Structure-guided design of a novel class of benzyl-sulfonate inhibitors for influenza virus neuraminidase

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Influenza NA (neuraminidase) is an antiviral target of high pharmaceutical interest because of its essential role in cleaving sialic acid residues from cell surface glycoproteins and facilitating release of virions from infected cells. The present paper describes the use of structural information in the progressive design from a lead binding ion (a sulfate) to a potent submicromolar inhibitor (K_i 0.13 μ M). Structural information derived from the X-ray structure of an NA complexed with several sulfate ions, in combination with results derived from affinity labelling and molecular modelling

studies, was used to guide design of potent sulfonic acid-based inhibitors. These inhibitors are structural fragments of the polysulfonate triazine dye Cibacron Blue 3GA and represent novel lead scaffolds for designing non-carbohydrate inhibitors for influenza neuraminidases.

Key words: benzyl-sulfonate inhibitor, Cibacron Blue 3GA (CB3GA), influenza, neuraminidase (NA), structure-guided design.

INTRODUCTION

Influenza is a highly contagious, acute viral respiratory disease that occurs seasonally in most parts of the world. Characteristics of epidemics are high attack rates, a short incubation period and the rapid progression of the disease through the population [1]. The hallmark of influenza is this epidemicity and the public health impact of influenza is dramatic. In the U.S.A. between 1977 and 1988, more than 10 000 deaths occurred in each of the seven epidemics, with more than 40 000 deaths occurring in two of these epidemics. The 1918 ‘Spanish Flu’ killed more than 500 000 people in the U.S.A. and a total of 20–50 million people worldwide [2]. Vaccination remains the primary method for prevention of influenza, but vaccine strains must be continually updated and their protective efficacy is limited in patients over 65 years of age, who are the major target group [3]. An alternative lies in antiviral drugs. Influenza NA (neuraminidase) has been proven as a valid therapeutic target for antiviral drugs due to its essential role in the viral replication cycle [4]. NA is thought to enhance viral mobility via hydrolysis of the α -(2,3)- or α -(2,6)-glycosidic linkage between a terminal sialic acid (Neu5Ac) residue and its adjacent carbohydrate moiety on the host receptor. These molecules with terminal Neu5Ac are also the target receptors for the viral HA (haemagglutinin) [5], the major surface glycoprotein on the viral particle surface. NA destroys these HA receptors allowing progeny virus particles budding from infected cell surfaces to be released [6,7].

NA is composed of a tetramer of identical 60 kDa glycosylated subunits. X-ray diffraction studies revealed a polypeptide fold of six β -sheets arranged like the blades of a propeller. Each sheet is composed of four antiparallel strands and has the topology of the letter ‘W’. The active site is centrally located on the NA subunit and lies on the propeller axis at the N-terminal ends of the first β -strand of each sheet [4].

The discovery of inhibitors of NA for the treatment of influenza infection has been an active area of research [8–13]. NA

inhibitors are a class of anti-influenza drugs used for both the prophylaxis and the treatment of influenza virus infections. Modern approaches for finding new leads for therapeutic targets are increasingly based on the three-dimensional structure of receptors [14,15]. The availability of crystal structures of inhibitor–NA complexes has enabled a detailed analysis of the structural basis for potent inhibition [16,17]. For example, with the aid of the X-ray crystal structure of NA complexed with sialic acid or 2,3-dehydrosialic acid, the nanomolar inhibitors zanamivir and oseltamivir were designed as enzyme–substrate mimics [17–19]. These compounds display high potency and specificity both *in vitro* and *in vivo* and are effective prophylactics. Both drugs were introduced into clinical practice in various parts of the world between 1999 and 2002, and share similar potency and specificity for all subtypes of influenza A and B virus NA [18,19].

The development of NA inhibitor-resistant influenza virus strains is a serious concern and there is an urgent need for research on newer anti-NA agents [1,2,9]. In the present paper, we describe a structure-guided approach to the design of a new class of non-carbohydrate benzyl-sulfonate inhibitors, and examine their binding features in the active-site pocket of influenza NA. Non-carbohydrate inhibitors give the advantages of high chemical and metabolic stability compared with dehydropyran-based inhibitors.

MATERIALS AND METHODS

Materials

Cyanuric chloride (2,4,6-trichlorotriazine), CB3GA (Cibacron Blue 3GA), 1-amino-4-bromo-2-methyl-4a,9a-dihydroanthracene-9,10-dione and all other reagents and chemicals were of analytical grade and obtained from Sigma-Aldrich Co. Crystalline BSA (fraction V) was obtained from Boehringer Mannheim.

Abbreviations used: ADH, alcohol dehydrogenase; CB3GA, Cibacron Blue 3GA; cyanuric chloride, 2,4,6-trichlorotriazine; HA, haemagglutinin; NA, neuraminidase; SBS, sulfate binding subsite.

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Expression and purification of NA

A/Beijing/262/95 H1N1 influenza NA was expressed in express-SF+® insect cells using the Baculovirus Expression Vector system (Protein Science Corporation) [20]. Purification of NA was carried out as described in [20].

Synthesis and purification of CB3GA and other analogues

Purification of CB3GA

Solid commercial CB3GA (purity 61.3 %, w/w) was purified to homogeneity in two stages according to the procedure described previously [21]. Briefly, 100 mg of CB3GA was dissolved in 20 ml deionised water by stirring at room temperature (25 °C). The solution was extracted twice with 20 ml diethyl ether, then the aqueous phase was concentrated (approx. 3-fold) on a rotary evaporator and finally the dye was precipitated by 60 ml of cold acetone. The precipitate was filtered through Whatman filter paper and dried overnight under reduced pressure. Dried dye was dissolved in 5 ml water/methanol (50:50, v/v) and filtered through a 0.45-µm-pore-size cellulose membrane filter (Millipore). The dye solution was applied on to a lipophilic Sephadex LH-20 column (2.5 cm × 30 cm) that had been equilibrated with water/methanol (50:50, v/v). The column was developed isocratically at a flow rate of 0.1 ml/min per cm. Fractions (5 ml) were collected and analysed by TLC, and those containing the pure dye were pooled and then concentrated (to approx. 60 % of the original volume) on a rotary evaporator under reduced pressure, before the product was lyophilized and stored at 4 °C in a desiccator.

Analysis of pure dye was performed by TLC and HPLC, whereas dye concentration was determined spectrophotometrically at 620 nm using a molar absorption coefficient (ϵ) of 12.6 litre · mol⁻¹ · cm⁻¹ [21]. Ascending TLC was performed on precoated plastic sheets with silica gel 60 (0.2 mm; Merck) using the solvent system of butanol-1/propanol-2/ethylacetate/water (2:4:1:3). HPLC analysis was carried out on a C18 reverse phase S5 ODS2 Spherisorb silica column (250 mm × 4.6 mm internal diameter). The starting solvent system was composed of 80 % (v/v) methanol and 20 % (v/v) water containing 0.1 % *N*-acetyltrimethylammonium bromide.

The hydrolysed analogue of 1-amino-4-[3-(2,4,6-dichlorotriazinylamino)-4-sulfophenylamino]anthraquinone-2-sulfonic acid was prepared by incubation in 0.1 M Na₂CO₃ at 25 °C for 1 h as described previously [22]. Purification of the analogue was achieved by preparative TLC on silica gel 60 plates, using the solvent system of butanol-1/propanol-2/ethylacetate/water (2:4:1:3). The blue band was scraped from the plate and the product was eluted from the silica gel with water. 1-Amino-4-[(4-amino-3-methylphenyl)amino]-2-methyl-4a,9a-dihydroanthracene-9,10-dione was synthesized as described previously [22]. All dyes were at least 98 % pure.

Synthesis of azido-CB3GA

Azido-CB3GA was synthesized by diazotization of the anthraquinone amino group of CB3GA using the NaNO₂/HCl system followed by nucleophilic displacement of the diazotized intermediate with NaN₃. To a cooled and stirred solution of 20 mg CB3GA in 1.5 ml of 3 M HCl, 10 mg NaNO₂ in 0.2 ml of water was added drop wise. Crushed ice was added to the reaction mixture to keep it cold and it was stirred continuously. Immediately after, a solution of sodium azide (12 mg in 0.5 ml water) was gradually added to the vigorously stirred mixture. Stirring and cooling of the solution in an ice bath was continued for 2 h. Purification of azido-CB3GA was achieved by preparative TLC on silica gel 60 plates using the solvent system of propanol/acetic acid/

water (4:1:1, by vol.). The red band was scraped from the plate and the product was eluted from the silica gel with water. The purity of the product was confirmed by HPLC analysis using a C18 reverse phase S5 ODS2 Spherisorb silica column (250 mm × 4.6 mm internal diameter). The starting solvent system composed of 80 % (v/v) methanol and 20 % (v/v) water containing 0.1 % (w/v) *N*-acetyltrimethylammonium bromide. Azido-CB3GA was at least 98.4 % pure.

Assay of enzyme activity and protein

Enzyme assays were performed at pH 5.9 at 37 °C, according to the method published previously [23], using sialyl-lactose as the substrate. Observed reaction velocities were corrected for spontaneous reaction rates where necessary. All initial velocities were determined in triplicate in buffers equilibrated at constant temperature. Turnover numbers were calculated on the basis of one active site per subunit. Protein concentration was determined by the method of Bradford [24] using BSA (fraction V) as standard. Kinetic inhibition studies were carried out using sialyl-lactose as the substrate at pH 5.9 at 37 °C. No significant interference was observed from the inhibitors. Kinetic constants were deduced from a Dixon plot using the GraFit program (Erithacus Software Ltd., Leatherbarrow, 1998).

Difference spectroscopy

Difference spectral titrations were performed on a Perkin-Elmer Lambda16 double beam, double monochromator UV-VIS spectrophotometer at 37 °C. Enzyme solution (10.5 µM in 1 ml of 20 mM potassium phosphate buffer, pH 5.9) and enzyme solvent (1 ml of 20 mM potassium phosphate, pH 5.9) were placed in the sample and reference black-wall silica cuvettes (10 mm path-length) respectively, and the baseline difference spectrum was recorded in the range 750–500 nm. Identical volumes (2–5 µl) of 0.5 mM dye solutions were added to both cuvettes and the difference spectra recorded after each addition. The difference absorption at $\lambda_{\max}$ was measured relative to a zero-absorbance reference area at 750 nm. The data were analysed using the following equation [25]:

$$\Delta A = \frac{\Delta A_{\max}[\text{ligand}]}{K_D[\text{ligand}]} \quad (1)$$

where ΔA is the difference absorption at $\lambda_{\max}$ after each addition of dye-ligand, and $\Delta A_{\max}$ is the maximum difference absorption at $\lambda_{\max}$ at a saturated dye concentration. The data were fitted to equation 1 using non-linear fitting using the GraFit program.

Synthesis of triazine-based sulfonate analogues

Synthesis was carried out as described previously [26,27], with the following modifications: 1 mM cyanuric chloride was dissolved in 20 ml of ice-cold acetone/water (2:1, v/v) and added slowly to a solution of 1 mM *O*-aminobenzenesulfonic acid in 20 ml ice-cold acetone/water (2:1, v/v) for 20 min with constant stirring. During the course of the reaction the pH was maintained at 6–7 by the addition of 0.5 M NaHCO₃, and the temperature was held between 0 and 4 °C. The progress of the reaction was monitored by TLC [silica; chloroform/methanol/acetic acid (8:4:0.5, by vol.)] until no cyanuric acid was detectable (after approx. 2 h). On completion, 1 mM *O*-aminobenzenesulfonic acid was added to the reaction mixture and then heated to 40–50 °C for 24 h. The reaction was monitored by TLC [silica, chloroform/methanol/acetic acid (8:4:0.5, by vol.)]. During the course of the reaction the pH was maintained at 6–7 by the addition of 0.5 M NaHCO₃. On completion, the reaction product was run on silica

gel plates (20 cm × 20 cm) using chloroform/methanol/acetic acid (8:4:0.5, by vol.) as the solvent. The band corresponding to the bis-substituted triazine product was identified under UV light and excised. The bis-substituted product was eluted from silica by subsequent resuspension in 5 ml of acetone/water (1:1, v/v), repeated three times. The acetone was removed by evaporation and the remaining liquid by lyophilization. The pure product (purity > 95 %) was subjected to MS analysis. The same procedure was followed for the synthesis of other bis-substituted triazine derivatives.

Reaction of azido-CB3GA with NA

Recombinant NA (1 unit) was incubated at 4 °C in the dark with 20 μ M azido-CB3GA in 20 mM potassium phosphate buffer, pH 5.9. The solution (200 μ l) was placed 2 cm in front of a UV lamp and irradiated. After various times of irradiation, an aliquot (10–20 μ l) was diluted 10-fold with buffer and residual enzyme activity was determined. When 1 mM Neu5Ac was tested for its ability to protect against inactivation, it was pre-incubated with the enzyme for 10 min prior to the addition of azido-CB3GA.

Stoichiometry of azido-CB3GA binding to NA

Recombinant N1 NA in 0.1 M potassium phosphate buffer, pH 6, was inactivated with azido-CB3GA at 4 °C. After extensive washing with distilled water, the dye-inactivated enzyme was separated from the free dye by ultrafiltration (Amicon stirred cell 8050 carrying a Diaflo YM10 ultrafiltration membrane with a cut-off point of 10 kDa). Further separation was achieved by gel-filtration chromatography by applying the inactive dye-enzyme complex to a Sephadex G-25 column (9 cm × 1.6 cm) equilibrated with water, and 0.5 ml fractions were collected at a flow rate of 10 ml/h. The solution with dye-inactivated NA was then lyophilized and stored at –20 °C. The lyophilized enzyme covalent complex was dissolved in 8 M urea and the absorbance was determined spectrophotometrically at 520 nm using a molar absorption coefficient of 9.5 litre · mol^{–1} · cm^{–1}. The protein concentration was determined by the method of Lowry et al. [28]; no dye interference was observed in protein determinations.

Chymotryptic digestion of the azido-CB3GA covalent complex and peptide purification using HPLC

Lyophilized NA–azido-CB3GA covalent complex (100 μ g) was dissolved in 1 ml of 0.1 M HEPES/NaOH buffer, pH 7.0, and denatured by the addition of solid urea to a concentration of 8 M in solution. The denatured enzyme was treated with 10 mM dithiothreitol at 25 °C for 1 h, then *N*-ethylmaleimide was added to a final concentration of 15 mM and the solution incubated for a further 30 min at room temperature. The enzyme was then dialysed against 0.1 M ammonium bicarbonate buffer, pH 8.3. The enzyme was digested by 10 μ g of chymotrypsin, and after overnight incubation at 30 °C the mixture was lyophilized and stored dry at –20 °C. Separation of the resulting peptides was achieved on a C18 reverse phase S5 ODS2 Spherisorb silica column (250 mm × 4.6 mm internal diameter). Analysis was performed using a water/acetonitrile linear gradient containing 0.1 % trifluoroacetic acid (0–80 % acetonitrile over 80 min) at a flow rate of 0.5 ml/min and 0.5 ml fractions were collected. The eluents were monitored by spectrophotometry at both 220 nm and 520 nm.

Molecular docking

The complex of NA with sulfate ions (PDB code 2B8H) was crystallized from buffer with ammonium sulfate [29]. This struc-

ture was prepared for docking by removing all crystallographic water molecules, solvent molecules and ions, and adding hydrogen atoms to fulfil unsatisfied valencies using the SYBYL program (SYBYL 6.8 release, Tripos Inc., St. Louis, MO 63144, U.S.A.). The positions of the hydrogen atoms was adjusted manually to ensure correct hydrogen-bonding patterns, then the energy was minimized until the maximum gradient was less than 0.2 kJ/mol per Å (1 Å = 0.1 nm; with protein atoms held fixed). All-atom Kollman charges were assigned to protein atoms. Ionizable amino acids were modelled in their ionized state, with the exception of Asp¹⁵², which was neutral. The ligand was constructed manually and minimized, and Gasteiger charges assigned using the SYBYL program.

The DOCK [31] suite of programs was employed to predict the geometry of the ligand bound to the protein. Electrostatic and van der Waals energies were evaluated on a grid [32] with a 0.3 Å spacing in the region spanning all residues within 12 Å of the active-site Asp¹⁵². A distance-dependent dielectric ($\epsilon = 4r$) and an energy cut-off distance of 10 Å were used to evaluate the interaction energy between protein and ligand. The position of the sulfate-sulfur atoms in the X-ray crystal structure was used to define the docking spheres; matching of sphere centres with ligand sulfur atoms used a maximum distance tolerance of 0.5 Å. Ligand conformational flexibility was explored through torsion angle sampling and minimization. The highest-ranked docked conformation was subjected to energy minimization in SYBYL, with all protein atoms held fixed.

RESULTS AND DISCUSSION

Structure-guided leading ligand design

Analysis of the crystal structure of whale N9 NA (PDB code 2B8H) [29], determined in sulfate buffer, revealed that four sulfate ions are located at well-defined positions with sulfur atoms 6.0–7.6 Å apart (Figure 1A). SBS1 (sulfate binding subsite 1) is located in the position usually occupied by the substrate's carboxylate moiety and forms ionic interactions with the side chain guanidinium groups of the arginine residues at positions 119, 294 and 372 (numbering according to Figure 1B). One of the oxygen atoms of this sulfate anion also comes into close contact (approx. 3.2 Å) with a carboxylate oxygen atom of Asp¹⁵². Outside the substrate-binding site, but close to Asp¹⁵², is a second sulfate anion that forms a hydrogen bond with the N_{δ1} atom of His¹⁵¹ (SBS2). Extending further from the binding site there are two more sulfates (SBS3 and SBS4), the first interacting with the side chain atoms of Arg¹⁵³ and Asn²⁰¹, and the second forms a hydrogen bond with the N_ε of Trp⁴⁵⁸ from a neighbouring monomer. These SBSs represent novel binding sites that may be explored to generate chemical feature-based pharmacophore models of the binding site of this enzyme, since the SBSs are located at generally conserved positions overlapping the active site (Figure 1B).

In an effort to discover novel, non-carbohydrate inhibitors of influenza virus NA we hypothesized that compounds that contain sulfonate groups in appropriate positions, which are able to occupy the SBSs, might be bound tightly by the enzyme. A well-known polysulfonate molecule that acts as an inhibitor for several enzymes and proteins is CB3GA (compound 4 shown on Figure 2). CB3GA has been utilized in biochemical and enzymological [33] studies, and in protein purification [34]. It has been well established that CB3GA tends to bind preferentially to the active-site regions of globular proteins and mimic the binding of the naturally occurring anionic substrates and coenzymes such as NADH, ATP, coenzyme A, flavins and folate [21,22,25,35].

Figure 1 Structural analysis of NA

(A) The sulfate binding sites SBS1–4 of NA as observed from the 2.2 Å crystal structure (PDB code 2B8H; [29]). Sulfate anions are presented as thin rods and the side-chains of binding residues are displayed as heavy rods. (B) The sulfate binding residues of influenza A NAs of different subtypes (N1 to N9). * Indicates amino acids that form the sulfate binding sites SBS1–4. The partial alignments were produced using ESPript. Abbreviations for NAs are in parentheses: A/Beijing/262/95 (N1); A/chicken/California/9420/2001 (N2); A/turkey/California/6878/79 (N3); A/mink/Sweden/E12665/84 (N4); A/shearwater/Australia/1/72 (N5); A/sanderling/Delaware/1258/86 (N6); avian/FPV/Weybridge (N7); A/turkey/Canada/63 (N8); A/whale/Maine/1/84 (N9).

The specific interaction of sulfonic groups with protonated amino acid residues (arginine, histidine and lysine) on the biomolecules has been investigated recently by Friess and Zenobi [36], who

showed that the sulfonic groups of CB3GA bind preferentially to protonated amino acid residues (arginine, histidine and lysine) on the biomolecules to form noncovalent complexes, whereas other

Figure 2 The structure of CB3GA analogues used in the present study

Compound 1: 1-amino-4-bromo-9,10-dioxo-4a,9,9a,10-tetrahydroanthracene-2-sulfonic acid. Compound 2: 1-amino-4-[(4-amino-3-sulfophenyl)amino]-9,10-dioxo-4a,9,9a,10-tetrahydroanthracene-2-sulfonic acid. Compound 3: hydrolysed 1-amino-4-[3-(4,6-dichlorotriazin-2-ylamino)-4-sulfophenylamino]anthraquinone-2-sulfonic acid. Compound 4: Cibacron Blue 3GA. Compound 5: 2-[(4-chloro-6-[(2-sulfophenyl)amino]-1,3,5-triazin-2-yl)amino]benzenesulfonic acid. Compound 6: 3-[(4-chloro-6-[(3-sulfophenyl)amino]-1,3,5-triazin-2-yl)amino]benzenesulfonic acid. Compound 7: 4-[(4-chloro-6-[(4-sulfophenyl)amino]-1,3,5-triazin-2-yl)amino]benzenesulfonic acid. Compound 8: 3-[(4-anilino-6-chloro-1,3,5-triazin-2-yl)amino]benzenesulfonic acid. Compound 9: 6-chloro-*N,N'*-diphenyl-1,3,5-triazine-2,4-diamine.

sulfonates of simpler structure, such as naphthalene sulfonic acid derivatives and 1-anilino-naphthalene-8-sulfonic acid, were found to bind preferentially to arginine. The present paper determines whether sulfonate molecules [e.g. CB3GA and its fragments (compounds 1–5; Figure 2)] are able to occupy the SBSs and inhibit NA, with the potential that it may lead to a new family of sulfonate-substituted affinity ligands.

Investigation of the interaction of NA with CB3GA and its fragments by difference spectroscopy and kinetic inhibition studies

The interaction of CB3GA with N1 NA was assessed by difference spectroscopy in the 750–500 nm region by studying the perturbation of the absorption spectrum of this compound. In the presence of NA the absorption spectrum of the dye undergoes a red shift, producing difference spectra consisting of positive maximum at the 675 nm region. Figure 3(A) depicts original difference spectra. CB3GA displays two broad peaks (a 675 nm positive and a 590 nm negative) and an isosbestic point at 630 nm, following a shift from its original absorbance maximum (612 nm). The shape and the wavelengths that correspond to the maximum and minimum of the dye spectrum remained unchanged during titration experiments, furthermore, no time-dependent changes in absorbance were observed. These findings indicate no irreversible binding of dye to NA, and formation of a single type of complex. The increase in the absorbance at positive maximum after each addition of the dye exhibits a hyperbolic dependence on the concentration of dye, indicating the formation

of a dye–NA complex (Figure 3B). This phenomenon was useful for the calculation of the K_D for the dye–NA complexes. The results showed that NA interacts with CB3GA with high affinity, $2.1 \pm 0.2 \mu\text{M}$ (Table 1).

Subramanian [35] has shown that the shape of the spectrum describing the dye–enzyme complex is characteristic of the nature of the interaction. The difference spectra of the CB3GA in a high salt environment is characterized by positive maximum (at 690 nm) and negative double minima (at 630 and 585 nm), while the difference spectrum of the dye in binary aqueous solvents displayed a positive peak and a positive shoulder at approx. 655 and 610 nm respectively, with a small negative contribution below 550 nm. Figure 3(A) therefore appears to show that the anthraquinone chromogen of CB3GA is located in a rather hydrophobic environment. This observation is supported further by the molecular modelling studies.

To analyse which fragment of the CB3GA molecule is responsible, or necessary, for strong binding to NA, the ability of structural fragments of CB3GA (compounds 1–3 and 5; Figure 2) to bind to NA was investigated by kinetic inhibition analysis and the results are presented in Table 1. The results showed that each of the CB3GA fragments exhibits a different affinity for NA compared with the parent CB3GA. In general, all analogues are able to bind NA, however, the relative affinity of analogues differs by approx. 220-fold. The data presented in Table 1 points to the conclusion that smaller inhibitors (e.g. compound 5, the terminal fragment derived from the parent CB3GA) exhibits highest affinity for NA. The anthraquinone moiety (compound 1)

Figure 3 Difference spectra titrations

(A) Difference spectra of CB3GA (from top to bottom: 15, 5 and 2 μM CB3GA) in the presence of recombinant NA at pH 5.9 and 37°C. (B) The difference absorbance at 675 nm as a function of the total CB3GA concentration (2–20 μM) for NA.

Table 1 Binding characteristics of CB3GA and its fragments to recombinant NA

The structures of analogues are depicted in Figure 2. Inhibition constants (K_i) were determined by kinetic inhibition studies.

Compound	K_i (μM)
1	56.5 ± 4
2	12.5 ± 2
3	12.8 ± 1.9
4	$2.9 \pm 0.2^*$
5	0.28 ± 0.04
6	0.13 ± 0.05
7	17.1 ± 1.9
8	4.3 ± 0.7
9	No detectable binding

* The value of 2.1 ± 0.2 μM for compound 4 was determined by difference spectra titrations.

does not appear to contribute significantly to the binding affinity.

Localization of CB3GA binding site by photoaffinity labelling experiments

Inactivation of NA by azido-CB3GA

To characterize and locate precisely the CB3GA-binding site of NA, affinity-labelling experiments were performed. Affinity

Figure 4 Affinity labelling of NA

(A) The structure of azido-CB3GA. (B) Photoaffinity labelling of NA by azido-CB3GA at pH 5.9 and 4°C. Recombinant NA was incubated in the absence (filled rhomb) or in the presence of azido-CB3GA at concentration of 20 μM (filled square). At the times indicated, aliquots were withdrawn, diluted and assayed for enzymatic activity. Enzyme was also incubated in the presence of 20 μM azido-CB3GA and 1 mM Neu5Ac (open square).

labelling is a useful tool for the identification and probing of specific, catalytic and regulatory sites in purified enzymes and proteins [37,38]. In the present report, a new photoactive derivative of CB3GA (azido-CB3GA, Figure 4A) was synthesized and evaluated for its ability to react covalently with NA.

NA in the presence of azido-CB3GA was inactivated by irradiation. Figure 4(B) illustrates the decrease in the enzyme activity as a function of time of irradiation. As controls, incubation of enzyme with azido-CB3GA in the dark, or irradiation of the enzyme under the same conditions but without the reagent, caused no significant loss of enzyme activity. These results show that loss of enzyme activity was due to the reaction of enzyme and photoactivated reagent. The rate of inactivation under these conditions was determined to be 0.017 ± 0.002 per min. The inactivation of NA by azido-CB3GA was irreversible and enzyme activity could not be recovered by extensive dialysis or after gel-filtration chromatography on a Sephadex G-25 column in the absence or presence of 8 M urea.

To determine the stoichiometry of dye binding to NA, NA was completely inactivated by the dye and the dye–enzyme covalent complex was resolved from free dye by gel filtration on Sephadex G-25 and ultrafiltration. The molar ratio of [dye]/[NA active site] was determined by measuring the dye spectrophotometrically in urea solution, and the protein by the method of Lowry et al. [28]. The molar ratio of dye/NA active site was 0.94 ± 0.15 , using a subunit molecular mass of 51.7 kDa, showing that the dye reacts stoichiometrically with the enzyme and therefore indicates a specific interaction between dye and protein.

The ability of specific ligands (e.g. substrates and inhibitors) to prevent enzyme inactivation by an irreversible inhibitor is

Figure 5 Interaction of CB3GA with NA from docking calculations

The solvent accessible surface of the sulfate binding sites are coloured blue (SBS1), red (SBS2) and green (SBS3). The sulfate ions identified in the X-ray analysis are displayed as thin rods. Side-chains of specific residues (numbering according to 2B8H; [29]) contributing to the SBSs and interacting with the ligand are presented as heavy rods beneath the transparent surface. Atoms are coloured white (H), cyan (C), blue (N), red (O), yellow (S) and grey (Cl). The sulfur atoms of the ligand coincide with the sulfate sulfur atoms. The Figure was prepared using the DINO program (<http://www.dino3d.org>).

the usual criterion used in arguing for binding to site-directed modifications [21,22,25,37,38]. Figure 4(B) shows that the rate of enzyme inactivation by azido-CB3GA decreased in the presence of 1 mM Neu5Ac, indicating that the dye interacts with the enzyme at the substrate-binding site.

Isolation and analysis of peptides from NA modified by azido-CB3GA

Modified NA was subjected to chymotrypsin digestion followed by fractionation on a reverse-phase HPLC column. Essentially, a single red peak (azido-CB3GA-labelled peptide) was eluted from the column. The red peak was freeze-dried and subjected to amino acid analysis and sequencing. The overall recovery of modified peptide, based on the initial amount of modified enzyme was 36%. Edman sequence analysis of the labelled peptide gave the sequence EECSCYPDTGTVMCVCDNW, where X indicates that no phenylthiohydantoin derivative was detected in the cycle. By comparison with the amino acid sequence of NA, the X in the peptide was identified as Arg²⁹⁴ (numbering according to Figure 1B), indicating that the side chain of Arg²⁹⁴ is the group responsible for reacting with the azido group of the dye.

Mapping of the CB3GA binding site by molecular modelling studies

Molecular modelling studies were employed to provide a detailed picture of CB3GA interaction with NA. CB3GA was docked to the active site of NA with its three sulfate groups located in SBSs 1, 2 and 3. In addition to the interactions of the sulfate groups with the protein mentioned above for the individual sulfate groups, the anthraquinone moiety makes hydrophobic contacts with the aliphatic atoms of the side chains of Ala²⁴⁸, Thr²⁴⁹ and Asn³⁴⁸ (numbering according to Figure 1B). The final geometry of the bound ligand is presented in Figure 5. The results listed in Table 1 and from the molecular modelling studies suggest that the binding of CB3GA and its fragments to NA may be primarily achieved by the sulfonate moieties that provide the driving force for

positioning and recognition of the analogues. This conclusion is also supported by the crystal structures of CB3GA–horse liver ADH (alcohol dehydrogenase) [39] and CB3GA–glutathione S-transferase complexes [40]. The interaction of sulfonate groups of CB3GA with an arginine has been observed by Biellmann et al. [39]. Lowe et al. [41] have used these results to investigate the interaction of CB3GA and ADH in more detail. They found that different parts in the molecular structure of CB3GA exhibited completely different reactivities, except for two of the sulfonate groups [41]. The terminal sulfonate group as well as the sulfonate group in the linking diaminobenzene unit were always found to interact with two arginine residues of ADH. Later, Burton et al. [42] considered the role of the third sulfonate group bound to the anthraquinone-system. Too close proximity to the amino group inhibits a strong sulfonate dye–protein interaction. Burton et al. [42] also demonstrated the strong affinity of sulfonates to positively charged guanidino groups; almost no affinity between dye and protein remained after substitution of the guanidino with a trimethylammonium group. In the case of the CB3GA–glutathione S-transferase complex the sulfonic group linked on the anthraquinone ring interacts with the guanidyl group of Arg¹³ [40]. In the model of CB3GA docked to NA the distance between the anthraquinone amine and Arg²⁹⁴ is 3.4 Å. Thus, replacement of this amine with an azide would place the azide in close proximity to Arg²⁹⁴, in agreement with the photoaffinity labelling experiments that identified Arg²⁹⁴ as the reactive residue.

Analysis of NA inhibition by benzyl-sulfonate analogues

To analyse the contribution of the sulfonic group orientation (e.g. ortho-, meta-, para-) in compound 5, two additional analogues were synthesized and their inhibitory activity was evaluated for NA using kinetic analysis. The inhibition constants (K_i) obtained are summarized in Table 1. All analogues exhibited a competitive type of inhibition with respect to substrate, indicating that the analogues bind at the substrate-binding site of NA. Substitution on the triazine ring for 3-aminobenzyl-sulfonic acid formed compound 6 (Figure 2), which exhibited a higher affinity for NA compared with compound 5. On the other hand, the isomer bearing bis-substituted 4-aminobenzyl-sulfonic acid (compound 7) clearly displayed a higher K_i value corresponding to approx. 132-fold reduction in affinity compared with compound 6. The importance of the sulfonic group in the analogues was demonstrated by compounds 8 and 9. For example, compound 8, with a single sulfonic group, displayed a lower affinity for NA compared with compound 6, whereas compound 9 lacking both sulfonic groups did not show any appreciable binding.

Molecular modelling studies were also employed to provide a detailed picture of the interaction of compounds 5, 6 and 7 with NA. This showed that the analogues docked with the sulfonic groups occupying SBSs 1 and 2 (Figure 6), which is consistent with their function as competitive inhibitors of NA. This mode of binding differs from that predicted for CB3GA (Figure 5) in which the sulfonic groups of the bis-benzylsulfonate-triazine moiety bind SBSs 2 and 3. The chlorine of compound 7, although largely solvent-exposed, interacts with the hydrophobic pocket occupied by the anthraquinone of CB3GA. By contrast, the chlorine atoms of compounds 5 and 6 occupy a hydrophobic region that does not directly participate in substrate binding. The results from the inhibition studies (Table 1) in combination with the molecular modelling studies allows us to speculate on the origins of binding of sulfonic acid analogues to NA. The positioning of the analogues with NA may be primarily achieved by ionic interactions of the sulfonate moiety with the side chain guanidinium groups of the arginine residues at positions 119,

Figure 6 Modelling of benzyl-sulfonate analogues in the active site of NA

Left-hand panel: compound 5, 2-aminobenzyl-sulfonic acid. Middle panel: compound 6, 3-aminobenzyl-sulfonic acid. Right-hand panel: compound 7, 4-aminobenzyl-sulfonic acid. The colours of the surface of NA and atoms are the same as detailed in Figure 5. The sulfate ions observed in the X-ray structure are presented as thin rods.

294 and 372 (subsite AS1). These interactions may provide the driving force for positioning and recognition of the analogues. This is in line with the observation that compound 8, with a single sulfonic group, displays good affinity for NA, whereas compound 9, lacking both sulfonic groups, does not show any appreciable binding to NA. Compounds possessing two sulfonate groups will have a greater affinity than those with one or no sulfonate. Further support may be obtained from the work of Friess and Zenobi [36], who employed matrix-assisted laser-desorption/ionization MS to demonstrate that sulfonic acid derivatives bind to arginine with higher selectivity compared with other basic residues (e.g. lysine and histidine) of proteins. The high selectivity of sulfonate compounds for arginine is not fully understood. The principal arginine-sulfonate interaction is electrostatic in nature (a salt bridge), enhanced by ionic hydrogen-bonds that are especially favourable because of the near-perfect shape complementarities of the two bidentate binding partners [36].

Conclusions

The polysulfonate molecule CB3GA and several of its structural components were shown to bind to NA. Difference spectroscopy analysis indicated that these compounds bound reversibly to the substrate binding-site. CB3GA binds NA with high affinity, $2.1 \mu\text{M}$; a derivative lacking the anthraquinone moiety binds with K_i of $0.13 \mu\text{M}$.

Docking studies of CB3GA to NA confirmed high structural complementarities between the sulfate binding sites observed in the X-ray crystal structure and the sulfonates of the inhibitor. The anthraquinone component of the molecule was predicted to contact several hydrophobic residues on the rim of the active-site pocket, which was supported by the difference spectroscopy analysis. An azido derivative of CB3GA was shown to covalently bind specifically to an active-site arginine residue in excellent agreement with the predicted conformation of CB3GA with NA.

The concept of selective staining of animal tissue and microbial organisms by dyes performed by Ehrlich laid the foundations of present-day chemotherapy [43]. During the 1960s, CIBA introduced the Cibacron range of dyes, which have found use not only as dyes but also in biomedical research. Although CB3GA and its structural components exhibit wide specificity [21,35,39], they provide access to leading structures in the development of non-carbohydrate inhibitors to influenza NA.

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