Full Paper

Synthesis and Anti-HIV Activity Evaluation of Novel 2,4-Disubstituted 7-Methyl-1,1,3-trioxo-2,4-dihydro-pyrazolo-[4,5-*e*][1,2]thiadiazines

Xin-yong Liu[1], Ren-zhang Yan[1], Yan Wang[1], Peng Zhan[1], Erik De Clercq[2], Christophe Pannecouque[2], Myriam Witvrouw[2], Maria Teresa Molina[3], and Salvador Vega[3]

[1] Institute of Medicinal Chemistry, School of Pharmaceutical Sciences, Shandong University, Jinan, P. R. China
[2] Rega Institute for Medical Research, Katholieke Universiteit Leuven, Leuven, Belgium
[3] Instituto de Quimica Medica (CSIC), Madrid, Spain

A series of novel 2,4-disubstituted 7-methyl-1,1,3-trioxo-2,4-dihydro-pyrazolo[4,5-*e*] [1,2,4]thiadiazines (PTDs) was synthesized, structurally confirmed by spectral analysis, and evaluated for their anti-HIV activities by inhibition of HIV-induced cytopathogenicity in MT-4 cell culture. The results showed that some compounds exhibited inhibitory activity specifically against HIV-2 replication. The most active HIV-2 inhibitor was compound **7i** (R_1 = benzyl, R_2 = 4-*t*-butyl-benzyl) with an EC_{50} value of 18.7 μM and SI=15, which may provide a useful lead for further molecular optimization.

Keywords: Phosphoinositide 3-kinase / phospholipases / HIV-1 / HIV-2 / NNRTIs / Pyrazolo[4,5-*e*][1,2,4]thiadiazines

Received: October 21, 2007; accepted: December 12, 2007

DOI 10.1002/ardp.200700216

Introduction

Although the highly active, anti-retroviral therapy (HAART) combination regimen has dramatically decreased the morbidity and mortality from infection by HIV, the causative agent of acquired immunodeficiency syndrome (AIDS), remains one of the world's most serious health problems, causing millions of deaths each year [1]. Currently, three different classes of chemotherapeutic agents have been used in HAART to block the replication of HIV-1, namely reverse transcriptase inhibitors (RTIs), protease inhibitors (PIs), and one-fusion inhibitor (enfuvirtide).

Correspondence: Xinyong Liu, Institute of Medicinal Chemistry, School of Pharmaceutical Sciences, Shandong University, No.44 Wenhuaxi Road, Jinan 250012, P. R. China.
E-mail: xinyongl@sdu.edu.cn
Fax: +86 531 8838-2264

Abbreviations: reverse transcriptase inhibitor (RTI); nucleoside (and nucleotide) reverse transcriptase inhibitor (NRTI, NtRTI); non-nucleoside reverse transcriptase inhibitor (NNRTI); 2,4-disubstituted 1,1,3-trioxo-2,4-dihydro-thieno[3,4-*e*][1,2,4]thiadiazine (TTD); pyrazolo[4,5-*e*][1,2,4]thiadiazine (PTD)

HIV-1 reverse transcriptase (HIV-1 RT) that catalyzed several steps in the replication of HIV is an attractive target for the development of anti-AIDS drugs. Three classes of HIV-1 RTIs are currently available: nucleoside (and nucleotide) reverse transcriptase inhibitors (NRTIs, NtRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs). NNRTIs are structurally diverse compounds by specifically targeting an allosteric site of HIV-1 RT, approximately 10–15 Å from the polymerase active site, causing a distortion of the catalytic aspartate triad [2]. The efficacy of NNRTIs like that of other types of anti-AIDS agents has been limited by emergence of drug-resistant viral strains and overwhelming side effects [3, 4]. Therefore, discovery and development of new NNRTIs with more potent, less toxic, and broad-spectrum activity are urgently needed in medicinal research.

In recent years, studies aimed at the discovery of new NNRTIs; Vega and his colleagues reported that a series of 2,4-disubstituted-1,1,3-trioxo-2,4-dihydro-thieno[3,4-*e*]-[1,2,4]thiadiazines (TTDs) effectively inhibited the replication of a variety of HIV-1 strains at the reverse-transcription steps, including strains that are resistant to AZT, but not against HIV-2 (ROD) [5, 6]. The prototypes QM96521,

QM96539 and QM96639 (Fig. 1), containing a benzyl or 2-halogenated benzyl moiety at the N_2 position and a cyanomethyl chain linked to the N_4 position, were found to selectively inhibit HIV-1 (III-B) replication in MT and CEM cell cultures [6, 7]. The cross-resistance pattern of these compounds against other NNRTI-resistant, mutant HIV-1 strains and molecular modelling of the HIV-1 RT binding site were found both to be similar to those on nevirapine [6]. Furthermore, molecular modification by replacement of the N_4 cyanomethyl with a substituted benzyl group led to the discovery of a new precursor QM96625 (N_2-benzyl, N_4-2-chlorobenzyl, EC_{50} = 0.1 μM, SI > 1190; Fig. 1) [7].

The initial structure-activity relationship analysis together with the molecular modelling disclosed that double substitutions containing π-electrons groups at the N_2 and N_4 sites of TTDs were necessary for preserving anti-HIV-1 activity, which perfectly match the "butterfly-like" three-dimensional (3D) model proposed by Schafer *et al*. [8] for filling the space of the NNRTI binding pocket.

As a continuation of the study on the structure-activity relationships of TTDs, we undertook a study of the substituted pyrazolo[4,5-*e*][1,2,4]thiadiazines (PTDs) series, because of the known bio-isosterism of TTDs-PTDs [9]. In recent publications, we reported the regioselective synthesis of novel N_2- or N_4-monosubstituted 7-methylpyrazolo[4,5-*e*][1,2,4]thiadiazines, as well as the synthesis of N_2, N_4-disubstituted 7-methylpyrazolo[4,5-*e*][1,2,4]thiadiazines by an one-pot reaction [10, 11]. In this paper, in order to find potent HIV replication inhibitors, a new series of 2,4-disubstituted PTDs was designed, in which, the PTD nuclear ring was alkylated at the N_2 and N_4 positions by those substituents active against HIV-1 activity in the TTD series. Herein, we reported the synthesis of the novel PTD analogues and their anti-HIV activities in cell culture.

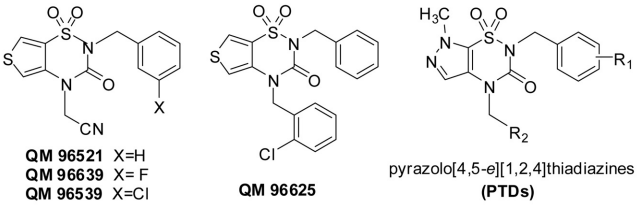
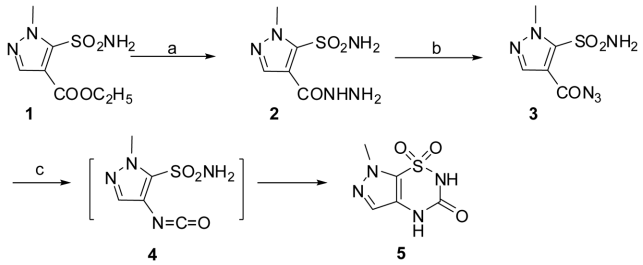
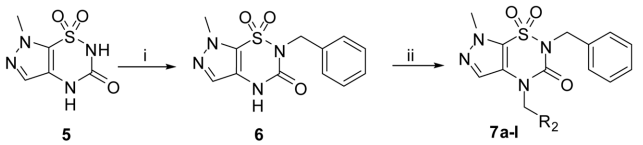


Figure 1. Lead compounds of 2,4-disubstituted-1,1,3-trioxo-2,4-dihydro-thieno[3,4-*e*][1,2,4]thiadiazines (TTDs) and newly designed 2,4-disubstituted-7-methylpyrazolo[4,5-*e*][1,2,4]thiadiazines (PTDs).



Reagents: (a) $N_2H_4 \cdot H_2O$/EtOH; (b) 2N HCl, $NaNO_2$, H_2O; (c) Δ/toluene

Scheme 1. Synthesis route of compounds **1 – 5**.



Reagents: (i) NaH/R_1CH_2X (1 : 1), DMF; (ii) NaH/R_2CH_2X (1 : 1), DMF

Scheme 2. Synthesis route of compounds **6** and **7a – l**.

Results and discussion

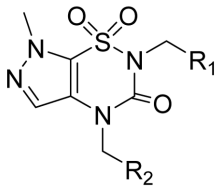
Chemistry

Similar to the synthesis of 1,1,3-trioxo-2,4-dihydro-thieno[3,4-*e*][1,2,4]thiadiazine (TTD), 1,1,3-trioxo-pyrazolo[4,5-*e*][1,2,4]thiadiazine (PTD, **5**) was synthesized starting from ethyl 1-methyl-5-sulfamoyl-1*H*-pyrazole-4-carboxylate **1** through hydrazinolysis, with excess hydrazine hydrate in the refluxed ethanolic solution to gave the 5-sulfamoylthiophene-4-carbohydrazide **2**, which was converted to the carbonyl azide **3** in almost quantitative yield by reaction with sodium nitrite in diluted hydrochloric acid at a temperature <10°C. Because of the potential explosion hazard of acyl azides, crude **3** was cautiously dried without further purification and then subjected to a Curtius rearrangement by heating in the refluxing dry toluene. The intermediate isocyanate **4** spontaneously cyclized to the desired 7-methyl-1,1,3-trioxo-2,4-dihydro-pyrazolo[4,5-*e*][1,2,4]thiadiazine (PTD, **5**) (Scheme 1) [5 – 7].

Compound **5** is a key intermediate for the preparation of N_2-monosubstituted derivatives **6**, from which different substituents can be introduced at the N_4 position by alkylation under the same conditions to obtain the target compounds of N_2, N_4-disubstituted 7-methyl-1,1,3-trioxo-2,4-dihydro-pyrazolo[4,5-*e*][1,2,4]thiadiazines (PTDs, **7a – l**, Scheme 2).

The N_2,N_4-disubstituted PTDs containing the same substituents were prepared starting from the nuclear ring **5** with two equivalents of sodium hydride in DMF solvent at a temperature <10°C, followed by addition of two equivalents of alkyl halides at 40 – 80°C for 2 – 8 h, achieving the N_2, N_4-disubstituted products **7m – r** in good yields (Scheme 3).

Table 1. Anti-HIV activities, cytotoxicities, and selectivity indices of 2,4-disubstituted 7-methyl-1,1,3-trioxo-2,4-dihydro-pyrazolo[4,5-*e*][1,2,4]thiadiazines (TTDs) **7a–r**.



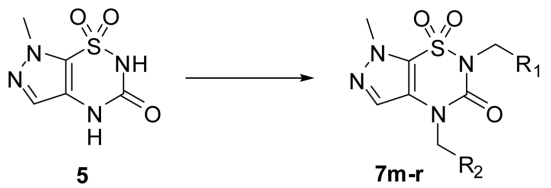
N°	R_1CH_2	R_2CH_2	EC_{50} (μM)[a]		CC_{50} (μM)[b]	SI[c]	
			HIV-1 III_B	HIV-2 ROD		HIV-1 III_B	HIV-2 ROD
7a	benzyl	CH_2CN	>251.4	>209.4	≥209.4	× 1	× 1
7b	benzyl	benzyl	>319.4	>327.2	≥319.4	× 1	× 1
7c	benzyl	2-Br-benzyl	>271.1	>271.1	>271.1	× 1	× 1
7d	benzyl	4-Br-benzyl	>6.7	>112.7	54.7	<1	<1
7e	benzyl	2-Cl-benzyl	>300.1	>300.1	>300.1	× 1	× 1
7f	benzyl	3-Cl-benzyl	>300.1	44.7	>300.1	× 1	>7
7g	benzyl	4-Cl-benzyl	>47.5	>56.2	53.9	<1	<1
7h	benzyl	2,4-Cl_2-benzyl	>277.2	>277.2	>277.2	× 1	× 1
7i	benzyl	4-(t-Bu)-benzyl	>285.4	18.7	>285.4	× 1	≥15
7j	benzyl	2-CN-benzyl	>265.4	>198.0	≥198.0	× 1	× 1
7k	benzyl	3-CN-benzyl	>307.1	>307.1	>307.1	× 1	× 1
7l	benzyl	4-CN-benzyl	>20.4	>23.8	24.2	<1	<1
7m	4-CH_3-benzyl	4-CH_3-benzyl	>170.5	>304.9	≥170.5	× 1	× 1
7n	2-Br-benzyl	2-Br-benzyl	>231.5	>231.5	>231.5	× 1	× 1
7o	4-(*t*-Bu)benzyl	4-(*t*-Bu)benzyl	>7.5	>5.3	7.1	<1	<1
7p	2,4-Cl_2-benzyl	2,4-Cl_2-benzyl	>253.0	38.1	>253.0	× 1	>7
7q	4-Cl-benzyl	4-Cl-benzyl	>6.7	>5.8	6.7	<1	<1
7r	4-Br-benzyl	4-Br-benzyl	>151.2	>151.2	161.6	<1	<1
QM96521[d]			0.9		502.7	559	
QM96625[d]			0.1		>119.0	>1190	
Nevirapine[d]			0.03		683	22767	
AZT[d]			0.0007		35.6	50587	

[a] Dose of compound required to achieve 50% protection of MT-4 cell from HIV (HIV-1 III_B, HIV-2 ROD)-induced cytotoxicity, as determined by the MTT method.

[b] CC_{50} : dose required to reduce the viability of mock-infected cells by 50%, as determined by the MTT method.

[c] SI: selectivity index (CC_{50}/EC_{50}); the SI values: 6 1 stand for =1 or <1. All data represent mean values for at least two separate experiments.

[d] The synthesis and antiviral properties of these compounds were previously described [5, 7].



Reagents: NaH/RCH_2X (2 : 2), DMF

Scheme 3. Synthesis route of compounds **7m–r**.

Anti-HIV evaluation

The activity and cytotoxicity of the newly designed and synthesized PTDs **7a-r** were tested for inhibition of HIV (HIV-1 III_B, HIV-2 ROD)-induced cytopathogenicity in MT-4 cells culture. The results are listed in Table 1. EC_{50} and CC_{50} for QM96521 and QM96625 are given for comparative purposes; AZT and nevirapine were used as the reference drugs.

As illustrated in Table 1, the new PTD derivatives, prepared by pyrazole substitution for the thiophene of the TTD derivatives, had completely lost their anti-HIV-1 activity, although the active substituents of TTDs were retained in the PTD molecules. Putatively, an increased hydrophilicity of the pyrazole-ring moiety, in comparison with the thiophene in the corresponding TTD analogues, did not fit for the requirement of a "butterfly like" model with its lipophilic center binding through van–der–Waals and π-π interactions with aromatic amino acid residues in the RT binding pocket [6]. This feature should be taken into account in the future for design of novel TTDs.

To our surprise, some PTD compounds, **7f**, **7i**, and **7p**, exhibited inhibitory activities specifically against HIV-2 (ROD) replication in MT-4 cell culture. The most active one was compound **7i** (R_1 = benzyl, R_2 = 4-*t*-butyl-benzyl) with an EC_{50} value of 18.7 μM and SI ≥ 15. Compounds **7f** and **7p** emerged as moderately active HIV-2 inhibitors with EC_{50} values in a similar range (44.7 μM and 38.1 μM, respectively).

HIV-2 infection had appeared predominantly in West Africa, but gradually spread to other parts of the world in recent years. *In-vitro* (laboratory) studies suggest that non-nucleoside reverse transcriptase for inhibitors (NNRTIs) are usually specific for HIV-1 and inactive against HIV-2. Therapeutic options for HIV-2 infection are therefore limited to nucleoside reverse transcriptase inhibitors (NRTIs) and protease inhibitors (PIs) [12]. There is insufficient data to recommend any specific drug or drug combinations for the treatment of HIV-2 infection [13]. PTD analogues could act as useful leads for further optimization, which will provide a stimulus for structure-based design of more potent anti-HIV-2 agents.

Conclusion

In conclusion, we designed and synthesized a series of novel 2,4-disubstituted-7-methyl-1,1,3-trioxo-2,4-dihydro-pyrazolo[4,5-*e*][1,2,4]thiadiazines (PTDs), which were structurally confirmed by IR, ^{1}H-NMR, ^{13}C-NMR, and MS spectral analysis and evaluated for their inhibition of HIV (HIV-1 III_B and HIV-2 ROD)-induced cytopathogenicity in MT-4 cell culture. The results indicated that PTD analogues derived from the TTDs by pyrazole-ring substitution for thiophene exhibited decreased anti-HIV-1 activity. Unexpected was the finding that PTD analogues were potential and specific HIV-2 inhibitors. The most active compound was compound **7i** (R_1 = benzyl, R_2 = 4-*t*-butyl-benzyl) with an EC_{50} value of 18.7 μM and SI=15, which provides a useful lead for further molecular optimization.

Experimental

Chemistry

All melting points were determined on a micro melting-point apparatus and are uncorrected. The ^{1}H- and ^{13}C-NMR spectra were obtained on a Brucker Avance-600 (600 MHz; Bruker Bioscience, Billerica, MA, USA). Chemical shifts are expressed in d units and TMS as internal reference. Infrared spectra (IR) were recorded with a Nexus 470FT-IR Spectrometer (Thermo Nicolet, Madison, WI, USA). Mass spectra were taken on a LC Autosampler Device: Standard G1313A instrument (Agilent, Palo Alto, CA, USA). Flash column chromatography was performed on column packed with silica gel 60 (230 – 400 mesh). Solvents were reagent grade and, when necessary, were purified and dried by standard methods. Concentration of the reaction solutions involved the use of rotary evaporator at reduced pressure.

*General procedure for the preparation of compounds N_2-benzyl, N_4-substituted 7-methyl-1,1,3-trioxo-2,4-dihydro-pyrazolo[4,5-e][1,2,4]thiadiazines **7a–l***

To a solution of 7-methyl-1,1,3-trioxo-2,4-dihydro-pyrazolo[4,5-*e*][1,2,4]thiadiazine (**5**, 1 eq) in dry DMF (1 mmol, 4 mL) was added sodium hydride (60% dispersion in mineral oil, 1 eq) in portions, under inert atmosphere (N_2) and keeping the temperature at 10°C. After 30 minutes stirring, the alkyl halide (1 eq) was added dropwise. The mixture was stirred at room temperature for 20 min and 30 – 50°C for 12 – 20 h (checked by TLC). After the solvent was evaporated off under reduced pressure, the crude product was purified by recrystallization from ethanol.

*2-Benzyl-4-cyanomethyl-7-methyl-1,1,3-trioxo-2,4-dihydro-pyrazolo[4,5-e][1,2,4]thiadiazine **7a***

Compound **5** reacted with chloroacetonitrile at 50°C for 20 h to give compound **7a**, which was purified by recrystallization from ethanol as brown solid (51%, mp. 176 – 178°C). IR (KBr, cm^{-1}): 1686 (C=O), 1333, 1197 (SO_2). ^{1}H-NMR ($CDCl_3$, 600 MHz) δ: 7.28 (s, 1H, PyH), 7.33 – 7.51 (m, 5H, PhH), 5.08 (s, 2H, NCH_2), 4.77 (s, 2H, NCH_2), 4.19 (s, 3H, CH_3). ^{13}C-NMR ($CDCl_3$, 150 MHz) δ: 148.4 (C=O), 134.5 (C-1′), 128.8, 128.4, 128.2, 123.9 (C-5), 123.6 (C-4a), 123.0 (C-7a), 113.0 (CN), 45.1 (N_2-CH_2), 39.2 (N_4-CH_2), 33.1 (CH_3). MS ($C_{14}H_{13}N_5O_3S$ 331.35): m/z (ESI) 332.4 [M + 1].

*2,4-Dibenzyl-7-methyl-1,1,3-trioxo-2,4-dihydro-pyrazolo[4,5-e][1,2,4]thiadiazine **7b***

Compound **5** reacted with benzyl bromide at 30°C for 12 h to give compound **7b**, which was purified by recrystallization from ethanol as white solid (72%, mp. 128 – 130°C). IR (KBr, cm^{-1}): 1668 (C=O), 1331, 1193 (SO_2). ^{1}H-NMR ($CDCl_3$, 600 MHz) δ: 7.16 (s, 1H, PyH), 7.51 (d, 2H, *J* = 8.50 Hz, PhH), 7.21 (d, 2H, *J* = 8.03 Hz, PhH), 7.29 – 7.37 (m, 6H, PhH), 5.13 (s, 2H, NCH_2), 5.07 (s, 2H, NCH_2), 4.12 (s, 3H, CH_3). ^{13}C-NMR ($CDCl_3$, 150 MHz) δ: 149.8 (C=O), 135.1, 135.9, 129.2, 128.9, 128.7, 127.1, 128.3, 128.2, 126.0 (C-5), 125.4 (C-4a), 123.2 (C-7a), 49.7 (N_2-CH_2), 44.9 (N_4-CH_2), 39.2 (CH_3). MS ($C_{19}H_{18}N_4O_3$ S 382.44): m/z (ESI) 383.4 [M + 1].

*2-Benzyl-4-(o-bromobenzyl)-7-methyl-1,1,3-trioxo-2,4-dihydro-pyrazolo[4,5-e][1,2,4]thiadiazine **7c***

Compound **5** reacted with 2-bromobenzyl bromide at 30°C for 12 h to give compound **7c**, which was purified by recrystallization from ethanol as white solid (71%, mp. 106 – 108°C). IR (KBr, cm^{-1}): 1692 (C=O), 1324, 1192 (SO_2). ^{1}H-NMR ($CDCl_3$, 600 MHz) δ: 7.11 (s, 1H, PyH), 7.60 (d, 1H, *J* = 7.87 Hz, PhH), 7.51 (d, 2H, *J* = 7.46 Hz, PhH), 6.91 (d, 1H, *J* = 7.57 Hz, PhH), 6.90 – 7.52 (m, 5H, PhH), 5.17 (s, 2H, NCH_2), 5.14 (s, 2H, NCH_2), 4.15 (s, 3H, CH_3). ^{13}C-NMR ($CDCl_3$, 150 MHz) δ: 148.3 (C=O), 135.3, 133.3, 129.2, 128.3, 132.9, 129.2, 127.9, 127.8, 126.9, 125.3 (C-5), 125.1 (C-4a), 122.9 (C-7a), 122.2, 49.1 (N_2-CH_2), 44.6 (N_4-CH_2), 38.9 (CH_3). MS ($C_{19}H_{17}BrN_4O_3S$ 461.33): m/z (ESI) 461.3 [M^+], 463.3 [M + 2].

2-Benzyl-4-(p-bromobenzyl)-7-methyl-1,1,3-trioxo-2,4-dihydro-pyrazolo[4,5-e][1,2,4]thiadiazine ***7d***

Compound **5** and 4-bromobenzyl bromide at 30°C for 12 h gave compound **7d**, which purified by recrystallization from ethanol as white solid (73%, mp. 168 – 170°C). IR (KBr, cm^{-1}): 1694 (C=O), 1330, 1149 (SO_2). ^{1}H-NMR (DMSO-d_6, 600 MHz) δ: 7.83 (s, 1H, PyH), 7.31 (d, 2H, *J* = 8.43 Hz, PhH), 7.21 (d, 2H, *J* = 8.42 Hz, PhH), 7.53 – 7.55 (m, 5H, PhH), 5.07 (s, 2H, NCH_2), 5.01 (s, 2H, NCH_2), 4.05 (s, 3H, CH_3). ^{13}C-NMR (DMSO-d_6, 150 MHz) δ: 148.9 (C=O), 135.6, 135.4, 131.7 131.4 130.0, 129.4, 126.2 (C-5), 125.5 (C-4a), 122.1 (C-7a), 121.0, 120.9, 48.2 (N_2-CH_2), 43.5 (N_4-CH_2), 38.1 (CH_3). MS ($C_{19}H_{17}BrN_4O_3S$ 461.33): m/z (ESI) 461.3 [M^+], 463.3 [M + 2].

2-Benzyl-4-(o-chlorobenzyl)-7-methyl-1,1,3-trioxo-2,4-dihydro-pyrazolo[4,5-e][1,2,4]thiadiazine ***7e***

Compound **5** reacted with 2-chlorobenzyl chloride at 45°C for 20 h to give compound **7e**, which was purified by recrystallization from ethanol as white solid (65%, mp. 104 – 106°C). IR (KBr, cm^{-1}): 1691 (C=O), 1326, 1193 (SO_2). ^{1}H-NMR ($CDCl_3$, 600 MHz) δ: 6.95 (s, 1H, PyH), 7.13 – 7.51 (m, 9H, PhH), 5.19 (s, 2H, NCH_2), 5.14 (s, 2H, NCH_2), 4.15 (s, 3H, CH_3). ^{13}C-NMR ($CDCl_3$, 150 MHz) δ: 149.3 (C=O), 132.5, 135.3, 128.6, 128.3, 131.8, 129.7, 129.0, 127.9, 127.2, 170.0, 125.3 (C-5), 124.9 (C-4a), 120.0 (C-7a), 46.6 (N_2-CH_2), 44.5 (N_4-CH_2), 38.9 (CH_3). MS ($C_{19}H_{17}ClN_4O_3S$ 416.88): m/z (ESI) 417.4 [M + 1].

2-Benzyl-4-(m-chlorobenzyl)-7-methyl-1,1,3-trioxo-2,4-dihydro-pyrazolo[4,5-e][1,2,4]thiadiazine ***7f***

Compound **5** reacted with 3-chlorobenzyl chloride at 45°C for 20 h to give compound **7f**, which was purified by recrystallization from ethanol as white solid (64%, mp. 106 – 108°C). IR (KBr, cm^{-1}): 1665 (C=O), 1320, 1194 (SO_2). ^{1}H-NMR ($CDCl_3$, 600 MHz) δ: 7.15 (s, 1H, PyH), 7.19 (s, 1H, PhH), 7.07 (d, 1H, *J* = 6.97 Hz, PhH), 7.20 – 7.51 (m, 7H, PhH), 5.12 (s, 2H, NCH_2), 5.03 (s, 2H, NCH_2), 4.14 (s, 3H, CH_3). ^{13}C-NMR ($CDCl_3$, 150 MHz) δ: 149.2 (C=O), 135.2, 136.6, 134.7, 130.0, 128.4, 128.3, 128.3, 128.1, 127.8, 126.8, 125.2 (C-5), 124.7 (C-4a), 122.7 (C-7a), 48.6 (N_2-CH_2), 44.4 (N_4-CH_2), 38.8 (CH_3). MS ($C_{19}H_{17}ClN_4O_3S$ 416.88): m/z (ESI) 417.4 [M + 1].

2-Benzyl-4-(p-chlorobenzyl)-7-methyl-1,1,3-trioxo-2,4-dihydro-pyrazolo[4,5-e][1,2,4]thiadiazine ***7g***

Compound **5** reacted with 4-chlorobenzyl chloride at 45°C for 20 h to give compound **7g**, which was purified by recrystallization from ethanol as white solid (65%, mp. 92 – 94°C). IR (KBr, cm^{-1}): 1671 (C=O), 1320, 1193 (SO_2). ^{1}H-NMR ($CDCl_3$, 600 MHz) δ: 7.28 (s, 1H, PyH), 7.13 – 7.50 (m, 9H, PhH), 5.11 (s, 2H, NCH_2), 5.02 (s, 2H, NCH_2), 4.13 (s, 3H, CH_3). ^{13}C-NMR ($CDCl_3$, 150 MHz) δ: 149.2 (C=O), 133.8, 135.2, 133.1, 127.8, 128.9, 128.5, 128.3, 128.1, 125.2 (C-5), 124.7 (C-4a), 122.7 (C-7a), 48.5 (N_2-CH_2), 44.4 (N_4-CH_2), 38.9 (CH_3). MS ($C_{19}H_{17}ClN_4O_3S$ 416.88): m/z (ESI) 417.4 [M + 1].

2-Benzyl-4-(2,4-dichlorobenzyl)-7-methyl-1,1,3-trioxo-2,4-dihydro-pyrazolo[4,5-e][1,2,4]thiadiazine ***7h***

Compound **5** reacted with 2,4-dichlorobenzyl chloride at 50°C for 20 h to give compound **7h**, which was purified by recrystallization from ethanol as white solid (63%, mp. 112 – 114°C). IR (KBr, cm^{-1}): 1697 (C=O), 1324, 1190 (SO_2). ^{1}H-NMR ($CDCl_3$, 600 MHz) δ: 7.12 (s, 1H, PyH), 7.49 (d, 2H, *J* = 6.98 Hz, PhH), 7.43 (d, 1H, *J* = 2.68 Hz, PhH), 7.14 (dd, 1H, *J* = 8.38 Hz, *J* = 2.09 Hz, PhH), 6.87 (d, 1H, *J* = 8.39 Hz, PhH), 7.32 – 7.37 (m, 3H, PhH), 5.13 (s, 2H, NCH_2), 5.12 (s, 2H, NCH_2), 4.15 (s, 3H, CH_3). ^{13}C-NMR ($CDCl_3$, 150 MHz) δ: 149.2 (C=O), 134.2, 135.2, 133.1, 129.4, 128.5, 128.3, 127.9, 127.8, 127.5, 125.1 (C-5), 124.7 (C-4a), 122.9 (C-7a), 46.1 (N_2-CH_2), 44.5 (N_4-CH_2), 38.9 (CH_3). MS ($C_{19}H_{16}Cl_2N_4O_3S$ 451.33): m/z (ESI) 451.3 [M^+].

2-Benzyl-4-(p-(t-butyl)chlorobenzyl)-7-methyl-1,1,3-trioxo-2,4-dihydro-pyrazolo[4,5-e][1,4]thiadiazine ***7i***

Compound **5** reacted with *p*-(*t*-butyl)chlorobenzyl chloride at 50°C for 20 h to give compound **7i**, which was purified by recrystallization from ethanol as white solid (55%, mp. 76 – 78°C). IR (KBr, cm^{-1}): 1690 (C=O), 1327, 1190 (SO_2). ^{1}H-NMR ($CDCl_3$, 600 MHz) δ: 7.20 (s, 1H, PyH), 7.15 (d, 2H, *J* = 8.26 Hz, PhH), 7.32 – 7.52 (m, 7H, PhH), 5.12 (s, 2H, NCH_2), 5.03 (s, 2H, NCH_2), 4.12 (s, 3H, CH_3), 1.30 (s, 9H, CH_3). ^{13}C-NMR ($CDCl_3$, 150 MHz) 150.8 (C=O), 131.5, 135.4, 149.2, 128.4, 128.2, 127.7, 126.5, 125.6, 125.5, 125.0 (C-4a), 122.5 (C-7a), 48.9 (N_2-CH_2), 44.3 (N_4-CH_2), 38.8 (CH_3), 34.3 (C), 31.0 (3C, CH_3). MS ($C_{23}H_{26}N_4O_3S$ 438.54): m/z (ESI) 439.5 [M + 1].

2-Benzyl-4-(o-cyanobenzyl)-7-methyl-1,1,3-trioxo-2,4-dihydro-pyrazolo[4,5-e][**1,2,4**]**thiadiazine** ***7j***

Compound **5** reacted with 2-cyanobenzyl chloride at 50°C for 20 h to give compound **7j**, which was purified by recrystallization from ethanol as white solid (45%, mp. 136 – 138°C). IR (KBr, cm^{-1}): 2224 (CN), 1690 (C=O), 1328, 1196 (SO_2). ^{1}H-NMR (DMSO-d_6, 600 MHz) d: 7.84 (s, 1H, PyH), 7.15 (d, 1H, *J* = 7.88 Hz, PhH), 7.26 – 7.89 (m, 8H, PhH), 5.29 (s, 2H, NCH_2), 5.02 (s, 2H, NCH_2), 4.09 (s, 3H, CH_3). MS ($C_{20}H_{17}N_5O_3S$ 407.45): m/z (ESI) 408.5 [M + 1].

2-Benzyl-4-(m-cyanobenzyl)-7-methyl-1,1,3-trioxo-2,4-dihydro-pyrazolo[4,5-e][1,2,4]thiadiazine ***7k***

Compound **5** reacted with 3-cyanobenzyl chloride at 50°C for 20 h to give compound **7k**, which was purified by recrystallization from ethanol as white solid (46%, mp. 208 – 810°C). IR (KBr, cm^{-1}): 2229 (CN), 1689 (C=O), 1331, 1192 (SO_2). ^{1}H-NMR ($CDCl_3$, 600 MHz) δ: 7.14 (s, 1H, PyH), 7.28 – 7.61 (m, 9H, PhH), 5.12 (s, 2H, NCH_2), 5.07 (s, 2H, NCH_2), 4.16 (s, 3H, CH_3). ^{13}C-NMR ($CDCl_3$, 600 MHz) δ: 144.4 (C=O), 130.3, 131.5, 126.9, 126.2, 125.4, 124.9, 123.2, 123.7, 123.6, 108.3, 120.3, 119.6, 118.1, 113.1 (CN), 43.7 (N_2-CH_2), 39.8 (N_4-CH_2), 34.2 (CH_3). MS ($C_{20}H_{17}N_5O_3S$ 407.45): m/z (ESI) 408.5 [M + 1].

2-Benzyl-4-(p-cyanobenzyl)-7-methyl-1,1,3-trioxo-2,4-dihydro-pyrazolo[4,5-e][1,2,4]thiadiazine ***7l***

Compound **5** reacted with 4-cyanobenzyl chloride at 50°C for 20 h to give compound **7l**, which was purified by recrystallization from ethanol as white solid (47%, mp. 148 – 150°C). IR (KBr, cm^{-1}): 2229 (CN), 1693 (C=O), 1325, 1196 (SO_2). ^{1}H-NMR ($CDCl_3$, 600 MHz) δ: 7.11 (s, 1H, PyH), 7.49 (d, 2H, *J* = 7.60 Hz, PhH), 7.28-7.63 (m, 7H, PhH), 5.11 (s, 2H, NCH_2), 5.10 (s, 2H, NCH_2), 4.15 (s, 3H, CH_3). ^{13}C-NMR ($CDCl_3$, 150 MHz) δ: 149.2 (C=O), 135.1, 139.9, 132.6, 128.5, 128.3, 127.9, 127.2, 111.9, 125.1 (C-5), 124.4 (C-4a), 123.0 (C-7a), 118.0 (CN), 48.7 (N_2-CH_2), 44.6 (N_4-CH_2), 38.9 (CH_3). MS ($C_{20}H_{17}N_5O_3S$ 407.45): m/z (ESI) 408.5 [M + 1].

*General procedure for the preparation of compounds N_2,N_4-disubstituted 7-methyl-1,1,3-trioxo-2,4-dihydro-pyrazolo[4,5-e][1,2,4]thiadiazines **7m–r***

To a solution of compound (**5**, 1 eq) in dry DMF (1 mmol, 4 mL) was added sodium hydride (60% dispersion in mineral oil, 2 eq) in portions, under inert atmosphere (N_2) and keeping the temperature at 10°C. After 60 minutes stirring, the alkyl halide (RX, 2 eq) was added dropwise. The mixture was stirred at room temperature for 20 min., and 30 – 80°C for 12 – 20 h (checked by TLC). After the solvent was evaporated off under reduced pressure, the crude product was purified by recrystallization from ethanol.

*2,4-Di-(p-methylbenzyl)-7-methyl-1,1,3-trioxo-2,4-dihydro-pyrazolo[4,5-e][1,2,4]thiadiazine **7m***

Compound **5** reacted with 4-methylbenzyl chloride at 60 – 70°C for 20 h, purification by recrystallization from ethanol gave compound **7m** as white solid (60%), mp. 116 – 118°C. IR (KBr, cm^{-1}): 1668 (C=O), 1331, 1192 (SO_2). ^{1}H-NMR (DMSO-d_6, 600 MHz) δ: 7.80 (s, 1H, PyH), 7.12 – 7.24 (m, 8H, PhH), 5.04 (s, 2H, NCH_2), 4.98 (s, 2H, NCH_2), 4.04 (s, 3H, CH_3), 2.27 (s, 3H, CH_3), 2.25 (s, 3H, CH_3). ^{13}C-NMR (DMSO-d_6, 150 MHz) δ: 148.6 (C=O), 137.1, 137.2, 133.2, 133.0, 129.4, 129.1, 128.0, 127.3, 126.3 (C-5), 125.5 (C-4a), 122.3 (C-7a), 48.4 (N_2-CH_2), 43.8 (N_4-CH_2), 39.0 (Py-CH_3), 20.8, 20.9 (2C, CH_3). MS ($C_{21}H_{22}N_4O_3S$ 410.49): m/z (ESI) 411.5 [M + 1].

*2,4-Di-(o-bromobenzyl)-7-methyl-1,1,3-trioxo-2,4-dihydro-pyrazolo[4,5-e][1,2,4]thiadiazine **7n***

Compound **5** reacted with 2-bromobenzyl bromide at 40 – 50°C for 15 h, purification by recrystallization from ethanol gave compound **7n** as white solid (81%), mp. 120 – 122°C. IR (KBr, cm^{-1}): 1697 (C=O), 1322, 1193 (SO_2). ^{1}H-NMR (DMSO-d_6, 600 MHz) δ: 7.79 (s, 1H, PyH), 7.67 (dd, 1H, *J* = 7.95 Hz, *J* = 1.10 Hz, PhH), 7.40 (d, 1H, *J* = 7.52 Hz, PhH), 7.03 (dd, 1H, *J* = 7.70 Hz, *J* = 1.23 Hz, PhH), 7.23 – 7.38 (m, 5H, PhH), 5.12 (s, 2H, NCH_2), 5.06 (s, 2H, NCH_2), 4.09 (s, 3H, CH_3). ^{13}C-NMR (DMSO-d_6, 150 MHz) δ: 148.7 (C=O), 134.4, 134.6, 133.2, 132.7, 129.7, 129.6, 128.3, 128.1, 127.7, 127.2, 126.4, 125.9 (C-5), 122.1, 122.0, 121.6, 49.7 (N_2-CH_2), 44.2 (N_4-CH_2), 39.2 (CH_3). MS ($C_{19}H_{16}Br_2N_4O_3S$ 540.23): m/z (ESI) 541.3 [M + 1].

*2,4-Di-(p-(t-butyl)benzyl)-7-methyl-1,1,3-trioxo-2,4-dihydro-pyrazolo[4,5-e][1,2,4]thiadiazine **7o***

Compound **5** reacted with 4-(*t*-butyl)benzyl chloride at 70 – 80°C for 20 h, purification by recrystallization from ethanol gave compound **7p** as white solid (65%), mp. 130 – 132°C. IR (KBr, cm^{-1}): 1695 (C=O), 1334, 1186 (SO_2). ^{1}H-NMR ($CDCl_3$, 600 MHz) δ: 7.19 (s, 1H, PyH), 7.44 (d, 2H, *J* = 8.33 Hz, PhH), 7.37 (d, 2H, *J* = 8.39 Hz, PhH), 7.32 (d, 2H, *J* = 8.33 Hz, PhH), 7.14 (d, 2H, *J* = 8.30 Hz, PhH), 5.09 (s, 2H, NCH_2), 5.03 (s, 2H, NCH_2), 4.12 (s, 3H, CH_3), 1.32 (s, 9H, CH_3), 1.30 (s, 9H, CH_3). ^{13}C-NMR ($CDCl_3$, 150 MHz) δ: 150.7 (C=O), 131.5, 132.3, 150.6, 149.2, 128.3, 126.5, 125.6, 125.5, 125.2, 125.0, 122.5 (C-4a), 122.0 (C-7a), 48.8 (N_2-CH_2), 44.0 (N_4-CH_2), 38.8 (CH_3), 34.3, 34.2 (2C), 31.0, 30.9 (6C, CH_3). MS ($C_{27}H_{34}N_4O_3S$ 494.65): m/z (ESI) 495.5 [M + 1].

*2,4-Di-(2,4-dichlorobenzyl)-7-methyl-1,1,3-trioxo-2,4-dihydro-pyrazolo[4,5-e][1,2,4]thiadiazine **7p***

Compound **5** reacted with 2,4-dichlorobenzyl chloride at 60 – 70°C for 20 h, purification by recrystallization from ethanol gave compound **7r** as white solid (73%), mp. 176 – 178°C. IR (KBr, cm^{-1}): 1698 (C=O), 1326, 1186 (SO_2). ^{1}H-NMR (DMSO-d_6, 600 MHz) δ: 7.82 (s, 1H, PyH), 7.69 (d, 1H, *J* = 2.12 Hz, PhH), 7.66 (d, 1H, *J* = 2.14 Hz, PhH), 7.31 (d, 1H, *J* = 8.47 Hz, PhH), 7.12 (d, 1H, *J* = 8.41 Hz, PhH), 7.38 – 7.43 (m, 2H, PhH), 5.13 (s, 2H, NCH_2), 5.08 (s, 2H, NCH_2), 4.08 (s, 3H, CH_3). ^{13}C-NMR (DMSO-d_6, 600 MHz) δ: 148.6 (C=O), 133.0, 133.2, 133.1, 132.7, 132.6, 132.2, 129.5, 129.4, 129.1, 128.9, 127.8, 127.7, 126.4 (C-5), 125.8 (C-4a), 122.0 (C-7a), 47.1 (N_2-CH_2), 41.4 (N_4-CH_2), 39.1 (CH_3). MS ($C_{19}H_{14}Cl_4N_4O_3S$ 520.22): m/z (ESI) 520.2 [M$^+$].

*2,4-Di-(p-chlorobenzyl)-7-methyl-1,1,3-trioxo-2,4-dihydro-pyrazolo[4,5-e][1,2,4]thiadiazine **7q***

Compound **5** reacted with 4-chlorobenzyl chloride at 50 – 60°C for 20 h, purification by recrystallization from ethanol gave compound **7q** as white solid (75%), mp. 146 – 148°C. IR (KBr, cm^{-1}): 1696 (C=O), 1332, 1190 (SO_2). ^{1}H-NMR (DMSO-d_6, 600 MHz) δ: 7.84 (s, 1H, PyH), 7.28 (d, 2H, *J* = 8.50 Hz, PhH), 7.37-7.42 (m, 6H, PhH), 5.09 (s, 2H, NCH_2), 5.03 (s, 2H, NCH_2), 4.05 (s, 3H, CH_3). ^{13}C-NMR (DMSO-d_6, 150 MHz) δ: 149.0 (C=O), 135.0, 135.3, 132.5, 132.6, 129.8, 129.2, 128.9, 128.6, 126.3 (C-5), 125.5 (C-4a), 122.2 (C-7a), 48.2 (N_2-CH_2), 43.4 (N_4-CH_2), 39.1 (CH_3). MS ($C_{19}H_{16}Cl_2N_4O_3S$ 451.33): m/z (ESI) 451.4 [M$^+$].

*2,4-Di-(p-bromobenzyl)-7-methyl-1,1,3-trioxo-2,4-dihydro-pyrazolo[4,5-e][1,2,4]thiadiazine **7r***

Compound **5** reacted with 4-bromobenzyl bromide at 40 – 50°C for 15 h, purification by recrystallization from ethanol gave compound **7o** as white solid (83%), mp. 182 – 184°C. IR (KBr, cm^{-1}): 1693 (C=O), 1329, 1189 (SO_2). ^{1}H-NMR (DMSO-d_6, 600 MHz) δ: 7.83 (s, 1H, PyH), 7.31 (d, 2H, *J* = 8.41 Hz, PhH), 7.21 (d, 2H, *J* = 8.38 Hz, PhH), 7.53 – 7.55 (m, 4H, PhH), 5.07 (s, 2H, NCH_2), 5.01 (s, 2H, NCH_2), 4.05 (s, 3H, CH_3). ^{13}C-NMR (DMSO-d_6, 150 MHz) 150.0 (C=O), 135.4, 135.6, 131.7 131.4 130.0, 129.4, 126.2 (C-5), 125.5 (C-4a), 122.1 (C-7a), 121.0, 120.9, 48.2 (N_2-CH_2), 43.5 (N_4-CH_2), 39.0 (CH_3). MS ($C_{19}H_{16}Br_2N_4O_3S$ 540.23): m/z (ESI) 541.2 [M + 1].

Anti-HIV activity assays

The anti-HIV activity and cytotoxicity were evaluated against wild type HIV-1 strain IIIB and HIV-2 (ROD) in MT-4 cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method [14] MT-4 cells were suspended in culture medium at 1×10^5 cells/mL and infected with HIV at a multiplicity of infection (MOI) of 0.02. Immediately after viral infection, 100 µL of the cell suspension were placed in each well of a flat-bottomed microtiter tray containing various concentrations of the test compounds. Stock solutions of the test compounds were prepared in DMSO at a concentration of 10 mg/mL. After four days of incubation at 37°C, the number of viable cells was determined using the MTT method. Compounds were tested in parallel for cytotoxic effects in uninfected MT-4 cells.

The 50% effective antiviral concentration (EC_{50}) was defined as the compound concentration required protecting 50% of the virus-infected cells against viral cytopathicity. The 50% cytotoxic concentration (CC_{50}) was defined as the compound concentration required reducing the viability of mock-infected cells by

50%. The symbol ">" is used to indicate the highest concentration at which the compounds were tested and still found to be non-cytotoxic. Average EC_{50} and CC_{50} values for at least two separate experiments are presented.#

The financial support from the National Natural Science Foundation of China (NSFC No.30772629 and 30371686), key project of The International Cooperation, Ministry of Science and Technology of China (2003DF000033) and Natural Science Foundation of Shandong Province (Y2003C11), is gratefully acknowledged.

The authors have declared no conflict of interest.

References

[1] AIDS Epidemic Update: December **2006**, *UNAIDS/WHO*, http: //www.unaids.org.

[2] E. De Clercq, *Antiviral. Res.* **2005**, *67*, 56–75.

[3] Z. Zhou, X. Lin, J. D. Madura, *Infect. Disord. Drug. Targets* **2006**, *6*, 391–413.

[4] M. A. Wainberg, J. P. Sawyer, J. S. Montaner, R. L. Murphy, *et al., Antivir. Ther.* **2005**, *10*, 13–28.

[5] M. E. Arranz, J. A. Dia, S. T. Ingate, M. Witvrouw, *et al.*, *J. Med. Chem.* **1998**, *41*, 4109–4117.

[6] M. Witvrouw, M. E. Arranz, C. Pannecouque, R. Declercq, *et al.*, *Antimicrob. Agents Chemother.* **1998**, *42*, 618–623.

[7] M. E. Arranz, J. A. Dia, S. T. Ingate, M. Witvrouw, *et al.*, *Bioorg. Med. Chem.* **1999**, *7*, 2811–2822.

[8] W. Schafer, W. G. Friebe, H. Leinert, H. Mertens, *et al.*, *J. Med. Chem.* **1993**, *36*, 726–732.

[9] G. P. Patani, E. J. LaVoie, *Chem. Rev.* **1996**, *96*, 3147–3176.

[10] X. Y. Liu, N. G. Chen, R. Z. Yan, W. F. Xu, *et al.*, *Heterocycles* **2006**, *68*, 1225–1232.

[11] X. Y. Liu, R. Z. Yan, N. G. Chen, W. F. Xu, *et al.*, *Molecules* **2006**, *11*, 827–836.

[12] E. De Clercq, *J. Med. Chem*. **1995**, *38*, 2491–2517.

[13] N. A. Smith, T. Shaw, N. Berry, C. Vella, *et al.*, *J. Infect.* **2001**, *42*, 126–133.

[14] R. Pauwels, J. Balzarini, M. Baba, R. Snoeck, *et al.*, *J. Virol. Methods* **1988**, *20*, 309–321.

