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Neuraminidase inhibitory activities of quaternary isoquinoline alkaloids from *Corydalis turtschaninovii* rhizome

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ABSTRACT

Clostridium perfringens is a Gram-positive spore-forming bacterium that causes food poisoning. The neuraminidase (NA) protein of *C. perfringens* plays a pivotal role in bacterial proliferation and is considered a novel antibacterial drug target. Based on screens for novel NA inhibitors, a 95% EtOH extract of *Corydalis turtschaninovii* rhizome showed NA inhibitory activity (68% at 30 μg/ml), which resulted in the isolation of 10 isoquinoline alkaloids; namely, palmatine (**1**), berberine (**2**), coptisine (**3**), pseudodehydrocorydaline (**4**), jatrorrhizine (**5**), dehydrocorybulbine (**6**), pseudocoptisine (**7**), glaucine (**8**), corydaline (**9**) and tetrahydrocoptisine (**10**). Interestingly, seven quaternary isoquinoline alkaloids **1**–**7** (IC_{50} = 12.8 ± 1.5 to 65.2 ± 4.5 μM) showed stronger NA inhibitory activity than the tertiary alkaloids **8**–**10**. In addition, highly active compounds **1** and **2** showed reversible non-competitive behavior based on a kinetic study. Molecular docking simulations using the Autodock 4.2 software increased our understanding of receptor–ligand binding of these compounds. In addition, we demonstrated that compounds **1** and **2** suppressed bacterial growth.

1. Introduction

Clostridium perfringens is a Gram-positive spore-forming bacterium found in the human gastrointestinal tract, marine sediment, insects and soil.[1–3] This pathogen causes gas gangrene and food poisoning in humans, as well as enterotoxemia and enteritis in livestock.[4,5] Food poisoning by *C. perfringens* in the USA and UK is the second most common foodborne disease. In the USA, 1 million cases of *C. perfringens* occur every year.[6] *C. perfringens* secretes over 15 toxins and enzymes, including the virulence factors encoded by the *nanH*, *I* and *J* genes, which can damage host tissue.[7,8]

Neuraminidase (EC. 3. 2. 1. 18, NA) from bacteria, named exo-α-silidase, hydrolyzes α-glycosidic linkages of terminal sialic acid residues in oligosaccharides, glycoproteins and glycolipids on host cells.[9,10] In fact, sialic acid, the *N*- or *O*-substituted derivatives of neuraminic acid from monosaccharides with a nine-carbon backbone, plays an important role in several cell–cell interactions and cell-molecule recognition processes and is a good source of nutrients for bacteria.[11,12] Bacteria can obtain sialic acid via two routes: de novo synthesis or scavenging from the host.[12–14] Neuraminidase secreted from bacteria catalyzes the hydrolysis of the terminal sialic acids of sialoglycoconjugates in diverse organisms, and free sialic acid is directly transported into the bacterial membrane.[14] Sialic acid is then used as an energy and carbon source for bacterial growth.[12,14] Moreover, neuraminidase plays a key role in the initial stages of bacterial infection, contributing to biofilm formation on glycoprotein-coated surfaces.[15,16] Recent studies have highlighted neuraminidase as a novel target for development of therapeutic agents to treat bacterial infections.

Therefore, we aimed to develop inhibitors of bacterial NA. Screening results of crude extracts from *Corydalis turtschaninovii* rhizomes demonstrated that the 95% EtOH extract at a concentration of 30 μg/ml had a 68% inhibitory effect on NA. Moreover, the inhibitory effects of berberine on viral NA, a quaternary isoquinoline alkaloid, found in this plant, have been reported.[17] Accordingly, seven quaternary and three tertiary isoquinoline alkaloids from the *C. turtschaninovii* rhizome were assessed for their activity against NA in vitro. The strong inhibitors (**1**, and **2**) were non-competitive based on kinetic studies, and an allosteric site may have been associated with the molecule-inhibitor interaction based on docking simulations. Polyphenols, including flavanones, isoflavones and pterocarpans are known to have inhibitory activity against NA, but the activities of alkaloids and their derivatives from natural sources remain unknown.[18–20]

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2. Results and discussion

2.1. Isolation and structural elucidation

Ethanol extract from *C. turtschaninovii* rhizome showed 68% inhibitory activity against NA at 30 μg/ml. The ethanol extract was subjected to column chromatography on a silica gel and C-18 column to yield compounds **1**–**10**. The chemical structures of isolated compounds **1**–**10** were identified by comparing their spectroscopic data (HPLC, ESI and HR-MS and 1D, 2D-NMR) with those published previously. The compounds were identified as palmatine (**1**),[22,23] berberine (**2**),[22] coptisine (**3**),[24] pseudodehydrocorydaline (**4**),[22,25] jatrorrhizine (**5**),[22] dehydrocorybulbine (**6**),[26] pseudocoptisine (**7**),[27] glaucine (**8**),[28] corydaline (**9**),[27] and tetrahydrocoptisine (**10**)[29] (Fig. 1).

2.2. Enzyme inhibition activity

To evaluate antibacterial activities, the inhibition by the isolated compounds of NA-catalyzed 4-methylumbelliferone formation from 4-methylumbelliferyl-α-D-*N*-acetyl neuraminic acid sodium salt hydrate was evaluated using fluorometeric determination with a SpectraMax M5 Multimode Reader (Molecular Devices, USA) in triplicate. Curcumin was used as a positive control. The results showed that palmatine (**1**) and berberine (**2**) showed strong inhibitory activity on NA with IC_{50} values of 12.8 ± 1.5 and 13.5 ± 2.3 μM, respectively. Coptisine (**3**), pseudodehydrocorydaline (**4**), jatrorrhizine (**5**) dehydrocorybulbine (**6**), pseudocoptisine (**7**), and glaucine (**8**) showed moderate inhibitory activity (IC_{50} 25.1 ± 0.8 to 96.9 ± 3.2 μM). In contrast, corydaline (**9**) and tetrahydrocoptisine (**10**) showed little effect, with 12.7 and 4.6%, respectively, inhibition of NA activity at 100 μM. Subsequently, compounds **1**–**10** suppressed viral NA activity. Viral NAs from rvH1N1 and H5N1 were inhibited by quaternary isoquinoline alkaloids **1**–**7** (Table 1). Among them, compound **3** showed IC_{50} values of 25.3 ± 0.4 and 26.4 ± 1.1 μM against rvH1N1 and H5N1, respectively, which were greater than the corresponding values of compound **2** (38.5 ± 1.2 and 32.2 ± 0.5 μM). Compound **2** was previously considered a viral NA inhibitor (Fig. 2).[17]

2.3. Enzyme kinetic study

The binding mechanisms of inhibitors were examined using enzyme-inhibitor kinetic studies. The data for highly active compounds **1** and **2** are shown in Figure 3. Plots of NA activity versus concentration of the two inhibitors yielded a group of straight lines passing through the origin, indicating that they were reversible inhibitors (Fig. 3A and D). Double reciprocal Michaelis–Menten and Dixon plots were generated by measurement of enzyme activity with various substrates at different concentrations. The results are presented in Figure 3. These compounds showed non-competitive behavior as indicated by their different V_{max} values and equal K_m values in the Michaelis–Menten plot (Fig. 3B and E). Additionally, the Dixon plots confirmed that the K_i of compounds **1** and **2** were 16.9 ± 1.5 and 11.9 ± 1.9 μM, respectively, (Fig. 3C and F).

2.4. Structure activity relationships (SARs)

Based on their inhibition of bacterial NA, this study explored the structure–activity relationships of isoquinoline alkaloids (**1**–**10**).

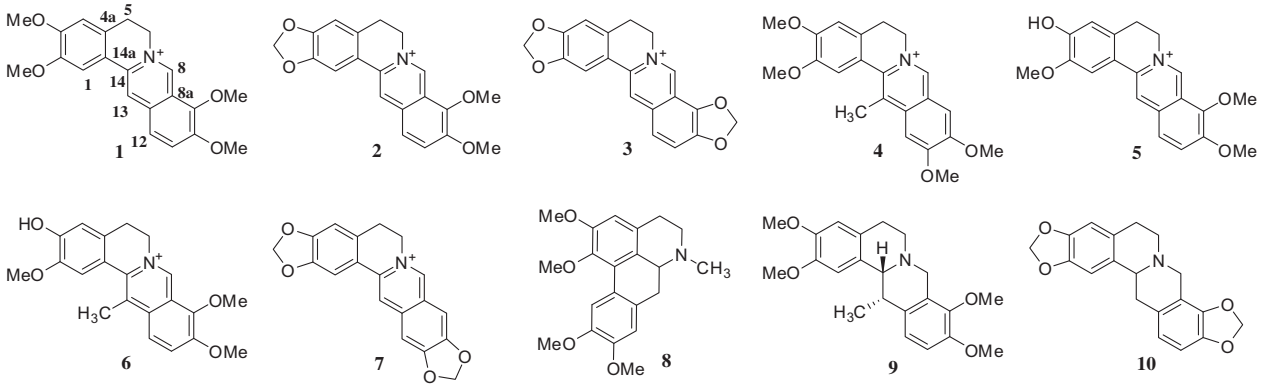

Figure 1. Structures of isolated compounds from the *C. turtschaninovii* rhizome.

Table 1
Neuraminidase inhibitory activities of isolated alkaloids (**1**–**10**)

Compound	Bacteria[d]	rvH1N1[d,e]	H5N1[d,f]	Compound	Bacteria[d]	rvH1N1	H5N1[d]
IC_{50}[a] (μM)							
1	12.8 ± 1.5	33.1 ± 2.0	29.8 ± 2.1	**6**	41.3 ± 3.5	80.5 ± 1.8	100.2 ± 0.9
2	13.5 ± 2.3	38.5 ± 1.2	32.2 ± 0.5	**7**	65.2 ± 4.5	125.7 ± 5.1	167.0 ± 1.3
3	25.1 ± 0.8	25.3 ± 0.4	26.4 ± 1.1	**8**	96.9 ± 3.2	N.D[c]	N.D[c]
4	32.6 ± 2.1	N.D[c]	83.6 ± 4.5	**9**	N.D[c]	N.D[c]	N.D[c]
5	37.0 ± 1.8	66.2 ± 4.2	76.3 ± 2.1	**10**	N.D[c]	N.D[c]	N.D[c]
Curcumin[b]	6.0 ± 2.5	10.0 ± 1.2	8.1 ± 1.5				

[a] All compounds examined in a set of duplicated or triplicated experiment.
[b] Positive control.
[c] Not detected.
[d] Neuraminidase.
[e] Tamiflu (IC_{50}: 20.5 ± 1.5 nM).
[f] Tamiflu (IC_{50}: 88.2 ± 2.9 nM).

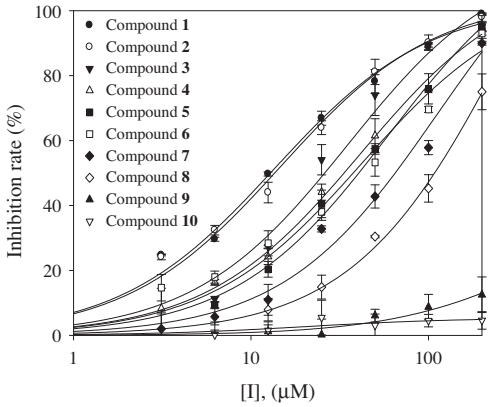

Figure 2. Effects of alkaloids (**1**–**10**) on the activity of neuraminidase from *C. perfringens* (*C. welchii*).

Quaternary isoquinoline alkaloids (**1**–**7**) showed stronger inhibition of NA than tertiary isoquinoline alkaloids (**8**–**10**). Among compounds **1**–**7**, structures with a hydroxyl group at C-3 showed low activity against NA. For example, compounds **5** (IC_{50}: 37.7 ± 1.8 μM) and **6** (IC_{50}: 41.3 ± 3.5 μM), which contain a free hydroxyl group at C-3, showed an NA inhibitory effect 3- to 3.5-fold lower than that of compound **1** (IC_{50}: 12.8 ± 1.5 μM). Finally, compounds substituted with free methoxyl groups instead of 1, 3-dioxolane at C-2, 3 and C-9, 10 increased NA inhibition based on comparisons with compounds **1** (IC_{50}, 12.8 ± 1.5 μM), **2** (IC_{50}, 13.5 ± 2.3 μM) and **3** (IC_{50}, 25.5 ± 0.8 μM).

2.5. Molecular simulation

Because compounds **1** and **2** exhibited strong inhibitory activities in vitro, we examined the binding mode of these compounds with their receptor (code: 2BF6 from protein data bank) using the docking software package AUTODOCK 4.2. Based on kinetic studies, rigid receptor-flexible ligand docking was performed to set up grids containing the full receptor for blind testing using the AutoDockTools. This revealed the low docking energy and allosteric binding site opportunities of binding forms between flexible ligands and activity sites (Fig. 4). Molecular simulation results showed a trend similar to the enzyme assays with binding energies for palmatine (−8.66 kcal/mol) and berberine (−8.27 kcal/mol). Compound **1** was closely associated with 15 amino acids in the receptor. Among them, GLU385, ASN386, ASN417 and ASN448 were individually linked by hydrogen bonding with 2.4, 2.3, 2.0, and 1.9 Å distances to palmatine (**1**). Compound **2** was proximal to 12 amino acids. Berberine (**2**) formed hydrogen bonds with ASN417 (2.12 Å) Table 2. Interestingly, ring A of the two ligands was close to TRY320 and HIS346 in the binding site (Fig. 4B and Fig. 45 in supporting data). Palmatine (**1**) interacted only via π–π stacking with HIS346, and berberine (**2**) exhibited π–π stacking with TRY320 and HIS346. Two ligands superimposed into the allosteric binding site are shown in Figure 4.

2.6. Antibacterial assay

To confirm the antibacterial activity of compounds **1** and **2**, their effects on *C. perfringens* growth in the presence and absence of the inhibitor were examined and expressed as MIC values (Table 3). Quality control standardization of the bioassay was

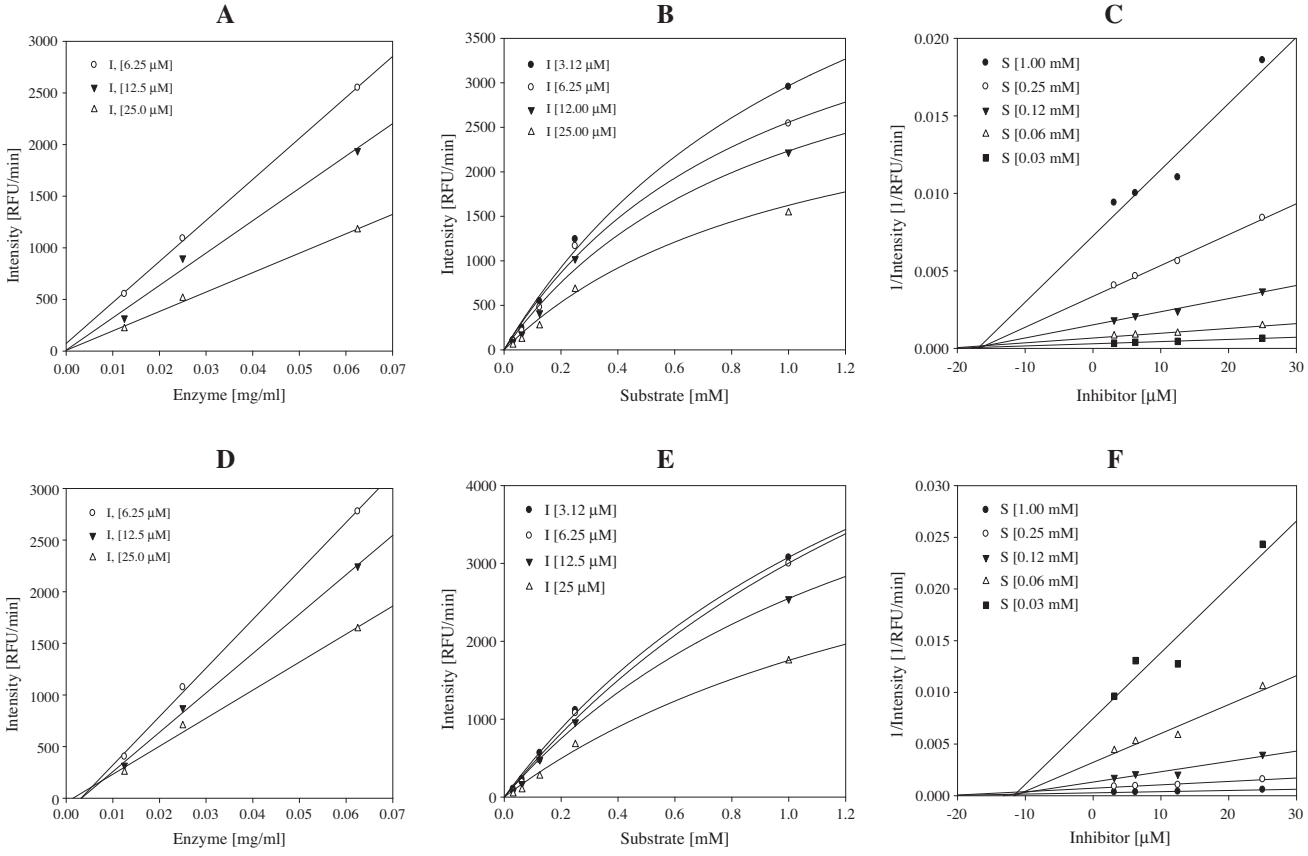

Figure 3. (A and D) The hydrolytic activity of neuraminidase as function of enzyme concentrations at various concentrations of compounds **1** and **2**. (B and E) Micheal–Menten plots of neuraminidase inhibition by compounds **1** and **2**. (C and F) Dixon plots of neuraminidase inhibition by compounds **1** and **2**.

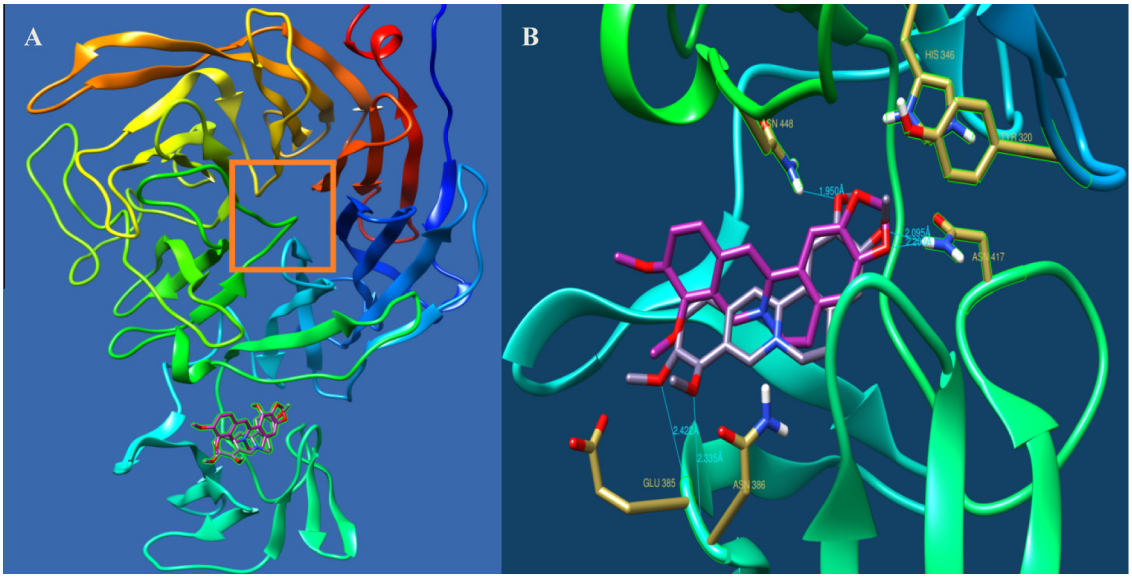

Figure 4. The best binding pose of two compounds **1** (silver) and **2** (pink) into the NA. (A) Orange box is the original activity site. (B) Complex of ligand-receptor became zoom.

Table 2
Hydrogen bonding between receptor and ligand

Ligand	Receptor[a]	Distance (Å)
Palmatin	GLU385	2.42
	ASN386	2.33
	ASN417	2.09
Berberin	ASN448	1.95
	ASN417	2.20

[a] Amino acid sequence number of receptor.

Table 3
Antibacterial activities of palmatine (**1**) and berberine (**2**)

Bacteria	MIC (μM)		
	Palmatine (**1**)	Berberine (**2**)	Ampicillin[a]
C. perfringens	44.7 ± 4.5	52.2 ± 4.7	300 ± 8.1 nM

[a] Positive control.

performed using ampicillin as a positive control, which had an MIC of 300 ± 8.1 nM. Compounds **1** and **2** (which showed considerable inhibition of bacterial NA) exhibited significant antibacterial activity against *C. perfringens*, with MICs of 44.7 ± 5.5 and 52.2 ± 4.7 μM, respectively.

3. Conclusion

We identified a novel inhibitor of NA from *C. perfringens*, a causative agent of food poisoning. Activity-guided fractionation of 95% ethanol extract from *C. turtschaninovii* rhizome led to the isolation of isoquinoline alkaloids **1**–**10**. Among them, palmatine (**1**) and berberine (**2**), which contain the quaternary isoquinoline alkaloid backbone, exhibited NA inhibitory activity. Previous reports have examined inhibitors of two *exo*-sialidases for bacterial and viral NAs because they have similar active sites and overall mechanism of action.[30,31] We also found similar results for viral and bacterial NA. In particular, coptisine (**3**) showed stronger inhibitory activity than berberine (**2**), which has been reported to be a viral NA inhibitor.[17] Isoquinoline alkaloids inhibited bacterial NA and suppressed viral activity. Based on the kinetic study and molecular simulations, the quaternary isoquinoline alkaloids **1** and **2** showed reversible non-competitive behavior in the ligand-receptor interaction for the inhibition of NA. In addition, the location for binding was predicted to be an allosteric site based on the optimal position of the ligands. Based on this information, the negatively charged amino acids ASN 417 and positively charged HIS 346 in the binding site, which are common residues of amino acid for hydrogen bonds and π–π stacking interactions with two ligands, provided little information for the development of new non-competitive NA inhibitors. Finally, palmatine (**1**) and berberine (**2**) suppressed bacterial growth and may thus represent potential novel antibacterial agents.

4. Materials and methods

4.1. General experimental procedures

NMR spectra were obtained on a JNM-ECA 400 and 600 MHz (JEOL, Tokyo, Japan) spectrometer using DMSO-d_6 and methanol-d_4 as solvents. HPLC analysis using the Chemstation software was performed using an Agilent 1200 series equipped with a binary pump and visible wavelength detector (VWD). The analytical column was a YMC-Pack amino column (250 × 4.6-mm i.d.). Molecular weight was determined using ESI MS (Esquire 6000, Bruker). Melting points were measured using an Electrothermal IA-92 00 instrument and chromatographic separations were performed by thin-layer chromatography (TLC) with commercially available glass plates pre-coated with normal and reverse-phase silica gel (Merck, Darmstadt, Germany). Spots were visualized under UV light (254 and 365 nm) and with Dragendorff reagent. Column chromatography was performed using 230–400-mesh silica gel (Kieselgel 60, Merck, Germany), RP-18 resins (ODS-A, 12 nm, S-150 μm; YMC), Sephadex LH-20 (Amersham Biosciences) and the MPLC system. Neuraminidase of *Clostridium perfringens* (SIGMA, N3001), curcumin (SGIMA, C1386), *p*-iodonitrotetrazolium violet (*p*-INT) (SIGMA I8377) and 4-methylumbellifery-a-D-N-acetylneuraminic acid sodium salt hydrate (SIGMA, M8639) were purchased from Sigma–Aldrich (ST. Louis, MO, USA). rvH1N1 and H5N1 were obtained from R&D Systems (ST. Paul, MO, USA) and Sino Biological Inc. (Beijing, P.R. China), respectively. *C. perfringens* was obtained from the Korean Culture Center of Microorganisms.

4.2. Plant material

C. turtschaninovii rhizomes were purchased from an herbal drug-store in Jeongeup city, Jeollabuk-do, Republic of Korea, in August 2012, and were identified by one of the authors (Prof. Young Ho Kim). A voucher specimen (CNU-13129) was deposited at the Herbarium of College of Pharmacy, Chungnam National University, Republic of Korea.

4.3. Isolation and Identification

C. turtschaninovii rhizomes (3.6 kg) were extracted with 95% EtOH (18 L) at room temperature over 7 days. After removing solvent under reduced pressure, a dark brownish residue (120 g) was obtained. The crude extract (120 g) was separated using silica-gel column chromatography with a gradient solvent system of $CHCl_3$/MeOH (20:1 → 0.5:1) to yield seven fractions (1–7). Fraction 1 (14 g) was subjected to MPLC using gradient elution with $CHCl_3$/MeOH (10:1 → 2:1) to yield four sub-fractions (1.1–1.4). Compounds **10** (20 mg) and **7** (30 mg) were isolated from fraction 1.3 (4 g) on a C-18 column eluted with H_2O/MeOH (10:1 → 0.1:1). Sub-Fraction 1.4 was separated using C-18 column chromatography with gradient elution of H_2O/MeOH (10:1 → 2.5:1) to afford compound **1** (45 mg). Fraction 3 (24 g) was subjected to silica gel column chromatography using gradient $CHCl_3$/MeOH (20:1 → 0.25:1) to yield seven sub-fractions (3.1–3.7). Sub-fraction 3.2 (2 g) was subjected to MPLC using gradient solvent ($CHCl_3$/MeOH, 20:1 → 0.1:1) to yield compounds **5** (13 mg) and **8** (32 mg). Sub-fraction 3.4 (12 g) was loaded on the MPLC system and eluted with $CHCl_3$/MeOH (10:1 → 0.5:1) to afford compounds **2** (7 mg), **4** (300 mg) and **9** (18 mg). Sub-fraction 3.6 (5 g) was chromatographed using a Sephadex LH-20 column and eluted with 100% MeOH to afford seven smaller fractions (3.6.1–3.6.7). Sub-fraction 3.6.5 (540 mg) was subjected to MPLC using gradient solvent ($CHCl_3$/MeOH, 20:1 → 0.25:1) to yield compounds **3** (5 mg) and **6** (8 mg).

4.4. Enzyme inhibition assay

All enzyme assays were performed as described previously using bacterial NA with minor modifications.[18] Briefly, each 50 μl of NA (0.1 mg/ml) in buffer and 20 μl of the respective ligands diluted from 100 μM to 6.25 μM dissolved in MeOH were mixed in 96-well plates containing 80 μl of 50 mM sodium acetate buffer (pH 5.0). Finally, 50 μl of 0.5 mM 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid sodium salt hydrate were added as a substrate. After starting the enzyme reaction at room temperature, 4-methylumbelliferone quantification intensity (RFU) was measured using fluorometric determination (excitation wavelength, 365 nm; emission wavelength, 450 nm) with a SpectraMax M^{2e} Multimode Reader (Molecular Devices, USA) for 10 min. The inhibition ratio was calculated using the equation:

$$\text{Inhibitory}\ (\%) = 100 - [(\Delta\text{ligand absorbance})/(\Delta\text{control absorbance})] \times 100$$

Curcumin was used as the positive control.

4.5. Molecular simulation

The 3D structure of the ligand was modeled using the GlycoBioChem PRODRG2 server and the flexible bonds of ligands were assigned with AutoDockTools. The structure of NA (PDB ID: 2BF6) was obtained from the protein data bank, after which the water, glycerol and *O*-sialic acid were removed from receptor complexes using Chimera. All hydrogen atoms and gasteiger charges in the receptor were added using AutoDockTools. Simulation studies were performed using the AUTODOCK 4.2 software according to the instructions provided on the Autodock Homepage (http://autodock.scripps.edu/). Briefly, to perform blind docking in AUTODOCK 4.2, the grid dimensions were establishing using grid center (X: 84.8, Y: −0.5, Z: 11.4), number of points (X: 150, Y: 200, Z: 150) and spacing (0.375 Å). Both ligand and receptor docking were performed using the Lamarckian Genetic Algorithm (Runs 50) after the default parameter settings generated by the AutoDockTools were used for docking, except that the maximum number of evals was set as long. The molecular simulation results are shown in Figure 4 and were prepared using Chimera.

4.6. Antibacterial activity assay

To confirm the inhibitory activity of compounds **1** and **2** on viable bacteria, *C. perfringens* was cultured in tryptic soy broth (TSB) at 37 °C with continuous shaking in an anaerobic environment for 24 h. Growth was monitored by measuring turbidity at 600 nm. Bacteria diluted 1:100 in TBS (198 μl) were cultured in the presence and absence of compounds **1** and **2** dissolved in DMSO (3.1–100 μM). A total of 40 μl of 1% *p*-INT was added to the each well of the plates, followed by incubation for 30 min. Absorbance was determined at 600 nm.[21]

4.7. Statistical analysis

All activity tests in the presence of inhibitors were performed in triplicate and results are presented as the means ± standard error of the mean (SEM). The results were subjected to analysis using Sigma Plot (SPP Inc., Chicago, IL, USA).

Acknowledgments

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A. Supplementary data

Supplementary data (HPLC, ^1H, ^{13}C NMR, UV, and ESI-MS spectra of compound **1**–**10**; Docking pose of compounds **1** and **2** into the allosteric site of NA) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.09.004.

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