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Design, Synthesis, Assessment, and Molecular Docking of Novel Pyrrolopyrimidine (7-deazapurine) Derivatives as Non-Nucleoside Hepatitis C Virus NS5B Polymerase Inhibitors

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Abstract

Hepatitis C virus (HCV) infection is highly persistent and presents an unmet medical need requiring more effective treatment options. This has spurred intensive efforts to discover novel anti-HCV agents. The RNA-dependent RNA polymerase (RdRp), NS5B of HCV, constitutes a selective target for drug discovery due to its absence in human cells; also, it is the centerpiece for viral replication. Here, we synthesized novel pyrrole, pyrrolo[2,3-*d*]pyrimidine and pyrrolo[3,2-*e*][1,2,4]triazolo[4,3-*c*]pyrimidine derivatives. The non-toxic doses of these compounds on Huh 7.5 cell line were determined and their antiviral activity against HCVcc genotype 4a was examined. Compounds **7j**, **7f**, **5c**, **12i** and **12f** showed significant anti HCV activity. The percent of reduction for the non-toxic doses of **7j**, **7f**, **5c**, **12i** and **12f** were 90 %, 76.7±5.8%, 73.3±5.8%, 70% and 63.3±5.8% respectively. The activity of these compounds was interpreted by molecular docking against HCV NS5B polymerase enzyme.

1. Introduction

Hepatitis C virus (HCV) is one of the major global health problems. HCV infection is responsible for 350,000 death cases annually [1]. HCV infection is particularly a national problem in Egypt. It is one of the top five death-causing diseases in the country. The incidence rate reaches 14.5 % among Egyptian population, which represents the highest prevalence of hepatitis C worldwide [2]. According to the Egyptian ministry of health, 1,000,000 new cases are identified each year [3]. Furthermore, some independent studies have reported that 500,000 new patients are added to HCV victims in Egypt each year, among them 70,000 are children [4].

There are six major genotypes of HCV and they differ in their geographical distribution. Genotype 1 is the most common HCV genotype around the world: the subtype 1a and 1b are prevalent in USA and Europe respectively, whereas, genotype 4 is the dominant HCV variant in the Egyptian population [5].

The standard of care (SOC) for chronic HCV infection is using the combination of pegylated interferon-α and ribavirin. This therapy is poorly tolerated, and there is variation in response among HCV genotypes. The sustained virological response (SVR) obtained by the combination therapy was markedly higher in patients infected with genotype 2 or genotype 3 than those infected with genotype 1 or genotype 4. Several studies have reported that interferon therapy is partially ineffective against genotype 4 which represent an obstacle in the treatment of Egyptian patients [6,7]. There are tremendous efforts to achieve interferon-free HCV-therapy through using direct-acting antivirals (DAAs) [8].

Replication of the HCV viral RNA is controlled by host factors as well as viral factors, and both can serve as drug targets. Possible direct targets in HCV are the viral NS3/4A protease, NS4B, NS5A, and NS5B polymerase [8]. Recently, FDA has approved different DAAs Anti-HCV: Telaprevir and Boceprevir, which are NS3/4A protease inhibitors, Daclatasvir and Ledispasvir as

NS5A inhibitors and Sofosbuvir, which is a nucleotide analog polymerase inhibitor. A new regime for HCV has been developed recently depending on the NS5B inhibitor "Sofosbuivir" in combination either with Ribavirin or Ribavirin and Interferon [1]. Other Interferon- and Ribavirin free-combinations are in clinical trials [9,10].

The HCV polymerase (NS5B) is an essential enzyme in the life cycle of the hepatitis C virus. The main role of NS5B is to assemble the replicase complex at the endoplasmic reticulum membrane and amplify the genetic material through RNA-dependent RNA polymerase (RdRp) activity. Due to its central role in the virus replication, and its absence in human body it became a preferable selective target during the design of anti-HCV drugs [11,12]. The NS5B RdRp shares the structural characteristics of nucleic acid polymerase. NS5B RdRp is composed of three domains: thumb, finger and palm. The catalytic site is located at the interface of the thumb and finger domains (**Figure 1**), which represents an interesting target for novel anti-HCV drugs [13].

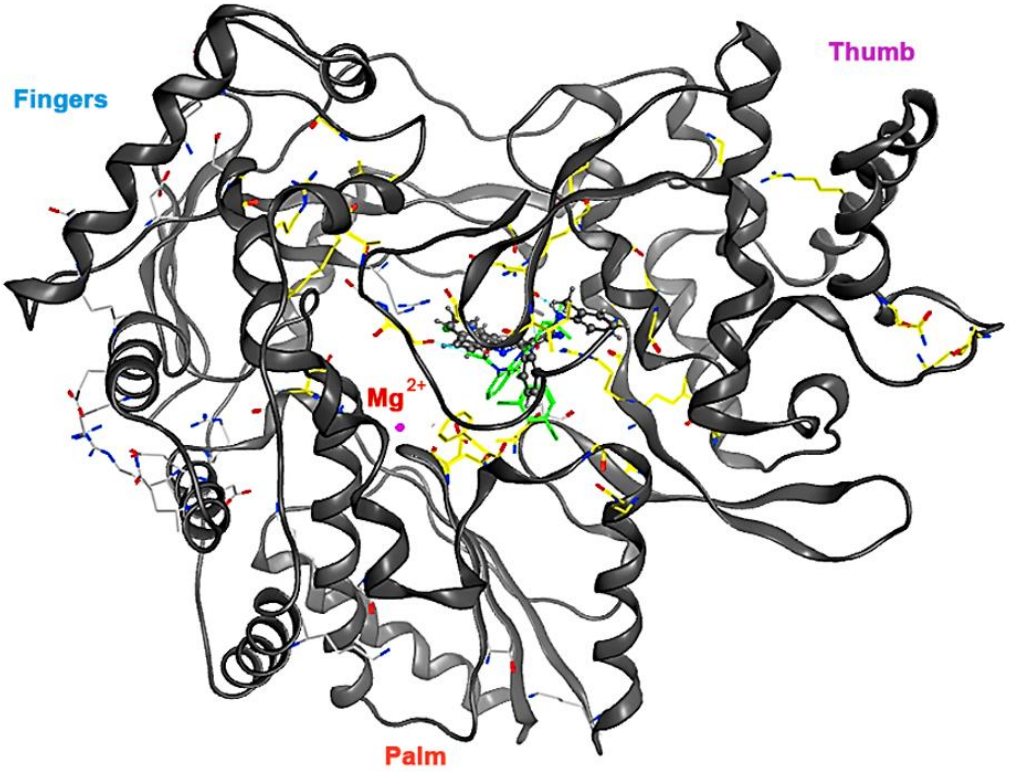
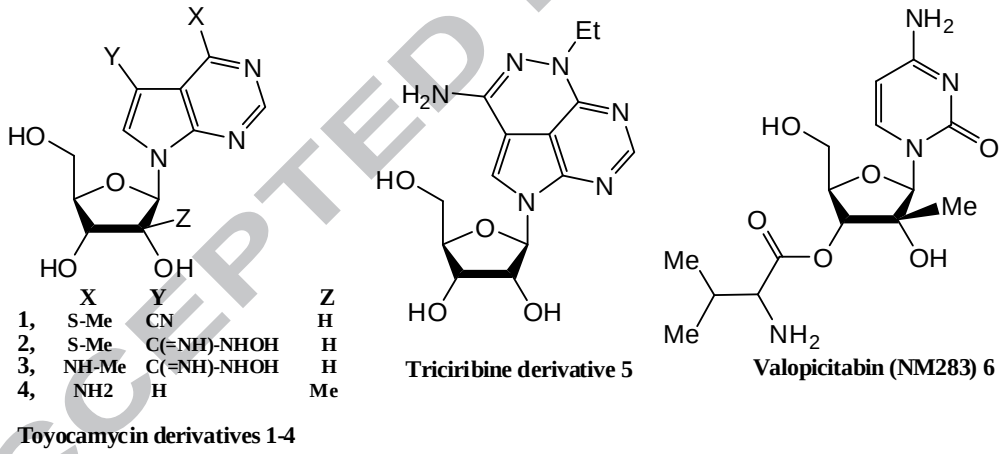


Figure 1: 3D structure of RdRp of HCV

The HCV NS5B polymerase inhibitors are classified as nucleoside inhibitors, e.g. toyocamycin, triciribine derivatives and Valopicitabine (**Figure 2a**) [14-23], and non-nucleoside inhibitors (NNIs), e.g. Deleobuvir, Tegobuvir, Filibuvir and sterobuvir (**Figure 2b**). Purine nucleoside analogs as pyrrolopyrimidine nucleosides are good examples of HCV NS5B polymerase inhibitors [16-23]. To date there is no pyrrolopyrimidine non-nucleosides that are acting as HCV NS5B polymerase inhibitors. The main aim of this work was to synthesize pyrrolopyrimidine derivatives as non-nucleoside purine analogs and evaluate their antiviral activity against HCV genotype 4. Finally, we will predict the binding mode of the synthesized compounds by using molecular docking against HCV NS5B polymerase.



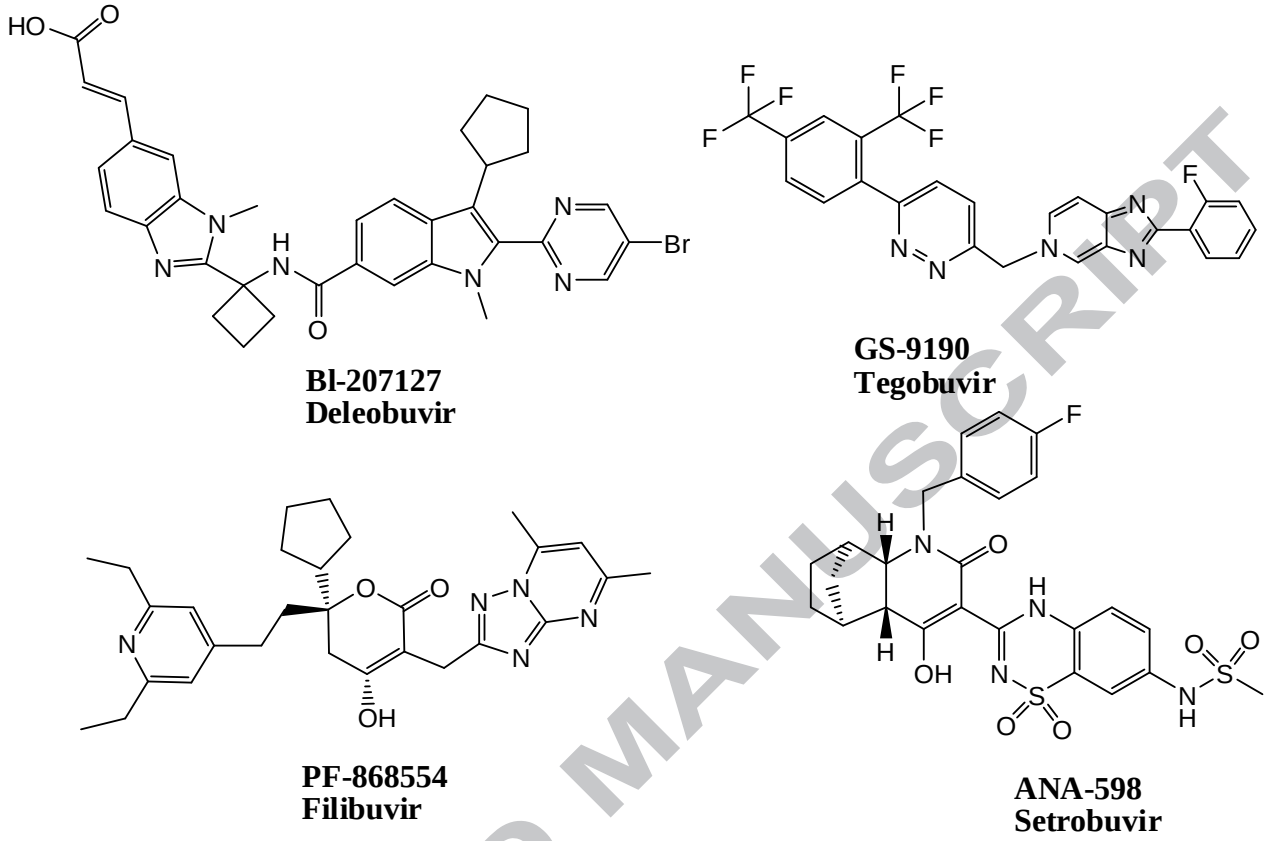
	X	Y	Z
1,	S-Me	CN	H
2,	S-Me	C(=NH)-NHOH	H
3,	NH-Me	C(=NH)-NHOH	H
4,	NH2	H	Me

Toyocamycin derivatives 1-4

Triciribine derivative 5

Valopicitabin (NM283) 6

Figure 2a: NS5B nucleoside inhibitors as anti HCV



Bl-207127
Deleobuvir

GS-9190
Tegobuvir

PF-868554
Filibuvir

ANA-598
Setrobuvir

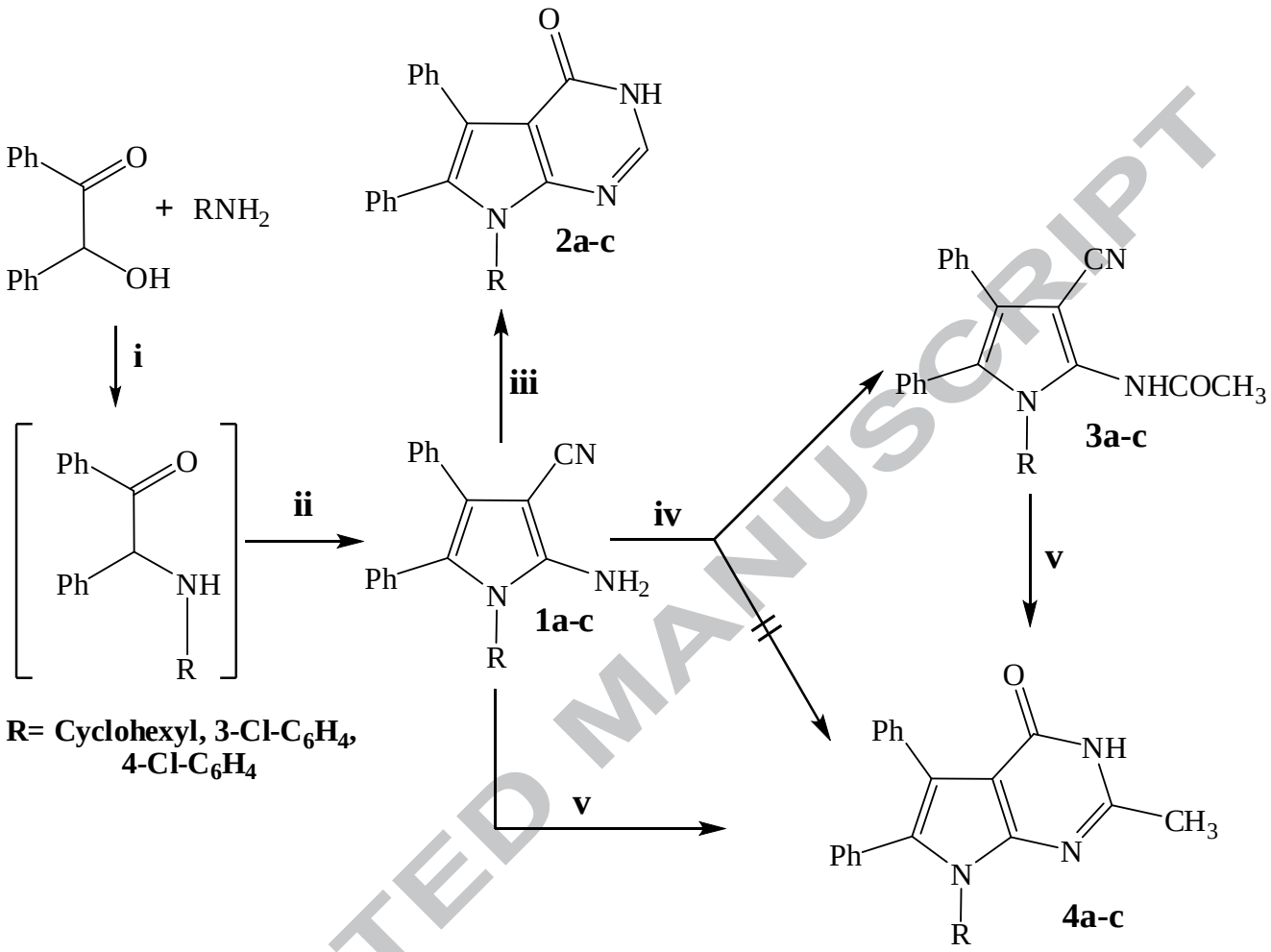
Figure 2b: Non-nucleoside NS5B inhibitors as anti HCV

2. Results and discussion

Chemical synthesis

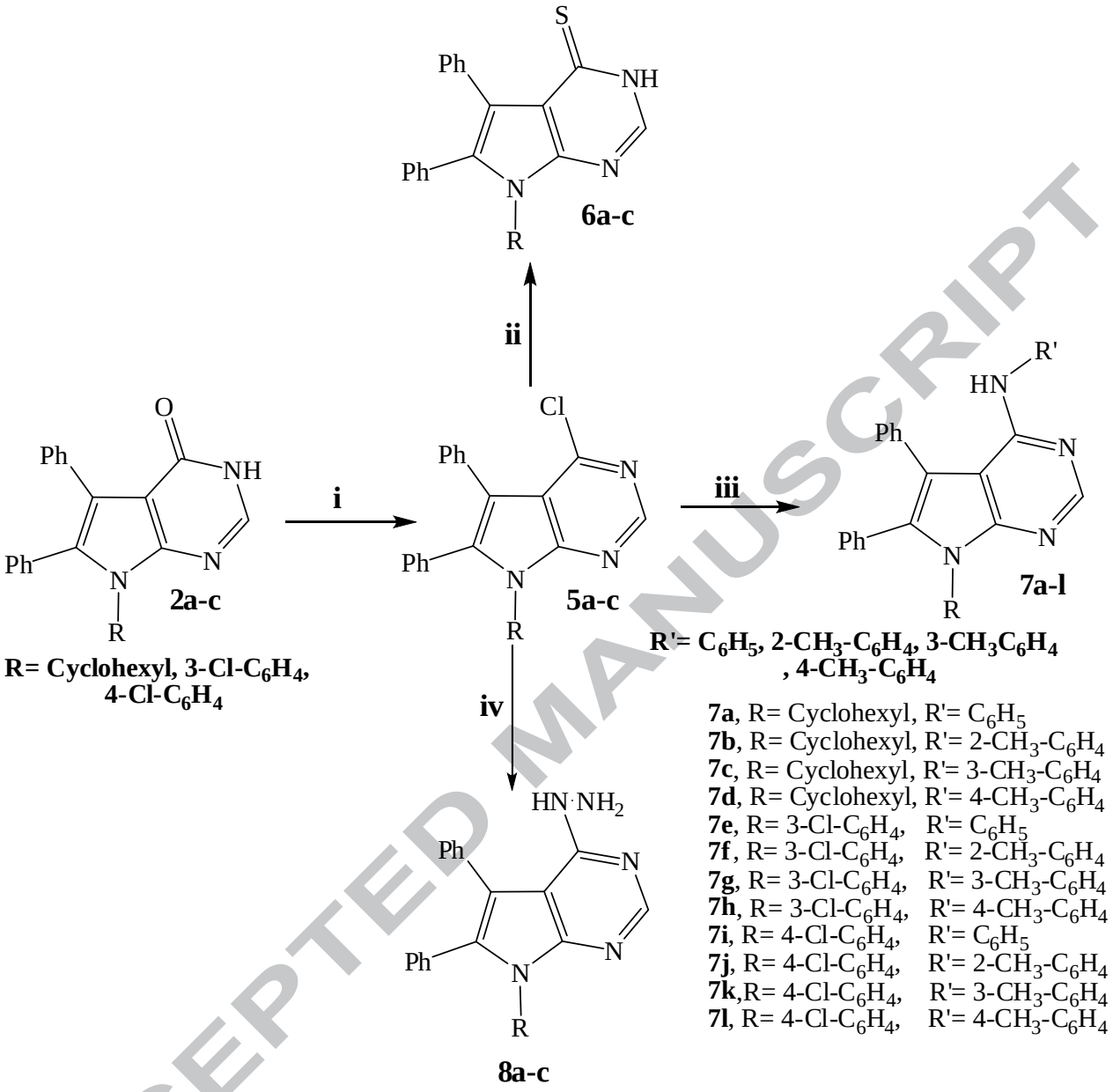
The synthesis of 2-aminopyrrole-3-carbonitriles requires the availability of α-aminoketones [24-30]. These are usually unstable compounds, obtained *in situ*, via either reaction of α-hydroxy ketones with amines in acidic media, reaction of α-halo ketones with amines, or from α-amino acids via Dakin-West reaction [31]. Our target pyrroles **1a-c** were synthesized via condensation of benzoin with aryl amines in refluxing toluene that resulted in the formation of α-aminoketone intermediate. The formed intermediate was condensed, without isolation, with malononitrile to produce 2-amino-pyrrole-3-carbonitriles derivatives **1a-c**.

Based on the importance of the pyrrolopyrimidine nucleoside analogs as HCV inhibitors [19-23], the chemical synthesis plan was to cyclize pyrroles **1a-c** to pyrrolopyrimidines using different reagents. Thus, Herein, pyrrolo[2,3-*d*]pyrimidines **2a-c** were prepared by condensation of 2-aminopyrrole-3-carbonitriles **1a-c** with formic acid. Although a literature survey indicated that the reaction of compounds analogous to **1a-c** with acetic anhydride gave the corresponding pyrimidine derivatives [32,33]**,** in our hands**,** condensation of **1a-c,** independently**,** with acetic anhydride gave the acetylated analogous **3a-c**. The structures of these compounds were proved by microanalysis and spectral data, where the IR spectra showed bands for NH, CN and C=O groups, in addition to ^{13}C NMR spectra that revealed CN and C=O groups, also, ^{1}H NMR and MS spectra were consistent with the structures. On the other hand, refluxing compounds **1a-c** with a mixture of acetic acid/HCl (3:1) gave the corresponding pyrrolo[2,3-*d*]pyrimidines **4a-c**. Unambiguously, **4a-c** were also obtained by refluxing of **3a-c** with a mixture of acetic acid/HCl (3:1) (**Scheme 1**). The IR spectra of compounds **4a-c** revealed bands for NH and C=O groups and the absence of CN group band, ^{1}H NMR and MS spectra were consistent with their structures.



Scheme 1: Synthesis of compounds 1a-c – 4a-c. (i) toluene, HCl; (ii) $CH_2(CN)_2$, pyridine; (iii) HCOOH; (iv)Ac_2O; (v) AcOH/HCl(3:1).

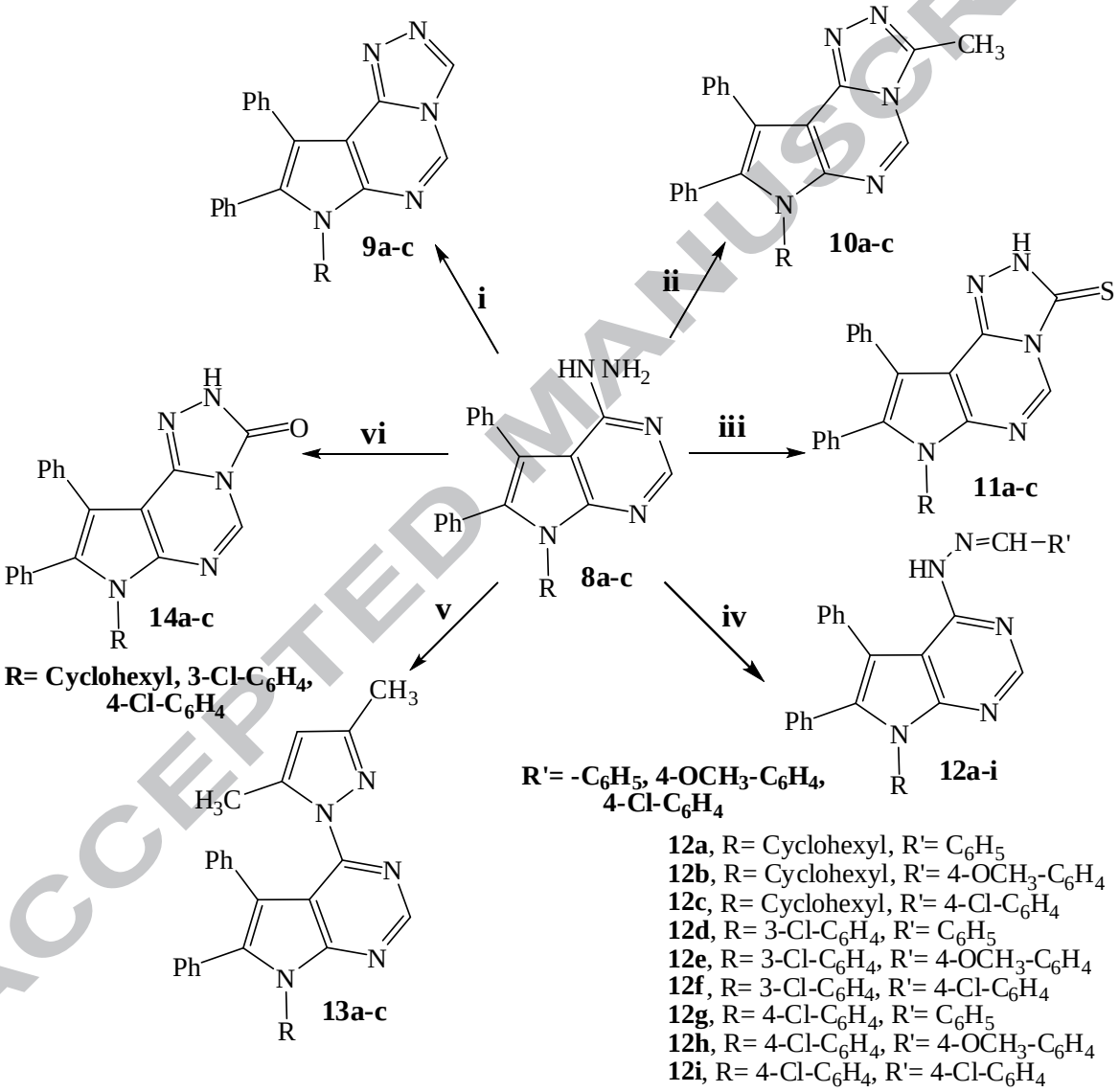
Pyrrolopyrimidines **2a-c** were converted to the corresponding 4-chloro derivatives **5a-c** by condensation with phosphorus oxychloride. 4-Chloropyrrolo[2,3-*d*]pyrimidines **5a-c** were reacted, independently, with thiourea, certain aryl amines or hydrazine hydrate to afford the respective pyrrolopyrimidine-4-thiones **6a-c,** 4-aryl amino **7a-l** and 4-hydrazino derivatives **8a-c** (**Scheme 2)**.



Scheme 2: Synthesis of compounds 5a-c – 8a-c. (i) $POCl_3$; (ii) $CS(NH_2)_2$, EtOH; (iii) $R'NH_2$,TEA, EtOH; (iv) $NH_2NH_2.H_2O$ (99%), EtOH.

4-Hydrazinopyrimidine derivatives were extensively used in many reports [34-43] as starting material for the synthesis of several fused tricyclic heterocycles. Analogously, in this work, we synthesized certain pyrrolo[3,2-*e*][1,2,4]triazolo[4,3-*c*]pyrimidine derivatives **9a-c**, **10a-c**, **11a-c** and **14a-c** by the reaction of 4-hydrazinopyrrolopyrimidines **8a-c** with formic acid, acetic anhydride,

carbon disulfide or ethyl chloroformate, respectively. Also, **8a-c** were reacted with aryl aldehydes in presence of triethylamine to afford 4-(2-arylhydrazinyl)pyrrolo[2,3-*d*]pyrimidines **12a-i** and finally they reacted with acetylacetone to yield 4-(3,5-dimethyl-1*H*-pyrazol-1-yl)pyrrolo[2,3-*d*]pyrimidines **13a-c** as revealed in **Scheme 3**. The structures of all synthesized compounds were substantiated by microanalysis and spectral data.



Scheme 3: Synthesis of compounds 9a-c – 14a-c. (i) HCOOH; (ii) Ac_2O; (iii) CS_2, EtOH; (iv) R'CHO, EtOH; (v) $(CH_3CO)_2CH_2$, EtOH; (vi) ClCOOEt, pyridine.

Antiviral activity and SAR analysis

Before determining the antiviral activity, the cytotoxicity of the synthesized compounds was assessed. For this purpose, we used the cell line Huh 7.5. The CC_{50} was determined using two different methods: first by counting the viable cells and secondly by examining the cell morphology (**Table 1**).

Table 1: CC_{50} and IC_{50} for the synthesized compounds

Compounds	CC_{50} (µM)	IC_{50}* for HCV genotype4 (µM)
1a	176	>150
1b	189	>150
1c	189	>150
2a	190	>150
2b	151	>150
2c	151	>150
3a	183	>150
3b	146	>150
3c	146	>150
4a	130	>150
4b	97	>150
4c	97	>150
5a	155	>150
5b	168	>150
5c	120	108.1±12.01
6a	130	>150
6b	145	>150
6c	121	>150
7a	158	>150
7b	153	>150

7c	131	>150
7d	153	>150
7e	148	>150
7f	144	92.4±10.3
7g	103	>150
7h	123	>150
7i	127	>150
7j	144	82.1±10.3
7k	1023	>150
7l	123	>150
8a	157	>150
8b	146	>150
8c	146	>150
9a	178	>150
9b	190	>150
9c	190	>150
10a	172	>150
10b	161	>150
10c	138	>150
11a	188	>150
11b	184	>150
11c	138	>150
12a	148	>150
12b	140	>150
12c	158	>150
12d	140	>150
12e	151	>150
12f	131	102.9±9.4
12g	140	>150
12h	113	>150
12i	131	93.6±9.4

13a	156	>150
13b	147	>150
13c	147	>150
14a	171	>150
14b	183	>150
14c	160	>150

*** IC_{50} is represented as the mean of 3 measurements ±SD**

The non-toxic doses of the synthesized compounds were measured to determine their activity against HCV genotype 4a. Only five compounds showed considerable antiviral activity. **Figure 3** showed the viral inhibition of compounds **5c**, **7f**, **7j, 12f** and **12i**. The CC_{50} for these compounds were **120**, **144**, **144**, **131** and **131** µM, respectively. It was observed that compound **7j** showed maximum antiviral activity (90%) with less toxicity, compound **7f** activity was (76.7±5.8%), compound **5c** activity was (73.3±5.8%), compound **12i** activity was (70%) and compound **12f** had the least activity (63.3±5.8%).

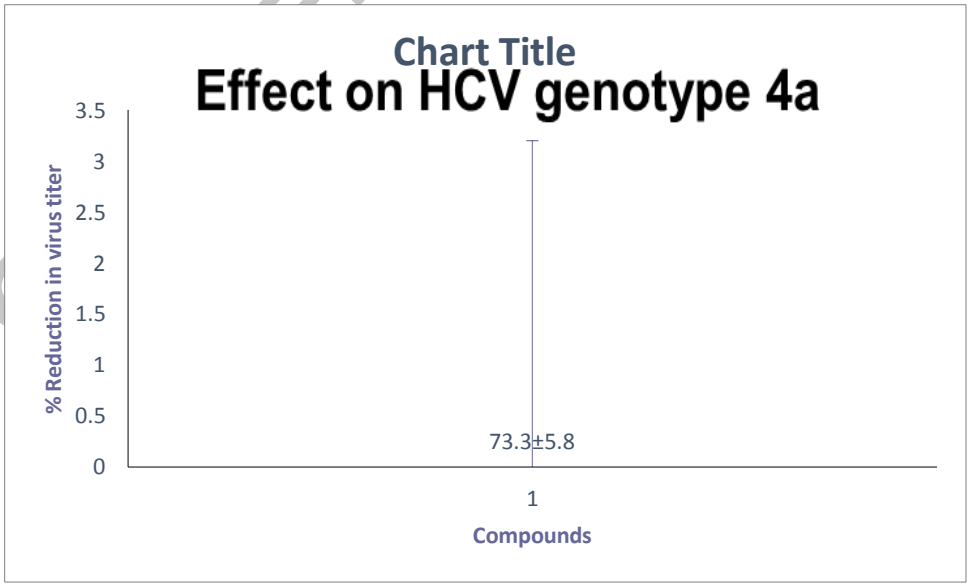


Figure 3: Antiviral activity of non-toxic doses of tested compounds 5c, 7f, 7j, 12f and 12i against HCVcc genotype 4a (number of determinations= 3).

It was observed that pyrrolo[2,3-*d*]pyrimidine analogues possessed significant anti-HCV activity when compared to the synthesized pyrrole derivatives **1a-c**, **3a-c** and pyrrolo[3,2-*e*][1,2,4]triazolo[4,3-*c*]pyrimidine derivatives **9a-c**, **10a-c**, **11a-c** and **14a-c** which did not exhibit any activity. Regarding SAR, the critical inspection of the biological activity results indicate that 5,6-diphenyl-7-(3- or 4-chlorophenyl)pyrrolo[2,3-*d*]pyrimidines with appropriate substitution (chloro or much better arylamino) at position 4 could be a useful scaffold as anti-HCV genotype 4a (compounds **5c**, **7f** and **7j**). Also, substitution at 4-position by arylamino group is essential for antiviral activity with comparative least toxicity as indicated by the effect of compounds **7j** and **7f**, **Table 1**. Extending the substituent at position-4, even replacing the aryl amino by aryl hydrazone function results in decreasing the antiviral activity with increasing cytotoxicity (compounds **12f** and **12i)**, **Table 1, Figure 4**. These results are in accordance with the results of molecular docking reported herein (next section), in addition to their consistency with the previous study [20].

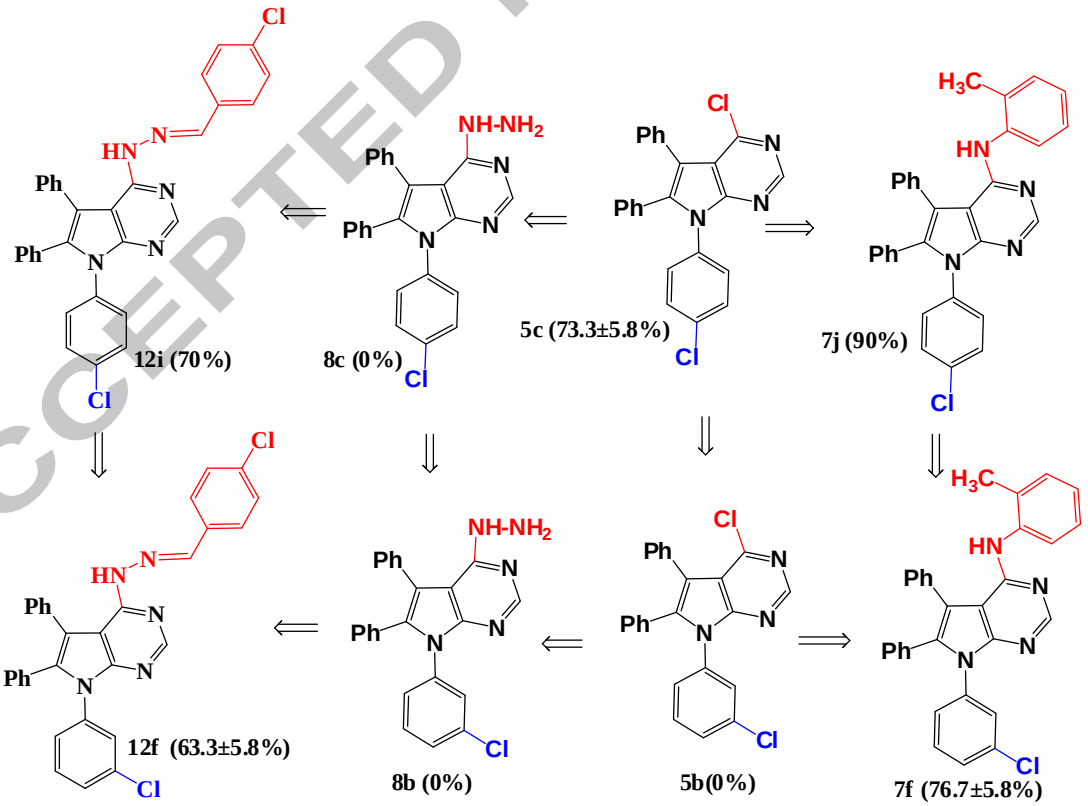


Figure 4: SAR of the newly synthesized Pyrrolopyrimidine derivatives.

Molecular docking

Many trials were done that revealed some potent inhibitors of HCV RdRp. Most of the reported inhibitors were in complex with the enzyme at distances may reach 30 Å from the palm domain within the groove between the thumb and palm (**Figure 1**) [45].

In this work a number of pyrrolo[2,3-*d*]pyrimidine derivatives were synthesized and screened for their HCV inhibitory activity. The binding modes of these compounds within the HCV RdRp catalytic site at the palm domain and the thumb region were computed. The docking results were compared to a reported substituted 3,3-dimethylhexahydro-1*H*-dibenzo[*b*,*e*][1,4]diazepin-1-one. According to the docking results, none of the docked compounds formed coordinate with Mg^{2+} found in the catalytic site. The placement of all poses was found to locate at 10.69 Å from the Mg^{2+}, which indicates that they are still close to the palm domain but at much better location than the previously reported inhibitors. The calculated entropy conformation score for all the ligands was neglected (0.00) or very low (1.40) which confirmed the strong binding within the docking site.

Clash penalty score, which indicates the quality of binding, was also calculated. It ranged from a low value for compound **2b** (1.41), compound **6b** (1.71), compound **7j** (2.00) and compound **5c** (2.27) and was found at its highest level for compound **4b** (11.36).

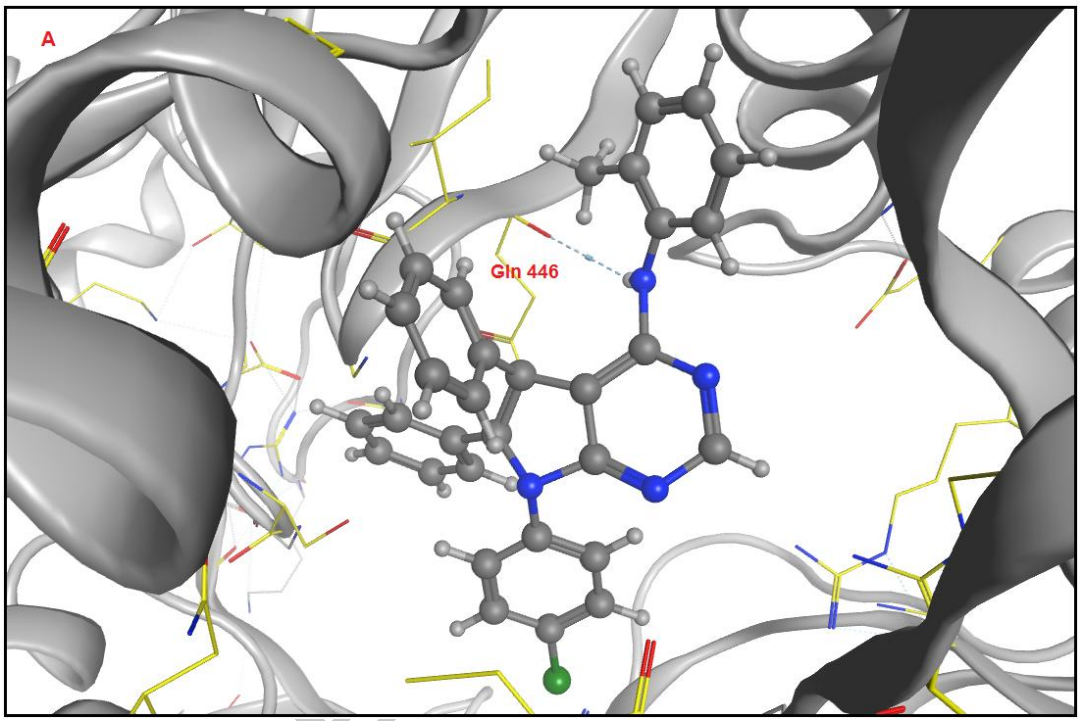
From all compounds with less ligand entropy conformation score and clash penalty score only compound **7j** showed low lipophilic contribution score (-13.97). As mentioned previously as the ligand entropy conformation score and clash penalty score are very low as the binding affinity is improved and that is very clear for the values of calculated affinities of compounds **7j**, **6b**, **5c**, and **2b** that were -24.92, -23.80, -23.4 and -23.27 kcal/mol respectively. This can interpret the activity of

compounds **7j** and **5c.** Compounds **12f** and **12i** were ranked just after **5c** according to their docking scores that were -22.43 and -22.31 kcal/mol respectively. They showed neglected entropy conformation score and relatively good clash score when compared to other active compounds (**Table 2**).

Table 2: Molecular model assessment scores

Compounds	Docking score [a] Kcal/mol	Lipo score [b]	Clash score [c]	Rot score [d]
1a	-16.59	-8.27	6.15	1.40
1b	-21.67	-10.89	4.09	0.00
1c	-18.33	-7.08	2.58	0.00
2a	-19.39	-12.01	5.04	1.40
2b	-23.27	-10.63	1.41	0.00
2c	-21.21	-12.47	4.85	0.00
3a	-19.51	-11.40	6.12	1.40
3b	-21.65	-13.21	9.04	0.00
3c	-21.31	-9.46	2.77	0.00
4a	-18.60	-9.78	2.86	1.40
4b	-20.22	-13.59	11.36	0.00
4c	-21.84	-11.84	4.44	0.00
5a	-15.08	-13.91	9.44	1.40
5b	-19.33	-11.80	4.77	0.00
5c	-23.42	-7.55	2.27	0.00
6a	-16.11	-10.57	3.46	1.40
6b	-23.80	-11.44	1.71	0.00
6c	-20.49	-11.22	5.21	0.00
7a	-19.15	-13.89	7.56	1.40
7b	-17.65	-11.18	4.89	1.40
7c	-16.10	-13.41	10.13	1.40
7d	-15.48	-8.89	5.23	1.40
7e	-21.38	-11.96	7.06	0.00
7f	-20.92	-14.49	3.21	0.00
7g	-20.66	-11.87	5.32	0.00
7h	-20.82	-11.83	6.77	0.00
7i	-19.64	-12.37	8.82	0.00
7j	-24.92	-13.97	2.00	0.00
7k	-21.80	-12.47	8.81	0.00
7l	-20.38	-12.77	9.49	0.00
12f	-22.43	-13.36	2.94	0.00
12i	-22.31	-12.68	3.04	0.00

Regarding the ligand-receptor interactions, we can find that compound **7j** showed a clear hydrogen bond with Gln 446 and that was confirmed by both MOE 2014.09 docking (**Figure 5A**) and Leadit 2.1.2 (**Figure 6C**).



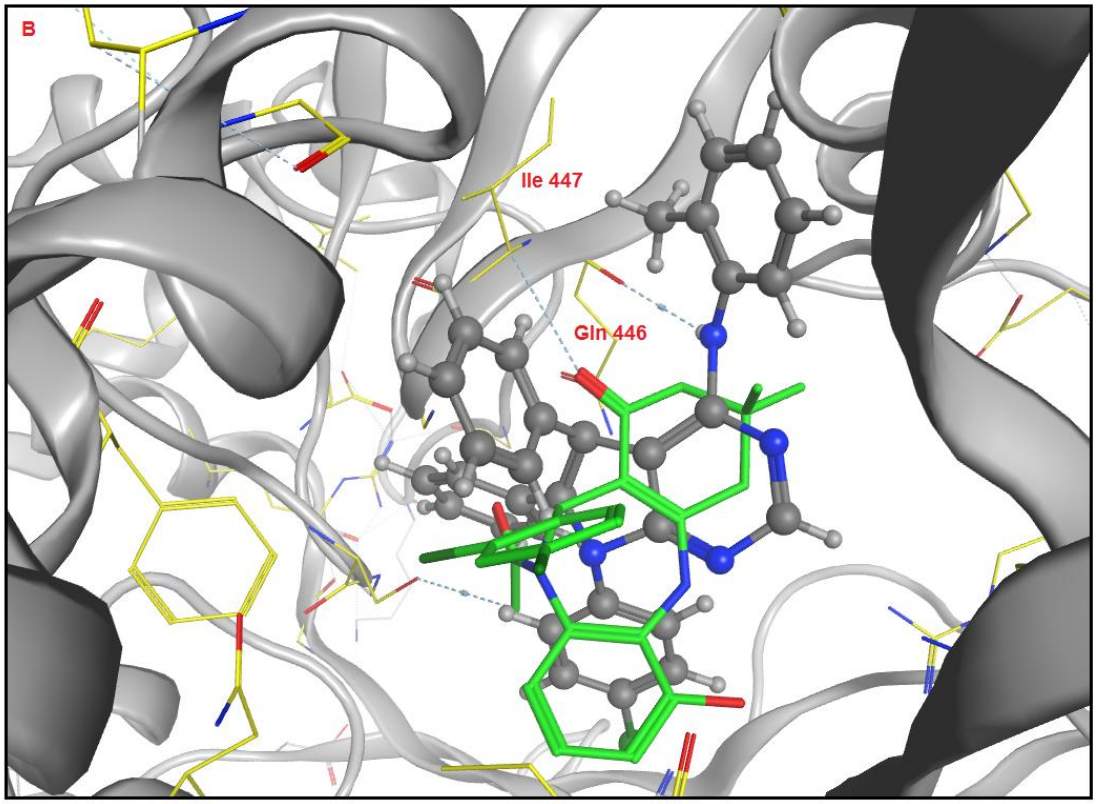


Figure 5: Molecular model for binding of 7J

A) Binding mode of compound **7j** forming a hydrogen bond with Gln 446. B) The placement of compound **7j** within the same site of the reported inhibitor.

By using Leadit 2.1.2 software, it was discovered that Gln 446 can have a unique binding with its C=O and NH_2 groups to form two different bonds with the aniline NH and the pyrimidine C=N group respectively. On the other hand compound **7f** showed a hydrogen bond with Gln 446 as well, where compound **5c** had a hydrogen bond with Tyr 448 within the same site (**Figure 6A,B**). Compounds **12f** showed two kinds of hydrogen bonds with Gly 449 (NH) and Gln 446 (C=O), while compound **12i** was involved in hydrogen bonds with Tyr 448 (NH) and Gly 449 (NH) as shown in (**Figure 6D, E**).

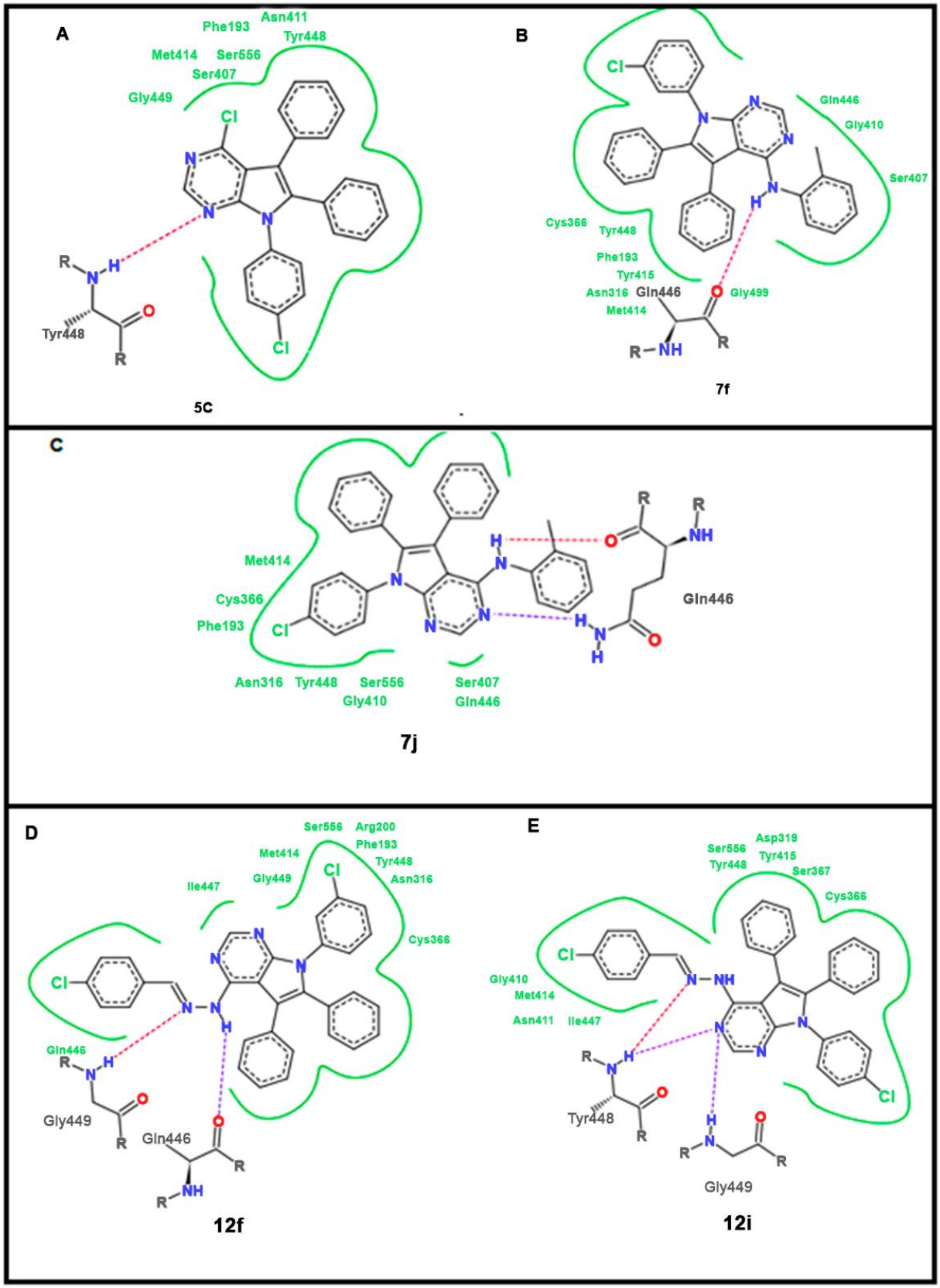


Figure 6: Suggested-binding modes of compounds 5c, 7f, 7j, 12f and 12i.

3. Conclusion

In this study, we synthesized novel pyrrole, pyrrolo[2,3-*d*]pyrimidine and pyrrolo[3,2-*e*][1,2,4]triazolo[4,3-*c*]pyrimidine derivatives and tested their activity towards HCV genotype 4a. Pyrrole and pyrrolo[3,2-*e*][1,2,4]triazolo[4,3-*c*]pyrimidine derivatives do not exhibit any activity against HCV genotype 4a, however, some pyrrolo[2,3-*d*]pyrimidine derivatives showed reasonable inhibition of the virus. The binding mode for the active compounds was predicted by a molecular docking study. Compound **7j** exhibited the highest potency as anti HCV which renders this molecule as a lead compound for drug discovery of anti-HCV agents.

4. Experimental

Chemistry

All chemicals used in this study were purchased from Merck (Darmstadt, Germany) as reagent grade. All melting points were uncorrected and determined using Electro-thermal IA 9100 apparatus (Shimadzu, Japan). We recorded the IR spectra as potassium bromide pellets on a Perkin-Elmer 1650 spectrophotometer (USA), Faculty of Science, Cairo University, Cairo, Egypt. The ^{1}H-NMR (300 MHz) and ^{13}C-NMR (75 MHz) spectra were determined by Varian Mercury spectrometer (Varian UK) and chemical shifts were expressed as ppm against TMS as internal reference (The Main Chemical warfare Laboratories, Almaza, Cairo, Egypt). A TSQ Quantum Access MAX triple quadrupole system was used for mass spectrometric analysis. Data acquisition and processing was performed using Thermo Scientific Xcalibur 2.1 software (Faculty of Pharmacy, Helwan University, Cairo, Egypt). Microanalyses were operated using Vario, Elmentar apparatus (Shimadzu, Japan) (Organic Microanalysis Unit, Faculty of Science, Cairo University, Cairo, Egypt). The progress of

reactions was monitored by TLC sheets pre coated with UV fluorescent silica gel (Merck 60 F254) using chloroform/methanol (3:1) and spots were visualized using UV-light and iodine vapor. All new compounds yielded spectral data consistent with the proposed structure and microanalysis within ±0.4% of the theoretical values. Compounds **1a,b-6a,b** and **7a-h** were prepared as we described before [44].

Preparation of 2-Amino-1-(4-chlorophenyl)-4,5-diphenyl-1H-pyrrole-3-carbonitrile (1c)

A mixture of benzoin (2.12 g, 10 mmol), 4-chloroaniline (1.27 g, 10 mmol) and conc. HCl (6-8 drops) in toluene (50 mL) was heated under reflux for 36 h then cooled. Malononitrile (0.66 g, 10 mmol) was added, followed by a catalytic amount of pyridine (1.5 mL) portion-wise and left to reflux until a solid mass was formed. The solvent was evaporated under reduced pressure and the residue was recrystallized from methanol to give compound **1c**.

Yield: 75%; m.p.: 290-292 ^{o}C; IR (KBr) υ (cm^{-1}): 3441, 3340 (NH_2), 2209 (C≡N); MS (ESI) m/z: 369.19 (M^+, ^{35}Cl, 5%), 371.23 (M^++2, ^{37}Cl, 1.58%); ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 5.39 (s, 2H, NH_2, D_2O exchangeable), 7.03-7.96 (m, 14H, Ar-H); Anal. Calcd for $C_{23}H_{16}ClN_3$ (369.86): C, 74.69; H, 4.36; N, 11.36%. Found: C, 74.42; H, 4.49; N, 11.61%.

Preparation of 7-(4-chlorophenyl)-5,6-diphenyl-3H-pyrrolo[2,3-d]pyrimidin-4(7H)-one (2c)

The appropriate aminopyrrole(**1c)** (3.699 g, 10 mmol) in formic acid (20 mL, 85%) was heated under reflux for 36 h, cooled, poured onto ice-water to give a precipitate **2c** which was filtered off, dried, and recrystallized from ethanol.

Yield: 71%; m.p.: > 300 ^{o}C; IR (KBr) υ (cm^{-1}): 3382 (NH), 1680 (C=O), 1587 (C=N); MS (ESI) m/z: 397.11 (M^+, ^{35}Cl, 2%), 399 (M^++2, ^{37}Cl, 0.62%); ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 6.99-7.91 (m, 14H, Ar-H), 8.25 (s, 1H, C2-H), 11.89 (s, 1H, NH, D_2O exchangeable); Anal.

Calcd for $C_{24}H_{16}ClN_3O$ (397.86): C, 72.45; H, 4.05; N, 10.56 %. Found: C, 72.19; H, 4.32; N, 10.72%.

Preparation of N-(1-(4-chlorophenyl)-3-cyano-4,5-diphenyl-1H-pyrrol-2-yl)acetamide (3c)

The appropriate aminopyrrole(**1c)** (3.699 g, 10 mmol) in acetic anhydride (40 mL) was heated under reflux for 5 h, then cooled, poured onto ice-water and neutralized with ammonia to give a precipitate, which was filtered off, dried, and recrystallized from methanol, to give compound **3c**.

Yield: 65%; m.p.: 210-212 ^{o}C; IR (KBr) υ (cm^{-1}): 3350 (NH), 2219 (C≡N), 1699 (C=O); MS (ESI) m/z: 411.52 (M^+, ^{35}Cl, 4%), 413.61(M^++2, ^{37}Cl, 1.36%); ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm): 1.90 (s, 3H, CH_3-CO), 7.07-7.91 (m, 14H, Ar-H), 11.40 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ (ppm): 21.74 (CH_3), 116.15 (C≡N), 124.04, 126.41,126.92, 127.81, 128.46, 128.65, 128.74, 128.89, 129.03, 129.39, 130.20, 131.37, 132.98, 134.48, 134.88, 135.89 (sp^2 carbon atoms), 171.28 (C=O); Anal. Calcd for $C_{25}H_{18}ClN_3O$ (411.88): C, 72.90; H, 4.40; N, 10.20%. Found: C, 72.60; H, 4.59; N, 10.45%.

Preparation of 7-(4-chlorophenyl)-2-methyl-5,6-diphenyl-3H-pyrrolo[2,3-d]pyrimidin-4(7H)-one (4c)

The appropriate aminopyrrole (**1c)** (3.699 g, 10 mmol) or its acetyl derivative (**3c**) (4.119 g, 10mmol) was refluxed, independently, in a mixture of acetic acid (15mL) and hydrochloric acid (5mL) for 5 h. The reaction mixture was cooled, poured onto ice and the formed solid was filtered off, dried and recrystallized from methanol to afford **4c**.

Yield: 68%; m.p.: 257-259 ^{o}C; IR (KBr) υ (cm^{-1}): 3395 (NH), 1707 (C=O), 1589(C=N); MS (ESI) m/z: 411.55(M^+, ^{35}Cl, 4%), 413.70(M^++2, ^{37}Cl, 1.32%); ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 2.32 (s, 3H, CH_3), 7.13-7.92 (m, 14H, Ar-H), 11.41 (s, 1H, NH, D2O exchangeable); Anal. Calcd for $C_{25}H_{18}ClN_3O$ (411.88): C, 72.90; H, 4.40; N, 10.20 %. Found: C, 73.21; H, 4.65; N, 10.36%.

Preparation of 4-chloro-7-(4-chlorophenyl)-5,6-diphenyl-7H-pyrrolo[2,3-d]pyrimidine (5c)

The appropriate pyrrolopyrimidinone (**2c)** (3.979 g, 10 mmol) was heated under reflux in phosphorus oxychloride (30 mL) for 15 h, then cooled and poured onto ice-water to yield precipitate, which was recrystallized from ethanol to give compounds **5c**.

Yield: 75%; m.p.: 252-254 ^{o}C; IR (KBr) υ (cm^{-1}): 3045, 2960 (CH), 1615 (C=C), 1571 (C=N); MS (ESI) m/z: 416.36 (M^{+}, ^{35}Cl, 3.2%), 418.31(M^{+}+2, ^{37}Cl, 2.13%), 420.4(M^{+}+4, Cl^{37}, 1.07%); ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 7.0-7.91 (m, 14H, Ar-H), 8.44 (s, 1H, C2-H); Anal. Calcd for $C_{24}H_{15}Cl_2N_3$ (416.30): C, 69.24; H, 3.63; N, 10.09 %. Found: C, 68.95; H, 3.79; N, 10.25%.

Preparation of 7-(4-chlorophenyl)-5,6-diphenyl-3H-pyrrolo[2,3-d]pyrimidine-4(7H)-thione (6c)

A mixture of 4-chloropyrrolopyrimidine (**5c)** (4.163 g, 10 mmol) and thiourea (1.5g, 0.02 mol) was heated under reflux in dry ethanol (30 mL) for 10 h, then cooled, poured onto ice-water, to give precipitate, which were filtered off, dried, and recrystallized from methanol to give compounds **6c**.

Yield: 70%; m.p.: 185-187 ^{o}C; IR (KBr) υ (cm^{-1}): 3375 (NH), 1585 (C=N), 1255(C=S); MS (ESI) m/z: 413.37 (M^{+}, ^{35}Cl, 2%), 415.34 (M^{+}+2, ^{37}Cl, 0.65%); ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 7.14-7.91 (m, 14H, Ar-H), 8.86 (s, 1H, C2-H), 10.15 (s, 1H, NH, D2O exchangeable); Anal. Calcd for $C_{24}H_{16}ClN_3S$ (413.92): C, 69.64; H, 3.90; N, 10.15 %. Found: C, 69.78; H, 4.11; N, 10.01%.

General procedure for the synthesis of 5,6-diphenyl-7-(4-chlorophenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-yl)-arylamine (7i-l):

A mixture of 4-chloropyrrolopyrimidine (**5c**) (4.163 g, 10 mmol), the appropriate amine (10 mmol) and few drops of triethylamine was heated under reflux in absolute ethanol for 8 h, then cooled and poured onto ice-water to give precipitate, which were filtered off, dried, and recrystallized from methanol to give compounds **7i-l.**

7-(4-Chlorophenyl)-N,5,6-triphenyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine (7i)

Yield: 61%; m.p.: 180-182 ^{o}C; IR (KBr) υ (cm^{-1}): 3435 (NH), 1607 (C=N); MS (ESI) m/z: 472 (M^{+}, ^{35}Cl, 3%), 474.21(M^{+}+2, ^{37}Cl, 0.9%); ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 6.90-7.90 (m, 19H, Ar-H), 8.44 (s, 1H, C2-H), 11.11 (s, 1H, NH, D_2O exchangeable); Anal. Calcd for $C_{30}H_{21}ClN_4$ (472.97): C, 76.18; H, 4.48; N, 11.85%. Found: C, 76.00; H, 4.71; N, 12.02%.

7-(4-Chlorophenyl)-5,6-diphenyl-N-o-tolyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine (7j)

Yield: 60%; m.p.: 210-212 ^{o}C; IR (KBr) υ (cm^{-1}): 3448 (NH), 1598 (C=N); MS (ESI) m/z: 487.22 (M^{+}, ^{35}Cl, 5%), 489.11(M^{+}+2, ^{37}Cl, 1.71%);^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 1.99 (s, 3H, CH_3), 6.88-7.91 (m, 18H, Ar-H), 8.38 (s, 1H, C2-H), 11.11 (s, 1H, NH, D_2O exchangeable); Anal. Calcd for $C_{31}H_{23}ClN_4$ (486.99): C, 76.46; H, 4.76; N, 11.50%. Found: C, 76.18; H, 4.54; N, 11.76%.

7-(4-Chlorophenyl)-5,6-diphenyl-N-m-tolyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine (7k)

Yield: 63%; m.p.: 202-204 ^{o}C; IR (KBr) υ (cm^{-1}): 3448(NH), 1598(C=N); MS (ESI) m/z: 485.22 (M^{+}-2H, ^{35}Cl, 2.8%) ; ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 2.10 (s, 3H, CH_3), 6.84-7.91 (m, 18H, Ar-H), 8.40 (s, 1H, C2-H), 11.67 (s, 1H, NH, D_2O exchangeable); Anal. Calcd for $C_{31}H_{23}ClN_4$ (486.99): C, 76.46; H, 4.76; N, 11.50%. Found: C, 76.70; H, 4.44; N, 11.29%.

7-(4-Chlorophenyl)-5,6-diphenyl-N-p-tolyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine (7l)

Yield: 65%; m.p.: 195-197 ^{o}C; IR (KBr) υ (cm^{-1}): 3448 (NH), 1598 (C=N); MS (ESI) m/z: 487.21 (M^{+}, ^{35}Cl, 6%), 489.25(M^{+}+2, ^{37}Cl, 1.9%); ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 2.25 (s, 3H, CH_3), 7.0-7.91 (m, 18H, Ar-H), 8.38(s, 1H, C2-H), 10.95 (s, 1H, NH, D_2O exchangeable); Anal. Calcd for $C_{31}H_{23}ClN_4$ (486.99): C, 76.46; H, 4.76; N, 11.50%. Found: C, 76.21; H, 4.95; N, 11.81%.

General procedure for the synthesis of 5,6-Diphenyl-4-hydrazinyl-7-substituted-7H-pyrrolo[2,3-d]pyrimidines (8a-c):

A mixture of 4-chloro pyrrolopyrimidine **5a-c** (10 mmol) and hydrazine hydrate (0.5 mL, 10 mmol) was heated under reflux in absolute ethanol for 8 h, cooled, poured onto ice water to give precipitates which were filtered off, dried, and recrystallized from methanol to give compounds **8a-c**.

7-Cyclohexyl-5,6-diphenyl-4-hydrazinyl-7H-pyrrolo[2,3-d]pyrimidine (8a)

Yield: 70%; m.p.: 202-204 ^{o}C; IR (KBr) υ (cm^{-1}): 3412-3322 (NH_2), 3237(NH), 1533 (C=N); MS (ESI) m/z: 383.35(M^+, 2%); ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 1.40-1.95 (m, 10H, cyclohexyl), 3.92 (m, 1H, CH-N cyclohexyl), 5.34 (s, 2H, NH_2 D_2O exchangeable), 7.12-7.69 (m, 10H, Ar-H), 8.12 (s, 1H, C2-H), 8.58 (s, 1H, NH, D_2O exchangeable); Anal. Calcd for $C_{24}H_{25}N_5$ (383.49): C, 75.17; H, 6.57; N, 18.26%. Found: C, 75.35; H, 6.82; N, 18.01%.

7-(3-Chlorophenyl)-5,6-diphenyl-4-hydrazinyl-7H-pyrrolo[2,3-d]pyrimidine (8b)

Yield: 72%; m.p.: 212-214 ^{o}C; IR (KBr) υ (cm^{-1}): 3427-3385 (NH_2), 3217(NH), 1558 (C=N); MS (ESI) m/z: 411.27 (M^+, ^{35}Cl, 4%), 413.50 (M^++2, ^{37}Cl, 1.4%);^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 6.28 (s, 2H, NH_2 D_2O exchangeable), 7.0-7.85 (m, 14H, Ar-H), 8.20 (s, 1H, C2-H), 8.40 (s, 1H, NH, D_2O exchangeable); Anal. Calcd for $C_{24}H_{18}ClN_5$ (411.89): C, 69.98; H, 4.40; N, 17.00%. Found: C, 69.71; H, 4.71; N, 16.83%.

7-(4-Chlorophenyl)-5,6-diphenyl-4-hydrazinyl-7H-pyrrolo[2,3-d]pyrimidine (8c)

Yield: 75%; m.p.: 220-222 ^{o}C; IR (KBr) υ (cm^{-1}): 3427-3385 (NH_2), 3217(NH), 1558 (C=N); MS (ESI) m/z: 411.22 (M^+, ^{35}Cl, 8%), 413.23 (M^++2, ^{37}Cl, 2.6%); ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 5.08(s, 2H, NH2 D_2O exchangeable), 7.0-7.79 (m, 14H, Ar-H), 7.90 (s, 1H, C2-H), 8.10 (s, 1H, NH, D_2O exchangeable); Anal. Calcd for $C_{24}H_{18}ClN_5$ (411.89): C, 69.98; H, 4.40; N, 17.00%. Found: C, 69.68; H, 4.19; N, 17.25%.

General procedure for the synthesis of 8,9-diphenyl-7-substituted-7H-pyrrolo[3,2-e][1,2,4]triazolo[4,3-c]pyrimidines (9a-c):

The appropriate hydrazine **8a-c** (10 mmol) was heated under reflux for 8 h in formic acid (20 mL, 85 %), cooled, poured onto ice water to give a precipitate which was filtered off, dried, and recrystallized from ethanol to yield compounds **9a-c**.

7-Cyclohexyl-8,9-diphenyl-7H-pyrrolo[3,2-e][1,2,4]triazolo[4,3-c]pyrimidine (9a)

Yield: 68%; m.p.: 280-282 ^{o}C; IR (KBr) υ (cm^{-1}): 1618 (C=N); MS (ESI) m/z: 394.49(M^{+}+1H, 8%); ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 1.41-1.96 (m, 10H, cyclohexyl), 3.82 (m, 1H, CH-N cyclohexyl), 7.17-7.70 (m, 10H, Ar-H), 8.16 (s, 1H, C3-H), 8.83 (s, 1H, C5-H); Anal. Calcd for $C_{25}H_{23}N_5$ (393.48): C, 76.31; H, 5.89; N, 17.80 %. Found: C, 76.49; H, 5.62; N, 18.07%.

7-(3-Chlorophenyl)-8,9-diphenyl-7H-pyrrolo[3,2-e][1,2,4]triazolo[4,3-c]pyrimidine (9b)

Yield: 70%; m.p.: 266-268 ^{o}C; IR (KBr) υ (cm^{-1}): 1566 (C=N); MS (ESI) m/z: 421.42 (M^{+}, ^{35}Cl, 2%), 423.39 (M^{+}+2, ^{37}Cl, 0.7%); ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 7.0-7.87 (m, 14H, Ar-H), 8.39 (s, 1H, C3-H), 8.90 (s, 1H, C5-H); Anal. Calcd for $C_{25}H_{16}ClN_5$ (421.88): C, 71.17; H, 3.82; N, 16.60%. Found: C, 71.36; H, 4.05; N, 16.36%.

7-(4-Chlorophenyl)-8,9-diphenyl-7H-pyrrolo[3,2-e][1,2,4]triazolo[4,3-c]pyrimidine (9c)

Yield: 72%; m.p.: 287-289 ^{o}C; IR (KBr) υ (cm^{-1}): 1566 (C=N); MS (ESI) m/z: 421.77 (M^{+}, ^{35}Cl, 2.9%), 423.80 (M^{+}+2, ^{37}Cl, 0.9%); ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 7.07-7.94 (m, 14H, Ar-H), 8.12 (s, 1H, C3-H), 8.84 (s, 1H, C5-H); Anal. Calcd for $C_{25}H_{16}ClN_5$ (421.88): C, 71.17; H, 3.82; N, 16.60%. Found: C, 71.01; H, 3.61; N, 16.89%.

General procedure for the synthesis of 8,9-Diphenyl-3-methyl-7-substituted-7H-pyrrolo[3,2-e][1,2,4]triazolo[4,3-c]pyrimidines (10a-c):

The appropriate hydrazine **8a-c** (10 mmol) was heated under reflux for 5 h in acetic anhydride (30 mL), cooled, poured onto ice water and neutralized with ammonia to give a precipitate which was filtered off, dried, and recrystallized from ethanol to yield compounds **10a-c**.

7-Cyclohexyl-8,9-diphenyl-3-methyl-7H-pyrrolo[3,2-e][1,2,4]triazolo[4,3-c]pyrimidine (10a)

Yield: 58%; m.p.: 260-262 ^{o}C; IR (KBr) υ (cm^{-1}): 1554 (C=N); MS (ESI) m/z: 407(M^{+}, 4%); ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 1.51-1.99 (m, 10H, cyclohexyl), 2.27 (s, 3H, CH_3), 3.93 (m, 1H, CH-N cyclohexyl), 7.13-7.65 (m, 10H, Ar-H), 8.64 (s, 1H, C5-H); Anal. Calcd for $C_{26}H_{25}N_5$ (407.51): C, 76.63; H, 6.18; N, 17.19%. Found: C, 76.89; H, 6.00; N, 17.35%.

7-(3-Chlorophenyl)-8,9-diphenyl-3-methyl-7H-pyrrolo[3,2-e][1,2,4]triazolo[4,3-c]pyrimidine (10b)

Yield: 60%; m.p.: 280-282 ^{o}C; IR (KBr) υ (cm^{-1}): 1612 (C=N); MS (ESI) m/z: 435.13 (M^{+}, ^{35}Cl, 4%), 437.21 (M^{+}+2, ^{37}Cl, 1.25%); ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 2.32 (s, 3H, CH_3), 7.0-7.79 (m, 14H, Ar-H), 8.68 (s, 1H, C5-H); Anal. Calcd for $C_{26}H_{18}ClN_5$ (435.91): C, 71.64; H, 4.16; N, 16.07%. Found: C, 71.39; H, 4.41; N, 16.31%.

7-(4-Chlorophenyl)-8,9-diphenyl-3-methyl-7H-pyrrolo[3,2-e][1,2,4]triazolo[4,3-c]pyrimidine (10c)

Yield: 59%; m.p.: 275-277 ^{o}C; IR (KBr) υ (cm^{-1}): 1612 (C=N); MS (ESI) m/z: 435.28 (M^{+}, ^{35}Cl, 7%), 437.30 (M^{+}+2, ^{37}Cl, 2.34%); ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 2.26 (s, 3H, CH_3), 7.01-7.90 (m, 14H, Ar-H), 8.80 (s, 1H, C5-H); Anal. Calcd for $C_{26}H_{18}ClN_5$ (435.91): C, 71.64; H, 4.16; N, 16.07%. Found: C, 71.89; H, 4.39; N, 15.85%.

General procedure for the synthesis of 8,9-Diphenyl-7-substituted-2H-pyrrolo[3,2-e][1,2,4]triazolo[4,3-c]pyrimidine-3(7H)-thiones (11a-c):

A mixture of the appropriate hydrazine **8a-c** (10 mmol) and carbon disulfide (0.8 mL, 10 mmol) was heated under reflux for 3 h in absolute ethanol (30 mL), cooled, poured onto ice water to give a precipitate which was filtered off, dried, and recrystallized from ethanol to yield compounds **11a-c**.

7-Cycloxexyl-8,9-diphenyl-2H-pyrrolo[3,2-e][1,2,4]triazolo[4,3-c]pyrimidine-3(7H)-thiones (11a)

Yield: 60 %; m.p.: 276-278 ^{o}C; IR (KBr) υ (cm^{-1}): 3287(NH), 1649(C=S),1595 (C=N); MS (ESI) m/z: 425(M^{+}, 4%); ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 1.29-1.88 (m, 10H, cyclohexyl), 3.93 (m, 1H, CH-N cyclohexyl), 7.11-7.83 (m, 10H, Ar-H), 8.12 (s, 1H, C5-H), 12.24 (s, 1H, NH, D_2O exchangeable); Anal. Calcd for $C_{25}H_{23}N_5S$ (425.55): C, 70.56; H, 5.45; N, 16.46%. Found: C, 70.81; H, 5.73; N, 16.20%.

7-(3-Chlorophenyl)-8,9-diphenyl-2H-pyrrolo[3,2-e][1,2,4]triazolo[4,3-c]pyrimidine-3(7H)-thiones (11b)

Yield: 61%; m.p.: 288-290 ^{o}C; IR (KBr) υ (cm^{-1}): 3314(NH), 1652(C=S), 1567 (C=N); MS (ESI) m/z: 453.44 (M^{+}, ^{35}Cl, 5%), 455.50 (M^{+}+2, ^{37}Cl, 1.7%); ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 7.0-7.90 (m, 14H, Ar-H), 8.25 (s, 1H, C5-H), 11.98 (s, 1H, NH, D_2O exchangeable); Anal. Calcd for $C_{25}H_{16}ClN_5S$ (453.95): C, 66.15; H, 3.55; N, 15.43%. Found: C, 66.32; H, 3.24; N, 15.56%.

7-(4-Chlorophenyl)-8,9-diphenyl-2H-pyrrolo[3,2-e][1,2,4]triazolo[4,3-c]pyrimidine-3(7H)-thiones (11c)

Yield: 63%; m.p.: 280-282 ^{o}C; IR (KBr) υ (cm^{-1}): 3314(NH), 1652(C=S), 1567 (C=N); MS (ESI) m/z: 453.61 (M^{+}, ^{35}Cl, 6%), 455.59 (M^{+}+2, ^{37}Cl, 2.1%); ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 6.99-7.83 (m, 14H, Ar-H), 8.03 (s, 1H, C5-H), 11.96 (s, 1H, NH, D_2O exchangeable); Anal. Calcd for $C_{25}H_{16}ClN_5S$ (453.95): C, 66.15; H, 3.55; N, 15.43%. Found: C, 66.41; H, 3.78; N, 15.64%.

General procedure for the synthesis of 4-(2-(Aryl)-hydrazinyl)-5,6-diphenyl-7-substituted-7H-pyrrolo[2,3-d]pyrimidines (12a-i):

A mixture of the appropriate hydrazine **8a-c** (10 mmol) and the appropriate aromatic aldehyde (10 mmol) was heated under reflux in absolute ethanol for 8 h, cooled, poured onto ice water to give precipitates which were filtered off, dried, and recrystallized from methanol to give compounds **12a-i**.

4-(2-Benzylidenehydrazinyl)-7-cyclohexyl-5,6-diphenyl-7H-pyrrolo[2,3-d]pyrimidines (12a)

Yield: 50%; m.p.: 253-255 ^{o}C; IR (KBr) υ (cm^{-1}): 3344(NH), 1586 (C=N); MS (ESI) m/z: 471 (M^{+}, 6%); ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 1.42-1.97 (m, 10H, cyclohexyl), 3.89 (m, 1H, CH-N cyclohexyl), 7.07-7.87 (m, 15H, Ar-H), 8.07 (s, 1H, C2-H), 8.86 (s, 1H, N=CH), 9.75 (s, 1H, NH, D_2O exchangeable); Anal. Calcd for $C_{31}H_{29}N_5$ (471.60): C, 78.95; H, 6.20; N, 14.85%. Found: C, 78.70; H, 6.47; N, 15.04%.

7-Cyclohexyl-5,6-diphenyl-4-(2-(4-methoxybenzylidene)hydrazinyl)-7H-pyrrolo[2,3-d]pyrimidines (12b)

Yield: 54%; m.p.: 270-272 ^{o}C; IR (KBr) υ (cm^{-1}): 3411(NH), 1611 (C=N); MS (ESI) m/z: 502.62 (M^{+}+1H, 15%); ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 1.41-1.99 (m, 10H, cyclohexyl), 3.8 (m, 1H, CH-N cyclohexyl), 4.04 (s, 3H, OCH3), 7.11-7.86 (m, 14H, Ar-H), 8.10 (s, 1H, C2-H), 8.57 (s, 1H, N=CH), 9.80 (s, 1H, NH, D_2O exchangeable); Anal. Calcd for $C_{32}H_{31}N_5O$ (501.62): C, 76.62; H, 6.23; N, 13.96 %. Found: C, 76.84; H, 6.59; N, 13.75%.

4-(2-(4-Chlorobenzylidene)hydrazinyl)-7-Cyclohexyl-5,6-diphenyl-7H-pyrrolo[2,3-d]pyrimidines (12c)

Yield: 52%; m.p.: 279-281 ^{o}C; IR (KBr) υ (cm^{-1}): 3339(NH), 1594 (C=N); MS (ESI) m/z: 506.60 (M^{+}, ^{35}Cl, 20%), (M^{+}+2, ^{37}Cl, 6.63%); ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 1.45-1.99 (m, 10H, cyclohexyl), 3.91 (m, 1H, CH-N cyclohexyl), 7.10-7.90 (m, 14H, Ar-H), 8.15 (s, 1H, C2-H), 8.64 (s, 1H, N=CH), 9.95 (s, 1H, NH, D_2O exchangeable); Anal. Calcd for $C_{31}H_{28}ClN_5$ (506.04): C, 73.58; H, 5.58; N, 13.84 %. Found: C, 73.31; H, 5.41; N, 13.59 %.

4-(2-Benzylidenehydrazinyl)-7-(3-chlorophenyl)-5,6-diphenyl-7H-pyrrolo[2,3-d]pyrimidines (12d)

Yield: 51%; m.p.: 276-278 ^{o}C; IR (KBr) υ (cm^{-1}): 3315(NH), 1619 (C=N); MS (ESI) m/z: 499.86 (M^{+}, ^{35}Cl, 10%), 501.99(M^{+}+2, ^{37}Cl, 3.25%); ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 6.99-7.91 (m, 19H, Ar-H), 8.15 (s, 1H, C2-H), 8.71 (s, H, N=CH), 10.03 (s, H, NH, D_2O exchangeable); Anal. Calcd for $C_{31}H_{22}ClN_5$ (499.99): C, 74.47; H, 4.43; N, 14.01 %. Found: C, 74.76; H, 4.69; N, 13.80 %.

7-(3-Chlorophenyl)-5,6-diphenyl-4-(2-(4-methoxybenzylidene)hydrazinyl)-7H-pyrrolo[2,3-d]pyrimidines (12e)

Yield: 53%; m.p.: 263-265 ^{o}C; IR (KBr) υ (cm^{-1}): 3350(NH), 1626 (C=N), 1230(C-O); MS (ESI) m/z: 529.35(M^{+}, ^{35}Cl, 7%), 531.40 (M^{+}+2, ^{37}Cl, 2.35%); ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 3.85 (s, 3H, OCH_3), 7.12-7.89 (m, 18H, Ar-H), 8.19 (s, 1H, C2-H), 8.62 (s, H, N=CH), 9.88 (s, H, NH, D_2O exchangeable); Anal. Calcd for $C_{32}H_{24}ClN_5O$ (530.02): C, 72.51; H, 4.56; N, 13.21 %. Found: C, 72.82; H, 4.25; N, 13.01 %.

4-(2-(4-Chlorobenzylidene)hydrazinyl)-7-(3-chlorophenyl)-5,6-diphenyl-7H-pyrrolo[2,3-d]pyrimidines (12f)

Yield: 55%; m.p.: 269-271 ^{o}C; IR (KBr) υ (cm^{-1}): 3290(NH), 1612 (C=N); MS (ESI) m/z: 534.34 (M^{+}, ^{35}Cl, 13%), 536.30 (M^{+}+2, ^{37}Cl, 8.7%), 538.37 (M^{+}+4, Cl^{37}, 4.35%); ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 7.0-7.95 (m, 18H, Ar-H), 8.26 (s, 1H, C2-H), 8.71 (s, H, N=CH), 10.01 (s, H, NH, D_2O exchangeable); Anal. Calcd for $C_{31}H_{21}Cl_2N_5$ (534.44): C, 69.67; H, 3.96; N, 13.10 %. Found: C, 69.91; H, 4.20; N, 13.29 %.

4-(2-Benzylidenehydrazinyl)-7-(4-chlorophenyl)-5,6-diphenyl-7H-pyrrolo[2,3-d]pyrimidines (12g)

Yield: 52%; m.p.: 284-286 ^{o}C; IR (KBr) υ (cm^{-1}): 3315(NH), 1619 (C=N); MS (ESI) m/z: 500 (M^{+}, ^{35}Cl, 9%), 502.1 (M^{+}+2, ^{37}Cl, 3%); ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 6.90-7.86 (m, 19H, Ar-H), 8.0 (s, 1H, C2-H), 8.69 (s, H, N=CH), 10.0 (s, H, NH, D_2O exchangeable); Anal. Calcd for $C_{31}H_{22}ClN_5$ (499.99): C, 74.47; H, 4.43; N, 14.01 %. Found: C, 74.60; H, 4.21; N, 14.31%.

7-(4-Chlorophenyl)-5,6-diphenyl-4-(2-(4-methoxybenzylidene)hydrazinyl)-7H-pyrrolo[2,3-d]pyrimidines (12h)

Yield: 56 %; m.p.: 277-279 ^{o}C; IR (KBr) υ (cm^{-1}): 3350(NH), 1626 (C=N), 1230(C-O); MS (ESI) m/z: 529.48 (M^{+}, ^{35}Cl, 5%), 531.5 (M^{+}+2, ^{37}Cl, 1.70%); ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 3.83 (s, 3H, OCH_3), 7.04-7.89 (m, 18H, Ar-H), 8.08 (s, 1H, C2-H), 8.54 (s, H, N=CH), 9.90 (s, H,

NH, D_2O exchangeable); Anal. Calcd for $C_{32}H_{24}ClN_5O$ (530.02): C, 72.51; H, 4.56; N, 13.21 %. Found: C, 72.73; H, 4.30; N, 13.47%.

4-(2-(4-Chlorobenzylidene)hydrazinyl)-7-(4-chlorophenyl)-5,6-diphenyl-7H-pyrrolo[2,3-d]pyrimidines (12i)

Yield: 54%; m.p.: 281-283 ^oC; IR (KBr) υ (cm^{-1}): 3290(NH), 1612 (C=N); MS (ESI) m/z: 534.56 (M^+, ^{35}Cl, 11%), 536.60 (M^++2, ^{37}Cl, 7.35%), 538.58 (M^++4, ^{37}Cl, 3.64%); ^1H NMR (DMSO-d6, 300 MHz) δ (ppm): 7.14-7.94 (m, 18H, Ar-H), 8.12 (s, 1H, C2-H), 8.72 (s, H, N=CH), 10.01 (s, H, NH, D_2O exchangeable); Anal. Calcd for $C_{31}H_{21}Cl_2N_5$ (534.44): C, 69.67; H, 3.96; N, 13.10 %. Found: C, 69.45; H, 3.70; N, 12.88%.

General procedure for the synthesis of 4-(3,5-dimethyl-1H-pyrazol-1-yl)-5,6-diphenyl-7-substituted-7H-pyrrolo[2,3-d]pyrimidines (13a-c):

A mixture of the appropriate hydrazine **8a-c** (10 mmol) and acetyl acetone (1 mL, 10 mmol) in absolute ethanol was heated under reflux for 6h, cooled, poured onto ice/water to give a precipitate which was filtered off, dried and recrystallized from ethanol to yield compounds **13a-c**.

7-Cyclohexyl-4-(3,5-dimethyl-1H-pyrazol-1-yl)-5,6-diphenyl-7H-pyrrolo[2,3-d]pyrimidines (13a)

Yield: 71%; m.p.: 269-271 ^oC; IR (KBr) υ (cm^{-1}): 1606 (C=N); MS (ESI) m/z: 447 (M^+, 8%); ^1H NMR (DMSO-d6, 300 MHz) δ (ppm): 1.41-1.96 (m, 10H, cyclohexyl), 2.42 (s, 3H, CH_3), 2.58 (s, 3H, CH_3), 3.93 (m, 1H, CH-N cyclohexyl), 6.84 (s, 1H, pyrazole), 7.10-7.74 (m, 10H, Ar-H), 8.90 (s, 1H, C2-H); Anal. Calcd for $C_{29}H_{29}N_5$ (447.57): C, 77.82; H, 6.53; N, 15.65 %. Found:C, 77.58; H, 6.25; N, 15.88 %.

7-(3-Chlorophenyl)-4-(3,5-dimethyl-1H-pyrazol-1-yl)-5,6-diphenyl-7H-pyrrolo[2,3-d]pyrimidines (13b)

Yield: 73%; m.p.: 275-277 ^oC; IR (KBr) υ (cm^{-1}): 1588 (C=N); MS (ESI) m/z: 475.88 (M^+, ^{35}Cl, 5%), 477.91 (M^++2, ^{37}Cl, 1.68%); ^1H NMR (DMSO-d6, 300 MHz) δ (ppm): 2.40 (s, 3H, CH_3), 2.60

(s, 3H, CH_3), 6.86 (s, 1H, pyrazole), 7.02-7.81 (m, 14H, Ar-H), 8.76 (s, 1H, C2-H); Anal. Calcd for $C_{29}H_{22}ClN_5$ (475.97): C, 73.18; H, 4.66; N, 14.71 %. Found:C, 73.41; H, 4.38; N, 14.93%.

7-(4-Chlorophenyl)-4-(3,5-dimethyl-1H-pyrazol-1-yl)-5,6-diphenyl-7H-pyrrolo[2,3-d]pyrimidines (13c)

Yield: 75 %; m.p.: 280-282 ^oC; IR (KBr) υ (cm^{-1}): 1588 (C=N); MS (ESI) m/z: 475 (M^+, ^{35}Cl, 10%), 477 (M^++2, ^{37}Cl, 3.5%); ^1H NMR (DMSO-d6, 300 MHz) δ (ppm): 2.40 (s, 3H, CH_3), 2.59 (s, 3H, CH_3), 6.87 (s, 1H, pyrazole), 7.06-7.82 (m, 14H, Ar-H), 8.79 (s, 1H, C2-H); Anal. Calcd for $C_{29}H_{22}ClN_5$ (475.97): C, 73.18; H, 4.66; N, 14.71 %. Found: C, 73.35; H, 4.82; N, 14.50%.

General procedure for the synthesis of 8,9-diphenyl-7-substituted-2H-pyrrolo[3,2-e][1,2,4]triazolo[4,3-c]pyrimidin-3(7H)-one (14a-c):

A solution of the appropriate hydrazine **8a-c** (10 mmol) in pyridine (10 mL) was cooled in ice bath and equimolar amount (1 mL, 10 mmol) of ethyl chloroformate was added portion wise. Then the mixture was heated under reflux for 5h, cooled, poured onto ice/water and neutralized with HCl to give a precipitate which was filtered off, dried and recrystallized from ethanol to yield compounds **14a-c**.

7-Cyclohexyl-8,9-diphenyl-2H-pyrrolo[3,2-e][1,2,4]triazolo[4,3-c]pyrimidin-3(7H)-one (14a)

Yield: 59%; m.p.: 261-263 ^oC; IR (KBr) υ (cm^{-1}): 3394(NH), 1659(C=O), 1585 (C=N); MS (ESI) m/z: 410.54 (M^+, 10%); ^1H NMR (DMSO-d6, 300 MHz) δ (ppm): 1.40-1.97 (m, 10H, cyclohexyl), 3.93 (m, 1H, CH-N cyclohexyl), 7.12-7.70 (m, 10H, Ar-H), 8.11 (s, 1H, C5-H), 12.06 (s, 1H, NH, D_2O exchangeable); Anal. Calcd for $C_{25}H_{23}N_5O$ (409.48): C, 73.33; H, 5.66; N, 17.10 %. Found: C, 73.09; H, 5.40; N, 17.35 %.

7-(3-Chlorophenyl)-8,9-diphenyl-2H-pyrrolo[3,2-e][1,2,4]triazolo[4,3-c]pyrimidin-3(7H)-one (14b)

Yield: 60 %; m.p.: 273-275 ^oC; IR (KBr) υ (cm^{-1}): 3402(NH), 1670(C=O), 1559 (C=N); MS (ESI) m/z: 438.44 (M^+ ^{35}Cl, 6.9%), 440(M^++2, ^{37}Cl, 2.3%); ^1H NMR (DMSO-d6, 300 MHz) δ (ppm): 7.04-

7.98 (m, 14H, Ar-H), 8.21 (s, 1H, C5-H), 12.04 (s, 1H, NH, D_2O exchangeable); Anal. Calcd for $C_{25}H_{16}ClN_5O$ (437.88): C, 68.57; H, 3.68; N, 15.99 %. Found: C, 68.29; H, 3.91; N, 16.20 %.

7-(4-Chlorophenyl)-8,9-diphenyl-2H-pyrrolo[3,2-e][1,2,4]triazolo[4,3-c]pyrimidin-3(7H)-one (14c)

Yield: 62%; m.p.: 278-280 ^oC; IR (KBr) υ (cm^{-1}): 3402(NH), 1670(C=O), 1559 (C=N); MS (ESI) m/z: 438.46 (M^+ ^{35}Cl, 24%), 440 (M^++2, ^{37}Cl, 8%); ^1H NMR (DMSO-d6, 300 MHz) δ (ppm): 7.07-7.94 (m, 14H, Ar-H), 8.05 (s, 1H, C5-H), 12.03 (s, 1H, NH, D_2O exchangeable); Anal. Calcd for $C_{25}H_{16}ClN_5O$ (437.88): C, 68.57; H, 3.68; N, 15.99 %. Found: C, 68.34; H, 3.40; N, 15.75%.

Antiviral screening

Cell culture

The cell line for Huh 7.5 was obtained from the Holding Company for Biological Products and Vaccines, VACSERA, Egypt. Cell line was routinely cultured in 96 multiwell plates (Greiner-Bio One, Germany) in DMEM (GIBCO BRL), at 37°C in a humidified incubator of 5% (v/v) CO_2.

Cytotoxicity test

The cytotoxicity test was performed according to methods reported [46,47]. Briefly, all samples (50 mg) were dissolved in 1 mL DMSO. Decontamination of samples was done by adding 24 µL of 100^x of antibiotic-antimycotic mixture to 1 mL of each sample. Then, bi-fold dilutions were done to 100 µL of original dissolved samples and 100 µL of each dilutions were inoculated in Huh 7.5 cell line to estimate the non-toxic dose of the tested samples. Cytotoxicity assay was done using cell morphology evaluation by inverted light microscope and cell viability test applying trypan blue dye exclusion method.

Cell morphology evaluation by inverted light microscopy

Huh 7.5 cell cultures ($2x10^5$ cells/mL) were prepared separately in 96-well tissue culture plates (Greiner-Bio one, Germany) and incubated for 24 h at 37°C in humidified 5% (v/v) CO2 atmosphere. Confluent cell monolayers were formed, the medium was removed from each well and replenished with 100µL of bi-fold dilutions of different samples tested prepared in DMEM (GIBCO BRL). For cell controls 100mL of DMEM without samples was added. All cultures were incubated at 37°C in a humidified 5%(v/v) CO2 atmosphere for 72 h. Cell morphology was observed daily for microscopically detectable morphological alterations, such as loss of confluence, cell rounding and shrinking, cytoplasm granulation an vacuolization. Morphological changes were scored [48].

Cell Viability Assay

It was done by trypan blue dye exclusion method [49]. Huh 7.5 cell cultures (2×10^5 cells/mL) were grown in 12-well tissue culture plates (Greiner-Bio one, Germany). After 24 h incubation, the same assay described above for tested samples cytotoxicity was followed by applying 100 µL of tested samples dilutions (bi-fold dilutions) per well. After 72 h, the medium was removed, cells were trypsinized and an equal volume of 0.4% (w/v) trypan blue dye aqueous solution was added to cell suspension. Viable cell were counted under the phase contrast microscope.

Determination of antiviral effect of tested samples against HCV genotype 4a replicon (ED-43/SG-feo (VYG) replicon)

ED-43/SG-feo (VYG) replicon of HCV genotype 4a was obtained from the lab. Of Prof. Dr. Charles Rice, The Rockefeller University, USA. 100 µL of non-toxic dose of the tested samples or PBS plus 20 µL of different doses of infectious HCV particles (1×10^3, 1×10^4, 1×10^5) was inoculated per well in Huh 7.5 cell lines. Change medium after one day incubation. HCV RNA was quantified as

initial titers and after treatment with the nontoxic doses of tested samples 72 h later using immunofluorescence technique [50].

Data Analysis

IC_{50} for each compound was obtained from dose-effect-curves. The IC_{50} is the concentration of the compound that causes 50% inhibition of the virus. The-dose-effect curve was plotted from the average of three assays with 5 concentrations within the inhibitory range of the compound.

Molecular Docking Studies with MOE 2014.09

All compounds were built and saved as moe using MOE 2014.09 [51]. Rigid receptor was used as a docking protocol. Both receptor-solvent were kept as a ‘’receptor’’. Triangle matcher was used as a placement method. Two rescoring were computed, rescoring 1 was selected as London dG. Rescoring 2 was selected as affinity. Force field was used as a refinement.

Molecular Docking Studies with Leadit 2.1.2

All compounds were built and saved as Mol2 using Leadit 2.1.2 [52]. The crystal structure of HCV RdRp enzyme complex with the inhibitor was downloaded from protein data bank (pdb code = 3GOL) [53]. The protein was loaded into Leadit 2.1.2 and the receptor components were chosen by selection of chain A as a main chain which in complexed with inhibitor. Binding site was defined by choosing the inhibitor as a reference ligand to which all coordinates were computed. Amino acids within radius 6.5 Å were selected in the binding site. All chemical ambiguities of residues left as default. Ligand binding was driven by enthalpy (classic Triangle matching). For scoring, all default settings were restored. Intra-ligand clashes were computed by using clash factor = 0.6. Maximum number of solutions per iteration = 200. Maximum of solution per fragmentation = 200. The base placement method was used as a docking strategy.

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Legends:

Scheme 1: Synthesis of compounds 1a-c – 4a-c.

(i) toluene, HCl; (ii) $CH_2(CN)_2$, pyridine; (iii) HCOOH; (iv)Ac_2O; (v) AcOH/HCl(3:1).

Scheme 2: Synthesis of compounds 5a-c – 8a-c.

(i) $POCl_3$; (ii) $CS(NH_2)_2$, EtOH; (iii) $R'NH_2$,TEA, EtOH; (iv) $NH_2NH_2.H_2O$ (99%), EtOH.

Scheme 3: Synthesis of compounds 9a-c – 14a-c.

(i) HCOOH; (ii) Ac_2O; (iii) CS_2, EtOH; (iv) R'CHO, EtOH; (v) $(CH_3CO)_2CH_2$, EtOH; (vi) ClCOOEt, pyridine.

Table 1: CC_{50} and IC_{50} for the synthesized compounds

Table 2: Molecular model assessment scores

Figure 1: 3D structure of RdRp of HCV
The figure shows the different structural domains of the RdRp: fingers, thumb and palm. It shows also the Mg^{2+} ion essential for enzymatic activity.

Figure 2: HCV antiviral drugs
Figure 2a shows NS5B nucleoside inhibitors as anti HCV drugs
Figure 2b shows NS5B non-nucleoside inhibitors as anti HCV drugs

Figure 3: Antiviral activity of non-toxic doses of tested compounds 5c, 7f, 7j, 12f and 12i against HCVcc genotype 4a. Activity was measured as viral inhibition. Each experiment was done in triplicate, error bars indicates the SE. **7j** was the most potent compound and show significantly different activity from **5c**, **7f, 12f** and **12i**. There was no significant difference in activity between **5c**, **7f, 12f** and **12i**. ** P > 0.01

Figure 4: SAR of the newly synthesized Pyrrolopyrimidine derivatives.
The replacement of 4-chloro group of compound **5c** with 4-(o-toloyl)-amino group (in compound **7j**) led to the maximum virus inhibition, while the replacement of 7-(4-chlorophenyl) (in compound **5c**) with 3-chlorophenyl (in compound **5b**) led to considerable loss of the activity. The replacement of 7-(4-chloro-phenyl) (in compounds **7j** and **12i**) with 7-(3-chloro-phenyl) (in compounds **7f** and **12f**) reduces the activity. The replacement of 4-(o-toloyl)-amino group (in compounds **7f** and **7j**) with 4-chlorobenzylidene-hydrazinyl group (in compounds **12f** and **12i**) reduces the activity.

Figure 5: Molecular model for binding of 7J

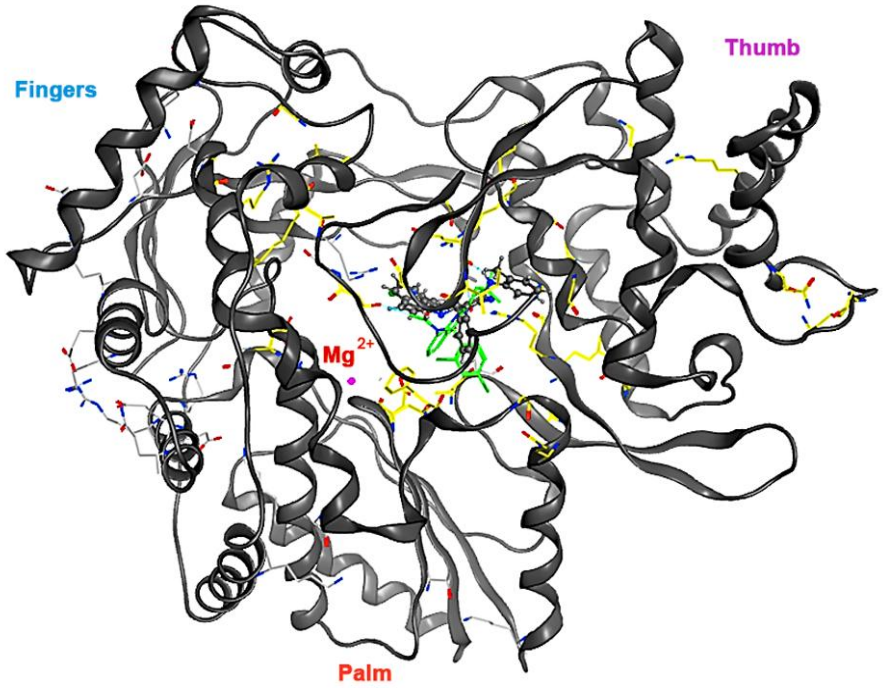
A) Binding mode of compound **7j** forming a hydrogen bond with Gln 446.

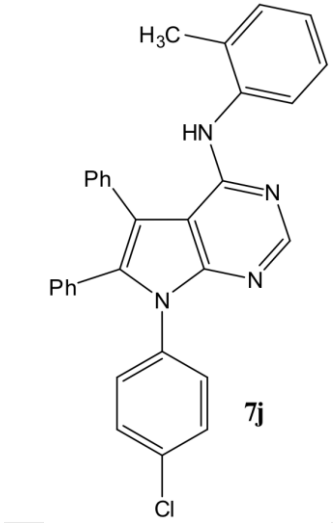
B) The placement of compound **7j** within the same site of the reported inhibitor.

Figure 6: Suggested-binding modes of compounds 5c, 7f, 7j, 12f and 12i.

A) The figure shows the binding of compound 5c. A hydrogen bond is formed with Tyr 448. B) The figure shows binding of compound 7f. A hydrogen bond is formed with Gln 446. C) The figure shows the binding of compound 7j. Two hydrogen bonds are formed with Gln 446. D) The figure shows the binding of compound 12f. Hydrogen bonds are formed with Gln 446 and Gly 449. E) The figure shows the binding of compound 12i. Hydrogen bonds are formed with Tyr 448.

Graphical Abstract





7j