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SEARCH FOR AGENTS THAT INCREASE THE LEVEL OF SIALIC ACID IN LOW-DENSITY LIPOPROTEINS

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Abstract: Background: Sialidase activity is responsible for the atherogenic modification of low-density lipoproteins (LDL). Sialidase-induced LDL desialylation triggers atherogenesis at the arterial cell level. Molecular docking was used to search for natural compounds exhibiting affinity for sialidase. Twenty flavonoids were identified that exhibited high thermodynamic affinity for this target, with the five leading compounds belonging to the class of catechins and their gallate derivatives. Epicatechin-3-gallate demonstrated the best binding affinity. In vitro experiments revealed that epigallocatechin gallate's inhibitory activity against neuraminidase was sixfold greater than that of avicularin, fully confirming the results of the virtual screening, as molecular docking placed avicularin in a group of agents that are potentially weaker sialidase inhibitors. At the same time, avicularin was found to have a pronounced activating effect on sialyltransferase, the enzyme responsible for LDL resialylation. Therefore, specific antiatherogenic agents that increase sialic acid levels in LDL particles were identified for the two pharmacological targets (enzymes).

Keywords: human sialidase (neuraminidase), sialyltransferase, molecular docking, flavonoids, epicatechin-3-gallate, epigallocatechin gallate, avicularin.

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INTRODUCTION

Despite the prevailing scientific understanding of the key role of oxidized low-density lipoproteins (LDL), another modification, namely LDL desialylation, has been shown to trigger atherogenesis at the arterial cell level [1]. LDL desialylation initiates a cascade of physical and chemical changes in the LDL particle that lead to multiple atherogenic modifications, including oxidation. However, oxidation occurs at the very late stages of this cascade, so the amount of oxidized LDL in the blood of patients with atherosclerosis is low compared to other forms of atherogenically modified LDL, and it does not significantly contribute to the atherogenic potential of LDL [1].

LDL desialylation is caused by sialidase activity circulating in the blood of patients [2, 3]. This activity represents a promising pharmacological target for the development of a new approach to anti-atherosclerotic therapy.

Using bioinformatic and chemoinformatic approaches, approximately 60 agents, including natural compounds, were previously identified for subsequent screening to evaluate their inhibitory activity [4]. Among these agents, the most interesting are naturally occurring substances (nutraceuticals), which are approved for use as ingredients in non-drug formulations, including dietary supplements (DS). This interest is driven by the need for long-term or even lifelong anti-atherosclerotic therapy.
The aim of this study was to conduct virtual screening of nutraceuticals and validate its results to identify the most effective inhibitors of sialidase activity.

Materials and methods

Protein Structure Preparation

The three-dimensional structure of human sialidase (NEU2 isoform) was downloaded from the Protein Data Bank (PDB). Crystallographic water molecules and cocrystallized ligands were removed from the structure. Polar hydrogen atoms were added, and atomic charges were assigned according to the force field. The enzyme's active site was determined based on the coordinates of the crystallographic substrate.

Ligand Preparation

The ligand library was compiled using data from the PubChem database [5]. The initial screening included compounds of natural origin. For each compound (Table 1), a three-dimensional structure was obtained, energy minimization was performed, and atomic types were assigned. PubChem identifiers were used to uniquely identify the molecules.

Molecular Docking

Docking was performed using AutoDock Vina [6]. Search parameters included a search space size sufficient to cover the enzyme's active site and an exhaustive search mode (exhaustiveness = 8). For each ligand, 10 conformers were generated, from which the pose with the lowest binding energy was selected. The docking protocol was validated by redocking the cocrystallized ligand, which yielded a root-mean-square deviation (RMSD) of less than 2.0 Å.

Enzyme Activity Assessment

The test compounds were assessed for their effect on the ability of Clostridium perfringens (C. welchii) neuraminidase type 6 to cleave the fluorogenic substrate 4-MUNANA (2'-4(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid) [7]. The amount of sialic acid was determined as a unit of activity. The acid released at a 1 mM concentration of the test compound in 1 minute, as well as a titrated aqueous solution of 4-MU (4-methylumbelliferone) at final concentrations of 0.3125–10 μM, were used to construct a calibration curve. To assess enzyme activity, 45 μl of 1 M neuraminidase was mixed with 5 μl of the test substance (final concentrations 0.3125–10 μM). 50 μl of a 300 μM 4-MUNANA substrate solution in MES buffer (33.3 mM MES-NaOH, 4 mM $CaCl_2$, pH 6.5) was added. The mixture was incubated at 37°C for 60 minutes in a Gnome incubator (DNA-Technology, Russia). The reaction was stopped by adding 100 μl of stop solution (0.824 M NaOH in absolute ethanol). Fluorometric measurements were performed using a VICTOR3™ plate reader (Perkin Elmer, USA) with excitation at 340 nm and emission at 460 nm.

To assess the effect of compounds on sialyltransferase activity, a coupled enzymatic assay based on the release of inorganic phosphate (Pi) by sialyltransferase (ST) was developed and used. The assay involves two sequential enzymatic steps: (1) ST catalyzes the transfer of sialic acid from CMP-sialic acid to an acceptor (asialofetuin), generating CMP; and (2) CMP is hydrolyzed by 5'-nucleotidase, releasing Pi, which is quantified colorimetrically. The reaction mixture in a final volume of 50 μl contained 5 mM asialofetuin, 5 mM CMP-sialic acid, 10 U/ml 5'-nucleotidase and 25 U/ml CT in a buffer solution (250 mM Tris, 100 mM $CaCl_2$, 100 mM $MnCl_2$, pH 8.0). 25 μl of sialyltransferase (α-2,6-sialyltransferase from Pasteurella multocida, 7.4 U/ml, MedChemExpress, USA, Cat. No. P-1059) were added to the mixture and incubated at 37 °C for 10 minutes. At the end of the incubation, the formed Pi was determined by adding malachite green-HCl reagent (0.44 mg/mL), consisting of ammonium molybdate $4H_2O$ in 4 N HCl and malachite green in 5% polyvinyl alcohol. After 10 minutes at room temperature, the absorbance of the resulting phosphomolybdate complex was measured at 620 nm using a Multiskan FC microplate reader (Thermo Fisher Scientific, model 1820). One unit of enzymatic activity was defined as the amount of enzyme required to release 1 μmol of CMP-sialic acid per minute. The Pi concentration was determined using a calibration curve using 1 mM KH_2PO_4 as a standard.

Results

Virtual screening

During the virtual screening, the binding of a number of natural compounds to the sialidase active site was analyzed. Table 1 presents the flavonoids that exhibited the lowest (most negative) binding energies, indicating high thermodynamic affinity for the target.

Table 1. Flavonoids that showed the lowest binding energy values for human sialidase.

PubChem ID	Compound	Binding energy, kcal/mol
65056	Epicatechin-3-Gallate	-6,923
107905	Epicatechin Gallate	-6,571
442827	Trifolirhizin	-6,27
72277	Epigallocatechin	-6,082
65064	Epigallocatechin Gallate	-6,058
5281605	Baicalein	-5,983
5317750	Glycitein	-5,959
5281803	4'-Methoxy-3',5,7-trihydroxyisoflavone	-5,867
44257483	glyceollidin I	-5,859
5281222	Butein	-5,82
9064	Catechin	-5,775
68071	(+)-Pinocembrin	-5,763
363863	3-Hydroxy-8,9-methylenedioxypterocarpane	-5,758
5317742	3-Methoxy-2-(3-methylbut-2-enyl)-6,11a-dihydro-[1]benzofuro[3,2-c]chromene-6a,9-diol	-5,709
182232	(+)-Epicatechin	-5,698
5280378	Formononetin	-5,665
5490064	Avicularin	-5,662
5280373	Biochanin A	-5,645

PubChem ID	Compound	Binding energy, kcal/mol
72276	Epicatechin	-5,642
5280961	Genistein	-5,64

Data analysis shows that the top five results belong to the class of catechins and their gallate derivatives. Epicatechin-3-gallate (PubChem 65056) demonstrated the best result (−6.923 kcal/mol). Compounds without a gallate group (e.g., catechin or epicatechin) showed slightly worse results (around −5.7 kcal/mol), indicating the importance of additional phenolic groups for interaction with the enzyme.

Verification of Virtual Screening Results

To verify the virtual screening results, the effect of one theoretically most effective inhibitor, epigallocatechin gallate, and one of the theoretically weakest inhibitors, avicularin, on sialidase activity was assessed.

Table 2. Inhibitory activity of epigallocatechin gallate and avicularin against neuraminidase.

Compound	PubChem CID *	MW	IC50 [μM]
Epigallocatechin gallate	65064	458.3	11.6
Avicularin	5490064	434.3	71.6

* https://pubchem.ncbi.nlm.nih.gov

As shown in Table 2, the IC_{50} of epigallocatechin gallate for inhibiting neuraminidase activity is 6.2 times lower than that of avicularin. This means that the inhibitory potency of epigallocatechin gallate is six times greater than that of avicularin. Thus, the experimental data fully confirmed the results of the virtual screening.

Sialyl acid levels in LDL may be increased by resialylating glycoconjugates. Since the substrate for sialidase and sialyltransferase is the same—sialic acid—the active sites of the enzymes should be similar, with potential differences in affinity. In other words, sialidase inhibitors may exhibit sialyltransferase activity. The effects of epigallocatechin gallate and avicularin on sialyltransferase activity were studied.

Table 3. Effect of epigallocatechin gallate and avicularin on sialyltransferase activity.

Compound	PubChem CID *	MW	Минимальная эффективная концентрация, μM	Повышение активности сиалилтрансферазы, %
Epigallocatechin gallate	65064	458.3	-	-
Avicularin	5490064	434.3	1,08	37,9

* https://pubchem.ncbi.nlm.nih.gov

As shown in Table 3, avicularin exerted a pronounced activating effect on sialyltransferase activity. In contrast, epigallocatechin gallate was not only ineffective but also a weak inhibitor of sialyltransferase (IC_{50} 530 μM). Thus, epigallocatechin gallate should be considered an effective sialidase inhibitor, and avicularin an effective sialyltransferase activator with a moderate inhibitory effect on sialidase activity.

DISCUSSION

The obtained data indicate that among the tested library of natural compounds, flavonoids exhibit the highest binding potential to human sialidase. The group of gallated catechins stands out in particular. The presence of a gallate residue at position 3 (as in epicatechin-3-gallate and epigallocatechin gallate) correlates with a decrease in binding energy of approximately 1.0–1.2 kcal/mol compared to non-gallated analogs. This can be explained by additional hydrogen bonds and π-π stacking interactions that the gallate group forms with amino acid residues in the active site (e.g., with aromatic tyrosine or tryptophan residues, characteristic of the substrate-binding pocket of sialidases).

However, it is important to note that flavonoids are not the only sialidase inhibitors. The chemical space of potential inhibitors includes sialic acid derivatives (e.g., DANA), cyclic guanidines, and other heterocyclic systems, which often exhibit higher affinity due to electrostatic interactions with the enzyme's catalytic triad. The binding energies obtained in this study (range -5.6 to -6.9 kcal/mol) are moderate. By comparison, known potent inhibitors of viral neuraminidase often exhibit values below -8.0 kcal/mol under similar docking conditions [8].

The advantages of the identified flavonoids include

their natural origin, low toxicity, and good bioavailability compared to synthetic analogs. However, the low selectivity of flavonoids may be a limiting factor, as they are capable of interacting with a wide range of target proteins.

A limitation of this study is the lack of molecular dynamics (MD) simulations to more accurately assess the stability of the complexes, as well as calculations of the binding energy (MM-GBSA/PBSA). Furthermore, future studies should expand the chemical screening space by including non-flavonoid classes of compounds (alkaloids, terpenoids, synthetic libraries) to eliminate the possibility of missing more effective molecular scaffolds.
The results of in vitro experimental studies conducted to verify the docking results allow two important conclusions.

First, the data on the sialidase inhibitory efficacy of flavonoids were fully consistent with the docking data. Second, avicularin was found to be highly effective in activating sialyltransferase, which increases the sialylation of glycoconjugates. Therefore, avicularin can be considered an agent capable of resialylating desialylated LDL, i.e., eliminating its atherogenicity by normalizing the sialic acid level in the LDL particle.
Thus, specific antiatherogenic agents that increase sialic acid levels and have different (opposite) mechanisms of action have been identified for two pharmacological targets. Potentially, a combination of these agents may have a synergistic effect when used for antiatherogenic therapy.

CONCLUSION

Molecular docking was used to screen natural compounds for sialidase activity inhibition. The search for such compounds is important for the potential suppression of sialidase activity in human blood, as it is this activity that initiates the conversion of native LDL into atherogenic forms.

1. It was found that among natural ligands, flavonoids, particularly gallated catechins, have the highest affinity for the enzyme's active site.
2. The leading compound was epicatechin-3-gallate (binding energy -6.923 kcal/mol).
3. A structure-function relationship was established: the presence of the gallate group significantly improves binding.
4. Despite the potential of flavonoids, the search for inhibitors should not be limited to this class of compounds.
5. The experimental in vitro data on sialidase inhibitory efficacy were fully consistent with the docking data.
6. An effective activator of sialyltransferase, avicularin, has been found, which can increase the level of sialic acid in LDL particles through resialization.

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