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Synthesis and evaluation of 2-pyridinylpyrimidines as inhibitors of HIV-1 structural protein assembly

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ABSTRACT

In an effort to identify an HIV-1 capsid assembly inhibitor with improved solubility and potency, we synthesized two series of pyrimidine analogues based on our earlier lead compound *N*-(4-(ethoxycarbonyl) phenyl)-2-(pyridine-4-yl)quinazoline-4-amine. In vitro binding experiments showed that our series of 2-pyridine-4-ylpyrimidines had IC_{50} values higher than 28 μM. Our series of 2-pyridine-3-ylpyrimidines exhibited IC_{50} values ranging from 3 to 60 μM. The congeners with a fluoro substituent introduced at the 4-*N*-phenyl moiety, along with a methyl at C-6, represent potent HIV capsid assembly inhibitors binding to the C-terminal domain of the capsid protein.

Human immunodeficiency virus type 1 (HIV-1) is the causative agent of acquired immunodeficiency syndrome (AIDS). Since the introduction of highly active antiretroviral therapy (HAART), the mortality of AIDS patients has declined, and the life expectancy of people with HIV infection has increased markedly. However, treatment success can be compromised by the unwanted side-effects of current medications and by selection of drug-resistant viruses. Therefore, there remains a demand for novel approaches and targets for therapeutic intervention in the viral life cycle.[1,2]

The HIV-1 virion is built up by assembly of approximately 2500 Gag polyprotein molecules that are organized inside the budding virions.[3] For the virus to mature, these polyproteins have to be specifically processed by the viral protease into final viral proteins, including matrix, nucleocapsid, small spacer peptides, and capsid (CA).[4–6] The latter possesses N- and C-terminal domains that are tethered by a flexible linker. The conical shape of the mature HIV capsid enclosing the viral genome stems from its architecture—the CA subunits form hexamers and pentamers, contributing to the overall fullerene-type structure.

At present, there is no HIV-1 assembly inhibitor on the market. There are few reports of non-peptidic compounds targeting CTD of CA.[7–11] The other reported compounds bind to the NTD of CA and some of them affect capsid assembly. Three independent binding sites have been defined in the NTD. Acylhydrazones, thioureas, benzodiazepines, benzimidazoles and the urea-based derivative CAP-1, bind to the hydrophobic pocket at the base of the helical bundle.[12–16] A second binding site interacts with the ligand PF-3450074.[17] A third ligand-binding site lies near the flexible cyclophilin A binding loop.[18] In general, the reported CA NTD-binding compounds exhibit mostly micromolar affinity towards CA in vitro and weak antiviral activity. However, several effective nanomolar inhibitors have been reported.[15,17]

The discovery by Sticht et al.[19] of a 12-mer peptide (CAI) that is capable of binding to the CA C-terminal domain (CTD) and inhibits assembly of immature and mature-like particles in vitro enabled the development of a competitive binding assay designed to select CTD ligands. This new screening assay for assembly inhibitors

Abbreviations: AIDS, acquired immunodeficiency syndrome; CA, capsid; CAI, capsid assembly inhibitor; CTD, C-terminal domain; HAART, highly active antiretroviral therapy; HIV, human immunodeficiency virus; ITC, isothermal titration calorimetry; NMR, nuclear magnetic resonance; TLC, thin layer chromatography; TBTU, *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(benzotriazol-1-yl)uronium tetrafluoroborate.

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0960-894X/

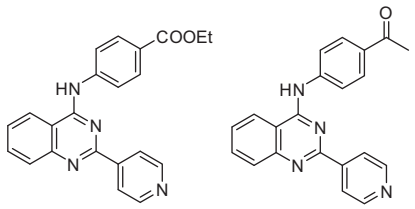
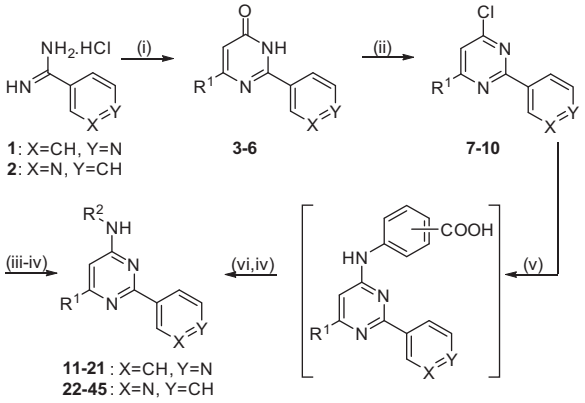


Figure 1. 4-Aminophenylquinazoline derivatives that bind to the C-terminal domain of the HIV-1 capsid protein.[7]



Scheme 1. Synthesis of pyrimidines **11**–**45**. R^1 = H, CH_3. The following reagents and conditions were used: (i) ethyl formylacetate sodium salt, ethanol, reflux or methyl acetoacetate, $NaHCO_3$, ethanol, reflux; (ii) neat $POCl_3$, 100 °C; (iii) aniline, HCl, dioxane, reflux or HCl, ethanol, water, reflux; (iv) HCl in dioxane; (v) aminobenzoic acid, HCl, ethanol, water, reflux; (vi) amine, TBTU, Et_3N, DMF.

utilizes an amplified luminescent proximity assay system (AlphaScreen). Briefly, interaction of CAI linked to donor beads with CA CTD on acceptor beads results in a strong luminescent signal originating from energy transfer from excited donor to acceptor beads. The presence of an effective assembly inhibitor causes the beads to separate, resulting in decreased luminescence.

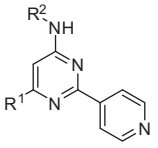
We previously identified a set of unique capsid assembly inhibitors binding to CA CTD from a high throughput screen.[7] Because the inhibitors compete with CAI, we proposed that they bind into a conserved hydrophobic groove in CA CTD.[20] We further optimized the identified hits to improve the inhibitory activity, resulting in the compounds shown in Figure 1. These compounds exhibit moderate affinity to the binding site in vitro, as determined by AlphaScreen assay and ITC.[7]

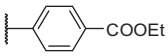
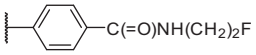
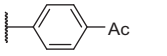
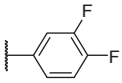
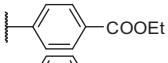
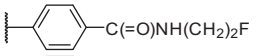
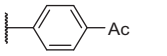
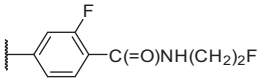
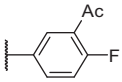
To improve the pharmacokinetics and binding of those quinazoline compounds, we searched for derivatives with superior solubility. From previous work,[7] it was obvious that the 4-*N*-phenyl and 2-pyridinyl moieties are crucial for binding. Therefore, we decided to replace the parent quinazoline scaffold with a smaller, and less hydrophobic, heterocyclic pyrimidine.

Here, we report evaluation of two series of *N*-4-phenyl-2-pyridinylpyrimidines that are substituted at the *N*-phenyl moiety. Pyridine-4-ylpyrimidines (compounds **11**–**21**) and pyridine-3-ylpyrimidines (compounds **22**–**45**), shown in Scheme 1, are bioisosteres that act as new classes of capsid assembly inhibitors binding to CA CTD.

We explored the structure–activity relationship (SAR) of these compounds with regard to substitution of the *N*-phenyl moieties. From previous work,[7] we concluded that the attachment of electron-withdrawing groups lead to the decrease of in vitro IC_{50} values determined by AlphaScreen assay. In particular, carbonyl and carboxyl functionalities were superior to sulfonamides and carboxamides, except for *N*-2-fluoroethylcarboxamide.

Table 1
Structure–activity relationship of the pyridine-4-yl derivatives



Compound	R^2	IC_{50} (μM)
11*		28 ± 3
12*		>100
13*		>100
14*		>100
15*		81 ± 8
16**		36 ± 3
17**		>100
18**		54 ± 5
19**		>100
20**		>100
21**		83 ± 7

The IC_{50} value was determined as the compound concentration sufficient for decreasing the AlphaScreen signal by 50%. For experimental details, see Supplementary materials.

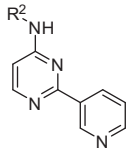
* 2,4-Disubstituted pyrimidine derivative (R^1 = H).

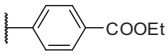
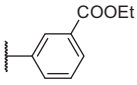
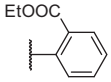
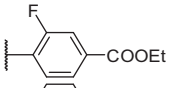
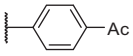
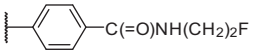
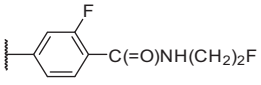
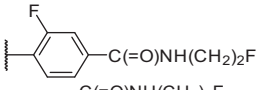
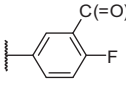
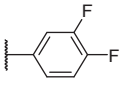
** 2,4,6-Trisubstituted pyrimidine derivative (R^1 = Me).

The compounds reported here were synthesized according to the general route outlined in Scheme 1. The commercially available 3-/4-cyanopyridines were first converted to corresponding amidines **1** and **2** by treatment with sodium methoxide and later with ammonium chloride.[21] The resulting amidines were either combined with the sodium salt of ethyl formylacetate, which was prepared by aldol condensation of ethyl acetate with ethyl formate, or with methyl acetoacetate to yield pyrimidinones **3**–**6**.[21,22] Subsequent treatment[23] with phosphorus oxychloride yielded 4-chloropyrimidines **7**–**10**, which were subjected to nucleophilic displacement of the chlorine atom by variously substituted anilines.[7,24] To prepare the carboxamide series (**13**, **17**, **20**, **28**–**31**, **39**–**44**), a different synthetic approach was used. An appropriate chloropyrimidine was first treated with aminobenzoic acid, and when TLC indicated its disappearance, the entire reaction mixture was evaporated to dryness and subjected to standard amidation using TBTU as a coupling agent. We found that this stepwise approach was superior to the nucleophilic aromatic substitution carried out with corresponding *N*-alkyl aminobenzamides in terms of yield and versatility. Finally, all prepared compounds were converted into the corresponding hydrochloride salts.

We started with preparation of pyridine-4-ylpyrimidines, which are congeners of the compounds shown in Figure 1. The first round of SAR studies revealed that ablation of the annulated benzene ring from the parent quinazoline led to a significant decrease

Table 2
Structure–activity relationship of the 2-(pyridine-3-yl) pyrimidine derivatives



Compound	R^2	IC_{50} (μM)
22		19 ± 1
23		21 ± 2
24		>100
25		30 ± 2
26		53 ± 8
27		39 ± 5
28		34 ± 4
29		43 ± 15
30		73 ± 12
31		68 ± 9
32		38 ± 5

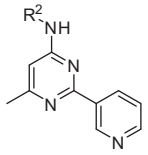
The IC_{50} value was determined as the compound concentration sufficient for decreasing the AlphaScreen signal by 50%. For experimental details, see Supplementary materials.

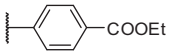
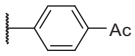
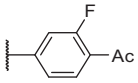
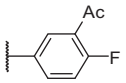
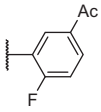
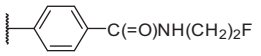
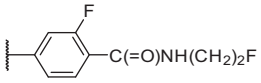
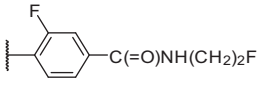
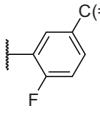
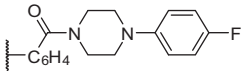
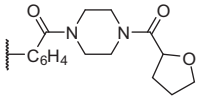
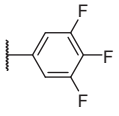
in inhibition potency, as evident from the IC_{50} values of compounds **11**–**14** (Table 1). We hypothesized that the lack of benzene ring in these compounds affected their lipophilicity, and therefore we attempted to compensate for the absence of a benzene ring by introduction of a methyl group at C-6 (**16**–**19**). However, this alteration did not lead to improved inhibition potency.

Next, we introduced an additional fluorine atom on the *N*-phenyl moiety of compounds **17** and **19**, because derivative **15** with a 3,4-difluorophenyl moiety revealed some, albeit weak, inhibition. The resulting compounds **20** and **21** did not benefit from the presence of a fluorine atom.

The second SAR round was performed with an isomeric scaffold possessing a pyridine-3-yl moiety (Table 2). Based on the inhibitory activities of carboxyl and carbonyl derivatives **22**–**23** and **26**–**27**, it is evident that this series has superior binding capabilities than that presented in Table 1. Furthermore, the solubility of all prepared pyridine-3-yl derivatives in aqueous systems was better than that of pyridine-4-yl congeners. The screening revealed that an ethyl ester moiety is more suitable than an acetyl moiety. Furthermore, the SAR data indicate that *meta* and *para* substituted compounds have similar potency, whereas *ortho* substituted ethoxycarbonyl derivative **24** was inactive. As seen with the first series, derivatives with an additional fluorine atom appeared to be less active (compare **22** and **28** with **25**, **29**, **30** and **31**).

Table 3
Structure–activity relationship of the 6-methyl-2-(pyridine-3-yl) pyrimidine derivatives



Compound	R^2	IC_{50} (μM)	EC_{50} (μM)	CC_{50} (μM)
33		21 ± 3	n.d.	n.d.
34		12 ± 2	50 ± 12	77 ± 39
35		10 ± 4	23 ± 1	64 ± 10
36		25 ± 3	n.d.	n.d.
37		9 ± 2	>100	>100
38		>100	n.d.	n.d.
39		8 ± 1	25 ± 1	44 ± 7
40		3 ± 1	20 ± 1	35 ± 3
41		15 ± 4	>100	>100
42		52 ± 6	n.d.	n.d.
43		20 ± 7	n.d.	n.d.
44		54 ± 8	n.d.	n.d.
45		22 ± 4	n.d.	n.d.

The IC_{50} value was determined as the compound concentration sufficient for decreasing the AlphaScreen signal by 50%. Anti-HIV-1 activity (EC_{50}) was calculated as the concentration of compound required to inhibit virus replication in MT-4 cells by 50%. The CC_{50} value represents the compound concentration reducing the cell viability by 50%. For experimental details, see Supplementary information. n.d. = not determined.

In the third SAR round Table 3, we intended to explore the effect of a methyl group introduced at C-6 of the pyrimidine core, similar to what was done with the first series (see **16**–**21**). The inhibition activity of ethyl ester derivative **33** was unaffected by the addition of a methyl substituent. Surprisingly, acetyl derivatives **34** and **35** appeared to be much more sensitive to the methyl introduction.

Their binding was significantly improved by the presence of a methyl moiety (compare **26** and **27** with **34** and **35**). Fluorinated acetyls **36**–**38** were of comparable potency or were weaker, which is in good agreement with the data shown in Table 2.

Introduction of 2-fluoroethylcarboxamide led to the discovery of compound **39**, which has one of the highest inhibition potencies found in this SAR cycle. To further explore the potential of the 2-fluoroethylcarboxamide derivative, we prepared three monofluorinated congeners. Introduction of the fluorine atom at the *meta* position (beside the carboxamide, see **40**) furnished the tightest ligand binding to CA CTD. In addition to primary amides, we also prepared two secondary amides containing a piperazine scaffold with a fluorophenyl (**43**) or carbonyl (**44**) moiety at N-4. Both compounds exhibited moderate micromolar binding to CA in the competitive assay. Trifluorophenyl derivative **45** had similar inhibitory activity as its congener **32** and displayed a one-order-of-magnitude weaker affinity to CA compared to **40**, the tightest binding compound reported here (Table 3).

The anti-HIV-1 activities (EC_{50}) of the compounds were determined by measuring inhibition of viral infectivity in MT-4 cells, in parallel with a cytotoxicity assay (for details, see the Supplementary material). Briefly, two-fold serial dilutions of each compound were added to MT-4 cells infected with the HIV-1 laboratory strain NL4-3 expressing human *Renilla* luciferase. Virus replication was assessed after 3 days by measuring luciferase activity. Cell toxicity was evaluated by XTT assay.

We observed weak but significant and reproducible antiviral activity for compounds **35**, **39** and **40**. These compounds also scored well in the competitive CA binding assay. As expected, binding to HIV CA *per se* is not sufficient to ensure antiviral activity. Many factors, including cell entry through the intact plasma membrane, serum protein binding and compound stability, can compromise the overall antiviral activity of the compounds. In addition, we detected slight cytotoxicity for some of the active compounds (see comparison of EC_{50} and CC_{50} values).

In summary, we have explored the SAR of pyridinylpyrimidines as in vitro HIV capsid ligands that bind to the CTD of the CA protein. We have described three series of pyridinylpyrimidines that exhibited improved activities upon substitution with methyl at C-6 along with introduction of a fluoro functionality at the *N*-4-phenyl moiety. Thus, our studies indicate that quinazoline, which was the central core in our previously reported inhibitors, can be replaced with a pyrimidine ring. Compounds containing the pyridine-3-yl moiety tended to have better solubility and proved to be superior CA binders compared to pyridine-4-ylpyrimidines. Compound **40**, with a 2-fluoroethyl carbamide motif, exhibited the tightest binding with a moderate activity against HIV-1 in tissue culture. Further studies focused on fluorinated pyridinylpyrimidines are needed to fully analyze the potential of this class of compounds as HIV assembly inhibitors.

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Supplementary data

Data contain procedures used for preparation of intermediates; synthesis of pyrimidines; general procedure for the synthesis of pyrimidine-4-amines; AlphaScreen methods; competition of soluble free CAI, scrambled control peptide CAIctrl, compounds **40** and **20** with CAI-biotin for binding to GST-CA measured by AlphaScreen; anti-HIV-1 and cytotoxicity testing; copies of ^{1}H and ^{13}C NMR spectra.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.06.039. These data include MOL files and InChiKeys of the most important compounds described in this article.

References and notes

1. De Clercq, E. *Adv. Pharmacol.* **2013**, *67*, 317.
2. Tintori, C.; Brai, A.; Fallacara, A. L.; Fazi, R.; Schenone, S. *Curr. Opin. Pharmacol.* **2014**, *18*, 1.
3. Konvalinka, J.; Krausslich, H. G.; Muller, B. *Virology* **2015**, 479.
4. Sundquist, W. I.; Krausslich, H. G. *Cold Spring Harbor Perspect. Med.* **2012**, *2*, a006924.
5. Briggs, J. A.; Krausslich, H. G. *J. Med. Biol.* **2011**, *410*, 491.
6. Ganser-Pornillos, B. K.; Yeager, M.; Pornillos, O. *Adv. Exp. Med. Biol.* **2012**, *726*, 441.
7. Machara, A.; Lux, V.; Kožíšek, M.; Grantz Šašková, K.; Štěpánek, O.; Kotora, M.; Parkan, K.; Pávová, M.; Glass, B.; Sehr, P.; Lewis, J.; Müller, B.; Krausslich, H. G.; Konvalinka, J. *J. Med. Chem.* **2016**, *59*, 545.
8. Machara, A.; Konvalinka, J.; Kotora, M. *ChemistrySelect* **2016**, *1*, 101.
9. Lampel, A.; Bram, Y.; Ezer, A.; Shaltiel-Kario, R.; Saad, J. S.; Bacharach, E.; Gazit, E. *ACS Chem. Biol.* **2015**, *10*, 1785.
10. Curreli, F.; Zhang, H.; Zhang, X.; Pyatkin, I.; Zagorodnikov, V.; Altieri, A.; Debnath, A. K. *Bioorg. Med. Chem.* **2011**, *19*, 77.
11. Thenin-Houssier, S.; de Vera, I. M. S.; Pedro-Rosa, L.; Brady, A.; Richard, A.; Konnick, B.; Opp, S.; Buffone, C.; Fuhrmann, J.; Kota, S.; Billack, B.; Pietka-Ottlik, M.; Tellinghuisen, T.; Choe, H.; Spicer, T.; Scampavia, L.; Diaz-Griffero, F.; Kojetin, D. J.; Valente, S. T. *Antimicrob. Agents Chemother.* **2016**, *60*, 2195.
12. Kelly, B. N.; Kyere, S.; Kinde, I.; Tang, C.; Howard, B. R.; Robinson, H.; Sundquist, W. I.; Summers, M. F.; Hill, C. P. *J. Mol. Biol.* **2007**, *373*, 355.
13. Tian, B.; He, M.; Tang, S.; Hewlett, I.; Tan, Z.; Li, J.; Jin, Y.; Yang, M. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 2162.
14. Li, J.; Tan, Z.; Tang, S.; Hewlett, I.; Pang, R.; He, M.; He, S.; Tian, B.; Chen, K.; Yang, M. *Bioorg. Med. Chem.* **2009**, *17*, 3177.
15. Lemke, Ch. T.; Titolo, S.; von Schwedler, U.; Goudreau, N.; Mercier, J. F.; Wardrop, E.; Faucher, A. M.; Coulombe, R.; Banik, S. S. R.; Fader, L.; Gagnon, A.; Kawai, S. H.; Rancourt, J.; Tremblay, M.; Yoakim, Ch.; Simoneau, B.; Archambault, J.; Sundquist, W. I.; Mason, S. W. *J. Virol.* **2012**, *86*, 6643.
16. Jin, Y.; Tan, Z.; He, M.; Tian, B.; Tang, S.; Hewlett, I.; Yang, M. *Bioorg. Med. Chem.* **2010**, *18*, 2135.
17. Blair, W. S.; Pickford, C.; Irving, S. L.; Brown, D. G.; Anderson, M.; Bazin, R.; Cao, J.; Ciaramella, G.; Isaacson, J.; Jackson, L.; Hunt, R.; Kjerrstrom, A.; Nieman, J. A.; Patrick, A. K.; Perros, M.; Scott, A. D.; Whitby, K.; Wu, H.; Butler, S. L. *PLoS Pathog.* **2010**, *6*, e1001220.
18. Goudreau, N.; Lemke, Ch. T.; Faucher, A. M.; Grand-Maitre, Ch.; Goulet, S.; Lacoste, J. E.; Rancourt, J.; Malenfant, E.; Mercier, J. F.; Titolo, S.; Mason, S. W. *ACS Chem. Biol.* **2013**, *8*, 1074.
19. Sticht, J.; Humbert, M.; Findlow, S.; Bodem, J.; Muller, B.; Dietrich, U.; Werner, J.; Krausslich, H. G. *Nat. Struct. Mol. Biol.* **2005**, *12*, 671.
20. Ternois, F.; Sticht, J.; Duquerroy, S.; Krausslich, H. G.; Rey, F. A. *Nat. Struct. Mol. Biol.* **2005**, *12*, 678.
21. Medwid, J. B.; Paul, R.; Baker, J. S.; Brockman, J. A.; Du, M. T.; Hallett, W. A.; Hanifin, J. W.; Hardy, R. A.; Tarrant, M. E.; Torley, L. W.; Wrenn, S. *J. Med. Chem.* **1990**, *33*, 1230.
22. Lesher, G. Y.; Singh, B.; Mielens, Z. E. *J. Med. Chem.* **1982**, *25*, 837.
23. Maurya, H. K.; Gupta, A. *RSC Adv.* **2014**, *4*, 22106.
24. Sirisoma, N.; Pervin, A.; Nguyen, B.; Crogan-Grundy, C.; Kasibhatla, S.; Tseng, B.; Drewe, J.; Cai, S. X. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 2305.