REVIEW

Institute of Medicinal Chemistry, School of Pharmaceutical Sciences, Shandong University, Ji'nan, Shandong, P. R. China

Pharmacophore model of influenza neuraminidase inhibitors - a systematic review

JIAN-ZHI GONG, YU LIU, WEN-FANG XU

Received April 29, 2009, accepted May 29, 2009

Wen-Fang XU, Institute of Medicinal Chemistry, School of Pharmaceutical Sciences, Shandong University, West Wenhua Road, 250012, Ji'nan, Shandong, P. R. China
drgongjianzhi@yahoo.com.cn

Pharmazie 64: 627–632 (2009) *doi: 10.1691/ph.2009.9620*

Recently, the worldwide spread of A/H5N1 avian influenza with high virulence has highlighted the potential threat of a human influenza pandemic. The viral surface glycoprotein, neuraminidase (NA), has been found to be a potential target to control influenza virus. With an understanding of the enzyme mechanism, the X-ray crystallographic structures of NA and its substrate or inhibitors, and progress in computational chemistry, the information about NA binding sites and pharmacophore models is derived from existing inhibitors and will serve as design guidelines of more potent agents against NA. This article gives a systematic review of the recent advances in this area.

1. Introduction

Influenza is a major respiratory infection associated with significant morbidity in the general population and mortality in elderly and high-risk patients (Service 1997). Recent global outbreaks of the highly pathogenic avian influenza H5N1 virus in birds and the increasing cases of bird to human transmission pose a pandemic threat to the public (Abdel-Ghafar et al. 2008). Subtypes of influenza virus are named based on the observed combinations of two viral surface membrane glycoproteins, hemagglutinin (HA) and neuraminidase (NA), with sixteen and nine types known to date, respectively (Fouchier et al. 2005). In the last century, three influenza pandemics, including H1N1 (1918), H2N2 (1957), and H3N2 (1968) cumulatively had caused 50 million deaths.

HA is involved in the attachment of viral particles to the sialic acid (SA, **1**) receptor on the cell surface, which mediates the virus entry (Stevens and Doni 2007). In contrast, NA promotes virus entry into host cells during the initial stage and facilitates the release of the newly formed virions from the infected cells at the final stage of viral replication (Gong et al. 2007). Additionally, the enzymatic active site of viral NA is highly conserved despite up to 75% sequence variation in amino acid sequence between influenza A and B. Hence, NA has been an attractive target for structure-based anti-influenza drug design (Liu et al. 2007). Several representative NA inhibitors (NAIs) are shown in Fig. 1.

With discovery of newly synthesized small molecular NAIs, it is more and more important for the establishment of a predictable pharmacophore model to guide the rational NAIs design. In this review, we will discuss the binding models of NA active site and quantitative structure-activity relationship (QSAR) studies on reported NAIs.

2. Binding models of NA active sites

2.1. Classic binding pockets

The X-ray crystal structure of influenza NA was first determined in 1983 (Colman et al. 1983; Varghese et al. 1983) and the high-resolution X-ray structures of NA A and NA B, complexed with SA were reported in 1992 (Varghese et al. 1992; Burmeister et al. 1992). Since then, structures of NA complexed with a variety of inhibitors have been reported (Varghese et al. 1995; Kim et al. 1997; Taylor et al. 1998; Babu et al. 2000; Wang et al. 2001). These structural studies provided a detailed understanding of the molecular interactions involved in the binding of various inhibitors to NA and formed the foundation for several successful rational drug design of NAIs. Overall, the highly conserved active site of NA contains a large number of polar and charged amino acid residues. Earlier crystallographic and ensuing structure-activity relationship (SAR) studies have revealed that the active site of NA can be effectively divided into four or five well-defined binding sites (Babu et al. 2000; Wang et al. 2001; Stoll et al. 2003).

In 2002, Wang proposed a modified model that could universally cover all prior work based on the previous "airplane" model (Wang 2002, Fig. 2). The positively charged Site 1 is comprised of three residues, Arg118, Arg292 and Arg371 and interacts with the carboxylate of SA *via* charge-charge interaction and hydrogen bonding. A cluster of negatively charged amino acid residues, Glu119, Glu227 and Asp151 makes up Site 2 and interact with the C-4 hydroxyl group of SA. The small hydrophobic pocket, Site 3, formed from the side chains of Trp 178 and Ile 222, accommodates the acetyl group of SA. Site 4, consisting of Glu276 and Glu277, binds to the glycerol side chain of SA. In the two-dimensional sense, Sites 3 and 1 are situated at the

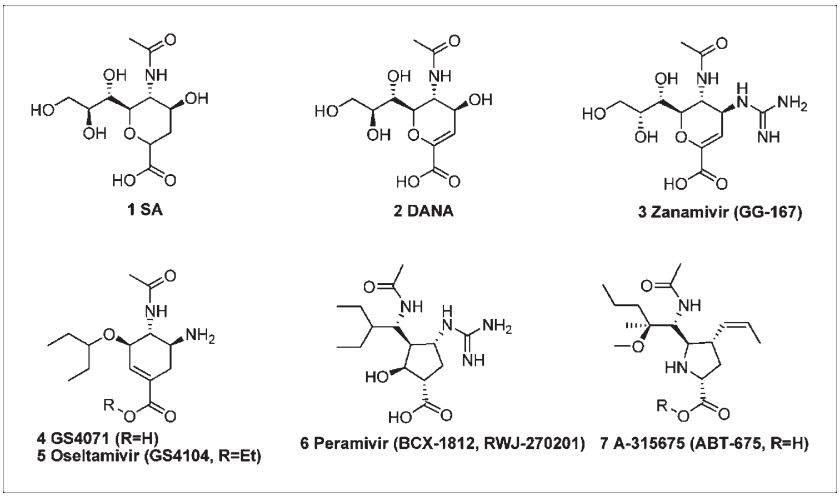

Fig. 1:
Chemical structures of sialic acid (SA) and several representative NA inhibitors (NAIs)

head and tail of the "airplane" respectively, separated by 9-10 Å or 6-7 single bond lengths, while Sites 2 and 4 are situated at two wings of the "airplane". The center of Site 2 is about 6 Å from Site 1 and about 4 Å from Site 3, while Site 4 is about 6 Å from Site 1 and 5 Å from Site 3. In the three-dimensional sense, these binding pockets are clearly off-set from the plane defined by the ring of the cyclic nucleus since aromatic (planar) mimics of zanamivir are poor inhibitors of NA (Chand et al. 1997; William et al. 1995; Singh et al. 1995). Literature data indicated that occupation of all four pockets is necessary for potent inhibition (von Itzstein et al. 1996; Taylor and von Itzstein 1994). Another hydrophobic binding site, Site 5, situated between Site 3 and Site 4, were also revealed. This site is defined by Ala246 and the side chain of Arg224 and appears quite extended. While not occupied by SA and zanamivir, it plays an important role in several potent inhibitors disclosed in recent years.

As predicted, based on the structure of NA active site, all the representative NAIs contain a carboxylate and an acetyl group that are separated by seven single bonds, consistent with the model of Fig. 2. For all these molecules, X-ray structures have confirmed that the carboxylate makes strong charge-charge interactions with the Arg triad of Site 1 and the methyl group of the acetyl moiety makes hydrophobic interaction with Site 3 of the NA active site (Varghese et al. 1995; Kim et al. 1997; Taylor et al. 1998; Babu et al. 2000; Wang et al. 2001).

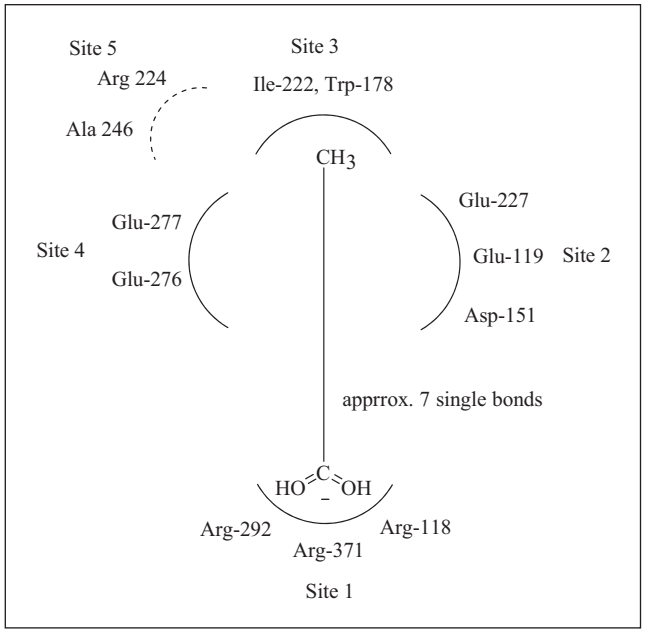

Fig. 2: Wang's working model of the NA active site shown in two-dimensional illustration

Both DANA (**2**) and zanamivir (**3**) are transition state analogues containing the glycerol side chain that binds to Site 4, as with the binding of SA. Reducing the polarity of DANA or zanamivir by replacing the glycerol side chain with a less polar moiety was an important objective toward the goal of designing orally active NAIs. In light of this, several research groups independently discovered that placing branched aliphatic groups into Site 4 can force Glu276 to undergo a conformational change by forming a salt bridge with Arg224 and exposing its side chain methylene groups, transforming Site 4 into a hydrophobic pocket (Kim et al. 1997; Taylor et al. 1998; Babu et al. 2000; Wang et al. 2001). However, as this conformational change of Glu276 involves much more extensive shift of amino acid residues and costs more energy for NA B than NA A (Taylor et al. 1998). Only compounds that present a highly steric demanding and rigid ligand to Site 4 can cause the Glu276 conformational change in NA B. Additionally, compounds that bind to Site 4 of NA B *via* this induced hydrophobic pocket consistently exhibit much weaker inhibition of NA B relative to NA A. In contrast, compounds that occupy Site 4 and Site 5 of NA B without the induced conformational change of Glu276 are often equipotent inhibitors of NA A and NA B, even if the binding with NA A does involve the induced conformational change of Glu276. This is exemplified by GS4071 (**4**), peramivir (**6**), and A-315675 (**7**) (Fig. 1).

Interestingly, the evolution of the ligand for Site 2 highlights the power of structure-based drug design (SBDD). DANA interacts with Site 2 *via* its C4-hydroxyl group, as with SA (Fig. 2). Replacement of the C4-hydroxyl by positively charged basic groups resulted in significantly more potent compounds by introducing a strong charge-charge interaction. Indeed, its guanidine analogue, zanamivir, is almost 1000-fold more potent [IC_{50} = 2-20 nM] than DANA. Similarly, the amino group of GS4071 and the guanidine group of peramivir make strong interactions with Site 2 and important contributions to binding.

However, placing a negatively charged carboxylate group into Site 1 and a positively charged group into Site 2 made these compounds (zanamivir, GS4071, and peramivir) zwitterions, which, predictably, had a detrimental effect on oral bioavailability. Excitingly, the discovery of Abbott research group (Maring et al. 2001) showed that the positive charge amino or guanidino group for Site 2 can be replaced with a neutral and hydrophobic moiety, which represents a major breakthrough. X-ray crystallographic studies has revealed that *cis*-propenyl group makes strong van der Waals interaction with the side chain methylene groups of Glu119, Glu227, Asp151 and Leu135 of the Site

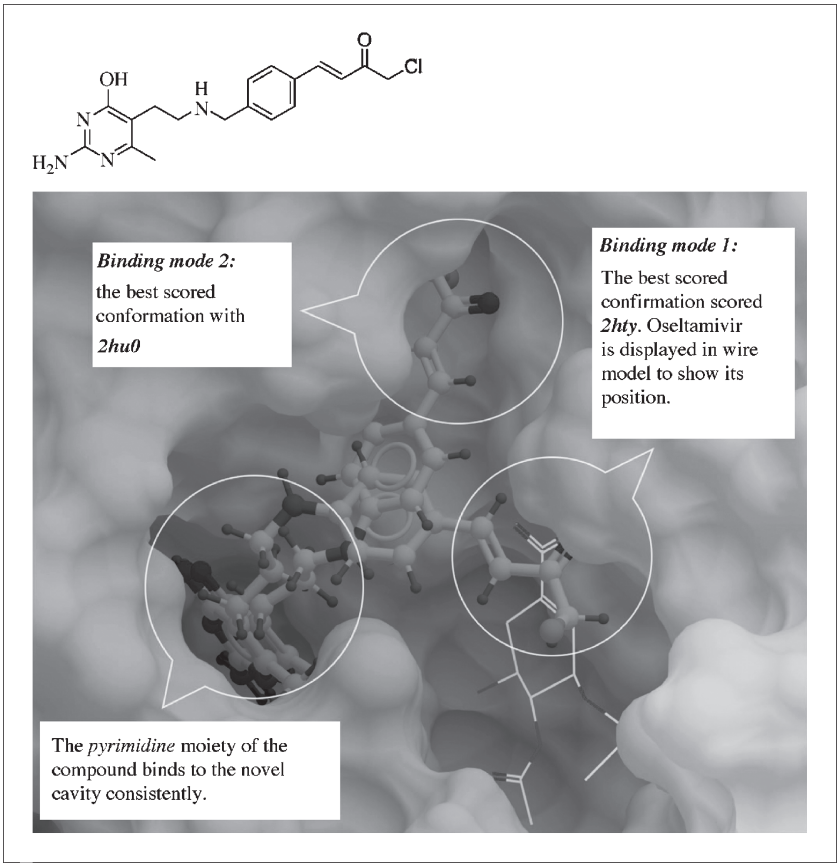

Fig. 3:
Predicted three-dimensional conformations of compound 8 bound to the open-form N1 neuraminidase. Oseltamivir is displayed as a thin wire to show its relative position to the docked compound 8. The pyrimidine moiety of compound 8 is deeply fitted in the new cavity, while the other end of the compound takes different conformations (An et al. 2009)

2 pocket. This novel discovery makes it possible to develop nonzwitterion NAIs with improved oral bioavailability.

2.2. Recently discovered 150-cavity

Until recently, the SBDD of NAIs has been carried out using the X-ray crystal structures of influenza A N2 and N9 and influenza B NA. In 2006, Russell and coworkers have described that influenza A virus NA could be divided into two distinct families: group 1 (N1, N4, N5, and N8 subtypes) and group 2 (N2, N3, N6, N7, and N9 subtypes). Excitingly, X-ray crystallography revealed that group-1 NAs contain an additional cavity adjacent to their active sites that closes on ligand binding, which is different from those of group-2 NAs. This observation provides new opportunities for directions in drug design of effective group-specific inhibitors (Russell et al. 2006). It is chemically possible to obtain new NAIs by adding extra substituent moieties to existing inhibitor skeletons.

Taking advantage of this 150 cavity, Du et al. did some computational chemistry work (Du et al. 2007). Six ligands were considered as the candidates for further experimental investigation. Ensemble-based virtual screening also revealed lots of potential novel antiviral compounds for avian influenza NA (Cheng et al. 2008). Furthermore, Serbian researchers designed two novel structures by functional modifications at position of the 4-amino group of oseltamivir in order to make polar contacts with the guanidinium side chain of Arg156, and thereby enhance the binding of a more potent inhibitor (Mitrasinovic 2009). Very recently, scientists from Hong Kong and Canada have identified a synthetic compound which appears to be able to stop the replication of influenza viruses, including the H5N1 bird flu virus (An et al. 2009). The predicted binding of compound **8** to the known H5N1 NA structure indicates a binding interface largely nonoverlapping with that of oseltamivir or zanamivir (Fig. 3). These results indicate that compound **8** or similar molecules would remain effective in the presence of virus mutations conferring resistance to either oseltamivir or zanamivir and also *vice versa*.

3. Quantitative structure-activity relationship (QSAR) models

With the amount of synthetic NAIs and the progress in computational chemistry over last two decades, many groups have derived optimized QSAR models with the purpose of proposing a presumable model for identifying effective inhibitors and designing new compounds.

In 1996, Taylor and von Itzstein used a molecular dynamics/energy-minimization protocol to analyze the structural and energetic effects of functional group substitution on the binding of a series of C4-modified DANA inhibitors (Taylor and von Itzstein 1996). Both methods showed definite trends in observed and calculated binding affinities; in both cases inhibitors with a positively charged C4 substituent formed the tightest binding to the enzyme, as observed experimentally.

Wall et al. (1999) applied the linear interaction energy (LIE) method to calculate the binding free energies of DANA analogs and influenza A NA (N2). This is a semiempirical technique for the calculation of free energy changes based on the simulation of only two states, (i) the solvated ligand and (ii) the ligand bound to the solvated protein. The final model gave a q^2 of 0.74 and contained van der Waals and electrostatic energy terms. This result was obtained without recourse to prior knowledge and was based solely on the information content of the data.

Two years later, Wang and Wade employed Comparative Binding Energy (COMBINE) analysis to deal with a series of 3D structures of NA-inhibitor complexes, and derived a predictive and robust QSAR model (Fig. 4) by considering the contributions of the protein residues and a key water molecule to the

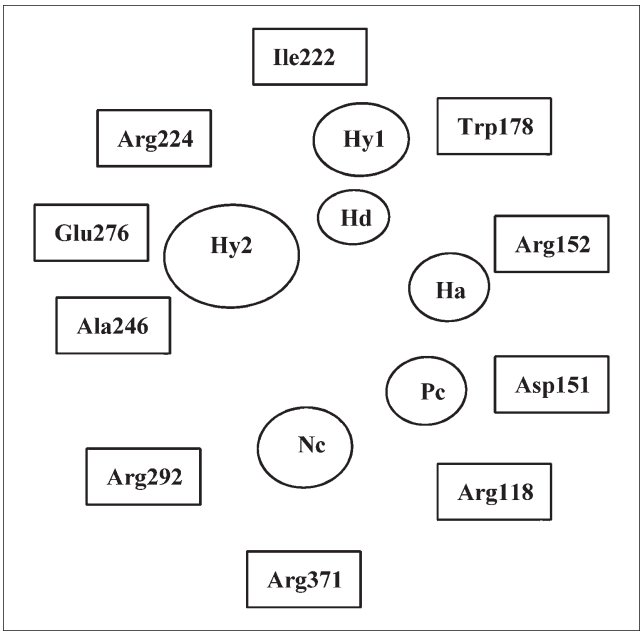

Fig. 4: Diagram of important structural features (◯) for a strong inhibitor and corresponding NA residues (□). Nc, negatively charged group; Pc, positively charged group; Ha, hydrogen bond acceptor; Hd, hydrogen bond donor; Hy1, small hydrophobic group; Hy2, large hydrophobic group (Wang and Wade 2001)

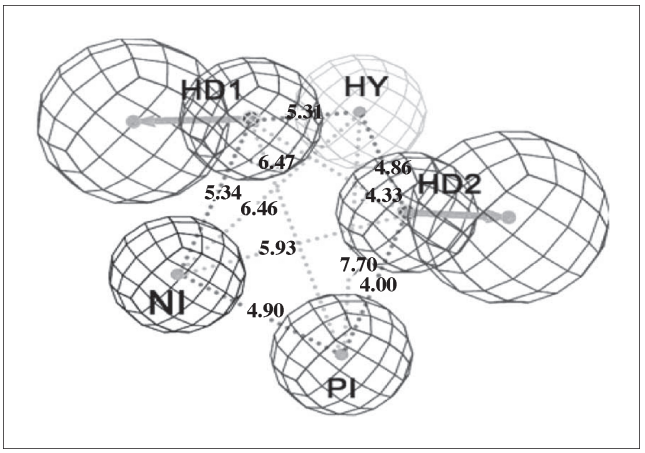

Fig. 5: Three dimensional pharmacophore model produced by Zhang et al. HY, hydrophobic group; HD1, hydrogen-bond donor 1; HD2, hydrogen-bond donor 2; PI, positive ionizable group; NI, negative ionizable group (Zhang et al. 2006)

electrostatic and van der Waals intermolecular interaction energies (Wang and Wade 2001). It is better to provide guidelines for structural modification of current inhibitors and the design of novel inhibitors in order to optimize inhibitory activity.

Yi et al. (2003) examined the binding model of five series of 37 NAIs, using molecular simulation method. The resulting conformation and orientation of the compounds were directly put into Comparative Molecular Similarity Analysis (CoMSIA) study to reveal the accordance of contour map with the distribution of amino acid residues in the binding site of NA. The result showed that the binding features of inhibitors with various scaffolds and different substituents to NA enzyme possessed an evident similarity. The CoMSIA method characterized by five force fields exhibits a statistical significance and a high predictability for activity. From the fraction of fields, it seemed that the hydrogen bond field conveyed significant contribution compared with the other fields. The derived robust QSAR model and its 3D contour map provided guidelines to building novel compounds with new scaffold and for structural optimization of current molecules.

In the following year, Steindl and Langer described the development of highly selective pharmacophore models for NAIs using the Catalyst software package (Steindl and Langer 2004). Models were generated in both an automated ligand-based approach and a structure-based approach, the latter providing more detailed information and accuracy in its description of ligand binding. The researchers concentrated on including multiple contributions (charge-charge interaction and hydrogen bond) of ligand functions and their importance for high binding affinity into the models. Through validation in virtual screening processes, the hypotheses returned very selective hit lists from 3-D databases containing NAIs. The models provide illustrations of the important interactions between the viral NA and its inhibitors and may serve as a preselection tool in drug development to check substances for their potential NA inhibitory activity, even before synthesis and further investigations are implemented.

Verma and Hansch (2006) developed seventeen QSAR for different sets of compounds, including benzoic acids, carbocyclic derivatives, cyclopentanes, isoquinolines, pyrrolidines, and miscellaneous compounds, to understand chemical-biological interactions governing their activities toward influenza NA. Of all the parameters, they found that hydrophobicity and molar volume play important role for the data sets.

To investigate whether knowledge of the ligand optimal charge distribution can facilitate more intuitive design of electrostatic interactions, researchers from the United States took a series of small-molecule influenza NAIs with known protein cocrystal structures and calculated the difference between the optimal and actual charge distributions (Armstrong et al. 2006). The differences from the electrostatic optimum correlates with the calculated electrostatic contribution to binding ($r^2 = 0.94$) despite small changes in binding modes caused by chemical substitutions, suggesting that the optimal charge distribution is a useful design goal. Those results suggest that charge optimization is useful in facilitating generation of compound ideas in lead optimization and also provide insight into design of NAIs.

Chinese scholars employed a ligand-based computational approach to identify molecular structure requirements as effective NAIs (Zhang et al. 2006). A highly predictive pharmacophore model was generated based on 22 training set compounds, which consists of five features, namely, one positive ionizable group, one negative ionizable group, one hydrophobic point, and two hydrogen-bond donors (shown in Fig. 5). This best hypothesis, 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 was successfully obtained. The application of the model showed great success in predicting the activities of 88 known NAIs in test set with a correlation coefficient of 0.818 with a cross-validation of 98% confidence level. Thus, this pharmacophore model should be reliable in identifying novel lead compounds with improved inhibitory activity through 3D database searches and useful to designing novel NAIs.

Nearly at the same time, to dig out helpful information for designing potent inhibitors with novel structures against NA, Zheng and other six Chinese researchers used automated docking, CoMFA (Comparative Molecular Field Analysis), CoMSIA, and HQSAR (Hologram Quantitative Structure Activity Relationship) methods to investigate the QSAR for 126 NAIs with great structural diversities and wide range of bioactivities against influenza A virus (Zheng et al. 2006). Based on the binding conformations discovered *via* molecular docking into the crystal structure of NA, CoMFA and CoMSIA models were successfully built with the cross-validated q^2 of 0.813 and 0.771, respectively. HQSAR which does not require 3D information of these compounds and could provide a detailed molecular fragment contribution to the inhibitory activity, was also carried out

as a complementary study. These models also show clearly how steric, electrostatic, hydrophobicity, and individual fragments affect the potency of NAIs. In addition, CoMFA and CoMSIA field distributions are found to be in well agreement with the structural characteristics of the corresponding binding sites. The final 3D-QSAR models and the information of the inhibitor-enzyme interaction are believed to be useful in developing novel potent NAIs.

Two Indian investigators developed a QSAR model with spatial, topological, electronic, thermodynamic and E-state indices on 30 thiourea analogues (Nair and Sobhia 2008). The model developed was validated by cross validation techniques, randomization and external test set prediction. The spatial and thermodynamic descriptors were found to play a major role in determining NA inhibitory activity. The shadow indices highlight the spatial importance and the atom type log *P* descriptors explain the hydrophobic contributions of different atom types, which must be taken into account for designing new NAIs.

Very recently, Li's group studied QSAR of 123 influenza NAIs utilizing three-dimensional holographic vector of atomic interaction field (3D-HoVAIF), which is a new method of QSAR for different sets of compounds to understand chemical-biological interactions governing their activities toward influenza NA (Li et al. 2009). HoVAIFA parameters, having clear physicochemical meaning and not considering the superposition of conformation, are of easy interpretation and can be calculated directly with more advantages in modeling stability and predictive ability than traditional methods of molecular characterization. Meanwhile the HoVAIFA parameters are easy to be obtained and the 3D-QSAR models independent of experimental data can be constructed only in the knowledge of molecular structures of the compounds. The QSAR models including classic electrostatic, steric and hydrophobic interactions have favorable stability and good predictive ability, it illustrates that HoVAIFA is an effective description methodology for characterization of the complex interactions of drug molecules.

Since the existence of two genetically distinct NA groups were demonstrated, lots of computational chemistry studies about group 1 NA, especially H5N1 have also been carried out.

In 2008, a Chinese team performed the binding interaction analysis between the active site of NA (N1 subtype) and its inhibitors by combining *ab initio* fragment molecular orbital (FMO) calculations and three-dimensional quantitative structure-activity relationship with comparative molecular field analysis (3D-QSAR CoMFA) modeling (Zhang et al. 2008). Initially, the 3D structure of N1 subtype of human influenza type A virus (N1hA) was built by homology modeling, employing the X-ray crystallographic structure of 2HU0 as the protein template. Subsequently, they analyzed and compared the receptor-ligand binding interactions in detail by performing FMO calculations based on docking oseltamivir into the active site of the N1hA homology model and the crystal complex structure for oseltamivir binding to the active site of 2HU0. Furthermore, a molecular docking-based 3D-QSAR CoMFA was performed on 27 well-known N1 inhibitors. Integration of the results from the 3D-QSAR CoMFA modeling, molecular surface property (electrostatic and steric) mapping of homology model, and binding interaction analysis has put forward a set of new receptor-ligand binding models and bioaffinity predictive models, which are valuable for rational design and virtual screening of more potent inhibitors of N1hA.

Structural bases for oseltamivir recognition by group-1 NA1, NA8 and group-2 NA9 were highlighted by the ScrewFit algorithm for quantitative structure comparison (Calligari et al. 2009). Oseltamivir binding to NA1 and NA8 affects the geometry of Glu119 and of regions Arg130-Ser160, Val240-Gly260, and Asp330-Glu382, leading to multiple NA conformations. Additionally, although NA1 and NA9 share almost the same oseltamivir-bound final conformation, they show some relevant differences as suggested by the ScrewFit algorithm. These results indicate that the design of new NAIs should take into account these family-specific effects induced on the whole structure of NAs.

4. Conclusions

The recent outbreaks of avian influenza virus, especially the H5N1 subtype, cause mounting fear that a mutated form of this virus may lead to a new human pandemic. In addition to universal vaccine development (Ehrlich et al. 2008), anti-influenza drug design has increasingly become a very active research filed in recent years (De Clercq and Neyts 2007).

The NAIs still represent a significant advance in anti-influenza chemotherapy. Compared to the M2 ion channel blockers, they have the advantages of broad spectrum of antiviral activity including all influenza A and B virus strains; lesser potential for emergence of clinically important drug-resistant viruses; better tolerability and documented efficacy in reducing respiratory events.

All the above information concerning the binding sites and QSAR models is derived from the studies on existing NAIs and will be valuable for future designing of NAIs. It is anticipated that more potent anti-influenza agents against NA shall be available soon.

Acknowledgements: This work was financially supported by the National Nature Science Foundation of China (Grant No. 36072541). The statements in this paper reflect the views of the authors and are based on work by numerous investigators in the anti-influenza field. The authors apologize for any unintended missed reference in this review.

References

Abdel-Ghafar AN, Chotpitayasunondh T, Gao Z, Hayden FG, Nguyen DH, de Jong MD, Naghdaliyev A, Peiris JS, Shindo N, Soeroso S, Uyeki TM (2008) Update on avian influenza A (H5N1) virus infection in humans. N Engl J Med 358: 261–273.

An J, Lee DC, Law AH, Yang CL, Poon LL, Lau AS, Jones, SJ (2009) A novel small-molecule inhibitor of the avian influenza H5N1 virus determined through computational screening against the neuraminidase. J Med Chem 52: 2667–2672.

Armstrong KA, Tidor B, Cheng AC (2006) Optimal charges in lead progression: a structure-based neuraminidase case study. J Med Chem 49: 2470–2477.

Babu YS, Chand P, Bantia S, Kotian P, Dehghani A, El-Kattan Y, Lin TH, Hutchison TL, Elliott AJ, Parker CD, Ananth SL, Horn LL, Laver GW, Montgomery JA (2000) BCX-1812 (RWJ-270201): Discovery of a novel, highly potent, orally-active and selective influenza neuraminidase inhibitor through structure-based drug design. J Med Chem 43: 3482–3486.

Burmeister WP, Ruigrok RW, Cusack S (1992) The 2.2 Å resolution crystal structure of influenza B neuraminidase and its complex with sialic acid. EMBO J 11: 49–56.

Calligari PA, Kneller GR, Giansanti A, Ascenzi P, Porrello A, Boced A (2009) Inhibition of viral group-1 and group-2 neuraminidases by oseltamivir: A comparative structural analysis by the ScrewFit algorithm. Biophys Chem 141: 117–123.

Chand P, Babu Y, Bantia CN, Cole B, Kotian PL, Laver WG, Montgomery JA, Pathak VP, Petty SL, Shrout DP, Walsh DA, Walsh GM (1997) Design and synthesis of benzoic acid derivatives as influenza neuraminidase inhibitors using structure-based drug design. J Med Chem 40: 4030–4052.

Cheng LS, Amaro RE, Xu D, Li WW, Arzberger PW, McCammon JA (2008) Ensemble-based virtual screening reveals potential novel antiviral compounds for avian influenza neuraminidase. J Med Chem 51: 3878–3894.

Colman PM, Varghese JN, Laver WG (1983) Structure of the catalytic and antigenic sites in influenza virus neuraminidase. Nature 303: 41–44.

De Clercq E, Neyts J (2007) Avian influenza A (H5N1) infection: targets and strategies for chemotherapeutic intervention. Trends Pharmacol Sci 28: 280–285.