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Human sialidase inhibitors: Design, synthesis, and biological evaluation of 4-acetamido-5-acylamido-2-fluoro benzoic acids

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ABSTRACT

Recent advances in the sialidase biology have clarified the role of human sialidases (NEU 1 to NEU4) in the development of various disease states such as cancer, diabetes and arteriosclerosis. Isoform selective human sialidase inhibitors could be a therapeutic tool or molecular probes for the exploration of the specific functions of human sialidases. In the present study, de novo design based virtual screening was performed to find a new class of human sialidase inhibitors using the experimental crystal structure of NEU2 isoform. A few of nitro benzene and fluoro benzoic acid were identified and a series of 4-acetamido-5-acylamido-2-fluoro benzoic acids were synthesized and, the inhibitory activity of all these compounds against all human sialidase enzymes was evaluated. All these compounds were found to have a poor inhibitory activity and only NEU2 showed more sensitivity to this series of compounds as compared to other isoforms. Molecular docking was performed to gain insight regarding the binding mode of these inhibitors and thereby provided valuable information for our study on the design of selective human sialidase inhibitors further.

1. Introduction

Sialidase enzymes are glycoside hydrolase enzymes (EC 3.2.1.18), which cleave the glycosidic linkages of sialic acid from various glycoproteins, glycopeptides, gangliosides, oligosaccharides and polysaccharides.[1] Sialidases are mainly involved in the metabolism of sialic acids and therefore regulate the cellular processes by modifying the sialic acid content in the cell. Sialidases are classified into two main types namely, *endo*-α-sialidase (E.C. 3.2.1.129) and *exo*-α-sialidase (EC 3.2.1.18), respectively.[2] *exo*-α-Sialidase is also known as α-*N*-acylneuraminate glycohydrolase or Neuramindase, which attacks the terminal sialic acid linkages. *exo*-α-Sialidases (hereafter sialidases) are widely distributed among the different classes of organisms such as viruses, bacteria, protozoa and vertebrates.[3] They are thought to be involved in various biological processes like cell growth, cell proliferation, cell differentiation, cell migration, cellular transport, signal transduction, antigen masking, inter-intra cell interactions and infection.[4] The most extensively studied sialidase is the viral sialidase, a drug target for the prevention of influenza infection.[5] Some of the bacterial (e.g., *Vibrio cholerae, Streptococcus pneumoniae*) and protozoic sialidases (e.g., *Trypanosoma cruzi, Trypanosoma brucei)* have been considered as important virulence factor and have also been discussed as potential targets for drug design.[6–9]

Several mammalian sialidases have been described at the molecular level. They have been demonstrated to differ from microbial sialidases in various aspects, especially in the presence of multi forms even in the single cell. At least four sialidase homologs have been identified in the human genome namely, intra lysosomal sialidase (NEU1), cytosolic (NEU2), plasma membrane associated sialidase (NEU3) and lysosomal or mitochondrial membrane associated sialidase (NEU4). Apart from their subcellular localization, these sialidases isoform (NEUs) also differ in their substrate specificities, immunological properties, and it has been suggested that distinct NEUs play specific role(s) in diverse cellular processes.[10] Unregulated NEUs expression has been speculated to be associated with various pathological conditions, for example, tumor progression, apoptosis suppression, metastasis, hyperinsulinemia, atheroma plaque formation, inflammation, etc.[11–14] Although the availability of genetically modified mice has recently allowed the functional characterization of individual NEUs in the diseases states like cancer, diabetes and arteriosclerosis, it is always desirable to have an inhibitor that selectively inhibits the tar-

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get isoform in the presence of others, so as to minimize the cross reactivity or potential side effects. So, there is an intense interest in identifying the isoform-selective inhibitors for human sialidase, and such tool inhibitors would facilitate the differential functional analysis of human sialidases in many vital cellular functions and potential therapeutic utilities of sialidase inhibitors.

Before embarking on the selectivity design, it is important to address the question of what key structurally and functionally important sites are that differentiate the target isoform protein from the others. Structural studies on various viral, bacterial, protozoic and human sialidases have revealed the common canonical six-blade β-propeller 3D arrangement in spite of their lower sequence similarity.[15] There are many conserved residues within the active sites of various sialidases, and are thought to be important for catalytic mechanism. These include the arginine triad and its close neighbor Tyr, and a pair of acidic residues (Asp/Glu) above and below the active site crevice. On the other hand, sialic acids (Neu5Ac, **1**) 5-*N*-acetyl or/and 6-glycerol binding groups in the active site are variable from sialidase to sialidase (Fig. 1a). In fact, this variation has been speculated to be responsible for the substrate/inhibitor specificity of sialidase from various sources.[16]

Free sialic acid **1** is itself the only poor inhibitor of all the sialidases (mM range) and its transition-state analogue, DANA (2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid, Neu5Ac2en, **2**) is available as a non-specific and high affinity inhibitor for most sialidases and inhibits the NEUs with IC_{50} values ~40–150 μM (Fig. 1b).[17] In our previous study, we have predicted structural similarities and differences at the active site amongst human sialidases by molecular modeling techniques using the crystal structure of NEU2.[18] Despite the high level of active site residues conservation, they seem to have differences in the amino acid residues that form the glycerol-binding pocket (specificity pocket). We reasoned that structure based approach in this regard might provide hints to exploit the non-selective inhibitor substituents that extend to the specificity pocket of the active site pocket or 'de novo design' for isoform selective inhibitor design. In our initial approach, we designed a series of C9 amide linked hydrophobic derivatives of DANA modified at glycerol side chain and followed by the synthesis and evaluation against all four NEUs gave us at least 100-fold selectivity for NEU1 over NEU2, NEU3, and NEU4 with **3** (GSC-649) (Fig. 1b).[19]

In the present approach, we applied structure based virtual screening approach to generate the novel inhibitor scaffolds, away from sugar framework, which are readily available or synthetically accessible and have desired molecular properties as well. The crystal structure of NEU2 has been used, as it is the only human sialidase which is experimentally resolved among human counterparts. First, structure based interaction sites were designed based on the interaction map generated by the LUDI program.[20] Then Ludi/CAP library was screened with selected interaction sites as three-dimensional (3D) queries. A set of fluoro-benzoic acids were predicted as potential fragments that could bind to sialidase active site. An extra amide linked various hydrophobic substituents were added to the fluoro-benzoic acids to hold out of glycerol-binding pocket for selectivity probing among NEUs. Here we report the design, synthesis and biological evaluation of a series of fluoro-benzoic acid derivatives as human sialidase inhibitors.

2. Results and discussion

2.1. Structure based de novo design/virtual screening

The structure directed approach is a rational starting point for the design of compounds that inhibit the target receptor or mimics the substrate of the receptor. This methodology uses the steric (shape and size) and chemical (charge and non-polar interactions) complimentaries of the target receptor. The methods include de novo design, virtual screening and fragment-based discovery. Ludi is a well-known de novo design program that uses a library of fragments and can be subjected to virtual screening protocols.[21] Ludi is absolutely geometric and avoids time-consuming potential energy calculations. Ludi calculates molecular interaction sites on a receptor and uses them to search for complementary compounds in the library of molecular fragments that should interact with receptor. As a part of the search and fit computation, Ludi also estimates the energy of each conformation searched from the fragments in the library, in the context of the receptor site. Ludi/CAP is a 3D Ludi library prepared from two Accelrys databases: Chemicals Available

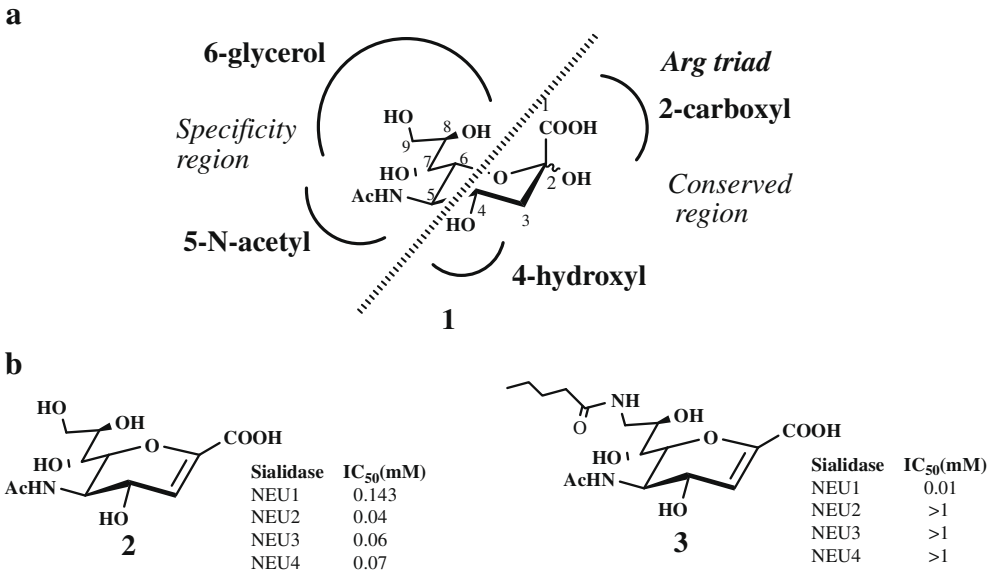


Figure 1. (a) Sialic acid **1** (Neu5Ac) and its four constituent groups required for binding and (b) structure of DANA **2 and** GSC-649 **3** with their inhibition data against human sialidases.[19]

for Purchase (CAP), a structural database of commercially available compounds, and CAPScreening, a database of compounds available from the suppliers of screening.[22] Known compounds retrieved from Ludi/CAP can be purchased as ligand reagents from the suppliers and can be tested either directly or with small modifications.

The crystal structure of human cytosolic sialidase NEU2, in complex with the inhibitor DANA (PDB code 1VCU) was taken as a starting-point.[23] Amino acid residues sites that complement DANAs four constituent groups, required for binding were chosen to represent the interaction sites. Ludi has identified several interaction features (HD—hydrogen-bond donors, HA—hydrogen-bond acceptors, and L—lipophilic areas) at the central part of the binding pocket (Fig. 2). The various representative structural features (7HA, 4HD, 4L) of twelve residues were selected to create 3D queries with four to eight features, which are then evaluated in an iterative process (Table 1). The combinations of features were selected in such a way that they could cover the active site volume and should allow the ligand to achieve the maximum interactions at the active of the enzyme. Instead of building all the possible combinations using the 10 available features, which would have led to a total of more than 1000 combinations, we manually designed about 25 3D queries, keeping conserved Arg304 in the middle of the arginine triad and specificity residue Leu217 as main residues, while maintaining the balance between the HBA, HBD and LIPO features.

We initiated virtual screening of Ludi/CAP database (78,175 compounds) to identify the compounds that satisfy the selected 3D structural queries. A total of 3437 hits possessing chemical matching with the 3D queries were retrieved and 914 fragments with high scores (>300) were retained. Since many of the hits were identified more than one 3D query, a careful manual selection was made not to duplicate hits. This operation yielded 214 unique compounds, which were chosen for a further analysis. The binding mode of selected hits was visually examined for their fit into the active site of NEU2 and those that bind in a convincing mode, particularly hits that had at least an interaction with the Arg triad, were considered. This resulted in a total of 14 compounds mainly fluoro benzoic acids and nitro benzene derivatives, which were taken to represent potential inhibitors (Fig. 3). Interestingly, benzoic acid derivatives are already known as sialidase inhibitors for viral and bacterial counter parts, and some of them showed effective IC50 of μmolar to several hundred μmolar concentrations.[24,25]

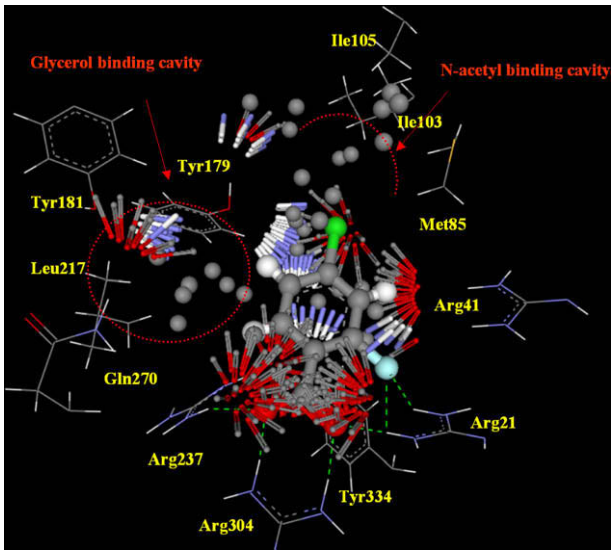
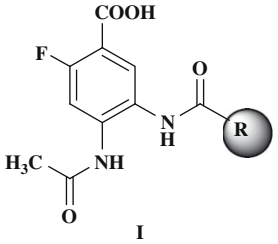


Figure 2. The interaction of map of NEU2 active site generated by the LUDI program. Hydrogen bond acceptors and donors are presented as green-gray and blue-gray sticks, respectively, and hydrophobic regions are displayed with gray spheres. This map also shows the interactions of a bound hit (**A**) with the active sites residues and the scope for the interactions at the glycerol binding and *N*-acetyl binding cavities.

Table 1
List of query features from selected atoms and their corresponding amino acid residues (HD—hydrogen-bond donors, HA—hydrogen-bond acceptors, and L—lipophilic areas)

Residue	Atom	Feature	Residue	Atom	Feature
Arg21	HH_{22}	HA_1	Tyr181	OH	HA_4, HD_2
Arg41	HH_{22}	HA_2	Leu217	CG	L_4
Met85	CG	L_1	Arg237	HH_{22}	HA_5
Ile103	CG	L_2	Glu270	OE_2	HD_3
Ile105	CG	L_3	Arg304	HH_{22}	HA_6
Tyr179	OH	HA_3, HD_1	Tyr334	OH	HA_7, HD_4

It is worth noting that *ortho*-fluoro group of the compounds could make an extra contacts with the conserved arginine triad of sialidase enzymes. An important fact is that the most sialidase inhibitors known until recently including the natural products, contain a typical carboxylic acid group and this fact reveals that carboxylic acid group is absolutely necessary for the sialidases inhibition. Hence, we reasoned that benzoic acid containing *ortho*-fluoro group could be a logical template to explore as a human sialidase inhibitors, moreover, there is no report of human sialidase inhibition with benzoic acid scaffold alone so far. As we aimed at specificity probing among human sialidases, we incorporated various N-amide linked derivatives into fluoro benzoic acid core structure, which is expected to make interactions in the glycerol group binding region (specificity pocket). Since the same region is primarily flanked by hydrophobic residues, various hydrophobic groups were designed to make hydrophobic contacts within this region. This fluoro benzoic acid ring was also incorporated with *N*-acetyl at *para*-position to mimic the conserved interactions at *N*-acetyl binding region.



I

2.2. Synthesis

We envisaged that 4-acetamido-2-fluoro benzoic acids having 5N-linked hydrophobic derivatives **I** could be easily synthesized by the following methods (Scheme 1). The commercially available 3-fluoro-4-methyl aniline **4** could be acetylated to give a 3-fluoro-4-methyl acetamide **5**, and then **5** can be oxidized to a 2-fluoro-4-acetamido benzoic acid **6**. Selective nitration at 5 position can afford a 5-nitro-2-fluoro-4-acetamido benzoic acid **7**, then subsequently it can be reduced and acylated with various hydrophobic acid chlorides or equivalents. The expected position of nitration in the benzene ring is 5, since it is *meta*- to carboxylic acid group that is *meta*-director and *para* to F, which is largely a para director. Accordingly, the starting material 3-fluoro-4-methyl aniline **4** was acetylated to give **5** in 96% yield using acetic anhydride in diisopropyl ether at 50 °C for 30 min. The crystalline compound **5** was then subjected to oxidation step using potassium dichromate/H_2SO_4 mixture. Oxidation was carried out by heating the oxidation mixture with **5** in water at 100 °C for 8 h, gave the compound **6** in

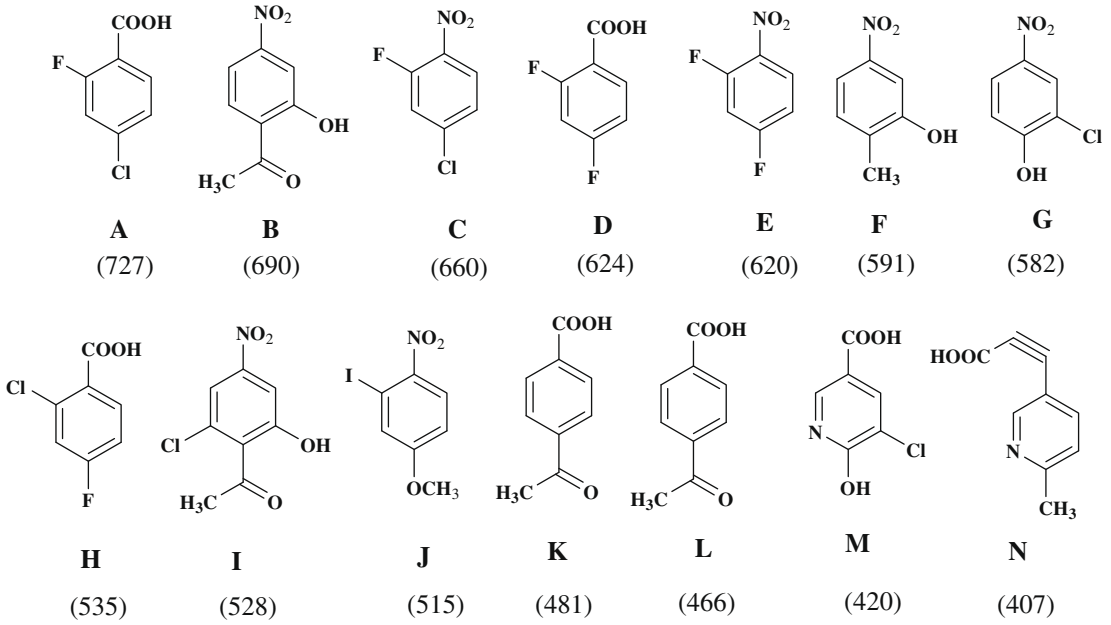
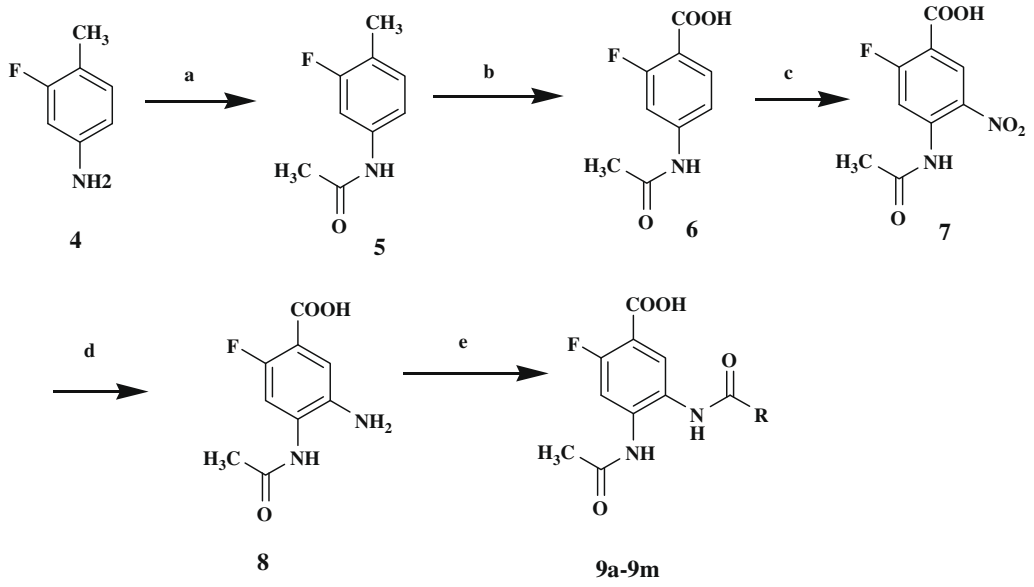


Figure 3. Hits obtained from Ludi/CAP library search. Individual Ludi score is given in the bracket.



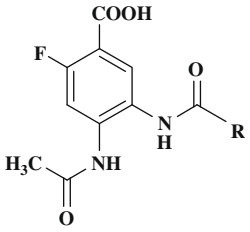
Scheme 1. Reagents and conditions: (a) $(CH_3CO)_2O$, diisopropylether, 50 °C, 30 min, 96% ; (b) $KMnO_4$, $MgSO_4$, H_2O 100 °C, 8 h, 73%; (c) HNO_3, −20 °C, 2 h, 69% ; (d) Pd/C, H_2, rt, ethylacetate/MeOH (1:1), 2d; (e) ROCl, satd $NaHCO_3$, THF/MeOH (1:1),1 h, 50–84% (two steps).

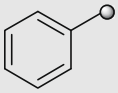
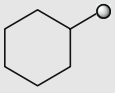
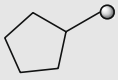
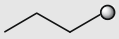
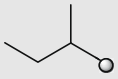
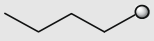
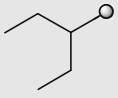
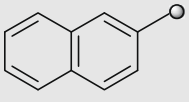
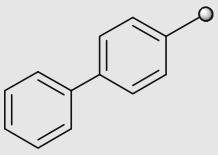
73% yield. The following nitration step gave some trouble in the beginning, but after some experimentation, we could achieve the selective nitration to give **7** in 69% yield. The condition employed for the nitration was the addition of compound in small amounts for 30 min to neat nitric acid, cooled to −20 °C. After having the nitrated compound in hand, reduction, followed by acylation was carried out. The hydrogenation of **7** in methanol and ethyl acetate (1:1) was done using Pd/C as a catalyst, gave the amine **8**, which was then converted to the respective amides. The condition used for the acylation is the addition of acyl chloride dissolved in THF to the stirring solution in satd $NaHCO_3$. This procedure gave the various hydrophobic 4-acetamido-5-acylamido-2-fluoro benzoic acids (**9a**–**9m**) in 50–84% yields.

2.3. Human sialidase inhibition

Inhibitory activities of a series of substituted 2-fluoro benzoic acids (**9a**–**9m**) against all four human sialidases were investigated. The percentage of inhibition at 1 mM concentration of inhibitors is given in Table 2. None of the compounds inhibited NEU1 at the tested concentrations, whereas some compounds inhibited NEU3 and NEU4 scarcely. NEU2 seems slightly more sensitive than the other isoforms to this series of compounds, as most of the compounds inhibited NEU2 in the range of 15–60% inhibition at 1 mM. The highest inhibition (%I = 60) was observed with compound **9m** which has N-amide linked bulky biphenyl group (IC_{50} of 0.552 ± 0.124 mM and K_i value of 0.307 ± 0.073 mM with

Table 2
Chemical structures of compounds **9a**–**9m** and their inhibitory activities against human sialidases: NEU1, NEU2, NEU3 and NEU4.



Compound	R	% of sialidase inhibition (at 1 mM)			
		NEU1[a]	NEU2	NEU3	NEU4
9a		–	37	23	8
9b		–	17	–	–
9c		–	17	–	6
9d		–	–	–	–
9e		–	20	–	–
9f		–	–	–	<5
9g		–	7	–	–
9h		–	25	13	<5
9i		–	15	–	11
9j		–	–	–	<5
9k		–	23	–	<5
9l		–	33	8	10
9m		–	60 (IC_{50} = 0.552 ± 0.124 mM)	8 (IC_{50} = >5 mM)	10 (IC_{50} = >5 mM)

[a] No inhibition.

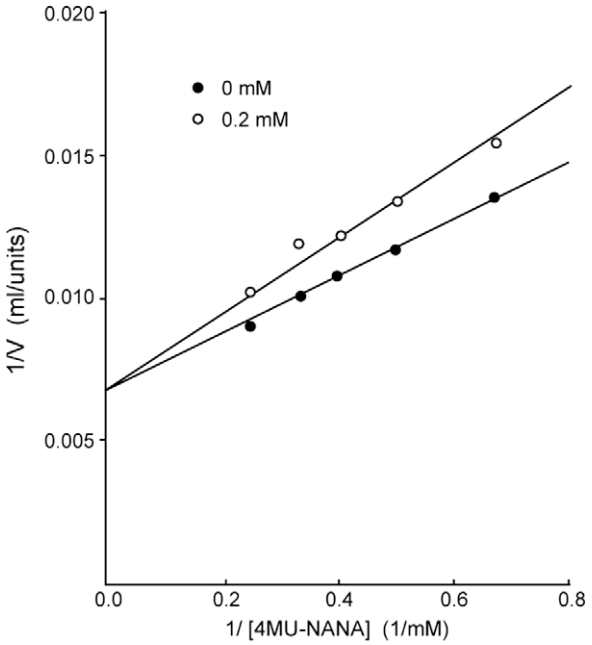


Figure 4. Lineweaver–Burk plot for the inhibition of the NEU2 activity by compound **9a** (K_i value of 0.307 ± 0.073 mM with 4MU-Neu5Ac as a substrate).

4MU-Neu5Ac as a substrate) (Fig. 4). Compound **9m** hardly inhibited NEU3 (%I = 8) and NEU4 (%I = 10) both with IC_{50} of >5 mM. Compounds **9a** (%I = 37) and **9l** (%I = 33) showed the modest inhibitory activity against NEU2 with the IC_{50} of 1.2 mM and 1.37 mM, respectively. The maximum inhibition of NEU3 was achieved with compound **9a** (%I = 23) and with the estimated IC_{50} of >2 mM. Taken together, about 10-fold of NEU2 selectivity was achieved with compound **9m** over other human sialidases.

2.4. Docking studies

In an attempt to understand the molecular basis of observed affinity of this series to NEU2, automated docking was performed using the DS LigandFit.[26] However, the docking study was not able to explain this binding affinity as binding poses were apparently in an irrational mode. This behavior might be due to the restricted access of bulky biphenyl group into the active site of NEU2, since LigandFit employs flexible docking of ligand into the fixed protein binding site. Thus, compounds (**9a** and **9m**) were manually docked into NEU2 active site in a proposed binding mode. A special emphasis was given to the interaction between carboxylic acid group of inhibitor and Arg304 of NEU2. The resulting complex was energetically refined to reduce the steric clashes and also to maximize the interactions. The binding poses of these compounds suggested that amide substituents could be tolerated in the glycerol-binding pocket of NEU2, but show a potential loss of a conserved H bond interaction with Arg234 and a steric bump of the same residue with H_6 of benzoic acid core ring. This is probably due to their conformation and restricted rotation along with the flat benzoic acid ring structure that could cause the overturning of the ligands, which could be an explanation for the observed weak inhibitory activity (Fig. 5). Interestingly, amino acid residue that located at the lip of the glycerol binding pocket is more flexible in NEU2 (Gln270), while in NEU3 and NEU4, it is substituted with comparatively steric bulk amino acid residues His277 and Trp274, respectively.[18] In the case of NEU1, this scenario is different as this subsite in NEU1 is majorly composed of acidic amino acids. These relative differences in the glycerol-binding pocket of human sialidases can be accounted for the observed marginal

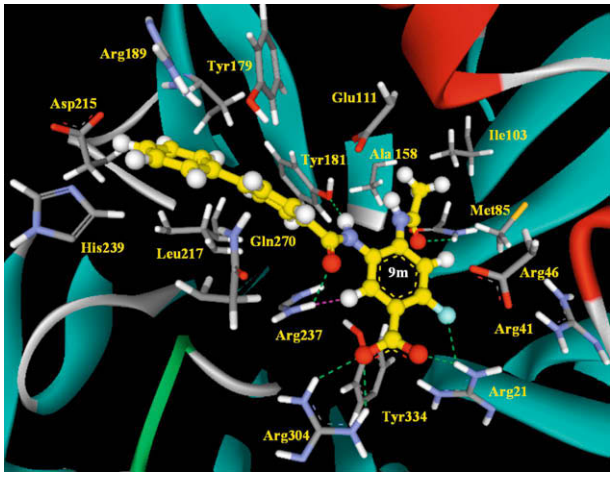


Figure 5. The predicted binding conformation of the inhibitor **9m** at NEU2 active site. Green and pink lines represent the hydrogen bonding and steric clash, respectively.

selectivity of 4-acetamido-5-acylamido-2-fluoro benzoic acids to NEU2 over other isoforms. Extended hydrophobic interactions of biphenyl carboxamido group at the active site of NEU2 can be reasoned for the increased binding affinity of **9m** over acetamido group (**9a**), as bulky groups are often known to exhibit better affinity and selectivity.

3. Conclusion

Structure-based de novo design/virtual screening enabled us to identify some nitro benzene and fluoro benzoic acids as potential inhibitors of human sialidase from Ludi/CAP database. 2-fluoro benzoic acid scaffold was selected and modified for the selectivity probing among human sialidases. The simple and straightforward method to access the various 4-acetamido-5-acylamido-2-fluoro benzoic acid analogues was developed. The synthesized compounds (**9a**–**9m**) were biologically evaluated against all four types of human sialidases. This series of compounds showed very weak inhibitory activities against human sialidases, except some derivatives inhibited NEU2 moderately as compared to other isoforms. Highest NEU2 inhibition (60% at 1 mM, IC_{50} of 0.552 ± 0.124 mM and K_i value of 0.307 ± 0.073 mM) was achieved with compound **9m** with ~10-fold selectivity. A docking investigation revealed that the conformational restriction of the current series of the derivatives which might disrupt the conserved interaction to the arginine triad of human sialidases could be a reason for their observed poor inhibitory activities. The inhibitory activity and isoform selectivity among human sialidases of current series can be improved by addressing the flexibility of the substituents at C5 of 4-acetamido-2-fluoro benzoic acid core structure. With this regard, the further efforts on synthesis and optimization are currently underway to expand the scope of this work. On the whole, we strongly believe that the opportunities and the limitations of the present study might be an interesting subject for the future inhibitor design.

4. Experimental

4.1. Synthesis

4.1.1. General

Reagents were purchased in the best quality available and the reagent grade solvents were dried over activated molecular sieves of appropriate size. Commercial grade solvents (CH_2Cl_2, hexane,

EtOAc, MeOH, acetone, $CHCl_3$, toluene) were purchased from Wako chemical company and used without any further purification. Reactions were monitored by thin layer chromatography (TLC) using Merck silica gel plates GF_{245}. Compounds were detected either with 254 nm UV light or nihydrin. Purification by normal phase chromatography was achieved by elution through columns of silica gel ~80 or 300 mesh size. ^1H, ^{13}C and ^{19}F nuclear magnetic resonance (NMR) spectra were recorded using a Jeol ECX NMR spectrometer. All NMR spectra were recorded at 400, 500 or 600 MHz. Chemical shifts are expressed as parts per million (ppm, δ) and are relative to the standard internal reference tetra methyl silane (TMS) (0.0δ). In order to confirm assignments, 2D NMR experiments were performed using the ^1H–^1H correlation spectroscopy (COSY) experiment for proton-proton interactions and the ^1H–^{13}C heteronuclear multiple quantum coherence (HMQC) experiment for proton–carbon interactions. Mass spectral data were obtained on a Bruker Daltonics microTOF-II in the positive or negative ion detection modes.

4.1.1.1. *N*-(3-Fluoro-4-methyphenyl) acetamide (5). To the solution of 3-fluoro-4-methyl aniline **(4)** (10 g, 80 mmol) in diisopropyl ether (50 mL) was added acetic anhydride (9.5 mL, 100 mmol) and heated at 50 °C for 30 min. Upon cooling, white needle shaped crystals were formed, filtered, and dried in vacuo to give **5** (12.8 g, 96%). Compound **5**: R_f = 0.5 (hexane/EtOAc 1:1). ^1H NMR (DMSO-d_6) δ 2.16 (3H, s, Ar-CH_3), 2.03 (3H, s, NAc), 7.17 (2H, m, H-5, H-4), 7.44 (1H, d, $J_{2,F}$ 13.0 Hz, H-2), 10.05 (1H, s, NH) (assignments were confirmed by ^1H–^1H COSY); ^{13}C NMR (DMSO-d_6) δ 13.6 (Ar-CH_3), 23.9 (NC(O)*Me*), 105.7 (C-2), 114.43 (C-6), 118.0 (C-4), 131.3 (C-5), 138.7 (C-1), 160.2 (C-3), 168.4 (N*C*(O)Me) (assignments were confirmed by ^1H–^{13}C HMQC); ^{19}F NMR (DMSO-d_6) δ −116.25. HRMS (ESI) calcd for $C_9H_{10}FNNaO$ (M + Na) 190.0644; found 190.0714.

4.1.1.2. 4-Acetamido-2-fluoro benzoic acid (6). To the solution of $KMnO_4$ (29.4 g) and $MgSO_4$ (20.4 g) in water was added compound **5** (10 g, 60 mmol) in portion wise, and the mixture was heated at 70–80 for 8 h. The reaction mixture was allowed to stand overnight and solid Na_2CO_3 (29 g) was added. The mixture was further stirred for 15 min and filtered. The colorless filtrate was cooled to 0 °C and acidified with 40 mL of concd HCl. The formed precipitate was filtered, dried and recrystallized from ethanol–water to give **6** (8.6 g, 73%) as off-white crystals. Compound **6**: R_f = 0.4 (5% MeOH in EtOAc). ^1H NMR (DMSO-d_6) δ 2.09 (3H, s, NAc), 7.33 (1H, dd, $J_{5,6}$ 8.8 Hz, $J_{5,3}$ 1.38 Hz, H-5), 7.66 (1H, t, $J_{6,F}$ and $J_{6,5}$ 8.8 Hz, H-6), 7.83 (1H, dd, $J_{3,F}$ 13.7 Hz, $J_{3,5}$ 1.38 Hz, H-3), 10.43 (1H, s, NH) 12.9 (1H, s, COOH) (assignments were confirmed by ^1H–^1H COSY); ^{13}C NMR (DMSO-d_6) δ 24.2 (NC(O)*Me*), 106.1 (C-3), 112.8 (C-1), 114.6 (C-5), 132.8 (C-6), 144.7 (C-4), 160.8 (C-2), 164.5 (*C*(O)OH), 169.2 (N*C*(O)Me) (assignments were confirmed by ^1H–^{13}C HMQC); ^{19}F NMR (DMSO-d_6) δ −107.73. HRMS (ESI) calcd for C9H7FNO3 (M−H$^+$) 196.0410; found 196.0465.

4.1.1.3. 4-Acetamido-2-fluoro-5-nitro benzoic acid (7). To the fuming nitric acid (50 mL) cooled at −20 °C was added compound **6** (4 g, 20 mmol) portion wise. After 2hrs the reaction mixture poured over ice (50 g) and water (50 mL) with stirring. The light yellow solid was collected by filtration and dried under reduced pressure to give the title compound (3.4 g, 69 %). Compound **7**: R_f = 0.5 (5% MeOH in EtOAc). ^1H NMR (DMSO-d_6) δ 2.17 (3H, s, NAc), 7.88 (1H, d, $J_{3,F}$ 12.8 Hz, H-3), 8.46 (1H, d, $J_{6,F}$ 7.3 Hz, H-6), 10.55 (1H, s, NH) 13.72 (1H, s, COOH) (assignments were confirmed by ^1H–^1H COSY); ^{13}C NMR (DMSO-d_6) δ 24.13 (NC(O)*Me*), 111.2 (C-3), 114.5 (C-1), 129.9 (C-6), 136.1 (C-4), 137.5 (C-5), 162.6 (C-2), 164.1 (*C*(O)OH), 169.2 (N*C*(O)Me) (assignments were confirmed by ^1H–^{13}C HMQC) ; ^{19}F NMR (DMSO-d_6) δ −99.56. HRMS (ESI) calcd for $C_9H_6FN_2O_5$ (M−H$^+$) 241.0261; found 241.0192.

4.1.1.4. 4-Acetamido-5-amino-2-fluoro benzoic acid (8). To the solution of compound **7** (242 mg, 1 mmol) in the mixture of ethyl acetate and methanol (1:1) (10 mL) was added 10% w/w palladium on carbon (25 mg) under nitrogen. The resulting suspension was hydrogenated at room temperature and ambient pressure for 2 days. The mixture was filtered through the Celite and the filtrate was concentrated to dryness under reduced pressure. The crude was used for subsequent reactions without further purification. Compound **8**: R_f = 0.5 (5% MeOH in EtOAc). HRMS (ESI) calcd for $C_9H_8FN_2O_3$ (M−H$^+$) 211.0519; found 211.0723.

4.1.2. General procedure V for the synthesis of 4-Acetamido-5-acylamido-2-fluoro benzoic acids (9a–9m)

To the solution of crude compound **8** in the mixture of ethyl acetate and methanol (1:1) (10 mL) was added saturated aq $NaHCO_3$ solution (10 mL) at rt. Acid chloride (1 mmol) dissolved in the THF (~500 μL) was added to the reaction, while maintaining the basic pH of the reaction mixture. Then the reaction mixture was stirred for 1–3 h. Then reaction mixture was adjusted to pH 5–6 using 2 N HCl and extracted with ethyl acetate (3 × 10 mL). The organic layers were combined and washed with water (10 mL), brine solution, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by either column chromatography (ethyl acetate/MeOH/H_2O) or recrystallized from ethyl acetate to give off-white to pure white powders.

4.1.2.1. 4,5-Diacetamido-2-fluoro benzoic acid (9a). Prepared according to General procedure V. Yield: (155 mg, 61 %).^1H NMR (DMSO-d_6) δ 2.07, 2.12 (2 × 3H, 2 × s, 2 × NAc), 7.81 (1H, d, $J_{3,F}$ 12.8 Hz, H-3), 7.92 (1H, d, $J_{6,F}$ 7.3 Hz, H-6), 9.43, 9.5 (2 × 1H, 2 × s, 2 × NH), 13.1 (1H, s, COOH) (assignments were confirmed by ^1H–^1H COSY); ^{13}C NMR (DMSO-d_6) δ 24.1, 24.6 (2 × NC(O)*Me*), 110.5 (C-3), 114.05 (C-1), 124.9 (C-5), 129.4 (C-6), 137.6 (C-4), 158.6 (C-2), 164.8 (*C*(O)OH), 169.6 (2 × N*C*(O)Me) (assignments were confirmed by ^1H–^{13}C HMQC) ; ^{19}F NMR (DMSO-d_6) δ −112.46. HRMS (ESI) calcd for $C_{11}H_{10}FN_2O_4$ (M−H$^+$) 253.0625; found 253.0841.

4.1.2.2. 4-Acetamido-5-benzamido-2-fluoro benzoic acid (9b). Prepared according to general procedure V. Yield: (68 mg, 215%). ^1H NMR (DMSO-d_6) δ 2.12 (3H, s, NAc), 7.52–7.63 (3H, m, H-3′, H-4′, H-5′), 7.83 (1H, d, $J_{3,F}$ 12.8 Hz, H-3), 7.98 (1H, d, $J_{6,F}$ 7.3 Hz, H-6), 8.01 (2H, d, H-2′, H-6′), 9.76, 9.89 (2 × 1H, 2 × s, 2 × NH), 13.17 (1H, s, COOH) (assignments were confirmed by ^1H–^1H COSY); ^{13}C NMR (DMSO-d_6) δ 24.8 (NC(O)*Me*), 110.8 (C-3), 114.1 (C-1), 124.9 (C-5), 128.4 (C-2′, C-6′), 128.8 (C-3′, C-5′), 130.7 (C-6), 132.3 (C-4), 134.6 (C-1), 138.5 (C-4), 159.1 (C-2), 166.3 (*C*(O)OH), 164.9 (N*C*(O) phenyl), 169.9 (N*C*(O)Me) (assignments were confirmed by ^1H–^{13}C HMQC); ^{19}F NMR (DMSO-d_6) δ −111.85. HRMS (ESI) calcd for $C_{16}H_{12}FN_2O_4$ (M−H$^+$) 315.0781; found 315.091.

4.1.2.3. 4-Acetamido-5-cyclohexanecarboxamido-2-fluoro benzoic acid (9c). Prepared according to general procedure V. Yield: (74 mg, 238%). ^1H NMR (DMSO-d_6) δ 1.15–1.45 (10H, 2 × m, H-2′, H-3′, H-4′, H-5′, H-6′), 2.11 (3H, s, NAc), 2.35–2.42 (1H, m, H-1′), 7.72 (1H, d, $J_{3,F}$ 12.8 Hz, H-3), 7.94 (1H, d, $J_{6,F}$ 7.9 Hz, H-6), 9.48, 9.31 (2 × 1H, 2 × s, 2 × NH), 13.11 (1H, s, COOH) (assignments were confirmed by ^1H–^1H COSY); ^{13}C NMR (DMSO-d_6) δ 24.8 (NC(O)*Me*), 25. 7 (C-3′, C-5′), 25. 9 (C-4′), 29. 5 (C-2′, C-6′), 44.7 (C-1′), 111.0 (C-3), 114.2 (C-1), 126.6 (C-5), 129.1 (C-6), 137.1 (C-4), 158.5 (C-2), 164.9 (*C*(O)OH), 169.5 (N*C*(O)Ac), 175.4 (N*C*(O) cyclohexyl) (assignments were confirmed by ^1H–^{13}C

HMQC) ; ^{19}F NMR (DMSO-d_6) δ −112.87. HRMS (ESI) calcd for $C_{16}H_{18}FN_2O_4$ ($M-H^+$) 321.1251; found 321.148.

4.1.2.4. 4-Acetamido-5-cyclopentanecarboxamido-2-fluoro benzoic acid (9d). Prepared according to general procedure V. Yield: (212 mg, 69%). ^{1}H NMR (DMSO-d_6) δ 1.52–1.90 (8H, 3 × m, H-2′, H-3′, H-4′, H-5′), 2.11 (3H, s, NAc), 2.84 (1H, p, H-1′), 7.72 (1H, d, $J_{3,F}$ 12.6 Hz, H-3), 7.94 (1H, d, $J_{6,F}$ 6.8 Hz, H-6), 9.37, 9.53 (2 × 1H, 2 × s, 2 × NH), 13.12 (1H, s, COOH) (assignments were confirmed by ^{1}H–^{1}H COSY); ^{13}C NMR (DMSO-d_6) δ 24.5 (NC(O)*Me*), 26. 1 (C-2′, C-4′), 30.4 (C-3′, C-5′), 45. 3 (C-1′), 111.8 (C-3), 113.9 (C-1), 125.3 (C-5), 128.3 (C-6), 137.2 (C-4), 158.4 (C-2), 164.4 (*C*(O)OH), 169.5 (N*C*(O)Ac), 175.5 (N*C*(O) cyclopentyl) (assignments were confirmed by ^{1}H–^{13}C HMQC) ; ^{19}F NMR (DMSO-d_6) δ −112.41. HRMS (ESI) calcd for $C_{15}H_{16}FN_2O_4$ ($M-H^+$) 307.1094; found 307.1201.

4.1.2.5. 4-Acetamido-5-cyclopropanecarboxamido-2-fluoro benzoic acid (9e). Prepared according to general procedure V. Yield: (162 mg, 58%). ^{1}H NMR (DMSO-d_6) δ 0.82–0.84 (4H, m, H-2′, H-3′), 1.84 (1H, p, H-1′), 2.12 (3H, s, NAc), 7.74 (1H, d, $J_{3,F}$ 12.8 Hz, H-3), 8.01 (1H, d, $J_{6,F}$ 7.9 Hz, H-6), 9.63, 9.71 (2 × 1H, 2 × s, 2 × NH), 13.09 (1H, s, COOH) (assignments were confirmed by ^{1}H–^{1}H COSY); ^{13}C NMR (DMSO-d_6) δ 8.0 (C-2′, C-3′), 15.05. 7 (C-1′), 24.5 (2 × NC(O)*Me*), 45. 3 (C-1′), 111.0 (C-3), 114.5 (C-1), 125.4 (C-5), 128.6 (C-6), 136.6 (C-4), 158.3 (C-2), 164.6 (*C*(O)OH), 169.6 (N*C*(O)Ac), 172.9 (N*C*(O) cyclopropyl) (assignments were confirmed by ^{1}H–^{13}C HMQC); ^{19}F NMR (DMSO-d_6) δ −113.1. HRMS (ESI) calcd for $C_{13}H_{12}FN_2O_4$ ($M-H^+$) 279.0781; found 279.0822.

4.1.2.6. 4-Acetamido-5-butyramido-2-fluoro benzoic acid (9f). Prepared according to general procedure V. Yield: (203 mg, 72%). ^{1}H NMR (DMSO-d_6) δ 0. 94 (3H, t, H-3′), 1.63 (2H, sex, H-2′), 2.11 (3H, s, NAc), 2.34 (1H, t, H-1′), 7.77 (1H, d, $J_{3,F}$ 12.6 Hz, H-3), 7.93 (1H, d, $J_{6,F}$ 6.8 Hz, H-6), 9.38, 9.50 (2 × 1H, 2 × s, 2 × NH), 13.08 (1H, s, COOH) (assignments were confirmed by ^{1}H–^{1}H COSY); ^{13}C NMR (DMSO-d_6) δ 14.1 (C-3′), 18.9 (C-2′), 23.9 (NC(O)*Me*), 38.3 (C-1′), 111.8 (C-3), 114.5 (C-1), 125.0 (C-5), 129.2 (C-6), 137.3 (C-4), 158.5 (C-2), 164.92 (*C*(O)OH), 169.6 (N*C*(O)Ac), 172.4 (N*C*(O) butyl) (assignments were confirmed by ^{1}H–^{13}C HMQC); ^{19}F NMR (DMSO-d_6) δ −112.62. HRMS (ESI) calcd for $C_{13}H_{14}FN_2O_4$ ($M-H^+$) 281.0938; found 281.1019.

4.1.2.7. 4-Acetamido-5-(2-methylbutanamido)-2-fluoro benzoic acid (9g). Prepared according to general procedure V. Yield: (175 mg, 59%). ^{1}H NMR (DMSO-d_6) δ 0.90 (3H, t, , H-3′), 1.11 (3H, d, H-1″), 1.38–1.68 (2H, 2 × m, H-2′), 2.10 (3H, s, NAc), 2.46 (1H, sex, H-1′), 7.71 (1H, d, $J_{3,F}$ 12.8 Hz, H-3), 7.94 (1H, d, $J_{6,F}$ 7.9 Hz, H-6), 9.39, 9.47 (2 × 1H, 2 × s, 2 × NH), 13.17 (1H, s, COOH) (assignments were confirmed by ^{1}H–^{1}H COSY); ^{13}C NMR (DMSO-d_6) δ 12.1 (C-3′), 17.6 (C-1″), 24.5 (NC(O)*Me*), 27.2 (C-2′), 42.1 (C-1′), 111.3 (C-3), 114.4 (C-1), 125.4 (C-5), 129.1 (C-6), 137.2 (C-4), 158.7 (C-2), 164.8 (*C*(O)OH), 169.4 (N*C*(O)Ac), 175.8 (N*C*(O) 2-methylbutyl) (assignments were confirmed by ^{1}H–^{13}C HMQC); ^{19}F NMR (DMSO-d_6) δ −112.68. HRMS (ESI) calcd for $C_{14}H_{16}FN_2O_4$ ($M-H^+$) 295.1094; found 295.1185.

4.1.2.8. 4-Acetamido-5-pentanamido-2-fluoro benzoic acid (9h). Prepared from **X** according to General procedure V. Yield: (228 mg, 77%). ^{1}H NMR (DMSO-d_6) δ 0. 91 (3H, t, H-4′), 1.35 (2H, sex, H-3′), 1.59 (2H, p, H-2′), 2.11 (3H, s, NAc), 2.36 (1H, t, H-1′), 7.77 (1H, d, $J_{3,F}$ 13.4 Hz, H-3), 7.92 (1H, d, $J_{6,F}$ 7.9 Hz, H-6), 9.37, 9.48 (2 × 1H, 2 × s, 2 × NH), 13.11 (1H, s, COOH) (assignments were confirmed by ^{1}H–^{1}H COSY); ^{13}C NMR (DMSO-d_6) δ 14.3 (C-4′), 22.3 (C-3′), 24.6 (NC(O)*Me*), 27.6 (C-2′), 36.1 (C-1′), 110.9 (C-3), 114.2 (C-1), 125.1 (C-5), 129.2 (C-6), 137.4 (C-4), 158.7 (C-2), 164.8 (*C*(O)OH), 169.6 (N*C*(O)Ac), 172.5 (N*C*(O) pentyl) (assignments were confirmed by ^{1}H–^{13}C HMQC); ^{19}F NMR (DMSO-d_6) δ −112.59. HRMS (ESI) calcd for $C_{14}H_{16}FN_2O_4$ ($M-H^+$) 295.1094; found 295.1238.

4.1.2.9. 4-Acetamido-5-isobutyramido-2-fluoro benzoic acid (9i). Prepared according to general procedure V. Yield: (226 mg, 80%). ^{1}H NMR (DMSO-d_6) δ 1.13 (6H, d, H-2′, H-2″), 2.11 (3H, s, NAc), 2.64 (1H, o, H-1′), 7.72 (1H, d, $J_{3,F}$ 13.4 Hz, H-3), 7.95 (1H, d, $J_{6,F}$ 7.9 Hz, H-6), 9.32, 9.49 (2 ×1H, 2 × s, 2 × NH), 13.09 (1H, s, COOH) (assignments were confirmed by ^{1}H–^{1}H COSY); ^{13}C NMR (DMSO-d_6) δ 19.9 (C-2′, C-2″), 24.5 (NC(O)*Me*), 35.0 (C-1′), 111.1 (C-3), 114.4 (C-1), 125.3 (C-5), 129.2 (C-6), 137.2 (C-4), 158.3 (C-2), 164.8 (*C*(O)OH), 169.5 (N*C*(O)Ac), 176.3 (N*C*(O) isobutyl) (assignments were confirmed by ^{1}H–^{13}C HMQC); ^{19}F NMR (DMSO-d_6) δ −112.74. HRMS (ESI) calcd for $C_{13}H_{14}FN_2O_4$ ($M-H^+$) 281.0938; found 281.1315.

4.1.2.10. 4-Acetamido-5-pivalamido-2-fluoro benzoic acid (9j). Prepared according to general procedure V. Yield: (189 mg, 64%). ^{1}H NMR (DMSO-d_6) δ 1.22 (9H, s, NC(O)C*Me*$_3$), 2.11 (3H, s, NAc), 7.51 (1H, d, $J_{3,F}$ 12.2 Hz, H-3), 7.89 (1H, d, $J_{6,F}$ 7.9 Hz, H-6), 8.94, 9.64 (2 × 1H, 2 × s, 2 × NH), 13.18 (1H, s, COOH) (assignments were confirmed by ^{1}H–^{1}H COSY); ^{13}C NMR (DMSO-d_6) δ 24.5 (NC(O)*Me*), 27.6 (NC(O)C*Me*$_3$), 39.8 (NC(O)C*Me*$_3$), 111.8 (C-3), 115.1 (C-1), 126.4 (C-5), 130.3 (C-6), 137.6 (C-4), 158.5 (C-2), 164.8 (*C*(O)OH), 169.5 (N*C*(O)Ac), 177.5 (NC(O)CMe$_3$) (assignments were confirmed by ^{1}H–^{13}C HMQC); ^{19}F NMR (DMSO-d_6) δ −112.71. HRMS (ESI) calcd for $C_{14}H_{16}FN_2O_4$ ($M-H^+$) 295.1094; found 295.1342.

4.1.2.11. 4-Acetamido-5-(2-ethylbutanamido)-2-fluoro benzoic acid (9k). Prepared according to general procedure V. Yield: (220 mg, 71%). ^{1}H NMR (DMSO-d_6) δ 0. 90 (6H, t, H-3′, H-3″), 1.50, 1.57 (2 × 2H, 2 × p, H-2′, H-2″), 2.09 (3H, s, NAc), 2.23 (1H, p, H-1′), 7.68 (1H, d, $J_{3,F}$ 12.8 Hz, H-3), 7.93 (1H, d, $J_{6,F}$ 8.2 Hz, H-6), 9.46, 9.50 (2 × 1H, 2 × s, 2 × NH), 13.21 (1H, s, COOH) (assignments were confirmed by ^{1}H–^{1}H COSY); ^{13}C NMR (DMSO-d_6) δ 11.6 (C-3′, C-3″), 23.9 (NC(O)*Me*), 24.9 (C-2′, C-2″), 49.3 (C-1′), 110.7 (C-3), 113.9 (C-1), 125.1 (C-5), 128.5 (C-6), 136.6 (C-4), 158.1 (C-2), 164.2 (*C*(O)OH), 169.7 (N*C*(O)Ac), 174.6 (N*C*(O) 2-ethylbutyl) (assignments were confirmed by ^{1}H–^{13}C HMQC); ^{19}F NMR (DMSO-d_6) δ −112.44. HRMS (ESI) calcd for $C_{15}H_{18}FN_2O_4$ ($M-H^+$) 309.1251; found 309.1491.

4.1.2.12. 4-Acetamido-5-(2-naphthamido)-2-fluoro benzoic acid (9l). Prepared according to general procedure V. Yield: (190 mg, 52%). ^{1}H NMR (DMSO-d_6) δ 2.13 (3H, s, NAc), 7.62–7.65 (2H, m, H-6′, H-7′), 7.83 (1H, d, $J_{3,F}$ 12.8 Hz, H-3), 7.99–8.08 (5H, m, H-6, H-3′, H-4′, H-5′, H-8′), 8.66 (1H, s, H-1′), 9.83, 10.13 (2 × 1H, 2 × s, 2 × NH), 13.21 (1H, s, COOH) (assignments were confirmed by ^{1}H–^{1}H COSY); ^{13}C NMR (DMSO-d_6) δ 24.0 (NC(O)*Me*), 110.5 (C-3), 114.1 (C-1), 124.5 (C-5), 126.8 (C-3′), 127.6 (C-1′, C-4′), 127.8 (C-5′, C-8′), 128.4 (C-6′), 128.9 (C-7′), 130.2 (C-6), 131.5 (C-10′), 132.0 (C-2′), 134.3 (C-9′), 137.5 (C-4), 157.8 (C-2), 163.7 (N*C*(O)Nap), 165.9 (*C*(O)OH), 169.4 (N*C*(O)Ac) (assignments were confirmed by ^{1}H–^{13}C HMQC); ^{19}F NMR (DMSO-d_6) δ −112.19. HRMS (ESI) calcd for $C_{20}H_{14}FN_2O_4$ ($M-H^+$) 365.0938; found 365.1213.

4.1.2.13. 4-Acetamido-5-(biphenyl-4-ylcarboxamido)-2-fluoro-benzoicacid (9m). Prepared according to general procedure V. Yield: (196 mg, 50%). ^{1}H NMR (DMSO-d_6) δ 2.13 (3H, s, NAc), 7.43 (1H, t, H-4″), 7.52 (2H, t, H-2″, H-6″), 7.77 (2H, d, H-3″, H-5″), 7.84–7.86 (3H, m, H-3, H-3′, H-5′), 7.99 (1H, d, $J_{6,F}$ 7.68 Hz, H-6), 8.11 (2H, d, H-2′, H-6′), 9.79, 9.96 (2 × 1H, 2 × s, 2 × NH),

13.21 (1H, s, COOH) (assignments were confirmed by ^{1}H–^{1}H COSY); ^{13}C NMR (DMSO-d_6) δ 24.5 (NC(O)*Me*), 110.1 (C-3), 114.5 (C-1), 126.5 (C-2″, C-6″), 126.9 (C-5, C-3′, C-5′), 128. 1 (C-1″),128.5 (C-2′, C-6′), 129.0 (C-1′), 130.2 (C-6), 132.9 (C-4″), 139.0 (C-4′), 143.2 (C-4), 158.2 (C-2), 164.3 (N*C*(O) biphenyl), 165.5 (*C*(O)OH), 169.4 (N*C*(O)Ac) (assignments were confirmed by ^{1}H–^{13}C HMQC); ^{19}F NMR (DMSO-d_6) δ −111.88. HRMS (ESI) calcd for $C_{22}H_{16}FN_2O_4$ ($M-H^+$) 391.1094; found 391.1164.

4.2. Molecular modeling

All computations and simulations were carried out on an Intel P4 based Microsoft windows 2000 workstation using Discovery Studio Modeling 1.1 and 1.2 Package (Accelrys).[27] The crystal structure of the human NEU2–DANA complex was obtained from the Brookhaven protein Data Bank (accession code: 1VCU, Chain B).[23] 4-morpholineethanesulfonic acid (MES) and all water molecules were removed from the protein structure. Hydrogen atoms were added and the atom types were assigned using the CHARMM forcefield. The protonation states of amino acid residues were adjusted to the dominant ionic forms at pH 7.4. The active site was defined as the collection of amino acids enclosed within 8 Å radius sphere centered on bound ligand DANA. The program LUDI was applied to design the potential fragments like compounds that bind NEU2 active site. The 15 structural features of 12 amino acid residues (Arg21, Arg41, Met85, Ile103, Ile105, Tyr179, Tyr181, Leu217, Arg237, Gln270, Arg 304, Tyr334) were used for the selection of structure based 3D queries (Tables 1 and 2). Ludi de novo receptor mode of program was used to generate interaction map and then a systematic search of Ludi/CAP library containing 78,175 compounds was performed with generated 3D queries. Ludi energy estimate3 was used to estimate the change in free energy upon the binding of the fragment to the receptor. Automated docking was attempted as reported elsewhere.[19] For the manual docking, structure of compounds **9a** and **9m** were built using Ludi/CAP search Hit **A**-NEU2 model complex as a template generated during Ludi/CAP search. The obtained complexes were then energetically minimized with 500–1000 iterations of 'in situ ligand minimization algorithm' using the SMART MINIMIZER program. A distance constraint was applied between carboxylic group of inhibitor and Arg304 of the conserved Arg triad in the NEU2 active site. Then binding poses were analyzed to understand the molecular interactions between the docked inhibitor and NEU2.

4.3. Biological assay

Sialidase inhibition activity was performed according to the previously reported procedures.[17] Inhibitory activities were carried out using the homogenates obtained from HEK-293 cells transiently transfected with the expression plasmid containing a full-length human sialidases cDNA. The cells were sonicated in 9 vol. of PBS, pH 7.4, containing 1 mM EDTA, 0.5 mM PMSF, 10 μg/ml leupeptin and 0.5 μg/ml pepstatin, and centrifuged at 1000 g for 10 min. The supernatant (crude extract) was used for sialidase assays. Briefly, the routine reaction mixture contained 10–20 nmol of substrate as bound sialic acid, 0.2 mg of BSA, 10 μmol of sodium acetate (pH 4.6) and 0.2 mg of Triton X-100. After incubation at 37 °C for 10–30 min, the amount of sialic acid released was determined fluorometrically. Sialidase activity with the 4MU-NeuAc (NEU1, NEU2, and NEU4) as substrate was assayed by spectrofluorometric measurement of the 4-methylumbelliferone released. In the case of NEU3, mixed ganglioside was used as a substrate and sialic acid released from GM3 were measured by fluorometric HPLC with malononitrile. One unit of sialidase was defined as the amount of enzyme cleaving 1 nmol of sialic acid/h. IC_{50} values from concentration-inhibition curves were calculated by means of non-linear regression analysis using Microsoft Excel 2000.

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