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Adenine and Deazaadenine Nucleoside and Deoxynucleoside Analogues: Inhibition of Viral Replication of Sheep MVV (In Vitro Model for HIV) and Bovine BHV-1

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Abstract—A series of N^6-cycloalkyl-2′,3′-dideoxyadenosine derivatives has been prepared by coupling of 2,6-dichloropurine to protected 2,3-dideoxyribose, followed by reaction with appropriate cycloalkylamines. Synthesized compounds, along with other purine nucleoside analogues previously synthesized in our laboratory, have been tested for their antiviral activity against Bovine herpesvirus 1 (BHV-1) and sheep Maedi/Visna Virus (MVV), the latter being an in vitro and in vivo model of Human Immunodeficiency Virus (HIV). All compounds showed good antireplicative activity against MVV, with the N^6-cycloheptyl-2′,3′-dideoxyadenosine (**5b**) being the most active [effective concentration (EC_{50}) causing 50% reduction of cytopatic effects (CPE) = 27 nM]. All compounds showed also a from low to very low cell toxicity, resulting in a cytotoxic dose 50 (CD_{50})/EC_{50} ratio in some cases higher than 1000.

Introduction

In spite of the numerous efforts to fight the viral diseases by vaccination, viral infections are still widespread and represent a continuous threat to the welfare of humans and animals. In the last decades, beside the development of new methods in the preparation of highly sophisticated immunizing products, a different strategy has been proposed to fight virus infections, that is the use of antiviral drugs.

The most appropriate and convenient way for testing the potential drugs for their antiviral activity appears to be the use of animal models, of course where a close analogy exists between viruses which are pathogenic for humans and those which affect animals. One example is represented by two lentiviruses: the first is identified as the Human Immunodeficiency Virus (HIV, causative agent of AIDS) and the second as the Maedi Visna Virus (MVV) of sheep.[1,2] The two viruses appear to be strikingly similar as far as biochemical and genomic properties are concerned.[3,4] A further example is given by the human and animal herpesviruses for their similarity in the biological and pathogenetic behaviour.[5,6]

For this study, we selected the MVV as a model for HIV and BHV-1 as a model for the human herpesviruses. The MVV is associated with chronic respiratory disease and/or mastitis and neurological distress in sheep and is responsible of serious economic losses when a flock becomes infected.[7] BHV-1 is considered one of the most widespread pathogenic agents of cattle and effects of infection caused by the virus usually vary from relatively mild illness to severe respiratory disease, but could result also in a number of other clinical conditions, such as conjunctivitis, genital lesions, abortions, enteritis and encephalitis. For this reason the persistence of the virus in herds represents a continuous threat to the welfare of the animals and the cause of significant economic losses for cattle industry.[8,9] In addition, serious problems to human health are caused by human herpesviruses, which mainly cause systemic, eye, and genital tract infections, leading even to the development of cancer.[10]

Most of the antiviral drugs that have been licensed so far for clinical use are members of the class of purine and pyrimidine nucleoside analogues, and a number of

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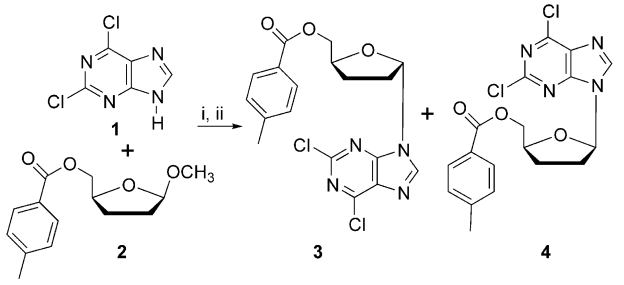
the most promising inhibitors of retrovirus replication are 2′3′-dideoxyanalogues.[11] In a previous paper we demonstrated the anti-HIV activity of N^6-cycloalkyl derivatives of 1-deazaadenine nucleosides.[12] Furthermore, our research group has a long expertise in synthesizing and testing purine nucleoside analogues, such as deazaadenosine derivatives,[13–16] N^6-substituted derivatives,[17] 2′-deoxy,[12,18] 3′deoxy,[19,20] and 2′,3′-dideoxy[12] analogues of adenosine, and various combinations of the above modifications.[12,17]



Scheme 1. Synthesis of 2,6-dichloro-9-(2,3-dideoxy-α-D-*glycero*-pentofuranosyl)-9H-purine and 2,6-dichloro-9-(2,3-dideoxy-β-D-*glycero*pentofuranosyl)-9H-purine: (i) HMDS, $(NH_4)_2SO_4$, Δ; (ii) CH_3CN, TMSTf.

Based on the fact that, as stated previously, many of the known nucleoside inhibitors of viral replication are dideoxypurine nucleosides, and the fact that we demonstrated the anti-lenti virus (anti-HIV) activity of N^6-cycloalkyl derivatives of 1-deazaadenine nucleosides,[12] in the present study we undertook the synthesis of 2,6-dichloro-2′,3′-dideoxypurine riboside **4** which is a versatile intermediate for the introduction of N^6-substituent on dideoxypurine nucleosides (Scheme 1). This dichloro derivative was obtained by coupling 2,6-dichloropurine to protected 2,3-dideoxyribose. From this compound we synthesized four N^6-cycloalkyl derivatives **5a**, **5b**, **6a**, **6b**, bearing small (C_3) or large (C_7) carbon ring on N^6, with or without a chlorine atom in the 2 position.

The synthesized compounds have been tested for their antireplicative activity on BHV-1 and on MVV, together with numerous related nucleosides, both from our chemical library (**7**–**23**, **28**–**31**) or from commercial sources (**24**–**27**) (Fig. 1). All compounds have also been

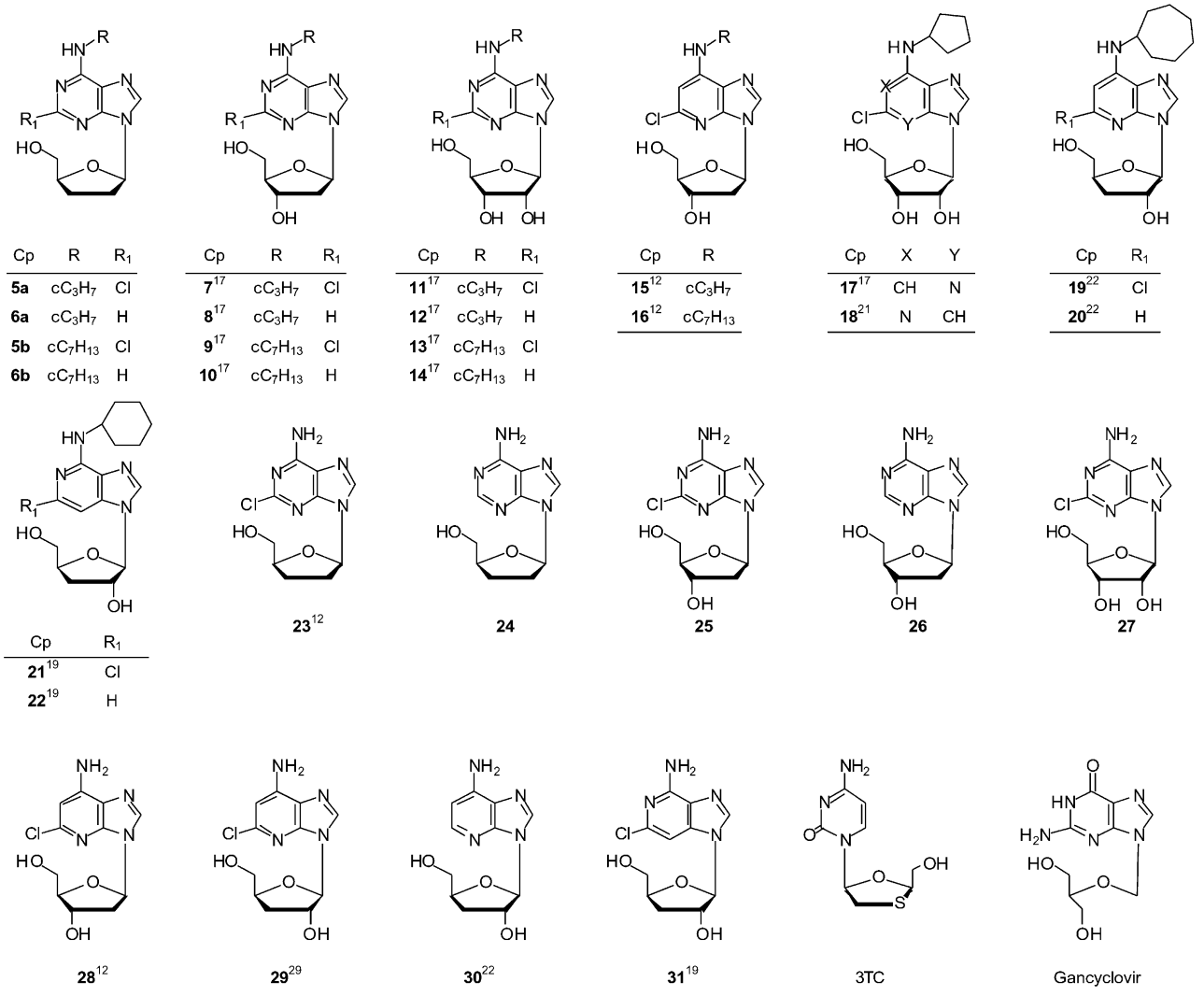


Figure 1. Structure of tested compounds.[21–23]

tested for their cytotoxicity on the cell lines used for virus replication.

Compounds **24** and **26** are substrates of Adenosine Deaminase (ADA), enzyme that converts adenine nucleosides into 6-hydroxypurine nucleosides by hydrolytic deamination.[24,25] They have been tested in all experiments both in absence and in presence of *erythro*-2-hydroxy-3-nonyladenine (EHNA), a reference ADA inhibitor, to assess the influence of deamination on their biological activity.

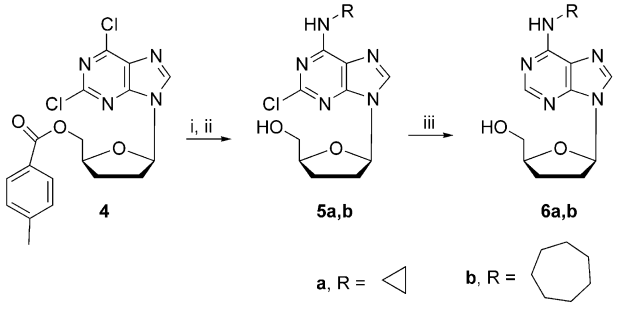
Results and Discussion

Chemistry

The synthesis of the N^6-cycloalkyl-2′,3′-dideoxyadenosine derivatives **5a**, **5b**, **6a**, **6b** was accomplished as reported in Schemes 1 and 2.

Commercially available 2,6-dichloropurine (**1**) was refluxed in hexamethyldisilazane (HMDS) added of a catalytic amount of ammonium sulfate; after work up in dry condition, the silylated derivative was coupled with 2,3-dideoxy-1-*O*-methyl-5-*O*-(4-methylbenzoyl)-D-*glycero*-pentofuranoside (**2**)[26] in CH_2Cl_2, using TMS–triflate as catalyst. After chromatographic purification the two anomers α (**3**) and β (**4**) were obtained in 3:2 ratio, respectively (Scheme 1). Similar coupling reactions have been already reported in literature, but from differently protected sugar, with diverse procedure and lower yield.[27,28]

The anomeric configuration of compounds **3** and **4** was assigned applying NOE difference spectroscopy. Saturation of H-1′ signal of **4** yielded 1% NOE of the H-4′ signal, establishing β-D configuration, while saturation of H-1′ signal of **3** gave no effect on the H-4′ signal, establishing α-D configuration. Moreover, this assignment is also in accordance with the empirical rule based on chemical shift differences between H-4′ and H-5′ (Δδ), which should be smaller for the β anomer, compared to the α one.[29] In the case of α-anomer **3**, Δδ was 0.49 ppm, whereas in the case of the β anomer **4** Δδ was 0 ppm.



Scheme 2. Synthesis of (2-chloro-)6-cycloalkylamino-9-(2,3-dideoxy-β-D-*glycero*pentofuranosyl)-9H-purines: (i) R-NH_2; (ii) NH_3/CH_3OH; (iii) Pd/C, H_2, OH^-.

The site of dideoxyribosylation was assigned to be N-9 on the purine ring based on UV and ^1H NMR spectral data. A strong NOE effect was observed on H-8 upon irradiation of H-1′, clearly excluding N-3 as the site of ribosylation.

Furthermore, the UV spectra of compounds **3** and **4**, at different pH value, were essentially indistinguishable and almost identical with that of 2,6-dichloro-9-methylpurine, previously reported in the literature, while being rather different from that of 2,6-dichloro-7-methylpurine.[30]

Compound **4** was reacted with the appropriate cycloalkylamine and then, without purification of the protected intermediate, with methanol saturated with ammonia. After usual work up and chromatographic purification compounds **5a** and **5b** were obtained in good yields (Scheme 2).

Catalytic dehalogenation of 2-chloroderivatives **5a** and **5b** was accomplished by shaking the nucleoside, dissolved in ethanol, in an atmosphere of hydrogen in the presence of a catalytic amount of palladium on carbon. After purification the 2-unsubstituted nucleosides **6a** and **6b** were obtained with yield of 73 and 63%, respectively.

Pharmacology

Synthesized compounds, along with other purine nucleosides previously synthesized in our laboratory (**7**–**23**, **28**–**31**), with some structurally related reference compounds (**24**–**27**) known for their antiviral and/or cytotoxic activity, and with reference anti-herpesviruses (Ganciclovir) or anti-lentiviruses (3TC) drugs (Fig. 1), have been tested on BHV-1 (strain IBR-LA), grown on bovine kidney cell line MDBK, and on MVV (strain KV1772)[31] replicating on sheep chondrocytes G81092. For both viruses, EC_{50} assays and titre reduction assays have been performed. All compounds have also been tested for their median cytotoxic dose (CD_{50}) on the cell lines used for virus replication.

Some of the compounds, in particular those with an intact and unsubstituted adenine moiety (**24** and **26**), are substrate for adenosine deaminase (ADA), enzyme that converts adenine nucleosides into 6-hydroxypurine nucleosides by hydrolytic deamination. Those compounds have been tested in all experiments both in presence and in absence of *erythro*-hydroxynonyladenine (EHNA), a reference ADA inhibitor, to assess if and how ADA activity can modify their biological actions.

Tables 1 and 2 report on biological results on BHV-1 and MVV assays, respectively.

BHV-1 inhibition. On BHV-1 all compounds showed an EC_{50} equal to or higher than 50 μM, whereas some of them were able to reduce the viral titre of (or close to) 3 log units (i.e., **6a**, **8**, **9**, **10**, **25**). This result is worth of

notice, given the fact that the same efficacy is shown by the reference compound Ganciclovir, an antiviral drug used in the therapy of human herpesvirus infections.

Almost all compounds showed to be cytotoxic against cells used for virus replication; only few of them had little or no toxicity. One of them is compound **6a** which, with a CD_{50} >400 μM, along with its previously underlined ability of reducing virus titre, is an interesting starting point for further developments of anti-BHV-1 drugs. From cytotoxicity data, the presence of a chlorine atom in the 2-position clearly raises activity against cell line, whereas the opposite effect can be seen, in many cases, for viral titre reduction (see, e.g., **5a** vs **6a** and **7** vs **8**).

The most active against the replication of BHV-1 of the tested nucleosides possess some structural analogies: an intact purine ring, and the lack of the hydroxyl group in position 2′. These observations can be useful in the optimization of anti-BHV-1 drugs.

Testing of compounds **24** and **26** both in presence and in absence of the inhibitor of ADA EHNA showed differences in biological activity only in the cytotoxicity test. In fact, compound **24** showed a lower toxicity when EHNA was present, whereas compound **26** had an opposite behavior. In antireplicative assays, the two compounds were almost inactive in both tests.

MVV inhibition. Table 2 shows reduction of virus titre and EC_{50} on MVV. It shows also CD_{50} on sheep chondrocytes, used for virus replication, and CD_{50}/EC_{50} ratio, which accounts for safety of the compounds when used as antivirals in vitro.

All compounds show a moderate to good ability of reducing virus titre, with the highest efficacy demonstrated by N^6-cycloheptyl-2-chloro-2′,3′-dideoxyadenosine (**6a**), with a reduction of 4 log units compared to the control. Many other nucleosides were able to reduce virus titre by 3 log units, demonstrating that MVV is overall more sensitive to tested compounds than BHV-1.

From anti-replicative and cytotoxicity data some interesting structure–activity relationships can be drawn. Assayed nucleosides possess high activity against MVV,

Table 1. Effect of compounds on bovine herpes virus (BHV-1, strain IBR-LA), grown on fetal kidney calf cell line MDBK

Compd	CD_{50}[a] (μM)	Viral titre[b]	EC_{50}[c] (μM)
5a	180±26	6.50	50
5b	315±46	7.50	>50
6a	>400	5.50	50
6b	244±22	7.50	>50
7	<50	7.50	>50
8	>400	5.74	50
9	130±11	5.50	50
10	320±33	5.50	50
11	359±50	7.50	>50
12	>400	7.50	>50
13	131±11	7.50	>50
14	>400	7.50	>50
15	185±35	7.50	>50
16	120±20	7.50	>50
17	180±30	7.50	>50
18	228±22	7.50	>50
19	191±28	7.50	>50
20	293±37	7.50	>50
21	255±41	7.50	>50
22	>400	7.50	>50
23	<50	7.50	>50
24	<50	7.50	50
24[d]	110±15	7.50	50
25	<50	5.50	50
26	250±30	7.50	>50
26[d]	<50	7.50	>50
27	<50	7.50	>50
28	105±15	7.50	>50
29	140±10	7.50	>50
30	260±20	8.50	>50
31	<50	7.50	>50
Ganciclovir	<50	5.50	50
Virus control		8.50	

[a]Concentration of compound able to give 50% of the maximal effect.
[b]Maximal dilution (negative log of concentration) at which the virus is still able to give cytopathic effects.
[c]Concentration of compound able to lower virus effects by 50%.
[d]Compound tested in presence of EHNA, inhibitor of adenosine deaminase.

Table 2. Effect of compounds on sheep maedi/visna lentivirus (MVV, strain KV1772) replicating on sheep chondrocytes G81092

Compd	CD_{50}[a] (μM)	Viral titre[b]	EC_{50}[c] (μM)	CD_{50}/EC_{50}
5a	180±30	4.50	0.41	439
5b	125±25	3.50	0.027	4630
6a	280±25	2.50	0.15	1867
6b	308±10	4.50	8.6	36
7	234±50	3.75	0.15	1560
8	312±30	4.50	1.5	208
9	98±25	3.75	0.27	363
10	108±33	5.50	1.5	72
11	225±25	4.75	15	15
12	105±20	6.50	15	7
13	100±20	6.50	0.15	667
14	126±30	5.50	15	8
15	236±45	4.50	2.7	87
16	230±55	4.75	1.41	163
17	260±26	4.75	0.5	520
18	205±25	4.75	1.5	137
19	145±45	3.50	0.15	967
20	210±45	3.75	0.5	420
21	270±40	3.50	1.5	180
22	>400	5.50	1.6	>250
23	195±45	4.50	0.15	1300
24	>400	3.75	0.41	>976
24[d]	180±50	3.50	0.027	6667
25	<50	4.50	1.5	<33
26	>400	4.75	1.5	>267
26[d]	>400	4.50	1.5	>267
27	<50	3.75	15	<3
28	>400	5.50	15	>27
29	>400	3.50	0.15	>2667
30	>400	4.00	4.1	>98
31	>400	5.50	15	>27
3TC	>300	<1.00	5	>60
Virus control		6.50		

[a]Concentration of compound able to give 50% of the maximal effect.
[b]Maximal dilution (negative log of concentration) at which the virus is still able to give cytopathic effects.
[c]Concentration of compound able to lower virus effects by 50%.
[d]Compound tested in presence of EHNA, inhibitor of adenosine deaminase.

with EC_{50} ranging from 15 μM to 27 nM. These data are definitely of interest, specially when compared to the 5 μM EC_{50} of the reference compound 2′,3′-dideoxy-3′-thiacytidine (3TC).

With the exception of 2′,3′-dideoxyadenosine (**24**), N^6-cycloalkyl derivatives **5**–**22** showed an average ability to inhibit virus replication higher than that of N^6-unsubstituted nucleosides **23**–**31**, suggesting that a substituent in this position is able to improve compound's activity. On the other hand, the same average difference can be seen in cytotoxicity assays. In fact, with few exceptions, N^6-unsubstituted compounds showed a CD_{50} higher than 400 μM, maximal assay concentration, whereas CD_{50} of N^6-cycloalkyl derivatives is ranging between 98 (**9**) and 312 (**8**) μM.

More in detail, *purine derivatives* (**5a**–**14**, **23**–**27**) data clearly show that a chlorine atom in the 2 position improves antireplicative activity, without a parallel increase in cytotoxicity (**5b** vs **6b**, **7** vs **8**, **9** vs **10**, **13** vs **14**). Also the size of the cycloalkyl substituent in N^6 modulates activity, with larger (cycloheptyl) ring bringing about in most cases higher efficacy than smaller (cyclopropyl) one (**5b** vs **5a**, **13** vs **11**, **16** vs **15**). Dideoxyribose clearly showed to be the sugar of choice for inhibiting viral replication (**5a**, **5b**, **6a**, **6b**, vs **7**–**10**, and vs **11**–**14**, **23** vs **25**, **24** vs **26**).

Looking at *deazapurine derivatives* (**15**–**22**, **28**–**31**), 1-deazanucleosides possess higher antireplicative activity than 3-deaza analogues (**17** vs **18**, **19** vs **21**, **29** vs **31**). Preferred sugar moiety is the 3′-deoxyribose (**19** vs **16**, **29** vs **28**). As already shown for purine derivatives, both N^6-cycloalkyl substitution (**16** vs **28**, **20** vs **30**, **21** vs **31**) and a chlorine atom in the 2 position (**19** vs **20**, **29** vs **30**) are able to increase activity of nucleosides.

The most active compounds proved to be **5b** (2-chloro-N^6-cycloheptyl-2′,3′-dideoxyadenosine) and **24** (2′,3′-dideoxyadenosine, when tested in presence of the ADA inhibitor EHNA), with a K_i for both of 27 nM. Those nucleosides show also the highest CD_{50}/ED_{50} ratio, of 4630 and 6667, respectively. Furthermore, compound **24**, when tested without ADA inhibitor, is about 15 times less active as anti-replicative drug ($EC_{50}=410$ nM), demonstrating that the inosine derivative formed by action of ADA possess a lower ability in inhibiting virus replication.

Conclusion

We have synthesized new N^6-cycloalkyl-2′,3′-dideoxy-derivatives of adenosine and tested them, together with other numerous purine nucleoside analogues, on BHV-1 (bovine herpes virus, very similar to human herpes viruses) and MVV (maedi-visna virus, sheep retrovirus, animal model for HIV) replication. Biological data demonstrated that tested compounds are very active in inhibiting MVV replication and possess a moderate cytotoxicity, resulting in antiviral nucleosides with cytotoxicity/antireplicative activity ratio in some cases well above 1000. These data can help in identifying a new class of anti-HIV compounds, and in finding therapeutics for viral infections of animals.

Experimental

Chemistry

Compounds **24**–**27** were purchased from Sigma-Aldrich s.r.l. (Milan, Italy); ^{1}H NMR spectra were obtained with a Varian VX 300 MHz spectrometer. UV spectra were recorded on a Varian Cary 1E UV–Visible spectrophotometer. Thin-layer chromatography (TLC) was carried out on precoated TLC plates with silica gel 60 F-254 (Merck). For column chromatography, silica gel 60 (Merck) was used. Elemental analysis were performed on a Fisons Instruments EA 1108 apparatus and results are within ±0.4% of theoretical values.

2,6-Dichloro-9-(2,3-dideoxy-α-D-*glycero*pentofuranosyl)-9H-purine (3) and 2,6-dichloro-3-(2,3-dideoxy-β-D-*glycero*pentofuranosyl)-9H-purine (4). To a suspension of 1.00 g (5.29 mmol) of 2,6-dichloropurine (**1**) in 20 mL of hexamethyldisilazane (HMDS) was added a catalytic amount of $(NH_4)_2SO_4$ and the mixture was heated at reflux for 5 h. The excess solvent was evaporated, the residue coevaporated three times with 15 mL of dry toluene, and then dissolved in 20 mL of dry methylene chloride. 2,3-Dideoxy-1-*O*-methyl-5-*O*-(4-methylbenzoyl)-D-*glycero*-pentofuranoside (**2**)[26] (1.524 g, 5.29 mmol) was added, the solution cooled on ice, and then 1.0 mL of TMS–triflate was added. The clear solution was stirred at rt overnight, added of 10 mL of a cold saturated solution of Na_2CO_3, then extracted with CH_2Cl_2 (3×20 mL). Organic layers were reunited, washed with cold water, dried (Na_2SO_4), filtered, and evaporated. The residue was purified on a flash chromatography column, eluting with a gradient of ethyl acetate in cyclohexane (15–85 to 20–80, v/v). Evaporation of the fractions containing the products afforded 0.785 g (1.93 mmol, 36%) of **3** (α anomer) and 0.523 g (1.28 mmols, 24%) of **4** (β anomer) as chromatographically pure thick oils.

(**3**): UV λ_{max} 274 (EtOH, ε 5500), 273 (pH = 1, ε 5850), 273 (pH = 12, ε 6300); ^{1}H NMR (DMSO-d_6): δ 2.01 (m, 1H, CH_2), 2,21 (m, 1H, CH_2), 2.36 (s, 3H, CH_3), 2.48–2.70 (m, 2H, CH_2), 4.17–4.40 (m, 2H, CH_2-5′), 4.78 (m, 1H, H-4′), 6.49 (dd, 1H, $J_1=5.8$ Hz, $J_2=4.2$ Hz, H-1′), 7.37 (d, 2H, $J=8.1$ Hz, 2 Ar–H), 7.91 (d, 2H, $J=8.1$ Hz, 2 Ar–H), 8.92 (s, 1H, H-8). Anal. calcd C 53.09, H 3.96, N 13.76; found C 52.73, H 3.81, N 13.88.

(**4**): UV λ_{max} 274 (EtOH, ε 5600), 274 (pH = 1, ε 5800), 273 (pH = 12, δ 6200); ^{1}H NMR (DMSO-d_6): δ 2.20 (m, 2H, CH_2), 2.35 (s, 3H, CH_3), 2.60 (m, 2H, CH_2), 4.30–4.50 (m, 3H, H-4′ and CH_2-5′), 6.32 (dd, 1H, $J_1=6.9$ Hz, $J_2=2.4$ Hz, H-1′), 7.24 (d, 2H, $J=8.1$ Hz, 2 Ar–H), 7.65 (d, 2H, $J=8.1$ Hz, 2 Ar–H), 8.83 (s, 1H, H-8). Anal. calcd C 53.09, H 3.96, N 13.76; found C 52.86, H 4.18, N 13.42.

2-Chloro-6-cyclopropylamino-9-(2,3-dideoxy-β-D-*glycero*-pentofuranosyl)-9H-purine (5a). Compound **4** (282 mg, 0.69 mmol) was added of 5 mL of cyclopropylamine. The solution was stirred at room temperature for 3 h, and then the excess of amine was evaporated. The residue was added of 20 mL of methanol saturated at 0 °C with ammonia, and stirred at rt in a tightly closed flask for 3 days. The solvent was then evaporated in vacuo, and the residue chromatographed on a silica gel column, eluting with $CHCl_3$–CH_3OH–CH_3CN (95:2.5:2.5, v/v). Evaporation of the fractions containing the product afforded 180 mg (0.58 mmol, 84%) of **5a** as chromatographically pure vitreous solid.

^{1}H NMR (DMSO-d_6): δ 0.58–0.83 (m, 4H, CH_2–CH_2 *cyclo*prop.), 2.04 (m, 2H, CH_2 rib.), 2.37 (m, 2H, CH_2 rib.), 2.99 (m, 1H, CH *cyclo*prop.), 3.42–3.69 (m, 2H, CH_2-5′), 4.12 (m, 1H, H-4′), 4.96 (t, 1H, $J=5.5$ Hz, OH), 6.18 (dd, 1H, $J_1=6.5$ Hz, $J_2=3.7$ Hz, H-1′), 8.40 (s, 1H, H-8), 8.45 (br s, 1H, NH). Anal. calcd C 50.41, H 5.21, N 22.61; found C 50.08, H 5.18, N 22.33.

2-Chloro-6-cycloheptylamino-9-(2,3-dideoxy-β-D-*glycero*-pentofuranosyl)-9H-purine (5b). Compound **4** (310 mg, 0.76 mmol) was added of 5 mL of cycloheptylamine. The solution was stirred at room temperature for 3 h, then the excess of amine was evaporated. The residue was added of 20 mL of methanol saturated at 0 °C with ammonia, and stirred at 40 °C in a tightly closed flask for 24 h. The solvent was then evaporated in vacuo, and the residue chromatographed on a silica gel column, eluting with $CHCl_3$–CH_3OH–CH_3CN (97:1.5:1.5, v/v). Evaporation of the fractions containing the product afforded 188 mg (0.51 mmol, 67%) of **5b** as a chromatographically pure vitreous solid.

^{1}H NMR (DMSO-d_6): δ 1.35–2.13 (m, 14H, 12H *cyclo*hept. + CH_2 rib.), 2.36 (m, 2H, CH_2 rib.), 3.44–3.70 (m, 2H, CH_2-5′), 4.11 (m, 1H, H-4′), 4.82 (m, 1H, CH *cyclo*hept.), 4.96 (t, 1H, $J=5.5$ Hz, OH), 6.16 (dd, 1H, $J_1=6.3$ Hz, $J_2=3.7$ Hz, H-1′), 8.20 (d, 1H, $J=8.5$ Hz, NH), 8.38 (s, 1H, H-8). Anal. calcd C 55.81, H 6.61, N 19.14; found C 55.45, H 6.83, N 18.77.

6-Cyclopropylamino-9-(2,3-dideoxy-β-D-*glycero*pentofuranosyl)-9H-purine (6a). Compound **5a** (140 mg, 0.45 mmol) was dissolved in 20 mL of ethanol and added of 1 mL of NaOH 2 N and a catalytic amount of Pd/C 10%; the suspension was stirred in a Parr apparatus under an atmosphere of H_2 (50 psi) for 5 h. The catalyst was filtered off, the solvent removed in vacuo, and the residue chromatographed on a silica gel column, eluting with $CHCl_3$–CH_3OH–CH_3CN (90:5:5, v/v). Evaporation of the fractions containing the product afforded 90 mg (0.33 mmol, 73%) of **6a** as chromatographically pure vitreous solid.

^{1}H NMR (DMSO-d_6): δ 0.58–0.80 (m, 4H, CH_2–CH_2 *cyclo*prop.), 2.06 (m, 2H, CH_2 rib.), 2.41 (m, 2H, CH_2 rib.), 3.05 (m, 1H, CH *cyclo*prop.), 3.45–3.70 (m, 2H, CH_2-5′), 4.12 (m, 1H, H-4′), 5.07 (t, 1H, $J=5.6$ Hz, OH), 6.24 (t, 1H, $J=5.0$ Hz, H-1′), 7.94 (br s, 1H, NH), 8.25 (s, 1H, H-2), 8.37 (s, 1H, H-8). Anal. calcd C 56.71, H 6.22, N 25.44; found C 56.59, H 6.18, N 25.16.

6-Cycloheptylamino-9-(2,3-dideoxy-β-D-*glycero*pentofuranosyl)-9H-purine (6b). Compound **5b** (150 mg, 0.41 mmol) was dissolved in 20 mL of ethanol and added of 1.5 mL of NaOH 2 N and a catalytic amount of Pd/C 10%; the suspension was stirred in a Parr apparatus under an atmosphere of H_2 (50 psi) for 7 h. The catalyst was filtered off, the solvent removed in vacuo, and the residue chromatographed on a silica gel column, eluting with $CHCl_3$-CH_3OH–CH_3CN (95:2.5:2.5, v/v). Evaporation of the fractions containing the product afforded 85 mg (0.26 mmol, 63%) of **6b** as chromatographically pure vitreous solid.

^{1}H NMR (DMSO-d_6): δ 1.40–2.08 (m, 14H, 12H *cyclo*hept. and CH_2 rib.), 2.42 (m, 2H, CH_2 rib.), 3.42–3.65 (m, 2H, CH_2-5′), 4.11 (m, 1H, H-4′), 4.26 (m, 1H, CH-*cyclo*hept.), 5.06 (t, 1H, $J=6.0$ Hz, OH), 6.22 (t, 1H, $J=5.0$ Hz, H-1′), 7.56 (d, 1H, $J=8.4$ Hz, NH), 8.18 (s, 1H, H-2), 8.34 (s, 1H, H-8). Anal. calcd C 61.61, H 7.60, N 21.13; found C 61.30, H 7.91, N 20.87.

Biology

Chemicals. All the chemicals used were reagent's grade or better. Eagle's minimum essential medium (EMEM) and Dulbecco's minimum essential medium (DMEM) were from Biospa Italia; antibiotics were from Sigma.

Foetal calf serum was from BioWhittaker (USA); Trypan blue and MTT were from Sigma (USA); microtiter plates from Nunc (Denmark).

Virus. MVV: The biological clone of increased neurovirulence KV 1772[31] was used. Stock viruses were prepared in cell line G81092 of sheep chondrocytes. The viral strain had a titre of $10^{6,50}$ $TCID_{50}$/0.025 mL ($TCID_{50}$ = virus dilution causing 50% of cytopathic effects).

The sheep chondrocytes cell line G81092 was grown in DMEM with antibiotics and 5% foetal calf serum.

BHV-1: Strain IBR-LA[32] of BHV-1 at its 52nd passage on Madin and Darby bovine cell line (MDBK)[33] was used. The viral strain had a titre of $10^{8,50}$ $TCID_{50}$/0.025 mL.

MDBK cells were grown in EMEM with antibiotics enriched with 5% foetal calf serum. The concentration of serum was reduced to 2% in both culture systems when cultures were subjected to virus inoculation.

Evaluation of cytotoxicity of tested compounds

Logarithmically growing chondrocyte cells were trypsinized and adjusted to 2×10^5 cells/mL in MEM supplemented with 2% foetal calf serum. Logarithmically growing MDBK cells were trypsinized and adjusted to 2×10^3 cells/mL in MEM supplemented with 10% foetal calf serum.

Aliquots of 0.1 mL were used to seed each well of 96-well plates; the plates were then incubated at 37 °C. 24 h later, the culture medium was removed, and fresh medium containing serial dilutions from 0.5 to 400 μM of each test compound was inoculated using three wells of the plate for each dilution and 100 μL of inoculum per well. The plates were incubated at 37 °C for 7 days.

To evaluate the cytotoxicity of the compounds under study the following methods were applied.

Light microscopy. Monolayers were prepared as above and incubated for 7 days with different concentrations of the compounds prepared as mentioned. Then the cells were fixed with ethanol and stained with the Giemsa method to facilitate the visualisation of cell morphology.

MTT method. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Thiazol blue) is a water-soluble tetrazolium salt yielding a yellowish solution when prepared in media or salt solutions lacking phenol red. Dissolved MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by dehydrogenases. This water insoluble formazan was solubilized using isopropanol and the dissolved material was characterised spectrophotometrically yielding absorbance as a function of concentration of converted dye. In contrast to dead cells, active mitochondrial dehydrogenases of living cells will cause this conversion.[34] The cells were prepared and compounds included as described above. A solution was prepared by dissolving 5 mg of MTT in 1 mL of medium without phenol red. The solution was filtered through a 0.2 μm filter and stored at 2–8 °C. The MTT solution was added to each culture in a volume of 100 μL per well and the plate was incubated at 37 °C for 3–4 h. At the end of the incubation period, the medium was removed and the converted dye was solubilized in acidic isopropanol (0.04–0.1 N HCl in absolute isopropanol). Absorbance of converted dye was measured at wavelength of 540 nm with background subtraction at 690 nm.

The cytotoxicity was expressed as the cytotoxic dose 50 (CD_{50}) which induces a 50% reduction of absorbance at 540 nm by the cells.

Virus titre reduction assay

One-day-old monolayers of cell cultures grown in 96 microtiter plates were used for the test. After removal of the medium from the plates, serial dilutions of virus from 10^{-1} to 10^{-8} were inoculated using three wells of the plate for each dilution, with an inoculum of 0.025 mL per well. The plates were then incubated at 37 °C for 60 min. Subsequently, test compounds were added at 50 μM concentration to each well. The plates were incubated in a CO_2 controlled incubator at 37 °C and were examined for cytopathic effects (CPE) at 7 days post infection for MVV and after 5 days for BHV-1, strain IBR-LA. Virus titres were calculated by the method of Reed and Muench.[35]

Cytopathic effects (CPE) inhibition assay.. For this test, 24 h monolayers grown in 96-well plates were also used. The wells of the plate were inoculated with 1×10^4 $TCID_{50}$ or with 3×10^5 $TCID_{50}$ of BHV-1 or MVV, respectively, in an inoculum of 0.025 mL per well.

After one h incubation at 37 °C, six wells of the plates were first inoculated with each of the following concentrations of the compounds: 0.005, 0.05, 0.5, 5, 50 μM in an inoculum of 0.025 mL per well.

Then, 0.05 mL of the culture medium was added to each well and the plates were incubated at 37 °C. The reading were made when the CPE were complete in the controls, that is after 5 (BHV-1) or 7 (MVV) days of incubation.

The antiviral activities of the compounds were expressed as the compound concentration that caused a 50% reduction of cytopathology (50% effective concentration, EC_{50}).

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