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Docking of Small Molecules with in Vivo Cardiovascular Activity into The Proteins – The Relationship with Side-Effects

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***Abstract*—Cardiac amyloidosis is a clinical pathology, usually of a genetically mediated nature, initiated by the formation of amyloid fibrils, that lead to death. The number of clinically used molecules for the treatment of cardiac amyloidosis is very limited with strong side effects (for example, tafamidis and diflunisal). In this study, the methods of molecular modeling and computer docking of ligands using ICM-pro (Molsoft LLS, USA) were used to evaluate for the first time the level of side binding of small molecules possessing *in vivo* cardiovascular activity into the ligand-binding domains of 136 proteins derived from protein database. The correlation between these data with the experimental data available for the investigated molecules has been discussed. Thus, this approach opens new tool for the selection of target compounds for the treatment of cardiac amyloidosis with selected mode of action and less side-effects, as well as may be the basis for the molecular docking in other software.**

Keywords—cardiac amyloidosis; molecular docking; small molecules; the active protein centres; mechanisms of ligand side binding; selective mode of action

I. INTRODUCTION

Currently, Group of protein-folding disorders with protein deposits are called amyloidosis and any organ may be involved. Several amyloidogenic proteins may be the source of deposits, and the disease prognosis is dependent on both the organ(s) involved and the amyloid type. Amyloid involvement of the heart, cardiac amyloidosis usually has the worst prognosis [1-4]. Cardiac amyloidosis may be due to myocardial deposition of transthyretin protein derived from the liver known as transthyretin cardiac amyloidosis (ATTR) or may be due to AL amyloidosis with myocardial deposition of immunoglobulin light-chain proteins derived from a clone of plasma cells [4].

Accumulation diseases including amyloidosis could mimic hypertrophic cardiomyopathy and lead to systolic and diastolic dysfunction, with increased myocardial stiffness and impaired myocardial compliance, reduced end-diastolic volume and reduced cardiac output contribution from infiltrated atria [5,6]. A characteristic feature of amyloidosis is the impairment of vital organ function by the deposition of extracellular fibrils corresponding to a collection of proteins with unstable tertiary structures, improperly coiled and/or misfolded [7]. Other specific aspects of these fibrils are that they are rigid, unbranched, linear, about 6-12 nm wide and indeterminate in length, insoluble or poorly soluble, and resistant to proteolytic degradation [8].

There are several sources of amyloid fibrils. The most common protein components of fibrils in cardiac amyloidosis are immunoglobulin light chains (AL) [9] and amyloid transthyretin (ATTR) [10]. The latter can either be familial due to an inherited pathogenic variant of the transthyretin gene or non-familial (ATTRwt) caused by an abnormal wild-type form of transthyretin, also known as senile cardiac amyloidosis [11].

The number of clinically used molecules for the treatment of cardiac amyloidosis is very limited, and all of them have toxicity and strong side effects. These drugs are presented by two small molecules, namely Diflunisal (5-(2,4-difluorophenyl) salicylic acid) and Tafamidis (2-(3,5-dichlorophenyl)-1,3-benzoxazole-6-carboxylic acid) which are clinically used for the treatment of cardiac amyloidosis not so long time [12].

Being nonsteroidal anti-inflammatory drug (NSAID), diflunisal was used for the treatment of inflammatory diseases, such as osteoarthritis, rheumatoid joint inflammation, essential dysmenorrhea, etc. for many years [13]. As it was demonstrated recently, this molecule stabilizes TTR tetrameric form [14], which has been confirmed in randomized clinical trials for patients with polyneuropathy. Main side-effects of diflunisal include serious gastrointestinal adverse effects, such as peptic ulcer disease, bleeding, kidney damage, digestive disorder, gastrointestinal distress, etc. [15].

Oral form of Tafamidis has the trade name Vydagel, which is used for patients with mutant ATTR, however this drug is not approved in the United States. The phase 3 of clinical trial investigating its usefulness in ATTR cardiomyopathy (both wild-type ATTR and mutant ATTR) has completed an enrollment [16, 17]. Main side-effects of Tafamidis include allergic reaction: hives, dyspnea, lips, tongue, or throat edemas, and also may harm an unborn baby [18, 19].

Along with other important tasks, such as design of highly effective and highly selective protein ligands and minimizing

their toxicity, the rational design of biologically active compounds requires taking into account and minimizing possible side effects associated with their use as drugs. Since there are tens of thousands of targets in living organisms in the form of various protein receptors, there are always some undesirable side effects due to the accidental binding of biologically active ligands with random receptors. All drug-like organic compounds have side effects, but their level may differ in many times. In addition, structural factors are known that can significantly weaken or, conversely, increase the level of side effects of the biologically active compounds studied. All this makes it possible not only to estimate in advance the possible level of side effects, but also to minimize their probability at the design stage. Another way to diminish side-effects is the development of drug delivery systems with targeted mode of action.

The complexity of the interactions of molecules used for cardiac amyloidosis treatment and the data about their interactions with proteins are not yet investigated.

In this work, the propensities of biologically active compounds bind to proteins from a representative set of 136 proteins from the PDBbind database were investigated using molecular modeling methods and docking of ligands into the active centers of proteins.

Recently we published the binding energy values of 10 small molecules obtained by molecular docking with random receptors from Protein Database [20]. Here we described the correlation between the data about binding with the receptors and side-effects of the small molecules, which demonstrated cardiovascular activity in animals models.

Diflunisal, a derivative of salicylic acid, is a nonsteroidal anti-inflammatory drug, and has analgesic, antipyretic and anti-inflammatory effects. Diflunisal has not been approved for the treatment of transthyretin familial amyloid polyneuropathy, despite the fact that its clinical efficacy has been proven [2], since it has a number of side effects, for example, it causes damage to kidney and liver functions, the development of cardiovascular diseases [3]. In addition, diflunisal is poorly soluble in water, which worsens its bio-distribution and leads to unfavorable pharmacokinetic profiles.

The known drug delivery systems of diflunisal were described in our recent review article [4].

Tafamidis is a drug used to slow the progression of the disease in adults with some forms of transthyretin amyloidosis. It can be used to treat both hereditary forms of familial amyloid cardiomyopathy and familial amyloid polyneuropathy, as well as "wild" type transthyretin amyloidosis, which was previously called senile systemic amyloidosis [1]. Tafamidis provides stabilization of the quaternary structure of the transthyretin protein. In patients with transthyretin amyloidosis, transthyretin breaks down and forms clusters called amyloid clusters that damage tissues, including nerve endings and the heart. Tafamidis was approved in the European Union in 2011 for the treatment of transthyretin amyloidosis with polyneuropathy and in Japan in 2013 [5]. Tafamidis is used in clinical practice for the treatment of transthyretin amyloidosis with cardiomyopathy. It was approved for the treatment of this form of the disease in the United States in 2019 and in the European Union in 2020. Two drugs have been approved in the USA: tafamidis meglumine (Vindakel) and tafamidis (Vindamax).

Tafamidis and diflunisal were selected for the introduction into the polymer matrix to ensure the prolonged action of the drugs, increase the therapeutic effectiveness of treatment and reduce the side effects by the increasing of the selectivity of action.

II. Materials and Methods

Molecular modeling and preparation of proteins and ligands (Table 1) as well as the docking of ligands into the active centers of 136 proteins from the PDBbind database were carried out according to the recently piblished procedures [21].

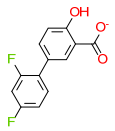
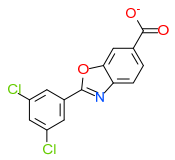
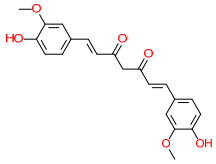
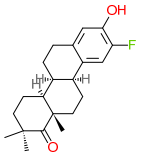
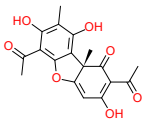
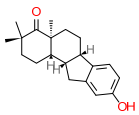
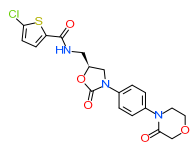
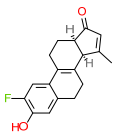
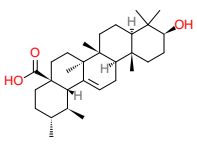
Diflunisal and tafamidis were chosen as molecules which are used in clinical practice for the treatment of cardiac amyloidosis.

Diflunisal has adverse effects such as: Increased liver function test (up to 15%); next side effects may occur up to 10% cases: Body fluid retention, Rash, Abdominal pain, Constipation, Diarrhea, Flatulence, Indigestion, Nausea, Dizziness, Headache, Insomnia, Tinnitus; and Edema (<1%), Hypertension, Myocardial infarction, Vasculitis (<1%), Erythema multiforme (<1%), Scaling eczema, Stevens-Johnson syndrome (<1%), Toxic epidermal necrolysis (<1%), Gastrointestinal hemorrhage (<1%), Gastrointestinal perforation (<1%), Inflammatory disorder of digestive tract, Agranulocytosis (<1%), Anemia (<1%), Thrombocytopenia (<1%), Hepatitis (<1%), Jaundice (<1%), Anaphylactoid reaction (<1%), Immune hypersensitivity reaction (<1%), Cerebrovascular accident, Impaired renal function disorder (<1%), Interstitial nephritis (<1%), Renal failure (<1%), Bronchospasm.

All mentioned numerous side effects include the interaction with many protein structures in the body, that results in a non-selective mode of therapeutic action.

In open DrugBank [Diflunisal: Uses, Interactions, Mechanism of Action | DrugBank Online] we found only few data about the interactions of diflunisal with Prostaglandin G/H synthase 2, Prostaglandin G/H synthase 1, UDP-glucuronosyltransferase 1-8, UDP-glucuronosyltransferase 1-9, Transthyretin, Serum albumin, Solute carrier family 22 member 6.

TABLE I. STRUCTURES OF SMALL MOLECULES OF DIFFERENT NATURE SELECTED FOR THIS STUDY

№	Structure	Known biological activity
1		Diflunisal. A nonsteroidal anti-inflammatory drug approved for the treatment of arthritis pain. Stabilizes the tetrameric form of transthyretin [14].
2		Tafamidis. Approved in Europe and Japan and in some countries for the treatment of mutant amyloidosis ATTR, which causes polyneuropathy [17].
3		Curcumin. It plays a protective role in the suppression of the cardiac hypertrophy development, heart failure, drug-induced cardiotoxicity, myocardial infarction, atherosclerosis, aortic aneurysm, stroke and diabetic cardiovascular complications [22].
4		2-Fluoro-17,17-dimethyl-D-homo-8-alpha-estrone. Prevents the deposition of excessive amounts of cholesterol in the aorta, reduces the content of triglycerides in blood serum in animal models [26].
5		Usnic acid. Reduces the frequency of adverse cardiovascular events [23].
6		16,16-Dimethyl-B-nor-D-homo-8-alpha estrone. Blocks excess cholesterol in the aorta of experimental animals. There is no hypertriglyceridemic effect [27].
7		Rivaroxaban (Xarelto). Prevents the formation of blood clots during atrial fibrillation. Rivaroxaban is more effective than warfarin in the reducing of the likelihood of ischemic strokes in patients with atrial fibrillation [25].
8		2-Fluoro-16-methyl-13α-estra-1,3,5(10),8(9),15-trien-17-on. It has hypolipidemic and cardioprotective activity, increases the HDL content in the blood without affecting the triglyceride level [28].
9		Ursolic acid. Inhibitor of cardiac marker enzymes, effects on the lipid profile [22].

Tafamidis [Tafamidis: Uses, Interactions, Mechanism of Action | DrugBank Online] interacts with ATP-binding cassette sub-family G member 2, Solute carrier family 22 member 6, Solute carrier family 22 member 8.

Because there are patients with cardiac amyloidosis without TTR mutations, we also choose the molecules which are available with known effects on heart parameters, that confirmed either in animal models or in clinical trials. Except of clinically used tafamidis and diflunisal, another type of molecules are natural compounds such as Curcumin, Usnic acid and Ursolic acid, as well as synthetic steroidal analogues were chosen based on the reported data about its influence on cardiovascular system. Curcumin plays a protective role in the suppression of the cardiac hypertrophy development, heart failure, drug-induced cardiotoxicity, myocardial infarction, atherosclerosis, aortic aneurysm, stroke and diabetic cardiovascular complications [22]. Usnic acid reduces the frequency of adverse cardiovascular events [23]. Ursolic acid is known as inhibitor of cardiac marker enzymes and has effects on the lipid profile [24]. Widely used Rivaroxaban (Xarelto) prevents the formation of blood clots during atrial fibrillation. Rivaroxaban is more effective than warfarin in the reducing of the likelihood of ischemic strokes in patients with atrial fibrillation [25]. Synthetic steroids in in vivo experiment demonstrated the prevention of the deposition of excessive amounts of cholesterol in the aorta, and the reduced content of triglycerides in blood serum in animal models, as well as have no hypertriglyceridemic effect [26-28]. It is important that these steroids have not or have significantly reduced hormonal activity, that also confirmed in animals models.

III. Results and Discussion

To study the binding mechanisms of ligands to proteins from the database of PDBbind 2018 complexes, a central, so-called "core" set of protein complexes with their native ligands was selected, obtained with the highest possible resolution using X-ray structural analysis methods [29]. From this set, complexes were selected in which the active centers had no structural breaks and gaps, and native ligands are corresponded to the Lipinsky rules [30-32].

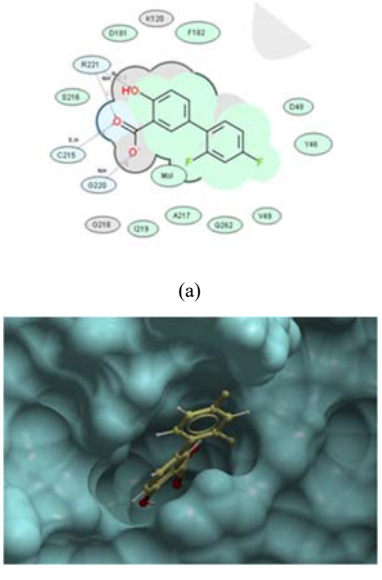
The final set of 136 protein complexes, according to the CF code (enzyme classification), contains enzymes of 4 main classes: CF2 Transferase, CF3 Hydrolase, CF4 Lyase (synthase), CF6 Ligase (synthase). Thus, since they are absent in the "cow set" of PDBbind obtained with high resolution, the random protein set we used does not include proteins from the classes of CF1 Oxidoreductase, CF5 Isomerase and CF7 Translocase.

The CF2 Transferase class contains 4 subclasses: transferring single-carbon groups (2.1.x.x), transferring sugar residues (2.4.x.x), transferring alkyl and aryl groups with the exception of the methyl residue (2.5.x.x), transferring phosphorus-containing residues (2.7.x.x). The CF3 Hydrolase class contains 5 subclasses: hydrolyzing ester bond (3.1.x.x), hydrolyzing sugars (3.2.x.x), hydrolyzing peptide bond (3.4.x.x), hydrolyzing non-peptide carbon-nitrogen bond (3.5.x.x), hydrolyzing acid-anhydride bonds (3.6.x.x). The KV4 class of Ligase (synthase) contains 3 subclasses: splitting carbon-carbon bonds (4.1.x.x), splitting carbon-oxygen bonds (4.2.x.x), splitting phosphorus-oxygen bonds (4.6.x.x). The class of CF6 Ligases (synthetases) contains 1 subclass: forming bonds between nitrogen and carbon (6.3.x.x). Also, the studied set of 136 studied structures (55 proteins) contains 18 structures (7 structural proteins) without the classification code CF [29].

As an example of the illustration of the results of docking ligands in the active center of proteins, Fig. 1 shows the structure with the lowest-energy (ICM-Score=-51.9) Diflunisal in the active center of human tyrosine protein phosphatase (PDB: 2HB1), which has very high binding constants.

The high binding level of Diflunisal in this active center is determined by several structural factors: a) a sufficiently deep cavity of the active center, which allows almost completely isolating the hydrophobic surface of the ligand from the solvent (water); b) the free volume and shape of the cavity of the active center correspond well to the size and shape of Diflunisal; and c) in the presented complex, all possible hydrogen bonds of the ligand inside the cavity of the active center of the protein.

Five compounds, namely Diflunisal, Tafamidis, Curcumin, Usnic acid and Rivaroxaban have a different tendency to bind in the active centers of proteins. Curcumin (~9.5%) and Diflunisal (~8.0%) have the highest level of side binding. Usnic acid, on the contrary, has a reduced level of side binding with proteins compared to the average (~0.7%). The remaining compounds (16,16-dimethyl-D-homoequilenin, 2-fluoro-17,17-dimethyl-D-homo-estrone, 16,16-Dimethyl-B-nor-D-homo-8alpha estrone, 2-fluoro-16-methyl-13α-estra-1,3,5(10),8(9),15-trien-17-on and Ursolic acid) showed a high level of the selectivity with the probability of the accidental binding << 0.7%.



(a)

(a)

Fig. 1. The structure of the lowest-energy (ICM-Score=-51.9) Diflunisal in the active center of human tyrosine protein phosphatase (PDB: 2HB1). Panel a) diagram showing the amino acid residues of the protein active center surrounding the ligand in the complex, as well as intermolecular hydrogen bonds between the protein and the ligand; b) the three-dimensional structure of this complex. Reproduced from the recently published data about the energy binding values [20].

According to the data obtained, both Tafamidis and Diflunisal have high binding level to two proteins: Tyrosine-protein phosphatase non-receptor type 1 (protein-tyrosine

phosphatase 1B, PTP1B); Peroxisome proliferator-activated receptor gamma (PPARG).

PTP1B interacts with BCAR1 [33], epidermal growth factor receptor [34,35], Grb2 [33,36] and IRS1 [37], Vascular endothelial growth factor Receptor-2 [38] and Vascular endothelial growth factor via PGC1-alpha/ERR-alpha [39], that indicate about many cascade functions involved by this interactions with diflunisal. PTP1B has clinical implications in the treatment of type 2 diabetes as well as cancer.

PPARG regulates fatty acid storage and glucose metabolism. The genes activated by PPARG stimulate lipid uptake and adipogenesis by fat cells. PPARG knockout mice are devoid of adipose tissue, thus PPARG acts as a regulator of adipocyte differentiation. PPARG increases insulin sensitivity by enhancing storage of fatty acids in fat cells (reducing lipotoxicity), by enhancing adiponectin release from fat cells, by inducing FGF21 [40] and by enhancing nicotinic acid adenine dinucleotide phosphate production through upregulation of the CD38 enzyme [41]. PPARG promotes anti-inflammatory M2 macrophage activation in mice [42]. Adiponectin induces ABCA1-mediated reverse cholesterol transport by activation of PPAR-γ and LXRα/β [43]. Abovementioned are is confirmed by the data about the combination use of Bezafibrate + diflunisal/ CRx-401 (developed by CombinatoRx manufacturer) presented in Part II Diabetes: All Drugs in Development (Curator: Stephen J Williams) [44].

Dipeptidyl-peptidase 4 (DPP-IV, adenosine deaminase complexing protein 2 or CD26) is an enzyme expressed on the surface of most cell types and is associated with immune regulation, signal transduction, and apoptosis. Furthermore, it suppresses in the development of some tumors [45-48].

Middle East respiratory syndrome coronavirus binds to DPP4. It is found on the surface of cells in the airways (such as the lungs) and kidneys, and it may be used for blocking the virus's entry into the cell [49]. The high binding of usnic acid with DPP-IV may explain broad spectrum activities of these natural compounds, including anti-viral properties of usnic acid [50].

The known Xarelto side-effects include: More common: Back pain, bleeding gums, bloody stools, bowel or bladder dysfunction, burning, crawling, itching, numbness, prickling, "pins and needles", or tingling feelings, coughing up blood, difficulty with breathing or swallowing, dizziness, headache, increased menstrual flow or vaginal bleeding, leg weakness, nosebleeds, numbness, paralysis, prolonged bleeding from cuts, red or black, tarry stools, red or dark brown urine, vomiting of blood or material that looks like coffee grounds; Less common: Fainting, pain in the arms or legs, wound secretion; Rare: Burning feeling while urinating, difficult or painful urination [51-54].

Found by molecular modelling interactions of Xarelto are CDR2 (cerebellar degeneration-related protein 2 [55], Hsp90. These data give a hint that the interaction with Hsp90, which is a key molecule for proteins functions, is more important, than the binding with Xa-receptor.

In the series of synthetic steroidal analogues, no any side effects have been detected in the toxicity experiments (rats) as well as during the evaluation of their activity against breast cancer in animal (rats) models, which correlate with the data of molecular docking. Taking into account that:

- steroid **4** (2-fluoro-17,17-dimethyl-D-homo-8-alpha-estrone) prevents the deposition of excessive amounts of cholesterol in the aorta, reduces the content of triglycerides in blood serum in animal models;

- steroid **6** (16,16-dimethyl-B-nor-D-homo-8-alpha estrone) blocks cholesterol excess in the aorta of experimental animals and has not hypertriglyceridemic effect;

- steroid **8** (2-fluoro-16-methyl-13α-estra-1,3,5(10),8(9),15-trien-17-on) has hypolipidemic and cardioprotective activity, increases the HDL content in the blood without affecting the triglyceride level;

they may be recommended for further investigations of its cardioprotective properties.

These results are in good correlation with available data for investigated compounds, and thus open suitable the way for the search of new molecules for the cardiac amyloidosis treatment.

IV. Conclusions and Future Perspectives

Based on the results of molecular docking into 134 proteins structures derived from PDB base of small molecules of different nature including clinically used Diflunisal and Tafamidis as well as such natural compounds as curcumin, usnic acid, ursolic acid, and synthetic steroids possessing cardiovascular activity in *in vivo* models it may be concluded:

1. Five compounds, namely Diflunisal, Tafamidis, Curcumin, Usnic acid and Rivaroxaban have a different tendency to bind in the active centers of random proteins.

2. Curcumin and Diflunisal have the highest level of side binding. Usnic acid, on the contrary, has a reduced level of side binding with random proteins compared to the average.

3. The other compounds (16,16-dimethyl-D-homoequilenin, 2-fluoro-17,17-dimethyl-D-homo-estrone, 16,16-Dimethyl-B-nor-D-homo-8alpha estrone, 2-fluoro-16-methyl-13α-estra-1,3,5(10),8(9),15-trien-17-on and Ursolic acid) showed a high level of selectivity with a probability of accidental binding less than << 0.7%.

Although ICM-pro (Molsoft LLS, USA) is well-known program used for molecular docking, the methodology for small molecules docking into proteins has been described for the first time.

The high ability of interactions of diflunisal and tafamidis with various proteins confirmed the side-effects of these compounds documented many times. The results of *in vivo* experiments confirm the modelling data obtained, and no any side effects were detected for synthetic steroids. This approach is a useful tool for the search of selective small molecules for the treatment of cardiac amyloidosis with less side-effects.

It is also the base for the further molecular docking studies of these small molecules to various proteins using other software like PyMOL, AutoDock, and Maestro-Schrodinger.

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