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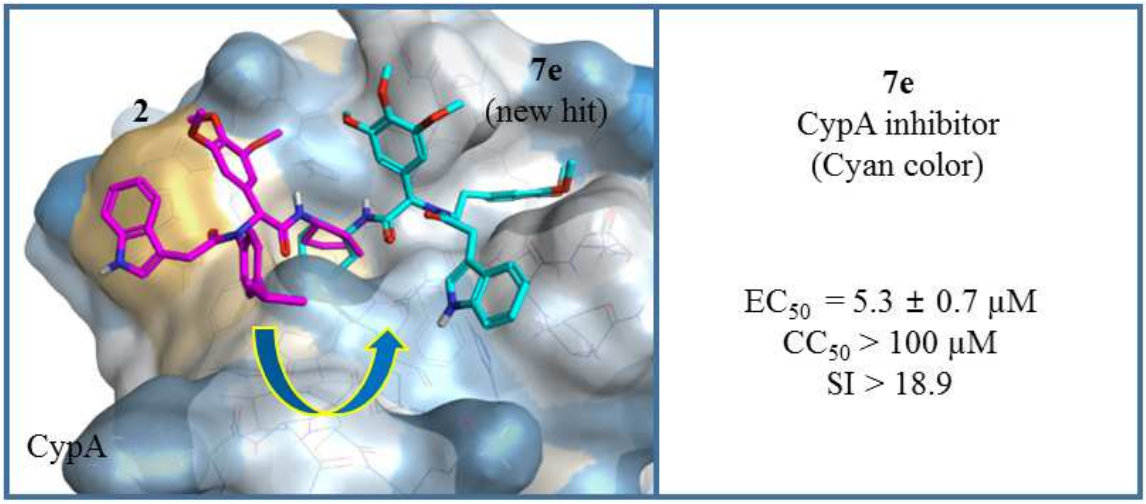
Graphical abstract



Molecular design, synthesis, and biological evaluation of bisamide derivatives as cyclophilin A inhibitors for HCV treatment

Jinhe Han [a,1], Hye Won Lee [b,1,†], Yifeng Jin [a], Daulat B. Khadka [a], Suhui Yang [a], Xiaoli Li [a], Meehyein Kim [b,**], Won-Jea Cho [a,*]

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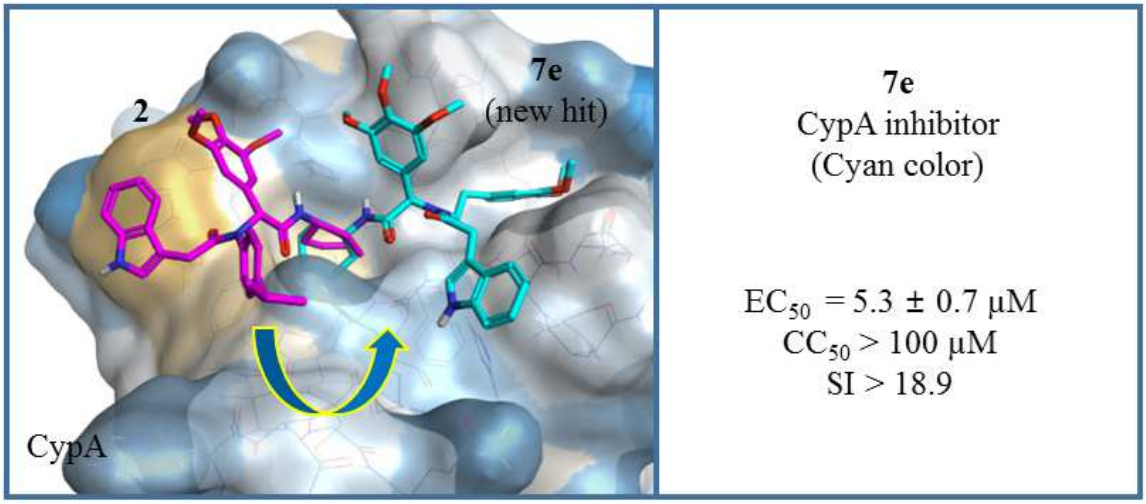
ABSTRACT

Hepatitis C virus (HCV) is a major cause of end-stage liver diseases. Direct-acting antivirals (DAAs), including inhibitors of nonstructural proteins (NS3/4A protease, NS5A, and NS5B polymerase), represent key components of anti-HCV treatment. However, some DAAs are associated with increased drug resistance and undesired side effects. Previous reports have shown that bisamides could be a novel class of cyclophilin A (CypA) inhibitors for treating HCV as a member of combinational therapies. To fully elucidate structure-activity relationships of bisamide derivatives and find a better hit compound with diverse binding modes, 16 biamides were designed with the help of docking program. They were then synthesized using one-pot four-component Ugi reaction. **7e** with selectivity index of more than 18.9 (50% effective concentration of 5.3 μM, but no cytotoxicity at 100 μM) and unique binding mode that could be dived into gatekeeper pocket was selected as a new hit compound. Surface plasmon resonance experiments revealed that **7e** is able to bind to CypA with a K_D of 3.66 μM. Taken together, these results suggest that **7e** as a CypA inhibitor could be used as an alternative anti-HCV agent in combinational therapy in the future.

Keywords:
Hepatitis C Virus, Cyclophilin A inhibitor, Bisamide, Ugi reaction, Molecular Modeling

Graphical abstract



Highlights

- A new group of bisamide derivatives, totally 16 compounds were designed and aimed to switch the binding mode against CypA.
- The EC_{50} and CC_{50} values of the newly synthesized compounds were helped to enrich the structure-activity relationships.
- Docking studies were proofed **7e** located into gatekeeper pocket with selectivity index of more than 18.9 (50% effective concentration of 5.3 μM, but no cytotoxicity at 100 μM).
- SPR results revealed that **7e** is able to bind to CypA with a K_D of 3.66 μM.

1. Introduction

Despite recent advances in treatment, hepatitis C virus (HCV) infection continues to be a burden of disease. Around 3% of the population are affected by HCV infection which leads to 300,000 deaths per year. Approximately 80% of infected patients develop chronic symptoms that can result in cirrhosis (27%) and hepatocellular carcinoma (25%) [1-3].

There are multiple strains of at least seven HCV genotypes (GTs) identified. Among them, GT 1a and GT 1b are well characterized clinically and molecular virologically. Different types of HCV infection need different treatment strategies [4, 5]. Before 2011, the standard treatment for HCV infection was mainly through a combination of pegylated interferon (pegIFN) alpha and ribavirin (RBV) for 24 to 48 weeks depending on HCV genotype [6]. This combination therapy can produce a sustained virologic response (SVR) rate of 40% to 50%. However, both regimens in this combination therapy have unwanted and intolerable side effects [7-9].

To improve the problem of side effects caused by the double combination therapy, telaprevir and boceprevir (NS3/4A protease inhibitors), the first two direct-acting antivirals (DAAs), were developed. When used in therapeutic combination, they could dramatically increase viral clearance rate by around 70% compared to the effect of double combination of pegIFN and ribavirin [10, 11]. However, these newly developed DAAs still possess several drawbacks including cumbersome dosing regimens, strict dietary requirements, and unfavorable adverse effect profiles. With the discovery of additional DAAs, therapy without using pegIFN became possible for curing all HCV genotypes while prominently improving unwanted side effects [12, 13].

In the next few years, several new DAAs were additionally developed and approved by the FDA as alternative regimens to supplement current combination therapies, including glecaprevir, grazoprevir (NS3/4A inhibitors), daclatasvir, and elbasvir (NS5A inhibitors) [14]. Combined DAAs therapy has higher efficacy than their monotherapy. Meanwhile, several recommended combination therapies such like sofosbuvir (an NS5B polymerase inhibitor) and simeprevir (an NS3 protease inhibitor) approved by FDA already put DAAs into the arsenal as new regimens to treat HCV [14]. However, ensuing drug-drug interaction has become an issue to be solved. For example, sofosbuvir plus pegIFN or RBV have been reported to cause the most common drug-drug interaction that leads patients to have fatigue, headache, nausea, insomnia, and anemia [15, 16]. To avoid flaws of current combination therapies including simultaneous viral mutations, drug-drug interactions, and/or drug resistance, a new class of anti-HCV agents with potential to replace existing regimen should be developed. In the present, we suggest that cyclophilin A (CypA) inhibitors could be one of acceptable options.

CypA plays critical roles in many biological signal networks. As a candidate of cellular peptidyl-prolyl cis-trans isomerase (PPIase), it is involved in cellular cycles such as protein folding and trafficking [17]. The first CypA inhibitors that showed antiviral activities were cyclosporine A (CsA) (Fig. 1) and sanglifehrin A, both of which were isolated from natural products [18]. Up to date, most of existing Cyp inhibitors are derived from these two natural products, sharing the same scaffold. Blockage of dephosphorylation-driven nuclear translocation of nuclear factor of activated T cells (NFAT) by CsA-CypA complex suggests that CsA can be an immunosuppressant, but not an anti-HCV agent [19]. Many non-immunosuppressive CsA analogs such as CPI-431-32

[20] and SCY-635 [21] have been developed. Due to disadvantages of such large CsA-like structures, including high risk

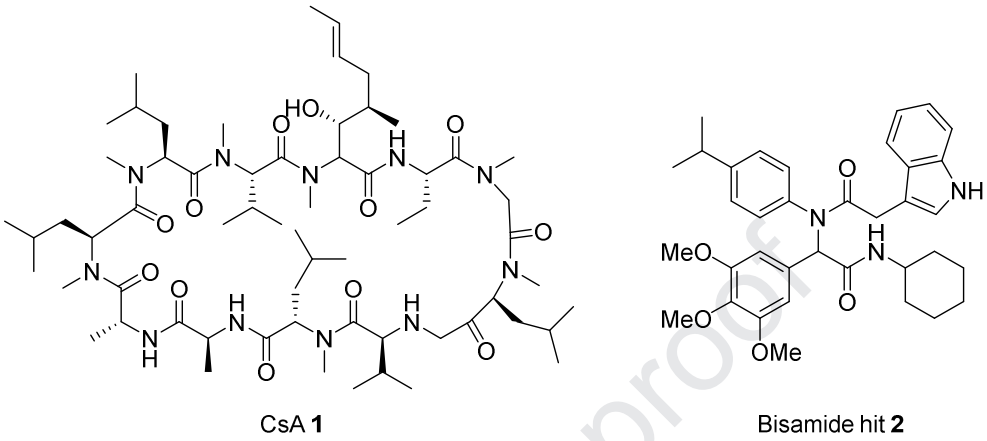


Fig. 1. Chemical structures of CsA **1** and bisamide hit **2**.

of having clinical safety profiles, complex synthetic route, and high cost [22], small synthetic molecules as new anti-HCV agents have been developed [20, 23-25].

Our previous research has reported that bisamide analogs as CypA inhibitors possess potent antiviral activity against HCV without cytotoxicity at a maximum concentration tested [26]. Bisamide hit **2** (Fig. 1) inhibited HCV replication but not mediated by immunosuppressive effects. The objective of the present study was to further investigate the structure-activity relationship of the bisamide analogs. New derivatives were synthesized and their anti-HCV properties were tested using a JFH1-derived infectious clone.

2. Results and Discussion

2.1. Design of bisamides

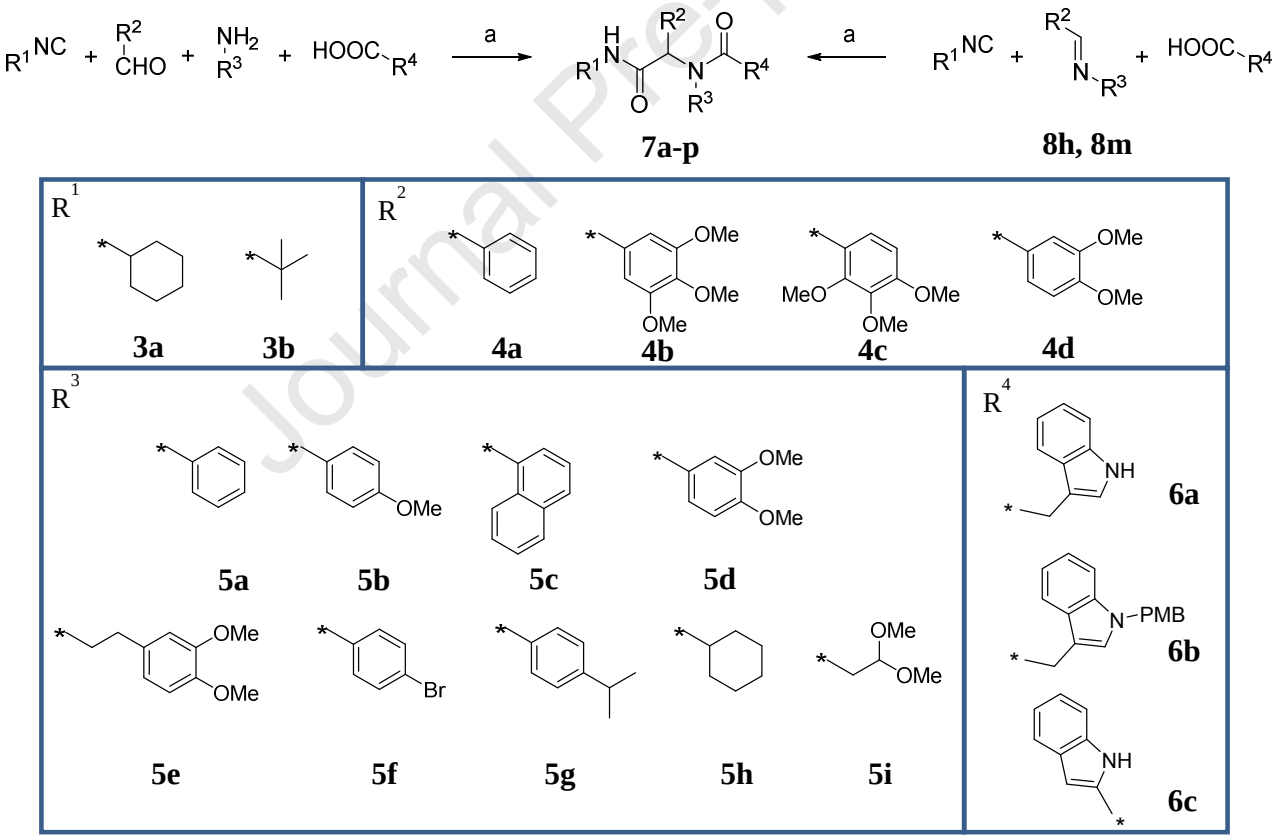
Our previous report has revealed that bis-amides can be novel CypA inhibitors by possessing selectivity without showing immunosuppressive effects. In addition, they are structurally highly different from current CsA-based inhibitors. Particularly, hit compound **2** had strong interactions with Arg55, Trp121, and Lys 125 of CypA through H-bonds by oxygen atoms of trimethoxyphenyl group as shown in our published report [26]. By investigating the binding mode of other bisamides reported in the previous studies, we found that the cyclohexyl moiety all occupied in the catalytic pocket. Meanwhile, hydrophobic interaction generated by the indolylmethyl moiety also seemed to be crucial for the binding affinity of bisamides derivatives to CypA.

Since our aim was to optimize bisamide structure by altering substituents so that a promising target compound could located in both of the two hydrophobic regions (the catalytic pocket and the gatekeeper pocket), the main strategy was to reserve the indole moiety and the cyclohexyl moiety while changing the rest of the compound. In this report, we mainly focused on bis-amides with di- or tri- methoxy groups as substitutions for R^2 moiety.

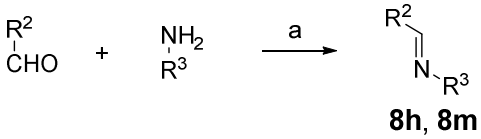
2.2. Chemistry

In the present study, bisamide derivatives were synthesized by the four-component Ugi reaction according to the first published report [27]. The possible mechanism is that the reaction starts from the formation of an imine between amine and aldehyde with a loss of one equivalent of water. The formed imine intermediate was protonated by the carboxylic acid to form iminium ion. Then nucleophilic addition between the iminium ion and isocyanide gives the nitrilium ion which is then reacted with carboxylic acid by another

nucleophilic addition. The final rearrangement called a Mumm rearrangement takes place with the transfer of acyl group from oxygen to nitrogen. Rearrangement is the only non-reversible reaction among all steps. It is considered a critical step that drives all reaction sequences. Because of the one-pot reaction property, the Ugi reaction gives the possibility to synthesize a large number of bisamide derivatives just by using various aldehydes, amines, isocyanides, and carboxylic acids. The reaction also has an easy workup procedure with high efficiency. Sixteen bis-amides were prepared by the Ugi reaction using methanol or 2,2,2-trifluoroethanol at room temperature (RT) or 55 °C (Schemes 1 and 2).



Scheme 1. Ugi multi-component reaction. (a) MeOH or 2,2,2-trifluoroethanol, room temperature or 55 °C.



8h, **8m**

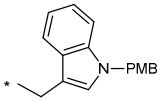
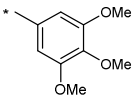
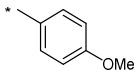
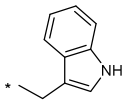
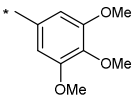
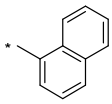
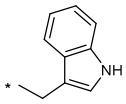
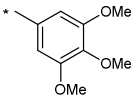
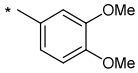
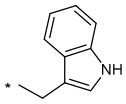
Scheme 2. Synthesis of imines **8h** and **8m**. (a) EtOH or MeOH or 2,2,2-trifluoroethanol, room temperature.

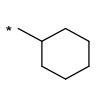
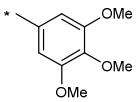
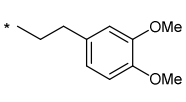
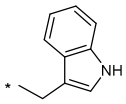
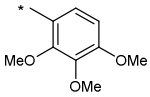
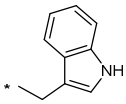
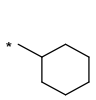
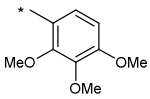
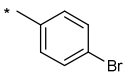
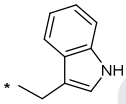
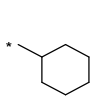
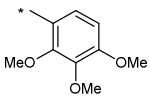
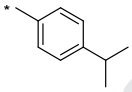
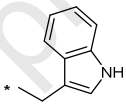
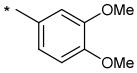
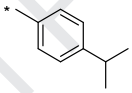
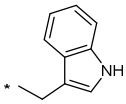
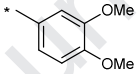
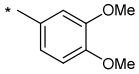
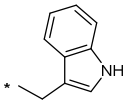
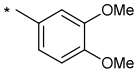
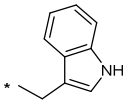
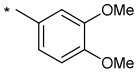
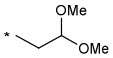
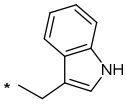
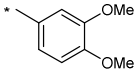
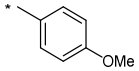
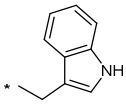
2.3. Evaluation of antiviral activity and cytotoxicity

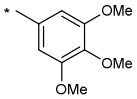
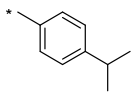
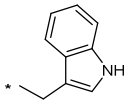
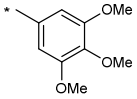
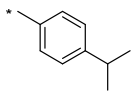
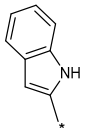
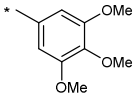
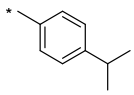
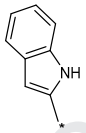
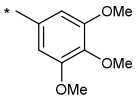
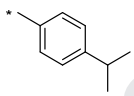
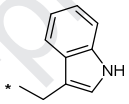
To test cytotoxicity, Huh7.5 cells were treated with increasing concentrations of compounds and their half maximal cytotoxic concentrations (CC_{50}) were determined by the standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In parallel, half-maximal effective concentrations (EC_{50}) were calculated by *Renilla* luciferase assay from Huh7.5 cells which were infected with the JFH1-Luc virus and treated with different concentrations of bisamides derivatives [28]. **7a** (Table 1) with a PMB substitution on the amino group of the indole moiety at R^4 showed no antiviral activity under its subtoxic concentrations (CC_{50} = 24.4 ± 7.2 μM) (Table 1). Similarly, all three bisamide derivatives with 2,3,4-trimethoxyphenyl at R^2 position showed cytotoxicities but no antiviral activity (Table 1). Some bisamide derivatives with 3,4-dimethoxyphenyl at R^2 position were less toxic compared to those with 2,3,4-trimethoxyphenyl at R^2 position (Table 1). Notably, **7j** and **7m** displayed selectivity index of 10.5 and 8.6, respectively. Given that the hit compound **2** had EC_{50} of 5.2 ± 0.7 μM and CC_{50} > 100 μM, it could be suggested that both compounds, **7j** (EC_{50} = 5.3 ± 0.6 μM; CC_{50} = 55.3 ± 2.4 μM) and **7m** (EC_{50} = 3.8 ± 0.7 μM; CC_{50} = 32.7 ± 5.9 μM), maintained comparable anti-HCV activity.

Finally, among the bisamide derivatives with 3,4,5-trimethoxyphenyl at R^2 position, five compounds (**7b**, **7d**, **7e**, **7o**, **7p**) showed selectivity indices over 5.0 (Table 1). It is noteworthy that **7e** (EC_{50}, 5.3 ± 0.7 μM; CC_{50}, >100 μM), showed promising antiviral activity and no cytotoxicity at a maximum concentration tested. However, by only switching the cyclohexyl group to tert-butyl at R^1 position of the compound, **7n** (EC_{50}, 6.3 ± 3.0 μM; CC_{50}, 13.9 ± 0.1 μM) exhibited lower selectivity index compared with **7e,** mainly due to increase in cytotoxicity rather than enhancement of its antiviral effect (Table 1). After changing the indolylmethyl group of **7n** to indolyl group, the resulting **7o** partially alleviated the cytotoxicity problem, without affecting anti-HCV activity.

Table 1. Anti-HCV activity and cytotoxicity of bisamides.

Compd	R^1	R^2	R^3	R^4	EC_{50}[a] (μM)	CC_{50}[b] (μM)	SI[c]
7a					>24.4	24.4 ± 7.2	n.d.[d]
7b					5.0 ± 2.4	44.8 ± 4.0	9.0
7c					9.4 ± 4.6	33.2 ± 1.6	3.4
7d					10.2 ± 2.9	56.7 ± 1.4	5.5

7e					5.3 ± 0.7	> 100.0	>18.9
7f					>18.8	18.8 ± 0.4	n.d.
7g					>7.2	7.2 ± 0.2	n.d.
7h					>5.9	5.9 ± 0.1	n.d.
7i					2.0 ± 0.6	6.9 ± 0.1	3.5
7j					5.3 ± 1.0	55.3 ± 2.4	10.5
7k					>14.6	14.6 ± 0.7	n.d.
7l					>54.5	54.5 ± 4.6	n.d.
7m					3.8 ± 0.7	32.7 ± 5.9	8.6

7n					6.3 ± 3.0	13.9 ± 0.1	2.3
7o					5.9 ± 3.3	36.7 ± 4.7	6.2
7p					2.0 ± 1.4	19.4 ± 3.0	9.7
2					5.2 ± 0.7	> 100.0	>19.2
Ribavirin	-	-	-	-	>100.0	16.3 ± 3.0	>6.1
INF-α[e]	-	-	-	-	>100.0	0.31 ± 0.1	>322.6

[a] The half-maximal effective concentration. [b] The half-maximal cytotoxic concentration; [c] Selectivity index, the ratio of CC_{50} to EC_{50}. [d] Not determined. All experiments were performed in triplicate. [e] Treatment concentration unit is ng/mL.

2.4. Structure and activity relationship

To identify preferential elements of the bis-amide compound as an anti-HCV agent, structure and activity relationship study in cells was investigated from its newly synthesized derivatives and structural priority in each group was summarized (Fig. 2).

According to our previous report [26], the indole moiety in the R^4 of the hit compound **2** can generate a π- π interaction with Trp121 based on docking study. When a para-methoxybenzyl (PMB) group was introduced to the nitrogen of the indolylmethyl group, **7a** showed cytotoxicity against the tested cell line. As for R^4 position, the carbon number between the indole moiety and adjacent amide also showed a significant impact on both cytotoxicity and antiviral activity. When the indolylmethyl group of the compound **2** was switched to the indolyl group to generate **7p**, it led to a 2.5-fold increase in anti-HCV activity (EC_{50} values of 5.2 and 2.0 μM, respectively). Meanwhile, its cytotoxicity was also over 5-fold enhanced (CC_{50} values of >100 and 19.4 μM, respectively). Innovation with the cyclohexyl moiety at the R^1 of **7p** to the t-butyl group, resulting in **7o** caused selectivity index to decrease over 1.5-fold (from >9.7 to 6.2), indicating that the cyclohexyl group at this position is more favorable than the t-butyl group.

Antiviral effects and cytotoxicity seemed to vary primarily depending on the substituent groups of the phenyl ring at the R^2 position. The introduction of one more methoxy group at ortho-position of R^2 resulted in loss of anti-HCV activity and showed strong cytotoxicity (Table 1). Among all newly synthesized bis-amides, **7i** (EC_{50}, 2.0 μM) showed best anti-HCV activity. At the same time, strong cytotoxicity was observed by **7i** (CC_{50}, 6.9 μM). It was related to the deletion of one of methoxy groups in meta-position.

The antiviral activity seems to be less influenced by the substitution groups of the phenyl ring at the R^3 position. The three compounds, **7b** (with a 4-methoxy substituent), **7c** (with a naphthalene moiety) and **7d** (with 3,4-dimethoxy substituents) showed similar antiviral activity ranging from 5.0 to 10.2 μM as well as comparable cytotoxicity ranging from 33.2 to 56.7 μM. Intriguingly, **7e** with more distance by inserting two carbons

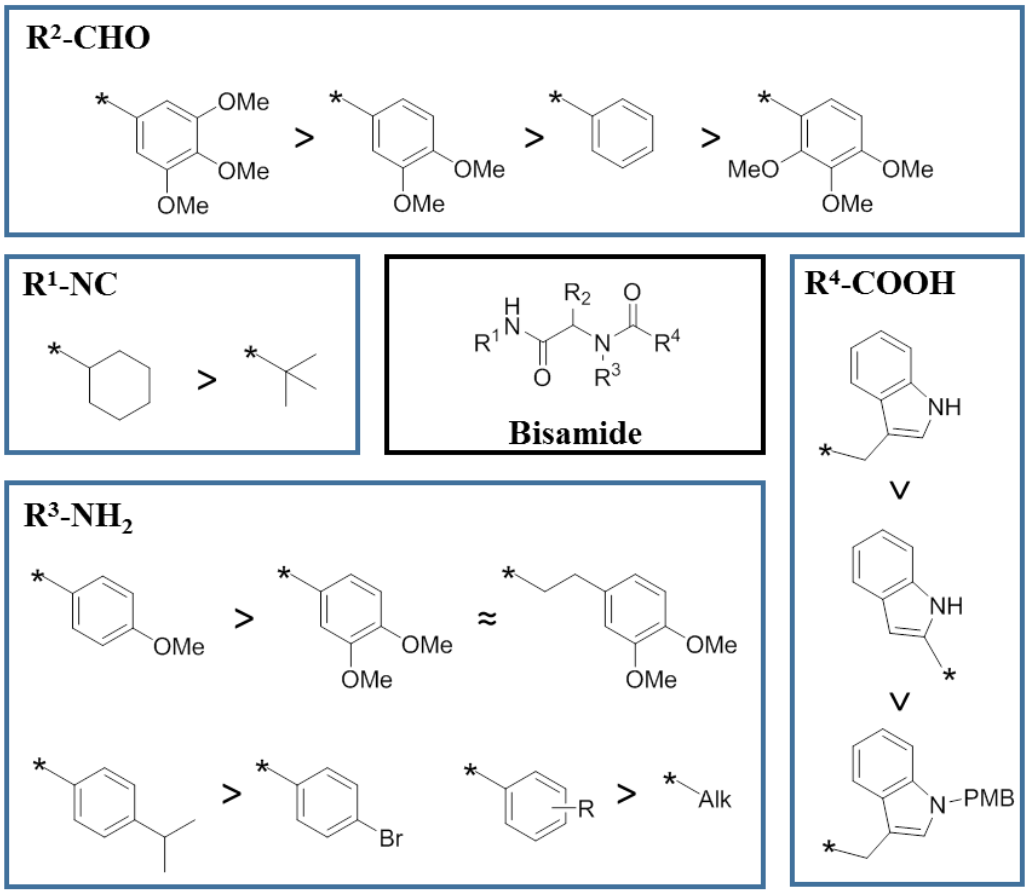


Fig. 2. Structure-activity relationship (SAR) of newly synthesized bisamides.

between the phenyl ring and the nitrogen dramatically reduced cytotoxicity (over 100 μM), subsequently enhancing its selectivity index over 18.9, as observed in the previous hit **2**. It might be related to the obviously computational binding mode change compared to that of bisamide with a substituted phenyl group at the R^3 position because of the massive structure difference. Accordingly, among the five hit compounds, **7b**, **7e**, **7j**, **7m**, and **7p, 7e** was selected as a new hit of CypA inhibitor.

2.5. Docking study

To figure out the potential of computational binding mode of the five potential active bisamide derivatives including **7b**, **7e**, **7j**, **7m**, and **7p** and the rest of the bisamide compounds, the binding mode with a reference to a tertiary structure of the CsA-CypA complex (PDB code: 1CWA) or the hit compound **2**-CypA complex were revealed in silico by utilizing Surflex-Dock. As a representative, binding modes of **7e** as well as CsA and hit **2** were visualized by using Flare docking program (Fig. 3).

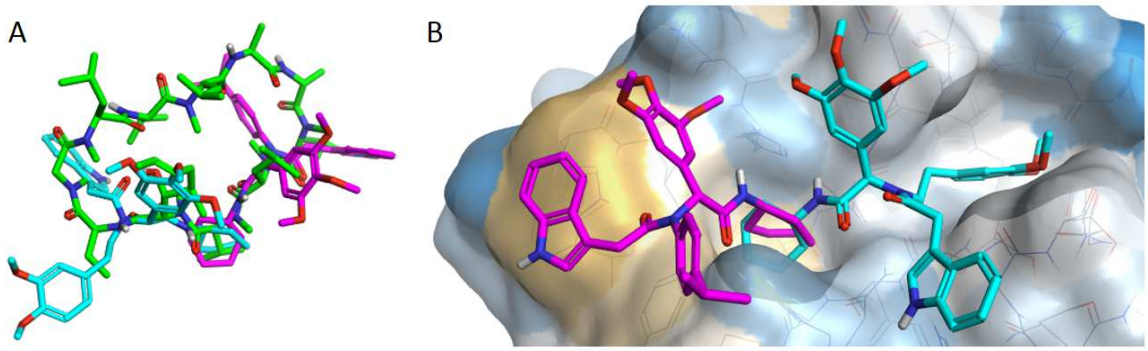


Fig. 3. A) Overlay of CsA (green), hit **2** (pink), and **7e** (cyan) bound to human CypA without protein structure. B) Overlay of hit **2** (pink), and **7e** (cyan) bound to human CypA, showing its active site with the hydrophobic pocket.

By analyzing the binding pattern of the newly synthesized analogs, surprisingly, a total of five compounds (**7c**, **7e**, **7j**, **7k**, and **7l**) showed more or less flip for the mode of the gatekeeper pocket (Fig.4 and Fig. 5A). And the rest 11 bisamides remained the similar binding mode compared with the hit **2** which had no interaction with the other hydrophobic pocket (gatekeeper pocket) (Fig. 5B).

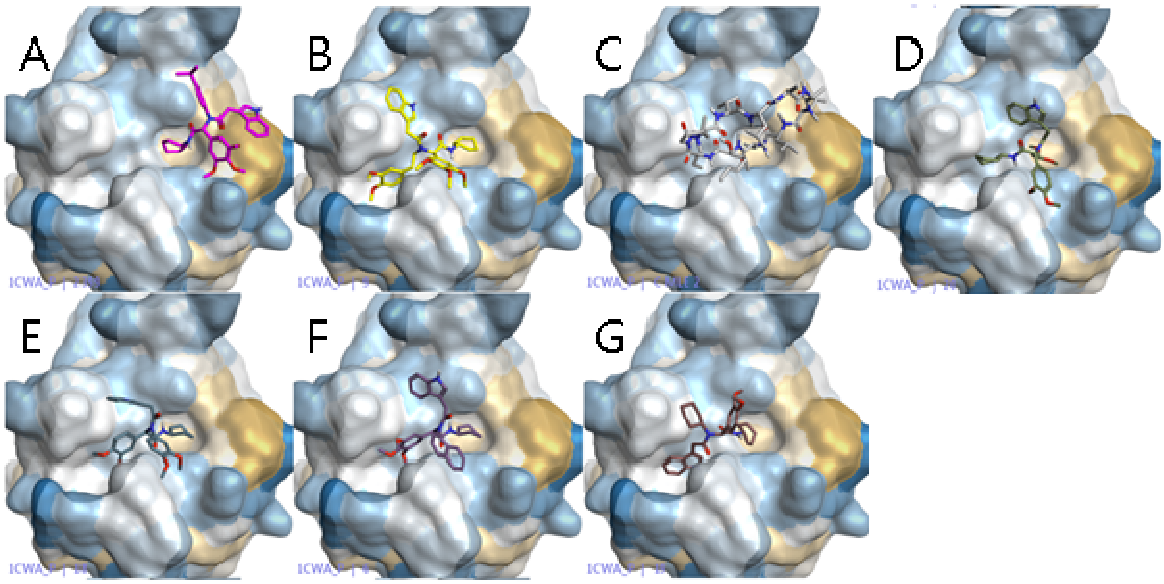


Fig. 4. Docking mode of biamides and CsA. A) hit **2**. B) **7e**. C) CsA. D) **7l**. E) **7j**. F) **7c**. G) **7k**.

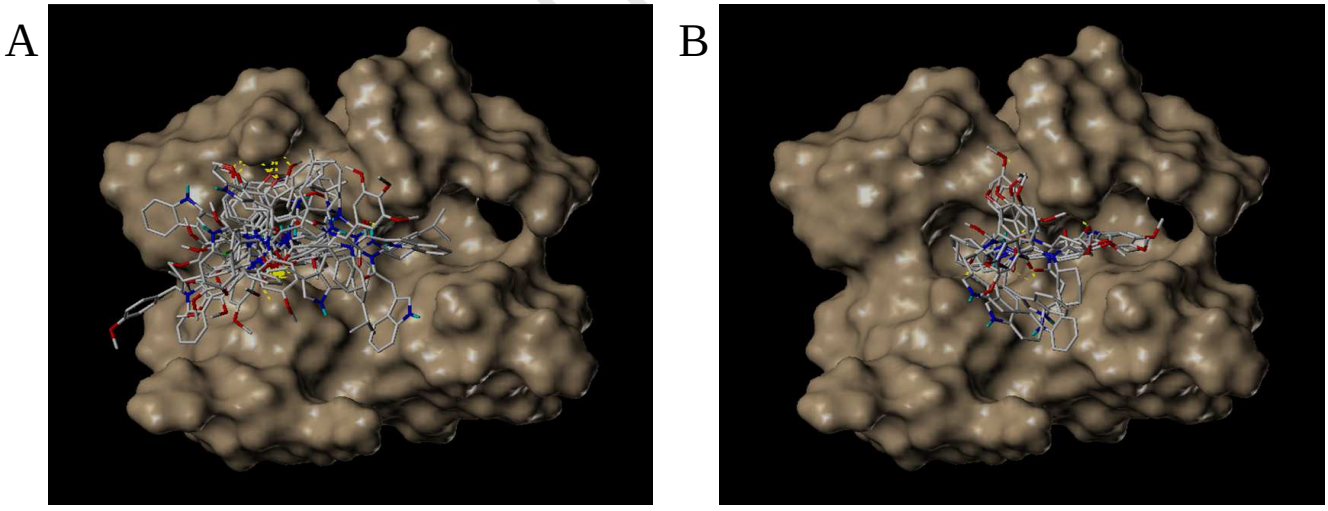


Fig. 5. Overlaid Docking modes of bisamide derivatives. A) the compounds failed to flip into gatekeeper pocket. B) the compounds succeeded to flip into gatekeeper pocket.

Among the docked compounds (Fig. 4.), **7e** was the only compound that showed antiviral activity (EC_{50}, 5.3 μM) and no cytotoxicity at the concentration of 100 μM. The docking results showed that binding modes of **7e** and hit compound **2** were obviously distinct, even though they share the catalytic pocket at the binding site (Fig. 3). The overlapping area was the cyclohexyl moiety that could have hydrophobic interaction with Phe113 of CypA. The extension of the carbon chain between the nitrogen and the phenyl ring at the R^3 position totally flipped the binding mode of **7e** from the catalytic site to the adjacent gatekeeper pocket. The hydrogen bond generated between the nitrogen of the indolylmethyl moiety and His54 of CypA increased the binding affinity (Fig. 6). The electrostatic charge of the dimethoxyphenylethyl moiety located in the gatekeeper can highly conform to its charge. The previous report [26] has revealed that this region is a highly attractive target pocket that can be used to develop isoform-specific CypA inhibitor because of residue distinction at position 103 in CyP isoforms. Ala103 participating in the formation of the gatekeeper pocket of CypA may contribute to the hydrophobic interaction with **7e**. It is surprising that trimethylphenyl moiety can also establish hydrophobic interaction with the vicinity amino acid Ala103 of CypA. This unique binding mode of **7e** potentially could make it an anti-HCV compound by blocking CypA.

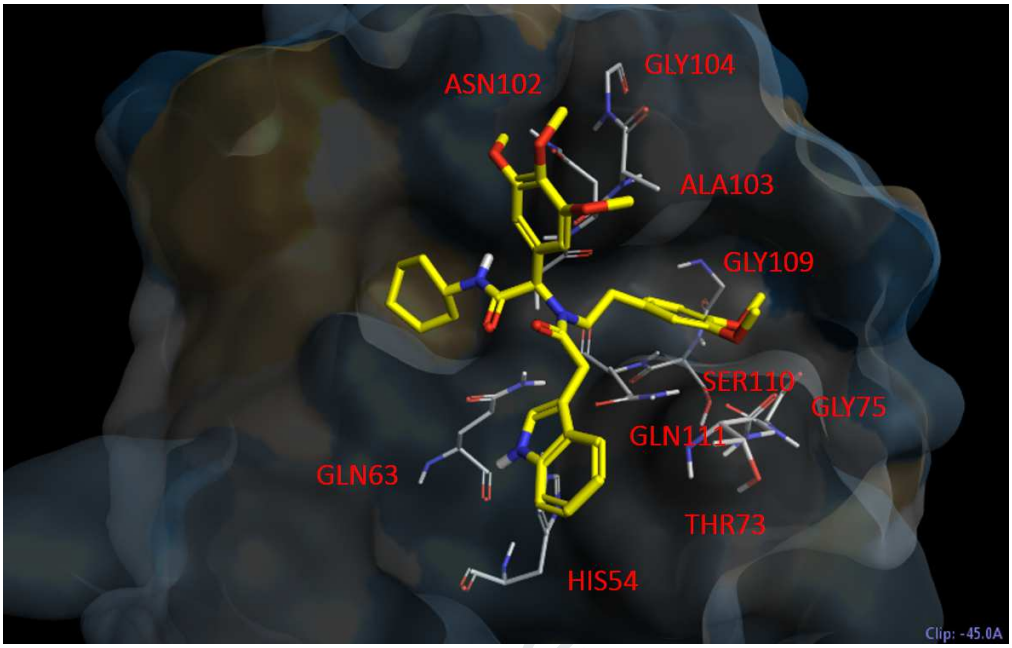


Fig. 6. Stereotype view of **7e** (yellow) bind to human CypA. Active site residues participating in the catalytic site and gatekeeper pocket are shown as ball and stick mode according to atom type (red: oxygen, blue: nitrogen, grey: carbon).

2.6. Surface Plasmon Resonance

The binding affinity of **7e** was evaluated by performing surface plasmon resonance (SPR). By testing different running concentration of **7e** (0.3125, 0.625, 1.25, 2.5, 5, 10, 20 μM), the kinetics parameters were obtained (Fig. 7). **7e** showed slow dissociation rate and large association rate ($K_a = 7.2 \times 10^2$ $M^{-1}S^{-1}$, $K_d = 2.6 \times 10^{-3}$ S^{-1}). The dissociation constant (K_D) was calculated from K_a and K_d (K_D [M] = K_d [s^{-1}] / K_a [$M^{-1}s^{-1}$]) that indicated that 7e is able to bind to CypA with a K_D of 3.66 μM.

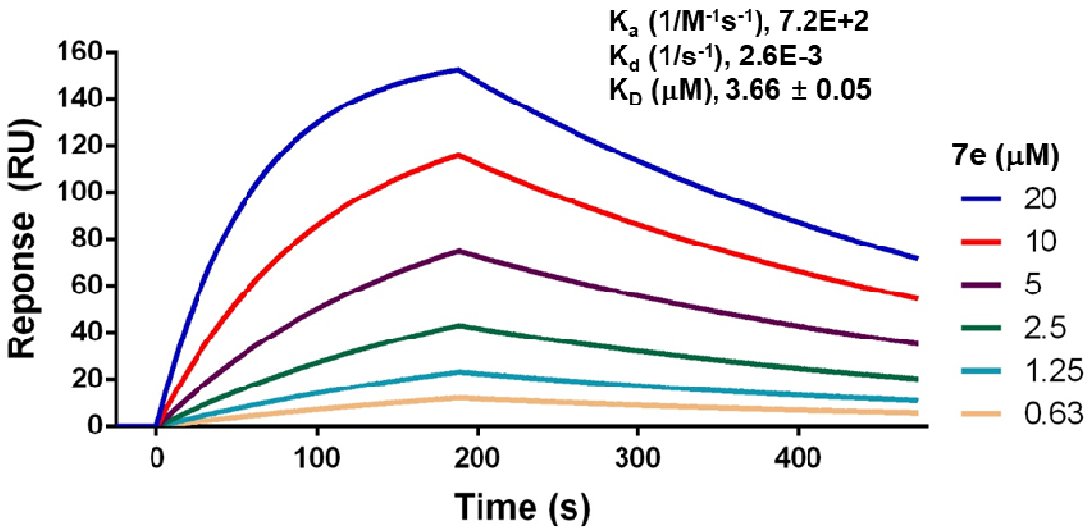


Fig. **7**. Relative response in resonance units showing interactions of **7e** with CypA at the different concentrations. The K_D value (mean ± S.D.) was calculated from three independent experiments.

3. Conclusion

By switching each of starting components in Ugi reaction, a new group of bisamides were synthesized with easy workup and promising yield for the purpose of finding analogs that could have interaction with the gatekeeper pocket of CypA protein. Meanwhile, the structure-activity relationship was further investigated after screening their cytotoxicities and anti-HCV activities. To identify new hit compounds among the newly synthesized compounds, docking study was carried out in parallel. As a result, five bisamides showed different binding modes that slightly shifted to the direction of the gatekeeper pocket. However, only **7e** generated hydrophobic interaction with the gatekeeper pocket. It also had better potential as specific CypA inhibitor than other

isoforms of Cyps. Thus, **7e** was selected as a new hit compound for further biological tests or pharmacokinetic studies prior to *in vivo* experiments. SPR binding assay confirmed that **7e** is able to bind to CypA with a K_D of 3.66 μM. As all data displayed, **7e** as a CypA inhibitor could be used as an alternative anti-HCV agent in combinational therapy in the future.

4. Experimental section

4.1. Chemistry

Melting points were determined by the capillary method with a MEL-TEMP capillary melting point apparatus. They were uncorrected. ^{1}H NMR was recorded with Varian Unity Plus 300 MHz at the Korea Basic Science Institute. Chemical shifts are reported in ppm downfield to tetramethylsilane (TMS) (δ = 0). Coupling constant (J) is shown in Hz. Data are reported in the following order: chemical shift, multiplicity (s, singlet; bs, broad singlet; b, broad; d, doublet; t, triplet; q, quartet; m, multiplet; dd, double doublet), coupling constant, and a number of protons. ^{13}C NMR was recorded with FT-NMR Spectrometer 400MHz in Chonnam Center for Research Facilities (CCRF). Mass spectra were obtained on a Shimadzu LCMS-2010 EV liquid chromatograph mass spectrometer using electron spray ionization (ESI) method. High-resolution mass spectrometry (HRMS) results were obtained on a Waters Synapt G2 high definition mass spectrometer. Elemental analyses were performed using a vario MICRO cube/Elementar/Germany elemental analyzer. Values obtained were within ± 0.3% of theoretical values. Column chromatography (gravity) was performed with a Merck silica gel 60 (70−230 mesh). Thin layer chromatography (TLC) was performed using plates

coated with silica gel 60 F254 (Merck). All chemical reagents were purchased from Sigma-Aldrich or Tokyo Chemical Industry Co., Ltd., Japan. They were used without further purification. Solvents were distilled prior to use. All reactions were conducted in oven-dried glassware with magnetic stirring.

Syntheses of bis-amides are outlined in Scheme 1. Bis-amides were prepared by employing an Ugi multi-component reaction. Conventional Ugi four-component condensation involves *in situ* formation of imine from an aldehyde **4** and an amine **5** which further reacts with isocyanide **3** and carboxylic acid **6** to an intermediate which in turn rearranges *via* an acyl transfer to yield desired bis-amides. Ugi reaction can also be carried out with preformed imine **8**, isocyanide **3,** and carboxylic acid **6** (Schemes 1 and 2).

4.1.1 *N*-Cyclohexyl-2-({2-[1-(4-methoxy-benzyl)-1H-indol-3-yl]-acetyl}[12]-phenyl-amino)-2-phenyl-acetamide (**7a**)

Procedure A: A solution of benzaldehyde **4a** (50 mg, 0.47 mmol) (70 mg, 0.35 mmol) and aniline **5a** (47 mg, 0.50 mmol) in methanol was stirred at RT for 6 h. To this solution, cyclohexyl isocyanide **3a** (51 mg, 0.47 mmol) and [1-(4-Methoxy-benzyl)-1H-indol-3-yl]-acetic acid **6b** (149 mg, 0.50 mmol) were added. The reaction mixture was heated at 55 °C overnight, evaporated to dryness, washed with saturated $NaHCO_3$ aqueous solution, and extracted with EtOAc. The organic phase was dried over anhydrous sodium sulfate and evaporated *in vacuo*. The resulting residue was purified to obtain bis-amide **7a** as a white solid (33 mg, 11%) after purification by column chromatography (*n*-hexane:EtOAc=3:1). Mp: 187.3-191.9 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.41 (d, *J* = 7.5 Hz, 2H), 7.23 – 7.00 (m, 14H), 6.82 – 6.79 (m, 3H), 6.06 (s, 1H), 5.73 (d, *J* = 7.2 Hz, 1H),

5.15 (s, 2H), 3.82 – 3.72 (m, 1H), 3.77 (s, 3H), 3.58 (s, 2H), 1.87 – 1.77 (m, 2H), 1.62 – 1.51 (m, 3H), 1.35 – 1.21 (m, 2H), 1.10 – 0.85 (m, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 170.7, 169.2, 159.0, 140.46, 136.1, 131.5, 130.6, 130.5, 129.1, 128.6, 128.2, 128.0, 127.8, 121.5, 119.4, 119.0, 114.3, 110.4, 108.6, 64.1, 55.5, 48.9, 48.4, 32.6, 32.5, 31.8, 25.1, 25.0. MS (ESI) *m/z* = 608 $(M+Na)^+$, 584 $(M-H)^-$.

4.1.2 *N*-Cyclohexyl-2-[(2-1H-indol-3-yl-acetyl)-(4-methoxy-phenyl)-amino]-2-(3,4,5-trimethoxy-phenyl)-acetamide (**7b**)

Procedure A was used with cyclohexyl isocyanide **3a** (56 mg, 0.51 mmol), 3,4,5-trimethoxybenzaldehyde **4b** (100 mg, 0.51 mmol), *p*-anisidine **5b** (77 mg, 0.63 mmol), 3-indoleacetic acid **6a** (186 mg, 1.06 mmol), and methanol to obtain bis-amide **7b** as an ivory solid (217 mg, 72%) after purification by column chromatography (*n*-hexane:EtOAc=3:1). Mp: 130.5-134.7 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.03 (s, 1H), 7.42 (d, *J* = 7.8 Hz, 2H), 7.31 (d, *J* = 8.1 Hz, 1H), 7.14 (t, *J* = 7.8 Hz, 1H), 7.04 (t, *J* = 8.1 Hz, 1H), 6.98 (s, 1H), 6.79 – 6.60 (m, 3H), 6.31 (s, 2H), 6.07 (s, 1H), 5.74 (d, *J* = 8.7 Hz, 1H), 3.77 (s, 4H), 3.74 (s, 3H), 3.62 – 3.54 (m, 8H), 1.19 – 1.80 (m, 2H), 1.66 – 1.53 (m, 3H), 1.36 – 1.23 (m, 2H), 1.10 – 0.99 (m, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): 171.2, 169.3, 158.7, 152.5, 137.4, 136.4, 133.3, 132.5, 131.6, 127.8, 124.2, 121.3, 119.1, 118.6, 113.7, 111.7, 108.9, 108.4, 64.1, 60.4, 56.2, 55.7, 48.4, 32.7, 32.5, 31.8, 25.7, 25.1, 25.0. MS (ESI) *m/z* = 608 $(M+Na)^+$, 584 $(M-H)^-$. Anal. Calcd for $C_{34}H_{39}N_3O_6 \cdot 0.1H_2O$: C, 69.51; H, 6.73; N, 7.51. Found: C, 69.57; H, 6.83; N, 7.23.

4.1.3 *N*-Cyclohexyl-2-[(2-1H-indol-3-yl-acetyl)-naphthale*n*-1-yl-amino]-2-(3,4,5-

trimethoxy-phenyl)-acetamide (**7c**)

Procedure A was used with cyclohexyl isocyanide **3a** (56 mg, 0.51 mmol), 3,4,5-trimethoxybenzaldehyde **4b** (100 mg, 0.51 mmol), 1-aminonapthalene **5c** (95 mg, 0.66 mmol), 3-indoleacetic acid **4a** (116 mg, 0.66 mmol), and methanol at RT to obtain bis-amide **7c** as a violet solid (62 mg, 20%) after purification by column chromatography (*n*-hexane:EtOAc=3:1). Mp: 206.5-215.1 °C (dec). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.99 (s, 1H), 7.87 (d, *J* = 8.4 Hz, 1H), 7.76 (t, *J* = 8.7 Hz, 2H), 7.62 (d, *J* = 6.6 Hz, 1H), 7.40 – 7.28 (m, 5H), 7.13 – 7.08 (m, 1H), 7.04 – 6.96 (m, 1H), 6.85 (d, *J* = 2.1 Hz, 1H), 6.29 (s, 2H), 5.96 (s, 1H), 5.77 (d, *J* = 8.1 Hz, 1H), 3.85 – 3.79 (m, 1H), 3.73 (s, 3H), 3.49 (s, 2H), 3.47 (s, 6H), 1.91 – 1.86 (m, 2H), 1.65 – 1.56 (m, 3H), 1.40 – 1.23 (m, 2H), 1.14 – 1.01 (m, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): 171.4, 169.5, 151.6, 137.1, 136.8, 136.3, 134.0, 132.1, 130.0, 129.8, 128.9, 128.2, 127.6, 127.1, 126.7, 126.4, 125.6, 124.3, 124.0, 121.3, 119.0, 119.0, 118.6, 111.6, 109.0, 108.7, 108.0, 65.0, 60.3, 60.2, 56.0, 55.7, 48.5, 32.5, 32.4, 31.7, 25.6, 25.1, 25.0. MS (ESI) *m/z* = 628 $(M+Na)^+$, 604 $(M-H)^-$.

4.1.4 *N*-Cyclohexyl-2-[(3,4-dimethoxy-phenyl)-(2-1H-indol-3-yl-acetyl)-amino]-2-(3,4,5-trimethoxy-phenyl)-acetamide (**7d**)

Procedure A was used with cyclohexyl isocyanide **3a** (55 mg, 0.50 mmol), 3,4,5-trimethoxybenzaldehyde **4b** (100 mg, 0.51 mmol), 4-aminoveratrole **5d** (97 mg, 0.63 mmol), 3-indoleacetic acid **6a** (133 mg, 0.76 mmol), and 2,2,2-trifluoroethanol at RT to obtain bis-amide **7d** as a light orange solid (239 mg, 76%) after purification by column chromatography (*n*-hexane:EtOAc=3:1). Mp: 113.5-115.6 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.99 (s, 1H), 7.39 – 7.37 (m, 1H), 7.31 (d, *J* = 7.8 Hz, 4H), 7.17 – 7.12 (m, 1H), 7.06 –

7.01 (m, 2H), 6.34 (s, 2H), 6.05 (s, 1H), 5.70 (d, *J* = 8.7 Hz, 1H), 3.82 (s, 4H), 3.77 (s, 4H), 3.63 – 3.61 (m, 9H), 3.40 – 3.31 (m, 1H), 1.92 – 1.81 (m, 2H), 1.68 – 1.60 (m, 3H), 1.35 – 1.23 (m, 2H), 1.18 – 1.03 (m, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): 171.2, 169.4, 152.5, 148.3, 136.4, 131.7, 127.7, 124.2, 123.8, 121.3, 119.0, 118.6, 115.4, 111.7, 111.2, 109.0, 108.5, 60.4, 56.2, 56.0, 55.5, 48.4, 39.5, 32.7, 32.5, 31.9, 25.7, 25.1, 25.0. MS (ESI) *m/z* = 638 $(M+Na)^+$, 614 $(M-H)^-$. Anal. Calcd for $C_{35}H_{41}N_3O_7 \cdot 0.65H_2O$: C, 67.00; H, 6.79; N, 6.70. Found: C, 67.12; H, 6.96; N, 6.76.

4.1.5 *N*-Cyclohexyl-2-[[2-(3,4-dimethoxy-phenyl)-ethyl]-(2-1H-indol-3-yl-acetyl)-amino]-2-(3,4,5-trimethoxy-phenyl)-acetamide (**7e**)

Procedure A was used with cyclohexyl isocyanide **3a** (55 mg, 0.50 mmol), 3,4,5-trimethoxybenzaldehyde **4a** (100 mg, 0.51 mmol), 3,4-dimethoxyphenethylamine **5e** (144 mg, 0.63 mmol), 3-indoleacetic acid **6a** (133 mg, 0.76 mmol), and 2,2,2-trifluoroethanol at RT to obtain bis-amide **7e** as a light yellow solid (18 mg, 5%) after purification by column chromatography (*n*-hexane:EtOAc=3:1). Mp: 177.7-179.4 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.11 (s, 1H), 7.60 (d, *J* = 8.1 Hz, 2H), 7.36 (d, *J* = 8.1 Hz, 2H), 7.22 – 7.08 (m, 3H), 6.70 (s, 2H), 6.42 – 6.39 (m, 1H), 5.93 (s, 1H), 5.75 (d, *J* = 8.4 Hz, 1H), 3.85 – 3.72 (m, 18H), 3.58 – 3.50 (m, 2H), 2.76 – 2.63 (m, 2H), 1.94 – 1.82 (m, 2H), 1.68 – 1.56 (m, 3H), 1.40 – 1.23 (m, 2H), 1.12 – 1.03 (m, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): 172.1, 169.0, 153.3, 153.1, 149.1, 147.7, 137.8, 136.6, 132.7, 131.6, 127.5, 124.6, 123.8, 121.6, 120.7, 119.0, 118.9, 112.5, 112.4, 111.9, 108.7, 107.1, 106.5, 61.2, 60.6, 56.3, 56.1, 56.0, 55.7, 48.3, 32.6, 32.4, 31.6, 25.6, 25.0. MS (ESI) *m/z* = 666 $(M+Na)^+$.

4.1.6 *N*-Cyclohexyl-2-[(2-1H-indol-3-yl-acetyl)-phenyl-amino]-2-(2,3,4-trimethoxy-phenyl)-acetamide (**7f**)

Procedure A was used with cyclohexyl isocyanide **3a** (55 mg, 0.50 mmol), 2,3,4-trimethoxybenzaldehyde **4c** (100 mg, 0.51 mmol), aniline **5a** (59 mg, 0.63 mmol), 3-indoleacetic acid **6a** (88 mg, 0.50 mmol), and 2,2,2-trifluoroethanol at RT to obtain bis-amide **7f** as a light yellow solid (75 mg, 26%) after purification by column chromatography (EtOAc). Mp: 108.5-112.3 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.04 (s, 1H), 7.38 (d, *J* = 7.8 Hz, 2H), 7.30 (d, *J* = 8.1 Hz, 1H), 7.15 – 7.10 (m, 4H), 7.05 – 6.98 (m, 3H), 6.67 (d, *J* = 8.7 Hz, 1H), 6.39 (s, 1H), 6.32 (d, *J* = 9.0 Hz, 1H), 5.64 (d, *J* = 8.1 Hz, 1H), 3.83 – 3.79 (m, 1H), 3.81 (s, 3H), 3.76 (s, 3H), 3.75 (s, 3H), 3.57 (s, 2H), 1.89 – 1.80 (m, 2H), 1.70 – 1.50 (m, 3H), 1.35 – 1.23 (m, 2H), 1.12 – 0.89 (m, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): 170.6, 169.6, 153.6, 152.1, 141.4, 136.4, 128.6, 128.0, 124.9, 124.1, 121.9, 121.3, 119.0, 118.6, 111.7, 109.0, 107.2, 61.1, 60.4, 58.8, 56.1, 48.6, 39.8, 32.8, 32.6, 31.8, 25.7, 25.3, 25.1. MS (ESI) *m/z* = 578 $(M+Na)^+$, 554 $(M-H)^-$.

4.1.7 2-[(4-Bromo-phenyl)-(2-1H-indol-3-yl-acetyl)-amino]-*N*-cyclohexyl-2-(2,3,4-trimethoxy-phenyl)-acetamide (**7g**)

Procedure A was used with cyclohexyl isocyanide **3a** (55 mg, 0.50 mmol), 2,3,4-trimethoxybenzaldehyde **4c** (100 mg, 0.51 mmol), 4-bromoaniline **5f** (108 mg, 0.63 mmol), 3-indoleacetic acid **6a** (88 mg, 0.50 mmol), and 2,2,2-trifluoroethanol at RT to obtain bis-amide **7g** as a white solid (163 mg, 50%) after filtration. Mp: 143.2-146.5 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.00 (s, 1H), 7.37 – 7.29 (m, 4H), 7.24 – 7.19 (m, 2H), 7.16 – 7.11 (m, 1H), 7.07 – 7.00 (m, 2H), 6.63 (d, *J* = 8.7 Hz, 1H), 6.37 – 6.34 (m, 2H), 5.51 (d, *J* = 8.4

Hz, 1H), 3.83 – 3.75 (m, 1H), 3.82 (s, 3H), 3.77 (s, 3H), 3.76 (s, 3H), 3.56 (s, 2H), 1.92 – 1.82 (m, 2H), 1.68 – 1.54 (m, 3H), 1.38 – 1.23 (m, 2H), 1.14 – 0.91 (m, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): 170.4, 169.5, 152.1, 140.1, 136.4, 131.5, 127.6, 124.8, 124.1, 121.7, 121.3, 121.0, 119.0, 118.6, 111.7, 109.8, 107.4, 61.1, 60.4, 58.7, 56.2, 48.6, 32.8, 32.6, 32.0, 25.7, 25.3, 25.1. MS (ESI) *m/z* = 656 $(M+Na)^+$. Anal. Calcd for $C_{33}H_{36}BrN_3O_5 \cdot 0.2H_2O$: C, 62.11; H, 5.75; N, 6.58. Found: C, 62.22; H, 5.87; N, 6.58.

4.1.8 *N*-Cyclohexyl-2-[(2-1H-indol-3-yl-acetyl)-(4-isopropyl-phenyl)-amino]-2-(2,3,4-trimethoxy-phenyl)-acetamide (**7h**)

Procedure B: A solution of 2,3,4-trimethoxybenzaldehyde **4c** (200 mg, 1.02 mmol) and 4-isopropylaniline **5g** (172 mg, 1.27 mmol) in ethanol was stirred at RT. The solid thus formed was filtered off to obtain imine. The imine (160 mg, 0.51 mmol) was then added to a solution of cyclohexyl isocyanide **3a** (56 mg, 0.51 mmol) and 3-indole acetic acid **6a** (112 mg, 0.64 mmol) in methanol and the reaction mixture was heated at 55 °C overnight. The mixture was evaporated to dryness, washed with saturated $NaHCO_3$ aqueous solution, and extracted with EtOAc. The organic phase was dried over anhydrous sodium sulfate and evaporated *in vacuo*. The resulting residue was purified by column chromatography using *n*-hexane:EtOAc (3:1) to obtain bis-amide **7h** as a light yellow solid (187 mg, 61%). Mp: 171.3-175.5 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.99 (s, 1H), 7.37 – 7.29 (m, 4H), 7.15 – 7.10 (m, 1H), 7.04 – 6.91 (m, 4H), 6.68 (d, *J* = 8.7 Hz, 1H), 6.35 – 6.31 (m, 2H), 5.67 (d, *J* = 8.1 Hz, 1H), 3.79 – 3.74 (m, 1H), 3.78 (s, 3H), 3.76 (s, 3H), 3.75 (s, 3H), 3.58 (s, 2H), 2.81 (heptet, *J* = 6.9 Hz, 1H), 1.88 – 1.80 (m, 2H), 1.66 – 1.52 (m, 3H), 1.26 – 1.19 (m, 2H), 1.16 (d, *J* = 6.9 Hz, 6H), 1.08 – 0.91 (m, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): 170.7,

169.5, 153.6, 148.0, 141.5, 138.4, 136.4, 130.5, 127.7, 126.3, 125.0, 124.1, 121.3, 119.0, 118.6, 111.6, 109.1, 107.2, 61.0, 60.3, 58.9, 56.2, 48.6, 33.4, 32.8, 32.6, 31.8, 25.7, 25.3, 25.1, 24.2, 24.1. MS (ESI) *m/z* = 620 $(M+Na)^+$, 596 $(M-H)^-$. Anal. Calcd for $C_{36}H_{43}N_3O_5 \cdot 1.4H_2O$: C, 69.41; H, 7.41; N, 6.75. Found: C, 69.12; H, 7.78; N, 6.73.

4.1.9 *N*-Cyclohexyl-2-(3,4-dimethoxy-phenyl)-2-[(2-1H-indol-3-yl-acetyl)-(4-isopropyl-phenyl)-amino]-acetamide (**7i**)

Procedure A was used with cyclohexyl isocyanide **3a** (66 mg, 0.60 mmol), 3,4-dimethoxybenzaldehyde **4d** (100 mg, 0.60 mmol), 4-isopropylaniline **5g** (101 mg, 0.75 mmol), 3-indoleacetic acid **6a** (210 mg, 1.20 mmol), and methanol at RT to obtain bis-amide **7i** as a white solid (176 mg, 51%) after purification by column chromatography (*n*-hexane:EtOAc=5:1). Mp: 163.7-168.4 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.01 (s, 1H), 7.37 – 7.29 (m, 3H), 7.16 – 7.10 (m, 2H), 7.05 – 6.98 (m, 4H), 6.76 (dd, *J* = 8.4, 1.8 Hz, 1H), 6.68 (d, *J* = 8.4 Hz, 1H), 6.47 (d, *J* = 2.1 Hz, 1H), 6.08 (s, 1H), 5.75 (d, *J* = 8.1 Hz, 1H), 3.82 (s, 3H), 3.80 – 3.74 (m, 1H), 3.58 (s, 2H), 3.50 (s, 3H), 2.84 (heptet, *J* = 6.9 Hz, 1H), 1.87 – 1.76 (m, 2H), 1.55 – 1.52 (m, 3H), 1.40 – 1.23 (m, 2H), 1.19 (d, *J* = 6.9 Hz, 6H), 1.10 – 0.87 (m, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): 170.1, 169.5, 148.7, 148.2, 148.1, 138.3, 136.4, 131.3, 128.4, 127.7, 126.4, 124.1, 123.4, 121.3, 119.0, 118.6, 114.3, 111.7, 111.4, 108.9, 63.7, 55.8, 55.7, 48.3, 39.7, 33.4, 32.6, 32.5, 31.9, 25.7, 25.1, 25.0, 24.3, 24.1. MS (ESI) *m/z* = 590 $(M+Na)^+$, 566 $(M-H)^-$.

4.1.10 *N*-Cyclohexyl-2-(3,4-dimethoxy-phenyl)-2-[(3,4-dimethoxy-phenyl)-(2-1H-indol-3-yl-acetyl)-amino]-acetamide (**7j**)

Procedure A was used with cyclohexyl isocyanide **3a** (66 mg, 0.60 mmol), 3,4-dimethoxybenzaldehyde **4d** (100 mg, 0.60 mmol), 4-aminoveratrole **5d** (115 mg, 0.75 mmol), 3-indoleacetic acid **6a** (210 mg, 1.20 mmol), and methanol at RT to obtain bis-amide **7j** as a light yellow solid (234 mg, 66%) after purification by column chromatography (*n*-hexane:EtOAc=3:1). Mp: 111.7-114.6 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.10 (s, 1H), 7.35 – 7.29 (m, 2H), 7.16 – 7.11 (m, 2H), 7.05 – 7.00 (m, 2H), 6.72 – 6.60 (m, 4H), 6.07 – 5.82 (m, 2H), 5.64 (d, *J* = 7.8 Hz, 1H), 3.87 – 3.75 (m, 7H), 3.61 (s, 3H), 3.60 (s, 3H), 3.32 (bs, 2H), 1.90 – 1.80 (m, 2H), 1.63 – 1.53 (m, 3H), 1.38 – 1.23 (m, 2H), 1.13 – 0.88 (m, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): 171.3, 148.3, 136.4, 133.3, 128.4, 127.7, 124.1, 123.7, 123.3, 121.3, 119.0, 118.6, 115.5, 114.4, 111.7, 111.4, 111.1, 109.1, 63.8, 55.9, 55.8, 55.7, 48.3, 32.7, 32.6, 31.9, 25.7, 25.1, 25.0. MS (ESI) *m/z* = 584 $(M-H)^-$.

4.1.11 *N*-Cyclohexyl-2-[cyclohexyl-(2-1H-indol-3-yl-acetyl)-amino]-2-(3,4-dimethoxy-phenyl)-acetamide (**7k**)

Procedure A was used with cyclohexyl isocyanide **3a** (66 mg, 0.60 mmol), 3,4-dimethoxybenzaldehyde **4d** (100 mg, 0.60 mmol), cyclohexylamine **5h** (75 mg, 0.75 mmol), 3-indoleacetic acid **6a** (210 mg, 1.20 mmol), and methanol at RT to obtain bis-amide **7k** as a white solid (181 mg, 56%) after purification by column chromatography (*n*-hexane:EtOAc=3:1). Mp: 110.3-114.8 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.92 – 10.84 (m, 1H), 8.06 (bs, 1H), 7.53 (bs, 1H), 7.32 – 6.73 (m, 6H), 6.39 (s, 1H), 5.56 (s, 1H), 3.84 – 3.77 (m, 2H), 3.70 (s, 3H), 3.49 (s, 3H), 3.16 (bs, 2H), 1.84 – 1.49 (m, 9H), 1.52 – 0.96 (m, 11H). ^{13}C NMR (100 MHz, DMSO-d_6): 171.9, 148.7, 136.6, 124.2, 121.6, 120.6, 119.6, 118.9, 111.8, 62.5, 55.9, 55.5, 48.2, 47.8, 39.2, 32.3, 31.4, 25.7, 25.5, 25.0, 24.5. MS (ESI)

m/z = 554 $(M+Na)^+$, 530 $(M-H)^-$.

4.1.12 *N*-Cyclohexyl-2-[(2,2-dimethoxy-ethyl)-(2-1H-indol-3-yl-acetyl)-amino]-2-(3,4-dimethoxy-phenyl)-acetamide (**7l**)

Procedure A was used with cyclohexyl isocyanide **3a** (66 mg, 0.60 mmol), 3,4-dimethoxybenzaldehyde **4d** (100 mg, 0.60 mmol), aminoacetaldehyde dimethyl acetal **5i** (79mg, 0.75 mmol), 3-indoleacetic acid **6a** (210 mg, 1.20 mmol), and methanol at RT to obtain bis-amide **7l** as a white solid (244 mg, 75%) after filtration. Mp: 182.9-183.7 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.88 (s, 1H), 7.88 (d, *J* = 7.5 Hz, 1H), 7.47 (d, *J* = 7.5 Hz, 1H), 7.34 (d, *J* = 8.1 Hz, 1H), 7.24 – 7.20 (m, 1H), 7.09 – 7.04 (m, 1H), 6.98 – 6.88 (m, 2H), 6.81 (d, *J* = 8.1 Hz, 1H), 6.74 (s, 1H), 5.94 (s, 1H), 3.74 (s, 3H), 3.62 (s, 3H), 3.54 – 3.45 (m, 4H), 3.17 – 3.09 (m, 2H), 3.15 (s, 3H), 3.07 (s, 3H), 1.68 – 1.51 (m, 5H), 1.26 – 1.05 (m, 5H). ^{13}C NMR (100 MHz, DMSO-d_6): 173.1, 169.3, 149.3, 149.2, 136.6, 129.1, 127.7, 123.9, 122.4, 121.5, 119.4, 119.0, 118.8, 113.6, 112.2, 111.8, 108.7, 104.8, 61.0, 56.1, 56.0, 55.9, 55.5, 55.4, 54.1, 48.2, 32.7, 32.5, 31.4, 25.6, 25.0, 24.9. MS (ESI) *m/z* = 560 $(M+Na)^+$, 536 $(M-H)^-$.

4.1.13 *N*-Cyclohexyl-2-(3,4-dimethoxy-phenyl)-2-[(2-1H-indol-3-yl-acetyl)-(4-methoxy-phenyl)-amino]-acetamide (**7m**)

Procedure B was used with cyclohexyl isocyanide **3a** (60 mg, 0.55 mmol), imine (formed by reaction of 3,4-dimethoxybenzaldehyde **4d** and *p*-anisidine **5b** in methanol) (150 mg, 0.55 mmol), 3-indoleacetic acid **6a** (121 mg, 0.69 mmol), and 2,2,2-trifluoroethanol at RT to obtain bis-amide **7m** as a white solid (235 mg, 76%) after

purification by column chromatography (EtOAc). Mp: 105.1-108.3 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.98 (s, 1H), 7.40 (d, *J* = 8.1 Hz, 2H), 7.31 (d, *J* = 8.1 Hz, 2H), 7.14 (t, *J* = 7.8 Hz, 1H), 7.06 – 7.01 (m, 2H), 6.72 – 6.53 (m, 5H), 6.09 (s, 1H), 5.66 (d, *J* = 9.0 Hz, 1H), 3.82 (s, 3H), 3.80 – 3.71 (m, 1H), 3.74 (s, 3H), 3.57 (s, 5H), 1.90 – 1.79 (m, 2H), 1.69 – 1.58 (m, 3H), 1.38 – 1.23 (m, 2H), 1.10 – 0.95 (m, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): 171.2, 169.6, 148.6, 148.3, 136.4, 133.3, 132.5, 128.4, 127.7, 124.1, 123.2, 121.3, 119.1, 118.6, 114.4, 113.8, 111.7, 111.4, 109.0, 63.7, 55.8, 55.7, 55.6, 48.3, 32.7, 32.5, 31.8, 25.7, 25.1, 25.0. MS (ESI) *m/z* = 578 $(M+Na)^+$.

4.1.14 *N*-tert-Butyl-2-[(2-1H-indol-3-yl-acetyl)-(4-isopropyl-phenyl)-amino]-2-(3,4,5-trimethoxy-phenyl)-acetamide (**7n**)

Procedure A was used with *tert*-butyl isocyanide **3b** (42 mg, 0.51 mmol), 3,4,5-trimethoxybenzaldehyde **4b** (100 mg, 0.51 mmol), 4-isopropylaniline **5g** (86 mg, 0.64 mmol), 3-indoleacetic acid **6a** (112 mg, 0.64 mmol), and 2,2,2-trifluoroethanol at RT to obtain bis-amide **7n** as a white solid (225 mg, 77%) after purification by column chromatography (*n-hexane*:EtOAc=3:1). Mp: 88.9-95.3 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.98 (s, 1H), 7.38 (d, *J* = 7.8 Hz, 2H), 7.31 (d, *J* = 7.8 Hz, 2H), 7.14 (t, *J* = 8.1 Hz, 1H), 7.03 (t, *J* = 7.8 Hz, 1H), 6.97 – 6.91 (m, 3H), 6.29 (s, 2H), 5.96 (s, 1H), 5.85 (s, 1H), 3.75 (s, 3H), 3.60 (s, 2H), 3.59 (s, 6H), 2.84 (heptet, *J* = 6.9 Hz, 1H), 1.30 (s, 9H), 1.19 (d, *J* = 6.9 Hz, 6H). ^{13}C NMR (100 MHz, DMSO-d_6): 170.8, 169.7, 152.5, 138.4, 136.4, 131.7, 131.3, 127.7, 126.3, 124.1, 121.3, 119.1, 118.6, 111.7, 109.0, 108.4, 64.3, 60.4, 56.1, 50.7, 33.5, 32.0, 28.9, 24.3, 24.2. MS (ESI) *m/z* = 594 $(M+Na)^+$.

4.1.15 1H-Indole-2-carboxylic acid [tert-butylcarbamoyl-(3,4,5-trimethoxy-phenyl)-methyl]-(4-isopropyl-phenyl)-amide (**7o**)

Procedure A was used with *tert*-butyl isocyanide **3b** (42 mg, 0.51 mmol), 3,4,5-trimethoxybenzaldehyde **4b** (100 mg, 0.51 mmol), 4-isopropylaniline **5g** (86 mg, 0.64 mmol), indole-2-carboxylic acid **6c** (103 mg, 0.64 mmol), and 2,2,2-trifluoroethanol at RT to obtain bis-amide **7o** as a white solid (130 mg, 45%) after filtration. Mp: 242.4-244.0 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 9.23 (s, 1H), 7.34 – 7.28 (m, 4H), 7.23 – 7.18 (m, 2H), 7.13 (bs, 2H), 6.98 (t, *J* = 6.9 Hz, 1H), 6.38 (s, 2H), 6.10 (s, 1H), 5.80 (s, 1H), 3.79 (s, 3H), 3.66 (s, 6H), 2.93 (heptet, *J* = 6.9 Hz, 1H), 1.37 (s, 9H), 1.27 (d, *J* = 6.9 Hz, 6H). ^{13}C NMR (100 MHz, DMSO-d_6): 169.6, 152.5, 149.0, 137.5, 136.0, 132.0, 131.0, 130.7, 127.3, 126.4, 124.0, 121.9, 120.0, 112.6, 108.8, 106.1, 65.4, 60.4, 56.2, 50.8, 33.6, 28.9, 24.4, 24.3. MS (ESI) *m/z* = 580 $(M+Na)^+$, 556 $(M-H)^-$.

4.1.16 1H-Indole-2-carboxylic acid [cyclohexylcarbamoyl-(3,4,5-trimethoxy-phenyl)-methyl]-(4-isopropyl-phenyl)-amide (**7p**)

Procedure A was used with cyclohexyl isocyanide **3a** (70 mg, 0.51 mmol), 3,4,5-trimethoxybenzaldehyde **4b** (100 mg, 0.51 mmol), 4-isopropylaniline **5g** (86 mg, 0.64 mmol), indole-2-carboxylic acid **6c** (103 mg, 0.64 mmol), and 2,2,2-trifluoroethanol at RT to obtain bis-amide **7p** as a white solid (120 mg, 40%) after filtration. Mp: 253.7-255.1 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 9.23 (s, 1H), 7.34 – 7.28 (m, 3H), 7.23 – 7.17 (m, 2H), 7.16 – 7.09 (m, 2H), 7.01 – 6.96 (m, 2H), 6.39 (s, 2H), 6.19 (s, 1H), 5.79 (d, *J* = 9.0 Hz, 1H), 3.90 – 3.83 (m, 1H), 3.80 (s, 3H), 3.65 (s, 6H), 2.93 (heptet, *J* = 6.9 Hz, 1H), 2.02 – 1.85 (m, 2H), 1.68 – 1.49 (m, 3H), 1.40 – 1.28 (m, 2H), 1.23 (d, *J* = 6.9 Hz, 6H), 1.19 – 1.05 (m,

3H). ^{13}C NMR (100 MHz, DMSO-d_6): 169.2, 152.6, 149.1, 138.0, 136.0, 132.0, 130.8, 130.7, 126.5, 124.0, 121.9, 120.0, 112.6, 108.9, 106.1, 65.2, 60.4, 56.2, 48.4, 33.6, 32.7, 32.7, 25.7, 25.1, 25.0, 24.4, 24.3. MS (ESI) *m/z* = 606 $(M+Na)^+$, 582 $(M-H)^-$.

4.2. EC_{50} and CC_{50} evaluation

Cell-Based antiviral and MTT assays were performed to evaluate EC_{50} and CC_{50} values of the synthesized compounds. To generate the recombinant virus, full-length RNA of JFH1-Luc, originating from a genotype 2a HCV clone, was *in vitro* transcribed from the plasmid pJFH1-Luc (a gift from Dr. Xulin Chen, Wuhan Institute of Virology, China). Naive Huh7.5 cells (grown to 70% confluence on 48-well plates) were cultured in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen). They were transfected with RNA transcript by electrophoresis and recombinant virus was harvested from the culture supernatants at day 30 after transfection. For antiviral analysis, fresh Huh7.5 cells were mock-infected or infected with the recombinant JFH1-Luc virus at a multiplicity of infection (MOI) of 0.004 for 5 h at 37 °C in the presence of serially (3-fold) diluted test compounds. On the third day post-infection, the cells were lysed to calculate EC_{50} by analyzing *Renilla* luciferase activity (Renilla luciferase assay system; Promega, Madison, WI, USA). In parallel, an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) (Sigma-Aldrich) assay was utilized to determine the cell viability (CC_{50}) of the mock-infected and chemical treated Huh7.5 cells as described previously [26].

4.3. Docking study

Docking studies were performed by using Sybyl-X 2.1.1 and Surflex-Dock. The structure of the CsA-CypA complex (PDB code: 1CWA) was retrieved from Protein Data Bank. Chain C and water molecule were removed and the ligand (CsA) was extracted from the complex. Hydrogens were added to the complex using Sybyl-X 2.1.1 and Surflex-Dock. Minimization was then carried out using MMFF94s force field with MMFF94 charges and a conjugate gradient method with distance-dependent dielectric constant and convergence to 0.01 kcal/mol·Å. Protomol, the binding site, was then generated based on the binding of CsA. Compounds needed to be docked were sketched and minimized using MMFF94s force field with MMFF94 charges. They were then kept in the Sybyl chemical database. The intended docking compound was docked into the generated protomol by the Surflex-Dock. Obtained docking results were visualized in FlareV2.0.

4.4 Surface Plasmon Resonance.

A SPR instrument (Reichert, SR7500DC system) was used to evaluate the direct target binding of hit compound **7e**. Immobilization of CypA protein on sample channel was contained three steps. 1-ethyl- 3-(3-dimethylaminopropyl)carbodiimide hydrochloride and N-hydroxysuccinimide were used for the activation of protein. Then protein stock was diluted with 10 mM sodium acetate which pH was 6.0. The quenching solution for the deactivation was 1 M ethanolamine (pH 8.5). A second reference was treated similarly except that protein was eliminated. All working running buffer was PBS with 5% DMSO. The association time was 3 min and the dissociation was 4 min. The Flow rate was 30

μL/min and regeneration solution was 50 mM NaOH. Kinetic parameters were obtained using Scrubber2.

Notes

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at ….

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Highlights

- A new group of bisamide derivatives, totally 16 compounds were designed and aimed to switch the binding mode against CypA.
- The EC_{50} and CC_{50} values of the newly synthesized compounds were helped to enrich the structure-activity relationships.
- Docking studies were proofed **7e** located into gatekeeper pocket with selectivity index of more than 18.9 (50% effective concentration of 5.3 μM, but no cytotoxicity at 100 μM).
- SPR results revealed that **7e** is able to bind to CypA with a K_D of 3.66 μM.