DESIGN, SYNTHESIS, BIOLOGICAL EVALUATION, AND IN SILICO STUDY OF NOVEL UREA DERIVATIVES AS INHIBITORS OF CARBONIC ANHYDRASE AND ACETYLCHOLINE ESTERASE

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ABSTRACT. This study aimed to synthesize novel unsymmetrical urea derivatives containing biologically active tryptamine and phenethylamines and investigate their inhibition effects on human carbonic anhydrase (hCA) I and II isozymes as well as acetylcholinesterase (AChE). For this purpose, five different N,N'-dialkyl urea derivatives 18-22 were synthesized from tryptamine and substituted 2-phenethylamine. According to the results of inhibition, all of the synthesized urea derivatives effectively inhibited both hCA (I - II) and AChE enzymes at micromolar concentrations. The IC_{50} values for these molecules were determined to be in the range of 1.214-2.281 µM for hCA-I, 0.888-2.984 µM for hCA-II, and 0.309-0.816 µM for AChE. The most potent inhibitors against hCA-I and hCA-II isozymes are compound 18 (IC_{50}: 1.214 µM; K_i: 0.984 ± 0.277 µM) and compound 19 (IC_{50}: 0.888 µM, K_i: 0.564 ± 0.019 µM), respectively. Furthermore, compound 20 (IC_{50}: 0.309 M and K_i: 0.470 ± 0.039 M) is the most effective AChE inhibitor. Molecular docking studies of compounds with the most effective inhibition were performed. The estimated binding energy values for compounds 18, 19, and 20 were calculated to be -7.81, -7.34, and -8.20 kcal/mol, respectively. Finally, the ADME studies were determined these compounds' physicochemical and pharmacokinetic descriptors and drug-like properties.

Keywords: biological activity, tryptamine, phenethylamine, toxicology, molecular docking, ADME

INTRODUCTION

N, N'-dialkyl, and N, N'-alkyl aryl ureas are very significant compounds having large application areas due to their physiological and pharmacological activities. For example 1: (a selective GSK-3β inhibitor) has been evaluated for the treatment of Alzheimer's disease[1][2], 2: eicosapentaenoic acid
derived regulator of cardiomyocyte contraction[3], 3: (a somatostatin agonist) is used for the treatment of Alzheimer’s disease and depression[1,2,4], 4: 1-(1-arylimidazolidine-2-ylidene)-3-(4-chlorobenzyl) urea as antiviral[5], 5: urotensin-II receptor agonists (ACT-058362)[6], 6: TRPV1 antagonists (A-425619)[7][8] (Figure 1).

Figure 1. Some biologically active N, N'-dialkyl and N, N'-alkyl aryl urea derivatives 1-6

On the other hand, in the mammalian brain, tryptamine, an indolamine metabolite of the essential amino acid tryptophan [9], has been demonstrated to activate amine-associated trace receptors and regulate glutamatergic, serotonergic, and dopaminergic system activity[10][11]. Furthermore, 2-phenethylamines are found in a wide range of biologically active compounds and drugs[12].

Carbonic anhydrase (EC 4.2.1.1., CA), a member of the metalloenzyme family, acts as a pH-regulating enzyme in all prokaryotic and eukaryotic life kingdoms[13]. It is known to have 7 different genetic families, namely α, β, γ, δ, ζ, η and θ CAs. α-CAs, which are found in a lot of organisms such as algae, green plants, protozoa, vertebrates, and some bacteria, are the most studied CA family in the world[14]. So far, 16 α-CA isozymes have been identified in terms of catalytic activity, sensitivity to different inhibitor classes, and subcellular localization (cytosolic, membrane-bound, mitochondrial, and saliva secretion).
CA s reversibly hydrate CO$_2$ (carbon dioxide) to HCO$_3^-$ (bicarbonate) and H$^+$ (proton) through a metal hydroxide nucleophilic mechanism[15]. Also, these isozymes play a crucial role in several metabolic processes such as bone resorption, fluid balance, calcification, edema, osteoporosis, glaucoma, tumor production, pH regulation, cancer, carboxylation reactions, and epilepsy[16].

Acetylcholine (ACh), an important substance for cholinergic neurotransmission in the peripheral and central nervous systems, is a molecule synthesized from choline at pre-synaptic terminals. This molecule is the most basic neurotransmitter that activates the muscles in the peripheral nervous system. Acetylcholinesterase (E.C.3.1.1.7., AChE), which is involved in the hydrolysis mechanism of ACh in living things, is an enzyme that breaks down acetylcholine into choline and acetate in synaptic spaces[17]. Alzheimer's disease (AD) is a dementia disease that occurs as a result of the decrease of neurotransmitters in the brain, such as ACh. AD, a neurodegenerative disorder, is associated with a decrease in the amount of acetylcholine in the brain[18]. In this disease, one of the most complex neuron degradation problems of the 21st century, important functions such as speaking ability, perception and judgment capacity, abstract thinking, and problem-solving are affected. Today, AChE inhibitors such as donepezil and rivastigmine are used to eliminate the symptoms of the disease[19–21].

As discussed above, N-N' dialkyl urea derivatives show significant biological activities. In this study, some new N-N' dialkyl urea derivatives 18-22 derived from tryptamine and phenethylamines were synthesized, and their inhibitory properties against hCA (I-II) and AChE were investigated.

RESULTS AND DISCUSSION

Chemistry

Isocyanates are important precursor intermediates for the synthesis of biologically active compounds. Synthesis of isocyanates occurs very easily with triphosgene in the literature[22]. Therefore, phenethylamines 7-11 were reacted with triphosgene to give isocyanate intermediates 12-16. Then, the reactions of isocyanates 12-16 with Tryptamine 17 afforded novel unsymmetrical ureas 18-22 in good yields (72%-78%) (Scheme 1). Elemental analysis, IR, $^1$H-NMR, and $^{13}$C-NMR were used to determine the chemical structures of synthesized compounds.
Biological Studies

Enzymes are generally used as the main target in the development of drugs. By designing suitable inhibitors for enzymes, they can be turned into potential drugs. In the presented study, the in vitro inhibitory effects of the novel synthesized unsymmetrical ureas 18-22 on the hCA-I, hCA-II, and AChE enzyme activities were evaluated.

hCA activity assay

Carbonic anhydrase inhibitors, which have been used clinically for many years, are potential therapeutic drugs for the treatment of many diseases, such as inflammation, neurological disorders, epilepsy, hypoxic tumors, glaucoma, obesity, arthritis, cancer, hemolytic anemia, and dental caries[23]. Dichlorophenamide, acetazolamide, dorzolamide, phentermine, sulpiride, methazolamide, sulthiame, brinzolamide, zonisamide, indisulam, topiramate, and ethoxzolamide molecules are some of the carbonic anhydrase inhibitors are used clinically[13][24]. Acetazolamide (AZA) from these molecules was used as a reference hCA inhibitor in this study. The previous studies have determined that a lot of synthesized molecules such as malononitrile, carbamate, sulfonamide, β-lactam, sulfamide, benzylsulfamide, bromophenol, phenolic, pyridazine, and pyrazoline derivatives were inhibited hCA-I and hCA-II[25-37]. Also, much research have displayed that urea derivatives exhibit effective inhibition, such as other hCAs inhibitor. In this context, novel phthalazine urea,
diphenyl urea, and diaryl urea derivatives were synthesized and determined inhibitory effects against hCA-I and hCA-II. These molecules demonstrated IC$_{50}$ values in the range of 5.28-153.15 µM for hCA-I and 5.51-16.96 µM for hCA-II [38-40]. In another study, Ozgeris et al. synthesized the novel urea derivatives incorporating dopaminergic 2-aminotetralin scaffolds. They discovered that these derivatives effectively inhibited both enzymes, with IC$_{50}$ values ranging from 7.65–9.95 nM for hCA-I and 9.27–74.69 nM for hCA-II[41].

Inhibition parameters (IC$_{50}$, K$_i$, and inhibition types) of N-N' dialkyl urea derivatives for cytosolic isoform hCA-I enzyme associated with hemolytic anemia disease were determined. As shown in Table 1, IC$_{50}$ values were found to be 1.214 µM for compound 18, 1.387 µM for compound 19, 1.486 µM for compound 20, 1.249 µM for compound 21, and 2.281 µM for compound 22. Inhibition constants (K$_i$) of the molecules 18-22 were calculated by means of Lineweaver–Burk graphs. These constants were found to be 0.984 ± 0.277 µM for compound 18, 1.422 ± 0.183 µM for compound 19, 1.426 ± 0.042 µM for compound 20, 1.244 ± 0.378 µM for compound 21, and 4.261 ± 1.287 µM for compound 22. Although compound 22 noncompetitively inhibited hCA-I, it competitively inhibited all other compounds.

According to the results, it was determined that all urea derivatives effectively inhibited the hCA-I enzyme at a level of µM. Compound 18 is the molecule with the most effective inhibition potential against hCA-I among all these. When the results obtained for hCA-I were compared with the results of the reference inhibitor (AZA), it was found that the inhibitor concentrations were the same (µM) and close to each other. Furthermore, selectivity indexes (SI) of novel urea derivatives 18-22 were displayed in Table 2. Among these compounds, compound 18 (Ks for hCA-I and hCA-II 0.984 ± 0.277 µM and 1.955 ± 0.581 µM, respectively) was determined as the most selective hCA-I inhibitor in these molecules with a SI value (hCA-II/hCA-I) of 1.987.

| Compound ID | IC$_{50}$ (µM) | K$_i$ (µM) | Inhibition Type | IC$_{50}$ (µM) | K$_i$ (µM) | Inhibition Type |
|-------------|----------------|-------------|-----------------|---------------|-------------|-----------------|
| 18          | 1.214          | 0.984 ± 0.277 | Competitive    | 2.975         | 1.955 ± 0.581 | Competitive     |
| 19          | 1.387          | 1.422 ± 0.183 | Competitive    | 0.888         | 0.564 ± 0.019 | Competitive     |
| 20          | 1.486          | 1.426 ± 0.042 | Competitive    | 1.130         | 0.591 ± 0.113 | Competitive     |
| 21          | 1.249          | 1.244 ± 0.378 | Competitive    | 2.984         | 2.075 ± 0.273 | Competitive     |
| 22          | 2.281          | 4.261 ± 1.287 | Noncompetitive | 1.935         | 1.389 ± 0.167 | Competitive     |
| AZA*        | 0.743          | 0.721 ± 0.101 | Noncompetitive | 0.267         | 0.239 ± 0.019 | Noncompetitive  |

* Acetazolamide (AZA) was used as a reference inhibitor for hCA-I and hCA-II enzymes.

hCA-II, another cytosolic isozyme, plays a vital role in various diseases such as renal tubular acidosis, edema, osteoporosis, epilepsy, and altitude sickness, especially glaucoma[24]. Inhibition results of synthesized
molecules on hCA-II are summarized in table 1. As can be seen from the table, IC$_{50}$ and K$_i$ values for the hCA-II enzyme are in the range of 0.888 to 2.983 µM, from 0.564 ± 0.019 to 2.075 ± 0.273 µM, respectively. All of the urea derivatives inhibited the hCA-II enzyme at the µM level. Compound 19 showed the strongest inhibition among these compounds with an IC$_{50}$ of 0.887 µM and a K$_i$ of 0.564 ± 0.019 µM. All of the compounds demonstrated competitive inhibition by binding to the active site of hCA-II. The molecules have higher inhibition values than standard inhibitors (AZA). However, inhibitor concentrations are low and at µM level.

According to our results, the novel synthesized N-N' dialkyl urea derivatives inhibited hCA-I and hCA-II enzymes more effectively than all other urea derivatives except novel urea derivatives incorporating dopaminergic 2-aminotetralin scaffolds.

As can be seen in table 2, compound 19 (K$_i$s for hCA-I and hCA-II 1.422 ± 0.183 µM and 0.564 ± 0.019 µM, respectively), with a SI value (hCA-II/hCA-I) of 0.397, was found to be the most selective inhibitor for hCA-II among these compounds.

**Table 2.** Selectivity index values for K$_i$ constants of the novel synthesized unsymmetrical ureas

| Compound ID | $K_i^a$ (TACb/AChE) | $K_i^a$ (AZAc/hCA-I) | $K_i^a$ (AZAc/hCA-II) | $K_i^a$ (hCA-II/hCA-I) |
|-------------|---------------------|----------------------|----------------------|-----------------------|
| 18          | 0.149               | 0.733                | 0.122                | 1.987                 |
| 19          | 0.091               | 0.507                | 0.424                | 0.397                 |
| 20          | 0.155               | 0.506                | 0.404                | 0.415                 |
| 21          | 0.054               | 0.580                | 0.116                | 1.668                 |
| 22          | 0.106               | 0.169                | 0.172                | 0.609                 |

*a The K$_i$ rates for the selectivity index; b Tacrine; c Acetazolamide.

**AChE activity assay**

Alzheimer’s disease is caused by malfunctions in various metabolic pathways. Neurotransmitters in the brain are reduced as a result of this condition. Acetylcholine is the neurotransmitter that has decreased the most[42]. For the treatment or prevention of the disease, the breakdown of acetylcholine should be reduced. This can be achieved by inhibition of AChE, which breaks down acetylcholine. Drugs designed for Alzheimer’s disease are used to control the level of acetylcholine, and drug design is carried out accordingly[43].
In recent years, the discovery of novel AChE inhibitors has been the focus of organic chemists and pharmacists in particular. The amino methyl, alkoxy methyl, phloroglucinol, benzimidazole, pyrazoles, bromophenol, imidazole, hydrazine, thiazolidindione, propanolamine, and quinazolinone derivatives were synthesized and investigated the inhibitory effect on AChE [44-55]. Moreover, many studies have examined the inhibitory effects of some urea derivatives on AChE. In this direction, new coumarylthiazole aryl urea, 1,3,4-thiadiazol-2-yl urea, and dopamine analogs incorporating urea were synthesized and investigated their inhibition effects against AChE. The compounds inhibited the AChE enzyme with IC$_{50}$ values in the range of 1.17 µM-4.30 mM [56-58].

In another study, Kurt et al. synthesized novel benzofuranylthiazole derivatives containing the aryl urea moiety. They determined that these derivatives inhibited AChE enzymes with IC$_{50}$ values in the range of 3.85-78.85 µM [59].

The inhibition effects of novel synthesized urea derivatives 18-22 against AChE were investigated using tacrine, which is a reference compound. In table 3, IC$_{50}$ values, K$_i$ constants, and inhibition types for AChE are demonstrated. IC$_{50}$ values and K$_i$ constants for these new urea derivatives were found to ranging between 0.309 to 0.816 µM and between 0.470 ± 0.039 to 1.349 ± 0.244, respectively. The results showed that all of the synthesized molecules exhibited an inhibitory effect against AChE. The molecule showing the highest inhibition effect was found to be compound 20, with an IC$_{50}$ value and K$_i$ constant of 0.309 and 0.470 ± 0.039 µM, respectively. The inhibition results were less than that of tacrine (IC$_{50}$: 0.249 µM) but very close to each other.

According to our results, the novel, synthesized unsymmetrical ureas inhibited the AChE enzyme more effectively than all other urea derivatives.

Table 3. The inhibition results of the novel unsymmetrical ureas on AChE enzyme

| Compound ID | IC$_{50}$ (µM) | K$_i$ (µM) | Inhibition Type |
|-------------|----------------|------------|----------------|
| 18          | 0.551          | 0.491 ± 0.029 | Competitive    |
| 19          | 0.576          | 0.809 ± 0.189 | Competitive    |
| 20          | 0.309          | 0.470 ± 0.039 | Competitive    |
| 21          | 0.605          | 1.349 ± 0.244 | Noncompetitive |
| 22          | 0.816          | 0.688 ± 0.025 | Competitive    |
| TAC*        | 0.249          | 0.073 ± 0.004 | Competitive    |

* Tacrine (TAC) was used as a reference inhibitor for AChE enzyme.
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Molecular docking

Compounds having the most effective inhibition on hCA-I, hCA-II, and AChE according to in vitro studies, were subjected to molecular docking studies to predict the interaction of compounds with receptor proteins. Binding interactions between molecules (compound 18 for hCA-I, compound 19 for hCA-II, and compound 20 for AChE) and enzymes were estimated using AutoDock 4.2.

Table 4. Results of molecular docking of urea derivatives with enzymes

| Compound ID | Enzyme | Estimated Free Energy of Binding (kcal/mol) | Estimated Inhibition K_i Constant (µM) |
|-------------|--------|---------------------------------------------|---------------------------------------|
| 18          | hCA-I  | -7.81                                       | 1.880                                 |
| 19          | hCA-II | -7.34                                       | 4.150                                 |
| 20          | AChE   | -8.20                                       | 0.984                                 |

The estimated binding energy value for the hCA-I receptor was calculated as −7.81 kcal/mol. The best binding poses and 2D ligand-receptor interaction diagrams of compound 18 are shown in Figure 2. In the interaction with hCA-I, compound 18 formed van der Waals interactions with Phe66, Ser65, Gln92, His67, Val62, Pro202, His200, Trp209, and His119 residues. The benzene ring of compound 18 showed a Pi-cation electrostatic interaction with His64 residue and had some hydrophobic interaction such as a pi-pi stacked and pi-pi T-shaped interaction with His94 residue, and pi-alkyl interactions with Ala121, Val143, and Leu198 residues. The urea moiety of compound 18 made a conventional hydrogen bond with Pro201 residue. Besides, the pyrrole ring of compound 18 formed a pi-donner conventional hydrogen bond with Thr199 residue and a pi-alkyl interaction with Leu198 residue.

Figure 2. Potential binding modes and 2D ligand-receptor interaction diagrams of compound 18 with hCA-I.
For the interaction of compound 19 with hCA-II receptor, estimated binding energy value was calculated to be -7.34 kcal/mol. Figure 3 demonstrates the optimal binding poses and 2D ligand-receptor interaction diagrams. In the interaction with hCA-II, compound 19 made van der Waals interactions with His4, Phe20, Thr199, His96, His119, Glu106, Trp208, His94, Val206, and Leu140 residues. The benzene ring of compound 19 formed pi-alkyl interactions with Pro201, Val121, and Val142 residues, a pi-sigma interaction with Leu197 residue. The urea moiety of compound 19 made a conventional hydrogen bond with Pro200 residue. The methoxy substituent on the benzene ring of 19 formed a carbon hydrogen bond with Pro200 residue, a pi-alkyl interaction with Trp5 residue, and a conventional hydrogen bond with Trp5 residue. Furthermore, the pyrrole ring of compound 19 formed a pi-cation interaction with Zn261 residue, a pi-alkyl interaction with Leu197 residue, an amide-pi stacked interaction with Thr198 residue, and a conventional hydrogen bond with Thr198 residue.

The estimated binding energy of the AChE receptor with compound 20 was determined to be -8.20 kcal/mol. Compound 20’s best binding poses and 2D ligand-receptor interaction diagrams are demonstrated in Figure 4. In the interaction with AChE, compound 20 formed van der Waals interactions with Ile287, Arg289, Phe288, Tyr334, Tyr70, Gly441, Glu199, Gly118, and Gly119 residues. The benzene ring of compound 20 made a pi-pi stacked interaction with Trp84 residue. The benzylic carbon of compound 20 showed a pi-sigma interaction with Phe330 residue. The urea moiety of compound 20 made a conventional hydrogen bond with Tyr121 residue. The methoxy substituent on the benzene ring of 20 formed conventional hydrogen bond with Ser200, His440.
residues, and pi-alkyl interactions with Phe331, Phe290, and His440 residues. The pyrrole ring of compound 20 formed a pi-pi stacked interaction with Trp279 residue and a pi-pi T-shaped interaction with Tyr121 residue. Also, the urea-bound methyl of compound 20 made a carbon hydrogen bond with Asp72 residue. As seen from Table 4, estimated inhibition constants (K_i) for hCA-I, hCA-II, and AChE were predicted to be 1.88 µM, 4.15 µM, and 0.984 µM, respectively.

Figure 4. Potential binding modes and 2D ligand-receptor interaction diagrams of compound 20 with AChE.

The ADME analysis for novel synthesized unsymmetrical ureas was carried out. As summarized in Table 5, all urea derivatives showed no deviations from Lipinski's rule of five, including the number of hydrogen bond acceptors, account molecular weight, mILogP (lipophilicity descriptor), number of rotatable bonds, and number of hydrogen bond donors. The molecular weights of all compounds were found to be lower than 500 g/mol. The logP (octanol-water partition coefficient) value calculated for all molecules was determined to be within the range (≤4.15) given according to Lipinski’s rule. It has been determined that the total values of the effects of polar structures such as hydrogen, oxygen, and nitrogen on the molecular surface area (Topological Polar Surface Area: TPSA) are lower than 90 Å. So, the SwissADME estimated that these urea derivatives could pass the BBB and be substrates for P-gp efflux. Also, gastrointestinal (GI) absorptions of all molecules are found to be high. Finally, the synthetic accessibility (sa) calculated scores ranged from 2.23 to 2.65, demonstrating that compounds in the present study agree with synthetic observations [60–63].
Table 5. Results of *in silico* ADME analysis for the novel synthesized unsymmetrical ureas

| Properties                        | Rules                  | Compounds   |
|-----------------------------------|------------------------|-------------|
|                                   |                        | 18  19  20  21  22 |
| Molecular Weight (g/mol)          | <500                   | 307.39 337.42 337.42 337.42 367.44 |
| Num. rotatable bonds              | <10                    | 8 9 9 9 10 |
| Number of H-bond acceptors        | <10                    | 1 2 2 2 3 |
| Number of H-bond donors           | <5                     | 3 3 3 3 3 |
| TPSA (Å²)                         | 20<TPSA<130            | 56.92 66.15 66.15 66.15 75.38 |
| ABS %                             | 89.4                   | 86.2 86.2 86.2 86.2 82.9 |
| miLogP                            | ≤4.15                  | 3.51 3.52 3.54 3.56 3.15 |
| nviolations                       | ≤1                     | 0 0 0 0 0 |
| GI absorption                     | High                   | High High High High High |
| BBB permeant                      | Yes                    | Yes Yes Yes Yes Yes |
| P-gp substrate                    | Yes                    | Yes Yes Yes Yes Yes |
| Log K_{p} (skin permeation) cm/s  | (-9.7<log K_p<-3.5)    | -6.23 -5.96 -6.43 -6.43 -6.16 |
| Synthetic accessibility (sa)      | 2.23                   | 2.41 2.43 2.36 2.65 |

**CONCLUSIONS**

In this study, we synthesized a set of novel N-N' dialkyl urea derivatives 18-22 by starting from tryptamine, an indolamine metabolite of the essential amino acid tryptophan, and substituted 2-phenethylamines. *In vitro* and *in silico* studies confirmed the potential inhibitory effects of all synthesized urea derivatives on hCA-I and hCA-II isozymes and AChE. These compounds have displayed good inhibition at micromolar concentrations against three metabolic enzymes. The molecules exhibiting the strongest inhibition are compound 18 for hCA-I (IC_{50}: 1.214 µM), compound 19 for hCA-II (IC_{50}: 0.888 µM), and compound 20 for AChE (IC_{50}: 0.309 µM). In addition, physicochemical and pharmacokinetic descriptors and drug-like properties of unsymmetrical urea derivatives were evaluated and showed no deviations from Lipinski's rule of five. Based on our results, the synthesized novel N-N' dialkyl urea derivatives may be useful for the treatment of a lot of diseases such as retinal pathology, glaucoma, epilepsy, and brain edema for CA isozymes and AD for AChE after being supported by in vivo and further toxicity studies.
EXPERIMENTAL SECTION

Materials

All chemicals and analytical reagents used for synthesis, purification, and biological activity were obtained from Sigma-Aldrich and Merck KGaA. The characterization of the organic product was performed using a 400 MHz Bruker NMR instrument. An electrothermal IA9100 capillary melting point device was used to determine the melting point. On a Shimadzu IRTracer-100 spectrophotometer with a single-reflection ATR accessory, FT-IR spectra were acquired. A Leco CHNS-932 device was used for element analysis.

General procedure for the synthesis of Ureas 18-22.

2-Phenethylamine 7-11 (3 mmol) in CH$_2$Cl$_2$ (5 ml) was added dropwise to a solution of triphosgene (1 mmol) dissolved in CH$_2$Cl$_2$ (15 ml) at 0°C, then a solution of NaOH (3 mmol) prepared in 7 ml of water was added. The mixture was stirred at room temperature for 1 hour after the addition. The water layer was removed. 3X20 mL of water was used to wash the organic phase, which was then dried over magnesium sulfate. The filtrate was evaporated to obtain the isocyanate, which was then used without additional purification. In dry THF (10 ml), the isocyanate 12-16 (1 mmol) was dissolved or suspended and cooled in an ice bath. Tryptamine 17 (1 mmol) was dissolved in dry THF (5 ml) and slowly added to the reaction mixture. At room temperature, stirring was continued for 6 hours. After that, the organic phase evaporated. N, N'-dialkyl ureas 18-22 were obtained by recrystallization from (EtOAc/Hexane [4-1]).

1-(2-(1H-indol-3-yl)ethyl)-3-phenethylurea (18, C$_{19}$H$_{21}$N$_{3}$O)

The product 18 was obtained as a white powder; Yield: 670 mg, (72%); M.p.: 112-114 °C; IR: $\nu$$_{max}$ 3289, 3024, 2912, 2369, 1707, 1647, 1502, 1431, 1101, 743 cm$^{-1}$; $^1$H NMR (400 MHz, DMSO-d$_6$) $\delta$ 10.84 (br.s, 1H, NH), 7.56 (d, J = 7.8 Hz, 1H, ArH), 7.37 (d, J = 8.1 Hz, 1H, ArH), 7.32 – 7.25 (m, 2H, ArH), 7.24 – 7.17 (m, 3H, ArH), 7.14 (d, J = 2.1 Hz, 1H, ArH), 7.13 – 7.04 (m, 1H, ArH), 7.03 – 6.96 (m, 1H, ArH), 6.03 – 5.95 (m, 2H, NH), 3.33 (dd, J = 13.2, 6.9 Hz, 2H, CH$_2$), 3.26 (dd, J = 13.2, 7.0 Hz, 2H, CH$_2$), 2.82 (t, J = 7.2 Hz, 2H, CH$_2$), 2.70 (t, J = 7.2 Hz, 2H). $^{13}$C NMR (100 MHz, DMSO-d$_6$): $\delta$ = 158.17 (CO), 139.71 (C), 136.24 (C), 128.64 (2CH), 128.26 (2CH), 128.12 (2CH), 127.86 (2CH), 127.27 (2CH).
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127.24 (C), 125.94 (CH), 122.59 (CH), 120.88 (CH), 118.34 (C), 118.19 (CH), 111.99 (CH), 111.32 (CH), 40.97 (CH₂), 40.11 (CH₂), 36.19 (CH₂), 26.07 (CH₂). Anal. Calcd. for C₁₉H₂₁N₃O: C, 74.24; H, 6.89; N, 13.67; found: C, 74.22; H, 6.86; N, 13.70%.

1-(2-(1H-indol-3-yl)ethyl)-3-(2-methoxyphenethyl)urea (19, C₂₀H₂₃N₃O₂)

The product 19 was obtained as a white powder; Yield: 780 mg, (76%); M.p.: 96-98 °C; IR: v_max 3387, 2964, 2875, 2365, 1709, 1685, 1560, 1492, 1240, 1122, 752, 735 cm⁻¹; ¹H NMR (400 MHz, DMSO-d₆): δ = 10.85 (br.s, 1H, NH), 7.53 (d, J = 6.6 Hz, 1H, ArH), 7.33 (d, J = 6.8 Hz, 1H, ArH), 7.25 – 7.02 (overlapped signals, 4H, ArH), 7.00 – 6.81 (overlapped signals, 3H, ArH), 5.94 (br.s, 2H, NH), 3.75 (s, 3H, OCH₃), 3.28 (s, 2H, CH₂), 3.18 (s, 2H, CH₂), 2.77 (s, 2H, CH₂), 2.65 (s, 2H, CH₂). ¹³C NMR (100 MHz, DMSO-d₆): δ = 158.55 (CO), 157.67 (C), 136.72 (C), 130.49 (C), 127.90 (2CH), 127.73 (C), 123.08 (CH), 121.34 (CH), 120.70 (CH), 118.83 (C), 118.63 (CH), 112.47 (CH), 111.80 (CH), 111.04 (CH), 55.68 (OCH₃), 40.53 (CH₂), 40.5-39 (CH₂, overlapping with DMSO), 31.24 (CH₂), 26.62 (CH₂). Anal. Calcd. for C₂₀H₂₃N₃O₂: C, 71.19; H, 6.87; N, 12.45; found: C, 71.30; H, 6.80; N, 12.48%.

1-(2-(1H-indol-3-yl)ethyl)-3-(3-methoxyphenethyl)urea (20, C₂₀H₂₃N₃O₂)

The product 20 was obtained as a white powder; Yield: 800 mg, (78%); M.p.: 113-115 °C; IR: v_max 3366, 3292, 2363, 1830, 1654, 1602, 1560, 1460, 1261, 1166, 789, 750 cm⁻¹; ¹H NMR (400 MHz, DMSO-d₆): δ = 10.80 (br.s, 1H, NH), 7.52 (d, J = 7.3 Hz, 1H, ArH), 7.32 (d, J = 7.9 Hz, 1H), 7.17 (d, J = 6.2 Hz, 1H, ArH), 7.11 (s, 1H, ArH), 7.08 – 7.00 (overlapped signals, 1H, ArH), 7.00 – 6.91 (overlapped signals, 1H, ArH), 6.75 (s, 3H, ArH), 5.98 – 5.81 (m, 2H, NH), 3.71 (s, 3H, OCH₃), 3.32 – 3.13 (m, 4H, CH₂), 2.79 – 2.71 (m, 2H, CH₂), 2.66 – 2.60 (m, 2H, CH₂). ¹³C NMR (100 MHz, DMSO-d₆): δ = 159.69(CO), 158.47 (C), 141.82 (C), 136.70 (C), 129.75 (CH), 127.71 (C), 123.06 (CH), 121.35 (2CH), 118.82 (C), 118.63 (CH), 114.68 (CH), 112.45 (CH), 111.92 (CH), 111.78 (CH), 55.31 (OCH₃), 41.28 (CH₂), 40.53 (CH₂), 36.69 (CH₂), 26.61 (CH₂). Anal. Calcd. for C₂₀H₂₃N₃O₂: C, 71.19; H, 6.87; N, 12.45; found: C, 71.23; H, 6.84; N, 12.39%.
1-(2-(1H-indol-3-yl)ethyl)-3-(4-methoxyphenethyl)urea
(21, C20H23N3O2)
The product 21 was obtained as a white powder; Yield: 800 mg, (78%); M.p.: 118-120°C; IR: v_max 3368, 3292, 2361, 1845, 1707, 1603, 1560, 1459, 1263, 1167, 789, 748 cm⁻¹; ¹H NMR (400 MHz, DMSO-d₆): δ = 10.84 (br.s, 1H, NH), 7.53 (d, J = 8.0 Hz, 1H, ArH), 7.33 (d, J = 8.1 Hz, 1H, ArH), 7.15 - 7.03 (m, 4H, ArH), 6.96 (t, J = 7.4 Hz, 1H, ArH), 6.82 (d, J = 8.3 Hz, 2H, ArH), 5.96 (t, J = 5.3 Hz, 1H, NH), 5.91 (t, J = 5.3 Hz, 1H, NH), 3.69 (s, 3H, OCH₃), 3.28 (dd, J = 12.9, 6.6 Hz, 2H, CH₂), 3.18 (dd, J = 12.9, 6.5 Hz, 2H, CH₂). ¹³C NMR (100 MHz, DMSO-d₆): δ = 158.55 (CO), 158.04 (C), 136.72 (C), 132.08 (C), 130.07 (2CH), 127.73 (C), 123.08 (CH), 121.34 (CH), 118.83 (C), 118.64 (CH), 114.17 (2CH), 112.47 (CH), 111.80 (CH), 55.40 (OCH₃), 41.68 (CH₂), 40.53 (CH₂), 35.79 (CH₂), 26.61 (CH₂). Anal. Calcd. for C₂₀H₂₃N₃O₂: C, 71.19%; H, 6.87%; N, 12.45%; found: C, 71.26%; H, 6.85%; N, 12.38%.

1-(2-(1H-indol-3-yl)ethyl)-3-(3,4-dimethoxyphenethyl)urea
(22, C₂₁H₂₅N₃O₃)
The product 22 was obtained as a white powder; Yield: 840 mg, (75%); M.p.: 110-112°C; IR: v_max 3366, 3294, 2363, 1838, 1654, 1602, 1560, 1460, 1431, 1261, 852, 789, 772 cm⁻¹; ¹H NMR (400 MHz, DMSO-d₆): δ = 10.81 (br.s, 1H, NH), 7.53 (d, J = 7.7 Hz, 1H, ArH), 7.33 (d, J = 8.0 Hz, 1H, ArH), 7.11 (s, 1H, ArH), 7.05 (t, J = 7.4 Hz, 1H, ArH), 6.96 (t, J = 7.3 Hz, 1H, ArH), 6.82 (d, J = 8.1 Hz, 1H, ArH), 6.77 (s, 1H, ArH), 6.68 (d, J = 7.9 Hz, 1H, ArH), 5.93 (t, J = 5.2 Hz, 1H, NH), 5.86 (t, J = 5.3 Hz, 1H, NH), 3.71 (s, 3H, OCH₃), 3.69 (s, 3H, OCH₃), 3.28 (dd, J = 12.9, 6.6 Hz, 2H, CH₂), 3.20 (dd, J = 12.7, 6.4 Hz, 2H, CH₂), 2.77 (t, J = 7.1 Hz, 2H, CH₂), 2.59 (t, J = 7.0 Hz, 2H, CH₂). ¹³C NMR (100 MHz, DMSO-d₆): δ = 158.50 (CO), 149.02 (C), 147.56 (C), 136.71 (C), 132.66 (C), 127.72 (C), 123.06 (CH), 121.35 (CH), 120.89 (CH), 118.82 (CH), 118.63 (CH), 112.94 (CH), 112.47 (CH), 112.29 (CH), 111.78 (CH), 55.76 (OCH₃), 41.54 (CH₂), 40.52 (CH₂), 36.22 (CH₂), 26.62 (CH₂). Anal. Calcd. for C₂₁H₂₅N₃O₃: C, 68.64%; H, 6.86%; N, 11.44%; found: C, 68.68%; H, 6.83%; N, 11.46%.

Biological activity
hCA-I and hCA-II isoenzymes were isolated from human erythrocyte cells. As described in previous studies, the purification was performed with an affinity column using sulfanilamide as ligand for CNBr-activated-Sephase 4B[64]. The AChE enzyme isolated from Electrophorus electricus (Type V/S,
C2888) was purchased commercially from Sigma-Aldrich Chemie GmbH for inhibition studies. The potential inhibition effects of novel synthesized unsymmetrical ureas on hCA-I, hCA-II, and AChE were examined spectrophotometrically by the activity method introduced by Verpoorte et al. for hCAs (348 nm) and Ellman et al. for AChE (412 nm)[65,66]. In order to obtain the IC$_{50}$ value, activities for hCAs and AChE were measured at 5 different inhibitor (urea derivatives) concentrations. IC$_{50}$ values were determined from the % Activity-Concentration graphics drawn. Then, activity measurements were carried out at 5 different substrates (p-Nitrophenyl acetate (p-NPA) for hCAs, acetylthiocholine iodide for AChE) concentrations, and 3 different fixed inhibitor concentrations in order to determine the inhibition types and K$_i$ values. The inhibition types and K$_i$ values were determined from the plots of 1/V versus 1/[S].

**Molecular Docking Studies**

AutoDock 4.2 was used to investigate possible docking mechanisms between compounds and enzymes (hCA-I, hCA-II, and AChE)[67]. In docking calculations, the 3D crystallographic structures of hCA-I (PDB code: 3LXE)[68], hCA-II (PDB code: 3HKU)[69], and AChE (PDB code: 1EEA) [70] receptors were utilized. The pdb file formats for enzymes were downloaded from the Protein Data Bank (http: //www.rcsb.org/pdb). ChemDraw software was used to create three-dimensional structures of the derivatives as a sdf file, which was then converted to a pdb file using Avogadro software. Water molecules, metal ions not belonging to the binding pocket, and other superfluous atoms were removed from the protein. Then, the polar H atoms and the Kollman charge were added to the protein, and the missing atoms were repaired. For the calculation of the energetic map, a grid size of 80x80x80 points with a spacing of 0.375 Å was applied. Grid boxes for the active site of protein were centred as x=-13.198, y=36.883, z=37.807 for hCA-I, x=11.497, y=-0.002, z=16.104 for hCA-II, and x=11.244, y=28.066, z=37.217 for AChE. To identify the proper binding locations, orientations, and conformations of ligands, the Lamarckian genetic algorithm was applied. The results docking were analyzed with Discovery Studio Visualizer.

**ADME (absorption, excretion, metabolism, and distribution) properties**

Physicochemical and pharmacokinetic descriptors, and drug-likeness properties of unsymmetrical urea derivatives were evaluated using the SwissADME web tool. In this context, The parameters such as molecular
weight (MW), percentage absorption (%ABS), the number of hydrogen bonds (n-OHNH donors), logarithm of partition coefficient of the compound between n-octanol and water (miLog P), topological polar surface area (TPSA), the number of hydrogen bond acceptors (n-ON acceptors), and the number of rotatable bonds (n-ROTB) were determined[71,72].

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