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Inhibitory potency of flavonoid derivatives on influenza virus neuraminidase

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ABSTRACT

The constant risk of emerging new influenza virus strains that are resistant to established inhibitors like oseltamivir leaves influenza neuraminidase (NA) a prominent target for drug design. The inhibitory activity of several flavonoid derivatives was experimentally tested in comparison to oseltamivir for the NA expressed by the seasonal influenza virus strains A/California/7/09 (A(H1N1)pdm09), A/Perth/16/09 (A(H3N2)), and B/Brisbane/60/08. IC_{50} values of polyphenols confirmed moderate inhibition in the μM range. Structurally, the amount and site of glycosylation of tested flavonoids have no significant influence on their inhibitory potency. In a pharmacophore-based docking approach the structure–activity relationship was evaluated. Molecular dynamics simulations revealed highly flexible parts of the enzyme and the contribution of salt bridges to the structural stability of NA. The findings of this study elucidate the impact of flavonoids on viral neuraminidase activity and the analysis of their modes of action provide valuable information about the mechanism of NA inhibition.

Licensed antiviral drugs against influenza A and B target mainly the viral surface protein neuraminidase (NA) to inhibit viral spread in the human body. All inhibitors targeting the viral M2 protein are ineffective against all circulating influenza A viruses due to viral resistance mutations and naturally do not affect type B viruses. Neuraminidase activity enables release of progeny virions from a host cell by cleaving terminal sialic acid from glycoproteins on the host cell surface. Influenza virus NAs are structurally and phylogenetically divided into group 1 (N1, N4, N5, and N8) and group 2 NAs (N2, N3, N6, N7, and N9).[1] Their main difference is the existence of an active site cavity located beneath loop-150 in group 1 NAs while group 2 neuraminidases exist only in the closed conformation.[1–3] As an exception of group 1, neuraminidase of the A(H1N1)pdm09 influenza A viruses was shown to exhibit a closed loop-150 conformation as well.[4] Since important amino acids in the active site of neuraminidases are relatively well conserved among the two groups, they embody an interesting target for anti-influenza drug design. The discovery of two well-established anti-influenza agents that mimic the natural ligand of neuraminidases (sialic acid) is among the first success stories of structure-based drug design: zanamivir (Relenza®) and oseltamivir (Tamiflu®).[5] Nevertheless, genetic mutations confer drug resistance such as the NA H274Y change that rendered seasonal H1N1 virus strains circulating until the appearance of the A(H1N1) pandemic in 2009 resistant to oseltamivir.[6] Due to the dynamic emergence of new resistant strains, the search for new drugs against influenza remains crucial.

Flavonoids are ubiquitous secondary metabolites of plants and are therefore part of common human nutrition. Today, there are over 5000 flavonoids identified from plant sources and the daily intake is estimated to range from few to several hundred milligrams.[7] Flavonoids exhibit a broad range of pharmacologic activities including gastroprotective, and anti-oxidant effects,[8] as well as anti-cancer, anti-inflammatory, and anti-bacterial behavior.[9] Flavonoids are furthermore reported to influence the central nervous system (CNS) by crossing the blood–brain barrier resulting in anxiolytic or anti-convulsive effects, amongst others.[10] In the past years, flavonoids have been repeatedly shown to act as anti-influenza agents by modulating NA activity.[11–16] For example, a recent study indicates that the extract of *Rhodiola rosea* roots which also contains the flavonol kaempferol exhibits neuraminidase (H1N1) inhibitory activity.[12] To date, only a few structure–activity relationship (SAR) studies have been published so far.[17–20] This concept of bioactive compounds interacting with multiple targets and thereby influencing one or more physiological pathways, is referred to as poly- or network

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pharmacology.[21,22] Showing polypharmacological behavior, the use of flavonoids as classical drugs in medicine seems to be of subordinate importance due to reduced selectivity. Nevertheless, about 80% of registered antiviral compounds (between 1981 and 2010) belong to or are derived from the class of natural products, including oseltamivir.[23,24] Therefore, studying the inhibitory activity and the mode of action of natural compounds remains important.

In the present study, the inhibitory potency of six flavonoid derivatives on NA activity (Fig. 1, **2**–**7**) was experimentally assessed in comparison to established NA-inhibitor oseltamivir (Fig. 1, **1**) on influenza A/California/7/09 (A(H1N1)pdm09), A/Perth/16/09 (A(H3N2)) and influenza B/Brisbane/60/08 virus. To further investigate their structure–activity relationship, all experimentally tested compounds were docked into homology models of neuraminidases of A(H1N1)pdm09, A(H3N2) and Influenza B, that were validated in molecular dynamics simulations. Putative binding modes were determined in a pharmacophore-based procedure. The aim of the study was to investigate the correlation between inhibitory potency and three-dimensional structure of flavonoid derivatives on viral neuraminidases to provide detailed information on significant interaction patterns for structure-based drug design or lead optimization approaches for new inhibitors of influenza NA.

Our study was initiated by experimentally assessing a total of 22 natural substances including polyphenols, flavonoids and alkaloids (Table S1) for their inhibitory potency on NA activity expressed by the three influenza virus strains A/California/7/09 (A(H1N1)pdm09), A/Perth/16/09 (A(H3N2)) and B/Brisbane/60/08 in comparison to established NA-inhibitor oseltamivir. The assay was based on Potier et al. (1979) using MUNANA fluorometric assay in the absence or presence of oseltamivir and polyphenols, respectively, detecting activity of viral neuraminidases (details provided in the supporting information). This analysis identified six flavonoid derivatives with moderate inhibitory activity which included the flavonoles kaempferol (**2**), quercitrin (**3**), isoquercitrin (**4**), hyperoside (**5**), and rutin (**6**), and the flavone luteolin-7-glucoside (**7**) (Fig. 1, **2**–**7**, Fig. S1, Table 1). The inhibitory potency of oseltamivir in the low nM range was consistent with published values.[25–27] Although detected IC_{50} values of flavonoid derivatives were significantly higher compared to oseltamivir, the experimental data were in agreement with previously published inhibition values in the low μM range.[12,13,15,17,18,28] Even though neuraminidases of three different influenza viruses were tested, the IC_{50} values of polyphenols showed no significant differences among themselves. Interestingly, there was no relationship between the inhibitory potency and the structure of flavonoid derivatives regarding the place of glycosylation (C7- and C3-glycosylation of **3**, **4**, **5**, **6**, compared to **7**) nor the amount of glycosylation (double- and single-glycosylation of **6** compared to **3**, **4**, **5**, and **7**). Given the relatively high standard deviations, detected IC_{50} values are relatively homogeneous, showing no differences between the three different NA subtypes nor the various flavonoid derivatives. The most predominant exception is isoquercitrin (**4**), which represents the weakest inhibitor displaying IC_{50} values of about 370–470 μM. Compared to structurally related compounds **3** and **5**, isoquercitrin differs only by the type of glycosylation: rhamnoside in **3**, glucoside in **4**, and galactoside in **5**. Significantly, the six substances showed no cytotoxicity over a wide range of concentrations as judged by the MTT test on MDCK cells, a cell line widely used for the propagation influenza viruses (Table S2).[29]

To further investigate the structure-activity relationship between experimentally tested flavonoids and neuraminidase subtypes, a molecular modeling study was performed. The workflow involved the creation of homology models of neuraminidases expressed by the influenza A/California/7/09, A/Perth/16/09 and B/Brisbane/60/08 strains and their validation via molecular dynamics simulations. Experimentally tested compounds were docked into the active sites of NA subtypes and their binding modes were evaluated in a pharmacophore-based approach.

For structural investigation, homology models were built for NA of the A/Perth/16/09 (A(H3N2)), and B/Brisbane/60/08 strains using PDB crystal structures 4GZP an 3K3A, respectively. PDB crystal structure 3TI6 was used as basic structure for NA subtype A/California/7/09 due to complete sequence identity. Comparison of amino acid sequences between the three viral NAs showed minor identity regarding the whole protein (between 28 and

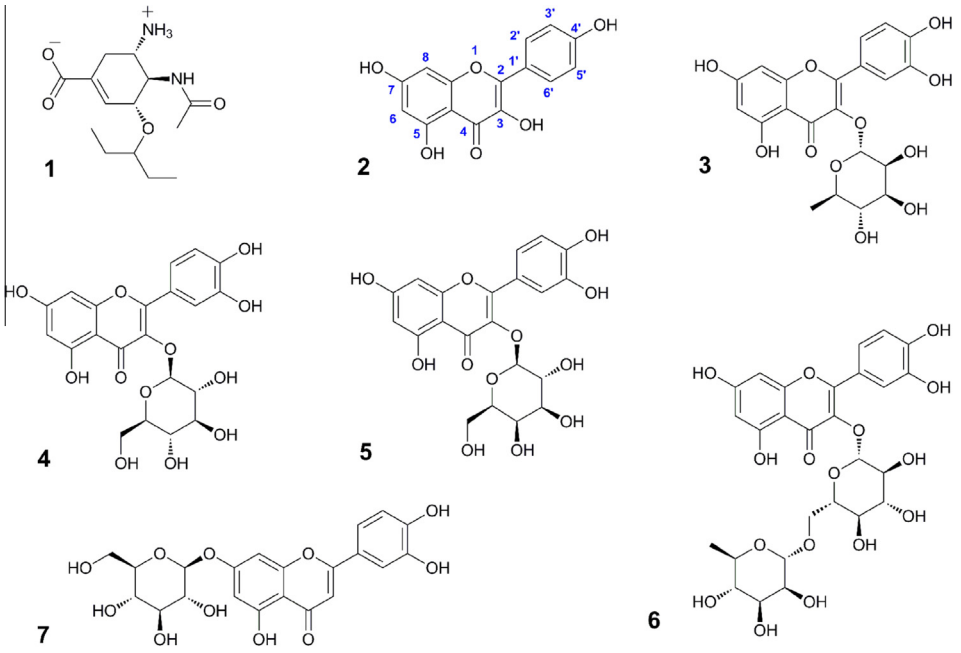

Figure 1. Molecular structures of investigated compounds (**1**) oseltamivir carboxylate, (**2**) kaempferol, (**3**) quercitrin, (**4**) isoquercitrin, (**5**) hyperoside, (**6**) rutin, and (**7**) luteolin-7-glucoside.