Mutations in surface protein of swine flu: A major problem for H1N1 inhibitor

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ABSTRACT :
The emergence of new mutant strains of influenza virus like H1N1, H3N2, H1N9, H7N9, etc. in every season of flu is mainly due to frequent mutation in eight genes of flu virus. This cause influenza virus spreads worldwide and become pandemic in 2009. Every year 36000 peoples are infected from flu. Around 123397 people have been tested itself in India in 2010. Drug for influenza treatment now has been resistance due to mutation in receptor of neuraminidase. Mutation is the major problem for designing inhibitor against influenza virus. Number of strains produced due genetic reassortment and mutant property of virus. This makes the different strains of hemagglutinin (H) and neuraminidase (N). Total eight gene of influenza virus, out of these only few gene involve for mutation continuously in which may be some beneficial or may some insensitive to flu. Gene mutation causes specific changes in sequence of the amino acids, and changes the conformation of other concerned residues which causing the unfitting binding site for influenza inhibitor. Number of other mutation associates with specific mutation like H274Y, N294S, I223R, E119V, Q136K and S31N etc. are responsible for changing conformation of binding site. Some mutation of surface protein of virus may cause negative or positive effect on binding site. Some induced mutation also helps in antiviral activity. This review highlights the mutation which causes resistance and changing residues in the binding site of in neuraminidase, hemagglutinin, and M2- channel protein which would give the pathway for designing rational drug. Fig.**2**" - Mechanism of drug-resistance and mutation in the neuraminidase amino acid (H274Y, R292K, N294S, or E119V. A shows the interaction of oseltamivir with wild type neraminidase receptor. B showing the interaction of oseltamivir with mutant type (274Y), and C showing the mechanism of mutation, how E276 rotate and make pocket for OST, and binds to R224. Histidine replaced by Tyrosine (bulkier residue) which changes the other residues conformation like E276, R292 so that E276 not rotate and oseltamivir not properly bind.
Keywords: Hemagglutinin, H274Y, Influenza virus, Mutation, Neuraminidase.

INTRODUCTION:

The emergence of influenza drug resistance is a major public health concern. Bird flu, avian flu and swine flu, have inherent property of mutation. The reassortment of genetic segments in different host species form different subtypes of influenza viruses frequently, which may generate new strains which may cause flu as epidemic or pandemic. Due to genetic assortment, they developed new subtypes which cause seasonal flu in humans [1, 2]. Inhibitors like (NAIs), such as oseltamivir (OST) and zanamivir, M2-channal inhibitors, were the drugs of choice against influenza A or B. Substitutions of amino acids in mainly Neuraminidase (NA), hemagglutinin (HA), and M2- channel protein cause mutation which leads to resistance for all anti-influenza drugs. There are two major classes of antivirals available for the treatment and prevention of influenza, the M2 inhibitors and the neuraminidase inhibitors (NAIs). Due to high mutation rate and generation of new strains of influenza virus, it is a major issue for developing current therapeutics, even vaccine is also ineffective due to antigenic drift and shift which causes point mutation. Avian influenza viruses carrying molecular markers for resistant which are also responsible for point mutations which arise due to natural fluctuations and triple reassortment [3].

On the basis of surface proteins, influenza virus has been classified in two types, A and B. Both have negative sense of RNA virus with a low fidelity of RNA polymerase [4]. Influenza gene is divided into the eight gene segments (hemagglutinin [HA], nucleoprotein [NA], matrix [M1], matrix [M2], M-protein channel [MA], polymerase basic 1 and 2 [PB], polymerase acidic [PA], and non-structural protein [NS] genes). They are all wrapped around central core which contains single strand RNA. On the basis

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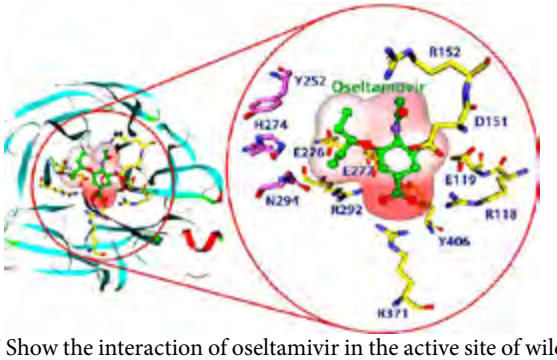
of phylogenetic, again neuraminidase divides into two groups, group A includes subtypes (N1, N4, N5, and N8) and group B includes (N2, N3, N6, N7, and N9) [5].

Influenza A is responsible for more morbidity and mortality than other types. It causes 10-20% of world's population and 250,000- 500,000 deaths per year throughout the world. During the decade of surveillance a significant increase in drug resistance was noted, from 0·4% in 1994–1995 to 12·3% in 2003–2004 [3]. This increase in the proportion of resistant viruses was 61% in 2004, and above 90% in 2009 and it became 100% till 2010 against Tamiflu (Oseltamivir carboxylate) [3, 6]. In Taiwan 1187 positive cases were tested for the H274Y substitution in the neuraminidase (NA) N2 gene that confers resistance to oseltamivir [7]. Drug oseltamivir resistance has conferred due to single point missense mutation from histidine to tyrosine at position 274 (H274Y). This change is different location in different strains at 273 in type B, 274 in type N2 and 275 in type A N1 amino acids numbering. All the viruses have mutation in NA gene and confirming OST resistance and cross resistance against other antivirals [7, 8].

Studies showed that NA amino acids sequences from America, Asia, Europe, Oceania and Mexico have similar molecular distribution patterns and among them only selected amino acids of NA affected by mutations [9]. Molecular distribution patterns of amino acids may change in each strains of virus. So that position of mutation in every strain may different in each strains of influenza virus. Some position of amino acid is also responsible for change in the NA active site in other continental which is not related to resistance but helps to conformational change in other amino acid. Many amino acids substitution is related to virus confer OST-resistance may be due to variation in seasonal influenza virus [10]. Mutation affects the residues to compensate the molecular change so that cause the adverse condition for any inhibitors. Some mutation in strains may help for new strains, direct or indirect involvement in resistance, reduce the susceptibility to inhibitors and some are interferes the inhibitor as well as residues. In wild type influenza proteins amino acids are surrounded with stable and perfect isomer to inhibitors and any change in amino acids of surroundings may disturbs the binding site, result cause the resistance to drugs [11].



"Fig.1" Show the interaction of oseltamivir in the active site of wild type neuraminidase (NA1). AAs R118, D151, R152, E276, R292 and 371, make the catalytic site for OST in fig.1B [12].

Among these surroundings amino acids of active sites, only some of amino acids are responsible for mutation, which causes conformational change to other AAs and caused improper binding [13]. 150-loop cavity which formed by 147- 156 in N2 numbering an open conformation adjacent to active site, also novel target for other NAI [14]. 150-loop NA active site, T148I substitution have major role in reduced NA activity towards inhibitor, OST have efficacy that open the loop [15]. Due to mutation in 150-loop the residue, R152 loss the interaction with OST but it strongly interact with 156 residue of loop-150 [16, 14]. Interaction shows that closed conformations of NA enzyme have strong interaction with OST with R156 and these conformations may be used for design of future inhibitors.

Mechanism of resistance:

According to the influenza Virus Sequence Database, NA sequence E119V, R292K H274Y and N294S were associated with resistance to oseltamivir in H3N2 and H275Y and I223R were potentially associated with oseltamivir and zanamivir resistance in H1N1 and H5N1 influenza type A. Mutation H273Y in influenza type B, also associated with resistance to oseltamivir and paramivir, but sensitive to zanamivir [17, 18, 19, 8]. In 2006 H5N1-NA resistance is due to change in bulkier residues at position Y274 which replaced the smaller side chain residue H274. Tyrosine (Y) have different side chain and larger in size then histidine which change the conformer of active sites. This alterations disturb the orientation of other residues like E276 (fig-2 C) come closer to binding site which is unable to form salt bridge with arginine 224 [20, 21, 8]. This causes improper fitness of OST in active site. Due to mutation and substitution of bulkier residue tyrosine (Y), change the orientation of Glu276 which cause less hydrogen bond forming with OST. The hydrophobic nature of binding pocket of OST is changes, resultant shrinkage of hydrophobic pocket and it makes unfavourable for OST in the active site of neuraminidase and this makes mutant structure in viral neuraminidase. Phenylalanine (F), Tyrosine (Y) like high hydrophobic residues in place of smaller residues like Histidine (H) reduced the susceptibility of OST due to bulkier substitution.

...Study showed that H274Y decreases the amount of neuraminidase that reaches the cell surface and that this defect can be stabilized by V234M and R222Q secondary mutations that also restore viral fitness and confirming the H274Y support the OST-resistance growth. V241I, V234M, R222Q, D344N, D354G, and N369K mutation compensate the defect of NA level and help to reaches cell surface and produced new strains of NA, some are compensate negative effects with the H275Y [23, 24, 25].

Mutation were unlikely to be caused by other viral mutations based on genetic sequences like L607V, K660R, F103S, W104G, but not such sensitive to OST [26]. Common mutations in N1 are detected at N294S and H274Y, while the E119V R292K and N294S (fig-2) mutations are

mostly found in the N2 and N9 subtypes. R152K, D198N, I222V, and R371K are specific mutation found in the influenza B-type [8, 27]**.** It has found that R292K NA mutation confers resistance to zanamivir, peramivir, and oseltamivir. Mutation in R292K, N294S, I223R and H274Y confer high level resistance in N1 as well as N2 they all are single nucleotide polymorphism [7]. R292K is the most common NA mutation in subtypes N2 and N9 whereas N294S mutation also associated with N1 (H1N1, H5N1) and N2 (H3N2) [28]. I223R mutation with the H275Y in N1- NA gives the synergetic effect on IC50 which caused contraction of active site of the enzyme which potentiate the resistance or reduced susceptibility to antiviral drugs [18]. In the novel strains like H7N9, H3N2 and H1N9 virus also reduced the sensitivity of NAIs against influenza flu [24, 25]. H274Y, N294S and R292K are the inhibiting reorientation of Glu276 which was unable to interact with Arg224 and prevent pocket formation for binding and they associated with OST resistance [19]. E119V occurs with I222V mutation also give greater change in IC50 value as Compared with susceptible virus, that's interferes with OST molecules because water molecule binds with OST side and valine (V) molecule at active site [26, 27]. Recently it was found that E119G mutation in NA confers that both direct and indirect affect the drug binding and reduce the affinity to inhibitor, it may cause cross resistance against zanamivir, paramivir [29, 30]. All these substitutions are associated with catalytic residues in the active site of the neuraminidase protein**.** From NA inhibition assay it had been confirmed that zanamivir selected residues 119 and 292, and oseltamivir select residue at 274 and 292 are mutant which acquired by virus [30].

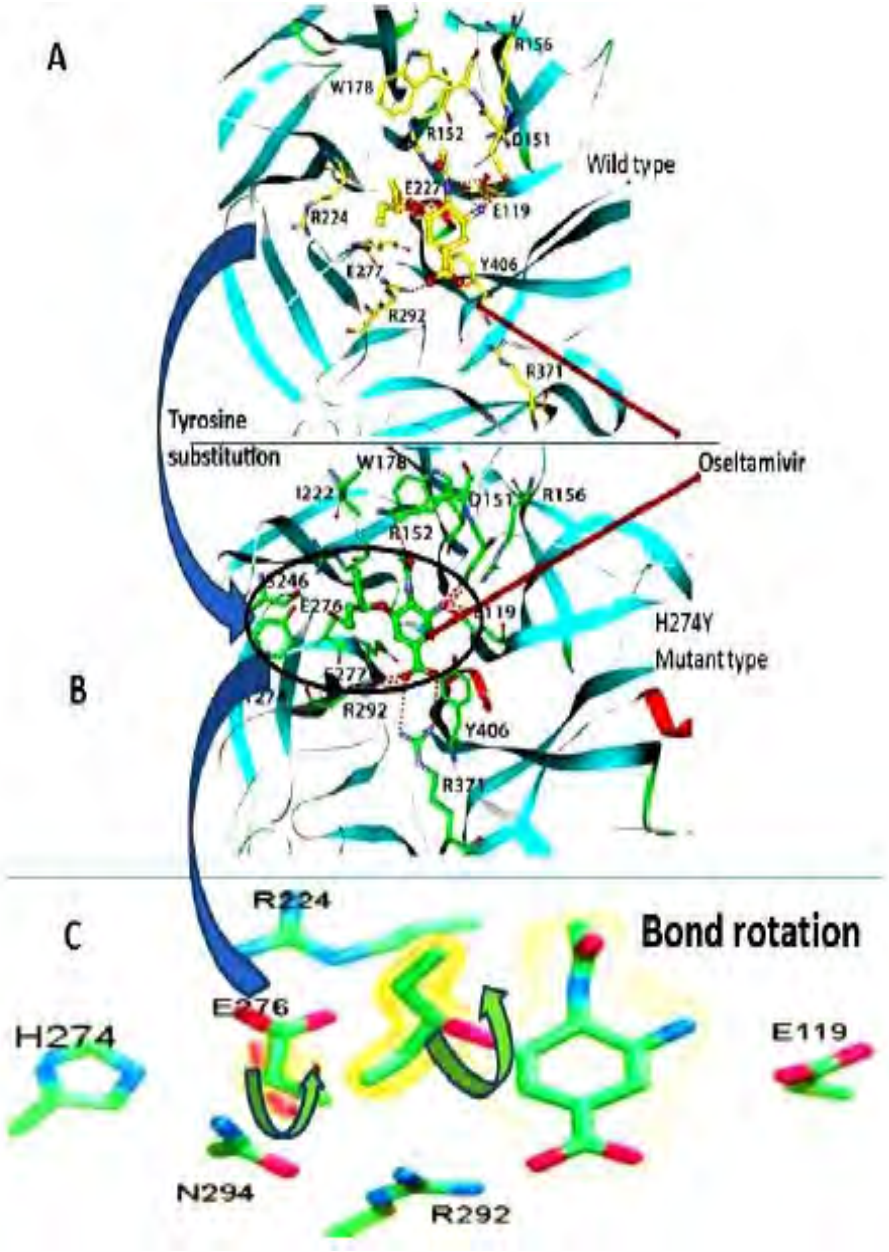


Fig.**2** - Mechanism of drug-resistance and mutation in the neuraminidase amino acid (H274Y, R292K, N294S, or E119V. A shows the interaction of oseltamivir with wild type neuraminidase receptor. B showing the interaction of oseltamivir with mutant type (274Y), and C showing the mechanism of mutation, how E276 rotate and make pocket for OST, and binds to R224. Histidine replaced by Tyrosine (bulkier residue) which changes the other residues conformation like E276, R292 so that E276 not rotate and oseltamivir not properly bind.

In fig-2B, its find that in binding pocket only few amino acids involve for interactions with inhibitor rather all the amino acids interact with each other for stabilization of binding pocket, favourable residues include R118, E119, D151, R152, W178, S179, I222, R224, E227, S246, E276, E277, R292, N294, R371, and Y406, so that makes format for active site [12]. Mutation in amino acids disturbs the frame of pocket and pocket size which caused the improper for inhibitors.

Recently it was found that mutation S247N is highly resistance to oseltamivir when along with H274Y it highly pandemic clinically. S247N also change the active site for zanamivir with I223R. A new combination of NA mutations Q313R and I427T caused resistance to both oseltamivir and zanamivir [30, 31]. All the changes in in residue take place in presence of antiviral drugs which cause adverse environments for virus; resulting changes in genes for survive and save from adverse condition. These survivals for fittest condition in virus gene cause monomer-monomer interface impede to NA inhibitors mutation in gene and result changes in proteins and produce new potent strains with alterations of surface protein (H and N). Studies show that even in the absence of oseltamivir used, there is rapid wide spread of H1N1 resistance strains transmission takes place [32, 33, 34, 35].

In fig-2, its find that in binding pocket only few amino acids involve for interactions with inhibitor rather all the amino acids interact with each other for stabilization of binding pocket, so that makes format for active site. Mutation in amino acids disturbs the frame of pocket and pocket size which caused the improper for inhibitors.

Table-**1:** Showing the all effective mutation with substitute amino-acid at specific position and effect of mutation on enzymes and related drugs resistance. Mutation (in bold) show highly frequent in influenza strains.

Mutation	Amino acid change	**Location**	**Replaced by**	**Effect of mutation**
H275Y H274Y H273Y	Histidine (H)	274	Tyrosine (Y)	Cause oseltamivir resistance to H1N1. Same amino acids change in binding site in influenza type A N1, N2 and influenza type B respectively.
N294S	Aspargine (N)	294	Serine (S)	Antagonistic Effect the OST resistance in H3N2.
I222R	Isoleusine(I)	222	Arginine (R)	Reduced the susceptibility to OST and zanamvir to NA in H1N1 with H274Y act synergistically.
Q313R I427T	Glutamine Isoleucine	313 427	Arginine Threonine	Increase the resistance to both oseltamivir and zanamivir.
V241I V234M D344N D354G N369K	Valine Aspartic acid Aspartic acid Valine Asperginine	241 234 344 354 369	isoleucine Methienine Aspergi-nine Glycine Lysine	Helps new strains of NA with compensate the defect of OST mutation. Helps for new virions. They cause negative effects with H275Y and reduce the fitness of virus.
S247N	Serine (S)	247	Asperginine (N)	Recent find mutation in H1N1 pandemic, highly resistance to OST.
R371K	Arginine (R)	371	Lysine	Associated with resistance of OST/ Zanamivir
E119V E119G	Glutamic Acid (E)	119 119	Valine (V) Glycine	Cause Zanamivir as well as OST resistance in N2-subtypes of NA. Direct and indirect influence drug binding to NA receptor.
T148I	Threonine	148	Isoleucine	Reduced the NA activity to OST by 50 %.
E276 R224	glutamic acid Arginine	276 224	No change	Rotation takes place when OST bind and Glu276 make bond with Arg224 to form pocket for OST.it is main source key for mutation in H274
R292K	Arginine (R)	292	Lysine (K)	All NA inhibitors resistance in N9- subtypes of NA, it confer high level resistance even in novel strains of swine
L607V,	Leucine (L)	607	Valine (V)	Mark on mutation but not sensitive for OST
K660R	Lysine (k)	660	Arginine (R)	Not effective, but mark on mutation
F103S,	Phenylalanine(F)	103	Serine (S)	Not effective, but mark on mutation

Q136K	Glutamate (Q)	136	Lysine (K)	Mutation in NA which confer zanamvir resistance and it give negative effect on viral growth
Q138R, P139S G140R	Glutamine (Q) Proline (P) Glycine (G)	138 139 140	Arginine (R) Serine (S) Arginine (R)	Monomer – monomer interaction show single mutation, which interfere OST and Zanamivir binding.
D187E	Aspartic acid (D)	187	Glutamic acid	In HA, Decrease virus affinity to linked with NAI to OST.
Y252H Q248G	Tyrosine (Y) Glutamate (Q)	252 248	Histidine (H) Glycine (G)	Hypothesis that both mutation increased the affinity to NA with OST
T220S, Q223R E275V, T333A, D239G	Threonine (T) Glutamate (Q) Glutamic acid(E), Threonine (T) Aspartic acid(D)	220 223 275 333 239	Serine (s) Arginine Valine (V) Alanine (A) Glycine (G)	Mutation in HA but not interfere the antiviral resistance. Decrease the viral affinity to NAI.
S31N	Serine	31	Aspareginine	Mutation takes place in M2- channel, cause amantadine resistance.
K660R, L607V, V292I	Lycine (l) Leucine (L) Valine (V)	660 607 292	Arginine (R) Valine (V) Isoleucine (I)	Mutation in PB2 protein but not affect resistance.
F103S	Phenylalanine (F)	103	Serine (S)	Mutation in non-structural protein but no any effect on resistance.
W104G	Tryptophan (W)	104	Glycine (G)	Mutation in nucleoprotein but no any effect on anti-viral resistance.

Other study showed that mutation in NA of H1N1 strains like Q136K, Q138R, P139S, G140R shows the monomer-monomer interaction in NA which alter the hydrogen bond interaction with R156 and D151 with zanamvir which confer zanamvir resistance. But research show that Q136K benefitted for the H274Y mutation NA by reduced the enzymatic activity as well as reduced NA levels in viral particles. It shows the key site that can affect the susceptibility of neuraminidase inhibitors. When Q136K mutation and H274Y mutation had introduced together give negative effect on virus growth. This mutation increase the 86 fold IC50 against zanamvir as wild type virus [35, 36]. Even in H5N1 the Y252H and Q248G mutation with the H274Y substitution in NA has been hypothesized to be responsible for an increased the affinity of NA for oseltamivir. Y252H mutation does not affect the binding energy and interaction of oseltamivir with N1 but it theoretically important for oseltamivir resistance.

Tyr is a larger side chain amino acid residue and it interacts with Arg224 and Glu276 by hydrogen bonding but not interacts with OST. It changes the other orientation of adjacent residues, which cause the shrinkage of active site [13, 37].

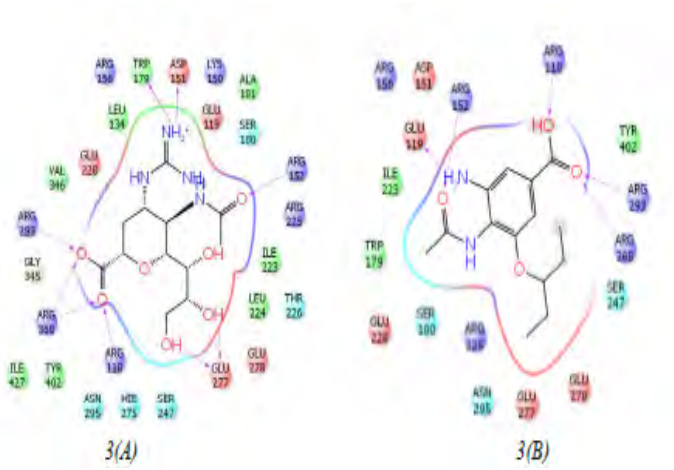


Figure 3, Interaction of Zanamivir (3A) and oseltamivir (3B) with resistant neuraminidase receptor PDB id- 4B7Q. No any amino acids are the best pose for OST, but Asp151, Arg152 Arg156, Ser179, Ser180, Arg225, Glu228, Glu277, Glu278, Arg293, Arg368 and Tyr402 (according to N1-subtype numbering) binding site for the inhibitors. Other hand Ser180, Ile223, Ser247, His275, Asp295, and Try402 show stabilization effects on docked site.

The new mutant sequence (PDB- 4B7Q) which are resistant against existing inhibitors but they do not bind the proper

as wild type NA receptor, so these are not effective against new strains. Structure based drug design (SBDD) and homology modelling help for the new ligands against the new mutant sequence.

Hemagglutinin

Mutation in Hemagglutinin (HA) subtypes (H1- H16) which circulates in human population through antigenic drift and shift. It also forms numbers of strains like H1N1, H1N2, H2N2, H3N2, H4N6, H10H8, H5N1, H5N7, etc. from HA. HA protein play important role to attachments, penetration and neutralization of the host antibody response [39]. Several amino acid substitutions in the HA1 domain from 2004 to 2009: takes place like D35N, T82K, Y94H, K141E, N125D, D187E, R189K, R209K, D222G, Q223R and E274K. Hemagglutinin residue 223 plays a critical role in the binding affinity of the galactose moiety of sialosaccharides for the receptor binding site of HA [40, 41]. Mutation D187E and Q223R of HA would decrease virus affinity for the 2, 6-linked receptor because the salt-bridge between E187 and R223 would lead to narrowing of the receptor binding pocket. But Q223R mutants showed low rates of human-to-human transmission, whereas D187E mutation shows the human to avian type receptor switch. In 2004- 2008 presence of NA H274Y mutation with the HA proteins mutation at residues 82, 141, or 189 promotes virus replication so that new virions are produced [42]. In 2009 H1N1 pandemic HA receptor showed mutation D222G and Q223R to be critical for receptor binding and to cause a shift from alpha 2, 6 to alpha 2, 3 sialic acid receptor specificity [41, 42, 43].

M2 channel

M2 protein in virus helps the transfer of viral RNA to nucleus to the human cells. It has two type pores, first is ion channel, second one is lipid face pore. Ion channel have more affinity to the amentadine and rimantidine [44]. More than 7000 Influenza A field isolates were screened for specific amino acid substitutions in the M2 gene known to confer drug resistance towards amantadine rimantadine [2].Among them V27A L26F and S31N mutant shows 98-100 % against the M2- channel inhibitors like amantadine and rimantadine. S31N mutant in M-2 channel has frequent, vulnerable effect on limited size, polarity, and dynamic nature of its amantadine-binding site changes so that it's more vulnerable effect on vaccine formation, as well as in 27 position of H1N1 [46]. But other mutation like V27A and L26F are less frequent. Till there is no effective vaccines developed against these influenza strains [47].

Several mutations at **surface protein**, HA (T220S, E275V, T333A, D239G), PB2 (K660R, L607V, V292I), Non-structural protein (F103S), and Nucleoprotein (W104G) were identified but none of them were likely to result in anti-viral resistance [46, 47]. Non-structural protein plays important role in identified mutation, spread of species, viral tropism, and infectivity in human. So these proteins have not vital role in influenza so that is not targeted as inhibitors.

Mutation problems:

Mutation in surface protein neuraminidase is major problem for design of drugs for influenza virus. This is due to change in one amino acid at active site it changes all adjacent amino acids and result resistance. The conserved residues that interact with NAIs are under selective pressure, but only a few have been linked to resistance. Literatures analysed that NA active site consist of four conserved binding site and 12 residues, site-1 have positive charge Arg118, 227, and 371, site-2 negatively charged Glu-151, 119 and 227, site-3 Ile222 Trp178 and trp406 and site-4 consist of Glu276, and 277. The amino acid R118, D151, R152, E227,, R224, E276, R371, and Y406 that directly interact with the NAls but have not reported to confer resistance to NAIs. They are form the active site or pocket for inhibitors. Acquainted mutant H274Y, N294S, I223R, E119G are the not directly interact with NAI, they are locate distant from active site but they are effect the binding mode of inhibitors. They are only associated with resistance. In combination with Y274, I117V, I119V, I223V mutation give synergetic effect on oseltamivir resistance whereas addition to N294S to Y275 give antagonistic effect on oseltamivir resistance [12, 48, 49].

Some accessories mutations helps virus for infection and the majority of mutations to NAIs are caused by mutations within the NA gene itself. Changes to the HA gene and M2-gene can also lead to decreased susceptibility to NAIs. These changes to turn decrease the need for NA activity substitutions, to more infectivity and new strains for virulence. But research say that induced mutation may help as unfavourable conditions and act as inhibitor for NA. In Table -1 showing that all possible important mutations which takes place in surface enzyme and M2-ion channel of influenza with its amino acids and its position. Overall mutation H274Y, N294S, I223R, E119V, E276V, S247N, Q136K, and S31N are the main frequent mutation which takes place in all types if surface protein as shown in table-1 as bold letters.

Pharmacophore model of existing molecule is also give great contribution to understanding the interaction with side chain of NA to NAI. In OST structure C1- position interacts the guanidine of arginine 118, 292, 371 of NA C4-position should be in range of electrostatics interaction and maintain positive group with Asp151, Glu119, and Glu227, like guanidine group in cyclopentene ring and dihydro-pyran derivatives. But C5-position having acetaimido group unchangeable but it could be substituted by bulky hydrophilic segment so that binding pocket is NA can be filled. C6-position formed hydrophilic region which formed the bond side chain of Glu277, Ser179, Arg156, Glu277, Arg292 [23, 38].

For new lead compounds the best pharmacophore characterised by five features, namely, one positive ionisable group, one negative ionisable group, one hydrophobic

point, and two hydrogen-bond donors, has a correlation coefficient of 0.902, a root mean square deviation of 1.392, and a cost difference of 72.88, suggesting that a highly predictive pharmacophore model for highly conserved domain [19]. From this pharmacophore condition develop new lead for the resistant active site

CONCLUSIONS:

There are so many mutations takes place in both surface glycoprotein neuraminidase hemagglutinin and M2- protein but only selected alteration mark the susceptibility of antiviral drugs. Result show that the position 274, 294, 276, 277, 223 and 119 of NA has more effect active site and also predisposed for mutation. Same as in HA residue 223 and 187 position and at 31 positions in M2 has more susceptible than other and help the high resistance towards antiviral drugs. This study, showed that, most frequent oseltamivir-resistant NA mutations including E119V, H274Y, R292K, I223R and N294S which impact more susceptible for substitution in all strains and it give the susceptibility profile to a novel neuraminidase inhibitor and significant resistance to oseltamivir. 150-loop may also take as future target for drug design. Pharmacophore model of OST and active site give proper information about the mutation and inhibitors for the NA. . New anti-influenza drugs may inhibit the oseltamivir-resistant strains such as H274Y mutant and urgently need to battle against the pandemic influenza.

There should be further research for targeting this important mutation by help of structure-based or ligand-based method and design the drug against resistant flu strains.

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