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In Search for the Low-Molecular-Weight Ligands of Human Serum Albumin That Affect Its Affinity for Monomeric Amyloid β Peptide

Evgenia I. Deryusheva [1,*], Marina P. Shevelyova [1], Victoria A. Rastrygina [1], Ekaterina L. Nemashkalova [1], Alisa A. Vologzhannikova [1], Andrey V. Machulin [2], Alija A. Nazipova [1], Maria E. Permyakova [1], Sergei E. Permyakov [1] and Ekaterina A. Litus [1]

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Abstract: An imbalance between production and excretion of amyloid β peptide (Aβ) in the brain tissues of Alzheimer's disease (AD) patients leads to Aβ accumulation and formation of noxious Aβ oligomers/plaques. A promising approach to AD prevention is reduction of free Aβ level by directed enhancement of Aβ binding to its natural depot, human serum albumin (HSA). We previously demonstrated the ability of specific low-molecular-weight ligands (LMWLs) of HSA to improve its affinity for Aβ. Here we develop this approach by bioinformatic search for the clinically approved AD-related LMWLs of HSA, followed by classification of the candidates according to the predicted location of their binding sites on HSA surface, ranking of the candidates, and selective experimental validation of their impact on HSA affinity for Aβ. The top 100 candidate LMWLs were classified into the five clusters. The specific representatives of the different clusters exhibit dramatically different behavior, with 3- to 13-fold changes in equilibrium dissociation constants for the HSA-Aβ40 interaction: prednisone favors HSA-Aβ interaction, mefenamic acid shows the opposite effect, while levothyroxine exhibits the bidirectional effects. Overall, the LMWLs of HSA chosen here provide a basis for drug repurposing for AD prevention, and for search for the medications promoting AD progression.

Keywords: Alzheimer's disease; amyloid β peptide; human serum albumin; low-molecular-weight ligand; protein-ligand interaction; surface plasmon resonance spectroscopy

1. Introduction

Human serum albumin (HSA) is the predominant protein in blood plasma, accounting for approximately 60% of the total protein content [1]. HSA is a 66 kDa protein, containing 585 amino acid residues organized into three domains [2]. It possesses seven fatty acid (FA) binding sites and the two major sites specific to small molecules [3,4]. HSA exhibits a remarkable ability to bind and transport through the bloodstream numerous endogenous and exogenous ligands [5,6]. Thereby HSA serves as a depot and delivery vehicle for small molecules in the blood, such as FAs, hormones, bilirubin, hemin, drugs, etc. [6–9]. The long plasma half-life of HSA (12.7-18.2 days [2]) is widely used to prolong the half-life of therapeutic peptides/proteins by their covalent modification with FAs or other substances with high affinity for HSA [10]. Some of them are used for treatment of diabetes mellitus (type 1 and 2) and/or obesity [11,12]: insulin detemir (Levemir®), insulin degludec (Tresiba®), liraglutide (Victoza®/Saxenda®), and semaglutide (Ozempic®, Rybelsus®, Wegovy®).

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HSA is a natural depot for amyloid β peptide (Aβ) [13,14], one of the key factors in the development of Alzheimer's disease (AD) [15,16]. HSA binds about 90% of Aβ in blood serum [17] and, according to various estimates, from 40% to 94% in cerebrospinal fluid [18]. HSA inhibits Aβ aggregation and lowers the risk of AD and its progression [19–23]. As a component of the interstitial fluid, HSA is presented in the intercellular space of the brain parenchyma [24]. Although HSA is primarily synthesized in the liver, brain microglial cells also synthesize and secrete HSA, especially upon their stimulation with Aβ or lipopolysaccharide [25]. Finally, HSA is included in amyloid deposits (plaques) in the brain of AD patients [26]. These facts indicate significance of HSA in Aβ metabolism and potential of HSA usage for therapy of AD.

Preliminary clinical trials confirm the effectiveness of AD treatment by replacement of the patient's serum albumin with its pharmacological preparation via plasmapheresis (plasma exchange (PE)) [19,23]. This approach is related with a risk of cardiovascular and respiratory complications, anaphylactoid reactions, infections, and hemorrhage [27]. The necessity to live with a catheter inserted in the chest and metabolic alterations related to PE can trigger psychiatric symptoms in the PE-treated AD patients [23]. Nevertheless, this approach is considered relatively safe, but requires participation of the highly trained medical personnel [23,28,29].

The potentially less harmful approach is to increase HSA affinity for Aβ via allosteric action of endogenous or exogenous HSA ligands, initially demonstrated for linoleic and arachidonic acids [30]. We further showed the even more pronounced effects for serotonin [31] and ibuprofen [32]. Moreover, ibuprofen enhances HSA ability to inhibit Aβ fibrillation [32]. These data are in line with clinical observations and the results derived from animal models of AD [33–35].

Despite the first encouraging results, a systematic search of the HSA ligands that can affect its interaction with Aβ has not been yet been carried out. To fill this gap, in the present work, we systematically search for the clinically approved low-molecular-weight HSA ligands related to AD progression that could modify HSA affinity for Aβ, and selectively test the candidate ligands from this perspective after their careful structural systematization and ranking.

2. Material and Methods

2.1. Materials

Recombinant human Aβ40 was expressed in *E. coli* and purified as previously described [32]. Catalytic core of ubiquitin carboxyl-terminal hydrolase 2 (Usp2-cc) was prepared mainly as described in ref. [36]. FA-free HSA prepared under non-denaturing conditions [37] was from Merck (cat. #126654, Darmstadt, Germany). Protein concentrations were measured spectrophotometrically at pH 7.4–8.0 and calculated using molar extinction coefficients at 280 nm estimated according to ref. [38]: 34,445 $M^{-1}cm^{-1}$ for HSA, 41,370 $M^{-1}cm^{-1}$ for Usp2-cc and 1,490 $M^{-1}cm^{-1}$ for Aβ40.

Propranolol (cat. #P913470), levothyroxine (L-thyroxine) (J62606.03), prednisone (PHR1042), warfarin (A2250), and mefenamic acid (M4267) were at least 98% pure and purchased from Macklin, Thermo Fisher Scientific (Waltham, Massachusetts, U.S.) and Sigma-Aldrich (Burlington, Massachusetts, U.S.) (for the last three compounds), respectively. Ultra-grade Tris and 2-mercaptoethanol (2-ME) were purchased from Amresco® LLC (Vienna, Austria). Urea, imidazole, sodium chloride, sodium hydroxide, sodium dodecyl sulfate (SDS), DL-dithiothreitol (DTT), and glycerol were from Panreac AppliChem (Darmstadt, Germany). Ethylenediaminetetraacetic acid (EDTA), acetonitrile, N-Ethyl-N′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC), N-hydroxysulfosuccinimide sodium salt (sulfo-NHS), and polyethylene glycol sorbitan monolaurate (TWEEN®) 20 were from Sigma-Aldrich (St. Louis, MO, USA). Calcium chloride was from Fluka (Buchs, Switzerland). Ethanolamine and Profinity™ IMAC resin were bought from Bio-Rad Laboratories (Hercules, USA). Hydrochloric acid was from Sigma Tec LLC (Moscow, Russia). Dimethyl sulfoxide (DMSO) was from Helicon (Moscow, Russia). Trifluoroacetic acid (TFA) was purchased from Fisher Scientific (Pittsburgh, Pennsylvania, U.S.). Potassium chloride and sodium azide were from Dia-M (Moscow, Russia). Acetic acid and ammonium hydroxide were from Chimmed and Component-reaktiv (Moscow, Russia).

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2.2. Bioinformatic Selection and Structural Analysis of the Therapeutic Low-Molecular-Weight HSA Ligands Associated with AD

The general workflow used for selection of the clinically approved LMWL of HSA related to AD and for their structural analysis is shown in **Figure 1**.

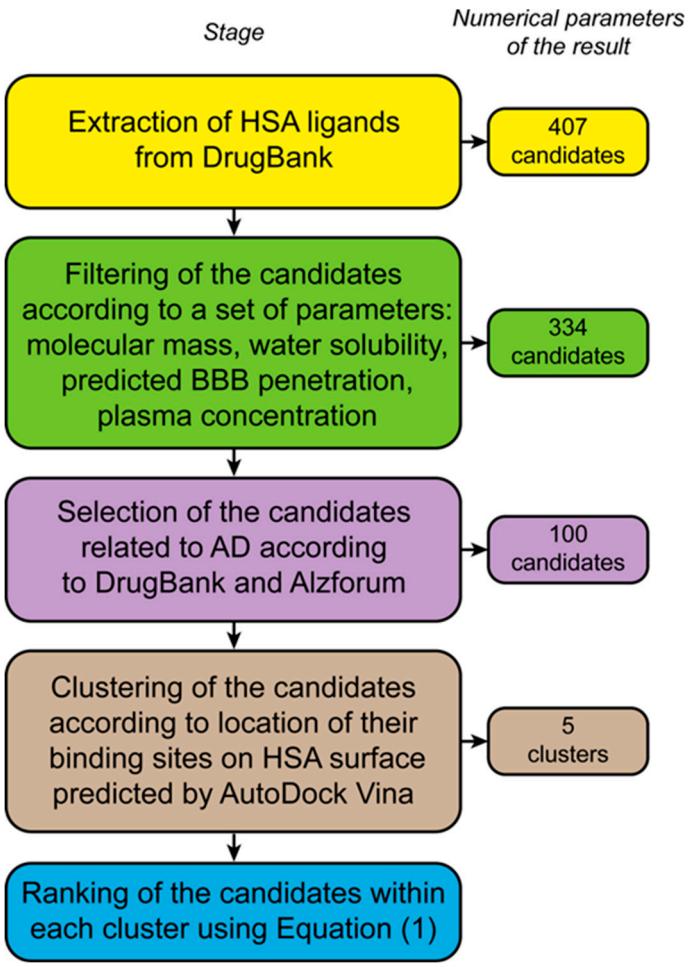

Figure 1. The scheme of the bioinformatic search of the AD-related therapeutic HSA ligands, their filtering, structural analysis and ranking.

The list of the substances directly related to HSA (UniProt [39] (https://www.uniprot.org, accessed on 14 March 2024) ID P02768) was extracted from DrugBank (https://go.drugbank.com/bio_entities/BE0000530, accessed on 10 June 2023), a database containing chemical, pharmacological and pharmaceutical data on the clinically approved endogenous and exogenous substances [40,41]. The compounds with molecular mass (average mass, field "Structure"), above 900 Da and less than 100 Da [42] were excluded from consideration. The resulting low-molecular-weight ligands (LMWLs) of HSA were filtered according to the following requirements:

- water solubility of the ligand (Experimental Water Solubility / Calculated Water Solubility, field "Properties" of DrugBank) should exceed 1 μM to ensure the possibility of efficient HSA loading with the ligand;
- blood-brain barrier (BBB) penetration of the ligand (the field "Predicted ADMET Features" of DrugBank) should exceed 50% to ensure its efficient transfer from the bloodstream into the brain;
- plasma ligand concentration (manually collected from Pubmed (https://pubmed.ncbi.nlm.nih.gov, accessed on 14 March 2024) and DrugBank (field "Absorption") should exceed 0.5 nM, which corresponds to the total plasma Aβ40 concentration [20].

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The FAs were excluded from further consideration, since they had been studied in the previous works [30].

The association of the resulting LMWLs with AD was examined for each of them using the query «"Alzheimer's disease" + "substance name"» in the Alzforum online resource (https://www.alzforum.org/papers, accessed on 10 October 2023). The substances with less than 2 references on Alzforum were excluded from the further analysis, resulting in a total number of the candidates of 100.

To classify the selected LMWLs according to expected location of their binding sites on HSA surface, molecular docking of HSA and the ligands was performed using AutoDock Vina [43] (https://vina.scripps.edu, accessed on 14 March 2024). The crystal structure of HSA was taken from Protein Data Bank (PDB) [44] (https://www.rcsb.org, accessed on 14 March 2024): chain A of entry 1UOR. AutoDockTools software (https://autodocksuite.scripps.edu/adt/, accessed on 14 March 2024) was used for preparation of the PDB structure for the docking process, including removal of water molecules and addition of lacking hydrogen atoms. The three-dimensional structures of the LMWLs were taken from PubChem server (https://www.ncbi.nlm.nih.gov/pccompound, accessed on 14 March 2024) in a structure-data format (filename extension .sdf) and converted to PDB format using PyMOL (https://pymol.org/, accessed on 14 March 2024). In the case of stereoisomers, the biologically active (predominantly R) isomer was used for the modeling (for ibuprofen and for warfarin the more active (S)-isomer and the both enantiomers were used). The docking model corresponding to the lowest energy of the HSA-ligand complex was chosen. The protein-ligand complexes were visualized using PyMOL v.1.6. The HSA-ligand interactions were analyzed using the protein-ligand interaction profiler PLIP [45] (https://plip-tool.biotec.tu-dresden.de/plip-web/plip/index, accessed on 14 March 2024). The numbering of the amino acid residues corresponds to PDB entry 1UOR.

Dynamic time warping algorithms implemented in the 'dtw' library [46] written in R language (https://www.r-project.org/, accessed on 14 March 2024) were used to hierarchically cluster the AD-associated LMWLs based on the predicted location of their binding sites on HSA molecules. The rank of a ligand in a cluster, *R*, was calculated for all clusters using the **Equation (1)**:

$$R = \sum_1^a \left(\frac{f_{aa}}{n} * k \right), \quad (1)$$

where f_{aa} is a frequency of occurrence of an amino acid in the binding site within the cluster, calculated as a number of ligands of the cluster whose binding site contains this amino acid; a is a number of amino acids of the cluster, calculated as a sum of all non-repetitive amino acids forming binding sites for all ligands of the cluster; n is a number of ligands in the cluster; k is a coefficient equal to 0 if the amino acid is absent in the binding site, and equal to 1 if the amino acid is present in the binding site.

The aforementioned algorithms for search, collection, alignment, representation and analysis of the data were implemented using the Python 3 (https://www.python.org/, accessed on 14 March 2024) programming language in PyCharm v.2018 environment (https://www.jetbrains.com/pycharm/, accessed on 14 March 2024). The specialized Python libraries (Requests, BeautifulSoup) were used to form HTTP requests and parse the web pages, search and collect the data into a local database.

2.3. Preparation of Recombinant Aβ

The human Aβ40 samples were pretreated prior to experimental studies essentially as described in ref. [32]. The freeze-dried Aβ40 samples were dissolved in neat TFA at a concentration of 0.5 mg/mL, followed by sonication for 30 s and TFA evaporation using an Eppendorf Concentrator plus. The dried Aβ40 samples were dissolved in DMSO at a concentration of 2 mg/mL and stored at -20 °C.

2.4. Solubility of HSA Ligands

Solubility of propranolol, prednisone, warfarin, levothyroxine, and mefenamic acid in 20 mM HEPES(Tris)-HCl, 150 mM NaCl, pH 7.4 buffer was determined by sequential addition of the ligand with constant stirring until appearance of the precipitate. DMSO (for prednisone, levothyroxine and

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propranolol) or ethanol (for warfarin and mefenamic acid) was added to the solution at a concentration of up to 4-5% (v/v) to improve solubility of the ligand.

2.5. Surface Plasmon Resonance Studies

Surface plasmon resonance (SPR) measurements of HSA interaction with monomeric Aβ40 were performed at 25 °C using a Bio-Rad ProteOn™ XPR36 instrument mainly according to ref. [30]. Ligand (50 μg/mL Aβ40 in 10 mM sodium acetate, pH 4.5 buffer) was immobilized on a ProteOn GLH sensor chip surface by amine coupling up to 10,000–14,000 resonance units, RUs. The remaining activated amine groups on the chip surface were blocked by 1 M ethanolamine solution. The noncovalently bound Aβ40 molecules were washed off the chip surface with a 0.5% SDS water solution until stabilization of the SPR signal. Analyte (2.5-40 μM HSA) in the running buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.4) in the presence/absence of prednisone (2.5 mM)/warfarin (1 mM)/mefenamic acid (250 μM)/levothyroxine (15 μM)/propranolol (1 mM) was passed over the chip at a rate of 30 μL/min for 300 s, followed by flushing the chip with the running buffer for 2,400 s. The sensor chip surface was regenerated by passage of 0.5% SDS water solution for 100 s. The kinetic and equilibrium association/dissociation constants for the HSA-Aβ40 interaction in the absence of the ligands were determined with or without addition of 5% DMSO/ethanol.

The kinetic SPR data were corrected for baseline drift and non-specific binding, and described using a heterogeneous ligand model (**Equation (2)**) (Aβ40 and HSA serve as a ligand (L) and an analyte (A), respectively):

$$L_1 + A \underset{K_{D1}}{\overset{k_{a1}}{\underset{k_{d1}}{\rightleftarrows}}} L_1A \qquad L_2 + A \underset{K_{D2}}{\overset{k_{a2}}{\underset{k_{d2}}{\rightleftarrows}}} L_2A \tag{2}$$

where k_a and k_d are kinetic association and dissociation constants, respectively; K_D are equilibrium dissociation constants. The k_a, k_d and K_D values were estimated using Bio-Rad ProteOn Manager™ v.3.1 software (Bio-Rad Laboratories, Inc.). The estimates were performed for each analyte concentrations, followed by their averaging (standard deviations are indicated). The free energy change accompanying HSA-Aβ40 interaction (ΔG) was calculated as follows: $\Delta G_i = -RT \ln(55.3/K_{Di})$, i=1,2.

3. Results

3.1. Bioinformatic Selection of the Therapeutic Low-Molecular-Weight HSA Ligands Associated with AD

DrugBank database [40,41] was used as a source of the clinically approved HSA ligands related to AD. For each of the HSA ligands we have collected a set of the characteristics necessary for their further filtering: molecular mass values of experimental and theoretical water solubility, BBB penetration. Information on plasma concentrations of the substances was manually collected from Pubmed and DrugBank. The association of the substances with AD was assessed by the number of the relevant literature sources found on the Alzforum online resource. The filtering of the candidates by a set of parameters: molecular mass above 100 Da and less than 900 Da, water solubility above 1 μM, predicted BBB penetration exceeding 50%, plasma concentration above 0.5 nM, and more than 1 reference on Alzforum, resulted in 100 LMWLs of HSA associated with AD (**Table S1**), after removal of the FAs studied in our previous works [9,30,47,48]. The candidate compounds belong to different drug classes, including such common ones as antidiabetic drugs (rosiglitazone), non-steroidal anti-inflammatory drugs (ibuprofen, meloxicam), neuroleptics (risperidone), vitamins (vitamin A, thiamine), antibiotics (tetracycline, ampicillin), and hormones (testosterone, estradiol).

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3.2. Classification and Ranking of the Selected LMWLs According to the Expected Location of Their Binding Sites on HSA

To predict location of the binding sites for the 100 AD-related LMWLs on HSA surface, three-dimensional structures of the HSA-LMWL complexes were built using Auto Dock Vina [43]. Note that the binding sites were previously experimentally localized by X-RAY only for a few members of our panel, namely ibuprofen [3], warfarin [3], indomethacin [3], halothane [49], propofol [49]. The binding sites predicted for these ligands coincide with those localized experimentally for all ligands, except for ibuprofen (the predicted site is located nearby the secondary binding site). The resulting binding sites were hierarchically classified into five clusters based on the dynamic time warping algorithms as implemented in the R language library 'dtw', used for classification and clustering of general number series (**Table S2**) [46]. **Figure 2** illustrates location of the key residues constituting these clusters on HSA molecule. The cluster I is located between HSA subdomains IIA and IIB and is predicted to be specific for the 34 AD-associated LMWLs. The clusters II (subdomain IIIA), III (subdomain IB) and V (subdomain IIA) are predicted to bind 24, 13 and 28 ligands, respectively. Levothyroxine (DB00451) was attributed to a distinct cluster IV located between the cleft and subdomain IB.

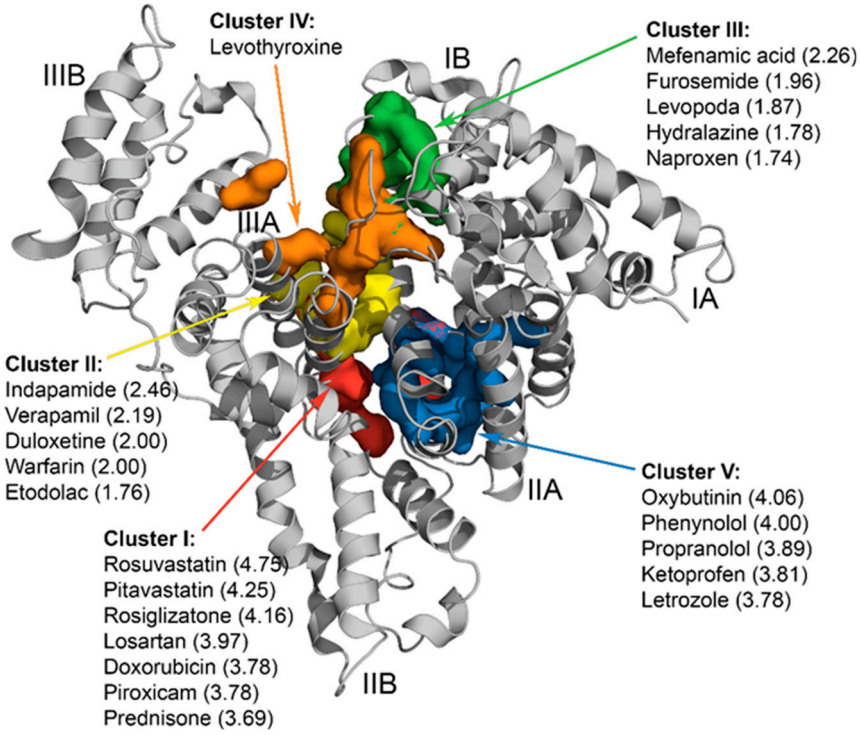

Figure 2. Predicted location of the key HSA residues of the binding sites for the AD-related LMWLs clustered using the R language library 'dtw'. The ligands of the individual clusters with highest *R* values (**Equation (1)**) are indicated (see **Table S2** for the complete list), along with the *R* values (shown in the parentheses). The subdomains IA, IB, IIA, IIB, IIIA and IIIB are marked.

The amino acid residues most commonly involved in recognition of the ligands (the residues with highest f_{aa} values, see **Equation (1)**, **Figure 2**):

- Cluster I: P447 (f_{aa} = 84.4%), R222 (78.1%), K444 (62.5%), D451 (59.4 %), E292 (46.9%);
- Cluster II: Y452 (f_{aa} = 40.5%), K436 (32.4%), K195 (32.4%), A191 (27.0%) and K432 (27.0%);
- Cluster III: R117 (f_{aa} = 39.0%), E141 (34.8%), R145 (30.4%), E86, P118 (26%) and Y140 (26%);
- Cluster IV: R145, E425, N109, Q459, H146, R114, K525, R186;
- Cluster V: E292 (f_{aa} = 55.6%), R257 (47.2%), K199 (44.4%), R218 (41.7%), L219 (38.9%).

To select the most representative ligands in each cluster, the ranks of the ligands within each cluster (*R*) were calculated according to **Equation (1)**, followed by choice of the ligands with high *R* values (**Figure 2**). The resulting ligands are most often recognized by the aforementioned residues, thereby serving as the most representative candidates for further experimental examination of their

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influence on HSA-Aβ interaction. Among them, only one candidate from each cluster was taken for further consideration, with an emphasis on the well-studied, socially significant drugs (**Table 1**).

Table 1. Information on the AD-associated LWMLs selected for experimental examination of their influence on HSA-Aβ interaction.

Predicted cluster on HSA molecule	Drug	Discovery date	Drug class	Application area	Equilibrium association constant for the drug-HSA interaction	Calculated occupancy of HSA binding sites for 2.5-40 μM HSA	Drug concentration used for the SPR studies
I	Prednisone	1950	Corticosteroid	Transplantology; treatment of allergy, inflammation, infection, cancer, endocrine, autoimmune conditions	$K = 10^3$ M^{-1} [50]	71%	2.5 mM
II	Warfarin	1945	Antithrombotic agent	Thromboembolism treatment	$K_1 = 2\times10^5$ M^{-1} $K_2 = 5\times10^4$ M^{-1} [51]	site1: 99-99.5%; site2: 98%	1 mM
III	Mefenamic acid	1961	non-steroidal anti-inflammatory agent	Analgesia, treatment of inflammation и fever	$K_1=4\times10^5$ M^{-1} [52] $K_2 = 1\times10^5$ M^{-1} [53]	95-96%	250 μM
IV	Levothyroxine	1914	Thyroid hormone	treatment thyroid diseases including hypothyroidism and cancer	$K = 10^5$ M^{-1} (4 sites) [54]	9-41%	15 μM
V	Propranolol	Early 1960s	Beta blockers	Cardiology, including hypertension and myocardial infarction	$K = 10^4$ M^{-1} (2 sites) [55]	90-91%	1 mM

3.3. Experimental Validation of the Ability of the Top-Ranked AD-Related LMWLs to Affect HSA Affinity for Aβ40

The effect on HSA interaction with Aβ40 of the following selected representatives of the clusters I-V was studied using SPR spectroscopy: prednisone (cluster I), warfarin (cluster II), mefenamic acid (cluster III), levothyroxine (cluster IV), propranolol (cluster V). The ligand concentrations were chosen to ensure their efficient binding to HSA, based on the literature data on HSA affinity for the ligands (**Table 1**). With the exception of levothyroxine, the estimated occupancy of HSA binding sites exceeds 70%. Due to the low solubility of levothyroxine in the assay buffers (15 μM), the achievable occupancy of HSA binding sites ranges from 9% to 41%. The resulting SPR sensograms for 20/40 μM HSA and their description using the heterogeneous ligand model (**Equation (2)**) are shown in **Figure 3**. The respective averaged kinetic and equilibrium dissociation/association constants are represented in **Table 2**. The most pronounced ligand-binding induced changes in HSA affinity for Aβ40 in the presence of ethanol are observed for mefenamic acid with an increase in the K_D values by a factor of 3-4. This effect is clearly seen in the scale of free energy changes accompanying the HSA-Aβ40 interaction (**Figure 4**). Similarly, the most marked changes in the presence of DMSO are observed for levothyroxine (3-fold increase in the K_{D1} value and 12-fold decrease in the K_{D2}) and prednisone (decrease in the K_{D2} value by a factor of 13) (see **Figure 4, Table 2**).

Taken together, the SPR data demonstrate the ability of some representative members of the specific clusters (namely, I, III and IV) to affect HSA affinity for Aβ40. Meanwhile, one should expect various ligand- and cluster-specific effects, as exemplified by ibuprofen (cluster II [32]) and serotonin (cluster V [31]).

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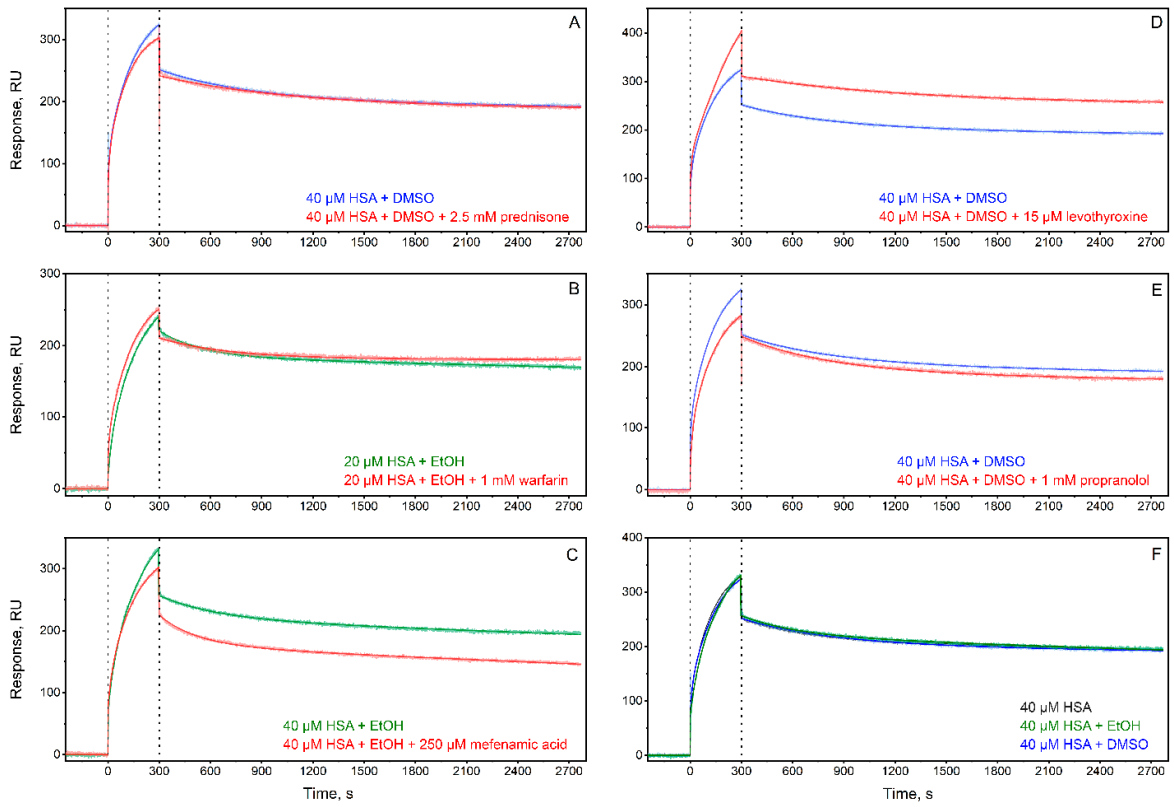

Figure 3. The kinetics of HSA association (0-300 s) with the Aβ40 monomer immobilized on SPR chip's surface by amine coupling, as well as the dissociation of their complex (300-2700 s), monitored by SPR spectroscopy in the presence (panels **A-E**) or absence (**F**) of the HSA ligands shown in **Table 1**: 2.5 mM prednisone (panel **A**), 1 mM warfarin (**B**), 250 μM mefenamic acid (**C**), 15 μM levothyroxine (**D**), 1 mM propranolol (**E**) (20 mM Tris-HCl, 150 mM NaCl, pH 7.4; 25 °C). The experimental curves are described within the heterogeneous ligand model (**Equation (2)**) (see **Table 2** for the fitting parameters).

Table 2. Parameters of HSA-Aβ40 interaction in the presence/absence of the HSA ligands shown in **Table 1** determined by SPR technique using the heterogeneous ligand model (**Equation (2)**).

Ligand/Additive	$k_{a1}\times10^{2}$, $M^{-1}s^{-1}$	$k_{d1}\times10^{-4}$, s^{-1}	$K_{D1}\times10^{-7}$, M	$k_{a2}\times10^{2}$, $M^{-1}s^{-1}$	$k_{d2}\times10^{-4}$, s^{-1}	$K_{D2}\times10^{-6}$, M
Without ligand + DMSO	2.7±2.6	0.216±0.007	0.64±0.48	8.9±9.0	26±13	5.0±3.4
Without ligand + ethanol	5.6±4.7	0.27±0.20	1.2±1.6	13±8	34±11	3.2±1.5
Prednisone + DMSO	2.73±0.96	0.11±0.05	0.38±0.05	36±8	13.6±1.2	0.38±0.05
Warfarin + ethanol	7.9±3.7	0.50±0.39	0.57±0.43	24±28	32±11	2.9±1.9
Mefenamic acid + ethanol	3.3±1.6	0.97±0.56	3.6±2.1	3.6±3.4	31±12	13±10
Levothyroxine + DMSO	0.575±0.011	0.109±0.006	1.90±0.14	27.0±1.6	11.20±0.12	0.41±0.02
Propranolol + DMSO	22±31	0.204±0.010	0.45±0.55	25±34	18.8±0.2	2.7±2.6

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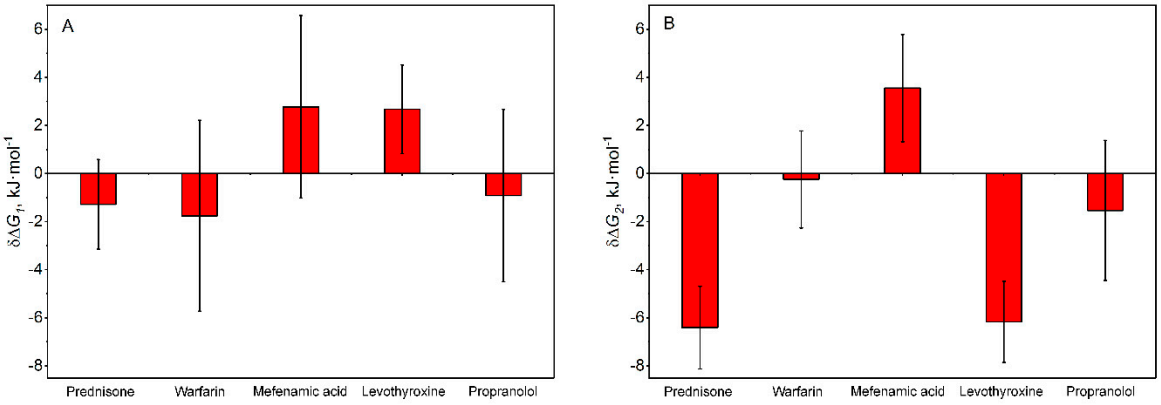

Figure 4. The changes in the free energy changes accompanying HSA-Aβ40 interaction ($\delta\Delta G_i$, i=1,2) induced by addition of the specific HSA ligands (see **Table 1**), calculated from the SPR data shown in **Table 2** ($\Delta G_i = -RT \ln(55.3/K_{Di})$, i=1,2). Panel A corresponds to K_{D1}, panel B – K_{D2}.

4. Discussion

The approved medications are a valuable source of the substances potentially suited for therapy of both common and rare diseases within the approach known as drug repurposing or repositioning [56]. For example, tetracyclines and some polyphenols are able to interfere with aggregation of several unrelated amyloidogenic proteins, such as α-synuclein (associated with Parkinson's disease), amyloid polypeptide (type-2 diabetes), and transthyretin (senile systemic amyloidosis, familial amyloid polyneuropathy and cardiomyopathy) [57–60]. Similarly, some of the approved drugs have the potential to worsen certain disorders. In the present work, we systematically searched for the approved medications able to affect specificity to Aβ of HSA as a major natural depot for Aβ in the blood/CSF [18], with a focus on the HSA ligands associated with AD. In our previous works we identified several such HSA ligands [30–32], but here we used a general bioinformatic approach that allowed us to get a panel of 100 drug candidates (**Table S1**). They were grouped into five clusters according to the predicted location of their binding sites on HSA molecule, followed by ranking of the candidates within each cluster (**Table S2**). The massive involvement of polar and charged amino acid residues in the predicted binding sites of the ligands on HSA (**Table S2**) indicates potential dependence of the ligand-HSA interactions on pH and ionic strength of a solution.

The developed panel of 100 LMWLs of HSA represents a valuable source of the approved drugs with the potential to affect AD progression due to their ability to modulate HSA-Aβ interaction. To probe this suggestion, one representative member from each cluster was selected (**Table 1**) for experimental validation of their ability to affect Aβ40 binding to HSA, focusing on their outstanding clinical value: prednisone, warfarin, levothyroxine, propranolol are included in the 100 most commonly prescribed medications in USA according to the ClinCalc DrugStats Database (https://clincalc.com/DrugStats/Top300Drugs.aspx, according to data for 2021; accessed on 14 March 2024). The clinical data (**Table 3**) [61–67], as well as the results derived from animal models and in vitro studies [68–70], evidence an association of the selected drugs with AD. Meanwhile, the influence of the candidates on Aβ metabolism and HSA interaction with Aβ has not been reported to date.

The clusters III and IV overlap with the Aβ-binding site predicted for HSA [32] and confirmed in ref. [71] (the region between domains IB and IIIB), while the remaining clusters are more or less close to it. Therefore, the ligands belonging to these clusters may directly or allosterically affect HSA-Aβ40 interaction. This suggestion is supported by the data presented in **Table 3**: several members of the different clusters exert different effects on HSA affinity to Aβ. For instance, mefenamic acid (cluster III) favors dissociation of HSA-Aβ40 complex, while prednisone (cluster I) promotes this interaction. Furthermore, the effects for ligands within the same cluster may be opposite, as exemplified by prednisone and risperidone.

We have previously shown that ibuprofen [32] and serotonin [31] favor HSA interaction with Aβ (**Table 3**). The analogous effect is shown here for prednisone (**Table 2**), indicating its potential

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value in prevention of AD onset. Meanwhile, in a randomized, placebo-controlled multicenter trial low-dose prednisone did not show behavioral improvements compared with the placebo group [72]. At the same time, the use of high-doze intrathecal corticosteroids has been proposed as a promising approach to AD prevention [62]. On the contrary, the substances that prevent HSA interaction with Aβ, such as mefenamic acid and risperidone (**Table 3**), should be considered as potentially harmful with regard to stimulation of AD progression. Apparently, *in vitro* and clinical studies are needed to establish the relevance for AD in these cases. In any case, identification of the drugs that affect HSA-Aβ interaction is important for further studies of their impact on AD, regardless of the direction of the effect.

Table 3. Summary of the effect of the AD-related LMWLs of HSA on its interaction with Aβ and data on the role of these ligands in AD progression.

Cluster	HSA ligand	Effect of the ligand on HSA affinity for Aβ	Relevance for AD progression
I	prednisone	↑ †	decline of AD biomarkers in non-AD patients after taking prednisone [61,62]; lack of effect in the treatment of AD patients [72]
	risperidone	↓ [73]	reduces psychosis and favors functioning in elderly patients with psychosis of AD and mixed dementia [74]
II	ibuprofen	↑ [32]	reduces the risk of AD progression [34]
III	mefenamic acid	↓ †	not available
IV	levothyroxine	bidirectional effect †	hypothyroidism, thyroiditis and hyperthyroidism are more common among AD patients [75]; lowered levothyroxine level in cerebrospinal fluid of AD patients [65]
V	serotonin	↑ [31]	modulates Aβ level in the central nervous system of AD patients [35]

† See **Table 2;** ↑ - promote interaction; ↓ - prevent interaction.

5. Conclusions

Our previous works have revealed the precious feature of the specific HSA ligands, such as ibuprofen [32] and serotonin [31], to improve HSA affinity for Aβ, which may contribute to prevention of AD. Here we extended search for the substances with a similar effect to all approved drugs. The careful filtering of the DrugBank according to several rational criteria, followed by selection of the candidates relevant to AD, gave rise to a panel of 100 top-ranked LMWLs. Although molecular docking studies enabled their classification depending on location of their binding sites on HSA molecule, the SPR data do not reveal clear regularities regarding the ability of the ligands belonging to different clusters to affect HSA-Aβ interaction. Thus, all 100 candidate ligands are of potential value in this respect.

Among the LMWLs studied in this work, prednisone has the most pronounced favorable effect on HSA affinity for monomeric Aβ, with a 13-fold decrease in the equilibrium dissociation constant. Meanwhile, HSA prevents Aβ fibrillation [20] via binding not only monomeric Aβ, but also its oligomeric forms and protofibrils [76–78], indicating the need to verify ability of the candidate ligands to favor the inhibition of Aβ fibrillation by HSA. The ability of LMWLs to modulate the HSA effects on the Ab fibril formation process has been previously shown for warfarin, palmitic acid, cholesterol, and ibuprofen [32,79]. Moreover, some of the HSA ligands may also inhibit Aβ fibrillation, as shown for serotonin [80]. Thus, search for the optimal therapeutic ligand involves extensive experimental studies and filtering of the candidates in accord with the abovementioned requirements. However,

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the present work provides a basis for these studies aimed at repurposing of the approved drugs for prevention and treatment of AD. Additional clinical trials should be performed for the ligands that prevent HSA-Aβ interaction (for instance, mefenamic acid and risperidone) to rule out the possibility of stimulation of Aβ depositions.

Supplementary Materials: The following supporting information can be downloaded at: Preprints.org, **Figure S1**: Scheme of the hierarchical classification of the AD-related low-molecular-weight HSA ligands according to the predicted location of their binding sites on HSA molecule; **Table S1**: List of the therapeutic low-molecular-weight HSA ligands associated with AD; **Table S2**: List of the AD-related low-molecular-weight HSA ligands, hierarchically classified according to the predicted location of their binding sites on HSA molecule.

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