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IN SILICO DESIGN OF AMINO ACIDS-FUNCTIONALIZED IMIDAZOLES AS INHIBITORS OF DENGUE VIRUS NS3 AND NS5 PROTEINS

DISEÑO *IN SILICO* DE IMIDAZOLES FUNCIONALIZADOS CON AMINOÁCIDOS COMO INHIBIDORES DE LAS PROTEÍNAS NS3 Y NS5 DEL VIRUS DEL DENGUE

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ABSTRACT

252 amino acid-functionalized imidazoles were designed to evaluate their potential as inhibitors using computational techniques. An analysis of the ADME+T profiles indicates that compounds derived from the polar amino acids Ser and Thr are the most favorable as drug candidates. Of these compounds, four (Ser-A22, Thr-A22, Ser-A35 and Thr-A35) stand out as candidates for inhibitors of proteins NS3 in their helicase domain and NS5 in their methyltransferase domain. For the NS2B-NS3Pro and NS5RdRp domains, the results are not encouraging. An analysis of the electron densities of these four compounds by DFT showed similarity with reference inhibitors in terms of frontier orbitals, reactivity and electrostatic potential surfaces, which explains the strength of the binding of these compounds with viral proteins.

Keywords: drug design; molecular docking; ADME+T; DFT; dengue virus; amino acid-functionalized imidazoles.

RESUMEN

Se diseñaron 252 imidazoles funcionalizados con aminoácidos para evaluar sus posibilidades como inhibidores, mediante técnicas computacionales. Un análisis de los perfiles ADME+T indica, que los compuestos derivados de los aminoácidos polares Ser y Thr, son los más favorables como candidatos a fármacos. De estos compuestos, cuatro (Ser-A22, Thr-A22, Ser-A35 y Thr-A35) resaltan como candidatos a inhibidores de las proteínas NS3 en su dominio helicasa y NS5 en su dominio metiltransferasa. Para los dominios NS2B-NS3Pro y NS5RdRp los resultados no son alentadores. Un análisis de las densidades electrónicas de estos cuatro compuestos mediante DFT, arrojó la similitud con inhibidores de referencia en cuanto a orbitales de frontera, reactividad y superficies de potencial electrostático, lo cual explica la fortaleza de la unión de estos compuestos con las proteínas virales.

Palabras clave: diseño de fármacos; acoplamiento molecular; ADME+T; DFT; virus del dengue; imidazoles funcionalizados con aminoácidos.

INTRODUCTION

Dengue virus (DENV) is the most prevalent arthropod-borne virus affecting humans today. Its main vectors are mosquitoes of the species *Aedes aegypti* and *Aedes albopictus*. The group of viruses consists of four serotypes that manifest with similar symptoms. It causes a spectrum of diseases, which range according to an updated WHO guideline in 2009 into: probable dengue, dengue with warning signs and severe dengue.[1]

Dengue viruses have evolved rapidly as they spread around the world, and genotypes associated with increased virulence have spread throughout Asia and the Americas.[2] In Cuba, the first DENV epidemic was caused by DENV-1 in 1977. Then, in 1981, Cuba reported the region's first "dengue hemorrhagic fever" epidemic, caused by DENV-2. In 2001-2002, a new epidemic caused by DENV-3 was reported, and small outbreaks of DENV-3 and DENV-4 occurred in subsequent years. Today, all four serotypes coexist.[3]

The differential diagnosis of dengue is difficult: it can be confused with other arboviral infections (Zika, Chikungunya, Oropouche fever),[4,5] malaria, influenza, measles, rubella and other exanthematous diseases.[6] There are currently no specific treatments or cures for dengue. The only commercial dengue vaccine, CYD-TDV "Dengvaxia" from Sanofi Pasteur, has been approved in some countries. However, its use is limited to patients between 9 and 45 years of age who reside in hyperendemic regions and who have been previously infected with DENV.[1,7]

Current treatment options are supportive and aim to limit complications and symptom severity, such as: fluid therapy,[8] administration of analgesics and antipyretics, single platelet donations[9] or recombinant human (rh) IL-11.[10] The ideal characteristics of a therapeutic agent against dengue would be to have pan-serotype activity, capable of rapidly resolving symptoms, be well tolerated with minimal toxicity, be easily distributed on a large scale, have minimal interaction with other medications, and be tolerable for adults, children, infants, pregnant women, and patients with comorbidities.[11,12]

The viral particle has a single-stranded RNA genome of positive polarity, within a capsid with icosahedral symmetry, and surrounded by an external envelope composed of three proteins E (envelope), prM (precursor membrane) and C (capsid). The remaining seven proteins of the genome are non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) and facilitate viral replication inside infected cells.[13]

Since NS5 is the most conserved protein, it is therefore a target of choice for designing a pan-serotype antiviral compound. Crystal structures of the NS5 protein show two domains: Methyltransferase (MTase) and RNA-dependent polymerase (RdRp). The MTase domain can be subdivided into three subdomains, of which the one of greatest pharmacological interest is the C-terminal side, generally occupied by the molecule S-adenosyl-L-methionine (SAM) or the byproduct of methylation reactions S-adenosyl-L-homocysteine (SAH).[14] The RdRp domain allows processive RNA polymerization, both of which are recommended targets for the inhibition of viral replication.[15]

On the other hand, the bipartite protein NS2B-NS3 protease/NS3 helicase is a fundamental enzyme for flavivirus replication and polyprotein processing. This protein is also highly conserved among flaviviruses.[16] The NS3 protease (NS2B-NS3Pro) is a trypsin-like serine protease that has been shown to harbor a classical serine protease catalytic triad composed of the residues His51, Asp75, and Ser135.[17] The activating cofactor NS2B is a prerequisite for the catalytic activity of NS2B-NS3Pro.[18] Instead, the combined activities of a polynucleotide-stimulated helicase and a nucleoside triphosphatase (NTPase) in the C-terminal domain are required to fuse secondary structures prior to the initiation of RNA synthesis and to unwind duplex RNA, either to separate double-stranded RNA (dsRNA) intermediates formed during viral RNA synthesis or as a translocase that can remove proteins bound to viral RNA.[19] Dengue viruses with altered helicase activities lose the ability to replicate, demonstrating that the helicase activity of NS2B-NS3Pro is not a reliable indicator of the ability to replicate. the importance of the NS3Hel domain in the life cycle and that inhibitors or modulators of these enzymes are potentially of interest as therapeutic agents.[20]

Multiple approaches have been used to find effective inhibitors against dengue viral proteins, leading to the design of a large number of compounds with proven activities both *in silico* and *in vitro*.[21]

These strategies often include the study of natural products,[22] the design of peptides and pseudopeptides[23] and the de novo design of compounds, with an important presence of heterocyclic rings, as well as polar functional groups: amino, carboxyl, nitro, etc.[24,25] However, most of

these are discarded in the different phases of clinical trials, mainly due to toxicity problems or lack of activity in vivo.[26]

Our research group has extensive experience in the design, synthesis and *in silico* study of tri- and tetra-substituted imidazole compounds. However, due to the *in vitro* toxicity of these compounds[27] a design strategy has been followed that involves functionalization with amino acid fragments in search of a decrease in toxicity, more possibilities of interaction with target proteins and greater solubility in water.

MATERIALS AND METHODS

Design of amino acid functionalized imidazoles

252 imidazoles functionalized with amino acids of apolar or weakly polar nature have been designed (Figure 1).

Screening by ADME+T

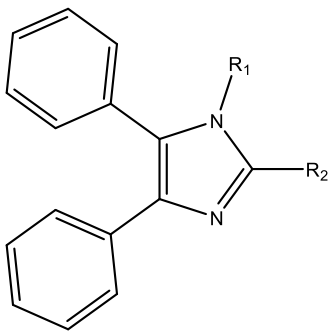
ADME+T screening was performed in the online software ADMETlab3.0, which is composed of a set of high-quality prediction models trained using a multi-task graph attention framework. ADMETlab3.0 integrates 77 prediction models, 59 classification models, and 18 T models, and enables the implementation of the calculation and prediction of 21 physicochemical properties, 20 medicinal chemistry measures, 34 ADME endpoints, 36 toxicity endpoints, and 8 toxicophore rules.[28]

Since ADMETlab3.0 estimates 119 parameters that provide information about the possible performance of the designed drug, it is therefore necessary to establish those whose fulfillment is vital for the correct functioning of the drug candidate. This is why the following parameters have been chosen:

- Carrier-mediated transport (Pgp inhibitor, Pgp substrate).
- Solubility in aqueous media and in a wide pH range (logS, logP, logD7.4), to evaluate the permeability of the cell membrane, since DENV non-structural proteins act inside the cell.
- Plasma protein binding (PPB) and the fraction of unbound drug (Fu). Polar or acidic drugs, such as most of the designed compounds, affect both parameters. This is why negative results are expected in these parameters, which is not necessarily a negative point, since for drugs that act inside the cells, a balance is established between bound, free and intracellular drug, so plasma proteins act as a kind of release system that protects the drug from metabolism and reduces undesirable effects.[29]
- Acute toxicity (LD50, ROA, FDAMDD), since an antiviral for DENV would be used for a period of time less than 14 days (infection time), acute toxicity would be of greater importance than long-term forms of toxicity.
- Chemical promiscuity (PAINS, NMR alarm rule, BMS rule). These parameters indicate which compounds react in a non-selective manner within the organism and with laboratory tests, generating false positives.

Computing resources

The calculations were performed primarily using the high-performance computing capabilities of the Universidad de Oriente, Cuba cluster (HPC-UO) (https://portal.uo.hpc.cu/website/ (access date: March 24, 2024).



R_1: N-terminal residues of Val, Tyr, Phe, Ser, Thr, Trp y Cys
R_2: -C_6H_5 (**A01**), -CH_3 (**A02**), -H (**A03**), -2-C_4H_3O (**A04**), -C_6H_4-p-$N(CH_3)_2$ (**A05**), -C_6H_4-p-NO_2 (**A06**), -C_6H_4-m-NO_2 (**A07**), -C_6H_4-o-NO_2 (**A08**), -C_6H_4-p-OH (**A09**), -C_6H_4-m-OH (**A10**), -C_6H_4-o-OH (**A11**), -C_6H_4-o-Cl (**A12**), -C_6H_4-m-Cl (**A13**), -C_6H_4-p-Cl (**A14**), -C_2H_5 (**A15**), -C_3H_7 (**A16**), -$C(CH_3)_3$ (**A17**), -$CH(CH_3)_2$ (**A18**), -C_6H_4-o-CH_3 (**A19**), -C_6H_4-m-CH_3 (**A20**), -C_6H_4-p-CH_3 (**A21**), -CH_2-C_6H_5 (**A22**), -CH=CH-C_6H_5 (**A23**), -C_6H_4-o-OCH_3 (**A24**), -C_6H_4-m-OCH_3 (**A25**), -C_6H_4-p-OCH_3 (**A26**), -C_6H_4-p-Br (**A27**), -C_6H_4-m-Br (**A28**), -C_6H_4-o-Br (**A29**), -C_6H_4-p-CN (**A30**), -C_5H_{11} (**A31**), -CH_2-$CH(CH_3)_2$ (**A32**), -C_6H_3-(4-OH)-3-OCH_3 (**A33**), -C_6H_3-(4-$OCOCH_3$)-3-OCH_3 (**A34**), -C_6H_4-p-COOH (**A35**), -CH_2-CH_2-C_6H_5 (**A36**)

Fig. 1- Designed amino acid-functionalized imidazoles

Note: The ID codes of each compound are formed by joining the code of the amino acid residue R_1 with the aldehyde residue R_2 separated by a short dash "-", for example, Val-A01 refers to the compound formed by the amino acid residue valine and the R_2 (-C_6H_5) residue.

Procedure for generating 3D structures of ligands for molecular docking

To obtain the structures of the designed amino acid-functionalized imidazoles, we started from their SMILES codes and used the OpenBabel 3.1.1[30] program to first generate the 2D coordinates and make the hydrogens explicit in *.cdx structures. Then, the 3D coordinates are generated in *.pdb format by applying the ("--best") option, which includes: 250 steepest descent geometry optimization steps with the MMFF94 force field, 200 iterations of a weighted rotor conformational search (optimizing each conformer with 25 steepest descent steps), and 250 conjugate gradient geometry optimization steps.

The obtained structures are reoptimized using the molecular mechanics method with the MMFF94 (Merck Molecular Force Field) force field until convergence and the *.pdbqt files, generated in AutoDockTools version 1.5.6[31] adding the Gasteiger charges, eliminating the non-polar hydrogens and assigning the AD4 atom type.

Preparation and selection of proteins for molecular docking

Protein preparation was carried out using the UCSF Chimera software package version 1.11.1[32] to remove crystallization residues and AutoDockTools version 1.5.6[31] to add Kollman charges, add polar hydrogens and assign the AD4 atom type.

A large number of structures for these proteins are available in the Protein Data Bank, of which not all meet the necessary, or at least desirable, requirements to be used as receptors in molecular docking calculations. Of the 99 available structures, they were exhaustively analyzed based on the following criteria: resolution of the reported structure, which is recommended to be less than 2Å;[33] presence of co-crystallized ligand, or failing that, report in the literature of the active site, variety of serotypes and bibliographic reports of its use in molecular docking. After analyzing these criteria, 10 of them were s elected (Table 1), which cover three of the four serotypes.

Table 1- Proteins chosen as DENV targets

Serotypes	1	2	3
Helicase-NS3		2BHR[34]	2WHX[35]
Methyltransferase-NS5	5IKM[36]	1L9K,[37]	4CTJ[38]
Polymerase-NS5		5K5M[39]	3VWS[40]
Protease-NS2B/NS3	3L6P[41]	2FOM[42]	3U1J[43]

Table 2 shows the characteristics of the proteins (organism, resolution and co-crystallized ligands). Not all structures have co-crystallised ligands, but in all cases, the amino acid residues of the active site are reported. In the case of MTases, only the ligand of the SAM binding site has been considered. For all cases, a study of the residues linked to the active site is necessary for a correct interpretation of the results, so they were studied according to the bibliographic reports (Table 3).

The docking study was performed using the Autodock software suite version 4.2.[31] For the generation of the grid with the AutoGrid4 software, a size of 60x60x60 points was chosen with a selected spacing of 0,375 from a central point of the active site (Table 4) considering the geometry of the protein as rigid. In the case of the structures that do not present a co-crystallized ligand, the center was taken on one of the atoms of an amino acid residue of the active site reported in the literature.

Table 2- Characteristics of the selected proteins

PDBID	Organism	Classification	Resolution (Å)	Co-crystallized ligands
2FOM	DENV-2	Protease	1,50	-
3L6P	DENV-1	Protease	2,20	-
3U1J	DENV-3	Protease	1,80	-
2BHR	DENV-2	Helicase	2,80	-
2WHX	DENV-3	Helicase	2,20	Adenosine-5'-diphosphate (ADP)
1L9K	DENV-2	Methyltransferase	2,40	SAH
4CTJ	DENV-3	Methyltransferase	1,47	SAM
5IKM	DENV-1	Methyltransferase	1,90	SAM
3VWS	DENV-3	Polymerase	2.10	5-{[;(4-chlorophenyl)sulfonyl];amino}-2-methyl-1-benzofuran-3-carboxylic acid (VWS)
5K5M	DENV-2	Polymerase	2.01	5-[;5-(3-hydroxyprop-1-in-1-yl)thiophen-2-yl];-2,4-dimethoxy-n-[;(3-methoxyphenyl)sulfonyl];benzamide (68T)

Table 3- Active site amino acid residues

	Active site amino acid residues
2FOM[42]	Gln86, Asn84, Asp82, Gly83, Asp75, Phe85, Asn152, Gly153, Gly151, His51, Ser135, Tyr161, Tyr150, Asp129, Tyr130
3L6P[41]	Ile75, His101(His51), Arg104, Gly105, Pro117, Trp119, Asp125 (Asp75), Tyr129, Gly130, Gly131, Trp133, Phe135, Trp139, Pro152, Gln160, Pro163, Gly174, Asp179, Gly183, Ser185 (Ser135), Tyr200, Gly201, Asn202, Gly203, Val212, Ser213, Ile215, Gln217
3U1J[43]	Ile30, Phe31, Thr34, His51, Val52, Asp75, Pro102, Asp129, Phe130, Pro132, Gly133, Ser135, Asn152, Gly153, Tyr161,
2BHR[34]	Arg457, Arg460, Arg610, Arg463, Gly196, Arg287, Gln254, Gln456, His287, Glu285, Glu224, Ala286, Asp223, Asp284, Lys37, Lys199, Thr200, Ala197, Gly198
2WHX[35]	Asp179, Ala197, Arg202, Glu173, Gly172, Tyr83, Glu169, Lys88
1L9K[37]	Thr104, Lys105, Val132, Ile147, Asp131, Gly106, Glu111, Tyr219, Ser56, Gly86, Trp87
4CTJ[38]	Gly106, Gly107, Pro108, Gly109, His110, Glilu112, Pro115, Met116, Ser117, Thr121
5IKM[36]	Phe133, Val152, Asp131, Lys130, Lys105, Thr104, His110, Ile147, Cys82, Gly83, Gly85, Gly86, Gly58, Ser56, Trp87, Asp156
3VWS[40]	Lys401, Ile797, Thr605, Gly604, Tyr606, Val603, Phe412, Val411, Thr413, Phe398, Phe485, Trp795, Asn405, Asn492
5K5M[39]	Ser710, His711, His798, Cys709, Ala799, His801, Lys800, Glu802, Trp803, His513, Met765, Leu512, Tyr766, Thr793, Thr784, Trp795, Met761, Ser796, Arg737, Arg729

Table 4- Active site coordinates for the selected proteins

PDBID	X	Y	Z
2FOM	-8,777	8,665	12,447
3L6P	-23,527	-19,216	27,165
3U1J	-7,583	-22,34	-15,129
2BHR	16,218	48,354	50,298
2WHX	19,271	-3,594	10,483
1L9K	15,468	-41,626	1,289
4CTJ	31,633	41,838	-5,505
5IKM	6,01	26,963	24,873
3VWS	32,109	68,109	17,691
5K5M	-15,018	-44,031	-21,029

To validate the docking results, several reported inhibitors were used as a comparison standard and the co-crystallized ligands were redocked.

Molecular Electrostatic Surface Potential (MESP)

The computational study of the electronic surface of the six molecules with the greatest prospects as inhibitors of nonstructural proteins of the dengue virus was carried out using the GAUSSIAN09 software[44] employing the density functional theory (DFT), with the hybrid functional B3LYP[45] and 6-31+G(d,p) basis set. The deduced structure was confirmed to be of minimum energy because of the absence of imaginary wave numbers on the calculated vibrational spectrum. The global reactivity parameters and the molecular electrostatic potential (MESP) surface were calculated, using the GaussView 6.1.1 software for visualization.[46]

RESULTS AND DISCUSSION

ADME+T screening

After applying the ADME+T analysis, according to the established criteria, of the initial design of 252 compounds, only 20 compounds met the vital parameters, which is consistent with the fact of the difficulty in finding effective inhibitors. The ADME+T screening of the compounds of the design with the selected parameters is described below.

Solubility and cell membrane penetration

The LogS parameter assessing solubility in aqueous systems affects 34 compounds. The presence of amino acid residues N-Tyr and N-Val is correlated with the LogS parameter, as is the presence of aldehyde residues: hexanal (A31), isovaleraldehyde (A32) and vanillin isobutyrate (A34). As for membrane penetration, assessed by the octanol/water partition coefficient (LogP) and the influence of pH (LogD7.4), 187 and 80 compounds respectively show a negative trend. All compounds with poor results in the LogD7.4 parameter also present adverse results in LogP. Both parameters are correlated with the presence of the amino acid residues N-Tyr, N-Phe, N-Tpr and N-Val and the aldehyde residues: 4-chlorobenzaldehyde (A14), 4-bromobenzaldehyde (A27), hexanal (A31), cinnamaldehyde (A23), 3-phenylpropanal (A36), 4-methoxybenzaldehyde (A21), 3-methoxybenzaldehyde (A20), 3-chlorobenzaldehyde (A13), vanillin isobutyrate (A34), isovaleraldehyde (A32), and 3-bromobenzaldehyde (A28).

Blood transport

The designed compounds do not present adverse results regarding plasma protein binding (PPB), blood volume distribution (VDss), fraction of drug not bound to plasma (Fu), nor are they substrates of the polyglycoprotein (Pgp-sub). However, 143

compounds are potential inhibitors of polyglycoprotein (Pgp-inh), where the amino acid residues N-Tpr, N-Val and N-Phe and those of aldehyde residues: cinnamaldehyde (A23), vanilin (A33), 2-methoxybenzaldehyde (A24), 4-methoxybenzaldehyde (A26), 2-chlorobenzaldehyde (A12), 3- chlorobenzaldehyde (A13), isobutyrate (A34), 3-bromobenzaldehyde (A28), 4-methylbenzaldehyde (A21), 2-bromobenzaldehyde (A29) and 3-phenylpropanal (A36) are the ones with the greatest negative contribution.

Acute toxicity

Among the acute toxicity indicators, neither the oral lethal dose (LD50_oral) nor the acute toxicity in rats (ROA) show adverse results, but the maximum recommended daily dose (FDAMMD) does, which affects 54 compounds, with an incidence of the amino acid residues N-Thr and N-Tpr and of aldehyde residues: 2-bromobenzaldehyde (A29), 3-phenylpropanal (A36), cinnamaldehyde (A23), 3-bromobenzaldehyde (A28), 4-bromobenzaldehyde (A27), p-formylbenzoic acid (A35) mainly.

Chemical promiscuity

One of the most important promiscuity indicators is the pan-reactivity assay (PAINS), which shows negative results for all compounds presenting the p-dimethylaminobenzaldehyde residue (A05). No compound violates the BMS rule, but 147 compounds react with thiol groups (Alarm_NMR). The amino acid residue with the greatest impact on the Alarm_NMR parameter is N-Phe, with 33 of the 36 compounds designed with this residue being reactive. The amino acid residues N-Tyr, N-Val and N-Tpr also have a negative influence, as well as the aldehyde residues: 2-methoxybenzaldehyde (A19), 2-methylbenzaldehyde (A19), 4-methylbenzaldehyde (A24), 4-nitrobenzaldehyde (A08), formaldehyde (A03), trimethylacetaldehyde (A17), 2-hydroxybenzaldehyde (A09), butyraldehyde (A16), acetaldehyde (A02), benzaldehyde (A01) and cinnamaldehyde (A23).

In general, only the aldehyde residues: benzaldehyde (A01), acetaldehyde (A02), formaldehyde (A03), furfural (A04), propanal (A15), butanal (A16), trimethylacetaldehyde (A17), isobutyraldehyde (A18), 2-phenylacetaldehyde (A22), cinnamaldehyde (A23), hexanal (A31), isovaleraldehyde (A32), p-formylbenzoic acid (A35); bound to the amino acids Ser and Thr have an ideal, or at least acceptable, ADME+T behavior. A molecular docking study was performed on these compounds against non-structural proteins of various DENV serotypes to evaluate their effectiveness as potential inhibitors of viral replication.

Molecular docking

Table 5 summarizes the results of the molecular docking study. It is noteworthy that for the NS5RdRp proteins only three of the compounds studied present results in the same order as Neosilyhermin B for the DENV-3 serotype (3VWS) and none for DENV-2 (5K5M). For the rest of the proteins, at least some of the compounds in the design present results in the same order as the reference inhibitors.

For NS2B-NS3Pro proteins, none of the designed compounds surpasses the inhibitors Balsacone B or Ivermectin B1a, however, several compounds have comparable values with a rather heterogeneous behavior according to the serotype. Among the compounds that stand out are Ser-A16 and Ser-A17 for serotype 1, Ser-A02, Thr-A02, Ser-A03 and Thr-A03 for serotype 3 and Thr-A01, Thr-A04 and Thr-A22 for serotype 2. This heterogeneous behavior contrasts with the high degree of conservation of the NS3 protein among the different DENV serotypes (77 %),[47] which deserves to be reviewed in more depth, for which the compound Thr-A03 was taken as an example (Figure 2).

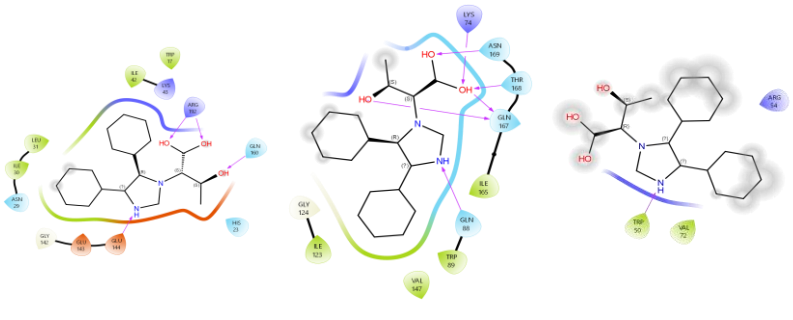


Fig. 2- Ligand interaction diagram of Thr-A03 with: (A) DENV-1 NS2B-NS3Pro (3L6P); (B) DENV-3 NS2B-NS3Pro (3U1J); (C) DENV-2 NS2B-NS3Pro (2FOM)

compounds to present an inhibitory activity worth taking into account, since the interaction with the main residues of the active site of the protein is practically null. Although, despite what has been discussed above, there could be inhibition, the heterogeneity of the results makes it difficult to find real possibilities of use as a possible antiviral, since the compound chosen would depend on the serotype.

Table 5- Docking scores (kcal/mol) against the selected proteins

	NS5MTasa			NS3Hel		NS2B-NS3Pro			NS5RdRp	
	DEV 1	**DEV 2**	**DEV 3**	**DEV 2**	**DEV 3**	**DEV1**	**DEV2**	**DEV3**	**DEV2**	**DEV3**
	5IKM	**1L9K**	**4CTJ**	**2BHR**	**2WHX**	**3L6P**	**2FOM**	**3U1J**	**5K5M**	**3VWS**
Ser-A01	-10,02	-5,09	-7,47	-4,11	-3,90	-4,66	-3,79	-5,02	-5,60	-5,95
Thr-A01	-9,97	-5,18	-6,75	-3,83	-3,84	-4,44	-4,47	-4,72	-4,76	-5,36
Ser-A02	-9,10	-4,71	-7,47	-4,21	-3,67	-5,10	-3,53	-5,84	-5,32	-5,60
Thr-A02	-8,71	-4,91	-6,94	-4,37	-3,76	-4,84	-3,73	-5,84	-4,94	-5,52
Ser-A03	-8,71	-4,51	-7,25	-4,17	-3,84	-4,86	-3,43	-5,78	-5,21	-5,53
Thr-A03	-8,60	-4,76	-7,00	-4,27	-3,92	-4,76	-3,19	-5,87	-4,66	-5,33
Ser-A04	-9,63	-5,18	-7,28	-4,08	-3,79	-4,63	-4,38	-4,82	-4,91	-5,26
Thr-A04	-9,61	-5,45	-7,03	-4,02	-3,98	-4,24	-4,47	-4,90	-4,54	-5,07
Ser-A15	-9,16	-4,63	-7,35	-4,06	-3,57	-5,16	-3,91	-4,58	-5,07	-5,55
Thr-A15	-9,05	-4,74	-7,11	-4,16	-3,72	-4,81	-3,61	-5,34	-4,56	-5,02
Ser-A16	-9,56	-4,66	-7,38	-4,01	-3,53	-5,20	-3,46	-5,39	-5,11	-5,43
Thr-A16	-9,31	-4,87	-6,88	-4,14	-3,48	-5,07	-3,48	-5,06	-4,64	-5,45
Ser-A17	-9,80	-4,73	-7,38	-4,28	-3,55	-5,23	-3,50	-4,83	-5,11	-4,96
Thr-A17	-9,85	-4,83	-6,65	-4,55	-3,36	-5,07	-4,14	-5,03	-4,79	-5,13
Ser-A18	-9,48	-4,68	-7,48	-4,18	-3,74	-4,96	-4,00	-4,63	-4,99	-5,59
Thr-A18	-9,45	-4,85	-6,77	-4,45	-3,84	-4,88	-3,85	-4,65	-5,14	-4,82
Ser-A22	-10,68	-5,42	-7,69	-4,40	-4,42	-5,17	-4,10	-4,95	-5,16	-6,24
Thr-A22	-10,69	-5,60	-7,45	-4,54	-4,39	-4,48	-4,53	-5,31	-4,69	-6,27
Ser-A23	-9,93	-5,67	-8,37	-4,90	-3,91	-5,11	-2,67	-5,56	-5,67	-5,32
Thr-A23	-10,11	-5,75	-7,52	-4,98	-4,10	-5,10	-3,60	-5,47	-5,31	-5,18
Ser-A31	-9,94	-4,75	-7,39	-4,09	-3,69	-5,19	-4,13	-4,80	-5,11	-5,92
Thr-A31	-9,40	-4,99	-6,93	-4,11	-3,78	-5,12	-3,51	-4,93	-4,58	-5,34
Ser-A32	-9,69	-5,01	-7,26	-4,22	-3,61	-5,28	-3,40	-5,24	-5,51	-5,43
Thr-A32	-9,72	-4,75	-6,92	-4,35	-3,50	-5,03	-3,80	-5,34	-5,17	-5,30
Ser-A35	-11,25	-5,91	-8,24	-5,89	-6,78	-4,39	-2,84	-5,71	-6,62	-6,01
Thr-A35	-11,26	-5,81	-8,14	-5,62	-6,67	-4,72	-3,09	-5,80	-6,70	-6,55
Sinefugin	-10,44	-5,45	-7,60	-	-	-	-	-	-	-
Ivermectin B1a	-9,61	-5,15	-7,37	-4,23	-3,78	-4,53	-4,49	-5,02	-	-
ADP	-	-	-	-4,68	-5,13	-	-	-	-	-
Balsacone B	-	-	-	-	-	-5,62	-4,98	-6,09	-	-
Neosilyhermin B	-	-	-	-	-	-	-	-	-8,38	-7,12

On the other hand, the NS3 protein cannot be completely ruled out, since its NS3Hel domain exhibits a completely different behavior. Four of the designed compounds (Ser-23, Thr-23, Ser-A35 and Thr-A35) present better docking scores than the ADP inhibitor for serotype 2, while Ser-A35 and Thr-A35 present better docking scores for serotype 3 (Figure 3).

ADP has multiple possibilities for hydrogen bonding with the amino group of adenine, as well as with both phosphate groups. Since ADP is a polar compound, it is logical to think that the compounds designed with greater polarity are those that present the best results. Both compounds (Ser-A35 and Thr-A35) have in common the possibility of establishing hydrogen bonds both with the carboxyl group of the aldehyde residue, as well as with the carboxyl and hydroxyl groups of the amino acid residue, which is why they present docking scores even higher than ADP.

In the DENV-3 NS3Hel protein, Ser-A35 shares with ADP the interactions with Gly198, Lys201, Thr200, Lys199, Arg463, Gly196, and Ala197, of which the interactions with Gly198, Lys201, Thr200, Arg463, and Gly196 are hydrogen bonds, and adds a hydrogen bond with the residue Asn416. Something similar occurs with DENV-2 NS3Hel, where Thr-A35 shares with ADP the interactions with the amino acid residues Tyr394, Glu230, Lys201, Thr200, Lys199, Arg463, and Asn416, where the great majority are by hydrogen bonds. In contrast, Ser-A23 and Thr-A23

compounds, which also present higher docking scores than ADP, compensate for the lack of hydrogen bonding possibilities through the aldehyde residue with multiple hydrophobic interactions originating from the π clouds of the aromatic rings. Common to Ser-A23 and ADP are interactions with Tyr394, Glu230, Lys201, Thr200, Lys199, Arg463, Asn416, and Arg398.

However, the most remarkable results of the designed compounds are obtained against NS5MTase proteins, where several of the compounds present docking scores better than or comparable to the inhibitors sinefugin and ivermectin B1a (Figure 4). The compounds Ser-A22, Thr-A22, Ser-A23, Thr-A23, Ser-A35 and Thr-A35 present very encouraging results, which coincide in part with those analyzed in the enzyme NS3Hel, as well as the nature of the interactions. Ser-A23 has hardly any interactions in common with the inhibitor sinefugin (Lys180 and Gly148), since they are compounds of very different polarity. Something similar occurs and should occur with Thr-A22, but the flexibility of the aldehyde fragment allows it a greater diversity of conformations in the active site, for which reason it interacts in common with sinefugin with the amino acid residues Phe133, Val132, Thr104, Lys105, Gly148, and Ile147.

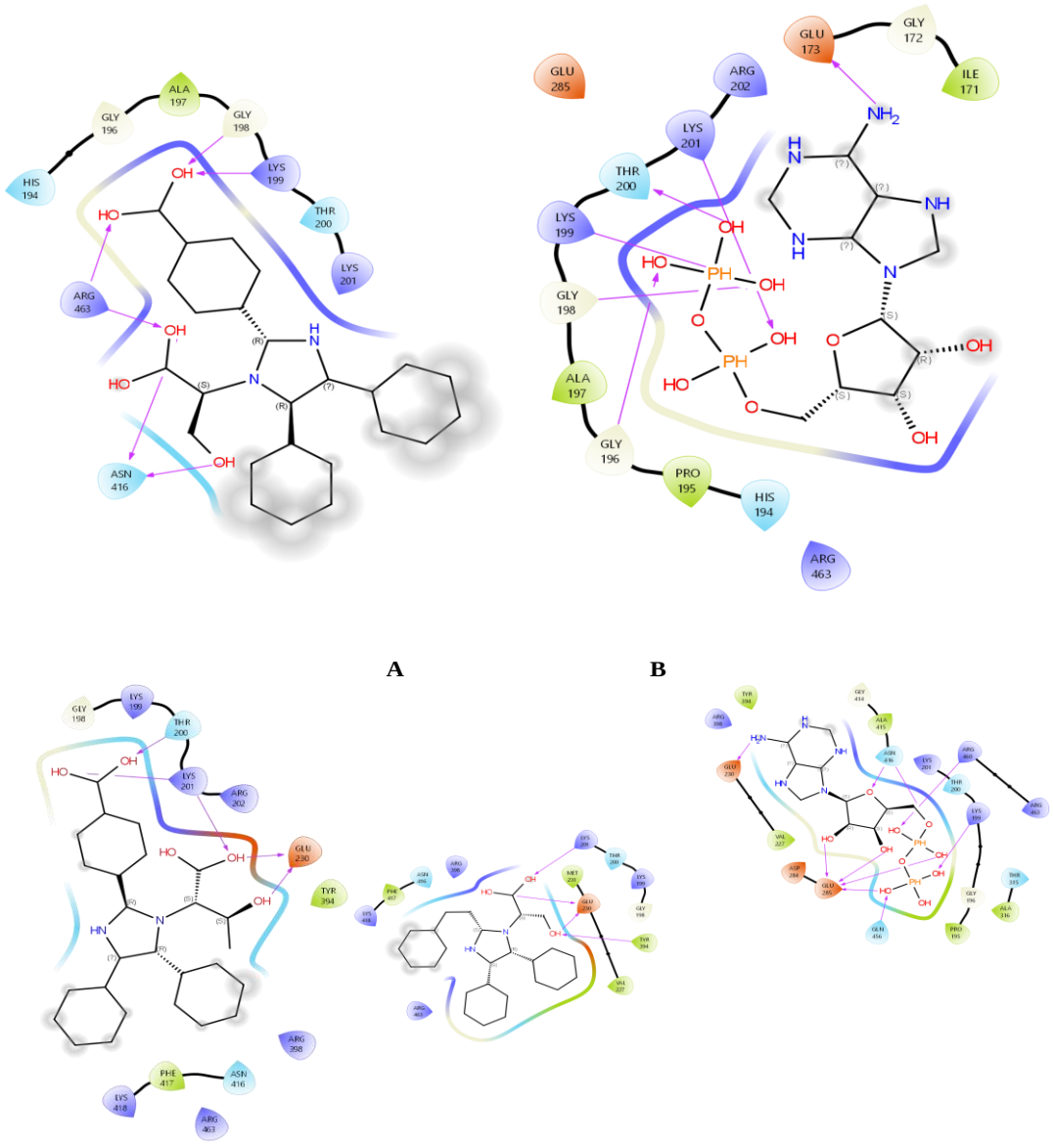


Fig. 3- Ligand interaction diagram of DENV-3 NS3Hel (2HWX) with (A) Ser-A35; and (B) ADP; and DENV-2 NS3Hel (2BHR) with (C) Thr-A35; (D) Ser-A23; and (E) ADP

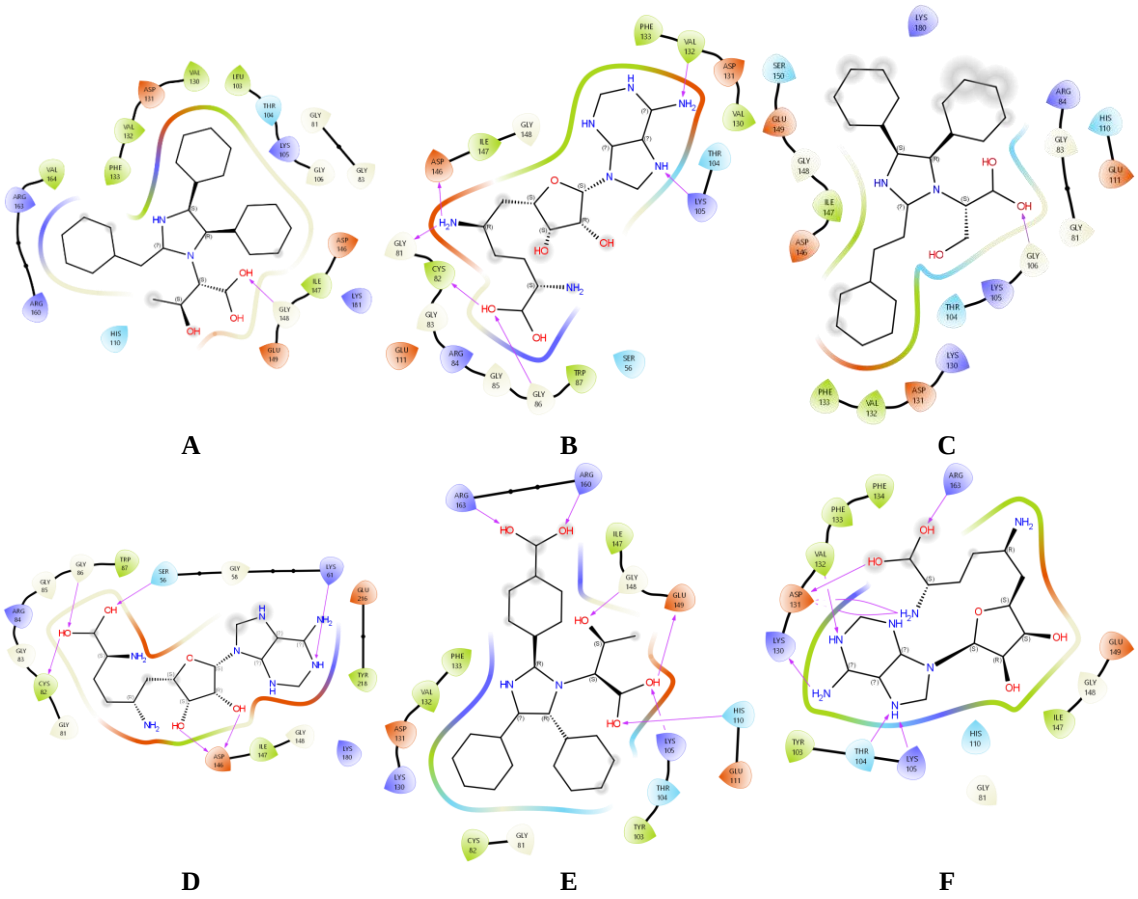


Fig. 4- Ligand interaction diagram of DENV-2 NS5MTasa (1L9K) with: (A) Thr-A22; (B) Sinefugin; DENV-3 NS5MTasa (4CTJ) with: (C) Ser-A23; and (D) Sinefugin; DENV-1 NS5MTasa o (5IKM) with: (E) Thr-A35; and (F) Sinefugin

As expected, the compound Thr-A35 is the one that behaves most similarly to sinefugin. Both share interactions with Glu149, Arg163, Phe133, Thr104, Gly81, Lys105, Val132, Ile147, and Gly148 and present a large number of hydrogen bonds. It is obvious that, although a large number of hydrophobic interactions can be established in the active site of these enzymes, hydrophilic interactions have a great weight in the stability, as well as the flexibility of the compounds. It is valid to point out the great similarity of behavior for the three serotypes and the presence of interactions with the same residues in proteins of different serotypes, which is an example of the greater possibility of developing an antiviral with panserotype effect by selecting this protein as a target.

Since the results for NS5RdRp are not very encouraging and those for NS3Pro are heterogeneous, and given the similarity in behaviour between NS5MTase and NS3Hel, the compounds Ser-A22, Thr-A22, Ser-A23, Thr-A23, Ser-A35 and Thr-A35 can be generally defined as the most promising for development as drug candidates. However, their ADME+T profiles need to be carefully evaluated.

ADME+T profile of promising compounds

Tabla 6 shows the pharmacokinetic, pharmacodynamic and toxicological parameters with the greatest disadvantages for the promising compounds.

As can be seen, there are difficulties in absorption in the body (PAMPA, MDCK) and at the same time, in crossing the blood-brain barrier (BBB), which is not necessary to guarantee the inhibition of DENV and can generate unwanted effects. They can also cause interference in HTS assays by being fluorescent (Blue and Green fluorescence). They are substrates of the CYP2C9 enzyme (CYP2C9-sub), so they must be metabolized by it. Being inhibitors of the bile salt export pump, multidrug resistance protein 1 and organic anion transporting polypeptide 1B1 (BSEP, MRP1, OATP1B3) can cause undesirable drug-drug interactions.

Table 6-. Poor ADME+T parameters for the compounds

ADME+T parameter	Treshold	Ser-A22	Ser-A23	Ser-A35	Thr-A22	Thr-A23	Thr-A35
Aggregators	<0,7	0,455	0,977	0,122	0,562	0,991	0,131
BBB	>0,7	0,525	0,725	0,013	0,32	0,624	0,002
Blue_fluorescence	<0,7	0,161	0,788	0,808	0,141	0,792	0,823
BSEP	<0,7	1	0,999	1	1	0,999	1
CYP2C9-sub	<0,7	0,998	1	1	0,998	1	0,997
DILI	<0,7	0,818	0,628	0,972	0,795	0,725	0,981
Fsp3	>0,3	0,12	0,077	0,08	0,154	0,111	0,115
Genotoxicity	<0,7	0,813		0,911	0,843		0,913
Green_fluorescence	<0,7	0,498	0,872	0,579	0,632	0,977	0,788
GSK	≠ 0	0	1	1	1	1	1
H-HT	<0,7	0,718	0,858	0,869	0,71	0,86	0,853
LM-human	>0,3	0,012	0	0	0,052	0	0
logP	<3	2,754	3,598	2,75	2,576	3,295	2,387
MCE-18	>45	42	42	46	44	44	48
MDCK	>-5,7	-4,514	-4,645	-4,689	-4,374	-4,588	-4,75
MRP1	<0,7	0,954	0,967	0,999	0,982	0,983	0,999
Nephrotoxicity-DI	<0,7	0,906	0,902	0,969	0,879	0,859	0,949
Neurotoxicity-DI	<0,7	0,754	0,813	0,776	0,799	0,859	0,813
NR-AhR	<0,7	0,019	0,847	0,091	0,01	0,769	0,033
NR-PPAR-gamma	<0,7	0,358	0,71	0,767	0,053	0,456	0,168
OATP1B3	<0,7	0,839	0,861	0,898	0,848	0,737	0,963
Ototoxicity	<0,7	0,701	0,825	0,918	0,73	0,846	0,915
PAMPA	<0,7	0,89	0,874	0,996	0,824	0,816	0,895
QED	>0,67	0,483	0,449	0,407	0,457	0,417	0,386
Skin_Sensitization		0	Aldehyde residue	0	0	Aldehyde residue	0
Respiratory	<0,7	0,55	0,277	0,457	0,728	0,527	0,691
SkinSen	<0,7	0,929	0,995	0,448	0,755	0,978	0,225
SR-ARE	<0,7	0,011	0,876	0,011	0,002	0,863	0,004
SR-MMP	<0,7	0,253	0,898	0,27	0,28	0,913	0,155
Toxicophores		Imidazole ring	Imidazole ring	Imidazole ring	Imidazole ring	Imidazole ring	Imidazole ring

The other difficulties regarding the ADME+T parameters are manageable. The Fsp3, MCE-18, GSK, and QED parameters are related to pharmacological or chemical-physical desirability parameters, such as the low number of sp3 carbons in relation to aromatic carbons, or the molar mass greater than 400 Da, which can be an indication in the search for derivative compounds and is not necessarily an indicator of pharmacological inefficiency. Regarding toxicity, these compounds present negative results regarding the parameters: Genotoxicity, Nephrotoxicity-DI, Neurotoxicity-DI, Ototoxicants, Respiratory, SkinSen. However, all of these are long-term damages, which is unlikely in a drug intended for acute viral infections that do not exceed fourteen days.

However, compounds derived from cinnamaldehyde (A22) exhibit a somewhat different ADMET behavior. The two most worrying parameters in these compounds are the ethanol/water partition coefficient (LogP), which is vital for membrane penetration, and colloidal aggregation (Aggregator), which can cause enzyme denaturation and nonspecific inhibition. In addition, these compounds present major toxicological problems, which is why they are not recommended as drug candidates. The cinnamaldehyde residue provides conjugation to the final structure of the amino acid-functionalized imidazole, which worsens the relationship between aromatic carbons and sp3 carbons. It is logical then that the toxicological parameters worsen. Nevertheless, these structures could be taken as a basis for future designs, since their affinities for the NS5MTase and NS3Hel enzymes are not negligible. That is, four compounds (Ser-A23, Ser-A35, Thr-A23 and Thr-A35) combine a favorable behavior in terms of the ADME+T parameters and docking score values to be considered as possible drug candidates through the inhibition of the NS5MTase and NS3Hel enzymes.

Frontier Orbitals, Global Chemical Reactivity Parameters

The compounds Ser-A22, Thr-A22, Ser-A35 and Thr-A35 were studied by DFT. The frontier orbitals and global reactivity parameters were calculated for these compounds. The same procedure was followed for the inhibitors ADP and SAH as a comparison criterion (Table 7).

It is significant that all the global reactivity parameters of the highlighted compounds resemble those of the reference compounds, although these have somewhat higher Gap and Ionization potential values. The HOMO orbitals of the designed compounds are located on the aromatic rings of the aldehyde residues that replace the imidazole in position 2, while the LUMO orbitals are located on the amino acid residues (Figure 5). This is a reflection of the importance of these residues in the interaction mode with the active site of proteins, since these fragments are the ones that provide the greatest number of interactions and practically all the hydrogen bonding, as could be observed in the analysis of the interactions in the ligand-protein complexes.

The reference compounds have a slightly different behavior, since both frontier orbitals are located on the adenine ring and not on the phosphate esters in ADP and homocysteine in SAH, which participate in the HOMO-2 orbital. However, this does not mean that there are no interactions of these fragments with the active site of the protein, but that the designed compounds present greater accessibility to interact with the active site of the protein. An analysis of the MESP (Figure 6) allows us to observe how in the designed compounds the positive charge density is located on the hydrogen of the carboxyl groups, while the negative charge density is centered on the oxygens of the carbonyl groups and, in part, on the electron cloud of the aromatic rings.

Compounds Ser-A35 and Thr-A35 have two carboxyl groups in addition to a hydroxyl group, all three with the potential to form hydrophilic bonds, which is similar to the multiple binding possibilities presented by the reference compounds. Compounds Ser-A22 and Thr-A22 also present these characteristics, although to a lesser degree. However, they compensate for this with another element in common with the reference compounds, which is a high electron density on the aromatic rings (adenine in the case of the reference compounds and benzene rings in the designed compounds). That is, although geometrically the designed compounds hardly have any elements in common with the reference compounds, their electronic surfaces do share common characteristics, which in some way justifies their behavior as possible inhibitors of the viral proteins NS3Hel and NS5MTase.

Table 7-. Frontier Orbitals and Global Chemical Reactivity Parameters of the promising compounds

	Ser-A22	**Thr-A22**	**Ser-A35**	**Thr-A35**	**ADP**	**SAH**
Total energy, TE (eV)	-35407,10	-36477,07	-39468,97	-40538,87	-57117,39	-44855,49
E_{HOMO} (eV)	-5,65	-5,64	-5,81	-5,83	-6,33	-6,39
E_{LUMO} (eV)	-1,31	-1,30	-2,10	-2,11	-1,47	-1,36
Gap, ΔE (eV)	4,34	4,34	3,71	3,71	4,87	5,02
Dipole moment, μ (Debye)	4,70	4,90	3,47	5,12	4,79	3,50
Ionization potential, I (eV)	5,65	5,64	5,81	5,83	6,33	6,39
Electron affinity, A	1,31	1,30	2,10	2,11	1,47	1,36
Electronegativity, χ	3,48	3,47	3,95	3,97	3,90	3,88
Chemical Potential, μ	-3,48	-3,47	-3,95	-3,97	-3,90	-3,88
Hardness, η	2,17	2,17	1,85	1,86	2,43	2,51
Electrophilicity index, ω	2,79	2,77	4,21	4,25	3,12	2,99
Softness, σ	0,46	0,46	0,54	0,54	0,41	0,40
Nucleophilicity index, N	0,36	0,36	0,24	0,24	0,32	0,33
Maximum charge transfer, ΔN_{max}	1,60	1,60	2,13	2,14	1,60	1,54
Global softness, S	0,23	0,23	0,27	0,27	0,21	0,20

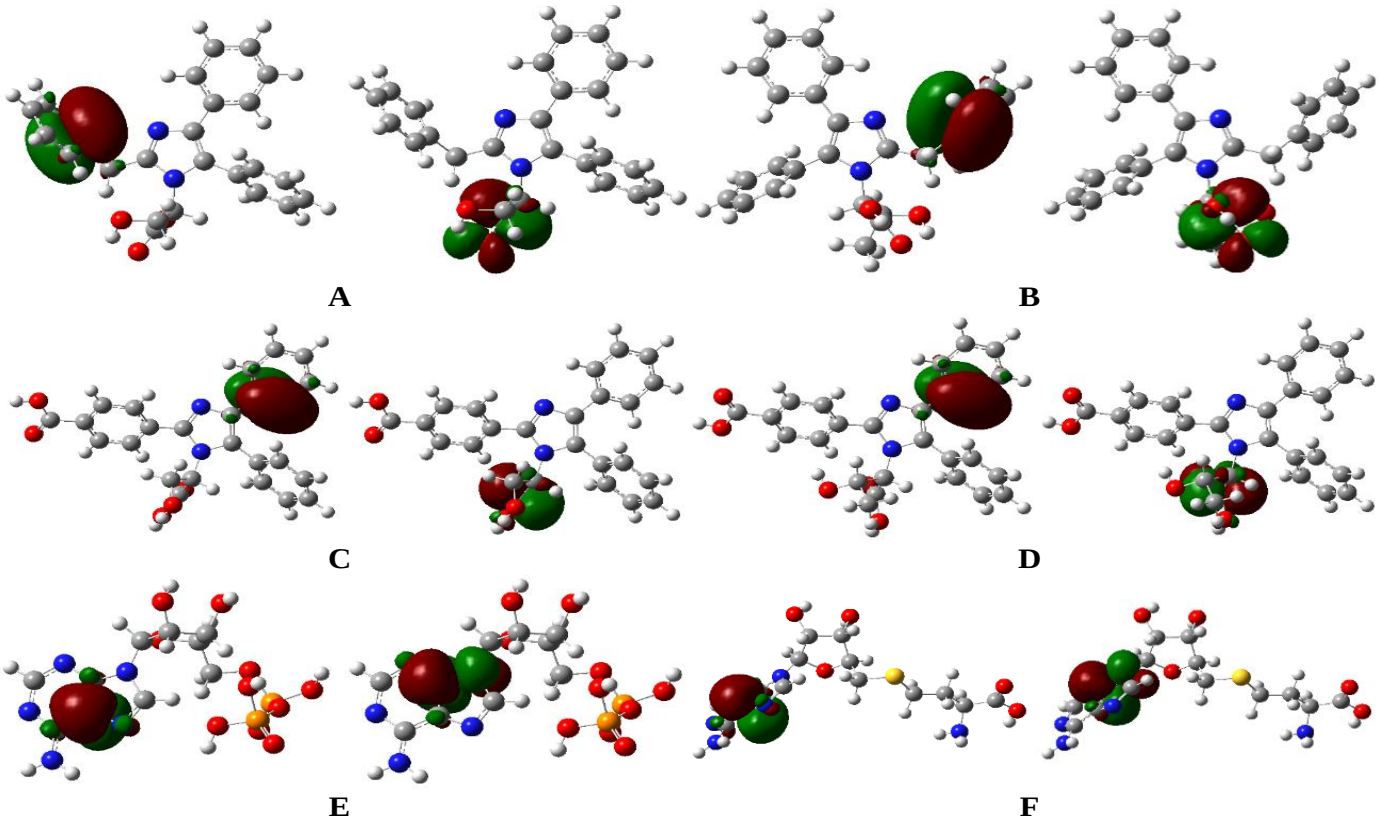


Fig. 5 - Frontier orbitals (HOMO and LUMO) of promising compounds: (A) Ser-A22; (B) Thr-A22; (C) Ser-A35; (D) Thr-A35; (E) ADP; and (F) SAH

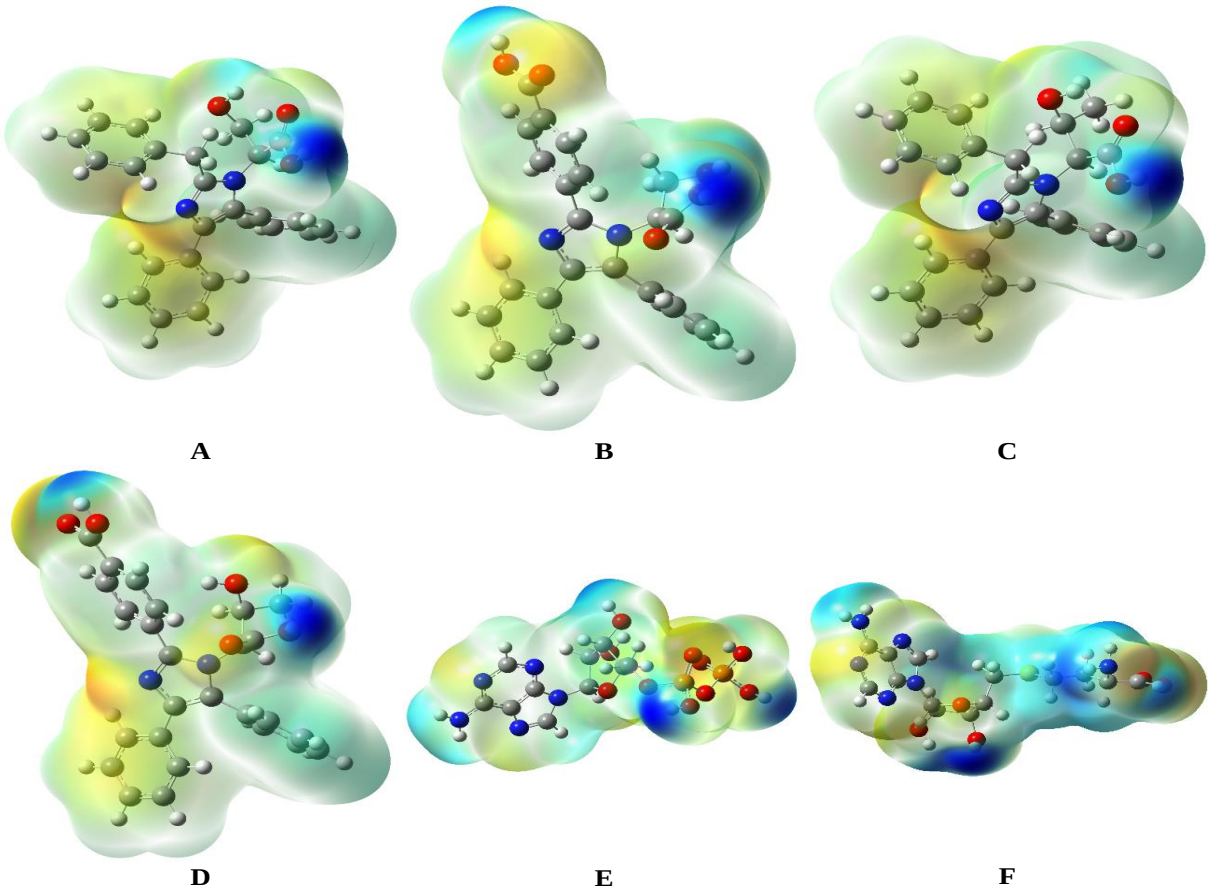


Fig. 6 - MESP. (A) Ser-A22; (B) Thr-A22; (C) Ser-A35; (D) Thr-A35; (E) ADP; and (F) SAH. $-7{,}5\cdot10^{-2}$ rojo, $7{,}5\cdot10^{-2}$ azul

CONCLUSIONS

Compounds designed with Ser and Thr amino acids present an adequate ADME+T profile. Viral proteins NS3 and NS5 of DENV serotypes 1, 2 and 3 were studied by molecular docking. The complexes formed by NS3Hel and NS5MTase with these compounds show docking score values in the same order, and even in higher orders than reference inhibitors and natural substrates. Four compounds (Ser-A22, Thr-A22, Ser-A35 and Thr-A35) stand out as the most promising in terms of their ADME+T profiles and docking score values, which show them as direct drug candidates. Regarding Ser-A23 and Thr-A23, with adverse ADME+T profiles, they could be taken as a basis for the design of new compounds seeking to maintain the affinity for viral proteins and improve their pharmacological profile. Compounds Ser-A22, Thr-A22, Ser-A35 and Thr-A35 show reactivity parameters similar to those of the SAH and ADP inhibitors. Their frontier orbitals are located on aldehyde and amino acid residues, which explains their low binding energies with viral proteins. A study of the MESP of these compounds shows electronic similarities with the reference inhibitors, which explains their similar behavior in the formation of ligand-protein complexes.

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INTEREST CONFLICT

The authors express that there are no conflicts of interest in the submitted manuscript.

AUTHOR'S CONTRIBUTION

Yonatan Mederos Nuñez: conceptualization, methodology, formal analysis, investigation, writing-original draft, and visualization.
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