chloro-5-(trifluoromethyl)pyridine-3-carbaldehyde (3.55 mmol, 1 eq.) in 4 mL of anhydrous MeOH at 0 °C. The mixture was stirred at 0 °C for 1 h before it was quenched with water. The combined mixture was concentrated to remove the MeOH and the resulting aqueous solution was extracted twice with EtOAc. The combined organic layers were washed with brine, dried over MgSO₄, filtered and concentrated. The resulting intermediate (0.59 mmol, 1 eq.) was then reacted using **procedure A** and *tert*-butyl piperazine-1-carboxylate for 20 h, the crude was purified with a gradient cyclohexane/EtOAc 100:0 to 70:30 to give **13b** as a yellow oil. Yield: 88%; LC-MS (ESI) *m/z* [M+H]⁺ = 362; ¹H NMR (300 MHz, CD₂Cl₂), δ (ppm): 2.50 (s, 9H), 3.23–3.26 (m, 4H), 3.57–3.60 (m, 4H), 4.73 (s, 2H), 8.00 (d, *J* = 2.0 Hz, 1H), 8.49 (d, *J* = 2.0 Hz, 1H).

9.1.2.15. [2-piperazin-1-yl-5-(trifluoromethyl)-3-pyridyl]methanol; hydrochloride (13). Using **procedure B**, starting with compound **13b** (0.44 mmol) and 9 eq. HCl 4 M for 2.5 h. The residue was purified by reverse phase flash chromatography (H₂O/MeOH, 90:10 to 0:100) to give **13** as a white powder. Yield: 59%; LC-MS (ESI) *m/z* [M+H]⁺ = 262; ¹H NMR (300 MHz, DMSO-*d*₆), δ (ppm): 3.18–3.24 (m, 4H), 3.45–3.51 (m, 4H), 4.50 (d, *J* = 5.1 Hz, 2H), 5.67 (t, *J* = 5.5 Hz, 1H), 8.05 (d, *J* = 2.1 Hz, 1H), 8.52 (d, *J* = 1.4 Hz, 1H), 9.40 (brs, 2H); ¹³C NMR (75 MHz, DMSO-*d*₆), δ (ppm): 42.6, 46.0, 58.4, 118.8 (q, ²*J*_{CF} = 32 Hz), 124.4 (q, ¹*J*_{CF} = 272 Hz), 128.3, 133.9 (q, ³*J*_{CF} = 3.2 Hz), 142.8 (q, ³*J*_{CF} = 4.4 Hz), 160.8; HRMS (TOF, ES⁺) *m/z* [M+H]⁺: calcd. for C₁₁H₁₅F₃N₃O 262.1167, found 262.1162.

9.1.2.16. tert-butyl 4-[5-iodo-3-(trifluoromethyl)-2-pyridyl]piperazine-1-carboxylate (14a). Using **procedure A**, starting with 2-chloro-5-iodo-3-(trifluoromethyl)pyridine (0.7 mmol) and *tert*-butyl piperazine-1-carboxylate (4.5 eq.) in 2 mL of MeCN at 80 °C for 96 h. The solvent

was evaporated and the residue was diluted with EtOAc. The organic layer was washed twice with aqueous HCl 1 N. The aqueous layer was then extracted once with EtOAc. The combined organic layers were washed with brine, dried over MgSO₄, and evaporated under reduced pressure to give **14a** as a light-yellow powder. Yield: 89%; LC-MS (ESI) *m/z* [M+H]⁺ = 458; ¹H NMR (300 MHz, CD₂Cl₂), δ (ppm): 1.45 (s, 9H), 3.20–3.26 (m, 4H), 3.48–3.54 (m, 4H), 8.16 (qd, *J* = 0.4, 2.3 Hz, 1H), 8.58 (*J* = 0.7, 2.2 Hz, 1H).

9.1.2.17. 1-[5-iodo-3-(trifluoromethyl)-2-pyridyl]piperazine; hydrochloride (14). Using **procedure B**, starting with compound **14a** (0.17 mmol) and 10 eq. HCl 4 M for 16 h to give **14** as a white powder. Yield: 65%; LC-MS (ESI) *m/z* [M+H]⁺ = 358; ¹H NMR (300 MHz, DMSO-*d*₆), δ (ppm): 3.10–3.24 (m, 4H), 3.34–3.46 (m, 4H), 8.37 (d, *J* = 2.0 Hz, 1H), 8.76 (d, *J* = 2.0 Hz, 1H), 9.52 (s, 2H); ¹³C NMR (75 MHz, DMSO-*d*₆), δ (ppm): 42.5, 47.1, 85.5, 117.5 (q, ²*J*_{CF} = 32 Hz), 122.8 (q, ¹*J*_{CF} = 273 Hz), 144.8 (q, ³*J*_{CF} = 4.9 Hz), 157.27, 157.31; HRMS (TOF, ES⁺) *m/z* [M+H]⁺: calcd. for C₁₀H₁₂F₃N₃ 358.0028, found 358.0024.

9.1.2.18. tert-butyl 4-(3-chloro-5-methoxycarbonyl-2-pyridyl)piperazine-1-carboxylate (15a). Using **procedure A**, starting with methyl 5,6-dichloropyridine-3-carboxylate (4.1 mmol) and *tert*-butyl piperazine-1-carboxylate (1.5 eq.) in 8 mL of MeCN at 80 °C for 16 h. The solvent was evaporated and the residue dissolved in EtOAc. The organic layer was washed with aqueous HCl 1 N. The aqueous layer was then washed three times with EtOAc. The combined organic layers were washed with brine, dried over MgSO₄, and evaporated under reduced pressure. The crude was purified by flash chromatography (cyclohexane/EtOAc 100:0 to 80:20) to give **15a** as a light orange powder. Yield: 99%; LC-MS (ESI) *m/z* [M+H]⁺ = 356; ¹H NMR (300 MHz, CD₂Cl₂), δ (ppm): 1.46 (s, 9H), 3.46–3.51 (m, 4H), 3.52–3.57 (m, 4H), 3.87 (s, 3H), 8.13 (d, *J* = 1.9 Hz, 1H), 8.71 (d, *J* = 1.9 Hz, 1H).

9.1.2.19. methyl 5-chloro-6-piperazin-1-yl-pyridine-3-carboxylate; hydrochloride (15). Using **procedure B**, starting with compound **15a** (0.7 mmol) and 10 eq. HCl 4 M for 72 h to give **15** as a white powder. Yield: 82%; LC-MS (ESI) *m/z* [M+H]⁺ = 256; ¹H NMR (300 MHz, DMSO-*d*₆), δ (ppm): 3.17–3.24 (m, 4H), 3.71 (t, *J* = 4.9 Hz, 4H), 3.85 (s, 3H), 8.16 (d, *J* = 2.0 Hz, 1H), 8.71 (d, *J* = 2.0 Hz, 1H), 9.55 (brs, 2H); ¹³C NMR (75 MHz, DMSO-*d*₆), δ (ppm): 42.4, 45.0, 52.3, 119.8, 120.1, 139.5, 147.2, 158.9, 164.2; HRMS (TOF, ES⁺) *m/z* [M+H]⁺: calcd. for C₁₁H₁₅ClN₃O₂ 256.0853, found 256.0847.

9.1.2.20. tert-butyl 4-(3-chloro-5-ethoxycarbonyl-2-pyridyl)piperazine-1-carboxylate (16a). Compound **15a** (0.28 mmol, 1 eq.) was added to a sodium ethoxide solution 21% in ethanol (5 eq.). The mixture was stirred at 70 °C for 20 h. This step led to the formation of the corresponding carboxylic acid. The reaction mixture was evaporated under reduced pressure and used without purification in the next step. The carboxylic acid was dissolved in anhydrous DMF (1 mL) and potassium carbonate (2 eq.) was added. The reaction mixture was stirred 5 min at r. t. and then iodoethane (55 eq.) was added. The reaction mixture was stirred at 100 °C for 16 h and then cooled to r. t. The mixture was poured into a separating funnel with EtOAc. The organic layer was washed twice with HCl 1 N, once with brine, dried over MgSO₄ and then evaporated under reduced pressure. The residue was purified by flash chromatography (cyclohexane/EtOAc 100:0 to 90:10) to give compound **16a** as a colorless oil. Yield: 40%; LC-MS (ESI) *m/z* [M+H]⁺ = 370; ¹H NMR (300 MHz, CD₂Cl₂), δ (ppm): 1.40 (t, *J* = 7.1 Hz, 3H), 1.50 (s, 9H), 3.50–3.54 (m, 4H), 3.57–3.61 (m, 4H), 4.37 (q, *J* = 7.1 Hz, 2H), 8.17 (d, *J* = 2.0 Hz, 1H), 8.76 (d, *J* = 2.0 Hz, 1H).

9.1.2.21. ethyl 5-chloro-6-piperazin-1-yl-pyridine-3-carboxylate; hydrochloride (16). Using **procedure B**, starting with compound **16a** (0.09 mmol) and 30 eq. HCl 4 M for 3 h to give **16** as a white powder. Yield:

quantitative; LC-MS (ESI) m/z $[M+H]^+$ = 270; 1H NMR (300 MHz, DMSO- d_6), δ (ppm): 1.32 (t, J = 7.1 Hz, 3H), 3.18–3.24 (m, 4H), 3.70–3.73 (m, 4H), 4.32 (q, J = 7.1 Hz, 2H), 8.16 (d, J = 2.0 Hz, 1H), 8.71 (d, J = 2.0 Hz, 1H), 9.60 (brs, 2H); ^{13}C NMR (75 MHz, DMSO- d_6), δ (ppm): 14.6, 42.8, 45.5, 61.5, 120.2, 120.8, 139.9, 147.6, 159.4, 164.1; HRMS (TOF, ES+) m/z $[M+H]^+$: calcd. for $C_{12}H_{17}ClN_3O_2$ 270.1009, found 270.0999.

9.1.2.22. 6-(4-tert-butoxycarbonylpiperazin-1-yl)-5-chloro-pyridine-3-carboxylic acid (17). Compound **15a** (4.86 mmol, 1 eq.), NaOH (8 eq.) and 1.5 mL water were stirred in 20 mL MeOH at 65 °C for 1 h. The reaction was diluted with water and then acidified with an aqueous solution of HCl 1 N. The aqueous layer was extracted thrice with EtOAc. The combined organic layers were washed with brine, dried over $MgSO_4$ and evaporated under reduced pressure to give compound **17** as a white solid. Yield: quantitative; LC-MS (ESI) m/z $[M+H]^+$ = 342; 1H NMR (300 MHz, DMSO- d_6), δ (ppm): 1.41 (s, 9H), 3.39–3.50 (m, 8H), 8.09 (d, J = 2.0 Hz, 1H), 8.66 (d, J = 2.0 Hz, 1H), 13.19 (s, 1H).

9.1.2.23. tert-butyl 4-[3-chloro-5-(methylcarbamoyl)-2-pyridyl]piperazine-1-carboxylate (18a). Using **procedure C**, starting with tert-butyl 4-(3-chloro-5-isopropoxycarbonyloxy-carbonyl-2-pyridyl)piperazine-1-carboxylate (0.5 mmol), methanamine 2 M in THF (1.5 eq.) and TEA (2.0 eq.) for 2.5 h to give **18a** as a colourless oil. Yield: 79%; LC-MS (ESI) m/z $[M+H]^+$ = 355; 1H NMR (300 MHz, CD_2Cl_2), δ (ppm): 1.46 (s, 9H), 2.94 (d, J = 4.9 Hz, 3H), 3.38–3.44 (m, 4H), 3.52–3.58 (m, 4H), 6.16 (s, 1H), 7.99 (d, J = 2.1 Hz, 1H), 8.48 (d, J = 2.1 Hz, 1H).

9.1.2.24. 5-chloro-N-methyl-6-piperazin-1-yl-pyridine-3-carboxamide; hydrochloride (18). Using **procedure B**, starting with compound **18a** (0.40 mmol) and 10 eq. HCl 4 M for 16 h to give **18** as a white powder. Yield: 43%; LC-MS (ESI) m/z $[M+H]^+$ = 255; 1H NMR (300 MHz, DMSO- d_6), δ (ppm): 2.76 (d, J = 4.7 Hz, 3H), 3.13–3.26 (m, 4H), 3.55–3.66 (m, 4H), 8.22 (d, J = 2.1 Hz, 1H), 8.66–8.74 (m, 2H), 9.56 (brs, 2H); ^{13}C NMR (75 MHz, DMSO- d_6), δ (ppm): 26.2, 42.5, 45.3, 120.4, 125.1, 138.0, 145.3, 157.9, 163.7; HRMS (TOF, ES+) m/z $[M+H]^+$: calcd. for $C_{11}H_{16}ClN_4O_3$ 255.1013, found 255.1019.

9.1.2.25. tert-butyl 4-(3-chloro-5-nitro-2-pyridyl)piperazine-1-carboxylate (19a). Using **procedure A**, starting with 2,3-dichloro-5-nitro-pyridine (1.0 mmol) and tert-butyl piperazine-1-carboxylate (1.5 eq.) in 1.5 mL of MeCN at 50 °C for 1 h. The reaction mixture was filtered and the solid rinsed with MeCN to give **19a** as a yellow powder. Yield: 87%; LC-MS (ESI) m/z $[M-H]^-$ = 341; 1H NMR (300 MHz, CD_2Cl_2), δ (ppm): 1.46 (s, 9H), 3.53–3.59 (m, 4H), 3.63–3.69 (m, 4H), 8.34 (d, J = 2.4 Hz, 1H), 8.95 (d, J = 2.4 Hz, 1H).

9.1.2.26. tert-butyl 4-(5-amino-3-chloro-2-pyridyl)piperazine-1-carboxylate (19). Compound **19a** (0.93 mmol, 1 eq.), $tBuOK$ (1.2 eq.) and bis(pinacolato)diboron (3.1 eq.) were heated at 110 °C in $iPrOH$ for 4 h. The reaction was evaporated under reduced pressure. The residue was dissolved in EtOAc and washed twice with water. The organic layer was then washed with brine, dried over $MgSO_4$ and evaporated under reduced pressure. Finally, the crude was purified by flash chromatography (cyclohexane/EtOAc 100:0 to 60:40) to give compound **19** as an orange oil. Yield: 84%; LC-MS (ESI) m/z $[M+H]^+$ = 313; 1H NMR (300 MHz, CD_2Cl_2), δ (ppm): 1.45 (s, 9H), 3.03–3.08 (m, 4H), 3.50–3.55 (m, 4H), 3.59 (brs, 2H), 7.06 (d, J = 2.6 Hz, 1H), 7.70 (d, J = 2.6 Hz, 1H).

9.1.2.27. tert-butyl 4-(5-acetamido-3-chloro-2-pyridyl)piperazine-1-carboxylate (20a). Compound **19** (0.12 mmol, 1 eq.) was stirred in 480 μL acetic anhydride (43 eq.) for 3 days at room temperature. The reaction was washed with saturated $NaHCO_3$ and extracted with EtOAc. The organic layers were combined, washed with brine, dried over $MgSO_4$ and evaporated under reduced pressure. The product was purified by

flash chromatography (DCM/MeOH 100:0 to 95:5) to give compound **20a** as a pale red oil. Yield: 38%; LC-MS (ESI) m/z $[M+H]^+$ = 355; 1H NMR (300 MHz, CD_2Cl_2), δ (ppm): 1.46 (s, 9H), 2.13 (s, 3H), 3.16–3.23 (m, 4H), 3.50–3.58 (m, 4H), 7.37 (s, 1H), 8.09 (d, J = 2.5 Hz, 1H), 8.15 (d, J = 2.5 Hz, 1H).

9.1.2.28. N-(5-chloro-6-piperazin-1-yl-3-pyridyl)acetamide; hydrochloride (20). Using **procedure B**, starting with compound **20a** (0.05 mmol) and 10 eq. HCl 4 M for 48 h to give **20** as a white powder. Yield: 84%; LC-MS (ESI) m/z $[M+H]^+$ = 255; 1H NMR (300 MHz, DMSO- d_6), δ (ppm): 2.05 (s, 3H), 3.15–3.24 (m, 4H), 3.36–3.42 (m, 4H), 8.19 (d, J = 2.3 Hz, 1H), 8.41 (d, J = 2.2 Hz, 1H), 9.40 (s, 2H), 10.47 (s, 1H); ^{13}C NMR (75 MHz, DMSO- d_6), δ (ppm): 23.7, 42.6, 45.9, 121.5, 129.5, 132.4, 136.6, 152.3, 168.8; HRMS (TOF, ES+) m/z $[M+H]^+$: calcd. for $C_{11}H_{16}ClN_4O$ 255.1013, found 255.1016.

9.1.2.29. tert-butyl 4-[3-chloro-5-(methanesulfonamido)-2-pyridyl]piperazine-1-carboxylate (21a). Compound **19** (0.47 mmol, 1 eq.) and methanesulfonyl chloride (1.5 eq.) were stirred at r.t. in 1 mL of anhydrous pyridine for 2 h. Water was added and the product was extracted with EtOAc. The combined organic layers were washed with brine, dried over $MgSO_4$ and evaporated under reduced pressure. The crude was purified by flash chromatography (cyclohexane/EtOAc 100:0 to 60:40) to give compound **21a**. Yield: quantitative; LC-MS (ESI) m/z $[M+H]^+$ = 391; 1H NMR (300 MHz, CD_2Cl_2), δ (ppm): 1.46 (s, 9H), 2.99 (s, 3H), 3.24–3.30 (m, 4H), 3.52–3.58 (m, 4H), 6.52 (s, 1H), 7.66 (d, J = 2.5 Hz, 1H), 8.07 (d, J = 2.5 Hz, 1H).

9.1.2.30. N-(5-chloro-6-piperazin-1-yl-3-pyridyl)methane sulfonamide; hydrochloride (21). Using **procedure B**, starting with compound **21a** (0.47 mmol) and 10 eq. HCl 4 M for 4 h to give **21** as a white powder. Yield: 22%; LC-MS (ESI) m/z $[M+H]^+$ = 291; 1H NMR (300 MHz, DMSO- d_6), δ (ppm): 3.03 (s, 3H), 3.13–3.23 (m, 4H), 3.38–3.47 (m, 4H), 7.70 (d, J = 2.4 Hz, 1H), 8.15 (d, J = 2.4 Hz, 1H), 9.51 (s, 2H), 10.04 (s, 1H); ^{13}C NMR (75 MHz, DMSO- d_6), δ (ppm): 39.6, 42.5, 45.8, 121.8, 130.9, 132.0, 138.8, 153.7; HRMS (TOF, ES+) m/z $[M+H]^+$: calcd. for $C_{10}H_{16}ClN_4O_2S$ 291.0683, found 291.0688.

9.1.2.31. tert-butyl 4-[3-chloro-5-(hydroxymethyl)-2-pyridyl]piperazine-1-carboxylate (22a). **Synthetic pathway 1:** In a tube, (5,6-dichloro-3-pyridyl)methanol (3.00 mmol, 1 eq.), tert-butyl piperazine-1-carboxylate (1.5 eq.), $Pd(OAc)_2$ (2 mol%), BINAP (3 mol%) and $tBuONa$ (1.4 eq.) were added in 3 mL dry toluene under argon. The mixture was heated at 110 °C for 8 h and then the solvent was evaporated under reduced pressure. The residue was purified by flash chromatography (cyclohexane/EtOAc 100:0 to 50:50) and by preparative HPLC (water + 0.1% HCOOH/MeCN + 0.1% HCOOH 80:20 to 0:100) to give compound **22a** as a white powder. Yield: 31%.

Synthetic pathway 2: A tube containing compound **15a** (0.70–1.50 mmol, 1 eq.) was purged under argon for 15 min. Then, 3–6 mL dry tetrahydrofuran were added and the reaction was cooled at 0 °C. $LiBH_4$ 2.0 M in THF (4 eq.) was added, the reaction was allowed to warm up at room temperature and was stirred for 3 days. The reaction mixture was quenched with EtOAc and washed twice with water. The aqueous layer was then extracted with EtOAc. The combined organic layers were washed with brine, dried over $MgSO_4$ and evaporated under reduced pressure. The crude was purified by flash chromatography (cyclohexane/EtOAc 100:0 to 50:50) to give compound **22a** as a white powder. Yield: 62%.

LC-MS (ESI) m/z $[M+H]^+$ = 328; 1H NMR (300 MHz, CD_2Cl_2), δ (ppm): 1.46 (s, 9H), 3.24–3.29 (m, 4H), 3.52–3.57 (m, 4H), 4.60 (s, 2H), 7.66 (d, J = 2.1 Hz, 1H), 8.13 (d, J = 2.1 Hz, 1H).

9.1.2.32. (5-chloro-6-piperazin-1-yl-3-pyridyl)methanol; hydrochloride (22). Using **procedure B**, starting with compound **22a** (0.18 mmol)

and 15 eq. HCl 4 M for 16 h to give **22** as a white powder. Yield: 70%; LC-MS (ESI) m/z $[M+H]^+$ = 228; 1H NMR (300 MHz, DMSO- d_6), δ (ppm): 3.17–3.20 (m, 4H), 3.45–3.48 (m, 4H), 4.45 (s, 2H), 7.80 (d, J = 2.0 Hz, 1H), 8.18 (d, J = 2.0 Hz, 1H), 9.61 (s, 2H); ^{13}C NMR (75 MHz, DMSO- d_6), δ (ppm): 42.5, 45.7, 59.5, 121.7, 134.0, 138.1, 144.1, 155.7; HRMS (TOF, ES+) m/z $[M+H]^+$: calcd. for $C_{10}H_{15}ClN_3O$ 228.0904, found 228.0904.

9.1.2.33. tert-butyl 4-(3-chloro-5-phenoxy-carbonyl-2-pyridyl)piperazine-1-carboxylate (23a). Using **procedure D**, starting with compound **17** (1.01 mmol, 1 eq.), phenol (1.5 eq.) and K_2CO_3 (2.5 eq.) for 16 h. A precipitate was formed and was recuperated by filtration, rinsed with water and part of the product was recrystallized in MeCN. The MeCN filtrate was evaporated, diluted with EtOAc, washed once with an aqueous solution of HCl 1 N, three times with a saturated solution of $NaHCO_3$, once with brine and dried over $MgSO_4$. The combined organic layers were evaporated under reduced pressure and the crude was purified by flash chromatography (cyclohexane/EtOAc 100:0 to 80:20) to give compound **23a** as a white solid. Yield: 78%; LC-MS (ESI) m/z $[M+H]^+$ = 418; 1H NMR (300 MHz, CD_2Cl_2), δ (ppm): 1.47 (s, 9H), 3.57 (s, 8H), 7.18–7.23 (m, 2H), 7.26–7.33 (m, 1H), 7.41–7.48 (m, 2H), 8.28 (d, J = 2.0 Hz, 1H), 8.88 (d, J = 2.0 Hz, 1H).

9.1.2.34. Phenyl 5-chloro-6-piperazin-1-yl-pyridine-3-carboxylate; 2,2,2-trifluoroacetic acid (23). In a flask containing compound **23a** (0.18 mmol, 1 eq.) in 5.4 mL of dry DCM, TFA (40 eq.) was added. The mixture was stirred at room temperature for 1.5 h. The reaction was evaporated under reduced pressure and the solid was rinsed twice with DCM and twice with diethyl ether to give compound **23** as a white solid. Yield: 95%; LC-MS (ESI) m/z $[M+H]^+$ = 318; 1H NMR (300 MHz, DMSO- d_6), δ (ppm): 3.28 (t, J = 4.8 Hz, 4H), 3.73 (t, J = 4.9 Hz, 4H), 7.26–7.36 (m, 3H), 7.45–7.52 (m, 2H), 8.36 (d, J = 2.0 Hz, 1H), 8.89 (d, J = 2.0 Hz, 1H), 8.97 (brs, 2H); ^{13}C NMR (75 MHz, DMSO- d_6), δ (ppm): 42.6, 45.2, 119.5, 119.6, 121.8, 126.2, 129.6, 140.1, 147.9, 150.3, 159.2, 162.5; HRMS (TOF, ES+) m/z $[M+H]^+$: calcd. for $C_{16}H_{17}ClN_3O_2$ 318.1009, found 318.1010.

9.1.2.35. tert-butyl 4-(5-benzyloxycarbonyl-3-chloro-2-pyridyl)piperazine-1-carboxylate (24a). Using **procedure D**, starting with compound **17** (0.50 mmol, 1 eq.), benzyl alcohol (1.5 eq.) and TEA (2 eq.) for 16 h. The crude was purified by flash chromatography (cyclohexane/EtOAc 100:0 to 90:10) and preparative HPLC (water + 0.1% HCOOH/MeCN + 0.1% HCOOH 80:20 to 0:100) to give compound **24a** as a colorless oil. Yield: 50%; LC-MS (ESI) m/z $[M+H]^+$ = 432; 1H NMR (300 MHz, CD_2Cl_2), δ (ppm): 1.46 (s, 9H), 3.46–3.57 (m, 8H), 7.33–7.47 (m, 5H), 8.14 (d, J = 2.0 Hz, 1H), 8.75 (d, J = 2.0 Hz, 1H).

9.1.2.36. benzyl 5-chloro-6-piperazin-1-yl-pyridine-3-carboxylate; hydrochloride (24). Using **procedure B**, starting with compound **24a** (0.25 mmol) and 15 eq. HCl 4 M for 5.5 h to give **24** as a white powder. Yield: 17%; LC-MS (ESI) m/z $[M+H]^+$ = 332; 1H NMR (300 MHz, DMSO- d_6), δ (ppm): 3.18–3.26 (m, 4H), 3.66–3.76 (m, 4H), 5.35 (s, 2H), 7.31–7.51 (m, 5H), 8.18 (d, J = 2.0 Hz, 1H), 8.75 (d, J = 2.0 Hz, 1H), 9.38 (brs, 2H); ^{13}C NMR (75 MHz, DMSO- d_6), δ (ppm): 42.5, 45.1, 66.4, 119.8, 120.1, 128.0, 128.2, 128.5, 135.9, 139.5, 147.3, 159.0, 163.6; HRMS (TOF, ES+) m/z $[M+H]^+$: calcd. for $C_{17}H_{19}ClN_3O_2$ 332.1166, found 332.1146.

9.1.2.37. 2-phenylethyl 5-chloro-6-piperazin-1-yl-pyridine-3-carboxylate; hydrochloride (25). Using **procedure C**, starting with tert-butyl 4-(3-chloro-5-isopropoxycarbonyloxycarbonyl-2-pyridyl)piperazine-1-carboxylate (0.5 mmol), 2-phenylethanol (1.2 eq.) and $tBuOK$ (1.6 eq.) for 4 h. The crude was purified by flash chromatography (cyclohexane/EtOAc 100:0 to 90:10). The obtained ester was deprotected using **procedure B** with 10 eq. HCl 4 M for 10 h to give compound **25** as a white

solid. Yield: 37%; LC-MS (ESI) m/z $[M+H]^+$ = 346; 1H NMR (300 MHz, DMSO- d_6), δ (ppm): 3.02 (t, J = 6.7 Hz, 2H), 3.19–3.22 (m, 4H), 3.69–3.72 (m, 4H), 4.47 (t, J = 6.6 Hz, 2H), 7.19–7.32 (m, 5H), 8.08 (d, J = 1.9 Hz, 1H), 8.64 (d, J = 1.9 Hz, 1H), 9.53 (brs, 2H); ^{13}C NMR (75 MHz, DMSO- d_6), δ (ppm): 34.3, 42.4, 45.0, 65.5, 119.7, 120.1, 126.4, 128.4, 128.9, 138.0, 139.4, 147.1, 158.9, 163.5; HRMS (TOF, ES+) m/z $[M+H]^+$: calcd. for $C_{18}H_{21}ClN_3O_2$ 346.1322, found 346.1328.

9.1.2.38. tert-butyl 4-[3-chloro-5-(3-phenylpropoxycarbonyl)-2-pyridyl]piperazine-1-carboxylate (26a). Using **procedure D**, starting with compound **17** (0.50 mmol, 1 eq.), 3-phenylpropan-1-ol (1.5 eq.) and TEA (2 eq.) for 2.5 h. The crude was purified by preparative HPLC (water + 0.1% HCOOH/MeCN + 0.1% HCOOH 80:20 to 0:100) to give compound **26a** as a colorless oil. Yield: 68%; LC-MS (ESI) m/z $[M+H]^+$ = 460; 1H NMR (300 MHz, CD_2Cl_2), δ (ppm): 1.46 (s, 9H), 2.02–2.13 (m, 2H), 2.77 (t, J = 7.5 Hz, 2H), 3.46–3.58 (m, 8H), 4.30 (t, J = 6.5 Hz, 2H), 7.14–7.33 (m, 5H), 8.09 (d, J = 2.0 Hz, 1H), 8.72 (d, J = 2.0 Hz, 1H).

9.1.2.39. 3-phenylpropyl 5-chloro-6-piperazin-1-yl-pyridine-3-carboxylate; hydrochloride (26). Using **procedure B**, starting with compound **26a** (0.35 mmol) and 10 eq. HCl 4 M for 4 h to give **26** as a colorless oil. Yield: 93%; LC-MS (ESI) m/z $[M+H]^+$ = 360; 1H NMR (300 MHz, DMSO- d_6), δ (ppm): 1.97–2.06 (m, 2H), 2.72 (t, J = 7.4 Hz, 2H), 3.18–3.24 (m, 4H), 3.71 (t, J = 4.8 Hz, 4H), 4.26 (t, J = 6.3 Hz, 2H), 7.13–7.31 (m, 5H), 8.09 (d, J = 2.0 Hz, 1H), 8.70 (d, J = 2.0 Hz, 1H), 9.54 (brs, 2H); ^{13}C NMR (75 MHz, DMSO- d_6), δ (ppm): 29.7, 31.6, 42.4, 45.1, 64.4, 119.8, 120.3, 125.9, 128.3 (2 ^{Ar}CH), 139.4, 141.2, 147.2, 159.0, 163.7; HRMS (TOF, ES+) m/z $[M+H]^+$: calcd. for $C_{19}H_{23}ClN_3O_2$ 360.1479, found 360.1471.

9.1.2.40. 2-aminoethyl 5-chloro-6-piperazin-1-yl-pyridine-3-carboxylate; dihydrochloride (27). Using **procedure C**, starting with tert-butyl 4-(3-chloro-5-isopropoxycarbonyloxycarbonyl-2-pyridyl)piperazine-1-carboxylate (0.37 mmol), tert-butyl *N*-(2-hydroxyethyl)carbamate (1.2 eq.) and $tBuOK$ (1.6 eq.) for 4.5 h. The crude was purified by flash chromatography (cyclohexane/EtOAc 100:0 to 70:30). The obtained ester was deprotected using **procedure B** with 10 eq. HCl 4 M for 9 h to give compound **25** as a colorless oil. Yield: 33%; LC-MS (ESI) m/z $[M+H]^+$ = 285; 1H NMR (300 MHz, DMSO- d_6), δ (ppm): 3.19–3.24 (m, 6H), 3.70–3.74 (m, 4H), 4.43–4.46 (m, 2H), 8.38 (brs, 3H), 8.44 (d, J = 2.0 Hz, 1H), 8.86 (d, J = 2.0 Hz, 1H), 9.57 (brs, 2H); ^{13}C NMR (75 MHz, DMSO- d_6), δ (ppm): 37.8, 42.4, 45.1, 61.7, 119.6, 119.8, 140.0, 147.9, 159.1, 163.5; HRMS (TOF, ES+) m/z $[M+H]^+$: calcd. for $C_{12}H_{18}ClN_4O_2$ 285.1118, found 285.1117.

9.1.2.41. tert-butyl 4-[5-[4-[2-(tert-butoxycarbonylamino)ethyl]phenoxy]carbonyl-3-chloro-2-pyridyl]piperazine-1-carboxylate (28a). Using **procedure D**, starting with compound **17** (0.50 mmol, 1 eq.), tert-butyl *N*-[2-(4-hydroxyphenyl)ethyl]carbamate (1.5 eq.) and K_2CO_3 (2.5 eq.) for 16 h. The crude was purified by flash chromatography (cyclohexane/EtOAc 100:0 to 70:30) to give compound **28a** as a colorless oil. Yield: 46%; LC-MS (ESI) m/z $[M+H]^+$ = 561; 1H NMR (300 MHz, CD_2Cl_2), δ (ppm): 1.42 (s, 9H), 1.47 (s, 9H), 2.81 (t, J = 7.0 Hz, 2H), 3.36 (q, J = 6.8 Hz, 2H), 3.57 (s, 8H), 4.64 (brs, 1H), 7.11–7.16 (m, 2H), 7.24–7.29 (m, 2H), 8.26 (d, J = 2.1 Hz, 1H), 8.87 (d, J = 2.0 Hz, 1H).

9.1.2.42. [4-(2-aminoethyl)phenyl] 5-chloro-6-piperazin-1-yl-pyridine-3-carboxylate; dihydrochloride (28). Using **procedure B**, starting with compound **28a** (0.21 mmol) and 10 eq. HCl 4 M for 16 h to give **28** as a white powder. Yield: 97%; LC-MS (ESI) m/z $[M+H]^+$ = 361; 1H NMR (300 MHz, DMSO- d_6), δ (ppm): 2.89–3.11 (m, 4H), 3.17–3.28 (m, 4H), 3.77 (t, J = 4.8 Hz, 4H), 7.25 (d, J = 8.5 Hz, 2H), 7.36 (d, J = 8.6 Hz, 2H), 8.13–8.20 (m, 3H), 8.32 (d, J = 2.1 Hz, 1H), 8.87 (d, J = 2.0 Hz, 1H), 9.59 (brs, 2H); ^{13}C NMR (75 MHz, DMSO- d_6), δ (ppm): 32.3, 40.3, 42.4, 45.0, 119.3, 119.6, 122.0, 129.8, 135.4, 140.0, 147.9, 149.1,

159.3, 162.6; HRMS (TOF, ES+) m/z $[M+H]^+$: calcd. for $C_{18}H_{22}ClN_4O_2$ 361.1431, found 361.1422.

9.1.2.43. *tert-butyl 4-[5-[3-[(tert-butoxycarbonylamino)methyl]phenoxy]carbonyl-3-chloro-2-pyridyl]piperazine-1-carboxylate (29a)*. Using **procedure D**, starting with compound **17** (1.00 mmol, 1 eq.), *tert-butyl N-[(3-hydroxyphenyl)methyl]carbamate* (1.6 eq.) and K_2CO_3 (2.5 eq.) for 20 h. The crude was purified by flash chromatography (cyclohexane/EtOAc 100:0 to 80:20) to give compound **29a** as a white solid. Yield: 71%; LC-MS (ESI) m/z $[M+H]^+$ = 547; 1H NMR (300 MHz, CD_2Cl_2), δ (ppm): 1.44 (s, 9H), 1.47 (s, 9H), 3.57 (s, 8H), 4.33 (d, J = 6.3 Hz, 2H), 5.02 (br s, 1H), 7.08–7.14 (m, 2H), 7.21 (d, J = 7.7 Hz, 1H), 7.40 (t, J = 7.9 Hz, 1H), 8.26 (d, J = 2.1 Hz, 1H), 8.87 (d, J = 2.0 Hz, 1H).

9.1.2.44. *[3-(aminomethyl)phenyl] 5-chloro-6-piperazin-1-yl-pyridine-3-carboxylate; dihydrochloride (29)*. Using **procedure B**, starting with compound **29a** (0.26 mmol) and 20 eq. HCl 4 M for 16 h to give **29** as a light yellow powder. Yield: 88%; LC-MS (ESI) m/z $[M+H]^+$ = 347; 1H NMR (300 MHz, DMSO- d_6), δ (ppm): 3.17–3.29 (m, 4H), 3.74–3.84 (m, 4H), 4.06 (q, J = 5.7 Hz, 2H), 7.31 (td, J = 1.8, 7.6 Hz, 1H), 7.43–7.56 (m, 3H), 8.33 (d, J = 2.0 Hz, 1H), 8.59 (brs, 3H), 8.89 (d, J = 2.0 Hz, 1H), 9.66 (brs, 2H); ^{13}C NMR (75 MHz, DMSO- d_6), δ (ppm): 41.7, 42.4, 45.0, 119.0, 119.6, 122.0, 122.3, 126.7, 129.8, 135.9, 140.0, 147.9, 150.3, 159.3, 162.5; HRMS (TOF, ES+) m/z $[M+H]^+$: calcd. for $C_{17}H_{20}ClN_4O_2$ 347.1275, found 347.1270.

9.1.2.45. *3-[[tert-butyl(dimethyl)silyl]oxymethyl]phenol (30a)*. To a stirred solution of 3-(hydroxymethyl)phenol (1.05 mmol, 1 eq.) and imidazole (1.3 eq.) in anhydrous DMF (0.8 mL) was added dropwise at 0 °C *tert-butyl-chloro-dimethyl-silane* (1.05 eq.) dissolved in 0.2 mL of DMF. The resulting mixture was stirred under an argon atmosphere at room temperature for 1 h. The mixture was quenched with water and extracted with EtOAc. The combined organic layers were washed with brine, dried over $MgSO_4$, and filtered. The organic solvent was removed under reduced pressure. The resulting residue was purified by flash chromatography (cyclohexane/EtOAc, 100:0 to 70:30) to give the desired product as a brown oil, which was directly used in the next step. Yield: 67%; LC-MS (ESI) m/z $[M+H]^+$ = 237.

9.1.2.46. *tert-butyl 4-[5-[3-[[tert-butyl(dimethyl)silyl]oxymethyl]phenoxy]carbonyl-3-chloro-2-pyridyl]piperazine-1-carboxylate (30b)*. Compound **17** (0.35 mmol, 1 eq.), HBTU (1.3 eq.), HOBT (0.3 eq.) and K_2CO_3 (1.5 eq.) were stirred in 1.5 mL DCM at room temperature for 10 min. In parallel, 3-[[*tert-butyl(dimethyl)silyl*]oxymethyl]phenol (1.5 eq.) was stirred at room temperature for 10 min with K_2CO_3 (1.5 eq.) in 1.5 mL of DCM. Then, the solution of 3-[[*tert-butyl(dimethyl)silyl*]oxymethyl]phenol and K_2CO_3 was added to the reaction mixture. It was allowed to stir at room temperature for 16 h. The reaction mixture was then quenched with water and extracted with DCM. The organic layer was washed twice with water, once with brine, dried over $MgSO_4$ and evaporated under reduced pressure. The crude was purified by flash chromatography (cyclohexane/EtOAc 100:0 to 95:5) to give compound **30b** as a colorless oil. Yield: 63%; LC-MS (ESI) m/z $[M+H]^+$ = 562; 1H NMR (300 MHz, CD_2Cl_2), δ (ppm): 0.12 (s, 6H), 0.94 (s, 9H), 1.47 (s, 9H), 3.57 (s, 8H), 4.77 (s, 2H), 7.07 (dd, J = 8.2, 2.2 Hz, 1H), 7.17 (s, 1H), 7.24 (d, J = 7.8 Hz, 1H), 7.39 (t, J = 7.9 Hz, 1H), 8.28 (d, J = 2.0 Hz, 1H), 8.88 (d, J = 2.0 Hz, 1H).

9.1.2.47. *tert-butyl 4-[3-chloro-5-[3-(hydroxymethyl)phenoxy]carbonyl-2-pyridyl]piperazine-1-carboxylate (30c)*. TBAF 1 M in THF (1.1 eq.) was added to a solution of compound **30b** (0.26 mmol, 1 eq.) in 1 mL anhydrous THF. The reaction was allowed to stir at room temperature for 2.25 h. The reaction was quenched with water and extracted with EtOAc. The organic layer was then washed twice with water, washed with brine, dried over $MgSO_4$ and evaporated under reduced pressure.

The crude was purified by flash chromatography (cyclohexane/EtOAc 100:0 to 70:30) to give compound **30c** as a colorless oil. Yield: 58%; LC-MS (ESI) m/z $[M+H]^+$ = 448; 1H NMR (300 MHz, CD_2Cl_2), δ (ppm): 1.46 (s, 9H), 3.47–3.52 (m, 4H), 3.53–3.57 (m, 4H), 5.28 (s, 2H), 5.58 (brs, 1H), 6.81 (ddd, J = 1.0, 2.5, 8.1 Hz, 1H), 6.91 (t, J = 2.2 Hz, 1H), 6.97 (d, J = 7.6 Hz, 1H), 7.25 (t, J = 7.9 Hz, 1H), 8.15 (d, J = 2.0 Hz, 1H), 8.76 (d, J = 2.1 Hz, 1H).

9.1.2.48. *[3-(hydroxymethyl)phenyl] 5-chloro-6-piperazin-1-yl-pyridine-3-carboxylate; hydrochloride (30)*. Using **procedure B**, starting with compound **30c** (0.15 mmol) and 20 eq. HCl 4 M for 24 h to give **30** as a white powder. Yield: 74%; LC-MS (ESI) m/z $[M+H]^+$ = 348; 1H NMR (300 MHz, DMSO- d_6), δ (ppm): 3.17–3.24 (m, 4H), 3.68–3.75 (m, 4H), 5.29 (s, 2H), 6.72–6.87 (m, 3H), 7.17 (t, J = 7.5 Hz, 1H), 8.16 (d, J = 2.0 Hz, 1H), 8.74 (d, J = 2.0 Hz, 1H), 9.45–9.62 (m, 3H); ^{13}C NMR (75 MHz, DMSO- d_6), δ (ppm): 42.9, 45.4, 66.6, 114.8, 115.4, 118.7, 120.2, 120.4, 130.0, 137.5, 139.8, 147.6, 157.7, 159.3, 163.9; HRMS (TOF, ES+) m/z $[M+H]^+$: calcd. for $C_{17}H_{19}ClN_3O_3$ 348.1115, found 348.1118.

9.1.2.49. *tert-butyl 4-[5-[[4-[2-(tert-butoxycarbonylamino)ethyl]phenyl]carbamoyl]-3-chloro-2-pyridyl]piperazine-1-carboxylate (31a)*. Using **procedure D**, starting with compound **17** (0.30 mmol, 1 eq.), *tert-butyl N-[2-(4-aminophenyl)ethyl]carbamate* (1.5 eq.) and TEA (2 eq.) for 1 h. The product precipitated, was filtered and rinsed with small amounts of cold EtOAc to give compound **31a** as a white solid. Yield: 70%; LC-MS (ESI) m/z $[M+H]^+$ = 560; 1H NMR (300 MHz, CD_2Cl_2), δ (ppm): 1.41 (s, 9H), 1.46 (s, 9H), 2.77 (t, J = 7.1 Hz, 2H), 3.33 (q, J = 6.6 Hz, 2H), 3.44–3.49 (m, 4H), 3.54–3.59 (m, 4H), 4.61 (br s, 1H), 7.20 (d, J = 8.5 Hz, 2H), 7.55 (d, J = 8.5 Hz, 2H), 7.81 (br s, 1H), 8.10 (d, J = 2.1 Hz, 1H), 8.62 (d, J = 2.2 Hz, 1H).

9.1.2.50. *N-[4-(2-aminoethyl)phenyl]-5-chloro-6-piperazin-1-yl-pyridine-3-carboxamide; dihydrochloride (31)*. Using **procedure B**, starting with compound **31a** (0.12 mmol) and 10 eq. HCl 4 M for 5 h to give **31** as a white powder. Yield: 99%; LC-MS (ESI) m/z $[M+H]^+$ = 360; 1H NMR (300 MHz, DMSO- d_6), δ (ppm): 2.83–3.05 (m, 4H), 3.18–3.25 (m, 4H), 3.66 (t, J = 4.5 Hz, 4H), 7.24 (d, J = 8.4 Hz, 2H), 7.75 (d, J = 8.5 Hz, 2H), 8.14–8.19 (m, 3H), 8.42 (d, J = 2.0 Hz, 1H), 8.84 (d, J = 2.1 Hz, 1H), 9.61 (brs, 2H), 10.47 (s, 1H); ^{13}C NMR (75 MHz, DMSO- d_6), δ (ppm): 32.4, 39.9, 42.4, 45.2, 120.1, 120.6, 125.2, 128.8, 132.8, 137.5, 138.5, 146.0, 158.1, 162.3; HRMS (TOF, ES+) m/z $[M+H]^+$: calcd. for $C_{18}H_{23}ClN_5O$ 360.1591, found 360.1592.

9.1.2.51. *tert-butyl 4-[5-[[4-[2-(tert-butoxycarbonylamino)ethyl]benzoyl]amino]-3-chloro-2-pyridyl]piperazine-1-carboxylate (32a)*. Using **procedure D**, starting with 4-[2-(*tert-butoxycarbonylamino*)ethyl]benzoic acid (1.5 eq.), compound **19** (0.50 mmol, 1 eq.) and TEA (3 eq.) for 48 h. The crude product was purified by reverse phase chromatography (MeOH/H₂O 10:90 to 100:0) to give compound **32a** as a light-yellow solid. Yield: 25%; LC-MS (ESI) m/z $[M+H]^+$ = 560; 1H NMR (300 MHz, CD_2Cl_2), δ (ppm): 1.41 (s, 9H), 1.46 (s, 9H), 2.86 (t, J = 6.9 Hz, 2H), 3.22–3.26 (m, 4H), 3.37 (q, J = 6.7 Hz, 2H), 3.53–3.58 (m, 4H), 4.61 (brs, 1H), 7.34 (d, J = 8.2 Hz, 2H), 7.81 (d, J = 8.2 Hz, 2H), 7.86 (brs, 1H), 8.23 (d, J = 2.5 Hz, 1H), 8.30 (d, J = 2.4 Hz, 1H).

9.1.2.52. *4-(2-aminoethyl)-N-(5-chloro-6-piperazin-1-yl-3-pyridyl)benzamide; dihydrochloride (32)*. Using **procedure B**, starting with compound **32a** (0.11 mmol) and 20 eq. HCl 4 M for 36 h to give **32** as a white powder. Yield: quantitative; LC-MS (ESI) m/z $[M+H]^+$ = 360; 1H NMR (300 MHz, DMSO- d_6), δ (ppm): 2.98–3.10 (m, 4H), 3.17–3.25 (m, 4H), 3.41–3.47 (m, 4H), 7.43 (d, J = 8.2 Hz, 2H), 8.01 (d, J = 8.2 Hz, 2H), 8.22–8.27 (m, 3H), 8.39 (d, J = 2.3 Hz, 1H), 8.70 (d, J = 2.2 Hz, 1H), 9.49 (brs, 2H), 10.68 (s, 1H); ^{13}C NMR (75 MHz, DMSO- d_6), δ (ppm): 32.8, 39.8, 42.6, 45.9, 121.3, 128.2, 128.8, 130.9, 132.27, 132.30, 138.0, 141.7, 152.7, 165.3; HRMS (TOF, ES+) m/z $[M+H]^+$: calcd. for

$C_{18}H_{23}ClN_5O$ 360.1591, found 360.1583.

9.1.2.53. *tert-butyl 4-[5-[[3-[(tert-butoxycarbonylamino)methyl]phenyl]carbamoyl]-3-chloro-2-pyridyl]piperazine-1-carboxylate (33a)*. Using **procedure D**, starting with compound **17** (0.43 mmol, 1 eq.), *tert-butyl N-[(3-aminophenyl)methyl]carbamate* (1.6 eq.) and TEA (2 eq.) for 16 h at 50 °C. The crude product was purified by flash chromatography (cyclohexane/EtOAc 100:0 to 60:40) to give compound **33a** as a light orange solid. Yield: 69%; LC-MS (ESI) m/z $[M+H]^+$ = 546; 1H NMR (300 MHz, CD_2Cl_2), δ (ppm): 1.44 (s, 9H), 1.46 (s, 9H), 3.44–3.51 (m, 4H), 3.53–3.60 (m, 4H), 4.29 (d, J = 6.2 Hz, 2H), 5.02 (brs, 1H), 7.08 (d, J = 7.7 Hz, 1H), 7.33 (t, J = 8.0 Hz, 1H), 7.50–7.58 (m, 2H), 7.84 (s, 1H), 8.10 (d, J = 2.2 Hz, 1H), 8.63 (d, J = 2.2 Hz, 1H).

9.1.2.54. *N-[3-(aminomethyl)phenyl]-5-chloro-6-piperazin-1-yl-pyridine-3-carboxamide; dihydrochloride (33)*. Using **procedure B**, starting with compound **33a** (0.29 mmol) and 30 eq. HCl 4 M for 30 h to give **33** as an off-white powder. Yield: 92%; LC-MS (ESI) m/z $[M+H]^+$ = 346; 1H NMR (300 MHz, DMSO- d_6), δ (ppm): 3.14–3.27 (m, 4H), 3.62–3.72 (m, 4H), 3.98 (q, J = 5.7 Hz, 2H), 7.29 (d, J = 7.7 Hz, 1H), 7.40 (t, J = 7.8 Hz, 1H), 7.74 (d, J = 8.4 Hz, 1H), 7.94 (s, 1H), 8.44 (d, J = 2.0 Hz, 1H), 8.55 (brs, 3H), 8.86 (d, J = 2.0 Hz, 1H), 9.70 (brs, 2H), 10.64 (s, 1H); ^{13}C NMR (75 MHz, DMSO- d_6), δ (ppm): 42.3, 42.4, 45.2, 120.1, 120.6, 121.1, 124.4, 125.0, 128.9, 134.5, 138.5, 139.1, 146.1, 158.2, 162.5; HRMS (TOF, ES+) m/z $[M+H]^+$: calcd. for $C_{17}H_{21}ClN_5O$ 346.1435, found 346.1440.

9.1.2.55. *tert-butyl N-[[3-[(5,6-dichloro-3-pyridyl)oxymethyl]phenyl]methyl]carbamate (34a)*. 5,6-dichloropyridin-3-ol (0.44 mmol, 1 eq.), *tert-butyl N-[(3-bromomethyl)phenyl]methyl]carbamate* (2 eq.) and K_2CO_3 (2.5 eq.) were heated at 80 °C in DMF (1.2 mL) for 1 h. The reaction was diluted with EtOAc and washed twice with water. The organic layer was then washed with brine, dried over $MgSO_4$ and evaporated under reduced pressure. The crude was purified by flash chromatography (cyclohexane/EtOAc 100:0 to 80:20) and by preparative HPLC (MeCN 0.1% formic acid/water 0.1% formic acid 10:90 to 100:0) to give compound **34a** as a colorless oil. Yield: 74%; LC-MS (ESI) m/z $[M+H]^+$ = 383; 1H NMR (300 MHz, CD_2Cl_2), δ (ppm): 1.44 (s, 9H), 4.31 (d, J = 6.2 Hz, 2H), 4.98 (brs, 1H), 5.09 (s, 2H), 7.26–7.40 (m, 4H), 7.43 (d, J = 2.8 Hz, 1H), 8.05 (d, J = 2.8 Hz, 1H).

9.1.2.56. *tert-butyl 4-[5-[[3-[(tert-butoxycarbonylamino)methyl]phenyl]methoxy]-3-chloro-2-pyridyl]piperazine-1-carboxylate (34b)*. In a tube, intermediate **34a** (0.12 mmol, 1 eq.), *tert-butyl piperazine-1-carboxylate* (1.8 eq.), $Pd(OAc)_2$ (4 mol%), BINAP (4 mol%), and *t*BuONa (1.7 eq.) were purged under argon for 15 min. Then, dry toluene (0.2 mL) was added and the mixture was heated at 110 °C overnight. The reaction mixture was filtered on a plug of celite and rinsed with EtOAc. It was concentrated under reduced pressure and the residue was purified by flash chromatography (cyclohexane/EtOAc 100:0 to 70:30) to give compound **34b** as a colorless oil. Yield: 67%; LC-MS (ESI) m/z $[M+H]^+$ = 533; 1H NMR (300 MHz, CD_2Cl_2), δ (ppm): 1.44 (s, 9H), 1.46 (s, 9H), 3.10–3.16 (m, 4H), 3.51–3.57 (m, 4H), 4.30 (d, J = 6.1 Hz, 2H), 5.00–5.08 (m, 3H), 7.24–7.39 (m, 5H), 7.95 (d, J = 2.7 Hz, 1H).

9.1.2.57. *[3-[(5-chloro-6-piperazin-1-yl-3-pyridyl)oxymethyl]phenyl]methanamine; dihydrochloride (34)*. Using **procedure B**, starting with compound **34b** (0.08 mmol) and 20 eq. HCl 4 M for 20 h to give **34** as an off-white powder. Yield: 67%; LC-MS (ESI) m/z $[M+H]^+$ = 333; 1H NMR (300 MHz, DMSO- d_6), δ (ppm): 3.13–3.22 (m, 4H), 3.31–3.37 (m, 4H), 3.98–4.06 (m, 2H), 5.16 (s, 2H), 7.42–7.55 (m, 3H), 7.59 (brs, 1H), 7.72 (d, J = 2.5 Hz, 1H), 8.10 (d, J = 2.5 Hz, 1H), 8.57 (brs, 3H), 9.55 (brs, 2H); ^{13}C NMR (75 MHz, DMSO- d_6), δ (ppm): 42.0, 42.6, 46.1, 70.2, 122.7, 126.0, 127.9, 128.4, 128.7, 128.8, 133.5, 134.5, 136.6, 151.2, 151.3; HRMS (TOF, ES+) m/z $[M+H]^+$: calcd. for $C_{17}H_{22}ClN_4O$

333.1482, found 333.1483.

9.1.2.58. *tert-butyl 4-[5-[[3-[(tert-butoxycarbonylamino)methyl]phenoxy]methyl]-3-chloro-2-pyridyl]piperazine-1-carboxylate (35a)*. Intermediate **22a** (0.27 mmol, 1 eq.) was dissolved in DCM (1 mL), cooled at 0 °C and methanesulfonyl chloride (2 eq.) was added. The reaction mixture was allowed to warm up at r.t. and was stirred for 2 h. The reaction was quenched with water and extracted with DCM. In parallel, *tert-butyl N-[(3-hydroxyphenyl)methyl]carbamate* (1.2 eq.) and cesium carbonate (1.3 eq.) were heated at 70 °C in dry DMF (1.3 mL). After 10 min, a solution of the previous intermediate in dry DMF (1 mL) was added and the reaction was heated for 4 h. The DMF was evaporated under reduced pressure, the residue dissolved in EtOAc and washed twice with an aqueous solution of HCl 1 N. The organic layer was then washed with brine, dried over $MgSO_4$ and evaporated under reduced pressure. The crude was purified by flash chromatography (cyclohexane/EtOAc 100:0 to 70:30) to give **35a** as a light-yellow solid. Yield: 49%; LC-MS (ESI) m/z $[M+H]^+$ = 533; 1H NMR (300 MHz, CD_2Cl_2), δ (ppm): 1.44 (s, 9H), 1.46 (s, 9H), 3.27–3.32 (m, 4H), 3.52–3.59 (m, 4H), 4.26 (d, J = 6.1 Hz, 2H), 4.97 (s, 2H), 6.82–6.92 (m, 3H), 7.26 (t, J = 8.0 Hz, 1H), 7.72 (d, J = 2.1 Hz, 1H), 8.22 (d, J = 2.1 Hz, 1H).

9.1.2.59. *[3-[(5-chloro-6-piperazin-1-yl-3-pyridyl)methoxy]phenyl]methanamine; dihydrochloride (35)*. Using **procedure B**, starting with compound **35a** (0.14 mmol) and 20 eq. HCl 4 M for 24 h to give **35** as a white powder. Yield: 79%; LC-MS (ESI) m/z $[M+H]^+$ = 333; 1H NMR (300 MHz, DMSO- d_6), δ (ppm): 3.17–3.25 (m, 4H), 3.47–3.54 (m, 4H), 3.98 (q, J = 5.8 Hz, 2H), 5.10 (s, 2H), 7.00–7.10 (m, 2H), 7.24 (s, 1H), 7.34 (t, J = 7.9 Hz, 1H), 7.97 (d, J = 1.9 Hz, 1H), 8.37 (d, J = 2.0 Hz, 1H), 8.48 (brs, 3H), 9.41 (brs, 2H); ^{13}C NMR (75 MHz, DMSO- d_6), δ (ppm): 42.0, 42.5, 45.6, 65.8, 114.6, 115.5, 121.4, 121.5, 128.2, 129.8, 135.7, 139.2, 145.7, 156.6, 158.1; HRMS (TOF, ES+) m/z $[M+H]^+$: calcd. for $C_{17}H_{22}ClN_4O$ 333.1482, found 333.1469.

9.1.2.60. *tert-butyl 4-[3-chloro-5-(3-methyl-1,2,4-oxadiazol-5-yl)-2-pyridyl]piperazine-1-carboxylate (36a)*. To a mixture of intermediate **17** (0.29 mmol, 1 eq.), *N*-hydroxyacetamide (1 eq.) and TEA (5 eq.) in 150 μ L EtOAc, was added dropwise T3P 50% in EtOAc (2.5 eq.). The reaction was heated at 80 °C for 16 h. The reaction was diluted with EtOAc, washed twice with water and twice with a saturated solution of $NaHCO_3$. The organic layer was then washed with brine, dried over $MgSO_4$ and evaporated under reduced pressure. The crude was purified by flash chromatography (cyclohexane/EtOAc 100:0 to 80:20) to give compound **36a** as a white solid. Yield: 76%; LC-MS (ESI) m/z $[M+H]^+$ = 380; 1H NMR (300 MHz, CD_2Cl_2), δ (ppm): 1.46 (s, 9H), 2.42 (s, 3H), 3.50–3.60 (m, 8H), 8.23 (d, J = 2.1 Hz, 1H), 8.82 (d, J = 2.0 Hz, 1H).

9.1.2.61. *5-(5-chloro-6-piperazin-1-yl-3-pyridyl)-3-methyl-1,2,4-oxadiazole; hydrochloride (36)*. Using **procedure B**, starting with compound **36a** (0.22 mmol) and 10 eq. HCl 4 M for 16 h to give **36** as a white powder. Yield: 75%; LC-MS (ESI) m/z $[M+H]^+$ = 280; 1H NMR (300 MHz, DMSO- d_6), δ (ppm): 2.41 (s, 3H), 3.22–3.27 (m, 4H), 3.69–3.75 (m, 4H), 8.37 (d, J = 2.1 Hz, 1H), 8.88 (d, J = 2.1 Hz, 1H), 9.18 (brs, 2H); ^{13}C NMR (75 MHz, DMSO- d_6), δ (ppm): 11.2, 42.6, 45.2, 114.5, 120.4, 137.9, 145.5, 158.6, 167.6, 172.2; HRMS (TOF, ES+) m/z $[M+H]^+$: calcd. for $C_{12}H_{15}ClN_5O$ 280.0965, found 280.0967.

9.1.2.62. *tert-butyl 4-[3-chloro-5-(5-methyl-1,3,4-oxadiazol-2-yl)-2-pyridyl]piperazine-1-carboxylate (37a)*. Intermediate **17** (0.14 mmol, 1 eq.), acetohydrazide (1.4 eq.), TEA (5 eq.) and T3P 50% in EtOAc (3.5 eq.) were put into reaction in 500 μ L EtOAc at 80 °C. The reaction was stopped after 48 h in total. It was diluted with EtOAc, washed twice with water and twice with $NaHCO_3$ sat. The organic layer was then washed with brine, dried over $MgSO_4$ and evaporated under reduced pressure. The crude product was purified by flash chromatography (cyclohexane/

EtOAc 100:0 to 60:40) to give compound **37a** as a white solid. Yield: 43%; LC-MS (ESI) m/z $[M+H]^+$ = 380; 1H NMR (300 MHz, CD_2Cl_2), δ (ppm): 1.46 (s, 9H), 2.57 (s, 3H), 3.44–3.50 (m, 4H), 3.53–3.59 (m, 4H), 8.18 (d, J = 2.1 Hz, 1H), 8.73 (d, J = 2.1 Hz, 1H).

9.1.2.63. *2-(5-chloro-6-piperazin-1-yl-3-pyridyl)-5-methyl-1,3,4-oxadiazole (37)*. Using **procedure B**, starting with compound **37a** (0.06 mmol) and 30 eq. HCl 4 M for 40 h. The residue was purified by reverse phase chromatography (MeOH/ H_2O 10:90 to 100:0) to give **37** as a white powder. Yield: 62%; LC-MS (ESI) m/z $[M+H]^+$ = 280; 1H NMR (300 MHz, DMSO- d_6), δ (ppm): 2.58 (s, 3H), 3.10–3.16 (m, 4H), 3.56–3.62 (m, 4H), 8.25 (d, J = 2.0 Hz, 1H), 8.75 (d, J = 2.1 Hz, 1H); ^{13}C NMR (75 MHz, DMSO- d_6), δ (ppm): 10.6, 43.3, 46.4, 114.5, 120.7, 136.6, 143.9, 158.3, 161.3, 164.0; HRMS (TOF, ES+) m/z $[M+H]^+$: calcd. for $C_{12}H_{15}ClN_5O$ 280.0965, found 280.0964.

9.1.2.64. *2,2,2-trichloroethyl 4-(5-acetyl-3-chloro-2-pyridyl)piperazine-1-carboxylate (38a)*. A mixture of 1-(5,6-dichloro-3-pyridyl)ethanone (1.02 mmol, 1 eq.), 2,2,2-trichloroethyl piperazine-1-carboxylate; hydrochloride (1.5 eq.), TEA (3 eq.) in 4.5 mL of MeCN was stirred for 6 h at 80 °C. The mixture was diluted with EtOAc, washed with an aqueous solution of HCl 1 N and with brine. The organic layer was then dried over $MgSO_4$ and evaporated under reduced pressure. The crude was purified by flash chromatography (cyclohexane/EtOAc 100:0 to 80:20) to give compound **38a** as a white solid. Yield: 79%; LC-MS (ESI) m/z $[M+H]^+$ = 416; 1H NMR (300 MHz, CD_2Cl_2), δ (ppm): 2.52 (s, 3H), 3.55–3.61 (m, 4H), 3.64–3.78 (m, 4H), 4.80 (s, 2H), 8.12 (d, J = 2.1 Hz, 1H), 8.70 (d, J = 2.1 Hz, 1H).

9.1.2.65. *2,2,2-trichloroethyl 4-[5-(2-bromoacetyl)-3-chloro-2-pyridyl]piperazine-1-carboxylate (38)*. Compound **38a** (0.47 mmol, 1 eq.) and phenyltrimethylammonium tribromide (1 eq.) were heated at 50 °C in 2.5 mL of a mixture THF/MeOH 4:1 for 5 h. The solvent was evaporated under reduced pressure, the reaction mixture was dissolved in 2 mL of THF and the reaction was stirred at room temperature with aqueous HCl 0.1 M (4 eq.) for 3.5 h. The reaction was diluted with EtOAc and washed with water. The organic layer was washed with brine, dried over $MgSO_4$ and evaporated under reduced pressure. The product was purified by flash chromatography (cyclohexane/EtOAc 100:0 to 80:20) to give compound **38** as a light-yellow solid. Yield: 68%; LC-MS (ESI) m/z $[M+H]^+$ = 494; 1H NMR (300 MHz, CD_2Cl_2), δ (ppm): 3.63–3.77 (m, 8H), 4.38 (s, 2H), 4.80 (s, 2H), 8.14 (d, J = 2.1 Hz, 1H), 8.72 (d, J = 2.1 Hz, 1H).

9.1.2.66. *2,2,2-trichloroethyl 4-[3-chloro-5-(2-methylthiazol-4-yl)-2-pyridyl]piperazine-1-carboxylate (39a)*. Compound **38** (0.22 mmol, 1 eq.) and thioacetamide (1.3 eq.) were heated at reflux in 1.5 mL of absolute ethanol for 3 h. The reaction was diluted with EtOAc and washed twice with water. The organic layer was then washed with brine, dried over $MgSO_4$ and evaporated under reduced pressure. The crude was purified by flash chromatography (cyclohexane/EtOAc 100:0 to 80:20) to give compound **39a** as a colorless oil. Yield: 77%; LC-MS (ESI) m/z $[M+H]^+$ = 471; 1H NMR (300 MHz, CD_2Cl_2), δ (ppm): 2.74 (s, 3H), 3.38–3.42 (m, 4H), 3.65–3.79 (m, 4H), 4.80 (s, 2H), 7.34 (s, 1H), 8.14 (d, J = 2.1 Hz, 1H), 8.66 (d, J = 2.1 Hz, 1H).

9.1.2.67. *4-(5-chloro-6-piperazin-1-yl-3-pyridyl)-2-methyl-thiazole (39)*. Zinc dust (10 eq.) and acetic acid (10 eq.) were added to a solution of compound **39a** (0.20 mmol, 1 eq.) in 1.8 mL THF. The reaction was allowed to stir at room temperature for 20 h. Petroleum ether was added to make the product precipitate. The solid was recuperated by filtration. It was solubilized in EtOAc and washed with a saturated solution of $NaHCO_3$. The organic layer was then washed with brine, dried over $MgSO_4$ and evaporated under reduced pressure to give compound **39** as an off-white solid. Yield: 53%; LC-MS (ESI) m/z $[M+H]^+$ = 295; 1H

NMR (300 MHz, DMSO- d_6), δ (ppm): 2.71 (s, 3H), 2.82–2.87 (m, 4H), 3.20–3.25 (m, 4H), 7.99 (s, 1H), 8.24 (d, J = 2.1 Hz, 1H), 8.75 (d, J = 2.1 Hz, 1H); ^{13}C NMR (75 MHz, DMSO- d_6), δ (ppm): 18.9, 45.4, 50.0, 114.0, 121.3, 125.0, 136.0, 143.3, 149.8, 157.2, 166.2; HRMS (TOF, ES+) m/z $[M+H]^+$: calcd. for $C_{13}H_{16}ClN_4S$ 295.0784, found 295.0807.

9.1.2.68. *2,2,2-trichloroethyl 4-[3-chloro-5-(2-methyloxazol-4-yl)-2-pyridyl]piperazine-1-carboxylate (40a)*. A mixture of compound **38** (0.26 mmol, 1 eq.), acetamide (1.4 eq.) and silver triflate (2.1 eq.) in 1 mL of EtOAc was protected from light and heated at 70 °C for 20 h. The mixture was then cooled to room temperature and diluted with 1 mL EtOAc. 1 mL of brine was added and the mixture was stirred at room temperature overnight. The silver salts were removed by filtration and the resulting biphasic solution was transferred to a separatory funnel and the layers separated. The organic layer was then washed with water, $NaHCO_3$ sat., HCl 1 N and water. Finally, it was washed with brine, dried over $MgSO_4$ and evaporated under reduced pressure. The crude was purified by flash chromatography twice (cyclohexane/EtOAc 100:0 to 80:20 and DCM/EtOAc 100:0 to 80:20) to give compound **40a** as a yellow solid. Yield: 34%; LC-MS (ESI) m/z $[M+H]^+$ = 455; 1H NMR (300 MHz, CD_2Cl_2), δ (ppm): 2.48 (s, 3H), 3.34–3.42 (m, 4H), 3.63–3.81 (m, 4H), 4.79 (s, 2H), 7.83 (s, 1H), 7.96 (d, J = 2.1 Hz, 1H), 8.49 (d, J = 2.1 Hz, 1H).

9.1.2.69. *4-(5-chloro-6-piperazin-1-yl-3-pyridyl)-2-methyl-oxazole; hydrochloride (40)*. Zinc dust (20 eq.) and acetic acid (20 eq.) were added to a solution of compound **40a** (0.09 mmol, 1 eq.) in 0.8 mL THF. The reaction was allowed to stir at room temperature for 36 h. Petroleum ether was added to make the product precipitate. The solid was recuperated by filtration. It was solubilized in EtOAc and washed with a saturated solution of K_2CO_3 . The aqueous layer was then extracted with EtOAc twice. The combined organic layers were washed with brine, dried over $MgSO_4$ and evaporated under reduced pressure. 200 μ L of HCl 4 M in 1,4-dioxane were added to the residue. The solid was recuperated by filtration to give compound **40** as a white solid. Yield: 29%; LC-MS (ESI) m/z $[M+H]^+$ = 279; 1H NMR (300 MHz, DMSO- d_6), δ (ppm): 2.46 (s, 3H), 3.16–3.26 (m, 4H), 3.49–3.56 (m, 4H), 8.15 (d, J = 2.0 Hz, 1H), 8.55 (s, 1H), 8.61 (d, J = 2.0 Hz, 1H), 9.47 (s, 2H); ^{13}C NMR (75 MHz, DMSO- d_6), δ (ppm): 13.5, 42.5, 45.6, 121.9, 123.0, 135.38, 135.41, 135.8, 142.4, 155.9, 161.9; HRMS (TOF, ES+) m/z $[M+H]^+$: calcd. for $C_{13}H_{16}ClN_4O$ 279.1013, found 279.0995.

9.1.2.70. *tert-butyl 4-(3-chloro-5-cyano-2-pyridyl)piperazine-1-carboxylate (41a)*. Using **procedure A**, starting with 5,6-dichloropyridine-3-carbonitrile (3.54 mmol) and *tert*-butyl piperazine-1-carboxylate (1.5 eq.) in 6 mL MeCN for 1 h at 50 °C. The mixture was diluted with EtOAc, washed with HCl 1 N and with brine. The organic layer was then dried over $MgSO_4$ and evaporated under reduced pressure to give compound **41a** as a light-yellow powder. Yield: 98%; LC-MS (ESI) m/z $[M+H]^+$ = 323; 1H NMR (300 MHz, CD_2Cl_2), δ (ppm): 1.46 (s, 9H), 3.54 (s, 8H), 7.77 (d, J = 2.0 Hz, 1H), 8.39 (d, J = 1.9 Hz, 1H).

9.1.2.71. *tert-butyl 4-[3-chloro-5-[(Z)-N'-hydroxycarbamimidoyl]-2-pyridyl]piperazine-1-carboxylate (41)*. Intermediate **41a** (3.47 mmol, 1 eq.), hydroxylamine hydrochloride (1.5 eq.) and TEA (1.6 eq.) were heated at reflux in 8 mL of ethanol absolute for 1 h. The solvent was evaporated under reduced pressure and the residue dissolved in EtOAc. It was washed with water twice. The organic layer was then washed with brine, dried over $MgSO_4$ and evaporated under reduced pressure to give **41** as a white solid. Yield: 95%; LC-MS (ESI) m/z $[M+H]^+$ = 356; 1H NMR (300 MHz, CD_2Cl_2), δ (ppm): 1.46 (s, 9H), 3.32–3.38 (m, 4H), 3.53–3.57 (m, 4H), 4.81 (brs, 2H), 6.75 (brs, 1H), 7.86 (d, J = 2.1 Hz, 1H), 8.38 (d, J = 2.1 Hz, 1H).

9.1.2.72. *tert-butyl 4-[3-chloro-5-(5-methyl-1,2,4-oxadiazol-3-yl)-2-pyridyl]piperazine-1-carboxylate (42a)*. Using **procedure E**, starting with 0.30 mmol of intermediate **41** and acetic acid for 24 h. The crude was purified by flash chromatography (cyclohexane/EtOAc 100:0 to 70:30) to give **42a** as a white solid. Yield: 33%; LC-MS (ESI) m/z $[M+H]^+$ = 380; 1H NMR (300 MHz, CD_2Cl_2), δ (ppm): 1.46 (s, 9H), 2.63 (s, 3H), 3.41–3.46 (m, 4H), 3.54–3.59 (m, 4H), 8.22 (d, J = 2.0 Hz, 1H), 8.78 (d, J = 2.0 Hz, 1H).

9.1.2.73. *(3-(5-chloro-6-piperazin-1-yl-3-pyridyl)-5-methyl-1,2,4-oxadiazole; hydrochloride) (42)*. Using **procedure B**, starting with compound **42a** (0.10 mmol) and 10 eq. HCl 4 M for 16 h. The residue was purified by reverse phase chromatography (MeOH/ H_2O 10:90 to 100:0) to give **42** as a white powder. Yield: 66%; LC-MS (ESI) m/z $[M+H]^+$ = 280; 1H NMR (300 MHz, DMSO- d_6), δ (ppm): 2.68 (s, 3H), 3.22–3.27 (m, 4H), 3.62–3.67 (m, 4H), 8.26 (d, J = 2.1 Hz, 1H), 8.79 (d, J = 2.0 Hz, 1H), 9.15 (brs, 2H); ^{13}C NMR (75 MHz, DMSO- d_6), δ (ppm): 12.0, 42.6, 45.3, 117.5, 121.1, 137.1, 144.4, 158.1, 164.9, 177.8; HRMS (TOF, ES+) m/z $[M+H]^+$: calcd. for $C_{12}H_{15}ClN_5O$ 280.0965, found 280.0964.

9.1.2.74. *tert-butyl 4-[5-[5-[(tert-butoxycarbonylamino)methyl]-1,2,4-oxadiazol-3-yl]-3-chloro-2-pyridyl]piperazine-1-carboxylate (43a)*. Using **procedure E**, starting with 0.50 mmol of intermediate **41** and Bocglycine for 24 h. The crude was purified by flash chromatography (cyclohexane/EtOAc 100:0 to 70:30) to give **43a** as a white solid. Yield: 27%; LC-MS (ESI) m/z $[M+H]^+$ = 495; 1H NMR (300 MHz, CD_2Cl_2), δ (ppm): 1.46 (s, 18H), 3.43–3.48 (m, 4H), 3.54–3.59 (m, 4H), 4.59 (d, J = 6.0 Hz, 2H), 5.27 (brs, 1H), 8.22 (d, J = 2.0 Hz, 1H), 8.79 (d, J = 2.0 Hz, 1H).

9.1.2.75. *([3-(5-chloro-6-piperazin-1-yl-3-pyridyl)-1,2,4-oxadiazol-5-yl]methanamine; dihydrochloride) (43)*. Using **procedure B**, starting with compound **43a** (0.14 mmol) and 10 eq. HCl 4 M for 16 h. The residue was purified by reverse phase chromatography (MeOH/ H_2O 10:90 to 100:0) to give **43** as an off-white powder. Yield: 46%; LC-MS (ESI) m/z $[M+H]^+$ = 295; 1H NMR (300 MHz, DMSO- d_6), δ (ppm): 3.19–3.26 (m, 4H), 3.67–3.71 (m, 4H), 4.57 (brs, 2H), 8.28 (d, J = 2.0 Hz, 1H), 8.81 (d, J = 2.0 Hz, 1H), 9.10 (brs, 3H), 9.59 (brs, 2H); ^{13}C NMR (75 MHz, DMSO- d_6), δ (ppm): 34.6, 42.5, 45.2, 116.6, 121.1, 137.2, 144.6, 158.5, 165.0, 174.5; HRMS (TOF, ES+) m/z $[M+H]^+$: calcd. for $C_{12}H_{16}ClN_6O$ 295.1074, found 295.1074.

9.1.2.76. *tert-butyl 4-[5-[5-[2-(tert-butoxycarbonylamino)ethyl]-1,2,4-oxadiazol-3-yl]-3-chloro-2-pyridyl]piperazine-1-carboxylate (44a)*. Using **procedure E**, starting with 0.50 mmol of intermediate **41** and 3-(tert-butoxycarbonylamino)propanoic acid for 16 h. The crude was purified by flash chromatography (cyclohexane/EtOAc 100:0 to 70:30) to give **44a** as a white solid. Yield: 66%; LC-MS (ESI) m/z $[M+H]^+$ = 509; 1H NMR (300 MHz, CD_2Cl_2), δ (ppm): 1.41 (s, 9H), 1.46 (s, 9H), 3.12 (t, J = 6.3 Hz, 2H), 3.42–3.48 (m, 4H), 3.54–3.65 (m, 6H), 5.07 (brs, 1H), 8.23 (d, J = 2.1 Hz, 1H), 8.81 (d, J = 2.1 Hz, 1H).

9.1.2.77. *(2-[3-(5-chloro-6-piperazin-1-yl-3-pyridyl)-1,2,4-oxadiazol-5-yl]ethanamine; dihydrochloride) (44)*. Using **procedure B**, starting with compound **44a** (0.33 mmol) and 10 eq. HCl 4 M for 16 h to give **44** as a white solid. Yield: 92%; LC-MS (ESI) m/z $[M+H]^+$ = 309; 1H NMR (300 MHz, DMSO- d_6), δ (ppm): 3.18–3.26 (m, 4H), 3.28–3.45 (m, 4H), 3.65–3.70 (m, 4H), 8.32 (d, J = 2.0 Hz, 1H), 8.40 (brs, 3H), 8.83 (d, J = 2.0 Hz, 1H), 9.59 (brs, 2H); ^{13}C NMR (75 MHz, DMSO- d_6), δ (ppm): 24.3, 35.6, 42.4, 45.2, 117.2, 121.0, 137.3, 144.6, 158.2, 164.8, 177.3; HRMS (TOF, ES+) m/z $[M+H]^+$: calcd. for $C_{13}H_{18}ClN_6O$ 309.1231, found 309.1231.

9.1.2.78. *tert-butyl 4-[5-[5-[3-(tert-butoxycarbonylamino)propyl]-1,2,4-oxadiazol-3-yl]-3-chloro-2-pyridyl]piperazine-1-carboxylate (45a)*. Using

procedure E, starting with 0.50 mmol of intermediate **41** and 4-(tert-butoxycarbonylamino)butanoic acid for 16 h. The crude was purified by flash chromatography (cyclohexane/EtOAc 100:0 to 70:30) and by reverse phase chromatography (water/MeOH 90:10 to 0:100) to give **45a** as a white solid. Yield: 41%; LC-MS (ESI) m/z $[M+H]^+$ = 523; 1H NMR (300 MHz, CD_2Cl_2), δ (ppm): 1.41 (s, 9H), 1.46 (s, 9H), 2.04 (p, J = 7.2 Hz, 2H), 2.98 (t, J = 7.5 Hz, 2H), 3.24 (q, J = 6.6 Hz, 2H), 3.42–3.46 (m, 4H), 3.54–3.59 (m, 4H), 4.77 (brs, 1H), 8.22 (d, J = 2.0 Hz, 1H), 8.80 (d, J = 2.0 Hz, 1H).

9.1.2.79. *(3-[3-(5-chloro-6-piperazin-1-yl-3-pyridyl)-1,2,4-oxadiazol-5-yl]propan-1-amine; dihydrochloride) (45)*. Using **procedure B**, starting with compound **45a** (0.21 mmol) and 10 eq. HCl 4 M for 16 h to give **45** as a white solid. Yield: quantitative; LC-MS (ESI) m/z $[M+H]^+$ = 323; 1H NMR (300 MHz, DMSO- d_6), δ (ppm): 2.11 (p, J = 7.5 Hz, 2H), 2.92–2.98 (m, 2H), 3.12–3.27 (m, 6H), 3.63–3.71 (m, 4H), 8.15 (brs, 3H), 8.26 (d, J = 2.0 Hz, 1H), 8.79 (d, J = 2.0 Hz, 1H), 9.48 (brs, 2H); ^{13}C NMR (75 MHz, DMSO- d_6), δ (ppm): 23.0, 23.7, 37.9, 42.5, 45.2, 117.4, 121.0, 137.2, 144.5, 158.2, 164.8, 179.8; HRMS (TOF, ES+) m/z $[M+H]^+$: calcd. for $C_{14}H_{20}ClN_6O$ 323.1387, found 323.1388.

9.2. Biology

9.2.1. *E. coli* strains construction

9.2.1.1. *Strains and media*. *E. coli* BW25113, its unmarked Δ acrB derivative and the *acrB* point mutants producing AcrB-Ala446Pro or AcrB-Ser450Pro have been described in Plé et al. 2022 [21]. Bacterial strains were cultured on LB broth (BD, DIFCO) at 30 or 37 °C. Bacterial antibiotic susceptibility tests were performed in cation-adjusted Mueller-Hinton broth (CAMHB; BD Difco) at 37 °C.

9.2.1.2. *acrB site directed mutagenesis*. The *acrB* wild-type gene was PCR amplified from the BW25113 chromosome with primer RH782 (ATCACCTACGCGCTATCTT) and RH783 (CGCAGCAGGTAAGCA GTT) and cloned into pCR-BluntII-TOPO to obtain pEP1537. This plasmid was submitted to site directed mutagenesis with primers RH1271 (ACGGATCCTTATCGTCCAATTCGCCAAAGACTTG) and RH1272 (CAAGTCTTTGGCGAATTGGACGATAAGGATCGCGT) to generate pEP1541 encoding AcrB-Glu947Gln; with primers RH1273 (CCTTATCGTCCAATTCGCCAAAACCTTGATGGATAAAGAAGG) and RH1274 (CCTTCTTTATCCATCAAGTTTTGGCGAATTGGACGATAAGG) to generate pEP1542 encoding AcrB-Asp951Asn. Both plasmids were able to complement the BW25113 Δ acrB strain for SDS resistance indicating they encode functional mutant AcrB proteins.

9.2.1.3. *acrB chromosomal mutants construction*. Each *acrB* allele was introduced into the BW25113 chromosome by Red-recombination as described in Plé et al. 2022 [21]. Briefly, arabinose induced BW25113 Δ acrB (pEP1436) electrocompetent cells were prepared at 30 °C and transformed with the *acrB* allele PCR products obtained using primers RH782 and RH783 on templates pEP1541 or pEP1542. Recombinants with a functional AcrB were selected by plating on LB agar containing 0.1% SDS and at 37 °C (to ensure pEP1436 loss). The correct insertion of each *acrB* allele was confirmed by sequencing of the *acrB* locus. The resulting strains were named BW25113 AcrB-Glu947Gln and BW25113 AcrB-Asp951Asn.

9.2.2. Minimum inhibitory concentration and EC_{90} determination

Antibiotics panel MICs experiments were carried out according to the CLSI guidelines [39]. Overnight cultures were diluted to an OD_{600} = 0.001 in Cation Adjusted Muller-Hinton Broth (CAMHB, Difco) followed by addition of efflux pump inhibitors (EPIs) at a final concentration of 100 μ M. Afterwards, 50 μ L of bacterial suspension were transferred to a 384-well flat-bottomed microtitre plate (Greiner-BIO, France) where a

dose response of the antibiotics of interest were added by serial dilutions (range of 0–128 mg L⁻¹) using a D300e-Tecan dispenser (Tecan, France). Plates were incubated at 37 °C for up to 20 h. Bacterial viability was evaluated using the resazurin microtitre assay (REMA) and measured by fluorescence (POLARstar Omega, BMG Labtech: Ex: 530 nm Em: 590 nm). MICs were defined as the concentration that prevented 90% of resazurin turnover compared to the non-treated bacteria. The MIC values are the average of four biological experiments with two technical repeats each.

EC₉₀ was defined as the compound concentration that allowed a sub-inhibitory dose of pyridomycin (8 µg/mL) to prevent 90% of resazurin turnover compared to the non-treated bacteria. The EC₉₀ values are the average of at least two independent experiments. All compounds were also tested in the absence of pyridomycin to determine their potential intrinsic antibacterial activity. Bacterial suspensions, incubation and plate readouts were carried out as described above.

9.3. Physico-chemical and pharmacokinetic properties

These experiments were performed using a UPLC Acquity I-class coupled with a Xevo TQD mass spectrometer (Waters) under MRM detection. Source parameters were set as follows: capillary 0.5 kV, desolvation temperature 600 °C, source temperature 150 °C, cone gas flow 50 L/h, desolvation gas flow 1200 L/h. Transitions monitored were as follows: compound **2**, 323.94–153.94 (polarity ES⁺, cone voltage 38 V, collision energy 34 eV); compound **29**, 347.09–154.88 (polarity ES⁺, cone voltage 30 V, collision energy 22 eV); compound **44**, 309.08–248.98 (polarity ES⁺, cone voltage 40 V, collision energy 26 eV).

9.3.1. Solubility

5 µL of a 10 mM of compound solution in DMSO was diluted either in 245 µL of PBS pH 7.4 (triplicate) or in 245 µL of MeOH or MeCN (triplicate twice). After gently stirring 24 h at room temperature, the solutions were centrifuged for 5 min at 4000 rpm and filtered over 0.45 µm filters (Millex-LH Millipore), excepted one of the MeOH or MeCN triplicate. Then, 10 µL of each solution were diluted in 490 µL of MeOH or MeCN were transferred in matrix tubes for LC-MS/MS analysis. The solubility was determined according to the following formula: Solubility (µM) = [AUC_(filtered PBS)/AUC_(not filtered MeOH or MeCN)] × 200.

The test was validated if: [AUC_(not filtered MeOH or MeCN) - AUC_(filtered MeOH or MeCN)]/AUC_(not filtered MeOH or MeCN) ≤ 10%

9.3.2. Plasma stability

Incubations were performed in duplicate in Eppendorf tubes. Mouse female plasma was pre-incubated 10 min at 37 °C before the addition of test compounds to a final concentration of 10 µM (0.1% DMSO). Aliquots were removed at 0, 15, 30, 60, 120, 240 and 360 min after compound addition, and the reaction was stopped by adding nine volumes of ice-cold MeCN or MeOH containing an internal standard. After centrifugation (10 min at 12 000 rpm), the supernatants were transferred in matrix tubes for LC-MS/MS analysis. The degradation half-life (t_{1/2}) values were calculated from the non-linear regression analysis of the degradation time course data using Xlfit® software from IDBS.Ltd.

9.3.3. Microsomal stability

To determine compound microsomal stability, liver microsomes from female mice (CD-1, Corning) were used. All incubations were performed in duplicate in a shaking water bath at 37 °C. The incubation mixtures contained 1 µM compound with 1% MeOH used as a vehicle, mouse liver microsomes (0.3 mg of microsomal protein per mL), 5 mM MgCl₂, 1 mM NADP, 5 mM glucose 6-phosphate, 0.4 U/mL glucose 6-phosphate dehydrogenase, and 50 mM potassium phosphate buffer (pH 7.4) in a final volume of 0.5 mL. Aliquots were removed at 5, 10, 20, 30, and 40 min after microsome addition, and the reaction was stopped by adding four volumes of ice-cold MeCN or MeOH containing an

internal standard. Propranolol, known as a high hepatic clearance drug in rodents, was used as a quality-control compound for the microsomal incubations. The samples were centrifuged for 10 min at 12 000 rpm and the supernatants were transferred in matrix tubes for LC-MS/MS analysis. Each compound was quantified by converting the corresponding analyte/internal standard peak area ratios to percentage drug remaining, using the initial ratio values in control incubations as 100%.

Intrinsic clearance (CL_{int}) is expressed as µL/min/mg proteins. CL_{int} = (dose/AUC_∞)/[microsomes] where dose is the initial concentration of product in the incubate (1 µM), AUC_∞ is the area under the concentration-time curve extrapolated to infinity and [microsomes] is the concentration in microsomes expressed in mg/µL.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Marion Flipo has patent Gram-negative bacteria efflux pump inhibitors pending to Inserm transfert. Ruben Hartkoorn has patent Gram-negative bacteria efflux pump inhibitors pending to Inserm transfert. Nina Compagne has patent Gram-negative bacteria efflux pump inhibitors pending to Inserm transfert. Nicolas Willand has patent Gram-negative bacteria efflux pump inhibitors pending to Inserm transfert. Anais Vieira Da Cruz has patent Gram-negative bacteria efflux pump inhibitors pending to Inserm transfert. Juan-Carlos Jimenez-Castellanos has patent Gram-negative bacteria efflux pump inhibitors pending to Inserm transfert.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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