Design, synthesis and activity of hindered nucleoside analogs with anti-*Flaviviridae* activity, and their interaction with RdRp

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During a preliminary screening of a representative library of nucleoside analogs, by us designed and synthesized, two adenine derivatives AD1 and AD2 showed an anti-*Flaviviridae* activity comparable to that of Ribavirin. These results encouraged us to proceed with the design and development of a new class of hindered nucleoside analogs, displaying a significant anti-BVDV activity, predictive of their anti-HCV potential. In this work we present the major results, outcoming from a jointed effort of computer-aided molecular design, organic synthesis and biological testing.

Keywords: RdRp, HCV, Flaviviridae, nucleoside analogs, antivirals, computer-aided drug design

1 INTRODUZIONE

Flaviviridae are enveloped, positive single-stranded RNA viruses. This virus family contains three genera: *Hepacivirus* (hepatitis C virus [HCV]), *Flavivirus* (e.g., Yellow fever virus [YFV], Dengue fever virus [DENV], Japanese encephalitis virus [JEV], Tick-borne encephalitis virus [TBEV]), and *Pestivirus* (Bovine viral diarrhea virus [BVDB], Classical swine fever virus [CSFV], and Border disease virus [BDV]). Although viruses belonging to different genera have different biological properties, and do not show serological cross-reactivity, great similarity in terms of virion morphology, genome organization, and presumed replication strategies have been noted [1].

A number of these viruses are the etiological agents for important worldwide human deadly pathologies. As an example, notwithstanding the existence of a vaccine against YFV, this virus still causes nearly 30000 annual deaths, while HCV, as recently estimated by the World Health Organization (WHO), infects up to 3% of world population, thus generating over 170 million chronic carriers at risk of developing cirrhosis and/or hepatocellular carcinoma (HCC). Presently, the solely therapeutic agents available for facing this plague is a combination of Ribavirin with α-interferon (pristine or pegylated) [2]; this cure, however, is expensive, is associated with many side effects (especially after prolonged therapy), and is effective in only a subset of patients (only 15-20% of patients have a sustained virological response). Accordingly, there is a serious demand for a more (cost-)effective treatment and/or immunoprophylaxis against HCV.

With the wider aim to identify new compounds active against the *Flaviviridae* virus family, a structure-activity relationship (SAR) study was activated, starting from a representative library of nucleoside analogs deriving from our previous work [3-5]. Among the screened compounds, two adenine analogs AD1 and AD2 were found endowed with highly significant inhibitory activity against *Flaviviridae*, at least comparable, if not superior to that of Ribavirin (see Figure 1 and Table 1). In view of this important result, we decided to investigate thoroughly the compounds AD1 and AD2. In particular, in a first phase we considered AD2 as a template for a new generation of compounds, modified at the sugar portion by introducing different moieties. Concomitantly, preliminary molecular modeling investigations and combination

studies with known inhibitors have been carried out, in order to verify the interaction with the protein target, the RNA-dependent RNA-polymerase (RdRp), at a molecular level.

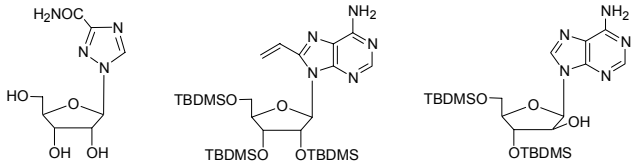

Fig. 1. Structure of Ribavirin (left), AD1 (middle) and AD2 (right).

Table 1. Cytostatic and antiviral activity of AD1 and AD2 in cell culture experiments, compared to Ribavirin.

Compound	BVDV	
	[a]CC_{50}	[b]EC_{50}
Ribavirin	40	2
AD1	>100	20
AD2	>100	10

[a]Compound concentration (μM) required to reduce the viability of mock-infected BT monolayers by 50%. [b]Compound concentration (μM) required to achieve protection of BT monolayers from the BVDV induced cytopathogenicity.

2 MODELING/EXPERIMENTAL DETAILS

2.1 Chemistry

With the structure of the two lead compounds as stating points, we investigated ribo-, 2'-deoxy-ribo-, and arabino-furanosyl derivatives of adenine. Resorting to computer-aided SARs, based on simple rational concepts (e.g., different sterical parameters, nature of bonding groups, and synthetic availability), different moieties were introduced at the hydroxyl functions of the sugar ring. In particular, TBMDS, DPMS, TBDPS, TIPDS, piranyl and triphenyl methyl groups were considered. All compounds were synthesized according to different procedures, reported in details in our papers [3-5].

2.2 Biology

Cellular cytotoxicity was tested over exponentially growing human $CD4^+$ lymphocytes (MT-4), baby hamster kidney (BHK-21), and Madin Darby bos kidney (MDBK). Cell viability was determined after 96 hrs at 37° by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method [4]. Activity against YFV, DENV and West Nile virus [WNV] was based on inhibition of virus-induced cytopathogenicity in acutely infected BHK-21 cells. Activity against BVDV was based on inhibition of virus-induced cytopathogenicity in acutely-infected MDBK cells. After 3-day incubation at 37°C, the number of viable cells was determined by the MTT method.

2.3 Molecular modeling

All simulations were run on a cluster of Silicon Graphics Octane R12K and performed using the program packages AutoDock 3.0 [6], AMBER 6.0 [7], and in-house developed codes. The starting 3D model of the RdRp was based on its X-ray crystallographic structure [8]. The all-atom force field of Cornell et al. [9] was applied in all calculations. After generation of the model structures of all nucleoside analogs, a conformational search was carried out using a well-validated combined molecular mechanics/dynamics simulated annealing (MDSA) protocol [10-14]. The electrostatic charges for the geometrically optimized nucleoside analogs were obtained by quantum mechanical calculations [15]. Each best drug/RdRp complex resulting from the docking procedure was further refined in the AMBER suite using the quenched molecular dynamics method [12,13]. Extended molecular dynamics simulations at 298 K were conducted to both qualify and quantify the interactions between the enzyme and the nucleoside analogs, using the MM/PBSA method [16].

3 RESULTS AND DISCUSSION

3.1 Biological activity

Notwithstanding the preliminary nature of these data, the biological activity of all tested compounds can be considered of great interest, as the vast majority of the molecules designed and synthesized were non cytotoxic (i.e., $CC_{50} > 100$) and endowed with activity against different *Flaviviridae*. In particular, significant potency and selectivity could be attributed to the hindering substituents at the sugar portion of ribo- and arabino-furanosyl nucleosides. Among the silyl moieties, the most effective in conferring inhibitory activity was the TBDPS in both arabino- and ribo- series; of the non-silyl groups, triphenylmethyl was the most active, but only in the arabino-furanosyl series. In particular, the substitution of the 5'-position induced

significant activity against BVDV and YFV, although associated to high cytotoxicity in BHK cells. The presence of a second trityl group at N6-position gave another compound that maintained the anti-BVDV activity and was endowed with a lesser cytotoxicity. All adenine active compounds presented an arabino- or ribo-nucleoside structure, thus suggesting mechanistic aspect of 2'-position connected to the inhibitory activity. These important results, currently under further investigations, imply that an essential requisite for the activity against *Flaviviridae* is the specific sugar structure linked to the presence of the bulky groups on hydroxyl functions.

3.2 Molecular modeling

Molecular modeling led to the identification of an allosteric binding site at the enzyme surface. As an example of the results obtained from the application of the lengthy procedure describe in the preceding paragraph, the interaction of compound **23** (i.e. AD2 disubstituted in 2' and 5' with TBDPS, CC_{50} >100, EC_{50} 11) with the enzyme is briefly described. The compound occupies the central portion of a long cleft (30Åx10Åx10Å), that extends nearly the entire length of the thumb surface, and eventually connects with the finger domain (see Figure 2).

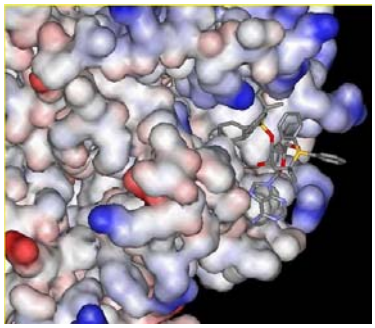

Fig. 2. Interaction of compound 23 with the allosteric binding site on the surface of RdRp.

The most intimate interactions involve the phenyl and *tert*-butyl bulky substituents on the sugar ring, which fit into a hydrophobic region, lined by the side chains of M423, W528, L419, Y477 and R422 (alkyl portion of the side chin). One edge of another phenyl ring of **23** rests on the side chains of M423 and L419, while a further phenyl moiety is surrounded by a side chain of L497, and W500. The base is aptly lined by the polar residues S476, S478, P479 and Y477 (see Figure 3). The above description of RdRp-inhibitor interactions applies, with minor modifications, to the other inhibitors.

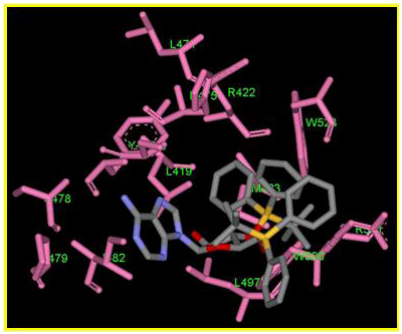

Fig. 3. Detailed interaction between compound 23 and the residues delimitating the allosterica binding site.

It is interesting to note that: 1) interaction of these inhibitors with HCV RdRp results only in minimal changes in overall protein architecture; 2) the inhibitor binding site may retain a high degree of 3D similarity across RdRp enzymes derived from known HCV genotypes. Accordingly, RdRp inhibitors that interact with this binding site have the potential to function as selective, broad-spectrum anti-HCV agents.

4 CONCLUSIONS

In conclusion, the present investigation, resulting from a jointed effort of computer-aided drug design, synthetic chemistry and biology conducted starting from two lead compounds, AD1 and AD2, has led to the discovery of a novel class of agents active against *Flaviviridae*. These nucleoside like compounds are characterized by hindering substituents at positions 3',5' or 2',5' or 5' of the sugar portion, and act as non competitive inhibitors of RdRp. This behavior resemble that of the known allosteric inhibitors of reverse transcriptase (RT) TSAO-T [17], that also possess an hindered substituents pattern at the sugar portion of thymidine. Of particular interest is the fact that the degree of selectivity on the different viruses was found connected with different substituents and the pattern of substitution, thus envisaging the possibility to design selective agents. In our opinion these results open new perspectives in the development of new anti-*Flaviviridae* agents endowed with selectivity on the different members of this family of viruses. In particular, these results are predictive of a possible activity as anti-HCV agents, that awaits further confirmation from *in vivo* experiments, which are currently performed by

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