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Hepatitis C Viral Polymerase Inhibition Using Directly Acting Antivirals: A Computational Approach

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Abbreviations: NS5B-Non-Structural 5B protein; RdRp-RNA dependent RNA polymerase; SVR-Sustained Viral Response; DAA -Direct Acting Antivirals; PEG-Poly Ethylene Glycol; HCV-Hepatitis C Virus; NI-Nucleotide Inhibitor; NNI-Non-Nucleotide Inhibitor; HOMO-Highest Occupied Molecular Orbital; LUMO-Lowest Unoccupied Molecular Orbital; PHYRE-Protein Homology/anologY Recognition Engine; SCOP-Structural Classification of Proteins; SAVES-Structure Analysis and Verification Server; QSAR-Quantitative Structure-Activity Relationship.

INTRODUCTION

Hepatitis C Virus (HCV)

Hepatitis C virus, as it appears from its name, is a liver- affecting virus. HCV is a blood prone virus that was first discovered in 1989 by Choo and coworkers. The virus was termed Non-A Non-B hepatitis. HCV can develop liver cirrhosis and reduce the functionality of liver in patients. Developed hepatocellular carcinoma has been recorded in some HCV patients after long periods of

chronic hepatitis viral infection. Today more than 200 million people worldwide are infected with chronic liver diseases that lead to liver cirrhosis and development of hepatocellular carcinoma [1-4]. The worldwide HCV prevalence is around 3% of the population. Egypt has the highest ratio of chronic liver disease prevalence that affects about 14% of the population most of which (90%) belongs to the genotype 4a [2, 5-9].

HCV is a small virus from the Flaviviridae family. It consists of RNA as the genetic material enveloped by protein capsid [6]. HCV genome is approximately 9600 base pairs single stranded RNA that encodes a polyprotein consisting of about 3000 amino acid residues. The polyprotein then cleaved by both viral and host cell proteases to 10 proteins (Figure 1) some of which are part of the structure of the virus [core, E1, E2 and p7], which are called structural proteins. Others have specific functions in viral replication [NS2, NS3, NS4A, NS4B, NS5A and NS5B]. These are termed Non-Structural **(NS)** proteins [2,3,6,10].

HCV genome is characterized by high mutation rate. About seven six main genotypes are present to date (1, 2, 3, 4, 5 and 6). The nucleotide sequence differs by 31% to 34% among the different genotypes. Genotypes are further classified into subtypes; more than 100 subtypes are present to date (1a, 1b, 2a, 2b etc...). The sequence similarity among different HCV subtypes in each individual genotype is about 90%. HCV circulates in infected patient's blood in the form of a number of different but closely related variants called quasi-species [11].

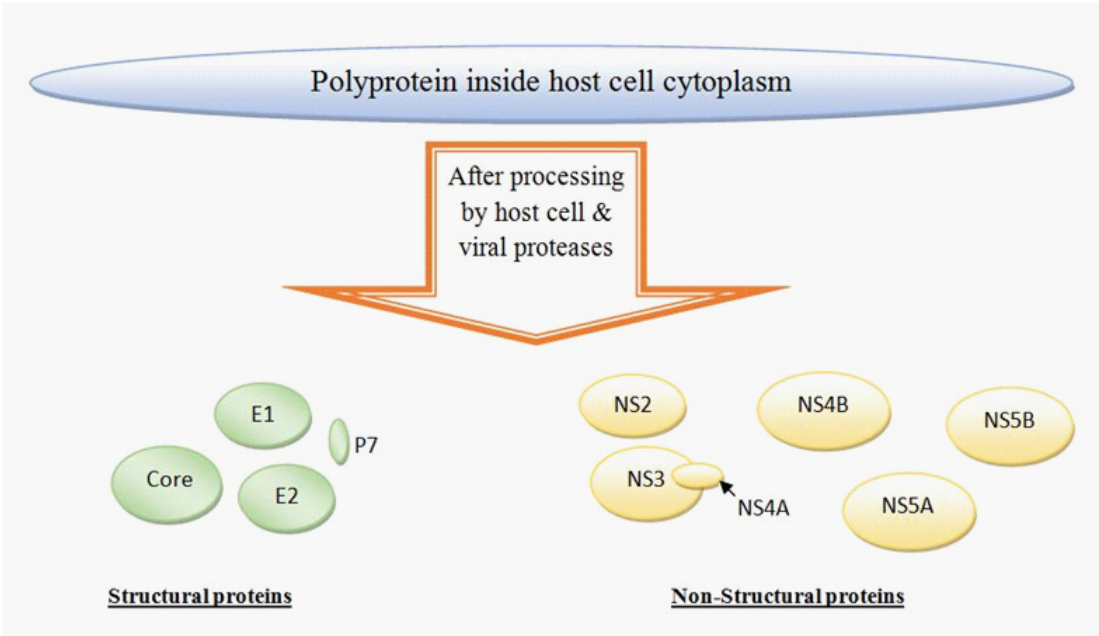


Figure 1: HCV polyprotein before and after cleavage by viral and host cell proteases.

HCV RNA-Dependent RNA Polymerase (RdRp)

HCV RNA dependent RNA polymerase **(RdRp)** is a part of the NS5B protein. It plays an important role in viral replication cycle. NS5B represents an excellent target for selective HCV inhibitors because its biochemical activity is limited to the RNA viruses with no effect on mammalian cells [12].

NS5B (Figure 2) is 68 KDa tail-anchored protein with an alpha helical trans-membrane domain consisting of 21C-terminal amino acids [13]. The domain architecture of NS5B RdRp is the same as other polymerases consisting of thumb, fingers and palm domains resembling the right hand [14,15]. The palm domain contains two consecutive metal binding aspartates that form the active site motif GDD (G88, D89 and D90) and carry out the nucleotidyl transfer reaction. Fingers and thumb domains regulate nucleic acid binding. Beside the active site, several other pockets that act as allosteric binding sites [12,15,16].

Due to the high mutation rate characterizing HCV genome, the production of efficient DAAs that inhibit NS5B RdRp protein remains challenging. To resolve this problem and improve the viral response a combination therapy was suggested by many authors with different drugs having different binding modes of action [4, 17].

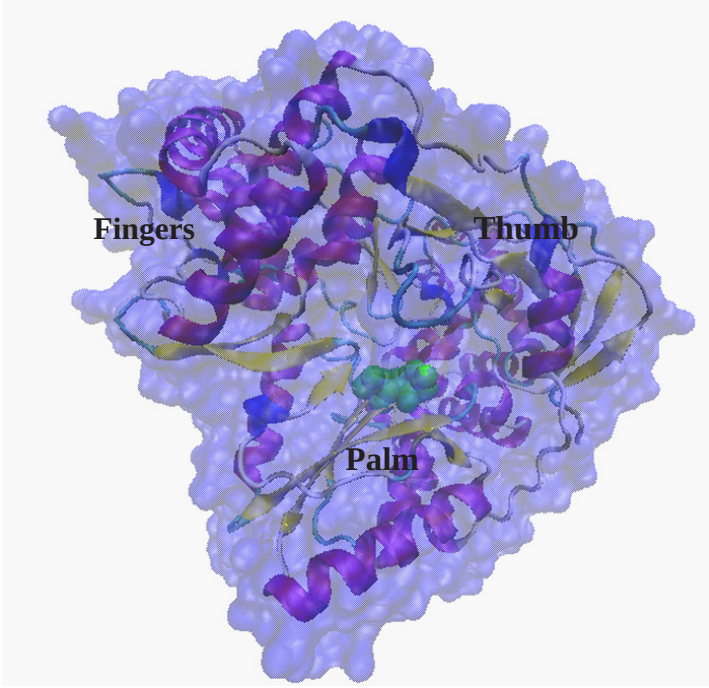


Figure 2: Structure of the protein NS5B polymerase from PDB file 4AEP downloaded from the Protein Data Bank. The active site motif GDD of the RdRp part is represented by green Van Der Waal **(VDW)** spheres. The figure was generated using Visulaising Molecular Dynamics **(VMD)** software.

HCV Treatment

Rational drug therapy

The rational drug therapy for HCV until five years ago was only the double therapy. It is a combination of Pegylated PEG related interferon alpha and the wide range antiviral Ribavirin **(PEG-IFN/RBV)**. This regimen gave varying Sustained Virologic Response **(SVR)** rates that depend on the viral genotype. SVR is up to 80% in genotypes 2 and 3, 60-70% for genotype 4 but only 40-50% in genotype 1 [9,18]. Unfortunately, the combination therapy is expensive and not tolerated by some patients. Interferon develops diversities of side effects that may lead to stopping the medication in some cases [6,10,19].

Due to the above-mentioned reasons, researchers started to direct their attention to interferon-free regimens. They worked on drugs that directly act on specific proteins that are important in viral replication. These types of drugs are called Direct Acting Antivirals **(DAAs)**.

Direct Acting Antivirals (DAAs)

The use of Direct Acting Antiviral **(DAA)** drugs that act on specific viral and/or host cell proteins gives good results in many cases. In the year, 2011, the FDA approved two drugs for the treatment of HCV genotype 1 in combination with interferon alpha and Ribavirin. The two approved drugs (Telaprevir and Boceprevir) are DAAs that target NS3 serine protease domain of the NS3 protein of HCV [20]. Sofosbuvir is a nucleotide NS5B polymerase inhibitor that was approved by FDA in December 2013 as a free drug or in combination with interferon against genotype 1.

For almost all therapies that were developed to act on the viral proteins, drug resistance occurred due to the high mutation rate induced by the nature of HCV (a single stranded RNA virus). One can mix a cocktail of DAAs to overcome resistance, putting into consideration the toxicity of the mixed drugs [21].

There are two types of DAAs against HCV NS5B polymerase. The first type is called Nucleotide Inhibitors **(NIs)** in which the nucleotide-like analogue is introduced into HCV NS5B polymerase active site to stop the polymerization process. NIs are classified into two subtypes; sugar modified nucleotide analogues and nitrogenous base modified analogous. Some drugs that act on NS5B polymerase are not related to the structure of nucleotides. These drugs called Non-Nucleotide Inhibitors **(NNIs)**. This is the second type of DAAs against HCV NS5B polymerase.

NIs are successful candidates in the treatment of HIV and herpes viruses. NIs compete with the nucleotides (natural substrate: Adenine, Guanine, Cytosine and Uracil) on HCV polymerase active site. They are, generally, prodrugs that are activated by phosphorylation inside the host cell. Once an NI becomes attached to the polymerase active site it stops the polymerization process, hence they are termed chain terminator inhibitors. They can also interfere with the cellular proliferative machinery [12, 22]. IDX-184, R7128 and Sofosbuvir (Figure 3) are examples of NIs against HCV.

These drugs are now either under clinical trials phases II (IDX-184 and R7128) or already approved (Sofosbuvir), [4,12,21,23-25]. These DAAs give good results in terms of increasing the SVR rate when administered in combination with rational regimen of double therapy [10, 26].

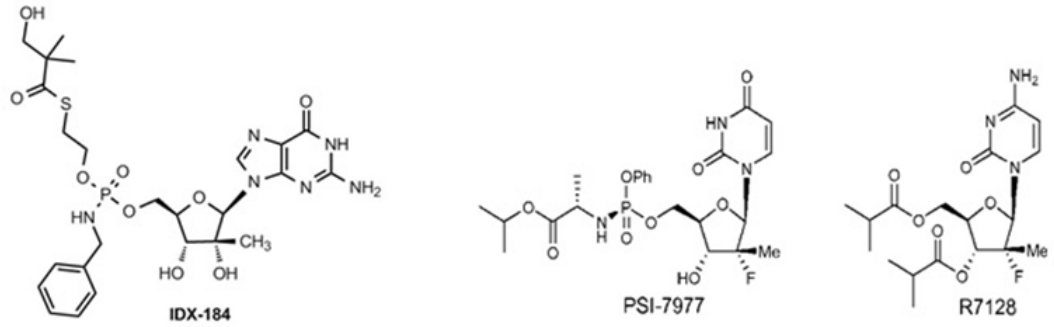


Figure 3: The structures of some nucleotide inhibitors, Sofosbuvir (PSI-7977), IDX-184 and R7128.

Molecular Modeling

Molecular modeling can be simply considered as a range of computerized techniques. These are based on the basic laws of physics and experimental data which can be used either to analyze molecules (number and types of atoms, bond, bond lengths, angles and dihedral angles) or molecular systems (nucleophilicity, electrophilicity and electrostatic potentials). Moreover,

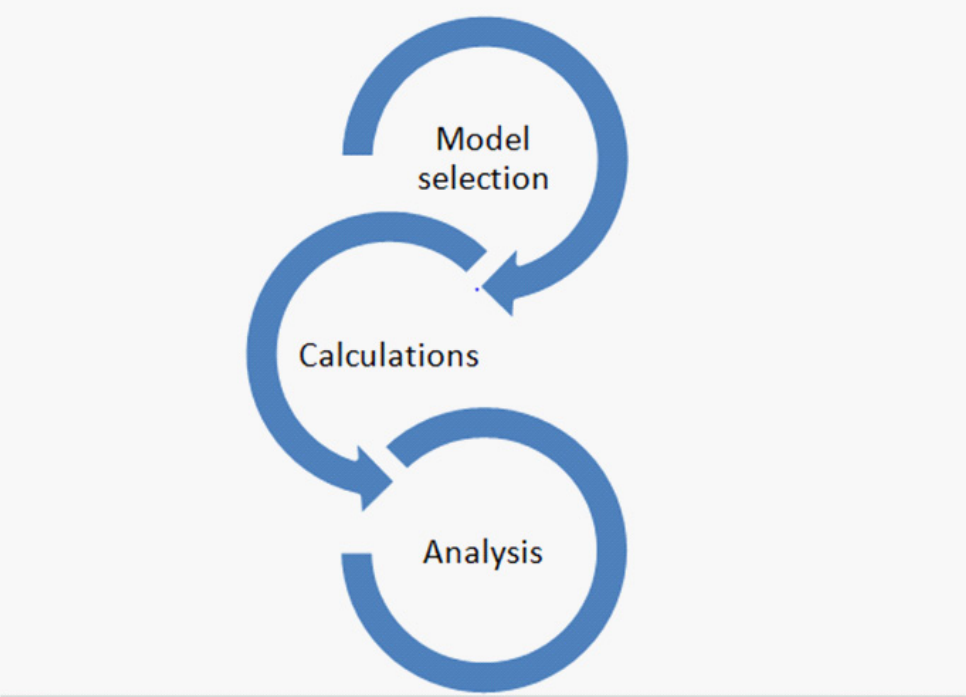
it can predict molecular and biological properties which are useful in the understanding of structure-activity relationship and in rational drug design [27].

As concluded in the scheme below, molecular modeling consists of three stages: The model selection, with which the calculations will be carried out. This step is governed by the complexity of the system and the computational time requirement. One may make calculations using Molecular Mechanics **(MM)**, Quantum Mechanics **(QM)** or hybrid MM/QM. If working on small molecules or peptides, one may use QM or semi-empirical QM [28]. If working on large molecules (like proteins or DNA), one should use an MM model. If working on the active site of large molecules, one may use the hybrid MM/QM model.

The selection of the calculation type is the second stage. Different calculations are possible depending on the goal of the experiment. Among the very large number of available calculations are; geometry optimization, vibrational spectra calculation, NMR spectra calculation and single point energy calculations.

The final and most important stage is the analysis of results. This stage will provide answers about the problem that might not be solvable by experimental work. One should perform extensive

analysis of the results in order to reach valuable conclusions about the investigated system. To get better results one should select the most appropriate model for calculations. This depends on the complexity of system and the available computational power [29].



Molecular Modeling scheme

The discovery of new drugs is the main target for pharmacologists and medicinal chemists. Drugs are chemical substances that can be used both for treatment and for diagnosis of a disease. In addition; it can prevent the development of disease in humans, animals and plants. The function of drugs is to inhibit or to enhance certain physiological functions. The biological and pharmacological effects of drugs are either helpful or harmful for living organisms. Drugs interact with specific targets in living organisms such as enzymes, receptors, nucleic acids, channels or other biological macromolecules [30]. The discovery of new pharmacological compounds requires the design and synthesis of drug, studying its physicochemical and biophysical properties in addition to its pharmaceutical functions. These studies improve drug safety and biological activity while reducing adverse side effects. The development of drugs has several strategies. These strategies involve either a change in the shape of the drug in order to fit into its active site receptor or a change in its pharmaceutical properties, which include Absorption, Distribution, Metabolism and Excretion **(ADME)**. These strategies require the synthesis of large number of compounds and substitutions that consume time and money.

Quantitative Structure-Activity Relationship **(QSAR)** is a technique that quantifies the relationship between a physicochemical property of a drug and its biological activity. QSAR is useful

for optimizing the groups that modulate the potency of a drug. It is based on the determination of mathematical equations that express the biological activities in terms of molecular descriptors such as the logarithm of partition coefficient (log P), steric constituent constant (Es) and molar refractivity. QSAR also may make use of structural indexes obtained by quantum mechanics such as Highest Occupied Molecular Orbital **(HOMO)** energies, Lowest Unoccupied Molecular Orbital **(LUMO)** energies, total dipole moments, charge, molecular polarizability, electronegativity and frontier orbital energies [25,27,30-33].

QSAR descriptors are not universal and depend on the nature of chemical structures or process involved. Once a correlation between structure and activity is found for a compound or group of compounds, the computer can be used to make screening in order to select structures with the desired properties. It is possible to select the most promising compounds to synthesize and test in the laboratory. A combination of QSAR and molecular modeling approach is the key for success in Computer Aided Drug Design **(CADD)** and to understand drug-receptor interactions [33].

CASE STUDY DESCRIPTION

In the following section, the use of molecular modeling combined with QSAR to study the binding of different drugs (Nucleotide Inhibitors) to NS5b RdRp of HCV from different genotypes will extensively illustrated. Moreover, a comparison between the binding energies of these drugs and native nucleotides using the same technique is also presented.

Steps of Computer Aided Drug Design (CADD)

Protein sequence analysis

Sequence comparison and analysis is important in rational drug design. As shown in previous work [25,33] the amino acid sequence around the active site moiety GDD and the surrounding environment is conserved (5 Å region around the GDD motif). Since the structure of the active site of polymerase is conserved among different HCV genotypes, it would be possible to target the active site in different genotypes with the same inhibitor. However, some studies on NS5B RdRp with NIs show different results for different genotypes. This may be due to the effect of mutations on the cavity at the active site. These mutations don't occur in the active site environment but aside from it and probably lead to drug disability to inhibit the protein or at least lead to decreased inhibition [4,10,34].

There are different methods that can be used for sequence comparison. For example, using Visualizing Molecular Dynamics **(VMD)** software, sequence alignments may be carried out for the sequences with the help of Clustal W program in multiseq extension or using the web based service of CLUSTALW 2 [25, 33].

Homology modeling

Homology modeling, also called comparative protein modeling or knowledge-based modeling, is the process by which a 3-dimentional model of a target sequence being built based

on a homologue of experimentally solved structure (experimental processes include X-ray crystallography, solution Nuclear Magnetic Resonance **[NMR]** and Electron Microscopy **[EM]**). Homology modeling relies on the identification of one or more known protein structures likely to resemble the structure of the query sequence and on the production of an alignment that maps residues in the query sequence to residues in the template sequence [35]. The sequence alignment and template structure are then used to produce a structural model of the target [36].

A target (or query) sequence is the primary sequence of a protein whose structure has to be modeled. When first loaded in the workspace, it is provisionally drawn as a long helix. A template structure, or simply a template, is an experimentally solved structure used as a scaffold to model the structure of the target sequence. Template sequence is the primary sequence of a template. The quality of the homology model is dependent on the quality of the sequence alignment and template structure. The approach can be complicated by the presence of alignment gaps that indicate a structural region present in the target but not in the template, and by structure gaps in the template that arise from poor resolution in the experimental procedure (usually X-ray crystallography) used to solve the structure.

After modeling, one should check the models for errors using different mechanisms including 3D structure related properties (such as bond angles, length, Ramachandran plots). This process is called "Protein Model Validation". On the web, there are several servers built for helping researchers to check their structures for errors. Structural Analysis and Verification Server **(SAVES)** is one of them, in which the protein three-dimensional structure **(PDB file)** is uploaded and checked by built-in programs which check the PDB file for errors where each program produces its own result. Based on the given results one can judge the validity of his own protein model. For example, SAVES server contains the program PROCHECK which check the stereo-chemical parameters such as Ramachandran plots, main and side chain parameters, residue properties, G-factor dihedrals, main chain bond angle, and bond length[37]. The programVERIFY-3D checks the residual environment [38]. ERRAT program generates overall quality factor of the protein model [39]. PROVE program checks atomic volumes and calculates the atomic Z-score[40]. The program WHATCHECK gives a report for almost all parameters of the uploaded protein structure PDB file [41].

Drug activation

Some drugs are converted to its active form inside target cells. In this study, phosphorylation of Sofosbuvir, IDX-184, R7128 and Ribavirin was performed in silico to become in its active tri-phosphate form [25, 33]. After in silico phosphorylation, the activated Nucleotide Inhibitors **(NIs)** are energy minimized using mechanical chemistry calculation method **(MM3)** followed by the semi-empirical quantum mechanics calculation method **(PM3)**. The use of a low level method (classical mechanical method **(MM3)**) for an initial energy minimization reduces the time of calculation needed by the higher level method **(PM3)** for energy minimization. Infrared

vibrational spectrum is then calculated at PM3 level in order to ensure that an active form of the drug is real (no negative vibrations). After optimization, infrared vibrational spectrum calculation is performed in order to ensure the structures being real.

QSAR descriptors calculation

In some previous studies, Quantitative Structure Activity Relationship **(QSAR)** descriptors are calculated for selected DAA drugs (Sofosbuvir, IDX-184 and R7128) and Ribavirin in addition to their parent nucleotide tri-phosphates (Guanine, Cytosine and Uracil) for comparison [25, 33]. QSAR calculations are carried out at PM3 level using computational chemistry integrated platform software SCIGRESS [42]. The calculated descriptors are: dipole moment, the logarithm of partition coefficient (Log P), electron affinity, molar refractivity, ionization potential, solvent accessible surface area, volume, total energy, heat of formation, Highest Occupied Molecular Orbitals **(HOMO)**, Lowest Unoccupied Molecular Orbitals **(LUMO)** and frontier energy gap (ΔE = LUMO - HOMO).

Drug-protein interaction

The final and very important step in rational drug design is the study of drug-protein interaction. The drug in its active form may form covalent or non-covalent bonds with the active site amino acid or the amino acid around the active site cavity. Some hydrophobic or Van der Waal's Walls interactions may stabilize the drug in the protein cavity. The interaction potency between the drug and the protein is the factor that one can depend on when comparing different drugs against specific protein.

One example of drug/protein interaction is the interaction between IDX-184 and NS5B protein model (Figure 4). A hydrogen bond is formed between the drug in its active form (Tri-phosphate) and the amino acid S59 of the polymerase active site environment. In addition, H-bonds are formed between active site environment's amino acids. Another force of interaction arises from weak interactions formed between the two Mg^{+2} ions and both of the triphosphates' oxygen and the two aspartic acids (active site amino acids) oxygen atoms.

Figure 4: The interaction between IDX-184 and the active site of NS5b RdRp D89 and D90 showing the formation of H-bond between the drug and S59 in addition to the coordination bonds between the two Mg+2 and the oxygen atoms of both D89, D90 of the protein and Phosphate group in the drug.

CONCLUSION

Molecular modeling represents a promising technique that had a very high momentum of development in the last decade. This progress was in fact, related to the rapid improvement in the hardware of new computers in the market. In addition, software improvement provided a second source making molecular modeling the best choice in different areas of research.

In presented case study, Computer Aided Drug Design CADD was utilized in order to provide an insight about the binding of Sofosbuvir, IDX-184, R7128 and Ribavirin to HCV NS5b active site. The results showed diversity among different drugs and different genotypes. These findings emerged from both QSAR calculated parameters and interaction energies calculated for the binding of the drugs to the active site of the polymerase. These results were in agreement with the experimental data obtained from patients infected by different genotypes of HCV, where different responses to the same drug were recorded.

CADD results implied that IDX-184 represented a promising drug against all studied genotypes. Also, all of the studied drugs (Sofosbuvir, IDX-184 and R7128) were able to interact more effectively with viral polymerase than ribavirin of the dual therapy. Hence, these drugs were better than ribavirin in competing the nucleotides for binding to HCV polymerase.

GLOSSARY

Active site environment: Describes a 5$\AA$ region around the active site motif GDD. This active site environment complex includes 12 amino acids (including GDD motif), two Mg^{+2} ions and one of the Nucleotides, NIs or ribavirin.

Frontier energy gap: The difference in energy between HOMO and LUMO. it is high in more stable structures.

Heat of formation: The change in enthalpy accompanying the formation of one mole of a compound from its elements in their natural and stable states, under standard condition of one atmosphere at a given temperature.

Ionization potential: It is the energy required to ionize an atom. High values of the ionization potential means a more stable structure.

Sustained Virologic Response: It is defined as aviremia (Lack of virus in the blood plasma) 24 weeks after completion of antiviral therapy for chronic hepatitis C virus infection.

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