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ScienceDirect
European Journal of Medicinal Chemistry 44 (2009) 1801–1807
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
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Preliminary communication

Anti-HIV evaluation of benzo[*d*]isothiazole hydrazones

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Received 1 February 2008; received in revised form 13 March 2008; accepted 23 May 2008
Available online 9 July 2008

Abstract

The synthesis and the anti-HIV-1 activity of novel benzo[*d*]isothiazole hydrazones are reported. Target compounds tested in MT-4 cells cultures for their anti-HIV properties against wild type HIV-1 and HIV strains carrying clinically relevant mutations (EFV^R, Y181C and K103/Y181C) showed good activity against wild type HIV-1 and against the EFV^R mutant. In terms of SAR the relevant result was that, in the class of benzisothiazole hydrazones, the benzo[*d*]isothiazol-3(2*H*)-one moiety (compounds **1** and **4**) is an essential structural requirement for the anti-retroviral activity.
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Keywords: Benzo[*d*]isothiazole hydrazones; Benzo[*d*]isothiazol-3-one derivatives; Antiviral activity; Anti-HIV-1 activity

1. Introduction

The major drugs for the therapy of acquired immunodeficiency syndrome (AIDS) fall into three families: the nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), the non-nucleoside reverse transcriptase inhibitors (NNRTIs), and the protease inhibitors (PIs) [1–3]. Moreover, recently three new drugs targeted at human immunodeficiency virus (HIV) entry, cell fusion and integration, have been approved [4,5]. Highly active antiretroviral therapy (HAART) regimens, which are based on triple or quadruple combinations of NRTIs, NNRTIs and PIs, although reducing HIV to very low levels, are unable to extinguish the infection. Thus, chronic treatments are necessary, which lead to the emergence of drug-resistant mutant strains [6,7]. Therefore, new anti-HIV agents that are effective against the emerging drug-resistant viral strains are urgently needed.

Our research group has been involved in programs aimed at more widely exploring the biological activity of benzisothiazole derivatives, that can be considered as structural bioisosters of naturally occurring nucleotides, such as adenine and guanine. Interestingly, compounds sharing a benzisothiazole skeleton have shown biological activity [8,9]. Namely, derivatives endowed with antimicrobial [10], antipsychotic [11] and anti-human leukocyte elastase [12] activities have been reported. Moreover, a number of substituted 2-benzisothiazolones also turned out to possess anti-HIV activity [13].

We recently studied a new class of benzisothiazole derivatives bearing a hydrazone function at positions 2 or 3 of the isothiazole nucleus [14]. Many of them showed a very good antimicrobial activity, and some turned out to be active activity against penicillin-resistant staphylococci [15,16]. The whole spectrum of biological activities of hydrazone derivatives has been recently reviewed and examples of compounds endowed with anti-HIV properties have been reported [17].

Starting from these preliminary remarks, and taking into account that new leads can emerge from the rational screening of synthetic heterocyclic compounds, new derivatives belonging to the class of benzisothiazole hydrazones were considered for anti-HIV activity.

Herein, we report the synthetic procedures and the results of cell-based evaluations of cytoxicity and anti-HIV-1 activity of 94 benzisothiazole hydrazones, **1a–1v**, **2a–2u**, **3a–3o**,

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doi:10.1016/j.ejmech.2008.05.030

4a–4u, **5a–5o**, and of two of their starting benzisothiazole hydrazides, **1** and **2** (Figs. 1 and 2; Schemes 1 and 2).

Given the multitude of enzymes and steps of the virus multiplication cycle targeted by anti-HIV agents, in order to evidence any potential antiviral activity, we chose to evaluate our compounds in cell-based, rather than in cell-free enzymatic assays; in fact, in our opinion, the former are better suited for optimization studies of a given lead compound.

With the aim to establish the key structural requirements for the anti-HIV activity of benzisothiazole hydrazones, our synthetic strategy was centred on: (1) variation of the alkylidene/arylidene hydrazono group, (2) variation of the substituent group and position on the benzylidene ring, (3) modification of the position of the hydrazone moiety on the isothiazole nucleus and (4) shortening/lengthening of the bridge between the supporting heterocycle and the hydrazone function.

2. Chemistry

The benzisothiazole hydrazones described in this paper were prepared by following the general procedure outlined in Schemes 1 and 2. Briefly, all the benzisothiazole hydrazones were obtained through condensation of the amino intermediates, namely the cyclic (Scheme 1) or acyclic hydrazides (Scheme 2) prepared according to the method previously reported by us, with the appropriate aryl- or alkyl- aldehyde [14–16].

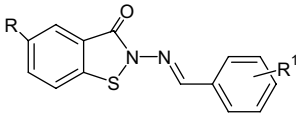
Compounds **1**, **1a–1n**, **1q–1u**, **2**, **2a–2t**, **3a–3n**, **4a**, **4e–4l**, and **5a–5n** were re-synthesized as previously described by us [14–16,18]. The new compounds **1o**, **1p**, **1v**, **2u**, **3o**, **4b–4d**, **4m–4o**, **4u** and **5o** were synthesized in 55–84% yield, through the same procedure, and then characterized and analysed by elemental analysis; their analytical IR and ^{1}H NMR spectral data were consistent with the assigned structures. In accordance with our previous experimental observations on analogous compounds, it should be noted that the ^{1}H NMR spectra of the benzo[*d*]isothiazole hydrazones **3o** and **5o** evidenced resonance signals attributable, in DMSO-d_6 solution at room temperature, to the presence of two conformers [18].

3. Biological results and discussion

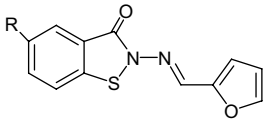
The activities of the title benzisothiazole hydrazones against HIV-1 wt and its clinically relevant NNRTI resistant mutants (EFV^R, Y181C and K103/Y181C) were established by determining their ability to prevent the virus-induced cytopathogenicity in MT-4 cells, as described elsewhere [20]. The results, detected for compounds of series **1** and **4**, are reported in Table 1. Results were compared with the antiviral activity of efavirenz, the most active anti-HIV NNRTI used in the clinic.

The results obtained with compounds **2**, **3** and **5** are not shown because none of them displayed antiretroviral activity at non-cytotoxic concentrations. Interestingly, compounds belonging to the above series (**2**, **3** and **5**) showed cytotoxicity for MT-4 cells at micromolar concentrations; therefore, they had been investigated as potential antiproliferative agents and their properties already reported [18].

The results of cell-based assays are expressed as CC_{50} (compound concentration required to reduce the viability of mock-infected cells by 50%), or EC_{50} (compound concentration required to achieve 50% protection of MT-4 cells from the HIV-1 induced cytopathogenicity). As shown in Table 1, among the benzisothiazol-3-one derivatives **1**, **1a–1v** and **4a–4u**, eleven compounds inhibited the cytopatic effect of HIV-1 wt at non-cytotoxic concentrations. The best EC_{50} values were 11 and 4.4 μM for **1a** and **1d**, respectively.



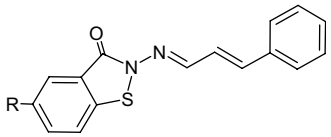
1a-1s (R=H), **4a-4r** (R=CH_3)



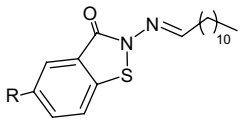
1t (R=H), **4s** (R=CH_3)

1	R^1
a	H
b	2-F
c	3-F
d	4-F
e	2-Cl
f	3-Cl
g	4-Cl
h	2-NO_2
i	3-NO_2
j	4-NO_2
k	2,4-$(NO_2)_2$
l	2-OH
m	3-OH
n	4-OH
o	2,4-$(OH)_2$
p	3,4-$(OH)_2$
q	3,4-O-CH_2-O
r	4-OCH_3
s	3-OCH_3,4-OH

4	R^1
a	H
b	2-F
c	3-F
d	4-F
e	2-Cl
f	3-Cl
g	4-Cl
h	2-NO_2
i	3-NO_2
j	4-NO_2
k	2,4-$(NO_2)_2$
l	2-OH
m	3-OH
n	4-OH
o	3,4-$(OH)_2$
p	3,4-O-CH_2-O
q	4-OCH_3
r	3-OCH_3,4-OH

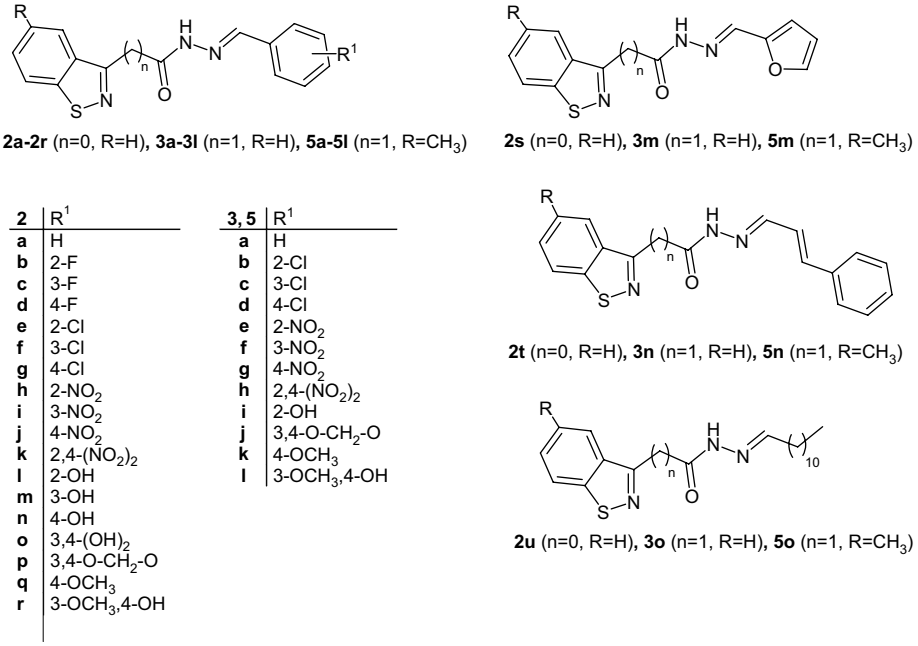


1u (R=H), **4t** (R=CH_3)



1v (R=H), **4u** (R=CH_3)

Fig. 1. Chemical structures of the benzo[*d*]isothiazole hydrazones **1a–1v** and **4a–4u**.



2	R^1
a	H
b	2-F
c	3-F
d	4-F
e	2-Cl
f	3-Cl
g	4-Cl
h	2-NO_2
i	3-NO_2
j	4-NO_2
k	2,4-$(NO_2)_2$
l	2-OH
m	3-OH
n	4-OH
o	3,4-$(OH)_2$
p	3,4-O-CH_2-O
q	4-OCH_3
r	3-OCH_3,4-OH

3, 5	R^1
a	H
b	2-Cl
c	3-Cl
d	4-Cl
e	2-NO_2
f	3-NO_2
g	4-NO_2
h	2,4-$(NO_2)_2$
i	2-OH
j	3,4-O-CH_2-O
k	4-OCH_3
l	3-OCH_3,4-OH

Fig. 2. Chemical structures of the benzo[*d*]isothiazole hydrazones **2a**–**2u**, **3a**–**3o** and **5a**–**5o**.

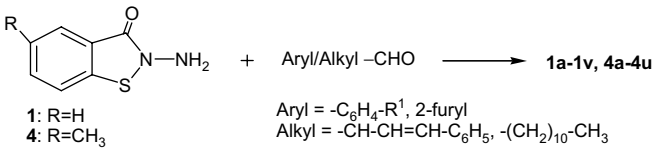
Interestingly, nine compounds (**1a**, **1c**, **1g**, **1i**, **1n**, **4d**, **4g**, **4i**, **4m**) displayed more potent activity (low micromolar range) against the EFV^R mutant than the wt strain. The same was true for compound **1i** against Y181C, while none of the test compounds showed better activity against the K183N/Y181C mutant than against the wt virus. The two starting benzisothiazole hydrazides **1** and **2** gave no significantly different results with respect to their hydrazono derivatives, being inactive at 17 μM (Table 1) and 100 μM (data not shown), respectively.

It is not easy to rule out the influence exerted by the modulation of the different hydrazono moieties that we have examined to acquire preliminary SAR information; however, it resulted clear that, in the class of the benzisothiazole hydrazones, the benzo[*d*]isothiazol-3(2*H*)-one moiety (series **1** and **4**) is an essential structural requirement for the antiretroviral activity. On the other hand, the hydrazones of the benzo[*d*]isothiazol-3-yl- series (**2**, **3** and **5**) display a potential antiproliferative activity [18]. In accordance with the results of our previous conformational analysis [18], we suggest that **1** and **4** (active as antiretroviral compounds), made by two flat structures connected by three flexible bonds, fit in the classical NNRTI butterfly model [19]. Our next step will be of further proving that the RT is the specific molecular target of the active benzisothiazol-3-one hydrazones and of exploring the structural requirements aimed at rationally modifying the chemical scaffold to obtain more potent derivatives.

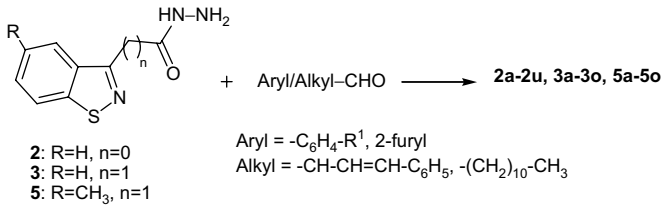
4. Conclusion

New analogues of benzisothiazole hydrazones were synthesized and tested *in vitro* against HIV-1 wt and clinically relevant NNRTI resistant mutants. A number of compounds belonging to the benzo[*d*]isothiazol-3(2*H*)-one series, **1** and **4**, showed antiretroviral activity. Compounds **1a** and **1d** showed good activity against HIV-1 wild type, while compounds **1a**, **1c**, **1g**, **1i**, **1n**, **4d**, **4g**, **4i** and **4m** showed good activity against the EFV^R mutant. Only one compound, **1i**, showed significant activity against the Y181C mutant. Unfortunately, no significant improvement in antiretroviral potency was observed when test compounds were compared to EFV.

Further studies are in progress to elucidate the mechanism of action of the active benzisothiazol-3-one hydrazones and to correlate their molecular structure with their anti-HIV-1 activity.



Scheme 1. The synthesis of the benzo[*d*]isothiazole hydrazones **1a**–**1v** and **4a**–**4u**. Reaction conditions: $EtOH/H_2O/HCl/CH_3COONa$, rt or 70 °C, 60 min.



Scheme 2. The synthesis of the benzo[*d*]isothiazole hydrazones **2a**–**2u**, **3a**–**3o** and **5a**–**5o**. Reaction conditions: $EtOH/H_2O/HCl/CH_3COONa$, rt or 70 °C, 60 min.

Table 1
Cytotoxicity and anti-HIV activity of compounds **1**, **1a–1v** and **4a–4u**[a]

Compound	CC_{50}[b] [μM]		EC_{50}[c] [μM]		
	MT-4	Wt	EFV^{Rd}	Y181C	K103N/Y181C
1	17	>17	>17	>17	>17
1a	17	**11**	**8.6**	>17	>17
1b	20	≥11	>20	>20	>20
1c	15	>15	**9**	>15	>15
1d	17	**4.4**	≥17	>17	>17
1e	47	20	24	26	>47
1f	>100	100	68	≥100	≥100
1g	26	>26	**2.9**	≥11	>26
1h	68	19	20	20	33
1i	>100	36	**11**	**13**	57
1j	>100	45	33	29	36
1k	>100	>100	>100	>100	>100
1l	18	>18	>18	>18	>18
1m	15	>15	>15	>15	>15
1n	18	>18	**8.4**	>18	>18
1o	21	>21	>21	>21	>21
1p	44	>44	>44	>44	>44
1q	17	>17	>17	>17	>17
1r	19	>19	>19	>19	>19
1s	15	>15	>15	>15	>15
1t	18	>18	>18	>18	>18
1u	18	>18	>18	>18	>18
1v	17	>17	>17	>17	>17
4a	44	≥33	≥33	≥33	>44
4b	18	>18	>18	>18	>18
4c	30	>30	>30	>30	>30
4d	15	>15	**7.3**	>15	>15
4e	>100	82	79	100	>100
4f	59	>59	25	>59	>59
4g	57	24	**11**	22	33
4h	>100	>100	>100	>100	>100
4i	>100	48	**18**	32	83
4j	>100	>100	72	≥100	>100
4k	>100	>100	>100	>100	>100
4l	30	>30	>30	>30	>30
4m	16	>16	**8.4**	>16	>16
4n	23	>23	≥23	>23	>23
4o	49	>49	>49	>49	>49
4p	>100	33	20	33	47
4q	55	>55	>55	>55	>55
4r	16	>16	>16	>16	>16
4s	18	>18	>18	>18	>18
4t	19	>19	>19	>19	>19
4u	19	>19	>19	>19	>19
EFV	30	0.002	3	0.008	0.3

Bold values refer to the most significant results. They can be converted in plain style values.

[a] Data represent mean values for the independent determinations.

[b] Compound dose required to reduce the viability of mock-infected cells by 50% as determined by the MTT method.

[c] Compound dose required to achieve 50% protection of MT-4 cells from HIV-1 induced cytopathogenicity, as determined by the MTT method.

[d] EFV^R = K103 + V179D + P225H mutant.

5. Experimental

5.1. Chemistry

Melting points (°C) were determined with a Buchi 512 apparatus and are uncorrected. New compounds were analysed in the analytical laboratory of the Dipartimento Farmaceutico, Università di Parma, on a ThermoQuest (Italia) FlashEA 1112 Elemental Analyser, for C, H, N, and S. The values found for C, H, N, S were always ±0.4% of the theoretical ones. IR spectra, such as KBr pellets, were recorded on a Jasco FT-IR 300E spectrophotometer (Jasco Ltd., Tokyo, Japan); wave numbers in the IR spectra are given in cm^{-1}. ^1H NMR spectra of the newly synthesized compounds, in DMSO-d_6 solutions, were recorded on a Bruker AC 300 instrument at 298 K. Chemical shifts are reported as δ (ppm) relative to TMS as internal standard; coupling constants *J* are expressed in Hertz. The reactions were followed by TLC on F_{254} silica-gel precoated sheets (Merck) and the purified compounds each showed a single spot. Solvents, unless otherwise specified, were of analytical reagent grade or of the highest quality commercially available. Synthetic starting material, reagents and solvents were purchased from Aldrich Chemical Co.

5.1.1. General procedure for synthesis of new benzisothiazole hydrazones *1o*, *1p*, *1v*, *2u*, *3o*, *4b–4d*, *4m–4o*, *4u*, *5o*

The appropriate hydrazide (5 mmol) was poured into water (70 mL), under stirring, and hydrochloric acid was added up to acidic pH; the suspension was then buffered with sodium acetate, and ethanol (20 mL) was added. The suitable aldehyde (5.7 mmol), dissolved in ethanol (10 mL), was dropped into the mixture and the reaction was left, while stirring, at room temperature (60 min) for compounds **4b–4d**, **4m–4o**, or heated at 70 °C (60 min), and then cooled at room temperature, for compounds **1o**, **1p**, **1v**, **2u**, **3o**, **4u** and **5o**. The resulting crude product was filtered, washed with water and recrystallised.

*5.1.1.1. 2-(2,4-Dihydroxybenzylideneamino)-benzo[d]isothiazol-3(2H)-one (**1o**).* Yield: 68%; mp 274–276 °C dec. (EtOH). TLC: eluent = CH_2Cl_2/MeOH 9/1. IR(KBr) ν: 3328 (O–H), 3070–3035 (aromatic C–H) 2920, 2842 (aliphatic C–H), 1668 (C=O), 1602 (N=C) cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6, δ ppm): 10.55 (s, 1H, OH); 10.10 (s, 1H, OH); 8.38 (s, 1H, CH); 8.02 (d, 1H, $J = 8.1$ Hz, H-4); 7.95 (d, 1H, $J = 7.9$ Hz, H-7); 7.76 (td, 1H, $J = 0.9$ Hz, $J = 7.9$ Hz H-5); 7.58 (d, 1H, $J = 8.1$ Hz, H-6′); 7.49 (td, 1H, $J = 0.9$ Hz, $J = 7.9$ Hz H-6); 6.40–6.37 (m, 2H, H-3′, H-5′). Anal. Calcd. for $C_{14}H_{10}N_2O_3S$ (286.31): C, 58.73; H, 3.52; N, 9.78; S, 11.20. Found: C, 58.68; H, 3.60; N, 9.52; S, 10.91.

*5.1.1.2. 2-(3,4-Dihydroxybenzylideneamino)-benzo[d]isothiazol-3(2H)-one (**1p**).* Yield: 83%; mp 251–252 °C (EtOH). TLC: eluent = CH_2Cl_2/MeOH 9/1. IR(KBr) ν: 3531 (O–H), 3320–3010 (bonded O–H), 2920, 2852 (aliphatic C–H), 1666 (C=O), 1598 (N=C) cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6, δ ppm): 9.43 (br s, 1H, OH); 9.36 (br s, 1H, OH); 8.31 (s, 1H, CH); 8.01 (d, 1H, $J = 8.7$ Hz, H-4); 7.95 (d, 1H, $J = 8.7$ Hz, H-7); 7.76 (t, 1H, $J = 8.2$ Hz, H-5); 7.49 (t, 1H, $J = 8.0$ Hz, H-6); 7.34 (s, 1H, $J = 1.8$ Hz, H-2′); 7.13 (dd, 1H, $J = 1.8$ Hz, $J = 7.9$ Hz, H-6′); 6.83 (d, 1H, $J = 8.1$ Hz, H-5′). Anal. Calcd for $C_{14}H_{10}N_2O_3S$ (286.31):

C, 58.73; H, 3.52; N, 9.78; S, 11.20. Found: C, 58.47; H, 3.81; N, 9.38; S, 10.81.

*5.1.1.3. 2-(Dodecylideneamino)-benzo[d]isothiazol-3(2H)-one (**1v**).* Yield: 72%; mp 38–40 °C (Ligroin). TLC: eluent = Petroleum ether/AcOEt 1/1. IR(KBr) ν: 3062 (aromatic C–H) 2930, 2858 (aliphatic C–H), 1675 (C=O), 1605 (N=C) cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6, δ ppm): 7.97 (d, 1H, $J = 8.2$ Hz, H-4); 7.92 (d, 1H, $J = 7.8$ Hz, H-7); 7.74 (t, 1H, $J = 7.4$ Hz, H-5); 7.58 (t, 1H, $J = 5.0$ Hz, CH); 7.46 (t, 1H, $J = 7.4$ Hz, H-6); 2.43–2.36 (m, 2H, CH_2); 1.55–1.22 (m, 18H, 9 CH_2); 0.82 (t, 3H, CH_3). Anal. Calcd. for $C_{19}H_{28}N_2OS$ (332.50): C, 68.63; H, 8.49; N, 8.42; S, 9.64. Found: C, 69.04; H, 8.86; N, 8.10; S, 9.98.

*5.1.1.4. N′-Dodecylidenebenzo[d]isothiazole-3-carboxydrazide (**2u**).* Yield: 55%; mp 65–67 °C (Ligroin). TLC: eluent = Petroleum ether/AcOEt 1/1. IR(KBr) ν: 3190 (N–H), 3055 (aromatic C–H) 2918, 2850 (aliphatic C–H), 1662 (C=O), 1623 (N=C) cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6, δ ppm): 11.90 (s, 1H, NH); 8.71 (d, 1H, $J = 8.1$ Hz, H-4); 8.29 (d, 1H, $J = 8.1$ Hz, H-7); 7.88 (t, 1H, $J = 5.5$ Hz, CH); 7.68–7.56 (m, 2H, H-5, H-6); 2.24 (td, 2H, $J = 7.5$ Hz, $J = 5.5$ Hz CH_2); 1.44 (quint, 2H, $J = 6.9$ Hz CH_2); 1.24–1.19 (m, 16H, 8 CH_2); 0.80 (t, 3H, $J = 6.6$ Hz CH_3). Anal. Calcd. for $C_{20}H_{29}N_3OS$ (359.53): C, 66.81; H, 8.13; N, 11.69; S, 8.92. Found: C, 67.16; H, 8.42; N, 11.46, S, 9.24.

*5.1.1.5. 2-(Benzo[d]isothiazol-3-yl)-N′-dodecylideneacetohydrazide (**3o**).* Yield: 83%; mp 119–120 °C (Ligroin-EtOH). TLC: eluent = Petroleum ether/AcOEt 1/1. IR(KBr) ν: 3218 (N–H), 3080 (aromatic C–H) 2923, 2862 (aliphatic C–H), 1663 (C=O), 1575 (N=C) cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6, δ ppm): 11.39 and 11.13 (2 s, 1H, NH); 8.19–8.14 (m, 1H, H-4); 8.07 (d, 1H, $J = 8.1$ Hz, H-7); 7.61–7.53 (m, 1H, H-5); 7.53–7.45 (m, 2H, H-6, CH); 7.33 (t, 1H, $J = 5.2$ Hz, CH); 4.35 and 4.04 (2 s, 2H, CH_2); 2.20–2.09 (m, 2H, CH_2); 1.43–1.36 (m, 2H, CH_2); 1.22–1.20 (m, 16H, 8 CH_2); 0.84–0.80 (m, 3H, CH_3). Anal. Calcd. for $C_{21}H_{31}N_3OS$ (373.56): C, 67.52; H, 8.36; N, 11.25; S, 8.58. Found: C, 67.16; H, 8.62; N, 11.48; S, 8.92.

*5.1.1.6. 2-(2-Fluorobenzylideneamino)-5-methylbenzo[d]isothiazol-3(2H)-one (**4b**).* Yield: 68%; mp 172–174 °C (EtOH). TLC: eluent = CH_2Cl_2/EtOH 95/5. IR(KBr) ν: 3065–3015 (aromatic C–H) 2960, 2920, 2845 (aliphatic C–H), 1675 (C=O), 1601 (N=C) cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6, δ ppm): 8.28 (s, 1H, CH); 7.99–7.89 (m, 2H, H-6′, H-7); 7.80 (s, 1H, 4); 7.63–7.52 (m, 2H, H-3′, H-5′); 7.39–7.32 (m, 2H, H-4′, H-6); 2.43 (s, 3H, CH_3). Anal. Calcd. for $C_{15}H_{11}FN_2OS$ (286.33): C, 62.92; H, 3.87; N, 9.78; S, 11.20. Found: C, 62.58; H, 4.11; N, 9.38; S, 11.60.

*5.1.1.7. 2-(3-Fluorobenzylideneamino)-5-methylbenzo[d]isothiazol-3(2H)-one (**4c**).* Yield: 56%; mp 190–191 °C (EtOH). TLC: eluent = CH_2Cl_2/EtOH 98/2. IR(KBr) ν: 3068 (aromatic C–H), 2980–2845 (aliphatic C–H), 1691 (C=O), 1601 (N=C) cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6, δ ppm): 8.29 (s, 1H, CH); 7.91 (d, 1H, $J = 8.1$ Hz, H-7); 7.81 (s, 1H, H-4); 7.71 (d, 1H, $J = 7.5$ Hz, H-6′); 7.66–7.51 (m, 3H, H-2′, H-4′, H-6); 7.33 (t, 1H, $J = 8.4$ Hz, H-5′); 2.43 (s, 3H, CH_3). Anal. Calcd. for $C_{15}H_{11}FN_2OS$ (286.33): C, 62.92; H, 3.87; N, 9.78; S, 11.20. Found: C, 63.00; H, 4.15; N, 9.48; S, 11.42.

*5.1.1.8. 2-(4-Fluorobenzylideneamino)-5-methylbenzo[d]isothiazol-3(2H)-one (**4d**).* Yield: 63%; mp 193–194 °C (EtOH). TLC: eluent = CH_2Cl_2/EtOH 95/5. IR(KBr) ν: 3070–3052 (aromatic C–H), 2973, 2918, 2860 (aliphatic C–H), 1666 (C=O), 1603 (N=C) cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6, δ ppm): 8.30 (s, 1H, CH); 7.95–7.90 (m, 3H, H-2′, H-6′, H-7); 7.80 (s, 1H, H-4); 7.62 (d, 1H, H-6); 7.37–7.31 (m, 2H, H-3′, H-5′); 2.43 (s, 3H, CH_3). Anal. Calcd. for $C_{15}H_{11}FN_2OS$ (286.33): C, 62.92; H, 3.87; N, 9.78; S, 11.20. Found: C, 63.02; H, 4.08; N, 9.46; S, 11.38.

*5.1.1.9. 2-(3-Hydroxybenzylideneamino)-5-methylbenzo[d]isothiazol-3(2H)-one (**4m**).* Yield: 65%; mp 228–230 °C (Dioxane). TLC: eluent = CH_2Cl_2/MeOH 95/5. IR(KBr) ν: 3182 (O–H), 2918, 2843 (aliphatic C–H), 1664 (C=O), 1610 (N=C) cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6, δ ppm): 9.68 (s, 1H, OH); 8.18 (s, 1H, CH); 7.89 (d, 1H, $J = 8.1$ Hz, H-7); 7.80 (s, 1H, H-4); 7.62 (d, 1H, $J = 8.1$ Hz, H-6); 7.32–7.26 (m, 3H, H-6', H-2′, H-5′); 6.90–6.87 (m, 1H, H-4′); 2.43 (s, 3H, CH_3). Anal. Calcd. for $C_{15}H_{12}N_2O_2S$ (284.33): C, 63.36; H, 4.25; N, 9.85; S, 11.28. Found: C, 63.17; H, 4.32; N, 9.55; S, 11.19.

*5.1.1.10. 2-(4-Hydroxybenzylideneamino)-5-methylbenzo[d]isothiazol-3(2H)-one (**4n**).* Yield: 70%; mp 242–243 °C (Dioxane). TLC: eluent = CH_2Cl_2/EtOH 95/5. IR(KBr) ν: 3181 (O–H), 3015 (aromatic C–H), 2963, 2888, 2850 (aliphatic C–H), 1658 (C=O), 1596 (N=C) cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6, δ ppm): 10.05 (s, 1H, OH); 8.19 (s, 1H, CH); 7.87 (d, 1H, $J = 8.1$ Hz, H-7); 7.78 (s, 1H, H-4); 7.69 (d, 2H, $J = 8.4$ Hz, H-2′, H-6′); 7.49 (d, 1H, $J = 8.1$ Hz, H-6); 6.86 (d, 2H, $J = 8.7$ Hz, H-3′, H-5′); 2.43 (s, 3H, CH_3). Anal. Calcd. for $C_{15}H_{12}N_2O_2S$ (284.33): C, 63.36; H, 4.25; N, 9.85; S, 11.28. Found: C, 63.06; H, 4.25; N, 9.82; S, 11.18.

*5.1.1.11. 2-(3,4-Dihydroxybenzylideneamino)-5-methylbenzo[d]isothiazol-3(2H)-one (**4o**).* Yield: 62%; mp 240–243 °C (Dioxane). TLC: eluent = CH_2Cl_2/EtOH 9/1. IR(KBr) ν: 3459 (OH) 3350–3002 (bonded O–H), 2955, 2920, 2843 (aliphatic C–H), 1664 (C=O), 1586 (N=C) cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6, δ ppm): 9.51 (s, 1H, OH); 9.35 (s, 1H, OH); 8.09 (s, 1H, CH); 7.86 (d, 1H, $J = 8.1$ Hz, H-7); 7.78 (d, 1H, $J = 1.2$ Hz, H-4); 7.49 (dd, 1H, $J = 1.2$ Hz, $J = 8.1$ Hz, H-6); 7.34 (d, 1H, $J = 2.1$ Hz, H-2′); 7.17 (dd, 1H, $J = 1.8$ Hz, $J = 8.1$ Hz, H-6′); 6.81 (d, 1H, $J = 8.1$ Hz, H-5′); 2.43 (s, 3H, CH_3). Anal. Calcd. for $C_{15}H_{12}N_2O_3S$

(300.33): C, 59.99; H, 4.03; N, 9.33; S, 10.68. Found: C, 59.51; H, 4.25; N, 8.91; S, 10.29.

*5.1.1.12. 2-(Dodecylideneamino)-5-methylbenzo[d]isothiazol-3(2H)-one (**4u**).* Yield: 84%; mp 77–78 °C (Ligroin). TLC: eluent = Petroleum ether/AcOEt 1/1. IR(KBr) ν: 3073 (aromatic C–H) 2952, 2858 (aliphatic C–H), 1665 (C=O), 1604 (N=C) cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6, δ ppm): 7.85 (d, 1H, $J = 8.1$ Hz, H-7); 7.74 (s, 1H, H-4); 7.58–7.54 (m, 2H, H-6, CH); 2.50 (m, 2H, CH_2); 2.41 (s, 3H, CH_3); 1.58–1.23 (m, 18H, 9 CH_2); 0.84 (t, 3H, CH_3). Anal. Calcd. for $C_{20}H_{30}N_2OS$ (346.53): C, 69.32; H, 8.73; N, 8.08; S, 9.25. Found: C, 69.70; H, 9.08; N, 7.65; S, 9.19.

*5.1.1.13. 2-(5-Methylbenzo[d]isothiazol-3-yl)-N′-dodecylideneacetohydrazide (**5o**).* Yield: 80%; mp 112–113 °C (Ligroin). TLC: eluent = Petroleum ether/AcOEt 1/1. IR(KBr) ν: 3205 (N–H), 3064 (aromatic C–H) 2918, 2850 (aliphatic C–H), 1668 (C=O), 1560 (N=C) cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6, δ ppm): 11.37 and 11.12 (2 s, 1H, NH); 8.06–8.01 (m, 1H, H-7); 7.94 and 7.86 (2 s, 1H, H-4); 7.52 (t, 1H, $J = 5.4$ Hz, CH); 7.46–7.41 (m, 1H, H-6); 7.33 (t, 1H, $J = 5.1$ Hz, CH); 4.31 and 4.00 (2 s, 2H, CH_2); 2.49 and 2.46 (2s, 3H, CH_3); 2.22–2.10 (m, 2H, CH_2); 1.43–1.36 (m, 2H, CH_2); 1.23–1.20 (m, 16H, 8 CH_2); 0.84–0.80 (m, 3H, CH_3). Anal. Calcd. for $C_{22}H_{33}N_3OS$ (387.58): C, 68.18; H, 8.58; N, 10.84; S, 8.27. Found: C, 68.54; H, 8.97; N, 10.46; S, 8.56.

5.2. Antiviral assay procedures

Compounds were solubilized in DMSO at 100 mM and then diluted in culture medium.

5.2.1. Cells and viruses

MT-4, C8166, and H9/IIIB cells were grown at 37 °C in a 5% CO_2 atmosphere in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 100 IU/mL penicillin G, and 100 μg/mL streptomycin. Cell cultures were checked periodically for the absence of mycoplasma contamination with a MycoTect Kit (Gibco). Human immunodeficiency viruses type 1 (HIV-1, IIIB strain) was obtained from supernatants of persistently infected H9/IIIB cells. The HIV-1 stock solutions had titers of 4.5×10^6 50% cell culture infectious dose ($CCID_{50}$)/mL. The Y181C mutant (NIH N119) was derived from an AZT-sensitive clinical isolate passage initially in CEM and then in MT-4 cells in the presence of nevirapine (10 μM). The double mutant K103N + Y181C (NIH A17) was derived from the IIIB strain passaged in H9 cells in the presence of BI-RG 587 (1 μM). The K103R + V179D + P225H mutant (EFV^R) was derived from an IIIB strain passage in MT-4 cells in the presence of efavirenz (up to 2 μM). Y181C, K103N + Y181C and K103R + V179D + P225H (EFV^R) stock solutions had titers of 1.2×10^8, 2.1×10^7 and 4.0×10^7 $CCID_{50}$/mL, respectively.

5.2.2. HIV titration

Titration of HIV was performed in C8166 cells by the standard limiting dilution method (dilution 1:2, four replica wells per dilution) in 96-well plates. The infectious virus titer was determined by light microscope scoring of syncytia after 4 days of incubation. Virus titers were expressed as $CCID_{50}$/mL.

5.2.3. Anti-HIV assays

The activity of test compounds against multiplication of wild type HIV-1, Y181C, K103N + Y181C and K103R + V179D + P225H (EFV^R) in acutely infected cells was based on inhibition of virus-induced cytopathogenicity in MT-4 cells. Briefly, an amount of 50 μL of culture medium containing 1×10^4 cells was added to each well of flat-bottom microtiter trays containing 50 μL of culture medium with or without various concentrations of test compounds. Then, an amount of 20 μL of HIV suspensions containing the appropriate amount of $CCID_{50}$ to cause complete cytopathogenicity at day 4, was added. After incubation at 37 °C, cell viability was determined by the 3-(4,5-dimethylthiazol-1-yl)-2,5-diphenyltetrazolium bromide (MTT) method [20]. The cytopathogenicity of test compounds was evaluated in parallel with their antiviral activity and was based on the viability of mock-infected cells, as monitored by the MTT method.

Acknowledgements

Financial support from Italian MUR (Ministero dell'Università e della Ricerca) is gratefully acknowledged. We are grateful to CIM (Centro Interdipartimentale Misure) of the University of Parma for NMR instrumental facilities.

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