Rational development of neuraminidase inhibitor as novel anti-flu drug

Anand Chintakrindi, Charlotte D'souza and Meena Kanyalkar*

Prin. K.M. Kundnani College of Pharmacy, Cuffe Parade, Mumbai - 400 005, India

Abstract: The highly pathogenic influenza virus has caused many human fatalities and poses an increasing pandemic threat. Neuraminidase inhibitors such as oseltamivir and zanamivir have been widely used in the treatment and have gained remarkable success. Although, they are effective in prevention of influenza; the concern for drug resistance still remains a question. Recently, the availability of crystal structures of the enzyme gave a new trend to the structure based drug designing of neuraminidase inhibitors. The article reviews a detailed understanding of the structural features within neuraminidase enzyme which turnouts to be crucial for future drug development. In depth analysis for the newly proposed spots within the 150 and 430-loop regions in N1 makes it distinguishable among the subtypes. Further we have discussed the various computational studies carried out in optimizing the designing of neuraminidase inhibitors thereby providing new clues to modify the currently available drugs.

Keywords: Avian Influenza, H1N1, H5N1, Neuraminidase, Oseltamivir, Zanamivir.

1. INFLUENZA

Influenza is a serious public health problem that causes severe illnesses and deaths for higher risk populations. There are three types of influenza – A, B and C. Type A influenza viruses are further typed into subtypes according to different kinds and combinations of virus surface proteins. According to WHO (World Health Organization) among many subtypes of influenza A viruses, currently influenza A (H1N1), A (H3N2), A (H5N1) and A (H9N2) subtypes are circulating among humans [1], [2]. Out of several strains, H5N1 is most virulent and dangerous due to high rate of mutability. Currently there are two ways to reduce the impact of influenza: vaccination or antiviral drug therapy. However, there are limitations associated with both. The development of vaccines to prevent or treat influenza infection has been greatly hindered by the high mutability of the virus [3]. Such mutations can create havoc during the pandemic. It might not be possible to supply vaccines in adequate amount while antiviral drugs can meet the need of the pandemic disaster. However, currently available antiviral therapy such as M2 inhibitors and Neuraminidase (NA) inhibitors is highly effective but it may show resistance if exposed for prolong period of time. The highly conserved active site of NA and its role in influenza virus replication has made it interesting target for the development of newer inhibitors. In recent years, tremendous progress has been made in developing NA inhibitors. Recently the availability of newer crystal structures of NA has given a new direction to the drug design of NA inhibitors. The focus of current article is to review and provide the insight of the NA in order to develop potential NA inhibitors for the treatment of avian influenza or bird flu. This will deal mainly with the discovery of structural differences in NA that has given a new way for structure based drug designing of NA inhibitors.

*Address correspondence to this author at the Plot 23, Jote Joy Building, Rambhau Salgaonkar Marg, Cuffe Parade, Mumbai 400005, India; Tel: 91-22-22164368, 22164387; Fax: 91-22-22165282;
E-mail: meenatul@gmail.com

1.1. Structure of Avian Influenza Virus

The influenza viruses are RNA viruses of orthomyxoviride family and are further classified into three distinct subtypes – A, B and C. Influenza A virus infect many species including birds and mammals while influenza subtype B and C virus infect essentially human. There are two major surface antigenic glycoprotein Hemagglutinin (HA) and Neuraminidase (Sialidase, NA) [4, 5] found in both influenza virus A and B subtypes. HA is responsible for binding to the terminal sialic acid of the receptor leading to the attachment and subsequent penetration of virus into the target cell [6]. NA is responsible for cleaving the terminal sialic acid from the receptor in order to release the newly formed virions from the infected cell. It also helps in the transport of the virus through the mucus of the respiratory tract. The distinct antigenic properties of different HA and NA molecules [7] are used to classify influenza type A viruses into subtypes: sixteen for Hemagglutinin (H1-H16) and nine for NA (N1-N9). NA enzymes are phylogenetically categorized into two groups: group-1 includes N1, N4, N5, and N8 while group-2 includes N2, N3, N6, N7, and N9 [8]. Though active residues are largely conserved across both groups, different NA subtypes exhibit varied drug susceptibility [9].

1.2. Antiviral Therapy Against Avian Influenza

Currently there are two classes of anti-influenza drugs available for the treatment of influenza viruses, the M2 proton channel inhibitors (adamantanes) (Fig. **1**) and the NA inhibitors (Fig. **2**). The adamantane derivatives, amantidine (Fig. **1a**) and rimantadine (Fig. **1b**) are widely available and quite economical, act by blocking the M2 ion channel of the virus to inhibit the early stages of viral replication. One of the main advantages of the NA inhibitors over the adamantanes are their activity against both influenza A and

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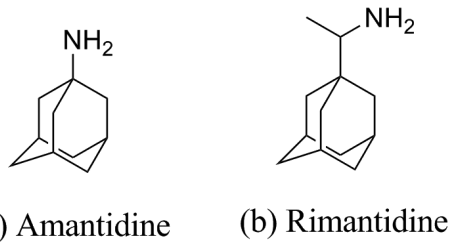

Fig. (1). Structures of M2 proton channel inhibitors – Adamantanes.

influenza B, improved safety profile, and lower potential for inducing resistance [10]. Fortunately, viruses that develop resistance to the adamantanes remain susceptible to NA inhibitors. The adamantanes show limited scope due to lack of activity against influenza virus B and unwanted side effects [11].

2. FOCUS ON NA INHIBITORS

2.1. Transition State Analogs

NA inhibitors have great potential as sensitive anti influenza drugs. They affect viral replication thus progress of infection. During the phases of development of synthetic NA inhibitors, one of the approaches was to synthesize analogs of sialic acid (Fig. **2a**), which is an endogenous ligand. The ability of transition-state analogs of sialic acid to inhibit the NA was first known in the 1970s [12-14], but the design of highly effective inhibitors became possible when analysis of the three-dimensional structure of influenza NA [15] disclosed the location and structure of the catalytic site. The sialic acid analog, DANA (Fig. **2b**) one of the most potent synthetic inhibitors for NA, mimics the transition state [16]. The use of the DANA–NA complex as a starting point developed many compounds having chemically simpler cyclic templates [17] and resulted into various commercially successful drugs such as Zanamivir (Fig. **2c**) [18] and Oseltamivir (Fig. **2d**) [19]. The replacement of hydroxyl group in DANA with a guanidine group resulted in Zanamivir, an extremely potent NA [20] inhibitor effective against both influenza A and B, administered by oral inhalation and delivering the drug directly to the respiratory tract. Being a transition state analog it closely mimic the natural substrate, fitting into the active site pocket and engaging the protein in the most energetically favorable interaction [16-18]. The poor bioavailability that forces its administration by nasal route and some side effects such as bronchospasm limits its usage. Oseltamivir is the next commercially accepted drug, which is an orally active NA inhibitor, for both treatment and prophylaxis of influenza. Oseltamivir carboxylate is an ester prodrug converted to an active free carboxyl form by hepatic hydrolysis upon administration. It was developed through modifications to the sialic acid framework (including the addition of a lipophilic side chain) that allow the drug to be used orally [21]. Oseltamivir is also an effective inhibitor of both A and B form of NA, with milder side effects such as nausea and vomiting. Due to high rate of mutability of the virus, oseltamivir is reported to have resistance [22]. Peramivir (Fig. **2e**) is another drug, structurally different in cyclic framework from the existing influenza NA inhibitors, found to be more potent and highly specific NA inhibitor [23]. The oral formulation of Peramivir was abandoned due to poor bioavailability. It has been developed as an injectable formulation by BioCryst pharmaceutical which is in Phase III trials [24]. The other inhibitor made by Abott labs is A315675 (Fig. **2f**), also undergoing phase III trials in North America and Europe [25]. Laninamivir (Fig. **2g**) and CS-8958 (Fig. **2h**), an octanoyl ester prodrug of laninamivir are novel NA inhibitors; developed by Daiichi Sankyo Co., Ltd., Tokyo, Japan. Laninamivir has shown *in vitro* NA inhibitory activity against various influenza A and B viruses, including subtypes N1 to N9 and oseltamivir resistant viruses [26]. A single dose of CS-8958 conferred a more potent and long-lasting protective effect to mice against H5N1 influenza viruses than that of oseltamivir phosphate [27].

Many series of other NA inhibitors (Fig. **3**) have been developed from a variety of scaffolds such as pyrrolidines [28], cyclopentanes [29], tetrahydrofurans [30], benzenes [31], furan, [32], acyl(thio)urea and thiadiazolopyrimidine [33] using rational drug design.

2.2. Natural Compounds as NA Inhibitors

Recently some reports have indicated NA inhibitory activity of the compounds derived from the natural source (Fig. **4**). Polyhydroxylated xanthones from *Cudrania tricuspidiata* showed to be particularly effective inhibitors of bacterial NA (*Clostridium welchii*). Kinetic analysis of these species has unveiled that they are all competitive, slow binding inhibitors [34]. However flavones and flavanones isolated from the same species [35] and flavanols obtained from roots of *Rhodiola rosea* are reported as noncompetitive inhibitors of bacterial NA (*Clostridium perfringens*). Noncompetitive inhibition of NA from recombinant influenza virus A (rvH1N1) by these flavanols is also reported [36]. Chalcones, flavanoids and coumarins from the roots of *Glycyrrhyza uralensis* shows similar activity [37]. C-Methylated Flavonoids from *Cleistocalyx operculatus* [38], oligostilbenes for *Vitis amurensis* [39], and chalcones from *Glycyrrhiza inflata* [40] shows noncompetitive inhibition of NA from novel influenza A virus (H1N1/09). These natural compounds show lesser NA inhibitory activity when compared with commercially available drugs. But due to their non competitive nature of inhibition they can be valuable in solving problems of drug resistance. Thus, all these natural compounds offer novel scaffolds to be developed as newer NA inhibitors. The binding mode of these compounds needs to be explored which will add a new dimension in design of NA inhibitors.

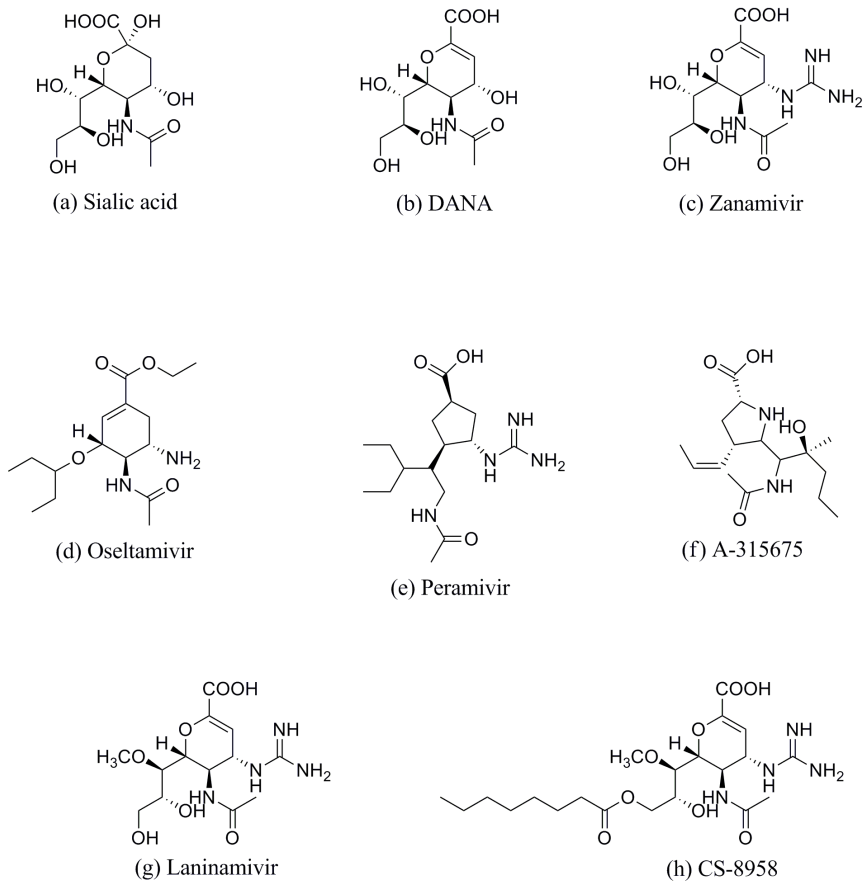

Fig. (2). Structures of transition state analogs.

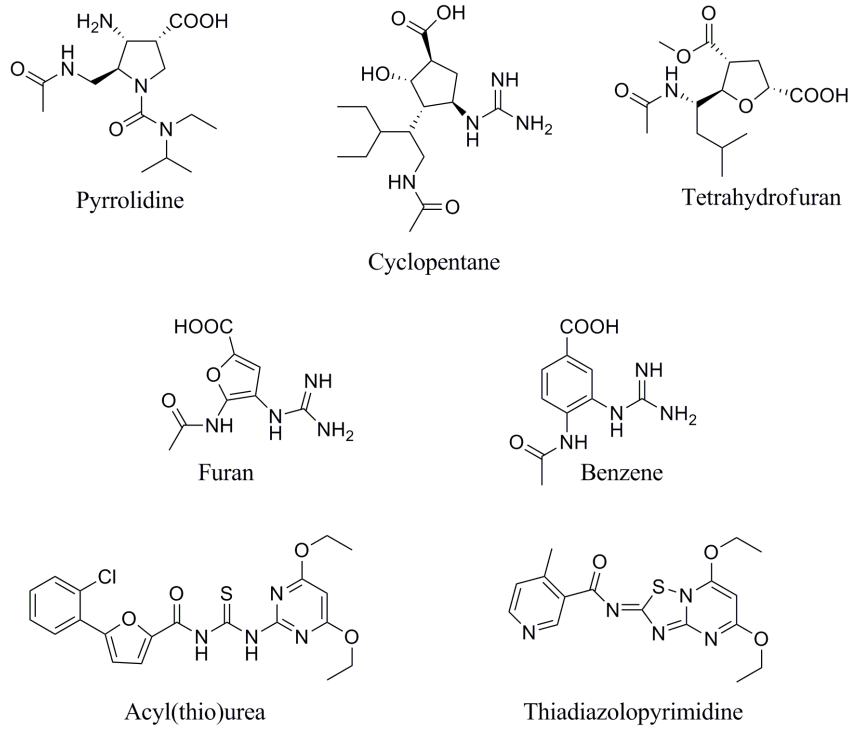

Fig. (3). Structures of different synthetic scaffolds as NA inhibitors.

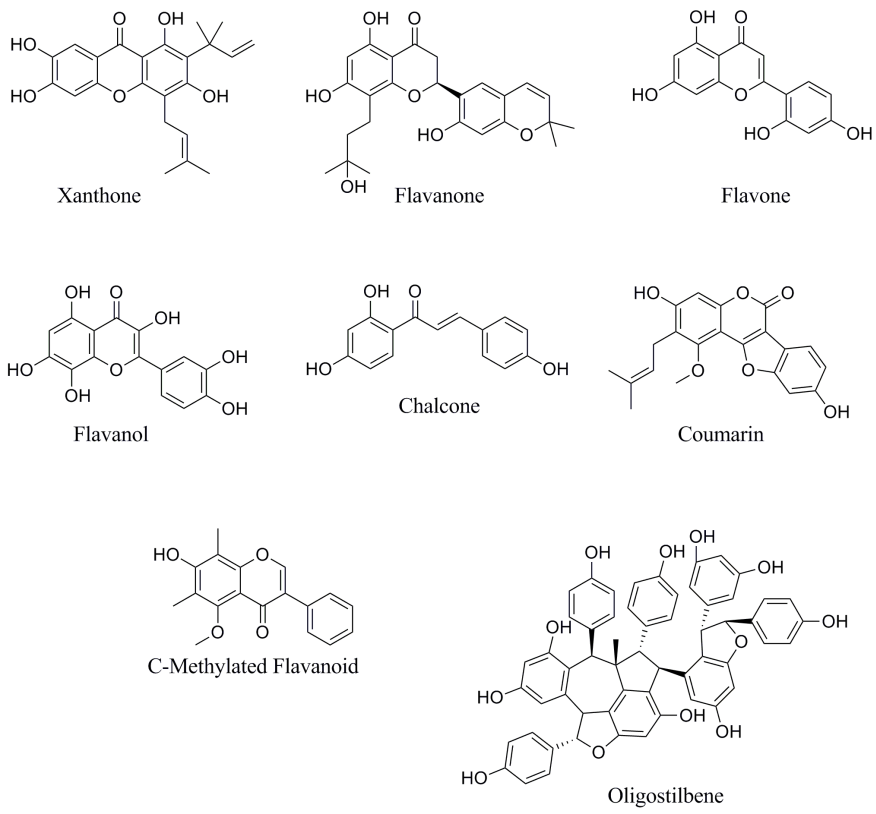

Fig. (4). Structures of some naturally occurring NA inhibitors.

3. NEURAMINIDASE (NA) ENZYME

Study of reported competitive and noncompetitive NA inhibitors drives our focus towards structure of NA. It is necessary to explore the structure of NA and its active site to develop a novel NA inhibitor.

3.1. Structure of NA and Its Active Site

Influenza virus NA is a homotetramer and an excellent target for structure based drug design due to its highly conserved active pocket across all influenza viral strains [41], rendering a broad-spectrum, anti-influenza agents possible. The active site of NA consists of four main well-conserved binding locations. Depending on these locations importance of four functional groups (carboxylic, acetamido, guanidine/ amine and glycerol/hydrophobic) on the main framework has been reported [42]. The site 1 (S1) consists of Arg 118, Arg 292 and Arg 371 which is positively charged and interacts with carboxylate part of sialic acid and NA inhibitors. The negatively charged site 2 (S2) consists of Glu 119, Glu 227 and Asp 151 which interacts with guanidine or amino group of the NA inhibitors. The site 3 (S3) contains a small hydrophobic region formed by Tyr 178 and Ile 222 that accommodates the acetyl group of NA inhibitors. The site 4 (S4) consists of Glu 276 and Glu 277 that binds to the glycerol or hydrophobic part of the inhibitors. The S3 and S1 are situated 9 -10 A° apart from each other. Based on this model of active site of NA, many scientists have developed competitive inhibitors targeting each site to understand its significance in the activity.

3.2. Structural Comparison between N1 and N9 – NA

The development of previous NA inhibitors such as oseltamivir and zanamavir was done by using the crystal structure of NA subtypes like N9 and N2 from the phylogenetically categorized group-2 of NA. The crystal structure of these NA - inhibitor complexes had simmilar 3D-structure of the active site thus it was assumed that all subtypes of NA may have similar topology and may bind to the active site in the same manner [8]. The assumption was mainly based on the perception that amino acid sequences of the active sites are essentially the same for all the subtypes. Thus it was presumed that drug developed using N9 crystal structure may also have comparable activity against N1. In depth study of the crystal structure of N1, N4 and N8 neuraminidases revealed that the active sites of these enzymes have a very different 3D structure from that of N9 and N2. The N1 neuraminidase has a small segment called the 150-loop or cavity (residues 147–152) which creates a hollow pocket that does not exist in the N2 and N9. This loop is formed by the amino acid segment "GT**VK**DR", while its corresponding segment in the N9 enzyme is ''GT**IH**DR''. The replacement of isoleucine by valine in N1 makes conformational change that creates150 loop which open at the conserved active pocket. It was reported by Russell [8], that the 150-loop of N1 enzyme has Val 149 residue whose C^{α} atom is about 7A° distance away from corresponding isoleucine residue in N9 enzyme.

Another difference between N1 and N9 NA is the carboxylic acid of Asp151 which points away from the

active site in N1 that result in an open conformation of 150-loop in contrast to N9 enzyme. Similarly, Glu119 also has different conformation in N1 enzyme [8], compared with N9 enzyme. The difference in position of two Asp151 and Glu119 acidic residues in N1 increases the width of the active site cavity by about 5A°. These amino acid side chains are found to be essential for the interaction with NA inhibitors. The conformation of 150-loop and the position of Gln136 determine the extent of the 150-loop. This residue forms a hydrogen bond with the main-chain carbonyl residue of the 150-loop in N9 but unable to make hydrogen bond in N1. It is also accounted [43] that the motion in the 150-loop is coupled to an outward movement in the adjacent strand-loop-strand segment comprising of residues Arg 430 – Thr 439 called the "430-loop". This leads to the expansion of the active site cavity even further, increasing the solvent accessible surface area. Together, the action of the 150-loop and 430-loop (Fig. **5**) causes substantial topological changes to occur directly within the active site pocket of N1. Thus these two regions 150-loop and 430-loop can provide support for developing high affinity drugs. However, interestingly the crystal structure of NA from the 2009 pandemic H1N1influenza strain indicates that it lacks the 150 loop in its active site. In contrast to other characterized N1-NA, which are all members of group 1, 2009 H1N1neuraminidase active site resembles to N9-NA, which belongs to group 2 [44].

3.3. Binding of Inhibitors to N1-NA

Recently few crystal structures of NA inhibitors in complex with N1-NA have been reported. The 150-loop forms one part of the enzyme active site is able to exist in at least two stable conformations. Studies showed that N1, N4 and N8 binds to drugs either in open or closed conformation of the 150-loop as observed in crystal structure 2HTY and 2HU4 respectively [8]. It was reported that initially oseltamivir binds to "open" form of NA. Upon binding, it gradually undergoes a conformational change that results in the "closed" form. As the inhibitor assumes tighter binding interaction, it leads to the conformational changes at Asp151 and Glu119 both gets oriented towards the inhibitor. Consequently the size of the 150-loop decrease and now overall conformation resembles that of N9 type. The closed conformation of the loop is actually higher in energy than the initial open conformation but access to open conformation is an energetically advantageous interaction with the inhibitor. This also indicates importance of open conformation with 150-loop in the design of newer NA inhibitors. In order to enhance the affinity of new inhibitor for N1, efforts should be directed to be accessible towards 150-loop open conformation, as it is energetically favorable. But once it binds tightly to this binding pocket, conformational change should be such that it can attain similar structure as that of the closed conformation. This complexity in the design of newer NA must be considered as it is evident from the findings of 2009 pandemic structure that it does not have 150-cavity.

3.4 Resistance of Inhibitors Towards NA

Recently there have been several reports [45] of resistance towards H5N1 with H274Y mutation which has been isolated from humans treated with oseltamivir. Resistance has been associated with mutations at N294S, H274Y and R292K of the neuraminidase. It consequently allows the drug-resistant virus to survive and propagate. Such emergence of oseltamivir-resistant strains towards the H5N1 is matter of immediate concern. Various mutations in the enzyme may limit the accessibility of oseltamivir during binding to the receptor. Structural analyses by Varghese et al [46] predicted much earlier that oseltamivir structure might

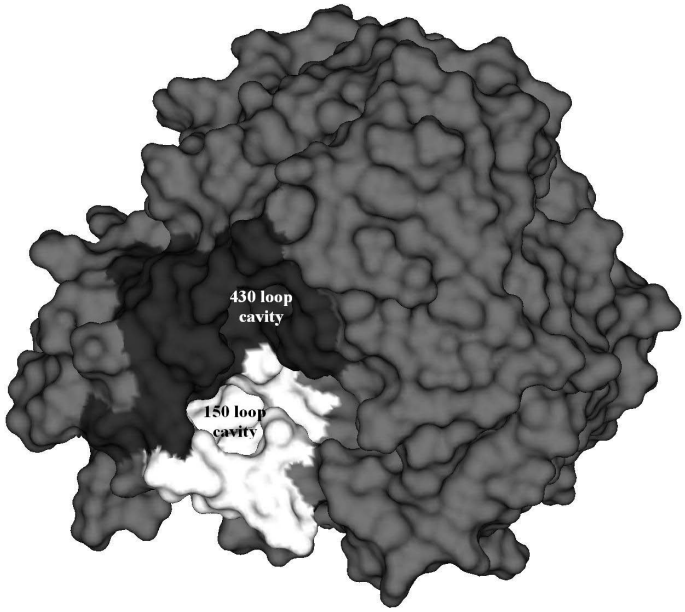

Fig. (5). Newly explored cavities 150-loop (white) and 430- loop (black) of H5N1-NA.

facilitate the development of resistance. The binding of oseltamivir to NA requires accommodating the bulky side chain in the active site. Molecular level analysis showed that to accommodate bulky side chain, the amino acid Glu 276 must rotate and bond with Arg 224 to form a pocket. But zanamivir does not require any reorientation of amino acids in the active pocket, thus still remains sensitive towards the drug. Incidentally, it was reported that neuraminidase from oseltamivir-resistance H5N1 viruses retains susceptibility towards zanamivir [47] suggesting that zanamivir is better than oseltamivir at binding to the active pocket. Though zanamivir is active against the oseltamivir-resistant H5N1 (H274Y variant) [48], it has low systemic availability following administration, including low drug levels in the lower respiratory tract (where most of the replication of current H5N1 viruses seems to take place) [49].

Some studies also point out different views on above observation about resistance. For the N294S mutant, the salt bridge between Glu 276 and Arg 224 is maintained, and the Glu 276 is not a key factor. Instead, there are at least two other structural features related to the weaker binding of the N294S-oseltamivir carboxylate complex. One is that the main-chain carbonyl of Tyr 347 flips out to form a hydrogen bond with Arg 292, and the other is that the Ser 294 residue in the N294S mutant locates farther from the binding site than does Asn 294 in the WT complex [50].

Zanamivir resistance has been associated with mutation Q136K in H1N1. The mutation has no effect on oseltamivir susceptibility but caused approximately a 300-fold and a 70-fold reduction in zanamivir and peramivir susceptibility, respectively. Mutation of Gln 136 to a Lys would break down the hydrogen bonding network formed with Asp151 and Arg156, possibly leading to increased mobility of Arg156 and Asp151, disturbing the hydrogen bonds formed between guanidino moiety of zanamivir and the latter two residues. Similarly, peramivir would be affected by the mutation since it has the same guanidino moiety as zanamivir. In contrast, oseltamivir does not form any interactions with Arg 156 or the Asp151 backbone and hence would be unaffected by the mutation. [51]. Thus the careful observation of mutant NA can articulate the requirement of structural framework, which can be a stepping stone in the development of novel resistance free drug.

4. STRUCTURE AIDED DRUG DESIGN AGAINST H5N1

Approach in search for NA inhibitor undergone phase wise transitions, starting from development of N-substituted oxamic acid [52] to transition state analogs of sialic acid [53] to the latest based on three dimensional structural insights on catalytic site of the receptor enzyme. According to Kim *et al* [21] influenza A and B NA despite of their complete homology in active site had quite different hydrophobic interactions with the lipophilic side chains. This different lipophilic environment in the active cavity of influenza A and B NA is the main reason for different drug efficacy to type A and type B influenza viruses. The same reason might be true in causing the resistance of H5N1 influenza virus to some currently available drugs.

A structure-activity relationship study is crucial to understand the drug-resistance of H5N1 virus for some existing NA inhibitors. The drug-resistant of H5N1 was investigated by means of structure-activity relationship between NA and three inhibitors, i.e., DANA, zanamivir and oseltamivir. The study indicates that a partially lipophilic pocket Ala 248 and Thr 249 in N9-NA is replaced by two hydrophilic residues Ser 227 and Asn 228 in the H5N1-NA respectively. Additionally, two lipophilic residues Ala 323 and Tyr 324 in the H5N1-NA are replaced by two hydrophilic residues Asn 347 and Asn 348 in the N9-NA, leading to the formation of a new lipophilic pocket [48]. Hence the original lipophilic environment is destroyed which further leads to changes in interaction between H5N1-NA and inhibitors. These findings explains how some of H5N1 strains bear high resistance for existing NA inhibitors along with a change in the lipophilic environment around the active site and must be taken into account while designing effective inhibitors.

Two recent studies, one experimental [8] and the other computational [43], exposed the flexibility within the 150- and 430-loop binding regions of N1, and these findings were conjectured to provide new opportunities for drug design. It revealed the fact that the motion in the 150-loop is coupled to motion in the neighboring 430-loop, which expands the active site cavity even further. It was hypothesized that the inhibitors that are able to exploit these two loops may potentially exhibit increased oral bioavailability and less structural susceptibility to structural mutations in N1 than currently available N1 inhibitors.

Several research groups demonstrated that four substituents of amino group, glycerol side chain, carboxylic acid and acetamide moiety on five to six member ring scaffold made important contribution to NA inhibitor activity. The potent inhibition of NA was determined by the relative position of above groups around the central ring. All studies so far revealed to focus on attachment of a new chemical group with a proper shape, size and electronic charge for fitting into the active site. The change at amino group and glycerol side chain showed large impact on selectivity towards N1 NA inhibition, as it can fit into the additional pocket opened by 150-loop. With the help of computational docking studies it was highlighted that the amino group of oseltamivir when changed to NHC ($=NH_2^+$) NH_2 and NHC ($=N–CH_4^+$) NH_2, it interacted with the 150-cavity by providing the charged group for additional binding to the negative site S2 [54]. In addition, the C8-pentyl group of oseltamivir was changed to partial hydrophobic and hydrophilic group in order to adjust its changed environment. The length, size of branching and geometry of alkyl groups intensely influenced the inhibitory activity.

An additional report [42] in this context suggested that when oseltamivir, zanamivir and peramivir embedded in the N1 and were compared it showed major difference in the structures of the guanidium and the bulky groups. This is due to the fact that the first side chain lies within the cavity formed by the 150-loop which exist only for group 1 NA (N1, N4, N5, N8). Also the conformation of the bulky hydrophobic side chain relates directly to the ligand in inhibiting the N1 enzymes. Peramivir is a novel NA inhibitor

designed by structure based approach. In comparison with oseltamivir and zanamivir, peramivir is reported to interact tightly via its –OH group with Asp151 residue located in 150-loop region and fit better to the NA pocket. The flexibility and steric effect of the bulky hydrophobic group lead to the rearrangement of the surrounded residues that is the negatively charged side chains of Glu 276, which was shifted and rotated to form hydrogen bonds with the positively charged Arg 224.

The studies also indicated that molecular volume may be crucial factor in the designing of drug against the 150-loop. The molecular docking results of modified oseltamivir obtained by appending glycyl, prolyl, benzyl and acetyl moiety on amino group [55] indicated that increasing the molecular volume on the amino group, changes the intermolecular interaction to a large extent depending on the size of the substituents. Addition of a small group such as glycyl ($–COCH_2NH_2$) with its amino group charged at physiological pH enhances the interaction with the receptor site and will possibly increase its inhibitor activity. On the other hand bulky groups like benzyl ($–COC_6H_5$) is not suitable substituent, as it bump into the receptor surface, shifting the locus of the binding, and will probably lead to a lower activity. Thus for improvement in antiviral activity, the introduction of functional moieties at the amino group, with proper shape; size, electronic charge and lipophilicity are critical. It was also observed that esterification of carboxylic group lead to a prodrug oseltamivir which is the only orally active NA inhibitor so far. In most studies acetamido group has not been modified extensively, and small alterations had not showed significant change in terms of activity, potency, enhanced susceptibility to virus or improvement in pharmacokinetic parameters. Several modification on the ring scaffold resulted in the mixed results. Because of poor bioavailability of zanmamivir and poor inhibitory activity of benzoic acid derivatives several groups decided to investigate cyclohexene analogs. It was demonstrated that the double bond position in the design of cyclohexene NA inhibitor plays an important role [56]. Compilation of all results ultimately emphasize that each combination of substituent had unique steric and electronic interaction with each other which influenced the overall activity of the compound with the active site. Several research groups have now started to explore these new cavities to find newer NA inhibitors which interact with residues of the 150 and 430 cavities. One such study involved development of chlorogenic acid as potential H5N1-NA inhibitor. Docking study of chlorogenic acid revealed interactions with Thr 439 of 430 cavity [57]. Ensemble-based virtual screening against H5N1-NA revealed potential novel antiviral compounds for avian influenza NA. These compounds target the catalytic cavity as well as the newly identified 150- and 430-cavities [58]. The insight into the 150- and 430- loop and the new cavity formation provided the new direction in structure based drug design of N1 inhibitors.

CONCLUSIONS

The influenza virus subtype H5N1 is of great concern because of its high virulence and mutation rate, and some strains have already acquired resistance to the currently available anti-influenza drugs such as oseltamivir and zanamavir. Thus exploring the H5N1 structure indicates that even though the classic flu drugs are effective against N1 flu viruses, the unknown 'pocket' could be exploited to design a drug that would be even more effective against H5N1-like viruses. In the new study, researchers report that the some pockets actually can have internal shapes substantially different than previously believed. The new insight into structural understanding of such loops could be valuable in efforts to design novel and more effective anti-flu drugs. Drugs capable of fitting more snugly into the cavity could yield a class of NA inhibitors that are more effective against H5N1-like flu viruses. The studies in future should focuses on designing drugs that has more binding potential towards H5N1 and reduce the resistance towards inhibitor.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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