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Studies of non-nucleoside HIV-1 reverse transcriptase inhibitors. Part 2: Synthesis and structure–activity relationships of 2-cyano and 2-hydroxy thiazolidenebenzenesulfonamide derivatives

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Abstract—In a previous study, we described the structure–activity relationships (SARs) for a series of thiazolidenebenzenesulfonamide derivatives. These compounds were found to be highly potent inhibitors of the wild type (WT) and Y181C mutant reverse transcriptases (RTs) and modest inhibitors of K103N RT. These molecules are thus considered to be a novel class of non-nucleoside HIV-1 RT inhibitors (NNRTIs). In this paper, we have examined the effects of substituents on both the thiazolidene and benzenesulfonamide moieties. Introduction of a 2-cyanophenyl ring into these moieties significantly enhanced anti-HIV-1 activity, whereas a 2-hydroxyphenyl group endowed potent activity against RTs, including K103N and Y181C mutants. Among the series of molecules examined, **10l** and **18b** (YM-228855), combinations of 2-cyanophenyl and 4-methyl-5-isopropylthiazole moieties, showed extremely potent anti-HIV-1 activity. The EC_{50} values of **101** and **18b** were 0.0017 and 0.0018 μM, respectively. These values were lower than that of efavirenz (**3**). Compound **11g** (YM-215389), a combination of 2-hydroxyphenyl and 4-chloro-5-isopropylthiazole moieties, proved to be the most active against both K103N and Y181C RTs with IC_{50} values of 0.043 and 0.013 μM, respectively.

1. Introduction

Reverse transcriptase (RT) is a key enzyme, which plays an essential and multifunctional role in the replication of human immunodeficiency virus type 1 (HIV-1) and thus considered to be an attractive target for inhibition of HIV-1 replication.[1] Non-nucleoside reverse transcriptase inhibitors (NNRTIs), a group of structurally diverse compounds, have been reported to directly inhibit the enzyme in an allosteric fashion by binding to a pocket near the polymerase active site.[2] To date, many classes of NNRTIs have been identified, and three inhibitors, nevirapine, delavirdine, and efavirenz, have been approved for the treatment of HIV-1 infection. However, NNRTI-containing regimens are compromised by rapid emergence of drug-resistant strains

Keywords: Thiazolidenebenzenesulfonamide; Non-nucleoside HIV-1 reverse transcriptase inhibitor; YM-215389.
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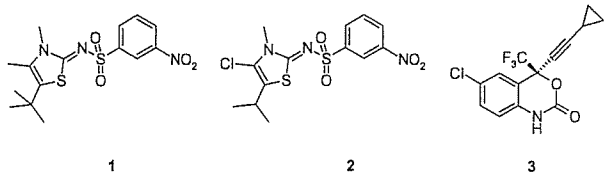


Figure 1. Structures of thiazolidenebenzenesulfonamide derivatives (**1**, **2**) and efavirenz (**3**).

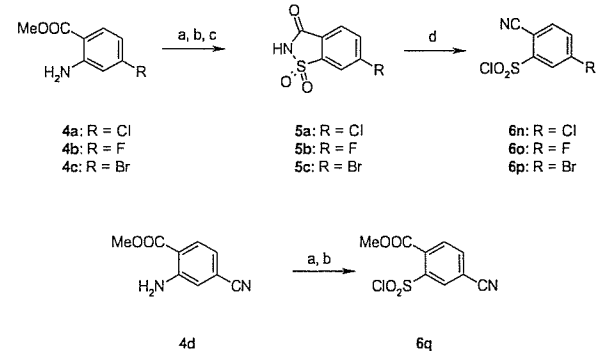
carrying the amino acid mutations surrounding the NNRTI binding pocket.

The mutation of tyrosine to cysteine at position 181 in HIV-1 RT (Y181C) following treatment with nevirapine or delavirdine has been documented in cell culture experiments.[3] Furthermore, the mutation of lysine to asparagine at position 103 (K103N) is frequently observed in patients who do not respond to the treatment with either NNRTI alone or in combination with other inhibitors.[4] The newest NNRTI, efavirenz (**3**) has been shown significant clinical efficacy in combination with both protease-containing and protease-sparing regimens.[5] Although the majority of patients receiving efavirenz-containing regimens show a sustained antiviral response, more than 90% of the viruses isolated from the patients whose viral loads have rebounded after an initial drug response have the K103N mutation.[6]

Our previously determined structure–activity relationships (SARs) for a series of thiazolidenebenzenesulfonamide derivatives and docking studies have suggested the importance of a bulky 5-alkyl group on the thiazolidene ring for potent inhibitory activity against Y181C RT.[7] In addition, we found that 3-nitrobenzenesulfonamide derivatives (**1**, **2**) possess potent activity against the wild type (WT) and Y181C RTs, but that their activity against K103N RT was not satisfactory. In this study, we have explored the SARs of substituents in a series of thiazolidenebenzenesulfonamides, in order to identify novel NNRTIs that are capable of inhibiting both K103N and Y181C RT activity and HIV-1 replication (Fig. 1).

2. Chemistry

A series of benzenesulfonamide derivatives (**1**, **10a–q**, **11a–g**, **16a**, **17a**, **18a,b**, **19**, **20**) was synthesized as shown in Schemes 1–4. Cyanobenzenesulfonylchlorides **6n–p** were prepared from their corresponding substituted methyl anthranilates (Scheme 1). Sandmeyer reactions of anthranilates **4a–c** with ammonia provided saccharins **5a–c**.[8] Treatment of saccharins **5a–c** with PCl_5 afforded 2-cyanobenzenesulfonylchlorides **6n–p**. Compound **4d** was converted to the methoxycarbonyl-substituted sulfonylchloride by a one-pot reaction (**6q**). Condensation of 2-aminothiazoles **8a–c** with the substituted sulfonylchlorides **6a–r**, followed by selective methylation on the thiazolidene ring of compounds **9a–s**, afforded the desired thiazolidenesulfonamide derivatives **1** and **10a–r** (Scheme 2).[7] The demethylation of the methoxy derivatives (**10f–h**, **j**, **k**, **o**, **p**) using BBr_3 provided the corresponding phenol analogues (**11a–g**).



Scheme 1. Reagents and conditions: (a) $NaNO_2$, HCl/AcOH; (b) SO_2, CuCl, $CuCl_2$/AcOH–H_2O; (c) NH_3 aq; (d) PCl_5.

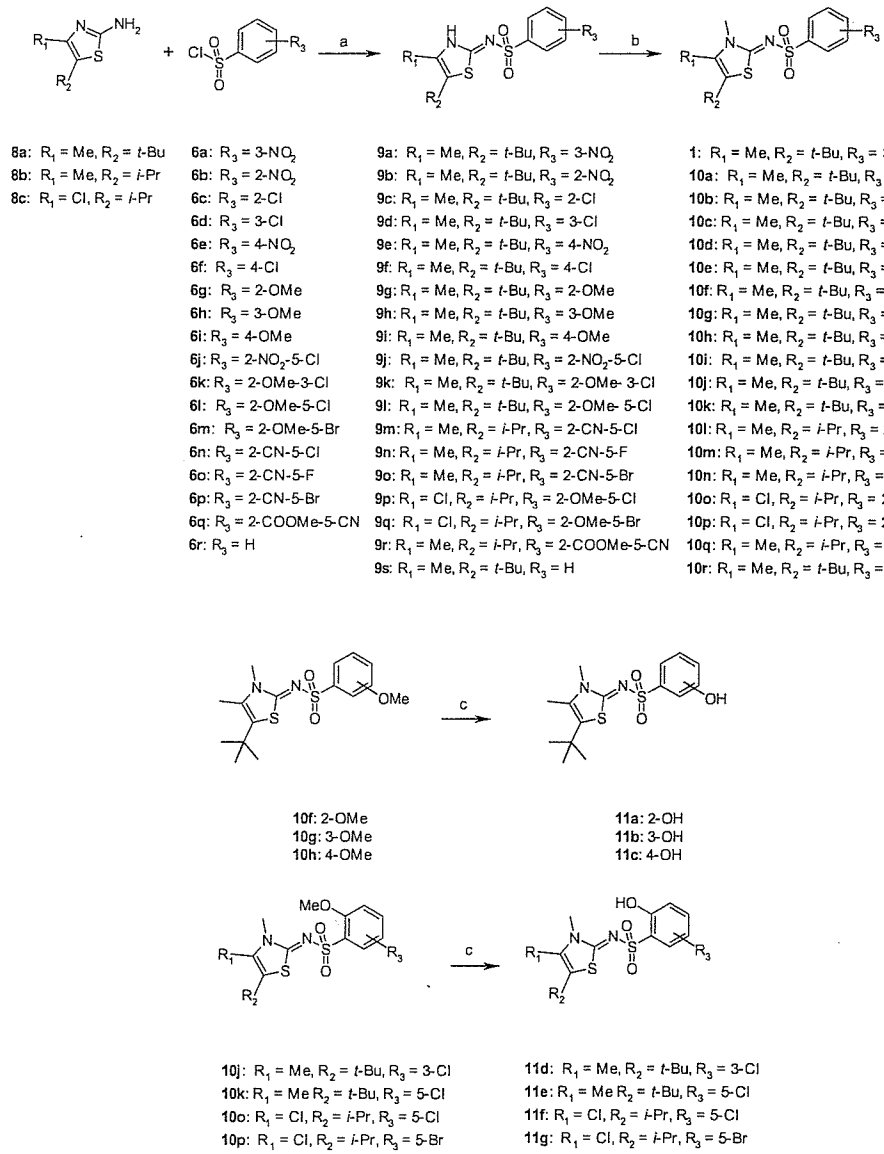
As shown in Scheme 3, the nitro compound **10i** was converted into aniline **12** by catalytic hydrogenation. Aniline **12** was reacted with acetyl chloride or methanesulfonyl chloride to provide acetamide **13** and methanesulfonamide **14**, respectively. The triflates **15a** and **15b** were prepared from the corresponding phenol analogues (**11e,f**). A palladium-catalyzed carbon monoxide insertion with triflate **15a** afforded the methoxycarbonyl derivative **16**.[9] Hydrolysis of the ester derivatives (**16**, **10q**) followed by amidation gave the carbamoyl derivatives (**17a,b**). The cyano derivatives **18a** and **18b** were obtained by dehydration of compounds **17a** and **17b**, respectively.

For the synthesis of 2-cyanobenzenesulfonamide derivatives (**19**, **20**), we have efficiently applied palladium-catalyzed cyanation of the aryl triflates with a combination of $Pd(dba)_2$, dppf, $Zn(CN)_2$ and Zn powder (Scheme 4).[10] Mono- and di-cyano compounds (**19**, **20**) were obtained by controlling the amount of $Zn(CN)_2$. Use of 0.6 mol equiv of $Zn(CN)_2$, which provided 1.2 equiv of cyanide anion, gave the mono-cyano compound **19** through reaction at the triflate group only. With use of 1.6 mol equiv of $Zn(CN)_2$, the major product was the di-cyano compound **20**.

3. Results and discussion

Tables 1–3 summarize the inhibitory activities against the WT, Y181C, and K103N RTs and HIV-1 replication of thiazolidenebenzenesulfonamide derivatives carrying different substituents on the phenyl ring, or at the 4-position on the thiazolidene ring, or both.

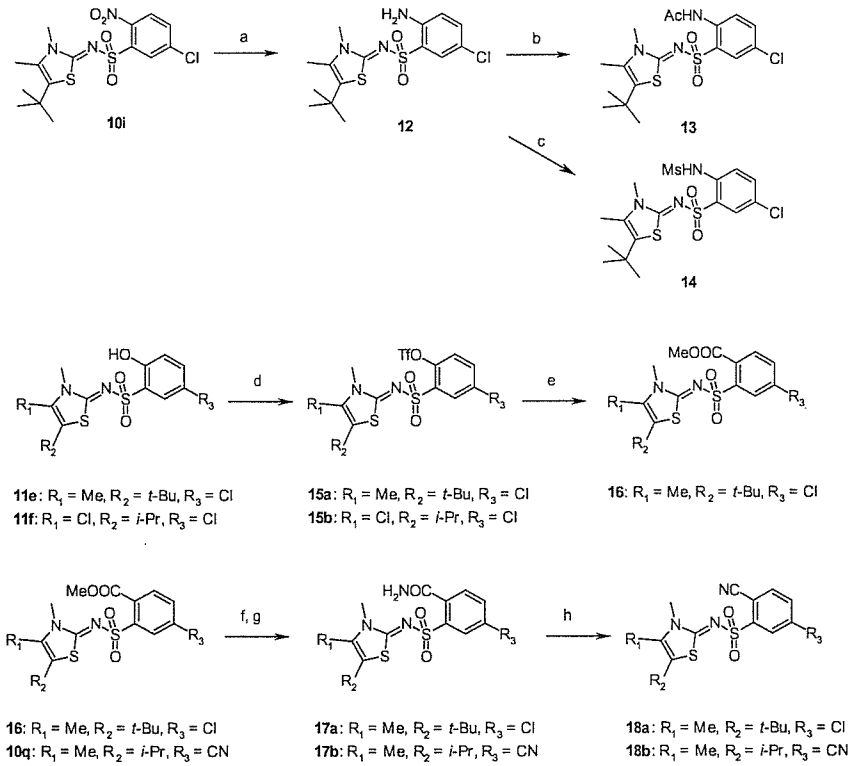
We first investigated the effect on the inhibitory activity of substituents on the benzene ring, as shown in Table 1. The RT inhibitory activity of the substituted benzenesulfonamide analogues varied considerably with different substituents. Substituents at the *meta*-position were favorable for the inhibition of RT and HIV-1 replication, and compounds that had a nitro (**1**) or chloro (**10c**) group were most potent against all RTs. These



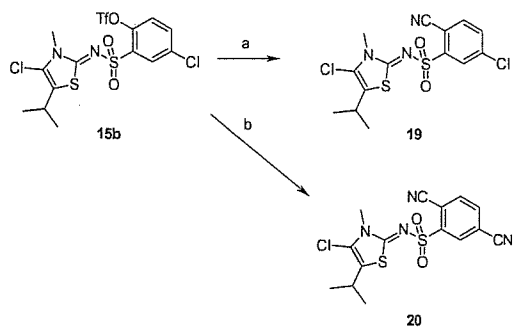
Scheme 2. Reagents and conditions: (a) Py; (b) MeI, NaH/THF; (c) BBr_3/CH_2Cl_2.

nitro and chloro substituents also resulted in more potent anti-HIV-1 activity, and compounds **1** and **10c** showed anti-HIV-1 activity with EC_{50} values of 0.085 and 0.20 μM, respectively. In contrast, *ortho*- and *para*-substituted compounds were essentially inactive against the WT RT, with an exception of the *ortho*-hydroxy compound **11a**, which showed a lower IC_{50} value than that of the unsubstituted compound **10r**. Compound **11a** also exhibited moderate anti-HIV-1 activity (EC_{50} = 2.3 μM). Therefore, we concluded that the substitution of a chloro or nitro group at the *meta*-position or a hydroxy group at the *ortho*-position on the benzene ring was favorable for RT inhibition.

We next focused on combinations of an *ortho*-substituent and a *meta*-chloro group, as shown in Table 2. Although the 2-hydroxy-3-chloro derivative (**11d**) was somewhat less active against the WT RT (IC_{50} = 6.3 μM), substitution at the 2-position on a 5-chlorophenyl ring (**11e**, **12**, **18a**), resulted in an enhancement of activity against the RTs. The introduction of an amino group at the 2-position of the phenyl ring (**12**) resulted in a significant improvement of anti-HIV-1 activity but reduced activity against K103N and Y181C RTs, when compared with **10c**. On the other hand, compound **11e** was about 10-fold more potent against the WT and K103N RTs, and 4-fold more potent against Y181C RT, as compared to compound **10c**. The cyano derivative **18a** possessed the most potent antiviral activity (EC_{50} = 0.0083 μM) with a therapeutic index (TI) of >960, but it showed no inhibition of K103N RT. Although the amino and cyano compounds (**12**, **18a**) showed less potent activity against WT RT than the hydroxy compound **11e**, these compounds possessed more potent anti-HIV-1 activity than **11e**. We cannot explain the exact reason for this phenomenon. One possibility,



Scheme 3. Reagents and conditions: (a) H_2, Pd–C/EtOH–THF; (b) AcCl, DMAP/Py; (c) MsCl, Et_3N/THF; (d) Tf_2O, 2,6-lutidine/CH_2Cl_2; (e) CO, MeOH, $Pd(OAc)_2$, dppp, Et_3N/DMF; (f) NaOH aq, THF/MeOH, (g) NH_4Cl, WSC·HCl, *i*-Pr_2NEt, HOBt/DMF; (h) $POCl_3$/DMF.



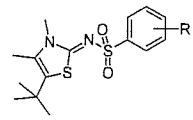
Scheme 4. Reagents and conditions: (a) $Zn(CN)_2$ (0.6 equiv), Zn, $Pd(dba)_2$, dppf/DMA; (b) $Zn(CN)_2$ (1.6 equiv), Zn, $Pd(dba)_2$, dppf/DMA.

however, is that the increase in lipophilicity caused by the substitution of the hydroxy group to the amino or cyano group potentiated their cell membrane permeability, which resulted in the increase of anti-HIV-1 activity. We also have to consider other possibilities, such as that the introduction of these groups allow compound stability to be maintained under the assay conditions, or that they acquire the other anti-viral mechanism (inhibition of HIV-protease, integrase, RNaseH, or virus adsorption).

On the other hand, replacement of the cyano group with other electron-withdrawing groups, such as nitro (**10i**), methoxycarbonyl (**16**) and carbamoyl (**17a**), led to loss of RT inhibition. Substitution of the cyano group with an acetamide or methanesulfonamide group (**13**, **14**), which are known to be bioisosteres of the phenolic hydroxy group, was also detrimental to inhibition with all RTs. Thus, concerning the 5-chlorophenyl derivatives, the introduction of a hydroxy, amino, or cyano group at the 2-position markedly enhanced the inhibition of HIV-1 replication.

We previously reported that compounds with 5-isopropyl-4-methyl- and 4-chloro-5-isopropyl-substituted thiazolidene moieties had increased activity against the WT and Y181C RTs.[7] On the basis of the SARs described in Table 2, we synthesized new compounds with a combination of 2-cyanophenyl or 2-hydroxyphenyl moiety and 5-isopropyl-4-methyl or 4-chloro-5-isopropyl thiazolidene moiety (**10l–n**, **11f,g**, **18b**, **19**, **20**; Table 3). Among these, compound **11f**, having both 2-hydroxy-5-chlorophenyl and 4-chlorothiazolidene moieties, was a more potent inhibitor of all the RT enzymes, compared to compound **11e**. In addition, compound **11g** (YM-215389), which has 5-bromophenyl ring, showed significantly more potent activity against all the RTs, compared to compound **11f**. Compound **11g** also exhibited strong anti-HIV-1 activity, with an EC_{50} value of 0.037 μM, and the TI value of **11g** exceeded 680. With the exception of compound **10m**, the 2-cyanophenyl derivatives (**10l**, **10n**, and **18b**), which all have 5-isopropyl-4-methylthiazolidene moieties, exhibited extremely potent anti-HIV-1 activity (EC_{50} = 0.0017–0.0021 μM), with TIs ranging from 6100 to >15,000. Interestingly,

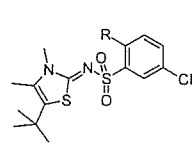
Table 1. In vitro activities of mono-substituted benzenesulfonamide derivatives

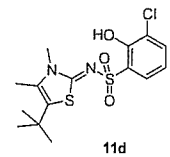


Compounds	R	IC_{50}[a] (μM)			EC_{50}[b] (μM)	CC_{50}[c] (μM)	TI[d]
		WT	K103N	Y181C			
1	3-NO_2	0.27	13	0.066	0.085	>25	>290
10a	2-NO_2	>50	>50	36	>25	>25	—
10b	2-Cl	>10	>10	>10	NT[e]	NT[e]	—
10c	3-Cl	0.30	11	0.044	0.20	>25	>125
10d	4-NO_2	>10	>10	>10	NT[e]	NT[e]	—
10e	4-Cl	>10	>10	>10	NT[e]	NT[e]	—
10f	2-OMe	50	>50	NT[e]	10	>25	>3
10g	3-OMe	8.8	>50	NT[e]	11	>25	>2
10h	4-OMe	>10	>10	>10	>25	>25	—
10r	H	4.9	>50	5.1	>25	>25	—
11a	2-OH	1.6	30	0.41	2.3	>25	>11
11b	3-OH	8.8	>50	14	>25	>25	—
11c	4-OH	>10	>10	>10	>25	>25	—

[a] Compound concentration required to achieve 50% inhibition of recombinant HIV-1 RT activities.
[b] Compound concentration required to achieve 50% protection of MT-4 cells from HIV-1 induced CPE, as determined by the MTT method.
[c] Compound concentration required to reduce the viability of mock-infected MT-4 cells, as determined by the MTT method.
[d] Therapeutic index (CC_{50}/EC_{50}).
[e] NT: not tested.

Table 2. In vitro activities of 2-substituted 5-chlorobenzenesulfonamide derivatives





11d

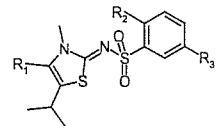
Compounds	R	IC_{50}[a] (μM)			EC_{50}[b] (μM)	CC_{50}[c] (μM)	TI[d]
		WT	K103N	Y181C			
10c	H	0.30	11	0.044	0.20	>25	>125
10i	NO_2	2.4	>50	NT[e]	0.36	22	61
11d		6.3	>50	NT[e]	11	>25	>2
11e	OH	0.032	1.1	0.011	0.026	>25	>960
12	NH_2	0.19	17	0.11	0.025	>25	>1000
13	NHCOMe	>50	>50	>50	2.7	>25	>9
14	$NHSO_2$ Me	>50	>50	>50	>25	>25	—
16	COOMe	>10	>10	10	>25	>25	—
17a	$CONH_2$	>10	>10	>10	NT[e]	NT[e]	—
18a	CN	0.18	>50	0.069	0.0083	8	>960

[a] Compound concentration required to achieve 50% inhibition of recombinant HIV-1 RT activities.
[b] Compound concentration required to achieve 50% protection of MT-4 cells from HIV-1 induced CPE, as determined by the MTT method.
[c] Compound concentration required to reduce the viability of mock-infected MT-4 cells, as determined by the MTT method.
[d] Therapeutic index (CC_{50}/EC_{50}).
[e] NT: not tested.

the 2-cyanophenyl and 5-isopropyl-4-methylthiazolidene derivatives, **10l** and **18b** (YM-228855), exhibited strong anti-HIV-1 activity, with EC_{50} values of 0.0017 and 0.0018 μM, respectively, both of which were more potent than that of efavirenz (EC_{50} = 0.0027 μM). The replacement of the 5-chloro or 5-cyano group on the phenyl ring with a 5-bromo group (**10n**) was tolerable for anti-HIV-1 activity, but this derivative was found to be a modest inhibitor of K103N RT.

We also investigated the substitution of the methyl group at the 4-position of **10l** with a chloro group (**19**, **20**), anticipating further increase in RT inhibitory activity and anti-HIV-1 activity. However, this attempt gave slightly less potent compounds than their methyl counterparts. Among the compounds shown in Table 3, compound **11g** was the most potent inhibitor against the WT, Y181C, and K103N RTs, with IC_{50} values of 0.0043, 0.043, and 0.013 μM, respectively, and

Table 3. In vitro activities of 5-isopropylthiazolidenesulfonamide derivatives



Compounds	R_1	R_2	R_3	IC_{50}[a] (μM)			EC_{50}[b] (μM)	CC_{50}[c] (μM)	TI[d]
				WT	K103N	Y181C			
10l	Me	CN	Cl	0.011	5.9	0.20	0.0017	>25	>15000
10m	Me	CN	F	0.092	>10	2.1	0.0077	>25	>3200
10n	Me	CN	Br	0.018	3.9	0.26	0.0021	>25	>12,000
11f	Cl	OH	Cl	0.0094	0.17	0.021	0.027	>25	>930
11g (YM-215389)	Cl	OH	Br	0.0043	0.043	0.013	0.037	>25	>680
18b (YM-228855)	Me	CN	CN	0.012	4.1	0.47	0.0018	11	6100
19	Cl	CN	Cl	0.0090	3.7	0.091	0.0047	3.8	810
20	Cl	CN	CN	0.0095	3.0	0.21	0.0036	22	6100
1				0.27	13	0.066	0.085	>25	>290
2				0.077	6.9	0.13	0.048	24	500
Efavirenz (3)				0.0069	0.021	0.0040	0.0027	8.5	3200

[a] Compound concentration required to achieve 50% inhibition of recombinant HIV-1 RT activities.
[b] Compound concentration required to achieve 50% protection of MT-4 cells from HIV-1 induced CPE, as determined by the MTT method.
[c] Compound concentration required to reduce the viability of mock-infected MT-4 cells, as determined by the MTT method.
[d] Therapeutic index (CC_{50}/EC_{50}).

accompanying potent anti-HIV-1 activity (EC_{50}: 0.037 μM). Consequently, the discovery of an effective compound against the WT, K103N, and Y181C mutant RTs as well as HIV-1 replication has been made by the exploration of the optimum combination of substituents on both the thiazole and phenyl rings. This compound is referred to as YM-215389. Further improvement of anti-HIV-1 properties in this series of compounds and their potential use as anti-HIV-1 agents will be reported in due course.

4. Conclusion

In this paper, the synthesis and SARs of thiazolidenebenzenesulfonamide derivatives have been described. An interesting aspect of this study is that both potency and spectrum of thiazolidenebenzenesulfonamides varied, depending on the number and position of the substituents on the phenyl ring. It was found that the combination of a hydroxy or cyano group at the 2-position on the phenyl ring with a 5-isopropylthiazolidene ring improved the inhibitory activities against RT enzymes and HIV-1 replication. The cyano derivatives (**10l** and **18b**) showed extremely potent anti-HIV-1 activity, with EC_{50} values of 0.0017 and 0.0018 μM, respectively. These values were significantly better than that of efavirenz (**3**). However, the activity of the cyano derivatives against the K103N mutant RT was insufficient. Compound **11g** (YM-215389) possessed the most potent activity against the WT, K103N, and Y181C RTs, with IC_{50} values of 0.0043, 0.043, and 0.013 μM, respectively. This compound also strongly inhibited HIV-1 replication in cell cultures (EC_{50} = 0.037 μM). Because of their excellent potency, these thiazolidenebenzenesulfonamide derivatives may have potential and should be further pursued as next-generation NNRTIs.

5. Experimental

5.1. Chemistry

Melting points were determined on a Yanaco micro-melting apparatus or Büchi B-545 melting point apparatus and are uncorrected. Proton magnetic resonance (^{1}H NMR) spectra were obtained in $CDCl_3$ or dimethylsulfoxide-d_6 (DMSO-d_6) using a JEOL JNM-EX90, JNM-EX400, JNM-GX500, or JNM-A500 spectrometer. Chemical shifts were expressed in δ (ppm) values with tetramethylsilane as an internal standard (in NMR description, s: singlet, d: doublet, t: triplet, m: multiplet, br: broad peak). Mass spectra (MS) were recorded on a JEOL JMS-DX300 or a HITACHI M-80 mass spectrometer. Elemental analysis was carried out on Yanaco MT-3 or MT-5 CHN analyzer and a Yokogawa IC7000S Ion Chromatoanalyzer. Chromatographic separations were performed using a silica gel column (Merck Kieselgel 60). Analytical thin-layer chromatography (TLC) was carried out on precoated glass plates (Merck Kieselgel 60F254).

The following known materials were prepared as described in the literature: (**6j**)[11] or obtained from commercial suppliers (**6a–i**, **6r**). And the preparation of **1**, **8a–c**, **9a** was described in our previous report.[7]

5.1.1. 6-Chloro-1,2-benzisothiazol-3(2*H*)-one-1,1-dioxide (5a). To solution of **4a** (7.42 g, 40 mmol) in acetic acid (45 mL) and concentrated hydrochloric acid (90 mL) was added sodium nitrite (2.90 g, 42 mmol) in water (12 mL) at −5 °C and the solution was stirred at −5 °C for 1 h. To a mixture of copper(II) chloride (5.38 g, 40 mmol) and copper(I) chloride (3.96 g, 40 mmol) in acetic acid (120 mL) and concentrated hydrochloric acid (15 mL), SO_2 gas was bubbled at −5 °C. The suspension of prepared diazonium salt was

added dropwise to the mixture at −10 °C and stirred at room temperature for 3 h. The reaction mixture was poured into water and 28% aqueous ammonia solution (500 mL) was added under ice-bath cooling. The resulting mixture was extracted with chloroform and washed with saturated aqueous sodium hydrogen carbonate solution. The organic layer was dried over anhydrous sodium sulfate and solvent was removed under reduced pressure to give **5a** (3.90 g, 45%) as a colorless powder. ^{1}H NMR (DMSO-d_6) δ: 7.97 (3H, m, benzene), 8.43 (1H, br s, NH); FAB-MS *m/z*: 218 (M^++1).

The following compounds were obtained in the same manner.

5.1.2. 6-Fluoro-1,2-benzisothiazol-3(2*H*)-one-1,1-dioxide (5b). 31% yield; ^{1}H NMR (DMSO-d_6) δ: 7.57 (1H, m, benzene), 7.82 (2H, m, benzene), 7.90 (1H, m, NH); FAB-MS *m/z*: 200 (M^-−1).

5.1.3. 6-Bromo-1,2-benzisothiazol-3(2*H*)-one-1,1-dioxide (5c). 56% yield; ^{1}H NMR (DMSO-d_6) δ: 7.80 (1H, br s, NH), 7.87 (1H, d, J = 8.3 Hz, benzene), 8.09 (1H, dd, J = 1.5, 8.3 Hz, benzene), 8.51 (1H, d, J = 1.5 Hz, benzene); FAB-MS *m/z*: 263 (M^++1).

5.1.4. 5-Chloro-2-cyanobenzenesulfonyl chloride (6n). A mixture of **5a** (3.90 g, 18.0 mmol) and phosphorus pentachloride (22.4 g, 90.0 mmol) was heated to 120 °C and stirred for 7 h. The reaction mixture was poured into ice-water. The resulting mixture was extracted with ethyl acetate and washed with saturated aqueous sodium hydrogen carbonate solution. The organic layer was dried over anhydrous sodium sulfate and solvent was removed under reduced pressure to give **6n** (2.89 g, 68%) as a pale yellow powder. This crude product was used for next step without further purification.

The following compounds were obtained in the same manner.

5.1.5. 5-Fluoro-2-cyanobenzenesulfonyl chloride (6o). 54% yield; ^{1}H NMR (DMSO-d_6) δ: 7.40 (1H, dt, J = 3.6, 11.2 Hz, benzene), 7.59 (1H, dd, J = 3.6, 12.0 Hz, benzene), 7.92 (1H, dd, J = 6.8, 11.2 Hz, benzene); EI-MS *m/z*: 219 (M^+).

5.1.6. 5-Bromo-2-cyanobenzenesulfonyl chloride (6p). Without isolation.

5.1.7. Methyl 2-chlorosulfonyl-4-cyanobenzoate (6q). Sodium nitrite (5.10 g, 73.5 mmol) in water (25 mL) was added to solution of **4d** (12.3 g, 70 mmol) in concentrated hydrochloric acid (120 mL) at −5 °C and the solution was stirred at −5 °C for 1.5 h. To a mixture of copper(II) chloride dihydrate (2.60 g, 15.0 mmol) in acetic acid (200 mL), SO_2 gas was bubbled at −5 °C. The suspension of prepared diazonium salt was added dropwise to the mixture at −10 °C and stirred at room temperature for 3 h. The reaction mixture was poured into water. The resulting precipitate was collected by filtration and washed with water. The precipitate was dried under reduced pressure to give **6q** (19.0 g, quantitative) as a colorless powder. ^{1}H NMR (DMSO-d_6) δ: 3.75 (3H, s, CH_3 of COOMe), 7.52 (1H, d, J = 8.1 Hz, benzene), 7.87 (1H, dd, J = 1.5, 8.1 Hz, benzene), 8.03 (1H, d, J = 1.5 Hz, benzene); EI-MS *m/z*: 259 (M^+).

5.1.8. *N*-(5-*tert*-Butyl-4-methyl-1,3-thiazol-2-yl)-2-nitrobenzenesulfonamide (9b). A solution of **8a**. hydrochloride (8.00 g, 38.7 mmol) in pyridine (100 mL) was added **6b** (10.3 g, 46.4 mmol) and the solution was stirred at room temperature for 12 h. The reaction mixture was poured into water. The resulting mixture was extracted with ethyl acetate and washed with saturated aqueous sodium hydrogen carbonate solution, 1 M hydrochloric acid and brine. The organic layer was dried over anhydrous sodium sulfate and solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography (ethyl acetate–hexane) to give **9b** (10.89 g, 79%) as an orange solid. ^{1}H NMR (DMSO-d_6) δ: 1.30 (9H, s, *t*-Bu), 2.18 (3H, s, 4-Me), 7.80 (2H, m, benzene), 7.87 (1H, m, benzene), 8.03 (1H, m, benzene), 12.65 (1H, br s, NH); FAB-MS *m/z*: 356 (M^++1).

The following compounds were obtained in the same manner.

5.1.9. *N*-(5-*tert*-Butyl-4-methyl-1,3-thiazol-2-yl)-2-chlorobenzenesulfonamide (9c). 34% yield; ^{1}H NMR (DMSO-d_6) δ: 1.30 (9H, s, CH_3 of *t*-Bu), 2.16 (3H, s, 4-Me), 8.03 (2H, d, J = 8.8 Hz, benzene), 8.36 (2H, d, J = 8.8 Hz, benzene), 12.58 (1H, br s, NH); FAB-MS *m/z*: 345 (M^++1).

5.1.10. *N*-(5-*tert*-Butyl-4-methyl-1,3-thiazol-2-yl)-3-chlorobenzenesulfonamide (9d). 99% yield; ^{1}H NMR (DMSO-d_6) δ: 1.29 (9H, s, CH_3 of *t*-Bu), 2.16 (3H, s, 4-Me), 7.58 (1H, t, J = 7.8 Hz, benzene), 7.67 (1H, br d, J = 7.8 Hz, benzene), 7.74 (1H, br s, benzene), 7.75 (1H, br d, J = 7.8 Hz, benzene), 12.46 (1H, br s, NH); FAB-MS *m/z*: 345 (M^++1).

5.1.11. *N*-(5-*tert*-Butyl-4-methyl-1,3-thiazol-2-yl)-4-nitrobenzenesulfonamide (9e). 85% yield; ^{1}H NMR (DMSO-d_6) δ: 1.30 (9H, s, CH_3 of *t*-Bu), 2.16 (3H, s, 4-Me), 8.03 (2H, d, J = 8.8 Hz, benzene), 8.36 (2H, d, J = 8.8 Hz, benzene), 12.58 (1H, br s, NH); FAB-MS *m/z*: 356 (M^++1).

5.1.12. *N*-(5-*tert*-Butyl-4-methyl-1,3-thiazol-2-yl)-4-chlorobenzenesulfonamide (9f). 78% yield; ^{1}H NMR (DMSO-d_6) δ: 1.29 (9H, s, CH_3 of *t*-Bu), 2.15 (3H, s, 4-Me), 7.60 (2H, d, J = 7.5 Hz, benzene), 7.78 (2H, d, J = 7.5 Hz, benzene), 12.42 (1H, br s, NH); FAB-MS *m/z*: 345 (M^++1).

5.1.13. *N*-(5-*tert*-Butyl-4-methyl-1,3-thiazol-2-yl)-2-methoxybenzenesulfonamide (9g). 50% yield; ^{1}H NMR (DMSO-d_6) δ: 1.31 (9H, s, CH_3 of *t*-Bu), 2.15 (3H, s, 4-Me), 3.73 (3H, s, MeO), 7.03 (1H, t, J = 7.5 Hz, benzene), 7.13 (1H, d, J = 7.5 Hz, benzene), 7.50 (2H, m, benzene), 12.14 (1H, br s, NH); FAB-MS *m/z*: 341 (M^++1).

5.1.14. *N*-(5-*tert*-Butyl-4-methyl-1,3-thiazol-2-yl)-3-methoxybenzenesulfonamide (9h). 61% yield; ^{1}H NMR (DMSO-d_6) δ: 1.29 (9H, s, CH_3 of *t*-Bu), 1.98 (3H, s, 4-Me), 3.80 (3H, s, MeO), 7.14 (1H, dd, J = 2.5, 8.3 Hz, benzene), 7.26 (1H, t, J = 2.5 Hz, benzene), 7.36 (1H, br d, J = 7.8 Hz, benzene), 7.45 (1H, t, J = 7.8 Hz, benzene), 12.34 (1H, br s, benzene); FAB-MS *m/z*: 341 (M^++1).

5.1.15. *N*-(5-*tert*-Butyl-4-methyl-1,3-thiazol-2-yl)-4-methoxybenzenesulfonamide (9i). Without isolation.

5.1.16. *N*-(5-*tert*-Butyl-4-methyl-1,3-thiazol-2-yl)-5-chloro-2-nitrobenzenesulfonamide (9j). 41% yield; ^{1}H NMR (DMSO-d_6) δ: 1.30 (9H, s, CH_3 of *t*-Bu), 2.20 (3H, s, 4-Me), 7.91 (1H, dd, J = 2.0, 8.3 Hz, benzene), 7.98 (1H, d, J = 2.0 Hz, benzene), 7.99 (1H, d, J = 8.8 Hz, benzene), 12.76 (1H, br s, NH); FAB-MS *m/z*: 390 (M^++1).

5.1.17. *N*-(5-*tert*-Butyl-4-methyl-1,3-thiazol-2-yl)-3-chloro-2-methoxybenzenesulfonamide (9k). Without isolation.

5.1.18. *N*-(5-*tert*-Butyl-4-methyl-1,3-thiazol-2-yl)-5-chloro-2-methoxybenzenesulfonamide (9l). 94% yield; ^{1}H NMR (DMSO-d_6) δ: 1.32 (9H, s, CH_3 of *t*-Bu), 2.17 (3H, s, 4-Me), 3.74 (3H, s, MeO), 7.18 (1H, d, J = 8.8 Hz, benzene), 7.59 (1H, dd, J = 2.9, 8.8 Hz, benzene), 7.73 (1H, d, J = 2.9 Hz, benzene), 12.30 (1H, br s, NH); FAB-MS *m/z*: 375 (M^++1).

5.1.19. 5-Chloro-2-cyano-*N*-(5-isopropyl-4-methyl-1,3-thiazol-2-yl)benzenesulfonamide (9m). 13% yield. ^{1}H NMR (DMSO-d_6) δ: 1.14 (6H, d, J = 6.8 Hz, CH_3 of *i*-Pr), 2.06 (3H, s, 4-Me), 3.11 (1H, heptet, J = 6.8 Hz, CH of *i*-Pr), 7.88 (1H, dd, J = 2.5, 8.3 Hz, benzene), 8.00 (1H, d, J = 2.5 Hz, benzene), 8.09 (1H, d, J = 8.3 Hz, benzene), 12.83 (1H, br s, NH); FAB-MS *m/z*: 356 (M^++1).

5.1.20. 5-Fluoro-2-cyano-*N*-(5-isopropyl-4-methyl-1,3-thiazol-2-yl)benzenesulfonamide (9n). 20% yield; ^{1}H NMR (DMSO-d_6) δ: 1.14 (6H, d, J = 6.9 Hz, CH_3 of *i*-Pr), 2.06 (3H, s, 4-Me), 3.11 (1H, heptet, J = 6.9 Hz, CH of *i*-Pr), 7.67 (1H, dt, J = 1.9, 8.8 Hz, benzene), 7.82 (1H, dd, J = 1.9, 8.8 Hz, benzene), 8.16 (1H, dd, J = 5.4, 8.8 Hz, benzene), 12.82 (1H, br s, NH); FAB-MS *m/z*: 340 (M^++1).

5.1.21. 5-Bromo-2-cyano-*N*-(5-isopropyl-4-methyl-1,3-thiazol-2-yl)benzenesulfonamide (9o). 27% yield from **8b**; ^{1}H NMR (DMSO-d_6) δ: 1.14 (6H, d, J = 6.8 Hz, CH_3 of *i*-Pr), 2.06 (3H, s, 4-Me), 3.11 (1H, heptet, J = 6.8 Hz, CH of *i*-Pr), 8.01 (2H, m, benzene), 8.13 (1H, d, J = 1.4 Hz, benzene), 12.84 (1H, br s, NH).; FAB-MS *m/z*: 400 (M^++1).

5.1.22. *N*-(4-Chloro-5-isopropyl-1,3-thiazol-2-yl)-5-chloro-2-methoxybenzenesulfonamide (9p). 22% yield; ^{1}H NMR ($CDCl_3$) δ: 1.25 (6H, d, J = 7.0 Hz, CH_3 of *i*-Pr), 2.67 (1H, br s, NH), 3.16 (1H, heptet, J = 7.0 Hz, CH of *i*-Pr), 3.87 (3H, s, MeO), 6.94 (1H, d, J = 8.8 Hz, benzene), 7.46 (1H, dd, J = 2.4, 8.8 Hz, benzene), 7.94 (1H, d, J = 2.4 Hz, benzene); FAB-MS *m/z*: 381 (M^++1).

5.1.23. *N*-(4-Chloro-5-isopropyl-1,3-thiazol-2-yl)-5-bromo-2-methoxybenzenesulfonamide (9q). 59% yield;. ^{1}H NMR (DMSO-d_6) δ: 1.20 (6H, d, J = 6.8 Hz, CH_3 of *i*-Pr), 3.12 (1H, heptet, J = 6.8 Hz, CH of *i*-Pr), 3.76 (3H, s, MeO), 7.14 (1H, d, J = 9.0 Hz, benzene), 7.79 (1H, dd, J = 2.6, 9.0 Hz, benzene), 7.87 (1H, d, J = 2.6 Hz, benzene); FAB-MS *m/z*: 427 (M^++1).

5.1.24. Methyl 4-cyano-2-{[(5-isopropyl-4-methyl-1,3-thiazol-2-yl)amino]sulfonyl}benzoate (9r). 68% yield; ^{1}H NMR (DMSO-d_6) δ: 1.15 (6H, d, J = 6.9 Hz, CH_3 of *i*-Pr), 2.07 (3H, s, 4-Me), 3.11 (1H, heptet, J = 6.9 Hz, CH of *i*-Pr), 3.80 (3H, s, CH_3 of COOMe), 7.78 (1H, d, J = 7.8 Hz, benzene), 8.14 (1H, dd, J = 1.4, 7.8 Hz, benzene), 8.28 (1H, d, J = 1.4 Hz, benzene), 12.59 (1H, br s, NH); FAB-MS *m/z*: 380 (M^++1).

5.1.25. *N*-(5-*tert*-Butyl-4-methyl-1,3-thiazol-2-yl)benzenesulfonamide (9s). 61% yield; ^{1}H NMR (DMSO-d_6) δ: 1.28 (9H, s, CH_3 of *t*-Bu), 2.14 (3H, s, 4-Me), 7.54 (3H, m, benzene), 7.78 (2H, m, benzene), 12.32 (1H, br s, NH); FAB-MS *m/z*: 311 (M^++1).

5.1.26. *N*-(5-*tert*-Butyl-3,4-dimethyl-1,3-thiazol-2(3*H*)-ylidene)-2-nitrobenzenesulfonamide (10a). To a solution of **9b** (10.89 g, 30.6 mmol) in tetrahydrofuran (100 mL) was added sodium hydride (60% dispersion in mineral oil: 1.47 g, 36.8 mmol) and iodomethane (5.7 mL, 91.8 mmol) under ice-bath cooling. The solution was warmed to room temperature and stirred for 12 h. The reaction mixture was poured into ice-water and extracted with ethyl acetate. The organic layer was washed with brine, and then dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure and the residue was purified by silica gel column chromatography (chloroform) and recrystalized from methanol to give **10a** (7.01 g, 62%) as a yellow powder. Mp 138–139 °C. ^{1}H NMR ($CDCl_3$) δ: 1.36 (9H, s, CH_3 of *t*-Bu), 2.28 (3H, s, 4-Me), 3.45 (3H, s, 3-Me), 7.61 (3H, m, benzene), 8.25 (1H, m, benzene); FAB-MS *m/z*: 370 (M^++1). Anal. Calcd for $C_{15}H_{19}N_3O_4S_2$: C, 48.76; H, 5.18; N, 11.37; S, 17.36. Found: C, 48.76; H, 4.99; N, 11.38; S, 17.69.

The following compounds were obtained in the same manner.

5.1.27. *N*-(5-*tert*-Butyl-3,4-dimethyl-1,3-thiazol-2(3*H*)-ylidene)-2-chlorobenzenesulfonamide (10b). 86% yield; mp 153–155 °C (ethyl acetate–benzene).^{1}H NMR ($CDCl_3$) δ: 1.33 (9H, s, CH_3 of *t*-Bu), 2.27 (3H, s, 4-Me), 3.47 (3H, s, 3-Me), 7.46 (3H, m, benzene), 8.23 (1H, m, benzene); FAB-MS *m/z*: 359 (M^++1). Anal. Calcd for $C_{15}H_{19}ClN_2O_2S_2$: C, 50.20; H, 5.34; N, 7.81; S, 17.87; Cl, 9.88. Found: C, 50.15; H, 5.17; N, 7.82; S, 17.80; Cl, 9.75.

5.1.28. *N*-(5-*tert*-Butyl-3,4-dimethyl-1,3-thiazol-2(3*H*)-ylidene)-3-chlorobenzenesulfonamide (10c). 90% yield;

mp 138–139 °C (chloroform). ^{1}H NMR (DMSO-d_6) δ: 1.31 (9H, s, CH_3 of *t*-Bu), 2.28 (3H, s, 4-Me), 3.42 (3H, s, 3-Me), 7.57 (1H, dd, J = 7.8, 8.3 Hz, benzene), 7.66 (1H, ddd, J = 1.0, 2.0, 8.3 Hz, benzene), 7.79 (1H, br s, benzene), 7.80 (1H, br d, J = 7.8 Hz, benzene); FAB-MS *m/z*: 359 (M^++1). Anal. Calcd for $C_{15}H_{19}ClN_2O_2S_2Cl$: C, 50.20; H, 5.34; N, 7.81; S, 17.87; Cl, 9.88. Found: C, 50.01; H, 5.26; N, 7.76; S, 18.00; Cl, 10.04.

5.1.29. *N*-(5-*tert*-Butyl-3,4-dimethyl-1,3-thiazol-2(3*H*)-ylidene)-4-nitrobenzenesulfonamide (10d). 50% yield; mp 184–185 °C (ethyl acetate–hexane). ^{1}H NMR ($CDCl_3$) δ: 1.37 (9H, s, CH_3 of *t*-Bu), 2.27 (3H, s, 4-Me), 3.46 (3H, s, 3-Me), 8.15 (2H, dt, J = 2.4, 8.8 Hz, benzene), 8.29 (2H, dt, J = 2.4, 8.8 Hz, benzene); FAB-MS *m/z*: 370 (M^++1). Anal. Calcd for $C_{15}H_{19}N_3O_4S_2$: C, 48.76; H, 5.18; N, 11.37; S, 17.36. Found: C, 48.70; H, 4.98; N, 11.51; S, 17.43.

5.1.30. *N*-(5-*tert*-Butyl-3,4-dimethyl-1,3-thiazol-2(3*H*)-ylidene)-4-chlorobenzenesulfonamide (10e). 50% yield; mp 157–158 °C (ethyl acetate–hexane). ^{1}H NMR ($CDCl_3$) δ: 1.36 (9H, s, CH_3 of *t*-Bu), 2.26 (3H, s, 4-Me), 3.43 (3H, s, 3-Me), 7.41 (2H, dt, J = 2.4, 8.8 Hz, benzene), 7.91 (2H, dt, J = 2.4, 8.8 Hz, benzene); FAB-MS *m/z*: 359 (M^++1). Anal. Calcd for $C_{15}H_{19}ClN_2O_2S_2$: C, 50.20; H, 5.34; N, 7.81; S, 17.87; Cl, 9.88. Found: C, 50.01; H, 5.10; N, 7.87; S, 17.86; Cl, 9.89.

5.1.31. *N*-(5-*tert*-Butyl-3,4-dimethyl-1,3-thiazol-2(3*H*)-ylidene)-2-methoxybenzenesulfonamide (10f). 20% yield; mp 197–198 °C (diethyl ether–hexane). ^{1}H NMR (DMSO-d_6) δ: 1.33 (9H, s, CH_3 of *t*-Bu), 2.28 (3H, s, 4-Me), 3.37 (3H, s, 3-Me), 3.73 (3H, s, MeO), 7.03 (1H, t, J = 7.9 Hz, benzene), 7.14 (1H, d, J = 8.3 Hz, benzene), 7.52 (1H, dt, J = 1.5, 7.9 Hz, benzene), 7.79 (1H, dd, J = 1.5 Hz, benzene); FAB-MS *m/z*: 355 (M^++1). Anal. Calcd for $C_{16}H_{22}N_2O_3S_2 \cdot 0.1CHCl_3$: C, 52.77; H, 6.08; N, 7.65; S, 17.50; Cl, 2.90. Found: C, 52.84; H, 5.98; N, 7.59; S, 17.56; Cl, 2.50.

5.1.32. *N*-(5-*tert*-Butyl-3,4-dimethyl-1,3-thiazol-2(3*H*)-ylidene)-3-methoxybenzenesulfonamide (10g). 76% yield; mp 192–193 °C (diethyl ether). ^{1}H NMR (DMSO-d_6) δ 1.31 (9H, s, CH_3 of *t*-Bu), 2.27 (3H, s, 4-Me), 3.40 (3H, s, 3-Me), 3.81 (3H, s, MeO), 7.14 (1H, ddd, J = 1.0, 2.5, 7.8 Hz, benzene), 7.29 (1H, t, J = 2.5 Hz, benzene), 7.39 (1H, br d, J = 7.8 Hz, benzene), 7.44 (1H, t, J = 7.8 Hz, benzene); FAB-MS *m/z*: 355 (M^++1). Anal. Calcd for $C_{16}H_{22}N_2O_3S_2$: C, 54.21; H, 6.26; N, 7.90; S, 18.09. Found: C, 54.31; H, 6.25; N, 7.86; S, 18.17.

5.1.33. *N*-(5-*tert*-Butyl-3,4-dimethyl-1,3-thiazol-2(3*H*)-ylidene)-4-methoxybenzenesulfonamide (10h). 81% yield from **8a**; mp 181–183 °C (ethyl acetate–hexane). ^{1}H NMR ($CDCl_3$) δ: 1.34 (9H, s, CH_3 of *t*-Bu), 2.24 (3H, s, 4-Me), 3.42 (3H, s, 3-Me), 3.87 (3H, s, MeO), 6.92 (2H, d, J = 8.6 Hz, benzene), 7.91 (2H, d, J = 8.6 Hz, benzene); FAB-MS *m/z*: 355 (M^++1). Anal. Calcd for $C_{16}H_{22}N_2O_3S_2$: C, 54.21; H, 6.26; N, 7.90; S, 18.09. Found: C, 54.23; H, 6.18; N, 7.89; S, 17.98.

5.1.34. *N*-(5-*tert*-Butyl-3,4-dimethyl-1,3-thiazol-2(3*H*)-ylidene)-5-chloro-2-nitrobenzenesulfonamide (10i). 49% yield; mp 204–205 °C (acetonitrile). ^{1}H NMR (DMSO-d_6) δ: 1.33 (9H, s, CH_3 of *t*-Bu), 2.31 (3H, s, 4-Me), 3.45 (3H, s, 3-Me), 7.91 (1H, dd, J = 1.9, 8.3 Hz, benzene), 7.98 (1H, d, J = 8.3 Hz, benzene), 8.01 (1H, d, J = 1.9 Hz, benzene); FAB-MS *m/z*: 404 (M^++1). Anal. Calcd for $C_{15}H_{18}ClN_3O_4S_2$: C, 44.60; H, 4.49; N, 10.40; S, 15.88; Cl, 8.78. Found: C, 44.44; H, 4.41; N, 10.34; S, 16.03; Cl, 8.82.

5.1.35. *N*-(5-*tert*-Butyl-3,4-dimethyl-1,3-thiazol-2(3*H*)-ylidene)-3-chloro-2-methoxybenzenesulfonamide (10j). 60% yield from **8a**; ^{1}H NMR (DMSO-d_6) δ: 1.22 (9H, s, CH_3 of *t*-Bu), 2.22 (3H, s, 4-Me), 3.35 (3H, s, 3-Me), 3.73 (3H, s, MeO), 7.38 (1H, dd, J = 7.8, 8.3 Hz, benzene), 7.77 (1H, dd, J = 1.5, 8.3 Hz, benzene), 7.88 (1H, dd, J = 1.5, 7.8 Hz, benzene); FAB-MS *m/z*: 388 (M^++1).

5.1.36. *N*-(5-*tert*-Butyl-3,4-dimethyl-1,3-thiazol-2(3*H*)-ylidene)-5-chloro-2-methoxybenzenesulfonamide (10k). 81% yield; ^{1}H NMR (DMSO-d_6) δ: 1.34 (9H, s, CH_3 of *t*-Bu), 2.29 (3H, s, 4-Me), 3.38 (3H, s, 3-Me), 3.73 (3H, s, MeO), 7.19 (1H, d, J = 8.8 Hz, benzene), 7.59 (1H, dd, J = 2.5, 8.8 Hz, benzene), 7.73 (1H, d, J = 2.5 Hz, benzene); FAB-MS *m/z*: 388 (M^++1).

5.1.37. 5-Chloro-2-cyano-*N*-(5-isopropyl-3,4-dimethyl-1,3-thiazol-2(3*H*)-ylidene)benzenesulfonamide (10l). 63% yield; mp 180–182 °C. ^{1}H NMR (DMSO-d_6) δ: 1.15 (6H, d, J = 6.9 Hz, CH_3 of *i*-Pr), 2.19 (3H, s, 4-Me), 3.22 (1H, heptet, J = 6.9 Hz, CH of *i*-Pr), 3.48 (3H, s, 3-Me), 7.88 (1H, dd, J = 1.9, 8.3 Hz, benzene), 8.02 (1H, d, J = 1.9 Hz, benzene), 8.10 (1H, d, J = 8.3 Hz, benzene); FAB-MS *m/z*: 370 (M^++1). Anal. Calcd for $C_{15}H_{16}ClN_3O_2S_2$: C, 48.71; H, 4.36; N, 11.36; S, 17.34; Cl, 9.58. Found: C, 48.43; H, 4.24; N, 11.33; S, 17.45; Cl, 9.38.

5.1.38. 5-Fluoro-2-cyano-*N*-(5-isopropyl-3,4-dimethyl-1,3-thiazol-2(3*H*)-ylidene)benzenesulfonamide (10m). 81% yield; mp 184–186 °C (methanol–chloroform). ^{1}H NMR (DMSO-d_6) δ: 1.14 (6H, d, J = 6.8 Hz, CH_3 of *i*-Pr), 2.19 (3H, s, 4-Me), 3.20 (1H, heptet, J = 6.8 Hz, CH of *i*-Pr), 3.49 (3H, s, 3-Me), 7.66 (1H, dt, J = 2.9, 8.3 Hz, benzene), 7.85 (1H, dd, J = 2.5, 8.3 Hz, benzene), 8.16 (1H, dd, J = 5.3, 8.8 Hz, benzene); FAB-MS *m/z*: 354 (M^++1). Anal. Calcd for $C_{15}H_{16}FN_3O_2S_2$: C, 50.97; H, 4.56; N, 11.89; S, 18.15; F, 5.38. Found: C, 51.05; H, 4.60; N, 11.76; S, 18.09; F, 5.63.

5.1.39. 5-Bromo-2-cyano-*N*-(5-isopropyl-3,4-dimethyl-1,3-thiazol-2(3*H*)-ylidene)benzenesulfonamide (10n). 56% yield; mp 179–181 °C (isopropanol). ^{1}H NMR (DMSO-d_6) δ: 1.15 (6H, d, J = 6.9 Hz, CH_3 of *i*-Pr), 2.18 (3H, s, 4-Me), 3.22 (1H, heptet, J = 6.9 Hz, CH of *i*-Pr), 3.48 (3H, s, 3-Me), 8.01 (2H, m, benzene), 8.14 (1H, d, J = 1.0 Hz, benzene); FAB-MS *m/z*: 414 (M^++1). Anal. Calcd for $C_{15}H_{16}BrN_3O_2S_2$: C, 43.48; H, 3.89; N, 10.14; S, 15.48; Br, 19.28. Found: C, 43.50; H, 3.70; N, 10.07; S, 15.51; Br, 18.91.

5.1.40. 5-Chloro-*N*-(4-chloro-5-isopropyl-3-methyl-1, 3-thiazol-2(3*H*)-ylidene)-2-methoxybenzenesulfonamide (10o). 60% yield; ^{1}H NMR (DMSO-d_6) δ: 1.23 (6H, d, J = 6.8 Hz, CH_3 of *i*-Pr), 3.20 (1H, heptet, J = 6.8 Hz, CH of *i*-Pr), 3.43 (3H, s, 3-Me), 3.74 (3H, s, MeO), 7.22 (1H, d, J = 8.8 Hz, benzene), 7.63 (1H, dd, J = 2.5, 8.8 Hz, benzene), 7.76 (1H, d, J = 2.4 Hz, benzene); FAB-MS *m/z*: 395 (M^++1).

5.1.41. 5-Bromo-*N*-(4-chloro-5-isopropyl-3-methyl-1, 3-thiazol-2(3*H*)-ylidene)-2-methoxybenzenesulfonamide (10p). 73% yield; ^{1}H NMR ($CDCl_3$) δ: 1.26 (6H, d, J = 6.8 Hz, CH_3 of *i*-Pr), 3.22 (1H, heptet, J = 6.8 Hz, CH of *i*-Pr), 3.51 (3H, s, 3-Me), 3.82 (3H, s, MeO), 6.85 (1H, d, J = 8.8 Hz, benzene), 7.55 (1H, dd, J = 2.4, 8.8 Hz, benzene), 8.16 (1H, d, J = 2.4 Hz, benzene); FAB-MS *m/z*: 439 (M^++1).

5.1.42. Methyl 4-cyano-2-{[(5-isopropyl-3,4-dimethyl-1,3-thiazol-2(3*H*)-ylidene)amino]sulfonyl}benzoate (10q). 37% yield; mp 224–226 °C (isopropanol–diethyl ether). ^{1}H NMR (DMSO-d_6) δ: 1.15 (6H, d, J = 6.9 Hz, CH_3 of *i*-Pr), 3.22 (1H, heptet, J = 6.9 Hz, CH of *i*-Pr), 2.19 (3H, s, 4-Me), 3.44 (3H, s, 3-Me), 3.81 (3H, s, MeO), 7.78 (1H, d, J = 7.8 Hz, benzene), 8.14 (1H, dd, J = 1.5, 7.8 Hz, benzene), 8.33 (1H, d, J = 1.5 Hz, benzene); FAB-MS *m/z*: 394 (M^++1). Anal. Calcd for $C_{17}H_{19}N_3O_4S_2$: C, 51.89; H, 4.87; N, 10.68; S, 16.30. Found: C, 51.65; H, 4.79; N, 10.72; S, 16.03.

5.1.43. *N*-(5-*tert*-Butyl-3,4-dimethyl-1,3-thiazol-2(3*H*)-ylidene)benzenesulfonamide (10r). 95% yield; mp 188–189 °C (ethyl acetate–hexane).^{1}H NMR (DMSO-d_6) δ: 1.31 (9H, s, CH_3 of *t*-Bu), 2.26 (3H, s, 4-Me), 3.40 (3H, s, 3-Me), 7.54 (3H, m, benzene), 7.82 (2H, m, benzene); FAB-MS *m/z*: 325 (M^++1). Anal. Calcd for $C_{15}H_{20}N_2O_2S_2$: C, 55.53; H, 6.21; N, 8.63; S, 19.77. Found: C, 55.38; H, 6.32; N, 8.55; S, 19.79.

5.1.44. *N*-(5-*tert*-Butyl-3,4-dimethyl-1,3-thiazol-2(3*H*)-ylidene)-2-hydroxybenzenesulfonamide (11a). Under argon atmosphere, boron tribromide (0.17 mL, 1.71 mmol) was added dropwise to a solution of **10f** (200 mg, 0.57 mmol) in dichloromethane (20 mL) at −78 °C and stirred at the same temperature for 30 min. The mixture was warmed to room temperature and stirred for 30 min. The reaction mixture was poured into saturated aqueous sodium hydrogen carbonate solution and extracted with chloroform. The organic layer was washed with brine, then dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure and the residue was recrystalized from methanol to give **11a** (167 mg, 86%) as a colorless crystals. Mp 201–202 °C. ^{1}H NMR (DMSO-d_6) δ: 1.31 (9H, s, CH_3 of *t*-Bu), 2.27 (3H, s, 4-Me), 3.39 (3H, s, 3-Me), 6.93 (1H, dd, J = 2.5, 7.8 Hz, benzene), 7.21 (1H, m, benzene), 7.23 (1H, br d, J = 7.8 Hz, benzene), 7.31 (1H, t, J = 8.3 Hz, benzene), 9.95 (1H, s, OH); FAB-MS *m/z*: 341 (M^++1). Anal. Calcd for $C_{15}H_{20}N_2O_3S_2 \cdot 0.2H_2O$: C, 52.36; H, 5.98; N, 8.14; S, 18.64. Found: C, 52.45; H, 5.83; N, 7.94; S, 18.34.

The following compounds were obtained in the same manner.

5.1.45. *N*-(5-*tert*-Butyl-3,4-dimethyl-1,3-thiazol-2(3*H*)-ylidene)-3-hydroxybenzenesulfonamide (11b). 87% yield; mp 145–146 °C (diethyl ether–hexane). ^{1}H NMR (DMSO-d_6) δ: 1.32 (9H, s, CH_3 of *t*-Bu), 2.27 (3H, s, 4-Me), 3.38 (3H, s, 3-Me), 6.88 (2H, m, benzene), 7.36 (1H, dt, J = 1.4, 7.3 Hz, benzene), 7.70 (1H, dd, J = 1.4, 7.8 Hz, benzene), 10.12 (1H, s, OH); FAB-MS *m/z*: 341 (M^++1). Anal. Calcd for $C_{15}H_{20}N_2O_3S_2$: C, 52.92; H, 5.92; N, 8.23; S, 18.84. Found: C, 52.78; H, 5.70; N, 8.16; S, 18.84.

5.1.46. *N*-(5-*tert*-Butyl-3,4-dimethyl-1,3-thiazol-2(3*H*)-ylidene)-4-hydroxybenzenesulfonamide (11c). 60% yield; mp 234–236 °C (ethyl acetate–hexane). ^{1}H NMR (DMSO-d_6) δ: 1.31 (9H, s, CH_3 of *t*-Bu), 1.99 (3H, s, 4-Me), 3.37 (3H, s, 3-Me), 6.84 (2H, d, J = 8.6 Hz, benzene), 7.63 (1H, d, J = 8.6 Hz, benzene), 10.22 (1H, br s, OH); FAB-MS *m/z*: 341 (M^++1). Anal. Calcd for $C_{15}H_{20}N_2O_3S_2$: C, 52.92; H, 5.92; N, 8.23; S, 18.84. Found: C, 52.68; H, 5.70; N, 8.02; S, 18.45.

5.1.47. *N*-(5-*tert*-Butyl-3,4-dimethyl-1,3-thiazol-2(3*H*)-ylidene)-3-chloro-2-hydroxybenzenesulfonamide (11d). 64% yield; mp 147–148 °C (methanol). ^{1}H NMR (DMSO-d_6) δ: 1.32 (9H, s, CH_3 of *t*-Bu), 2.29 (3H, s, 4-Me), 3.41 (3H, s, 3-Me), 6.98 (1H, t, J = 7.8 Hz, benzene), 7.59 (1H, dd, J = 1.5, 7.8 Hz, benzene), 7.69 (1H, dd, J = 1.5, 7.8 Hz, benzene), 9.93 (1H, br s, OH); FAB-MS *m/z*: 375 (M^++1). Anal. Calcd for $C_{15}H_{19}ClN_2O_3S_2$: C, 48.05; H, 5.11; N, 7.47; S, 17.11; Cl, 9.46. Found: C, 47.94; H, 5.07; N, 7.32; S, 17.16; Cl, 9.38.

5.1.48. *N*-(5-*tert*-Butyl-3,4-dimethyl-1,3-thiazol-2(3*H*)-ylidene)-5-chloro-2-hydroxybenzenesulfonamide (11e). 71% yield; mp 147–149 °C (ethyl acetate–hexane). ^{1}H NMR (DMSO-d_6) δ: 1.32 (9H, s, CH_3 of *t*-Bu), 2.28 (3H, s, 4-Me), 3.38 (3H, s, 3-Me), 6.92 (1H, d, J = 8.7 Hz, benzene), 7.41 (1H, dd, J = 3.0, 8.7 Hz, benzene), 7.65 (1H, d, J = 3.0 Hz, benzene), 10.56 (1H, br s, OH); FAB-MS *m/z*: 375 (M^++1). Anal. Calcd for $C_{15}H_{19}ClN_2O_3S_2$: C, 48.05; H, 5.11; N, 7.47; S, 17.11; Cl, 9.46. Found: C, 48.04; H, 5.09; N, 7.61; S, 17.24; Cl, 9.23.

5.1.49. 5-Chloro-*N*-(4-chloro-5-isopropyl-3-methyl-1, 3-thiazol-2(3*H*)-ylidene)-2-hydroxybenzenesulfonamide (11f). 53% yield; mp 132–133 °C (diethyl ether). ^{1}H NMR (DMSO-d_6) δ: 1.21 (6H, d, J = 6.8 Hz, CH_3 of *i*-Pr), 3.18 (1H, heptet, J = 6.8 Hz, CH of *i*-Pr), 3.43 (3H, s, 3-Me), 6.88 (1H, d, J = 8.8 Hz, benzene), 7.56 (1H, dd, J = 2.4, 8.8 Hz, benzene), 7.79 (1H, d, J = 2.4 Hz, benzene), 10.86 (1H, br s, OH); FAB-MS *m/z*: 381 (M^++1). Anal. Calcd for $C_{13}H_{14}Cl_2N_2O_3S_2$: C, 40.95; H, 3.70; N, 7.35; S, 16.82; Cl, 18.60. Found: C, 40.94; H, 3.49; N, 7.36; S, 16.75; Cl, 18.39.

5.1.50. 5-Bromo-*N*-(4-chloro-5-isopropyl-3-methyl-1, 3-thiazol-2(3*H*)-ylidene)-2-hydroxybenzenesulfonamide (11g). 41% yield; mp 135–137 °C (diethyl ether). ^{1}H

NMR (DMSO-d_6) δ: 1.21 (6H, d, J = 6.8 Hz, CH_3 of *i*-Pr), 3.18 (1H, heptet, J = 6.8 Hz, CH of *i*-Pr), 3.43 (3H, s, 3-Me), 6.93 (1H, d, J = 8.8 Hz, benzene), 7.44 (1H, dd, J = 2.9, 8.8 Hz, benzene), 7.67 (1H, d, J = 2.9 Hz, benzene), 10.85 (1H, br s, OH); FAB-MS *m/z*: 425 (M^++1). Anal. Calcd for $C_{13}H_{14}ClBrN_2O_3S_2$: C, 36.67; H, 3.31; N, 6.58; S, 15.06; Cl, 8.33; Br, 18.77. Found: C, 36.67; H, 3.36; N, 6.54; S, 15.04; Cl, 8.49; Br, 18.53.

5.1.51. 2-Amino-*N*-(5-*tert*-butyl-3,4-dimethyl-1,3-thiazol-2(3*H*)-ylidene)-5-chlorobenzenesulfonamide (12). To a suspension of **10i** (960 mg, 2.38 mol) in ethanol (10 mL) and tetrahydrofuran (30 mL) was added 10% palladium–charcoal. The reaction mixture was stirred at room temperature for 1.5 h under hydrogen atmosphere. The suspension was filtered through the Celite pad and evaporated. The residue was purified with recrystalization from isopropanol–diethyl ether to give **12** (548 mg, 62%) as a brown powder. Mp 163–164 °C. ^{1}H NMR (DMSO-d_6) δ: 1.31 (9H, s, CH_3 of *t*-Bu), 2.27 (3H, s, 4-Me), 3.41 (3H, s, 3-Me), 5.94 (2H, br s, NH2), 6.79 (1H, d, J = 8.8 Hz, benzene), 7.24 (1H, dd, J = 2.4, 8.8 Hz, benzene), 7.49 (1H, d, J = 2.4 Hz, benzene); FAB-MS *m/z*: 374 (M^++1). Calcd for $C_{15}H_{20}ClN_3O_2S_2$: C, 48.18; H, 5.39; N, 11.24; S, 17.15; Cl, 9.48. Found: C, 48.43; H, 5.36; N, 11.10; S, 17.05; Cl, 9.19.

5.1.52. *N*-(2-{[(5-*tert*-Butyl-3,4-dimethyl-1,3-thiazol-2(3*H*)-ylidene)amino]sulfonyl}-4-chlorophenyl)acetamide (13). A solution of **12** (330 mg, 0.88 mmol), *N*,*N*-dimethylaminopyridine (110 mg, 0.88 mmol) and acetyl chloride (0.13 mL, 1.76 mmol) in pyridine (7 mL) was stirred at room temperature for 5 h. The reaction mixture was evaporated and diluted with ethyl acetate. The solution was washed with 1 M hydrochloric acid, saturated aqueous sodium hydrogen carbonate solution and brine, then dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure and the residue was purified by silica gel column chromatography (chloroform–methanol) and recrystalized from acetonitrile to give **13** (92 mg, 25%) as a colorless powder. Mp 194–195 °C. ^{1}H NMR (DMSO-d_6) δ: 1.28 (9H, s, CH_3 of *t*-Bu), 2.12 (3H, s, 4-Me), 2.28 (3H, s, CH_3 of Ac), 3.41 (3H, s, 3-Me), 7.63 (1H, dd, J = 2.5, 8.8 Hz, benzene), 7.81 (1H, d, J = 2.5 Hz, benzene), 8.11 (1H, d, J = 8.8 Hz, benzene), 9.24 (1H, br s, NH); FAB-MS *m/z*: 416 (M^++1). Calcd for $C_{17}H_{22}ClN_3O_3S_2$: C, 49.09; H, 5.23; N, 10.10; S, 15.42; Cl, 8.52. Found: C, 49.10; H, 5.25; N, 10.06; S, 15.49; Cl, 8.46.

5.1.53. *N*-(5-*tert*-Butyl-3,4-dimethyl-1,3-thiazol-2(3*H*)-ylidene)-5-chloro-2-[(methylsulfonyl)amino]benzenesulfonamide (14). A solution of **12** (200 mg, 0.54 mmol), triethylamine (0.15 mg, 1.08 mmol), and methanesulfonyl chloride (0.062 mL, 0.83 mmol) in tetrahydrofuran (4 mL) was stirred at room temperature for 1 h. The reaction mixture was evaporated and diluted with ethyl acetate. The solution was washed with 1 M hydrochloric acid, saturated aqueous sodium hydrogen carbonate solution and brine, then dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure and the residue was purified by silica gel column chromatography (ethyl acetate–toluene) and recrystalized from methanol to give **14** (135 mg, 56%) as a colorless powder. Mp 165–166 °C. ^{1}H NMR (DMSO-d_6) δ: 1.32 (9H, s, CH_3 of *t*-Bu), 2.29 (3H, s, 4-Me), 3.24 (3H, s, CH_3 of Ms), 3.44 (3H, s, 3-Me), 7.62 (1H, d, J = 8.8 Hz, benzene), 7.69 (1H, dd, J = 2.4, 8.8 Hz, benzene), 7.82 (1H, d, J = 2.4 Hz, benzene), 8.68 (1H, br s, NH); FAB-MS *m/z*: 452 (M^++1). Calcd for $C_{16}H_{22}ClN_3O_4S_3$: C, 42.51; H, 4.91; N, 9.30; S, 21.28; Cl, 7.84. Found: C, 42.51; H, 4.87; N, 9.29; S, 21.41; Cl, 7.66.

5.1.54. 2-{[(5-*tert*-Butyl-3,4-dimethyl-1,3-thiazol-2(3*H*)-ylidene)amino]sulfonyl}-4-chlorophenyl trifluoromethanesulfonate (15a). A solution of **11e** (1.00 g, 2.67 mmol), 2,6-lutidine (0.47 mL, 4.01 mmol), and *N*,*N*-dimethylaminopyridine (33 mg, 0.27 mmol) in dichloromethane (15 mL) was added trifluoromethanesulfonic anhydride (0.68 mL, 4.01 mmol) under ice-bath cooling. The solution was stirred at the same temperature for 30 min. The reaction mixture was poured into water and extracted with chloroform. The organic layer was washed with saturated aqueous potassium hydrogen sulfate and brine, then dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure and the residue was recrystalized from diethyl ether to give **15a** (877 mg, 64%) as a colorless powder. This crude product was used for next steps without further purification.

5.1.55. 4-Chloro-2-{[(4-chloro-5-isopropyl-3-methyl-1,3-thiazol-2(3*H*)-ylidene)amino]sulfonyl}phenyl trifluoromethanesulfonate (15b). Compound **15b** was obtained from **11f** in the same manner as described in the synthesis of **15a** quantitative. ^{1}H NMR (DMSO-d_6) δ: 1.18 (6H, d, J = 6.8 Hz, CH_3 of *i*-Pr), 3.17 (1H, heptet, J = 6.8 Hz, CH of *i*-Pr), 3.48 (3H, s, 3-Me), 7.60 (1H, s, J = 8.8 Hz, benzene), 7.88 (1H, dd, J = 2.4, 8.8 Hz, benzene), 8.01 (1H, d, J = 2.4 Hz, benzene); FAB-MS *m/z*: 513 (M^++1).

5.1.56. Methyl 2-{[(5-*tert*-butyl-3,4-dimethyl-1,3-thiazol-2(3*H*)-ylidene)amino]sulfonyl}-4-chlorobenzoate (16). Under argon atmosphere, **15a** (25.4 g, 50.1 mmol) was dissolved in *N*,*N*-dimethylformamide (280 mL) and methanol (140 mL). Triethylamine (12.9 mL, 92.6 mmol), 1,3-bis(diphenylphosphino)propane (1.74 g, 4.2 mmol), palladium acetate (0.95 g, 4.2 mmol) was added and carbon monoxide gas was bubbled. The reaction mixture was stirred at 70 °C for 2.5 h under carbon monoxide atmosphere. The mixture was evaporated and diluted with ethyl acetate. The organic layer was washed with 1 M hydrochloric acid and brine, then dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure and the residue was purified by silica gel column chromatography (ethyl acetate–toluene) and recrystalized from isopropanol–diethyl ether to give **16** (7.32 g, 42%) as a colorless powder. Mp 142–143 °C. ^{1}H NMR (DMSO-d_6) δ: 1.32 (9H, s, CH_3 of *t*-Bu), 2.30 (3H, s, 4-Me), 3.43 (3H, s, 3-Me), 3.78 (3H, s, CH_3 of COOMe), 7.61 (d, J = 8.3 Hz, benzene), 7.75 (1H, dd, J = 2.0, 8.3 Hz, benzene), 7.91 (1H,

d, $J = 2.0$ Hz, benzene); FAB-MS *m/z*: 417 (M^++1). Calcd for $C_{17}H_{21}ClN_2O_4S_2$: C, 48.97; H, 5.08; N, 6.72; S, 15.38; Cl, 8.50. Found: C, 49.02; H, 4.95; N, 6.78; S, 15.43; Cl, 8.47.

5.1.57. 2-{[(5-*tert*-Butyl-3,4-dimethyl-1,3-thiazol-2(3*H*)-ylidene)amino]sulfonyl}-4-chlorobenzamide (17a). To a solution of **16** (100 mg, 0.248 mmol) in *N*,*N*-dimethylformamide (2 mL), 1-hydroxybenzotriazole (50 mg, 0.372 mmol), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (WSC·HCl, 71 mg, 0.372 mmol), *N*,*N*-diisopropylethylamine (0.17 mL, 0.992 mmol), and ammonium chloride (27 mg, 0.496 mmol) was added. The solution was stirred at room temperature for 2.5 h. The reaction mixture was poured into water and extracted with chloroform. The organic layer was washed with 1 M hydrochloric acid and brine, then dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure and the residue was purified by silica gel column chromatography (methanol–chloroform) and recrystalized from diethyl ether to give **17a** (71 mg, 71%) as a colorless powder. Mp 196–198 °C. ^{1}H NMR (DMSO-d_6) δ: 1.31 (9H, s, CH_3 of *t*-Bu), 2.28 (3H, s, 4-Me), 3.42 (3H, s, 3-Me), 7.47 (1H, d, $J = 8.3$ Hz, benzene), 7.61 (2H, br s, NH_2), 7.68 (1H, dd, $J = 2.0$, 8.3 Hz, benzene), 7.87 (1H, d, $J = 2.0$ Hz, benzene); FAB-MS *m/z*: 402 (M^++1). Anal. Calcd for $C_{16}H_{20}ClN_3O_3S_2$: C, 47.81; H, 5.02; N, 10.45; S, 15.96; Cl, 8.82. Found: C, 47.72; H, 4.78; N, 10.42; S, 15.82; Cl, 8.91.

5.1.58. 4-Cyano-2-{[(5-isopropyl-3,4-dimethyl-1,3-thiazol-2(3*H*)-ylidene)amino]sulfonyl}benzamide (17b). Compound **17b** was obtained from **10q** in the same manner as described in the synthesis of **17a**. 68% yield. ^{1}H NMR (DMSO-d_6) δ: 1.15 (6H, d, $J = 6.8$ Hz, CH_3 of *i*-Pr), 2.17 (3H, s, 4-Me), 3.21 (1H, heptet, $J = 6.8$ Hz, CH of *i*-Pr), 3.43 (3H, s, 3-Me), 7.61 (1H, d, $J = 8.3$ Hz, benzene), 7.72 (2H, br d, $J = 6.3$ Hz, NH_2), 8.02 (1H, dd, $J = 1.9$ Hz, benzene), 8.27 (1H, d, $J = 1.9$ Hz, benzene); FAB-MS *m/z*: 379 (M^++1).

5.1.59. 2,5-Dicyano-*N*-(5-isopropyl-3,4-dimethyl-1,3-thiazol-2(3*H*)-ylidene)benzenesulfonamide (18b). Phosphorous oxychloride (0.3 mL, 3.3 mmol) and *N*,*N*-dimethylformamide (50 μL, 0.66 mmol) was added to a solution of **17b** (250 mg, 0.66 mmol) in chloroform (30 mL). The reaction mixture was refluxed for 24 h. The reaction mixture was poured into ice-water and extracted with chloroform. The organic layer was washed with saturated aqueous sodium hydrogen carbonate and brine, then dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure and the residue was purified by silica gel column chromatography (methanol–chloroform) to give **18b** (52 mg, 22%) as a yellow powder. Mp 222–224 °C. ^{1}H NMR (DMSO-d_6) δ: 1.15 (6H, d, $J = 6.8$ Hz, CH_3 of *i*-Pr), 2.19 (3H, s, 4-Me), 3.22 (1H, heptet, $J = 6.8$ Hz, CH of *i*-Pr), 3.49 (3H, s, 3-Me), 8.28 (2H, m, benzene), 8.42 (1H, br s, benzene); FAB-MS *m/z* : 361 (M^++1). Anal. Calcd for $C_{16}H_{16}N_4O_2S_2$: C, 53.31; H, 4.47; N, 15.54; S, 17.79. Found: C, 53.15; H, 4.39; N, 15.70; S, 17.74.

5.1.60. *N*-(5-*tert*-Butyl-3,4-dimethyl-1,3-thiazol-2(3*H*)-ylidene)-5-chloro-2-cyanobenzenesulfonamide (18a). Compound **18a** was obtained from **17a** in the same manner as described in the synthesis of **18b**. 67% yield; mp 160–162 °C. ^{1}H NMR (DMSO-d_6) δ: 1.32 (9H, s, CH_3 of *t*-Bu), 2.30 (3H, s, 4-Me), 3.49 (3H, s, 3-Me), 7.88 (1H, dd, $J = 2.0$, 8.3 Hz, benzene), 8.02 (1H, d, $J = 2.0$ Hz, benzene), 8.09 (1H, d, $J = 8.3$ Hz, benzene); FAB-MS *m/z*: 384 (M^++1). Anal. Calcd for $C_{16}H_{18}ClN_3O_2S_2$: C, 50.06; H, 4.73; N, 10.95; S, 16.70; Cl, 9.23. Found: C, 49.84; H, 4.63; N, 10.75; S, 16.67; Cl, 9.15.

5.1.61. 5-Chloro-*N*-(4-chloro-5-isopropyl-3-methyl-1,3-thiazol-2(3*H*)-ylidene)-2-cyanobenzenesulfonamide (19). Under argon atmosphere, **15b** (3.64 g, 7.1 mmol) was dissolved in *N*,*N*-dimethylformamide (100 mL) and palladium tris(dibenzylideneacetone)dipalladium (408 mg, 0.71 mmol), 1,1′-bis(diphenylphosphino)ferrocene (787 mg, 14.2 mmol), zinc powder (56 mg, 0.85 mmol), and zinc(II) cyanide (500 mg, 4.3 mmol) was added. The reaction mixture was stirred at 130 °C for 2.5 h under argon atmosphere. The mixture was evaporated and diluted with ethyl acetate. The organic layer was washed with 2 M aqueous ammonia solution, water, and brine, then dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure and the residue was purified by silica gel column chromatography (ethyl acetate–toluene) and recrystalized from isopropanol to give **19** (1.38 g, 50%) as a pale yellow powder. Mp 181–182 °C (isopropanol). ^{1}H NMR (DMSO-d_6) δ: 1.20 (6H, d, $J = 6.8$ Hz, CH_3 of *i*-Pr), 3.18 (1H, heptet, $J = 6.8$ Hz, CH of *i*-Pr), 3.32 (3H,s, 3-Me), 7.92 (1H, dd, $J = 1.9$, 8.3 Hz, benzene), 8.04 (1H, d, $J = 1.9$ Hz, benzene), 8.12 (1H, d, $J = 8.3$ Hz, benzene); FAB-MS *m/z*: 390 (M^++1). Anal. Calcd for $C_{14}H_{13}Cl_2N_3O_2S_2$: C, 43.08; H, 3.36; N, 10.77; S, 16.43; Cl, 18.17. Found: C, 43.03; H, 3.23; N, 10.70; S, 16.34; Cl, 18.21.

5.1.62. *N*-(4-Chloro-5-isopropyl-3-methyl-1,3-thiazol-2(3*H*)-ylidene)-2,5-dicyanobenzenesulfonamide (20). Compound **20** was obtained from **15b** in the same manner as described in the synthesis of **19** with 1.6 mol equiv of $Zn(CN)_2$. 60% yield. Mp 205–207 °C (isopropanol). ^{1}H NMR ($CDCl_3$): 1.27 (6H, d, $J = 6.8$ Hz, CH_3 of *i*-Pr), 3.22 (1H, heptet, $J = 6.8$ Hz, CH of *i*-Pr), 3.60 (3H, s, 3-Me), 7.87 (1H, dd, $J = 1.9$, 8.3 Hz, benzene), 7.92 (1H, d, $J = 8.3$ Hz, benzene), 8.47 (1H, d, $J = 1.9$ Hz, benzene); FAB-MS *m/z*: 381 (M^++1). Anal. Calcd for $C_{15}H_{13}ClN_4O_2S_2$: C, 47.30; H, 3.44; N, 14.71; S, 16.84; Cl, 9.31. Found: 47.15; H, 3.52; N, 15.00; S, 16.65; Cl, 9.35.

5.2. Pharmacology

5.2.1. In vitro RT inhibition assay. A expression plasmide, pG280, encoding HIV-1 RT proteins as LacZ fusion proteins were used for the expression of the WT RT and mutated RTs.[12] The single amino acid-substituted RTs (K103N RT and Y181C RT) were constructed using pG280 from a Quikchange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Recombinant

RT enzymes were expressed in *E. coli*. UTX81 and purified by the scheme described by Saitoh et al.[12] In vitro RT assays were conducted according to the previously described method with the following modifi cations.[13] Test compounds and 0.01 unit of recombinant HIV-1 RT (either wild type or mutant) were incubated in a reaction mixture (50 μL), containing 50 mM Tris–HCl (pH 8.4), 100 mM KCl, 10 mM $MgCl_2$, 0.1% Triton X-100, 2 mM dithiothreitol, 0.01 OD_{260} of poly(rC)/oligo(dG)$_{12-18}$, and 1 μCi of [1′,2′-^{3}H]dGTP (33 Ci/mmol) at 37 °C for 1 h. The reaction was stopped with 200 μL of 5% cold trichloroacetic acid. The precipitated materials were analyzed for radio activity using a scintillation counter (Aloka Co., Ltd., Tokyo, Japan).

5.2.2. Cells and viruses. MT-4 cells[14] and HIV-1$_{IIIB}$ were used for the anti-HIV-1 assays. MT-4 cells were grown and maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin G (100 units/mL), and gentamicin (20 mg/mL). MT-4 cells and HIV-1$_{IIIB}$ were obtained from Rational Drug Design Laboratories (Fukushima, Japan).

5.2.3. Anti-HIV-1 assay. Determination of the antiviral activity of the test compounds against HIV-1$_{IIIB}$ replication was based on the inhibition of virus-induced cytopathicity in MT-4 cells. Briefly, MT-4 cells were suspended in culture medium at 1×10^5 cells/mL and infected with virus at a multiplicity of infection (MOI) of 0.02. Immediately after virus infection, the cell suspension (100 μL) was brought into each well of a flat-bottomed microtiter tray containing various concentrations of the test compounds. After a 5-day incubation at 37 °C, the number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method.[15] The HTS of our compound library was also performed using the MTT assay against HIV-1$_{IIIB\text{-}R}$.[14] The anti-HIV-1 activity and cytotoxicity of test compounds were expressed as EC_{50} and CC_{50}, respectively. EC_{50} is the concentration of a test compound that was able to achieve 50% protection of MT-4 cells from HIV-1 induced CPE. CC_{50} is the concentration of a test compound that reduced viable cell number by 50% in mock-infected cells. The therapeutic index (TI) is the ratio of CC_{50} to EC_{50}.

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RNase S complex bearing arginine-rich peptide and anti-HIV activity

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Basic peptide-mediated protein delivery into living cells is becoming recognized as a potent approach for the understanding of cellular mechanisms and drug delivery. We have prepared the conjugates of the S-peptide (1–15) derived from RNase S with membrane-permeable basic peptides, octaarginine and the human immunodeficient virus (HIV)-1 Rev (34–50). The RNase S complexes, formed among these S-peptide (1–15)–basic peptide conjugates and the S-protein and having a dissociation constant in the range of 10^{-5} M, efficiently penetrated into the HeLa cells. These RNase S complexes exerted an anti-HIV replication activity. The time-of-drug-addition assay suggested that the site of action for these complexes would reside in the stages between the viral entry into the cells and reverse transcription. The present study exemplified the applicability of the arginine-rich peptides to the intracellular targeting of non-covalent protein complexes and supramolecular assemblies for the research in chemical and cellular biology. Copyright © 2004 John Wiley & Sons, Ltd.

Keywords: **arginine-rich peptide; drug delivery; protein design; cell membrane; anti-HIV activity; protein transduction; HIV-1 Tat; HIV-1 Rev; RNase**

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INTRODUCTION

Basic peptide-mediated protein delivery into living cells has been emphasized as a novel and promising technology for the understanding and modulation of cellular events with therapeutic potential (for reviews see Futaki *et al.*, 2003; Snyder and Dowdy, 2004; Wright *et al.*, 2003; Vivès, 2003). Among the peptides having such an activity is the arginine-rich peptide derived from HIV-1 Tat (positions 48–60; Vivès *et al.*, 1997); HIV-Rev (34–50) and octaarginine (R_8) peptides show an equivalent ability (Futaki *et al.*, 2001a, 2003; Suzuki *et al.*, 2002; Wright *et al.*, 2003). Although these peptides are highly hydrophilic, they easily translocate through cell membranes. By chemical conjugation or genetic fusion with the Tat segment, many proteins, as well as oligonucleotides, chelating molecules and magnetic beads, successfully entered cells while retaining their biological activity.

On the other hand, there are many non-covalent protein complexes or supramolecular assemblies that have great potential for intracellular applications. Although delivery of the Tat-biotin–avidin complex has been reported (Lee and Pardridge, 2001), the binding is quite strong and substantially irreversible. It has never been demonstrated whether a protein assembly with a dissociation constant in the range 10^{-5}–10^{-6} M is able to cross the membrane with retention of its structure.

In this report, we clearly show that such a non-covalent protein assembly was successfully introduced into cells using the RNase S bearing an arginine-rich segment as a model. We also describe how these protein complexes exerted anti-HIV activity. These internalized RNase S complexes retained their non-covalent assembly structure, suggesting minimal structural change in protein structure or unfolding is required for basic-peptide mediated protein translocation into cells.

METHODS

Preparation of arginine peptide–S-peptide conjugates

Preparation of the Rev-S peptide conjugate has already been reported (Futaki *et al.*, 2001b). R_8–S peptide conjugate was prepared similarly to the Rev-S peptide. Briefly, N-terminal thioglycolated basic peptide segments and the N-terminal

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Abbreviations used: AZT, 3′-azido-3′-deoxythymidine; DS, dextran sulfate; EC_{50}, 50% effective concentration; EC_{90}, 90% effective concentration; ESIMS, electrospray ionization mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; HIV, human immunodeficient virus; LTR, long terminal repeat; MAGI, multinuclear activation of the galactosidase indicator; MALDI-TOFMS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; α-MEM, alpha-minimum essential medium; PBS, phosphate buffered saline; RRE, Rev response element; TAR, *trans*-activation responsive region; Tris, tris(hydroxymethyl)aminomethane.

chloroacetylated S-peptide (1–15) segment were prepared by Fmoc-solid-phase peptide synthesis followed by conjugation with each other by reacting these segments overnight in 6 M guanidine HCl–0.1 M tris(hydroxymethyl)aminomethane (Tris) (pH 8.0). The fidelity of the peptides was ascertained by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS) [calculated for $(M+H)^+$, 3111.6; found, 3111.5].

Rhodamine labeling

Rhodamine labeling of the S-protein was conducted by incubating the S-protein (Sigma; 1 mg) with tetramethylrhodamine succinimidyl ester (Molecular Probes) (45 μg, 1 eq.) in PBS (100 μl) at room temperature for 1 h. The mixture was applied to a Sephadex G-25 column (1 × 5 cm) and eluted with 1 M acetic acid. The first major peak was collected and lyophilized to yield 0.7 mg of a mixture of the non-labeled, mono-labeled, and di-labeled S-proteins at a ratio of 7:2.5:1. These ratios were estimated from the peak areas of the electrospray ionization mass spectrometry (ESIMS) performed on an Applied Biosystems API-3000. R_8 peptide (NH_2-R_8-$CONH_2$) was similarly labeled with tetramethylrhodamine and purified by HPLC.

Preparation of the leucine zipper peptide derived from GCN4 and its conjugates

Peptide **4** was constructed with Fmoc-solid-phase peptide synthesis, followed by treatment with trifluoroacetic acid-ethanedithiol (95:5) and HPLC purification [MALDI-TOFMS: calculated for $(M+H)^+$, 4081.7; found, 4081.7]. Peptide **4** was labeled with 5-maleimidofluorescein diacetate (Sigma) to give peptide **5** as reported (Futaki *et al.*, 2001a) [MALDI-TOFMS: calculated for $(M+H)^+$, 4593.1; found, 4593.0]. Conjugate **6** was prepared by cross-linking the peptide **4** with the R_8 peptide (NH_2-R_8-$CONH_2$), using *N*-(4-maleimidobutyryloxy)succinimide ester as reported (Futaki *et al.*, 2001a) [MALDI-TOFMS: calculated for $(M+H)^+$, 5513.4; found, 5513.5].

Cell culture

Human cervical cancer-derived HeLa cells were maintained in alpha-minimum essential medium (α-MEM) (Invitrogen) with 10% heat-inactivated calf serum (Invitrogen). Cells were grown on 60 mm dishes and incubated at 37 °C under 5% CO_2 to approximately 70% confluence. A sub-culture was performed every 3–4 days.

Protein internalization and microscopic observation

For each assay, 2×10^5 cells were plated on 35 mm glass-bottomed dishes (Iwaki) and cultured for 48 h. After complete adhesion, the culture medium was changed. Prior to incubating with cells, the S-peptide–arginine peptide conjugate was mixed with the tetramethylrhodamine-labeled S-protein in a molar ratio of 2:1 and allowed to stand at room temperature for 10 min. The stipulated protein concentration was based on that of the added S-protein. The cells were then incubated at 37 °C for 1 h with the fresh medium (200 μl) containing the protein complexes (10 μM). For comparison, cells were treated with tetramethylrhodamine-labeled R_8 peptide (1 μM) at 37 °C for 1 h. Cells were then washed five times with phosphate-buffered saline (PBS). Distribution of the fluorescence-labeled peptides was analyzed using a confocal scanning laser microscope LSM 510 (Zeiss) equipped with a × 40 lens without fixing cells.

Flow cytometry

To analyze the internalization of the RNase S complexes **1** and **3** by FACS, 1.5×10^5 HeLa cells in a fresh culture medium (1.5 ml) were plated on a 12-well microplate (Iwaki) and cultured for 48 h. After complete adhesion, the culture medium was exchanged (with α-MEM containing 10% heat-inactivated calf serum) and the cells were incubated with S-peptide–R_8 peptide conjugate or the S-peptide (1–15) mixed with tetramethylrhodamine-labeled S-protein in a molar ratio of 2:1 and allowed to stand for 10 min. The protein concentration (10 μM) was based on that of the added S-protein. The 1 mol of the S protein was found to be labeled with 0.43 mol of tetramethylrhodamine by ESIMS. Tetramethylrhodamine-labeled R_8 peptide (4.3 μM) was therefore used for the comparison of the internalization efficiency. The cells were incubated at 37 °C for 1 h with fresh medium (400 μl) containing the protein complexes prior to washing for 2 × 3 min with PBS. The cells were treated with 0.01% trypsin (Invitrogen, 400 μl) at 37 °C for 10 min prior to adding 600 μl of PBS. The cells were centrifuged at 2000 rpm for 5 min and, after removing the supernatant, they were washed with 1 ml of PBS and centrifuged at 2000 rpm for 5 min. After repeating this washing cycle, the cells were suspended in 1.5 ml of fresh culture medium and subjected to fluorescence analysis using flow cytometry. This was performed with a FACScalibur (BD Biosciences) flow cytometer using 488 nm laser excitation and a 564–606 nm emission filter. Usually tetramethylrhodamine is not used for FACS analysis because of its low excitability at 488 nm, but the dye was used here to analyze the internalization efficiency under the condition as close as that used for microscopic observation.

MAGI assay

The assay was conducted with some modification using the viral preparation titrated as previously reported (Kodama *et al.*, 2001). Briefly, target cells (HeLa CD4-LTR/β-gal; 10^4 cells/well), which both express high levels of CD4 and contain a single integrated copy of a β-galactosidase gene under the control of a truncated HIV-1 long terminal repeat (LTR), were plated in 96-well flat microtitier culture plates. On the following day, the medium was aspirated, and the cells were infected with HIV-1 (III_B) (70 MAGI units/well) and cultured in the presence of various concentrations of the protein complexes in fresh medium. Forty-eight hours after

viral exposure, all the blue cells in each well were counted. All experiments were performed in triplicate.

RESULTS AND DISCUSSION

Design of RNase S complexes bearing arginine-rich segments

RNase S is a protein complex comprising a non-covalent assembly of two segments, the S-peptide (positions 1–20 of bovine pancreatic RNase A) and the S-protein (positions 21–124). These respective segments do not retain the RNase activity by themselves, but when mixed together they fold into a complex and exert RNase activity. Interestingly, the S-peptide and the S-protein form an active structure even when the S-peptide is conjugated with other exogenous proteins (Karpeisky *et al.*, 1994).

The design of RNase S bearing an arginine-rich peptide is illustrated in Fig. 1(A). Octaarginine (R_8), and Rev (34–50) were employed as membrane-permeable peptides (Futaki *et al.*, 2001a). These peptides were conjugated with the

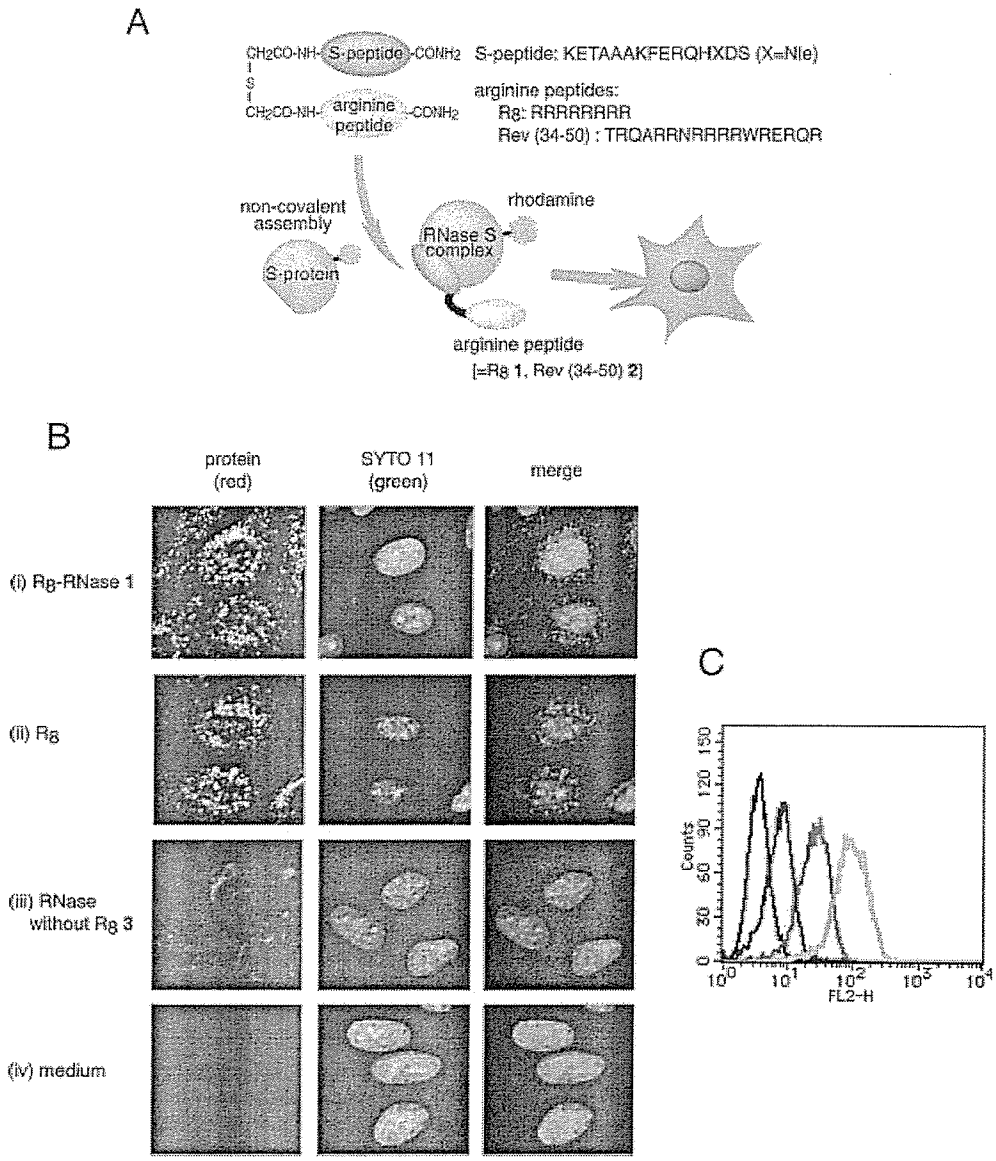

Figure 1. (A) The schematic representation of RNase S complexes bearing arginine-rich segments, and (B) confocal laser microscopic observations of HeLa cells treated at 37 °C for 1 h with the rhodamine-labeled R_8 peptide (1 μM), RNase S bearing the R_8 peptide **1** (10 μM), and RNase S without an arginine-rich segment **3** [the complex of the S protein with S-peptide (1–15) (NH_2-KETAAAKFERQHXDS-$CONH_2$, X = norleucine), 10 μM], respectively. Note that the rhodamine was attached only on the S-protein in the case of RNase complexes. Nucleus was stained with SYTO (no. 11; 5 μM). (C) FACS analysis of internalization efficiency of RNase S complexes **1** and **3**. HeLa cells were treated with RNase S complexes bearing R_8 peptide (**1**, red), RNase S complex without R_8 peptide (**3**, blue) and R_8 peptide (orange), respectively, as described in the methods. Control cells (untreated) are shown in black.

S-peptide (1–15), the segment of which sufficiently forms a complex with the S-protein without loss of the RNase activity (Karpeisky *et al.*, 2004). The S-protein was labeled with the tetramethylrhodamine for microscopic observation.

Internalization of the RNase S complexes bearing arginine-rich peptides was assessed in HeLa cells. Figure 1(B) shows confocal microscopic observation after incubation of the protein complex (10 μM) with HeLa cells in the presence of medium at 37 °C for 1 h. Internalization of the complex was clearly observed in almost all the cells treated with RNase S bearing the respective arginine-rich peptides and resulted in intracellular distributions similar to those of the R_8 peptide [Fig. 1(B) (i) and (ii)]. On the other hand, much less efficient internalization was observed for RNase S without the arginine-rich peptides [a complex of the S-protein and S-peptide (1–15), NH_2-KETAAAKFERQH XDS-$CONH_2$ (X = norleucine, Nle)] **3** [Fig. 1(B) (iii)]. Note that the fluorescent probe was attached only on the S-protein, suggesting that the S-protein was internalized with retention of its complex structure. FACS analysis showed that, by conjugation with R_8 peptide, the internalization efficiency of the RNase complex **1** was almost five times as much as that for the RNase complex without having an arginine segment **3**. On the other hand, that for complex **1** was approximately 25% of that for the R_8 peptide [Fig. 1(C)]. When the cells were treated with a 1:1 mixture of the S-peptide–R_8 conjugate and the S-protein, the internalization efficiency was about 80% of that of the 2:1 mixture described above. Co-incubation of the complex **3** with two equivalents of R_8 peptide (NH_2-R_8-$CONH_2$, 20 μM) gave no increase in the cellular uptake of **3** (data not shown).

Recently, internalization of HIV-1 Tat (48–60) has become interpreted as being mediated by an endocytic process (Richard *et al.*, 2003; Vivès, 2003). Although detailed mechanisms of Tat entry awaits clarification, translocation of the peptides from endocytic vesicular compartments to the cytosol would be necessary for the delivered molecules to work in living cells.

Anti-HIV replication activity of the RNase S complexes

The Rev segment was originally identified as an RNA-binding peptide which recognizes a specific structure of the RNA derived from HIV (Tan and Frankel, 1995). Moreover, we have previously shown that the RNase S bearing the Rev peptide cleaved an RNA corresponding to the Rev binding site (the Rev response element, RRE; Futaki *et al.*, 2001b). It has also been reported that a peptide composed of nine residues of arginine (R_9) also binds to the *trans*-activation responsive region (TAR) of HIV-1 RNA (Calnan *et al.*, 1991). Therefore, it may be possible that the R_8 segment could bind to the TAR or other specific sites of the viral RNA. These facts motivated us to examine the anti-HIV activity of the RNase S bearing these arginine-rich segments. The activity of RNase S complexes bearing the R_8 and Rev peptides, represented as the concentration that blocks HIV-1 replication by 50% (EC_{50}), was 0.67 and 1.7 μM, respectively [Fig. 2(A)], determined by the multinuclear activation of the galactosidase indicator (MAGI) assay (Kodama *et al.*, 2001). In this assay, HIV-1 infection in HeLa CD4/LTR-β-gal cells leads to the activation of

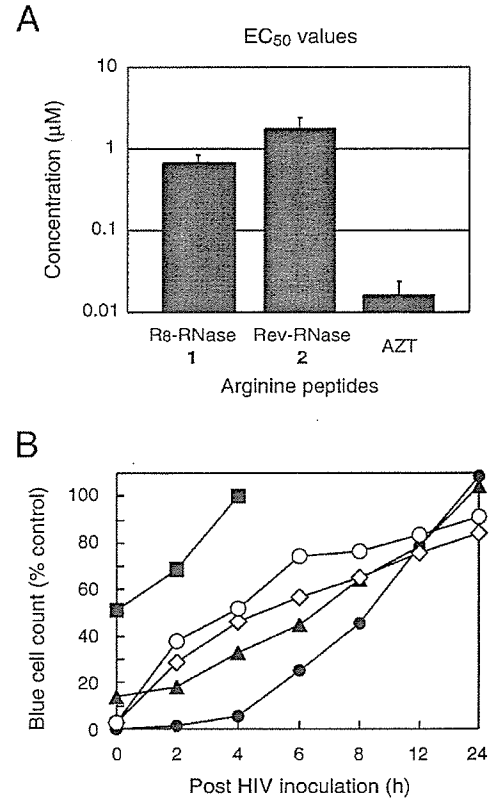

Figure 2. (A) Anti-HIV replication activity of RNase S complexes bearing arginine-rich peptides **1** and **2** in MAGI assay (Kodama *et al.*, 2001). HIV-1 infection in HeLa CD4/LTR-β-gal cells leads to the activation of β-galactosidase gene, which is visualized through the formation of blue cells in 48 h of culture. The anti-HIV activity is demonstrated by the decrease in the number of blue cells. EC_{50} of RNase S without having an arginine segment **3** was judged to be >100 μM. (B) Anti-HIV activity of RNase S complexes **1** and **2** in time-of-drug-addition assays. The RNase S complexes were added to the MAGI (HeLa CD4/LTR-β-gal) cells at the indicated points after viral inoculation, and blue cells produced were counted at the completion of the 48 h period of incubation. Protein complexes at the concentration corresponding to the EC_{90} (MAGI assay) were employed: RNase S complex bearing R_8 **1** (open circle, 7 μM) and Rev **2** (open diamond, 10 μM), respectively. DS (solid square, 200 ng/ml), AZT (solid triangle, 500 nM), MKC442 (solid circle, 500 nM).

β-galactosidase gene, which is visualized through the formation of blue cells after 48 h of culture. The anti-HIV activity is demonstrated by the decrease in the number of blue cells. Although the activity of the RNases was lower than that of 3′-azido-3′-deoxythymidine (AZT; EC_{50} value 0.016 μM), these RNase complexes actually exerted anti-HIV activity. These facts clearly indicated that these intracellular RNase S complexes retained their active structure. On the other hand, the EC_{50} of the RNase complex without the arginine segment **3** was >100 μM.

An oligoarginine peptide, acetyl-NH-(D-Arg)$_9$-$CONH_2$ (ALX40-4C) has been reported to inhibit interaction of HIV-1 gp120 and CXCR4, which is used as a co-receptor for T-tropic HIV, and the eventual viral entry to the cells (Doranz *et al.*, 1997). We have confirmed that the R_8 peptide also has a similar activity to prevent the viral entry to the cells using syncytium formation assay (Mitsuya *et al.*, 1998;

data not shown). In order to elucidate the interaction stage of these RNase S complexes with the HIV replicative cycle, a time-of-drug-addition experiment (De Clercq *et al.*, 1992) was conducted [Fig. 2(B)]. HeLa CD4-LTR/β-gal cells were infected with HIV-1 (III_B) (70 MAGI units/well) at time zero. The test protein complexes at concentrations that block the HIV-1 replication by 90% (EC_{90}; obtained from MAGI assay) were then added at the indicated time points (0, 2, 4, 6, 8, 12 or 24 h). Blue cells produced were counted at the completion of the 48 h period of incubation. If complexes were added before the viral replication cycle reached their target replication stage, they were effective without significant loss of activity. In comparison of the delay time for the sample with that for the drug of known mechanism, the site of action could be estimated [Fig. 2(B)]: dextran sulfate (DS) (MW 5000) inhibited the adsorption of HIV on the cell surface, which showed activity only when it was added early after viral exposure. MKC442 is a non-nucleoside reverse transcriptase inhibitor, which directly binds and inhibits reverse transcriptase, and the addition was effective up to ~4 h after viral inoculation. AZT is a nucleoside reverse transcriptase inhibitor. Activation of the drug requires its phosphorylation in the cells. Thus, addition of AZT was effective up to ~2 h after viral inoculation.

The effect of RNase S bearing these arginine-rich peptides decreased more slowly than that of DS and faster than MKC442. The result suggested that the action stage of these RNase S complexes is not the adsorption of the virus on the cell surface as in the case of R_8 peptide, but should reside after this stage and before the start of reverse transcription. Thus, the RNase complexes showed anti-HIV activity only while the virus stayed in the RNA form. Considering the difference in translocation efficiency (~5 fold) and EC_{50} values (>100 fold) between the RNase complexes with and without arginine segments, it would be possible that the arginine segments not only accelerated the internalization of the RNase complexes but also increased their affinities to HIV RNA.

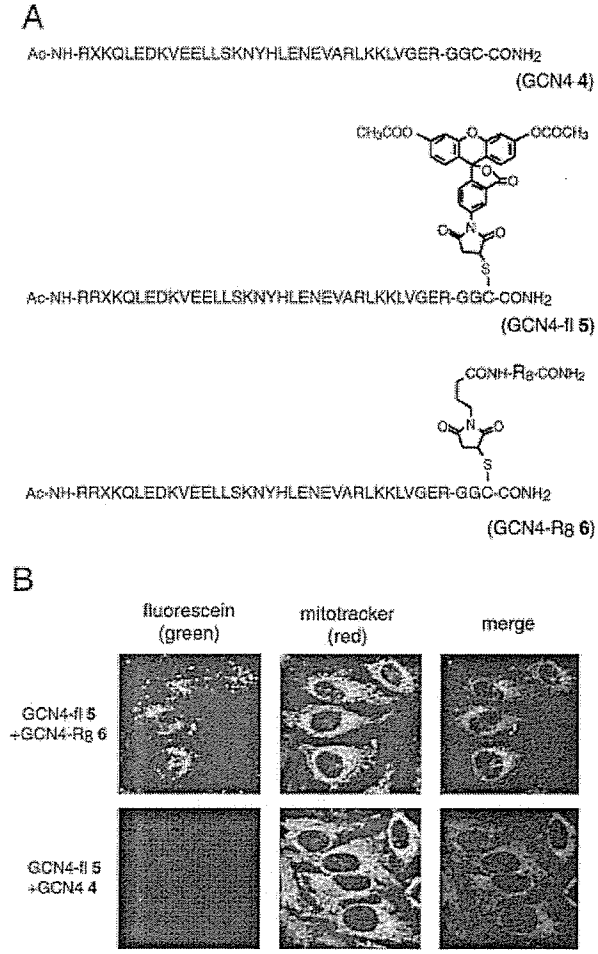

Figure 3. (A) Structures of GCN4 leucine–zipper segments bearing fluorescein (**5**) and R_8 (**6**) moieties (X = norleucine). (B) Internalization of GCN4 leucine zipper complex into HeLa cells. The cells were incubated with the peptide mixture for 3 h and subjected to confocal microscopic observation. MitoTracker (Molecular Probes; 0.5 μM) was used to outline the shapes of the cells.

General applicability of this approach to the delivery of other non-covalent protein complexes

The dissociation constant of the RNase S bearing the R_8 segment was determined to be 1.8×10^{-5} M using the procedure of Woodfin and Massey (1968). This value is very close to that for RNase S without the arginine peptide (2.5×10^{-5} M), suggesting that conjugation with the arginine-peptides had little effect on the stability of the RNase complexes. This result indicates that even a non-covalent complex with a dissociation constant in this range can go through the membranes while retaining its structure. Similar results were obtained from the study using a leucine-zipper segment of yeast transcription factor GCN4. This leucine zipper peptide has been well characterized to form a homodimer with a dissociation constant of 4.7×10^{-7} M (Wendt *et al.*, 1994).

For the assessment of internalization of the leucine–zipper complex, the peptide segment **4** shown in Fig. 3(A) was synthesized. The peptide design was based on GCN4-p1C peptide, which has already been shown to form a stable homodimer (O'Shea *et al.*, 1989). The C-terminus cysteine of peptide **4** was labeled with 5-maleimidofluorescein diacetate, which fluoresces after hydrolysis of the acetyl moieties by cellular esterases (Guilbault and Kramer, 1964), to give peptide **5**. Alternatively, peptide **4** was cross-linked with the R_8 peptide (NH_2-R_8-$CONH_2$) using *N*-(4-maleimidobutyryloxy)succinimide ester to give conjugate **6**.

Internalization of the GCN4 leucine–zipper peptides with the aid of R_8 was observed by confocal microscopy after treating HeLa cells with a mixture of **5** and **6** (20 μM each) at 37 °C for 1 h [Fig. 3(B)]. Significant internalization was not observed for the cells treated with a mixture of peptides **4** and **5** (20 μM each). In the above experiments, the homodimer of each peptide segment should also be formed. However, because the efficiency of internalization for the homodimer of **5** was estimated to be very low by the latter experiments, it was judged that only the complex of segments **5** and **6** was observable in the cells. These results clearly showed that the fluorescein-labeled GCN4 peptide

was efficiently brought into the cells with the help of the GCN4-R_8 peptide.

CONCLUSIONS

In this report, we have demonstrated that non-covalent protein complexes were efficiently incorporated into the cells while retaining their active structure. The results suggested that many supramolecules having dissociation constants in this range can be introduced into cells using this strategy. This opens new technological avenues for studies of mechanism and control of cellular functions.

Acknowledgments

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Attempt to reduce cytotoxicity by synthesizing the L-enantiomer of 4′-C-ethynyl-2′-deoxypurine nucleosides as antiviral agents against HIV and HBV

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We investigated the potential of 4′-C-substituted nucleosides for the treatment of HIV-1 and HBV. Of the nucleosides we prepared, several 4′-C-ethynyl-2′-deoxypurine nucleosides showed the most potent anti-HIV activity. However, two candidates, 4′-C-ethynyl-2′-deoxyguanosine and 9-(2-deoxy-4-C-ethynyl-β-D-*ribo*-pentofuranosyl)-2,6-diaminopurine, were very toxic during *in vivo* study. On the other hand, lamivudine (3TC) is known to show remarkable activity against HIV and HBV with lower cytotoxicity. Therefore, we attempted to synthesize the L-enantiomer of 4′-C-ethynyl-2′-deoxypurine nucleosides in 20–21 steps. These methods consisted of preparing 4-C-ethynyl-L-sugar, starting from D-arabinose and then condensing the L-sugar derivative with 2,6-diaminopurine. 4′-C-Ethynyl-2′-deoxyguanosine was also prepared by enzymatic deamination from the 2,6-diaminopurine derivative. The compounds' antiviral activity against HIV and HBV was then evaluated. Unfortunately, they demonstrated no activity and no cytotoxicity.

Keywords: 4′-C-ethynyl-2′-deoxy nucleosides (4′-EdNs), L-enantiomer, lamivudine, anti-HIV-1 agent, anti-HBV agent

Introduction

A number of nucleosides with unnatural L-sugars that exhibit antiviral activity and no cytotoxicity have recently been discovered by Chu *et al.* They have reported on one of the most potent antiviral drugs, lamivudine (3TC) (Beach *et al.*, 1992). Consequently, these L-nucleosides are expected to lead to the development of more potent and less toxic antiviral nucleoside drugs.

We synthesized 4′-*C*-ethynyl-2′-deoxycytidine **1** and reported its anti-HIV activity to have an EC_{50} value of 0.0048 μM. However, this compound also showed potent cytotoxicity (IC_{50}=0.92 μM)(Ohrui *et al.*, 2000) (Figure 1). These results prompted us to synthesize the L-enantiomer of 4′-*C*-ethynyl-2′-deoxycytidine **2**; unfortunately, it did not show any activity against human immunodeficiency virus (HIV)-1 up to 100 μM (Kohgo *et al.*, 2001) (Figure 1).

During an exploration of novel nucleoside reverse transcriptase inhibitors (NRTIs), we selected 9-(2-deoxy-4-*C*-ethynyl-β-D-*ribo*-pentofuranosyl)-2,6-diaminopurine **3** and 4′-*C*-ethynyl-2′-deoxyguanosine **5** as anti-HIV agents because of their high biological activity. Interestingly, compound **3** was also active against hepatitis B virus (HBV), with an excellent EC_{50} value. However, they showed high levels of toxicity in mice (Ashida, Yamasa Corporation, personal comunication). Therefore, we attempted to synthesize the L-enantiomers of 4′-EdNs (**3** and **5**), which were designated as compounds **4** and **6**, in order to reduce cytotoxicity without losing biological activity (Figure 2).

As mentioned above, the L-enantiomers of **1** completely lost biological activity. However, we are still interested in making L-enantiomers of 4′-EdNs (**3** and **5**) in order to elucidate the structure-activity relationship of these nucleosides.

Materials and methods

General method for chemistry

All melting points were determined on a Yanagimoto MP-500 D micro melting point apparatus and are uncorrected.