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New tools in nucleoside toolbox of tick-borne encephalitis virus reproduction inhibitors

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ABSTRACT

Design and development of nucleoside analogs is an established strategy in the antiviral drug discovery field. Nevertheless, for many viruses the coverage of structure-activity relationships (SAR) in the nucleoside chemical space is not sufficient. Here we present the nucleoside SAR exploration for tick-borne encephalitis virus (TBEV), a member of *Flavivirus* genus. Promising antiviral activity may be achieved by introduction of large hydrophobic substituents in the position 6 of adenosine or bulky silyl groups to the position 5′. Introduction of methyls to the ribose moiety does not lead to inhibition of TBEV reproduction. Possible mechanisms of action of these nucleosides include the inhibition of viral entry or interaction with TBEV non-structural protein 5 methyltransferase or RNA-dependent RNA polymerase domains.

Nucleoside and nucleotide analogs and derivatives often show significant antiviral activity. Numerous successful antiviral drugs are based on these molecules.[1] Whereas a large body of information is available for such viruses as human immunodeficiency virus (HIV), hepatitis C virus (HCV), influenza A virus, efficiency of nucleosides against other viruses remains poorly studied. For rational design of antivirals it is required to know which structural features are important for the inhibition of reproduction of these viruses.

Tick-borne encephalitis virus (TBEV) is a member of *Flavivirus* genus, which includes enveloped viruses borne by blood-sucking arthropods, namely ticks and mosquitoes. These viruses pose a threat for public health all over the world. Mosquito-borne flaviviruses (dengue virus (DENV), West Nile virus, Japanese encephalitis virus, yellow fever virus, Zika virus, etc.) are mostly spread in tropical regions, while tick-borne flaviviruses, including TBEV, are common in temperate climates of Europe, Russia, and northern Asia. Up to 10,000 clinical cases of tick-borne encephalitis are registered annually,[2] and about one-third of them lead to prolonged sequelae with different degrees of severity. Genome of flaviviruses is represented by 11 kb (+)RNA, and its replication is performed by NS5 protein containing RNA-dependent RNA polymerase (RdRp) and methyltransferase (MTase) domains.[3] These enzymes are common targets of nucleoside-based antivirals.[4] Moreover, RdRp sequences are rather conserved among RNA viruses, and flavivirus RdRp is similar to HCV one, opening a possibility to expand the knowledge from one virus to another.[3]

Until 2015, there was no information on nucleoside inhibitors of TBEV replication, and limited data were available for other classes.[5,6] Notably, well-known broad-spectrum antiviral drug ribavirin is not efficient as a TBEV reproduction inhibitor.[7–9] Then it was found that close analogs of nucleosides, e.g., 7-deaza-2′-*C*-methyladenosine (7DCMA), inhibited replication of TBEV at micromolar concentrations in an NS5-dependent manner,[7] and nucleosides with large hydrophobic substituents in the position 5 of nucleobase (RAFIs) showed TBEV entry inhibition at two-digit nanomolar concentrations, presumably due to interaction with the viral membrane.[10] In a follow-up study, SAR was

Abbreviations: 7DCMA, 7-deaza-2′-*C*-methyladenosine; DENV, dengue virus; EV-A71, enterovirus A71; MTase, methyltransferase; NS5, non-structural protein 5; RAFI, rigid amphipathic fusion inhibitor; RdRp, RNA-dependent RNA polymerase; SAH, S-adenosyl-L-homocysteine; TBEV, tick-borne encephalitis virus.

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explored around 7DCMA to show that 2′-*C*-methylation of common nucleosides was the most efficient approach, whereas 2′-*O*-methylation, 3′-*O*-methylation, and 3′-dehydroxylation did not lead to nucleosides with improved anti-TBEV effect.[8] Another adenosine analog NITD008 (7-deaza-2′-*C*-ethynyladenosine) was shown to inhibit TBEV reproduction along with three less common tick-borne flaviviruses at a micromolar level.[11]

Despite numerous nucleoside analogs had been tested on mosquito-borne flaviviruses, there were no systematic structure-activity studies, and only islands of structure-activity relationship data for particular compound series were available from different laboratories (Fig. 1). Tritylation[12–15] or silylation[16,17] of sugar moiety were found to be especially attractive strategies of achieving antiflaviviral effect for nucleosides. On the contrary, acyclic nucleoside analogs were shown to be ineffective against flavivirus reproduction in cells.[15,18] Nucleobase was varied in a rather conservative manner, mostly by changing of a hydrogen bonding or electron density pattern by swapping nitrogen and carbon atoms.[19,20]

In this study we expand the anti-TBEV nucleoside SAR by variation of substituents in the position N^6 of adenosine along with examples of N^2 substituted guanosines, N^4 substituted cytidine, polar nucleobase ribosides, and 3′-*C*-methylated nucleosides. The study of N^6 substituted adenosines was particularly inspired by the high potency of N^6-benzyladenosine and N^6-furfuryladenosine against Lassa and Marburg viruses revealed in high-throughput screening campaigns[21,22] and by activity of compounds from this series against enterovirus A71.[23,24]

The previously published procedures were used for the synthesis of N^6-substituted adenosines **1** and **2b–j**,[23,24] 3′-*C*-methylnucleosides **3**[25,26], **2a**, **4a**, **4b**,[27] **5** and **6**[28] (Table 1). Synthesis and characterization data for compounds **1e–k** are provided in the Supplementary Information.

Anti-TBEV activity was assessed in the plaque reduction assay in PEK (porcine embryo kidney) cells using the method described earlier.[10] Samples of the compounds were incubated with TBEV (strain Absettarov) for one hour and then added to the cell monolayer, achieving a final 50 μM concentration for each compound. Compounds that showed a 50% decrease of plaque count at 50 μM concentration were used for the subsequent cell toxicity (CC_{50}) and antiviral efficiency (EC_{50}) determination. The results are given in Table 1.

Cell toxicity of all the active molecules in the series was not observed in 50 μM concentrations after 24 h incubation, but chronic (7 d) toxicity was observed for N^6-(2-pyrenylmethyl)adenosine **1h** and N^6-benzyl-5′-*O*-trityladenosine **1i**. As these molecules are very hydrophobic, further modifications are required to achieve the optimal pharmacokinetics.

Most compounds of the series did not show TBEV reproduction inhibition *in vitro*. That suggests that the decoration of nucleobase amino groups as well as 3′-*C*-methylation of ribose do not usually lead to the emergence of this kind of activity. Nevertheless, the anti-TBEV activity gradually increased with the increase of N^6 substituent size from methyl to 2-pyrenylmethyl: whereas methyladenosine **2a** and benzyladenosine **1a** were not active, anthracenylmethyl adenosine **1g** was definitely active, and N^6-(2-pyrenylmethyl)adenosine **1h** showed clear antiviral effect on a micromolar level (on par with previously reported nucleosides[7,8]), though being rather cytotoxic after 7 days incubation. Removal of ribose from **1a**, giving benzyladenine **1l**, did not lead to any activity. Due to clear dependence of the efficiency on the size of aromatic substituent, the activity of **1h** may be attributed at least partially to the interaction with the viral membrane, analogously to RAFIs.[10]

Possible mechanism of action of the identified TBEV inhibitors could be related to the interaction with NS5 protein, which consists of MTase and RdRp domains, or cell entry inhibition.

Similar substituted nucleosides showed inhibition of flavivirus NS5 activities in the enzymatic assays.[12,16,17,29] We employed

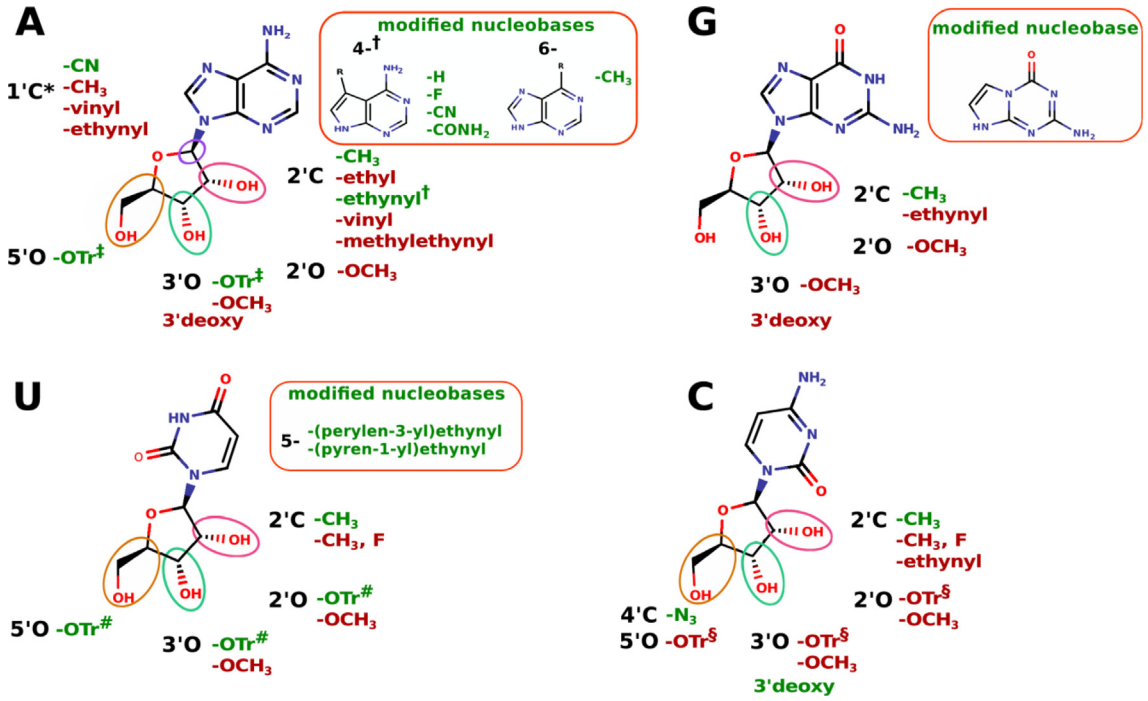

Fig. 1. Antiflaviviral activity of nucleosides according to literature.[7,8,10–15,20,36,37] Modifications of the nucleoside scaffold leading to antiviral activity are colored green, the ones not introducing activity – red. *1′-*C*-analogs were assessed as 4-aza-7,9-dideazaadenosine derivatives. †4-Substituted adenosines were highly toxic in PS cells. 4-Substituted 2′-*C*-ethynyl derivatives were active with moderate toxicity in another assay. ‡*O*-tritylated analogs were tested only for fludarabine and inosine. #*O*-tritylated analogs with various substituents; compounds with trityl moieties in 3′-*O* and 5′-*O* positions were the most active. §*O*-tritylated analogs were tested for a series of cytidine derivatives either unprotected at the base moiety or N^4-benzoylated.