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Antiviral activity of novel 2′-fluoro-6′-methylene-carbocyclic adenosine against wild-type and drug-resistant hepatitis B virus mutants

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ABSTRACT

Novel 2′-fluoro-6′-methylene-carbocyclic adenosine (**9**) was synthesized and evaluated its anti-HBV activity. The titled compound demonstrated significant antiviral activity against wild-type as well as lamivudine, adefovir and double lamivudine/entecavir resistant mutants. Molecular modeling study indicate that the 2′-fluoro moiety by a hydrogen bond, as well as the van der Waals interaction of the carbocyclic ring with the phenylalanine moiety of the polymerase promote the positive binding, even in the drug resistant mutants.

Chronic hepatitis B virus (HBV) infection is one of the leading causes of morbidity and mortality worldwide. Chronic infection with HBV occurs in approximately 350 million of the world population, including 1.7 million in the USA.[1] HBV infection can persist for the life of the host, often leading to severe consequences such as liver failure, cirrhosis and eventually hepatocellular carcinoma, resulting in annually 0.5–1.2 million deaths worldwide.[2] HBV is an incomplete double-stranded DNA virus. Its DNA replication is unique because it includes a reverse transcription step. The HBV DNA polymerase/reverse transcriptase is an essential and multifunctional enzyme, which operates as a DNA polymerase/reverse transcriptase, an RNAse H, through coordinating the assembly of viral nucleocapsids, as well as catalyzing the generation of DNA primers.[3] Nucleoside analogues can suppress HBV replication by inhibiting the viral polymerase/reverse transcriptase. The pivotal role of nucleoside/nucleotide analogues such as lamivudine, adefovir, telbivudine, entecavir, clevudine, and tenofovir has been demonstrated by their therapeutic efficacy in clinical practice. However, long-term therapy with these drugs is often associated with viral resistance, which significantly compromises the clinical application of these agents. For example, the extensive use of lamivudine resulted in the emergence of mutants that are resistant to the anti-HBV activity; 24% after a 1-year therapy, increasing to over 70% after 4 years of therapy. Adefovir has been used for the patients, who develop lamivudine-resistant mutants, however, a significant number of patients (29% after 5 years of use) also develop the adefovir resistant mutant (N236T).

Entecavir is a carbocyclic 2′-deoxyguanosine analog that demonstrates potent anti-HBV activity[4] and is recommended for patients with the wild-type strain as well as for those patients harboring lamivudine-resistant strains.[5] However, a recent study by Tanaka and his co-workers suggest that the viral breakthrough was observed in the lamivudine-refractory group in 4.9% of patients at baseline and increase to 14.6%, 24% and 44.8% at weeks 48, 96 and 144, respectively.[6]

In view of the fact that currently adefovir and entecavir are the most prescribed drugs for the treatment of chronic HBV infection, it is critical to discover the agents that do not confer cross-resistance with the adefovir and lamivudine/entecavir-mutants for the future treatment of drug resistant patients. In this report we try to demonstrate that our newly discovered compound **9** may potentially play a significant role for that purpose.

Carbocyclic nucleosides are an interesting class of compounds in which the methylene group replaces the oxygen atom of a furanose ring. As a consequence, the glycosidic bond is resistant to nucleoside phosphorylase as well as nucleoside hydrolase, which makes the carbocyclic nucleosides more stable towards metabolic

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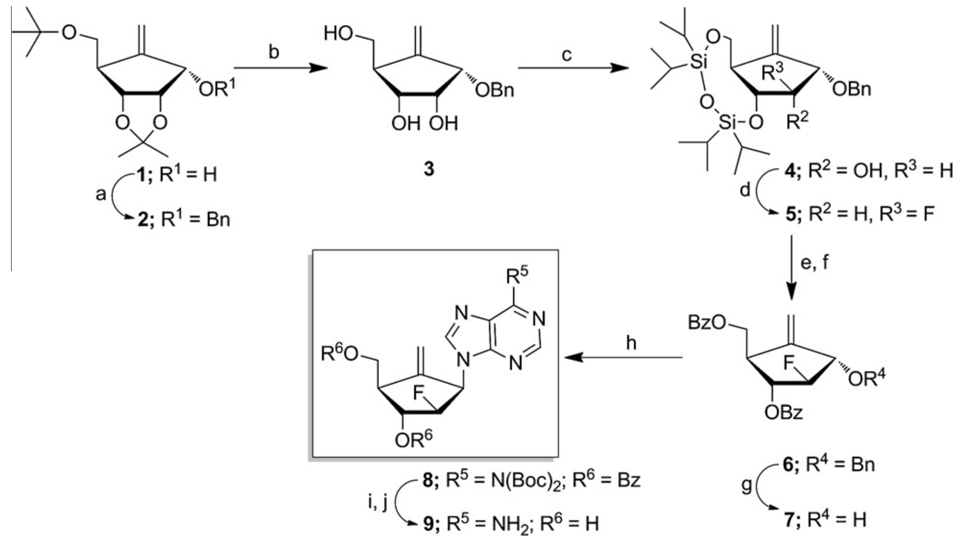

Scheme 1. Synthesis of target compound **9**. Reagents and conditions: (a) NaH, BnBr, DMF, 0 °C; (b) TFA/H_2O (2:1), 50 °C; (c) $TIDPSCl_2$/imidazole, DMF, 0 °C; (d) DAST, CH_2Cl_2, rt; (e) TBAF/AcOH, THF, rt; (f) BzCl, pyridine, rt; (g) BCl_3, CH_2Cl_2, −78 °C; (h) *N,N*-dibocprotected adenine, DIAD, Ph_3P, THF, 0 °C; (i) TFA, CH_2Cl_2, rt; (j) DIBAL-H, CH_2Cl_2, −78 °C.

degradation.[7] Due to these features, carbocyclic nucleosides have received much attention as potential chemotherapeutic agents.[8] Carbovir and entecavir are examples of results of these efforts.

It is also well known that incorporation of a fluorine atom at the 2′-position of nucleosides can increase the stability of the glycosyl bond towards chemical and metabolic degradation.[9,10] A fluorine substitution on the carbocyclic sugar moiety has been proven to be useful in producing effective antiviral agents as demonstrated by our group in 2′-fluoro-5-methyl-β-L-arabinofuranosyluracil (L-FMAU or clevudine)[11] as well as in clofarabine.[12]

In view of the 2′-F substitution[9,10] as well the introduction of an exocyclic double bond to carbocyclic nucleosides,[4] which have been beneficial for anti-HBV activity such as in entecavir, 2′-fluoro-6′-methylene-carbocyclic adenosine or (+)-9-[(1*R*,2*R*,3*R*,4*R*)-2-fluoro-3-hydroxy-4-(hydroxymethyl)-5-methylenecyclopentan-1-yl] adenine **9** was synthesized and evaluated for its antiviral activity against wild-type HBV as well as adefovir, lamivudine and lamivudine/entecavir (double)-resistant mutants in vitro.

The synthesis of the target nucleoside **9** commenced with compound **7** as the key intermediate (Scheme 1). Compound **1** was synthesized according to the reported procedure from our group.[13] The allylic hydroxyl group of **1** was protected with a benzyl group and subsequent deprotection of the acetonide and the *t*-butyl group of compound **2** gave **3** in 86% yield. The 3, 5-hydroxy groups of **3** were selectively protected with 1,3-dichloro-1,1,2,2-tetraisopropyl disilazine to give **4** in 95% yield. Transformation of the 2-β-hydroxyl group to 2-α-fluoro was accomplished by treating the alcohol **4** with DAST to give 47% yield of compound **5**. However, debenzylation of **5** was unsuccessful under the Birch reduction or the Lewis acid (BCl_3) conditions. Therefore, the silyl group of **5** was removed by using tetrabutyl ammonium fluoride (TBAF/HOAc) to yield 82% of a diol, which was re-protected by benzoyl chloride in pyridine to give the fully protected intermediate **6** in 86% yield. The compound **6** was then treated with BCl_3 at −78 °C to obtain the key intermediate **7** in 76% yield. *N,N*-diboc protected adenine was synthesized according to the reported protocol in literature[14] and condensed with **7** to obtain **8** in 51% yield. The deprotection of the Boc group was carried out by TFA to afford 82% yield. Eventually, the treatment of DIBAL-H gave the target compound **9**[15] in 76% yield.

The synthesized nucleoside **9** was evaluated for its antiviral activity against wild-type HBV as well as adefovir, lamivudine and lamivudine/entecavir-drug resistant mutants in vitro,[16] and the results are summarized in Table 1. As the compound **9** is a derivative of an adenine analog, we directly compared its antiviral activity to that of adefovir instead of entecavir (a guanine analog) although the carbocyclic moiety is similar to that of entecavir. Furthermore, compound **9**, an adenine analogue, can interact with the thymidine moiety in the DNA template–primer site while entecavir interacts with the cytosine moiety at the same site in the active site. Thus, the base moiety is the major deciding factor, not the sugar moiety in determining the mode of action.

The target compound **9** demonstrated a significant antiviral in vitro activity against wild-type (WT) HBV with an EC_{50} value of 1.5 μM. The antiviral potency was similar to that of adefovir, while being 7-fold less potent than lamivudine. However, the concentration of the compound **9** required to inhibit 90% (EC_{90}) of wild-type HBV is 4.5 μM, which is 1.5-fold more potent than adefovir (EC_{50} 7.1 μM; Table 1).

The compound **9** also showed excellent activity against both lamivudine and adefovir resistant HBV mutants.[17] Particularly, the compound **9** showed a 4.5-fold enhanced potency of EC_{50} (1.7 μM) and a 7.8-fold more favorable EC_{90} (4.6 μM) against adefovir mutant rtN236T. For lamivudine mutants, rtM204V and rtM204I, the compound **9** showed an EC_{50} value of 1.8 versus 1.6 μM for adefovir, and 1.0 versus 1.9 μM for compound **9** and adefovir, respectively, while in the EC_{90} value, compound **9** demonstrated more favorable anti-HBV activity for both mutants, rtM204 V (4.7 vs 7.0 μM) and rtM204I (5.0 vs 8.0 μM). For mutant rtL180M, the antiviral activity of compound **9** was similar to that of lamivudine in the EC_{50} 2.1 versus 1.5 μM, while the compound **9** exhibited a 4.3-fold increased antiviral activity in the EC_{90} value (5.1 vs 22.0 μM).

Compound **9** was also evaluated against the lamivudine double mutant, rtL180M/rtM204V, and it exhibited the EC_{50} 2.2 μM that was equal to the adefovir, while the EC_{90} value of 5.5 μM of compound **9** was more effective than that of adefovir (8.5 μM). In addition, deamination studies with adenosine deaminase from calf thymus indicated that the compound **9** was completely stable.[18]

In preliminary studies, compound **9** was also evaluated against lamivudine/entecavir double resistant clone (L180M + S202I + M202V), in which compound **9** demonstrated significant anti-HBV activity (EC_{50} 0.67 μM) against the mutant. In the case of lamivudine and entecavir, there are significant decrease in their

Table 1
In vitro anti-HBV activity against adefovir, lamivudine and entecavir drug-resistant mutants in the intracellular HBV DNA replication assay[16,17]

Strains	Compound **9** (μM)				Adefovir (μM)			Lamivudine (μM)			Entecavir (μM)		
	EC_{50}[b]	EC_{90}[c]	CC_{50}[d,e]	Fold resistance[f] (EC_{90})	EC_{50}	EC_{90}	Fold resistance (EC_{90})	EC_{50}	EC_{90}	Fold resitance (EC_{90})	EC_{50}	EC_{90}	CC_{50}
Wild Type	1.5	4.5	>100	–	1.3	7.1	–	0.2	0.6	–	0.008	0.033	28
rtM204V	1.8	4.7	>100	1.0	1.6	7.0	1.0	>100	>100	>166	NT[h]	NT	NT
rtM204I	1.0	5.0	>100	1.1	1.9	8.0	1.1	>100	>100	>166	NT	NT	NT
rtL180M	2.1	5.1	>100	1.1	5.5	7.7	1.1	1.5	22.0	36.7	NT	NT	NT
rtLM/rtMV[a]	2.2	5.5	>100	1.2	2.1	8.5	1.2	>100	>100	>166	NT	NT	NT
rtN236T	1.7	4.6	>100	1.0	7.8	36.0	5.1	0.2	0.9	1.5	NT	NT	NT
rtLM/rtMV/rtSG[g]	0.67	NT	NT	–	NT	NT	–	>500[i]	NT	–	1.20[j]	NT	NT

[a] rtLM/rtMV = rtL180M/rtM204V double mutant.
[b] Effective concentration required to inhibit 50% of HBV-DNA.
[c] Concentration required to reduce infectious virus titer by 90%.
[d] The > sign indicates that the 50% inhibition was not reached at the highest concentration tested.
[e] The drug concentration required to reduce the cellular viability by 50% as assayed by an MTT assay.
[f] Fold resistance = (mutant EC_{90})/(wt EC_{90}).
[g] rtLM/rtMV/rtSG = rtL180M/rtM204V/rtS202G.
[h] NT = not tested.
[i] Ref. 19.
[j] Ref. 20.

antiviral potency (EC_{50} > 500 and 1.2 μM, respectively) as shown in Table 1.[19,20]

It was of interest to know how the compound **9** demonstrated the favorable anti-HBV activity in comparison to that of adefovir. Therefore, molecular modeling studies were conducted to obtain the insight of the molecular mechanism of compound **9** by using the Schrodinger suite.[21] The homology model of HBV RT was constructed based on the published X-ray crystal structure of HIV reverse transcriptase (PDB code: 1RTD),[22] which was previously used for molecular mechanism studies of several anti-HBV nucleosides.[23] In the homology model of HBV polymerase, the relative position of α-, β- and γ-phosphates of compound **9** with respect to the catalytic triad were assumed to occupy the similar position to the dNTP in the crystal structure of the HIV-1 RT-DNA–dNTP complex. The molecular docking[24] of compound **9** shows that the triphosphate forms all the network of hydrogen bonds with the active site residues, S85, A86, A87, R41, K32 (Fig. 1a). The γ-phosphate of compound **9** retains a critical H-bonding with the OH of S85 with connection of hydrogen bonds between S85 and N236. Generally, the N236T mutant loses the hydrogen bond to S85, which results in destabilization of the S85 to γ-phosphate interaction, thus causes resistance. However, compound **9** (as its triphosphate) maintains a critical H-bonding with S85 (Fig. 1b) similar to that as observed in wild type HBV (Fig. 1a).

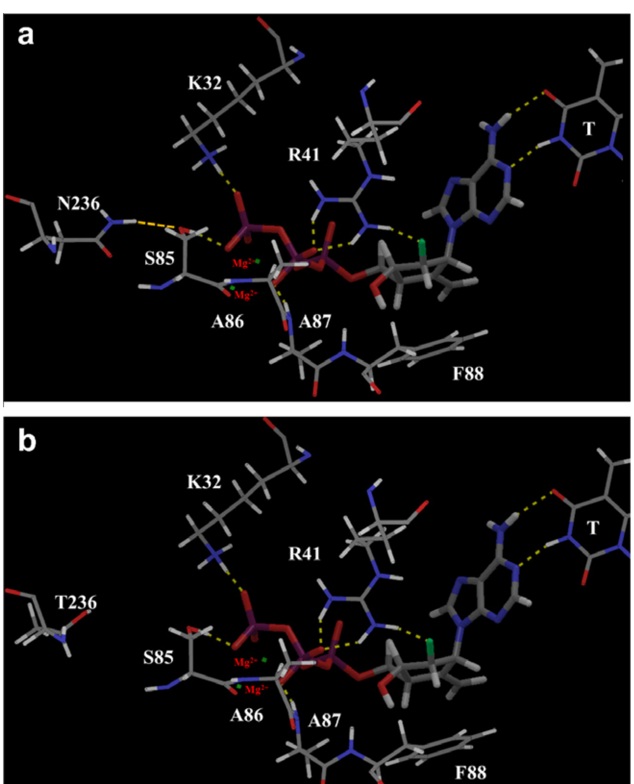

Figure 1. Binding mode and van der Waals interaction of compound **9** (a) in wild-type HBV and (b) in N236T adefovir mutant HBV. Yellow dotted lines are hydrogen bonding interactions (<2.5 Å).

The carbocyclic ring with an exocyclic alkene of compound **9** occupies the hydrophobic pocket (residues F88, L180 and M204) and makes the favorable van der Waals interaction with F88 (Fig. 1a and b). The 2′-fluorine substituent in the carbocyclic ring of compound **9** appears to promote an additional binding with R41 as shown in Figure 1a and b, which corroborates with the antiviral activity of compound **9** shown in Table 1. Overall, the modeling studies can qualitatively explain the favorable anti-HBV activity of the newly discovered compound **9** in WT (Fig. 1a) as well as against adefovir resistant mutant, N236T (Fig. 1b). These modeling studies are qualitative, and therefore, more quantitative calculation is warranted in the future.

In summary, a novel carbocyclic adenosine derivative **9** was synthesized, and evaluated for its anti-HBV activity. From these studies, the target nucleoside demonstrated significant anti-HBV activity against both the wild-type as well as the major nucleoside-resistant HBV mutants (adefovir and lamivudine), including the lamivudine/entecavir double mutant. In view of these promising anti-HBV activities, further biological and biochemical studies of the nucleoside **9** is warranted to assess the full potential as an anti-HBV agent.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.08.113.

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15. *Compound* ***9***: $[\alpha]_D^{25}$ + 151.80° (c 0.23, CH_3OH); mp 215–217 °C; UV (H_2O) λ_{max} 259.0 nm (e 14,000, pH 2), 260.0 nm (e 15,600, pH 7), 260.0 nm (e 15,600, pH 11); ^1H NMR (500 MHz, CD_3OD) d 8.26 (s, 1H), 8.10 (d, *J* = 2.5 Hz, 1H), 5.90 (d, *J* = 2.5 Hz, 1H), 5.46 (s, 1H), 5.01–4.89 (m, 2H), 4.46–4.42 (m, 1H), 391–3.81 (m, 2H), 2.81 (br, 1H); ^{19}F NMR (500 MHz, CD_3OD) δ −192.93 (m); ^{13}C NMR (125 MHz, CD_3OD) d 155.9, 152.5, 149.9, 146.0, 141.1, (d, J = 5.3 Hz), 117.8, 111.7, 109.8, 95.9 (d, *J* = 184 Hz) 72.9 (d, *J* = 23.6 Hz), 61.7, 57.5 (d, *J* = 17.4 Hz), 51.0; Anal. Calcd For $C_{12}H_{14}FN_5O_2$: C, 51.61; H, 5.05; N, 25.08; Found C, 51.74; H, 5.09; N, 24.92.
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