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Article

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Novel Hits in the Correction of ΔF508-Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Protein: Synthesis, Pharmacological, and ADME Evaluation of Tetrahydropyrido[4,3-d]pyrimidines for the Potential Treatment of Cystic Fibrosis

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KEYWORDS. Cystic Fibrosis, Correctors, CFTR, ΔF508-CFTR, Ussing Chamber, ADME, drug-like

ABSTRACT. Cystic Fibrosis (CF) is a lethal genetic disease caused by mutations of the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) with a prevalence of

the ΔF508 mutation. Whereas the detailed mechanisms underlying disease have yet to be fully elucidated, recent breakthroughs in clinical trials have demonstrated that CFTR dysfunction can be corrected by drug-like molecules. Based on this success, a screening campaign was carried out, seeking new drug-like compounds able to rescue ΔF508-CFTR that led to the discovery of a novel series of correctors based on a tetrahydropyrido[4,3-d]pyrimidine core. These molecules proved to be soluble, cell-permeable and active in a disease relevant functional-assay. The series was then further optimized with emphasis on biological data from multiple cell systems while keeping physico-chemical properties under strict control. The pharmacological and ADME profile of this corrector series hold promise for the development of more efficacious compounds to be explored for therapeutic use in CF.

INTRODUCTION

Cystic Fibrosis (CF) is an autosomal recessive genetic disease with a lethal outcome, mostly affecting the lungs together with a variety of organs throughout the body such as pancreas and meconium ileus.[1] Around 1700 different mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) have been identified as causing CF.[2] CFTR is a cAMP-regulated chloride channel expressed in epithelial membranes of airways, intestinal tracts, pancreas duct, testes and sweat glands. Due to anion flow blockade, water movement slows and abnormally thick mucus clogs the ducts leading to severe bacterial infections.[1]

CF affects 1 in 2500 live births in caucasians,[3] and about two-thirds of all CF cases can be attributed to the deletion of phenylalanine 508 (ΔF508) in the CFTR protein.[4] The ΔF508 mutant is largely unable to exit the endoplasmic reticulum, presumably due to a folding defect, and only a small fraction of the synthesized protein reaches the plasma membrane, giving trafficking defect.[5,6] However, when localized to the plasma membrane by pharmacological treatment or

low-temperature incubation, the ΔF508 protein shows an accelerated rate of internalization and an intrinsic low channel activity (gating defect) compared to wild type CFTR.[4]

Until recently, therapies available to CF patients were not designed to correct the basic defect, but focused on preventing and treating its consequences: the airway bacterial colonization, the mucociliary clearance deficit, the intestinal malabsorption, and included anti-inflammatory, antibiotics, vitamins and enzymes as nutrition supplements.[7] These agents have improved the quality and duration of patients' lives leading to a median survival rate of about 40 years.[8] Recently, however, the impressive growth of research around CFTR malfunctioning has started to deliver new small molecules therapies addressing the root cause of CF by acting on the faulty gene or faulty protein.

In recent years, a number of small molecules have progressed into clinical trials targeting defective CFTR protein (Figure 1). Usually, the term 'potentiators' refers to compounds able to interfere with the gating defect, whereas 'correctors' are those drugs acting on the mistrafficking of mutated CFTR. Ivacaftor (1), initially called VX-770 (Vertex Pharmaceuticals) is a potentiator for the G551D mutation that has shown a significant improvement in patient lung function in Phase III study.[9] This drug is the first FDA-approved medicine targeting the basic defect in CF, though the patients bearing the G551D mutation, who will benefit from this therapy, represent only 4% of the CF population. More recently (December 2014), the FDA approved Ivacaftor for use in patients with CF with one of the following ten mutations: R117H, G551D, G178R, S549N, S549R, G551S, G1244E, S1251N, S1255P or G1349D, expanding the number of patients who can be treated by it.

Another investigational drug from Vertex, the corrector lumacaftor (**2**, VX-809), acts on the most common mutation ΔF508. [10] The molecule did not give outstanding results as a single agent

in a Phase II trial and that formed the basis for combination studies (Phase III trial) of ivacaftor together with lumacaftor or together with VX-661 (**3**, Phase II trial), a second corrector developed by Vertex.[11] The combination is intended to work synergistically by first correcting the defect and allowing CFTR to be tracked up to the surface, and then potentiating the protein activity by prolonging channel opening. Results from Phase III trials of the combination drug for people with two copies of the ΔF508 mutation ages 12 and older demonstrated significant improvements in lung function and other key measures of the disease,[12] and lumacaftor in now part of an approved drug combination (ORKAMBI™) with ivacaftor. Another compound, ataluren (**4**, PTC124; PTC Therapeutics) is aimed at treating patients with the CFTR 'nonsense mutation' that affects 10% of CF patients.[13] Ataluren is in Phase III after encouraging results showing improvements in chloride channel activity and positive trends in lung function.

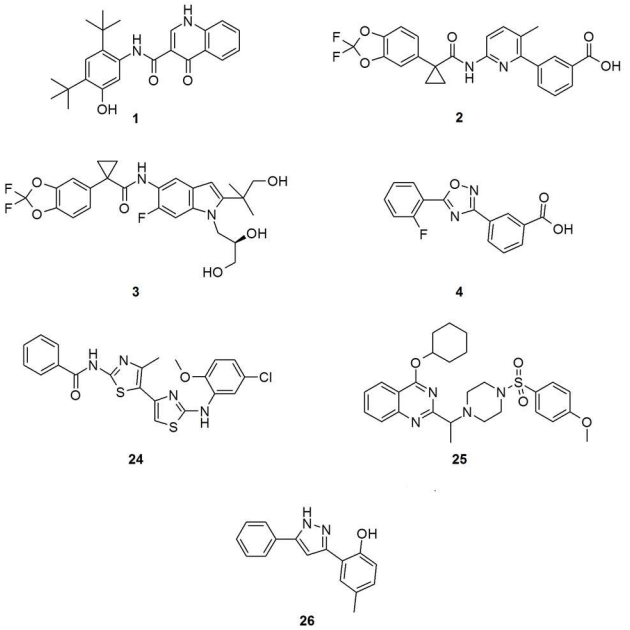


Figure 1. Small molecules currently in clinical trials for CF or used as benchmark in this study.

Given these data, the identification of small molecules able to halt disease progression in the vast majority of the CF population (i.e. carrying ΔF508 mutation) by restoring CFTR

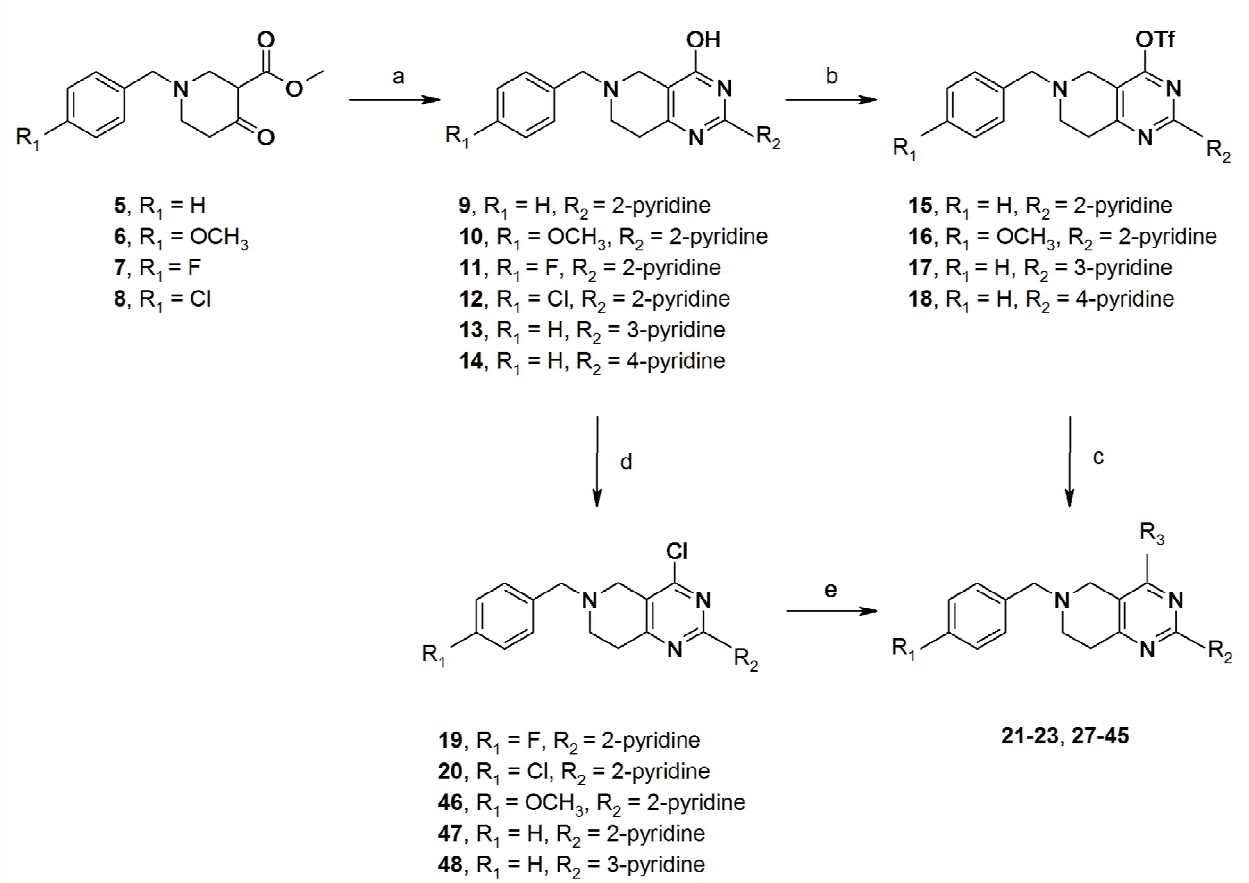
malfunction at the folding or trafficking level still represents a challenging objective. The potentiator ivacaftor showed the success of a small molecule approach to the gating defect of mutated CFTR. However, when targeting ΔF508-CFTR with a potentiator, only a low level of mutant protein could be modulated at the cell surface. A preferred option would be to use a corrector compound targeting earlier stages of malfunctioning, i.e. allowing CFTR to properly fold and traffic to the cell surface, considering that all other liabilities (degradation, instability, low activity) are a direct consequence of the folding defect.

Bearing the above factors in mind we embarked on a drug discovery program with the aim of identifying new disease-modifying molecules targeting the most common ΔF508 CFTR mutation. In particular, we focused on compounds acting as correctors. Herein we describe the discovery, synthesis, pharmacological and ADME characterization of a novel corrector series based on the tethrahydropyridopyrimidine scaffold. Compounds were optimized against multiple cell models whilst aiming to maintain a good physico-chemical profile. Although moderately potent, the new series showed promising overall profile in terms of in vitro pharmacology and ADME properties and represent a promising starting point for lead discovery.

CHEMISTRY

Several tetrahydropyridopyrimidines were identified as ΔF508-CFTR correctors during a screening campaign (Figure 2). The most interesting compounds were synthesized as depicted in Scheme 1.

Scheme 1



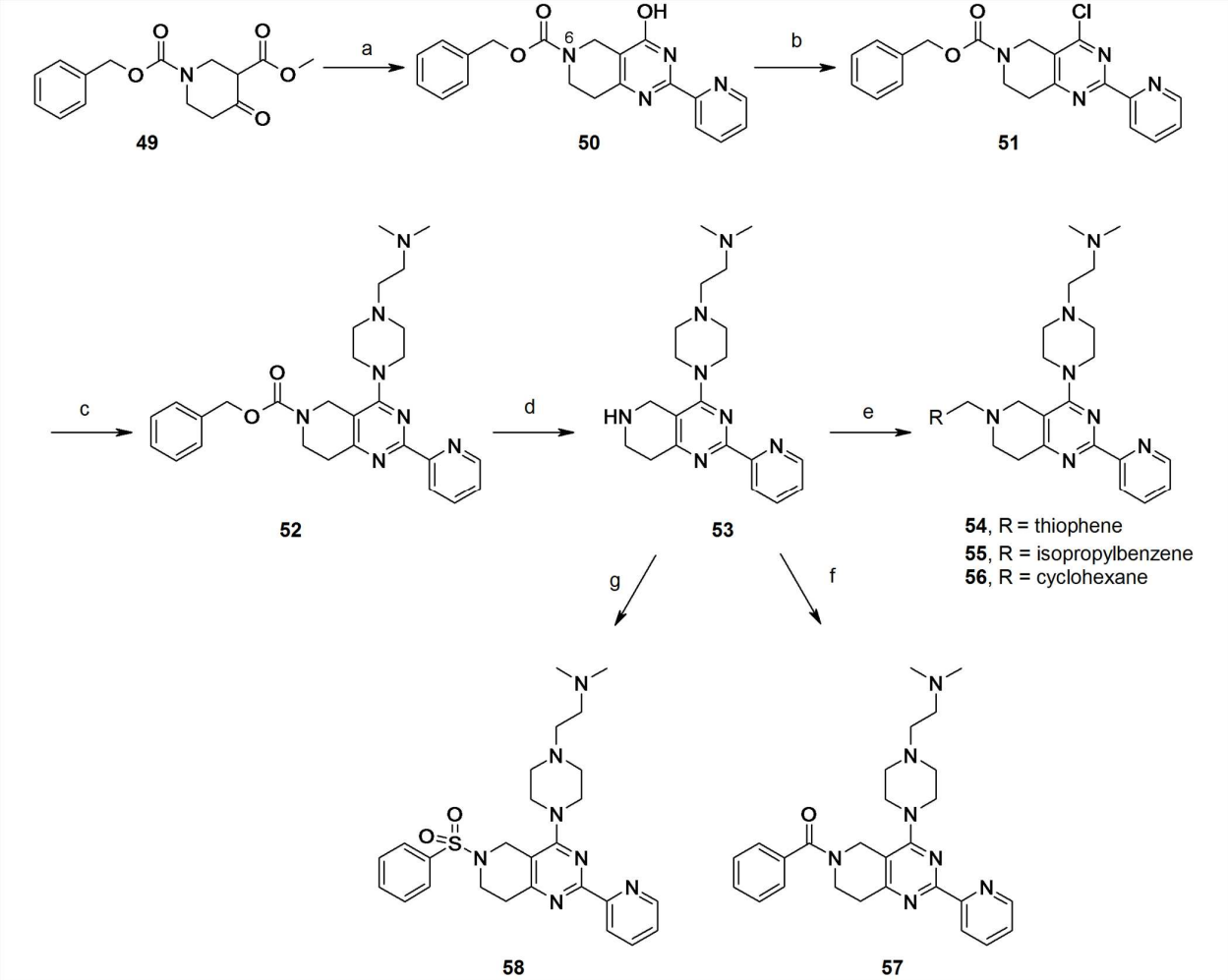
Reagents and conditions: (a) *p*-substituted-methyl-1-benzyl-4-oxopiperidine-3-carboxylate, amidinopyridine hydrochloride, $NaOCH_3$, EtOH, 80 °C, 2.5 h; (b) triflic anhydride, pyridine, -20 °C, then room temperature 2h; (c) amine, K_2CO_3, dioxane, 90 °C, overnight; (d) $POCl_3$, toluene, 130 °C, 2 h; (e) amine, Et_3N, DMF, 90 °C, overnight.

The 2-pyridinyltetrahydropyrido[4,3-d]pyrimidine core[14] in compound **9-14** was obtained by thermal condensation of commercially available piperidone **5-8** in the presence of sodium methoxide and amidinopyridine in ethanol. Activation of the hydroxyl group as a triflate using trifluoromethanesulfonic anhydride in pyridine followed by reaction with a primary or secondary amine afforded the corresponding 4-substituted tetrahydropyrido[4,3-d]pyrimidines **21-23, 27-28, 31-35** and **41** in moderate yields. An alternative activation of the hydroxyl group was

identified by reaction of compounds **9-13** in the presence of phosphoryl chloride in refluxing toluene, yielding the 4-chloro derivative in good yields (compounds **19-20** and **46-48**). The 4-chloro intermediate underwent nucleophilic substitution with the appropriate amines in DMF in the presence of triethylamine to yield the final compounds **29**, **30**, **36-40,** and **42-45**.

In order to explore the activity of the substituent at the 6-position (Scheme 2) of the tetrahydropyrido[4,3-d]pyrimidine core, commercially available **49** was reacted in the same reaction sequence as described in Scheme 1 including ring closure with 2-amidinopyridine to obtain **50,** followed by activation with $POCl_3$ (compound **51**) and subsequent nucleophilic substitution with dimethylaminoethylpiperazine to give compound **52**.

Scheme 2



Reagents and conditions: (a) 2-amidinopyridine hydrochloride, $NaOCH_3$, EtOH, 80 °C, 2.5 h; (b) $POCl_3$, CH_3CN, 60 °C, 2 h; (c) amine, K_2CO_3, CH_3CN, 90 °C, overnight ; (d) H_2, 5% Pd/C, EtOH, rt, 24 h, then at 50 °C, 24 h; (e) aldehyde, titanium (IV) isopropoxide, HOAc , 1,2-dichloroethane, rt, 1h, then sodium triacetoxyborohydride (STAB), rt, overnight; (f) benzoic acid, O-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium-hexafluorophosphate (HATU), DIPEA, CH_3CN, rt, 1 h, then amine (compound **53**), rt, 48 h (g) compound **53**, Et_3N, DCM, rt, 10 min, then benzenesulfonyl chloride, rt 2 h.

Derivative **52** was deprotected by catalytic hydrogenation affording the free amine **53**, which was reacted with the corresponding aldehyde under reductive conditions to afford products **54-56** or employed in the coupling with the benzoic acid and the benzenesulfonyl chloride to yield respectively the final products **57** and **58**.

RESULTS AND DISCUSSION

In an effort to discover small drug-like molecules able to target the root cause of the disease and to correct ΔF508-CFTR, a screening campaign was carried out on 6000 compounds. The screening library was defined through a diverse selection approach aimed at sampling the available chemical space in a corporate compound collection consisting of about 70,000 unique compounds. *In silico* molecular properties were carefully checked and filtered as described below.

Beside lumacaftor, which is the result of a drug discovery process that produced a clinical candidate, other known correctors such as Corr4a[15] and VRT-325[16] (**24** and **25**, respectively, Figure 1) are routinely used as reference compounds for pharmacological studies although they hold a sub-optimal physico-chemical profile, in particular in terms of solubility or permeability (see Table 1).

In order to expedite the drug discovery process and maximize the probability of finding high quality hits, a series of filtering rules were applied to take out molecules with an undesired profile. A 'drug-likeness' filter was used to remove molecules with unsuitable calculated physicochemical properties as defined by the widely applied Lipinski's rule of five and Veber's rule.[17,18] Moreover, in order to avoid complex stereoisomers mixtures, the number of chiral centers with defined and undefined stereochemistry was set not to exceed 3 and 1, respectively.

Molecules having experimentally determined aqueous solubility lower than 20 μM were removed, while for those molecules without experimental solubility data, we applied an in house generated insolubility model to filter out compounds predicted to have aqueous solubility lower than 40 μM.[19] Molecules containing highly reactive groups or expected to be interferent or promiscuous were discarded.[20] Finally, lipophilic molecules bearing more than 4 aromatic rings and flat compounds, which are often associated with low aqueous solubility and poor developability, were removed.[21] Having applied these filters, a subset of about 6000 compounds were tested according to the following screening cascade.

FRT cells co-expressing ΔF508-CFTR and a fluorescent YFP halide sensor were used as the primary screening system allowing for mode of action (corrector vs. potentiator) determination.[22] Our main aim was the identification of corrector compounds; however, since compounds with both potentiator and corrector mode of action (dual-acting compounds) have been reported, [23-26] we opted for testing our compound set in both assay modes.

To identify correctors, compounds were tested at 2 and 20 μM in order not to lose slightly less active compounds. Normalization was performed for each plate with respect to negative (DMSO, 0%) and positive control (**24**, 100%). Moreover, as corrector identification is usually challenging we decided to use a relatively low 30% cutoff value for considering a compound worthy of further characterization. Thus, 115 compounds acting as correctors with an efficacy higher than 30% at 2 μM or 20 μM with respect to **24** were selected. They were first clustered and 42 representative structures were characterized by concentration-response curve determination in ΔF508-CFTR FRT cells co-expressing YFP. Thirty-six compounds were finally confirmed with EC_{50}s ranging in [0.2, 17] μM and efficacy from 30% to 100%.

In potentiator mode, a single concentration of 10 μM was used to test compounds. Normalization was done according to negative (forskolin, 0%) and positive (genistein, 100%) controls and a 40% cutoff value was applied to select potentiators; a total of 242 compounds were selected for further investigation. Compounds were clustered according to their chemical structure to select 123 representative molecules to be characterized by concentration-response curve determination in ΔF508-CFTR FRT cells co-expressing YFP. Eighty-six compounds were confirmed as potentiators with K_d ranging from 0.1 to 18 μM and efficacy from 30% to 100%.

Two dual-acting compounds were identified and characterized by concentration-response curves in both corrector and potentiator assays. They were close structural analogues of VRT-532 (**26**, Figure 1),[27] a tool compound known to exhibit both corrector and potentiator functions. These observations, while confirming the validity of our screening cascade, led us to abandon the series, which does not offer any novelty, and to focus our attention on the corrector series.

Corrector compounds with confirmed EC_{50} were evaluated in a series of early in vitro ADME assays to assess aqueous solubility at pH 3 and pH 7.4, passive permeability in a Parallel Artificial Membrane Permeability Assay (PAMPA), and stability upon incubation with recombinant cytochrome h3A4. Compounds with an appropriate balance of activity and early ADME properties underwent further in vitro profiling consisting of counter screening on FRT null cells to detect unspecific activity signals. Electrophysiology was also done by Ussing chamber experiments on active compounds measuring transepithelial Cl^- currents in FRT cells expressing ΔF508-CFTR to corroborate the data obtained using the fluorescent assay. At this point, the tetrahydropyrido[4,3-d]pyrimidine series was identified as ΔF508-CFTR correctors with an overall promising profile for both pharmacological and physico-chemical parameters (Figure 2 and Table 1).

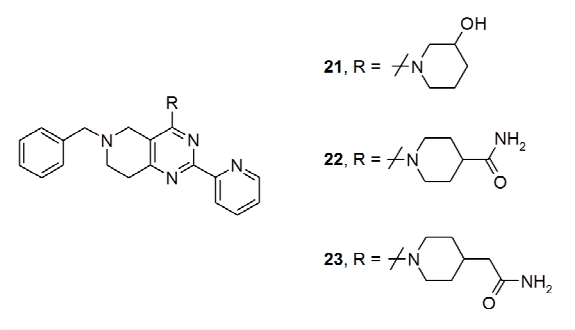


Figure 2. Hit series identified in screening campaign

Table 1. In vitro activity data in FRT cells and ADME profile for compounds **21**-**23** and known corrector compounds **24**, **25**, and **2**

Compound	ClogP	MPSA Å^2	ΔF508-CFTR[a] EC_{50} μM (Emax %)	WT-CFTR[b] Emax %	Ussing ΔF508-CFTR[c] Emax %	Sol[d] μM	Perm[e]	Met Stab[f] %
21	2.8	65.4	2.8 (62.9)	97.8	68.3	247	High	25
22	1.7	88.2	1.4 (56.6)	102.9	74.6	250	High	71
23	0.9	91.5	2.8 (57.9)	94.0	30.1	207	High	57
24	6.2	132.6	1.1 (113.8)	115.3	100	1	No result	55
25	5.9	93.2	2.2 (92.9)	Not tested	41.4	1	No result	13
2	5.7	97.8	2.6 (66.7)	Not tested	75	126	Medium	95

[a]Values for EC_{50} and Emax are obtained from dose–response relationships of YFP fluorescence experiments. [b]Data are normalized according to DMSO (100%), all compounds are tested at 10 μM. Data reported for biological assays are expressed as mean values from 2 or 3 independent experiments. [c]Data are normalized according to DMSO (0%) and **24** at 10 μM (100%), **25** tested at 10 μM and our compounds were tested at 15 μM. [d]Solubilities were determined at pH 7.4 at pseudothermodynamic equilibrium. [e]Permeability is based on measuring the permeation rate of

the test article through an artificial membrane. [f]Metabolic stability was determined as percentage remaining after incubation for 1 h with recombinant hCYP3A4.

The three compounds were found to have micromolar corrector activity on FRT cells carrying the ΔF508-CFTR mutation. Despite being less efficacious with respect to the reference compounds, they showed a promising physico-chemical profile, being highly soluble and permeable. Metabolic stability versus Cyp3A4 was generally good; only compound **21** showed some liability that was viewed as acceptable as a hit of a screening campaign.

Ussing chamber experiment on ΔF508-CFTR FRT epithelia further confirmed activity of compounds **21-23**, with compounds **21** and **22** being able to significantly decrease the CFTR-dependent Cl^- current.

Finally, compounds **21-23** did not exert any effect on cells expressing wild-type CFTR thus confirming their specificity of action on malfunctioning CFTR cell system.

All these data were rather encouraging and the series was deemed to be worthy of further development. At that point we turned our attention on having compounds acting on multiple cell models.

It has been reported that correctors may be strongly affected by the cell background and many correctors have been found to work in one cell type only.[28] Two different modes of action have been proposed for correctors where they could act as pharmacological chaperones able to directly bind to CFTR and allow its proper folding, or proteostasis regulators which modulate other proteins involved in CFTR maturation. While the former hypothesis implies that correctors should work in any cell type, the latter paves the way to the possibility of having cell-type dependent correctors. In order to identify compounds with a more general mechanism of action and avoid cell-type specific compounds, we introduced testing in ΔF508-CFTR transfected A549

cells, which are of human lung epithelial origin, as a second *in vitro* pharmacologic tool for further characterizing correctors.

As shown in Table 2, two out of three original hits showed some activity on A549 cells. Although the efficacy was very partial, these data further prompted us to deepen the investigation on the series. Medicinal chemistry efforts were thus focused on generating compounds with significant biological activity in both FRT and A549 cell systems while maintaining the optimal physico-chemical profile. Compounds were deemed of interest when they possessed an efficacy higher than 30% with respect to **24** in both cell lines.

In the initial phase of structure-activity relationship (SAR) study on the series, we identified three main regions to explore corresponding to the pyridine ring in the 2-position (A), the amine in 4-position (B) and the *N*-substitution on 6-position (C) of the tetrahydropyrido[4,3-d]pyrimidine core (Figure 3).

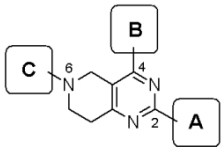
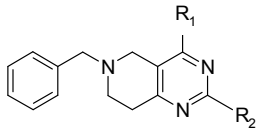
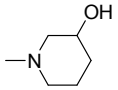
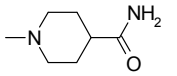
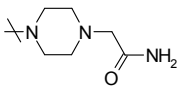
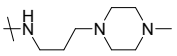
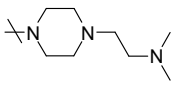
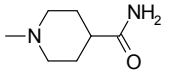
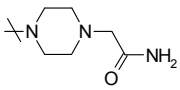
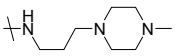
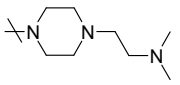
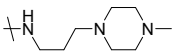
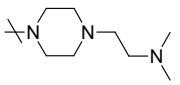


Figure 3. The three regions explored in our SAR studies.

On the basis of results obtained from the original three hit compounds (**21**, **22, 23**) the first set of analogues synthesized explored the effect of the pyridine ring on position 2- of the core structure. Table 2 contains examples of synthesized compounds bearing a benzyl group in position 6- and various amines in 4-position of the tetrahydropyrido[4,3-d]pyrimidine core.

Table 2. In vitro Activity data and ADME profile for compounds **21-23, 27-34**.



Cpd	R_1	R_2	ClogP	MPSA Å^2	ΔF508-CFTR[a] EC_{50} μM (Emax %)		Sol[b] μM	Perm[c]	Met Stab[d] %
					FRT	A549			
21		2-pyridine	2.8	65.4	2.8 (62.9)	0.4 (30.6)	247	High	25
22		2-pyridine	1.7	88.2	1.4 (56.6)	7.1 (34.4)	250	High	71
23		2-pyridine	0.9	91.5	2.8 (57.9)	inactive	207	High	57
27		2-pyridine	2.4	60.4	3.1 (53.4)	inactive	180	High	100
28		2-pyridine	3.6	51.6	1.5 (41.3)	11.7 (57)	218	High	100
29		3-pyridine	1.4	88.2	inactive	inactive	224	High	22
30		3-pyridine	0.7	91.5	inactive	inactive	156	High	17
31		3-pyridine	2.2	60.4	inactive	inactive	234	High	15
32		3-pyridine	3.4	51.6	inactive	inactive	247	High	41
33		4-pyridine	2.2	60.4	inactive	inactive	250	High	13
34		4-pyridine	3.4	51.6	inactive	inactive	250	High	21

[a]Values for EC_{50} and Emax are obtained from dose–response relationships of YFP fluorescence experiments. Data reported for biological assays are expressed as mean values from 2 or 3

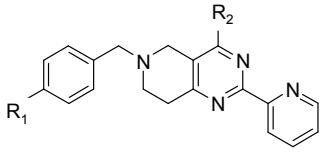
independent experiments.. [b]Solubilities were determined at pH 7.4 at pseudothermodynamic equilibrium. [c]Permeability is based on measuring the permeation rate of the test article through an artificial membrane. [d]Metabolic stability was determined as percentage remaining after incubation for 1 h with recombinant hCYP3A4.

Two of the newly synthesized compounds (**27** and **28**) proved active in the FTR cell assay with efficacy ranging from 41 to 53%. They were all characterized by a 2-pyridine substituent. Among these, only compound **28** bearing the dimethylaminoethylpiperazine substituent in 4-position was active on both cell lines, with higher efficacy on A549 (57%) with respect to FRT cells.

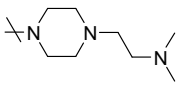
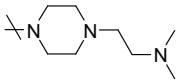
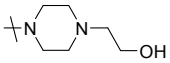
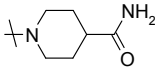
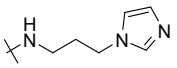
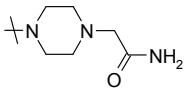
In general, the molecules had excellent solubility and passive permeability while metabolic stability towards cytochrome 3A4 was more variable. 3-Pyridyl and 4-pyridyl derivatives proved inferior to the 2-pyridyl analogues not only for biological activity but also for metabolic stability as clearly showed by going from **28** to **32** and **34** or from **27** to **31** and **33**. Taken together, these data indicate the 2-pyridyl substitution is essential for both activity and ADME properties.

With the aim of a multiparameter optimization of the molecules, we then decided to: i. investigate the metabolic stability by inserting simple substitutions on 4-position of the benzyl group, which is a known labile site, while keeping the 2-pyridyl substituent fixed, and ii. expand the initial selection of amines on 4-position of the pyridopirimidine core (Table 3).

Table 3. In vitro activity data and ADME profile for compounds **35-40**.



Cpd	R_1	R_2	ClogP	MPSA	ΔF508-CFTR[a] EC_{50} μM	Sol[b]	Perm[c]	Met Stab[d]

				Å^2	(Emax %)		μM		%
					FRT	A549			
35	-OCH_3		3.5	60.9	inactive	inactive	229	High	70
36	-F		3.8	51.6	inactive	inactive	231	High	82
37	-OCH_3		2.5	77.9	0.4 (106.9)	inactive	124	High	97
38	-OCH_3		1.6	97.5	3.5 (86.6)	inactive	224	Medium	78
39	-OCH_3		3.1	81.0	0.2 (40.6)	inactive	217	High	28
40	-OCH_3		0.7	100.7	2.3 (35.9)	10.9 (36.8)	217	Medium	79

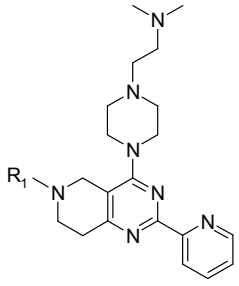
[a]Values for EC_{50} and Emax are obtained from dose–response relationships of YFP fluorescence experiments. Data reported for biological assays are expressed as mean values from 2 or 3 independent experiments.. [b]Solubilities were determined at pH 7.4 at pseudothermodynamic equilibrium. [c]Permeability is based on measuring the permeation rate of the test article through an artificial membrane. [d]Metabolic stability was determined as percentage remaining after incubation for 1 h with recombinant hCYP3A4.

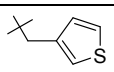
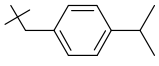
The *p*-OMe substituent gave the highest activity values observed on the FRT cell line with compounds **37** and **38** showing efficacy values >85%, but were completely inactive on the A549 cell line. One example (compound **40**) showed weak activity on both cell lines with values around 35%. The introduction of the *p*-OMe substituent also exerted some effect on the physico-chemical profile of the molecules: compounds **38** and **40,** direct analogues of primary hits **22** and **23**, benefited from the para-substitution in terms of stability versus cytochrome 3A4. On the other hand, passive permeability was not optimal for compounds **38** and **40**: this trend was in line

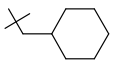
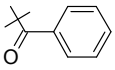
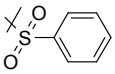
with ClogP and MPSA values in this array of molecules, i.e. the compounds with lower permeability were characterized by a lower ClogP and higher MPSA values.

Maintaining activity across the series on the A549 cell line proved to be more difficult than attaining consistent response of this class of compounds on the FRT cell line. In fact, in all cases compounds active on the A549 cell line showed activity also on the FRT cells. This trend encouraged us to select the dimethylaminoethylpiperazine substituent, which showed the highest level of activity on the A549 cell line (compound **28**), and explore the left handside of the molecule, switching from benzyl to different type of substituents on 6-position (Table 4). Unexpectedly, none of the compounds synthesized in this mini array showed activity on the A549 cell line, whereas some of these maintained activity on FRT.

Table 4. In vitro activity data and ADME profile for compounds **54**-**58**.



Cpd	R_1	ClogP	MPSA Å^2	ΔF508-CFTR[a] (Emax %)	EC_{50} μM	Sol[b] μM	Perm[c]	Met Stab[d] %
				FRT	A549			
54		3.3	79.9	0.2 (79.8)	inactive	235	High	66
55		5.0	51.6	0.3 (57.2)	inactive	186	High	58

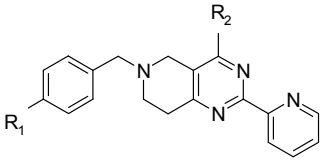
56		4.5	51.6	0.9 (41)	inactive	191	High	65
57		2.7	68.7	inactive	inactive	244	Medium	100
58		3.0	94.2	inactive	inactive	195	High	84

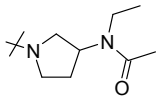
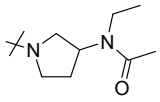
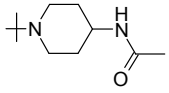
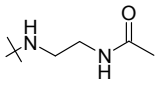
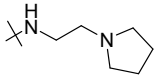
[a]Values for EC_{50} and Emax are obtained from dose–response relationships of YFP fluorescence experiments. Data reported for biological assays are expressed as mean values from 2 or 3 independent experiments.. [b]Solubilities were determined at pH 7.4 at pseudothermodynamic equilibrium. [c]Permeability is based on measuring the permeation rate of the test article through an artificial membrane. [d]Metabolic stability was determined as percentage remaining after incubation for 1 h with recombinant hCYP3A4.

This set of molecules completed the preliminary SAR around the scaffold: 2-pyridyl substitution on position 2- of the tetrahydropyrido[4,3-d]pyrimidine core and a substituted benzyl group in position 6- proved almost essential to find activity on both cell lines. The amine on position 4- of the core structure could accommodate various groups which were active mostly on FRT cells, but with the best result in both cell lines obtained only by cyclic or linear aliphatic amines with hydrogen bond acceptor/donor groups located at the far end of the moiety.

With these observations in mind, we proceeded to the synthesis of a focused set of compounds, selecting *p*-substituted benzyl groups at position 6 and a new set of amines in position 4. Indeed in this table (Table 5) we found a more robust set of results with three compounds (**43-45**) showing activity on both cell lines with EC_{50}s in the low-to-submicromolar range. In particular compound **43** showed the best combination of activity observed among this series both in terms of EC_{50} (0.7 μM and 1.9 μM on FTR and A549 respectively) and Emax values (>60% on both assays). Compounds **44** and **45** showed a lower activity but an improved metabolic stability with respect to **43**.

Table 5. In vitro activity data and ADME profile for compounds **41**-**45**.



Cpd	R_1	R_2	Clog P	MPSA Å^2	ΔF508-CFTR[a] EC_{50} μM (Emax %)		Sol[b] μM	Perm[c]	Met Stab[d] %
					FRT	A549			
41	OCH_3		3.0	74.7	0.3 (36.9)	inactive	218	High	61
42	Cl		3.8	65.5	inactive	inactive	223	High	3
43	Cl		2.4	74.3	0.7 (65.6)	1.9 (61.7)	198	High	25
44	F		2.7	83.0	0.2 (73.8)	0.4 (44.3)	226	High	80
45	F		4.4	57.2	0.2 (40)	0.5 (40.6)	196	High	71

[a]Values for EC_{50} and Emax are obtained from dose–response relationships of YFP fluorescence experiments. Data reported for biological assays are expressed as mean values from 2 or 3 independent experiments.. [b]Solubilities were determined at pH 7.4 at pseudothermodynamic equilibrium. [c]Permeability is based on measuring the permeation rate of the test article through an artificial membrane. [d]Metabolic stability was determined as percentage remaining after incubation for 1 h with recombinant hCYP3A4.

In order to further investigate the extent of metabolic stability of the compounds, intrinsic clearance (CL_{int}) was determined for the most promising correctors (**22**, **27**, **43**).

In vitro measurement of CL_{int} in human hepatic microsomes was used to estimate the stability towards CYP450-dependent metabolism. Results are reported in Table 6 together with additional data for a summary of the ADME *in vitro* profile including permeability in the MDCK cell line and plasma stability.

Table 6. ADME in vitro profile of most interesting compounds.

Cpd	CL_{int}[a] µL/min/mg	MDCK AB[b] 10^{-6} cm/s	MDCK BA/AB[c]	Plasma Stability 3 h; rat[d]
22	36.5	0.4	52.3	85
27	30.1	1.8	18.1	101
43	97.5	3.1	12.1	93.2

[a]In vitro intrinsic clearance from human liver microsomal stability assay. [b]MS-based quantification of apical → basolateral transfer rate of a test compound at 10 µM across contiguous monolayers of MDCK (Madin−Darby canine kidney) cells. [c]Ratio of (basolateral → apical) to (apical → basolateral) transfer rate of a test compound at 10 µM across contiguous monolayers of MDCK cells. [d]Plasma stability was determined as percentage remaining after incubation for 3 h with fresh rat plasma.

Intrinsic clearance in the presence of human hepatic microsomes for compounds **22** and **27**, although not optimal, was considered acceptable (ca. 30 µL/min/mg). Compound **43,** confirmed the low metabolic data observed with CYP3A4 also in the intrinsic clearance assay. Interestingly, while no particular liabilities were highlighted in terms of passive permeability, data from an MDCK cell line pointed out a poor ability of compounds to enter cellular barriers (MDCK AB) and a clear indication of a role for efflux transporters (MDCK BA/AB > 3). Improving active

efflux for the series will be one of the major goals for the next round of optimization. Finally, stability towards the hydrolysis by plasma circulating enzymes appears to be optimal.

Chemistry

General Methods. Nuclear magnetic resonance spectra were recorded using a Varian Mercury Plus 400 MHz spectrometer equipped with a PFG ATB broadband probe. UPLC-MS analyses were run using a Acquity Waters UPLC equipped with a Waters SQD (ES ionization) and Waters Acquity PDA detector, using a column BEH C18 1.7 μm, 2.1 mm × 50 mm. Basic method: gradients were run using 0.1% NH_4HCO_3 water/acetonitrile 95/5 and acetonitrile with a gradient 95/5 to 15/85, flow: 0.8 mL/min over 3 min. Acid method: gradients were run using 0.05% formic acid water/acetonitrile 95/5 and acetonitrile with a gradient 95/5 to 100, flow: 0.8 mL/min over 3 min as stated in the examples. Retention times were expressed in minutes. Temperature: 40 °C. UV detection at 215 nm and 254. ESI+ detection in the 80−1000 m/z range. HPLC-MS analysis were run using a Waters 2795 separation module equipped with a Waters Micromass ZQ (ES ionization) and Waters PDA 2996, using a X-Bridge C18 3.5 μm 2.10 x 50 mm column. Basic method: gradients were run using 0.1% ammonia/water and acetonitrile with gradient 85/15 to 5/95, flow 0.8 ml/min over 5/10 minutes. Acid method: gradients were run using 0.1% formic acid/water and 0.1% formic acid/acetonitrile with gradient 95/15 to 5/95, flow 0.8 ml/min over 5/10 minutes. Temperature: 40 °C. UV Detection at 215 nm and 254. ESI+ detection in the 80-1000 m/z range. Preparative HPLC was run using a Waters 2767 system with a binary gradient module Waters 2525 pump and coupled to a Waters Micromass ZQ (ES) or Waters 2487 DAD, using a X-Bridge C18 5 μm, 19 mm × 150 mm, using a 0.1% formic acid/ water and 0.1% formic acid/methanol flow: 17 mL/min. Alternatively in basic condition it was used 0.1%

ammonia/water and methanol flow: 17 mL/min. The purity of compounds submitted for screening was > 95% as determined by integrating at 215 nm the peak area of the LC chromatograms. To further support the purity statement, all compounds were also analyzed at a different wavelengths (254 nm), and total ion current (TIC) chromatogram and NMR spectra were used to further substantiate results. HRMS data were obtained through an LTQ-Orbitrap mass spectrometer via direct infusion of a 1 μM sample in acetonitrile 30%/water 69.9%/formic acid 0.1% (flow: 7 μL/min). The LTQ-Orbitrap instrument was calibrated prior to analysis with the manufacturer's positive ion Calibration Solution in the mass range 100−2000 m/z. Each determination was the combination of five successive scans acquired at 100.000 fwhm resolution. Elemental composition calculations and isotopic pattern simulations were executed using the specific tool included in the QualBrowser module of Xcalibur (Thermofisher Scientific, release 2.0.7) software, using a tolerance of 5 ppm. All TLC analyses were performed on silica gel (Merck 60 F254) and spots revealed by UV visualization at 254 nm and $KMnO_4$ or ninhydrin stain.

General procedure A for the synthesis of 6-(*p*-substituted-benzyl)-2-pyridin-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-ols (9-14 and 50). To a solution of *p*-substituted-1-benzyl-4-oxopiperidine-3-carboxylic acid methyl ester (3.18 g, 12 mmol, 1 equiv) in EtOH (50 mL) was added NaOMe (24 mmol, 2 equiv) followed by amidinopyridine hydrochloride (12 mmol, 1 equiv). The reaction mixture was heated to 80 °C for 2.5 h, then cooled to rt. The solvent was removed under reduced pressure, and a saturated solution of ammonium chloride was added to the residue. The insoluble material was filtered off, washed with H_2O (60 mL) and then ether (20 mL) and concentrated in vacuo to give a solid, which was not further purified.

6-Benzyl-2-pyridin-2-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-ol (9). 5 was prepared according to general procedure A, using **5** (1.8 g, 7.37 mmol) and 2-amidinopyridine hydrochloride (1.16 g, 7.37 mmol) to afford 6-benzyl-2-pyridin-2-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-ol **(9)** as an orange solid (1.54 g, yield: 66%) which was used in the next step without further purification.

^{1}H NMR (400 MHz, d_6-DMSO) δ 8.74 – 8.67 (m, 1H), 8.29 – 8.24 (m, 1H), 8.07 – 7.96 (m, 1H), 7.66 – 7.57 (m, 1H), 7.39 – 7.32 (m, 4H), 7.32 – 7.23 (m, 1H), 3.70 (s, 2H), 3.27 (s, 2H), 2.74 (s, 4H).

Mass (ES) *m/z* 319 (M+1). HPLC (acid method, 3 min); R_t = 1.44 min.

6-(4-Methoxybenzyl)-2-pyridin-2-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-ol (10). 10 was prepared according to general procedure A, using **6** (4.3 g, 15.5 mmol) and 2-amidinopyridine hydrochloride (12.44 g, 15.5 mmol) to afford 6-(4-methoxybenzyl)-2-pyridin-2-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-ol **(10)** as an orange solid (5.3 g, yield: 98%) which was used in the next step without further purification.

^{1}H NMR (400 MHz, d_6-DMSO) δ 11.95 (s, 1H), 8.75 – 8.65 (m, 1H), 8.32 – 8.22 (m, 1H), 8.08 – 7.95 (m, 1H), 7.67 – 7.57 (m, 1H), 7.27 (d, J = 8.6 Hz, 2H), 6.91 (d, J = 8.6 Hz, 2H), 3.75 (s, 3H), 3.62 (s, 2H), 3.24 (s, 2H), 2.72 (s, 4H).

Mass (ES) *m/z* 349 (M+1).

6-(4-Fluorobenzyl)-2-pyridin-2-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-ol (11). 11 was prepared according to general procedure A, using **7** (3.18 g, 12 mmol) and 2-

amidinopyridine hydrochloride (1.89 g, 12 mmol) to afford 6-(4-fluorobenzyl)-2-pyridin-2-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-ol **(11)** as a brown solid (2.23 g, yield: 56%) which was used in the next step without further purification.

^{1}H NMR (400 MHz, d_6-DMSO) δ 11.32 – 10.57 (m, 1H), 8.74 – 8.69 (m, 1H), 8.32 – 8.25 (m, 1H), 8.06 – 7.97 (m, 1H), 7.64 – 7.57 (m, 1H), 7.45 – 7.34 (m, 2H), 7.24 – 7.10 (m, 2H), 3.68 (s, 2H), 3.25 (s, 2H), 2.73 (s, 4H).

Mass (ES) *m/z* 337 (M+1).

6-(4-Chlorobenzyl)-2-pyridin-2-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-ol (12). 12 was prepared according to general procedure A, using **8** (1.4 g, 4.98 mmol) and 2-amidinopyridine hydrochloride (785 mg, 4.98 mmol) to afford 6-(4-chlorobenzyl)-2-pyridin-2-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-ol **(12)** as solid (1.4 g, yield: 80%) which was used in the next step without further purification.

^{1}H NMR (400 MHz, d_6-DMSO) δ 11.69 (s, 1H), 8.77 – 8.66 (m, 1H), 8.36 – 8.21 (m, 1H), 8.13 – 7.94 (m, 1H), 7.72 – 7.57 (m, 1H), 7.49 – 7.30 (m, 4H), 3.69 (s, 2H), 3.27 (s, 2H), 2.73 (s, 4H).

Mass (ES) *m/z* 353 (M+1).

6-Benzyl-2-pyridin-3-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-ol (13). 13 was prepared according to general procedure A, using **5** (1.5 g, 6 mmol) and 3-amidinopyridine hydrochloride (950 mg, 6 mmol) to afford 6-benzyl-2-pyridin-3-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-ol **(13)** as orange solid (1.46 g, yield: 76%) which was used in the next step without any further purification.

^{1}H NMR (400 MHz, d_6-DMSO) δ 11.69 (s, 1H), 9.62 – 9.55 (m, 1H), 8.72 – 8.68 (m, 1H), 8.68 – 8.61 (m, 2H), 7.47 – 7.28 (m, 5H), 3.69 (s, 2H), 3.27 (s, 2H), 2.73 (s, 4H).

Mass (ES) *m/z* 319 (M+1). UPLC (basic method); R_t = 0.19 min.

6-Benzyl-2-pyridin-4-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-ol (14). 14 was prepared according to general procedure A, using **5** (1.5 g, 6 mmol) and 4-amidinopyridine hydrochloride (950 mg, 6 mmol) to afford 6-benzyl-2-pyridin-4-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-ol **(14)** as orange solid (1.9 g, yield: 100%) which was used in the next step without any further purification.

^{1}H NMR (400 MHz, d_6-DMSO) δ 8.65 – 8.57 (m, 2H), 8.12 – 8.05 (m, 2H), 7.41 – 7.14 (m, 5H), 3.73 – 3.61 (m, 2H), 3.60 – 3.33 (m, 2H), 2.77 – 2.57 (m, 4H).

Mass (ES) *m/z* 319 (M+1). UPLC (basic method); R_t = 0.30 min.

General procedure B for the synthesis of trifluoro-methanesulfonic acid 6-(*p*-substitutedbenzyl)-2-pyridin-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl esters (15-18). To a solution of 6-(*p*-substitutedbenzyl)-2-pyridin-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-ol (1 equiv, 2.38 mmol) in pyridine (20ml) was added trifluoromethanesulfonic anhydride (1 equiv, 2.85 mmol) dropwise at -20 ^{0}C. The reaction mixture was slowly warmed to room temperature and then stirred for 2 h. The reaction was poured into water and the solution was filtered. A brown oil (low-melting wax) was recovered which was dissolved in CH_3OH and evaporated under vacuum to give the desired product.

Trifluoromethanesulfonic acid 6-benzyl-2-pyridin-2-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl ester (15). Prepared following general procedure B using **9** (2 g, 6.28 mmol) and trifluoromethanesulfonic anhydride (1.26 mL, 7.53 mmol). The product **15** (1.9 g, yield: 67%) was obtained as a brown solid by precipitation from the reaction mixture by adding H_2O and was used in the next step without further purification.

Mass (ES) *m/z* 481 (M+1). UPLC (basic method); R_t = 1.61 min.

Trifluoromethanesulfonic acid 6-(4-methoxybenzyl)-2-pyridin-2-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl ester (16).

16 was prepared following general procedureB using**10** (4.8 g, 13.8 mmol) and trifluoromethanesulfonic anhydride (2.78 mL, 16.56 mmol). The product **16** (1.8 g, yield: 30%) was obtained as a brown solid by precipitation from the reaction mixture by adding H_2O and was used in the next step without further purification.

Mass (ES) *m/z* 481 (M+1).

Trifluoromethanesulfonic acid 6-benzyl-2-pyridin-3-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl ester (17). 17 was prepared following general procedure B using **13** (1.9 g, 6 mmol) and trifluoromethanesulfonic anhydride (1.21 mL, 7.2 mmol). The product **17** (2.1 g, yield: 78%) was obtained as a brown solid by precipitation from the reaction mixture by adding H_2O and was used in the next step without further purification.

Mass (ES) *m/z* 451 (M+1). UPLC (basic method); R_t = 1.64 min.

Trifluoromethanesulfonic acid 6-benzyl-2-pyridin-4-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl ester (18). 18 was prepared following general procedureB using **14** (1.4 g, 4.4 mmol) and trifluoromethanesulfonic anhydride (0.9 mL, 5.28 mmol). The product **18** (1 g, yield: 51%) was obtained as a brown solid by precipitation from the reaction mixture by adding H_2O and was used in the next step without further purification.

Mass (ES) *m/z* 451 (M+1). UPLC (basic method); R_t = 2.09 min.

General procedure C for the synthesis of 6-(*p*-substitutedbenzyl)-4-chloro-2-pyridin-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidines (19-20, 46-48 and 51). To a solution of 6-(*p*-substitutedbenzyl)-2-pyridin-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-ol (1 equiv, 0.9 mmol, 300 mg) in toluene, phosphoryl chloride (2 equiv, 1.8 mmol, 0.16 µL) was added and the mixture was heated at reflux for 2 h. The reaction was allowed to cool to room temperature, the mixture was poured into water and the excess of $POCl_3$ was quenched with 1 N NaOH. The product was extracted into DCM (3 x 10 mL) and the combined organic layers were dried over Na_2SO_4. Evaporation of volatiles provided the desired product, which was used in the next step without any further purification.

4-Chloro-6-(4-fluorobenzyl)-2-pyridin-2-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidine (19). By use of general procedure C, reaction of **11** (1.18 g, 3.5 mmol) with phosphoryl chloride (0.64 mL, 7 mmol) gave the chlorinated derivative (850 mg, yield: 69%) as a brown solid.

^{1}H NMR (400 MHz, d_6-DMSO) δ 8.78 – 8.70 (m, 1H), 8.38 – 8.30 (m, 1H), 8.03 – 7.94 (m, 1H), 7.59 – 7.52 (m, 1H), 7.49 – 7.38 (m, 2H), 7.26 – 7.14 (m, 2H), 3.78 (s, 2H), 3.64 (s, 2H), 3.02 (t, J = 5.8 Hz, 2H), 2.84 (t, J = 5.8 Hz, 2H).

Mass (ES) *m/z* 355 (M+1).

4-Chloro-6-(4-chlorobenzyl)-2-pyridin-2-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidine (20). Prepared according to general procedure C, by reaction of **12** (300 mg, 0.85 mmol) with phosphoryl chloride (0.15 mL,1.7 mmol) to give the title compound **20** (300 mg, yield: 100%) as a brown solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.83 – 8.77 (m, 1H), 8.47 – 8.39 (m, 1H), 7.87 – 7.75 (m, 1H), 7.41 – 7.32 (m, 1H), 7.33 – 7.28 (m, 4H), 3.72 (s, 2H), 3.67 (s, 2H), 3.12 (t, J = 5.8 Hz, 2H), 2.82 (t, J = 5.8 Hz, 2H).

Mass (ES) *m/z* 372 (M+1).

4-Chloro-6-(4-methoxybenzyl)-2-pyridin-2-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidine (46). By use of general procedure C, reaction of **10** (3 g, 8.62 mmol) with phosphoryl chloride (1.57 mL,17.24 mmol) gave the chlorinated derivative (3 g, yield: 100%) as a brown solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.87 – 8.80 (m, 1H), 8.51 – 8.44 (m, 1H), 7.89 – 7.79 (m, 1H), 7.27 (d, J = 8.6 Hz, 2H), 6.91 (d, J = 8.6 Hz, 2H), 3.79 (s, 2H), 3.73 (s, 2H), 3.15 (t, J = 5.8 Hz, 2H), 2.86 (t, J = 5.8 Hz, 2H).

Mass (ES) *m/z* 368 (M+1).

6-Benzyl-4-chloro-2-pyridin-2-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidine (47). General procedure C was applied. Compound **9** (2.1 g, 7 mmol) was reacted with phosphoryl chloride (6 mL, 62 mmol, 9 equiv) in CH_3CN (20 mL) at 60 °C overnight. After work up, the crude was

purified by flash chromatography on silica gel (EtOAc/cyclohexane 70:30) to give the chlorinated derivative (1.5 g, yield: 66%) as a brown solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.87 – 8.80 (m, 1H), 8.51 – 8.44 (m, 1H), 7.89 – 7.79 (m, 1H), 7.44 – 7.28 (m, 6H), 3.79 (s, 2H), 3.73 (s, 2H), 3.15 (t, J = 5.8 Hz, 2H), 2.86 (t, J = 5.8 Hz, 2H).

Mass (ES) *m/z* 337 (M+1).

6-Benzyl-4-chloro-2-pyridin-3-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidine (48). General procedure C was applied. Compound **13** (2.4 g, 8 mmol) was reacted with phosphoryl chloride (3 mL, 34 mmol, 5 equiv) in CH_3CN (20 mL) at 60 °C for 2 h. After work up, the crude was purified by flash chromatography on silica gel (EtOAc/cyclohexane 30:70) to give the chlorinated derivative (0.7 g, yield: 28%) as a brown solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 9.62 – 9.55 (m, 1H), 8.72 – 8.68 (m, 1H), 8.68 – 8.61 (m, 2H), 7.47 – 7.28 (m, 5H), 3.79 (s, 2H), 3.70 (s, 2H), 3.06 (t, J = 5.8 Hz, 2H), 2.86 (t, J = 5.8 Hz, 2H).

Mass (ES) *m/z* 337 (M+1).

General procedure D for the synthesis of 1-(6-*p*-substitutedbenzyl-2-pyridin-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl)-amine (21-23, 27-28, 31-35, 41 and 52). A mixture of the intermediate (compounds **15-18**) (0.32 mmol) obtained by the above described procedure (General ProcedureB), the amine (0.481 mmol, 1.5 equiv) and K_2CO_3 (0.962 mmol, 3 equiv) in anhydrous 1,4-dioxane (4 mL) was stirred at 90 °C overnight in a pressure tube. The solvent was evaporated, the residue was dissolved in water and extracted with EtOAc. The organic phase was dried over Na_2SO_4, filtered and concentrated. The crude residue was purified by column

chromatography (silica gel) using a 2 M NH_3 solution in CH_3OH/DCM gradient followed by preparative basic HPLC column to yield the desired compound as a solid.

1-(6-Benzyl-2-pyridin-2-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl)-piperidin-3-ol (21). The general procedure D for the synthesis of compound **21** was followed, starting with trifluoromethanesulfonic acid 6-benzyl-2-pyridin-2-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl ester **(15)** (100 mg, 0.22 mmol, 1 equiv) and 3-hydroxypiperidine (0.33 mmol, 1.5 equiv). The crude material was purified by SiO_2 column (gradient from 100% DCM to DCM - 2 M methanolic ammonia 99:1) affording 51 mg (yield: 58%) of the title product.

^{1}H NMR (400 MHz, d_6-DMSO) δ 8.70 – 8.66 (m, 1H), 8.30 – 8.24 (m, 1H), 7.95 – 7.86 (m, 1H), 7.49 – 7.42 (m, 1H), 7.38 – 7.32 (m, 4H), 7.31 – 7.24 (m, 1H), 4.90 (d, J = 4.4 Hz, 1H), 3.86 – 3.74 (m, 1H), 3.70 (s, 2H), 3.65 – 3.50 (m, 2H), 3.45 (s, 2H), 3.04 – 2.94 (m, 1H), 2.92 – 2.75 (m, 5H), 1.94 – 1.67 (m, 2H), 1.50 – 1.29 (m, 2H).

Mass (ES) *m/z* 402 (M+1). UPLC (acid method); R_t = 0.75 min. HRMS: calcd for $C_{24}H_{27}N_5O$ + H^+, 402.22884; found (ESI, $[M + H]^+$ obsd), 402.22856.

1-(6-Benzyl-2-pyridin-2-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl)-piperidine-4-carboxylic acid amide (22). The general procedure D for the synthesis of compound **22** was followed, starting with the compound **15** (110 mg, 0.24 mmol, 1 equiv) and isonipecotamide (0.36 mmol, 1.5 equiv). The crude material was purified by SiO_2 column (gradient from 100% DCM to DCM - 2 M methanolic ammonia 99:1) affording 30 mg (yield: 29%) of the title compound.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.84 – 8.76 (m, 1H), 8.43 – 8.35 (m, 1H), 7.86 – 7.75 (m, 1H), 7.43 – 7.28 (m, 6H), 5.44 (d, J = 59.9 Hz, 2H), 3.92 (d, J = 13.3 Hz, 2H), 3.77 (s, 2H), 3.58 (s,

2H), 3.21 – 3.08 (m, 2H), 3.08 – 2.95 (m, 2H), 2.90 (s, 2H), 2.49 – 2.33 (m, 1H), 1.98 (d, J = 13.3 Hz, 2H), 1.90 – 1.73 (m, 2H).

Mass (ES) *m/z* 429 (M+1). UPLC (basic method); R_t = 1.03 min. HRMS: calcd for $C_{25}H_{28}N_6O$ + H^+, 429.23974; found (ESI, $[M + H]^+$ obsd), 429.23934.

2-[4-(6-Benzyl-2-pyridin-2-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl)-piperazin-1-yl]-acetamide (23). The general procedure D for the synthesis of compound **23** was followed, starting with the compound **15** (260 mg, 0.57 mmol, 1 equiv) and 2-piperazin-1-yl-acetamide hydrochloride (0.85 mmol, 1.5 equiv). The crude material was purified by SiO_2 column (gradient from 100% DCM to DCM - 2 M methanolic ammonia 98:2) affording 30 mg (yield: 12%) of the title product.

^{1}H NMR (400 MHz, d_6-DMSO) δ 8.70 – 8.66 (m, 1H), 8.30 – 8.24 (m, 1H), 7.94 – 7.86 (m, 1H), 7.50 – 7.42 (m, 1H), 7.39 – 7.32 (m, 4H), 7.32 – 7.24 (m, 2H), 7.19 – 7.12 (m, 1H), 3.69 (s, 2H), 3.48 – 3.40 (m, 6H), 2.93 – 2.85 (m, 4H), 2.84 – 2.76 (m, 2H), 2.54 – 2.50 (m, 4H).

Mass (ES) *m/z* 444 (M+1). UPLC (basic method); R_t = 1.01 min. HRMS: calcd for $C_{25}H_{29}N_7O$ + H^+, 444.25064; found (ESI, $[M + H]^+$ obsd), 444.25049.

(6-Benzyl-2-pyridin-2-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl)-[3-(4-methyl-piperazin-1-yl)-propyl]-amine (27). The general procedure D for the synthesis of compound **27** was followed, starting with the compound **15** (117 mg, 0.26 mmol, 1 equiv) and 1-(3-aminopropyl)-4-methylpiperazine (0.4 mmol, 1.5 equiv). The crude material was purified by SiO_2 column (gradient from 100% DCM to DCM - 2 M methanolic ammonia 96:4) affording 29 mg (yield: 24%) of the title compound.

^{1}H NMR (400 MHz, d_6-DMSO) δ 8.68 – 8.63 (m, 1H), 8.28 – 8.20 (m, 1H), 7.91 – 7.83 (m, 1H), 7.46 – 7.33 (m, 5H), 7.32 – 7.23 (m, 1H), 6.81 (t, J = 5.6 Hz, 1H), 3.75 (s, 2H), 3.56 – 3.44 (m, 2H), 3.38 (s, 2H), 2.73 (s, 4H), 2.47 – 2.16 (m, 10H), 2.14 (s, 3H), 1.80 – 1.66 (m, 2H).

Mass (ES) *m/z* 458 (M+1). UPLC (basic method); R_t = 1.14 min. HRMS: calcd for $C_{27}H_{35}N_7$ + H^+, 458.30267; found (ESI, $[M + H]^+$ obsd), 458.30225.

{2-[4-(6-Benzyl-2-pyridin-2-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl)-piperazin-1-yl]-ethyl}-dimethyl amine (28). The general procedure D for the synthesis of compound **28** was followed, starting with the compound **15** (117 mg, 0.26 mmol, 1 equiv) and 1-[2-(dimethylamino)ethyl]piperazine (0.4 mmol, 1.5 equiv). The crude material was purified by SiO_2 column (gradient from 100% DCM to DCM - 2 M methanolic ammonia 96:4) affording 12 mg (yield: 10%) of the title compound.

^{1}H NMR (400 MHz, d_6-DMSO) δ 8.71 – 8.59 (m, 1H), 8.30 – 8.19 (m, 1H), 7.95 – 7.77 (m, 1H), 7.49 – 7.40 (m, 1H), 7.39 – 7.18 (m, 5H), 3.68 (s, 2H), 3.41 (br s, 2H), 3.39 – 3.33 (m, 4H), 3.28 (s, 2H), 2.92 – 2.73 (m, 4H), 2.47 – 2.41 (m, 4H), 2.38 (br s, 2H), 2.15 (s, 6H).

Mass (ES) *m/z* 458 (M+1). UPLC (acid method); R_t = 1.28 min. HRMS: calcd for $C_{27}H_{35}N_7$ + H^+, 458.30267; found (ESI, $[M + H]^+$ obsd), 458.30228.

(6-Benzyl-2-pyridin-3-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl)-[3-(4-methyl-piperazin-1-yl)-propyl]-amine (31). The general procedure D for the synthesis of compound **31** was followed, starting with the compound **17** (100 mg, 0.22 mmol, 1 equiv) and 1-(3-aminopropyl)-4-methylpiperazine (0.33 mmol, 1.5 equiv). The crude material was purified by

SiO_2 column (gradient from 100% DCM to DCM - 2 M methanolic ammonia 96:4) affording 18 mg (yield: 18%) of the title compound.

^{1}H NMR (400 MHz, d_6-DMSO) δ 9.45 – 9.38 (m, 1H), 8.67 – 8.60 (m, 1H), 8.59 – 8.52 (m, 1H), 7.50 – 7.43 (m, 1H), 7.42 – 7.33 (m, 4H), 7.32 – 7.22 (m, 1H), 6.91 (t, J = 5.2 Hz, 1H), 3.74 (s, 2H), 3.56 – 3.46 (m, 2H), 3.36 (s, 2H), 2.73 (s, 4H), 2.45 – 2.17 (m, 10H), 2.14 (s, 3H), 1.79 – 1.68 (m, 2H).

Mass (ES) *m/z* 458 (M+1). UPLC (basic method); R_t = 1.28 min. HRMS: calcd for $C_{27}H_{35}N_7$ + H^+, 458.30267; found (ESI, $[M + H]^+$ obsd), 458.30240.

{2-[4-(6-Benzyl-2-pyridin-3-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl)-piperazin-1-yl]-ethyl}-dimethyl amine (32). The general procedure D for the synthesis of compound **32** was followed, starting with the compound **17** (100 mg, 0.22 mmol, 1 equiv) and 1-[2-(dimethylamino)ethyl]piperazine (0.33 mmol, 1.5 equiv). The crude material was purified by SiO_2 column (gradient from 100% DCM to DCM - 2 M methanolic ammonia 96:4) affording 16 mg (yield: 16%) of the title compound.

^{1}H NMR (400 MHz, d_6-DMSO) δ 9.47 – 9.33 (m, 1H), 8.72 – 8.42 (m, 2H), 7.56 – 7.46 (m, 1H), 7.41 – 7.17 (m, 5H), 3.69 (s, 2H), 3.46 – 3.36 (m, 6H), 2.93 – 2.74 (m, 4H), 2.48 – 2.44 (m, 4H), 2.43 – 2.28 (m, 4H), 2.14 (s, 6H).

Mass (ES) *m/z* 458 (M+1). UPLC (basic method); R_t = 1.48 min. HRMS: calcd for $C_{27}H_{35}N_7$ + H^+, 458.30267; found (ESI, $[M + H]^+$ obsd), 458.30230.

(6-Benzyl-2-pyridin-4-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl)-[3-(4-methylpiperazin-1-yl)-propyl]-amine (33). The general procedure D for the synthesis of

compound **33** was followed, starting with the compound **18** (117 mg, 0.26 mmol, 1 equiv) and 1-(3-aminopropyl)-4-methylpiperazine (0.4 mmol, 1.5 equiv). The crude material was purified by SiO_2 column (gradient from 100% DCM to DCM - 2 M methanolic ammonia 96:4) affording 17 mg (yield: 14%) of the title compound.

^{1}H NMR (400 MHz, d_6-DMSO) δ 8.67 (dd, J = 4.5, 1.6 Hz, 2H), 8.16 (dd, J = 4.5, 1.6 Hz, 2H), 7.43 – 7.32 (m, 4H), 7.32 – 7.24 (m, 1H), 6.95 (t, J = 5.5 Hz, 1H), 3.74 (s, 2H), 3.56 – 3.48 (m, 2H), 3.37 (s, 2H), 2.80 – 2.70 (m, 4H), 2.46 – 2.20 (m, 8H), 2.14 (s, 3H), 1.80 – 1.66 (m, 2H).

Mass (ES) *m/z* 458 (M+1). UPLC (basic method); R_t = 1.35 min. HRMS: calcd for $C_{27}H_{35}N_7$ + H^+, 458.30267; found (ESI, $[M + H]^+$ obsd), 458.30222.

{2-[4-(6-Benzyl-2-pyridin-4-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl)-piperazin-1-yl]-ethyl}-dimethyl amine (34). The general procedure D for the synthesis of compound **34** was followed, starting with the compound **18** (117 mg, 0.26 mmol, 1 equiv) and 1-[2-(dimethylamino)ethyl]piperazine (0.4 mmol, 1.5 equiv). The crude material was purified by SiO_2 column (gradient from 100% DCM to DCM - 2 M methanolic ammonia 96:4) affording 32 mg (yield: 27%) of the title compound.

^{1}H NMR (400 MHz, d_6-DMSO) δ 8.69 (dd, J = 4.5, 1.6 Hz, 2H), 8.16 (dd, J = 4.5, 1.6 Hz, 2H), 7.38 – 7.32 (m, 4H), 7.32 – 7.25 (m, 1H), 3.69 (s, 2H), 3.47 – 3.38 (m, 6H), 2.94 – 2.74 (m, 4H), 2.49 – 2.44 (m, 4H), 2.43 – 2.29 (m, 4H), 2.14 (s, 6H).

Mass (ES) *m/z* 458 (M+1). UPLC (basic method); R_t = 1.56 min. HRMS: calcd for $C_{27}H_{35}N_7$ + H^+, 458.30267; found (ESI, $[M + H]^+$ obsd), 458.30225.

(2-{4-[6-(4-Methoxy-benzyl)-2-pyridin-2-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl]-piperazin-1-yl}-ethyl)-dimethyl amine (35). The general procedure D for the synthesis of compound **35** was followed, starting with the compound **16** (100 mg, 0.20 mmol, 1 equiv) and 1-[2-(dimethylamino)ethyl]piperazine (0.30 mmol, 1.5 equiv). The crude material was purified by SiO_2 column (gradient from 100% DCM to DCM - 2 M methanolic ammonia 97:3) affording 12 mg (yield: 12%) of the title compound.

^{1}H NMR (400 MHz, d_6-DMSO) δ 8.68 – 8.63 (m, 1H), 8.27 – 8.21 (m, 1H), 7.93 – 7.84 (m, 1H), 7.47 – 7.40 (m, 1H), 7.24 (d, J = 8.6 Hz, 2H), 6.89 (d, J = 8.6 Hz, 2H), 3.73 (s, 3H), 3.60 (s, 2H), 3.43 – 3.33 (m, 6H), 2.93 – 2.70 (m, 4H), 2.47 – 2.42 (m, 4H), 2.41 – 2.27 (m, 4H), 2.12 (s, 6H).

Mass (ES) *m/z* 488 (M+1). UPLC (acid method); R_t = 0.59 min. HRMS: calcd for $C_{28}H_{37}N_7O$ + H^+, 488.31324; found (ESI, $[M + H]^+$ obsd), 488.31314.

N-{1-[6-(4-methoxybenzyl)-2-pyridin-2-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl]-pyrrolidin-3-yl}-N-ethyl-acetamide (41). The general procedure D for the synthesis of compound **41** was followed, starting with the compound **16** (100 mg, 0.20 mmol, 1 equiv) and 3-(N-acetyl-N-ethylamino)pyrrolidine (0.31 mmol, 1.5 equiv). The crude material was purified by SiO_2 column (gradient from 100% DCM to DCM - 2 M methanolic ammonia 98:2) followed by preparative basic HPLC column affording 20 mg (yield: 20%) of the title compound.

^{1}H NMR (400 MHz, d_6-DMSO) δ 8.73 – 8.56 (m, 1H), 8.31 – 8.14 (m, 1H), 7.96 – 7.74 (m, 1H), 7.50 – 7.33 (m, 1H), 7.30 – 7.23 (m, 2H), 6.96 – 6.84 (m, 2H), 4.81 – 4.30 (m, 1H), 4.03 – 3.36 (m, 12H), 3.21 – 2.52 (m, 5H), 2.20 – 1.83 (m, 5H), 1.18 – 0.91 (m, 3H).

Mass (ES) *m/z* 487 (M+1). UPLC (acid method); R_t = 0.83 min. HRMS: calcd for $C_{28}H_{34}N_6O_2$ + H^+, 487.28160; found (ESI, $[M + H]^+$ obsd), 487.28162.

General procedure E for the synthesis of 1-(6-*p*-substitutedbenzyl-2-pyridin-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl)-amine (29-30, 36-40, 42-45). The chloro intermediate (obtained with General Procedure C) (200 mg, 0.56 mmol, 1 equiv) was treated with the corresponding amine (0.84 mmol, 1.5 equiv) and Et_3N (1.12 mmol, 157 µL, 2 equiv) in DMF (3 mL). The mixture was heated at 90 °C overnight in a pressure tube, cooled, concentrated and partitioned between CH_2Cl_2 and 1 M NaOH. The layers were separated and the aqueous layer was extracted with CH_2Cl_2. The combined CH_2Cl_2 layers were dried ($MgSO_4$), filtered, and concentrated in vacuo. The residue was purified by column chromatography on silica gel (gradient from DCM - 2 M methanolic ammonia 99.5:0.5 to 98:2) and by preparative basic HPLC column to provide the desired product.

1-(6-Benzyl-2-pyridin-3-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl)-piperidine-4-carboxylic acid amide (29). The general procedure E for the synthesis of compound **29** was followed, starting with the compound **48** (172 mg, 0.51 mmol, 1 equiv) and isonipecotamide (0.77 mmol, 1.5 equiv). The purification of crude material afforded 80 mg (**yield**: 36%) of the title compound.

^{1}H NMR (400 MHz, d_6-DMSO) δ 9.46 – 9.39 (m, 1H), 8.70 – 8.62 (m, 1H), 8.61 – 8.51 (m, 1H), 7.56 – 7.46 (m, 1H), 7.39 – 7.32 (m, 4H), 7.31 – 7.22 (m, 2H), 6.79 (s, 1H), 3.91 (d, J = 13.2 Hz, 2H), 3.71 (s, 2H), 3.44 (s, 2H), 3.01 – 2.93 (m, 2H), 2.92 – 2.73 (m, 4H), 2.44 – 2.31 (m, 1H), 1.83 – 1.73 (m, 2H), 1.66 – 1.52 (m, 2H).

Mass (ES) *m/z* 429 (M+1). HPLC (basic method, 10 min); R_t = 2.50 min. HRMS: calcd for $C_{25}H_{28}N_6O + H^+$, 429.23974; found (ESI, $[M + H]^+$ obsd), 429.23990.

2-[4-(6-Benzyl-2-pyridin-3-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl)-piperazin-1-yl]-acetamide (30). The general procedure E for the synthesis of compound **30** was followed, starting with the compound **48** (172 mg, 0.51 mmol, 1 equiv) and 2-piperazin-1-yl-acetamide hydrochloride (0.77 mmol, 1.5 equiv). The purification of crude material afforded 38 mg (**yield**: 17%) of the title compound.

^{1}H NMR (400 MHz, CD_3OD) δ 9.45 – 9.38 (m, 1H), 8.75 – 8.67 (m, 1H), 8.62 – 8.56 (m, 1H), 7.57 – 7.49 (m, 1H), 7.42 – 7.28 (m, 5H), 3.76 (s, 2H), 3.59 – 3.51 (m, 4H), 3.48 (s, 2H), 3.04 (s, 2H), 3.03 – 2.98 (m, 2H), 2.96 – 2.88 (m, 2H), 2.65 – 2.57 (m, 4H).

Mass (ES) *m/z* 444 (M+1). HPLC (basic method, 10 min); R_t = 2.52 min. HRMS: calcd for $C_{25}H_{29}N_7O + H^+$, 444.25064; found (ESI, $[M + H]^+$ obsd), 444.25051.

(2-{4-[6-(4-Fluorobenzyl)-2-pyridin-2-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl]-piperazin-1-yl}-ethyl)-dimethyl amine (36). The general procedure E for the synthesis of compound **36** was followed, starting with the compound **19** (200 mg, 0.56 mmol, 1 equiv) and 1-[2-(dimethylamino)ethyl]piperazine (0.84 mmol, 1.5 equiv). In this case the reaction quench was performed with water. The purification of crude material afforded 17.5 mg (**yield**: 7%) of the title compound.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.83 – 8.78 (m, 1H), 8.43 – 8.35 (m, 1H), 7.83 – 7.74 (m, 1H), 7.38 – 7.29 (m, 3H), 7.09 – 6.98 (m, 2H), 3.68 (s, 2H), 3.53 – 3.42 (m, 6H), 3.11 (t, J = 6.1 Hz, 2H), 2.83 (t, J = 6.1 Hz, 2H), 2.66 – 2.46 (m, 8H), 2.34 (s, 6H).

Mass (ES) *m/z* 476 (M+1). UPLC (acid method); R_t = 0.59 min. HRMS: calcd for $C_{27}H_{34}N_7F$ + H^+, 476.29325; found (ESI, $[M + H]^+$ obsd), 476.29295.

2-{4-[6-(4-Methoxybenzyl)-2-pyridin-2-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl]-piperazin-1-yl}-ethanol (37). The general procedure E for the synthesis of compound **37** was followed, starting with the compound **46** (261 mg, 0.71 mmol, 1 equiv) and 1-(2-hydroxyethyl)piperazine (1.06 mmol, 1.5 equiv). In this case the reaction quench was performed with water. The purification of crude material afforded 13.5 mg (yield: 4%) of the title compound.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.83 – 8.77 (m, 1H), 8.43 – 8.36 (m, 1H), 7.84 – 7.75 (m, 1H), 7.37 – 7.30 (m, 1H), 7.29 – 7.26 (m, 1H), 7.26 – 7.24 (m, 1H), 6.93 – 6.85 (m, 2H), 3.81 (s, 3H), 3.71 – 3.63 (m, 4H), 3.55 – 3.42 (m, 6H), 3.11 (t, J = 6.1 Hz, 2H), 2.83 (t, J = 6.1 Hz, 2H), 2.67 – 2.53 (m, 6H).

Mass (ES) *m/z* 461 (M+1). HPLC (basic method, 10 min); R_t = 2.23 min. HRMS: calcd for $C_{26}H_{32}N_6O_2$ + H^+, 461.26595; found (ESI, $[M + H]^+$ obsd), 461.26573.

1-[6-(4-Methoxybenzyl)-2-pyridin-2-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl]-piperidine-4-carboxylic acid amide (38). The general procedure E for the synthesis of compound **38** was followed, starting the compound **46** (261 mg, 0.71 mmol, 1 equiv) and isonipecotamide (1.06 mmol, 1.5 equiv). In this case the reaction quench was performed with water. The purification of crude material afforded 22.3 mg (yield: 7%) of the title compound.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.83 – 8.77 (m, 1H), 8.44 – 8.35 (m, 1H), 7.83 – 7.74 (m, 1H), 7.36 – 7.30 (m, 1H), 7.30 – 7.26 (m, 2H), 6.92 – 6.85 (m, 2H), 5.39 (d, J = 65.8 Hz, 2H), 4.00 –

3.87 (m, 2H), 3.82 (s, 3H), 3.66 (s, 2H), 3.49 (s, 2H), 3.10 (t, J = 6.1 Hz, 2H), 3.04 – 2.93 (m, 2H), 2.83 (t, J = 6.1 Hz, 2H), 2.49 – 2.33 (m, 1H), 2.03 – 1.92 (m, 2H), 1.90 – 1.70 (m, 2H).

Mass (ES) *m/z* 459 (M+1). HPLC (basic method, 10 min); R_t = 2.18 min. HRMS: calcd for $C_{26}H_{30}N_6O_2 + H^+$, 459.25030; found (ESI, $[M + H]^+$ obsd), 459.24992.

(3-Imidazol-1-ylpropyl)-[6-(4-methoxybenzyl)-2-pyridin-2-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl]-amine (39). The general procedure E for the synthesis of compound **39** was followed, starting with the compound **46** (500 mg, 1.36 mmol, 1 equiv) and 1-(3-aminopropyl)-imidazole (2.04 mmol, 1.5 equiv). In this case the reaction quench was performed with water. The purification of crude material afforded 22.4 mg (yield: 4%) of the title compound.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.84 – 8.77 (m, 1H), 8.33 – 8.27 (m, 1H), 7.85 – 7.76 (m, 1H), 7.50 (s, 1H), 7.36 – 7.33 (m, 1H), 7.31 (d, J = 8.6 Hz, 2H), 7.09 – 7.04 (m, 1H), 6.96 – 6.93 (m, 1H), 6.90 (d, J = 8.6 Hz, 2H), 4.29 (t, J = 5.8 Hz, 1H), 4.06 (t, J = 6.8 Hz, 2H), 3.82 (s, 3H), 3.71 (s, 2H), 3.66 (dd, J = 12.8, 6.8 Hz, 2H), 3.28 (s, 2H), 3.04 (t, J = 5.8 Hz, 2H), 2.83 (t, J = 5.8 Hz, 2H), 2.22 – 2.15 (m J = 6.8 Hz, 2H).

Mass (ES) *m/z* 456 (M+1). HPLC (basic method, 10 min); R_t = 2.60 min. HRMS: calcd for $C_{26}H_{29}N_7O + H^+$, 456.25064; found (ESI, $[M + H]^+$ obsd), 456.25027.

2-{4-[6-(4-Methoxybenzyl)-2-pyridin-2-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl]-piperazin-1-yl}-acetamide (40). The general procedure E for the synthesis of compound **40** was followed, starting with the compound **46** (500 mg, 1.36 mmol, 1 equiv) and 2-piperazin-1-yl-

acetamide hydrochloride (2.04 mmol, 1.5 equiv). In this case the reaction quench was performed with water. The purification of crude material afforded 13 mg (yield: 2%) of the title compound.

^{1}H NMR (400 MHz, d_6-DMSO) δ 8.68 – 8.63 (m, 1H), 8.28 – 8.20 (m, 1H), 7.91 – 7.83 (m, 1H), 7.46 – 7.33 (m, 5H), 7.32 – 7.23 (m, 1H), 6.81 (t, J = 5.6 Hz, 1H), 3.75 (s, 2H), 3.56 – 3.44 (m, 2H), 3.38 (s, 2H), 2.73 (s, 4H), 2.47 – 2.16 (m, 10H), 2.14 (s, 3H), 1.80 – 1.66 (m, 2H).

Mass (ES) *m/z* 474 (M+1). HPLC (basic method, 10 min); R_t = 2.40 min. HRMS: calcd for $C_{26}H_{31}N_7O_2 + H^+$, 474.26120; found (ESI, $[M + H]^+$ obsd), 474.26126.

N-{1-[6-(4-Chlorobenzyl)-2-pyridin-2-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl]-pyrrolidin-3-yl}-N-ethyl-acetamide (42). The general procedure E for the synthesis of compound **42** was followed, starting with the compound **20** (100 mg, 0.27 mmol, 1 equiv) and 3-(N-acetyl-N-ethylamino)pyrrolidine (0.40 mmol, 1.5 equiv). In this case the reaction quench was performed with water. The purification of crude material afforded 41 mg (yield: 31%) of the title compound.

^{1}H NMR (400 MHz, d_6-DMSO) δ 8.69 – 8.61 (m, 1H), 8.29 – 8.20 (m, 1H), 7.91 – 7.82 (m, 1H), 7.46 – 7.41 (m, 1H), 7.40 – 7.34 (m, 4H), 4.77 – 4.36 (m, 1H), 3.94 – 3.57 (m, 7H), 3.53 – 3.35 (m, 2H), 3.31 – 3.06 (m, 1H), 2.94 – 2.71 (m, 3H), 2.71 – 2.53 (m, 1H), 2.20 – 1.91 (m, 5H), 1.18 – 0.94 (m, 3H).

Mass (ES) *m/z* 491 (M+1). HPLC (basic method, 10 min); R_t = 2.90 min. HRMS: calcd for $C_{27}H_{31}N_6OCl + H^+$, 491.23206; found (ESI, $[M + H]^+$ obsd), 491.23190.

N-{1-[6-(4-Chlorobenzyl)-2-pyridin-2-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl]-piperidin-4-yl}-acetamide (43). The general procedure E for the synthesis of compound **43** was

followed, starting with the compound **20** (100 mg, 0.27 mmol, 1 equiv) and 4-acetamidopiperidine (0.40 mmol, 1.5 equiv). In this case the reaction quench was performed with water. The purification of crude material afforded 66 mg (yield: 52%) of the title compound.

^{1}H NMR (400 MHz, d_6-DMSO) δ 8.73 – 8.62 (m, 1H), 8.33 – 8.23 (m, 1H), 7.96 – 7.87 (m, 1H), 7.83 (d, J = 7.7 Hz, 1H), 7.48 – 7.43 (m, 1H), 7.43 – 7.35 (m, 4H), 3.93 – 3.75 (m, 3H), 3.70 (s, 2H), 3.44 (s, 2H), 3.10 – 2.93 (m, 2H), 2.94 – 2.71 (m, 4H), 1.88 – 1.70 (m, 5H), 1.52 – 1.30 (m, 2H).

Mass (ES) *m/z* 477 (M+1). UPLC (acid method); R_t = 0.86 min. HRMS: calcd for $C_{26}H_{29}N_6OCl$ + H^+, 477.21641; found (ESI, $[M + H]^+$ obsd), 477.21608.

N-{2-[6-(4-Fluorobenzyl)-2-pyridin-2-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl]-aminoethyl}-acetamide (44). The general procedure E for the synthesis of compound **44** was followed, starting with the compound **19** (110 mg, 0.31 mmol, 1 equiv) and N-(2-aminoethyl)acetamide (0.46 mmol, 1.5 equiv). In this case the reaction quench was performed with water. The purification of crude material afforded 9.4 mg (yield: 7%) of the title compound.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.81 – 8.69 (m, 1H), 8.46 – 8.35 (m, 1H), 7.88 – 7.76 (m, 1H), 7.45 (br s, J = 4.4 Hz, 1H), 7.40 – 7.31 (m, 3H), 7.11 – 6.90 (m, 2H), 5.34 (t, J = 5.3 Hz, 1H), 3.85 – 3.65 (m, 4H), 3.65 – 3.44 (m, 2H), 3.34 (s, 2H), 3.04 – 2.91 (m, 2H), 2.91 – 2.68 (m, 2H), 1.90 (s, 3H).

Mass (ES) *m/z* 421 (M+1). UPLC (acid method); R_t = 0.70 min. HRMS: calcd for $C_{23}H_{25}N_6OF$ + H^+, 421.21466; found (ESI, $[M + H]^+$ obsd), 421.21481.

[6-(4-Fluorobenzyl)-2-pyridin-2-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl]-(2-pyrrolidin-1-yl-ethyl)-amine (45). The general procedure E for the synthesis of compound **45** was followed, starting with the compound **19** (110 mg, 0.31 mmol, 1 equiv) and 1-(2-aminoethyl)-pyrrolidine (0.46 mmol, 1.5 equiv). In this case the reaction quench was performed with water. The purification of crude material afforded 9.8 mg (yield: 6%) of the title compound.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.82 – 8.75 (m, 1H), 7.82 – 7.73 (m, 1H), 7.39 – 7.33 (m, 2H), 7.33 – 7.28 (m, 1H), 7.09 – 6.98 (m, 2H), 5.19 (br s, J = 4.2 Hz, 1H), 3.72 (s, 2H), 3.71 – 3.64 (m, 2H), 3.32 (s, 2H), 3.06 – 2.96 (m, 2H), 2.87 – 2.68 (m, 4H), 2.59 – 2.48 (m, 4H), 1.84 – 1.66 (m, 4H).

Mass (ES) *m/z* 433 (M+1). UPLC (acid method); R_t = 0.65 min. HRMS: calcd for $C_{25}H_{29}N_6F$ + H^+, 433.25105; found (ESI, $[M + H]^+$ obsd), 433.25121.

4-Hydroxy-2-pyridin-2-yl-7,8-dihydro-5H-pyrido[4,3-d]pyrimidine-6-carboxylic acid benzyl ester (50). 50 was prepared according to general procedure A, using the commercially available compound **49** (2.1 g, 7.3 mmol) and 2-amidinopyridine hydrochloride (1.14 g, 7.3 mmol) to afford **50** as off white solid (2.4 g, yield: 91%) which was used in the next step without further purification.

^{1}H NMR (400 MHz, d_6-DMSO) δ 12.21 (s, 1H), 8.78 – 8.68 (m, 1H), 8.35 – 8.22 (m, 1H), 8.12 – 7.95 (m, 1H), 7.70 – 7.56 (m, 1H), 7.45 – 7.25 (m, 5H), 5.14 (s, 2H), 4.32 (br s, 2H), 3.71 (br s, 2H), 2.81 – 2.68 (m, 2H).

Mass (ES) *m/z* 363 (M+1).

4-Chloro-2-pyridin-2-yl-7,8-dihydro-5H-pyrido[4,3-d]pyrimidine-6-carboxylic acid benzyl ester (51). The general procedure C was followed to obtain the compound **51**. The reaction of **50** (200 mg, 0.55 mmol) with phosphoryl chloride (0.1 mL, 1.1 mmol, 2 equiv) in CH_3CN (3 mL) was heated at 60 °C for 2 h to give the chlorinated derivative (209 mg, yield: 100%) as a brown solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.87 – 8.76 (m, 1H), 8.53 – 8.37 (m, 1H), 7.93 – 7.76 (m, 1H), 7.51 – 7.30 (m, 6H), 5.20 (s, 2H), 4.69 (s, 2H), 3.92 – 3.76 (m, 2H), 3.13 (br s, 2H).

Mass (ES) *m/z* 381 (M+1).

4-[4-(2-Dimethylamino-ethyl)-piperazin-1-yl]-2-pyridin-2-yl-7,8-dihydro-5H-pyrido[4,3-d]pyrimidine-6-carboxylic acid benzyl ester (52). The general procedure D for the synthesis of compound **52** was followed, starting with the compound **51** (1.5 g, 4 mmol, 1 equiv) and 1-[2-(dimethylamino)ethyl]piperazine (5.9 mmol, 1.5 equiv) in CH_3CN (30 mL). The residue was purified by flash chromatography on silica gel (gradient from 100% DCM to 30% MeOH/DCM) to provide 1.85 g (yield: 84%) of the title compound.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.84 – 8.77 (m, 1H), 8.46 – 8.39 (m, 1H), 7.85 – 7.77 (m, 1H), 7.42 – 7.29 (m, 6H), 5.17 (s, 2H), 4.56 (s, 2H), 3.83 (t, J = 6.3 Hz, 2H), 3.52 (br s, 4H), 3.13 (br s, 2H), 2.65 (br s, 4H), 2.60 – 2.39 (m, 4H), 2.29 (s, 6H).

Mass (ES) *m/z* 502 (M+1).

Dimethyl-{2-[4-(2-pyridin-2-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl)-piperazin-1-yl]-ethyl}-amine (53). A suspension of compound **52** (1 equiv, 3.1 g, 6.3 mmol) and Pd/C (5 percent) in EtOH (30 mL) was hydrogenated at 4 atm hydrogen pressure for 1 day at rt and for 1

day al 50 °C. The catalyst was filtered off and the filtrate was evaporated down in vacuo. 4.5 g of a colorless oil was recovered (yield: 100%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.81 – 8.73 (m, 1H), 8.43 – 8.36 (m, 1H), 7.83 – 7.72 (m, 1H), 7.37 – 7.28 (m, 1H), 3.85 (s, 2H), 3.53 – 3.45 (m, 4H), 3.24 (t, J = 6.2 Hz, 2H), 3.04 (t, J = 6.2 Hz, 2H), 2.66 – 2.61 (m, 4H), 2.59 – 2.46 (m, 4H), 2.30 (s, 6H).

Mass (ES) *m/z* 368 (M+1).

General procedure F for the synthesis of {2-[4-(6-substituted-2-pyridin-2-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl)-piperazin-1-yl]-ethyl}-dimethyl amines (54-56). A solution of compound **53** (0.32 mmol, 1 equiv), aldehyde (0.65 mmol, 2 equiv), titanium(IV) isopropoxide (0.7 mmol, 2.2 equiv) and acetic acid (560 µl, 9.81 mmol) in DCE (3 ml) was stirred at rt for 1 h. $NaBH(OAc)_3$ (1.63 mmol, 5 equiv) was added and the reaction mixture was allowed to stir for overnight. The mixture was diluted with 1 N NaOH and extracted with DCM, the combined organic layers were dried and concentrated. The resulting residue was purified on a SCX cartridge followed by preparative HPLC column, giving the title compound as a white solid.

Dimethyl-{2-[4-(2-pyridin-2-yl-6-thiophen-3-yl-methyl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl)-piperazin-1-yl]-ethyl}-amine (54). The general procedure F for the synthesis of compound **54** was followed, starting with the compound **53** (120 mg, 0.33 mmol, 1 equiv) and 3-thiophenecarboxyaldehyde (0.65 mmol, 2 equiv). The resulting residue was purified on a SCX cartridge followed by basic preparative HPLC column, giving 47 mg (yield: 31%) of the title compound.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.83 – 8.76 (m, 1H), 8.45 – 8.35 (m, 1H), 7.85 – 7.73 (m, 1H), 7.34 – 7.32 (m, 1H), 7.32 – 7.29 (m, 1H), 7.21 – 7.14 (m, 1H), 7.12 – 7.05 (m, 1H), 3.74 (s, 2H), 3.58 – 3.40 (m, 6H), 3.11 (t, J = 6.1 Hz, 2H), 2.84 (t, J = 6.1 Hz, 2H), 2.68 – 2.39 (m, 8H), 2.28 (s, 6H).

Mass (ES) *m/z* 464 (M+1). HPLC (basic method, 10 min); R_t = 2.50 min. HRMS: calcd for $C_{25}H_{33}N_7S + H^+$, 464.25909; found (ESI, $[M + H]^+$ obsd), 464.25899.

(2-{4-[6-(4-Isopropylbenzyl)-2-pyridin-2-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl]-piperazin-1-yl}-ethyl)-dimethyl amine (55). The general procedure F for the synthesis of compound **55** was followed, starting with the compound **53** (120 mg, 0.3 mmol, 1 equiv) and cuminaldehyde (0.65 mmol, 2 equiv). The resulting residue was purified on a SCX cartridge followed by acid preparative HPLC column, giving 52 mg (yield: 32%) of the title compound.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.83 – 8.76 (m, 1H), 8.42 – 8.34 (m, 1H), 7.83 – 7.74 (m, 1H), 7.36 – 7.30 (m, 1H), 7.29 – 7.26 (m, 2H), 7.23 – 7.18 (m, 2H), 3.68 (s, 2H), 3.52 – 3.45 (m, 6H), 3.11 (t, J = 6.1 Hz, 2H), 2.99 – 2.87 (m, 1H), 2.84 (t, J = 6.1 Hz, 2H), 2.65 – 2.53 (m, 8H), 2.36 (s, 6H), 1.28 – 1.24 (m, 6H).

Mass (ES) *m/z* 500 (M+1). HPLC (basic method, 10 min); R_t = 2.50 min. HRMS: calcd for $C_{30}H_{41}N_7 + H^+$, 500.34962; found (ESI, $[M + H]^+$ obsd), 500.34994.

{2-[4-(6-Cyclohexylmethyl-2-pyridin-2-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl)-piperazin-1-yl]-ethyl}-dimethyl amine (56). The general procedure F for the synthesis of compound **56** was followed, starting with the compound **53** (120 mg, 0.33 mmol, 1 equiv) and

cyclohexanecarboxyladehyde (0.65 mmol, 2 equiv). The resulting residue was purified on a SCX cartridge followed by basic preparative HPLC column, giving 26 mg (yield: 17%) of the title compound.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.83 – 8.76 (m, 1H), 8.43 – 8.35 (m, 1H), 7.83 – 7.75 (m, 1H), 7.36 – 7.29 (m, 1H), 3.56 – 3.48 (m, 4H), 3.43 (s, 2H), 3.11 (t, J = 6.1 Hz, 2H), 2.78 (t, J = 6.1 Hz, 2H), 2.69 – 2.61 (m, 4H), 2.61 – 2.47 (m, 4H), 2.41 – 2.23 (m, 8H), 1.99 – 1.64 (m, 5H), 1.63 – 1.50 (m, 1H), 1.33 – 1.10 (m, 3H), 1.03 – 0.80 (m, 2H).

Mass (ES) *m/z* 464 (M+1). HPLC (basic method, 10 min); R_t = 3.58 min. HRMS: calcd for $C_{27}H_{41}N_7 + H^+$, 464.34962; found (ESI, $[M + H]^+$ obsd), 464.34938.

{4-[4-(2-Dimethylamino-ethyl)-piperazin-1-yl]-2-pyridin-2-yl-7,8-dihydro-5H-pyrido[4,3-d]pyrimidin-6-yl}-phenyl methanone (57). O-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium-hexafluorophosphate (HATU) (1.23 mmol, 1.1 equiv), DIPEA (0.47 mL, 2.7 mmol, 2.2 equiv) were added to benzoic acid (1.23 mmol, 1.1 equiv) in CH_3CN (2 mL) and the activation of the acid occurred in 1 h at rt. Then, compound **53** (400 mg, 1.1 mmol, 1 equiv) was added to the resulting solution and the reaction mixture was stirred for 48 h at rt. The reaction mixture was purified by NH_2-column, SCX-column, column chromatography on silica gel (gradient DCM - 2 M methanolic ammonia) and by basic preparative HPLC to obtain 12 mg (yield: 2%) of the title compound **(57)**.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.87 – 8.71 (m, 1H), 8.48 – 8.35 (m, 1H), 7.91 – 7.72 (m, 1H), 7.55 – 7.30 (m, 6H), 5.08 – 4.24 (m, 2H), 4.20 – 2.94 (m, 8H), 2.91 – 2.37 (m, 8H), 2.27 (s, 6H).

Mass (ES) *m/z* 472 (M+1). HPLC (basic method, 10 min); R_t = 2.17 min. HRMS: calcd for $C_{27}H_{33}N_7O + H^+$, 472.28194; found (ESI, $[M + H]^+$ obsd), 472.28152.

{2-[4-(6-Benzenesulfonyl-2-pyridin-2-yl-5,6,7,8-tetrahydropyrido[4,3-d]pyrimidin-4-yl)-piperazin-1-yl]-ethyl}-dimethyl amine (58). The compound **53** (100 mg, 0.3 mmol, 1 equiv) and TEA (0.046 mL, 0.32 mmol) was dissolved in DCM (2 mL), after ten minutes benzenesulfonyl chloride (0.3 mmol, 1 equiv) was added to the resulting solution and the reaction mixture was stirred for 2 h at rt. To the reaction mixture 1 N NaOH was added, the aqueous phase was extracted with DCM and the combined organic layers were dried over Na_2SO_4 and were concentrated. The residue was purified by SCX-column and column chromatography on silica gel (gradient DCM - 2 M methanolic ammonia) to give 20 mg (yield: 15%) of the title compound.

^{1}H NMR (400 MHz, CD_3OD) δ 8.68 – 8.67 (m, 1H), 8.36 – 8.34 (m, 1H), 7.97 – 7.93 (m, 1H), 7.81 – 7.79 (m, 2H), 7.56 – 7.49 (m, 4H), 4.28 (s, 2H), 3.65 (t, J = 6.5 Hz, 2H), 3.57 – 3.54 (m, 4H), 3.16 (br s, 2H), 2.98 (t, J = 6.5 Hz, 2H), 2.81 – 2.74 (m, 12H).

Mass (ES) *m/z* 508 (M+1). HPLC (basic method, 10 min); R_t = 2.61 min. HRMS: calcd for $C_{26}H_{33}N_7O_2S + H^+$, 508.24892; found (ESI, $[M + H]^+$ obsd), 508.24867.

Cell culture. Clonal populations of Fischer rat thyroid (FRT) and A549 cells stably coexpressing human CFTR and the high-sensitivity halide-sensing yellow fluorescent protein (YFP)-H148Q/I152L[19] were generated as described previously.[26] FRT cells were cultured in Coon's modified Ham's F-12 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. A549 cells were cultured in Dulbecco's modified Eagle's medium (DMEM)-Ham's F-12 (1:1) medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. For

fluorescence assays of CFTR activity, FRT and A549 cells were plated (50,000 cells/well) on clear-bottomed 96-well black microplates (Corning Life Sciences, Acton, MA). For Ussing chamber experiments, FRT cells were seeded into Snapwell permeable supports (Corning Life Sciences) at 500,000 cells per insert.

Fluorescence assay for CFTR activity. FRT and A549 cells ex- pressing F508del-CFTR were grown at 37°C (90% humidity; 5% CO_2) for 18–24 h and then incubated for 18–24 h at 27°C (to rescue mutant CFTR to the plasma membrane, for potentiator testing) or at 37°C with medium containing test compounds (for corrector testing). At the time of assay, the cells were washed with PBS (containing 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$, and 0.5 mM $MgCl_2$) and then incubated for 25 min with PBS containing forskolin (20 μM) plus test compounds (for potentiator testing) or plus genistein (50 μM; for corrector testing). Cells were then transferred to a microplate reader (FluoStar Optima; BMG Labtech, Offenburg, Germany) for CFTR activity determination. The plate reader was equipped with high-quality excitation (HQ500/20X: 500 ± 10 nm) and emission (HQ535/30M: 535 ± 15 nm) filters for YFP (Chroma Technology, Brattleboro, VT). Each assay consisted of a continuous 14-s fluorescence reading with 2 s before and 12 s after injection of an iodide-containing solution (PBS with Cl^- replaced by I^-; final I^- concentration 100 mM). Data were normalized to the initial background-subtracted fluorescence. To determine I^- influx rate, the final 11 s of the data for each well were fitted with an exponential function to extrapolate initial slope (dF/dt).

Transepithelial current measurements. Short-circuit current measurements were performed on the same FRT cells coexpressing CFTR and YFP-H148Q/I152L used for the fluorescence assay for CFTR activity. Experiments on FRT cells were performed 7–10 days after seeding. Snapwell inserts were mounted in a self-contained Ussing chamber system (vertical diffusion

chamber; Corning Life Sciences). Transepithelial currents were measured with a transepithelial Cl^- gradient. Accordingly, the basolateral solution contained 130 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM Na-Hepes (pH 7.3), and 10 mM glucose. For the apical side, this solution was modified by replacing half of the NaCl with sodium gluconate and increasing CaCl2 to 2 mM to compensate for calcium buffering caused by gluconate. During experiments, solutions in both chambers were continuously bubbled with air. The hemichambers were connected to DVC-1000 voltage clamps (World Precision Instruments, Sarasota, FL) via Ag/AgCl electrodes and 1 M KCl agar bridges. Transepithelial currents were digitized with PowerLab 4/25 data acquisition systems and stored on Macintosh computers. All measurements were performed at 37°C.

Solubility Assay. Standard and sample solutions were prepared from a 10 mM DMSO stock solution using an automated dilution procedure. For each compound, three solutions were prepared: one to be used as standard and the other two as test solutions. Standard: 250 μM standard solution in acetonitrile/buffer, with a final DMSO content of 2.5% (v/v). Test sample for pH 3.0: 250 μM sample solution in acetic acid 50 mM, pH = 3, with a final DMSO content of 2.5% (v/v). Test sample for pH 7.4: a 250 μM sample solution in ammonium acetate buffer 50 mM, pH = 7.4, with a final DMSO content of 2.5% (v/v). The 250 μM product suspensions/solutions in the aqueous buffers were prepared directly in Millipore MultiScreen-96 filter plates (0.4 μm PTCE membrane) and sealed. Plates were left for 24 h at room temperature under orbital shaking to achieve “pseudo-thermodynamic equilibrium” and to presaturate the membrane filter. Product suspensions/solutions were then filtered using centrifugation, diluted 1:2 with the same buffer solution, and analyzed by UPLC/UV/TOF-MS, using UV detection at 254 nm for quantitation. Solubility was calculated by comparing the sample and standard UV

areas: $S = (A_{smp} \times FD \times C_{st}) / A_{st}$, where S was the solubility of the compound (μM), A_{smp} was the UV area of the sample solution, FD was the dilution factor (2), C_{st} was the standard concentration (250 μM), and A_{st} was the UV area of the standard solution.

Metabolic Stability Assay. Compounds in 10 mM DMSO solution were added to an incubation mixture in a 96-well microplate containing 20 pmol/mL of hCYP3A4. The mixture was split in two aliquots: one receiving a NADPH regenerating system, the other an equal amount of phosphate buffer. The final substrate concentration was 1 μM along with 0.25% of organic solvent. Incubation proceeded for 1 h at 37 °C and was stopped by addition of acetonitrile to precipitate proteins. Metabolic stability was given as the percent remaining following incubation with cofactor (NADPH) with reference to the incubation mixture without NADPH: % remaining = $area_{NADPH} \times 100/area_{ctrl}$ where $area_{ctrl}$ was the MS peak area of the sample solution without NADPH and $area_{NADPH}$ was the MS area of the sample solution with NADPH. The % CV obtained was typically within 10%.

Permeability Assay. The assay was run in a PAMPA filter plate onto which is deposited a mixture of porcine pig brain lipids in dodecane (2% w/v), and compounds (10 μM in HBSS + Hepes buffer pH = 7.4) were added to the donor chamber and incubated for 4 h at 37 °C and 80% humidity. Warfarin was used in each well as control for membrane integrity. Concentrations of reference t(0), donor, and acceptor solutions were measured by UPLC-MS-TOF. The passive permeability was calculated according to the following expression:

$$C_A(t) = \left(\frac{M}{V_D + V_A}\right) + \left(C_A(0) - \frac{M}{V_D + V_A}\right)^{-P_e A\left(\frac{1}{V_D} + \frac{1}{V_A}\right)}$$

where M refers to the total amount of drug in the system minus the amount of sample lost in membrane (and surfaces), $C_A(t)$ was the concentration of the drug in the acceptor well at time t, $C_A(0)$ was the concentration of the drug in the acceptor well at time 0, V_A was the volume of the acceptor well, V_D was the volume of the donor well, P_e was the effective permeability, A was the membrane area, and t was the permeation time. Compounds were defined as low, medium, or highly permeable following the following classification: $>10 \times 10^{-6}$ cm/s, high (passive permeability was unlikely to be limiting for passive diffusion); between 2 and 10×10^{-6} cm/s, medium (permeability may be limiting in the case of low solubility, high metabolic turnover rate or active secretion); between 0 and 2×10^{-6} cm/s, low (high risk that permeability was limiting for passive diffusion).

In Vitro Intrinsic Clearance. Test compounds were incubated separately at 1 μM concentration in 100 mM phosphate buffer (pH 7.4) and 1 mM EDTA with 0.2 mg/mL human hepatic microsomal protein. The enzymatic reaction was initiated by addition of a NADPH regenerating system (final concentrations: 2 mM β-nicotinamide adenine dinucleotide phosphate (NADP) + 10 mM glucose-6- phosphate (G6P) + 0.4 U/mL glucose-6-phosphate dehydrogenase (G6PDH)). Reactions were terminated at regular time intervals (0 – 5 – 10 – 20 – 40 min) by adding an equal volume of acetonitrile. All incubations were performed in duplicate. Verapamil as positive control for the assay was incubated in parallel under the same conditions. Samples were analyzed by LC-MS. Substrate depletion data (peak area at different time points) were fitted to a monoexponential decay model (eq 1), with a 1/y weighting, $C_{(t)} = C_0 e^{-kt}$, where C_0 was the substrate concentration in the incubation media at time 0 and k was the terminal rate constant. Under the assumption that the concentration of 1 μM was far below the K_m of the test compound, the *in vitro* CL_{int} was calculated by dividing the elimination constant (k) for the

microsomal protein concentration (PMS), expressed in mg/μL, to obtain CL_{int} in units of μL/min/mg protein: CL_{int} = k/PMS = μL/min/mg protein. Compounds were defined as low, medium, or highly metabolized based on the in vitro Clint values: <1.8, low; 1.8 – 49.2, medium; >49.2, high.

MDCK Cellular Permeability. Madin-Darby canine kidneys were maintained in tissue culture flasks in EMEM with Glutamax added with 1% MEM, penicillin (100 U/mL), streptomycin (100 ug/mL), and 10% FBS. Five days before the permeability experiment, the cells were split and placed on permeable cell culture inserts (24-well Millipore) at a density of 250000 cells/well. Transepithelial electrical resistance (TEER) was measured for each well before incubation using an EVOMX instrument (WPI) to ensure that the monolayer was confluent and the tight junctions intact. A TEER > 70 Ohm × cm^2 was considered suitable for experimentation. Compounds (10 μM in HBSS-Hepes buffer) were added in duplicate to the donor chamber and buffer to the acceptor chamber (alternatively apical and basolateral) and incubated for 2 h at 37 °C under gentle agitation. Standards with a high (antipyrin), low (cimetidine), and medium (warfarin) permeability were incubated in the same plate under the same conditions. An aliquot (100 μL) from each well (both apical and basolateral) at time 0 and 120 min was filtered and analyzed by LC-MS. Following incubation, the cell monolayer was washed and incubated with Lucifer Yellow, a fluorescent probe with low permeability, to verify monolayer integrity after incubation. The apparent permeability (P_{app}) in cm/sec was calculated using the following equation in both directions (apical-to-basolateral and basolateral-to-apical): $P_{app} = dC \times V_r / dt \times A \times C_0$ (where V_r was the volume (mL) of the receiver chamber, A was the surface area of the cell culture insert, and dt was the time in seconds). The mass balance in both directions was estimated by the following equation: mass balance = [(final donor mass + mass

transferred) / initial donor mass]. The efflux ratio was calculated by comparing P_{app} B→A with P_{app} A→B. A high efflux ratio was an indication of the compound being a substrate for efflux transporters. The alert threshold was an efflux ratio >3.

Plasma Stability. Compounds were incubated at 10 μM in 100 % of fresh plasma from rat. After 0, 30 and 3h of incubation at 37 °C, the reactions were quenched by adding of 3 volumes of cold acetonitrile. The quenched mixtures were centrifuged at 3000 g for 15 minutes for protein precipitation and the surnatants were diluted 1:10 with water before LC-MS/MS analysis. Plasma stability results, calculated as average of two replicates, are obtained by comparing the peak area at the different time points (PA_t, 30min and 3h) with that at time 0 (PA_0) and are reported as percentage remaining: % remaining = (PA_t / PA_0)*100.

CONCLUSIONS

The field of cystic fibrosis research has seen significant breakthroughs in recent years, with a number of molecules now available to patients that address the root causes of the pathology. This has been accompanied by significant advances in CF basic science which have deepened our understanding of the mechanisms behind the disease. On the other hand, the underlying mechanism of action for compounds acting as correctors has yet to be fully elucidated, e.g. whether they act directly on mutated CFTR protein or on a target not yet identified. This generally makes the development of correctors a challenging exercise, causing the SAR to be built on functional assays without the support of specific target-driven data.

In this paper we described a concise approach which leads to the discovery of a new series of compounds showing activity on two different cell line assays, acting as ΔF508-CFTR correctors. This series has been expanded with the synthesis of analogs and a SAR has been established with

clear indication for a follow-up optimization. The benzyl group in position 6 as well as the 2-pyridine moiety in position 2 appears essential to activity with the possibility to substitute the former to further modulate both biological activity and metabolic stability. Cyclic or linear aliphatic amines bearing terminal hydrogen bond acceptor/donor feature in position 4 are also required.

Alongside the activity as ΔF508-CFTR correctors, newly synthesized compounds were also assessed for water solubility, metabolic stability, and permeability. In general this series demonstrated good solubility in water. Compounds were highly permeable in the passive permeability assay with still some active efflux issues to be resolved in a subsequent multi-parameter optimization program. The metabolic stability versus P450 3A4 isoform proved quite variable, with several compounds classified as unstable (<20% remaining). Analogously, preliminary intrinsic clearance evaluation indicated no serious issues for further progression of the series.

Although the extent of corrector activity is still sub-optimal with respect to other known correctors, we believe that the current series represent a robust starting point worth of further investigation.

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

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ABBREVIATIONS

CF, Cystic Fibrosis; CFTR, Cystic Fibrosis Transmembrane Regulator; YFP, Yellow Fluorescent Protein; FRT, Fischer Rat Thyroid; ACN, acetonitrile; B/P, brain to plasma ratio; HPLC, high pressure liquid chromatography; MDCK, Madin Darby canine kidney; P450, cytochrome P450; Rt, retention time; UPLC, ultraperformance liquid chromatography.

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