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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.

Keywords

antivirals; nucleosides; Flavivirus; tick-borne encephalitis.

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.

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 sequela with different degree 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, which gives 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), inhibit 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) show TBEV entry inhibition at two-digit nanomolar concentrations, presumably due to interaction with the viral membrane.[10] In a follow-up study, SAR was 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 on a micromolar level.[11]

Despite numerous nucleoside analogs tested on mosquito-borne flaviviruses, there have been no systematic structure-activity studies, and only islands of structure-activity relationship data for particular compound series are 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]

[Figure 1 should be placed here]

Figure 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. 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 5-aza-cytidine.

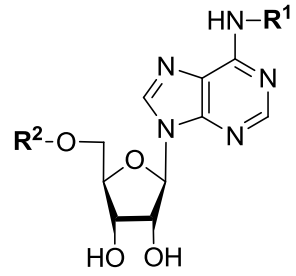
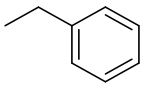
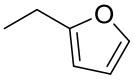
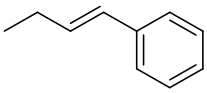
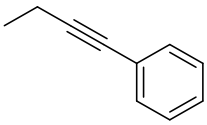
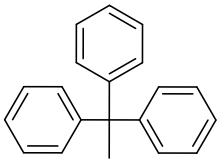
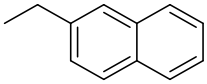
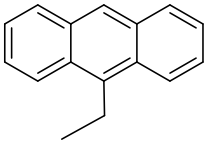
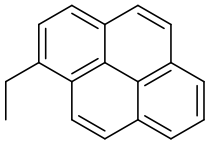
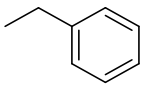
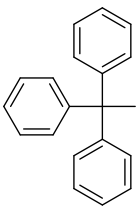
In this study we expand the anti-TBEV nucleoside SAR by variation of substituents in the position N^6 of adenosine along with examples of N^2 substituted guanosines, N^4 substituted cytidine, polar nucleobase ribosides, and 3'-C-methylated nucleosides. The study of N^6 substituted adenosines was particularly inspired by 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]

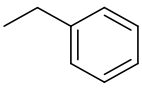
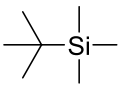
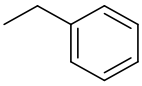
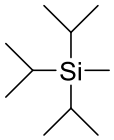
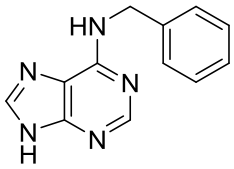
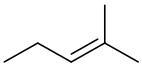
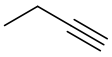
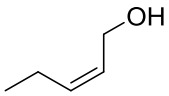
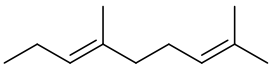
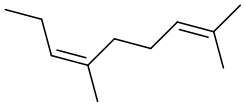
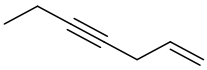
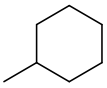
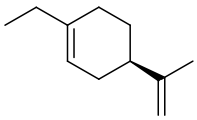
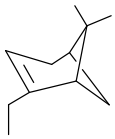
The previously published procedures were used for the synthesis of N^6-substituted adenosines **1** and **2b-j**,[23,24] 3'-C-methylnucleosides **3**[25,26], **2a**, **4a**, **4b**,[27] **5** and **6**[28] (Table 1); synthesis and characterization data for compounds **1e-k** are provided in the Supplementary 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 the final 50 µM concentration for each compound. Compounds that showed the 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.

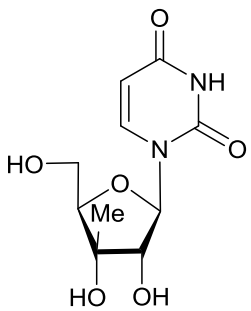
Table 1.

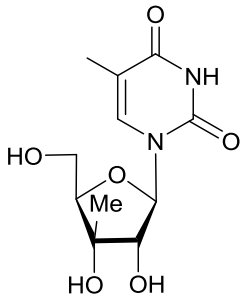
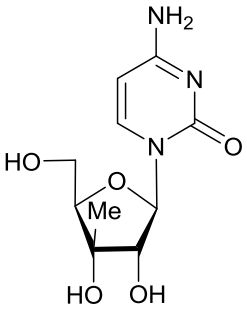
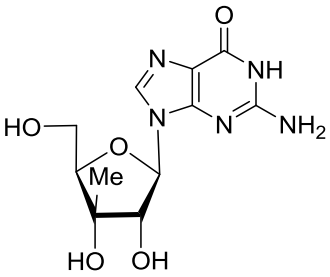
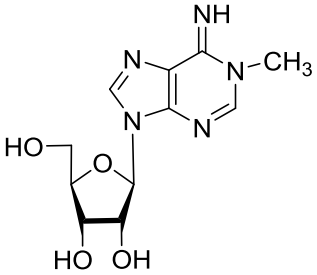
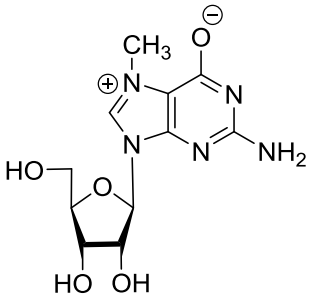
Anti-TBEV activity and cytotoxicity of nucleosides.

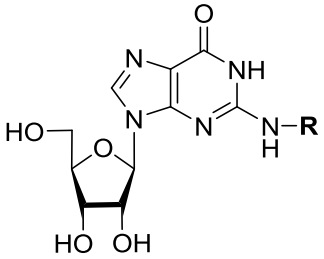
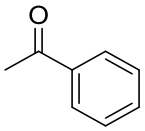
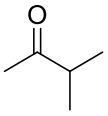
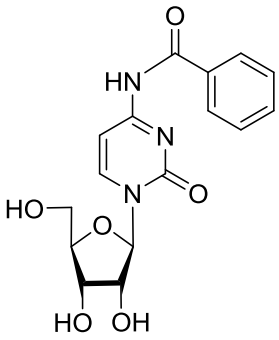
***N*^{6}-Substituted adenosines** 						
No.	**Compound name**	**Substituent**		**CC_{50} (24h), μM**	**CC_{50} (7d), μM**	**EC_{50}, μM**
		R^1	**R^2**			
1a	*N*^{6}-Benzyladenosine		H	>50	>50	>50
1b	*N*^{6}-Furfuryladenosine		H	ND	ND	>50
1c	*N*^{6}-(*E*)-(3-Phenyl-2-propen-1-yl)adenosine		H	ND	ND	>50
1d	*N*^{6}-(3-phenyl-2-propin-1-yl)-adenosine		H	ND	ND	>48
1e	*N*^{6}-Trityladenosine		H	ND	ND	>50
1f	*N*^{6}-(β-Naphthylmethyl)-adenosine		H	ND	ND	>50
1g	*N*^{6}-(9-Anthranylmethyl)-adenosine		H	>50	>50	15±2
1h	*N*^{6}-(1-Pyrenylmethyl)-adenosine		H	>50	9	6±1
1i	*N*^{6}-Benzyl-5'-*O*-trityladenosine			>50	9	2±1

1j	N^6-Benzyl-5′-*O*-*tert*-butyldimethylsilyl-adenosine			>50	>50	20±10
1k	N^6-Benzyl-5′-*O*-triisopropylsilyladenosine			>50	>50	5±4
1l	N^6-Benzyladenine			>50	>50	>50
2a	N^6-Methyladenosine	CH_3	H	ND	ND	>50
2b	N^6-Isopentenyladenosine		H	ND	ND	>50
2c	N^6-Propargyladenosine		H	ND	ND	>50
2d	N^6-(*Z*)-(4-Hydroxy-2-butenyl)adenosine		H	ND	ND	>50
2e	N^6-Neryladenosine		H	ND	ND	>50
2f	N^6-Geranyladenosine		H	ND	ND	>50
2g	N^6-(5-Hexene-2-yne-1-yl)adenosine		H	ND	ND	>50
2h	N^6-Cyclohexyladenosine		H	ND	ND	>50
2i	N^6-(*S*)-(-)-Perillyladenosine		H	ND	ND	>50
2j	N^6-(1*R*)-(-)-Myrtenyladenosine		H	ND	ND	>50

3'-C-Methylnucleosides

No.	Compound name	Structure	CC_{50} (24h), μM	CC_{50} (7d), μM	EC_{50}, μM
3a	3'-C-Methyluridine		ND	ND	>50

3b	3'-C-Methyl-2'-hydroxythymidine		ND	ND	>50
3c	3'-C-Methylcytidine		ND	ND	>50
3d	3'-C-Methylguanosine		ND	ND	>50
Polar hydrophilic nucleosides					
No.	**Compound name**	**Structure**	**CC_{50} (24h), μM**	**CC_{50} (7d), μM**	**EC_{50}, μM**
4a	1*N*-Methyladenosine		ND	ND	>50
4b	2-Amino-9-ribosyl-7-methyl-9H-purin-7-ium-6-olate		ND	ND	>50

N^2-Substituted guanosines					
					
No.	**Compound name**	**R**	**CC_{50} (24h), μM**	**CC_{50} (7d), μM**	**EC_{50}, μM**
5a	N^2-Benzoylguanosine		ND	ND	>50
5b	N^2-Isobutyrylguanosine		ND	ND	>50
N^4-Substituted cytidine					
6	N^4-Benzoylcytidine		ND	ND	>50

*ND — not determined (the compound was ineffective at 50 μM concentration and further investigation was not performed)

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 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, anthracenylmethyladenosine **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 toxic 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 homology modelling of TBEV NS5 and molecular docking as the tools that could allow us to assess the relevance of this mode of action to our compounds.

The model of full-length TBEV NS5 was built in Modeller 9.15[30] based on the Japanese encephalitis virus NS5 template (PDB ID 4K6M[31]). The SAH molecule in MTase domain was retained, and RdRp domain was in the conformation similar to other flavivirus RdRps in complex with inhibitors. Docking of the ligand conformations generated with OMEGA[32] was performed using FRED and HYBRID.[33]

Modification of N^6-benzyladenosine with bulky protective groups on 5'-hydroxyl group is in line with the previous findings for mosquito-borne flaviviruses[12-17]: compounds **1i-k** efficiently inhibit reproduction of TBEV. This modification is compatible with NS5 MTase domain interaction shown previously for similarly substituted compounds.[17,29] Docking results show the possibility of realization of binding modes consistent with this hypothesis (Fig. 2A).

[Figure 2 should be placed here]

Figure 2. Docking of active molecules into binding sites of TBEV NS5 model. A) Methyltransferase binding site. S-Adenosyl-L-homocysteine molecule is shown with cyan carbons, **1j** — with green carbons. B) RdRp binding site. TBEV NS5 model is red, X-ray structures of DENV3 RdRp with inhibitors are overlapped (5IQ6, brown; 5K5M, violet). Example docking poses of **1h** (gray carbons) and **1g** (light blue carbons) are shown. The figure was prepared in VIDA 4.3.0[38].

Inhibition of flaviviral NS5 RdRp by bulky hydrophobic nucleosides was also previously shown.[12,16] We used information available from X-ray structures of DENV RdRp complexed with non-nucleoside inhibitors (PDB IDs: 5IQ6[34], 5K5M[35]) to assess the possibility of interaction between our molecules and TBEV RdRp model. The generated binding modes overlap with both known inhibitor binding sites, suggesting that the interaction is theoretically possible. The large volume of the binding site is consistent with the lack of activity of smaller nucleosides: only pyrene and anthracene are large enough to occupy a significant portion of the binding site and prevent RNA binding (Fig. 2B). Thus, both NS5 domains may contribute to the antiviral activity of these compounds.

The role of identified compounds in entry inhibition needs to be evaluated in additional studies, for example, time-of-addition test.

The molecules presented here provide important novel information for further development of flavivirus reproduction inhibitors based on the nucleoside scaffold. Large aromatic substituents are shown to be the most efficient when introduced into the nucleobase of adenosine. Modification of 5' hydroxyl with bulky hydrophobic groups proved its feasibility for flavivirus reproduction inhibition. Docking simulation suggests that the interaction with NS5 protein is quite possible for the studied compounds, as well as the inhibition of TBEV reproduction on the entry phase. Further mechanism of action studies for these N^6-substituted adenosines should provide reasonable details for the design of efficient TBEV antivirals.

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Figure 1

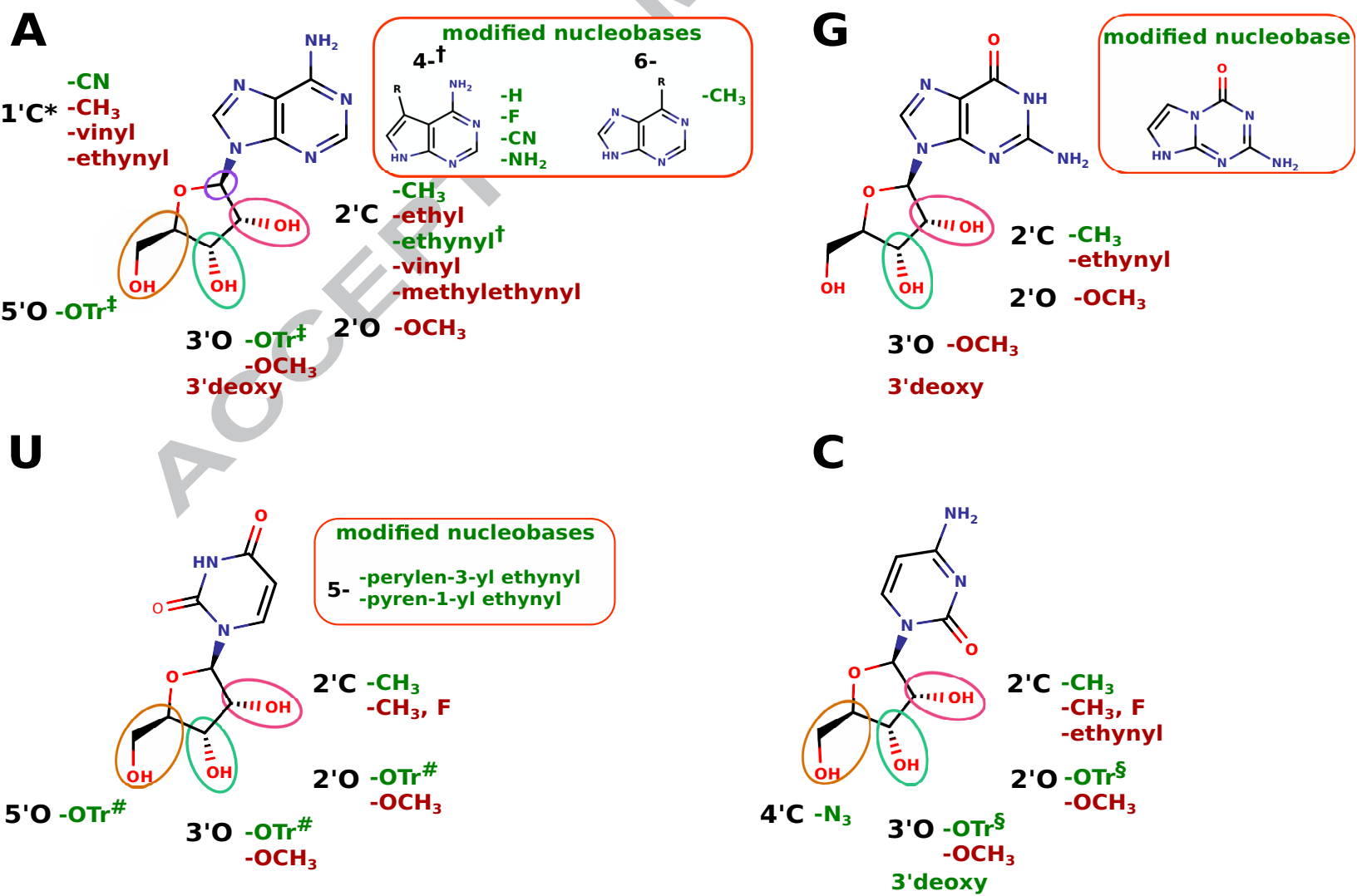
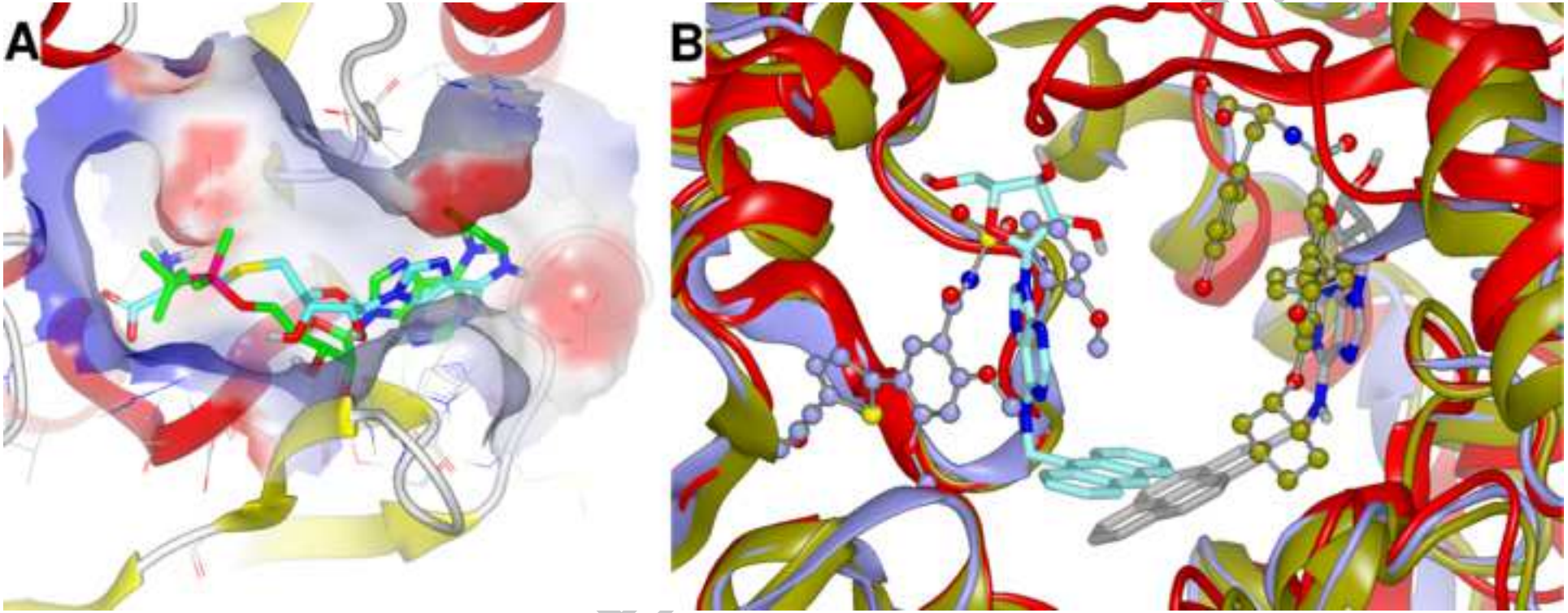


Figure 2



Graphical abstract