SUPPLEMENTARY MATERIAL

Synthesis, characterization and biological evaluation of 1,3-thiazolidine-4-ones derived from (2S)-2-benzoylamino-3-methylbutanohydrazide hydrazones

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Results and Discussion

Biological Evaluation

Table S1. Anti-HIV activity and cytotoxicity of compounds **14-33**.

Compound	Strain	IC_{50} (µg/ml)	CC_{50} (µg/ml)	SI	Compound	Strain	IC_{50} (µg/ml)	CC_{50} (µg/ml)	SI
14	III_B	>70.5	=70.5	<1	**24**	III_B	>77.4	=77.4	<1
	ROD	>77.1	=77.1	<1		ROD	>83.4	=83.4	<1
15	III_B	>76.1	=76.1	<1	**25**	III_B	>65.3	=65.3	<1
	ROD	>89.4	=89.4	<1		ROD	>77	=77	<1
16	III_B	>69.1	=69.1	<1	**26**	III_B	>73.9	=73.9	<1
	ROD	>73.5	=73.5	<1		ROD	>71.5	=71.5	<1
17	III_B	>66.9	=66.9	<1	**27**	III_B	>69	=69	<1
	ROD	>79.4	=79.4	<1		ROD	>78.3	=78.3	<1
18	III_B	>100.7	=100.7	<1 or X1	**28**	III_B	>61.1	=61.1	<1
	ROD	>102.8	=102.8	<1 or X1		ROD	>73.7	=73.7	<1
19	III_B	>115	=115	<1	**29**	III_B	>70.3	=70.3	<1
	ROD	>125	>125	X1		ROD	>73.3	=73.3	<1
20	III_B	>59.3	=59.3	<1	**30**	III_B	>108.9	=108.9	<1 or X1
	ROD	>72.3	=72.3	<1		ROD	>116	=116	<1 or X1
21	III_B	>68.3	=68.3	<1	**31**	III_B	>57.7	=57.7	<1
	ROD	>84.4	=84.4	<1		ROD	>70	=70	<1
22	III_B	>93.6	=93.6	<1	**32**	III_B	>74.7	=74.7	<1
	ROD	>113.5	=113.5	<1 or X1		ROD	>93.3	=93.3	<1
23	III_B	>68.3	=68.3	<1	**33**	III_B	>13.4	=13.4	<1
	ROD	>82.6	=82.6	<1		ROD	>13.5	=13.5	<1

CC_{50}: 50% Cytotoxic concentration, as determined by measuring cell viability with the colorimetric formazan-based MTT assay.

IC_{50}: 50% Inhibitor concentration, as determined by measuring cell viability with the colorimetric formazan-based MTT assay.

SI: Selectivity index.

Table S2. Anti-Feline Corona Virus (FIPV) and anti-Feline Herpes Virus activity and cytotoxicity of compounds 1**4-33** in CRFK cell cultures.

Compound	CC_{50}[a] (µM)	EC_{50}[b] (µM)	
		Feline Corona Virus (FIPV)	Feline Herpes Virus
14	65.6	>20	>20
15	>100	>100	>100
16	>100	>100	>100
17	>100	>100	>100
18	>100	>100	>100
19	>100	>100	>100
20	>100	>100	>100
21	88.9	>20	>20
22	>100	>100	>100
23	>100	>100	>100
24	72.1	>20	>20
25	38.5	>20	>20
26	>100	>100	>100
27	83.2	>20	>20
28	72.4	>20	>20
29	>100	>100	>100
30	>100	>100	>100
31	40.0	>20	>20
32	>100	>100	>100
33	43.1	>20	>20
24	72.1	>20	>20
UDA (µg/ml)	>100	1.6	2.6
HHA (µg/ml)	>100	0.8	2.7
Ganciclovir	>100	>100	2.9

[a]CC_{50}: 50% Cytotoxic concentration, as determined by measuring the cell viability with the colorimetric formazan-based MTS assay.

[b]EC_{50}: 50% Effective concentration, or concentration producing 50% inhibition of virus-induced cytopathic effect, as determined by measuring the cell viability with the colorimetric formazan-based MTS assay.

Table S3. Antiviral activity and cytotoxicity of compounds 1**4-33** in HEL cell cultures.

Compound	Minimum cytotoxic concentration[a] (μM)	EC_{50}[b] (μM)				
		Herpes simplex virus-1 (KOS)	Herpes simplex virus-2 (G)	Vaccinia virus	Vesicular stomatitis virus	Herpes simplex virus-1 TK^-KOSACV^r
14	>100	>100	>100	>100	>100	>100
15	>100	>100	>100	>100	>100	>100
16	>100	>100	>100	>100	>100	>100
17	>100	>100	>100	>100	>100	>100
18	>100	>100	>100	>100	>100	>100
19	>100	>100	>100	>100	>100	>100
20	>100	>100	>100	>100	>100	>100
21	>100	>100	>100	>100	>100	>100
22	>100	>100	>100	>100	>100	>100
23	100	>20	>20	>20	>20	>20
24	>100	>100	100	>100	>100	>100
25	100	>20	>20	>20	>20	>20
26	≥100	>100	>100	>100	>100	>100
27	>100	>100	>100	>100	>100	>100
28	>100	>100	>100	>100	>100	>100
29	>100	>100	>100	>100	>100	>100
30	>100	>100	>100	>100	>100	>100
31	100	>20	>20	>20	>20	>20
32	>100	>100	>100	>100	>100	>100
33	100	>20	>20	>20	>20	>20
Brivudin	>250	0.04	10	2	>250	50
Ribavirin	>250	50	50	5	>250	150
Acyclovir	>250	0.4	0.4	146	>250	50
Ganciclovir	>100	0.03	0.03	>250	>100	0.8

[a] Required to cause a microscopically detectable alteration of normal cell morphology.
[b] Required to reduce virus-induced cytopathogenicity by 50%.

Table S4. Antiviral activity and cytotoxicity of compounds 1**4-33** in HeLa cell cultures.

Compound	Minimum cytotoxic concentration[a] (μM)	EC_{50}[b] (μM)		
		Vesicular stomatitis virus	Coxsackie virus B4	Respiratory syncytial virus
14	>100	100	>100	>100
15	>100	100	>100	>100
16	>100	>100	>100	>100
17	>100	>100	>100	>100
18	>100	>100	>100	>100
19	>100	>100	>100	>100
20	>100	>100	>100	>100
21	100	>20	>20	>20
22	>100	>100	>100	>100
23	≥100	>100	>100	>100
24	>100	100	>100	>100
25	100	>20	>20	>20
26	>100	>100	>100	>100
27	>100	>100	>100	>100
28	>100	>100	>100	>100
29	>100	>100	>100	>100
30	>100	>100	>100	>100
31	100	>20	>20	>20
32	>100	>100	>100	>100
33	100	>20	>20	>20
Brivudin	>250	>250	>250	>250
(S)-DHPA	>250	146	>250	>250
Ribavirin	>250	2	146	10

[a] Required to cause a microscopically detectable alteration of normal cell morphology.
[b] Required to reduce virus-induced cytopathogenicity by 50%.

Table S5. Antiviral activity and cytotoxicity of compounds 1**4-33** in Vero cell cultures.

Compound	Minimum cytotoxic concentration[a] (μM)	EC_{50}[b] (μM)				
		Parainfluenza-3 virus	Reovirus-1	Sindbis virus	Coxsackie virus B4	Punta Toro virus
14	100	>20	>20	>20	>20	>20
15	>100	>100	>100	>100	>100	>100
16	>100	>100	>100	>100	>100	>100
17	>100	>100	>100	>100	>100	>100
18	>100	>100	>100	>100	>100	>100
19	>100	100	>100	>100	>100	>100
20	>100	100	>100	>100	>100	>100
21	≥100	>100	>100	>100	>100	>100
22	>100	>100	>100	>100	>100	>100
23	>100	>100	>100	>100	>100	>100
24	>100	>100	>100	>100	>100	>100
25	100	>20	>20	>20	>20	>20
26	>100	>100	>100	>100	>100	>100
27	>100	>100	>100	>100	>100	>100
28	>100	>100	>100	>100	100	>100
29	>100	50	50	50	>100	>100
30	100	>20	>20	>20	>20	>20
31	100	>20	>20	>20	>20	>20
32	>100	>100	>100	>20	>20	>100
33	100	>20	>20	>20	>20	>20
Brivudin	>250	>250	>250	>250	>250	>250
(S)-DHPA	>250	50	>250	>250	>250	>250
Ribavirin	>250	50	>250	>250	>250	150

[a] Required to cause a microscopically detectable alteration of normal cell morphology.
[b] Required to reduce virus-induced cytopathogenicity by 50%.

Table S6. Anti-VZV and Anti-Cytomegalovirus and cytotoxicity of compounds 1**4-33** in HEL cell cultures.

Compound	*Cytomegalovirus*			*Varicella-zoster* virus			
	Antiviral activity EC_{50} (μM) [a]	Cytotoxicity (μM)		Antiviral activity EC_{50} (μM) [a]		Cytotoxicity (μM)	
	AD-169 strain	Cell morphology (MCC)[b]	Cell growth (CC_{50}) [c]	TK^+VZV OKA strain	TK^-VZV 07/1 strain	Cell morphology (MCC)[b]	Cell growth (CC_{50}) [c]
14	>100	>100	>100	34.9	32.1	>100	>100
15	>100	>100	>100	>100	>100	>100	>100
16	>100	>100	>100	>100	>100	>100	>100
17	>100	>100	>100	>100	>100	>100	>100
18	>100	>100	>100	>100	>100	>100	>100
19	>100	>100	>100	>100	>100	>100	>100
20	>20	100	>100	>100	>100	>100	>100
21	>100	>100	>100	>100	76.5	>100	>100
22	>100	>100	>100	>100	>100	>100	>100
23	>100	>100	>100	>20	>100	≥100	>100
24	>100	>100	>100	31.4	20	≥100	>100
25	>20	100	61.1	>20	>20	100	61.1
26	>100	>100	>100	>100	>100	>100	>100
27	>100	>100	>100	>100	>100	>100	>100
28	100	>100	>100	>100	64.5	>100	>100
29	>100	>100	>100	>100	>100	>100	>100
30	>20	100	54.2	>20	>20	100	54.2
31	>20	100	68.1	>20	>20	100	68.1
32	100	>100	>100	>100	66.9	>100	>100
33	>20	100	48.3	>20	>20	100	48.3
Ganciclovir	12.6	1575	508	N.D[d]	N.D[d]	N.D[d]	N.D[d]
Cidofovir	1.1	254	211	N.D[d]	N.D[d]	N.D[d]	N.D[d]
Acyclovir	N.D[d]	N.D[d]	N.D[d]	1.8	57.8	>220	N.D[d]
Brivudin	N.D[d]	N.D[d]	N.D[d]	0.0075	115.6	>150	N.D[d]

[a] Effective concentration required to reduce virus plaque formation by 50%. Virus input was 100 plaque forming unit (PFU).

[b] Minimum cytotoxic concentration that causes a miscroscopically detectable alteration of cell morphology.

[c] Cytotoxic concentration required to reduce cell growth by 50%.

[d] Not determined

Table S7. Anti-HCV NS5B RdRp activity of compounds **14-32**.

Compound	% Inhibition[a]	Compound	% Inhibition[a]
14	13.3	**23**	16.8
16	6.2	**25**	19.7
17	12.1	**26**	20.9
18	4.1	**27**	23.1
19	NI	**28**	27.0
20	8.4	**30**	22.8
21	14.6	**31**	21.7
22	NI	**32**	25.7

[a] Percent inhibition was determined at 100 μM concentration of the indicated compound and represents an average of at least two independent measurements in duplicate. NI.: no inhibition.

Table S8. Antimicrobial activity of compounds **14-32** using microdilution method (MIC, μg/ml)

Compound	Microorganisms and Minimal Inhibitory Concentration (μg/ml)								
	Ec	**Pa**	**Sa**	**MRSA**	**Kp**	**Bs**	**Ca**	**Se**	**Ef**
14	625	625	>5000	1250	1250	5000	625	2500	2500
16	5000	5000	625	1250	5000	2500	625	2500	2500
17	1250	625	1250	1250	1250	1250	625	2500	2500
18	>5000	>5000	625	2500	1250	>5000	625	>5000	2500
19	625	1250	1250	1250	1250	5000	625	2500	2500
20	1250	2500	1250	1250	5000	2500	625	2500	2500
21	1250	>5000	1250	1250	1250	>5000	625	2500	2500
22	1250	1250	625	1250	1250	1250	625	2500	2500
23	1250	1250	1250	1250	2500	2500	625	2500	5000
25	>5000	>5000	625	1250	>5000	>5000	5000	2500	5000
26	>5000	1250	625	1250	5000	>5000	1250	2500	5000
27	5000	2500	625	2500	1250	2500	625	2500	2500
28	1250	5000	1250	1250	2500	1250	1250	2500	2500
30	1250	>5000	1250	1250	>5000	>5000	1250	2500	2500
31	625	1250	1250	1250	>5000	>5000	1250	2500	2500
32	625	1250	1250	2500	1250	1250	625	2500	2500
DMSO	1250	1250	2500	1250	1250	1250	625	2500	2500
Ciprofloxacin	<0.12	<0.12	1	1	<0.12	<0.12	-	<0.12	1
Fluconazole	-	-	-	-	-	-	0.5	-	-

Ec*: Escherichia coli* ATCC 25922, Pa: *Pseudomonas aeruginosa* ATCC 27853, Sa: *Staphylococcus aureus* ATCC 25923, MRSA: Methicillin resistant *Staphylococcus aureus* ATCC 43300, Kp: *Klebsiella pneumoniae* ATCC 4352, Bs: *Bacillus subtilis* ATCC 6633, Ca: *Candida albicans* ATCC 10231, Se*: Staphylococcus epidermidis* ATCC 12228, Ef: *Enterococcus faecalis* ATCC 29212.

Experimental

In Vitro Antiviral Assays

Inhibition of HIV-induced cytopathicity in MT-4 cells

Evaluation of the antiviral activity of the compounds against *HIV-1 strain III_B* and *HIV-2 strain (ROD)* in MT-4 cells was performed using the MTT assay as previously described. [1, 2] Stock solutions (10 x 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 2000 robot (Beckman instruments, Fullerton, CA). Untreated control HIV-and mock-infected cell samples were included for each samples.

HIV-1(IIIB) [3] or *HIV-2 (ROD)* [4] stock (50 μl) at 100-300 $CCID_{50}$ (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 [5] were centrifuged for 5 minutes at 1000 rpm and the supernatant was discarded.

The MT-4 cells were resuspended at 6 x 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.

The MTT assay is based on the reduction of yellow coloured 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Acros Organics, Geel, Belgium) by mitochondrial dehydrogenase of metabolically active cells to a blue-purple formazan that can be measured spectrophotometrically. The absorbances were read in an eight-channel computer-controlled photometer (Multiscan Ascent Reader, Labsystems, Helsinki, Finland), at two wavelenghths (540 and 690 nm). All data were calculated using the median OD (optical density) value of tree wells. The 50% cytotoxic concentration (CC_{50}) was defined as the concentration of the test compound that reduced the absorbance (OD540) of the mock-infected control sample by 50%. The concentration achieving 50% protection from the cytopathic effect of the virus in infected cells was defined as the 50% effective concentration (EC_{50}).

Antiviral assays

The antiviral assays, other than HIV-1, were based on inhibition of virus-induced cytopathicity in HEL cells (*HSV-1(KOS), HSV-1(TK$^-$KOSACV^r), HSV-2(G), Vaccinia virus, Vesicular stomatitis virus*), Hela cells (*Vesicular stomatitis virus, Respiratory syncytial virus, Coxsackie B4 virus*) and Vero cells (*Parainfluenza-3 virus, Reovirus-1, Sindbis virus, Coxsackie B4 virus, Punta Toro virus*), following previously established procedures. [6-8] Briefly, confluent cell cultures in microtiter 96-well plates were inoculated with 100 CCID50 of virus, 1 CCID50 being the virus dose required to infect 50% of the cell cultures. After a 1-h virus adsorption period, residual virus was removed, and the cell cultures were incubated in the presence of varying concentrations 400, 200, 100, … µg/ml) of the test compounds. Viral cytopathicity was recorded as soon as it reached completion in the control virus-infected cell cultures that had not been treated with the test compounds.

NS5B inhibition assay

The biological activity of the compounds against NS5B polymerase were evaluated in a reaction buffer containing 20 mM Tris-HCl (pH 7.0), 100 mM NaCl, 100 mM sodium glutamate, 0.1 mM DTT, 0.01% BSA, 0.01% Tween-20, 5% glycerol, 20 U/mL of RNase Out, 0.25 µM of poly rA/U_{12}, 25 µM UTP, 2 µCi [∞-^{32}P]UTP, 300 ng of NS5BCΔ21 and 1.0 mM $MnCl_2$ with or without inhibitors (100 µM) in a total volume of 25 µL for 1 h at 30ºC as previously described. [9, 10] Reactions were terminated by the addition of ice-cold 5% (v/v) trichloroacetic acid (TCA) containing 0.5 mM pyrophosphate. Reaction products were precipitated on GF-B filters and quantified on a liquid scintillation counter. NS5B activity in the presence of DMSO control was set at 100% and that in the presence of the compounds was determined relative to this control.

Antimicrobial evaluation

The newly synthesized derivatives **14-32** were screened for their in vitro antibacterial activity against *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, *Klebsiella pneumoniae* ATCC 4352, *Bacillus subtilis* ATCC 6633, *Staphylococcus epidermidis* ATCC 12228, methicillin resistant *Staphylococcus aureus* (MRSA) ATCC 43300 and antifungal activity against *Candida albicans* ATCC 10231. The minimal inhibitory concentrations (MIC) of these compounds were determined using microdilution technique using Mueller Hinton Broth (Difco, Detroit, USA) for bacteria, RPMI-1640 medium modified (Sigma) for the yeast strain. Ciprofloxacin (64- 0.06 µg/ml) was used as reference powder for bacteria and fluconazole (64- 0.06 µg/ml) for the yeast. Serial two fold dilutions of synthesized derivatives and DMSO ranging from 5000 to 4.9 µg/ml were prepared in media. The inoculum was prepared using 4-6 hr broth culture of each bacteria and yeast strains adjusted and diluted in broth media to give a final concentration of $5x10^5$ cfu/ml for bacteria and 1-5 $x10^3$ cfu/ml for yeast in the test tray. The trays are covered and placed in plastic bags to prevent evaporation. The trays containing Mueller Hinton Broth were incubated at 35 ºC for 18-24 hr and the trays containing RPMI-1640 medium were incubated at 35 ºC for 24- 48 hr. The MIC was defined as the lowest concentration of compound giving complete inhibition of visible growth. [11, 12]

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