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Antiviral activities of 5-chlorobenzotriazole derivatives

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Abstract

In this study, we designed and synthesized a series of new 5-chlorobenzotriazole derivatives. Compounds were tested for cytotoxicity and antiviral activity in cell-based assays against several positive single-stranded RNA viruses: BVDV, YFV, CVB-5, and Sb-1; two negative single-stranded RNA viruses: RSV and VSV; a double-stranded RNA virus (Reo-1); and two DNA viruses: VV and HSV-1. *N*-[4-(5-Chloro-2*H*-benzo[*d*][1,2,3]triazol-2-yl)phenyl]-3,4,5-trimethoxybenzamide turned out to be the most potent compound against bovine viral diarrhea virus (BVDV). Its activity was shown to be comparable to the activity of the reference drug NM 107 (EC₅₀ 3.0 vs. 1.7 μ M). It is going to be used as a lead compound for future antiviral drug developments.

Graphical abstract



Keywords 5-Chloro-phenyl-benzotriazoles · Antiviral activity · Drug research · Heterocycles · Respiratory syncytial virus

Introduction

Viruses are small infectious obligate parasites that need a host cell to reproduce. Over the years, viruses have developed many strategies to be able to elude the immune response of the host, so that, notwithstanding the everimproving antiviral drug development, viruses continue causing many diseases in humans.

The first viruses were discovered in the last years of the nineteenth century, but only 70 years later, it was possible to begin to treat viral infections. In the 1960s, several

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² Department of Biomedical Sciences, University of Cagliari, Cittadella Universitaria, Monserrato, 09042 Cagliari, Italy compounds, originally synthesized as potential anticancer agents, proved to be active against different viruses. Later on, the discovery of the antiviral activity of drugs like ribavirin and acyclovir opened the way for antiviral drug development research, whose efforts are principally focused on searching for nucleotides or nucleosides analogues, because most antiviral compounds are inhibitors of viral helicase and/or polymerase enzymes [1].

Nowadays, many DNA and RNA viruses are known to infect humans and cause several serious diseases [2], but there are still no fool proof drugs against them. Antivirals can be differentiated into two groups: immunomodulators and antiviral agents. Antiviral agents inhibit viral replication by targeting different stages of the viral life cycle, such as viral adsorption; virus–host cell fusion; viral uncoating; viral nucleic acids (DNA or RNA) synthesis. Antivirals inhibit the enzymes involved in these processes and stop or restrict viral maturation [3]. Antivirals can also target host proteins which are relevant for viral replication, such as endoplasmic reticulum (ER) alpha-glucosidases involved in the viral glycoprotein folding quality control [4]. Both virus- and host-targeting antiviral agents need to inhibit viral replication without affecting the host organism, and they need to be non-toxic; for example, they cannot be carcinogenic, allergenic, toxic, or mutagenic.

In the last 10 years, we have established an antiviral drug development research program, and have synthesized several molecules with powerful antiviral activity [5–9]. Indeed, as it is well known, scaffolds such as benzimidazoles [10, 11] and benzotriazoles [12] allow developing molecules with different biological activities, including antiviral activity. Following on from these encouraging results, we tried to streamline and simplify these molecules [13–15].

Encouraged by these evidences, we have now prepared a series of 5-chlorobenzotriazole-benzamides (compounds 5–10, Scheme 2; compound 15, Scheme 3); 5-chlorobenzotriazole-phenyl-ureas (11, 12); and 5-chlorobenzotriazole-benzamines (compounds 13, 14, Scheme 3), with the aim of evaluating the selectivity of the compounds against representative DNA viruses, positive- and negative-sense single-stranded RNA viruses, and double-stranded RNA viruses. All compounds were also tested for cytotoxicity in the cell lines sustaining the alternative virus replication.

Results and discussion

Chemistry

The mixture of intermediates 5-chloro-2-(4-nitrophenyl)-2*H*-benzo[*d*][1,2,3]triazole (3a),5-chloro-1-(4-nitrophenyl)-1*H*-benzo[d][1,2,3]triazole (**3b**), 6-chloro-1-(4-nitrophenyl)-1*H*-benzo[d][1,2,3]triazole (**3c**) was prepared as reported in the literature [16] (Scheme 1) starting from the condensation of 4-chloronitrobenzene (2) with 5-chlorobenzotriazole (1) in DMF with the presence of Cs_2CO_3 used as a base, to obtain a mixture of the 1(2)(3)-pnitrophenyl benzotriazole derivatives **3a–3c**. The reduction of the mixture of three isomers **3a–3c** by methylhydrazine in ethanol (at 100 °C in autoclave for 46 h) gave the mixture of compounds 4a-4c. Chromatography on silica gel (petroleum ether/ethyl acetate 8/2) allowed the separation of the three isomers. The N2 isomer derivatives appeared to be the most promising molecules; therefore, 4-(5-chloro-2*H*-benzo[*d*][1,2,3]triazol-2-yl)aniline (4a) was isolated in 43% yield. The isomers 4b and 4c, as well as the isomer 4a, were purified and tested in antiviral cell assays for their potential antiviral activity, as well.

The series of *N*-[4-(5-chloro-2*H*-benzo[*d*][1,2,3]triazol-2yl)phenyl]carboxamides, 1-[4-(5-chloro-2*H*-benzo[*d*][1,2,3] triazol-2-yl)phenyl]-3-alkylureas, and 4-(5-chloro-2*H*-benzo [*d*][1,2,3]triazol-2-yl)-*N*-alkylanilines and *N*-[4-(5-chloro-2*H*benzo[*d*][1,2,3]triazol-2-yl)phenyl]acetamides were prepared, as reported in Schemes 2 and 3, by condensation of compound 4a with the suitable substituted benzoyl chlorides in dimethylformamide (DMF), at reflux for 2 h (compounds 5– 10); or with substituted isocyanates in dichloromethane (DCM) at room temperature for 24 h (compounds 11, 12); or with dimethyl sulfate in DMF at 100 °C for 2 h (compound 13); or with diethyl sulfate in DMF at 100 °C for 5 h (compound 14); or with acetic anhydride in DMF at 60 °C for 1 h (compound 15), respectively.

The purification of compounds **5–15** was achieved by flash chromatography using an appropriate mixture of solvents as eluents. The amide-BT derivatives **5–10** were isolated in the range of 13-27.3% yield and the ureido-BT derivatives **11**, **12** were obtained in the range of 38-50% yield. The *N*-alkyl derivatives **13**, **14** and the acetamide-derivative **15** were obtained in the range of 76-66% yield.

Anti-viral assays

All derivatives considered in this work (Table 1) were evaluated for antiviral activity against RNA and DNA viruses (Table 2). Among single-stranded, positive RNA viruses (ssRNA⁺), we considered a Flavivirus (Yellow Fever Virus, YFV) and a Pestivirus (Bovine Viral Diarrhea Virus, BVDV); and two Picornaviruses (Coxsackie virus type-5, CVB-5, and Poliovirus type-1, Sabin strain, Sb-1). Among single-stranded, negative RNA viruses (ssRNA⁻), a paramyxoviridae (respiratory syncytial virus, RSV) and a Rhabdoviridae (Vesicular Stomatitis Virus, VSV) were selected as representatives. Among double-stranded RNA (dsRNA) viruses, a Reoviridae family member (Reo-1) was included. Finally, two representatives of DNA virus families were also included: Herpes Simplex Virus type-1, HSV-1 (Herpesviridae), and Vaccinia Virus, VV (Poxviridae). NM 107 (2'-C-methylcytidine), NM 108 (2'-C-methylguanosine), ribavirin, 6-azauridine, ACV (acyclovir), pleconaril, and mycophenolic acid were used as reference inhibitors (Table 2).

Conclusions

As far as the antiviral activity is concerned, none of compounds turned out to be active against Reo-1, YFV, CVB-5, Sb-1, VSV, VV, and HSV-1 viruses. Some compounds showed activity against RSV (**4a**, **4b**, **13**, and **14**) and BVDV (**4a** and **6**) in the range of EC₅₀ 10–35 and $3-14 \mu$ M, respectively.

We recall that human respiratory syncytial virus (RSV) is one of the most important causes of lower respiratory tract infections in infants and young children and of pneumonia and bronchiolitis in adults [17, 18]. Furthermore, RSV is one of the main causes of respiratory



problems in the elderly and in immuno-compromised patients [19, 20]. For these reasons, new molecules with anti-RSV activity are especially desirable. Therefore, compound **4b** showing a good antiviral activity accompanied by high selectivity and devoid of cytotoxicity towards all the cell lines used is a very promising molecule. The molecule has a simple chemical structure and will be used as lead compound in future drug development efforts centered around the insertion of alkyl groups on the amino nitrogen (the latter modifications have proved useful in derivatives of **4a** as shown in this paper).

Bovine viral diarrhea virus (BVDV) causes a range of clinical manifestations in bovines, including respiratory problems, abortion, and teratogenesis [21, 22], and impacts significantly on the livestock industry. *N*-[4-(5-Chloro-2*H*-benzo[*d*][1,2,3]triazol-2-yl)phenyl]-3,4,5-trimethoxyben-zamide (**6**) shows an excellent activity against BVDV, comparable to that of the reference drug NM 107 (EC₅₀ 3.0 vs. 1.7 μ M), and displays complete selectivity of action and lack of cytotoxicity. Compound **6**, therefore, proves to be an excellent candidate for further drug development, in particular for in vivo assays.

Scheme 2



Table 1 List of compounds 5-12

Compound	\mathbb{R}^1	\mathbb{R}^2	R ³	Compound	R^4
5	Н	NO_2	Н	11	Cyclohexyl
6	OCH ₃	OCH ₃	OCH ₃	12	Propyl
7	Н	OCH ₃	Н		
8	Н	CF ₃	Н		
9	Н	CH ₃	Н		
10	Н	CN	Н		

Experimental

NMR spectra were recorded on a Bruker AVANCE III 400 Nanobay instrument for ¹H at 400 MHz and for ¹³C at 100 MHz, using TMS as internal standard. The chemical shifts values (δ) are reported in parts per million (ppm) and coupling constants (*J*) are expressed in Hertz (Hz). Signal multiplicities are represented by: s (singlet), d (doublet), dd (double doublet), t (triplet), q (quadruplet), and m (multiplet). A Kofler heating bench was used to determine the melting point of each compound. GC–MS analyses were performed

Table 2	Antiviral activity	and cytotoxicity	of 5-chlorobenzotriazoles	(4a–4c and 5–15)
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Comps	^a MDBK CC ₅₀ /µM	^b BVDV EC ₅₀ /μM	^c BHK 21 CC ₅₀ /µМ	^d YFV EC ₅₀ /μl	^d Reo-1 M	^e Vero-76 CC ₅₀ /μM	^f CVB-5 EC ₅₀ /μM	^f Sb-1	^f VSV, VV, HSV-1	^f RSV
4a	54	14	30	> 30	> 30	> 100	> 100	> 100	> 100	10
4b	> 100	> 100	77	> 77	> 77	> 100	> 100	> 100	> 100	30
4c	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
5	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
6	> 100	3	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
7	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
8	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
9	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
10	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
11	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
12	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
13	70	> 70	46	> 46	> 46	> 100	> 100	> 100	> 100	21
14	75	> 75	94	> 94	> 94	> 100	> 100	> 100	> 100	35
15	80	> 80	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 32
References										
NM 107	> 100	1.8	> 100	1.4						
NM 108	55	15								
Ribavirin			> 100	46		10				1.1
6-Azauridine						> 100			HSV-1 (2.4)	
ACV						80	0.05	2		
Pleconaril						20			VV (1.4)	
Mycophenolic acid	> 100	1.7	> 100	1.4						

Data represent mean values \pm SD for three independent determinations. For values where SD is not shown, variation among duplicate samples was less than 15%

The bold values are the most interesting results of this antiviral screening, are highlighted the EC_{50} values that are comparable with the EC_{50} of the reference drugs

^aCompound concentration (µM) required to reduce the viability of mock-infected MDBK cells by 50%, as determined by the MTT method

 b Compound concentration (μ M) required to achieve 50% protection of MDBK cells from the BVDV-induced cytopathogenicity, as determined by the MTT method

^cCompound concentration (μ M) required to reduce the viability of mock-infected BHK monolayers by 50%, as determined by the MTT method ^dCompound concentration (μ M) required to achieve 50% protection of BHK cells from YFV and Reo-1 induced cytopathogenicity, as determined by the MTT method

^eCompound concentration (μ M) required to reduce the viability of mock-infected VERO-76 monolayers by 50%

^fCompound concentration (µM) required to reduce the plaque number of the indicated virus by 50% in VERO-76 monolayers

using an Agilent Model 6850 series gas chromatograph system coupled to an Agilent Technologies 5973 network mass selective detector. The chromatographic separations were performed using an HP-5 (5% Phenyl, 95% methyl polysiloxane), 30 m × 0.20 mm i.d., fused-silica capillary column of 0.25 μ m film thickness. The injector temperature was 250 °C and the splitless injection mode was used. The oven temperature program was 80 °C (held for 3 min) to 300 °C at 10 °C/min (held for 15 min). The MS operating conditions were performed on the AB SCIEX 4000 QTRAP[®]LC–MS/MS system by direct injection (50 mm³) and detected in positive polarity.

Flash chromatography was performed using 70–230 mesh (Merck silica gel 60). Light petroleum refers to the fraction with b.p. 40–60 °C. The progress of the reactions, the $R_{\rm f}$, and the purity of the final compounds were monitored by TLC using Merck F-254 commercial plates. In all cases, elemental chemical composition was within $\pm 0.4\%$ of the theoretical value.

The intermediates 5-chloro-2-(4-nitrophenyl)-2*H*-benzo[*d*][1,2,3]triazole (**3a**) and 4-(5-chloro-2*H*-benzo[*d*][1,2,3]triazol-2-yl)aniline (**4a**) were synthesized according to procedure previously reported by us [13]. 5-Chlorobenzotriazole (**1**) and 4-chloronitrobenzene (**2**) were commercially available (Sigma-Aldrich).

5-Chloro-2-(4-nitrophenyl)-2H-benzo[d][1,2,3]triazole (3a, $C_{12}H_7CIN_4O_2$) A mixture of 1.0 g *p*-chloro-nitrobenzene 6.35 mmol), 1.2 g *p*-chloro-benzotriazole (2, (1, 7.62 mmol), and 3.10 g Cs₂CO₃ (9.52 mmol) in 10 cm³ DMF was stirred and heated at 140 °C for 5 h. Progress of the reaction was monitored by TLC (diethyl ether/petroleum ether, 7/3). The reaction mixture was poured onto about 250 cm³ of crushed ice. After all ice melted, the solid was filtered off and washed with H₂O. The product was suspended in 200 cm³ MeOH, stirred for 30 min, and left in suspension overnight. The precipitate was filtered off and dried in an oven (45 °C). The crude 3 was a mixture of three isomers. Compound 3a was isolated by flash chromatography (petroleum ether/diethyl ether, 3/1) in 20% vield (60 mg). Yellow solid; m.p.: 218.8–220.0 °C; R_f = 0.67 (petroleum ether/diethyl ether, 3/1); ¹H NMR (400 MHz, DMSO- d_6): δ = 7.59 (d, 1H, J = 12 Hz, CH-6), 8.16 (d. 1H. J = 8.0 Hz, CH-5), 8.28 (s. 1H, CH-3), 8.53 (dd, 4H, J = 8.0 Hz, J = 20 Hz, CH-2',5', CH-3',6') ppm; ¹³C NMR (100 MHz, DMSO- d_6): $\delta = 117.52$ (CH-2' + CH 6'), 120.44 (CH-3' + CH 5'), 121.28 (CH-4), 125.62 (CH-6), 129.85 (CH-7), 133.17 (C-Cl), 143.24 (C-1'), 143.61 (C-7a), 145.28 (C-3a), 147.44 (C-NO₂) ppm; GC-MS: calc. m/z = 274.02 (C₁₂H₇ClN₄O₂, [M]⁺), found $m/z = 274 ([M]^+)$ at 23.472 min (Scan 6460).

Synthesis of compounds 4a–4c A mixture of 1.5 g of compounds 3a–3c (5.46 mmol) and 3.75 cm³ methylhydrazine (71.65 mmol) in 120 cm³ EtOH was stirred and heated in an autoclave at about 100 °C for 46 h. The solvent was removed under reduced pressure to obtain the crude mixture 4a–4c as a yellow solid. The TLC (petroleum ether/ethyl acetate, 7/3) confirmed that the crude 4a–4c is a mix of three isomers. Their separation was performed by flash chromatography (petroleum ether/ethyl acetate, 4/1) to give 4a in 43% yield, 4b in 22% yield, and 4c in 35% yield.

4-(5-Chloro-2*H***-benzo[***d***][1,2,3]triazol-2-yl)aniline (4a, C_{12}H_9-ClN₄) Pale yellow solid; m.p.: 161.3–162.1 °C; R_f = 0.34 (petroleum ether/ethyl acetate, 7/3); ¹H NMR (400 MHz, DMSO-***d***₆): \delta = 5.77 (s, 2H, NH₂), 6.74 (d, 2H, J = 8.0 Hz, CH-3',5'), 7.46 (d, 1H, J = 8.0 Hz, CH-5), 7.96 (d, 2H, J = 8.0 Hz, CH-2',6'), 8.01 (d, 1H, J = 8.0 Hz, CH-6), 8.11 (s, 1H, CH-3) ppm; ¹³C NMR (100 MHz, DMSO-***d***₆): \delta = 113.70 (CH-2' + CH 6'), 116.66 (CH-3' + CH 5'), 119.44 (CH-4), 121.61 (CH-6), 127.53 (C-1'), 128.56 (CH-7), 131.01 (C–Cl), 142.56 (C-7a), 144.33 (C-3a), 150.52 (C-NH₂) ppm; GC–MS: calc.** *m/z* **= 244.05 (C₁₂H₉ClN₄, [M]⁺), found** *m/z* **= 244 ([M]⁺) at 23.498 min (Scan 7198).**

4-(5-Chloro-1*H***-benzo[***d***][1,2,3]triazol-1-yl)aniline (4b, C_{12}H_9-ClN₄) Pale yellow solid; m.p.: 125.6–126.2 °C; R_f = 0.21**

(petroleum ether/ethyl acetate, 7/3); ¹H NMR (400 MHz, DMSO- d_6): δ = 5.63 (s, 2H, NH₂), 6.78 (d, 2H, J = 8.8 Hz, CH-3',5'), 7.60 (d, 2H, J = 8.8 Hz, CH-2',6'), 7.80 (d, 1H, J = 8.8 Hz, CH-6), 8.17 (d, 1H, J = 8.8 Hz, CH-7), 8.27 (s, 1H, CH-4) ppm; ¹³C NMR (100 MHz, DMSO- d_6): δ = 115.64 (CH-2' + CH 6'), 117.43 (CH-3' + CH 5'), 120.83 (CH-4), 122.94 (CH-6), 124.12 (CH-7), 127.41 (C-1'), 129.08 (C-7a), 135.33 (C-Cl), 146.65 (C-3a), 149.81 (C-NH₂) ppm; LC-MS: calc. m/z = 244.05 (C₁₂H₉ClN₄, [M]⁺), found m/z = 245.1 ([M+H]⁺).

4-(6-Chloro-1*H***-benzo[***d***][1,2,3]triazol-1-yl)aniline (4c, C₁₂H₉-ClN₄) Pale yellow solid; m.p.: 166.7–168.0 °C; R_f = 0.18 (petroleum ether/ethyl acetate, 7/3); ¹H NMR (400 MHz, DMSO-***d***₆): \delta = 5.66 (s, 2H, NH₂), 6.78 (d, 2H,** *J* **= 8.8 Hz, CH-3',5'), 7.43 (d, 2H,** *J* **= 8.8 Hz, CH-2',6'), 7.49 (d, 1H,** *J* **= 8.8 Hz, CH-4), 7.82 (s, 1H, CH-7), 8.16 (d, 1H,** *J* **= 8.8 Hz, CH-5) ppm; ¹³C NMR (100 MHz, DMSO-***d***₆): \delta = 115.78 (CH-2' + CH 6'), 118.18 (CH-3' + CH 5'), 120.61 (CH-7), 122.81 (CH-5), 125.04 (CH-4), 128.14 (C-1'), 130.52 (C-7a), 134.90 (C–Cl), 142.19 (C-3a), 149.80 (C-NH₂) ppm; LC–MS: calc.** *m/z* **= 244.05 (C₁₂H₉ClN₄, [M]⁺), found** *m/z* **= 245.1 ([M+H]⁺).**

N-[4-(5-Chloro-2*H*-benzo[*d*][1,2,3]triazol-2-yl)phenyl]-4-ni-

trobenzamide (5, C₁₉H₁₂ClN₅O₃) A mixture of 130 mg 4-nitrobenzovl chloride (0.68 mmol) in 1.5 cm³ DMF and 140 mg compound 4a (0.57 mmol) in 2.5 cm³ DMF was stirred at 120 °C. The reaction was complete after 2 h. Cold H_2O (100 cm³) was poured to the reaction mixture. The precipitate was filtered off and dried in an oven overnight. The crude obtained was purified by flash chromatography (petroleum ether/ethyl acetate, 7.5/1.5) to give 5 in 27.3% yield (60 mg). Pale yellow solid; m.p.: 272.8–275.1 °C; $R_f = 0.32$ (petroleum ether/ethyl acetate, 7.5/1.5); ¹H NMR (400 MHz, DMSO- d_6): $\delta = 7.54$ (d, 1H, J = 12 Hz), 8.09–8.12 (m, 3H), 8.22–8.24 (m, 3H), 8.34 (d, 2H, J = 8.0 Hz), 8.41 (d, 2H, J = 8.0 Hz), 10.90 (s, 1H, NH) ppm; ¹³C NMR (100 MHz, DMSO- d_6): $\delta = 117.15$ (CH-2' + CH 6'), 120.01 (CH-3' + CH 5'), 120.93 (CH-4), 121.15 (CH-6), 123.59 (CH-3" + CH 5"), 128.61 (CH-2" + CH 6"), 129.31 (CH-6), 132.01 (C-Cl), 135.09 (C-1'), 140.06 (C-1"), 140.21 (C-NH), 142.98 (C-7a), 144.70 (C-3a), 149.28 (C-NO₂), 164.19 (CO) ppm; GC-MS: calc. $m/z = 393.06 (C_{19}H_{12}ClN_5O_3, [M]^+)$, found m/z $z = 393 ([M]^+)$ at 24.582 min (Scan 4520).

N-[4-(5-Chloro-2H-benzo[d][1,2,3]triazol-2-yl)phenyl]-3,4,5-

trimethoxybenzamide (6, $C_{22}H_{19}CIN_4O_4$) A mixture of 140 mg 3,4,5-trimethoxybenzoyl chloride (0.57 mmol) in 2.0 cm³ DMF and 160 mg compound **4a** (0.57 mmol) in 3.0 cm³ DMF was stirred at 120 °C. The reaction was complete after 2 h. Cold H₂O (100 cm³) was poured onto

the reaction mixture. The precipitate was filtered off and dried in an oven overnight. The crude obtained was purified by flash chromatography (petroleum ether/ethyl acetate, 6/3) to give 5 in 20% yield (50 mg). White solid; m.p.: 222.3–224.5 °C; $R_f = 0.40$ (petroleum ether/ethyl acetate, 6/3); ¹H NMR (400 MHz, DMSO- d_6): $\delta = 3.75$ (s, 3H, OCH_3-4''), 3.90 (s, 6H, 2 × $OCH_3-3'',5''$), 7.32 (s, 2H, $2 \times CH-2'', 6''$, 7.54 (d, 1H, J = 8 Hz, CH-5), 8.06 (d, 2H, J = 8.0 Hz, 2 × CH-2',6'), 8.10 (d, 1H, J = 12 Hz, CH-6), 8.21 (s, 1H, CH-3), 8.32 (d, 2H, J = 12 Hz, $2 \times$ CH-3',5'), 10.45 (s, 1H, NH) ppm; 13 C NMR (100 MHz, DMSO- d_6): $\delta = 56.12 (2 \text{ CH}_3), 60.13 (\text{CH}_3), 105.42 (\text{CH}-2'' + \text{CH} 6''),$ 117.12 (CH-2' + CH 6'), 119.97 (CH-3' + CH 5'), 120.84 (CH-4), 121.15 (CH-6), 128.54 (CH-7), 129.68 (C-1"), 131.95 (C-1'), 134.74 (C-Cl), 140.46 (C-NH), 140.53 (C-7a), 142.95 (C-4"), 144.68 (C-3a), 152.65 (CH-3" + CH 5"), 164.35 (CO) ppm; LC–MS: calc. m/z = 438.10 (C₂₂₋ $H_{19}ClN_4O_4$, $[M]^+$), found m/z = 439.3 ($[M+H]^+$).

N-[4-(5-Chloro-2H-benzo[d][1,2,3]triazol-2-yl)phenyl]-4-

methoxybenzamide (7, C₂₀H₁₅ClN₄O₂) A mixture of 630 mg 4-methoxybenzoyl chloride (3.68 mmol) in 8.0 cm^3 DMF and 450 mg compound 4a (1.84 mmol) in 8.0 cm³ DMF was stirred at 120 °C. The reaction was complete after 1 h. Cold H₂O (100 cm³) was poured onto the reaction mixture. The precipitate was filtered off and washed with water several times. The filtered was dried in an oven overnight. The crude obtained was purified by flash chromatography (petroleum ether/ethyl acetate, 1/1) to give 7 in 13% yield (40 mg). White solid; m.p.: 259.2–261.8 °C; $R_f = 0.32$ (petroleum ether/ethyl acetate, 2/3); ¹H NMR (400 MHz, DMSO- d_6): $\delta = 3.66$ (s, 3H, OCH₃), 7.08 (d, 2H, J = 8.0 Hz, CH-3", 5"), 7.54 (d, 1H, J = 8.0 Hz, CH-5), 8.01 (d, 2H, J = 8.0 Hz, CH-3',5'), 8.10 (m, 3H, CH-2',6', CH-6), 8.21 (s, 1H, CH-3), 8.30 (d, 2H, J = 8.0 Hz, Ch-2",6"), 10.43 (s, 1H, NHCO) ppm; ¹³C NMR-APT (100 MHz, DMSO- d_6): $\delta = 55.46$ (CH₃), 113.68 (CH-3" + CH 5"), 117.13 (CH-2' + CH 6'), 119.97 (CH-3' + CH 5'), 120.82 (CH-4), 120.52 (CH-6), 126.56 (C-1"), 128.86 (CH-2" + CH 6"), 129.74 (CH-7), 131.92 (C-1'), 134.53 (C-Cl), 140.80 (C-NH), 142.95 (C-7a), 144.68 (C-3a), 162.12 (C-4"), 165.16 (CO) ppm; GC-MS: calc. $m/z = 378.08 (C_{20}H_{15}CIN_4O_2, [M]^+)$, found m/z $z = 378 ([M]^+)$ at 43.878 min (Scan 9661).

N-[4-(5-Chloro-2*H*-benzo[*d*][1,2,3]triazol-2-yl)phenyl]-4-(trifluoromethyl)benzamide (8, $C_{20}H_{12}ClF_3N_4O$) A mixture of 800 mg 4-(trifluoromethyl)benzoyl chloride (3.84 mmol) in 9.0 cm³ DMF and 470 mg compound 4a (1.92 mmol) in 8.0 cm³ DMF was stirred at 120 °C. The reaction was complete after 2 h. Cold H₂O (100 cm³) was poured onto the reaction mixture. The precipitate was filtered off and washed with water several times. The filtered was dried in an oven overnight. The crude obtained was purified by flash chromatography (petroleum ether/ethyl acetate, 1/1) to give 8 in 20% yield (160 mg). White solid; m.p.: 262.6–264.2 °C; $R_f = 0.15$ (petroleum ether/ethyl acetate, 8/2); ¹H NMR (400 MHz, DMSO- d_6): $\delta = 7.54$ (d, 1H, J = 8 Hz, CH-5), 7.95 (d, 2H, J = 8 Hz, CH-3', 5'), 8.09-8.10 (m, 3H, CH-2',6', CH-6), 8.18-8.21 (m, 3H, CH-3, CH-3", 5"), 8.33 (d, 2H, J = 8 Hz, CH-2", 6"), 10.80 (s, 1H, NHCO) ppm; 13 C NMR-APT (100 MHz, DMSO- d_6): $\delta = 117.15$ (CH-2' + CH 6'), 120.00 (CH-3' + CH 5'), 120.91 (CH-4), 121.10 (CH-6), 125.43 (CH-7), 128.70 (CH-2" + CH 6"), 126,130.06 (CH-3" + CH 5"), 131.71 (CF₃), 132.00 (C-1'), 134.99 (C-Cl), 138.40 (C-4"), 140.20 (C-1"), 141.01 (C-NH), 142.98 (C-7a), 144.70 (C-3a), 164.69 (CO) ppm; GC-MS: calc. m/z = 416.06 (C₂₀H₁₂ ClF_3N_4O , $[M]^+$), found m/z = 416 ($[M]^+$) at 28.777 min (Scan 5402).

N-[4-(5-Chloro-2H-benzo[d][1,2,3]triazol-2-yl)phenyl]-4-

methylbenzamide (9, C20H15CIN4O) 4-Toluoyl chloride $(0.44 \text{ cm}^3, 3.34 \text{ mmol})$ was added to the solution of 400 mg compound 4a (1.67 mmol) in 15.0 cm³ DMF. The reaction mixture was stirred at 120 °C. The reaction was complete after 2 h. Cold H₂O (100 cm³) was poured onto the reaction mixture. The precipitate was filtered off and washed with water several times. The filtered was dried in an oven overnight. The crude obtained was purified by flash chromatography in eluent gradient (from petroleum ether/ethyl acetate, 1/1 to ethyl acetate) to give 9 in 15% yield (50 mg). White solid; m.p.: 271.8–273.0 °C; $R_{f_{r}}$ = 0.18 (petroleum ether/ethyl acetate, 4/1); ¹H NMR (400 MHz, DMSO- d_6): $\delta = 2.41$ (s, 3H, CH₃), 7.37 (d, 2H, J = 8.0 Hz, CH-3",5"), 7.53 (d, 1H, J = 8.0 Hz, CH-5), 7.92 (d, 2H, J = 8.0 Hz, CH-3',5'), 8.09 (m, 3H, CH-2',6', CH-6), 8.20 (s, 1H, CH-3), 8.30 (d, 2H, J = 8.0 Hz, CH-2",6"), 10.50 (s, 1H, NHCO) ppm; 13C NMR (100 MHz. DMSO- d_6): $\delta = 21.02$ (CH₃), 117.13 (CH-2' + CH 6'), 119.98 (CH-3' + CH 5'), 120.83 (CH-4), 120.91 (CH-6), 127.79 (CH-2" + CH 6"), 128.53 (CH-3" + CH 5"), 128.96 (CH-7), 131.70 (C-1'), 131.93 (C-4"), 134.63 (C-Cl), 140.67 (C), 141.92 (C-7a), 142.95 (C-NH), 144.68 (C-3a), 165.64 (CO) ppm; GC–MS: calc. m/z = 362.09 (C₂₀₋ $H_{15}CIN_4O$, $[M]^+$), found m/z = 362 ($[M]^+$) at 24.840 min (Scan 5152).

N-[4-(5-Chloro-2H-benzo[d][1,2,3]triazol-2-yl)phenyl]-4-

cyanobenzamide (10, C_{20}H_{12}CIN_5O) A mixture of 550 mg 4-cyanobenzoyl chloride (3.34 mmol) in 7.0 cm³ DMF and 400 mg compound **4a** (1.67 mmol) in 8.0 cm³ DMF was stirred at 120 °C. The reaction was complete after 2 h. Cold H₂O (100 cm³) was poured onto the reaction mixture. The precipitate was filtered off and washed with water several times. The filtered was dried in an oven overnight. The crude obtained was purified by flash chromatography in gradient method of eluents (from petroleum ether/ethyl

acetate, 1/1 to ethyl acetate) to give **10** in 25% yield (150 mg). White solid; m.p.: 281.9–283.1 °C; $R_f = 0.12$ (petroleum ether/ethyl acetate, 4/1); ¹H NMR (400 MHz, DMSO- d_6): $\delta = 7.53$ (d, 1H, J = 12 Hz), 8.05–8.11 (m, 5H), 8.15 (d, 2H, J = 8 Hz), 8.21 (s, 1H), 8.33 (d, 2H, J = 8 Hz) ppm; ¹³C NMR (100 MHz, DMSO- d_6): $\delta = 114.06$ (CN), 118.26 (C–CN), 117.15 (CH-2' + CH 6'), 120.00 (CH-3' + CH 5'), 120.92 (CH-4), 121.11 (CH-6), 128.35 (CH-2'' + CH 6''), 128.61 (CH-7), 132.51 (CH-3'' + CH 5''), 132.09 (C-1'), 135.04 (C–CI), 138.60 (C-4''), 140.11 (C-1''), 140.88 (C-NH), 142.56 (C-7a), 144.71 (C-3a), 164.46 (CO) ppm; GC–MS (EI): calc. m/z = 373.07 (C₂₀H₁₂ClN₅O, [M]⁺), found m/z = 371 ([M–2]⁺) at 24.840 min (Scan 5152).

1-[4-(5-Chloro-2H-benzo[d][1,2,3]triazol-2-yl)phenyl]-3-cy-

clohexylurea (11, C₁₉H₂₀ClN₅O) A solution of 130 mg compound 4a (0.531 mmol) in 6.5 cm³ DCM at 0 °C was treated with 90 cm³ cyclohexyl isocyanate (0.69 mmol) and strongly stirred as the temperature slowly rose to room temperature. After stirring at room temperature for 24 h, the reaction was finished and a white solid suspension was produced. To the resulting suspension, hexane was added to yield more precipitate. The solid material was collected by filtration to provide the compound 11 in 50% yield (60 mg). White solid; m.p.: 297.0–298.5 °C; $R_{\rm f} = 0.56$ (petroleum ether/ethyl acetate, 3/2); ¹H NMR (400 MHz, DMSO- d_6): $\delta = 1.21$ (m, 3H, CH₂-3", CH₂-4"a), 1.32 (m, 2H, CH₂-5"), 1.55 (m, 1H, CH₂-4"b), 1.68 (m, 2H, CH₂-2"), 1.82 (m, 2H, CH₂-6"), 3.50 (s, 1H, CH-1"), 6.23 (d, 1H, J = 8.0 Hz, CONH-cyclohexyl), 7.51 (d, 1H, J = 8.0 Hz, CH-5), 7.65 (d, 2H, J = 8.0 Hz, CH-3',5'), 8.07 (d, 1H, J = 8.0 Hz, CH-6), 8.16 (m, 3H, CH-2',6', CH-3), 8.72 (s, 1H, NHCO) ppm; ¹³C NMR (100 MHz, DMSO- d_6): $\delta = 24.31$ (2 CH₂), 25.19 (CH₂), 32.82 (2 CH₂), 47.69 (CH), 117.01 (CH-2' + CH 6'), 117.87 (CH-3' + CH 5'), 119.83 (CH-4), 121.04 (CH-6), 128.23 (CH-7), 131.65 (C-1'), 132.67 (C-Cl), 142.12 (C-7a), 142.82 (C-3a), 144.57 (C4'-NH), 154.07 (C=O) ppm; LC-MS (in MeOH): calc. m/z = 369.13 (C₁₉H₂₀ClN₅O, [M]⁺), found $m/z = 369.1 ([M]^+).$

1-[4-(5-Chloro-2H-benzo[d][1,2,3]triazol-2-yl)phenyl]-3-

propylurea (12, C₁₆H₁₆ClN₅O) A solution of 150 mg compound **4a** (0.613 mmol) in 10 cm³ DCM at 0 °C was treated with 120 cm³ propyl isocyanate (1.226 mmol) and strongly stirred as the temperature slowly rose to room temperature. After stirring at room temperature for 24 h, the reaction resulted finished and a white solid suspension was produced. To the resulting suspension, hexane was added to yield more precipitate. The solid material was collected by filtration to provide the compound **12** in 38% yield (60 mg). White solid; m.p.: 304.5–306.1 °C; $R_{\rm f.} = 0.57$ (petroleum ether/ethyl acetate, 3/2); ¹H NMR

(400 MHz, DMSO- d_6): $\delta = 0.89$ (t, 3H, J = 8.0 Hz, CH₃), 1.45 (m, 2H, CH₂CH₂CH₃), 3.08 (m, 2H, CH₂CH₂CH₂CH₃), 6.30 (m, 1H, CON<u>H</u>-propyl), 7.51 (d, 1H, J = 12.0 Hz, CH-5), 7.67 (d, 2H, J = 12.0 Hz, CH-3',5'), 8.07 (d, 1H, J = 8.0 Hz, CH-6), 8.16 (m, 3H, CH-2',6', CH-3), 8.83 (s, 1H, N<u>H</u>CO) ppm;¹³C NMR-APT (100 MHz, DMSO- d_6): $\delta = 21.30$ (CH₃), 22.88 (CH₂), 40.88 (CH₂), 117.00 (CH-2' + CH 6'), 117.93 (CH-3' + CH 5'), 119.83 (CH-4), 121.01 (CH-6), 128.22 (CH-7), 131.65 (C-1'), 132.67 (C-Cl), 142.18 (C-7a), 144.82 (C-3a), 144.56 (C4'-NH), 154.90 (CO) ppm; LC-MS (in MeOH): calc. m/z = 329.10(C₁₆H₁₆ClN₅O, [M]⁺), found m/z = 330.1 ([M+H]⁺).

4-(5-Chloro-2H-benzo[d][1,2,3]triazol-2-yl)-N-methylaniline (13, $C_{13}H_{11}CIN_4$) A solution of 500 mg compound 4a (2.05 mmol) in 5 cm³ DMF was treated with 0.22 cm³ dimethyl sulfate (2.30 mmol) and heated at 100 °C and strongly stirred. The reaction finished after 2 h. By cooling down, a white solid suspension was produced. The precipitate was filtered off and washed with diethyl ether several times. The filtered was dried in an oven overnight. The pure product 13 was obtained in 76% yield (380 mg). White solid; m.p.: 302.5-304.1 °C; $R_{f} = 0.47$ (petroleum ether/ethyl acetate, 3/2); ¹H NMR (400 MHz, DMSO- d_6): $\delta = 2.67$ (s, 3H, CH₃), 6.72 (s, 1H, NH), 6.81 (d, 2H, *J* = 8.8 Hz, CH-3', CH-5'), 7.46 (d, 1H, *J* = 8.8 Hz, CH-7), 8.05-7.99 (m, 3H, CH-6, CH-2', CH-6'), 8.11 (s, 1H, H-4) ppm;¹³C NMR-APT (100 MHz, DMSO- d_6): $\delta = 28.96$ (CH₃), 110.59 (CH-3' + CH 5'), 121.35 (CH-2' + CH 6'), 123.44 (CH-4), 125.25 (C-1'), 128.88 (CH-6), 132.65 (CH-7), 135.12 (C-Cl), 144.46 (C-7a), 147.50 (C-3a), 151.20 (C-NH) ppm; LC-MS (in MeOH): calc. m/z = 258.07 $(C_{13}H_{11}CIN_4, [M]^+)$, found $m/z = 259.1 ([M+H]^+)$.

4-(5-Chloro-2H-benzo[d][1,2,3]triazol-2-yl)-N,N-diethylani-

line (14, C₁₆H₁₇ClN₄) A solution of 500 mg compound 4a (2.05 mmol) in 5 cm³ DMF was treated with 0.38 g diethyl sulfate (0.32 cm³, 2.50 mmol), heated at 100 °C and strongly stirred. The reaction resulted finished after 5 h. By cooling down, a brown solid suspension was produced. The precipitate was filtered off and washed with diethyl ether several times. The filtered was dried in an oven overnight. The crude product was purified by crystallization with acetone and pure product 14 was obtained in 66% yield (400 mg). Brown solid; m.p.: 279.2–280.1 °C; $R_f = 0.53$ (petroleum ether/ethyl acetate, 3/2); ¹H NMR (400 MHz, DMSO- d_6): $\delta = 1.12$ (s, 3H, CH₂-CH₃), 3.73 (m, 2H, CH_2CH_3), 6.76 (d, 2H, J = 8.8 Hz, CH-3'-5'), 7.46 (d, 1H, J = 8.8 Hz, CH-7), 8.11–7.96 (m, 3H, CH-6, CH-2',6'), 8.16 (s, 1H, CH-4) ppm; ¹³C NMR-APT (100 MHz, DMSO- d_6): $\delta = 12.98$ (2 CH₃), 46.23 (2 CH₂), 110.75 (CH-3' + CH 5'), 122.43 (CH-2' + CH 6'), 123.46 (CH-4), 128.33 (C-1'), 130.55 (CH-6), 133.76 (CH-7), 136.12 (C-Cl), 146.68 (C-7a), 148.31 (C-3a), 150.10 (C-NH) ppm; LC-MS (in MeOH): calc. m/z = 300.11 (C₁₆H₁₇ClN₄, [M]⁺), found m/z = 301.1 ([M+H]⁺).

N-[4-(5-Chloro-2H-benzo[d][1,2,3]triazol-2-yl)phenyl]acet-

amide (15, C₁₄H₁₁ClN₄O) A portion of 500 mg of compound 4a (2.05 mmol) was added to 10 cm^3 of acetic anhydride (90 mmol, 9.25 g), heated at 60 °C, and strongly stirred. The reaction resulted finished after 1 h and monitored by TLC (petroleum ether/ethyl acetate, 7/3). By cooling down, a white solid suspension was produced. The precipitate was filtered off and washed with water several times and diethyl ether. The filtered was dried in an oven overnight. The pure product was obtained in 66% yield (510 mg). White solid; m.p.: 241.2–243.0 °C; $R_{\rm f} = 0.39$ (petroleum ether/ethyl acetate, 3/2); ¹H NMR (400 MHz, DMSO- d_6): $\delta = 2.11$ (s, 1H, CH₃), 7.51 (d, 2H, J = 9.2 Hz, CH-7), 7.86 (d, 1H, J = 8.4 Hz, CH-3'-5'), 8.08 (d, 1H, J = 9.2 Hz, CH-6), 8.18 (s, 1H, CH-4), 8.23 (d, 2H, J = 8.4 Hz, CH-2',6'), 10.29 (s, 1H, NH) ppm; ¹³C NMR-APT (100 MHz, DMSO- d_6): $\delta = 23.88$ (CH₃), 120.15 (CH-3' + CH 5'), 121.20 (CH-2' + CH 6'), 124.66 (CH-4), 129.77 (CH-6), 131.61 (CH-7), 132.82 (C-1'), 133.99 (C-Cl), 145.51 (C-7a), 148.13 (C-3a), 148.76 (C-NH), 169.55 (CO) ppm; LC–MS (in MeOH): calc. m/z = 286.06 $(C_{14}H_{11}CIN_4O, [M]^+)$, found $m/z = 287.1 ([M+H]^+)$.

Test compounds

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

Cells and viruses

Cell lines were purchased from American Type Culture Collection (ATCC). The absence of mycoplasma contamination was checked periodically by the Hoechst staining method. Cell lines supporting the multiplication of RNA and DNA viruses were the following: Madin-Darby Bovine Kidney (MDBK) [ATCC CCL 22 (NBL-1) Bos taurus]; Baby Hamster Kidney (BHK-21) [ATCC CCL 10 (C-13) Mesocricetus auratus]; Monkey kidney (Vero-76) (ATCC CRL 1587 Cercopithecus aethiops). Viruses were purchased from American Type Culture Collection (ATCC) except Yellow Fever Virus (YFV), and Human Immunodeficiency Virus type-1 (HIV-1). Viruses representative of positive-sense single-strand RNA (ssRNA⁺) group used were: (1) Retroviridae family: the laboratory strain HIV-1 IIIB wild type, obtained from the supernatant of the persistently infected H9/IIIB cells (NIH 1983); (2) Flaviviridae family: YFV [strain 17-D vaccine (Stamaril Pasteur J07B01)] and Bovine Viral Diarrhea Virus (BVDV) [strain NADL (ATCC VR-534)] (compounds were evaluated in vitro for antiviral activity against viruses representative

of only two of the three genera of the Flaviviridae family. i.e., Flaviviruses (Yellow Fever Virus, YFV) and Pestiviruses (Bovine Viral Diarrhea Virus, BVDV), as Hepaciviruses can hardly be used in routine cell-based assays); (3) Picornaviridae family: Human Coxsackievirus type B5 (CVB-5) strain (ATCC[®] VR-1036AS/HOTM) and Human Poliovirus type-1 Sabin (Sb-1) [strain Chat (ATCC VR-1562)]. Virus representative of a negative-sense singlestrand RNA (ssRNA⁻) group used was: Vesicular Stomatitis Virus (VSV) [strain Indiana Lab (ATCC VR-158)] and Human Respiratory Syncytial Virus (RSV) [strain A2 (ATCC VR-1540)]. A virus representative of a double-strand RNA (dsRNA) group used was: reovirus type-1 [strain 3651 (SV-12, simian virus 12) (ATCC VR-214)]. Viruses representatives of DNA group used were: (1) Poxviridae family: Vaccinia Virus (VV) [strain Elstree-Lister Vaccine (ATCC VR-1549)]; (2) Herpesviridae family: Human Herpesvirus 1 (HSV-1) [strain KOS (ATCC VR-1493)].

Cytotoxicity assays

Cytotoxicity assays were run in parallel with antiviral assays. MDBK and BHK cells were seeded at an initial density of 6×10^5 and 1×10^6 cells/cm³ in 96-well plates, respectively, in culture medium [Minimum Essential Medium with Earle's salts (MEM-E) with L-glutamine, supplemented with 10% horse serum and 1 mM sodium pyruvate (for MDBK cells) or with 10% fetal bovine serum (FBS) (for BHK cells), and 0.025 g/dm³ kanamycin]. Cell cultures were then incubated at 37 °C in a humidified, 5% CO₂ atmosphere in the absence or presence of serial dilutions of test compounds. Cell viability was determined after 48-96 h at 37 °C by the 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide (MTT) method [23]. Vero-76 cells were seeded at an initial density of 4×10^5 cells/cm³ in 24-well plates, in culture medium [Dulbecco's Modified Eagle Medium (D-MEM) with L-glutamine, supplemented with fetal bovine serum (FBS), and 0.025 g/dm³ kanamycin]. Cell cultures were then incubated at 37 °C in a humidified, 5% CO₂ atmosphere in the absence or presence of serial dilutions of test compounds. Cell viability was determined after 48-96 h at 37 °C by the crystal violet staining method.

Antiviral assays

Compound activity against YFV and Reo-1 was based on inhibition of virus-induced cytopathogenicity in BHK-21 cells acutely infected with an m.o.i. of 0.01. Compounds activity against BVDV was based on inhibition of virusinduced cytopathogenicity in MDBK cells acutely infected with an m.o.i. of 0.01. Briefly, BHK and MDBK cells were seeded in 96-well plates at a density of 5×10^4 and 3×10^4 cells/well, respectively, and were allowed to form confluent monolayers by incubating overnight in growth medium at 37 °C in a humidified CO₂ (5%) atmosphere. Cell monolayers were then infected with 50 mm³ of a proper virus dilution in maintenance medium (MEM-E with L-glutamine, supplemented with 0.5% inactivated FBS, 1 mM sodium pyruvate and 0.025 g/dm³ kanamycin) to give an m.o.i of 0.01. After 2 h, 50 mm³ of maintenance medium, without or with serial dilutions of test compounds, were added. After a 3–4 day incubation at 37 °C, cell viability was determined by the MTT method.

Compound activity against CVB-5, Sb-1, VSV, VV, HSV-1, and RSV was determined by plaque reduction assays in infected Vero-76 cell monolayers. To this end, Vero-76 cells were seeded in 24-well plates at a density of 2×10^5 cells/well and were allowed to form confluent monolayers by incubating overnight in growth medium (D-MEM with L-glutamine and 4500 mg/dm³ D-glucose, supplemented with 10% FBS and 0.025 g/dm³ kanamycin) at 37 °C in a humidified CO₂ (5%) atmosphere. Then, monolayers were infected for 2 h with 250 mm³ of proper virus dilutions to give 50-100 PFU/well. Following removal of unadsorbed virus, 500 mm³ of maintenance medium (D-MEM medium with L-glutamine and 4500 mg/ dm³ D-glucose supplemented with 1% inactivated FBS and 0.75% methyl-cellulose), without or with serial dilutions of test compounds were added. Cultures were incubated at 37 °C for 2 (Sb-1 and VSV), 3 (CVB-5, VV and HSV-1), or 5 days (RSV), and then fixed with PBS containing 50% ethanol and 0.8% crystal violet, washed, and air-dried. Plaques were then counted and EC₅₀ (50% effective concentration) was calculated by linear regression technique.

Linear regression analysis: viral and cell growth at each drug concentration was expressed as percentage of untreated controls and concentrations resulting in 50% (EC_{50} and CC_{50}) growth inhibition.

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