Full Paper

Synthesis and Antiviral Activity of New Substituted Methyl [2-(arylmethylene-hydrazino)-4-oxo-thiazolidin-5-ylidene]acetates

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A series of new methyl [2-(arylmethylene-hydrazono)-4-oxo-thiazolidin-5-ylidene]acetates (**5a–o**) were synthesized via cyclocondensation of thiosemicarbazones (**3a–o**) with dimethyl but-2-ynedioate (**4**) in good yields under solvent-free conditions. The environmentally friendly solvent-free protocol overcomes the limitations associated with the customary protracted solution phase synthesis and afforded the title compounds in a few minutes. Compounds **5b–i** and **5h–o** were evaluated for their antiviral activity against the replication activity of HIV-1 and HIV-2 in MT-4 using the MTT assay. The same compounds were also evaluated *in vitro* for their selective antiviral activity against hepatitis C virus (HCV) in the Huh 5-2 replicon system (type 1b, Con1 strain).

Keywords: Anti-HCV activity / Anti-HIV activity / Non-nucleoside inhibitors (NNRTIs) / Thiazolidin-4-ones

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Introduction

The global spread and fatal prognosis of human immunodeficiency virus (HIV-1) infection, the causative agent in the transmission and the development of acquired immune deficiency syndrome (AIDS) [1], emphasize the urgent need for effective antiretroviral therapies. Combinations of RT nucleoside inhibitors (NRTIs), RT non-nucleoside inhibitors (NNRTIs) and protease inhibitors (PIs), clinically used for the treatment of HIV infections, dramatically decrease viral load in most infected persons, but resistance to the currently available hemotherapeutics invariably emerges [2]. Consequently, there is a high medical need to develop novel, selective, potent, safe, inexpensive antiviral agents, also effective against mutant strains of HIV.

Thiazole and thiazolidines are very useful intermediates/subunits for the development of molecules of pharmaceutical or biological interest [3–6] including antibacterial [7–9], anti-inflammatory [10], antiprotozoal [11, 12], anticonvulsant [13], anticancer [14, 15] properties, as effective antihypertensive agent (e.g.: β-(hydroxyethyl)thiazolidines **1**; Fig. 1) [16], and renin inhibitor [17]. In recent years, many research groups have been engaged in the development of new non-nucleoside RT inhibitors (NNRTI) having thiazole backbone such as thiazolo[3,4-*a*]benzimidazoles (TBZs) and their analogs as potent anti-HIV agents [18–21], and 1-(2,6-difluorophenyl)-thiazolo[3,4-*a*]benzimidazole **2** (Fig. 1) is one example of TBZs with a highly potential inhibitory of HIV-1-induced cytopathic effect in a variety of human cell lines, meanwhile it inhibited the replication of various strains of HIV-1 including azidovudine resistant strain (G910-6) [21]. Monforte et al. have reported the synthesis of 2,3-diaryl-1,3-thiazolidin-4-ones [22] as potent anti-HIV-1 agents, meanwhile Rawal et al. [23] have synthesized 2-(2,6-dibromophenyl)-3-heteroaryl-1,3-thiazolidin-4-ones which effectively inhibited HIV-1 replication at 20–320 nM. Phenethylthiazolethiourea (PTTA) compounds [24] have been reported as a new class of HIV RT inhibitors. Further, some derivatives of thiazoles exhibited remarkable activity against herpes simplex virus (HSV) [25]. A brief review of thiazoles associated with large number of biological activities is presented by Siddiqui et al. [26] and D'hooghe and De Kimpe [27]. In addition, some thiazole derivatives showed their potency as selective cyclooxygenase-

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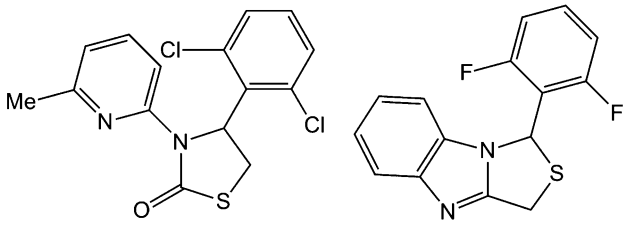


Figure 1. Structures of compounds **1** and **2**. **1**: 4-(2,4-Dichlorophenyl)-3-(6-methylpyridin-2-yl)thiazolidin-2-one. **2**: Thiazolobenzimidazole (TBZ).

2 [28], as well as bacterial DNA gyrase B [29] inhibitors, while some organosilicon-containing thiazole derivatives have been reported as potential lipoxygenase inhibitors and anti-inflammatory agents [30]. We have recently shown that Schiff base derivatives of some thiazolidines are potential urease inhibitors [31].

In continuation of our attempts in searching for new anti-HIV agents [32] and on the basis of above promising biological results, we considered thiazolidines and their analogs particularly interesting to optimize the synthetic approaches to our antiviral agents. In this study, we prepared a new series of thiazolidines with evaluation of their anti-HIV and anti-hepatitis virus (HCV) activity.

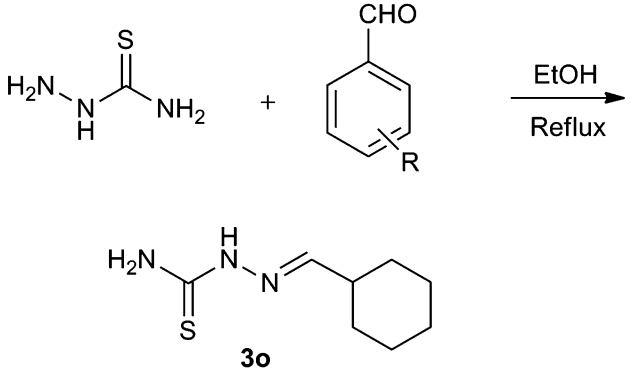
Results and discussion

Chemistry

A new series of thiazole derivatives, incorporating two important pharmacophores (thiazole and imino/hydrazono group), were synthesized by condensation of thiosemicarbazones with dimethyl acetylenedicarboxylate **4**. The first step of the synthesis involved the preparation of a series of thiosemicarbazones **3a–o** by acid catalyzed condensation of thiosemicarbazide with a range of substituted aromatic aldehydes. IR spectra of the synthesized hydrazide derivatives revealed the presence of C=S stretching at 1602 cm^{-1} (C=N), 1590 (Ar–C=C), in addition to stretching at 3385 and 3229 cm^{-1}, indicating the presence of NH_2 and NH, respectively. The ^1H NMR spectra of **3a–o** showed a singlet at $\sim \delta$ 8.11 ppm, assigned to azomethine (CH=N) protons while the singlets at $\sim\delta$ 10.40 and 7.50 ppm were attributed for NH and NH_2 protons, respectively. In the ^{13}C NMR spectra, the signals at $\sim\delta$ 179.0 and 153 ppm were assigned to C=S and C=N carbon atoms, respectively (Scheme 1).

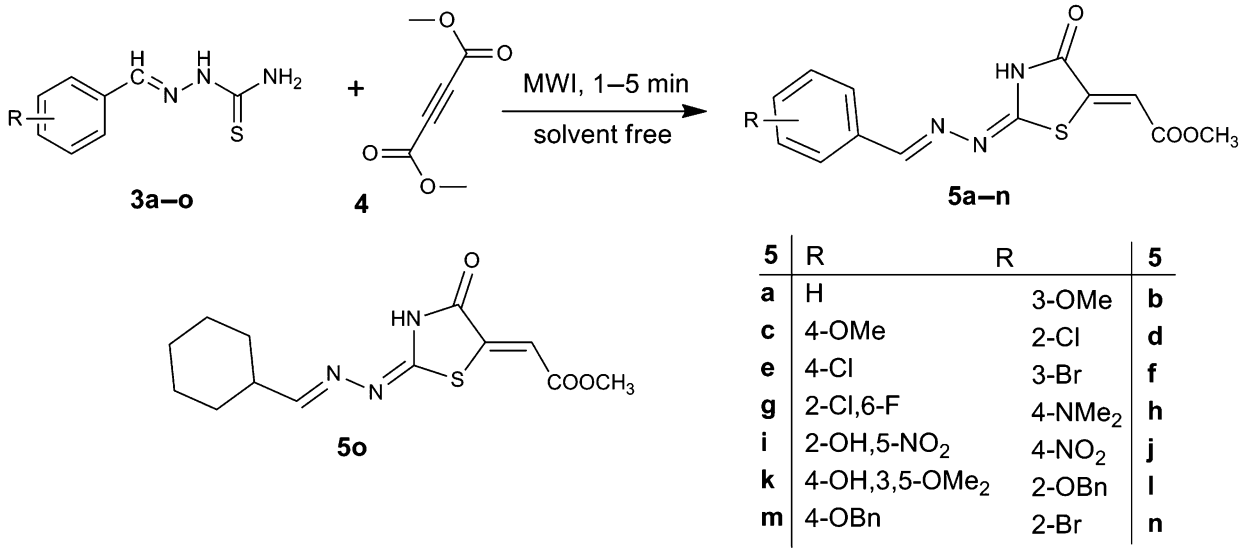
Dimethyl acetylenedicarboxylate **4** is a highly electrophilic reagent and widely employed as a dienophile in cycloaddition reactions, such as the Diels–Alder reaction and behaving as Michael acceptor in organic transformations. Next, treatment of an equimolar mixture of thiosemicarbazones **3a–o** with **4**, in the absence of any solvent or catalyst, afforded thiazolinones **5a–o** in good to excellent yields (Scheme 2).

The structures of **5a–o** were assigned on the basis of their ^1H and ^{13}C NMR, and mass spectra. In the ^1H NMR spectra of **5a–o**, the down-field singlets in the region δ 13.02–8.05 ppm were assigned to NH ring proton, while the azomethine protons ($C^2_{thiazol}H$=N) appeared in the region δ 9.35–7.48 ppm. The olefinic protons (CH=C) resonated in the region δ 6.72–6.40 ppm, while the singlets in the region δ 3.90–3.78 ppm were attributed to the protons acetoxy group. In the ^{13}C NMR spectra, carbonyl carbon atoms of the



	R	R	
3a	H	3-OMe	**3b**
3c	4-OMe	2-Cl	**3d**
3e	4-Cl	3-Br	**3f**
3g	2-Cl,6-F	4-NMe_2	**3h**
3i	2-OH,5-NO_2	4-NO_2	**3j**
3k	4-OH-3,5-OMe_2	2-OBz	**3l**
3m	4-OBz	2-Br	**3n**

Scheme 1. Synthesis of 1-(substituted benzylidene)thiosemicarbazides **3a–o**.



5	R	R	5
a	H	3-OMe	b
c	4-OMe	2-Cl	d
e	4-Cl	3-Br	f
g	2-Cl,6-F	4-NMe_2	h
i	2-OH,5-NO_2	4-NO_2	j
k	4-OH,3,5-OMe_2	2-OBn	l
m	4-OBn	2-Br	n

Scheme 2. Synthesis of methyl [(2-arylmethylene)-hydrazino)-4-oxo-thiazolidin]-acetates **5a–o**.

thiazole ring and the acetoxy group appeared in the regions δ 174.0–170.1 ppm and δ 168.5–164.5 ppm, respectively, whereas C-2 of the thiazole ring resonated in the region δ 159.4–156.3 ppm. The resonances in the region δ 149.6–145.2 ppm were assigned to azomethine carbon atoms attached to the aromatic rings (Ar*C*H=N), except those of compounds **5i** and **5l** which appeared at δ 160.3 and 158.4 ppm, respectively. Olefinic carbon atoms ($C_{thiazol}{}^5$=*C*H) were observed in the region δ 141.9–137.9 ppm, while C-5 of the thiazole ring appeared in the region δ 133.6–131.1 ppm. Aromatic carbon atoms and carbons of the other substituents were fully assigned (cf. Experimental section).

Compound **5e** was selected for further NMR studies. In the gradient-selected HMBC spectrum [33] of **5e**, the olefinic proton (CH=C) at δ_H = 8.14 ppm showed two $^2J_{C,H}$ couplings: one to C-5 of the thiazole ring at δ_C 131.4 ppm and the other coupling was with the carbonyl carbon atom of the ester group at δ_C 167.5 ppm, respectively. A $^3J_{C,H}$ between the same proton (CH=C) with carbonyl carbon atom of the thiazole ring at δ_C 171.0 ppm was observed. Furthermore, a $^2J_{C,H}$ coupling between azomethine proton at δ_H 6.60 ppm and aromatic carbon atom (C-1) at δ_C 136.8 ppm was assigned (Fig. 2).

Mass spectral data and elemental analyses are also in accordance with the proposed structure of compounds **5a–o**.

Bioactivities

In vitro anti-HIV assay

Compounds **5a–i** and **5k–o** were evaluated for their *in vitro* anti-HIV-1 (strain III_B) and anti-HIV-2 (strain ROD) activity and monitored by the inhibition of the virus induced cytopathic effect in the (MT-4) cells, based on MTT assay [34]. The results are summarized in Table 1, in which the data for nevirapine (BOE/BIRG587) [35, 36] and azidothymidine (DDN/AZT) [37] were included for comparison. Compound **5i** was found to be the only compound in the series inhibiting HIV-1 and HIV-2 replication in cell cultures with EC_{50} of >0.088 μM, but no selectivity was witnessed (SI < 1). However, implantation of hydroxy and nitro in 2 and 5 positions of the phenyl group considerably increased the anti-HIV activity, in comparison to the effectiveness of other functional groups.

In vitro anti-HCV assay

Compounds **5b–i** and **5k–o** were evaluated for their *in vitro* selective antiviral activity against hepatitis C virus in the Huh 5-2 replicon system (type 1b, Con1 strain). The results are shown in Table 2.

The establishment of stable HCV subgenomic replicon systems [38, 39] in the human hepatoblastoma cell line Huh7 has provided a useful system for the development of new antiviral approaches against HCV [40–43]. The overriding aims of the new therapeutic strategies are higher efficacy

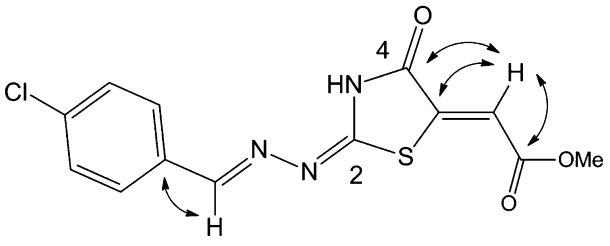


Figure 2. $J_{C,H}$ correlations in the HMBC NMR spectrum of **5e**.

Table 1. *In vitro* anti-HIV-1[a] and HIV-2[b] activity and cytotoxicity of some new 1,3-thiazolidin-4-ones.

Entry	HIV-1 (III_B) EC_{50} (μM)[c]	HIV-2 (ROD) EC_{50} (μM)[c]	CC_{50} (μM)[d]	SI[e] (III_B)	SI[e] (ROD)
5b	>61.30	>61.30	61.30	<1	<1
5c	>72.10	>72.10	72.10	<1	<1
5d	>78.70	>78.70	78.70	<1	<1
5e	>4.23	>4.23	4.23	<1	<1
5f	>11.15	>11.15	11.15	<1	<1
5g	>52.70	>52.70	52.70	<1	<1
5h	>87.25	>87.25	87.25	<1	<1
5i	>0.088	>0.088	0.088	<1	<1
5k	>14.50	>14.50	14.50	<1	<1
5l	>14.35	>14.35	14.35	<1	<1
5m	>97.45	>97.45	97.45	<1	<1
5n	>13.45	>13.45	13.45	<1	<1
5o	>57.53	>57.53	57.53	<1	<1
Nevirapine	0.050	>4.00	>4.00	>80	<1
AZT	0.0022	0.00094	>25	>11,363	>26,596

[a] Anti-HIV-1 activity measured with strain III_B.
[b] Anti-HIV-2 activity measured with strain ROD.
[c] Compound concentration required to achieve 50% protection of MT-4 cells from the HIV-1 and 2-induced cytopathogenic effect.
[d] Compound concentration that reduces the viability of mock-infected MT-4 cells by 50%.
[e] SI: selectivity index (CC_{50}/EC_{50}).

associated with shortened duration of treatment, favorable mode of administration, and thus improved tolerability and adherence.

However, compounds **5d** and **5g** showed EC_{50} of 3.15 and 3.26 μM with CC_{50} of 29.2 μM and >50 μM, resulting in a selectivity index of 9.27 and >15.4, in addition to inhibition of 89.8 and 77%, respectively.

Even though for many compounds an EC_{50} was obtained with selectivity index (SI) up to >15.4 (e.g. compound **5g**) (Table 2), none of the compounds matched the selection criteria of a selective inhibitor of virus replication in this assay (i.e. >70% inhibition at concentrations that do not elicit an anti-metabolic effect on the host cells).

Conclusion

In conclusion, we report the *in vitro* antiviral activity against HIV-1 (III_B strain) and HIV-2 (ROD strain) and HCV (type 1b, Con1 strain) of methyl [2-(arylmethylene-hydrazono)-4-oxo-thiazolidin-5-ylidene]-acetates **5a–i** and **5j–o**. The first results

Table 2. *In vitro* anti-HCV-1b activity, cytotoxicity, and inhibition% of some thiazolidin-4-ones.

Entry	CC_{50} μM	EC_{50} μM	SI	% Inh	at []
5b	>50	=8.28	=6.04	76.1	34
5c	=36.9	=14.7	=2.52	64.9	31.7
5d	=29.2	=31.5	=9.27	89.8	12.8
5e	>50	=18	>2.78	80.2	48.9
5f	=23.2	=2.95	=7.88	71.8	9.6
5g	>50	=3.26	>15.4	77	20.9
5h	>50	=14.4	=3.47	78.8	41.6
5i	=5.86	=1.94	=3.01	64.5	4.79
5k	=26.2	=22.2	=1.18	54.1	31.4
5l	=23.9	=3.9	=6.11	82.8	12.7
5m	>50	=12.8	>3.91	81.5	50
5n	=25.7	=8.8	=9.27	89.9	12.8
5o	=29.9	=16.5	=1.81	62.6	30.1

CC_{50} = 50% cytostatic/cytotoxic concentration (concentration at which 50% adverse effect is observed on the host cell).
EC_{50} = 50% effective concentration (concentration at which 50% inhibition of virus replication is observed).
SI = Selectivity index (CC_{50}/EC_{50}).
[] = Concentration [μM].

showed that **5i** has antiviral properties against HIV with $EC_{50} = 0.088$ μM. Although no selectivity was observed (SI < 1) for **5i**, it was the agent of choice for further pharmacological evaluation. In addition, **5d** and **5g** exhibited anti-HCV activity ($EC_{50} = 3.15$ and 3.26 μ with SI = 9.27 and >15.4, respectively, but none of these compounds matched the selection criteria of a selective inhibitor of virus replication in this assay. However, introduction of hydroxyl and nitro groups in positions 2 and 5 of the phenyl group, respectively, considerably increased the anti-HIV but left the anti-HIV activity intact (compound **5i**).

Experimental

General

Melting points are uncorrected and were measured on a digital Gallenkamp (SANYO) model MPD BM 3.5 apparatus. Nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR) spectra were recorded on a Varian Gemini 300 [300 MHz (^{1}H), 75 MHz (^{13}C)] spectrometer or Bruker NMR [300 MHz (^{1}H)] spectrometer, using $CDCl_3$ as the solvent or otherwise specified, and chemical shifts are reported in parts per million downfield from the TMS internal standard or as described. LCMS were performed on an Agilent 1200 series LC system. All compounds were purified by thick layer chromatography using silica gel from Merck.

*General method for the preparation of methyl 2-(2-(arylidenehydrazino)-4-oxo-thiazolidin-5-ylidene)acetates **5a–o***

A thoroughly mixed uniform mixture of substituted benzylidene thiosemicarbazides (**3a–o**) (1.0 mmol) and dimethyl acetylenedicarboxylate **4** (142 mg, 1.0 mmol) was stirred for 1–5 min under MWI. The progress of reaction was monitored by TLC (hexane/ethyl acetate 4:1). After the reaction was completed, the solution was diluted with EtOH and the mixture was filtered. The precipitate was recrystallized from EtOH to afford the esters (**5a–o**) in good yields.

*Methyl (2-(2-benzylidenehydrazino)-4-oxo-thiazolidin-5-ylidene)acetate **5a***

From **3a** (179 mg). Yield: 268 mg (93%) as a yellow solid, m.p.: 203°C. ^{1}H NMR (DMSO-d_6): δ 9.05 (s, 1H, NH), 8.42 (s, 1H, CH=N), 7.51–7.80 (m, 5H, Ar-H), 6.60 (s, 1H, CH=C), 3.80 (s, 3H, CO_2Me). ^{13}C NMR (DMSO-d_6): δ 170.1 ($C_{thiazol}$=O), 165.7 (CO_2Me), 156.3 ($C_{thiazol,}^2$), 145.2 (Ar*C*H=N), 140.2 (C_5=*C*H), 135.2 (C_{arom}^1), 132.2 ($C_{thiazol}^5$), 131.2, 128.3, 126.5 (C_{arom}), 53.3 (CO_2*Me*). LC–MS $[M+H]^+$ calcd. for $C_{14}H_{13}N_3O_4S$ 289.05, found 290.08. Anal. calcd. for $C_{13}H_{11}N_3O_3S$ (289.31): C, 53.97; H, 3.83; N, 14.52. Found: C, 54.03; H, 3.87; N, 14.61.

*Methyl 2-(2-(3-methoxybenzyledine)hydrazino)-4-oxo-thiazolidin-5-ylidene)acetate **5b***

From **3b** (209 mg). Yield: 298 mg (93%) as a yellow solid, m.p.: 192°C. ^{1}H NMR (DMSO-d_6): δ 8.05 (s, 1H, NH), 7.48 (s, 1H, CH=N), 7.41 (d, 2H, *J* = 8.5 Hz, Ar-H), 7.01 (s, 1H, Ar-H), 6.70 (s, 1H, CH=C), 3.91 (s, 3H, 3-*Me*O-Ph), 3.85 (s, 3H, CO_2*Me*). ^{13}C NMR (DMSO-d_6): δ 172.3 ($C_{thiazol}$=O), 166.7 (CO_2Me), 160.2 (C_{arom}-*O*Me), 158.4 ($C_{thiazol}^2$), 149.2 (Ar*C*H=N), 139.2 (C_5=*C*H), 135.4 (C_{arom}^1), 131.1 ($C_{thiazol}^5$), 121.3 (C_{arom}^6), 116.4, (C_{arom}^4), 111.5 (C_{arom}^2), 56.3 (C_{arom}-OMe), 53.3 (CO_2*Me*). LC–MS $[M+H]^+$ calcd. for $C_{14}H_{13}N_3O_4S$ 319.06; found 320.16. Anal. calcd. for $C_{14}H_{13}N_3O_4S$ (319.34): C, 52.66; H, 4.10; N, 13.16. Found: C, 52.61; H, 4.17; N, 13.10.

*Methyl 2-(2-(4-methoxybenzylidene)hydrazino)-4-oxo-thiazolidin-5-ylidene)acetate **5c***

From **3c** (180 mg). Yield: 218 mg (79%) as a yellow solid, m.p.: 198°C. ^{1}H NMR (DMSO-d_6): δ 11.29 (s, 1H, NH), 8.07 (s, 1H, CH=N), 7.72 (s, 2H, *J* = 8.5 Hz, Ar-H), 6.93 (d, 2H, *J* = 8.5 Hz, Ar-H), 6.65 (s, 1H, CH=C), 3.37 (s, 3H, 4-*Me*O-Ph), 3.81 (s, 3H, s, CO_2*Me*); ^{13}C NMR (DMSO-d_6): δ 172.3 ($C_{thiazol}$=O), 166.7 (CO_2Me), 158.5 ($C_{thiazol}^2$ + C_{arom}-*OMe*), 149.1 (Ar*C*H=N), 139.4 (C_5=*C*H), 131.2 ($C_{thiazol}^5$), 128.2 (C_{arom}^1), 127.3 (C_{arom}^2 + C_{arom}^6), 114.5 (C_{arom}^3 + C_{arom}^5), 55.3 (C_{arom}-OMe), 54.3 (CO_2*Me*). LC–MS ($M+H^+$) calcd. for $C_{14}H_{13}N_3O_4S$ 319.06; found 320.13; Anal. calcd. for $C_{14}H_{13}N_3O_4S$ (319.34): C, 52.66; H, 4.10; N, 13.16%. Found: C, 52.80; H, 4.01; N, 13.01.

*Methyl 2-(2-(2-chlorobenzylidene)hydrazino)-4-oxothiazolidin-5-ylidene)acetate **5d***

Form **3d** (213 mg). Yield: 290 mg (90%) as a yellow solid, m.p.: 198°C. ^{1}H NMR (DMSO-d_6): δ 10.45 (s, 1H, NH), 8.14 (s, 1H, CH=N), 7.78 (d, 2H, *J* = 7.4 Hz, Ar-H), 7.66–7.70 (m, 2H, Ar-H), 6.60 (s, 1H, CH=C), 3.81 (s, 3H, CO_2Me). ^{13}C NMR (DMSO-d_6): δ 171.0 ($C_{thiazol}$=O), 167.5 (CO_2Me), 159.3 ($C_{thiazol}^2$), 146.5 (Ar*C*H=N), 141.9 (C_5=*C*H), 136.8 (C_{arom}^1), 133.8 (C_{arom}–Cl), 133.8 (C_{arom}^4), 131.2 ($C_{thiazol}^5$), 129.8, 129.1, 122.6 (3 × C_{arom}), 52.7 (CO_2*Me*). LC–MS $[M+H]^+$ calcd. for $C_{13}H_{10}BrN_3O_3S$ 323.01; found 324.12. Anal. calcd. for $C_{13}H_{10}ClN_3O_3S$ (368.21): C, 42.41; H, 2.74; N, 11.41. Found: C, 42.80; H, 2.69; N, 11.21.

*Methyl 2-(2-(4-chlorobenzylidene)hydrazino)-4-oxothiazolidin-5-ylidene)acetate **5e***

Form **3e** (200 mg). Yield: 220 mg (73%) as a yellow solid, m.p.: 198°C. ^{1}H NMR (DMSO-d_6): δ 9.35 (s, 1H, NH), 8.10 (s, 1H, CH=N), 7.80 (d, 2H, *J* = 7.5 Hz, Ar-H), 7.70 (d, 2H, *J* = 7.5 Hz, Ar-H), 6.70 (s, 1H, CH=C), 3.80 (s, 3H, CO_2Me). ^{13}C NMR (DMSO-d_6): δ 171.2 ($C_{thiazol}$=O), 167.5 (CO_2Me), 159.4 ($C_{thiazol}^2$), 146.5 (Ar*C*H=N), 141.8 (C_5=*C*H), 137.8 (C_{arom}–Cl), 133.2 (C_{arom}^1 + $C_{thiazol}^5$), 129.8, 122.6 (4 × C_{arom}), 53.7 (CO_2*Me*). LC–MS $[M+H]^+$ calcd. for $C_{13}H_{10}BrN_3O_3S$ 323.03; found 324.10. Anal. calcd. for $C_{13}H_{10}ClN_3O_3S$ (368.21): C, 42.41; H, 2.74; N, 11.41. Found: C, 42.80; H, 2.69; N, 11.21.

*Methyl 2-(2-(3-bromobenzylidene)hydrazino)-4-oxo-thiazolidin-5-ylidene)acetate **5f***

From **3f** (258 mg). Yield: 301 mg (82%) as a yellow solid, m.p.: 201°C. ^{1}H NMR (DMSO-d_6): δ 11.55 (s, 1H, NH), 8.24 (s, 1H, CH=N), 7.73 (d, 2H, *J* = 7.4 Hz, Ar-H), 7.61 (d, 1H, *J* = 7.5 Hz, Ar-H), 7.39 (d, 1H, *J* = 7.3 Hz, Ar-H), 6.61 (s, 1H, CH=C), 3.83 (s, 3H, CO_2Me). ^{13}C NMR (DMSO-d_6): δ 174.0 ($C_{thiazol}$=O), 168.5 (CO_2Me), 158.3 ($C_{thiazol}^2$), 149.5 (Ar*C*H=N), 139.9 (C_5=*C*H), 138.8 (C_{arom}^1), 133.9 (C_{arom}^4), 133.9 (C_{arom}^2), 131.8 ($C_{thiazol}^5$), 129.8 (2 × C_{arom}), 122.6 (C_{arom}-Br), 52.1 (CO_2*Me*). LC–MS $[M+H]^+$ calcd. for $C_{13}H_{10}BrN_3O_3S$ 366.96; found 367.59. Anal. calcd. for $C_{13}H_{10}BrN_3O_3S$ (368.21): C, 42.41; H, 2.74; N, 11.41. Found: C, 42.80; H, 2.69; N, 11.21.

Methyl 2-(2-(2-chloro-6-fluorobenzylidene)hydrazino)-4-oxo-thiazolidin-5-ylidene)acetate ***5g***

From **3g** (232 mg). Yield: 270 mg (79%) as a yellow solid, m.p.: 211°C. ^{1}H NMR (DMSO-d_6): δ 11.70 (s, 1H, NH), 8.29 (s, 1H, CH=N), 7.36–7.45 (m, 3H, Ar-H), 6.72 (s, 1H, CH=C), 3.78 (s, 3H, CO_2Me). ^{13}C NMR (DMSO-d_6): δ 174.0 ($C_{thiazol}$=O), 165.7 (CO_2Me + C_{arom}-F), 158.3 ($C^2_{thiazol}$), 149.6 (Ar*C*H=N), 139.9 (C_5=*C*H), 138.8 (C_{arom}^1), 133.9 (C_{arom}^4), 133.9 (C_{arom}^2), 131.9 ($C_{thiazol}^5$), 129.8 (2 × C_{arom}), 122.6 (C_{arom}–Br), 52.1 (CO_2*Me*). LC–MS $[M+H]^+$ calcd. for $C_{13}H_9ClFN_3O_3S$ 341.00; found 342.13. Anal. calcd. for $C_{13}H_9ClFN_3O_3S$ (341.5): C, 45.69; H, 2.65; N, 12.30. Found: C, 45.71; H, 2.60; N, 12.17.

Methyl 2-(2-(3-(dimethylamino)benzylidene)hydrazino)-4-oxo-thiazolidin-5-ylidene)acetate ***5h***

From **3h** (150 mg). Yield: 170 mg (76%) as a yellow solid, m.p.: 215°C. ^{1}H NMR (DMSO-d_6): δ 8.34 (s, 1H, s, CH=N), 7.62 (d, 2H, J = 8.4 Hz, Ar-H), 6.78 (d, 2H, J = 8.4 Hz, Ar-H), 6.61 (s, 1H, CH=C), 3.79 (s, 3H, CO_2Me), 3.01 (s, 6H, NMe_2). ^{13}C NMR (DMSO-d_6): δ 172.5 ($C_{thiazol}$=O), 166.5 (*C*O_2Me), 156.4 ($C_{thiazol}^2$), 151.8 (C_{arom}^4-NMe_2), 147.4 (Ar*C*H=N), 138.7 (C_5=*C*H), 134.9, 134.5 (C_{arom}^2 + C_{arom}^6), 133.6 ($C_{thiazol}^5$), 118.4 (C_{arom}^1), 111.3 (2 × C_{arom}), 55.1 (CO_2*Me*), 41.4 (NMe_2). LC–MS $[M+H]^+$ calcd. for $C_{15}H_{16}N_4O_3S$ 332.09; found 333.23. Anal. calcd. for $C_{15}H_{16}N_4O_3S$ (332.38): C, 54.20; H, 4.85; N, 16.86. Found: 54.08; H, 4.92; N, 16.91.

Methyl 2-((2-(2-hydroxy-4-nitrobenzylidene)hydrazino)-4-oxo-thiazolidin-5-ylidene)acetate ***5i***

From **3i** (100 mg). Yield: 112 mg (77%), as a yellow solid, m.p.: 226°C. ^{1}H NMR (DMSO-d_6): δ 12.40 (s, 1H, NH), 8.72 (s, 1H, CH=N), 8.58 (s, 1H, Ar-OH), 7.66 (d, 2H, J = 5.4 Ar-H), 7.10 (s, 1H, Ar-H), 6.72 (s, 1H, CH=C), 3.90 (s, 3H, CO_2Me). ^{13}C NMR (DMSO-d_6): δ 173.5 ($C_{thiazol}$=O), 164.5 (*C*O_2Me), 161.3 (C_{arom}^2-OH), 160.3 (Ar*C*H=N), 158.4 ($C_{thiazol}^2$), 151.7 (C_{arom}^3-NO_2), 139.8 (C_5=*C*H), 131.5 ($C_{thiazol}^5$), 124.6 (C_{arom}^5), 124.6 (C_{arom}^6), 118.4 (C_{arom}^1), 111.3 (C_{arom}^3), 54.1 (CO_2*Me*). LC–MS $[M+H]^+$ calcd. for $C_{13}H_{10}N_4O_6S$ 350.03; found 351.09. Anal. calcd. for $C_{13}H_{10}N_4O_6S$ (350.31): C, 44.57; H, 2.88; N, 15.99. Found: 44.61; H, 2.92; N, 16.01.

Methyl 2-(-2-(4-nitrobenzylidene)hydrazino)-4-oxothiazolidin-5-ylidene)acetate ***5j***

Form **3j** (224 mg). Yield: 270 mg (81%), as a yellow solid, m.p.: 210°C. ^{1}H NMR (DMSO-d_6): δ 9.35 (s, 1H, CH=N), 7.60 (d, 2H, J = 8.4 Hz, Ar-H), 6.78 (d, 2H, J = 8.5 Hz, Ar-H), 6.60 (s, 1H, CH=C), 3.79 (s, 3H, CO_2Me). ^{13}C NMR (DMSO-d_6): δ 170.5 ($C_{thiazol}$=O), 167.5 (*C*O_2Me), 159.4 ($C_{thiazol}^2$), 151.8 (C_{arom}^3-NO_2), 149.4 (Ar*C*H=N), 137.9 (C_5=*C*H + C_{arom}^1), 131.8 ($C_{thiazol}^5$), 134.9, 134.5, 127.5, 123.3 (C_{arom}), 53.1 (CO_2*Me*). LC–MS $[M+H]^+$ calcd. for $C_{13}H_{10}N_4O_5S$ 334.03; found 335.13. Anal. calcd. for $C_{13}H_{10}N_4O_5S$ (334.31): C, 46.71; H, 3.02; N, 16.76. Found: C, 47.01; H, 3.12; N, 16.82.

Methyl 2-(2-(3,5-dimethoxybenzylidene)hydrazino)-4-oxo-thiazolidin-5-ylidene)acetate ***5k***

From **3k** (100 mg). Yield: 121 mg (85%) as a yellow solid, m.p.: 196°C (dec.). ^{1}H NMR (DMSO-d_6): δ 9.03 (s, 1H, NH), 7.52 (s, 1H, CH=N), 7.11 (s, 1H, Ar-H), 6.53 (s, 1H, Ar-H), 6.40 (s, 1H, CH=C), 3.81 (s, 6H, 2× C_{arom}-OMe), 3.70 (s, 3H, CO_2Me), 2.90 (br s., 1H, OH). ^{13}C NMR (DMSO-d_6): δ 173.3 ($C_{thiazol}$=O), 167.6 (*C*O_2Me), 162.2 (C_{arom}^3-OMe + C_{arom}^5-OMe), 157.4 ($C_{thiazol}^2$), 147.4 (Ar*C*H=N), 140.8 (C_5=*C*H + C_{arom}^4-OH), 134.9 (C_{arom}^1), 134.2 ($C_{thiazol}^5$), 103.5, 102.1 (C_{arom}^2 + C_{arom}^6), 57.3 (2 × C_{arom}-*OMe*), 53.3 (CO_2*Me*). LC–MS $[M+H]^+$ calcd. for $C_{15}H_{15}N_3O_5S$ 365.36; found 366.09. Anal. calcd. for $C_{15}H_{15}N_3O_6S$ (365.36): C, 49.31; H, 4.14; N, 11.50. Found. C, 50.01; H, 4.19; N, 11.41.

Methyl 2-(2-(2-(benzyloxy)benzylidene)hydrazino)-4-oxo-thiazolidin-5-ylidene)acetate ***5l***

From **3l** (105 mg). Yield: 98 mg (67%) as a yellow solid, m.p.: 203°C. ^{1}H NMR (DMSO-d_6): δ 12.73 (s, 1H, NH), 8.55 (s, 1H, CH=N), 8.11 (s, 1H, Ar-H), 7.87 (s, 1H, Ar-H), 7.36–7.49 (m, 5H, Ar-H), 7.10–6.98 (m, 2H, Ar-H), 6.67 (s, 1H, CH=C), 5.23 (s, 2H, OCH_2), 3.82 (s, 3H, CO_2Me). ^{13}C NMR (DMSO-d_6): δ 173.5 ($C_{thiazol}$=O), 165.8 (*C*O_2Me), 162.3 (C_{arom}^2), 158.4 ($C_{thiazol}^2$ + Ar*C*H=N), 139.7 (C_5=*C*H), 134.7 ($C_{thiazol}^5$), 136.3, 132.3, 128.9, 127.7, 127.1, 123.7, 121.1, 114.3 (C_{arom}), 72.3 (OCH_2), 53.1 (CO_2*Me*). LC–MS [$[M+H]^+$ calcd. for $C_{20}H_{17}N_3O_4S$ 395.09; found 396.19. Anal. calcd. for $C_{20}H_{17}N_3O_4S$ (395.43): C, 60.75; H, 4.33; N, 10.63. Found: C, 60.77; H, 4.31; N, 10.67.

Methyl 2-(2-(4-methoxybenzylidene)hydrazino)-4-oxo-thiazolidin-5-ylidene)acetate ***5m***

From **3m** (120 mg). Yield:137 mg (82%) as a yellow solid, m.p.: 217°C. ^{1}H NMR (DMSO-*d*): δ 13.02 (s, 1H, NH), 8.72 (s, 1H, CH=N), 8.63 (s, 1H, Ar-H), 8.51 (s, 1H, Ar-H), 8.15–8.35 (m, 5H, Ar-H), 7.72–7.83 (m, 2H, Ar-H), 6.70 (s, 1H, CH=C), 5.20 (s, 2H, OCH_2), 3.80 (s, 3H, CO_2Me); ^{13}C NMR (DMSO-d_6): δ 172.5 ($C_{thiazol}$=O), 165.8 (*C*O_2Me), 162.3 (C_{arom}^4), 157.4 ($C_{thiazol}^2$), 146.4 (Ar*C*H=N), 140.7 (C_5=*C*H), 136.4 (C_{arom}), 131.3 ($C_{thiazol}^5$), 129.4, 128.9, 127.7, 127.1, 126.3, 114.3 (C_{arom}), 70.3 (OCH_2), 54.1 (CO_2*Me*). LC–MS $[M+H]^+$ calcd. for $C_{20}H_{17}N_3O_4S$ 395.05; found 396.15. Anal. calcd. for $C_{20}H_{17}N_3O_4S$: C, 60.75; H, 4.33; N, 10.63. Found: C, 60.83; H, 4.29; N, 10.67.

Methyl 2-(2-(2-bromobenzylidene)hydrazino)-4-oxo-thiazolidin-5-ylidene)acetate ***5n***

From **3n** (137 mg). Yield: 150 mg (77%) as a yellow solid, m.p.: 203°C. ^{1}H NMR (DMSO-d_6): δ 11.55 (s, 1H, N), 8.24 (s, 1H, CH=N), 7.73 (d, 2H, J = 7.4 Hz, Ar-H), 7.61 (d. 1H, J = 7.5 Hz, Ar-H), 7.39 (d, 1H, J = 7.3 Hz, Ar-H), 6.61 (s, 1H, CH=C), 3.83 (s, 3H, CO_2Me). ^{13}C NMR (DMSO-d_6): δ 173.0 ($C_{thiazol}$=O), 168.5 (*C*O_2Me), 158.3 ($C_{thiazol}^2$), 149.5 (Ar*C*H=N), 139.9 (C_5=*C*H), 135.4 (C_{arom}^1), 133.8 (C_{arom}^3), 131.8 ($C_{thiazol}^5$), 130.2, 127.8, 127.1 (3 × C_{arom}), 121.6 (C_{arom}-Br), 52.1 (CO_2*Me*). Anal. calcd. for $C_{13}H_{10}BrN_3O_3S$: C, 42.41; H, 2.74; N, 11.41. Found: C, 42.38; H, 2.76; N, 11.31.

Methyl 2-(2-(cyclohexylmethylene)hydrazino)-4-oxo-thiazolidin-5-ylidene)acetate ***5o***

From **3o** (98 mg). Yield: 110 mg (70%) as a yellow solid, m.p.: 225°C. ^{1}H NMR (DMSO-d_6): δ 8.50 (s, 1H, NH), 7.80 (s, 1H, CH=N), 6.40 (s, 1H, CH=C), 3.77 (s, 3H, CO_2Me), 2.40 (m, 1H, $C_{cyclohexane}^1$), 1.13–1.49 (m, 10H, CH_2-cyclohexane). ^{13}C NMR (DMSO-d_6): δ 172.0 ($C_{thiazol}$=O), 166.5 (*C*O_2Me), 163.5 (cyclohexan-*C*H=N), 158.4 ($C_{thiazol}^2$), 138.9 (C_5=*C*H), 131.5 ($C_{thiazol}^5$), 29.8, 25.6 (5 × CH_2-cyclohexane), 54.1 (CO_2*Me*). LC–MS $[M+H]^+$ calcd. for $C_{13}H_{17}N_3O_3S$ 295.09; found 296.59. Anal. calcd. for $C_{13}H_{17}N_3O_3S$ (295.36): C, 52.86; H, 5.80; N, 14.23. Found: C, 52.91; H, 5.81; N, 14.17.

Antiviral assays

In vitro anti-HIV assay

Evaluation of the antiviral activity of the compounds **6–15** and **16–19** against HIV-1 strain III_B and HIV-2 strain (ROD) in MT-4 cells

was performed using the MTT assay as previously described [35, 36, 44]. Briefly, stock solutions (10× final concentration) of test compounds were added in 25 μL volumes to two series of triplicate wells so as to allow simultaneous evaluation of their effects on mock and HIV-infected cells at the beginning of each experiment. Serial 5-fold dilutions of test compounds were made directly in flat-bottomed 96-well microtiter trays using a Biomek 3000 robot (Beckman instruments). Untreated control HIV and mock-infected cell samples were included for each sample. HIV-1(III_B) [45] or HIV-2 (ROD) [46] stock (50 μL) at 100–300 $CCID_{50}$ (50% cell culture infectious dose) or culture medium was added to either the infected or mock-infected wells of the microtiter tray. Mock-infected cells were used to evaluate the effect of test compound on uninfected cells in order to assess the cytotoxicity of the test compound. Exponentially growing MT-4 cells [47] were centrifuged for 5 min at 1000 rpm and the supernatant was discarded. The MT-4 cells were resuspended at 6×10^5 cells/mL and 50 μL volumes were transferred to the microtiter tray wells. Five days after infection, the viability of mock and HIV-infected cells was examined spectrophotometrically by the MTT assay.

Anti-HCV assay

Huh 5.2 cells, containing the hepatitis C virus genotype 1b I389luc-ubi-neo/NS3-3′/5.1 replicon [48] were sub-cultured in DMEM supplemented with 10% FCS, 1% non-essential amino acids, 1% penicillin/streptomycin and 2% geneticin at a ratio of 1:3 to 1:4, and grown for 3–4 days in 75 cm^2 tissue culture flasks. One day before addition of the compound, cells were harvested and seeded in assay medium (DMEM, 10% FCS, 1% non-essential amino acids, 1% penicillin/streptomycin) at a density of 6500 cells/well (100 μL/well) in 96-well tissue culture microtiter plates for evaluation of anti-metabolic effect and CulturPlate (Perkin–Elmer) for evaluation of the antiviral effect. The microtiter plates were incubated overnight (37°C, 5% CO_2, 95–99% relative humidity), yielding a non-confluent cell monolayer.

The evaluation of the anti-metabolic as well as antiviral effect of each compound was performed in parallel. Four-step, 1-to-5 compound dilution series were prepared for the first screen, to collect data for a more detailed dose-response curve, an eight-step, 1-to-2 dilution series was used. Following assay setup, the microtiter plates were incubated for 72 h (37°C, 5% CO_2, 95–99% relative humidity). For the evaluation of anti-metabolic effects, the assay medium was aspirated, replaced with 75 μL of a 5% MTS solution in phenol red-free medium and incubated for 1.5 h (37°C, 5% CO_2, 95–99% relative humidity). Absorbance was measured at a wavelength of 498 nm (Safire^2, Tecan), and optical densities (OD-values) were converted to percentage of untreated controls. For the evaluation of antiviral effects, assay medium was aspirated and the cell monolayers were washed with PBS. The wash buffer was aspirated, and 25 μL of Glo Lysis Buffer (Promega) was added allowing for cell lysis to proceed for 5 min at room temperature. Subsequently, 50 μL of Luciferase Assay System (Promega) was added, and the luciferase luminescence signal was quantified immediately (1000 ms integration time/well, Safire^2, Tecan). Relative luminescence units were converted into percentage of untreated controls.

The EC_{50} and EC_{90} (values calculated from the dose-response curve) represent the concentrations at which 50 and 90% inhibition, respectively, of viral replication is achieved. The CC_{50} (value calculated from the dose-response curve) represents the concentration at which the metabolic activity of the cells is reduced by 50% as compared to untreated cells.

A concentration of compound is considered to elicit a genuine antiviral effect in the HCV replicon system when the anti-replicon effect is well above the 70% threshold at concentrations where no significant anti-metabolic activity is observed [48].

Cells and HCV viruses

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