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Exploring naphthyl-carbohydrazides as inhibitors of influenza A viruses

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Abstract

A library of hydrazone derivatives was synthesized to target non-structural protein 1 of influenza A virus (NS1) as a means to develop anti-influenza drug leads. The lead compound 3-hydroxy-*N*-[(*Z*)-1-(5,6,7,8-tetrahydronaphthalen-2-yl)ethylideneamino]naphthalene-2-carboxamide, which we denoted as “HENC”, was identified by its ability to increase the melting temperature of the effector domain (ED) of the NS1 protein, as assayed using differential scanning fluorimetry. A library of HENC analogs was tested for inhibitory effect against influenza A virus replication in MDCK cells. A systematic diversification of HENC revealed the identity of the R group attached to the imine carbon atom significantly influenced the antiviral activity. A phenyl or cyclohexyl at this position yielded the most potent antiviral activity. The phenyl containing compound had antiviral activity similar to that of the active form of oseltamivir (Tamiflu), and had no detectable effect on cell viability.

Keywords

Carbohydrazone; Influenza; Hemagglutinin; Plaque; Real time PCR

1. Introduction

Influenza A virus is a virulent pathogen that causes outbreaks of respiratory disease on an annual basis, and is responsible for periodic pandemics that usually result in increased mortality [1]. The development of new antivirals is of great importance [2]. Four antiviral compounds are currently available, two (amantadine and rimantadine) are directed at the viral M2 ion channel protein, and two (oseltamivir and zanamivir) are directed against the viral neuraminidase [3,4]. However, influenza strains are developing resistance to these currently available antivirals, highlighting the need for novel inhibitors for influenza A virus.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ejmech.2013.10.063>.

Our group has been interested in identifying inhibitors of the influenza A virus non-structural protein 1 (NS1), a validated drug target [5]. NS1 has an *N*-terminal domain that binds double stranded RNA (dsRNA), and a *C*-terminal effector domain (ED) that interacts with several host proteins, specifically including those involved in the host cell innate immune response [6]. In particular, an X-ray structure has revealed the mode of interaction between the NS1 ED and the cellular target CPSF30. That interaction involves the binding of three aromatic rings, residues Y97, F98, and F102 from CPSF30, into a hydrophobic pocket on NS1 [7].

2. Initial approach

Recently, a high throughput screen for potential inhibitors of NS1 produced four compounds that appeared to suppress its function [8]. We carried out docking experiments which suggested that one of these compounds, NSC125044 ((*E*)-*N'*-(2,3-dihydro-1*H*-inden-5-yl)methylene-2-hydroxybenzohydrazide), could interact with the CPSF30 binding pocket in the NS1 ED. Our hypothesis was that this compound would bind to this ED pocket. To measure binding to the protein we employed differential scanning fluorimetry, which detects an increase in the melting temperature of a protein caused by the binding of a compound to the protein. However, NSC125044 did not increase the thermal stability of the ED (Fig. 1). An inspection of commercially available compounds using SciFinder provided a variety of related carbohydrazide compounds, several of which did increase the thermal stability of the effector domain. The most effective compound was CID 5762446, 3-hydroxy-*N*-[(*Z*)-1-(5,6,7,8-tetrahydronaphthalen-2-yl)ethylideneamino]naphthalene-2-carboxamide (HENC).

We then measured the antiviral activity of HENC. Antiviral activity was determined by measuring the effect of HENC and other compounds on the multiple cycle growth of influenza A/Udorn/72 (Ud) virus [9]. MDCK cells were infected with the Ud virus with a multiplicity of infection (moi) of 0.001 plaque forming units (PFU)/cell. After 1 h absorption, chemicals were added at the indicated concentrations in media containing 1% DMSO. Virus production at various time points after infection was measured in three ways: (1) plaque assays of aliquots of the media; (2) hemagglutinin (HA) assays of the aliquots of the media; and (3) quantitative RT-PCR measurements of the amount of one of the influenza A virus virion RNAs (vRNAs), specifically the vRNA encoding neuraminidase [10-12]. The quantitative RT-PCR reactions were carried out in duplicate, and converted to the amount of virus produced by normalizing the results to the quantitative RT-PCR results obtained at the beginning of the infection, which corresponds to the amount of virus added to the cells to initiate infection. The results of multiple experiments established that the quantitative RT-PCR results closely match the HA and plaque assay results. The HA and quantitative RT-PCR results yielded results more rapidly than the plaque assay results, and hence were used for most of the screening. The plaque assays were used to verify the HA and quantitative RT-PCR results. Fig. 2 shows that HENC at 30 μ M inhibits Ud virus by approximately 100-fold.

3. Chemistry

Such carbohydrazone derivatives like HENC have not been explored for their inhibitory activity against influenza virus. We therefore began a systematic diversification of the carbohydrazone derivatives to define which structural motifs were crucial to influenza virus antiviral activity. HENC is commercially available but its analogs are not, and due to the high price and limited number of suppliers, we decided to synthesize HENC as well as its analogs. The synthesis involves the esterification of 2-hydroxy 3-naphthoic acid with ethanol and catalytic amount of H_2SO_4 in refluxing conditions for 8 h (Scheme 1). The ester was then transformed to the hydrazide with the application of hydrazine at refluxing conditions in ethanol for 8 h. The hydrazide was then condensed with a previously synthesized ketone to form the ketonehydrazide derivatives. The ketones were synthesized by Friedel Crafts acylation. During NMR characterization, we found that a handful of molecules described in this paper either showed broad signals or duplicate peak patterns with different intensity ratios. This is attributed to the rapid or slow interconversion between the *E* and *Z* isomers of the inhibitors. The structure activity relationship study discussed in this paper with HENC and its derivatives is shown in Table 1.

The low solubility of HENC in most organic solvents creates a challenge for pharmaceutical applications. Hence, we introduced polar functional groups, such as hydroxyl on the periphery of the naphthalene ring (at 4 and 7 positions, Scheme 1). Unfortunately, the molecules become cytotoxic. We also incorporated ethylamine and propylamine groups in place of the R group to enhance the solubility. However these molecules showed no inhibitory activity.

4. Results and discussions

Fig. 3 shows a structure activity relationship (SAR) study of HENC and its analogs at 4 and 8 μM concentrations in the HA assay when titrated against influenza A virus produced in MDCK cells. The assay was carried out for a total time period of 48 h by analyzing aliquots at different times, such as 24, 36 and 48 h. The positive control in the graph indicates the production of virus particles in the absence of any inhibitors. HENC showed complete absence (below the detection limit) of any virus particles at 8 μM concentration at the 24 h time point. However, as the time progresses from 36 to 48 h, the antiviral activity of HENC decreases. At lower concentration (4 μM) and at 24 h, HENC showed activity as a potential inhibitor for influenza A viruses. After introducing a smaller ring size in place of cyclohexyl, such as cyclopentyl (**1**), the compound (only at 8 μM) showed good activity as an inhibitor at 24 h time point, whereas in other time points the molecule showed no activity. The presence of a phenyl ring (**8**) in place of cyclohexyl makes the molecule completely inactive. Hence we can conclude that the presence of the cyclohexyl ring is crucial for the antiviral activity.

Introducing an additional polar functional group, such as hydroxyl, at the 2-position of the tetrahydronaphthalene ring in **6**, led to similar activities with HENC at both lower and higher concentrations (4 and 8 μM). However, **6** showed better activity than HENC at the 24 h time point for the 4 μM concentration. However, the antiviral activity of **6** is reversed at

higher concentration (8 μ M) at the 36 and 48 h time points compared to HENC. Hence at higher concentrations, the presence of an extra hydroxyl group adjacent to the carbonyl group in the tetrahydro-naphthalene ring reduces the antiviral activity. Upon omitting both the hydroxyl groups, the molecule (**7**) completely loses antiviral properties. From these observations, we can conclude that the presence of an extra hydroxyl group at the 2-position of the tetrahydronaphthalene ring does not change the activity of HENC to a significant extent. However, the presence of a hydroxyl group at the 2-position in the naphthalene ring is important for the molecule to be active as an inhibitor for influenza A virus. Also, replacing the methyl group with hydrogen (**9**) makes HENC completely inactive. From this study, we can conclude that the presence of tetrahydronaphthalene ring, an alkyl group in place of R, and a hydroxyl group at the 2-position in the naphthalene ring are the important factors for the antiviral activity of HENC.

We also explored the importance of the naphthalene and tetrahydronaphthalene rings on both sides of HENC by replacing them with different aromatic rings (Fig. 4). At the same time we also investigated the effect of placing a hetero-atom such as bromine (**3**) on the naphthalene ring as well as the effect of a bigger alkyl substituent such as cyclopropyl (**5**) in place of methyl. Comparing HENC with **2** reveals that changing the naphthalene to a benzene ring makes the inhibitor completely inactive. On the other hand, comparing HENC with **4** indicates that omitting the cyclohexyl group makes the inhibitor completely inactive as well. Hence, the presence of the naphthalene ring and the tetrahydronaphthalene unit on the both sides of HENC are key features of the active inhibitor structure. Introducing a polar group such as bromine on the periphery of the naphthalene ring (**3**) reduces the inhibitory activity of HENC to a great extent. **3** shows antiviral activity only at higher concentrations (8 μ M) at 24 h time point whereas at longer time points (36 and 48 h), the molecule is completely inactive. However, one interesting observation was obtained by comparing **4** with **5**. Previously we have observed that omitting the cyclohexyl ring from the tetrahydronaphthalene ring makes the molecule (**4**) completely inactive. Also by replacing methyl with a cyclopropyl group in the same structural motif of **4**, **5** retains some antiviral property at shorter time point (24 h) at both lower (4 μ M) and higher (8 μ M) concentrations. Hence, the presence of a cyclopropyl unit (**5**) in place of methyl must be responsible for retaining inhibitory activity. From these observations we can conclude that the presence of a bigger alkyl group in place of methyl has a profound effect on the antiviral activity of these carbonyl derivatives.

From the previous discussions, we can conclude that the presence of a hydroxyl group at the 2-position in the naphthalene ring, an alkyl substituent in place of R, naphthalene and tetrahydronaphthalene rings connected *via* a carbonyl group, are the key features of the active inhibitor. Changing the R group from methyl to a bigger cyclopropyl group increases the antiviral activity. Hence our next goal was to analyze the effect of different substituents in place of the R group, by changing the electronic and the steric properties of the various aliphatic and aromatic units.

A comparison of R groups in terms of electronic and steric properties towards antiviral activity at 4 and 8 μ M concentrations in the HA assay when titrated against influenza A virus produced in the MDCK cells is shown in Fig. 5. When the R group is phenyl (**18**), the

derivative of HENC showed the highest activity as no sign of virus particles were observed in both 4 and 8 μM concentrations at all the time points. Whereas, in the real time qPCR assay (see Supporting informations for details about RT qPCR assay) at 24, 36 and 48 h time points, for both 4 and 8 μM concentrations, the viral RNA titer count is 10^3 times less than the positive control. Hence, high antiviral activity of **18** is supported by both assays. Changing the R group from phenyl to a cyclohexyl (**15**), retains equal activity in the HA assay at higher concentration (8 μM) at all the time points. However, at the 36 h time point and 4 μM concentration there was a tiny virus titer observed for **15**. Similar observation was obtained from RT-qPCR assay for **15** as well. Hence it can be concluded from both the assays that by changing the R group from an aromatic ring to an aliphatic ring system slightly reduces the activity of the inhibitor. If we further reduce the size of the R group to a cyclopentyl group (**14**), only at higher concentration (8 μM) does the inhibitor show similar activity as that of **18** and **15** in both assays. Whereas at lower concentrations (4 μM), the inhibitory effect of **14** is less than **15** at 36 and 48 h time points as observed in both assays. Further, by reducing to a cyclopropyl group, compound **13** showed very similar activity to **15** at higher concentrations (8 μM). Hence, in summary, the aromatic ring (**18**) in place of R showed the best antiviral activity. Whereas for aliphatic rings; cyclohexyl and cyclopropyl showed promising antiviral activity. Also reducing the size of the R group from cyclohexyl to cyclopropyl reduces the antiviral property of the carbonylhydrazide derivatives.

After changing the R group to a linear aliphatic chain such as *n*-propyl (**11**), we observed no sign of virus particles at 8 μM concentrations in the HA assay at all the time points. However, at 4 μM and longer time points (48 h), the molecule seems to lose its activity. The RT-qPCR assay supports this observation as well. The antiviral activity of linear chain *n*-propyl analog of HENC (**11**) is very similar to its cyclic counterpart (**13**) at higher concentrations (8 μM). Next, we compared the antiviral property by varying the size of the R group by incorporating different linear or branched aliphatic units, such as *iso*-propyl (**12**) and ethyl (**10**). With *iso*-propyl as the R group, **12** showed similar activity as that of *n*-propyl counterpart (**11**) in both assays at 24 h time points at both concentrations. However, the activity of **12** decreased with time as observed in both assays at both concentrations. With ethyl as the R group, **10** showed antiviral activity only at higher concentration (8 μM). On the other hand, HENC with a methyl group as R, showed better inhibitory activity than ethyl (**10**) or *iso*-propyl (**12**), but lower than that of *n*-propyl. In summary, the inhibitory activity is very dependent on the size of the R group. Among all the linear chain aliphatic substituents as the R group, *n*-propyl showed the most promising activity.

It is clear from both assays that the antiviral activity of the linear chain aliphatic groups in place of R are less than the cyclic groups at both lower and higher concentrations at all the time points. In order to compare the effect of polar substituents with the aliphatic and aromatic units, we incorporated 3-chloropropyl (**16**) and *N,N'*-dimethylamino propyl (**17**) groups in place of R. However, neither **16** nor **17** showed any antiviral activity at longer time points (36 and 48 h) and at lower concentration (4 μM). However, only at higher concentration (8 μM), **16** showed low antiviral activity. Hence, introduction of polar functional groups reduces the antiviral property of these inhibitors to a large extent. When we replace the R group with hydrogen, we observe a dramatic loss of activity at longer time

points (36 and 48 h) and very little activity was observed at the 24 h time point. This experiment proves the importance of an alkyl or an aryl group at the R position. The presence of a phenyl ring showed the best inhibitory activity among all the molecules tested in this experiment.

We then compared the most potential inhibitor (**18**) with the active form of Tamiflu (Fig. 6) [13]. Tamiflu pro-drug or oseltamivir is a neuraminidase inhibitor [14]. However, the ester form of Tamiflu is an inactive chemical which is converted to the active carboxylic acid form by hydrolysis [14]. Oseltamivir serves as a competitive inhibitor of the activity of the viral neuraminidase (NA) enzyme upon sialic acid, which is found on glycoproteins on the surface of the normal host cells [15]. By blocking the activity of the enzyme, oseltamivir prevents new virus particles from being released from the infected cells [15]. Single cycle growth curve of Influenza A virus production in MDCK cells at high multiplicity of infection (MOI) showed that the active form of Tamiflu is slightly more active than **18** up to 6.5 h time point, however, as the time progresses **18** showed better antiviral activity than the active form of Tamiflu.

To determine whether compound **18** is cytotoxic, we employed the CellTiter-Glo Luminescent Cell Viability assay (Promega), which measures the number of viable cells based on quantitating the amount of ATP in the cells. The luminescent signal is proportional to the amount of ATP in the lysed cells. MDCK cells were infected with Ud virus at low multiplicity either in the absence or presence of 4 μ M **18** (Fig. 7). Cells were lysed at the indicated times after infection. **18** did not reduce the amount of luminescence. We concluded that **18** did not reduce the number of viable cells during the 48 h of Ud virus infection.

5. Conclusion

We have successfully designed and synthesized different analogs of HENC which show appreciable inhibitory activity towards influenza A viruses (Fig. 8). From our experimental results, it is clear that the presence of a naphthalene ring and a tetrahydronaphthalene ring connected via a carbonyl linkage and the presence of a hydroxyl group at the 2-position in the naphthalene ring are crucial factors for antiviral activity. The presence of a phenyl ring in place of the R group showed the most promising activity, while reducing the size of the ring or introducing an aliphatic chain reduces the inhibitory activity. Our effort to improve the solubility of these inhibitors in aqueous solvent by introducing polar functional groups on the periphery of the naphthalene and tetrahydronaphthalene ring as well as in place of the R group reduced the activity of the inhibitor. Our future goal is to explore other aromatic and heterocyclic rings in place of R.

6. Experimental protocols

6.1. Synthesis

All reagents used were of commercial quality and were obtained from Aldrich Chemical Co. and Fisher Scientific. They were used as received. NMR spectra were recorded in their respective solvents on a Varian spectrometer using solvent as reference. Chemical shifts are given in parts per million (ppm). Signals are reported as m (multiplet), s (singlet), d

(doublet), t (triplet), q (quartet), bs (broad singlet), bm (broad multiplet). LC–MS data was recorded on an Agilent 6130 Quadrupole instrument. High resolution mass spectrometry was performed with a Varian 9.4T QFT-ESI ICR system. All solvents were removed by rotary evaporation under vacuum using a standard rotovap equipped with a dry ice condenser. All filtrations were performed with a vacuum. Flash chromatography was performed using Sorbtech 60 Å 230 × 400 mesh silica gel. All the molecules reported in this paper were synthesized by the same synthetic protocol as described in the paper. Here we are just reporting the complete characterization of the final compounds and synthetic protocol for the final step.

6.1.1. (E)-3-Hydroxy-*N'*-(1-(5,6,7,8-tetrahydronaphthalen-2-yl) ethylidene)-2-naphthohydrazide (HENC)—A mixture of 3-hydroxy-2-naphthohydrazide (0.42 g, 2.1 mmol) and 1-(5,6,7,8-tetrahydronaphthalen-2-yl)-ethanone (0.35 mL, 2.1 mmol) were refluxed for 20 h in ethanol under nitrogen atmosphere. The reaction mixture was filtered and washed with warm ethanol to afford light yellow color solid product (yield 81%). ¹H NMR (300 MHz, mixture of chloroform-*d* and methanol-*d*₄) δ ppm: 1.78 (m, 4H, CH), 2.28 (s, 3H, CH), 2.78 (m, 4H, CH), 7.16 (d, 1H, *J* = 6.2 Hz, CH), 7.24 (t, 1H, *J* = 6.4 Hz, CH), 7.30 (s, 1H, CH), 7.52 (t, 1H, *J* = Hz, CH), 7.58 *J* = 6.4 (d, 1H, *J* = 6.2 Hz, CH), 7.60 (s, 1H, CH), 7.78 (d, 1H, *J* = 6.2 Hz, CH), 7.98 (d, 1H, *J* = 6.2 Hz, CH), 8.62 (s, 1H, CH).

¹³C NMR (75 MHz, mixture of chloroform-*d* and methanol-*d*₄) δ ppm: 22.09, 23.01, 23.08, 29.08, 29.09, 111.02, 119.08, 124.00, 125.01, 126.00, 126.02, 127.03, 128.00, 128.01, 128.03, 128.05, 128.08, 130.03, 132.00, 137.04, 137.09, 139.02, 139.03, 139.04, 155.00. LC–MS (ESI+): *t*_R = 9.41 min (acetonitrile and water in 1:1 ratio was used as an eluent), *m/z* 359.1, purity 95%; [M + 1]⁺; HRMS (TOF, ESI) *m/z*: calculated for C₂₃H₂₃N₂O₂: 359.17540, found: 359.17510.

6.1.2. (E)-*N'*-(1-(2,3-Dihydro-1H-inden-5-yl)ethylidene)-3-hydroxy-2-naphthohydrazide (1)—A mixture of 3-hydroxy-2-naphthohydrazide (0.42 g, 2.1 mmol) and 1-(2,3-dihydro-1H-inden-5-yl)-ethanone (0.34 mL, 2.1 mmol) were refluxed for 20 h in ethanol under nitrogen atmosphere. The reaction mixture was filtered and washed with warm ethanol to afford light yellow color solid product (yield 78%). ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm: 2.06 (q, 2H, *J* = 14.8 Hz, CH), 2.48 (s, 3H, CH), 3.43 (m, 4H, CH), 7.29 (d, 1H, *J* = 7.9 Hz, CH), 7.37 (t, 1H, *J* = 8.4 Hz, CH), 7.38 (s, 1H, CH), 7.46 (t, 1H, *J* = 8.4 Hz, CH), 7.65 (d, *J* = 7.9 Hz, CH), 7.75 (s, 1H, CH), 7.76 (d, 1H, *J* = 7.9 Hz, CH), 7.98 (d, *J* = 7.9 Hz, CH), 8.62 (s, 1H, CH). ¹³C NMR (100 MHz, DMSO-*d*₆) δ ppm: 14.08, 18.54, 25.04, 32.16, 56.08, 110.64, 120.78, 122.28, 123.86, 124.09, 124.74, 125.69, 127.18, 128.25, 128.92, 129.00, 132.18, 143.97, 145.43, 152.77, 152.79, 161.45. LC–MS (ESI+): *t*_R 9.39 min (acetonitrile and water in 1:1 ratio was used as an eluent), *m/z* 345.2, purity 98%; [M + 1]⁺; HRMS (TOF, ESI) *m/z*: calculated for C₂₂H₂₁N₂O₂: 345.15975, found: 345.15970.

6.1.3. (E)-3-Hydroxy-*N'*-(1-(3-hydroxy-5,6,7,8-tetrahydronaphthalen-2-yl)ethylidene)-2-naphthohydrazide (6)—A mixture of 3-hydroxy-2-naphthohydrazide (0.42 g, 2.1 mmol) and 1-(3-hydroxy-5,6,7,8-tetrahydronaphthalen-2-yl)ethanone (0.39 mL, 2.1 mmol) were refluxed for 20 h in ethanol under nitrogen atmosphere. The reaction

mixture was filtered and washed with warm ethanol to afford light yellow color solid product (yield 72%). ^1H NMR (400 MHz, DMSO- d_6) δ ppm: 1.71 (m, 4H, CH), 2.42 (s, 3H, CH), 2.69 (m, 4H, CH), 6.62 (s, 1H, CH), 7.33 (s, 1H, CH), 7.35 (s, 1H, CH), 7.34–7.37 (m, 2H, CH), 7.52 (t, 1H, $J = 4.2$ Hz, CH), 7.76 (d, $J = 7.9$ Hz, CH), 7.97 (d, 1H, $J = 7.9$ Hz, CH), 8.59 (s, 1H, CH). ^{13}C NMR (100 MHz, DMSO- d_6) δ ppm: 13.22, 22.49, 22.94, 28.01, 28.74, 110.59, 116.70, 117.14, 120.31, 123.85, 125.72, 126.68, 127.08, 128.31, 128.55, 128.87, 132.02, 135.82, 140.49, 152.86, 156.16, 161.78, 163.01. LC–MS (ESI+): $t_R = 7.01$ min (acetonitrile and water in 1:1 ratio was used as an eluent), m/z 375.2, purity 99%; $[\text{M} + 1]^+$; HRMS (TOF, ESI) m/z : calculated for $\text{C}_{23}\text{H}_{23}\text{N}_2\text{O}_3$: 375.17032, found: 375.16983.

6.1.4. (*E*)-*N'*-(1-(5,6,7,8-Tetrahydronaphthalen-2-yl)ethylidene)-2-naphthohydrazide (7)—

A mixture of 3-hydroxy-2-naphthohydrazide (0.42 g, 2.1 mmol) and 1-(5,6,7,8-tetrahydronaphthalen-2-yl)ethanone (0.36 mL, 2.1 mmol) were refluxed for 20 h in ethanol under nitrogen atmosphere. The reaction mixture was filtered and washed with warm ethanol to afford light yellow color solid product (yield 76%). ^1H NMR (400 MHz, DMSO- d_6) δ ppm: 1.76 (m, 4H, CH), 2.36 (s, 3H, CH), 2.66 (m, 4H, CH), 7.12 (m, 1H, CH), 7.44–7.59 (m, 3H, CH), 7.93–8.07 (dt, 2H, CH), 8.43 (s, 1H, CH), 10.81 (s, 1H, CH). ^{13}C NMR (100 MHz, DMSO- d_6) δ ppm: 14.60, 22.61, 22.69, 28.64, 28.87, 123.61, 124.63, 126.75, 126.99, 127.63, 127.69, 127.90, 128.06, 128.70, 128.89, 131.39, 132.04, 134.23, 135.29, 136.48, 138.36, 155.73, 163.84. LC–MS (ESI+): $t_R = 9.8$ min (acetonitrile and water in 1:1 ratio was used as an eluent), m/z 343.2, purity 97%; $[\text{M} + 1]^+$; HRMS (TOF, ESI) m/z : calculated for $\text{C}_{23}\text{H}_{23}\text{N}_2\text{O}_1$: 343.18049, found: 343.18026.

6.1.5. (*E*)-3-Hydroxy-*N'*-(1-(naphthalene-2-yl)ethylidene)-2-naphthohydrazide (8)—

A mixture of 3-hydroxy-2-naphthohydrazide (0.42 g, 2.1 mmol) and 1-(naphthalene-2-yl)ethanone (0.36 mL, 2.1 mmol) were refluxed for 20 h in ethanol under nitrogen atmosphere. The reaction mixture was filtered and washed with warm ethanol to afford light yellow color solid product (yield 77%). ^1H NMR (400 MHz, DMSO- d_6) δ ppm: 2.48 (s, 3H, CH), 7.34–7.36 (m, 2H, CH), 7.38 (t, 1H, $J = 4.2$ Hz, CH), 7.49–7.57 (m, 2H, CH), 7.77 (d, 1H, $J = 7.9$ Hz, CH), 7.93–8.16 (m, 4H, CH), 8.19 (d, 1H, $J = 11.6$ Hz, CH), 8.35 (s, 1H, CH), 8.66 (s, 1H, CH). ^{13}C NMR (100 MHz, DMSO- d_6) δ ppm: 18.55, 110.69, 120.82, 123.69, 123.88, 125.71, 126.53, 126.58, 126.94, 127.19, 127.52, 127.77, 128.31, 128.58, 128.96, 132.31, 132.77, 133.33, 135.27, 135.78, 151.99, 152.83, 161.56. LC–MS (ESI+): $t_R = 9.1$ min (acetonitrile and water in 1:1 ratio was used as an eluent), m/z 355.1, purity 98%; $[\text{M} + 1]^+$; HRMS (TOF, ESI) m/z : calculated for $\text{C}_{23}\text{H}_{19}\text{N}_2\text{O}_2$: 355.14410, found: 355.14423.

6.1.6. (*E*)-3-Hydroxy-*N'*-((5,6,7,8-tetrahydronaphthalen-2-yl) methylene)-2-naphthohydrazide (9)—

A mixture of 3-hydroxy-2-naphthohydrazide (0.42 g, 2.1 mmol) and 5,6,7,8-tetrahydro naphthalene-2-carbaldehyde (0.34 mL, 2.1 mmol) were refluxed for 20 h in ethanol under nitrogen atmosphere. The reaction mixture was filtered and washed with warm ethanol to afford light yellow color solid product (yield 77%). ^1H NMR (300 MHz, DMSO- d_6) δ ppm: 1.68 (m, 4H, CH), 2.68 (m, 4H, CH), 7.00 (d, 1H, $J = 6.2$ Hz, CH), 7.22 (s, 1H, CH), 7.24 (s, 1H, CH), 7.35–7.45 (m, 3H, CH), 7.56 (d, 1H, $J = 6.2$ Hz, CH), 7.68 (d, 1H, $J = 6.2$ Hz, CH), 8.16 (s, 1H, CH), 8.42 (s, 1H, CH). ^{13}C NMR (75 MHz,

DMSO-*d*₆) δ ppm: 23.01, 23.09, 28.07, 29.03, 29.07, 111.09, 117.01, 124.00, 125.00, 125.09, 126.07, 128.02, 128.05, 128.09, 130.01, 130.06, 131.00, 132.03, 137.05, 137.09, 141.00, 150.00, 155.02, 166.00. LC–MS (ESI+): t_R = 9.2 min (acetonitrile and water in 1:1 ratio was used as an eluent), m/z 345.2, purity 98%; $[M + 1]^+$; HRMS (TOF, ESI) m/z : calculated for C₂₂H₂₁N₂O₂: 345.15975, found: 345.15946.

6.1.7. (*E*)-2-Hydroxy-*N'*-(1-(5,6,7,8-tetrahydronaphthalen-2-yl)ethylidene)benzohydrazide (2)

—A mixture of 2-hydroxybenzohydrazide (0.32 g, 2.1 mmol) and 1-(5,6,7,8-tetrahydro naphthalen-2-yl)ethanone (0.36 mL, 2.1 mmol) were refluxed for 20 h in ethanol under nitrogen atmosphere. The reaction mixture was filtered and washed with warm ethanol to afford light yellow solid product (yield 71%). In ¹H NMR, each peak appeared twice with an intensity ratio of 4:1. It is believed to be due to the presence of the two isomers *E* and *Z*. The intensity ratio number (as 4 or 1) is indicated at the end in the parenthesis for each peak. ¹H NMR (300 MHz, mixture of chloroform-*d* and methanol-*d*₄) δ ppm (intensity ratio 4): 1.75 (m, 4H, CH), 2.30 (s, 3H, CH), 2.75 (m, 4H, CH), 6.89–6.94 (d, 1H, *J* = 3.9 CH), 6.96 (d, 1H, *J* = 7.06–7.10 (d, 1H, *J* = 3.9 Hz, CH), 7.36–7.42 (m, 2H, CH), 7.55 (bs, 2H, CH). ¹³C NMR (75 MHz, mixture of chloroform-*d* and methanol-*d*₄) μ ppm (intensity ratio 4): 22.92, 22.93, 29.25, 29.29, 29.35, 117.98, 118.07, 118.25, 123.18, 123.74, 127.05, 127.31, 129.26, 130.36, 134.35, 134.40, 137.31, 139.68, 154.75, 159.76. LC–MS (ESI+): t_R = 7.78 min (acetonitrile and water in 1:1 ratio was used as an eluent), m/z 309.15, purity 98%; $[m + 1]^+$; HRMS (TOF, ESI) m/z : calculated for C₁₉H₂₁N₂O₂: 309.1575, found: 309.1596.

¹H NMR (300 MHz, mixture of chloroform-*d* and methanol-*d*₄) δ ppm (intensity ratio 1): 1.77 (m, 4H, CH), 2.42 (s, 3H, CH), 2.75 (m, 4H, CH), 6.77–6.79 (m, 1H, *J* = 3.9 Hz, CH), 6.97 (d, 1H, CH), 7.06–7.10 (d, 1H, CH), 7.24 (d, 2H, CH), 7.35 (m, 1H, CH), 7.45 (bs, 1H, CH). ¹³C NMR (75 MHz, mixture of chloroform-*d* and methanol-*d*₄) δ ppm (intensity ratio 1): 22.74, 22.78, 24.96, 26.44, 29.56, 113.01, 113.53, 115.06, 118.51, 119.05, 125.21, 125.36, 126.09, 130.36, 134.41, 139.03, 143.29, 160.39, 169.79.

6.1.8. (*E*)-6-Bromo-3-hydroxy-*N'*-(1-(5,6,7,8-

tetrahydronaphthalen-2-yl)ethylidene)-2-naphthohydrazide (3)—A mixture of 6-bromo-3-hydroxy-naphthohydrazide (0.59 g, 2.1 mmol) and 1-(5,6,7,8-tetrahydro naphthalen-2-yl)ethanone (0.36 mL, 2.1 mmol) were refluxed for 20 h in ethanol under nitrogen atmosphere. The reaction mixture was filtered and washed with warm ethanol to afford light yellow solid product (yield 68%). ¹H NMR (300 MHz, mixture of chloroform-*d* and methanol-*d*₄) δ ppm: 1.68 (m, 4H, CH), 2.22 (s, 3H, CH), 2.62 (m, 4H, CH), 6.96 (d, 1H, *J* = 3.9 Hz, CH), 7.10 (d, 1H, *J* = 3.9 Hz, CH), 7.24 (s, 1H, CH), 7.40 (s, 1H, CH), 7.50 (m, 2H, CH), 7.81 (s, 1H, CH), 7.89 (s, 1H, CH) 8.16 (s, 1H, CH). ¹³C NMR (75 MHz, mixture of chloroform-*d* and methanol-*d*₄) δ ppm: 13.87, 22.63, 22.70, 28.66, 28.88, 110.66, 117.74, 116.18, 116.49, 122.12, 123.67, 127.02, 127.02, 128.03, 128.37, 128.94, 134.17, 135.05, 136.56, 138.41, 152.87, 153.13, 161.06. LC–MS (ESI+): t_R = 4.4 min (acetonitrile and water in 1:1 ratio was used as an eluent), m/z 437.09, purity 96%; $[M + 1]^+$; HRMS (TOF, ESI) m/z : calculated for C₂₃H₂₂BrN₂O₂: 437.08563, found: 437.08592.

6.1.9. (E)-3-Hydroxy-*N'*-(1-phenylethylidene)-2-naphthohydrazide (4)—A

mixture of 3-hydroxy-2-naphthohydrazide (0.42 g, 2.1 mmol) and acetophenone (0.25 mL, 2.1 mmol) were refluxed for 20 h in ethanol under nitrogen atmosphere. The reaction mixture was filtered and washed with warm ethanol to afford white solid product (yield 68%). ¹H NMR (300 MHz, mixture of chloroform-*d* and methanol-*d*₄) δ ppm: 2.60 (s, 3H, CH), 7.21 (s, 1H, CH), 7.20–7.40 (m, 5H, CH), 7.54 (d, 1H, CH), 7.80 (m, 2H, CH), 7.95 (d, 1H, CH), 8.64 (s, 1H, CH). ¹³C NMR (75 MHz, mixture of chloroform-*d* and methanol-*d*₄) δ ppm: 15.00, 111.08, 119.02, 124.51, 124.82, 126.00, 126.10, 127.00, 128.00, 128.04, 128.09, 129.02, 129.06, 130.00, 132.06, 133.05, 136.08, 164.00. LC–MS (ESI+): *t*_R 5.8 min (acetonitrile and water in 1:1 ratio was used as an eluent), *m/z* 305.13, purity 96%; [M + 1]⁺; HRMS (TOF, ESI) *m/z*: calculated for C₁₉H₁₇N₂O₂: 305.12845, found: 305.12859.

6.1.10. (E)-*N'*-(Cyclopropyl(phenyl)methylene)-3-hydroxy-2-naphthohydrazide (5)—A

mixture of 3-hydroxy-2-naphthohydrazide (0.42 g, 2.1 mmol) and cyclopropyl phenyl methanone (0.31 mL, 2.1 mmol) were refluxed for 20 h in ethanol under nitrogen atmosphere. The reaction mixture was filtered and washed with warm ethanol to afford white solid product (yield 69%). ¹H NMR (300 MHz, mixture of chloroform-*d* and methanol-*d*₄) δ ppm: 0.91–1.00 (m, 4H, CH), 1.21–1.40 (m, 4H, CH), 2.05 (m, 1H, CH), 7.20–7.55 (m, 5H, CH), 7.40 (s, 1H, CH), 7.43 (m, 1H, CH), 7.62 (d, 1H, CH), 7.80 (d, 1H, CH), 7.95 (s, 1H, CH), 9.2 (s, 1H, CH). ¹³C NMR (75 MHz, mixture of chloroform-*d* and methanol-*d*₄) δ ppm: 5.00, 10.08, 17.05, 112.00, 124.72, 124.66, 125.50, 126.10, 128.09, 128.72, 128.63, 128.55, 128.46, 128.09, 129.01, 129.44, 129.36, 129.10, 129.98, 130.00, 130.76. LC–MS (ESI+): *t*_R = 5.9 min (acetonitrile and water in 1:1 ratio was used as an eluent), *m/z* 331.1, purity 96%; [M + 1]⁺; HRMS (TOF, ESI) *m/z*: calculated for C₂₁H₁₉N₂O₂: 331.14410, found: 331.14420.

6.1.11. (E)-3-Hydroxy-*N'*-(phenyl(5,6,7,8-tetrahydronaphthalen-2-yl)methylene)-2-naphthohydrazide (18)—A

mixture of 3-hydroxy-2-naphthohydrazide (0.42 g, 2.1 mmol) and phenyl(5,6,7,8-tetrahydronaphthalen-2-yl)methanone (0.49 mL, 2.1 mmol) were refluxed for 20 h in ethanol under nitrogen atmosphere. The reaction mixture was filtered and washed with warm ethanol to afford yellow solid product (yield 58%). ¹H NMR (300 MHz, mixture of chloroform-*d* and methanol-*d*₄, shows a 1:1 mixture of the *E* and *Z* isomers) δ ppm: 1.64–1.90 (m, 8H, CH), 2.6–2.95 (m, 8H, CH), 6.95–7.05 (t, 4H, CH), 7.03–7.13 (d, 4H, CH), 7.20–7.40 (m, 10H, CH), 7.50 (m, 4H, CH), 7.60–7.70 (m, 4H, CH), 8.02 (s, 1H, CH), 8.18 (s, 1H, CH). ¹³C NMR (75 MHz, mixture of chloroform-*d* and methanol-*d*₄) δ ppm: 22.65, 22.67, 29.96, 30.05, 111.78, 112.00, 124.16, 124.20, 125.90, 126.01, 126.05, 127.98, 128.00, 129.01, 129.41, 129.50, 129.81, 130.20, 130.83, 130.95, 131.24, 131.78, 137.31, 137.96, 138.00, 139.71, 140.00, 140.21, 156.71. LC–MS (ESI+): *t*_R = 9.64 min (acetonitrile and water in 1:1 ratio was used as an eluent), *m/z* 421.2, purity 96%; [M + 1]⁺; HRMS (TOF, ESI) *m/z*: calculated for C₂₈H₂₅N₂O₂: 421.19105, found: 421.19107.

6.1.12. (E)-*N'*-Cyclohexyl(5,6,7,8-tetrahydronaphthalen-2-yl)methylene-3-hydroxy-2-naphthohydrazide (15)—A

mixture of 3-hydroxy-2-naphthohydrazide (0.42 g, 2.1 mmol) and cyclohexyl(5,6,7,8-tetrahydronaphthalen-2-yl)methanone (0.51 mL, 2.1

mmol) were refluxed for 20 h in ethanol under nitrogen atmosphere. The reaction mixture was filtered and washed with warm ethanol to afford yellow solid product (yield 61%). ^1H NMR (300 MHz, mixture of chloroform-*d* and methanol-*d*₄) δ ppm: 1.00–1.43 (m, 6H, CH), 1.60–1.94 (m, 8H, CH), 2.60 (m, 1H, CH), 2.80 (m, 4H, CH), 6.84–6.96 (s, 2H, CH), 7.10–7.35 (m, 3H, CH), 7.40 (s, 1H, CH), 7.60 (m, 2H, CH), 7.88 (s, 1H, CH). ^{13}C NMR (75 MHz, mixture of chloroform-*d* and methanol-*d*₄) δ ppm: 22.80, 23.14, 26.00, 26.43, 26.51, 29.91, 29.99, 30.06, 30.90, 46.81, 111.06, 118.00, 124.10, 124.20, 124.30, 126.42, 127.31, 128.16, 128.62, 129.00, 130.05, 130.57, 136.38, 138.43, 139.08, 154.40, 164.22, 165.00. LC–MS (ESI⁺): t_{R} = 9.24 min (acetonitrile and water in 1:1 ratio was used as an eluent), m/z 427.2, purity 97%; $[\text{M} + 1]^+$; HRMS (TOF, ESI) m/z : calculated for $\text{C}_{28}\text{H}_{31}\text{N}_2\text{O}_2$: 427.23800, found: 427.23781.

6.1.13. (*E*)-*N'*-(Cyclopropyl(5,6,7,8-tetrahydronaphthalen-2-yl) methylene)-3-hydroxy-2-naphthohydrazide (13)—A mixture of 3-hydroxy-2-naphthohydrazide (0.42 g, 2.1 mmol) and cyclopropyl(5,6,7,8-tetrahydronaphthalen-2-yl)methanone (0.42 mL, 2.1 mmol) were refluxed for 20 h in ethanol under nitrogen atmosphere. The reaction mixture was filtered and washed with warm ethanol to afford a light yellow solid product (yield 71%). ^1H NMR (300 MHz, DMSO-*d*₆ in ^1H NMR, each peak appeared broad. It might be due to the presence of the two isomers *E* and *Z* in 1:1 ratio). δ ppm: 0.60 (m, 2H, CH), 1.2 (m, 2H, CH), 1.78 (m, 4H, CH), 1.89 (m, 1H, CH), 2.78 (m, 4H, CH), 7.02 (s, 1H, CH), 7.10 (d, 1H, CH), 7.15 (s, 1H, CH), 7.30 (m, 1H, CH), 7.50 (m, 2H, CH), 7.79 (d, 1H, CH), 8.01 (d, 1H, CH), 8.69 (s, 1H, CH). ^{13}C NMR (75 MHz, DMSO-*d*₆) δ ppm: 6.54, 8.73, 22.43, 22.61, 22.67, 28.64, 28.85, 110.59, 120.74, 123.82, 124.63, 127.23, 127.69, 128.27, 128.98, 129.65, 130.47, 132.83, 134.18, 135.77, 136.19, 137.79, 138.00, 154.57, 160.66. LC–MS (ESI⁺): t_{R} = 9.24 min (acetonitrile and water in 1:1 ratio was used as an eluent), m/z 385.2, purity 97%; $[\text{M} + 1]^+$; HRMS (TOF, ESI) m/z : calculated for $\text{C}_{25}\text{H}_{25}\text{N}_2\text{O}_2$: 385.19105, found: 385.19069.

6.1.14. (*E*)-3-Hydroxy-*N'*-(1-(5,6,7,8-tetrahydronaphthalen-2-yl) butylidene)-2-naphthohydrazide (11)—A mixture of 3-hydroxy-2-naphthohydrazide (0.42 g, 2.1 mmol) and 1-(5,6,7,8-tetrahydronaphthalen-2-yl)butan-1-one (0.42 mL, 2.1 mmol) were refluxed for 20 h in ethanol under nitrogen atmosphere. The reaction mixture was filtered and washed with warm ethanol to afford a light yellow solid product (yield 70%). ^1H NMR (300 MHz, DMSO-*d*₆) In ^1H NMR, each peak appeared twice with an intensity ratio of 5:1. It is believed to be due to the presence of the two isomers *E* and *Z* with the intensity ratio 5:1. ^1H NMR (300 MHz, DMSO-*d*₆) δ ppm (intensity ratio 5): 1.01 (t, 3H, J = 4.9 Hz, CH), 1.57 (sx, 2H, J = 9.9 Hz, CH), 1.73 (m, 4H, CH), 2.72 (m, 2H, CH), 2.75 (m, 4H, CH), 7.09 (d, 1H, J = 7.9 Hz, CH), 7.34 (t, 1H, J = 5.2 Hz, 1H), 7.36 (s, 1H, CH), 7.49 (t, 1H, J = 7.9 Hz, CH), 7.55 (s, 1H, CH), 7.56 (d, 1H, J = 6.8 Hz, CH), 7.75 (d, 1H, J = 5.6 Hz, CH), 7.96 (d, 1H, J = 5.6 Hz, CH), 8.63 (s, 1H, CH), 11.63 (bs, 1H, OH). ^{13}C NMR (75 MHz, DMSO-*d*₆) δ ppm: 14.10, 19.24, 22.64, 22.72, 28.68, 28.84, 28.92, 110.70, 120.74, 123.75, 123.95, 125.73, 126.98, 127.19, 127.32, 128.29, 128.96, 129.03, 132.54, 134.25, 135.76, 136.68, 138.36, 152.40, 161.02. LC–MS (ESI⁺): t_{R} 9.20 min (acetonitrile and water in 1:1 ratio was used as an eluent), m/z 387.2, purity 96%; $[\text{M} + 1]^+$; HRMS (TOF, ESI) m/z : calculated for $\text{C}_{25}\text{H}_{27}\text{N}_2\text{O}_2$: 387.20670, found: 387.20645.

¹H NMR (300 MHz, DMSO-*d*₆) δ ppm (intensity ratio 1): 0.87 (t, 3H, *J* = 4.9 Hz, CH), 1.42 (sx, 2H, *J* = 9.9 Hz, CH), 1.75 (m, 4H, CH), 2.58 (t, 2H, *J* = 5.6 Hz, CH), 2.80 (m, 4H, CH), 7.06 (d, 1H, *J* = 7.9 Hz, CH), 7.17 (s, 1H, CH), 7.18 (d, 1H, *J* = 4.2 Hz, CH), 7.29 (t, 1H, *J* = 5.2 Hz, CH), 7.44 (t, 1H, *J* = 7.56 Hz, 7.9 Hz, CH), (s, 1H, CH), 7.65 (d, 1H, *J* = 3.9 Hz, CH), 7.90 (d, 1H, *J* = 7.9 Hz, CH), 8.56 (s, 1H, CH). ¹³C NMR (75 MHz, DMSO-*d*₆) δ ppm: 13.53, 19.59, 22.49, 22.53, 28.75, 28.84, 28.91, 110.38, 120.51, 123.75, 125.60, 125.72, 127.19, 127.26, 128.19, 128.92, 129.76, 131.19, 132.75, 135.67, 137.77, 138.04, 157.78, 160.80, 161.31.

6.1.15. (*E*)-*N'*-(Cyclopentyl(5,6,7,8-tetrahydronaphthalen-2-yl) methylene)-3-hydroxy-2-naphthohydrazide (14)—A mixture of 3-hydroxy-2-naphthohydrazide (0.42 g, 2.1 mmol) and 1-(5,6,7,8-tetrahydronaphthalen-2-yl)butan-1-one (0.48 mL, 2.1 mmol) were refluxed for 20 h in ethanol under nitrogen atmosphere. The reaction mixture was filtered and washed with warm ethanol to afford a light yellow solid product (yield 70%). ¹H NMR (300 MHz, mixture of chloroform-*d* and methanol-*d*₄): 1.41–1.92 (bm, 12H, CH), 2.68–2.86 (bm, 4H, CH), 3.10 (m, 1H, CH), 6.83–6.96 (m, 2H, CH), 7.13–7.30 (m, 3H, CH), 7.40 (m, 1H, CH), 7.47–7.70 (m, 3H, CH). ¹³C NMR (75 MHz, mixture of chloroform-*d* and methanol-*d*₄): 23.80, 24.37, 26.17, 29.35, 30.00, 30.73, 31.05, 46.24, 48.44, 113.05, 117.98, 124.91, 125.00, 126.02, 126.93, 127.32, 128.68, 129.01, 129.97, 130.55, 130.94, 132.47, 137.78, 138.79, 139.11, 143.32, 156.00. LC–MS (ESI+): *t*_R = 9.29 min (acetonitrile and water in 1:1 ratio was used as an eluent), *m/z* 413.2, purity 96%; [*m* + 1]⁺; HRMS (TOF, ESI) *m/z*: calculated for C₂₇H₂₉N₂O₂: 413.22235, found: 413.22221.

6.1.16. (*E*)-3-Hydroxy-*N'*-(2-methyl-1-(5,6,7,8-tetrahydronaphthalen-2-yl)propylidene)-2-naphthohydrazide (12)—A mixture of 3-hydroxy-2-naphthohydrazide (0.42 g, 2.1 mmol) and 2-methyl-1-(5,6,7,8-tetrahydronaphthalen-2-yl)propan-1-one (0.42 mL, 2.1 mmol) were refluxed for 20 h in ethanol under nitrogen atmosphere. The reaction mixture was filtered and washed with warm ethanol to afford a light yellow solid product (yield 76%). ¹H NMR (300 MHz, mixture of chloroform-*d* and methanol-*d*₄): 1.21 (d, 6H, *J* = 3.2 Hz, CH), 1.90 (m, 4H, CH), 2.83 (m, 4H, CH), 3.04 (m, 1H, CH), 6.93–7.01 (m, 2H, CH), 7.20–7.38 (m, 3H, CH), 7.41–7.56 (m, 2H, CH), 7.62–7.71 (d, 1H, *J* = 3.2 Hz, CH), 9.22 (s, 1H, CH). ¹³C NMR (75 MHz, mixture of chloroform-*d* and methanol-*d*₄): 19.23, 20.04, 22.89, 29.27, 29.39, 35.11, 36.64, 112.43, 123.96, 124.02, 125.39, 126.25, 126.60, 126.92, 127.19, 127.59, 128.63, 129.19, 129.30, 130.36, 133.66, 138.88, 139.28, 142.85, 156.44. LC–MS (ESI+): *t*_R = 9.39 min (acetonitrile and water in 1:1 ratio was used as an eluent), *m/z* 387.20, purity 95%; [*M* + 1]⁺; HRMS (TOF, ESI) *m/z*: calculated for C₂₅H₂₇N₂O₂: 387.20670, found: 387.20663.

6.1.17. (*E*)-3-Hydroxy-*N'*-(1-(5,6,7,8-tetrahydronaphthalen-2-yl) propylidene)-2-naphthohydrazide (10)—A mixture of 3-hydroxy-2-naphthohydrazide (0.42 g, 2.1 mmol) and 1-(5,6,7,8-tetrahydronaphthalen-2-yl)propan-1-one (0.39 mL, 2.1 mmol) were refluxed for 20 h in ethanol under nitrogen atmosphere. The reaction mixture was filtered and washed with warm ethanol to afford a light yellow solid product (yield 76%). The ¹H NMR showed similar peaks with 4:1 intensity ratio due to the presence of the *E* and the *Z* isomers. ¹H NMR (300 MHz, mixture of chloroform-*d* and methanol-*d*₄, intensity ratio 4):

1.16 (t, 3H, $J = 6.2$ Hz, CH), 1.76–1.82 (m, 4H, CH), 2.65–2.84 (m, 6H, CH), 7.12 (d, 1H, $J = 6.4$ Hz, CH), 7.36 (d, 1H, $J = 7.9$ Hz, CH), 7.51 (t, 1H, $J = 6.4$ Hz, CH), 7.56 (s, 1H, CH), 7.57 (d, 1H, 1H, $J = 5.9$ Hz, CH), 7.76 (d, $J = 7.9$ Hz, CH), 8.00 (d, 1H, $J = 5.9$ Hz, CH), 8.64 (s, 1H, CH), 11.62 (s, 1H, OH). ^{13}C NMR (75 MHz, mixture of chloroform- d and methanol- d_4 , intensity ratio 4): 10.29, 22.59, 22.67, 28.63, 28.87, 28.89, 110.67, 120.88, 123.64, 123.88, 125.67, 126.94, 127.24, 128.23, 128.91, 129.04, 132.36, 133.83, 135.69, 136.66, 138.34, 152.50, 156.51, 161.21. LC–MS (ESI+): t_R 9.29 min (acetonitrile and water in 1:1 ratio was used as an eluent), m/z 373.2, purity 96%; $[m + 1]^+$; HRMS (TOF, ESI) m/z : calculated for $\text{C}_{24}\text{H}_{25}\text{N}_2\text{O}_2$: 373.19105, found: 373.19095.

^1H NMR (300 MHz, mixture of chloroform- d and methanol- d_4 , intensity ratio 1): 1.04 (t, 3H, $J = 5.9$ Hz, CH), 1.77 (m, 4H, CH), 2.60 (q, 2H, $J = 8.0$ Hz, CH), 2.77 (m, 4H, CH), 7.03 (m, 1H, CH), 7.19 (s, 1H, CH), 7.24 (d, 1H, CH), 7.32 (m, 1H, CH), 7.47 (m, 1H, CH), 7.68 (s, 1H, CH), 7.69 (d, 1H, CH), 7.98 (d, 1H, CH), 8.62 (s, 1H, CH), 10.94 (bs, 1H, OH). ^{13}C NMR (75 MHz, mixture of chloroform- d and methanol- d_4 , intensity ratio 1): 11.15, 22.45, 22.48, 28.69, 28.71, 31.11, 110.38, 120.57, 123.75, 123.91, 125.96, 127.12, 128.14, 128.20, 128.90, 129.71, 131.10, 132.68, 135.60, 137.70, 137.96, 152.20, 160.64.

6.1.18. (*E*)-*N'*-(4-Chloro-1-(5,6,7,8-tetrahydronaphthalen-2-yl) butylidene)-3-hydroxy-2-naphthohydrazide (16)—A mixture of 3-hydroxy-2-naphthohydrazide (0.42 g, 2.1 mmol) and 4-chloro-1-(5,6,7,8-tetrahydronaphthalen-2-yl)butan-1-one (0.49 mL, 2.1 mmol) were refluxed for 20 h in ethanol under nitrogen atmosphere. The reaction mixture was filtered and washed with warm ethanol to afford a light yellow solid product (yield 71%). ^1H NMR (400 MHz, $\text{DMSO}-d_6$): 1.71 (m, 4H, CH), 2.49 (m, 2H, CH), 2.68 (m, 2H, CH), 2.72 (m, 2H, CH), 4.65 (m, 4H, CH), 7.13 (d, 1H, $J = 7.8$ Hz, CH), 7.25 (s, 1H, CH), 7.27 (t, 1H, CH), 7.45 (t, 1H, CH), 7.62 (d, 1H, $J = 7.8$ Hz, CH), 7.70 (s, 1H, CH), 7.76 (d, 1H, $J = 3.9$ Hz, CH), 7.87 (d, 1H, $J = 3.9$ Hz, CH), 8.73 (s, 1H, CH). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): 17.47, 17.58, 22.10, 22.28, 29.07, 29.92, 62.11, 111.69, 122.35, 124.12, 125.92, 127.17, 127.18, 127.31, 129.30, 129.55, 130.36, 131.77, 131.79, 131.98, 137.50, 138.93, 148.49, 154.73, 166.76. LC–MS (ESI+): $t_R = 9.10$ min (acetonitrile and water in 1:1 ratio was used as an eluent), m/z 385.2, purity 98%; $[M + 1]^+$; HRMS (TOF, ESI) m/z : calculated for $\text{C}_{25}\text{H}_{25}\text{N}_2\text{O}_2$: 385.19105, found: 385.19085.

6.1.19. (*E*)-*N'*-(4-(Dimethylamino)-1-(5,6,7,8-tetrahydronaphthalen-2-yl)butylidene)-3-hydroxy-2-naphthohydrazide (17)—A mixture of 3-hydroxy-2-naphthohydrazide (0.42 g, 2.1 mmol) and 4-(dimethylamino)-1-(5,6,7,8-tetrahydronaphthalen-2-yl) butan-1-one (0.51 mL, 2.1 mmol) were refluxed for 20 h in ethanol under nitrogen atmosphere. The reaction mixture was filtered and washed with warm ethanol to afford a light yellow solid product (yield 61%). ^1H NMR (400 MHz, $\text{DMSO}-d_6$): 1.80 (s, 3H, CH), 2.00 (m, 2H, CH), 2.17 (m, 6H, CH), 2.33 (m, 2H, CH), 2.79 (m, 4H, CH), 3.05 (m, 2H, CH), 7.09 (d, 1H, $J = 3.9$ Hz, CH), 7.34 (t, 1H, $J = 4.2$ Hz, CH), 7.40 (s, 1H, CH), 7.50 (t, 1H, $J = 4.2$ Hz, CH), 7.60 (d, 1H, $J = 3.9$ Hz, CH), 7.69 (s, 1H, CH), 7.72 (d, 1H, $J = 3.9$ Hz, CH), 7.79 (d, 1H, $J = 3.9$ Hz, CH), 8.21 (s, 1H, CH). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): 22.72, 23.01, 23.10, 23.52, 24.17, 29.33, 29.47, 44.19, 55.62, 112.15, 118.03, 123.42, 123.90, 126.19, 127.04, 126.77, 127.55, 128.28, 128.32, 129.23,

133.08, 136.93, 137.43, 139.73, 156.45, 158.12, 167.45. LC-MS (ESI+): t_R = 6.10 min (acetonitrile and water in 1:1 ratio was used as an eluent), m/z 430.2, purity 98%; $[M + 1]^+$; HRMS (TOF, ESI) m/z : calculated for $C_{27}H_{32}N_3O_2$: 430.24890, found: 430.24887.

6.2. Biology

6.2.1. RT-PCR assay—An RT-qPCR assay was employed to measure the concentration of viral particles in aliquots of the supernatant surrounding cells infected with Influenza A (strain A/Udorn/307/1972 H3N2). The primer/probe set detects segment 6 of Influenza A. The probe is labeled with Fluorescein on the 5' end and Black Hole Quencher 1 on the 3' end. The oligonucleotides were ordered from Integrated DNA Technologies (Coralville, Iowa, USA) and have the following sequences:

FluA.Fw AAGGCTGGGCCTTTGACGAT

FluA.Rev AGGTGTGGACCAACCACCAA

FluA.Probe/56-FAM/ACGTGTGGATGGGAA-GAACGATCAGCGAGGA/3BHQ_1/

Supernatant samples were reverse transcribed in duplicate using M-MuLV Reverse Transcriptase (NEB) and the FluA.Fw primer (1.25 μ M concentration) for 12 h at 37 C. The reverse transcribed products were amplified in duplicate on MicroAmp Optical 384-Well Reaction plates (Applied Biosystems, Foster City, CA, USA) using FastStart Universal Probe Master with ROX (Roche, Indianapolis, IN, USA), FluA.Fw (0.8 μ M concentration), FluA.Rev (0.8 μ M concentration), FluA.Probe (0.24 μ M concentration), and fluorescence was recorded on a 7900HT Fast Real-Time PCR System (Applied Biosystems). The PCR protocol was as follows: 2 min at 50 C, 10 min at 95 C, and then 50 cycles of 95 C for 15 s and 60 C for 1 min. For any single time-course compound comparison, all the reverse transcription and qPCR reactions were performed using a single master mix to which either supernatant or reverse-transcribed product was added, respectively. Viral RNA concentrations are reported relative to the smallest concentration of viral RNA that was detected in each experiment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- [1]. Khanna MP, Kumar K, Choudhary BK, Vijayan VK. Emerging influenza virus: a global threat. *J. Biosci.* 2008; 33:475–482. [PubMed: 19208973]
- [2]. Lew W, Chen X, Kim CU. Discovery and development of GS4104 (oseltamivir): an orally active influenza neuraminidase inhibitor. *Curr. Med. Chem.* 2000; 7:663–672. [PubMed: 10702632]
- [3]. (a) Van der Vries E, Schutten M, Boucher CA. The potential for multidrug-resistant influenza, *Curr. Opin. Infect. Dis.* 2011; 24:599–604. (b) Leonov H, Astrahan P, Krugliak M, Arkin IT.

How do aminoadamantanes block the influenza M2 channel, and how does resistance develop. *J. Am. Chem. Soc.* 2011; 133:9903–9911. [PubMed: 21534619]

- [4]. (a) Noah DL, Twu KY, Krug RM. Cellular antiviral responses against influenza A viruses are countered at the post-transcriptional level by the viral NS1A protein via its binding to a cellular protein required for the 3' end processing of cellular pre-mRNAs. *Virology*. 2003; 307:386–395. [PubMed: 12667806] (b) McKimm-Breschkin JL, Rootes C, Mohr PG, Barrett S, Streltsov VA. In vitro passaging of a pandemic H1N1/09 virus selects for viruses with neuraminidase mutations conferring high-level resistance to oseltamivir and peramivir, but not to zanamivir. *J. Antimicrob. Chemother.* 2012; 67:1874–1883. [PubMed: 22563014]
- [5]. (a) Min JY, Krug RM. The primary function of RNA binding by the influenza A virus NS1 protein in infected cells: inhibiting the 2' oligo (A) synthetase/RNase L pathway. *Proc. Natl. Acad. Sci. U. S. A.* 2006; 103:7100–7105. [PubMed: 16627618] (b) Hale BG, Randall RE, Ortin J, Jackson D. The multifunctional NS1 protein of influenza A viruses. *J. Gen. Virol.* 2008; 89:2359–2376. [PubMed: 18796704] (c) Twu KY, Kuo RL, Marklund J, Krug RM. The H5N1 influenza virus NS genes selected after 1998 enhance virus replication in mammalian cells. *J. Virol.* 2007; 81:8112–8121. [PubMed: 17522219]
- [6]. (a) Li Y, Yamakita Y, Krug RM. Regulation of a nuclear export signal by an adjacent inhibitory sequence: the effector domain of the influenza virus NS1 protein. *Proc. Natl. Acad. Sci. U. S. A.* 1998; 95:4864–4869. [PubMed: 9560194] (b) Aramini JM, Li-Chung M, Zhou L, Schander CM, Hamilton K, Amer BR, Mack TR, Lee HW, Ciccocanti CT, Zhao L. Dimer interface of the effector domain of non-structural protein 1 from influenza virus: an interface with multiple functions. *J. Biol. Chem.* 2011; 286:26050–26060. [PubMed: 21622573]
- [7]. (a) Xia S, Monzingo AF, Robertus J. Structure of NS1A effector domain from the influenza A/udorn/72 virus, *Acta Crystallogr. Sect. D: Biol. Crystallogr.* 2009; 65:11–17. (b) Basu D, Walkiewicz MP, Frieman M, Baric RS, Auble DT, Engel DA. Novel influenza virus NS1 antagonists block replication and restore innate immune function. *J. Virol.* 2009; 83:1881–1891. [PubMed: 19052087]
- [8]. (a) Niesen FH, Berglund H, Vedadi M. The use of differential scanning fluorimetry to detect ligand interactions that promotes protein stability. *Nat. Protoc.* 2007; 2:2212–2221. [PubMed: 17853878] (b) Park H, Lee S. Determination of the active site protonation state of b-secretase from molecular dynamics simulation and docking experiment: implications for structure based inhibitor design. *J. Am. Chem. Soc.* 2003; 125:16416–16422. [PubMed: 14692784]
- [9]. (a) Hsiang TY, Zhou L, Krug RM. Roles of phosphorylation of specific serines and threonines in the NS1 protein of human influenza A viruses. *J. Virol.* 2012; 86:10370–10376. [PubMed: 22787231] (b) Zebedee SL, Lamb RA. Influenza A virus M2 protein: monoclonal antibody restriction of virus growth and detection of M2 in virions. *J. Virol.* 1988; 62:2762–2772. [PubMed: 2455818]
- [10]. (a) Otawa K, Hirakata Y, Kaku M, Nakai Y. Bacteriophage control of vancomycin-resistant enterococci in cattle compost. *J. Appl. Microbiol.* 2012; 113:499–507. [PubMed: 22702478] (b) Gronowicz E, Coutinho A, Melchers F. A plaque assay for all cells secreting Ig of a given type or class. *Eur. J. Immunol.* 1976; 6:588–590. [PubMed: 789096]
- [11]. (a) Camara B, Kamar N, Bonafe J, Danjoux M, Suc B, Rostaing L. Siphilis hepatitis and liver transplantation. *Med. Maladies Infect.* 2007; 37:121–123. (b) Murphy BR, Phelan MA, Nelson DL, Yarchoan R, Tierney EL, Alling DW, Chanock RM. Hemagglutinin-specific enzyme-linked immunosorbent assay for antibodies to influenza A and B viruses. *J. Clin. Microbiol.* 1981; 13:554–560. [PubMed: 7240388]
- [12]. (a) Wang CYT, Arden KE, Greer R, Sloots TP, Mackay IM. A novel duplex real time PCR for HP1V-4 detects co-circulation of both viral stereotypes among ill children during 2008. *J. Clin. Virol.* 2012; 54:83–85. [PubMed: 22361219] (b) Schmittgen TD, Zakrajsek BA. Effect of experimental treatment on house keeping gene expression: validation by real-time, quantitative RT-PCR. *J. Biochem. Biophys. Methods.* 2000; 46:69–81. [PubMed: 11086195]
- [13]. (a) Rimmelzwaan GF, Baars M, Claas ECJ, Osterhaus ADME. Comparison of RNA hybridization, hemagglutination assay, titration of infectious virus and immunofluorescence as methods for monitoring influenza virus replication in vitro. *J. Virol. Methods.* 1998; 74:57–66. [PubMed: 9763129] (b) Sullivan B, Carrera I, Drouin M, Hudlicky T. Symmetry-based design

for the chemo enzymatic synthesis of oseltamivir (Tamiflu) from ethyl benzoate. *Angew. Chem. Int. Ed.* 2009; 48:4229–4231.

- [14]. (a) Ward P, Small I, Smith J, Suter P, Dutkowski R. Oseltamivir (Tamiflu) and its potential for use in the event of influenza pandemic. *J. Antimicrob. Chemother.* 2005; 55:5–21. (b) Mita T, Fukuda N, Roca FX, Kanai M, Shibasaki M. Second generation catalytic asymmetric synthesis of Tamiflu: allylic substitution route. *Org. Lett.* 2007; 9:259–262. [PubMed: 17217279]
- [15]. (a) Le MQ, Kiso M, Someya K, Sakai TY, Nguyen HT, Nguyen HLK, Pham DN, Ngyen HH, Yamada S, Muramoto Y, Horimoto T, Takada A, Goto H, Suzuki T, Suzuki Y, Kawaoka Y. Avian flu: isolation of drug-resistant H5N1 virus. *Nature.* 2005; 437:1108. [PubMed: 16228009] (b) Treanor JJ, Hayden FG, Vrooman PS, Barbarash R, Bettis R, Riff D, Singh S, Kinnersley N, Ward P, Mills RG. Efficacy and safety of the oral neuramidase inhibitor oseltamivir in treating acute influenza; a randomized controlled trial. *J. Am. Med. Assoc.* 2000; 283:1016–1024.

Fig. 1. Melting temperature shift on fluorescence (left) (broken line for HENC and solid line for NSC125044) and in differential of the fluorescence curve (right) (white circles for HENC and black circles for NSC125044).

Fig. 2.
Comparison of antiviral activity of HENC with control at 30 µM.

Fig. 3.
Inhibition of influenza A virus production in MDCK cells by HENC and its analogs (shown at the top).

Fig. 4.
Inhibition of influenza A virus production in MDCK cells by HENC and its analogs (shown at the top).

Fig. 5.
Inhibition of influenza A virus production in MDCK cells by HENC and its analogs (shown at the top).

Fig. 6. Comparison of **18** and the active form of Tamiflu *via* single cycle growth curve on Influenza virus A production in MDCK cells.

Fig. 7.
The effect of **18** on cell viability during infection with Ud virus at low multiplicity.

Fig. 8.
Summary of the SAR study.

Scheme 1.
General synthesis of HENC and its analogs.

Table 1

HENC and its analogs.

x	y	z	m	n	R	Compound name
OH	Ph	Cy	H	H	Me	HENC
OH	Ph	Cp	H	H	Me	1
OH	None	Cy	H	H	Me	2
OH	Ph	Cy	Br	H	Me	3
OH	Ph	None	H	H	Me	4
OH	Ph	None	H	H	Cypr	5
OH	Ph	Cy	H	OH	Me	6
H	Ph	Cy	H	H	Me	7
OH	Ph	Ph	H	H	Me	8
OH	Ph	Cy	H	H	H	9
OH	Ph	Cy	H	H	Et	10
OH	Ph	Cy	H	H	<i>n</i> -Pr	11
OH	Ph	Cy	H	H	<i>i</i> -Pr	12
OH	Ph	Cy	H	H	Cypr	13
OH	Ph	Cy	H	H	Cp	14
OH	Ph	Cy	H	H	Cy	15
OH	Ph	Cy	H	H	Cl- ^t Bu	16
OH	Ph	Cy	H	H	DMA- ^t Bu	17
OH	Ph	Cy	H	H	Ph	18

Cy = cyclohexyl, Cp = cyclopentyl, Cy-pr = cyclopropyl, DMA-But = dimethylamino *tert*-butyl, Cypr = cyclopropyl, DMA = dimethylamino, ^tBu = *tert*-butyl.