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Design and synthesis of purine analogues as highly specific ligands for FcyB, a ubiquitous fungal nucleobase transporter

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Key words: *Aspergillus nidulans*; purine transporters; structure activity relationships; combiglide; imidazo[4,5-*c*]pyridine; deazaadenine

Abstract: In the course of our study on fungal purine transporters, a number of new 3-deazapurine analogues have been rationally designed, based on the interaction of purine substrates with the *Aspergillus nidulans* FcyB carrier, and synthesized following an effective synthetic procedure. Certain derivatives have been found to specifically inhibit FcyB-mediated [^{3}H]-adenine uptake. Molecular simulations have been performed, suggesting that all active compounds interact with FcyB through the formation of hydrogen bonds with Asn163, while the insertion of hydrophobic fragments at position 9 and *N*6 of 3-deazaadenine enhanced the inhibition.

1. Introduction

Fungal pathogens, and especially *Aspergillus fumigatus*, constitute an emerging threat due to the increasing number of immunosuppressed patients.[1,2] Most present day antifungals are rather hydrophobic compounds which enter fungal cells via non-facilitated diffusion and target enzymes involved in the synthesis of the plasma membrane or the cell wall.[3,4] The most common of such antifungals include azoles, polyenes and echinocardins. Due to the mechanism of non-specific cellular uptakes, these antifungals are associated with side effects and mediocre efficiency. In addition, resistance to these antifungals arises frequently due to genetic mechanisms activating efflux by ABC xenobiotic exporters, or the overproduction or modification of their target.[5-7] An alternative category of antifungals is exemplified by 5-fluorocytosine (5-FC). This highly efficient antifungal pyrimidine analogue is incorporated in fungal cells by specific transporters and metabolically converted to the highly cytotoxic 5-fluorouracil.[3] The apparent absence of 5-FC transporters in human cells makes this antifungal little, if not at all, cytotoxic for humans. In addition, several fungi seem to use many transporters for 5-FC uptake, so that mutation in a single gene does not confer full resistance to the drug.[8,9]

Rather surprisingly, emerging knowledge on fungal transporters has not been rationally exploited to date in relationship to the identification of novel antifungals. Ideally, a drug recognized by a fungal transporter, but not by host transporters, as is the case of 5-FC, will also have a highly targeted antifungal potential. As a step towards the rational design of novel antifungals, we study structure-function relationships in nucleobase/nucleoside transporters in *A. nidulans*, a genetically tractable fungus, where we have identified, cloned and fully characterized all 7 major transporters, catalyzing the uptake of purines, pyrimidines, nucleosides and purine analogues, namely UapA, UapC, AzgA, FurD, FurA, FcyB and CntA.[10-12] The characterization of these seven transporters has allowed the construction, through standard reverse and classical genetics, of a 'master mutant' strain named Δ7, where all seven transporter genes are deleted.[13] The genetic re-introduction of any selected nucleobase transporter gene in Δ7 allows the direct and rigorous functional assessment of the corresponding transporter in a 'clean' genetic background.[9]

In this work, we investigated whether we could rationally design, based on previously theoretical models describing purine-transporter interactions,[9,14-16] and synthesize analogues which will be recognized by a single specific nucleobase transporter of *A. nidulans*. More specifically, we wanted to test whether we can design and synthesize purine analogues recognized solely by FcyB. We have chosen FcyB as this transporter is not only ubiquitously present in all fungi but has also very similar transport kinetics and substrate specificity with AzgA, both recognizing at the μM range and transporting efficiently all salvageable purines (adenine, guanine and hypoxanthine). Thus, being able to distinguish substrates/ligands of these two functionally similar transporters would be a rigorous test for investigating the feasibility of future efforts for rational drug design, related to specific fungal transporters. As earlier observations showed that purine analogues substituted at position 3 of the purine ring could still be recognized efficiently by FcyB,[17] 3-deazaadenine was selected as a primary compound in hit-lead campaign against FcyB. Our results show that, indeed, it is possible to identify purine analogues highly specific solely for FcyB. The importance of these findings is apparent for the future design of highly-targeted antifungals recognized by specific fungal, but not by host, transporters.

2. Results and Discussion

2.1. Rational design of new 3-deaza analogues

Starting from the FcyB homology model structure previously constructed on the on the Sodium-Hydantoin Transporter Mhp1 template and validated by site mutation experiments,[15] we explored the possibilities to modify 3-deazaadenine considering the binding site of the substrate occluded structure based on the Mhp1 benzyl-hydantoin permease from *M. liquefaciens*. The resulted low energy docking poses of 3-deazaadenine within the substrate binding site of the transporter is shown in Figure 1. Two different orientations within the cavity of similar calculated interaction energy have been considered. In the first (Fig. 1A) 3-deazaadenine interacts with FcyB in a very similar way to adenine, through a bidentate H bond that is formed between Asn163 amide group and ligand sites C6-NH_2 and *N*1 (original purine numbering). In the second (Fig. 1B) 3-deazaadenine interacts with FcyB forming H bonds through C6-NH_2 and *N*7, with Asn163 as well.

According to these theoretical models two major directions can be explored as targeting regions, depicted as I and II in Figure 1 with red arrows. The first lies in the upper end of transmembrane segment 1 (TMS1) among Val84 (TMS1), Ala162, Val166 (TMS3) and Glu397 (TMS9). The second one is a hydrophobic pocket lying near Pro353 (TMS8), Trp77 (TMS1) and Tyr262 (TMS6). In order to explore those sites, 1 and 4 substituted 3-deazaadenine analogs were designed using combiglide algorithm as implemented on Schrodinger Suite 2014.

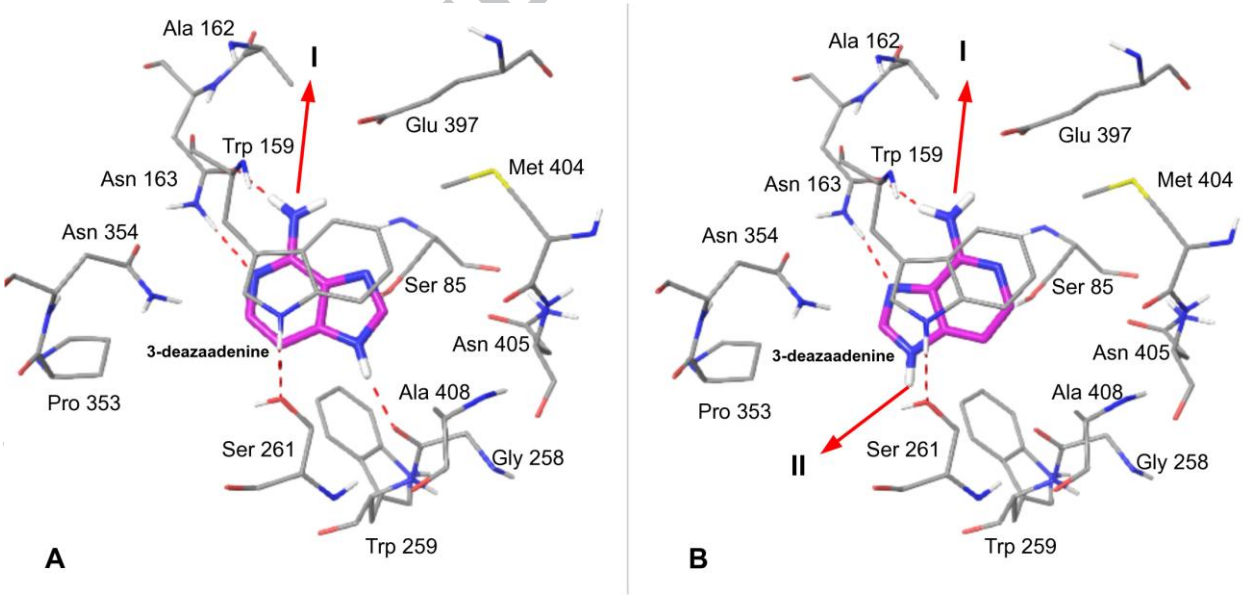


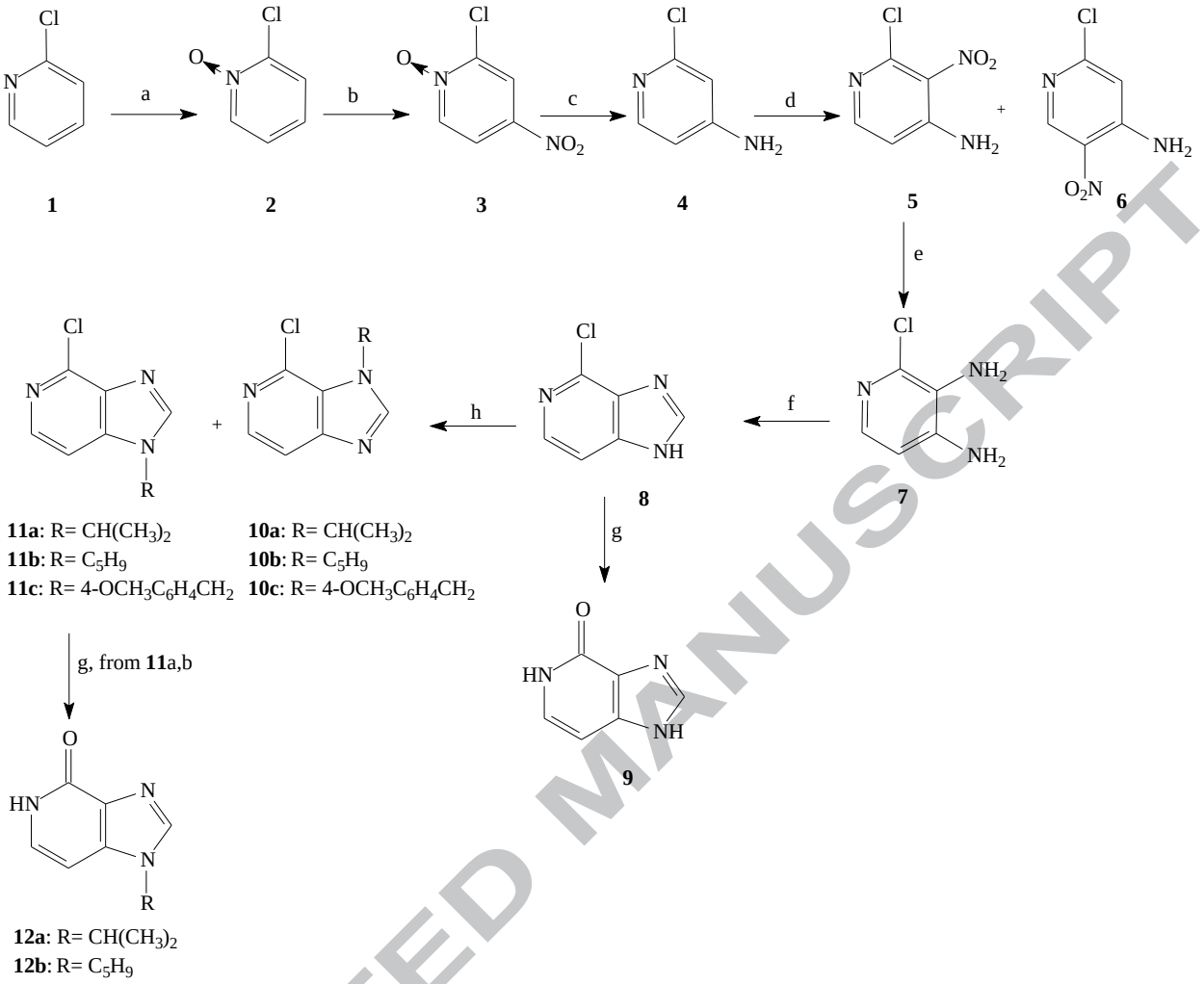
Figure 1. Global minima structures of FcyB in complex with 3-deaza-adenine. Major directions I and II for substitution are shown with red arrows.

A virtual in-house library containing 100 fragments was considered for probable modifications, using 0 to 3 methylene groups as spacers, resulting 1600 virtual molecules for *in-silico* evaluation. The resulted molecules were ranked based on GlideScore following a Virtual Screening procedure. The 40 high ranked ligands (~3Kcal/mol from global minimum) were selected as input for Induced Fit Docking. The output structures were then carefully inspected for their theoretical interactions inside the binding pocket to select the most potent substrates.

4-Methylpiperazine, 2-(dimethylamino)ethylamine and benzylamine groups appeared to be suitable modifications at position 4 of imidazopyridine to target Glu397 or Glu401 as well as more hydrophobic residues in region I. A morpholine group has been considered as alternative to piperazine to balance the hydrophobic part of the region. Isopropyl and cyclopentyl groups were best fitted within the binding pocket directed to the second targeted region II and appeared to be the most adequate moieties to be buried inside the first hydrophobic pocket near Trp77 and Ala80. Finally, the 3,5-dimethoxyphenoxy substitution was also investigated since the resulted molecule showed a different but interesting binding mode. The volume of 3,5-dimethoxyphenoxy group forces the molecule to be flipped. The *N*1-H forms hydrogen bond with Glu397, while the deazapurine core forms π-π stacking with Trp159 and the benzyl group with Trp259.

2.2. Chemistry

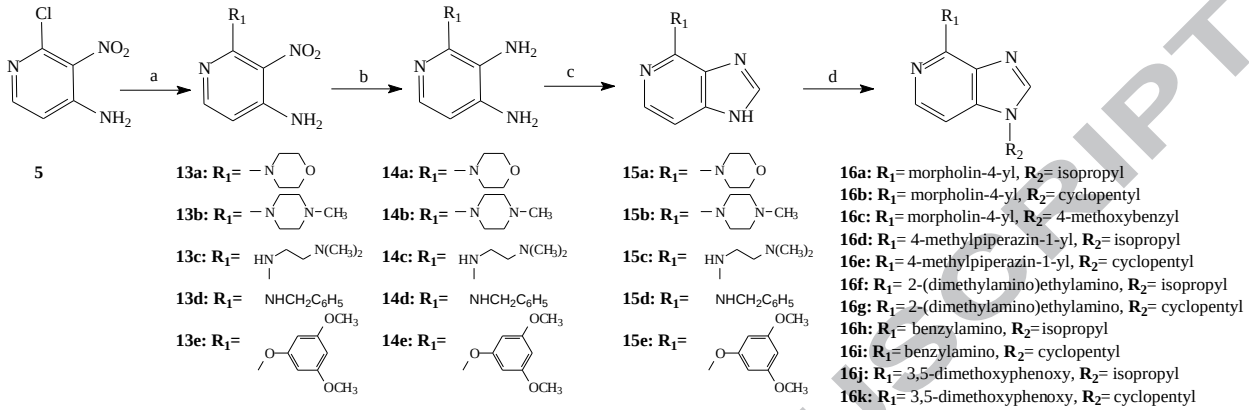
For the preparation of the target compounds, 2-chloropyridine (**1**, Scheme 1) was used as starting material, which underwent successively *N*-oxidation,[18] nitration and reduction of both the *N*-oxide and the nitrogroup to afford 4-amino-2-chloropyridine (**4**).[19] This compound was nitrated to result into the nitroderivatives **5** and **6**.[20] The selected 3-nitroderivative **5** was then reduced with the use of tin(II) chloride and the resulting diaminopyridine **7** was ring-closed upon reaction with triethylorthoformate to give the imidazopyridine **8**.[21] Acidic hydrolysis of **8** provided the hypoxanthine analogue **9**. On the other hand, compound **8** was treated with the suitable alkyl or arylhalide to provide the regio isomers **10a-c** and **11a-c** which were chromatographically isolated and identified using nOe spectral data (correlation peak of the protons of the substituent at position 1 with H-7, in the case of compounds **11**). Then, derivatives **11a,b** were hydrolyzed to provide the corresponding imidazopyridinones **12a,b**.



Scheme 1. Reagents and conditions: a) *m*-CPBA, CH_2Cl_2, r.t.; b) $HNO_{3\ (fuming)}$, $H_2SO_{4\ (98\%)}$, 90 ˚C; c) Fe, $HCl_{\ (36\%)}$, EtOH, reflux; d) i) $HNO_{3\ (fuming)}$, $H_2SO_{4\ (98\%)}$, 0 ˚C, ii) $H_2SO_{4\ (98\%)}$, 75 ˚C; e) $SnCl_2{\cdot}H_2O$, $HCl_{\ (36\%)}$, 50 ˚C; f) triethyl orthoformate, $HCl_{\ (36\%)}$, r.t.; g) $HCl_{\ (36\%)}$, EtOH, H_2O, reflux; h) i) K_2CO_3, DMF, r.t., ii) isopropyl bromide (for **10a**, **11a**) or cyclopentyl bromide (for **10b**, **11b**) or 4-methoxybenzyl chloride (for **10c**, **11c**), r.t.

Attempts to substitute the 4-chloro group of compounds **11** with suitable nucleophiles, either by refluxing compounds **11** with excess of the amine in the presence of ethanol or 2-ethoxyethanol as the solvent, or by refluxing compounds **11** in dioxane in the presence of a palladium catalyst (tris(dibenzylideneacetone)dipalladium, $Pd_2(dba)_3$) and a ligand (2-dicyclohexylphosphino-2',4',6'-triisopropylbiphenyl, X-Phos) in basic conditions (cesium carbonate), were not successful since they resulted in extremely low yields of the target compounds. Consequently, we have modified the synthetic methodology in order to insert the suitable substituents at an earlier stage. Thus, the nitropyridine **5** reacted with a number of primary or secondary amines as well as with a substituted phenol (Scheme 2), to provide in high

yield the intermediate nitropyridines **13a-e**. These derivatives were reduced and the resulting diamines **14a-e** were cyclized without further purification to give the imidazopyridines **15a-e**.



Scheme 2. Reagents and conditions: a) amine, EtOH, reflux (for **13a-13d**) or Cs_2CO_3, 3,5-dimethoxyphenol, THF, reflux (for **13e**); b) H_2, Pd/C, EtOH, 33-55psi, r.t.; c) triethyl orthoformate, HCl (36%), r.t.; d) i) K_2CO_3, DMF, r.t., ii) isopropyl bromide (for **16a**, **16d, 16f, 16h, 16j**) or cyclopentyl bromide (for **16b**, **16e, 16g, 16i, 16k**) or 4-methoxybenzyl chloride (for **16c**), r.t.

From the cyclization of compound **14d**, we have also isolated the 7-amino-3-benzyl derivative **15d$_1$** (Figure 2) in 10% yield, which has obviously resulted upon ring-closure of 2 and 3 aminogroups of **14d**.

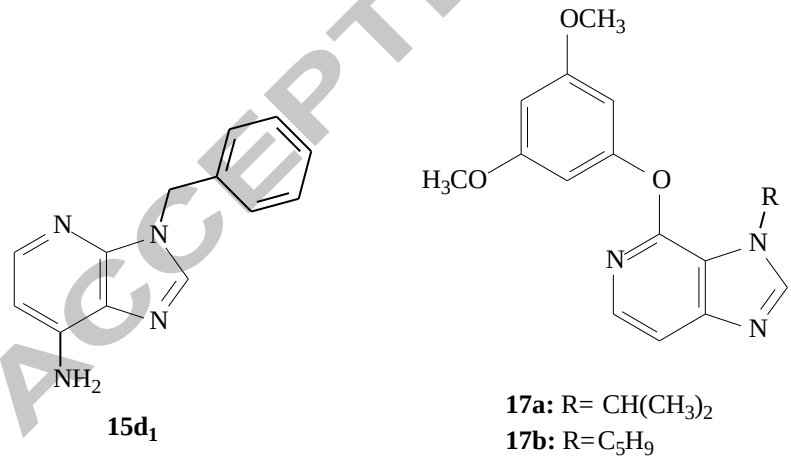
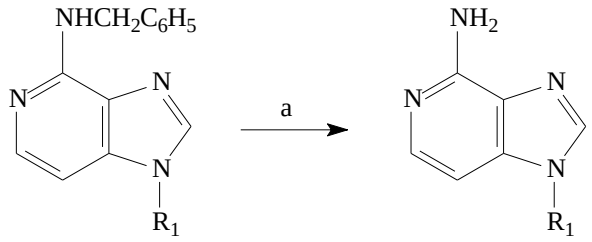


Figure 2. By-product from the cyclization of **14d** and regio-isomers of **16j-k**.

The appropriate alkyl-group was finally inserted at position 1 of the imidazopyridines **15**, resulting into the target compounds **16a-k**. In the case of the 4-(3,5-dimethoxyphenoxy) derivative **15e**, the regio-isomers **17a,b** (Figure 2), were isolated, together with the corresponding 1-substituted derivatives **16j,k**.

The 4-benzylaminoderivatives **15d**, **16h** and **16i**, were converted to the corresponding 4-aminoderivatives **18a-c**, upon treatment with ammonium formate in the presence of palladium on carbon as catalyst.

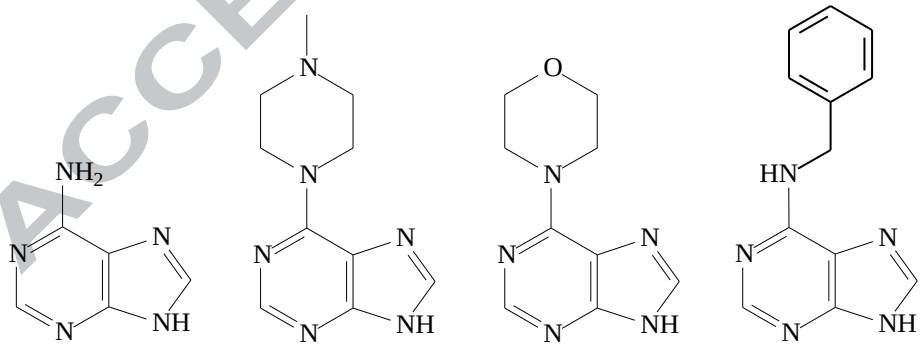


15d: $\mathbf{R_1}$= H
16h: $\mathbf{R_1}$= $CH(CH_3)_2$
16i: $\mathbf{R_1}$= C_5H_9

18a: $\mathbf{R_1}$= H
18b: $\mathbf{R_1}$= $CH(CH_3)_2$
18c: $\mathbf{R_1}$= C_5H_9

Scheme 3. Reagents and conditions: a) $HCOONH_4$, Pd/C, MeOH, reflux.

In parallel, and in order to extract more accurate structure-activity relationships, we have also included into the biological evaluation tests adenine (**19**), as well as a number of selected 6-aminosubstituted purines (**20-22**, Figure 3). Compounds **20-22** have been previously reported and were prepared from 6-chloropurine.[22, 23] We have synthesized those derivatives in almost quantitative yield, following a slightly modified and convenient procedure, by refluxing 6-chloropurine in ethanol with two equivalents of the suitable amine.



19 **20** **21** **22**

Figure 3. Adenine and aminosubstituted purines.

The prepared compounds were tested, by direct *in vivo* transport assays, for their potential to inhibit FcyB-mediated uptake of radiolabelled hypoxanthine or adenine.

2.3. Biological studies

2.3.1. Competition at FcyB and AzgA transporter by the synthesized derivatives

The new derivatives were tested as competitive inhibitors of FcyB-mediated ^{3}H-adenine uptake. As a control for testing whether these compounds are, as expected, specific for FcyB, we also tested the same purine analogues as competitive inhibitors of AzgA-mediated ^{3}H-adenine uptake. In both cases, assays were performed in strains expressing solely the transporter studied in each case, that is, in the absence of all other functionally relative nucleobase/nucleoside-related transporters. These strains (i.e. Δ7::FcyB and Δ7::AzgA), were constructed by selecting for genetic transformants of a Δ7 mutant strain, arising from single-copy integration events of plasmids carrying either the *fcyB* or the *azgA* gene. Transformant selection was based on standard complementation of auxotrophic markers, PCR and southern analysis (for details see Experimental procedures). In the case of FcyB, the transporter message is transcribed via the medium-strength promoter of the *uapA* gene, rather than its own native promoter, in order to achieve relatively higher protein expression levels necessary for uptake assays. The selected transformants could both grow on purines as sole nitrogen sources in the medium. As expected, Δ7::FcyB transformant was also sensitive to 5-FU, whereas the Δ7::AzgA transformant was sensitive to 8-azaguanine.

Δ7::FcyB and Δ7::AzgA strains were used to perform competitive inhibition assays of either FcyB-mediated or AzgA-mediated ^{3}H-adenine uptake, using the synthesized derivatives. Competition was carried out at 1000-fold excess inhibitors in order to identify compounds that have even low binding affinity for FcyB or AzgA. Results are shown in Figures 4 and 5 respectively. Six deazapurine analogues, **16e**, **16f**, **16g**, **16h**, **16i** and **18c** competed very efficiently FcyB-mediated ^{3}H-adenine uptake (i.e. <10% transport compared to the non-inhibited), but had no effect on AzgA-mediated ^{3}H-adenine uptake rate (compare results in Fig. 4 and 5). The same result was obtained when analogues were tested as inhibitors of FcyB-mediated or AzgA-mediated ^{3}H-hypoxanthine uptake (results not shown). These six analogues did not inhibit at all UapA, the third major purine transporter specific for xanthine or uric acid (results not shown).

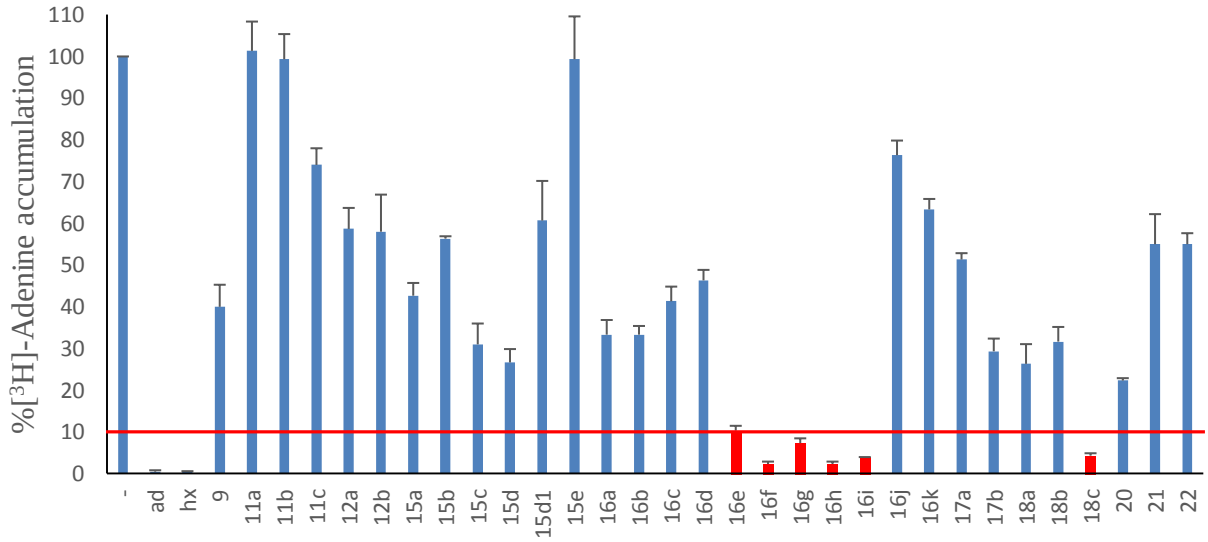


Figure 4. Competition of FcyB-mediated [^{3}H]-adenine uptake by 1000-fold excess (0.5 mM) of unlabeled purine analogues. Compounds exhibiting inhibitory activity more than 90% are depicted in red.

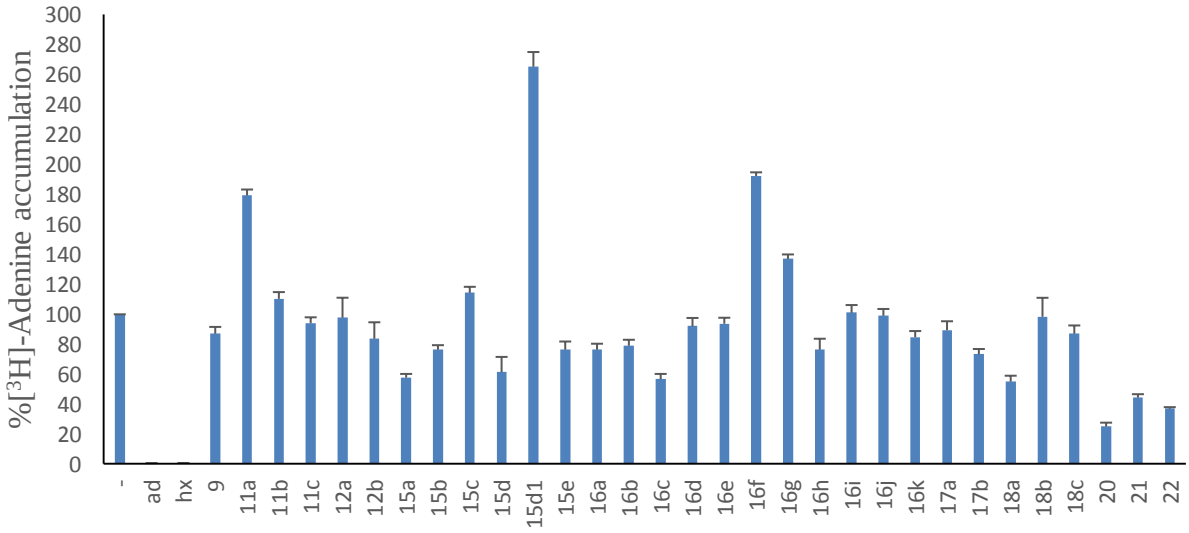


Figure 5. Competition of AzgA-mediated [^{3}H]-adenine uptake by 1000-fold excess (0.5 mM) of unlabelled purine analogues.

2.3.2. Kinetic characterization of competing analogues

We estimated the K_i values, using IC_{50} measurements,[13] of the six analogues, which specifically inhibit FcyB transport. Results are shown in Table 1. All K_is were in the 5-95 μM range, with analogue **16h** showing the highest affinity binding in FcyB (5 μM).

compound	K_i (μM)
16e	38±6
16f	72±7
16g	95±9
16h	5±1
16i	21±4
18c	18±2

Table 1. Substrate binding specificity profile of FcyB. Results are averages of at least three independent experiments with three replicates for each concentration point.

2.4. Structure-activity relationship study

From the data presented in Figure 4 it is obvious that only the 4-amino substituted compounds possess considerable biological activity. This is confirmed by the fact that 4-chlorosubstituted derivatives **11a-c**, as well as the corresponding imidazopyridinones **9** and **12a,b** are devoid of activity. A crucial structural feature of the most potent derivatives is the simultaneous 1- and 4- substitution since compounds **16** are considerably more active than their mono-substituted counterparts **15**, with **16h** and **16i** being the most active analogues. It seems that *N*1-cyclopentyl substitution is preferable over the corresponding *N*1-isopropyl substitution. Another important finding is that among the 4-alkylamino substituted derivatives the existence of 4-NH (compounds **16f-i**) is in favor of the transporter inhibitory activity. Concerning the remaining aminosubstituted derivatives, only the piperazine analogue **16e** possesses a certain degree of activity although moderate; however, this derivative bears a *N*1-cyclopentyl moiety as well. Finally, it should be noted that although the number of derivatives is limited, our data concerning adenine and 3-deazaadenine suggest that the 3-nitrogen is not crucial for this kind of protein ligand interaction, since the new imidazopyridines exhibited comparable activity with the purine derivatives **20-22**. However, this would request further investigation.

2.5. Molecular modeling

Molecular simulations suggest that active compounds can interact with FcyB through residue Asn163. This residue and Pro353 were shown to be irreplaceable for FcyB-mediated transport.[15] More specifically Asn163 proved to be critical for determining the substrate binding affinity and/or specificity of FcyB, without affecting protein stability. All active compounds interact with FcyB through the formation of hydrogen bonds with Asn163. The insertion of a hydrophobic fragment at position 1 of 3-deazaadenine (**18b**, **18c**) enhanced the inhibition, especially for the cyclopentyl moiety as already mentioned. Those analogs bind at the same position as adenine and the hydrophobic fragment is placed near hydrophobic region TMS1α. (data not shown). The docking structures of the most active compounds **16h** and **16f** form a

bidentate hydrogen bond (HB) via C4-NH and N3 of the ligand underlining the importance of the presence of NH at position C4 (Fig. 6). In both structures the isopropyl moiety is accommodated between Pro353, Trp77 and Tyr262 filling the space in the hydrophobic cavity. In the case of **16f** (Fig. 6B) an extra salt bridge appears possible between the tertiary amine positive charge and Glu397 carboxylate while in **16h** (Fig 6A) the phenyl group seems to exhibit hydrophobic interactions with Val84, Ala162, Val166. On all minima structures π-π stacking interactions with Trp159 and Trp259 are also very important.

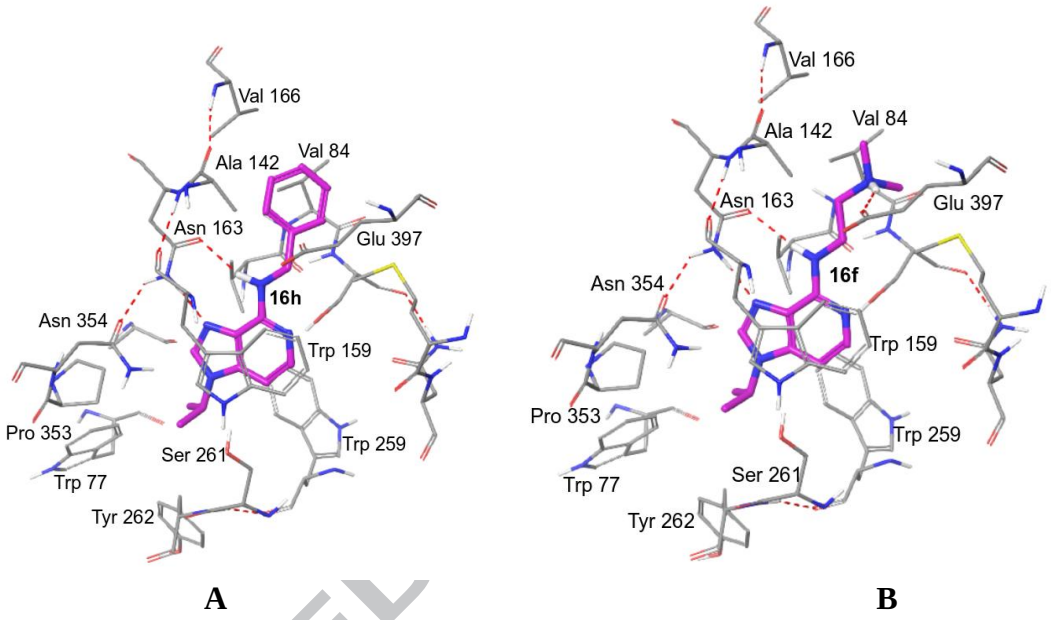


Figure 6. Minimum energy structures of FcyB in complex with compound **16h** (A) and **16f** (B). Hydrogen bonds are shown with red dashed lines.

3. Conclusion

In conclusion, we have designed and synthesized a number of 3-deazapurines substituted in the corresponding 6- and 9-positions of the original purine scaffold. These compounds were tested in substrate competition assays related to FcyB and AzgA transporters, both of which recognize and transport purines with high affinities at the low μM range. A number of the tested 3-deazapurines was found to be specific solely for FcyB. The K_i values of FcyB-specific substrates/ligands were determined. Given that the synthesized 3-deazapurines were designed rationally based on the interaction of purine substrates with FcyB, our results show that our approach can be used successfully to design substrates/ligands highly specific for a given nucleobase transporter. The importance of these findings is apparent for the future design of compounds recognized specifically by all fungal, but not by host, transporters.

4. Experimental section

4.1. Chemistry

4.1.1. General

Melting points were determined on a Büchi apparatus and are uncorrected. ^{1}H NMR spectra and 2D spectra were recorded on a Bruker Avance III 600 or a Bruker Avance DRX 400 instrument, whereas ^{13}C NMR spectra were recorded on a Bruker Avance III 600 or a Bruker AC 200 spectrometer in deuterated solvents and were referenced to TMS (δ scale). The signals of ^{1}H and ^{13}C spectra were unambiguously assigned by using 2D NMR techniques: ^{1}H^{1}H COSY, NOESY, HMQC, and HMBC. Mass spectra were recorded with a LTQ Orbitrap Discovery instrument, possessing an Ionmax ionization source. Flash chromatography was performed on Merck silica gel 60 (0.040–0.063 mm). Analytical thin layer chromatography (TLC) was carried out on precoated (0.25 mm) Merck silica gel F-254 plates. The purity of all the synthesized compounds was >95% as ascertained by elemental analysis. Elemental analyses were undertaken using a PerkinElmer PE 240C elemental analyzer (Norwalk, CT, U.S.) and the measured values for C, H, and N were within ±0.4% of the theoretical values.

4.1.2. General procedure for the synthesis of compounds 10a-c and 11a-c

Potassium carbonate (1.9 mmol) was added into a solution of compound **8** (1.3 mmol) in dry DMF (5 mL) under argon, and this mixture was stirred at room temperature for 20 min. Subsequently, the suitable halide (isopropyl bromide, cyclopentyl bromide, 4-methoxybenzyl chloride, 1.9 mmol) was added and the reaction was stirred at room temperature for 72h. Then, the solvent was removed in vacuo and water was added to the residue, followed by extraction with chloroform (3x50 mL). The combined organic extracts were dried over sodium sulfate, filtered and the solvent was removed under reduced pressure. The 1- and 3- substituted isomers were chromatographically separated.

4.1.2.1. 4-Chloro-3-isopropyl-3*H*-imidazo[4,5-*c*]pyridine (10a) and 4-chloro-1-isopropyl-1*H*-imidazo[4,5-*c*]pyridine (11a).

These derivatives were synthesized according to general procedure described above, upon reaction of compound **8** with isopropyl bromide. Purification was effected using a mixture of cyclohexane / ethyl acetate (7/3, v/v) as the eluent, to provide pure **10a** and **11a**.

Data for 10a: Yield 24%. White solid, mp 93 °C (Et_2O). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.16 (s, 1H, H-2), 8.14 (d, 1H, *J*=5.4Hz, H-6), 7.59 (d, 1H, *J*=5.4Hz, H-7), 5.38 (septet, 1H, *J*=6.6Hz, C<u>H</u>$(CH_3)_2$), 1.62 (d, 6H, *J*=6.6Hz, 2xCH_3). ^{13}C NMR (151 MHz, $CDCl_3$) δ 151.44 (C-7a), 143.62 (C-2), 140.88 (C-6), 133.61 (C-4), 127.91 (C-3a), 114.97 (C-7), 49.86 (<u>C</u>H$(CH_3)_2$), 23.92 (2xCH_3). HR-MS (ESI) *m/z*: Calcd for $C_9H_{11}ClN_3$: $[M1+H]^+$ =196.0636, found 196.0639. Anal. Calcd for $C_9H_{10}ClN_3$: C, 55.25; H, 5.15; N, 21.48. Found: C, 55.39; H, 5.21; N, 21.38.

Data for 11a: Yield 76%. White solid, mp 59 °C (*n*-pentane). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.05 (d, 1H, *J*=5.5Hz, H-6), 8.02 (s, 1H, H-2), 7.27 (d, 1H, *J*=5.5Hz, H-7), 4.59 (septet, 1H, *J*=6.6Hz, C<u>H</u>$(CH_3)_2$), 1.54 (d, 6H, *J*=6.6Hz, 2xCH_3). ^{13}C NMR (151 MHz, $CDCl_3$) δ 142.48 (C-4), 141.91 (C-6), 140.70 (C-2), 139.03 (C-7a), 137.44 (C-3a), 105.61 (C-7), 48.75 (<u>C</u>H$(CH_3)_2$),

22.29 (2xCH_3). HR-MS (ESI) *m/z*: Calcd for $C_9H_{11}ClN_3$: $[M1+H]^+$ =196.0636, found 196.0641. Anal. Calcd for $C_9H_{10}ClN_3$: C, 55.25; H, 5.15; N, 21.48. Found: C, 55.44; H, 5.23; N, 21.30.

4.1.2.2. 4-Chloro-3-cyclopentyl-3*H*-imidazo[4,5-*c*]pyridine (10b) and 4-chloro-1-cyclopentyl-1*H*-imidazo[4,5-*c*]pyridine (11b).

These derivatives were synthesized according to general procedure described above, upon reaction of compound **8** with cyclopentyl bromide. Purification was effected using a mixture of chloroform / methanol (100/1, v/v) as the eluent, to provide pure **10b** and **11b**.

Data for 10b: Yield 17%. White solid, mp 77-8 ˚C (Et_2O). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.20 (d, 1H, *J*=5.3Hz, H-6), 8.14 (s, 1H, H-2), 7.64 (d, 1H, *J*=5.3Hz, H-7), 5.50 (m, 1H, H-1'), 2.40-2.32 (m, 2H, cyclopentyl-H), 2.05-1.97 (m, 2H, cyclopentyl-H), 1.94-1.83 (m, 4H, cyclopentyl-H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 151.57 (C-4), 144.07 (C-6), 140.94 (C-2), 133.91 (C-3a), 128.46 (C-7a), 114.98 (C-7), 58.43 (C-1'), 34.06 (C-2', C-5'), 23.77 (C-3', C-4'). HR-MS (ESI) *m/z*: Calcd for $C_{11}H_{13}ClN_3$: $[M1+H]^+$ =222.0793, found 222.0797. Anal. Calcd for $C_{11}H_{12}ClN_3$: C, 59.60; H, 5.46; N, 18.96. Found: C, 59.75; H, 5.53; N, 18.78.

Data for 11b: Yield 76%. White solid, mp 87-8 ˚C (Et_2O). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.20 (d, 1H, *J*=5.6Hz, H-6), 8.05 (s, 1H, H-2), 7.33 (d, 1H, *J*=5.6Hz, H-7), 4.74 (m, 1H, H-1'), 2.36-2.30 (m, 2H, cyclopentyl-H), 2.06-1.99 (m, 2H, cyclopentyl-H), 1.97-1.92 (m, 2H, cyclopentyl-H), 1.89-1.83 (m, 2H, cyclopentyl-H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 142.71 (C-7a), 142.49 (C-2), 140.88 (C-6), 139.66 (C-4), 137.97 (C-3a), 105.87 (C-7), 57.79 (C-1'), 32.31 (C-2', C-5'), 23.73 (C-3', C-4'). HR-MS (ESI) *m/z*: Calcd for $C_{11}H_{13}ClN_3$: $[M1+H]^+$ =222.0793, found 222.0799. Anal. Calcd for $C_{11}H_{12}ClN_3$: C, 59.60; H, 5.46; N, 18.96. Found: C, 59.49; H, 5.40; N, 19.03.

4.1.2.3. 4-Chloro-3-(4-methoxybenzyl)-3*H*-imidazo[4,5-*c*]pyridine (10c) and 4-chloro-1-(4-methoxybenzyl)-1*H*-imidazo[4,5-*c*]pyridine (11c).

These derivatives were synthesized according to general procedure described above, upon reaction of compound **8** with 4-methoxybenzyl chloride. Purification was effected using a mixture of dichloromethane / methanol (100/1, v/v) as the eluent, to provide pure **10c** (yield 18%) and **11c** (yield 45%). Compound **11c** has previously been reported.[24]

Data for 10c: White solid, mp 133-4 ˚C (Et_2O). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.25 (d, 1H, *J*=4.5Hz, H-6), 8.10 (s, 1H, H-2), 7.70 (d, 1H, *J*=4.5Hz, H-7), 7.17 (d, 2H, *J*=8.5Hz, H-2', H-6'), 6.91 (d, 2H, *J*=8.5Hz, H-3', H-5'), 5.70 (CH_2), 3.81 (OCH_3). ^{13}C NMR (151 MHz, $CDCl_3$) δ 160.17 (C-4'), 150.16 (C-4), 146.74 (C-2), 141.65 (C-6), 128.93 (C-2', C-6'), 126.93 (C-3a), 126.74 (C-1'), 114.74 (C-3', C-5'), 114.62 (C-7a), 114.57 (C-7), 55.38 (OCH_3), 50.25 (CH_2). HR-MS (ESI) *m/z*: Calcd for $C_{14}H_{13}ClN_3O$: $[M1+H]^+$ =274.0742, found 274.0745. Anal. Calcd for $C_{14}H_{12}ClN_3O$: C, 61.43; H, 4.42; N, 15.35. Found: C, 61.62; H, 4.51; N, 15.17.

4.1.3. General procedure for the synthesis of compounds 12a-b.

Concentrated hydrochloric acid (1 mL) was added dropwise into a solution of the corresponding chloroderivative **11** (0.25 mmol) in a mixture of ethanol (2.0 mL) and water (2.0 mL), and this reaction mixture was refluxed for 72 h. Then the organic solvent was removed in vacuo, the residue was neutralized with sodium bicarbonate and extracted with ethyl acetate. The combined organic layers were dried over sodium sulfate and the solvent was removed under reduced pressure. The residue was purified by column chromatography (silica gel) to provide pure derivatives **12a** and **12b**.

4.1.3.1. 1-Isopropyl-1,5-dihydro-4*H*-imidazo[4,5-*c*]pyridin-4-one (12a).

This compound was synthesized according to general procedure described above, starting from **11a**. Purification was effected using a mixture of dichloromethane / methanol (9/1, v/v) as the eluent to provide pure **12a** as a beige solid, in 16% yield. Mp 122-3 °C (MeOH). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.15 (s, 1H, NH), 8.12 (s, 1H, H-2), 7.15 (d, *J*=6.5 Hz, 1H, H-6), 6.62 (d, *J*=6.5 Hz, 1H, H-7), 4.60 (septet, *J*=6.8 Hz, 1H, C<u>H</u>(CH$_3$)$_2$), 1.47 (d, *J*=6.8 Hz, 6H, CH$_3$). ^{13}C NMR (151 MHz, DMSO-d_6) δ 158.15 (C-4), 138.69 (C-2), 138.21 (C-7a), 131.50 (C-3a), 129.08 (C-6), 93.00 (C-7), 47.66 (<u>C</u>H(CH$_3$)$_2$), 22.46 (CH$_3$). HR-MS (ESI) *m/z*: Calcd for $C_9H_{12}N_3O$: $[M1+H]^+$ =178.0975, found 178.0980. Anal. Calcd for $C_9H_{11}N_3O$: C, 61.00; H, 6.26; N, 23.71. Found: C, 60.83; H, 6.20; N, 23.89.

4.1.3.2. 1-Cyclopentyl-1,5-dihydro-4*H*-imidazo[4,5-*c*]pyridin-4-one (12b).

This compound was synthesized according to general procedure described above, starting from **11b**. Purification was effected using a mixture of dichloromethane / methanol (95/5, v/v) as the eluent to provide pure **12b** as a beige solid, in 27% yield. Mp >250 °C (MeOH). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.17 (s, 1H, NH), 8.09 (s, 1H, H-2), 7.15 (d, 1H, *J*=7.1 Hz, H-6), 6.60 (d, 1H, *J*=7.1 Hz, H-7), 4.72 (m, 1H, H-1'), 2.17 (m, 2H, cyclopentyl-H), 1.84 (m, 4H, cyclopentyl-H), 1.69 (m, 2H, cyclopentyl-H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 158.06 (C-4), 138.96 (C-2), 138.73 (C-7a), 131.58 (C-3a), 129.08 (C-6), 92.98 (C-7), 56.50 (C-1'), 32.16 (C-2', C-5'), 23.46 (C-3', C-4'). HR-MS (ESI) *m/z*: Calcd for $C_{11}H_{14}N_3O$: $[M1+H]^+$ =204.1131, found 204.1138. Anal. Calcd for $C_{11}H_{13}N_3O$: C, 65.01; H, 6.45; N, 20.68. Found: C, 64.79; H, 6.28; N, 20.91.

4.1.4. General procedure for the synthesis of compounds 13a-d.

The suitable amine (6.3 mmol) was added into a solution of compound **5** (2.9 mmol) in absolute ethanol (10 mL) and this mixture was refluxed for 2h. Upon completion of the reaction, the organic solvent was removed under reduced pressure, water was added to the residue and the precipitate was filtered in vacuo, washed with water and air-dried, to provide the pure aminosubstituted derivatives **13a-d**.

4.1.4.1. 2-(Morpholin-4-yl)-3-nitropyridin-4-amine (13a).

This compound was synthesized according to general procedure described above in 80% yield, upon treatment of the chloroderivative **5** with morpholine. Yellow solid, mp 131-2 ˚C ($CHCl_3/Et_2O$). ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.67 (d, 1H, *J*=4.5Hz, H-6), 7.28 (brs, 2H, D_2O exch, NH_2), 6.25 (d, 1H, *J*=4.5Hz, H-5), 3.61 (m, 4H, H-2', H-6'), 3.25 (m, 4H, H-3', H-5'). ^{13}C NMR (151 MHz, DMSO-d_6) δ 154.75 (C-2), 151.11 (C-4), 148.60 (C-6), 119.37 (C-3), 103.35 (C-5), 65.89 (C-2', C-6'), 47.91 (C-3', C-5'). HR-MS (ESI) *m/z*: Calcd for $C_9H_{13}N_4O_3$: $[M1+H]^+$ =225.0982, found 225.0979. Anal. Calcd for $C_9H_{12}N_4O_3$: C, 48.21; H, 5.39; N, 24.99. Found: C, 48.05; H, 5.30; N, 25.14.

4.1.4.2. 2-(4-Methylpiperazin-1-yl)-3-nitropyridin-4-amine (13b).

This compound was synthesized according to general procedure described above in 73% yield, upon treatment of the chloroderivative **5** with *N*-methylpiperazine. Yellow solid, mp 166-7 ˚C (EtOAc). ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.65 (d, 1H, *J*=5Hz, H-6), 7.24 (brs, 2H, D_2O exch, NH_2), 6.20 (d, 1H, *J*=5Hz, H-5) 3.25 (m, 4H, H-2', H-6'), 2.32 (m, 4H, H-3', H-5'), 2.18 (s, 3H, NCH_3). ^{13}C NMR (151 MHz, DMSO-d_6) δ 154.75 (C-2), 151.05 (C-4), 148.61 (C-6), 119.22 (C-3), 102.87 (C-5), 54.36 (C-3', C-5'), 47.26 (C-2', C-6'), 45.68 (NCH_3). HR-MS (ESI) *m/z*: Calcd for $C_{10}H_{16}N_5O_2$: $[M1+H]^+$ =238.1299, found 238.1292. Anal. Calcd for $C_{10}H_{15}N_5O_2$: C, 50.62; H, 6.37; N, 29.52. Found: C, 50.82; H, 6.48; N, 29.31.

4.1.4.3. N^2-[2-(Dimethylamino)ethyl]-3-nitropyridine-2,4-diamine (13c).

This compound was synthesized according to general procedure described above in 77% yield, upon treatment of the chloroderivative **5** with *N,N*-dimethylethylenediamine. Yellow solid, mp 120-1 ˚C ($EtOAc/Et_2O$). ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.90 (t, 1H, *J*=4.6Hz, D_2O exch, NH), 8.09 (brs, 2H, D_2O exch, NH_2), 7.64 (d, 1H, *J*=5.8Hz, H-6), 6.09 (d, 1H, *J*=5.8Hz, H-5), 3.51 (q, 2H, *J*=6.1Hz, H-2'), 2.44 (t, 2H, *J*=6.1Hz, H-3'), 2.17 (s, 6H, $2xCH_3$). ^{13}C NMR (151 MHz, DMSO-d_6) δ 154.13 (C-4), 153.41(C-2), 151.32(C-6), 116.14(C-3), 101.13(C-5), 57.50 (C-3'), 45.13 ($2xCH_3$), 38.87 (C-2'). HR-MS (ESI) *m/z*: Calcd for $C_9H_{16}N_5O_2$: $[M1+H]^+$ =226.1299, found 226.1296. Anal. Calcd for $C_9H_{15}N_5O_2$: C, 47.99; H, 6.71; N, 31.09. Found: C, 48.26; H, 6.89; N, 30.78.

4.1.4.4. N^2-Benzyl-3-nitropyridine-2,4-diamine (13d).

This compound was synthesized according to general procedure described above in 96% yield, upon treatment of the chloroderivative **5** with benzylamine. Yellow solid, mp 111-2 ˚C (CH_2Cl_2/*n*-pentane). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.15 (m, 1H, D_2O exch, NH), 8.12 (brs, 2H, D_2O exch, NH_2), 7.62 (d, 1H, *J*=5.7Hz, H-6), 7.35-7.20 (m, 5H, phenyl-H), 6.11 (d, 1H, *J*=5.7Hz, H-5), 4.68 (d, 2H, *J*=5.7Hz, CH_2). ^{13}C NMR (151 MHz, DMSO-d_6) δ 153.81 (C-2), 153.16 (C-4), 150.90 (C-6), 139.58 (C-1'), 128.19 (C-3', C-5'), 127.14 (C-2', C-6'), 126.59 (C-4'), 116.01 (C-3), 101.34 (C-5), 44.10 (CH_2). HR-MS (ESI) *m/z*: Calcd for $C_{12}H_{13}N_4O_2$:

$[M1+H]^+$ =245.1033, found 245.1037. Anal. Calcd for $C_{12}H_{12}N_4O_2$: C, 59.01; H, 4.95; N, 22.94. Found: C, 58.88; H, 4.89; N, 23.13.

4.1.5. 2-(3,5-Dimethoxyphenoxy)-3-nitropyridin-4-amine (13e).

3,5-Dimethoxyphenol (450 mg, 2.9 mmol) and cesium carbonate (940 mg, 2.9 mmol) were added into a solution of compound **5** (500 mg, 2.9 mmol) in tetrahydrofuran (20 mL), under argon, and this mixture was heated at 70˚C for 12h. Upon completion of the reaction, the organic solvent was removed under reduced pressure, water was added to the residue and the precipitate was filtered in vacuo, washed with water and air-dried, to provide the pure phenoxy derivative **13e**, as a pale yellow solid in 89% yield. Mp 166-7 ˚C (EtOAc). ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.64 (d, 1H, *J*=5.7Hz, H-6), 7.43 (brs, 2H, D_2O exch, NH_2), 6.59 (d, 1H, *J*=5.7Hz, H-5), 6.35 (s, 1H, H-4'), 6.27 (s, 2H, H-2', H-6'), 3.71 (s, 6H, 2xOCH_3). ^{13}C NMR (151 MHz, DMSO-d_6) δ 160.89 (C-3', C-5'), 156.49 (C-2), 154.90 (C-4), 150.77 (C-1'), 147.39 (C-6), 120.61 (C-3), 108.63 (C-5), 99.98 (C-2', C-6'), 97.07 (C-4'), 55.43 (2xOCH_3). HR-MS (ESI) *m/z*: Calcd for $C_{13}H_{14}N_3O_5$: $[M1+H]^+$ =292.0928, found 292.0924. Anal. Calcd for $C_{13}H_{13}N_3O_5$: C, 53.61; H, 4.50; N, 14.43. Found: C, 53.78; H, 4.55; N, 14.37.

4.1.6. General procedure for the synthesis of aminoderivatives 14a-e.

A solution of the nitro derivatives **13a-e** (2.0 mmol) in absolute ethanol (60 mL) was hydrogenated in the presence of 10% Pd/C (90 mg) under a pressure of 33 psi for **14d** and 55 psi for the rest compounds, at room temperature for 4h. The solution was filtered through a celite pad to remove the catalyst and the filtrate was evaporated to dryness. The diaminoderivatives **14a-e** were used immediately to the next step, with no further purification.

4.1.7. General procedure for the synthesis of imidazopyridines 15a-e.

Concentrated hydrochloric acid (0.3 mL) was added dropwise into a suspension of the diamines **14a-e** (2.0 mmol) in triethyl orthoformate (5 mL), under argon, and this reaction mixture was stirred at room temperature for 14h. The excess of triethyl orthoformate was removed under reduced pressure, the residue was dissolved in methanol, neutralized with sodium bicarbonate and then purified by column chromatography (silica gel) to provide pure derivatives **15a-e**.

4.1.7.1. 4-(Morpholin-4-yl)-1*H*-imidazo[4,5-*c*]pyridine (15a).

This compound was synthesized according to the general procedure described above, starting from **14a**. Purification was effected using a mixture of dichloromethane / methanol (9/1, v/v) as the eluent to provide pure **15a** as a white solid, in 92% yield. The spectroscopic data of this derivative have already been referred.[25]

4.1.7.2. 4-(4-Methylpiperazin-1-yl)-1*H*-imidazo[4,5-*c*]pyridine (15b).

This compound was synthesized according to the general procedure described above, starting from **14b**. Purification was effected using a mixture of dichloromethane / methanol (9/1, v/v) as the eluent to provide pure **15b** as a white solid, in 99% yield. Mp 234-5 ˚C (EtOH/Et_2O). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.61 (brs, 1H, D_2O exch, NH), 8.10 (s, 1H, H-2), 7.76 (d, 1H, *J*=5.5Hz, H-6), 6.86 (d, 1H, *J*=5.5Hz, H-7), 4.05 (m, 4H, H-2', H-6'), 2.43 (m, 4H, H-3', H-5'), 2.22 (s, 3H, CH_3). ^{13}C NMR (151 MHz, DMSO-d_6) δ 151.45 (C-4), 139.90 (C-6), 139.55 (C-7a), 139.23 (C-2), 128.12 (C-3a), 99.48 (C-7), 54.97 (C-3', C-5'), 45.81 (C-2', C-6'), 45.96 (CH_3). HR-MS (ESI) *m/z*: Calcd for $C_{11}H_{16}N_5$: $[M1+H]^+$ =218.1400, found 218.1397. Anal. Calcd for $C_{11}H_{15}N_5$: C, 60.81; H, 6.96; N, 32.23. Found: C, 60.93; H, 7.02; N, 32.02.

4.1.7.3. 4-[(2-Dimethylamino)ethylamino]-1*H*-imidazo[4,5-*c*]pyridine (15c).

This compound was synthesized according to the general procedure described above, starting from **14c**. Purification was effected using a mixture of dichloromethane / methanol / triethylamine (8/2/0.5, v/v/v) as the eluent to provide pure **15c** as a white solid, in 60% yield. Mp 286-7 ˚C (MeOH/EtOAc). ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.08 (brs, 1H, D_2O exch, NH-1), 8.99 (brs, 1H, D_2O exch, N<u>H</u>CH_2), 8.46 (s, 1H, H-2), 7.70 (d, 1H, *J*=6.8Hz, H-6), 7.20 (d, 1H, *J*=6.8Hz, H-7), 4.08 (m, 2H, H-2'), 3.43 (m, 2H, H-3'), 2.86 (s, 6H, 2xCH_3). ^{13}C NMR (151 MHz, DMSO-d_6) δ 147.18 (C-4), 143.56 (C-2), 139.76 (C-7a), 129.59 (C-6), 126.26 (C-3a), 100.95 (C-7), 55.52 (C-3'), 42.98 (2xCH_3), 37.71 (C-2'). HR-MS (ESI) *m/z*: Calcd for $C_{10}H_{16}N_5$: $[M1+H]^+$ =206.1400, found 206.1393. Anal. Calcd for $C_{10}H_{15}N_5$: C, 58.51; H, 7.37; N, 34.12. Found: C, 58.68; H, 7.45; N, 33.83.

4.1.7.4. *N*-Benzyl-1*H*-imidazo[4,5-*c*]pyridin-4-amine (15d) and 3-benzyl-3*H*-imidazo[4,5-*b*]pyridin-7-amine (15d$_1$).

These compounds were obtained according to the general procedure described above, starting from **14d**. Purification was effected using a mixture of dichloromethane / methanol (100/2, v/v) as the eluent to provide pure **15d** (82% yield) and **15d$_1$** (10% yield) as white solids. The spectroscopic data of **15d** have already been referred.[26]

Data for 15d$_1$: Mp 168-9 ˚C (MeOH). ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.19 (s, 1H, H-2), 7.89 (d, 1H, *J*=5.5Hz, H-5), 7.34-7.25 (m, 5H, phenyl-H), 6.37 (d, 1H, *J*=5.5Hz, H-6), 6.33 (brs, D_2O exch, 2H, NH_2), 5.38 (s, 2H, CH_2). ^{13}C NMR (151 MHz, DMSO-d_6) δ 147.14 (C-3a), 146.77 (C-7), 144.77 (C-5), 140.37 (C-2), 137.64 (C-1'), 128.53 (C-2', C-6'), 127.50 (C-4'), 127.44 (C-3', C-5'), 122.68 (C-7a), 102.03 (C-6), 45.92 (CH_2). HR-MS (ESI) *m/z*: Calcd for $C_{13}H_{13}N_4$: $[M1+H]^+$ =225.1135, found 225.1131. Anal. Calcd for $C_{13}H_{12}N_4$: C, 69.62; H, 5.39; N, 24.99. Found: C, 69.51; H, 5.30; N, 25.11.

4.1.7.5. 4-(3,5-Dimethoxyphenoxy)-1*H*-imidazo[4,5-*c*]pyridine (15e).

This compound was synthesized according to the general procedure described above, starting from **14e**. Purification was effected using a mixture of dichloromethane / methanol (100/2, v/v) as the eluent to provide pure **15e** as a white solid, in 77% yield. Mp 211-2 ˚C (MeOH). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.35 (s, 1H, H-2), 7.81 (d, 1H, *J*=5.5Hz, H-6), 7.35 (d, 1H, *J*=5.5Hz, H-7), 6.38-6.36 (m, 1H, H-4'), 6.38-6.34 (m, 2H, H-2', H-6'), 3.73 (s, 6H, 2xOCH_3). ^{13}C NMR (151 MHz, DMSO-d_6) δ 161.08 (C-3', C-5'), 156.00 (C-1'), 154.19 (C-4), 142.70 (C-2), 141.08 (C-7a), 138.58 (C-6), 128.52 (C-3a), 104.68 (C-7), 99.88 (C-2', C-6'), 96.46 (C-4'), 55.50 (2xOCH_3). HR-MS (ESI) *m/z*: Calcd for $C_{14}H_{14}N_3O_3$: $[M1+H]^+$ =272.1030, found 272.1024. Anal. Calcd for $C_{14}H_{13}N_3O_3$: C, 61.99; H, 4.83; N, 15.49. Found: C, 62.22; H, 4.96; N, 15.30.

4.1.8. General procedure for the synthesis of compounds 16a-k and 17a-b.

These compounds were synthesized following an analogous synthetic procedure to the one described for the synthesis of compounds **10a-c** and **11a-c**, starting from the imidazopyridines **15a-e**.

4.1.8.1. 1-Isopropyl-4-(morpholin-4-yl)-1*H*-imidazo[4,5-*c*]pyridine (16a).

This compound was synthesized according to the general procedure described above, upon reaction of **15a** with isopropyl bromide. Purification was effected using a mixture of dichloromethane / methanol (100/1, v/v) as the eluent to provide pure **16a** as a white solid, in 53% yield. Mp 75-6 ˚C (*n*-pentane). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.93 (d, 1H, *J*=5.7Hz,H-6), 7.79 (s, 1H, H-2), 6.75 (d, 1H, *J*=5.7Hz, H-7), 4.52 (septet, 1H, *J*=6.8Hz, C<u>H</u>$(CH_3)_2$), 4.11 (m, 4H, H-3'', H-5''), 3.86 (m, 4H, H-2'', H-6''), 1.56 (d, 6H, J=6.8Hz, 2xCH_3). ^{13}C NMR (50 MHz, $CDCl_3$) δ 151.30 (C-4), 139.60 (C-7a), 138.73 (C-6), 137.26 (C-2), 128.78 (C-3a), 97.86 (C-7), 67.10 (C-2'', C-6''), 48.13 (<u>C</u>H$(CH_3)_2$), 47.22 (C-3'', C-5''), 22.55 (2xCH_3). HR-MS (ESI) *m/z*: Calcd for $C_{13}H_{19}N_4O$: $[M1+H]^+$ =247.1553, found 247.1558. Anal. Calcd for $C_{13}H_{18}N_4O$: C, 63.39; H, 7.37; N, 22.75. Found: C, 63.56; H, 7.51; N, 22.52.

4.1.8.2. 1-Cyclopentyl-4-(morpholin-4-yl)-1*H*-imidazo[4,5-*c*]pyridine (16b).

This compound was synthesized according to the general procedure described above, upon reaction of **15a** with cyclopentyl bromide. Purification was effected using a mixture of dichloromethane / methanol (98/2, v/v) as the eluent to provide pure **16b** as a white solid, in 90% yield. Mp 97-8 ˚C (EtOAc/*n*-pentane). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.92 (d, 1H, *J*=5.1Hz, H-6), 7.76 (s, 1H, H-2), 6.76 (d, 1H, *J*=5.1Hz, H-7), 4.62 (m, 1H, H-1'), 4.20 (m, 4H, H-3'', H-5''), 3.85 (m, 4H, H-2'', H-6''), 2.27-2.17 (m, 2H, cyclopentyl-H), 2.00-1.92 (m, 2H, cyclopentyl-H), 1.91-1.84 (m, 2H, cyclopentyl-H), 1.82-1.73 (m, 2H, cyclopentyl-H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 140.66 (C-4), 140.06 (C-7a), 139.92 (C-6), 137.46 (C-2), 129.04 (C-3a), 98.01 (C-7), 67.18 (C-2'', C-6''), 57.13 (C-1'), 46.96 (C-3'', C-5''), 32.36 (C-2', C-5'), 23.81 (C-3', C-4'). HR-MS (ESI) *m/z*: Calcd for $C_{15}H_{21}N_4O$: $[M1+H]^+$ =273.1710, found 273.1717. Anal. Calcd for $C_{15}H_{20}N_4O$: C, 66.15; H, 7.40; N, 20.57. Found: C, 66.38; H, 7.54; N, 20.31.

4.1.8.3. 1-(4-Methoxybenzyl)-4-(morpholin-4-yl)-1*H*-imidazo[4,5-*c*]pyridine (16c).

This compound was synthesized according to the general procedure described above, upon reaction of **15a** with 4-methoxybenzyl chloride. Purification was effected using a mixture of dichloromethane / methanol (100/1, v/v) as the eluent to provide pure **16c** as a pale yellow oil, in 32% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.87 (d, 1H, *J*=5.7Hz, H-6), 7.71 (s, 1H, H-2), 7.03 (d, 2H, *J*=8.7Hz, H-2', H-6'), 6.80 (d, 2H, *J*=8.7Hz, H-3', H-5'), 6.62 (d, 1H, *J*=5.7Hz, H-7), 5.13 (s, 2H, CH_2), 4.09 (m, 4H, H-3'', H-5''), 3.82 (m, 4H, H-2'', H-6''), 3.72 (s, 3H, OCH_3). ^{13}C NMR (50 MHz, $CDCl_3$) δ 159.58 (C-4'), 151.98 (C-4), 140.32 (C-6), 140.12 (C-7a), 139.66 (C-2), 128.84 (C-3a), 128.53 (C-2', C-6'), 126.97 (C-1'), 114.41 (C-3', C-5'), 97.63 (C-7), 67.14 (C-2'', C-6''), 55.26 (OCH_3), 48.37 (CH_2), 46.95 (C-3'', C-5''). HR-MS (ESI) *m/z*: Calcd for $C_{18}H_{21}N_4O_2$: $[M1+H]^+$ =325.1659, found 325.1664. Anal. Calcd for $C_{18}H_{20}N_4O_2$: C, 66.65; H, 6.21; N, 17.27. Found: C, 66.39; H, 6.04; N, 17.50.

4.1.8.4. 1-Isopropyl-4-(4-methylpiperazin-1-yl)-1*H*-imidazo[4,5-*c*]pyridine (16d).

This compound was synthesized according to the general procedure described above, upon reaction of **15b** with isopropyl bromide. Purification was effected using a mixture of dichloromethane / methanol (9/1, v/v) as the eluent to provide pure **16d** as a white solid, in 84% yield. Mp 168-9 °C (CH_2Cl_2). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.86 (d, 1H, *J*=5.7Hz, H-6), 7.73 (s, 1H, H-2), 6.66 (d, 1H, *J*=5.7Hz, H-7), 4.44 (septet, 1H, *J*=6.8Hz, C<u>H</u>$(CH_3)_2$), 4.13 (m, 4H, H-2'', H-6''), 2.54 (m, 4H, H-3'', H-5''), 2.28 (s, 3H, NCH_3), 1.49 (d, 6H, *J*=6.8Hz, 2xCH_3). ^{13}C NMR (151 MHz, $CDCl_3$) δ 152.09 (C-4), 140.13 (C-6), 139.36 (C-7a), 136.69 (C-2), 128.91 (C-3a), 97.44 (C-7), 55.23 (C-3'', C-5''), 47.90 (<u>C</u>H$(CH_3)_2$), 46.10 (NCH_3, C-2'', C-6''), 22.50 (2xCH_3). HR-MS (ESI) *m/z*: Calcd for $C_{14}H_{22}N_5$: $[M1+H]^+$ =260.1870, found 260.1876. Anal. Calcd for $C_{14}H_{21}N_5$: C, 64.84; H, 8.16; N, 27.00. Found: C, 64.64; H, 8.11; N, 27.21.

4.1.8.5. 1-Cyclopentyl-4-(4-methylpiperazin-1-yl)-1*H*-imidazo[4,5-*c*]pyridine (16e).

This compound was synthesized according to the general procedure described above, upon reaction of **15b** with cyclopentyl bromide. Purification was effected using a mixture of dichloromethane / methanol (9/1, v/v) as the eluent to provide pure **16e** as a white solid, in 45% yield. Mp 192-3 °C ($CHCl_3$/Et_2O). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.90 (d, 1H, *J*=5.7Hz, H-6), 7.75 (s, 1H, H-2), 6.73 (d, 1H, *J*=5.7Hz, H-7), 4.61 (m, 1H, H-1'), 4.17 (m, 4H, H-2'', H-6''), 2.61 (m, 4H, H-3'', H-5''), 2.35 (s, 3H, NCH_3), 2.25-2.15 (m, 2H, cyclopentyl-H), 1.98-1.91 (m, 2H, cyclopentyl-H), 1.89-1.82 (m, 2H, cyclopentyl-H), 1.81-1.73 (m, 2H, cyclopentyl-H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 152.08 (C-4), 140.17 (C-6), 139.97 (C-7a), 137.43 (C-2), 129.07 (C-3a), 97.86 (C-7), 57.23 (C-1'), 55.27 (C-3'', C-5''), 46.12 (NCH_3, C-2'', C-6''), 32.37 (C-2', C-5'), 23.91 (C-3', C-4'). HR-MS (ESI) *m/z*: Calcd for $C_{16}H_{24}N_5$: $[M1+H]^+$ =286.2026, found 286.2032. Anal. Calcd for $C_{16}H_{23}N_5$: C, 67.34; H, 8.12; N, 24.54. Found: C, 67.03; H, 8.02; N, 24.88.

4.1.8.6. 4-[2-(Dimethylamino)ethylamino]-1-isopropyl-1*H*-imidazo[4,5-*c*]pyridine (16f).

This compound was synthesized according to the general procedure described above, upon reaction of **15c** with isopropyl bromide. Purification was effected using a mixture of dichloromethane / methanol / triethylamine (85/15/5, v/v/v) as the eluent to provide pure **16f** as an orange colored oil, in 84% yield. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.86 (s, 1H, H-2), 7.85 (1H, *J*=6.1Hz, H-6), 6.71 (d, 1H, *J*=6.1Hz, H-7), 6.01 (brs, 1H, D_2O exch, NH), 4.54 (septet, 1H, *J*=6.7Hz, C<u>H</u>$(CH_3)_2$), 3.89 (t, 2H, *J*=6.1Hz, H-2'), 2.97 (t, 2H, *J*=6.1Hz, H-3'), 2.55 (s, 6H, $N(CH_3)_2$), 1.58 (d, 6H, *J*=6.7Hz, 2xCH_3). ^{13}C NMR (151 MHz, $CDCl_3$) δ 151.10 (C-4), 139.23 (C-6), 138.58 (C-2), 137.69 (C-3a), 127.79 (C-7a), 97.24 (C-7), 58.01(C-3'), 48.49 (<u>C</u>H$(CH_3)_2$), 44.55 ($N(CH_3)_2$), 38.07 (C-2'), 22.71 (2xCH_3). HR-MS (ESI) *m/z*: Calcd for $C_{13}H_{22}N_5$: $[M1+H]^+$ =248.1870, found 248.1874. Anal. Calcd for $C_{13}H_{21}N_5$: C, 63.13; H, 8.56; N, 28.31. Found: C, 63.31; H, 8.63; N, 28.02.

4.1.8.7. 1-Cyclopentyl-4-[2-(dimethylamino)ethylamino]-1*H*-imidazo[4,5-*c*]pyridine (16g).

This compound was synthesized according to the general procedure described above, upon reaction of **15c** with cyclopentyl bromide. Purification was effected using a mixture of dichloromethane / methanol (85/15, v/v) as the eluent to provide pure **16g** as an orange colored oil, in 81% yield. ^{1}H NMR (400 MHz, CD_3OD) δ 8.26 (s, 1H, H-2), 7.85 (d, 1H, *J*=6.3Hz, H-6), 7.10 (d, 1H, *J*=6.3Hz, H-7), 4.87 (m, 1H, H-1'), 3.97 (t, 2H, *J*=5.3Hz, H-2''), 3.46 (t, 2H, *J*=5.3Hz, H-3''), 3.01 (s, 6H, 2xCH_3), 2.34-2.26 (m, 2H, cyclopentyl-H), 2.03-1.96 (m, 2H, cyclopentyl-H), 1.96-1.90 (m, 2H, cyclopentyl-H), 1.86-1.79 (m, 2H, cyclopentyl-H). ^{13}C NMR (151 MHz, CD_3OD) δ 151.79 (C-4), 142.65 (C-2), 140.54 (C-7a), 137.73 (C-6), 128.62 (C-3a), 100.37 (C-7), 60.36 (C-3''), 59.04(C-1'), 44.06 ($N(CH_3)_2$), 39.29 (C-2''), 33.49 (C-2', C-5'), 25.00 (C-3', C-4'). HR-MS (ESI) *m/z*: Calcd for $C_{15}H_{24}N_5$: $[M1+H]^+$ =274.2026, found 274.2030. Anal. Calcd for $C_{15}H_{23}N_5$: C, 65.90; H, 8.48; N, 25.62. Found: C, 66.06; H, 8.53; N, 25.39.

4.1.8.8. *N*-Benzyl-1-isopropyl-1*H*-imidazo[4,5-*c*]pyridin-4-amine (16h).

This compound was synthesized according to the general procedure described above, upon reaction of **15d** with isopropyl bromide. Purification was effected using a mixture of dichloromethane / methanol (100/2, v/v) as the eluent to provide pure **16h** as a pale yellow oil, in 86% yield. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.91 (d, 1H, *J*=5.9Hz, H-6), 7.75 (s, 1H, H-2), 7.41 (d, 2H, *J*=7.2Hz, H-2', H-6'), 7.31 (t, 2H, *J*=7.2Hz, H-3', H-5'), 7.24 (t, 1H, *J*=7.2Hz, H-4'), 6.70 (d, 1H, *J*=5.9Hz, H-7), 5.90 (brs, 1H, D_2O exch, NH), 4.83 (d, 2H, *J*=5.6Hz, CH_2), 4.53 (septet, 1H, *J*=6.7Hz,C<u>H</u>$(CH_3)_2$), 1.58 (d, 6H, *J*=6.7Hz, 2xCH_3). ^{13}C NMR (151 MHz, $CDCl_3$) δ 151.74 (C-4), 140.74 (C-6), 139.77 (C-1'), 137.98 (C-2), 137.53 (C-7a), 128.63 (C-3', C-5'), 127.93 (C-2', C-6'), 127.21 (C-4', C-3a), 97.06 (C-7), 48.40 (<u>C</u>H$(CH_3)_2$), 45.20 (CH_2), 22.80 (2xCH_3). HR-MS (ESI) *m/z*: Calcd for $C_{16}H_{19}N_4$: $[M1+H]^+$ =267.1604, found 267.1610. Anal. Calcd for $C_{16}H_{18}N_4$: C, 72.15; H, 6.81; N, 21.04. Found: C, 72.29; H, 6.89; N, 20.79.

4.1.8.9. *N*-Benzyl-1-cyclopentyl-1*H*-imidazo[4,5-*c*]pyridin-4-amine (16i).

This compound was synthesized according to the general procedure described above, upon reaction of **15d** with cyclopentyl bromide. Purification was effected using a mixture of dichloromethane / methanol (100/2, v/v) as the eluent to provide pure **16i** as a pale yellow oil, in 52% yield. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.84 (d, 1H, *J*=5.9Hz, H-6), 7.66 (s, 1H, H-2), 7.34 (d, 2H, *J*=7.5Hz, H-2', H-6'), 7.24 (t, 2H, *J*=7.5Hz, H-3', H-5'), 7.17 (t, 1H, *J*=7.5Hz, H-4'), 6.64 (d, 1H, *J*=5.9Hz, H-7), 5.77 (brs, 1H, D_2O exch, NH), 4.77 (d, 2H, *J*=5.7Hz, CH_2), 4.57 (m, 1H, H-1''), 2.22-2.15 (m, 2H, cyclopentyl-H), 1.96-1.89 (m, 2H, cyclopentyl-H), 1.87-1.81 (m, 2H, cyclopentyl-H), 1.76-1.70 (m, 2H, cyclopentyl-H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 151.71 (C-4), 140.67 (C-6), 139.78 (C-1'), 138.64 (C-2), 137.99 (C-7a), 127.92 (C-2', C-6'), 127.20 (C-4'), 97.30 (C-7), 57.54 (C-1''), 45.20 (CH_2), 32.55 (C-2'', C-5''), 24.05 (C-3'', C-4''). HR-MS (ESI) *m/z*: Calcd for $C_{18}H_{21}N_4$: $[M1+H]^+$ =293.1761, found 293.1766. Anal. Calcd for $C_{18}H_{20}N_4$: C, 73.94; H, 6.89; N, 19.17. Found: C, 74.14; H, 6.98; N, 18.83.

4.1.8.10. 4-(3,5-Dimethoxyphenoxy)-1-isopropyl-1*H*-imidazo[4,5-*c*]pyridine (16j) and 4-(3,5-dimethoxyphenoxy)-3-isopropyl-3*H*-imidazo[4,5-*c*]pyridine (17a).

These compounds were synthesized according to the general procedure described above, upon reaction of **15e** with isopropyl bromide. Purification was effected using a mixture of dichloromethane / methanol (100/1, v/v) as the eluent to provide the pure isomers **16j** and **17a**.

Data for 16j: Yield 29%. White solid, mp 127-8 °C (Et_2O). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.00 (s, 1H, H-2), 7.95 (d, 1H, *J*=5.7Hz, H-6), 7.09 (d, 1H, *J*=5.7Hz, H-7), 6.44 (d, 2H, *J*=2.2Hz, H-2'', H-6''), 6.31 (t, 1H, *J*=2.2Hz, H-4'), 4.62 (septet, 1H, *J*=6.7Hz, C<u>H</u>$(CH_3)_2$), 3.75 (s, 6H, 2xOCH_3), 1.63 (d, 6H, *J*=6.7Hz, 2xCH_3). ^{13}C NMR (151 MHz, $CDCl_3$) δ 161.26 (C-3'', C-5''), 155.75 (C-1''), 155.61 (C-4), 140.72 (C-7a), 140.60 (C-2), 139.54 (C-6), 129.68 (C-3a), 102.49 (C-7), 100.30 (C-2'', C-6''), 97.41 (C-4''), 55.50 (2xOCH_3), 48.75 (<u>C</u>H$(CH_3)_2$), 22.76 (2xCH_3). HR-MS (ESI) *m/z*: Calcd for $C_{17}H_{20}N_3O_3$: $[M1+H]^+$ =314.1499, found 314.1503. Anal. Calcd for $C_{17}H_{19}N_3O_3$: C, 65.16; H, 6.11; N, 13.41. Found: C, 64.98; H, 6.02; N, 13.69.

Data for 17a: Yield 36%. White solid, mp 135-6 °C (Et_2O). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.10 (s, 1H, H-2), 7.92 (d, 1H, *J*=5.7Hz, H-6), 7.41 (d, 1H, *J*=5.7Hz, H-7), 6.36 (d,2H, *J*=2.2Hz, H-2'', H-6''), 6.33 (t, 1H, *J*=2.2Hz, H-4''), 5.15 (septet, 1H, *J*=6.7Hz, C<u>H</u>$(CH_3)_2$), 3.76 (s, 6H, 2xOCH_3), 1.64 (d, 6H, *J*=6.7Hz, 2xCH_3). ^{13}C NMR (151 MHz, $CDCl_3$) δ 161.55 (C-3'', C-5''), 155.38 (C-1''), 152.26 (C-4), 150.11 (C-7a), 142.53 (C-2), 138.71 (C-6), 119.97 (C-3a), 111.65 (C-7), 99.85 (C-2'', C-6''), 97.36 (C-4''), 55.55 (2xOCH_3), 49.96 (<u>C</u>H$(CH_3)_2$), 23.71 (2xCH_3). HR-MS (ESI) *m/z*: Calcd for $C_{17}H_{20}N_3O_3$: $[M1+H]^+$ =314.1499, found 314.1507. Anal. Calcd for $C_{17}H_{19}N_3O_3$: C, 65.16; H, 6.11; N, 13.41. Found: C, 65.34; H, 6.17; N, 13.24.

4.1.8.11. 1-Cyclopentyl-4-(3,5-dimethoxyphenoxy)-1*H*-imidazo[4,5-*c*]pyridine (16k) and 3-cyclopentyl-4-(3,5-dimethoxyphenoxy)-3*H*-imidazo[4,5-*c*]pyridine (17b).

These compounds were synthesized according to the general procedure described above, upon reaction of **15e** with cyclopentyl bromide. Purification was effected using a mixture of dichloromethane / methanol (100/1, v/v) as the eluent to provide the pure isomers **16k** and **17b**.

Data for 16k: Yield 44%. White solid, mp 165-6 °C (Et_2O). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.96 (s, 1H, H-2), 7.94 (d, 1H, *J*=5.7Hz, H-6), 7.09 (d, 1H, *J*=5.7Hz, H-7), 6.43 (d, 2H, *J*=2.2Hz, H-2", H-6"), 6.31 (t, 1H, *J*=2.2Hz, H-4"), 4.72 (m, 1H, H-1'), 3.76 (s, 6H, 2xOCH_3), 2.33-2.26 (m, 2H, cyclopentyl-H), 2.06-1.99 (m, 2H, cyclopentyl-H), 1.96-1.90 (m, 2H, cyclopentyl-H), 1.85-1.81 (m, 2H, cyclopentyl-H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 161.24 (C-3", C-5"), 156.77 (C-7a), 155.77 (C-1"), 155.54 (C-4), 141.18 (C-2), 139.47 (C-6), 129.73 (C-3a), 102.72 (C-7), 100.26 (C-2", C-6"), 97.37 (C-4"), 57.77 (C-1'), 55.47(2xOCH_3), 32.55 (C-2', C-5'), 24.00 (C-3', C-4'). HR-MS (ESI) *m/z*: Calcd for $C_{19}H_{22}N_3O_3$: $[M1+H]^+$ =340.1656, found 340.1662. Anal. Calcd for $C_{19}H_{21}N_3O_3$: C, 67.24; H, 6.24; N, 12.38. Found: C, 67.01; H, 6.14; N, 12.55.

Data for 17b: Yield 44%. White solid, mp 139-140 °C (Et_2O). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.06 (s, 1H, H-2), 7.91 (d, 1H, *J*=5.7Hz, H-6), 7.40 (d, 1H, *J*=5.7Hz, H-7), 6.36 (d, 2H, *J*=2.2Hz, H-2", H-6"), 6.33 (t, 1H, *J*=2.2Hz, H-4"), 5.23 (m, 1H, H-1'), 3.76 (s, 6H, 2xOCH_3), 2.33-2.26 (m, 2H, cyclopentyl-H), 2.06-1.99 (m, 2H, cyclopentyl-H), 1.91-1.84 (m, 2H, cyclopentyl-H), 1.80-1.74 (m, 2H, cyclopentyl-H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 161.51 (C-3", C-5"), 155.47 (C-1"), 152.42 (C-4), 150.24 (C-7a), 143.01 (C-2), 138.67 (C-6), 120.43 (C-3a), 111.62 (C-7), 99.79 (C-2", C-6"), 97.25 (C-4"), 59.23 (C-1'), 55.51 (2xOCH_3), 33.64 (C-2', C-5'), 23.86 (C-3', C-4'). HR-MS (ESI) *m/z*: Calcd for $C_{19}H_{22}N_3O_3$: $[M1+H]^+$ =340.1656, found 340.1664. Anal. Calcd for $C_{19}H_{21}N_3O_3$: C, 67.24; H, 6.24; N, 12.38. Found: C, 66.98; H, 6.09; N, 12.66.

4.1.9. General procedure for the synthesis of compounds 18b-c.

Ammonium formate (150 mg, 2.5 mmol) and 10% Pd/C (70 mg) were added into a solution of the benzylamines **16h** or **16i** (0.25 mmol) in methanol (5 mL), under argon, and this reaction mixture was refluxed for 48h. An additional amount (300 mg, 5.0 mmol) of ammonium formate was added and the reflux was continued for 48h. Upon completion of the reaction, the solution was filtered through a celite pad to remove the catalyst and the filtrate was evaporated to dryness. The crude product was purified by column chromatography (silica gel) to provide pure aminoderivatives **18b** and **18c**.

4.1.9.1. 1-Isopropyl-1*H*-imidazo[4,5-*c*]pyridin-4-amine (18b).

This compound was synthesized according to the general procedure described above, starting from **16h**. Purification was effected using a mixture of dichloromethane / methanol (9/1, v/v) as the eluent to provide pure **18b** as a pale yellow oil, in 43% yield. ^{1}H NMR (600 MHz, $(CD_3)_2CO$) δ 8.12 (s, 1H, H-2), 7.71 (d, 1H, *J*=6.0 Hz, H-6), 6.92 (d,1H, *J*=6.0 Hz, H-7), 6.29 (brs, 2H, D_2O exch, NH_2), 4.73 (septet, 1H, *J*=6.7 Hz, C<u>H</u>$(CH_3)_2$), 1.60 (d, 6H, J=6.7 Hz, 2xCH_3). ^{13}C NMR (151 MHz, $(CD_3)_2CO$) δ 153.01 (C-4), 140.53 (C-2), 139.13 (C-7a), 138.87 (C-6), 128.39 (C-3a), 98.48 (C-7), 49.18 (<u>C</u>H$(CH_3)_2$), 22.76 (2xCH_3). HR-MS (ESI) *m/z*: Calcd

for $C_9H_{13}N_4$: $[M1+H]^+$ =177.1135, found 177.1130. Anal. Calcd for $C_9H_{12}N_4$: C, 61.34; H, 6.86; N, 31.79. Found: C, 61.52; H, 6.93; N, 31.49.

4.1.9.2. 1-Cyclopentyl-1*H*-imidazo[4,5-*c*]pyridin-4-amine (18c).

This compound was synthesized according to the general procedure described above, starting from **16i**. Purification was effected using a mixture of dichloromethane / methanol (100/8, v/v) as the eluent to provide pure **18c** as a pale yellow oil, in 82% yield. ^{1}H NMR (600 MHz, $(CD_3)_2CO$) δ 8.12 (s, 1H, H-2), 7.72 (d, 1H, *J*=6.0 Hz, H-6), 6.95 (d,1H, *J*=6.0 Hz, H-7), 6.44 (brs, 2H, D_2O exch, NH_2), 4.87 (m, 1H, H-1'), 2.34-2.26 (m, 2H, cyclopentyl-H), 2.08-2.00 (m, 2H, cyclopentyl-H), 1.97-1.91 (m, 2H, cyclopentyl-H), 1.84-1.77 (m, 2H, cyclopentyl-H). ^{13}C NMR (151 MHz, $(CD_3)_2CO$) δ 152.81 (C-4), 141.24 (C-2), 139.75 (C-7a), 138.14 (C-6), 128.44 (C-3a), 98.76 (C-7), 58.28 (C-1'), 33.04 (C-2', C-5'), 24.64 (C-3', C-4'). HR-MS (ESI) *m/z*: Calcd for $C_{11}H_{15}N_4$: $[M1+H]^+$ =203.1291, found 203.1284. Anal. Calcd for $C_{11}H_{14}N_4$: C, 65.32; H, 6.98; N, 27.70. Found: C, 65.44; H, 7.04; N, 27.51.

4.2. Combinatorial Design-Molecular Docking Calculations

4.2.1. Ligand Enumeration - Preparation

Based on the scaffold of 3-deazaadenine, we enumerated a virtual combinatorial library using our in-house fragment library containing 200 chemical fragments. Moreover, we defined 2 positions for substitutions (1 and 4) combining 0 to 2 methylene groups as linkers between the fragment and the core. Thus the intensity of the new virtual chemical library enumerated 1200 compounds. All ligands were prepared using the ligprep module as implemented on Schrodinger Suite 2014. The geometries of the generated structures are optimized using a restricted version of the MacroModel™ computational program, bmin, or a short conformational search is performed to relax the structure into 3 dimensions while strongly encouraging chiral centers to adopt the proper chirality (if the structure is highly strained). For the mono 4-substitued analogues of 3-deaza adenine, both 1NH and 3NH tautomers were generated for further docking calculations.

4.2.2. Virtual Screening

The modeled structure of FcyB transporter is already described by our group,[15] and prepared for Virtual Screening using the protein preparation workflow as implemented on Schrodinger suite 2014. Glide energy grids were generated using the Receptor Grid Generation panel on Maestro software with default values. Glide software was utilized for virtual screening using the SP protocol. The enumerated database created passed through virtual screening and only the first 2% (40 structures) of ligands based on GlideScore stored for Induced Fit Docking.

4.2.3. Induced Fit Docking

All selected molecules for synthesis were passed through exhaustive molecular docking calculations using the IFD protocol (Induced Fit Docking protocol 2015–2, Glide version 6.4, Prime version 3.7,Schrödinger, LLC, 2015),[27,28] which is intended to circumvent the inflexible binding site and accounts for the side-chain or backbone movements, or both, upon ligand binding. In the first stage of the IFD protocol, softened-potential docking step, 20 poses per ligand were retained. In the second step, for each docking pose, a full cycle of protein refinement was performed, with Prime 3.7 (Prime, version 3.7, Schrödinger, LLC) on all residues having at least one atom within 8 Å of an atom in any of the 20 ligand poses. The Prime refinement starts with a conformational search and minimization of the side-chains of the selected residues and after convergence to a low-energy solution, an additional minimization of all selected residues (side-chain and backbone) is performed with the truncated-Newton algorithm using the OPLS parameter set and a surface Generalized Born implicit solvent model. The obtained complexes are ranked according to Prime calculated energy (molecular mechanics and solvation), and those within 30 kcal/mol of the minimum energy structure are used in the last step of the process, redocking with Glide 6.4 (Glide, version 6.4, Schrödinger, LLC, 2015) using standard precision and scoring. In the final round, the ligands used in the first docking step are redocked into each of the transporter structures retained from the refinement step. The final ranking of the complexes is done by a composite score which accounts for the transporter–ligand interaction energy (GlideScore) and solvation energies (Prime energy).

4.3. *Aspergillus* manipulations

Standard complete and minimal media (MM) for *A. nidulans* were used. Media and supplemented auxotrophies were at the concentrations given in http://www.fgsc.net. 10 mM $NaNO_3$ was used as a nitrogen source. Inhibitors are added in MM dissolved in DMSO at 500 μM. Transformations were performed as described previously.[29] Transformants of the Δ7 master mutant, arising from single-copy plasmid integration events of vectors carrying the either *fcyB* or the *azgA* gene, in addition to a wild-type *pabA1* selection marker, were obtained by complementation of the *pabA1* auxotrophy. The Δ7 master mutant contains total genetic deletion of all major nucleobase/nucleoside transporters (*ΔfcyB::argB ΔazgA, ΔuapA, ΔuapC::AfpyrG, ΔfurD::riboB ΔfurA::riboB, ΔcntA::riboB, pantoB100, pabA1*), in addition to *pabA1* auxotrophy. Confirmation of single copy integrations, introducing intact *fcyB* or *azgA* genes, was obtained by Southern and PCR analyses. *azgA*- and *fcyB*- containing vectors,[16,17] and the Δ7 mutant strain[13] have been described before. In the case of *fcyB*, transcription is driven by the *uapA* promoter for obtaining sufficient protein levels for functional assays.[17] AzgA transcription is driven by its nature promoter.

4.4. Transport assays

Transport assays for measuring the activity of purine transporters, such as FcyB, AzgA or UapA, is carried out in germinating conidiospores, as recently described in detail.[13] For transport competition assays, 0.5 μM of ^{3}H-radiolabelled substrate (adenine, hypoxanthine or xanthine) is added in a mix with 1000-fold excess 3-deazaadenine analogues (500 μM).[13] Assays are terminated after 1 min by freezing, immediate centrifugation and washing of cells. K_i values are estimated from IC_{50} measurements using the Cheng and Prussof equation [$K_i = IC_{50}/1+ [S]/K_m$, where [S] is the fixed concentration of radiolabeled substrate used] and analyzed by the GraphPad Prism software. All experiments are carried out at three times, with each assays performed in triplicate. Standard deviation in all cases is less than 30%. Radiolabeled purines used are: [2,8-^{3}H]-adenine 20.0 Ci/mmol, [2,8-^{3}H]-hypoxanthine 27.7 Ci/mmol or [8-^{3}H]-xanthine 22.8 Ci/mmol, all from Moravek Biochemicals.

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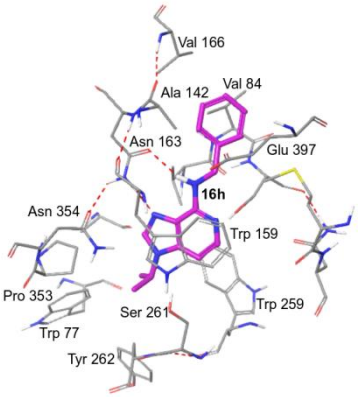

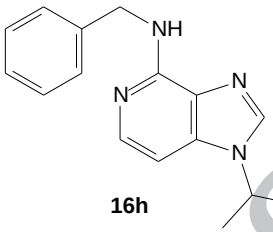

16h

specific inhibition of FcyB transporter
Ki (µM): 5±1