Identification of the Inhibition Effects of Some Natural Antiproliferative Agents on CA-I, CA-II, and AChE Activities Isolated from Human Erythrocytes by Kinetic and Molecular Docking Studies

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Abstract—Nowadays the determination of inhibitors of carbonic anhydrase isoenzymes (CAs) have become one of the main goals of drug design studies, and inhibitors of CAs have taken their place in clinical applications to be used in the treatment and diagnosis of many diseases from glaucoma to cancer. On the other hand, acetylcholinesterase (AChE) inhibitors are also the main target molecules for the treatment of Alzheimer's disease. However, the unwanted side effects of existing CA and AChE inhibitors necessitate the identification of new and selective inhibitors of these enzymes. In this study, we examined the inhibition effects of some natural antiproliferative agents on CA-I, CA-II, and AChE activities isolated from human erythrocytes. Betulinic acid (**I**) had the strongest inhibitory effect on esterase activity of hCA-I (IC_{50} 29.16 μM) and hCA-II (IC_{50} 31.82 μM). On the other hand, sanguinarine chloride (**VI**) had the strongest inhibitory effect (IC_{50}: 19.44 μM) on hAChE activity. Molecular modeling studies were also carried out to elucidate the inhibition mechanism of betulinic acid on hCA-I and hCA-II isoenzymes and sanguinarine chloride on the hAChE enzyme. We believe that the results we obtained in this study will contribute to the design of new and natural CA and AChE inhibitors.

INTRODUCTION

Carbonic anhydrases (CAs, E.C. 4.2.1.1), also known as carbonate dehydratases, are metalloenzymes that contain Zn^{2+} ions, which are commonly found in all organisms, and reversibly catalyze the dehydration reactions of carbon dioxide (CO_2). The reversible hydration reaction of carbon dioxide catalyzed by carbonic anhydrase is a simple but fundamental reaction for metabolism. Due to the bicarbonate anion and a proton produced as a result of the reaction, this reaction plays a central role in the regulation of intracellular and extracellular pH in various tissues and organs [1−3]. Apart from catalyzing the CO_2 hydration reaction, carbonic anhydrase enzyme is an enzyme that plays an important role in various pathological and physiological processes involved in many metabolic events such as acid-base balance, respiration, ion transport, ureagenesis, gluconeogenesis, lipogenesis, and electrolyte secretion [4, 5]. There are eight different genetic families of CA isoenzymes as:

α-, β-, γ-, δ-, ζ-, n-, θ-, and ι CAs [6, 7]. These eight CA gene families use metal ion as cofactor for their catalytic activity: α-, β-, δ-, n-, θ-CAs use Zn^{2+} and γ-CAs use Fe^{2+} as cofactors. At the same time, γ-CAs are active when Zn^{+2} and Co^{2+} ions are bound [7]. ζ-CAs use both Cd^{2+} and Zn^{2+} as cofactors [8−10]. Recently discovered ι CAs use Mn^{+2} and Zn^{2+} as cofactors for their activities [6]. They are the enzymes in monomeric structure of the α-class, which contain the isoenzymes that were first identified and most studied. Until now, sixteen α-CA isoenzymes have been identified in mammals. These isoenzymes differ in their catalytic activity, distribution to tissues, and their behavior towards inhibitors and activators [3, 11]. The isoenzymes on which the most scientific studies have been conducted are cytosolic CA-I and CA-II and tumor-associated CA-X and CA-XII isoenzymes. Various studies have shown that abnormal levels or activity changes of carbonic anhydrase isoenzymes are associated with various diseases [12]. Therefore, determining new carbonic anhydrase inhibitors and activators specific to CA isoenzymes for use in diagnosis and

treatment of various diseases in biomedical applications has become a major goal [13−15]. Today, inhibitors of carbonic anhydrase isoenzymes have taken their place in clinical applications to be used in the diagnosis and treatment of various diseases such as glaucoma, epilepsy, oedema [5, 16], obesity, anemia, osteoporosis, and cancer [5, 17]. In addition, recent studies show that CA activators may have pharmacological applications in neurological diseases such as Alzheimer's disease and aging [18, 19]. CA inhibitors used in the clinical applications (Acetazolamide, Methazolamide, Dorzolamide, Brinzolamide, etc.) are not selective against CA isoforms and have undesirable side effects [20, 21]. This has led researchers to discover new and isoenzyme-specific CA inhibitors, and studies in this area are gaining popularity day by day.

Acetylcholinesterase enzyme (AChE, E.C. 3.1.1.7) found in all peripheral and central nervous systems of humans and animals, catalyzes the hydrolysis of acetylcholine, an important neurotransmitter, to acetic acid and choline [22−24]. Alzheimer's disease (AD) occurs due to imbalances in the cholinergic pathway and as a result of decreased neurotransmitters in the brain. The neurotransmitter that decreases most in Alzheimer's is acetylcholine. Therefore, AChE inhibitors have been targeted molecules in the treatment of AD [3]. Today, inhibitors of AChE are important drug groups that have achieved a certain success rate in the treatment of AD. AChE inhibitor compounds such as donepezil, tacrine, huperzine A, galantamine and rivastigmine have been used as essential drugs in the treatment of AD [25] and identifying new AChE inhibitors for use in the treatment of AD is highly critical.

Herein, the inhibition effects of some natural antiproliferative compounds (Figure 1) (Betulinic acid (**I**), Biochanin A (**II**), 2-methoxyestradiol (**III**), plumbagin (**IV**), rhein (**V**), sanguinarine chloride (**VI**), budesonide (**VII**)) on human erythrocytes CA-I and CA-II isozymes and AChE activity were investigated under in vitro conditions. Additionally, molecular docking studies have been carried out to elucidate the binding modes, binding energies and inhibition mechanisms of antiproliferative compounds that show the best in vitro inhibition effect on hAChE, hCA-I and hCA-II enzymes.

RESULTS AND DISCUSSION

Inhibitors of carbonic anhydrase isoenzymes are remedy-expected molecules in diagnosing and treating many diseases from glaucoma to cancer [5]. Therefore, the identification of new, effective, isoenzyme-specific natural inhibitors contributes significantly to the discovery of CA inhibitor drugs that can be used in clinical applications. So, many researchers have aimed to identify new CA inhibitors and activators that can be obtained from natural sources [26, 27]. On the other hand, acetylcholinesterase inhibitors are the main focus of interest for scientists to discover

drugs to be used in the treatment of Alzheimer's disease [3, 25]. Considering this information, the inhibition effects of Betulinic acid (**I**), Biochanin A (**II**), 2-methoxyestradiol (**III**), plumbagin (**IV**), rhein (**V**), sanguinarine chloride (**VI**), and budesonide (**VII**) which are important natural molecules with many vital biological activities along with anticancer activity, on hCA-I, hCA-II and hAChE activities were investigated. For this aim, hCA-I and hCA-II were purifed from human erythrocyte with a yield of 67.70 and 75.77%, 160.49 and 187.50 purification fold, a specific activity of 1794.3 and 2096.22 EU/mg protein, respectively by using CNBr-activated Sepharose-4B-L-tyrosine-sulfanilamide affinity chromatography method. The hAChE enzyme was partially purified from human erythrocytes using DE-52 anion exchange chromatography with 75.22% yield, 10.53-fold, 0.02 EU/mg protein specific activity. After completing the purification of enzymes, the inhibition efficacy of the molecules was determined by the IC_{50} values representing the inhibitor concentration that reduced the enzyme's activity by half. IC_{50} values of (**I**), (**II**), (**IV**), (**V**), (**VI**), (**VII**) for hCA-I were found as 29.16, 46.66, 1155.2, 350, 77.77, 350 μM, respectively. IC_{50} values of (**I**), (**II**), (**IV**), (**V**), (**VI**), (**VII**) for hCA-II were found as 31.82, 74.64, 770.41, 402.25, 136.72, 807.75 μM, respectively. IC_{50} values of (**II**), (**III**), (**IV**), (**V**), (**VI**) for hAChE were found as 63.64, 38.8, 38.8, 21.87, 19.44 μM, respectively (Table 1). Low IC_{50} value indicate strong inhibition effect. Except for 2-methoxyestradiol (**III**), all molecules inhibited the esterase activity of hCA-I and hCA-II isoenzymes. Betulinic acid (**I**) had the strongest inhibitory effect on esterase activity of hCA-I (IC_{50} 29.16 μM) and hCA-II (IC_{50} 31.82 μM). Betulinic acid (**I**) is a natural pentacyclic triterpene found in the shells of plant species. It has a wide variety of biological activities such as antibacterial, antimalarial, antihelmintic, antioxidant, anti-human immunodeficiency virus and anti-angiogenic, along with antitumor activity. Due to its low toxicity, it attracts increasing attention [28, 29]. In recent study, it was found that the novel sulfamate conjugates of betulin and betulinic acid have very strong inhibition effects on the tumor-associated CA isoenzyme CA-IX activity [29]. On the other hand, the plumbagin (**VI**) had the weakest inhibitory effect for hCA-I (IC_{50} 1155.2 μM), while the budesonide (**VII**) showed the weakest inhibitory effect for hCA-II (IC_{50} 807.75 μM). The inhibitory effect of Rhein and budesonide on hCA-I was found to be equal. Also, Rhein (for hCA-I IC_{50} 350 μM, for hCA-II IC_{50} 402.25 μM) had a more inhibition effect on hCA-I than hCA-II. It was determined that biochanin A (for hCA-I IC_{50} 46.66 μM, for hCA-II IC_{50} 74.64 μM) and sanguinarine chloride (for hCA-I IC_{50} 77.77 μM, for hCA-II IC_{50} 136.72 μM) also had an inhibition effect on the esterase activity of hCA-I and hCA-II isoenzymes at micromolar levels. Both molecules had a stronger inhibition effect on
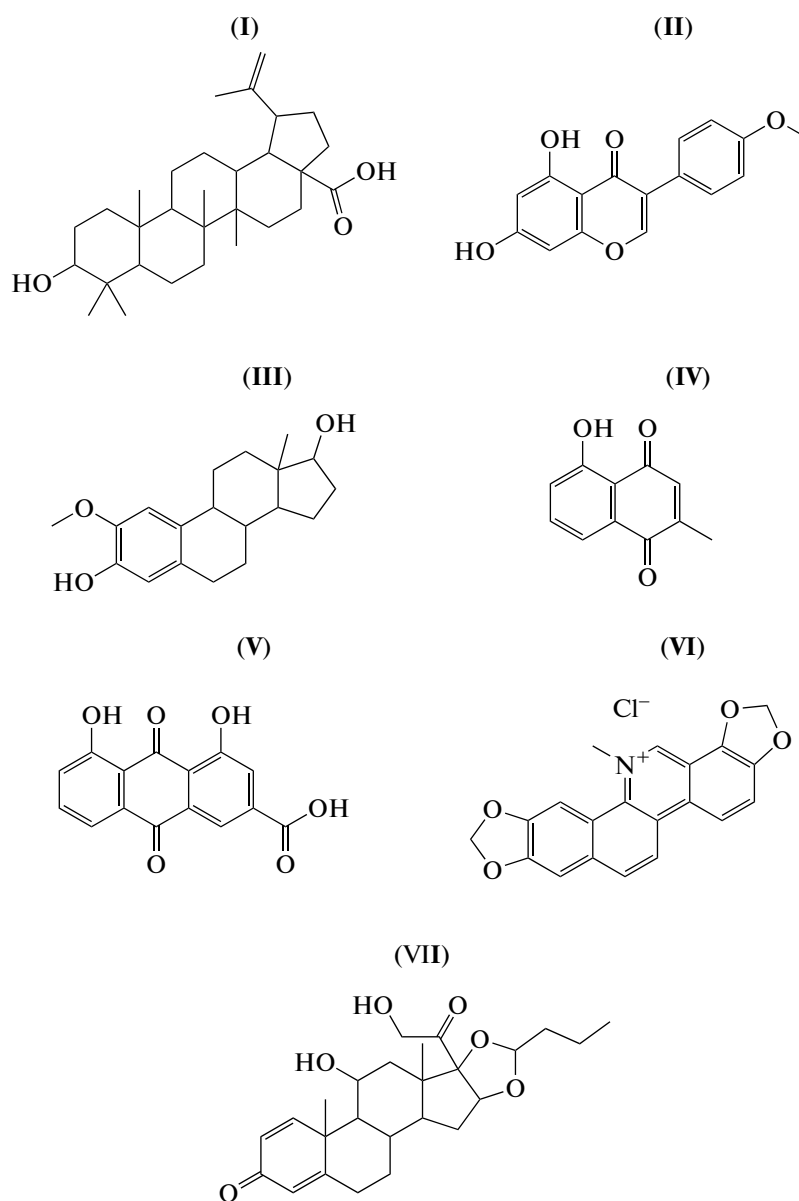
Fig. 1. The molecular structures of natural antiproliferative agents used in this study.

esterase activity of hCA-I than hCA-II (Table 1). In a previous study, the effect of Biochanin A on the hydratase activities of human CA I, II, IV, VI and XII isoenzymes were investigated, and the K_i values of Biochanin A for hCA-I, II were over 10000, 7078.5 nM for (**IV**), 371.5 nM for (**VII**). It was found to be 52.5 nM for (**XII**) [30]. When we compare the results, we obtained for Biochanin A in our study with this study, we can say that the inhibition effect of Biochanin A on esterase activity of hCA-I and hCA-II is more than its inhibition effect on hydratase activity. In addition, it was observed that compounds (**I**), (**II**), (**IV**), (**V**), (**VI**), (**VII**) had weaker inhibition power on hCA-I and hCA-II isoenzymes than acetazolamide, which is the reference inhibitor.

In addition, the inhibition effects of substances (**I**)−(**VII**) on the AChE enzyme isolated from human erythrocytes were investigated. Betulinic acid (**I**) and budesonide (**VII**) molecules did not show an inhibitory effect on AChE enzyme activity. Molecules (**II**)−(**VI**) were found to have an inhibition effect on hAChE enzyme activity at micromolar levels. Sanguinarine chloride (**VI**) had the strongest inhibitory effect (IC$_{50}$ 19.44 μM) on hAChE activity while Biochanin A (**II**) (IC$_{50}$: 63.64 μM) had the weakest inhibitory effect. The inhibition effect of 2-methoxyestradiol (**III**) and plumbagin (**IV**) on hAChE enzyme activity was found to be equal (for both IC$_{50}$ 38.8 μM). Rhein (**V**) had the second strongest inhibitory effect (IC$_{50}$ 21.87 μM)

Table 1. Inhibition results of antiproliferative agents used in this study for hCA-I, hCA-II and hAChE

Compounds	For hAChE		For hCA-I		For hCA-II	
	IC$_{50}$, μM	R^2	IC$_{50}$, μM	R^2	IC$_{50}$, μM	R^2
Betulinic acid (**I**)	–	–	29.16	0.9222	31.82 μM	0.9971
Biochain A (**II**)	63.64	0.9981	46.66	0.9838	74.64 μM	0.9830
2-Methoxyestradiol (**III**)	38.80	0.8958	–	–	–	–
Plumbagin (**IV**)	38.80	0.9290	1155.2	0.9817	770.41 μM	0.9573
Rhein (**V**)	21.87	0.9563	350	0.8887	402.25 μM	0.9528
Sanguinarine chloride (**VI**)	19.44	0.9308	77.77	0.9150	136.72 μM	0.9830
Budesonide (**VII**)	–	–	350	0.9834	807.75 μM	0.9732
Acetazolamide*	–	–	0.73	0.9525	0.27 μM	0.9776
Tacrine*	0.011	0.9509	–	–	–	–

hCA-I and hCA-II: human erythrocyte carbonic anhydrase isoenzymes I and II, hAChE: human erythrocyte acethylcholinesterase. * Acetazolamide used as a standard inhibitor for hCA-I and hCA-II isoenzymes and Tacrine* used as a standard inhibitor for hAChE.

Table 2. Molecular docking scores and predicted free binding energy (ΔG_{bind}) values of antiproliferative agents

Compounds	hAChE		hCA-I		hCA-II	
	IFD Docking Score	MM-GBSA ΔG_{bind}	IFD Docking Score	MM-GBSA ΔG_{bind}	IFD Docking Score	MM-GBSA ΔG_{bind}
Betulinic acid (**I**)	–	–	−10.622	−52.54	−8.935	−32.88
Acetazolamide	–	–	−7.879	−31.37	−8.864	−28.02
Sanguinarine chloride (**VI**)	−9.262	−53.47	–	–	–	–
Tacrine	−8.998	−73.78	–	–	–	–

after sanguinarine chloride (**VI**) for hAChE. In addition to, it was observed that (**II**), (**III**), (**IV**), (**V**), (**VI**) had weaker inhibition power on hAChE activity than tacrine, which is the reference inhibitor.

The receptor is assumed to be rigid in standard docking simulations, but in fact, most receptor binding sites change to match the form and binding mode of the ligand, a process known as induced-fit. The Induced Fit Docking (IFD) protocol accurately estimates receptor structural changes and ligand binding mode. The IFD protocol was used to assess the binding modes and interactions of betulinic acid, which has the best in vitro inhibition effect for hCA-I and hCA-II, and sanguinarine chloride, which has the best in vitro inhibition effect on the AChE enzyme. As positive control molecules, the well-known AChE inhibitor Tacrine, and well-known CA-I and CA-II inhibitor Acetazolamide were docked using the same protocol.

Molecular docking studies of antiproliferative agents, using Induced-Fit Docking (IFD) protocol, were carried out to gain a better comprehending of the ligand−protein interactions at the atomic level. For this purpose, betulinic acid, which has the best in vitro inhibition effect for hCA-I and hCA-II, and sanguinarine chloride compound, which has the best in vitro inhibition effect on the hAChE enzyme, were docked

into the respective enzymes. In addition, we used the Prime MM/GBSA module to calculate free binding energies to better understand the structural and thermodynamic factors involved in the hCA-I, hCA-II, and hAChE inhibitory activities of betulinic acid and sanguinarine chloride compounds. Induced-Fit Docking scores and MM-GBSA free binding energy results are summarized in Table 2.

The re-docking procedure was used to verify the docking procedure in this research. The co-crystal ligands 1YL (Dihydrotanshinone I), 3TV (2,3,5,6-tetrafluoro-4-(propylsulfanyl) benzenesulfonamide), and 51J (2-(but-2-yn)-1-ylsulfamoyl)-4-sulfamoyl-benzoic acid) were isolated from hAChE, hCA-I, and hCA-II and re-docked to the corresponding proteins for docking validation. The best pose of the 1YL, 3TV, and 51J were superimposed with the co-crystalized ligands after the re-docking phase, and the ligands' RMSD (Root Mean Square Deviation) were estimated to be 0.918, 1.40, and 1.87 Å, respectively (Fig. 2).

The results of IFD docking score (−9.262 kcal/mol) and MM-GBSA free binding energy (−53.47 kcal/mol) of sanguinarine chloride compound, which experimentally showed a potent inhibitory effect against AChE enzyme, confirmed the experimental findings. Compared to the positive control compound tacrine, the
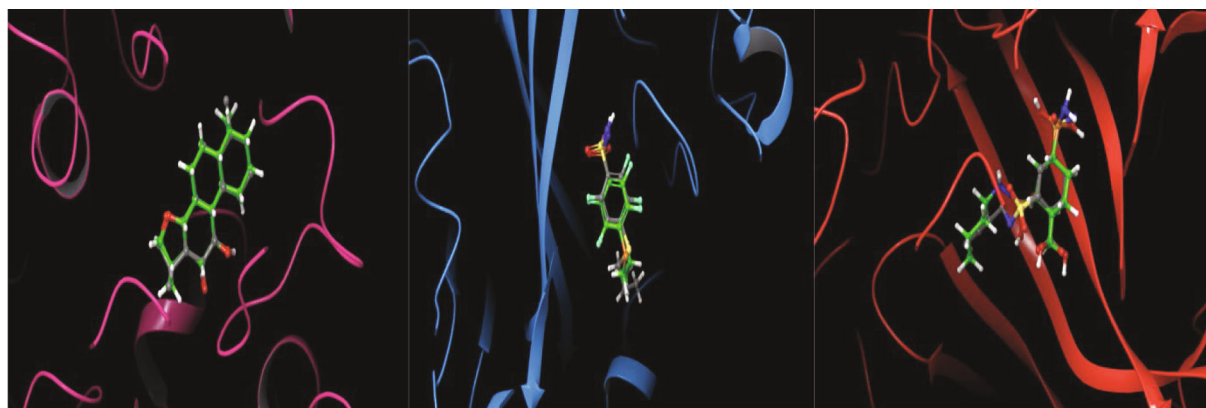
Fig. 2. hAChE (left) hCA-I (middle), and hCA-II (right) receptor docking validations. Grey ball and stick modeling depicts co-crystallized ligands, while green ball and stick modeling depicts docked ligands.

sanguinarine chloride compound scored higher, while the free binding energy was calculated lower (Table 3). The Peripheral Active Site (PAS) is a gorge at the rim of the human AChE active site. This active-site gorge includes 279−297 amino acid residues [31]. TRP286 at the PAS, in particular, has been shown to play an important role in the allosteric regulation of AChE activity [32]. The sanguinarine chloride compound established four π−π interactions with the TRP286 amino acid fraction at the PAS. It also made hydrogen bonding with the TYR72 and TYR124 fractions (with distances 2.09 and 1.98 Å, respectively) (Fig. 3).

The findings of IFD docking for the betulinic acid compound, which has impressive in vitro inhibitory action on both CA-I and CA-II enzymes, reveal that the compound is well positioned in the active site of the enzymes. The compound established a metal coordination bond and dual salt bridges through the carboxyl moiety with the Zinc ion, which is in the active site of carbonic anhydrases and is responsible for the carrying out of catalytic activity (Figs. 4, 5). The compound has also established a hydrogen bonding through the −OH moiety with ASN62 residue (with a distance 2.03 Å) at the CA-II catalytic active site (Fig. 5). Betulinic acid compound scored better against both enzymes (IFD docking scores −10.622 and −8.935 kcal/mol for CA-I and CA-II, respectively) compared to the positive control compound acetazolamide. The MM-GBSA calculations have been very successful in predicting the free binding energies (ΔG_{bind}) of betulinic acid compound based on its IFD docking scores against both enzymes, as can be seen in Table 3. So, it was determined that betulinic acid, which was experimentally determined to have very close IC_{50} values against CA-I and CA-II enzymes (29.16 and 31.82 μM, respectively), theoretically had similar inhibition mechanisms.

The QikProp tool of Maestro were used to forecast drug-likeness and pharmacokinetics. To assess druggable properties, we analyzed physiochemically

descriptors and pharmaceutically related properties of antiproliferative agents (Table 3). All of the antiproliferative agents had high partition coefficients (QPlogPo/w) ranging from 0,935 to 3.85, which were essential for drug distribution and absorption. The permeability of these compounds was measured by factor QPPCaco, which ranged from 281.278 to 9504.2, where QPPCaco was a potential apparent Caco-2 cell permeability in nm/sec, a critical factor for estimating cell permeability in biological membranes. QPPMDCK (cell permeable parameter) values of the compounds were between 3.242 to 5641.04, their percentage of human oral absorption values were between 48.228 to 100%, QPlogHERG (K^+ channel blockage) values were less than −5, and their water solubility (QPlogS) ranged between −1.311 to −5.748. The drug's capacity to cross the blood-brain barrier is shown by the QPlogBB parameter. The ability of the compounds to cross the blood-brain barrier means that they can be useful in the management of neurological disorders. In this respect, it is very important that the QPlogBB value of the compound sanguinarine chloride, which we showed in our study to be a good inhibitor of the AChE enzyme, which is an enzyme associated with neurological diseases, is within the recommended range. In addition, the CNS (central nervous system activity) value of the compound sanguinarine chloride was estimated to be +2 (active). In conclusion, all of the antiproliferative agents used in this study met all of the pharmacokinetic criteria for a drug-like substance and were shown to be beyond the appropriate range for clinical usage.

EXPERIMENTAL

Betulinic acid, Biochanin A, 2-methoxyestradiol, plumbagin, rhein, sanguinarine chloride, budesonide and all chemicals used in the purification steps and kinetic studies of AChE and CA isoenzymes were obtained from Sigma-Aldrich Co. (Steinheim, Germany).

Table 3. In Silico ADMET screening results of antiproliferative agents

	2-Methoxyestradiol	Betulinic acid	Biochanin A	Budesonide	Plumbagin	Rhein	Sanguinarine chloride	Recommended range
[1]mol MW	302.41	456.71	284.27	430.54	188.18	284.23	333.34	130.0–725.0
[2]donorHB	2	2	1	2	0	1	0	0.0–6.0
[3]accptHB	3.20	3.70	3.75	9.85	3.75	5.5	3.50	2.0–20.0
[4]QPlogPo/w	2.994	5.664	2.363	2.04	0.801	0.935	3.85	−2.0–6.5
[5]QPlogS	−4.073	−5.748	−3.153	−4.073	−1.311	−2.528	−4.01	−6.5–0.5
[6]QPlogHERG	−3.671	−1.309	−4.6810	−4.424	−3.737	−2.613	−4.27	Concern below −5
[7]QPPCaco	1288.1	489.947	581.81	281.278	557.16	7.643	9504.2	<25 poor, >500 great
[8]QPlogBB	−0.4130	−0.222	−0.7850	−1.327	−0.550	−1.892	0.5480	−3.0 − 1.2
[9]QPPMDCK	650.413	290.988	275.49	125.583	262.896	3.242	5641.04	<25 poor, >500 great
[10]%HOA	100	95.301	90.268	82.726	80.784	48.228	100	>80% is high <25% is poor
[11]CNS	0	−1	−1	−2	0	−2	2	−2 (inactive) to +2 (active)
[12]RuleOfFive	0	1	0	0	0	0	0	Maximum is 4
[13]RuleOfThree	0	1	0	0	0	1	0	Maximum is 3

[1] Molecular weight of the molecule. [2] Estimated number of hydrogen bonds that would be accepted by the solute from water molecules in an aqueous solution. [3] Estimated number of hydrogen bonds that would be donated by the solute to water molecules in an aqueous solution. [4] Predicted octanol/water partition coefficient. [5] Predicted aqueous solubility, log S. S in mol dm^{-3} is the concentration of the solute in a saturated solution that is in equilibrium with the crystalline solid. [6] Predicted IC_{50} value for blockage of HERG K^+ channels. [7] Predicted apparent Caco-2 cell permeability in nm./s. [8] Predicted brain/blood partition coefficient. [9] Predicted apparent MDCK cell permeability in nm./s. [10] Predicted human oral absorption on 0 to 100% scale. [11] Predicted central nervous system activity on a −2 (inactive) to +2 (active) scale. [12] Number of violations of Lipinski's rule of five. [13] Number of violations of Jorgensen's rule of three.
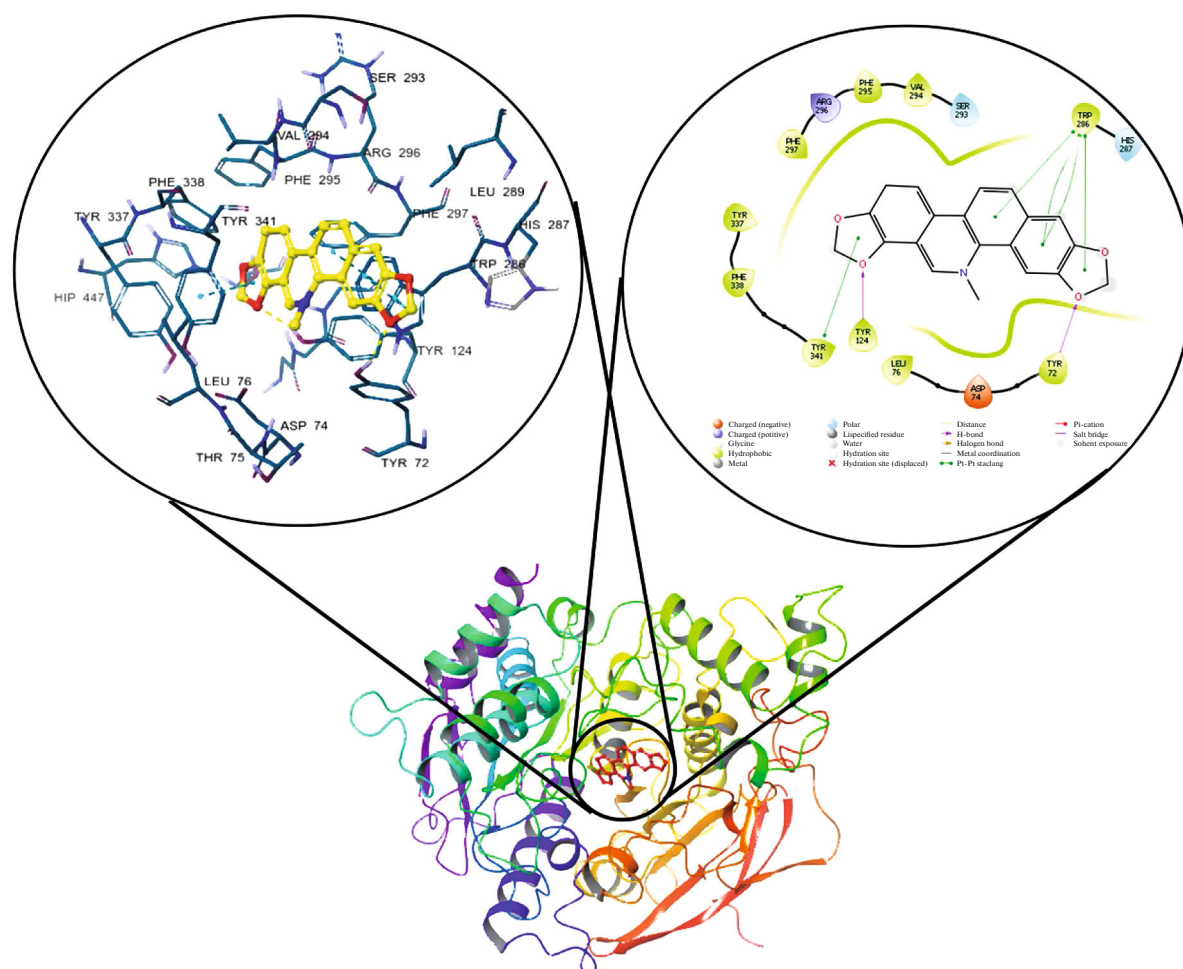
Fig. 3. 3D detailed binding mode and 2D ligand interactions of sanguinarine chloride with AChE receptor.

Purification Studies of CA-I, CA-II and AChE from Human Erythrocytes

The healthy human erythrocyte used in this study was obtained from the Atatürk University blood center. Carbonic anhydrase enzyme from human erythrocytes was purified using Sepharose-4B-L-Tyrosine sulfanilamide affinity chromatography method as in our previous studies [1, 2, 17]. During the purification process, quantitative protein determination in hemolysate and pure enzyme samples was determined by the Bradford method [33]. The purity of isoenzymes was checked by Laemmli's SDS-PAGE method [34]. Pure isoenzymes obtained after affinity chromatography were dialyzed against 0.05 M Tris-SO$_4$ (pH 7.4) buffer overnight at 4°C [1, 2, 17]. After dialysis, pure isoenzymes were separated into 1 mL fractions and stored at −80°C for use in kinetic studies.

In this study, the AChE enzyme was partially purified from human erythrocytes. For this purpose, erythrocytes were precipitated by centrifugation and washed 2−3 times with 0.9% NaCl solution. Erythrocytes were hemolysis by stirring with ice water and the waste cell membrane was separated from the hemolysate by centrifugation. All these operations were carried out at about 4°C. The pH of hemolysate was adjusted to 7.8 with K$_2$HPO$_4$ and applied to DE-52 anion exchange chromatography column equilibrated with 100 mM KH$_2$PO$_4$ (pH 7.5) buffer. The gel was washed with the same buffer. AChE was eluated with the increasing salt gradient. Active tubes were combined and dialyzed against the 20 mM KH$_2$PO$_4$ (pH 7.5) buffer [35]. It was then separated into 1 mL small fractions for use in kinetic studies and stored at −80°C.

In vitro Inhibition Studies

In this study, while investigating the inhibition effects of (**I−VII**) on the activities of hCA-I and hCA-II isoenzymes, the esterase activity method described by Verpoorte et al. [36] was used as in our previous studies [1−3, 17]. In this method, CA isoenzymes use the *p*-nitrophenyl acetate as substrate and hydrolyzes *p*-nitrophenyl acetate to *p*-nitrophenol and acetic
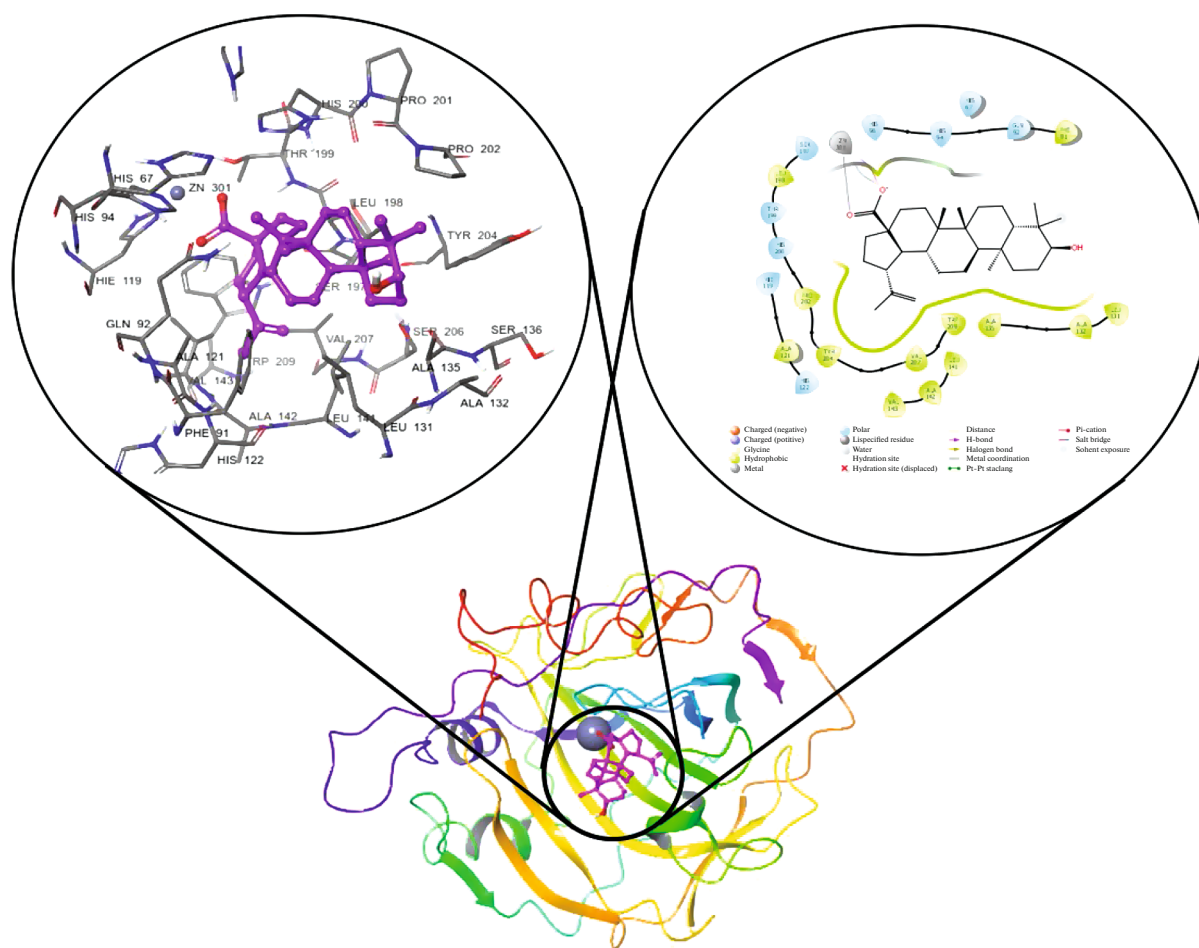
Fig. 4. 3D detailed binding mode and 2D ligand interactions of betulinic acid with hCA-I receptor.

acid. According to this method, in this study the formation of *p*-nitrophenol from *p*-nitrophenyl acetate was monitored by measuring the absorbance at 348 nm, 25°C for 3 min using a spectrophotometer. The enzyme unit was calculated using the absorption coefficient ($\varepsilon = 5.4 \times 10^3$ M^{-1} cm^{-1}) of *p*-nitrophenyl acetate at 348 nm.

While determining the inhibitory effects of (**I**−**VII**) on acetylcholine esterase activity, AChE activity was assayed at 436 nm with a spectrophotometer, according to Worek et al. (1999)'s method, a modified method of the Ellman procedure [37]. In this procedure, acetylthiocholine is hydrolyzed to thiocholine and acetic acid by the catalysis of the AChE enzyme. As a result of the interaction of 5,5-Dithiobis (2-nitrobenzoic) acid (DTNB) with thiocholine, a yellow colored compound, 5-thio-2 nitrobenzoic acid (TNB) is formed. The method is based on the measurement of the absorbance increases due to increased TNB concentration [38].

In inhibition studies, activity measurements were performed at least five different concentrations of each molecule to determine the IC$_{50}$ values of (**I**−**VII**) for

hCA-I, hCA-II and hAChE. Activity % versus inhibitor concentration plots were drawn for molecules that showing inhibition effect. IC$_{50}$ values were calculated from the equations of these curves. Also, acetazolamide was used as reference inhibitor for hCA-I and hCA-II isoenzymes and tacrine was used as reference inhibitor for hAChE enzyme.

Molecular Docking Studies

Molecular docking studies have been carried out to elucidate the binding modes, binding energies and inhibition mechanisms of antiproliferative compounds that show the best in vitro inhibition effect on hAChE, hCA-I and hCA-II enzymes. Molecular docking simulation studies, as shown in our previous study [39], were carried out using the Maestro 12.5 of the Schrödinger Molecular Modeling Suite package program [40]. The X-ray crystal structures of the AChE (PDB ID: 4M0E), CA I (PDB ID: 4WR7), and CA II (PDB ID: 5AML) receptors were retrieved from RCSB Protein Data Bank (PDB). Receptors were preprocessed and prepared at physiological pH using the
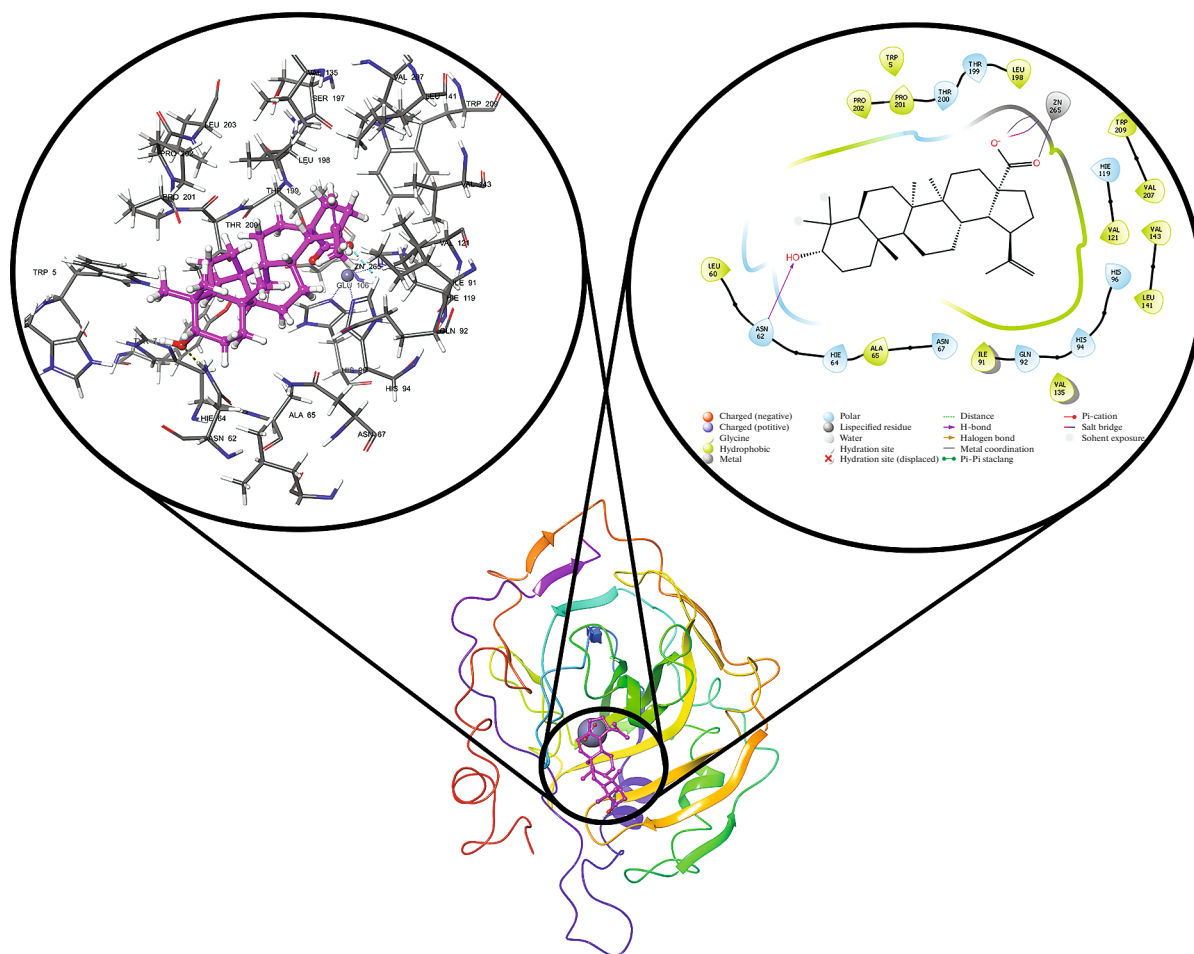
Fig. 5. 3D detailed binding mode and 2D ligand interactions of betulinic acid with hCA-II receptor.

Protein Preparation Wizard [41]. Optimization and minimization of the receptors was carried out using the OPLS3e force field. A grid box of 20 × 20 × 20 Å was created around the native ligands in protein crystal structures using the Receptor Grid Generation platform of Maestro. 3D drawing of ligands, determination of correct molecular geometries and protonation states at pH 7.0 ± 2.0 were performed with the LigPrep module. To perform molecular docking simulations, Glide Induced-Fit Docking (IFD) protocol [42] was used as stated in our previous study [39]. The re-docking method was used to verify the docking procedure. For this purpose, hAChE, hCA-I and hCA-II co-crystal ligands 1YL (Dihydrotanshinone I), 3TV (2,3,5,6-tetrafluoro-4-(propylsulfanyl) benzenesulfonamide), and 51J (2-(but-2-yn)-1-ylsulfamoyl)-4-sulfamoylbenzoic acid) was extracted from the protein crystal structures and subjected to re-docking. Then, using the Maestro Superposition panel, the co-crystal ligands and the best poses of each co-crystal ligands after docking were superimposed, and the Root Mean Square Deviation (RMSD) values were determined. An RMSD value of less than 2Å indicates the accuracy of the docking procedure [43].

Binding Free Energy Calculation Using Molecular Mechanics/Generalized Born Surface Area (MM/GBSA)

The Molecular Mechanics/Generalized Born Surface Area (MM/GBSA) approach combines molecular mechanics measurements with continuum solvation models to measure binding free energies (ΔG_{bind}) for macromolecules [44]. Prime/MM-GBSA which utilizes the OPLS3e force field and VSGB dissolvable model was used for calculations to the binding free energies of the protein-ligand complexes [45].

ADME Studies

The physicochemical descriptors, pharmacokinetic properties, and drug-likeness of the antiproliferative agents have also been determined through ADME (absorption, distribution, metabolism, and excretion) studies. QikProp panel of Maestro was used to assess the ADME properties of antiproliferative agents. QikProp calculates values by comparing the properties of a new molecule to those of 95% of existing drugs [40, 46].

CONCLUSIONS

In this study, the effects of some natural molecules (Betulinic acid (**I**), Biochanin A (**II**), 2-methoxyestradiol (**III**), plumbagin (**IV**), rhein (**V**), sanguinarine chloride (**VI**), budesonide (**VII**)) with known antiproliferative effects on hCA-I, hCA-II and AChE activities were investigated. It was determined that Betulinic acid showed strong inhibitory effect for hCA-I and hCA-II, while Sanquinarine Chloride had strong inhibitory effect for AChE. Obtained inhibition results were supported by molecular modeling results. We hope that these results will contribute to the studies on the determination of new, effective, natural CA and AChE inhibitors.

COMPLIANCE WITH ETHICAL STANDARDS

Healthy human erythrocytes used in this study were obtained from Atatürk University blood center. Other than that, it does not include any studies involving human participants by any of the authors, and does not include any animal studies performed by any author.

Conflict of Interests

The authors declare that they have no conflicts of interest.

REFERENCES

1. Alım, Z., Kılınç, N., Şengül, B., and Beydemir, Ş., *Chem. Biol. Drug Des.*, 2015, vol. 86, pp. 857–863.
https://doi.org/10.1111/cbdd.12561

2. Alım, Z., *J. Biochem. Mol. Toxicol.*, 2018, vol. 32, article ID e22194.
https://doi.org/10.1002/jbt.22194

3. Köksal, Z., Alım, Z., Bayrak, S., Gülçin, I., and Özdemir, H., *J. Biochem. Mol. Toxicol.*, 2019, vol. 33, article ID e22300.
https://doi.org/10.1002/jbt.22300

4. Guney, M., Çavdar, H., Sentürk, M., and Ekinci, D., *Bioorg. Med. Chem. Lett.*, 2015, vol. 25, pp. 3261–3263.
https://doi.org/10.1016/j.bmcl.2015.05.073

5. Kumar, S., Rulhania, S., Jaswal, S., and Monga, V., *Eur. J. Med. Chem.*, 2021, vol. 209, article ID 112923.
https://doi.org/10.1016/j.ejmech.2020.112923

6. Jensen, E.L., Clement, R., Kosta, A., Maberly, S.C., and Gontero, B., *Isme J.*, 2019, vol. 13, pp. 2094–2106.
https://doi.org/10.1038/s41396-019-0426-8

7. Pretea, S.D., Nocentinib, A., Supuran, C.T., and Capassoa, C., *J. Enzyme Inhib. Med. Chem.*, 2020, vol. 35, pp. 1060–1068.
https://doi.org/10.1080/14756366.2020.1755852

8. Supuran, C.T., *Expert Opin. Drug Discov.*, 2017, vol. 12, pp. 61–88.
https://doi.org/10.1080/17460441.2017.1253677

9. Bhatt, A., Mahon, B.P., Cruzeiro, V.W.D., Cornelio, B., Laronze-Cochard, M., Ceruso, M., Sapi, J., Rance, G.A., Khlobystov, A.N., Fontana, A., Roitberg, A., Supuran, C.T., and McKenna, R., *ChemBioChem*, 2017, vol. 18, pp. 213–222.
https://doi.org/10.1002/cbic.201600513

10. Alterio, V., Langella, E., Viparelli, F., Vullo, D., Ascione, G., Dathan, N.A., Morel, F.M.M., Supuran, C.T., Desimone, G., and Monte, S.M., *Biochimie*, 2012, vol. 94, pp. 1232–1241.
https://doi.org/10.1016/j.biochi.2012.02.013

11. Kalaycı, M., Turkeş, C., Arslan, M., Demir, Y., and Beydemir, S., *Arch. Pharm.*, 2020, vol. 354, article ID 2000282.
https://doi.org/10.1002/ardp.202000282

12. Pinard, M.A., Mahon, B., and McKenna, R., *Biomed. Res. Int.*, 2015, article ID 453543.
https://doi.org/10.1155/2015/453543

13. Hekman, M.C.H., Rijpkema, M., Aarntzen, E.H., Mulder, S.F., Langenhuijsen, J.F., Oosterwijk, E., Boerman, O.C., Oyen, W.J.G., and Mulders, P.F.A., *Eur. Urol.*, 2018, vol. 74, pp. 257–260.
https://doi.org/10.1016/j.eururo.2018.04.026

14. Andreucci, E., Ruzzolini, J., Peppicelli, S., Bianchini, F., Laurenzana, A., Carta, F., Supuran C.T., and Calorini, L., *J. Enzym Inhib. Med. Chem.*, 2019, vol. 34, pp. 117–123.
https://doi.org/10.1080/14756366.2018.1532419

15. Alterio, V., Kellner, M., Esposito, D., Liesche-Starnecker, F., Bua, S., Supuran, C.T., Monti, S.M., Zeidler, R., and DeSimone, G., *J. Mol. Biol.*, 2019, vol. 431, pp. 4910–4921.
https://doi.org/10.1016/j.jmb.2019.10.022

16. Turkes, C., Arslan, M., Demir, Y., Çoçajd, D., Nixhad, A.R., and Beydemir, S., *Bioorg. Chem.,* 2019, vol. 89, article ID 103004.
https://doi.org/10.1016/j.bioorg.2019.103004

17. Alım, Z., Köksal, Z., and Karaman, M., *Pharmacol. Rep.*, 2020, vol. 72, pp. 1738–1748.
https://doi.org/10.1007/s43440-020-00149-4

18. Temperini, C., Innocenti, A., Scozzafava, A., Mastrolorenzo, A., and Supuran, C.T., *Bioorg. Med. Chem. Lett.,* 2007, vol. 17, pp. 628–635.
https://doi.org/10.1016/j.bmcl.2006.11.027

19. Supuran, C.T., *Nat. Rev. Drug. Discov.,* 2008, vol. 7, pp. 168–181.
https://doi.org/10.1038/nrd2467

20. Scozzafava, A., Mastrolorenzo, A., and Supuran, C.T., *Expert Opin. Ther. Pat.*, 2004, vol. 14, pp. 667–702.
https://doi.org/10.1517/13543776.14.5.667

21. Supuran, C.T., and Scozzafava, A., *Bioorg. Med. Chem.,* 2007, vol. 15, pp. 4336–4350.
https://doi.org/10.1016/j.bmc.2007.04.020

22. Sever, B., Türkeş, C., Altıntop, M.D., Demir, Y., and Beydemir, S., *Int. J. Biol. Macromol.*, 2020, vol. 163, pp. 1970–1988.
https://doi.org/10.1016/j.ijbiomac.2020.09.043

23. Aydın, B.O., Anıl, D., and Demir, Y., *Arch. Pharm.*, 2021, vol. 354, article ID 2000330.
https://doi.org/10.1002/ardp.202000330

24. Bilginer, S., Gul, H.I., Anil, B., and Demir, Y., *Arch. Pharm.*, 2021, vol. 354, article ID 2000243.
https://doi.org/10.1002/ardp.202000243

25. Kucukoglu, K., Gul, H.I., Taslimi, P., Gulcin, I., and Supuran, C.T., *Bioorg. Chem.*, 2019, vol. 86, pp. 316–321.
https://doi.org/10.1016/j.bioorg.2019.02.008

26. Adem, S., Akkemik, E., Aksit, H., Guller, P., Tüfekci, A.R., Demirtas, I., and Ciftci, M., *Med. Chem. Res.*, 2019, vol. 28, pp. 711–722.
https://doi.org/10.1007/s00044-019-02329-1

27. Güller, P., Dağalan, Z., Güller, U., Çalışır, U., and Nişancı, B., *J. Mol. Struct.*, 2021, vol. 1239, p. 130498.
https://doi.org/10.1016/j.molstruc.2021.130498

28. Zhang, X., Hu, J., and Chen, Y., *Mol. Med. Rep.*, 2016, vol. 14, pp. 4489–4495.
https://doi.org/10.3892/mmr.2016.5792

29. Vanchanagiri, K., Emmerich, D., Bruschke, M., Bache, M., Seifert, F., Csuk, R., Vordermark, D., and Paschke, R., *Chem. Biol. Interact.*, 2018, vol. 25, pp. 12–23.
https://doi.org/10.1016/j.cbi.2018.02.014

30. Karioti, A., Ceruso, M., Carta, F., Bilia, A.R., and Supuran, C.T., *Bioorg. Med. Chem.,* 2015, vol. 23, pp. 7219–7225.
https://doi.org/10.1016/j.bmc.2015.10.018

31. Butini S., Campiani G., Borriello M., Gemma S., Panico A., Persico M., Catalanotti, B., Ros, S., Brindisi, M., Agnusdei, M., Fiorini, I., Nacci, V., Novellino, E., Belinskaya, T., Saxena, A., and Fattorusso, C., *J. Med. Chem.*, 2008, vol. 51, pp. 3154–3170.
https://doi.org/10.1021/jm701253t

32. Shi, J., Boyd, A.E., Radic, Z., and Taylor, P., *J. Biol. Chem.*, 2001, vol. 276, pp. 42196–42204.
https://doi.org/10.1074/jbc.M106896200

33. Bradford, M.M., *Anal. Biochem.*, 1976, vol. 72, pp. 248–254.
https://doi.org/10.1016/0003-2697(76)90527-3

34. Laemmli, U.K., *Nature*, 1970, vol. 227, pp. 680–685.
https://doi.org/10.1038/227680a0

35. Guller, U., Guller, P., and Çiftçi, M., *Altern. Ther. Health. Med.*, 2020, article ID 32619207.

36. Verpoorte, J.A., Mehta, S., and Edsall, J.T., *J. Biol. Chem.*, 1967, vol. 242, pp. 4221–4229.
https://doi.org/10.1016/S0021-9258(18)95800-X

37. Ellman, G.L., Courtney, K.D., Andres Jr, V., and Featherstone, R.M., *Biochem. Pharmacol.,* 1961, vol. 7, pp. 88–95.
https://doi.org/10.1016/0006-2952(61)90145-9

38. Worek, F., Mast, U., Kiderlen, D., Diepold, C., and Eyer, P., *Clin. Chim. Acta*, 1999, vol. 288, pp. 73–90.
https://doi.org/10.1016/S0009-8981(99)00144-8

39. Gök, N., Akıncıoğlu, A., Binici, E.E., Akıncıoğlu, H., Kılınç, N., and Göksu, S., *Arch. Pharm.,* 2021, vol. 354, article ID e2000496.
https://doi.org/10.1002/ardp.202000496

40. *Schrödinger Release 2020-3, Maestro*, Schrödinger, LLC, New York, NY, 2020.

41. Sastry, G.M., Adzhigirey, M., Day, T., Annabhimoju, R., and Sherman, W., *J. Comput. Aid. Mol. Des.,* 2013, vol. 27, pp. 221–234.
https://doi.org/10.1007/s10822-013-9644-8

42. Sherman, W., Day, T., Jacobson, M.P., Friesner, R.A., and Farid, R., *J. Med. Chem.*, 2006, vol. 49, pp. 534–553.
https://doi.org/10.1021/jm050540c

43. Sandor, M., Kiss, R., and Keseru, G.M., J. *Chem. Inf. Model.,* 2010, vol. 50, pp. 1165–1172.
https://doi.org/10.1021/ci1000407

44. Genheden, S. and Ryde, U., *Expert Opin. Drug Discov.*, 2015, vol. 10, pp. 449–461.
https://doi.org/10.1517/17460441.2015.1032936

45. *Prime*, Schrödinger, LLC, New York, NY, 2020.

46. *QikProp*, Schrödinger, LLC, New York, NY, 2020.