Two Birds with One Stone? Possible Dual-Targeting H1N1 Inhibitors from Traditional Chinese Medicine

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Abstract

The H1N1 influenza pandemic of 2009 has claimed over 18,000 lives. During this pandemic, development of drug resistance further complicated efforts to control and treat the widespread illness. This research utilizes traditional Chinese medicine database@Taiwan (TCM Database@Taiwan) to screen for compounds that simultaneously target H1 and N1 to overcome current difficulties with virus mutations. The top three candidates were de novo derivatives of xylopine and rosmaricine. Bioactivity of the de novo derivatives against N1 were validated by multiple machine learning prediction models. Ability of the de novo compounds to maintain CoMFA/CoMSIA contour and form key interactions implied bioactivity within H1 as well. Addition of a pyridinium fragment was critical to form stable interactions in H1 and N1 as supported by molecular dynamics (MD) simulation. Results from MD, hydrophobic interactions, and torsion angles are consistent and support the findings of docking. Multiple anchors and lack of binding to residues prone to mutation suggest that the TCM de novo derivatives may be resistant to drug resistance and are advantageous over conventional H1N1 treatments such as oseltamivir. These results suggest that the TCM de novo derivatives may be suitable candidates of dual-targeting drugs for influenza.

Introduction

The first global pandemic of the 21st century was announced by the World Health Organization (WHO) in 2009 due to the worldwide spread of influenza A subtype H1N1 (H1N1/09) [1]. More than 214 countries have reported laboratory confirmed cases, and more than 18,449 deaths have been recorded [2]. Currently, the neuraminidase inhibitor Tamiflu (oseltamivir) remains the primary drug prescribed to patients infected with H1N1/09 [3]. However, the emergence of drug resistant viral strains [4] and limited drug administration window [5] exemplifies the need for additional therapies.

Important constituents of influenza surface membrane proteins include hemagglutinin, neuraminidase, and the matrix protein 2 (M2) proton channel [6,7]. Hemagglutinin mediates the binding of viral particles to host cell surface sialic acid and the invasion of viruses into host cell [8–10]. Neuraminidase is responsible for the cleavage of sialic acid residues to promote the release of progeny viruses [11,12]. M2 proton channels are critical for viral mRNA incorporation into the virion and virus budding [13]. Over one hundred serological subtypes [14] have been identified through different combinations of the 16 hemagglutinin (H1–H16) and nine neuraminidase groups (N1–N9) currently known. The 3D-structure of M2 proton channels have recently been solved in both influenza A and B [15,16], allowing more in depth studies regarding its biological function and action mechanism [17–19]. These proteins have been used as targets for rational attempts to design drugs for influenza [20–27].

The H1N1/09 virus strain is a triple reassortant that contains gene segments from avian, swine and human influenza viruses [28]. In addition to antigenic shift that can lead to fundamental changes in influenza surface antigens, antigenic drift could reduce binding affinity of host antibodies to antigens [29,30]. A major challenge in influenza vaccine development is the rapid evolution of influenza viruses, causing vaccines to be easily outdated and reformulation necessary each year [31–33]. Although the H1N1/09 virus is susceptible to neuraminidase inhibitors, cases regarding oseltamivir-resistant viruses with neuraminidase mutation (such as H275Y) have been reported [34,35]. Given that influenza viruses have RNA genomes that are prone to changes, it is imperative to devise new therapies. Much effort has been made to investigate the mechanism and devise alternative drugs against the drug-resistance issue of H1N1 [36–40]. Developing inhibitors that target both H1 and N1 antigens can reduce resistance issues resulting from the mutation of a single target antigen.

Computational approaches have been widely applied to molecular biology and medicine [41–50]. Structure-based methods, including docking and MD simulation, are invaluable tools in
Author Summary

The influenza A subtype H1N1 (H1N1/09) pandemic raised public concerns due to drug resistance strains. Drug resistance occurs from conformational changes causing the original drug to lose binding ability and exhibit biological effects. The world’s largest TCM Database®Taiwan was employed to screen for potential leads that simultaneously bind to H1 and N1. Three de novo compounds derived from *Rosemarinus officinalis* and *Guatteria amplifolia* were identified as having dual binding properties to H1 and N1. Structural analysis indicated that the candidates bind to multiple residues in both H1 and N1. In addition, the de novo derivatives were predicted as bioactive using four different computational models. The compounds are validated as potent dual targeting influenza drug candidates through multiple validations. Key advantages of the candidates include (1) binding to H1 and N1 through multiple amino acids, and (2) not binding to known mutation residues in H1 or N1. Such advantages can reduce drug resistance caused by single point mutations. On a broader context, features important for successful H1N1 drug development are discussed in hopes of providing starting templates for drug development and improvements.

Figure 1. The flowchart of dual-target H1N1 experimental procedures. 
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Table 1. Docking results of top ten *de novo* derivatives.

| Docking | Name           | H1    | N1    | H1+N1 |
|---------|----------------|-------|-------|-------|
|         | Xylopine_2     | 144.183 | 139.023 | 283.206 |
|         | Rosmaricine_14 | 138.916 | 125.808 | 264.724 |
|         | Rosmaricine_15 | 135.671 | 121.759 | 257.430 |
|         | Rosmaricine_5  | 97.921  | 116.324 | 214.245 |
|         | Rosmaricine_16 | 96.528  | 113.750 | 210.278 |
|         | Rosmaricine_23 | 95.854  | 113.573 | 209.427 |
|         | Rosmaricine_12 | 95.520  | 113.207 | 208.727 |
|         | Rosmaricine_6  | 95.429  | 113.069 | 208.498 |
|         | Rosmaricine_21 | 95.233  | 112.762 | 207.995 |
|         | Rosmaricine_11 | 94.731  | 112.283 | 207.014 |
|         | Tamiflu®       | 48.545  | 87.794 | 137.482 |

*control.
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drug discovery and design. Computational docking is important for investigating ligand-protein interactions and elucidating binding mechanisms [51–57]. Since publication of the pioneer paper in 1977 [58], it has been established that low-frequency motions existing in proteins and DNA can help reveal dynamic mechanisms underlying fundamental biological functions [59–63]. NMR observation later confirmed such inferences and the findings were applied to medical treatments [64–67]. In recent years, application of molecular dynamics to investigate internal motions and biological functions of biomacromolecules has opened new frontiers. Vast amounts of information on molecular recognition and binding [68–71], conformations or conformational changes [72–75], molecular mechanisms of bioactivity and stability [76–79], and drug discovery [80–84] have been found. To understand interaction of drugs with proteins or DNA, consideration should be given not only to the static structures but dynamical information obtained by simulation through a dynamic process. In this regard, both docking and MD simulation were utilized in this study to provide comprehensive analysis protein-ligand interactions under static and dynamic conditions.

Much effort has been placed on developing new, effective influenza treatments, but most have focused on neuraminidase or M2 as the target protein [37,38,85–87]. To date, no hemagglutinin inhibitor is available. Traditional Chinese medicine (TCM) has been used extensively for finding effective drugs [88], and we...
have successfully designed novel medicinal compounds and identified potential drug leads through traditional Chinese Medicine Database@Taiwan (TCM@Taiwan) [89]. Preliminary studies conducted in this lab show potential for TCM compounds to serve as neuramindase and hemagglutinin inhibitors individually [90–95]. In view of the current needs for drugs effective against native and mutant H1N1/09 and our promising preliminary results, the present study integrates the concept of “dual targeting” with the aforementioned computational tools and TCM in the attempt to identify dual-targeting inhibitors of H1N1 that may be useful for drug development.

**Results/Discussion**

**Screening and Structure Analysis**

The experimental procedures and screening results after each filtering step are summarized in Figure 1. Among the 829 native TCM compounds, 81 docked into both H1 and N1 and were used for *de novo* evolution (Table S1). *De novo* compounds with dual binding capacities to H1 and N1 were ranked by combined DockScore and the top ten derivatives are listed in Table 1. Nine of the ten top ranking *de novo* compounds were derived from Rosmaricine, a natural compound isolated from *Rosmarinus officinalis* [96]. The remaining *de novo* compound was based on Xylopine, which is naturally found in *Guatteria amplifolia* [97].

The top three derivatives, Xylopine_2, Rosmaricine_14 and Rosmaricine_15, have in common a pyridinium addition to their native structure (Figure 2). The pyridinium addition could be the main explanation for higher DockScores of these three derivatives compared to their native compounds and the other derivatives. Rosmaricine_14 and Rosmaricine_15 differed by the number of fused rings, but the slight difference in DockScore suggests that addition of an acyclic ring has little influence on binding affinity.

**Characteristics of De Novo Product Binding Poses**

Docking of the *de novo* compounds back to the receptor provides insights to modifications that can be made to modulate or enhance molecular properties and also highlights important protein-ligand interactions. When docked into the N1 protein binding site, Xylopine_2 interacts with Asp151 via a protonated amino group and has pi and hydrogen bond (H-bond) interactions with Glu228, Glu225, Tyr226, Gln107, Tyr228, and Tyr320. Rosmaricine_14 and Rosmaricine_15 also interact with the same amino acids, but their orientations are different due to the presence of fused rings.

**Figure 5.** RMSD and total energy profiles of top three *de novo* derivatives and Tamiflu® in N1 complexes during 20 ns MD simulation. doi:10.1371/journal.pcbi.1002315.g005

**Figure 6.** Important locations for ligand-N1 stability (left) and relative spatial arrangements (right) within the N1 protein binding site. (A) Xylopine_2, (B) Rosmaricine_14, (C) Rosmaricine_15, and (D) Tamiflu®. Ligands are presented in cyan, amino acids in yellow, and hydrogen bond formations in white dashed lines. doi:10.1371/journal.pcbi.1002315.g006

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**Table 2.** N1 activity prediction (pIC₅₀) of top three *de novo* derivatives by MLR and SVM models.

| Name          | MLR     | SVM     |
|---------------|---------|---------|
| Xylopine_2    | 9.837   | 7.295   |
| Rosmaricine_14| 14.069  | 5.963   |
| Rosmaricine_15| 14.116  | 5.915   |
| Tamiflu®*     | 7.613   | 6.372   |

*control.

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interactions with Trp179 and Glu228, respectively (Figure 3A).
Rosmaricine_14 (Figure 3B) and Rosmaricine_15 (Figure 3C),
have interactions with Asp151 and Arg293 via the carbonyl
group and Glu228 through the 2-aminopyridinium group. Tamiflu® forms H-bond interactions with Arg156, Arg293 and
Arg368, but not with Asp151 or Glu228 (Figure 3D). Both
Asp151 and Glu228 have been reported as one of the major
residues in the N1 ligand binding site [98,99]. The ability of the
de novo derivatives to form interactions with both Asp151 and
Glu228 may account for the higher DockScores.

Binding of the top three de novo derivatives to H1 site is detailed
elsewhere [92]. The ability to bind with important H1 residues
Asp103 and Arg238 [100] indicates the dual targeting possibility of
the candidates.

Prediction of N1 Inhibition by Support Vector Machine (SVM) and Multiple Linear Regression (MLR)
The top ranking model generated by genetic function
approximation (GFA) includes the following descriptors: ES_ Sum_dssC, CHI_3_C, Kappa_1, Jurs_PNSA_1, and Jurs_RPC.
Utilizing these five descriptors, the MLR model established for the
neuraminidase inhibitors is:

$$pIC_{50} = 3.8919 - 1.0012 \times ES_{\text{Sum_dssC}}$$
$$+ 2.116 \times CHI_3_C$$
$$+ 0.50012 \times Kappa_1 - 0.029158 \times Jurs_{\text{PNSA_1}}$$
$$+ 2.0289 \times Jurs_{\text{RPC}}$$

Correlation between the observed and predicted activities of the
27 ligands are shown in Figure 4A. All values were within the 95%
prediction bands and the $r^2$ value = 0.8043. The SVM model was
constructed using identical molecular descriptors and ligands as
the MLR model. The $r^2$ value of the SVM model was 0.8605 and
the correlation between observed and predicted activities of 27
ligands are illustrated in Figure 4B.

Table 2 summarizes the $pIC_{50}$ values of Tamiflu® and the top
three candidates as predicted by the generated MLR and SVM
models. The predicted activity of Tamiflu® using the generated
MLR model ($pIC_{50} = 7.613$) is similar to observed bioactivity
values reported in the literature ($pIC_{50} = 7.823$) [101]. This
indicates that the generated MLR model is a good prediction
model. Predicted activity values using the SVM model indicate a
lower $pIC_{50}$ with regard to Tamiflu®. Nonetheless, both models
indicate that all TCM de novo derivatives are good candidates with
neuraminidase inhibitory activity. MLR and SVM models for
predicting hemagglutinin inhibitory activity were not established
due to the lack of available hemagglutinin inhibitor structures in
the literature.

Molecular Dynamics (MD) Simulation

Stability profile analysis. Root mean square deviation (RMSD)
and total energy results from MD are summarized in
Figure 5 and provide information on N1-ligand complex and
ligand stability. During the 20 ns simulation process, the RMSDs
of the four complexes ranged between 1.4–1.7 Å. Xylopine_2
stabilized after 15 ns, and the total energy of the complex
equilibrated at $-19,000$ kcal/mol. The ligand RMSD of
Rosmaricine_14 remained stable throughout the simulation, and
no evident changes in total energy were observed after 17 ns. The
RMSD and total energy of Rosmaricine_15 stabilized after 13 ns.
Fluctuations in ligand RMSD was observed in Tamiflu® for the
first 5 ns, but no changes were observed in ligand RMSD and total
energy from 5 ns until the end of MD. The larger ligand RMSD

| Name             | H-bond   | Ligand atom | Amino acid | H-bond occupancy |
|------------------|----------|-------------|------------|------------------|
| Xylopine_2       | H-bond_1 | H55         | Glu228 : OE2 | 98.23%          |
|                  | H-bond_2 | H57         | Glu228 : OE1 | 7.23%           |
|                  | H-bond_3 | H57         | Glu228 : OE2 | 2.73%           |
|                  | H-bond_4 | H66         | Glu228 : OE1 | 80.50%          |
|                  | H-bond_5 | H66         | Glu228 : OE2 | 35.50%          |
|                  | H-bond_6 | H57         | Leu224 : O  | 19.28%          |
| Rosmaricine_14   | H-bond_1 | O1          | Arg293 : HH22 | 4.70%         |
|                  | H-bond_2 | H66         | Glu119 : OE1 | 98.63%          |
|                  | H-bond_3 | H66         | Glu119 : OE2 | 5.38%           |
|                  | H-bond_4 | H55         | Glu228 : OE2 | 96.05%          |
| Rosmaricine_15   | H-bond_1 | O1          | Arg293 : HH22 | 99.80%         |
|                  | H-bond_2 | H48         | Asn344:OD1  | 78.10%          |
|                  | H-bond_3 | H53         | Glu228:OE2  | 34.00%          |
|                  | H-bond_4 | H64         | Thr226:O    | 45.95%          |
|                  | H-bond_5 | H55         | Thr226:O    | 26.50%          |
| Tamiflu®         | H-bond_1 | O3          | Arg368:HH22 | 82.50%          |
|                  | H-bond_2 | O10         | Arg118:HE   | 8.90%           |
|                  | H-bond_3 | O10         | Arg156:HH12 | 57.33%          |
|                  | H-bond_4 | H25         | Tyr402:OH   | 32.30%          |

Table 3. H-bond analysis of top three de novo derivatives and Tamiflu® in N1 during MD simulation.

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fluctuations and higher total energy observed for Xylopine_2 may be attributed to its spatial structure and docking characteristics. Xylopine_2 consists of a bulky xylopine and a 2-aminopyridinium residue linked through 1 in a way similar to that of cis conformations (Figure 6). It is well established that cis conformations are less stable than their trans counterparts, and thus may explain the higher total energy levels for Xylopine_2. In addition, Xylopine_2 binds to N1 though the aminopyridinium residue, allowing the xylopine structure more freedom to rotate and thereby increasing ligand RMSDs and total energy levels.

**H-Bond network during MD simulation.** The H-bond occupancy of each compound in N1 is summarized in Table 3. Xylopine_2 can easily form H-bond at Glu228, and the occupancy rate can reach as high as 98.23%. Rosmaricine_14 forms H-bonds at Glu119 and Glu228, with high occupancy rates of 98.63% and 96.05%. Rosmaricine_15 forms H-bonds with Arg293 and Asn344 (99.80% and 78.10%, respectively). Tamiflu primarily forms H-bonds at Arg368 (82.50%) and Arg156 (57.33%). High H-bond occupancy rates indicate that the compound can maintain stable binding during the 20 ns MD. Since occupancy is calculated from the designated cut-off distance of 2.5 Å, distance fluctuations exceeding the critical value can lead to underestimation of H-bond formations and skewed conclusions of candidate stability. Distance profiles of ligand atoms capable of forming H-bonds during MD are illustrated in Figure 7. The distance between Xylopine_2 and Glu228 was maintained between 2–5 Å (Figure 7A). The distance of Rosmaricine_14 and Glu119 and Glu 228 also remained between 2–3 Å (Figure 7B). From Table 3, Rosmaricine_15 and Tamiflu did not form high occupancy H-bonds with Tyr402. Intriguingly, bond distance profiles indicate that Tyr402 was one of the key amino acids for H-bond formation (Figure 7C, 7D). Tyr402 bond distance generally exceeded 2.5 Å in Rosmaricine_15, thus explaining the low occupancy rate in Table 3. For Tamiflu®, the distance in the first ns was between 3–4 Å and then decreased to 2–3 Å from 1–20 ns, thus accounting for the low occupancy rate as well. Despite the low occupancy rates, the bond distance profiles suggest that Tyr402 is an important N1 binding site for Rosmaricine_15 and Tamiflu®.

**Possible mechanism for protein-ligand interaction.** Insights to how ligand stabilization occurs within the protein binding site can be discerned from MD simulation. The H-bond formations with Leu224 at 2 and Glu228 at 3 and 4 “sandwich” the aminopyridinium and anchors Xylopine_2 (Figure 6A). However, the xylopine moiety remained unattached, causing strain to the compound and possibly contributing to large H-bond distance fluctuations (Figure 7A). At approximately 15 ns, attraction between Glu228 and 3 causes the terminal amine residue to torque towards Glu228 increasing the distance from Leu224...
As a result, the stable H-bond with Leu224 was lost, and an additional H-bond with Glu228 was formed from 2. At the end of MD simulation, the primary binding force for Xylopine_2 was H-bonds formed with Glu228. In contrast to Xylopine_2, multiple binding sites secured Rosmaricine_14 and 15 within the binding site as reflected by the small H-bond distance fluctuations compared to Xylopine_2 (Figure 7B, 7C). Rosmaricine_14 bound to Glu119, Glu228, and Arg293 through 6, 7, and 8 respectively (Figure 6B). The multiple attachment points anchor both the aminopyridine moiety and the rosmaricine moiety, reducing strain on the molecular structure as reflected by the low total energy and bond distance fluctuations. The increase in bond distance from Arg293 in Figure 7B starting at 3 ns was due to Arg293 intermolecular H-bond formation between the O atom and NH2 residue. Nonetheless, all H-bonds were maintained throughout the MD simulation, suggesting good stability of Rosmaricine_14. The mechanism for stability of Rosmaricine_15 is similar to that of Rosmaricine_14. In addition to H-bonds at 9 with Glu228 and 10 with Arg293 which are identical to Rosmaricine_14, H-bonds are formed at 11 with Glu228, 12 with Thr226, 13 with Asn262, and 14 with Tyr402 (Figure 6C). These additional anchor points stabilized residues that were available for rotation in Rosmaricine_14, further lowering the total energy of the compound (Figure 5C). Within the anchored ligand, twisting of 15 contributes to H-bond fluctuations at Glu228 such as

![Figure 8. Torsion angles of TCM candidates and Tamiflu in H1 and N1.](A) Xylopine_2, (B) Rosmaricine_14, (C) Rosmaricine_15 and (D) Tamiflu.H1N1 Inhibitors from Traditional Chinese Medicine. doi:10.1371/journal.pcbi.1002315.g008)
observed at 14 ns (Figure 7C). As a result of the torque at 15, 11 flips toward 12 and the stable H-bond with Glu228 is replaced by that of Thr226. For Tamiflu, H-bonds formed with Arg156 at 16, Arg368 at 17, and Tyr402 at 18 anchored the molecule in the binding site and limited structural fluctuations caused by the intermolecular H-bond between 17 and 19, and the “antler-like” structure 20 (Figure 6D). The stability of Tamiflu as a result of these binding anchors is reflected in the low total energy profile (Figure 5) and small H-bond distance fluctuations (Figure 7D).

The ability of the de novo derivatives to form stable H1-ligand complexes has also been assessed [92]. All de novo derivatives were capable of forming H-bonds at Glu83 and Asp103, the key binding sites on H1.

The torsion angles of flexible bonds in each candidate when in complex with H1 and N1 are summarized in Figure 8. In Xylopine_2, all monitored bonds were stable in H1 except for e (Figure 8A). The fluctuations could be attributed to the attraction between the amine group H atoms and Asp 103. When bound to N1, b was the primary location for torque changes in Xylopine_2. The recorded torsion angle changes at b support our previous speculation that the unattached xylopine moiety is a key source of instability for Xylopine_2. Torsion angle fluctuations of Rosmaricine_14 in both H1 and N1 were mainly due to rotations at g and j (Figure 8B). Such changes are expected as the H atoms on the amine group continuously rotate to form H-bonds with key amino acids. Bonds in Rosmaricine_15 (Figure 8C) exhibited similar characteristics to those in Rosmaricine_14. Rapid rotations at the amine groups 1 and o are visualized by the recorded angle trajectories. NAG (Figure 8D) and Tamiflu (Figure 8E) both have relatively stable intermolecular torsion changes. This indicates that the lower stability of NAG and Tamiflu in H1 and N1, respectively, are not due to instability of their ligand structures, but may be attributed to weaker or unstable ligand-protein affinities.

**Hydrophobic interactions.** Hydrophobic interactions also played a role in stabilizing ligands within H1 (Figure 9) and N1 (Figure 10) binding sites during MD. Due to differences in ligand structure and binding conformation, amino acids with which hydrophobic interactions were formed differed. In H1, amino acids involved in hydrophobic interactions included Pro82, Asp103, Asn104, Cys107, Cys153, and Pro154. More stabilizing interactions including H-bonds and hydrophobic interactions were observed in the N1 binding site (Figure 10). For the TCM candidates and Tamiflu, the spatial distribution of H-bonds coupled and hydrophobic interactions limits the free movement of ligands within N1, thus increasing stability of the N1-ligand complex.

**CoMFA and CoMSIA Analysis**

To further investigate docking features, CoMFA and CoMSIA models were built and validated using 27 neuraminidase inhibitors listed in Table S2. The PLS analyses results for CoMFA and CoMSIA models are shown in Table 4. The CoMFA model was generated using both steric and electrostatic fields and yielded a
non-cross validated $r^2$ value of 0.924 and a cross validated $q^2$ value of 0.524 with an optimal number of components as 5. The optimal CoMSIA model ($r^2 = 0.937$, $q^2 = 0.673$, ONC = 5) consisted of steric and hydrophobic fields, H-bond acceptors and donors. When compared against actual observed activities [102], both CoMFA and CoMSIA models had good predictability, predicting pIC$_{50}$ values that differed only marginally from the actual pIC$_{50}$ values of 24 compounds (Table 5).

The validated CoMFA and CoMSIA maps were used to assess ligand bioactivity. Contour of the de novo compounds at 20 ns MD simulation to the relative spatial positions of CoMFA and CoMSIA feature maps are shown in Figure 11. In Xylopine_2, Rosmaricine_14, and Rosmaricine_15, the H-bond between the 2-aminopyridinium group and Glu228 matched the electropositive group feature of the CoMFA model (Figure 11A,11C,11E) and the H-bond donor feature in CoMSIA model (Figure 11B,11D,11F). The hydrophobic benzene structures of Xylopine_2 matched the steric favoring region of the CoMFA map and the hydrophobic feature of the CoMSIA map. The carbonyl groups in Rosmaricine_14 and Rosmaricine_15 which formed H-bonds with Tyr402 satisfied the H-bond acceptor feature in the CoMSIA model. Tamiflu also contours to both CoMFA and CoMSIA models. The 3-methoxypentane group close to Arg293 and Asn344 matched the steric favoring region of CoMFA (Figure 11G) and the hydrophobic feature of CoMSIA (Figure 11H). This residue has similar characteristics to the 2-aminopyridinium group in the de novo derivatives. In addition, the N-methylacetamide group in Tamiflu, which forms H-bond

![Figure 10. Protein-ligand interactions of TCM candidates and Tamiflu® in N1.](image-url)

Table 4. CoMFA and CoMSIA analysis for N1 using PLS.

| Model | Fraction | ONC | $q^2_{cv}$ | $r^2$ | SEE | F     |
|-------|----------|-----|------------|-------|-----|-------|
| CoMFA | -        | 5   | 0.524      | 0.924 | 0.372 | 63.567 |
| CoMSIA| S        | 5   | 0.426      | 0.936 | 0.369 | 41.649 |
|       | E        | 5   | 0.255      | 0.871 | 0.626 | 12.652 |
|       | H        | 5   | 0.337      | 0.979 | 0.212 | 132.092 |
|       | D        | 5   | 0.220      | 0.737 | 0.750 | 7.940  |
|       | A        | 5   | 0.221      | 0.798 | 0.658 | 11.186 |
|       | S+E      | 5   | 0.200      | 0.925 | 0.401 | 34.912 |
|       | S+H      | 6   | 0.565      | 0.977 | 0.223 | 119.225 |
|       | S+H+D    | 5   | 0.570      | 0.906 | 0.435 | 34.000 |
|       | H+D+A    | 6   | 0.528      | 0.957 | 0.304 | 62.855 |
|       | S+H+D+A* | 5   | 0.673      | 0.937 | 0.357 | 53.360 |
|       | S+H+D+A  | 5   | 0.561      | 0.946 | 0.331 | 62.823 |

ONC: Optimal number of components.
SEE: Standard error of estimate.
F: F-test value.
S: Steric.
E: Electrostatic.
H: Hydrophobic.
D: Hydrogen bond donor.
A: Hydrogen bond acceptor.
*: optimum CoMSIA model.

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with Tyr402, is located near the H-bond donor feature in CoMSIA.

Though all compounds contoured to the N1 inhibitor features identified by CoMFA and CoMSIA, a critical difference was observed between Tamiflu and the TCM de novo derivatives. All compounds except Tamiflu formed H-bonds at Glu228. As Glu228 is a primary binding site of N1 [99], ability of the TCM de novo derivatives to maintain stable binding with Glu228 during MD simulation supports the potential of these compounds as drug alternatives to Tamiflu.

Due to the lack of reported H1 ligand bioactivities in the literature, direct assessment of bioactivity through construction of CoMFA and CoMSIA models was not possible. Alternatively, indirect support was provided by assessing the ability of de novo derivatives to maintain contour to the N1 CoMFA/CoMSIA maps while forming interactions at key residues in H1, Glu83 and Asp103 [92]. As illustrated in Figure 12 the TCM de novo derivatives docked into the H1 binding site and formed critical interactions at Glu83 and Asp103 without losing contour to the CoMFA and CoMSIA maps. These results suggest that not only were the TCM de novo derivatives capable of docking into both H1 and N1, but that biological activity was also predicted in both binding sites, thus it is possible to develop dual-targeting drugs from the selected de novo derivatives.

Important features for potential H1 and N1 inhibitors are summarized in Figure 13. For H1, a salt bridge with Glu83 and H-bond donor and/or electrostatic interactions with Asp103 are important characteristics that should be met. Potential inhibitors for N1 should have salt bridge and/or H-bond formation at Glu228 and interactions with Asp293. These features can be used to identify or design novel drugs for H1 and/or N1. In the case of the TCM de novo derivatives from this study, each compound could structurally fulfill the requirements of both H1 (Figure 13A,13B,13C) and N1 (Figure 13D,13E,13F) binding sites, thus supporting their potential as dual-targeting compounds.

In this research, we identified Xylopine_2, Rosmaricine_14, and Rosmaricine_15 as the top three de novo derivatives exhibiting binding affinity to H1 and N1. Addition of a pyridinium residue to the native structures of xylopine and rosmaricine contributes to bond formation at key residues in both H1 (Glu83, Asp103) and N1 (Glu228, Arg292). The de novo derivatives were predicted as

Table 5. Observed and predicted activities of 27 neuraminidase inhibitors by CoMFA and CoMSIA models.

| Compound | Observed pIC₅₀ * | Predicted pIC₅₀ | Residual | Predicted pIC₅₀ | Residual |
|----------|-----------------|----------------|----------|-----------------|---------|
| 1        | 5.20            | 5.21           | 0.01     | 4.97            | −0.23   |
| 2        | 5.70            | 6.00           | 0.30     | 5.85            | 0.15    |
| 3        | 6.74            | 6.69           | −0.05    | 6.40            | −0.34   |
| 4        | 6.52            | 6.44           | −0.08    | 6.42            | −0.10   |
| 5        | 6.70            | 6.75           | 0.04     | 6.67            | −0.03   |
| 6        | 8.00            | 8.36           | 0.36     | 7.82            | −0.18   |
| 7        | 9.00            | 8.75           | −0.25    | 8.31            | −0.69   |
| 8        | 7.80            | 7.82           | 0.02     | 8.62            | 0.82    |
| 9        | 6.82            | 6.93           | 0.11     | 7.00            | 0.18    |
| 10       | 9.00            | 8.94           | −0.06    | 8.93            | −0.07   |
| 11       | 5.70            | 5.45           | −0.25    | 5.57            | −0.13   |
| 12       | 6.65            | 6.54           | −0.11    | 6.48            | −0.17   |
| 13       | 5.66            | 5.81           | 0.15     | 5.81            | 0.15    |
| 14       | 7.66            | 7.88           | 0.22     | 7.59            | −0.07   |
| 15       | 7.22            | 7.34           | 0.12     | 7.16            | −0.06   |
| 16       | 6.28            | 6.23           | −0.05    | 6.68            | 0.40    |
| 17       | 5.82            | 5.76           | −0.06    | 6.08            | 0.25    |
| 18       | 7.00            | 6.58           | −0.42    | 6.76            | −0.24   |
| 19       | 4.60            | 4.79           | 0.19     | 4.84            | 0.24    |
| 20       | 8.74            | 8.58           | −0.16    | 8.80            | 0.06    |
| 21       | 8.34            | 8.40           | 0.06     | 8.30            | −0.04   |
| 22       | 5.51            | 5.55           | 0.04     | 5.38            | −0.13   |
| 23       | 5.47            | 5.49           | 0.02     | 5.39            | −0.08   |
| 24       | 5.64            | 5.49           | −0.15    | 5.96            | 0.32    |
| 25*      | 3.70            | 4.03           | −0.33    | 4.72            | −1.02   |
| 26*      | 5.43            | 5.23           | 0.20     | 5.56            | −0.13   |
| 27*      | 6.85            | 7.97           | −1.12    | 7.74            | −0.89   |

*: Adapted from [102].
*: test set.
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active by the SVM and MLR models, and contoured well to the 3D-QSAR models. The TCM de novo derivatives were able to maintain contour while forming key binding interactions in H1, thus providing indirect support for bioactivity in H1. The results of this study indicate that the TCM de novo derivatives not only can bind to, but can also exhibit biological activities in both H1 and N1.

Key binding locations of the de novo derivatives include Glu83 and Asp103 for H1, and Glu228 and Arg292 for N1. Mutations currently attributed to oseltamivir resistance are located at H275 and N295S of the NA [103]. Since the key binding locations of the TCM derivatives do not overlap with those causing oseltamivir resistance, derivatives will be able to bind to viruses that are currently resistant to Tamiflu®. In addition, the de novo derivatives do not bind to amino acids in H1 or N1 that are prone to mutation (Table 6, Table 7) [40,104], thus would likely be able to exert activity across a range of mutant H1N1 viruses. Last but not the least, multiple bond formations observed in MD provide additional insurance against possible mutations at key binding residues. In the case of a single point mutation, the de novo compounds will remain bound to the H1 and N1 sites through another key residue, therefore resisting the development of drug resistance in the virus. Based on the results and observations of this study, the TCM de novo derivatives may be attractive compounds for designing novel dual-target inhibitors for H1 and N1.
analysis (CoMSIA) models were constructed using SYBYL/C223 analysis (CoMFA) and comparative molecular similarities indices (C223) developed by PerkinElmer Inc., Cambridge, MA). Comparative molecular field analysis (CoMFA) and comparative molecular similarities indices (CoMSIA) were used to generate two-dimensional and three-dimensional structures of TCM compounds. The dynamics (MD) simulation were performed using Discovery Studio 2.5 (DS2.5; Accelrys Inc., San Diego, CA). The complexes were created with HARvard Molecular Mechanics (CHARMm) [105]. The All procedures were completed under the forcefield of Chemistry at HARvard Molecular Mechanics (CHARMm) [105]. The virtual screening process was performed using LigandFit. The conformational search method was based on the Monte Carlo algorithm. Rigid body minimization following initial ligand placement was completed using Smart Minimizer. Scoring functions used by LigandFit were DockScore. The top ten compounds with the highest DockScore were selected and ranked by the sum of their respective H1 and N1 DockScore. De novo evolution was used to generate schematic diagrams of protein-ligand interactions for each candidate and control in H1 and N1.

Materials and Methods

Software

Virtual screening, de novo derivative generation, and molecular dynamics (MD) simulation were performed using Discovery Studio Client v2.5.0.9164 (DS2.5; Accelrys Inc., San Diego, CA). The two-dimensional and three-dimensional structures of TCM compounds were generated using ChemBioOffice 2008 (PerkinElmer Inc., Cambridge, MA). Comparative molecular field analysis (CoMFA) and comparative molecular similarities indices analysis (CoMSIA) models were constructed using SYBYL® 8.3 package (Tripos Inc., St. Louis, MO).

Docking Analysis

Compounds from the TCM Database@Taiwan were docked to H1 and N1 protein active sites reported in our previous study [91]. All procedures were completed under the forcefield of Chemistry at HARvard Molecular Mechanics (CHARMm) [105]. The virtual screening process was performed using LigandFit. The conformational search method was based on the Monte Carlo algorithm. Rigid body minimization following initial ligand placement was completed using Smart Minimizer. Scoring functions used by LigandFit were DockScore. TCM compounds that docked into both H1 and N1 proteins were selected and then ranked by the sum of their H1 and N1 DockScore. Tamiflu® was used as the control for N1, and its N1 docking score was set as the minimum requirement. The top TCM compounds that passed the filtering were selected for de novo evolution.

De Novo Evolution and Lipinski’s Rule of Five

In de novo evolution, TCM compounds were placed into the H1 and N1 protein binding sites described previously, and Lipinski’s fragments were attached to the native structure. The new derivatives were generated in full evolution mode. Derivatives from de novo evolution were subjected to additional screening through Lipinski’s rule [106] to rule out orally unstable or pharmacologically inapplicable compounds. As de novo products generated for H1 and N1 proteins differed, all de novo products were re-docked to H1 and N1 proteins to assess binding affinity. De novo products that docked into both H1 and N1 proteins were selected and ranked by the sum of their respective H1 and N1 DockScore. The top ten compounds with the highest DockScore were selected for further structure-based analysis.

Bioactivity Prediction by SVM and MLR

The 27 neuraminidase inhibitors used, including 24 training set compounds and 3 test set compounds, were adapted from Zhang’s study [102]. Compounds were drawn using ChemBioOffice 2008 (PerkinElmer Inc., Cambridge, MA) and modified to physiological ionization using the Prepare Ligand function in DS 2.5. Bioactivity values (IC50) were also obtained from Zhang’s study though the original sources were not clarified, and converted to pIC50 (log[1/IC50]). Molecular descriptors of the compounds were calculated using Calculate Molecular Properties in DS 2.5 and the GFA was used to select the best representative molecular descriptors [107]. Utilizing the best representative molecular descriptors identified through GFA, MLR and SVM models were constructed using MATLAB (The Mathworks Inc., Natick, MA) and LibSVM [108], respectively, and used to predict the bioactivity of TCM de novo compounds.

MD Simulation

The MD simulation was performed using the Molecular Dynamics package of DS 2.5. The complexes were created with a 10 Å solvation shell of TIP3 water around the protein. Sodium cations were added to each system for neutralization. Minimization using Steepest Descent and Conjugate Gradient were performed at 500 cycles each. Each protein-ligand complex was gradually heated from 0K to 310K over 50 ps, followed by a 200 ps equilibration phase. The production stage was performed for 20 ns using NVT canonical ensemble and trajectory frames were saved every 20 ps. SHAKE algorithm was applied to immobilize all bonds involving hydrogen atoms throughout the MD simulation. Long-range electrostatics were treated with PME method. Time step was set to 2 fs for all MD stages. The temperature coupling decay time for the Berendsen thermal coupling method was 0.4 ps. Post processing of the trajectory was performed using Analyze Trajectory module. Torsion angles of each bond were also monitored through DS 2.5. LIGPLOT [109] was used to generate schematic diagrams of protein-ligand interactions for each candidate and control in H1 and N1.

CoMFA and CoMSIA Models

CoMFA and CoMSIA models were constructed through the partial least square (PLS) analysis using previously described neuraminidase inhibitors [102]. The optimal number of components was obtained from leave-one-out method to yield the highest $r^2$ and $q^2$ values in non-cross validation and cross-validation, respectively. Biological activities of the TCM de novo compounds were evaluated based on contour to the generated 3D-QSAR map.
Table 6. HA mutation points between the 1918 H1N1 and H1N1/09 viruses.

| Amino acid location | 130 | 144 | 145 | 146 | 154 | 155 | 158 | 163 | 169 | 183 | 185 | 196 | 200 | 202 | 203 | 217 | 220 | 222 | 224 | 225 | 226 | 228 |
|---------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| A/South_Carolina/1/18(H1N1) | K | E | T | T | S | Y | A | S | R | L | T | S | V | V | V | P | G | Y | Q | S | S | K | N | R | R | T |
| A/New_York/1/18(H1N1) | K | E | T | T | S | Y | A | S | R | L | T | S | V | V | V | P | G | Y | Q | S | S | K | N | R | R | T |
| A/Brevig_Mission/1/18 (H1N1) | K | E | T | T | S | Y | A | S | R | L | T | S | V | V | V | P | G | Y | Q | S | S | K | N | R | R | T |
| A/London/1/1918(H1N1) | K | E | T | T | S | Y | A | S | R | L | T | S | V | V | V | P | S | Y | Q | S | S | K | N | R | R | T |
| Japan_HA_Samples “I” | R | D | S | N | P | H | A | K | K | I | V | N | I | D | I | S | G | A | Q | F | T | R | S | K | K | K |
| Japan_HA_Samples “II” | R | D | S | N | P | H | A | K | K | I | V | N | I | D | I | S | G | A | Q | F | T | R | S | K | K | K |

| Amino acid location | 233 | 237 | 239 | 241 | 251 | 256 | 266 | 267 | 269 | 274 | 275 | 277 | 278 | 284 | 287 | 295 | 300 | 305 | 315 | 319 | 325 | 331 | 338 | 362 | 382 | 391 |
|---------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| A/South_Carolina/1/18(H1N1) | A | V | D | A | L | T | I | A | W | L | N | G | S | T | A | K | H | S | V | E | R | M | I | I | Q | G |
| A/New_York/1/18(H1N1) | A | V | G | A | L | T | I | A | W | L | N | G | S | T | A | K | H | S | V | E | R | M | I | I | Q | G |
| A/Brevig_Mission/1/18 (H1N1) | A | V | D | A | L | T | I | A | W | L | N | G | S | T | A | K | H | S | V | E | R | M | I | I | Q | G |
| A/London/1/1918(H1N1) | A | V | D | A | L | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| A/London/1/1919(H1N1) | A | I | G | A | L | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Japan_HA_Samples “I” | I | V | D | E | V | K | V | V | R | M | E | N | A | I | T | T | K | T | I | K | K | L | V | V | L | E |
| Japan_HA_Samples “II” | I | V | D | E | V | K | V | V | R | M | E | N | A | I | T | T | K | T | I | K | K | L | V | V | L | E |

Data compiled from Yang et al. (2011) [40] and Morlighem et al. (2011) [104].

- Information not provided in original research.

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### Table 7. NA mutation points between 1918 H1N1 and H1N1/09 viruses.

| Amino acid location | 13 | 16 | 17 | 19 | 20 | 21 | 34 | 40 | 45 | 46 | 53 | 74 | 75 | 79 | 80 | 81 | 84 | 94 | 105 | 126 | 149 | 163 |
|---------------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| A/Brevig_Mission/1/18 (H1N1) | I | V | V | I | I | S | V | T | H | P | I | V | V | D | A | T | I | T | I | G | H | V | V |
| Japan_HA_Samples “I” | V | T | I | M | A | N | I | L | Q | I | V | F | A | S | V | V | K | A | V | S | P | I | I |
| Japan_HA_Samples “II” | V | T | I | M | A | N | I | L | Q | I | V | F | A | S | V | V | K | A | V | S | P | I | I |
| A/Taiwan/5515/2005 (Clade 1) | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| A/Taiwan/2823/2008 (Clade 2A) | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| A/Taiwan/10092/2007 (Clade 2B-1) | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| A/Taiwan/6566/2006 (Clade 2B-2) | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| A/Taiwan/2832/2008 (Clade 2C-1) | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| A/Taiwan/2832/2008 (Clade 2C-2) | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

Data compiled from Yang et al. (2011) [40] and Morlighem et al. (2011) [104].

- Information not provided in original research.

Amino acid mutation contributing to oseltamivir-resistance (H275Y) are located at 275 and shown in bold.

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Supporting Information

Table S1  
TCM database screening results of H1 and N1.
(DOC)

Table S2  
Molecular structures of training and test set compounds for N1 CoMFA and CoMSIA modeling.  
(DOC)

Acknowledgments

V. Antiviral treatment for patients hospitalized with 2009 pandemic influenza A (H1N1). N Engl J Med 361: e110.
24. Layne SP, Monte AS, Taubenberger JK. (2009) Pandemic influenza: an inconvenient mutation. Science 323: 1560–1561.
25. Nicholas KG, Aoki FY, Osterhaus AD, Trottier S, Carewicz O, et al. (2000) Mechanism of drug inhibition and drug pharmacological characterization of inhibitors of amantadine-resistant mutants of influenza a virus. J Am Chem Soc 132: 17695–17697.
26. Nicholson KG, Aoki FY, Osterhaus AD, Trottier S, Carewicz O, et al. (2000) Mechanism of drug inhibition and drug pharmacological characterization of inhibitors of amantadine-resistant mutants of influenza a virus. J Am Chem Soc 132: 17695–17697.
27. Balannik V, Wang J, Ohigashi Y, Jing X, Magavern E, et al. (2009) Design and functional analysis of the influenza B proton channel. Nat Struct Mol Biol 16: 231–239.
28. Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, et al. (2009) Antigenic and genetic characteristics of swine-origin 2009 (H1N1) influenza viruses circulating in humans. Science 325: 197–201.
29. McDonald NJ, Smith CB, Cox NJ (2009) Pandemic influenza: an inconvenient mutation. Science 323: 1560–1561.
30. McDonnell MJ, Smith CB, Cox NJ (2009) Pandemic influenza: an inconvenient mutation. Science 323: 1560–1561.
31. Treanor J (2004) Influenza vaccine–Outmaneuvering antigenic shift and drift. The N Engl J Med 350: 218–220.
32. Kiihtrity Y, Nakano M, Inoue Y (2006) Frequency of amantadine-resistant Influenza A Virus isolated from 2001–02 to 2004–05 in Nara Prefecture. Jpn J Infect Dis 59: 197–199.
33. Cheng PK, Leung TW, Ho EC, Leung PC, Ng AY, et al. (2009) Oseltamivir-and amantadine-resistant influenza viruses A (H1N1). Emerg Infect Dis 15: 966–968.
34. World Health Organization (2009) Oseltamivir-resistant pandemic (H1N1) 2009 influenza virus. Weekly Epidemiological Record 84: 453–468. Available at http://www.who.int/wer/2009/wer444/en/index.html Accessed 06 August 2011.
35. Baranovich T, Saito R, Suzuki Y, Zarakes H, Dupat C, et al. (2010) Emergence of H27Y oseltamivir-resistant AH1N1 influenza viruses in Japan during the 2008–2009 season. J Clin Virol 47: 23–28.
36. Pelak RM, Chou JJ (2010) Hu channel drug resistance: a tale of two sites. Protein Cell 1: 246–258.
37. Wang J, Ma C, Feirin G, Carnevale V, Wang T, et al. (2011) Molecular dynamics simulation directed rational design of inhibitors targeting drug-resistant mutants of influenza a virus M2. J Am Chem Soc 133: 12834–12841.
38. Mai BK, Li MS (2011) Neuraminidase inhibitor R-125489–a promising drug for treating influenza virus: steered molecular dynamics approach. Biochem Biophys Res Commun 410: 680–691.
39. Leonov H, Astraphan P, Kruglik M, Arkin IT (2011) How do aminoacida- mantanes block the influenza a virus channel, and how does resistance develop? J Am Chem Soc 133: 9903–9911.
40. Yang JR, Lin YC, Huang YP, Su CH, Lo J, et al. (2011) Reassortment and mutations associated with emergence and spread of oseltamivir-resistant seasonal influenza A(H1N1) viruses in 2005–2009. PLoS One 6: e18177.
41. He Z, Zhang J, Shi XH, Hu LL, Kong X, et al. (2010) Predicting drug-target interaction networks based on functional groups and biological features. PLoS One 5: e9603.
42. Saven JG (2011) Computational protein design: engineering molecular diversity, nonnatural enzymes, nonbiological cofactor complexes, and membrane proteins. Curr Opin Chem Biol 15: 452–457.
43. Vanhee P, van der Sloot AM, Verschueren E, Serrano L, Rousseau F, et al. (2011) Computational design of peptide ligands. Trends Biotechnol 29: 231–239.
44. Morin A, Meiler J, Mizoue LS (2011) Computational design of protein-ligand features, subcellular locations and interaction networks. PLoS One 5: e9617.
45. Mosin A, Meiler J, Mizoue LS (2011) Computational design of protein-ligand interfaces: potential in therapeutic development. Trends Biotechnol 29: 159–166.
46. Peng T, Shi XH, Wang P, Pe Z, Feng K, et al. (2010) Analysis and prediction of the metabolic stability of proteins based on their sequential features, subcellular locations and interaction networks. PLoS One 5: e10972.
47. Wang P, Hu L, Liu G, Jiang N, Chen X, et al. (2011) Prediction of antimicrobial peptides based on sequence alignment and feature selection methods. PLoS One 6: e16176.
48. Chou KC (2004) Structural bioinformatics and its impact to biomedical science. Curr Med Chem 11: 2105–2134.
49. Chen CY, Chang YH, Bao DT, Huang HJ, Tsai FJ, et al. (2009) Discovery of potent inhibitors for phosphodiesterase 3 by virtual screening and pharmacophore analysis. Acta Pharmacologica Sinica 30: 1190–1194.
50. Chen CYC (2010) Bioinformatics, chemoinformatics, and pharmainformatics analysis of HER2/HSPSO dual-targeted inhibitors. Journal of the Taiwan Institute of Chemical Engineers 41: 143–149.
