Synthesis and primary antiviral activity evaluation of 3-hydrazono-5-nitro-2-indolinone derivatives

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Abstract

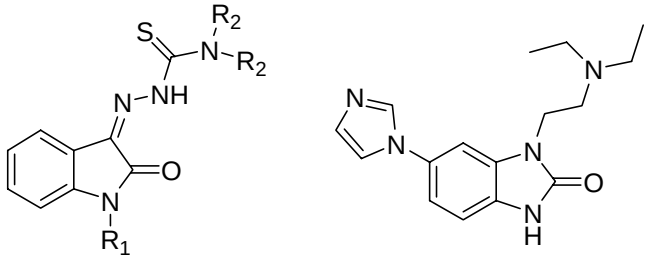
A series of 5-nitro-3-[(5-nonsubstituted/methyl-4-thiazolidinone-2-ylidene)hydrazono]-1*H*-2-indolinones (**3a-j** and **4a-h**) was synthesized by cyclization of 5-nitro-1*H*-indole-2,3-dione-3-thiosemicarbazones (**1a-k**) with ethyl bromoacetate or ethyl 2-bromopropionate. The primary antiviral activities of the new hydrazonoindolinone derivatives, the previously reported 5-nitro-1*H*-indole-2,3-dione-3-thiosemicarbazones (**1a-k**) and 1-morpholino/piperidinomethyl-5-nitro-indole-2,3-dione-3-thiosemicarbazones (**2a-l**) were evaluated against some pathogenic viruses in the Rega Institue for Medical Research, Belgium. Among the tested compounds, **1c**, **2b** and **3b** afforded some weak activity against the yellow fever virus (YFV) in vero cells, whereas compounds **2b**, **3a**, **3f**, **4e** and **4f** inhibited the growth of bovine viral diarrhea virus (BVDV) in MDBK CODA cells.

Keywords: 1*H*-2-Indolinones, 4-thiazolidinones, antiviral activity

Introduction

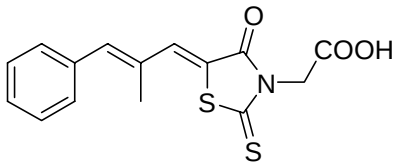
Isatin (1*H*-indole-2,3-dione) is a versatile lead molecule for designing potential antiviral agents. Several authors found that isatin-β-thiosemicarbazone (1*H*-indole-2,3-dione-3-thiosemicarbazone) and its N-Mannich bases were active against various viruses[1]. The first clinically approved antiviral agent, N-methylisatin-β-thiosemicarbazone (methisazone, **I**), and isatin-β-thiosemicarbazone (**II**) are active against poxviruses[2]. Antiviral properties of certain thiourea and semicarbazone derivatives have been reported in which the antiviral effect is attributed to the presence of an intact NHC(=S)NH grouping and NHC(=O)NH grouping[3]. The cyclic urea derivative (**III**), as a replicase inhibitor specific to the bovine viral diarrhea virus (BVDV)[4] has been recently identified. On the other hand, some original 4-thiazolidones are

undergoing different stages of clinical trials as potential antimicrobial, antiviral, anticancer and thrombolytic drugs. It has been reported that members of a series of 2,3-diaryl-1,3-thiazolidin-4-ones were highly effective in inhibiting the cytopathic effect of HIV-1 in human T-lymphocyte cells[5]. The 2-thioxo-4-thiazolidone derivative, epalrestat (**IV**) is a highly active aldose reductase inhibitor (Figure 1).



I R_1= CH_3, R_2= H
II R_1=R_2= H

III



IV

Figure 1

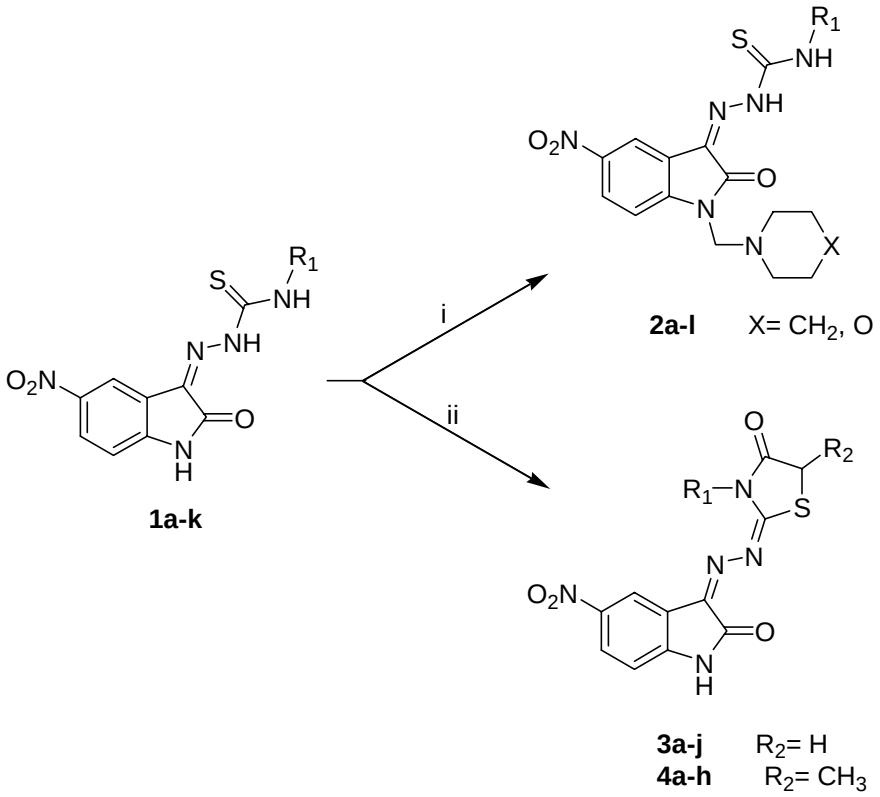
In view of these observations, we report here the synthesis of the new 5-nitro-3-[(5-nonsubstituted/ methyl-4-thiazolidinone-2-ylidene)hydrazono]-1*H*-2-indolinones (**3** and **4**) and evaluation of *in vitro* antiviral activity of these new compounds and some of the previously reported 5-nitro-1*H*-indole-2,3-dione-3-thiosemicarbazones (**1**) and 1-morpholino/ piperidinomethyl-5-nitro-indole-2,3-dione-3-thiosemicarbazones (**2**).

Results and Discussion

Treatment of ethyl bromoacetate or ethyl 2-bromopropionate with 5-nitro-1*H*-indole-2,3-dione-3-thiosemicarbazone **1a-k** in anhydrous ethanolic medium furnished the corresponding 5-nitro-3-[(5-nonsubstituted/methyl-4-thiazolidinone-2-ylidene)hydrazono]-1*H*-2-indolinones (**3a-j** and **4a-h**) (Scheme 1).

The structures of **3** and **4** were confirmed by analytical and spectral data (IR, ^{1}H NMR, ^{13}C NMR and EIMS) (Table 1). In the IR spectra of **3** and **4**, the C=S bands disappeared, a new C=O band (1756-1734 cm^{-1}) indicative of the thiazolidinone structure appeared in addition to the lactam C=O stretching (1705-1689 cm^{-1})[6]. ^{1}H-NMR spectra of **3** and **4** did not display the signals of the thiosemicarbazone protons and exhibited a singlet or two singlets resulting from the indole

NH (δ 11.28-11.43 ppm). The SCH_2 protons of **3** were observed as two singlets (δ 4.09-4.20 ppm and 4.11-4.24 ppm) due to anisotropic effect of carbonyl group. The CH_3 (δ 1.60-1.72 ppm) and the SCH (δ 4.47-4.58 ppm) protons of **4** resonated as a doublet and a quartet, respectively[7]. In EIMS spectrum, **3d**, **3f**, **3i**, **4c** and **4e** chosen as prototypes showed molecular ions with different intensity and the fragments peculiar to the indole and thiazolidinone moieties[8].



Scheme 1. Reagents and conditions: (i) piperidine/morpholine, HCHO, EtOH (ii) ethyl bromoacetate / ethyl 2-bromopropionate, anhyd. CH_3COONa, EtOH.

The new 3-hydrazono-5-nitro-2-indolinone derivatives **3a-j** and **4a-h** and the previously synthesized[9] 5-nitro-1*H*-indol-2,3-dion-3-thiosemicarbazones **1a-k** and N-Mannich bases **2a-l** were evaluated for in vitro antiviral activity against the yellow fever virus (YFV) (strain 17D) in vero cells and the bovine viral diarrhea virus (BVDV-strain NADL) in Madin-Dardy bovine kidney (MDBK CODA) cells in Rega Institue for Medical Research, Belgium[4,10,11]. For each compound, the 50% effective concentration (EC_{50}) and the minimal toxic concentration (MTC) or the 50% cytotoxic concentration (CC_{50}) were obtained. Ribavirin (Rib) was used as the standard in the tests. Both antiviral activity and cytotoxicity were determined by means of the MTS method. As can be seen in Table 2, some of the tested compounds afforded some weak activity against YFV, but at subtoxic concentrations. Among the compounds tested, the MTC

value of **1c**, **2b** and **3b** are 100 μg/mL and the EC_{50} of these compounds are 2.18, 11 and 2.52 μg/mL, respectively. The EC_{50} and the MTC values of ribavirin are 45 and >100 μg/mL in the tests against YFV, respectively. When these data are examined, it is observed that **1c**, **2b** and **3b** are more active, but more toxic than ribavirin. The most active compound against YFV was R_1-allyl substituted thiosemicarbazone derivative **1c**. In comparison with **1c**, formation of N-Mannich bases (**2b, 2c**) and 5-methyl-4-thiazolidinone derivatives(**4a**) caused a decrease in antiviral activity against YFV. 5-Nonsubstituted 4-thiazolidinone derivative (**3c**) exhibited no change in activity when compared to **1c**, but demonstrated higher toxicity, whereas compounds **2b**, **3a**, **3f**, **4e** and **4f** inhibited the growth of BVDV. The EC_{50} values of these compounds are 40, 50, 44, 13 and 48 μg/mL, respectively. The EC_{50} value of ribavirin is 40 μg/mL in the tests against BVDV. The results of **2b**, **3a**, **3f**, **4e** and **4f** were compared with ribavirin. Compound **4e** showed much higher antiviral activity than that of ribavirin. Among 5-nitro-3-hydrazono-2-indolinone derivatives tested against BVDV, 4-thiazolidinone derivatives **3** and **4** were more active than 3-thiosemicarbazones **1** and N-Mannich bases **2**. In addition, the presence of the methyl group at C-5 of the thiazolidinone ring also seems to have a significant impact on the resultant antiviral activity. Considering the anti-BVDV effect of substituents on the thiazolidinone ring, R_1-aryl substituted were more active than the corresponding alkyl substituted ones. In fact, R_1-(4-bromophenyl) substituted 5-methyl-4-thiazolidinone derivative **4e** showed the most favourable antiviral activity against BVDV. 3-Hydrazono-5-nitro-2-indolinone derivatives **1-4** were also evaluated against the hepatitis C virus in Huh-5-2 cells, the parainfluenza-3 virus, reovirus-1, sindbis virus, coxsackie virus B4 and punta toro virus in vero cells, herpes simplex virus-1, herpes simplex virus-2, vaccinia virus, vesicular stomatitis virus in human embryonic lung (HEL) cells and vesicular stomatitis virus, coxsackie virus B4 and respiratory syncytial virus in HeLa cells. Whereas, no specific antiviral effects (i.e. minimal antivirally effective concentration ≥5-fold lower than minimal cytotoxic concentration) were noted for any of the compounds against any of the viruses.

The obtained preliminary results suggest that some of these compounds might serve as potential candidates for antiviral agents.

Table 1. Formulas, physical constants and elemental analyses for **3** and **4**

Comp.	R_1	R_2	Yield (%)	m.p. °C	Formula (M.W.)	Analysis(calc./found) C	H	N
3a	CH_3	H	84	>300	$C_{12}H_9N_5O_4S.H_2O$	42.73	3.29	20.76
					(337.31)	43.13	3.53	21.13
3b	C_2H_5	H	96	>300	$C_{13}H_{11}N_5O_4S.½H_2O$	45.61	3.53	20.45
					(342.33)	45.46	3.87	20.64
3c	CH_2-CH=CH_2	H	99	>300	$C_{14}H_{11}N_5O_4S$	48.69	3.21	20.27
					(345.33)	48.43	3.67	19.71
3d	n-C_4H_9	H	99	>300	$C_{15}H_{15}N_5O_4S.½H_2O$	48.64	4.35	18.90
					(370.39)	48.63	4.40	18.58
3e	cycl-C_6H_{11}	H	95	>300	$C_{17}H_{17}N_5O_4S$	52.70	4.42	18.07
					(387.41)	52.44	4.59	18.10
3f	C_6H_5	H	88	>300	$C_{17}H_{11}N_5O_4S$	53.54	2.90	18.36
					(381.37)	54.10	3.10	18.28
3g	$C_6H_4CH_3$(4-)	H	99	>300	$C_{18}H_{13}N_5O_4S$	54.68	3.31	17.71
					(395.39)	54.82	3.35	17.84
3h	C_6H_4Br(4-)	H	96	>300	$C_{17}H_{10}BrN_5O_4S$	44.36	2.19	15.21
					(460.27)	43.72	2.33	15.30
3i	C_6H_4Cl(4-)	H	85	>300	$C_{17}H_{10}ClN_5O_4S$	49.10	2.42	16.84
					(415.81)	49.11	2.44	16.87
3j	C_6H_4F(4-)	H	92	>300	$C_{17}H_{10}FN_5O_4S$	51.12	2.52	17.53
					(399.36)	52.01	2.77	17.27
4a	CH_2-CH=CH_2	CH_3	98	238-40	$C_{15}H_{13}N_5O_4S$	50.13	3.64	19.48
					(359.36)	50.27	3.16	18.99
4b	n-C_4H_9	CH_3	100	253-5	$C_{16}H_{17}N_5O_4S$	51.19	4.56	18.65
					(375.40)	51.60	4.31	18.38
4c	C_6H_5	CH_3	94	>300	$C_{18}H_{13}N_5O_4S$	54.68	3.31	17.71
					(395.39)	55.13	3.08	17.53
4d	$C_6H_4CH_3$(4-)	CH_3	77	282-3	$C_{19}H_{15}N_5O_4S$	55.73	3.69	17.11
					(409.42)	55.80	3.31	17.03
4e	C_6H_4Br(4-)	CH_3	91	>300	$C_{18}H_{12}BrN_5O_4S$	45.58	2.54	14.76
					(474.29)	46.01	2.11	14.21
4f	C_6H_4Cl(4-)	CH_3	92	>300	$C_{18}H_{12}ClN_5O_4S$	50.29	2.81	16.29
					(429.83)	50.21	2.96	16.01
4g	C_6H_4F(4-)	CH_3	94	>300	$C_{18}H_{12}FN_5O_4S$	52.30	2.92	16.94
					(413.38)	52.67	2.55	16.56
4h	$C_6H_4NO_2$(4-)	CH_3	88	>300	$C_{18}H_{12}N_6O_6S$	49.09	2.74	19.08
					(440.39)	48.90	2.56	18.61

Table 2. Antiviral activity and cytotoxicity on YFV and BVDV of compounds **1-4**

Comp.	R_1	R_2	X	YFV (Vero)		BVDV (MDBK)	
				EC_{50} (μg/mL)	MTC (μg/mL)	EC_{50} (μg/mL)	CC_{50} (μg/mL)
1a	CH_3	-	-	> 100	100	> 50	> 50
1b	C_2H_5	-	-	1.96	20	> 50	> 50
1c	CH_2-CH=CH_2	-	-	**2.18**	**100**	> 50	> 50
1d	n-C_4H_9	-	-	< 0.8	20	> 50	> 50
1e	cycl-C_6H_{11}	-	-	< 0.8	20	> 5	5
1f	C_6H_5	-	-	< 0.8	4	> 50	> 50
1g	$C_6H_4CH_3$(4-)	-	-	1.96	20	> 17	17
1h	C_6H_4Br(4-)	-	-	< 0.8	20	> 17	17
1i	C_6H_4Cl(4-)	-	-	< 0.8	4	> 19	19
1j	C_6H_4F(4-)	-	-	< 0.8	4	> 20	20
1k	$C_6H_4NO_2$(4-)	-	-	4	20	> 22	22
2a	CH_3	-	O	> 100	100	> 20	20
2b	CH_2-CH=CH_2	-	O	**11**	**100**	**40**	**> 50**
2c	CH_2-CH=CH_2	-	CH_2	> 4	4	> 16	16
2d	n-C_4H_9	-	O	> 20	20	> 19	19
2e	cycl-C_6H_{11}	-	O	< 0.8	20	> 17	17
2f	cycl-C_6H_{11}	-	CH_2	< 0.8	20	> 21	21
2g	C_6H_5	-	O	2.18	20	> 50	> 50
2h	C_6H_5	-	CH_2	> 20	20	> 20	20
2i	$C_6H_4CH_3$(4-)	-	O	3.06	20	> 21	21
2j	$C_6H_4CH_3$(4-)	-	CH_2	1.59	20	> 19	19
2k	C_6H_4Cl(4-)	-	O	> 20	20	> 17	17
2l	C_6H_4Cl(4-)	-	CH_2	> 20	20	> 19	19
3a	CH_3	H	-	> 20	20	**50**	**> 50**
3b	C_2H_5	H	-	**2.52**	**100**	> 17	17
3c	CH_2-CH=CH_2	H	-	2.18	20	> 4	4
3d	n-C_4H_9	H	-	> 100	100	> 50	> 50
3e	cycl-C_6H_{11}	H	-	1.96	20	> 3	3
3f	C_6H_5	H	-	> 20	20	**44**	**> 50**
3g	$C_6H_4CH_3$(4-)	H	-	4	20	> 50	> 50
3h	C_6H_4Br(4-)	H	-	> 20	20	> 5	5
3i	C_6H_4Cl(4-)	H	-	1.52	20	> 2	2
3j	C_6H_4F(4-)	H	-	> 20	20	> 9	9
4a	CH_2-CH=CH_2	CH_3	-	> 20	20	> 50	> 50
4b	n-C_4H_9	CH_3	-	> 20	20	> 50	> 50
4c	C_6H_5	CH_3	-	> 20	20	> 7	7
4d	$C_6H_4CH_3$(4-)	CH_3	-	> 20	20	> 41	41
4e	C_6H_4Br(4-)	CH_3	-	1.96	4	**13**	**> 50**
4f	C_6H_4Cl(4-)	CH_3	-	1.59	20	**48**	**> 50**
4g	C_6H_4F(4-)	CH_3	-	> 4	4	> 3	3
4h	$C_6H_4NO_2$(4-)	CH_3	-	> 100	100	> 18	18
Rib.				45	> 100	40	

Experimental Section

General Procedures. M.p.'s were determined on a Büchi 530 apparatus and are uncorrected. Elemental analyses were performed on Carlo Erba 1106 elemental analyzers. IR spectra were recorded on KBr discs, using a Perkin Elmer 1600 FT-IR spectrometer. ^{1}H NMR and ^{13}C NMR spectra were obtained on Bruker AC 200 (200 and 50.3 MHz) spectrophotometer using DMSO-d_6. EIMS (70 eV) were recorded onVG Zab Spec.

Synthesis of 5-nitro-3-[(5-nonsubstituted/methyl-4-thiazolidinone-2-ylidene)hydrazono]-1*H*-2-indolinones (3a-j and 4a-h). To a suspension of an appropriate thiosemicarbazone **1** (2.5 mmol) in absolute ethanol (25 mL), anhydrous sodium acetate (10 mmol) and ethyl bromoacetate or ethyl 2-bromopropionate (2.5 mmol) were added. The reaction mixture was refluxed for 4 h, cooled, diluted with water and allowed to stand overnight. The yellow crystals obtained were filtered and purified by re-crystallization from ethanol.

3-[(3-n-Butyl-4-thiazolidinone-2-ylidene)hydrazono]-5-nitro-1*H*-2-indolinone (3d). IR (ν, cm^{-1}): 3208 (NH), 1734, 1689 (C=O), 1496, 1339 (NO_2). ^{1}H NMR (δ, ppm): 0.94 (t, *J*=7.2 Hz, 3H, CH_3), 1.47 (m, 2H, butyl), 1.75 (quin, *J*=7.4 Hz, 2H, butyl), 3.90 (t, *J*=7.3 Hz, 2H, butyl), 4.09, 4.11 (2s, 2H, SCH_2), 7.07 (d, *J*=8.6 Hz, 1H, indole C_7-H), 8.29 (dd, *J*=8.7, 2.3 Hz, 1H, indole C_6-H), 8.94 (d, *J*=2.3 Hz, 1H, indole C_4-H), 11.34 (s, 1H, indole NH). EIMS (m/z, %): 361 (M^+, 4), 331 (17), 277 (14), 191 (9), 171 (14), 156 (40), 133 (19), 117 (57), 78 (86), 63 (100).

5-Nitro-3-[(3-phenyl-4-thiazolidinone-2-ylidene)hydrazono]-1*H*-2-indolinone (3f). IR (ν, cm^{-1}): 3320 (NH), 1735, 1698 (C=O), 1524, 1341 (NO_2). ^{1}H NMR (δ, ppm): 4.20, 4.24 (2s, 2H, SCH_2), 6.99 (d, *J*=8.4 Hz, 1H, indole C_7-H), 7.42-8.20 (m, 7H, indole $C_{4,6}$-H and phenyl), 11.28 (br.s, 1H, indole NH). EIMS (m/z, %): 381 (M^+, 100), 351 (26), 281 (13), 191 (88), 190 (15), 135 (42), 118 (37), 77 (36).

3-[(3-(4-Chlorophenyl)-4-thiazolidinone-2-ylidene)hydrazono]-5-nitro-1*H*-2-indolinone (3i). IR (ν, cm^{-1}): 3296 (NH), 1734, 1705 (C=O), 1493, 1334 (NO_2). ^{1}H NMR (δ, ppm): 4.19, 4.22 (2s, 2H, SCH_2), 7.00 (d, *J*= 8.4 Hz, 1H, indole C_7-H), 7.52-8.22 (m, 6H, indole $C_{4,6}$-H and phenyl), 11.30 (br.s, 1H, indole NH). EIMS (m/z, %): 415 [M^+, 100 (417, 39)], 387 [33 (389, 8)], 225 [33 (227, 13)], 191 (9), 176 (21), 169 (30), 152 [26 (154, 9)], 132 (20), 118 (9).

3-[(3-Allyl-5-methyl-4-thiazolidinone-2-ylidene)hydrazono]-5-nitro-1*H*-2-indolinone (4a). IR (ν, cm^{-1}): 3179 (NH), 1734, 1698 (C=O), 1505, 1339 (NO_2). ^{1}H NMR (δ, ppm): 1.60 (d, *J*=7.3 Hz, 3H, CH_3), 4.47 (q, *J*=7.3 Hz, 1H, SCH), 4.52 (d, *J*=4.8 Hz, 2H, CH_2-CH=CH_2), 5.23, 5.33 (dd, *J*=17.5, 10.4 Hz, 2H, CH_2-CH=CH_2), 5.88- 6.07 (m, 1H, CH_2-CH=CH_2), 7.06 (d, *J*=8.7 Hz, 1H, indole C_7-H), 8.30 (dd, *J*=8.7, 2.3 Hz, 1H, indole C_6-H), 8.91 (d, *J*=2.3 Hz, 1H, indole C_4-H), 11.43 (s, 1H, indole NH).

5-Nitro-3-[(3-phenyl-5-methyl-4-thiazolidinone-2-ylidene)hydrazono]-1*H*-2-indolinone (4c). IR (ν, cm^{-1}): 3296 (NH), 1756, 1699 (C=O), 1519, 1350 (NO_2). ^{1}H NMR (δ, ppm):1.70 (d, *J*=7.2 Hz, 3H, CH_3), 4.55 (q, *J*=7.2 Hz, 1H, SCH), 6.99 (d, *J*=8.6 Hz, 1H, indole C_7-H), 7.50-7,66 (m, 5H, ar), 8.14-8.17 (m, 1H, indole C_6-H), 8.21 (d, *J*=2.5 Hz, 1H, indole C_4-H), 11.31 (s,

1H, indole NH). ^{13}C NMR (δ, proton decoupled): 19.30 (thiazolidin CH_3), 42.05 (thiazolidin C_5), 110.55 (indole C_7), 116.00 (indole C_{3a}), 122.88 (indole C_6), 127.59 (phenyl $C_{2,6}$), 128.78 (indole C_4), 129.16 (phenyl C_4), 129.32 (phenyl $C_{3,5}$), 135.00 (phenyl C_1), 142.00 (indole $C_{5,7a}$), 149.00 (indole C_3), 154.00 (thiazolidin C_2), 165.00 (indole C=O), 175.11 (thiazolidin C=O). EIMS (m/z, %): 395 (M^+, 100), 320 (52), 205 (86), 190 (6), 135 (48), 118 (36).

3-[(3-(4-Bromophenyl)-5-methyl-4-thiazolidinone-2-ylidene)hydrazono]-5-nitro-1*H*-2-indolinone (4e). IR (ν, cm^{-1}): 3273 cm^{-1} (NH), 1750, 1691 (C=O), 1490, 1328 (NO_2). ^{1}H NMR (δ, ppm): 1.68 (d, *J*=7.2 Hz, 3H, CH_3), 4.53 (q, *J*=7.2 Hz, 1H, SCH), 7.00 (d, *J*=8.4 Hz, 1H, indole C_7-H), 7.51 (d, *J*=8.6 Hz, 2H,ar), 7.80 (d, *J*=8.7 Hz, 2H,ar), 8.16-8.20 (m, 1H, indole C_6-H), 8.23 (d, *J*=2.5 Hz, 1H, indole C_4-H), 11.38 (s, 1H, indole NH). EIMS (m/z, %): 473 [M^+, 23 (475, 23)], 395 (96), 283 [16 (285, 17)], 205 (100), 192 (17), 135 (52), 118 (48).

5-Nitro-3-[(3-(4-nitrophenyl)-5-methyl-4-thiazolidinone-2-ylidene)hydrazono]-1*H*-2-indolinone (4h). IR (ν, cm^{-1}): 3314 cm^{-1} (NH), 1747, 1696 (C=O), 1516, 1349 (NO_2). ^{1}H NMR (δ, ppm): 1.72 (d, *J*=7.2 Hz, 3H, CH_3), 4.58 (q, *J*=7.2 Hz, 1H, SCH), 6.98 (d, *J*=8.7 Hz, 1H, indole C_7-H), 7.88 (d, *J*=9.1 Hz, 2H, ar), 7.98 (d, *J*=2.4 Hz, 1H, indole C_4-H), 8.18 (dd, *J*=8.7, 2.6 Hz, 1H, indole C_6-H), 8.49 (d, *J*=8.9 Hz, 2H, ar), 11.38 (s, 1H, indole NH).

Antiviral activity assays. Antiviral activity against the yellow fever virus (YFV), hepatitis C virus (HCV), the parainfluenza-3 virus, reovirus-1, sindbis virus, coxsackie virus B4, punta toro virus, herpes simplex virus-1, herpes simplex virus-2, vaccinia virus, vesicular stomatitis virus, coxsackie virus B4 and respiratory syncytial virus was determined essentially as described previously[11].

Antiviral activity assay for BVDV. MDBK cells were seeded at a density of 5 x 10^3 per well in 96-well cell culture plates in MEM-FCS. Following 24 h incubation at 37°C and 5% CO_2 medium was removed and 5-fold serial dilutions of the test compounds were added in a total volume of 100 µL, after which the cp BVDV virus inoculum [100 $CCID_{50}$ (50% cell culture infective dose) in 100 µL] was added to each well. This inoculum resulted in a greater than 90% destruction of the cell monolayer after 4-5 days of incubation at 37°C. Uninfected cells and cells receiving virus without compound were included in each assay plate. After 5 days, medium was removed, and 90 µL of MEM-FCS supplemented with 10 µL of MTS/PMS solution (Promega, Leiden, The Netherlands) was added to each well. Following a 2 h incubation period at 37°C, the optical density of each well was read at 498 nm in a microplate reader. The 50% effective concentration (EC_{50}) was defined as the concentration of compound of which 50% cell viability was protected from virus-induced cytopathic effect (CPE)[4].

Cytostatic assay for BVDV. MDBK cells were seeded at a density of 5 x 10^3 cells per well of a 96-well plate in MEM-FCS. 24 hours later, serial dilutions of the test compounds were added. Cells were allowed to proliferate for 3 days at 37°C, after which the cell number was determined by means of the MTS/PMS method (Promega). The 50% cytotoxic concentration (CC_{50}) was defined as the concentration that inhibited the proliferation of exponentially growing cells by 50%.

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References

1. (a) Teitz, Y.; Barko, N.; Abramoff, M.; Ronen, D.; *Chemotherapy* **1994**, *40*, 195. (b) Teitz, Y.; Ronen, D.; Vansover, A.; Stematsky, T.; Riggs, J. L. *Antiviral Res.* **1994**, *24*, 305. (c) Winter, J.; Hall, R.L.; Moyer, R.W. *Virology* **1995**, *211*, 462.
2. Sethi, M. L. *Foye's Principles of Medicinal Chemistry*, Lippincott Williams and Wilkins: Philadelphia, 2002, chapter 39.
3. (a) Zhang, H.; Vrang, L.; Bacbro, K.; Lind, P.; Sahlberg, C.; Unge, T.; Öberg, B. *Antiviral Res.* **1995**, *28*, 331. (b) Mishra, V.; Pandeya, S. N.; DeClercq, E.; Pannecouque, C.; Witvrouw, M. *Pharm. Acta Helv*. **1998**, *73*, 215.
4. Sun, J.-H.; Lemm, J. A.; O'Boyle, D. R.; Colonno, R.; Gao, M. *J. Virology* **2003**, *77*, 6753.
5. (a) Rao, A.; Carbone, A.; Chimirri, A.; De Clercq, E.; Monforte, A. M.; Monforte, P.; Pannecouque, C.; Zappala, M. *Il Farmaco* **2003**, *58*, 115. (b) Barreca, M. L.; Chimirri, A.; De Clercq, E.; De Luca, L.; Monforte, A. M.; Monforte, P.; Rao, A.; Zappalà, M. *Il Farmaco* **2003**, *58*, 259. (c) Rao, A.; Balzarini, J.; Carbone, A.; Chimirri, A.; De Clercq, E.; Monforte, A. M.; Monforte, P.; Pannecouque, C.; Zappalà, M. *Il Farmaco* **2004**, *59*, 33. (d) Rao, A.; Balzarini, J.; Carbone, A.; Chimirri, A.; De Clercq, E.; Monforte, A. M.; Monforte, P.; Pannecouque, C.; Zappalà, M. *Antiviral Res.* **2004**, *63*, 79.
6. (a) Petrov, I.; Grupce, O.; Stafilov, T. *J. Mol. Struct*. **1986**, *142*, 275. (b) Naumov, P.; Anastasova, F. *Spectr. Acta* **2001**, *57A*, 469. (c) Moustafa, M. A.; Bayomi, S. M.; El-Emam, A. A.; El-Kerdawy, M. M. *Sci. Pharm*. **1989**, *57*, 125.
7. Farghaly, A. M.; Habib, N. S.; Khalil, M. A.; El-Sayed, O. A.; Bistawroos, A. E. *Arch. Pharm. (Weinheim)* **1990**, *323*, 247.
8. (a) Omar, A.-M. M. E.; Eshba, N. H.; Salama, H. M. *Arch Pharm. (Weinheim)* **1984**, *317*, 701. (b) Varma, R. S.; Singh, A. P.; Singh, S. P. *Org. Mass Spectr*. **1992**, *27*, 17.
9. Karalı, N. *Eur. J. Med. Chem*. **2002**, *37*, 909.
10. Balzarini, J.; De Clercq, E.; Van Hemel, J.; Neyts, J.; Peys, E.; Ruttens, B.; Van der Eycken, J.; Vandenkerckhove, J. US Patent Appl. 20040209823, 514023000 (USPTO), A61K031/70 (Intl Class), 10/21/04; Davis, Brown, Koehn, Shors & Roberts, P.C. The Financial Center - Des Moines, IA, US.
11. (a) Leysen, P.; De Clercq, E.; Neyts, J.; *Clin. Microbiol. Rev*. **2000,** *13*, 67. (b) De Clercq, E.; Holy, A.; Rosenberg, I.; Sakuma, T.; Balzarini, J.; Maudgal, P. C. *Nature* **1986,** *323*,

464. (c) Balzarini, J.; Naesens, L.; Slachmuylders, J.; Niphuis, H.; Rosenberg, I.; Holy, A.; Schellekens, H.; De Clercq, E. *AIDS* **1991,** *5*, 21. (d) Neyts, J.; Meerbach, A.; McKenna, P.; De Clercq, E. *Antiviral Res.* **1996,** *30*, 125.