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Benzoic Acid Inhibitors of Influenza Virus Neuraminidase

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Abstract

A strategy was developed to design non-carbohydrate inhibitors of influenza virus neuraminidase. Using an iterative cycle of modeling, synthesis, biological testing and X-ray crystallography structure determination, a series of inhibitors based on benzoic acid were produced. The refined structures of three compounds complexed with neuraminidase are reported. The results demonstrate the success of this structure-based drug-design strategy.

Introduction

Influenza virus neuraminidase provides a target for design of active-site-specific inhibitors that can stop virus infection by inhibiting the activity of neuraminidase. Neuraminidase (NA) is one of the two major glycoproteins, the other one being hemagglutinin (HA), protruding from the surface of influenza virus. HA is responsible for attachment to the receptors (sialic acid) for influenza virus on the host cell, which are sialylated cell surface glycoconjugates. NA cleaves off the terminal sialic acids on the glycosylated HA during virus budding to facilitate virus release and probably helps virus spread through the circulation by further removing sialic acids from the cell surface (Palese & Schulman, 1974). NA is not an efficient enzyme and is present in fewer copies than HA so it does not destroy all sialic acid receptors. The activity of NA has been shown to be essential for influenza virus replication by the selection of a NA-deficient mutant of influenza virus which requires the presence of exogenous neuraminidase activity for growth (Liu & Air, 1993).

NA is a tetramer arranged around a fourfold rotation axis and the tetrameric heads can be released from the viral surface by proteolytic cleavage. The purified heads can be crystallized and still maintain full neuraminidase activity in the crystalline state. NA has been classified into nine subtypes for type A influenza virus according to their serological properties by the World Health Organization; but there are no NA subtypes in type B influenza virus. The amino-acid sequence homology among neuraminidases from both type A and B virus strains has been found to be approximately 30%. The crystal structure of several native NA heads as well as the complex of NA to α-sialic acid (NANA) have been reported (Varghese, Laver & Colman, 1983; Baker, Varghese, Laver & Colman, 1987; Varghese, McKimm-Breschkin, Caldwell, Kortt & Colman, 1992; Burmeister, Ruigrok & Cusack, 1992; Janakiraman, White, Laver, Air & Luo, 1994). The monomeric subunit of NA's from different strains of type A and B virus is all composed of six, four-stranded antiparallel β-sheets arranged as a right-handed propeller (Fig. 1). Most of the disulfide bridges are conserved. The active site is a shallow

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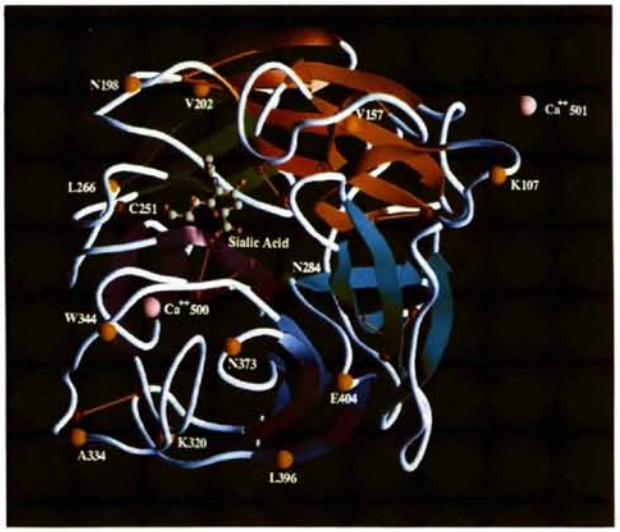


Fig. 1. A ribbon drawing of influenza virus neuraminidase monomer. The active site is indicated by the ball-and-stick model of the bound sialic acid. The orange spheres represent amino acids unique to B/Lee/40 neuraminidase.

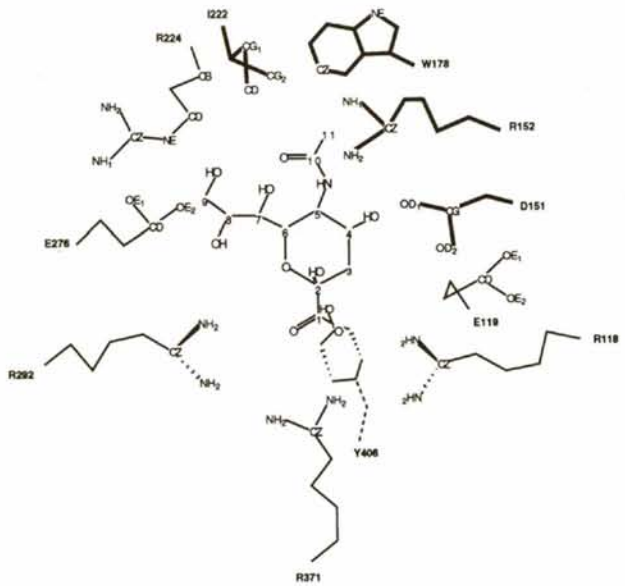


Fig. 2. The chemical structure of 4-guanidino DANA, DANA and 4-(acetylamino) benzoic acid.

pocket lined by a shell of 11 invariant amino acids that directly interact with the substrate. Surrounding the central active-site residues is a second shell of mostly conserved or homologous residues that are tightly associated with the central shell through an extensive hydrogen-bonding network. Fig. 2 is a cartoon drawing of the active-site residues in the central shell, which are numbered according to A/Tokyo/3/67 (N2). The position of sialic acid bound in the active site is also shown.

Early workers identified the compound 2-deoxy-2,3-dehydro-*N*-acetyl neuraminic acid (DANA) as a potent inhibitor ($K_i = 5.3 \times 10^{-6}\,M$) of neuraminidase and a possible transition-state analog of the enzyme reaction (Meindl, Bodo, Palese, Schulman & Tuppy, 1974). Crystallographic studies of DANA complexed to influenza virus neuraminidase have indeed shown that DANA binds in a similar fashion as NANA in the neuraminidase active site (Varghese *et al.*, 1992; Burmeister *et al.*, 1992, Bossart-Whitaker *et al.*, 1993; Janakiraman *et al.*, 1994). The structural feature making DANA a strong inhibitor is the double bond between atoms C2 and C3, which forces a planar conformation around atom C2, and thus mimicking the transition-state oxocarbonium ion. Further modifications of DANA at various positions increased the inhibitory activity of this class of compounds and the best DANA analog reported has a guanidino group replacing the OH4 group (von Itzstein *et al.*, 1993). The addition of a guanidino group at the C4 position decreases the K_i more than 1000-fold. By intranasal administration to ferrets, the compound was shown to be effective in reducing the symptoms of influenza virus infection and amount of virus shed. Our efforts have been focused on the design of a new class of orally administrable non-carbohydrate inhibitors of influenza virus neuraminidase (Fig. 3). These inhibitors have a potential to be developed as orally active anti-influenza agents.

Experiments

Chemical synthesis

The details of chemical synthesis of benzoic acid derivatives will be published elsewhere (Jedrzejas, Singh, Brouillette, Laver, Air & Luo, 1995*a*,*b*). Fig.

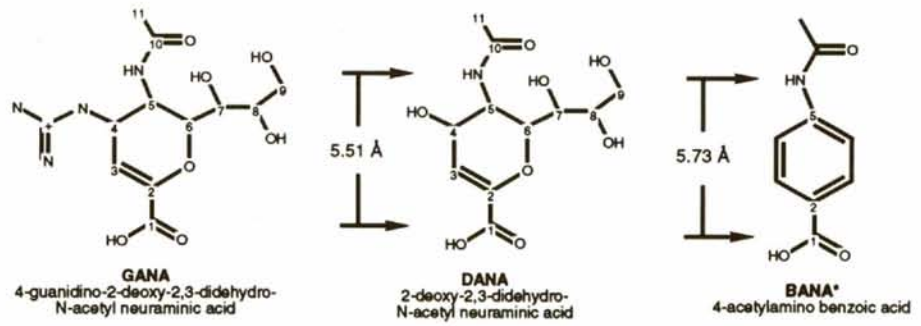


Fig. 3. A cartoon diagram of the active site of A/Tokyo/3/67 N2 neuraminidase interacting with sialic acid. Only the 11 universally conserved residues are presented.

4 shows the structures of the three compounds discussed in this work.

Inhibition assay

The *in vitro* assay for the inhibitory activity of the compounds on influenza virus neuraminidase utilizes two substrates, the fluorogenic 4-methylumbelliferyl-NANA (MUN) and the high molecular weight fetuin, in which case the released sialic acid is detected by the standard colorimetric assay (Aymard-Henry *et al.*, 1973; Lentz, Webster & Air, 1987). All inhibitors were tested with viruses containing N2 (A/Tokyo/3/67) and type B (B/Memphis/3/89) neuraminidases. The results are shown in Table 1.

Crystallographic structure determination

NA heads of A/Tokyo/3/67 (N2) NA were crystallized by the hanging-drop method with a buffer containing 0.1 *M* sodium phosphate, 0.15 *M* sodium chloride and 12.5% PEG 3350. The inhibitor compounds at a concentration of 5 m*M* were soaked into native crystals at room temperature. The soaking buffer consists of 0.10 *M* sodium phosphate, 0.15 *M* sodium chloride, 5% DMSO and 12.5% PEG 4000, pH 6.3.

The diffraction data were collected at room temperature on a SIEMENS multiwire area detector mounted on a Rigaku rotating-anode X-ray generator RU-200 using Cu $K\alpha$ radiation. A total of 400 contiguous frames of 0.15° oscillation were collected for each complex at a crystal-to-detector distance of 16 cm, $2\theta = 20°$. The data were processed using the *XENGEN* (Nicolet Instrument Co., 1987) package. All data were initially collected to 2.4 Å resolution and the data for NA–BANA 105 were recollected to 1.8 Å resolution. The new crystalline form of A/Tokyo/3/67 N2 has a space group of $C222_1$, $a = 120.97$, $b = 141.14$ and $c = 142.16$ Å (Jedrzejas *et al.*, 1995*a*,*b*). After modeling the compounds into the $||F_o| - |F_c||$ electron-density map, the complex structures were refined by using the *X-PLOR* Version 3.1 package (Brünger, 1992) using rigid-body, positional and simulated-annealing procedures. The topology and the parameter files for inhibitors were created based on literature values and a small molecule crystal structure of the 4-(acetylamino)-3-hydroxy-5-nitrobenzoic acid (BANA 105) inhibitor determined for this purpose (Jedrzejas, Singh, Air, Brouillette & Luo, 1995). The final statistics are tabulated in Table 2.

Table 1. *IC_{50} values* (m*M*) *of BANA inhibition of influenza virus neuraminidase by the in vitro assay (Jedrzejas, Singh, Brouillette, Laver, Air & Luo, 1995)*

	MUN		Fetuin	
Compound	N2	B/Mem/89	N2	B/Mem/89
DANA	0.015	0.015	N/D*	N/D
NANA	N/D	> 10	N/D	N/D
105	0.75	0.75	1	1
106	> 10	10	> 10	> 10
108	> 10	> 20	> 10	N/D

* N/D stands for not done.

Results and discussion

Evaluation of the NA–DANA complex structure (Bossart-Whitaker *et al.*, 1993; Janakiraman *et al.*, 1994) revealed that the carboxylate is held by four interactions to three different arginine residues (Arg118, 292, and 371). This three Arg to one COO^- interaction dominates the energetic contribution to the tight binding of DANA to NA. Additionally, the *N*-acetyl group occupies a hydrophobic pocket that is formed mainly by Ile222 and Trp178. Our design began with constructing a cyclic spacer which could place these two essential substituents in the proper position in the active site. The dimensions of the benzene ring suggested that it should be a sufficient spacer to generate a class of non-carbohydrate compounds for further elaboration as neuraminidase inhibitors. In addition, this spacer was chosen for its inherently simple stereochemistry (*i.e.* no chiral C atoms occur at side-chain to ring branches), relative conformational rigidity (the predicted positioning of side-chain functionality is simplified), relative ease of synthesis, possible oral activity and prospects for good metabolic disposition (many useful drugs are based upon benzene). Fig. 3 shows the geometry of DANA and a primitive benzoic acid analog, 4-(acetylamino) benzoic acid. The *meta* positions on the benzene ring, referenced

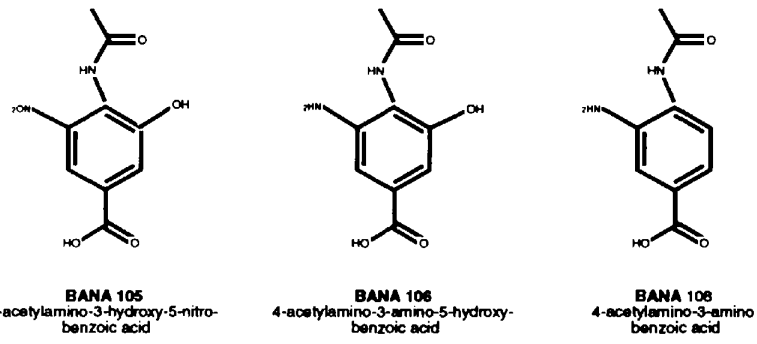


Fig. 4. The chemical structure of three benzoic acid derivatives, 4-(acetylamino)-3-amino benzoic acid (BANA 108), 4-(acetylamino)-3-amino-5-hydroxyl benzoic acid (BANA 106), and 4-(acetylamino)-3-hydroxyl-5-nitro benzoic acid (BANA 105). BANA stands for benzoic acid inhibitor of neuraminidase.

Table 2. *Crystallographic data for NA–BANA complexes (Jedrzejas, Singh, Brouillette, Laver, Air & Luo, 1995)*

Compound	No. of reflections	N2 unit cell ($C222_1$) (Å)	Resolution (Å)	*R* factor %	R.m.s. (bond length, δ Å)	R.m.s. (bond angle, δ °)
105	33264	$a = 119.93$ $b = 139.87$ $c = 140.01$	1.8	22.5	0.013	2.0
106	25898	$a = 120.35$ $b = 139.72$ $c = 140.23$	2.4	21.7	0.015	1.7
108	28488	$a = 121.88$ $b = 140.88$ $c = 141.49$	2.4	20.4	0.013	2.0

to the carboxylate group, are the first place where further modifications could be added since these correspond to the C4 and C6 positions of DANA.

Starting with 4-(acetylamino)-benzoic acid, a series of derivatives have been synthesized, three of which are shown in Fig. 4. These three compounds are not very strong inhibitors of influenza virus neuraminidase (Table 1), but their inhibitory activities are comparable to or better than that of sialic acid in our assays. The preliminary results indicated that the simple benzoic acid derivatives could bind to the active site since sialic acid has been shown to bind to the neuraminidase active site. The crystal structure of N2 (A/Tokyo/3/67) complexed with three benzoic acid compounds were determined as described in Experiments and a portion of the $\left|2|F_o| - |F_c|\right|$ map for the N2 complex with BANA 105 is shown in Fig. 5.

The three BANA compounds have similar orientations in the active site as shown in Fig. 6. The carboxylate group and the acetylamino group in all three compounds have the same contacts with the active-site residues as those of DANA. In addition, the aromatic ring may enhance the binding through aromatic interaction with the OH of Tyr406, the center of which is about 5.3 Å to

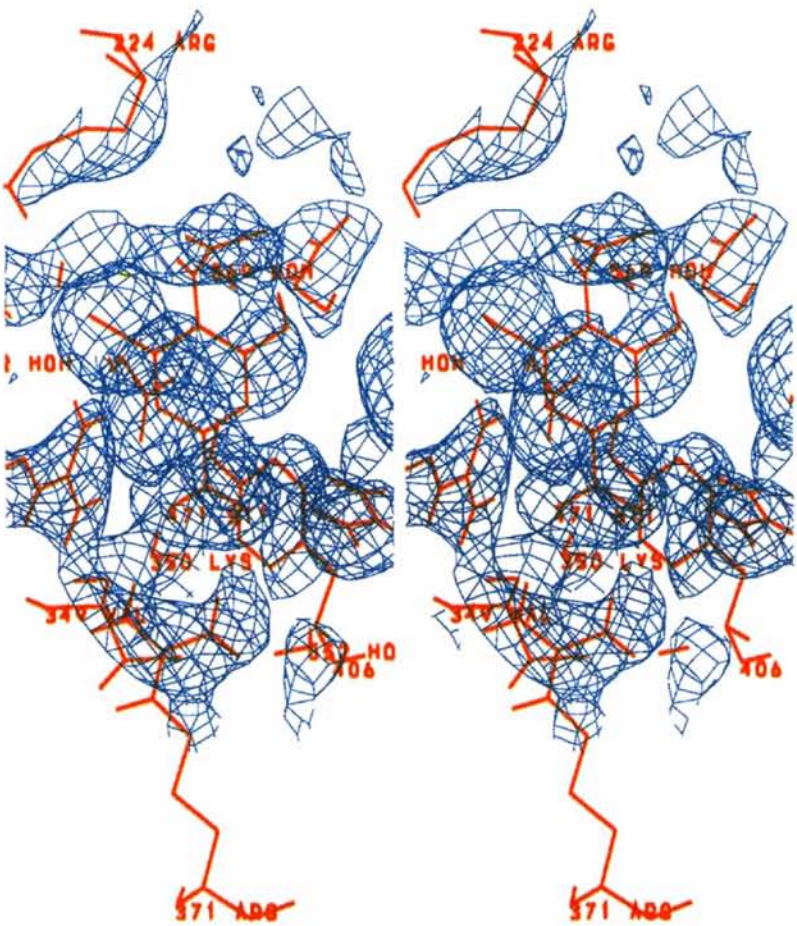
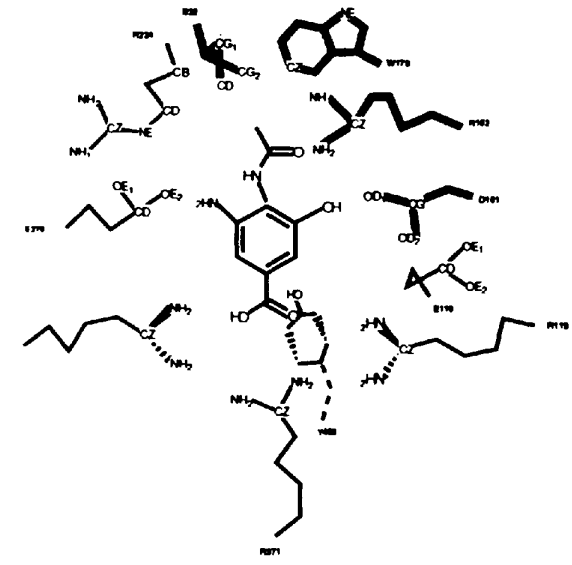


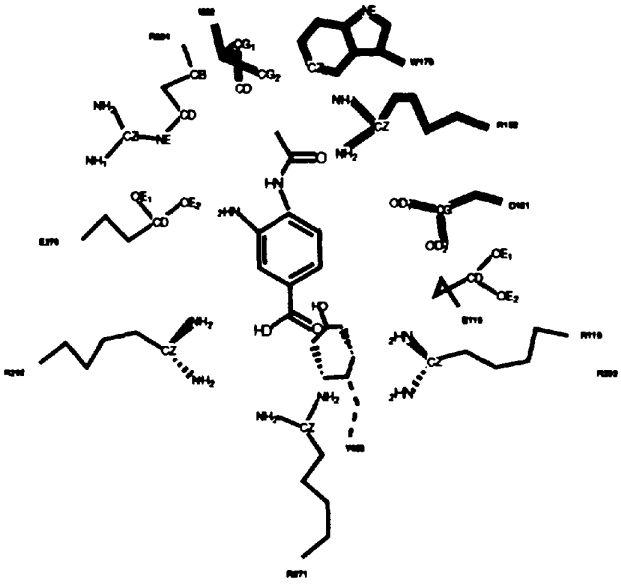
Fig. 5. The $\left|2|F_o| - |F_c|\right|$ electron-density map of the refined NA–BANA 105 complex structure at 2.4 Å resolution. The electron-density was contoured at 1σ level (Jedrzejas, Singh, Brouillette, Laver, Air & Luo, 1995).

the center of the BANA compounds. The NH_2 group of BANA 108 does not take the position of DANA OH4 in the complex. Instead, it is at the position of DANA glycerol group (C5), but making no contacts with any active-site residues. The benzene ring is rotated by about 10° relative to DANA. As a result, the NH_2 group is pointing away from the active site. It appears that the only interactions with the active site are through the carboxylate and acetylamino groups and the benzene-ring rotation is not determined by these interactions. In the BANA 106 complex, the NH_2 group takes the same position as in BANA 108, and the OH group at C4 is involved in hydrogen bonding with Asp151 and Glu113. This additional interaction eliminates the rotation of the benzene ring and results in a slight increase in IC_{50} of BANA 106 over that of BANA 108. The enhanced inhibitory activity of BANA 105, therefore, must be mostly due to the presence of the NO_2 group at the C5 position since it has the same OH hydrogen-bonding interaction as BANA 106. The contribution by the NO_2 group perhaps resides in an increase in acidity of the carboxylate group or the aromatic interaction through electron-attracting effects because it did not make any direct contacts with any active-site residues.

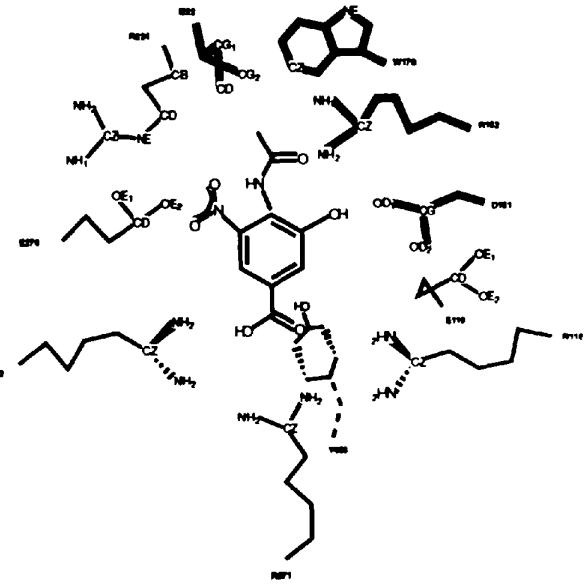
Once we determined the coordinates of NA complexes with these preliminary compounds, we began our next design stage with the aid of two computer programs, *GRID* (Wade, Clark & Goodford, 1993) and *DelPhi* (BIOSYM Technologies, Inc., 1990). The *GRID* program determines energetically favorable binding regions on the known structure for small probing groups such as $—NH_3^+$, $—CH_3$, $—COO^-$ and H_2O. Thus, it permits predictions of favorable changes to the ligand structure by modification of previously observed interactions, or incorporation of additional interactions, into the next generation of benzoic acid targets. A typical *GRID* map calculated by use of a $—NH_3^+$ group in the context of NA–BANA 105 complex is plotted partially in Fig. 7 with a contour level of $-8.5\,kcal\,mol^{-1}$. One of the pockets with a minimum of $-18\,kcal\,mol^{-1}$ is located under the acetylamino group at the C5 position of



BANA 106



BANA 108



BANA 105

Fig. 6. The interaction pattern of BANA inhibitors with the active site of N2 NA; (*a*) BANA 106; (*b*) BANA 108 and (*c*) BANA 105.

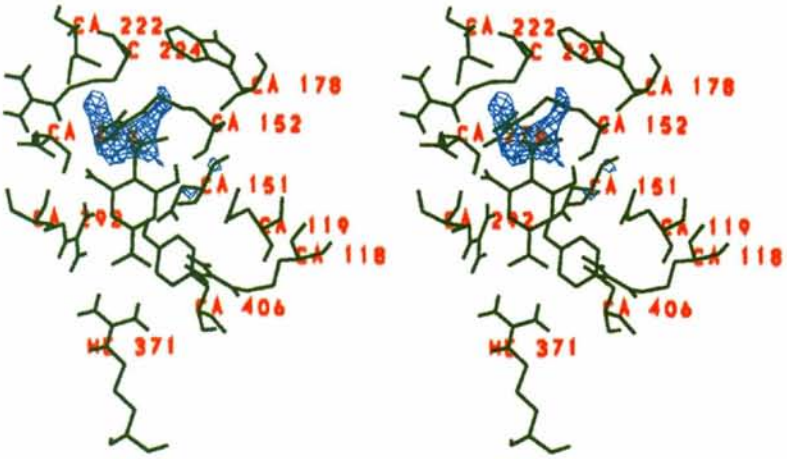


Fig. 7. The *GRID* map calculated using the coordinates of NA–BANA 105 complex and an NH_3^+ probe. The contour level is –8.5 kcal mol^{-1} and only the region close to the active site is plotted (Jedrzejas, Singh, Brouillette, Air & Luo, 1995).

the benzene ring, near residues Glu277 and Glu227. Addition of positively charged groups on the benzoic acid derivatives may enhance binding if these groups fit into those sites.

Attempts were made to estimate the K_i for the proposed new target molecules using *DelPhi* calculations. Sialic acid, DANA and the three BANA compounds were used as benchmarks to calibrate our calculations. The K_i values for sialic acid and DANA are known and the K_i values for BANA 105, 106 and 108 were calculated to be 5.6×10^{-5}, 3.8×10^{-4} and 7.1×10^{-3} *M*, respectively, based on their IC_{50} values (Table 1) (Jedrzejas *et al.*, 1995*a*,*b*). The charges of the compounds were calculated using the MOPAC 6.0 AM1 algorithm (Dewar, Zoebisch, Healy & Stewart, 1985) and those of the protein were taken from the Amber library.

The total free-energy change associated with inhibitor binding was calculated based on the scheme of Gilson & Honig (1988) in which,

$$\Delta G = \Delta G_s + \Delta G_{\text{inter}},$$

where ΔG_s is the change in the solvent interaction energies of the two molecules upon binding,

$$\Delta G_s = \Delta G_{\{s,1\}} + \Delta G_{\{s,2\}},$$

and ΔG_{inter} is the electrostatic interaction energy between the charges of the two molecules in the complex surrounded by solvent,

$$\Delta G_{\text{inter}} = \Delta G_{\{\text{complex}\}} - (\Delta G_{\{1,2\}} + \Delta G_{\{2,1\}}),$$

where 1 and 2 represent protein molecule and inhibitor, respectively. The calculated K_i values (Table 3) have an acceptable agreement with the experimental results in that it at least predicted the correct order with respect to their activity.

Overall, we have developed a working strategy to design target-specific inhibitors based on protein structure and with the aid of computer programs. Using iterative cycles of design, synthesis, complex structure determination and biological testing, we expect that some of the benzoic acid derivatives will become potent inhibitors of influenza virus neuraminidase.

Table 3. *K_i values of BANA compounds calculated by DelPhi (Jedrzejas, Singh, Brouillette, Air & Luo, 1994b)*

Compound	ΔG_s (kcal mol^{-1})	ΔG_{inter} (kcal mol^{-1})	ΔG (kcal mol^{-1})	K_i (mol)
DANA	1.01	−8.36	−7.35	4.8×10^{-6}
105	1.71	−7.58	−5.87	5.6×10^{-5}
106	2.33	−7.06	−4.73	3.8×10^{-4}
108	3.67	−6.64	−2.97	7.1×10^{-3}

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References

AYMARD-HENRY, M., COLEMAN, M. T., DOWDLE, W. R., LAVER, W. G., SCHILD, G. C. & WEBSTER, R. G. (1973). *Bull. WHO*, **48**, 199–202.

BAKER, A. T., VARGHESE, J. N., LAVER, W. G., COLMAN, P. M. (1987). *Proteins Struct. Funct. Genet.* pp. 111–117.

BIOSYM Technologies, Inc. (1990). *DelPhi* 2.0, BIOSYM Technologies, Inc., San Diego, CA 92121, USA.

BOSSART-WHITAKER, P., CARSON, M., BABU, Y. S., SMITH, C. D., LAVER, W. G., & AIR, G. (1993). *J. Mol. Biol.* **232**, 1069–1083.

BRÜNGER, A. T. (1992). *X-PLOR Version* 3.1. *A System for X-ray Crystallography and NMR*. New Haven and London: Yale Univ. Press.

BURMEISTER, W. P., RUIGROK, R. W. H. & CUSACK, S. (1992). *EMBO J.* **11**, 49–56.

DEWAR, M. J. S., ZOEBISCH, E. G., HEALY, E. F. & STEWART, J. J. P. (1985). *J. Am. Chem. Soc.* **107**, 3902–3909.

GILSON, M. K. & HONIG, B. (1988). *Proteins Struct. Funct. Genet.* **4**, 7–18.

ITZSTEIN, M. VON, WU, W.-Y., KOK, G. B., PEGG, M. S., DYASON, J. C., JIN, B., VAN PHAN, T., SYMTHE, M. L., WHITE, H. F., OLIVER, S. W., COLMAN, P. G., VARGHESE, J. N., RYAN, D. M., WOODS, J. M., BETHELL, R. C., HOTHAM, V. J., CAMERON, J. M. & PENN, C. R. (1993). *Nature (London)*, **363**, 418–423.

JANAKIRAMAN, M. N., WHITE, C. L., LAVER, W. G., AIR, G. M. & LUO, M. (1994). *Biochemistry*, **33**, 8172-8179.

JEDRZEJAS, M., SINGH, S., AIR, G. M., BROUILLETTE, W. J. & LUO, M. (1995). *Acta Cryst.* **C51**. In the press.

JEDRZEJAS, M., SINGH, S., BROUILLETTE, W. J, AIR, G. M. & LUO, M. (1995*a*). *Biochemistry*. In the press.

JEDRZEJAS, M., SINGH, S., BROUILLETTE, W. J., AIR, G. M. & LUO, M. (1995*b*). *J. Med. Chem.* Submitted.

LENTZ, M. R., WEBSTER, R. G. & AIR, G. M. (1987). *Biochemistry*, **26**, 5351–5358.

LIU, C. & AIR, G. M. (1993). *Virology*, **193**, 1–5.

MEINDL, P., BODO, G., PALESE, P., SCHULMAN, J. & TUPPY, H. (1974). *Virology*, **58**, 457–463.

Nicolet Instrument Co. (1987). *XENGEN: A Guide to Macromolecular X-ray Data Reduction Using the XENGEN Package for Nicolet Area Detector Systems*, Nicolet Instrument Co., Madison, WI, USA.

PALESE, P. & SCHULMAN, J. L. (1974). *Virology*, **59**, 490–498

VARGHESE, J. N., LAVER, W. G. & COLMAN, P. M. (1983). *Nature (London)*, **303**, 35–40.

VARGHESE, J. N., MCKIMM-BRESCHKIN, J. L., CALDWELL, J. B., KORTT, A. A. & COLMAN, P. M. (1992). *Proteins*, **14**, 327–332.

WADE, R. C., CLARK, K. J. & GOODFORD, P. J. (1993). *J. Med. Chem.* **36**, 140–147.