Antiviral Research 123 (2015) 216–223

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Catalytic mechanism and novel receptor binding sites of human parainfluenza virus type 3 hemagglutinin-neuraminidase (hPIV3 HN)

Victor A. Streltsov *, Pat Pilling, Susan Barrett, Jennifer L. McKimm-Breschkin *

CSIRO Manufacturing, 343 Royal Parade, Parkville, Victoria 3052, Australia

ARTICLE INFO

Article history:
Received 20 July 2015
Revised 24 August 2015
Accepted 26 August 2015
Available online 10 September 2015

Keywords:
Human parainfluenza virus type 3 (hPIV3)
Hemagglutinin-neuraminidase (HN)
structure
Catalytic mechanism
Receptor binding sites
Neuraminidase inhibitors

ABSTRACT

The human parainfluenza virus type 3 (hPIV3) hemagglutinin-neuraminidase (HN) has opposing functions of binding sialic acid receptors and cleaving them, facilitating virus release. The crystal structure of hPIV3 HN complexed with the substrate analogue difluorosialic acid (DFSA) revealed that catalysis by HN involves the formation of a covalently linked sialosyl-enzyme intermediate which was trapped along with a transition-state analogue resembling an oxocarbenium ion. This mechanism of enzyme catalysis was also confirmed in the crystal structure of the influenza N9 neuraminidase complexed with DFSA. Additionally, novel secondary receptor binding sites were identified in the hPIV3 HN–DFSA complex including one near the catalytic cavity which upon binding DFSA imposes subtle changes and may help the HN balance the opposing functions. Multiple receptor binding sites may increase avidity to facilitate cell binding and fusion promotion. The secondary receptor binding sites in the paramyxoviruses are so far unique to each virus type.

1. Introduction

The *Paramyxoviridae* family of viruses includes the human Parainfluenza viruses types 1–3 (hPIV1–3) causing acute respiratory infections in young children, the elderly, and the immunocompromised, and the avian Newcastle Disease Virus (NDV). These viruses have two membrane glycoproteins, the hemagglutinin-neuraminidase (HN), and the fusion (F) protein. The HN has multiple functions including receptor binding, cleavage of sialic acids, facilitating virus release and spread, as well as interacting with the F protein promoting membrane fusion (Porotto et al., 2003). Neuraminidase (NA) inhibitors including zanamivir, designed to target the influenza virus NA, and 2-deoxy-2,3-dehydro-N-acetyl neuraminic acid (DANA) can also inhibit not only the catalytic function of the HN, but also interfere with receptor binding (Porotto et al., 2004). Thus they have been used to probe whether these multiple functions are carried out at a single site, or at discrete sites. Structural studies of a thialoside inhibitor with the HN of NDV identified two distinct sites (Zaitsev et al., 2004). Functionally one site carries out receptor binding and catalysis, and the second site is involved in receptor binding and fusion promotion (Bousse and Takimoto, 2006; Porotto et al., 2004, 2006, 2012).

Our previous structural studies on hPIV3 HN (Lawrence et al., 2004) showed no distinct catalytic and receptor binding sites, however like NDV a novel neuraminic acid analogue may be needed to identify this elusive second site. The recently described 2,3-difluorosialic acid (DFSA) mechanism based inhibitors of influenza virus N2 NA (Vavricka et al., 2013), and human sialidase hNeu2 (Buchini et al., 2014) and hPIV3 (Dirr et al., 2015) showed that DFSA and its derivatives form covalent intermediates only, linked to a tyrosine in the NA active site, thus leading to inhibition of enzyme activity. Results showed that the influenza NA operates through a similar mechanism to bacterial and trypanosomal NAs (Watts et al., 2006) utilising a covalent sialosyl-enzyme intermediate. However, both the formation and hydrolysis of the glycosyl enzyme species are hypothesised to proceed *via* transition states with considerable oxocarbenium ion character, partially observed in DFSA based inhibition of the influenza virus N9 NA (Kim et al., 2013), but this has never been clearly demonstrated. The introduction of an electronegative fluorine atom adjacent to the anomeric position is expected to slow both the rates of glycosyl enzyme formation and breakdown. However, the inclusion of a reactive leaving group, such as fluoride, at the anomeric centre of the inhibitor serves to accelerate formation of the glycosyl enzyme intermediates with these inhibitors (Berkowitz et al., 2008; Watts et al., 2006).

Abbreviations: hPIV3, human parainfluenza virus type 3; DFSA, 2,3-difluorosialic acid; NA, neuraminidase; HN, hemagglutinin-neuraminidase.

* Corresponding authors.
E-mail addresses: victor.streltsov@csiro.au (V.A. Streltsov), pat.pilling@csiro.au (P. Pilling), susan.barrett@csiro.au (S. Barrett), mck245@csiro.au (J.L. McKimm-Breschkin).

http://dx.doi.org/10.1016/j.antiviral.2015.08.014
0166-3542/

We hypothesised that fluorinated substrate analogues, such as DFSA, which slow the rates of enzymatic reaction, are able to trap enzyme bound species resembling intermediates or "transition states", including a glycosyl-enzyme covalent intermediate as well as an oxocarbocation transition-state analogue, a sialosyl cation. We propose that an oxocarbocation analogue is stabilized through electrostatic interactions with the anionic elements of the substrate, leaving group and binding interactions that enforce a flattened sugar conformation. This promotes stabilizing hyperconjugative effects in the intermediate similar to that observed in ribozyme (Dinner et al., 2001) and uracil DNA glycosylase (Unrau and Bartel, 2003; Werner and Stivers, 2000).

Using a combination of enzyme inhibition assays and X-ray crystallography (at 2.5 Å resolution) we demonstrated that DFSA bound to hPIV3 HN, not only forming a covalently linked sialosyl-enzyme intermediate as recently demonstrated by others at 3 Å resolution (Dirr et al., 2015), but we also identified a trapped sialosyl cation transition-state analogue. Additionally, the structures of the HN–DFSA complex revealed novel secondary receptor binding sites for the ligand, which have remained elusive to other investigators. In this study, we confirmed the catalytic role of the conserved active-site tyrosine of parainfluenza hPIV3 HN and influenza N9 NA, and showed that both the PIV HN and the influenza NA may function through a mixed manner involving both a direct hydroxylation mechanism and a covalent enzyme-sialosyl intermediate co-existing in equilibrium.

2. Materials and methods

2.1. Inhibitors and HN inhibition assays

Zanamivir was provided by GSK (Stevenage, UK) and 2,3-difluorosialic acid (DFSA) was kindly provided by Dr. Stephen Withers (UBC, Canada) (Kim et al., 2013). DANA was from Sigma (USA). The fluorescent substrate 4-methylumbelliferyl N-α-D-neuraminic acid (MUNANA Carbosynth, Berkshire, UK) was used for enzyme assays. K_m values were calculated using GraphPad Prism as previously described (Barrett et al., 2011) with MUNANA concentrations ranging from 0.125 to 10 mM, using a pH 4.5 acetate buffer. Results are the means of duplicate experiments.

As the NA inhibitors (NAI) are described as slow binding inhibitors for influenza (Pegg and von Itzstein, 1994; von Itzstein et al., 1993), this means that preincubation with inhibitor is required for its maximum occupancy of the active site. A single time point has been routinely used for measuring the IC_{50}. However, we have shown that the IC_{50} and the K_i change with time due to slow binding or dissociation. Hence we have developed a simple 96-well based real time IC_{50} kinetics assay which demonstrates whether an inhibitor is fast or slow binding by comparing the IC_{50}s over 10 min periods for 60 min, with and without preincubation of NAI and virus (Barrett et al., 2011). This method has now demonstrated differences in kinetics for many different NAIs binding to wild type and mutant influenza NAs (McKimm-Breschkin and Barrett, 2015; McKimm-Breschkin et al., 2013a, 2013b; Oakley et al., 2010). Binding of DFSA to hPIV3 HN was evaluated in the IC_{50} kinetics assay using a pH 4.5 acetate buffer and 0.5 mM MUNANA (Barrett et al., 2011). Zanamivir and DANA were used as controls as they are known to bind to hPIV3 HN (Lawrence et al., 2004), although their binding kinetics have never been studied. Additionally the K_i was calculated for the 50–60′ time period as previously described (Barrett et al., 2011). Results are the mean of duplicate assays.

To determine if the inhibitors could interfere with receptor binding we tested them in a hemagglutination inhibition assay using turkey blood cells (more details of methods in Supplementary material).

2.2. Protein expression and purification

Purification methods for hPIV3 HN and influenza N9 NA proteins were as previously described by Lawrence et al. (2004) and Varghese et al. (1997), respectively. Brief details are given in Supplementary material.

2.3. Crystallization

The hPIV3 HN protein was concentrated to 4.4 mg/ml and co-crystallized with 10 mM DFSA dissolved in water by vapour diffusion hanging drops containing equal amounts (1 μl) of protein, well solution, and DFSA at 293 K. Crystals were grown from 0.1 M Hepes (pH 7.5), 1.0 M $(NH_4)_2SO_4$, 1.0 M Li_2SO_4. Crystals appeared after 1 week and were taken for X-ray data collection in 3 weeks. The influenza N9 NA was crystallized in potassium phosphate buffer (1.7 M pH 6.7) as previously described (Varghese et al., 1997). The N9–DFSA complex was prepared by soaking crystals for ~190 min at 4 °C in cryo-protectant solution containing crystallization well solution plus 20% glycerol and 2 mM concentration of inhibitor (Kim et al., 2013). Further details are in Supplementary material.

2.4. X-ray data collection, structure determination, and refinement

X-ray diffraction data sets for single crystals of hPIV3 HN and N9 NA with inhibitor complexes at 2.5 Å and 2.1 Å resolution, respectively (in well solution plus 15% (v/v) glycerol as cryo-protectant) were collected at −173 °C with 180°/360° (hPIV3 HN/N9 NA) rotation, 1° oscillation and 5 s/1 s (hPIV3 HN/N9 NA) exposure with wavelength of 1.0000/0.95369 Å (hPIV3 HN/N9 NA) using the Photon Factory BL5A beamline, Tsukuba, Japan, and the MX1 beamline at the Australian synchrotron, respectively. The data sets were processed with HKL2000 (Otwinowski and Minor, 1997). Further data collection statistics are given in Table 2. The positions of two hPIV3 HN and one N9 NA molecules were identified in the asymmetric units by PHASER (McCoy et al., 2007) molecular replacement using the structure of hPIV3 HN (PDB entry: 1V3C) (Lawrence et al., 2004) and the structure N9 (PDB entry: 1NNC) (Varghese et al., 1997) without ligands, respectively. The structures with protein molecules alone were refined and then the DFSA inhibitors were built into the observed residual electron densities. Initial refinement of the inhibitor position did not account for all the residual density observed in both hPIV3 HN and N9 NA protein structures. In particular there was slight negative residual density around the F3 atom and positive residual density in the region between the inhibitor and the Y530/Y406 (hPIV3 HN/N9 NA) residue. The continuous bridge of residual electron density between the C2 atom of the inhibitor and hydroxyl oxygen of the aromatic side chain of Y530/Y406 residue suggested the C–O covalent bond, however the refined bond distance was ~1.9 Å. Next, the covalent intermediate species (FSI) accompanied by unsaturated forms of fluorosialic acid as oxocarbenium ion transition-state analogues (DF4) were refined in both hPIV3 HN and N9 NA protein structures. The geometry of DF4 is very similar to that of DANA in which the carboxylate group is coplanar with the ring plane with considerable sp^2 hybridization at the anomeric carbon. Since a fluorine atom is refined at the chiral position, the double bond is between the ring oxygen and the anomeric carbon, with positive charge at the oxygen resembling an oxocarbonium ion transition state. Adjusting the occupancies of two bonded and non-bonded conformations the length of the covalent linkage for bonded species was refined to a chemically sensible value of ~1.4 Å. After that the corresponding LINK record was added to the PDB files to define this bond. The occupancies of two conformations were refined to 70% and 30% (hPIV3 HN for both chains A and