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Substituent effects on the binding of natural product anthocyanidin inhibitors to influenza neuraminidase with mass spectrometry

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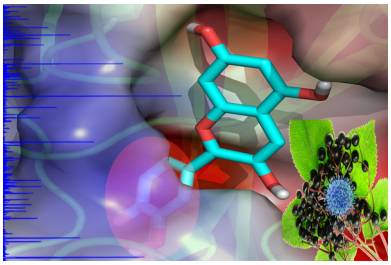
HIGHLIGHTS

- MALDI MS approach identifies differences in binding affinity of similar inhibitors.
- Relative reduction in ion signal is in accord with their inhibitory potential.
- Approach is a sensitive and high-throughput molecular screen of drug binding.

GRAPHICAL ABSTRACT



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ABSTRACT

The binding of three closely related anthocyanins within the 430-cavity of influenza neuraminidase is studied using a combination of mass spectrometry and molecular docking. Despite their similar structures, which differ only in the number and position of the hydroxyl substituents on the phenyl group attached to the chromenylium ring, subtle differences in their binding characteristics are revealed by mass spectrometry and molecular docking that are in accord with their inhibitory properties by neuraminidase inhibition assays. The cyanidin and delphinidin, with the greatest number of hydroxyl groups, bind more strongly and are better inhibitors than pelargonidin that contains a lone hydroxyl group at the 4′ position. The study demonstrates, for the first time, the sensitivity of the mass spectrometry based approach for investigating the molecular basis and relative affinity of antiviral inhibitors, with subtly different structures, to their target protein. It has broader application for the screening of other protein interactions more generally with reasonable high-throughput.

1. Introduction

Neuraminidase inhibitors are the only class of antiviral drugs currently approved for use against influenza virus infections [1]. They function by binding within the active site of the neuraminidase enzyme on the surface of the virus particle and in doing so inhibit its sialidase activity. This prevents progeny viruses from being detached from the host cell that, in turn, reduces the viral load and spread.

The first influenza neuraminidase inhibitors, zanamivir [2] and oseltamivir carboxylate [3], were designed based on the natural inhibitor to the enzyme, the dehydrated form of *N*-acetylneuraminic acid (Neu5Ac). Both have been widely administered against the influenza virus over the past decade with relatively few side effects [4,5]. No resistance amongst both type A and B influenza viruses was reported for nearly a decade and this was attributed to the highly conserved nature of the neuraminidase active site [2] as well as a loss of fitness observed in strains that developed drug resistant mutations in and around this region [6,7].

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In 2007, the unexpected emergence of oseltamivir resistant type A HxN1 strains were detected bearing the H274Y mutation [8,9]. By 2009, over 99% of all seasonal H1N1 influenza strains were resistant to oseltamivir and by the start of the subsequent year, these resistance mutant strains had completely displaced the susceptible wild-type strains in circulation [10]. These strains were also found to be resistant to the newer antiviral drug peramivir [11], approved for emergency use against the virus [12], due to the similar binding modes of the two inhibitors. Similarly, the 2009 swine-originating influenza virus pandemic strains also developed resistance to oseltamivir with more than 300 resistant strains reported by August 2010 [13]. Antiviral resistance was also identified among type A H3N2 and type B influenza seasonal viruses bearing the neuraminidase mutations E119V and I221T respectively [14,15].

Resistance to zanamivir and its long lasting analogue laninamivir has been found to be far less prevalent, though this may be partially attributed to their reduced administration. Zanamivir resistant strains have been observed among immunocompromised individuals [16,17] and the need for a new generation of neuraminidase inhibitors is of vital importance [18].

The drug resistance problem notwithstanding, there is an added complication associated with altered binding efficacies of the inhibitors to the various neuraminidase subtypes of type A viruses. This has been attributed to the open or closed conformation adopted within the so-called 150-loop comprising residues 147–152 and an adjacent cavity of the neuraminidase active site [19]. The loop may be in an open conformation that leads to formation of the 150-cavity or a closed conformation that lacks the cavity. This is of particular importance given that one of the major advantages of neuraminidase inhibitors, over earlier anti-influenza chemotherapies, is their broad activity across all viral subtypes. These unexpected changes in the neuraminidase active site structure, and their effect on inhibitor binding, has prompted the development of inhibitors that bind more remotely from the 150-loop and target other structurally conserved regions. This has led to an interest in the conserved 430-loop region adjacent to the active site as a target for novel antiviral compounds [20]. It has been reported that the 430-loop drives the specific 150-loop conformation [20]. Recent molecular dynamic simulation studies have revealed a great deal of conformational flexibility within the neuraminidase active site, which would allow for the design of innovative scaffolds that possess different binding modes to conventional neuraminidase inhibitors [21]. Furthermore, the extended conformational shifts identified by Amaro and colleagues in the 430-cavity [22,23] of the protein suggest several possibilities for the design of derivatives that would bind in its proximity.

Several classes of compounds derived from plant sources have been studied and evaluated for their neuraminidase binding and inhibition properties. Flavonoids are one such class of compounds that have been shown promise based on computational studies [24,25]. Anthocyanins are highly coloured pigments found in flowers and fruits, particularly berries, that represent one class of flavonoids. In a recent report, a novel mass spectrometry based assay, developed in this laboratory, was used in conjunction with molecular docking to show that the anthocyanin cyanidin-3-sambubiocide binds to N1 neuraminidase within the 430-loop region [26] (Fig. 1). The study also found that when bound, the compound inhibited the sialidase activity of the enzyme as identified in separate in vitro neuraminidase inhibition assays [26].

This study examines the contact residues important to this interaction, and examines some of the most important substituents of the glycan-free anthocyanidin needed to preserve it. It further demonstrates the ability of the mass spectrometric based assay to reliably study the binding location and affinity of subtly different antiviral inhibitors to their target protein.

2. Materials and methods

2.1. Neuraminidase, anthocyanidin and fluorescent substrate stocks

Recombinant influenza N1 neuraminidase based on a 2009 pandemic strain (A/California/04/2009) was purchased from Sino Biological Inc. (Beijing, China) as 100 μg lyophilized protein (73% purity) and reconstituted in 500 μL of Milli-Q purified water. Delphinidin chloride purchased from Sapphire Bioscience (Waterloo, NSW, Australia) and pelargonidin chloride purchased from Sigma–Aldrich (Castle Hill, NSW, Australia) were prepared as aqueous stock solutions (1 mg mL^{-1}). The neuraminidase fluorescent substrate 2′-(4-methylumbelliferyl)-α-D-*N*-acetyl neuraminic acid (MUNANA) was purchased from Sigma–Aldrich (Castle Hill,

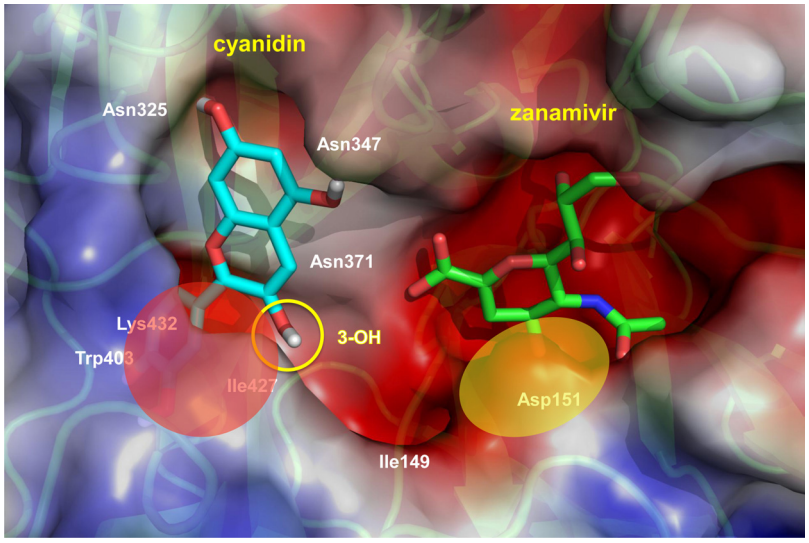

Fig. 1. Relative positions of the 150-cavity and the 430-loop within the active site of the influenza N1 neuraminidase showing the location of zanamivir (right) and cyanidin (left) and the 150-cavity (shaded yellow) and 430-cavity (shaded red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)