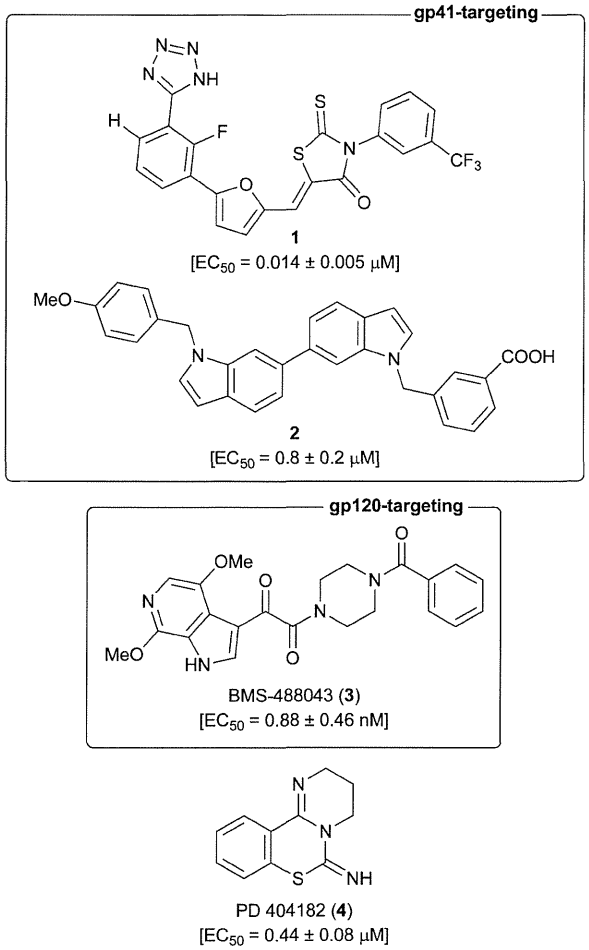
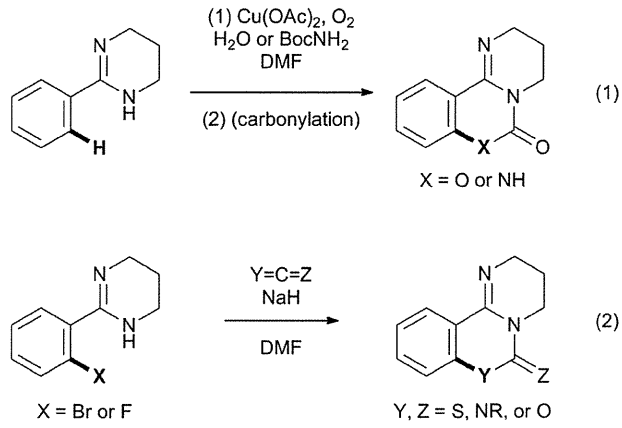


Fig. 1 Structures of newly reported anti-HIV compounds (**1**–**3**) targeting HIV-1 envelope proteins, and PD 404182 (**4**).

antimicrobial effects. In the course of our SAR investigations in this study, antiviral activities of **4** against HCV, HIV, and simian immunodeficiency virus were reported.[16,17] Although compound **4** exhibits virucidal effects at high concentrations, the mechanism of action and the target molecule remain ambiguous.[17]

Recently, we have established two independent approaches for the synthesis of PD 404182 derivatives (Scheme 1): C–H functionalization of 2-phenyl-1,4,5,6-tetrahydropyrimidine with water or *tert*-butylcarbamate in the presence of copper(II) acetate provides pyrimido[1,2-*c*][1,3]benzoxazine or pyrimido[1,2-*c*]-quinazoline in one or two step(s) (eqn (1), Scheme 1).[18] Alternatively, addition of 2-(2-halophenyl)-1,4,5,6-tetrahydropyrimidine to carbon disulfide, isocyanate, or isothiocyanate, and subsequent aromatic nucleophilic substitution (S_NAr) affords pyrimidobenzothiazines and -oxazines, and pyrimidoquinazolines (eqn (2), Scheme 1).[19] The derivatives obtained from these reactions were easily converted to the pyrimido[1,2-*c*][1,3]benzothiazin-6-imine scaffold. Our two synthetic methods provide a variety of PD 404182 derivatives from the corresponding benzaldehydes in a few steps and in good yields, facilitating the lead optimization process.[20] In this article, a SAR study of PD 404182 derivatives using these synthetic approaches is described.



Scheme 1 Our synthetic methods for PD 404182 derivatives.

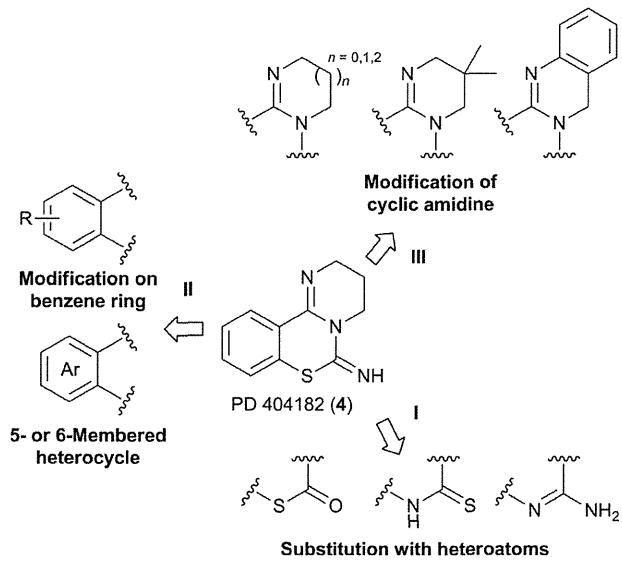


Fig. 2 Strategy for SAR study of PD 404182 (**4**).

Results and discussion

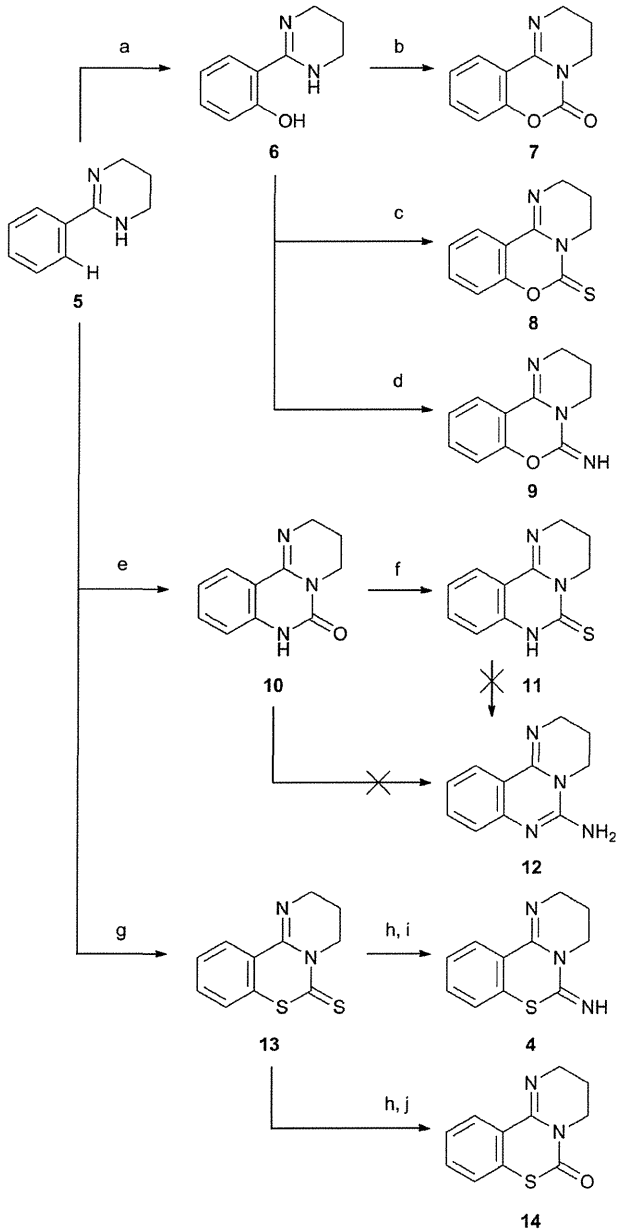
Strategy for the SAR study of PD 404182

PD 404182 consists of three components, namely a 1,3-thiazin-2-imine core, and left-fused benzene and cyclic amidine moieties (Fig. 2). In order to obtain detailed insights into the relationships between the compound structure and anti-HIV activity, we planned to investigate substituent effects on each component: (I) derivatives with various heteroatom (N, S, and O) arrangements on the 1,3-thiazin-2-imine core (Fig. 2); (II) pyrimido[1,2-*c*][1,3]-thiazin-6-imine derivatives fused with a substituted benzene ring or a five- or six-membered aromatic heterocycle; and (III) benzo-[*e*][1,3]thiazin-2-imine derivatives fused with a cyclic amidine ring with or without accessory alkyl or aryl groups.

Synthesis of pyrimido[1,2-*c*][1,3]benzothiazin-6-imines and related tricyclic heterocycles

Our investigation began with the synthesis of tricyclic heterocycles with different combinations of heteroatoms on the

1,3-thiazin-2-imine core. Previously, we reported syntheses of pyrimido[1,2-*c*][1,3]benzoxazine and pyrimido[1,2-*c*]quinazoline derivatives using copper(II)-mediated C–H functionalization;[18] this facilitates the introduction of oxygen or nitrogen functional groups at the *ortho*-position of 2-phenyl-1,4,5,6-tetrahydropyrimidine (**5**). Using compound **5** as a key starting material, a divergent approach was used for the preparation of a series of scaffolds (Scheme 2).



Scheme 2 Synthesis of PD 404182 derivatives with different combinations of heteroatoms. Reagents and conditions: (a) $Cu(OAc)_2$, H_2O, O_2, DMF, 130 °C, 69%; (b) triphosgene, TMEDA, CH_2Cl_2, 0 °C to rt, 70% [2 steps (a,b)]; (c) thiophosgene, Et_3N, CH_2Cl_2, 0 °C to rt, quant.; (d) BrCN, CH_2Cl_2, rt, 34%; (e) $Cu(OAc)_2$, $BocNH_2$, O_2, DMF, 130 °C, 53%; (f) Lawesson's reagent, xylene, reflux, 19%; (g) $Cu(OAc)_2$, CS_2, O_2, 1,4-dioxane, 130 °C, 11%; (h) NaOH, MeOH, H_2O, reflux; (i) BrCN, EtOH, reflux, 61% [2 steps (h,i)]; (j) triphosgene, Et_3N, CH_2Cl_2, 0 °C to rt, 65% [2 steps (h,j)].

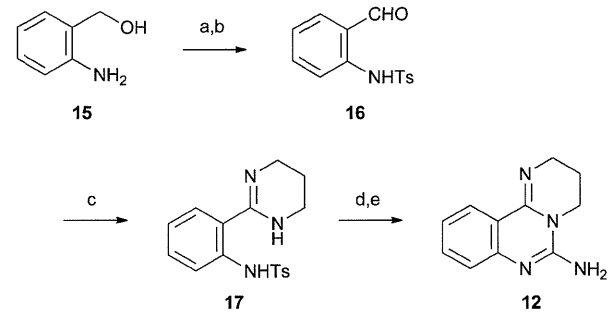
A one-pot reaction for $Cu(OAc)_2$-mediated C–H functionalization of **5** and subsequent treatment with triphosgene provided a 1,3-oxazin-2-one derivative **7** (Scheme 2). The same one-pot procedure using thiophosgene produced a trace amount of the desired thiocarbonyl derivative **8**; treatment of the purified intermediate **6** with thiophosgene provided the desired 1,3-oxazine-2-thione **8** in high yield. 1,3-Oxazin-2-imine **9** was obtained by the reaction of **6** with BrCN.

The copper-mediated C–N bond formation of compound **5** with *tert*-butylcarbamate followed by spontaneous intramolecular cyclization afforded a pyrimido[1,2-*c*]quinazolin-6-one scaffold **10**, as demonstrated in our previous report (Scheme 2).[18] Subsequent treatment with Lawesson's reagent led to formation of the thiocarbonyl derivative **11**. Since no hydrolysis of the carbonyl or thiocarbonyl group of compound **10** or **11** for construction of the 2-aminoquinazoline structure in **12** occurred, an alternative approach starting from 2-aminobenzyl alcohol **15** was used for the synthesis of the 2-aminoquinazoline derivative **12** (Scheme 3). After protection and PCC oxidation of **15**, oxidative amidination[21] provided 2-(*p*-tosylamino)phenyltetrahydropyrimidine **17**. Deprotection followed by BrCN-mediated cyclization of **17** provided the expected 2-aminoquinazoline derivative **12**.

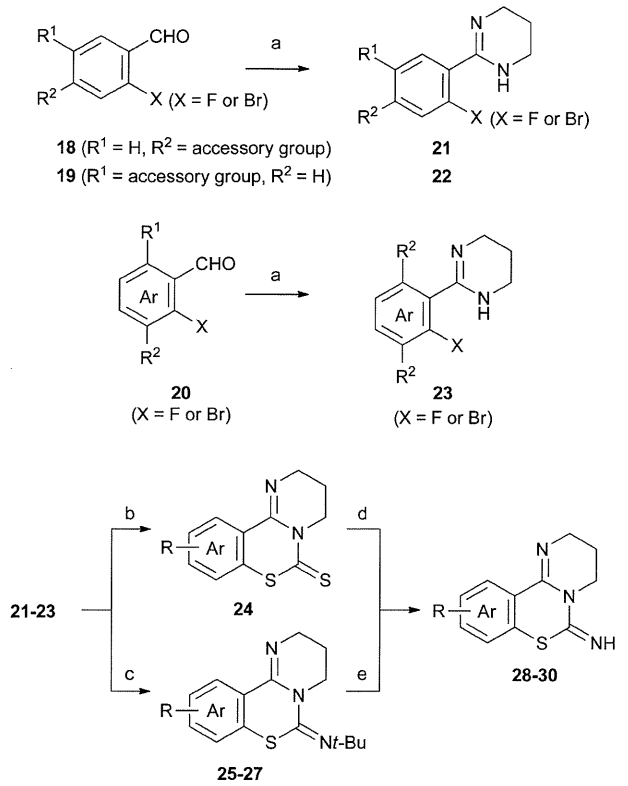
For the synthesis of pyrimido[1,2-*c*][1,3]benzothiazine derivatives, we adapted the C–H functionalization reaction for C–S bond formation (Scheme 2). After optimization of the reaction conditions, we found that exposure of compound **5** to CS_2 in the presence of $Cu(OAc)_2$ directly afforded a pyrimido[1,2-*c*][1,3]-benzothiazine-6-thione scaffold **13**. Hydrolysis of the thiocarbonyl group in **13** followed by treatment with BrCN or triphosgene provided 6-imino or 6-oxo derivatives (**4** or **14**), respectively.

Synthesis of pyrimido[1,2-*c*][1,3]thiazine derivatives with fused benzene and heterocycles

Pyrimido[1,2-*c*][1,3]thiazin-6-imine derivatives **28–30** with a series of fused ring systems were prepared by consecutive heterocumulene addition and S_NAr reactions (Scheme 4).[19] These reactions provide easy access to the construction of the 1,3-thiazin-2-imine derivatives and are more efficient than the diversity-oriented C–H functionalization approach. The oxidative amidination of aromatic aldehydes **18–20** with an accessory



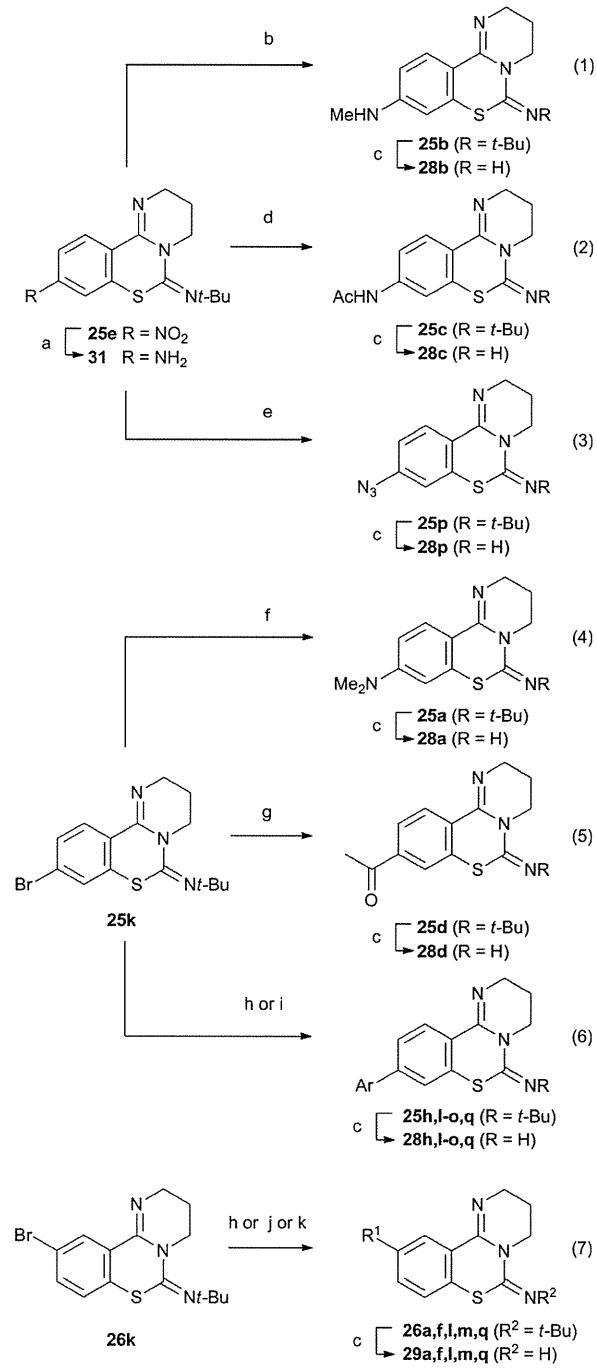
Scheme 3 Synthesis of 2-aminoquinazoline derivative **12**. Reagents and conditions: (a) *p*-TsCl, pyridine, $CHCl_3$, rt; (b) PCC, silica gel, CH_2Cl_2, rt, 80% [2 steps (a,b)]; (c) 1,3-propanediamine, I_2, K_2CO_3, *t*-BuOH, 70 °C, 98%; (d) conc. H_2SO_4, 100 °C, then NaOH, H_2O; (e) BrCN, EtOH, reflux, 66% [2 steps (d,e)].



Scheme 4 Synthesis of pyrimido[1,2-*c*][1,3]thiazin-6-imine derivatives fused with substituted benzene and heterocycles (**28–30**). Reagents and conditions: (a) 1,3-propanediamine, I_2, K_2CO_3, *t*-BuOH, 70 °C, 58–91%; (b) NaH, CS_2, DMF, 80 °C, 67%-quant.; (c) NaH or *t*-BuOK, *t*-BuNCS, DMF or DMAc, −20–80 °C, 28–95%; (d) (i) NaOH, MeOH, H_2O, reflux, (ii) BrCN, EtOH, reflux, 32–68%; (e) TFA, MS4Å, $CHCl_3$, reflux, 63–92%.

functional group afforded the corresponding 2-phenyltetrahydropyrimidine derivatives **21–23**. The pyrimido[1,2-*c*][1,3]thiazine-6-thione scaffold **24** was obtained by additions of **21f,g,i** or **23s,t,u** to carbon disulfide followed by S_NAr-type C–S bond formation. The desired 6-imino derivatives **28f,g,i** and **30s,t,u** were obtained *via* hydrolysis of the thiocarbonyl group of **24** followed by BrCN treatment. Alternatively, reactions of other 2-phenyltetrahydropyrimidines **21–23** with *tert*-butyl isothiocyanate afforded *N*-(*t*-Bu)-protected thiazinimine derivatives **25–27**, which were treated with TFA to provide the expected products **28–30**.

The intermediates **25e**, **25k**, and **26k** were subjected to further manipulations to obtain the functionalized derivatives (Scheme 5). The nitro group of **25e** was reduced by hydrogenation to form the 9-amino derivative **31**. Alkylation of **31** afforded the 9-(*N*-methylamino) derivative **25b** (eqn (1), Scheme 5). The 9-acetamide derivative **25c** was obtained by treatment of **31** with acetic anhydride (eqn (2), Scheme 5). Sandmeyer reaction of **31** gave the 9-azide derivative **25p** (eqn (3), Scheme 5). Me_2N- and MeO-substituted derivatives (**25a**, **26a**, and **26f**) were obtained by Me_2NH-mediated *N*-arylation[22] of the 9-bromo **25k** and 10-bromo derivatives **26k**, and NaOMe-mediated Ullmann coupling[23] of **26k**, respectively (eqn (4) and



Scheme 5 Synthesis of 9- or 10-substituted pyrimido[1,2-*c*][1,3]benzothiazin-6-imine derivatives. Reagents and conditions: (a) H_2, Pd/C, EtOH, rt, 88%; (b) NaOMe, $(CH_2O)_n$, MeOH, reflux, then $NaBH_4$, 91%; (c) TFA, MS4Å, $CHCl_3$, reflux, 37–95%; (d) Ac_2O, DMAP, Et_3N, CH_2Cl_2, rt, quant.; (e) $NaNO_2$, AcOH, H_2O, 0 °C, then NaN_3, 70%; (f) $Pd(OAc)_2$, *t*-Bu_3P, $NHMe_2$, THF, KO*t*-Bu, toluene, reflux, quant.; (g) 2-hydroxyethylvinylether, $Pd(OAc)_2$, 1,3-bis(diphenylphosphino)-propane, K_2CO_3, H_2O, 90 °C, 13% [2 steps (g,c)]; (h) R-$B(OH)_2$ or R-Bpin, $Pd(PPh_3)_4$, $PdCl_2(dppf)\cdot CH_2Cl_2$, K_2CO_3, toluene or 1,4-dioxane, EtOH, H_2O, reflux, 62–96%; (i) *n*-$BuB(OH)_2$, $Pd_2(dba)_3$, P(*t*-Bu)$_3$, $CsCO_3$, 1,4-dioxane, reflux, 6% (for **25h**); (j) Pd(P*t*-Bu)$_2$, $NHMe_2$, THF, KO*t*-Bu, toluene, 170 °C, 67% (for **26a**); (k) CuBr, NaOMe, MeOH, DMF, reflux, 40% (for **26f**).

(7), Scheme 5). The 9-acetyl derivative **25d** was obtained by Heck reaction[24] of **25k** with 2-hydroxyethyl vinyl ether (eqn (5), Scheme 5). Other derivatives with a variety of functional groups (**25h,l–o,q** and **26l,m,q**) were synthesized by Suzuki–Miyaura coupling reactions[25] of **25k** and **26k** with boronic acids or their pinacol esters (eqn (6) and (7), Scheme 5). Final deprotection of the *tert*-butyl group in **25** and **26** afforded the 9- or 10-substituted pyrimido[1,2-*c*][1,3]benzothiazine derivatives **28** and **29**, respectively.

Synthesis of benzo[*e*][1,3]thiazine derivatives with fused cyclic amidines

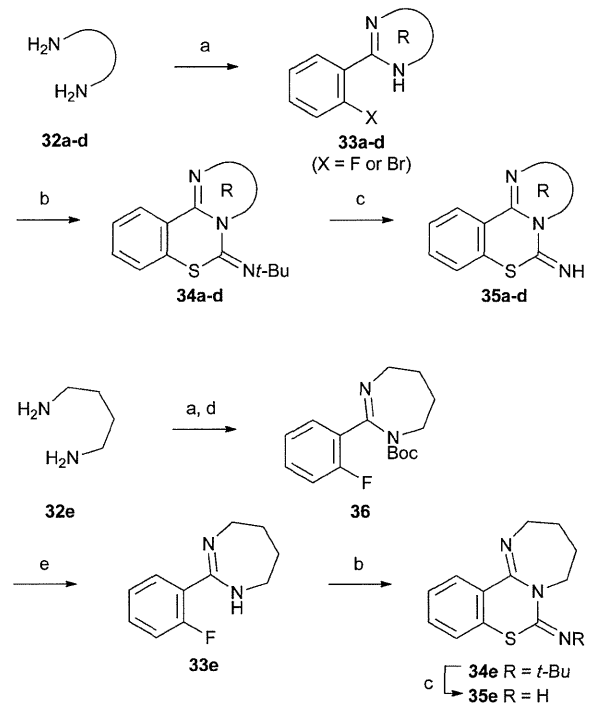
Benzo[*e*][1,3]thiazine derivatives with various ring-sized and/or modified cyclic amidine moieties **35** were also synthesized by the consecutive heterocumulene addition and S_NAr reactions (Scheme 6). Oxidative amidination using several diamines **32** proceeded efficiently to form five- or six-membered rings (**33a–d**). The same reaction for the seven-membered amidine (**33e**) was incomplete, but purification of the Boc-protected amidine **36** followed by subsequent deprotection of the Boc group gave the pure seven-membered amidine **33e**. The resulting amidines were converted to cyclic-amidine-fused benzo[*e*][1,3]-thiazin-2-imines **34** *via tert*-butyl isothiocyanate addition and an S_NAr reaction. TFA-mediated deprotection gave the expected derivatives **35**.

Structure–activity relationships of the central heterocyclic core in pyrimido[1,2-*c*][1,3]benzothiazines

Initially, the structural requirements of the 1,3-thiazin-2-imine core substructure in **4** (PD 404182) for anti-HIV activity were investigated (Table 1). The antiviral activities against the HIV-1_{IIIB} strain were evaluated using the MAGI assay. Substitution of the imino group in **4** with a carbonyl group (**14**) resulted in a significant decrease in anti-HIV activity (EC_{50} = 8.94 μM). Pyrimido[1,2-*c*][1,3]benzoxazines (**7–9**), pyrimido [1,2-*c*]quinazolines (**10–12**), and pyrimido[1,2-*c*][1,3]benzothiazine-6-thione (**13**), in which the 1-sulfur and/or 2-imino groups in **4** were modified, showed no activity. These results suggested that both the 1-sulfur atom and the 2-imino group are indispensable functional groups for the inhibitory activity against HIV infection, and may be involved in potential interactions with the target molecules.

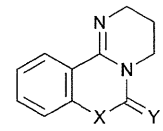
Structure–activity relationships of the benzene substructure in pyrimido[1,2-*c*][1,3]benzothiazine

A series of derivatives with modification of the benzene substructure in the pyrimido[1,2-*c*][1,3]benzothiazine were evaluated for anti-HIV activity (Table 2). The addition of positively charged *N,N*-dimethylamino (**28a**) and *N*-methylamino groups (**28b**) at the 9-position significantly decreased the anti-HIV activity. The 9-acetamide group (**28c**), which has hydrogen-bond donor/acceptor abilities, also attenuated the bioactivity. The acetyl (**28d**) and nitro (**28e**) groups, with hydrogen acceptor properties, induced slight decreases in the anti-HIV activity. In contrast, derivatives with less-polarized substituents (**28f–o** and



Scheme 6 Synthesis of benzo[*e*][1,3]thiazine derivatives with fused cyclic amidines. Reagents and conditions: (a) 2-fluorobenzaldehyde or 2-bromobenzaldehyde, I_2, K_2CO_3, *t*-BuOH, 70 °C, 68–79%; (b) NaH, *t*-BuNCS, DMF, rt −80 °C, 18–50%; (c) TFA, MS4Å, $CHCl_3$, reflux, 16–86%; (d) Boc_2O, Et_3N, DMAP, CH_2Cl_2, rt, 37% [2 steps (a,d)]; (e) TFA, CH_2Cl_2, reflux, 80%.

Table 1 SARs for 1,3-thiazin-2-imine core

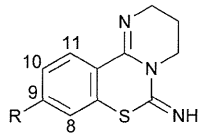
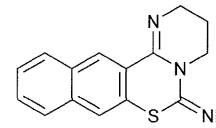
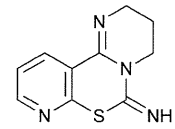
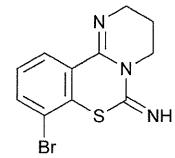
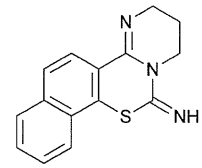
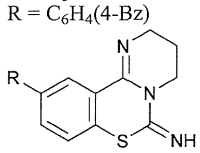
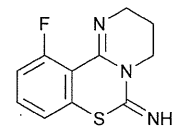
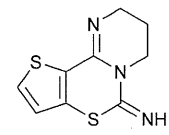


Compound	X	Y	EC_{50} (μM)[a]
4	S	NH	0.44 ± 0.08
7	O	O	>10
8	O	S	>10
9	O	NH	>10
10	NH	O	>10
11	NH	S	>10
12	NH	NH	>10
13	S	S	>10
14	S	O	8.94 ± 1.07

[a] EC_{50} values were the concentration that blocks HIV-1 infection by 50% and derived from three independent experiments.

28q) at this position generally reproduced the potent anti-HIV activity of **4**. In terms of the electron-donating or -withdrawing properties of the substituent groups on the benzene substructure, good correlations were not observed. For example, the electron-donating methoxy (**28f**), methyl (**28g**), and *n*-butyl groups (**28h**), and the electron-withdrawing fluoro (**28i**) and trifluoromethyl groups (**28j**) exhibited similar anti-HIV activities (EC_{50} =

Table 2 SARs for benzene part

Compound		EC_{50} (μM)[a]	Compound		EC_{50} (μM)[a]
			**30r**		0.56 ± 0.13
4	R = H	0.44 ± 0.08			
28a	R = NMe_2	4.74 ± 1.07			
28b	R = NHMe	>10			
28c	R = NHAc	>10	**30s**		2.55 ± 0.26
28d	R = COMe	1.44 ± 0.33			
28e	R = NO_2	1.13 ± 0.18			
28f	R = OMe	0.57 ± 0.09			
28g	R = Me	0.49 ± 0.10			
28h	R = *n*-butyl	0.44 ± 0.09			
28i	R = F	0.50 ± 0.07	**30k**		>10
28j	R = CF_3	0.53 ± 0.12			
28k	R = Br	0.25 ± 0.09			
28l	R = Ph	0.24 ± 0.04			
28m	R = vinyl	0.18 ± 0.05			
28n	R = styryl	0.25 ± 0.05			
28o	R = pentenyl	0.24 ± 0.11	**30t**		>10
28p	R = N_3	0.43 ± 0.06			
28q	R = C_6H_4(4-Bz)	0.53 ± 0.12			
			**30i**		1.68 ± 0.19
29a	R = NMe_2	2.12 ± 0.26			
29e	R = NO_2	3.00 ± 0.59			
29f	R = OMe	0.53 ± 0.04			
29g	R = Me	0.38 ± 0.04			
29k	R = Br	0.24 ± 0.05			
29l	R = Ph	0.24 ± 0.05	**30u**		>10
29m	R = vinyl	0.40 ± 0.09			
29q	R = C_6H_4(4-Bz)	0.67 ± 0.16			

[a] EC_{50} values were the concentration that blocks HIV-1 infection by 50% and derived from three independent experiments.

0.44–0.57 μM), indicating that the antiviral activity is independent of the electronic state of the 1,3-benzothiazin-2-imine core in forming potential π-stacking interaction(s) with the target molecules. Among the hydrophobic substituents at this position, bromo (**28k**), phenyl (**28l**), vinyl (**28m**), styryl (**28n**), and pentenyl groups (**28o**) induced inhibitory activity two or three times greater than that of **4** (EC_{50} = 0.18–0.25 μM). Modification with photoreactive azido (**28p**) and benzoylphenyl groups (**28q**) maintained the inhibitory activity; these could be used as probe molecules to identify the target molecule(s) of **4**.[26]

Similar SARs were observed for modification at the 10-position of pyrimido[1,2-*c*][1,3]benzothiazine. Addition of positively charged *N*,*N*-dimethylamino (**29a**) and polarized nitro groups (**29e**) reduced the anti-HIV activity (EC_{50} = 2.12 and 3.00 μM, respectively). Hydrophobic groups including methoxy (**29f**), methyl (**29g**), bromo (**29k**), phenyl (**29l**), vinyl (**29m**), and 4-benzoylphenyl (**29q**) (EC_{50} = 0.24–0.67 μM) had favorable effects on the bioactivity, suggesting potential hydrophobic interactions of these additional functional groups with the target molecule(s).

Further miscellaneous modifications of benzothiazine substructure were also investigated (Table 2). The naphtho[2,3-*e*][1,3]thiazine derivative **30r**, with a 9,10-fused benzene, exhibited anti-HIV activity equipotent to that of the parent **4** (EC_{50} = 0.56 μM). A 6-fold decrease in the anti-HIV activity of the pyridine-fused pyrido[3,2-*e*][1,3]thiazine derivative (**30s**) was observed (EC_{50} = 2.55 μM). In addition, introduction of 8-bromo (**30k**) and 8,9-fused benzene (**30t**, naphtho[2,1-*e*][1,3]-thiazine) substituents on benzothiazine resulted in a loss of activity, suggesting that modification at the 8-position was inappropriate for favorable interactions with the target molecule(s). The 11-fluoro derivative **30i** and thiophene-fused **30u**, which has a 5-6-6 framework (thieno[2,3-*e*][1,3]thiazine), exhibited four times lower and no inhibitory potencies, respectively.

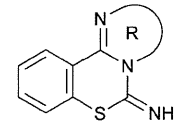
Structure–activity relationships of cyclic amidine part of pyrimido[1,2-*c*][1,3]benzothiazine

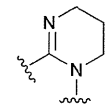
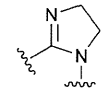
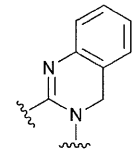
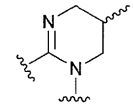
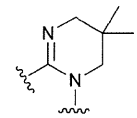
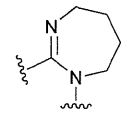
A SAR study of the top-right cyclic amidine substructure was carried out. The five-membered dihydroimidazole derivative **35a** had no anti-HIV activity (Table 3), suggesting that the five-membered ring may impair the critical interactions with the target molecule(s) *via* its small-sized ring strain or indirect effects on the thiazinimine core with a possibly altered conformation. Similarly, compound **35b** with the phenyl-fused dihydropyrimidine substructure showed lower inhibitory activity (EC_{50} = 3.78 μM). Appending one or two methyl groups on the six-membered pyrimidine (**35c** and **35d**) induced 1.5- to 2-fold higher inhibitory potencies (EC_{50} = 0.35 and 0.24 μM, respectively) compared with that of the parent compound **4**. In addition, compound **35e** with a seven-membered tetrahydro-1,3-diazepine substructure exhibited similar anti-HIV activity to that of **4** (EC_{50} = 0.31 μM).

Mechanistic studies of anti-HIV pyrimido[1,2-*c*][1,3]-benzothiazin-6-imines and related tricyclic heterocycles

To investigate the mechanism of action of PD 404182 derivatives, a time of drug addition study was carried out (Fig. 3). In this experiment, the anti-HIV activity profiles of **4**[27] and its derivatives **29k**[27] were compared with those of well-known anti-HIV agents such as an adsorption inhibitor (DS 5000),[28] fusion inhibitor [enfuvirtide (T-20)],[5] NRTI (AZT),[29] NNRTI (nevirapine),[30] and integrase inhibitor (raltegravir).[6] After inoculation of HeLa-CD4/CCR5-LTR/β-gal cells with HIV-1_{IIIB}, each anti-HIV-1 drug was added at a 90% inhibitory effect concentration at the indicated time points. The inhibitory effects on the infection were determined by counting the blue cells 48 h later. This investigation revealed that compound **4** (PD 404182) had an inhibitory profile in the early stage of viral infection similar to those of DS 5000 and enfuvirtide (Fig. 3). Identical profiles were observed for derivative **29k**.

Table 3 SARs for cyclic amidine



Compound		EC_{50} (μM)[a]
4		0.44 ± 0.08
35a		>10
35b		3.78 ± 1.39
35c		0.35 ± 0.09
35d		0.24 ± 0.04
35e		0.31 ± 0.06

[a] EC_{50} values were the concentration that blocks HIV-1 infection by 50% and derived from three independent experiments.

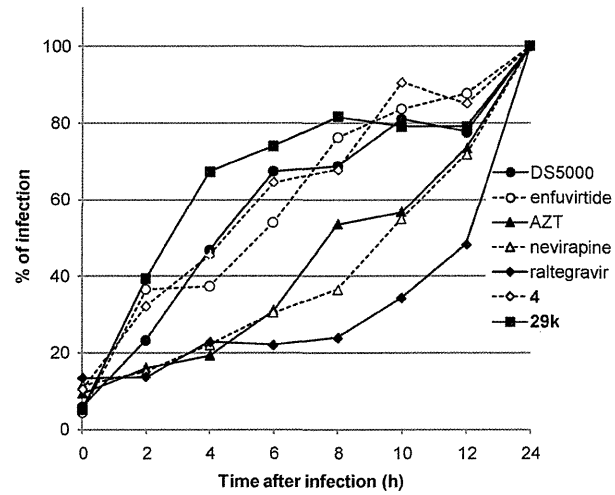


Fig. 3 Time of drug addition profiles for infection by HIV-1_{IIIB} strain of HeLa-CD4/CCR5-LTR/β-gal cells.

Table 4 Anti-HIV activity of **4** and **35d** against other HIV strains

Strain	EC_{50} (μM)[a]	
	4	**35d**
HIV-1_{NL4-3}	0.38 ± 0.06	0.25 ± 0.03
HIV-1_{BaL}	0.37 ± 0.06	0.16 ± 0.02
HIV-2_{EHO}	0.31 ±0.06	0.17 ± 0.03
HIV-2_{ROD}	0.30 ± 0.06	0.11 ± 0.03

[a] EC_{50} values were the concentration that blocks HIV infection by 50% and derived from three independent experiments.

To gain additional insights into the mechanism of action of PD 404182 derivatives, the antiviral activities against other HIV subtypes were evaluated (Table 4). Compound **4** was effective against not only HIV-1_{IIIB} but also other two HIV-1 strains (HIV-1_{NL4-3} and HIV-1_{BaL}) with similar potency. Both HIV-1_{IIIB} and HIV-1_{NL4-3} strains utilize CXCR4 as a coreceptor for entry, while HIV-1_{BaL} strain does CCR5, indicating that chemokine receptors CXCR4 and CCR5 are not the molecular targets of PD 404182 derivatives. The similar level of antiviral activity of **4** against HIV-2 (HIV-2_{EHO} and HIV-2_{ROD}), which is mainly distributed in West Africa, was observed. Highly potent inhibitory activities of a derivative **35d**[27] against these HIV strains were observed, as in the case of the SAR study of the HIV-1_{IIIB} strain discussed above. It has been well known that NNRTIs are not effective against HIV-2, highlighting that PD 404182 derivatives do not act as NNRTIs. Although PD 404182 derivatives and enfuvirtide showed similar anti-HIV-1 profile in the time of drug addition assay, HIV-2_{EHO} and HIV-2_{ROD} infection were affected by PD 404182 derivatives, in contrast with the less effective enfuvirtide,[31] suggesting that PD 404182 derivatives may not be directed at the HIV gp41 envelope protein. Recent reports have suggested that the antiviral activities of compound **4** against HIV, HCV, and pseudotype lentiviruses were derived from disruption of the structural integrities of virions.[17] Although the mechanism of action of PD 404182 derivatives is not fully understood at this stage, the unidentified biomolecule(s) in viruses or host cells, including envelope protein(s), lipid membranes and/or sugar chain(s), could be promising molecular targets for this new class of anti-HIV agents.

Conclusion

In conclusion, we have designed and synthesized PD 404182 derivatives for a novel series of anti-HIV agents. Comprehensive SAR studies demonstrated that the 6-6-6 fused pyrimido[1,2-*c*]-[1,3]benzothiazine scaffold and the heteroatom arrangement in the thiazinimine moiety are indispensable for the inhibitory activity of **4** (PD 404182) against HIV infection. Optimization studies of the benzene and cyclic amidine rings indicate that the introduction of a hydrophobic group on the benzene ring and the amidine group is more effective in improving the antiviral activity, giving potential favorable interaction(s) with the target molecule(s). In addition, PD 404182 derivatives could be promising agents for treatment of HIV-2 infection. We also revealed, using time of drug addition experiments, that PD 404182 derivatives prevent the HIV infection process at an early stage. For iterative molecular design of more effective derivatives based on binding modes, the identification of the target molecule(s) of PD 404182 derivatives is being investigated using derivatives such as **28p** and **28q**.

Experimental section

General

^{1}H NMR spectra were recorded using a JEOL AL-400 or a JEOL ECA-500 spectrometer. Chemical shifts are reported in δ (ppm) relative to Me_4Si as an internal standard. ^{13}C NMR spectra were referenced to the residual solvent signal. Exact mass (HRMS) spectra were recorded on a JMS-HX/HX 110A mass spectrometer. Melting points were measured by a hot stage melting point apparatus (uncorrected). For flash chromatography, Wakogel C-300E (Wako) or aluminium oxide 90 standardized (Merck) were employed. For preparative TLC, TLC Silica gel 60 F_{254} (Merck), TLC Aluminium oxide 60 F_{254} basic (Merck), or NH_2 Silica Gel 60 F_{254} Plate (Wako) were employed. For analytical HPLC, a Cosmosil 5C18-ARII column (4.6 × 250 mm, Nacalai Tesque, Inc., Kyoto, Japan) was employed with method A [a linear gradient of CH_3CN containing 0.1% (v/v) TFA] or method B [a linear gradient of CH_3CN containing 0.1% (v/v) NH_3] at a flow rate of 1 cm^3 min^{-1} on a Shimadzu LC-10ADvp (Shimadzu Corp., Ltd., Kyoto, Japan), and eluting products were detected by UV at 254 nm. The purity of the compounds was determined by combustion analysis or HPLC analysis as >95% unless otherwise stated.

General procedure of oxidative amidination: synthesis of 2-(3-bromo-2-fluorophenyl)-1,4,5,6-tetrahydropyrimidine (23k). To a solution of 3-bromo-2-fluorobenzaldehyde **20k** (0.71 g, 3.5 mmol) in *t*-BuOH (33 cm^3) was added propylenediamine (285.4 mg, 3.9 mmol). The mixture was stirred at 70 °C for 30 min, and then K_2CO_3 (1.45 g, 10.5 mmol) and I_2 (1.11 g, 4.4 mmol) were added. After being stirred at the same temperature for 3 h, the mixture was quenched with sat. Na_2SO_3. The organic layer was separated and concentrated. The resulting solid was dissolved in H_2O, and then pH was adjusted to 12–14 with 2N NaOH. The whole was extracted with $CHCl_3$. The extract was dried over $MgSO_4$. After concentration, the resulting solid was recrystallized from $CHCl_3$–*n*-hexane to give compound **23k** as colorless crystals (0.62 g, 69%): mp 99 °C; IR (neat) ν_{max}/cm^{-1}: 1624 (C=N); δ_H (400 MHz; $CDCl_3$; Me_4Si) 1.84–1.89 (2H, m, CH_2), 3.50 (4H, t, J = 5.7 Hz, 2 × CH_2), 5.13 (1H, br s, NH), 7.03 (1H, td, J = 8.0, 0.9 Hz, Ar), 7.54 (1H, ddd, J = 8.0, 6.4, 1.3 Hz, Ar) and 7.69 (1H, ddd, J = 8.0, 6.5, 1.3 Hz, Ar). δ_C (100 MHz; $CDCl_3$) 20.6, 42.1 (2C), 109.6 (d, J = 22.3 Hz), 125.1 (d, J = 4.1 Hz), 126.3 (d, J = 13.2 Hz), 129.8 (d, J = 3.3 Hz), 134.3, 150.8 and 156.3 (d, J = 248.3 Hz); δ_F (500 MHz; $CDCl_3$) −110.7; *Anal.* Calc. for $C_{10}H_{10}BrFN_2$: C, 46.72; H, 3.92; N, 10.90. Found: C, 46.64; H, 4.10; N, 10.93%.

General procedure of CS_2-mediated cyclization for pyrimido-[1,2-*c*][1,3]benzothiazine-6-thiones 24: synthesis of 3,4-dihydro-2*H*,6*H*-pyrimido[1,2-*c*]thieno[2,3-*e*][1,3]thiazin-6-thione (24u). To a mixture of 2-(3-bromothiophen-2-yl)-1,4,5,6-tetrahydropyrimidine **23u** (122.6 mg, 0.50 mmol) and NaH (40.0 mg,

1.0 mmol; 60% oil suspension) in DMF (1.7 cm^3) was added CS_2 (0.060 cm^3, 1.0 mmol) under an Ar atmosphere. After being stirred at 80 °C for 12 h, the mixture was concentrated. The residue was purified by flash chromatography over silica gel with *n*-hexane–EtOAc (8 : 2) to give the compound **24u** as a pale yellow solid (80.5 mg, 67%): mp 167 °C (from $CHCl_3$–*n*-hexane); IR (neat) ν_{max}/cm^{-1}: 1624 (C=N); δ_H (400 MHz; $CDCl_3$; Me_4Si) 2.04–2.10 (2H, m, CH_2), 3.68 (2H, t, J = 5.5 Hz, CH_2), 4.42 (2H, t, J = 6.1 Hz, CH_2), 6.76 (1H, d, J = 5.4 Hz, Ar) and 7.49 (1H, d, J = 5.4 Hz, Ar). δ_C (100 MHz; $CDCl_3$) 21.5, 45.0, 48.5, 122.3, 128.4, 130.8, 131.0, 141.7 and 189.7; HRMS (FAB): *m/z* Calc. for $C_9H_9N_2S_3$ $[M + H]^+$ 240.9928; found: 240.9936.

General procedure of *t*-BuNCS-mediated cyclization for *t*-Bu protected pyrimido[1,2-*c*][1,3]thiazin-6-imines 25–27, and 34: synthesis of *N*-(*tert*-butyl)-3,4-dihydro-9-nitro-2*H*,6*H*-pyrimido[1,2-*c*]-[1,3]benzothiazin-6-imine (25e). To a mixture of 2-(2-fluoro-4-nitrophenyl)-1,4,5,6-tetrahydropyrimidine **21e** (2.0 g, 8.96 mmol) and NaH (716.8 mg, 17.92 mmol; 60% oil suspension) in DMF (29.8 cm^3) was added *t*-BuNCS (2.28 cm^3, 17.92 mmol) under an Ar atmosphere. After being stirred at −20 °C to rt for 2 days, EtOAc was added. The resulting solution was washed with sat. $NaHCO_3$, brine, and dried over $MgSO_4$. After concentration, the residue was purified by flash chromatography over aluminium oxide with *n*-hexane–EtOAc (10 : 0 to 9 : 1) to give compound **25e** as a pale yellow solid (1.77 g, 62%): mp 152–153 °C (from $CHCl_3$–*n*-hexane); IR (neat) ν_{max}/cm^{-1}: 1604 (C=N), 1591 (NO_2), 1581 (C=N), 1523 (NO_2); δ_H (500 MHz; $CDCl_3$; Me_4Si) 1.39 (9H, s, 3 × CH_3), 1.91–1.96 (2H, m, CH_2), 3.66 (2H, t, J = 5.2 Hz, CH_2), 3.88 (2H, t, J = 5.7 Hz, CH_2), 7.97 (2H, dd, J = 9.7, 2.3 Hz, Ar), 8.01 (2H, d, J = 2.3 Hz, Ar) and 8.39 (1H, d, J = 9.2 Hz, Ar). δ_C (125 MHz; $CDCl_3$) 21.7, 30.0, 45.3, 45.5, 54.5, 119.9, 120.3, 130.0, 131.1, 132.8, 136.1, 146.5 and 148.5; HRMS (FAB): *m/z* Calc. for $C_{15}H_{19}N_4O_2S$ $[M + H]^+$ 319.1229; found: 319.1229.

General procedure of BrCN-mediated cyclization for pyrimido-[1,2-*c*][1,3]thiazin-6-imines 28 and 30: synthesis of 3,4-dihydro-9-methyl-2*H*,6*H*-pyrimido[1,2-*c*][1,3]benzothiazin-6-imine (28g). 3,4-Dihydro-9-methyl-2*H*,6*H*-pyrimido[1,2-*c*][1,3]benzothiazine-6-thione **24g** (62.1 mg, 0.25 mmol) was suspended in a 0.1 M solution of NaOH in MeOH–H_2O (9 : 1) (5 cm^3). After being stirred for 12 h under reflux, the mixture was concentrated. The residue was suspended in anhydrous EtOH (1 cm^3) and BrCN (53.0 mg, 0.50 mmol) was added under Ar atmosphere. After stirring for 2 h under reflux, the reaction mixture was quenched with 2 N NaOH. The whole was extracted with $CHCl_3$, and dried over $MgSO_4$. After concentration, the residue was purified by flash chromatography over aluminium oxide with *n*-hexane–EtOAc (9 : 1) to give the compound **28g** as colorless solid (39.2 mg, 68%): mp 121 °C (from $CHCl_3$–*n*-hexane); IR (neat) ν_{max}/cm^{-1}: 1620 (C=N), 1569 (C=N); δ_H (500 MHz; $CDCl_3$; Me_4Si) 1.94–1.99 (2H, m, CH_2), 2.32 (3H, s, CH_3), 3.67 (2H, t, J = 5.7 Hz, CH_2), 4.01 (2H, t, J = 6.3 Hz, CH_2), 6.84 (1H, s, Ar), 7.02 (1H, d, J = 8.6 Hz, Ar), 7.16 (1H, br s, NH) and 8.10 (1H, d, J = 8.6 Hz, Ar). δ_C (125 MHz; $CDCl_3$) 21.1, 21.1, 43.8, 44.9, 123.6, 124.1, 127.4, 128.6, 128.8, 141.1, 146.6 and 153.6; HRMS (FAB): *m/z* Calc. for $C_{12}H_{14}N_3S$ $[M + H]^+$ 232.0908; found: 232.0912.

General procedure of *tert*-butyl deprotection for pyrimido[1,2-*c*][1,3]benzothiazin-6-imines 28–30: synthesis of 3,4-dihydro-9-nitro-2*H*,6*H*-pyrimido[1,2-*c*][1,3]benzothiazin-6-imine (28e). TFA (1.5 cm^3) was added to a mixture of *N*-(*tert*-butyl)-3,4-dihydro-9-nitro-2*H*,6*H*-pyrimido[1,2-*c*][1,3]benzothiazin-6-imine **25e** (47.8 mg, 0.15 mmol) in $CHCl_3$ and molecular sieves 4 Å (225 mg, powder, activated by heating with Bunsen burner). After being stirred under reflux for 1.5 h, the mixture was concentrated. To a mixture of this residue in $CHCl_3$ was added dropwise Et_3N at 0 °C to adjust the pH to 8–9. The whole was extracted with EtOAc, and the extract was washed with sat. $NaHCO_3$, brine, and dried over $MgSO_4$. After concentration, the residue was purified by flash chromatography over aluminium oxide with *n*-hexane–EtOAc (19 : 1 to 1 : 1) to give compound **28e** as a pale yellow solid (24.9 mg, 63%): mp 170–172 °C (from $CHCl_3$–*n*-hexane); IR (neat) ν_{max}/cm^{-1}: 1620 (C=N), 1587 (NO_2), 1568 (C=N), 1523 (NO_2); δ_H (400 MHz; $CDCl_3$; Me_4Si) 1.97–2.03 (2H, m, CH_2), 3.74 (2H, t, J = 5.6 Hz, CH_2), 4.04 (2H, t, J = 6.2 Hz, CH_2), 7.41 (1H, br s, NH), 7.93 (1H, d, J = 2.2 Hz, Ar), 8.00 (1H, dd, J = 9.0, 2.2 Hz, Ar) and 8.42 (1H, d, J = 9.0 Hz, Ar). δ_C (100 MHz; $CDCl_3$) 20.8, 43.8, 45.2, 118.9, 120.5, 130.4, 130.8, 131.7, 145.1, 148.7 and 151.3; *Anal.* Calc. for $C_{11}H_{10}N_4O_2S$: C, 50.37; H, 3.84; N, 21.36. Found: C, 50.29; H, 4.03; N, 21.08%.

General procedure of Suzuki–Miyaura cross coupling for 9-aryl pyrimido[1,2-*c*][1,3]thiazine derivatives: synthesis of *N*-(*tert*-butyl)-3,4-dihydro-9-phenyl-2*H*,6*H*-pyrimido[1,2-*c*][1,3]benzothiazin-6-imine 25l. To a solution of 9-bromo-*N*-(*tert*-butyl)-3,4-dihydro-2*H*,6*H*-pyrimido[1,2-*c*][1,3]benzothiazin-6-imine **25k** (52.8 mg, 0.15 mmol) and phenylboronic acid (21.9 mg, 0.18 mmol) in a mixture of toluene (1.5 cm^3), EtOH (0.9 cm^3) and 1 M aq. K_2CO_3 (1.5 cm^3) was added $Pd(PPh_3)_4$ (6.9 mg, 4 mol%) and $PdCl_2(dppf)\cdot CH_2Cl_2$ (3.67 mg, 3 mol%). After being stirred at reflux for 1 h, the mixture was extracted with $CHCl_3$. The organic layers were dried over $MgSO_4$ and concentrated. The residue was purified by flash chromatography over aluminium oxide with *n*-hexane–EtOAc (10 : 0 to 9 : 1) to give the compound **25l** as colorless solid (44.8 mg, 85%): mp 122.5–124 °C (from $CHCl_3$–*n*-hexane); IR (neat) ν_{max}/cm^{-1}: 1592 (C=N); δ_H (500 MHz; $CDCl_3$; Me_4Si) 1.40 (9H, s, 3 × CH_3), 1.90–1.95 (2H, m, CH_2), 3.64 (2H, t, J = 5.4 Hz, CH_2), 3.89 (2H, t, J = 6.0 Hz, CH_2), 7.33–7.37 (2H, m, Ar), 7.41–7.44 (3H, m, Ar), 7.58 (2H, d, J = 6.9 Hz, Ar) and 8.25 (1H, d, J = 8.6 Hz, Ar). δ_C (125 MHz; $CDCl_3$) 21.9, 30.0 (3C), 45.1, 45.4, 54.2, 122.7, 124.8, 126.5, 127.0 (2C), 128.0, 128.8 (2C), 128.9, 129.5, 138.3, 139.4, 142.9 and 147.7; HRMS (FAB): *m/z* Calc. for $C_{21}H_{24}N_3S$ $[M + H]^+$ 350.1691; found: 350.1683.

Determination of anti-HIV activity

The sensitivity of three HIV-1 strains and two HIV-2 strains was determined by the MAGI assay.[32] The target cells (HeLa-CD4/CCR5-LTR/β-gal; 104 cells per well) were plated in 96 well flat microtiter culture plates. On the following day, the cells were inoculated with the HIV-1 (60 MAGI U per well, giving 60 blue

cells after 48 h of incubation) and cultured in the presence of various concentrations of the drugs in fresh medium. Forty-eight hours after viral exposure, all the blue cells strained with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) were counted in each well. The activity of test compounds was determined as the concentration that blocked HIV-1 infection by 50% (50% effective concentration [EC_{50}]). EC_{50} was determined by using the following formula:

$$EC_{50} = 10\text{^}[\log(A/B) \times (50 - C)/(D - C) + \log(B)],$$

wherein *A*: of the two points on the graph which bracket 50% inhibition, the higher concentration of the test compound, *B*: of the two points on the graph which bracket 50% inhibition, the lower concentration of the test compound, *C*: inhibitory activity (%) at the concentration *B*, *D*: inhibitory activity (%) at the concentration *A*.

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Structure–activity relationship study of pyrimido[1,2-*c*][1,3]benzothiazin-6-imine derivatives for potent anti-HIV agents

Tsukasa Mizuhara [a], Shinya Oishi [a,*], Hiroaki Ohno [a], Kazuya Shimura [b], Masao Matsuoka [b], Nobutaka Fujii [a,*]

[a] *Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan*
[b] *Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto 606-8507, Japan*

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ABSTRACT

3,4-Dihydro-2*H*,6*H*-pyrimido[1,2-*c*][1,3]benzothiazin-6-imine (PD 404182) is an antiretroviral agent with submicromolar inhibitory activity against human immunodeficiency virus-1 (HIV-1) and HIV-2 infection. In the current study, the structure–activity relationships of accessory groups at the 3- and 9-positions of pyrimido[1,2-*c*][1,3]benzothiazin-6-imine were investigated for the development of more potent anti-HIV agents. Several different derivatives containing a 9-aryl group were designed and synthesized using Suzuki–Miyaura cross-coupling and Ullmann coupling reactions. Modification of the *m*-methoxyphenyl or benzo[*d*][1,3]dioxol-5-yl group resulted in improved anti-HIV activity. In addition, the 2,4-diazaspiro[5.5]undec-2-ene-fused benzo[*e*][1,3]thiazine derivatives were designed and tested for their anti-HIV activities. The most potent 9-(benzo[*d*][1,3]dioxol-5-yl) derivative was two–threefold more effective against several strains of HIV-1 and HIV-2 than the parent compound, PD 404182.

1. Introduction

Highly active antiretroviral therapy, involving the co-administration of nucleoside reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI), and/or protease inhibitors, is a standard treatment regimen for human immunodeficiency virus (HIV) infections. This regimen suppresses the replication of HIV and controls disease progression in HIV-infected patients.[1,2] Unfortunately, however, an increasing number of patients with HIV infection/AIDS have failed to respond to the current antiretroviral therapeutics because of serious problems including the emergence of drug-resistant HIV variants[3] and drug-related adverse effects.[4] With this in mind, there is therefore a continuous need to develop novel anti-HIV drugs that are more effective against drug-resistant viruses and produce fewer adverse effects. Recently, a series of extensive studies led to the development of a series of novel antiretrovirals with new mechanisms of action for anti-HIV therapy, including a fusion inhibitor (enfuvirtide),[5–7] an integrase inhibitor (raltegravir),[8,9] and a CC chemokine receptor type 5 (CCR5) antagonist (maraviroc).[10,11] CXC chemokine receptor type 4 (CXCR4) antagonists,[12–16] CD4 mimics,[17–20] gp41-binding peptides[21–23] and small molecules[24–26] represent promising alternative anti-HIV agents.

3,4-Dihydro-2*H*,6*H*-pyrimido[1,2-*c*][1,3]benzothiazin-6-imine (PD 404182) (**1**) was previously reported as an antimicrobial agent that inhibited 3-deoxy-D-manno-octulosonic acid 8-phosphate synthase[27] and phosphopantetheinyl transferase (Fig. 1).[28,29] Following a recent random screening program using a multinuclear activation of a galactosidase indicator (MAGI) assay, compound **1** was identified as a new antiretroviral candidate with a high therapeutic index (CC_{50}/EC_{50} >200). The MAGI assay allows for the inhibitory activity of an early-stage HIV infection, including inhibition of the virus attachment and membrane fusion to host cells, to be effectively evaluated.[30] Compound **1** showed a similar antiviral profile in HIV-1 infection to DS 5000[31] (adsorption inhibitor) and enfuvirtide (fusion inhibitor). The virucidal effects of compound **1** against the human hepatitis C virus, HIV, and simian immunodeficiency virus have also been reported.[32,33] The mechanism of action for compound **1**, however, has not yet been fully understood.

In our previous structure–activity relationship (SAR) study of compound **1**,[34] a number of PD 404182 derivatives were designed and synthesized according to a series of facile synthetic procedures,[35,36] in which the tricyclic heterocycles related to PD 404182 were easily obtained in a few steps from benzaldehydes via C–H functionalization or aromatic nucleophilic substitution.

Abbreviations: CCR5, CC chemokine receptor type 5; CXCR4, CXC chemokine receptor type 4; MAGI, Multinuclear activation of a galactosidase indicator; NNRTI, Non-nucleoside reverse transcriptase inhibitors; NRTI, Nucleoside reverse transcriptase inhibitors.

* Corresponding authors. Tel.: +81 75 753 4551; fax: +81 75 753 4570.
E-mail addresses: soishi@pharm.kyoto-u.ac.jp (S. Oishi), nfujii@pharm.kyoto-u.ac.jp (N. Fujii).

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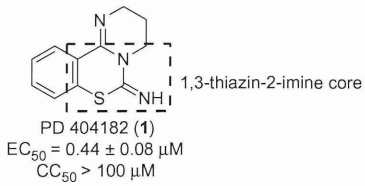


Figure 1. Structure of PD 404182.

The 6-6-6 fused pyrimido[1,2-*c*][1,3]benzothiazine scaffold and the heteroatom arrangement in the 1,3-thiazin-2-imine moiety are indispensable for the inhibitory activity of compound **1** against HIV infection (Fig. 1). Optimization studies indicated that the introduction of a hydrophobic group on the benzene ring and the cyclic amidine substructures effectively improved the antiviral activity by generating a potentially favorable interaction(s) with the target molecule(s). The most potent compounds identified were twofold more potent than PD 404182 and contained a phenyl group at 9-position of pyrimido[1,2-*c*][1,3]benzothiazine (compound **2**) or a geminal dimethyl group on the pyrimidine moiety (compound **3**) (Fig. 2).

In the current study, further structural optimization was conducted from the lead compounds **2** and **3** according to three approaches (Fig. 2), including the introduction of substituents on the 9-phenyl group (I), the substitution of the 9-phenyl group with fused arenes or heterocycles (II), and the modification of the cyclic amidine moiety (III). The anti-HIV profiles of the most potent derivative are also described.

2. Results and discussion

2.1. Synthesis of 9-aryl-3,4-dihydro-2*H*,6*H*-pyrimido[1,2-*c*][1,3]benzothiazin-6-imine derivatives

The 9-aryl-3,4-dihydro-2*H*,6*H*-pyrimido[1,2-*c*][1,3]benzothiazin-6-imine derivatives (**7** and **8**) were synthesized using a Suzuki–Miyaura cross-coupling reaction[37–39] of *N*-(*tert*-butyl)-protected bromide **4** with aryl boronic acid (pinacol ester) or by an Ullmann coupling[40] with pyrazole or imidazole (Scheme 1). Subsequent trifluoroacetic acid (TFA)-mediated deprotection of the *tert*-butyl groups afforded the desired biaryl-type derivatives.

2.2. Synthesis of spiropyrimidine-fused benzothiazinimine derivatives

The synthesis of the spiropyrimidine-fused derivatives started with the dialkylation of malononitrile with dihaloalkanes (**9**, **10**,

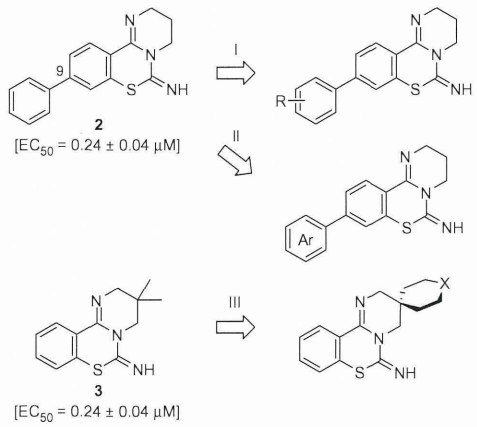
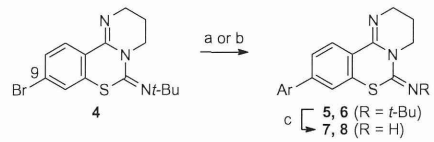
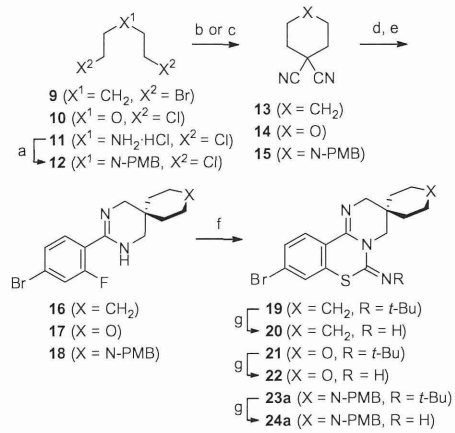


Figure 2. Strategy for the structural optimization of PD 404182 derivatives.



Scheme 1. Synthesis of 9-aryl-pyrimido[1,2-*c*][1,3]benzothiazin-6-imine derivatives. Reagents and conditions: (a) R-B(OH)$_2$ or R-Bpin, Pd(PPh$_3$)$_4$, PdCl$_2$(dppf)·CH$_2$Cl$_2$, K$_2$CO$_3$, toluene or 1,4-dioxane, EtOH, H$_2$O, reflux, 29%-quant.; (b) pyrazole or imidazole, CuCl, K$_2$CO$_3$, acetylacetone, NMP, 130 °C, 51–71% (for **6n** or **6o**); (c) TFA, MS4Å, CHCl$_3$, (MeOH), reflux, 34–94%.

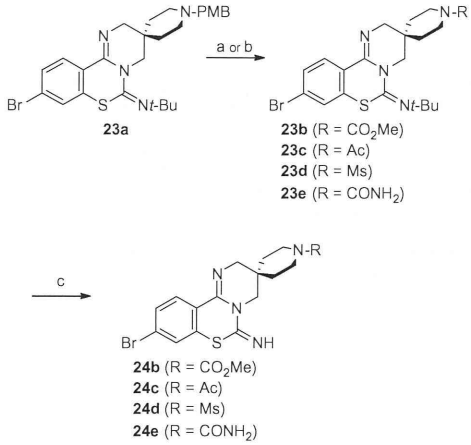


Scheme 2. Synthesis of spiropyrimidine-fused benzothiazinimine derivatives. Reagents and conditions: (a) (i) 4-methoxybenzoyl chloride, Et$_3$N, CH$_2$Cl$_2$, rt; (ii) LiAlH$_4$, Et$_2$O, rt, 75% (2 steps); (b) malononitrile, DBU, DMF, 50 °C, 8–60% (for **13** and **14**); (c) malononitrile, K$_2$CO$_3$, DMF, 65 °C, 85% (for **15**); (d) BH$_3$, THF, 0 °C to rt; (e) 4-bromo-2-fluorobenzaldehyde, I$_2$, K$_2$CO$_3$, *t*-BuOH, 70 °C, 11–62% [2 steps (d,e)]; (f) NaH, *t*-BuNCS DMF, rt −80 °C, 78–94%; (g) TFA, MS4Å, CHCl$_3$, reflux, 66–72%.

or **12**, Scheme 2). BH$_3$-mediated reduction of the alkylated malononitriles (**13–15**) followed by oxidative amidination[41] with 4-bromo-2-fluorobenzaldehyde gave the 2-phenyl-1,4,5,6-tetrahydropyrimidine derivatives (**16–18**). Subsequent exposure of compounds **16–18** to *tert*-butylisothiocyanate provided the tetracyclic compounds **19**, **21**, and **23a**. Deprotection of the *tert*-butyl groups in compounds **19**, **21**, and **23a** afforded the desired spiropyrimidine-fused benzothiazinimine derivatives (**20**, **22**, and **24a**). The substitution of the *p*-methoxybenzyl (PMB) group in compound **24a** was also attempted (Scheme 3). The treatment of compound **23a** with methyl chloroformate or acetyl chloride directly provided derivatives **23b** and **23c**, respectively. A two-step procedure, including the removal of the PMB group by treatment with 1-chloroethyl chloroformate followed by modification with mesyl chloride (MsCl) or trimethylsilyl isocyanate (TMSNCO) was used for the synthesis of the derivatives **23d** and **23e**, respectively, because the reaction of compound **23a** with MsCl and TMSNCO failed. Deprotection of the *tert*-butyl group in **23b–e** afforded the respective N-substituted derivatives **24b–e**.

2.3. Structure–activity relationships of 9-phenylpyrimido[1,2-*c*][1,3]benzothiazine derivatives

We initially examined substituent effects at the *para*-position of the 9-phenyl group of compound **2** (Table 1). The introduction of methoxycarbonyl (**7a**), cyano (**7b**), nitro (**7c**), and trifluoromethyl (**7d**) groups slightly reduced the anti-HIV activity (EC$_{50}$ = 0.44–0.81 μM), whereas a significant decrease in the anti-HIV activity was observed following the introduction of a carbamoyl group (**7e**) with hydrogen-bond donor/acceptor properties



Scheme 3. Synthesis of derivatives **24b–e** from **23a**. Reagents and conditions: (a) $ClCO_2Me$ or AcCl, CH_2Cl_2, 0 °C, 81–96% (for **23b** or **23c**); (b) (i) 1-chloroethyl chloroformate, Et_3N, CH_2Cl_2, 0 °C, then MeOH, reflux, (ii) MsCl or TMSNCO, (Et_3N), CH_2Cl_2, rt, 29–82% (2 steps, for **23d** or **23e**); (c) TFA, MS4Å, $CHCl_3$, reflux, 65–94%.

(EC_{50} = 8.71 μM). Compounds containing a hydrophobic group, including methoxy (**7f**, EC_{50} = 0.24 μM), methylthio (**7g**, EC_{50} = 0.20 μM), and trifluoromethoxy (**7h**, EC_{50} = 0.38 μM) groups showed similar levels of anti-HIV activity to that of compound **2**. These results indicated that the hydrophobic and electron donating properties of these substituents had a positive impacts on improving the anti-HIV activity.

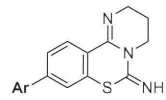
Similar SARs were observed following modifications at the *meta*-position of the 9-phenyl group (Table 1). For example, the addition of the electron-withdrawing methoxycarbonyl (**7i**), cyano (**7j**), and nitro (**7k**) groups resulted in a slight decrease in the anti-HIV activity (EC_{50} = 0.39–1.26 μM), whereas the hydrophilic (1-hydroxy)ethyl (**7l**, EC_{50} = 1.19 μM), acetamido (**7m**, EC_{50} >10 μM), mesylamido (**7n**, EC_{50} >10 μM), and hydroxy (**7o**, EC_{50} = 2.62 μM) groups led to a reduction or loss in the levels of anti-HIV activity. In contrast, the introduction of a methoxy group (**7p**) at the *meta*-position of the 9-phenyl group improved the inhibitory activity (EC_{50} = 0.15 μM). The more hydrophobic isopropoxy group (**7q**) maintained the anti-HIV activity of compound **2** (EC_{50} = 0.32 μM), whereas the introduction of a phenyl group (**7r**) led to a decrease in the inhibitory activity (EC_{50} = 1.35 μM).

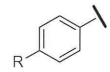
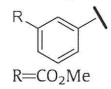
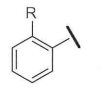
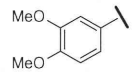
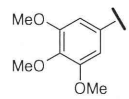
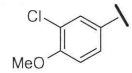
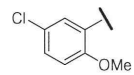
Similar anti-HIV activities to that of compound **2** were also exhibited by the *ortho*-methoxy (**7s**) and *ortho*-phenyl (**7t**) derivatives (EC_{50} = 0.41 and 0.32 μM, respectively), suggesting that the twisted conformation of the biaryl axis in the 9-aryl-modified PD 404182 derivatives can be tolerated and can interact with the target molecule(s).

To develop more potent anti-HIV agents, several compounds were designed with bis- and tris-modifications on the 9-phenyl group of compound **2** (Table 1). The introduction of the 3,4-dimethoxy (**7u**, EC_{50} = 0.27 μM) and 3,4,5-trimethoxy (**7v**, EC_{50} = 0.25 μM) groups did not alter the bioactivity. The Cl-modified derivatives **7w** and **7x** exhibited similar levels of potency to compound **2** (EC_{50} = 0.32 and 0.48 μM, respectively). Taken together, these results suggest that the hydrophobic property of the phenyl substituting group may provide the predominant contribution in any potential interaction with the target molecule(s).

We proceeded to investigate the impact of introducing a bicyclic aromatic group at the 9-position of the pyrimido[1,2-*c*][1,3]benzothiazine scaffold (Table 2). Modifications with a variety of 3,4-fused phenyl groups were investigated because the 2-naphthyl-modified analog (**8a**) exhibited slightly more potent

Table 1
Structure–activity relationships for biphenyl-type derivatives



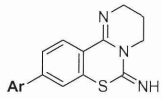
Compound	Ar	EC_{50}[a] (μM)
		
2	R=H	0.24 ± 0.04
7a	R=CO_2Me	0.81 ± 0.29
7b	R=CN	0.44 ± 0.10
7c	R=NO_2	0.46 ± 0.06
7d	R=CF_3	0.55 ± 0.16
7e	R=$CONH_2$	8.71 ± 0.82
7f	R=OMe	0.24 ± 0.04
7g	R=SMe	0.20 ± 0.06
7h	R=OCF_3	0.38 ± 0.06
		
7i	R=CO_2Me	0.39 ± 0.09
7j	R=CN	1.17 ± 0.27
7k	R=NO_2	1.26 ± 0.13
7l	R=$CH(OH)CH_3$	1.19 ± 0.19
7m	R=NHAc	>10
7n	R=NHMs	>10
7o	R=OH	2.62 ± 0.26
7p	R=OMe	0.15 ± 0.05
7q	R=Oi-Pr	0.32 ± 0.10
7r	R=Ph	1.35 ± 0.26
		
7s	R=OMe	0.41 ± 0.10
7t	R=Ph	0.32 ± 0.12
7u		0.27 ± 0.04
7v		0.25 ± 0.03
7w		0.32 ± 0.04
7x		0.48 ± 0.06

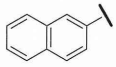
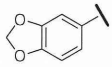
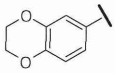
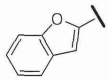
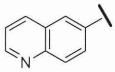
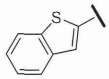
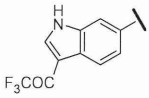
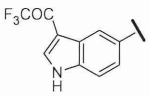
[a] EC_{50} values represent the concentration of compound required to inhibit the HIV-1 infection by 50% and were obtained from three independent experiments.

anti-HIV activity (EC_{50} = 0.20 μM) than that of the 1-naphthyl congener (**8b**, EC_{50} = 0.39 μM). Compound **8c**, which contained a benzo[*d*][1,3]dioxol-5-yl group, displayed inhibitory activity two-fold greater than that of compound **2** (EC_{50} = 0.15 μM), whereas the 2,3-dihydrobenzo[*b*][1,4]dioxin-6-yl derivative **8d** and quinolin-6-yl derivative **8e** exhibited less favorable effects (EC_{50} = 0.26 μM and 0.25 μM, respectively). The introduction of trifluoroacetylindolyl groups (**8f** and **8g**) resulted in no anti-HIV activity, and the compounds also showed unexpected levels of cytotoxicity.

The substitution of the 9-phenyl group with a variety of different heterocyclic substructures was also investigated (Table 2). Pyridine substitution (**8h** and **8i**) led to a slight reduction in the anti-HIV activity (EC_{50} = 0.45 μM and 0.54 μM, respectively), whereas the introduction of a furan (**8j**), benzofuran (**8k**), thiophene (**8l**), benzothiophene (**8m**), and pyrazole (**8n**) was well

Table 2
Structure–activity relationships for biaryl-type derivatives



Compound	Ar	EC_{50}[a] (μM)	Compound	Ar	EC_{50}[a] (μM)
8a		0.20 ± 0.06	**8h**		0.45 ± 0.07
8b		0.39 ± 0.12	**8i**		0.54 ± 0.04
8c		0.15 ± 0.03	**8j**		0.26 ± 0.02
8d		0.26 ± 0.07	**8k**		0.20 ± 0.03
8e		0.25 ± 0.04	**8l**		0.22 ± 0.07
			8m		0.26 ± 0.06
8f		>1.00[b]			
			8n		0.42 ± 0.08
8g		>1.00[b]	**8o**		5.12 ± 1.02

[a] EC_{50} values represent the concentration of compound required to inhibit the HIV-1 infection by 50% and were obtained from three independent experiments.
[b] Cytotoxicity was observed at 10 μM.

tolerated and had little impact on the activity relative to compound **2** (EC_{50} = 0.20–0.42 μM). It is worthy of note that the substitution of the 9-phenyl group with a basic imidazole moiety led to a significant reduction in the anti-HIV (**8o**, EC_{50} = 5.12 μM).

Taken together, these data led to the identification of two highly potent compounds **7p** and **8c** (EC_{50} = 0.15 μM), which contained *m*-methoxyphenyl and benzo[*d*][1,3]dioxol-5-yl groups, respectively. Furthermore, no cytotoxic effects were observed for these derivatives at 10 μM in the MAGI assay.

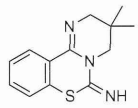
2.4. Structure–activity relationships of spiropyrimidine-fused benzothiazinimine derivatives

Several spiropyrimidine-fused derivatives were designed for the SAR study based on the geminal dimethylpyrimidine substructure **3** (Table 3).[42] Cyclohexane (**20**) and *N*-methoxycarbonylpiperidine (**24b**) derivatives exhibited the similar levels of anti-HIV activity to that of the parent dimethyl derivative **3**. In contrast, the tetrahydropyran (**22**) and *N*-(*p*-methoxybenzyl)piperidine (**24a**) derivatives exerted inhibitory activities that were five–sevenfold lower than that of the parent dimethyl derivative **3**. The *N*-acetyl- (**24c**), *N*-methanesulfonyl- (**24d**), and *N*-carbamoyl- (**24e**) piperidine derivatives also provided reduced levels of antiviral activity. With this in mind, the *N*-alkoxycarbonyl piperidine group was identified as a linkage for the introduction of additional functional group(s) to PD 404182 with potent anti-HIV activity (**24b**).

2.5. Anti-HIV profiles of the most potent derivative 8c

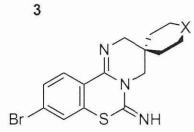
A time-of-drug addition study was carried out to further investigate the anti-HIV profile of the most potent derivative **8c** as an anti-HIV agent (Fig. 3). This assay has been used previously to

Table 3
Structure–activity relationships for spiropyrimidine-fused derivatives



EC_{50} = 0.24 ± 0.04 μM

3



Compound	X	EC_{50}[a] (μM)
20	CH_2	0.25 ± 0.01
22	O	1.73 ± 0.35
24a	N-PMB	1.45 ± 0.05
24b	N-CO_2Me	0.44 ± 0.02
24c	N-Ac	2.74 ± 0.15
24d	N-Ms	1.81 ± 0.43
24e	N-$CONH_2$	>10

[a] EC_{50} values represent the concentration of compound required to inhibit the HIV-1 infection by 50% and were obtained from three independent experiments.

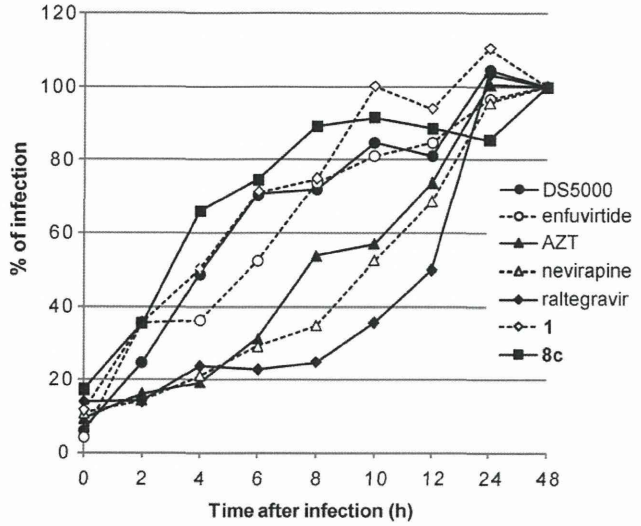


Figure 3. Time of drug addition profiles for infection by HIV-1_{IIIB} strain of HeLa-CD4/CCR5-LTR/β-gal cells.

Table 4
Anti-HIV activity of compounds **1** and **8c** against other HIV strains

Strains	EC_{50}[a] (μM)	
	1[34]	**8c**
HIV-1_{NL4-3}	0.38 ± 0.06	0.23 ± 0.09
HIV-1_{BaL}	0.37 ± 0.06	0.13 ± 0.05
HIV-2_{EHO}	0.31 ± 0.06	0.14 ± 0.02
HIV-2_{ROD}	0.30 ± 0.06	0.10 ± 0.04

[a] EC_{50} values represent the concentration of compound required to inhibit the HIV infection by 50% and were obtained from three independent experiments.

approximately determine which stage in the replication cycle of HIV-1 is inhibited by the compound. Two compounds (**1** and **8c**) were selected for testing in this assay together with five standard anti-HIV agents, including DS5000 (adsorption inhibitor),[31] enfuvirtide (fusion inhibitor),[6,7] AZT (NRTI),[43] nevirapine (NNRTI),[44,45] and raltegravir (integrase inhibitor).[8,9] The results revealed that the infection profile in the presence of compound **8c** was similar to that of DS5000 and enfuvirtide, suggesting that **8c** exerted its anti-HIV activity at the early stages of the viral infection, including the binding and fusion stage. This was similar to PD 404182, indicating that the bioactivity profile was not influenced by the newly appended functional group(s).

We also evaluated the antiviral activity of compounds **1** and **8c** against several HIV strains such as HIV-1_{NL4-3}, HIV-1_{BaL}, HIV-2_{EHO}, and HIV-2_{ROD}. This study enabled us to estimate the impact of the target molecules on the process of binding and fusion because these viruses have different susceptibilities[46] to different anti-HIV agents. These results implied that compounds **1** and **8c** exhibited their anti-HIV activity through different mechanisms from those of the known binding and fusion inhibitors including CCR5 antagonists, CXCR4 antagonists, and enfuvirtide. In addition, compound **8c** was two–threefold more effective against these HIV-1 and HIV-2 strains than PD 404182 (Table 4).

3. Conclusions

In conclusion, we have designed and synthesized a series of PD 404182 derivatives for the development of novel anti-HIV agents. The structural optimization study on the 9-position of pyrimido[1,2-*c*][1,3]benzothiazinimine identified two potent derivatives containing *m*-methoxyphenyl (**7p**) and benzo[*d*][1,3]dioxol-5-yl groups (**8c**) that exhibited threefold higher anti-HIV activity than that of PD 404182 (**1**). The common hydrophobic biaryl moiety is effective to improve the antiviral activity, providing potential interaction with the target molecule(s). In addition, we demonstrated that the most effective derivative, **8c**, inhibited viral infection against all of the HIV strains examined and acted at the early stage of the HIV infection. The design and synthesis of chemical probes based on these SAR data are being investigated to identify the target molecule(s).

4. Experimental

4.1. Synthesis

4.1.1. General methods

^1H NMR spectra were recorded using a JEOL AL-400 or a JEOL ECA-500 spectrometer. Chemical shifts are reported in δ (ppm) relative to Me_4Si ($CDCl_3$) or DMSO (DMSO-d_6) as internal standards. ^{13}C NMR spectra were referenced to the residual solvent signal. Exact mass (HRMS) spectra were recorded on a JMS-HX/HX 110A mass spectrometer. Melting points were measured by a hot stage melting point apparatus (uncorrected). For flash chromatography, Wakogel C-300E (Wako) or aluminium oxide 90 standardized (Merck) were employed. For preparative TLC, TLC silica gel 60 F_{254} (Merck) or TLC aluminium oxide 60 F_{254} basic (Merck) were employed. For analytical HPLC, a Cosmosil 5C18-ARII column (4.6 × 250 mm, Nacalai Tesque, Inc., Kyoto, Japan) was employed with method A [a linear gradient of CH_3CN containing 0.1% (v/v) TFA] or method B [a linear gradient of CH_3CN containing 0.1% (v/v) NH_3] at a flow rate of 1 mL/min on a Shimadzu LC-10ADvp (Shimadzu Corp., Ltd., Kyoto, Japan), and eluting products were detected by UV at 254 nm. The purity of the compounds was determined by combustion analysis or HPLC analysis as >95%.

4.1.2. General procedure of Suzuki–Miyaura cross coupling for 9-aryl pyrimido[1,2-*c*][1,3]thiazine derivatives 5 and 6: *N*-(*tert*-butyl)-3,4-dihydro-9-(4-methoxycarbonylphenyl)-2*H*,6*H*-pyrimido[1,2-*c*][1,3]benzothiazin-6-imine 5a

To a solution of bromide **4** (52.8 mg, 0.15 mmol) and 4-(methoxycarbonyl)phenylboronic acid (32.4 mg, 0.18 mmol) in a mixture of toluene (1.5 mL), EtOH (0.9 mL) and 1 M aq K_2CO_3 (1.5 mL) was added $Pd(PPh_3)_4$ (6.9 mg, 4 mol %) and $PdCl_2(dppf)\cdot CH_2Cl_2$ (3.7 mg, 3 mol %). After being stirred under reflux for 1 h, the mixture was extracted with $CHCl_3$. The organic layers were dried over $MgSO_4$ and concentrated. The residue was purified by flash chromatography over aluminum oxide with *n*-hexane/EtOAc (10:0/9:1) to give the compound **5a** as colorless solid (47.3 mg, 77%): mp 201–202 °C (from $CHCl_3$–*n*-hexane); IR (neat) cm^{-1}: 1719 (C=O), 1593 (C=N); ^1H NMR (400 MHz, $CDCl_3$) δ: 1.40 (s, 9H, 3 × CH_3), 1.90–1.96 (m, 2H, CH_2), 3.65 (t, *J* = 5.5 Hz, 2H, CH_2), 3.89 (t, *J* = 6.1 Hz, 2H, CH_2), 3.94 (s, 3H, CH_3), 7.36 (d, *J* = 1.7 Hz, 1H, Ar), 7.44 (dd, *J* = 8.5, 1.7 Hz, 1H, Ar), 7.65 (d, *J* = 8.2 Hz, 2H, Ar), 8.10 (d, *J* = 8.2 Hz, 2H, Ar), 8.28 (d, *J* = 8.5 Hz, 1H, Ar). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 21.9, 30.0 (3C), 45.2, 45.4, 52.1, 54.2, 123.0, 124.8, 127.0 (2C), 127.3, 129.1, 129.6, 129.8, 130.2 (2C), 138.0, 141.7, 143.8, 147.5, 166.8; HRMS (FAB): *m*/*z* calcd for $C_{23}H_{26}N_3O_2S$ $[M+H]^+$ 408.1746; found: 408.1748.

4.1.3. *N*-(*tert*-Butyl)-3,4-dihydro-9-(1*H*-pyrazol-1-yl)-2*H*,6*H*-pyrimido[1,2-*c*][1,3]benzothiazin-6-imine (6n)

To a solution of bromide **4** (52.8 mg, 0.15 mmol), pyrazole (12.3 mg, 0.18 mmol), CuCl (1.5 mg, 0.015 mmol) and K_2CO_3 (21.8 mg, 0.16 mol) in *N*-methylpyrrolidone (0.3 mL) was added

acetylacetone (3.8 μL, 0.038 mmol) under an Ar atmosphere. After being stirred at 130 °C for 19 h, EtOAc and brine were added. The organic layers were washed with H_2O, and dried over $MgSO_4$. After concentration, the residue was purified by flash chromatography over aluminum oxide with *n*-hexane/EtOAc (7:3) to give the title compound **6n** as colorless solid (39.8 mg, 71%): mp 132–133 °C (from $CHCl_3$–*n*-hexane); IR (neat) cm^{-1}: 1597 (C=N); ^1H NMR (400 MHz, $CDCl_3$) δ: 1.39 (s, 9H, 3 × CH_3), 1.90–1.96 (m, 2H, CH_2), 3.63 (t, J = 5.6 Hz, 2H, CH_2), 3.88 (t, J = 6.2 Hz, 2H, CH_2), 6.48 (dd, J = 2.7, 1.8 Hz, 1H, Ar), 7.47 (dd, J = 8.8, 2.2 Hz, 1H, Ar), 7.56 (d, J = 2.2 Hz, 1H, Ar), 7.73 (d, J = 1.8 Hz, 1H, Ar), 7.94 (d, J = 2.7 Hz, 1H, Ar), 8.28 (d, J = 8.8 Hz, 1H, Ar). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 21.8, 30.0 (3C), 45.0, 45.4, 54.2, 108.2, 114.3, 115.9, 125.4, 126.7, 129.9, 130.8, 137.7, 141.0, 141.7, 147.3; HRMS (FAB): *m/z* calcd for $C_{18}H_{22}N_5S$ $[M+H]^+$ 340.1596; found: 340.1598.

4.1.4. General procedure of *tert*-butyl deprotection for pyrimido[1,2-*c*][1,3]benzothiazin-6-imines (7, 8, 20, 22, and 24): 3,4-dihydro-9-(4-methoxycarbonylphenyl)-2*H*,6*H*-pyrimido[1,2-*c*][1,3]benzothiazin-6-imine (7a)

TFA (2.0 mL) was added to a mixture of *N*-(*tert*-butyl)-protected pyrimido[1,2-*c*][1,3]benzothiazin-6-imine **5a** (38.4 mg, 0.094 mmol) in small amount of $CHCl_3$ and MS4Å (300 mg, powder, activated by heating with Bunsen burner). After being stirred under reflux for 1 h, the mixture was concentrated. To a stirring mixture of the residue in $CHCl_3$ was added dropwise Et_3N at 0 °C to adjust pH to 8–9. The whole was extracted with EtOAc. The extract was washed with sat. $NaHCO_3$, brine, and dried over $MgSO_4$. After concentration, the residue was purified by flash chromatography over aluminum oxide with *n*-hexane/EtOAc (9:1/1:1) to give the title compound **7a** as colorless solid (27.3 mg, 83%): mp 185–186 °C (from $CHCl_3$–*n*-hexane); IR (neat) cm^{-1}: 1719 (C=O), 1619 (C=N), 1566 (C=N); ^1H NMR (500 MHz, $CDCl_3$) δ: 1.97–2.02 (m, 2H, CH_2), 3.71 (t, J = 5.7 Hz, 2H, CH_2), 3.94 (s, 3H, CH_3), 4.04 (t, J = 6.0 Hz, 2H, CH_2), 7.27 (d, J = 1.7 Hz, 1H, Ar), 7.46 (dd, J = 8.0, 1.7 Hz, 1H, Ar), 7.63 (d, J = 8.6 Hz, 2H, Ar), 8.10 (d, J = 8.6 Hz, 2H, Ar), 8.30 (d, J = 8.0 Hz, 1H, Ar). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 21.0, 43.8, 45.0, 52.2, 122.0, 125.1, 126.2, 126.9 (2C), 129.5, 129.6, 129.7, 130.2 (2C), 142.1, 143.4, 146.2, 153.0, 166.7; HRMS (FAB): *m/z* calcd for $C_{19}H_{18}N_3O_2S$ $[M+H]^+$ 352.1120; found: 352.1119

4.1.5. Bis(2-chloroethyl)-*N*-(4-methoxybenzyl)amine (12)

To a suspension of bis(2-chloroethyl)amine hydrochloride **11** (8.92 g, 50.0 mmol) in CH_2Cl_2 (300 mL) were added Et_3N (2.89 mL, 100.0 mmol) and 4-methoxybenzoyl chloride (6.77 mL, 50.0 mmol). After being stirred at rt for 2 h, the reaction mixture was washed with 1 N HCl, satd $NaHCO_3$, brine, and dried over $MgSO_4$. After concentration, the residue was dissolved in anhydrous Et_2O (250 mL) and $LiAlH_4$ (2.1 g, 55.0 mmol) was slowly added at 0 °C under an Ar atmosphere. After being stirred at rt overnight, the reaction mixture was quenched by addition of water, 2 N NaOH, and water. The mixture was dried over $MgSO_4$. After concentration, the residue was purified by flash chromatography over silica gel with *n*-hexane/EtOAc (19:1) to give the title compound **12** as colorless oil (9.88 g, 75%): ^1H NMR (400 MHz, $CDCl_3$) δ: 2.90 (t, J = 7.1 Hz, 4H, 2 × CH_2), 3.48 (t, J = 7.1 Hz, 4H, 2 × CH_2), 3.67 (s, 2H, CH_2), 3.80 (s, 3H, CH_3), 6.86 (d, J = 8.5 Hz, 2H, Ar), 7.24 (d, J = 8.5 Hz, 2H, Ar). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 42.0 (2C), 55.2, 56.2 (2C), 58.6, 113.8 (2C), 129.7 (2C), 130.7, 158.9; LRMS (FAB): *m/z* $[M+H]^+$ 262.

4.1.6. 1-(4-Methoxybenzyl)piperidine-4,4-dicarbonitrile (15)

To a solution of malononitrile (2.49 g, 37.7 mmol) in DMF (94.3 mL) was added K_2CO_3 (5.73 mg, 41.5 mmol). After being stirred at 65 °C for 2 h, a solution of chloride **12** (9.88 mg, 37.7 mmol) in DMF (37.7 mL) was added. After being stirred at same temperature for 5 h, EtOAc was added. The mixture was washed with 5% aq $NaHCO_3$, and dried over $MgSO_4$. After concentration, the residue was purified by flash chromatography over silica gel with *n*-hexane/EtOAc (2:1) to give the title compound **15** as yellow oil (8.13 g, 85%): IR (neat) cm^{-1}: 2248 (C≡N); ^1H NMR (400 MHz, $CDCl_3$) δ: 2.22 (t, J = 5.4 Hz, 4H, 2 × CH_2), 2.61 (br s, 4H, 2 × CH_2), 3.48 (s, 2H, CH_2), 3.80 (s, 3H, CH_2), 6.86 (d, J = 8.5 Hz, 2H, Ar), 7.19 (d, J = 8.8 Hz, 2H, Ar). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 31.1, 34.1 (2C), 48.5 (2C), 55.2, 61.9, 113.8 (2C), 115.4 (2C), 129.2, 130.1 (2C), 159.0; HRMS (FAB): *m/z* calcd for $C_{15}H_{18}N_3O$ $[M+H]^+$ 256.1450; found: 256.1454.

4.1.7. 3-(4-Bromo-2-fluorophenyl)-9-(4-methoxybenzyl)-2,4,9-triazaspiro[5.5]undec-2-ene (18)

To a solution of nitrile **15** (4.05 g, 15.9 mmol) in THF (39.8 mL) was added BH_3 in THF (79.5 mL, 79.5 mmol, 1.0 M) at 0 °C under an Ar atmosphere. The mixture was warmed to rt. After being stirred at 65 °C for 5 h, the reaction mixture was cooled to 0 °C, and 1 N HCl was added. After being stirred at rt for 1 h, the mixture was basified with 2 N NaOH. The whole was extracted with $CHCl_3$ and dried over $MgSO_4$. After concentration, the residue was dissolved in *t*-BuOH (159.0 mL) and 4-bromo-2-fluorobenzaldehyde (3.23 g, 15.9 mmol) was added. After being stirred at 70 °C for 30 min, K_2CO_3 (6.59 g, 47.7 mmol) and I_2 (5.05 g, 19.9 mmol) were added. After being stirred at same temperature for 3 h, the reaction mixture was quenched with sat. Na_2SO_3 until the iodine color almost disappeared. The reaction mixture was basified with 2 N NaOH. The whole was extracted with $CHCl_3$, and dried over $MgSO_4$. After concentration, the residue was purified by flash chromatography over aluminium oxide with EtOAc–MeOH (10:0/95:5) to give the title compound **18** as colorless solid (752 mg, 11%): mp 179–181 °C (from $CHCl_3$–*n*-hexane), IR (neat) cm^{-1}: 1630 (C=N); ^1H NMR (500 MHz, $CDCl_3$) δ: 1.45 (t, J = 5.4 Hz, 4H, 2 × CH_2), 2.35 (t, J = 5.4 Hz, 4H, 2 × CH_2), 3.16 (s, 4H, 2 × CH_2), 3.40 (s, 2H, CH_2), 3.73 (s, 3H, CH_3), 4.63 (s, 1H, NH), 6.78 (d, J = 8.6 Hz, 2H, Ar), 7.14–7.23 (m, 4H, Ar), 7.62 (t, J = 8.3 Hz, 1H, Ar). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 27.3, 32.8 (2C), 49.1 (2C), 51.4 (2C), 55.2, 62.7, 113.5 (2C), 119.4 (d, J = 27.3 Hz), 122.7 (d, J = 12.4 Hz), 123.7 (d, J = 9.9 Hz), 127.8 (d, J = 3.3 Hz), 130.2, 130.3 (2C), 131.7 (d, J = 4.1 Hz), 150.3 (d, J = 1.7 Hz), 158.6, 159.7 (d, J = 251.6 Hz); ^{19}F NMR (500 MHz, $CDCl_3$) δ: −114.6. HRMS (FAB): *m/z* calcd for $C_{22}H_{26}BrFN_3O$ $[M+H]^+$ 446.1243; found: 446.1237.

4.1.8. General procedure for *t*-BuNCS mediated cyclization: 9-bromo-*N*-(*tert*-butyl)-1′-(4-methoxybenzyl)-2*H*-spiro[benzo[*e*]pyrimido[1,2-*c*][1,3]thiazine-3,4′-piperidin]-6(4*H*)-imine (23a)

To a mixture of fluoride **18** (2.0 g, 4.48 mmol) and NaH (358.4 mg, 8.96 mmol; 60% oil suspension) in DMF (14.8 mL) was added *t*-BuNCS (1.14 mL, 8.96 mmol) under an Ar atmosphere. After being stirred at rt overnight, the reaction mixture was warmed to 60 °C. After being stirred at this temperature for 1 h, EtOAc was added. The resulting solution was washed with sat. $NaHCO_3$, brine, and dried over $MgSO_4$. After concentration, the residue was purified by flash chromatography over aluminum oxide with *n*-hexane/EtOAc (10:0/9:1) to give the title compound **23a** as colorless solid (2.28 g, 94%): mp 89–91 °C (from $CHCl_3$–*n*-hexane); IR (neat) cm^{-1}: 1577 (C=N); ^1H NMR (500 MHz, $CDCl_3$) δ: 1.37 (s, 9H, 3 × CH_3), 1.49–1.52 (m, 4H, 2 × CH_2), 2.40–2.46 (m, 4H, 2 × CH_2), 3.41 (s, 2H, CH_2), 3.47 (s, 2H, CH_2), 3.75 (s, 2H, CH_2), 3.80 (s, 3H, CH_3), 6.85 (d, J = 8.6 Hz, 2H, Ar), 7.22 (d, J = 8.6 Hz, 2H, Ar), 7.28–7.31 (m, 2H, Ar), 8.03 (d, J = 8.6 Hz, 1H, Ar). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 29.7, 29.9 (3C), 32.6 (2C), 49.2 (2C), 51.6, 54.3, 55.2, 55.5, 62.7, 113.6 (2C), 124.5, 126.3, 126.8, 129.2, 130.0, 130.1, 130.4 (2C), 130.9, 137.5, 146.3, 158.7; HRMS

(FAB): *m/z* calcd for $C_{27}H_{34}BrN_4OS$ $[M+H]^+$ 541.1637; found: 541.1633.

4.1.9. 9-Bromo-*N*-(*tert*-butyl)-1′-(methoxycarbonyl)-2*H*-spiro[benzo[*e*]pyrimido[1,2-*c*][1,3]thiazine-3,4'-piperidin]-6(4*H*)-imine (23b)

To the solution of *N*-(4-methoxybenzyl)piperidine **23a** (40.6 mg, 0.075 mmol) in CH_2Cl_2 (0.38 mL) was added methyl chloroformate (86.4 μL, 1.13 mmol) at 0 °C under an Ar atmosphere. After being stirred at same temperature for 30 min, the reaction mixture was concentrated. The residue was purified by flash chromatography over silica gel with *n*-hexane/EtOAc (1:1) to give compound **23b** as a colorless solid (29.2 mg, 81%): mp 157–158 °C (from *n*-hexane); IR (neat) cm^{-1}: 1699 (C=O), 1577 (C=N); ^1H NMR (400 MHz, $CDCl_3$) δ: 1.37 (s, 9H, 3 × CH_3), 1.46 (t, J = 5.6 Hz, 4H, 2 × CH_2), 3.44 (br s, 4H, 2 × CH_2), 3.56 (br s, 2H, CH_2), 3.70 (s, 3H, CH_3), 3.81 (s, 2H, CH_2), 7.29–7.33 (m, 2H, Ar), 8.05 (d, J = 8.5 Hz, 1H, Ar). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 29.9 (3C), 30.1, 32.2 (2C), 39.9 (2C), 50.8, 52.5, 54.3, 55.2, 124.7, 126.1, 126.8, 129.3, 130.0, 130.9, 137.7, 146.3, 155.9; HRMS (FAB): *m/z* calcd for $C_{21}H_{28}BrN_4O_2S$ $[M+H]^+$ 479.1116; found: 479.1115.

4.1.10. 9-Bromo-*N*-(*tert*-butyl)-1′-(methanesulfonyl)-2*H*-spiro[benzo[*e*]pyrimido[1,2-*c*][1,3]thiazine-3,4'-piperidin]-6(4*H*)-imine (23d)

To the solution of *N*-(4-methoxybenzyl)piperidine **23a** (54.2 mg, 0.10 mmol) in CH_2Cl_2 (0.5 mL) were added Et_3N (28.9 μL, 0.20 mmol) and 1-chloroethyl chloroformate (21.8 μL, 0.20 mmol) at 0 °C under an Ar atmosphere. After being stirred at same temperature for 30 min, the reaction mixture was concentrated. The residue was dissolved in MeOH (2.0 mL). After being stirred under reflux for 10 min, the reaction mixture was concentrated. The residue was dissolved in $CHCl_3$, and was washed with sat. $NaHCO_3$, brine, and dried over $MgSO_4$. After concentration, the residue was dissolved in CH_2Cl_2 (1.0 mL) and Et_3N (28.9 μL, 0.20 mmol) and methanesulfonyl chloride (15.5 μL, 0.20 mmol) was added at rt under an Ar atmosphere. After being stirred at rt for 10 min, the reaction mixture was washed with sat. $NaHCO_3$, brine, and dried over $MgSO_4$. After concentration, the residue was purified by flash chromatography over aluminum oxide with *n*-hexane/EtOAc (6:4) to give compound **23d** as a colorless solid (40.9 mg, 82%): mp 177 °C (from $CHCl_3$–*n*-hexane); IR (neat) cm^{-1}: 1577 (C=N), 1331 (NSO_2), 1155 (NSO_2); ^1H NMR (400 MHz, $CDCl_3$) δ: 1.38 (s, 9H, 3 × CH_3), 1.62 (t, J = 5.5 Hz, 4H, 2 × CH_2), 2.80 (s, 3H, CH_3), 3.21–3.27 (m, 2H, CH_2), 3.31–3.37 (m, 2H, CH_2), 3.46 (s, 2H, CH_2), 3.84 (s, 2H, CH_2), 7.29–7.33 (m, 2H, Ar), 8.05 (d, J = 8.5 Hz, 1H, Ar). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 29.8, 29.9 (3C), 32.0 (2C), 34.7, 42.0 (2C), 50.1, 54.4, 55.1, 124.8, 125.9, 126.9, 129.4, 130.0, 130.8, 137.9, 146.3; HRMS (FAB): *m/z* calcd for $C_{20}H_{28}BrN_4O_2S_2$ $[M+H]^+$ 499.0837; found: 499.0840.

4.2. Determination of anti-HIV activity

The sensitivity of three HIV-1 strains and two HIV-2 strains was determined by the MAGI assay. The target cells (HeLa-CD4/CCR5-LTR/β-gal; 10^4 cells/well) were plated in 96-well flat microtiter culture plates. On the following day, the cells were inoculated with the HIV-1 (60 MAGI U/well, giving 60 blue cells after 48 h of incubation) and cultured in the presence of various concentrations of the test compounds in fresh medium. Forty-eight hours after viral exposure, all the blue cells stained with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) were counted in each well. The activity of test compounds was determined as the concentration that blocked HIV-1 infection by 50% (50% effective concentration [EC_{50}]). EC_{50} was determined by using the following formula:

$$EC_{50} = 10^{\wedge}[\log(A/B) \times (50 - C)/(D - C) + \log(B)], \quad (1)$$

wherein

A: of the two points on the graph which bracket 50% inhibition, the higher concentration of the test compound,
B: of the two points on the graph which bracket 50% inhibition, the lower concentration of the test compound,
C: inhibitory activity (%) at the concentration B,
D: inhibitory activity (%) at the concentration A.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.08.030.

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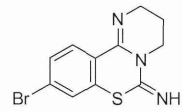
42. Because a 9-brominated derivative **25** exhibited comparable anti-HIV activity with compound **2** in our previous SAR study,[34] we employed compound **25** as a lead.



25

EC_{50} = 0.25 ± 0.09 μM

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Chemistry & Biology
Article

CXCR4 Stimulates Macropinocytosis: Implications for Cellular Uptake of Arginine-Rich Cell-Penetrating Peptides and HIV

Gen Tanaka,[1] Ikuhiko Nakase,[1] Yasunori Fukuda,[1] Ryo Masuda,[2] Shinya Oishi,[2] Kazuya Shimura,[4] Yoshimasa Kawaguchi,[1] Tomoka Takatani-Nakase,[5] Ülo Langel,[6] Astrid Gräslund,[7] Katsuya Okawa,[3,9] Masao Matsuoka,[4] Nobutaka Fujii,[2] Yasumaru Hatanaka,[8] and Shiroh Futaki[1,*]

[1]Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan
[2]Graduate School of Pharmaceutical Sciences
[3]Frontier Technology Center, Kyoto University Faculty of Medicine
Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan
[4]Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto 606-8507, Japan
[5]School of Pharmaceutical Sciences, Mukogawa Women's University, Nishinomiya, Hyogo 663-8179, Japan
[6]Department of Neurochemistry, Stockholm University, 106 92 Stockholm, Sweden
[7]Department of Biochemistry and Biophysics, Arrhenius Laboratories for Natural Sciences, Stockholm University, 106 91 Stockholm, Sweden
[8]Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Sugitani, Toyama 930-0194, Japan
[9]Present address: Drug Discovery Research Laboratories, Kyowa Hakko Kirin, Nagaizumi-cho, Suntou-gun, Shizuoka 411-8731, Japan
*Correspondence: futaki@scl.kyoto-u.ac.jp
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SUMMARY

CXCR4 is a coreceptor of HIV-1 infection in host cells. Through a photocrosslinking study to identify receptors involved in internalization of oligoarginine cell-penetrating peptides (CPPs), we found that CXCR4 serves as a receptor that stimulates macropinocytic uptake of the arginine 12-mer peptide (R12) but not of the 8-mer. We also found that stimulating CXCR4 with its intrinsic ligands, stromal cell-derived factor 1α and HIV-1 envelope glycoprotein 120, induced macropinocytosis. R12 had activity to prevent viral infection for HIV-1_{IIIB}, a subtype of HIV-1 that uses CXCR4 as a coreceptor for entry into susceptible cells, whereas the addition of a macropinocytosis inhibitor, dimethylamiloride, resulted in enhancement of viral infection. The present study shows that CXCR4 triggers macropinocytosis, which may have implications for the cellular uptake of oligoarginine CPPs and internalization of HIV.

INTRODUCTION

Recently developed intracellular delivery technology using cell-penetrating peptides (CPPs) has provided novel strategies for drug delivery, diagnostics, and chemical biology (Futaki, 2006; Wender et al., 2008). Conjugation of CPPs to molecules of interest leads to the efficient delivery of these molecules into cells, and successful modulation of cellular function has been reported. Arginine-rich CPPs, including oligoarginines and one derived from positions 48–60 of HIV-1 Tat protein (Tat peptide), are a representative CPP class (Futaki, 2006). Understanding the internalization methods of these CPPs and their conjugates should be beneficial for the design of more sophisticated and effective delivery systems. However, there remain many ambiguities regarding their methods of internalization.

It has been demonstrated by our group and others that macropinocytosis also plays an important part in the cellular uptake of arginine-rich CPPs to achieve high-efficiency intracellular delivery (Nakase et al., 2004; Wadia et al., 2004). Macropinocytosis is a transient, actin-driven fluid-phase endocytosis that involves membrane ruffling and the formation of large vacuoles called macropinosomes (Swanson and Watts, 1995; Conner and Schmid, 2003; Falcone et al., 2006). In most cells other than dendritic cells, macropinocytosis is not a constitutive endocytosis process but rather is activated by external stimuli such as epidermal growth factors and fibroblast growth factor 2 (FGF2) (Tkachenko et al., 2004). Ruffling of plasma membranes is induced by actin reorganization (Meier et al., 2002). Protrusion of the plasma membranes, followed by membrane fusion, leads to the formation of large endocytic vacuoles with a diameter that often exceeds 1 μm (Conner and Schmid, 2003). The importance of this endocytic pathway to the infection of various viruses has recently received attention (Mercer and Helenius, 2008, 2009).

On the other hand, CXCR4 is a CXC chemokine receptor (C-X-C chemokine receptor type 4) and is also a coreceptor for HIV-1 infection. Binding of HIV-1 envelope glycoprotein 120 (gp120) to CD4 host cells leads to further interactions with CXCR4 and the eventual fusion of viral and host cell membranes driven by the HIV-1 gp41 protein (Tamamura et al., 2005). CXCR4 is thus a promising target for anti-HIV agents (Schramm et al., 2000). Stromal cell-derived factor 1α (SDF-1α) is a natural CXCR4 ligand. Induction of CXCR4 endocytosis and actin polymerization by SDF-1α has been suggested (Rey et al., 2007; Yoder et al., 2008). It has been reported that SDF-1α induces rapid endocytosis and downmodulation of CXCR4 (Signoret et al., 1997; Orsini et al., 1999; Venkatesan et al., 2003). It has also been reported that treatment of cells with SDF-1α leads to actin polymerization (Rey et al., 2007; Yoder et al., 2008). However, although involvement of clathrin-dependent endocytosis has

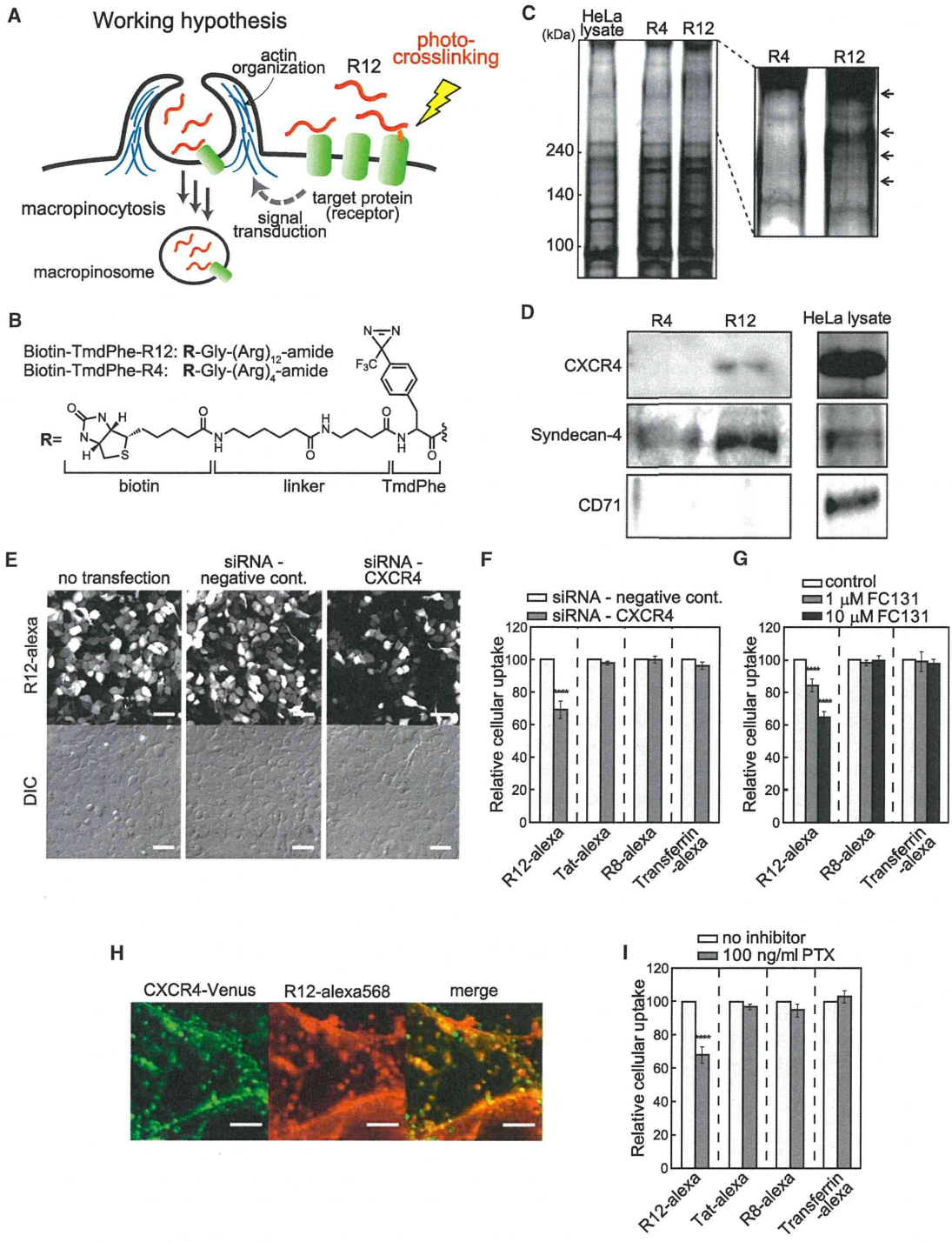

Figure 1. Identification of CXCR4 as a Potential Receptor that Contributes to Cellular Uptake of the Arginine R12 Peptide

(A) Outline of the photocrosslinking study to identify the cell-surface receptor for the R12 peptide.

(B) Structure of biotin-TmdPhe-R12, the photocrosslinking probe to identify the receptor responsible for R12 cellular uptake. Structures of other peptides used in this study are given in Figure S3.

(C) SDS-PAGE of the proteins obtained with magnetic spheres after photocrosslinking of HeLa cells with biotin-TmdPhe-R12 (0.5 μM) or biotin-TmdPhe-R4 (1.5 μM) for 3 min at 4°C. Arrows indicate specific bands observed for biotin-TmdPhe-R12-treated cells.

(D) Western blot analysis of the proteins obtained with magnetic spheres from biotin-TmdPhe-R12-treated cells yielded positive staining with the anti-CXCR4 antibody but not with the anti-CD71 antibody.