‘Flu’ and structure-based drug design

Rebecca C Wade

The threat of a catastrophic outbreak of influenza is ever present. Vaccines are only partially effective and the two compounds, amantidine and rimantidine, used clinically against influenza A cause side-effects and rapid viral resistance. Recent advances bring hope that specific and potent drugs against influenza may soon be available in the clinic. These compounds were designed to inhibit influenza neuraminidase (NA), one of the viral coat glycoproteins, using the crystal structure of NA which was first published in 1983. In this review, the application of structure-based drug design approaches to the design of anti-influenza agents targeted at NA and haemagglutinin (HA), the other viral surface glycoprotein, is discussed.

Address: Structural Biology Programme, European Molecular Biology Laboratory, Meyerhofstasse 1, 69012 Heidelberg, Germany.

E-mail: wade@embl-heidelberg.de

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The report [1] earlier this year of an orally bioavailable anti-neuraminidase agent, GS4104, entering clinical trials means that the day when anti-influenza tablets are readily available may not be too far off. The influenza virus’ rapid rate of antigenic variation is the reason that vaccines are only modestly effective, and each year bouts of influenza cause many deaths (influenza is one of the ten most common causes of death in the USA), much suffering and many days of work missed: reasons enough for the development of a good drug. Every so often the virus undergoes a major antigenic transformation resulting in a pandemic strain, such as the strain which killed more than 20 million people in 1918–1919. A pandemic can strike human and other populations, such as seal, horse or fowl, at any time. Efficient prevention of disaster requires a simply administered drug that is effective against all types of influenza with minimal side-effects and viral resistance. In this review, the steps followed towards the development of candidate drugs that may fulfil this role are charted. A crucial breakthrough in this process was the determination of the crystal structure of influenza NA [2] and its exploitation in structure-based drug design (SBDD). Thus, the ways in which SBDD has aided the design of antineuraminidase agents is examined, and the design of these compounds is compared with that of haemagglutinin inhibitors.

Three decades of antineuraminidase inhibitor discovery

The systematic search for antineuraminidase inhibitors started in the 1960s and the main steps in their development are outlined in Figure 1 (for detailed reviews see [3,4]). NA destroys cell receptors for the virus by cleaving the sialyl moiety, to which HA binds, from cell surface glycoconjugates and releasing sialic acid (Neu5Ac). The role of NA is not completely understood [5], but it is thought to facilitate the liberation of progeny virions from the cell surface, thereby preventing virus aggregation. NA may also assist the virus in penetrating mucosal secretions. The drug design goal, therefore, has been to develop potent NA inhibitors, first by random screening, then by mechanistic design of transition state analogues, and most recently by SBDD (Figure 2).

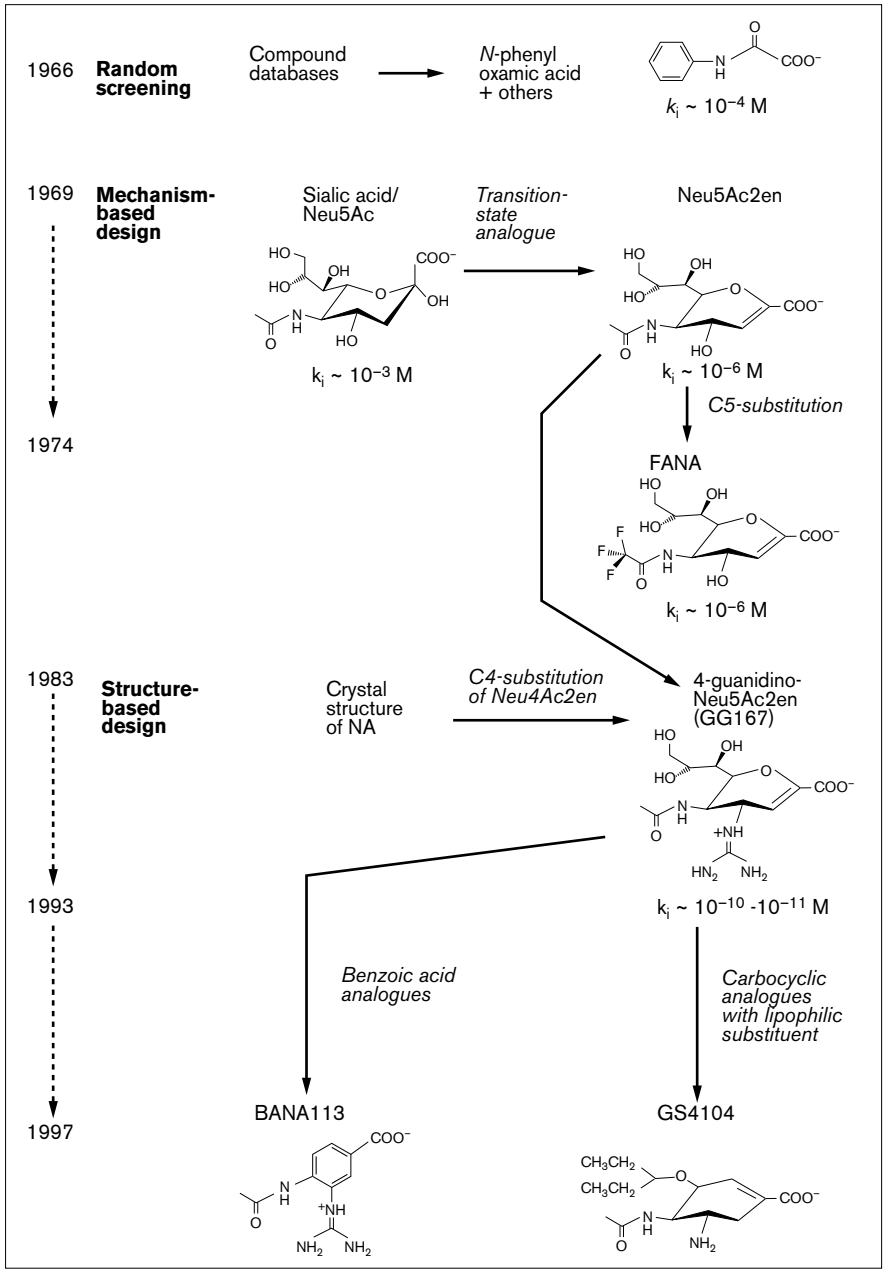
Pre-1983 and the NA structure determination

Random screening did not produce potent inhibitors of NA [6]. The classical mechanistic route to enzyme inhibitor design, namely designing a possible transition state analogue, resulted in a promising lead compound, 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (Neu5Ac2en), with an ~10^3-fold greater binding affinity than the product, Neu5Ac [7]. Not surprisingly, the affinity of Neu5Ac2en is similar for all types of sialidases, including mammalian ones. Improvements in potency and specificity were sought by synthesizing a number of Neu5Ac2en analogues but the most potent, a halogenated derivative (FANA), had only a micromolar k_i [8], and none of these analogues showed antiviral activity in animals.

Post-1983 and the NA structure determination: 1st generation compounds

Further progress was only made when crystal structures of influenza A NA [2,4,9] became available. The crystal structure revealed a tetrameric ‘head’, with each subunit possessing a β-sheet propeller structure and a surface depression surrounded by conserved residues which turned out to be the active site (Figure 3). von Itzstein *et al.* [10,11] probed the active site computationally using the GRID program [12,13] to detect energetically favourable binding sites for different functional groups. This analysis revealed a conserved pocket in the NA–Neu5Ac complex that could be filled by a basic substituent at the 4-OH position of Neu5Ac2en that would interact with Glu119 and Glu227 (Figure 4a; region A). As a result of this finding, 4-guanidino-Neu5Ac2en (GG167) was subsequently investigated and turned out to be very potent and specific against influenza A and B; this compound is now undergoing phase II clinical trials. Although resistant strains have been isolated in tissue culture, none has so far been identified in animals or in clinical trials. The resistant strains have a single point mutation (Glu119→Gly) in the 4-OH pocket, which results in a NA that is less stable than the parent NA but has the same specific activity [14].

Figure 1



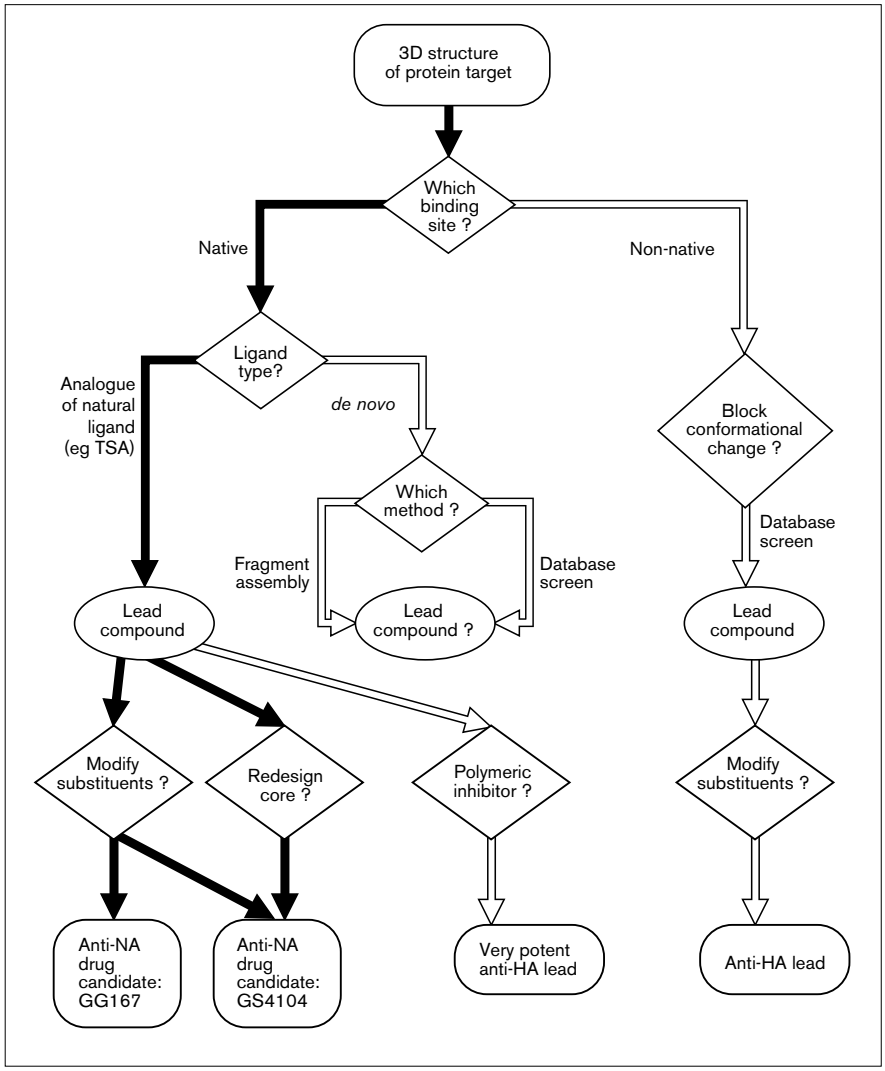
Schematic diagram showing the chronology of the main steps in the design of antineuraminidase inhibitors. k_i and IC_{50} values are given for the inhibition of neuraminidase activity. Compounds GG167 and GS4104 are now in clinical trials.

Crystal structures of the NA–4-guanidino-Neu5Ac2en complex [10,15] confirmed that 4-guanidino-Neu5Ac2en has the same binding mode as Neu5Ac2en and generally validated the SBDD. A deviation from the initial modelled structure was the orientation of the 4-guanidino substituent, which whilst engaging in a salt link to Glu119, did not form hydrogen bonds to it as designed. Subsequent modelling of the complex using refined protocols yielded structures close to that observed experimentally [15,16]. Moreover, from models of the complexes of a series of 4-OH-substituted Neu5Ac2en analogues, a correlation was obtained between experimental binding affinities and relative electrostatic binding free energies calculated using a continuum electrostatic model and neglecting changes in internal ligand energy [17].

Neu5Ac analogues with substituents chosen to exploit other favourable binding sites detected with the GRID program have been synthesized. For example, 4-*epi*-amino-Neu5Ac2en was designed to exploit region B in Figure 4a; it bound NA tenfold more tightly than Neu5Ac2en but tenfold less well than 4-amino-Neu5Ac2en, apparently

Figure 2

Schematic diagram showing SBDD approaches applied to the design of anti-influenza agents. The routes by which the best inhibitors have been derived are indicated: those shown by black arrows have led to drug candidates in clinical trials.

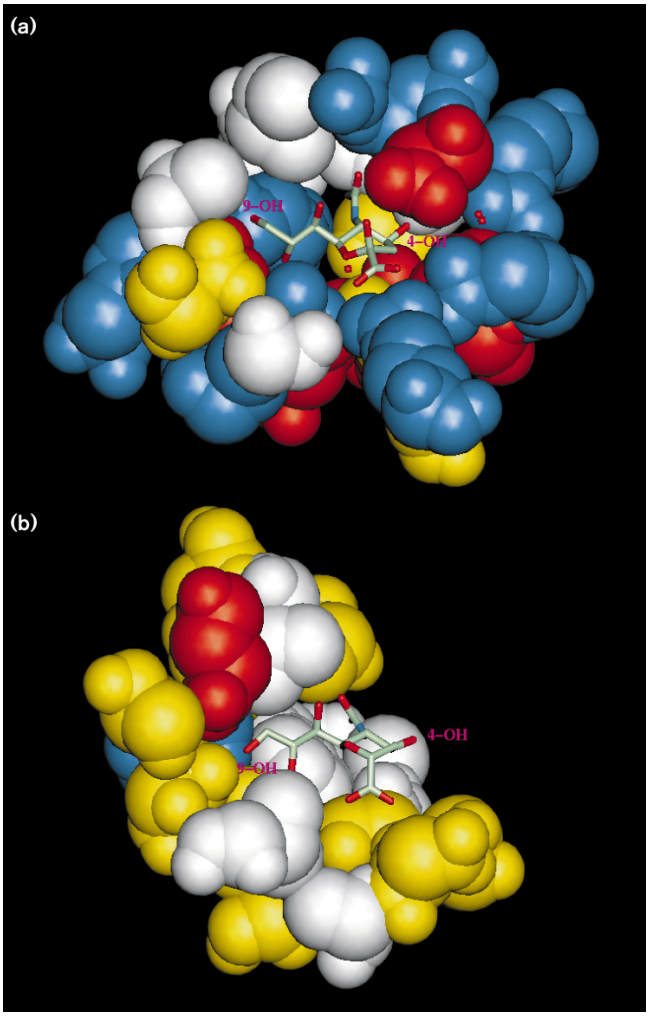


because it could only just fit into the binding site without causing too much distortion [11]. Improved binding by substituting a phosphonate instead of the carboxylate moiety of Neu5Ac was predicted [11] and independently shown to improve binding ten to 100-fold over that of Neu5Ac [18]. The equatorial phosphonate analogue appears to make more favourable interactions with the protein and to bind with a less distorted ring than Neu5Ac.

Post-1983 and the NA structure determination: 2nd generation compounds

GG167 must be inhaled or used as a nasal spray because of its poor oral bioavailability and rapid excretion. Thus further design studies have been aimed at deriving more stable noncarbohydrate compounds. Attempts to replace the pyranose ring of Neu5Ac2en with a flat benzene ring have so far resulted in less potent inhibitors [19,20], indicating that the shape of the strained pyranose ring and the orientation of the substituents on the ring may be important for high affinity. Benzoic acid analogues have, however, provided several interesting insights as regards SBDD. In particular, a benzoic acid analogue (BANA113; Figure 1) with a guanidino substituent designed to bind in the same pocket as that of GG167 actually bound in a different orientation with the guanidino group at the position of the glycerol group of Neu5Ac2en (region D; Figure 4a) [20]. Using the GRID program to locate substituents and the continuum electrostatics approach to compute binding constants, Jedrzejas *et al.* [21] estimated that the addition of a second guanidino group would increase binding affinity. Subsequent experimental data for a compound with two guanidino groups showed that it bound structurally as anticipated [22]. The addition of a second guanidino group, however, did not increase

Figure 3



The sialyl-binding sites in the crystal structures of **(a)** NA complexed with Neu5Ac and **(b)** HA complexed with sialyl lactose with only the sialyl moiety shown. All residues with atoms within 5 Å of any atom in the sialyl group are shown by van der Waals spheres coloured according to residue polarity: red, acidic; blue, basic; yellow, polar; white, nonpolar. The ligands are coloured according to atom type and water molecules in the NA-binding site are shown by small red spheres. Differences in the polarity and solvent-exposure of the NA and HA-binding sites can be observed.

binding affinity indicating that desolvation effects may have been underestimated.

Recently, carbocyclic analogues that closely mimic the ring structure of Neu5Ac2en were synthesized and found to have antineuraminidase activity [23]. By making the functional groups attached to the ring more lipophilic, Kim *et al.* [1]. derived an orally bioavailable compound, GS4104 (Figure 1), with potency exceeding that of GG167. This compound has now entered clinical trials and has shown activity against influenza A and B in animals. The design rationale used for GS4104 is summarized in Figure 5 and was validated crystallographically [1]. The crystal structure showed that the hydrophobic substituent introduced to improve lipophilicity packed well with hydrocarbon sidechains but forced the carboxylate group of Glu276 to reorient away from the binding pocket.

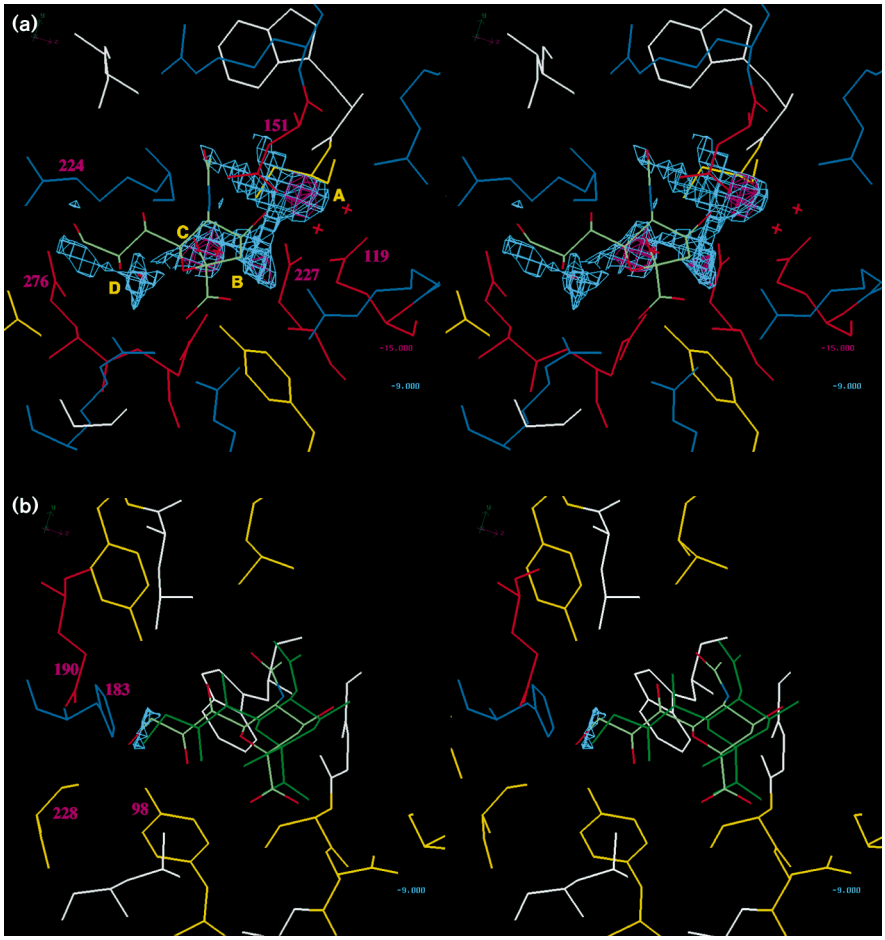
Comparison of the design of anti-influenza agents targeted at NA and HA

Both HA and NA bind sialic acid with affinities in the millimolar range. The crystal structure of HA [24] was published in 1981, shortly before that of NA, and shows a trimeric 'head-on-stalk' structure with, like NA, sialyl-binding sites in the 'head'. Despite these parallels, when similar design strategies to those employed for antineuraminidase inhibitors have been applied to HA, they have so far met with less success and other strategies have been employed to achieve potent inhibitors of haemagglutination. The reasons for these differences are discussed here.

Choice of binding site and ligand type: de novo *versus native*

HA is responsible for binding to cell receptors and for membrane fusion. Bodian *et al.* [25] used the DOCK program to identify a non-native binding site on the protein and, by means of database screening, uncovered promising lead compounds that stabilize the nonfusogenic conformation of HA. On the other hand, most inhibitor design against HA has been directed towards compounds which block virus attachment to cell receptors by binding in the highly conserved native Neu5Ac-binding site. As yet, no novel potent inhibitors which bind to this site have been reported as a result of *de novo* computer-aided design, although potential inhibitors have been suggested [26–28]. Instead, all reported inhibitors binding at the Neu5Ac-binding site have incorporated analogues of the native ligand. The native ligand, however, provides a better starting point for inhibitor design for NA than HA for several reasons. Firstly, for NA, knowledge that enzymes tend to bind transition state analogues tightly could be used to derive a lead compound (Neu5Ac2en) with reasonably high affinity. Its affinity is greater than that of Neu5Ac because it is not internally strained when bound and because binding is not opposed by an unfavourable anomeric ratio. (Both NA and HA bind the α anomer of Neu5Ac which has only 5% population in solution.) Secondly, Neu5Ac binds NA in a distorted boat conformation [29–31], whereas it binds HA in an unstrained chair conformation [32] and thus, as its binding affinities to HA and NA are very similar, makes less favourable interactions with HA. This can be understood from comparing the structures of the two complexes shown in Figure 3. The binding site in HA is shallower and has fewer charged residues. The functional groups on Neu5Ac make different interactions in the two proteins and, in particular, the carboxylate group is equatorial in NA and surrounded by three arginine residues while it is axial in HA,

Figure 4



The sialyl-binding sites of **(a)** NA and **(b)** HA. The colouring scheme is the same as used in Figure 3. The contours enclose energetically favourable binding regions for an amino probe computed with the GRID program (pink: −15; cyan: −9 kcal/mol). The regions identified in NA are: A, the most favourable region where the guanidino group of GG167 binds [15]; B, the region where the amino group of 4-*epi*-amino-Neu5Ac2en is designed to bind [11]; C, a region whose exploitation in ligand design has not yet been reported; D, the region where the guanidino group of BANA113 binds [19]. In HA, only one energetically favourable region is observed at these contour levels and is located near the 9-OH group of Neu5Ac where hydrogen bonds can be made to Glu190, Ser228 and Tyr98 [26]; this region was also detected by a HOOK/MCSS computation [28]. In (b), 9-amino-9-deoxy-Neu5Acα2Me (Neu5Acα2Me9N) is shown in dark green at its crystallographically observed position in its complex with HA: the amino group is shifted ~0.8 Å out of the binding site from the position of 9-OH [33] and lies less than 0.5 Å from the position of the GRID energy minimum for the amino probe. This energy minimum is at about −12 kcal/mol, whereas the closest energy minimum for a hydroxyl probe is at about −9 kcal/mol and, therefore, one might expect an amino substituted compound to bind better than a hydroxylated one. However, substitution of 9-OH of Neu5Acα2Me by an amino group resulted in an eightfold reduced binding affinity [33]. A reason for the reduced affinity, is that the GRID energy minimum for the amino probe is narrow with steep gradients. At the position of 9-NH_3 in Neu5Acα2Me9N, the GRID energy for the amino probe, while more favourable than for a hydroxyl probe, is 4 kcal/mol greater than at the energy minimum despite its proximity. At the position of 9-OH in Neu5Acα2Me, the relative GRID energies of the two probes are inverted and the hydroxyl group is favoured. Thus the GRID energy maps are consistent with the experimental observations at the 9-OH and 9-NH_3 positions, but the precise position of the energy minimum for the amino probe cannot be attained by the 9-NH_3 group in Neu5Acα2Me9N, probably because the positions of its other functional groups cannot be too unduly compromised. In contrast, the 4-OH binding pocket in NA has a large more energetically favourable region that can be utilized for improving binding more easily.

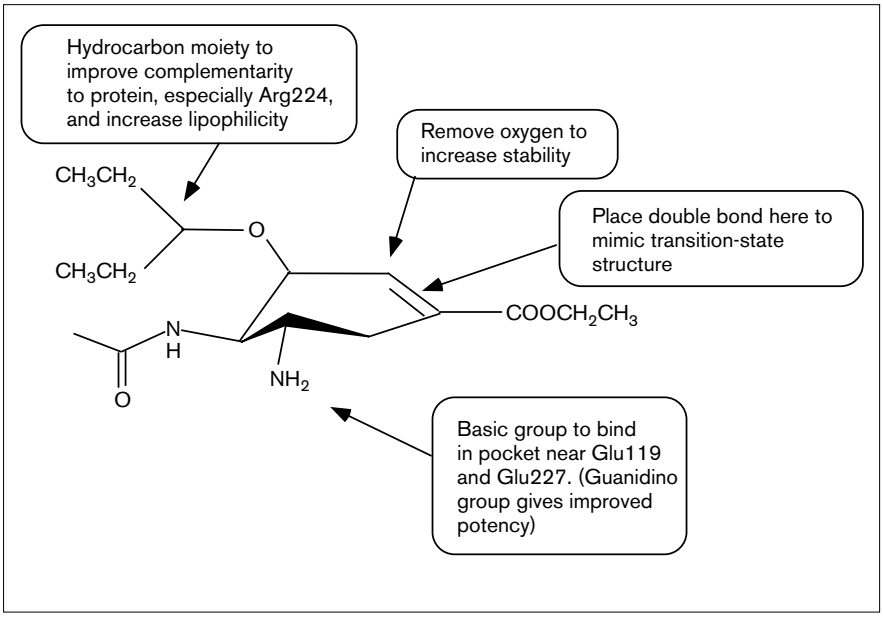
pointing into the binding site but lacking positively charged protein residues in its immediate vicinity. Finally, sialoside haemagglutination inhibitors must be designed to be resistant to inactivation by NA. On the other hand, it would be advantageous for NA inhibitors to bind to HA and thus inhibit cell attachment.

Improving binding by substituent modification

Many Neu5Ac analogues have been investigated for inhibition of haemagglutination [33,34], but the most potent monovalent sialoside analogue inhibitor reported to date has a K_D of 3.7 μM for HA [35]. This is a fluorescent molecule which has a large dansyl group attached at the 4-OH position by a flexible glycine linker. This position for the fluorophore was chosen because the structure of NA indicated that such a large group could not be accommodated at the 4-OH position and indeed, no cleavage of the glycosidic bonds by NA was observed. In contrast, in HA the 4-OH position is exposed to solution (Figure 3b) and the dansyl moiety has been shown crystallographically to point into solution [36].

For HA, an approximately 10^3-fold improvement in inhibition compared to the lead compound, Neu5Acα2Me, has been achieved by modifying substituents [35]; whereas for NA, the corresponding improvement compared to Neu5Ac2en is 10^4–10^5 fold. An explanation for this difference is provided by comparison of the GRID energy maps for an amino probe (see Figure 4 legend). In NA, the pocket for the 4-amino substituent is larger and

Figure 5



Design rationale for the derivation of GS4104, an orally bioavailable antineuraminidase agent.

much more energetically favourable than that for an amino substituent in HA. Thus the desolvation energy of an amino group can be overcome more easily and exploitation of the binding pocket is facilitated by its size, which provides more flexibility for attaining complementarity for other functional groups in the ligand.

Alternative strategy for HA: exploitation of polyvalency

HA binds sialosides with low affinity yet the influenza virus binds tightly to cells. This high affinity is achieved by the simultaneous binding of multiple HAs to sialoside cell receptors which is possible because of the high density of HA on the virion surface (~five times that of NA) and the high density of Neu5Ac on the mammalian cell surface. The same strategy has been exploited in influenza inhibitor design resulting in a number of multivalent sialoside-bearing inhibitors that are more potent than monovalent ones (see [37] and references therein). Polymeric haemagglutination inhibitors based on liposomes, poly(acrylic acid) or polyacrylamide, have reported potencies of up to ~10^{-11} M [37,38]. Potency is achieved in three ways: by the use of *C*-glycosides because these, unlike the natural *O*-glycosidic linkages, are resistant to cleavage by NA; by high-affinity binding through the entropic advantage of polyvalency; and by steric stabilization of the surface preventing HA from binding the cell receptors.

Concluding remarks

The success of the SBDD of antineuraminidase agents is very encouraging, showing that it is possible to improve binding by modifying substituents on a known ligand using structural information and computational techniques. The studies on NA and HA both show the ability of computational techniques to locate binding sites in proteins for functional groups, but point to short comings in the ability to estimate ligand binding affinities and further work is necessary to improve this. The successful approach against NA was, nevertheless, rather conservative, that is based on a transition-state analogue binding at a native binding site on the protein. *De novo* design techniques for deriving novel ligands, independently of known ligands, to bind to native or non-native binding sites are much less reliable. Both design strategies should benefit from recent advances in combinatorial chemistry as the complementary 'rational' and 'random' approaches to SBDD become integrated.

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