ABSTRACT: Carbonic Anhydrase-I (CA-I) is the most abundant CA isozyme expressed in human erythrocytes and the gastrointestinal (GI) tract. CA-I acts in promoting biocalcification. It is well known that inhibitors of carbonic anhydrase (CAIs) are widely used in the remedy of some diseases such as edema, glaucoma, idiopathic intracranial hypertension, and osteoporosis. So, in this study, it was aimed to analyze primer effects of 4-ethylresorcinol and 5-methylresorcinol on hCA-I and to clarify inhibition profiles of compounds. For this purpose, firstly hCA-I was isolated from human erythrocytes by affinity chromatography. Secondly, in vitro inhibition studies were performed and interactions between compounds and enzyme were explained via molecular docking study. Both 4-ethylresorcinol and 5-methylresorcinol inhibited the enzyme competitively with $K_i$ constant of 0.81±0.23 and 0.79±0.14 µM. According to molecular docking analysis estimated free energy of binding of compounds were predicted as -4.81 and -4.51 kcal.mol$^{-1}$ respectively.

Keywords: Binding energy, carbonic anhydrase-I, inhibition, in vitro, molecular docking, resorcinol
INTRODUCTION

Carbonic Anhydrase-I (CA-I) is one of the α-class CA isozymes that catalyzes the reversible conversion of carbon dioxide to bicarbonate and proton. CO₂, which is the result of oxidation metabolism, needs to be converted to water-soluble form in order to prevent cellular organelles and membrane from its damage (Aggarwal and McKenna, 2012; Alterio et al., 2012). CA-I is the cytosolic enzyme and is also largely expressed in human erythrocytes and the gastrointestinal (GI) tract. CA-I and CA-II are the only isoforms that are 62.3% identical and known to be present in erythrocytes (Supuran, 2008). The amount of CA-I is five times more than that of CA-II in erythrocyte cells, but its activity is half the total CA activity in these cells because it is less active (Supuran et al., 2003). Under normal physiological conditions, the hydration activity of CA-I decreases by 92%. Despite its low activity, the importance of CA-I is uncertain (Maren et al., 1976; Sly and Hu, 1995; Supuran et al., 2005). It is thought that in the absence of CA-II, CA-I may compensate for possible loss of CA-II. It was reported that in patients with CA-II deficiency CA-I was expressed a higher level in red blood cells, which compensated for the absence of CA-II (Sly et al., 1983). HCO₃⁻ which is the product of the reaction catalyzed by CA, rapidly binds Ca and forms calcium carbonate. CA-I can promote arthritis calcification, arthrosis fusion, and ossification by expediting calcium carbonate accumulation (Chang et al., 2012; Zheng et al., 2012).

Besides, in the case of breast cancer, the expression of CA-I increases at a high rate in cancerous tissue and blood and this situation causes calcification of the tumor tissue and suppression of apoptosis (Zheng et al., 2015). CA-I expression and cell calcification are suppressed when AZA (Acetazolamide), which is one of the most frequently used CA inhibitors in the clinical treatment of glaucoma, was applied to cancer cells (Chang et al., 2012; Zheng et al., 2015).

Considering the role of CA-I in promoting biocalcification, the aim of our study is to search for new CA-I inhibitors and draw attention to these inhibitors for clinical use. For this purpose, inhibition effects of 4-ethylresorcinol and 5-methylresorcinol (Figure 1) were investigated on hCA-I via both in vitro and in silico methods.

Resorcinols are known as well-rounded chemicals that can be easily used by chemists in many fields from medicine to industry to benefit living things. For example, mono-alkyl substituted resorcinols show great antiseptic properties and are widely used in a variety of therapeutic and agricultural applications. 2-alkyl substituted resorcinols have been used as a basic material in the synthesis of various pharmaceutical and agricultural reagents. Besides, 4-hexylresorcinol is used in cosmetic applications as well as pharmaceutical applications (Durairaj, 2005).

![Figure 1. Structure of compounds of which inhibition mechanisms were studied on hCA-I](image-url)
MATERIALS AND METHODS

Materials

Sepharose-4B, p-nitrophenyl acetate (PNF), dialysis bag and L-tyrosine were procured from Sigma Chem. Co. and all other chemicals from E. Merck AG. 5-methylresorcinol, and 4-ethylresorcinol were obtained from Sigma and Acros. The human erythrocyte was taken from the Turkish Red Crescent Blood Centre (Erzurum Branch).

Methods

Enzyme assay

CA-I was assayed according to its esterase activity put by Verpoorte et al. (1967). The carbonic anhydrase enzyme catalyzes the hydrolysis of p-nitrophenylacetate (PNF) to p-nitrophenol or p-nitrophenylate ion which gives maximum absorption at 348 nm.

Purification of hCA-I via affinity chromatography

Erythrocytes obtained from the Erzurum Turkish Red Crescent, were hemolysis by stirring with five volume of ice water and cell membrane waste was precipitated by the centrifugation method for 15 min at 10 000 rpm. Before hemolysate was loaded to the Sepharose 4B-L-tyrosine-sulfanylamide column the pH of it was step up to 8.7 by using Tris (Ekinci et al., 2007; Adem et al., 2019). Column was washed with 25 mM Tris/HCl buffer (pH 8.7) including 22 mM Na2SO4 to clean other proteins adsorbed to the colon. Then CA-I was eluted with 25 mM Na2HPO4 buffer (pH 6.3) containing 1 M NaCl. The active eluates were combined and dialyzed against the 50 mM Tris/SO4 buffer at pH 7.4. All experiments were carried out at 4°C and active enzyme solutions were stored at -20°C for use in inhibition studies.

Inhibition studies

For analyzing the in vitro inhibition effects of 4-ethylresorcinol and 5-methylresorcinol on hCA-I, activities of enzyme were assayed at five various concentrations of compounds. The activity determined in the inhibitor’s absence was taken consider as 100% activity. Activities in the presence of inhibitor were calculated and Activity%-[compound] graphs were plotted and the amount of inhibitor that reduced activity by 50% (IC50) was calculated for the compounds from these plots. Activities were assayed at three various compound concentrations and five various substrate concentrations, then 1/V-1/S values were calculated and Lineweaver-Burk graphs were created. Ki values and inhibition types were determined via these graphs (Lineweaver and Burk, 1934).

Molecular docking studies

Possible docking modes between molecules and hCA-I were studied by using the AutoDock4 (Morris et al., 2009). The crystal structures of hCA-I (PDB code: 3LXE) (Alterio et al., 2010) was used in docking calculations and its pdb file was downloaded from protein data bank (http://www.rcsb.org/pdb). To prepare the protein Autodock tool was used, water molecules and other unnecessary atoms were deleted, polar H atoms were added, missing atoms were checked, and Kollman charge was added. To calculate the energetic map, a grid spacing of 0.375 Å was employed. Pdb files of ligands were converted from sdf file obtained by ChemDraw through Avogadro software. Number of torsion of ligand was set. Then, pdbqt files were prepared and saved by using Autodock tool. The appropriate binding positions, orientations, and conformations of ligands were determined by using the Lamarckian genetic algorithm. The results files were analyzed using Protein-Ligand Interaction Profiler (PLIP) Support Server. AZA (Acetazolamide) was used as standard inhibitor for hCA-I.
RESULTS AND DISCUSSIONS

In respect of the reaction catalyzed by CAs, they play an important role in physiological functions such as respiration, pH and CO₂ homeostasis, electrolyte secretion and lipogenesis (Gulcin et al., 2016; Imran et al., 2016).

Up to now, it was also reported that so many compounds such as hydroxyl and phenolic compounds, tetra-pyridine-triazole-substituted phthalocyanines, some uracil derivatives, acridine bis-sulfonamides, 1,3-bis-chalcone derivatives, pyrazole derivatives, 5-methyl-2,4-dihydro-3H-1,2,4-triazole-3-one’s aryl Schiff base derivatives, ureido benzenesulfonamides, chalcone substituted benzenesulfonamides, chalcones derivatives bearing morpholine moiety, Schiff bases of sulfa drugs, and alicilaldehyde-N-methyltoluenesulfonylhydrazone were inhibited hCAs (Alyar and Adem, 2014; Esirden et al., 2015; Arslan et al., 2016; Salmas et al., 2016; Turkoglu et al., 2017; Alyar et al., 2018; Kursun Aktar et al., 2018; Lolak et al., 2019; Ozil et al., 2019; Turkan et al., 2019; Tutar et al., 2019; Arslan et al., 2020). Common CAIs such as acetazolamide (AAZ), celecoxib (CLX), ethoxzolamide (EZA), and methazolamide (MZA) have been reported as useful drugs in the treatment of many diseases such as glaucoma, edema, osteoporosis, idiopathic intracranial hypertension (Ahlskog et al., 2009).

In this research, inhibition effects of 4-ethylresorcinol and 5-methylresorcinol were examined on hCA-I through in vitro studies. Firstly, human erythrocytes CA-I isozyme was isolated via Sepharose-4B L-tyrosine-sulphanilamide affinity chromatography. Then inhibition studies were performed, enzyme activities were assayed considering the inhibition by reference to PNF on basis of esterase activity. As summarized in Table 1, compounds inhibit enzyme at micromolar level. In vitro inhibition results were found in agreement with previous results in the study regarding the effects of indole-1,2,3-triazole chalcone hybrids (Kᵢ was found in a range of 0.18 µM-5.5 µM) and pyrazole-3,4-dicarboxamides (Kᵢ was found in ranging from 0.11 µM to 1.66 µM) (Mert et al., 2016; Singh et al., 2020).

Table 1. In vitro inhibition results of 4-ethylresorcinol and 5-methylresorcinol on hCA-I

| Inhibitor            | IC₅₀ (µM) | R²     | Kᵢ (µM)         | Inhibition Type |
|----------------------|----------|--------|-----------------|----------------|
| 4-ethylresorcinol    | 1.27     | 0.900  | 0.81±0.23       | Competitive    |
| 5-methylresorcinol   | 1.12     | 0.955  | 0.79±0.14       | Competitive    |

The inhibitory constant (Kᵢ) of 4-ethylresorcinol and 5-methylresorcinol were found as 0.81±0.23 µM and 0.79±0.14 µM respectively. As seen from Figure 2, it was found that both compounds inhibited the enzyme as competitively; that is, inhibitors have competed with PNF in binding to the active site of the enzyme. It was concluded that the inhibitory effects of 4-ethylresorcinol and 5-methylresorcinol were higher than calix [4] azacrown substituted sulphonamides and some cardiac drugs (Argan et al., 2020; Oguz et al., 2020).

Inhibition mechanisms of CAIs were classified as five different mechanisms (Supuran, 2016, 2017). Since the inhibition type was found competitive inhibition, it was predicted from in vitro experiments that compounds inhibited hCA-I by binding to the active site such as 2-(benzylsulfonyl) benzoic acid (D’Ambrosio et al., 2015; Supuran, 2016).

To get insight the interactions between compounds and enzyme, molecular docking was also performed and summarized in Table 2. The estimated free energy of binding was calculated as -4.81, -4.51, and -5.75 kcal.mol⁻¹ for 4-ethylresorcinol, 5-methylresorcinol and AZA respectively.
Effects of 4-Ethyl Resorcinol and 5-Methylresorcinol on Human Carbonic Anhydrase-I and Molecular Docking Study

Figure 2. Activity%-[compound] graphs and Lineweaver-Burk graphs of 4-ethylresorcinol and 5-methylresorcinol

Table 2. Results of binding energies, and ligand interaction types of 4-ethylresorcinol and 5-methylresorcinol with hCA-I (PDB: 3LXE)

| Ligand               | Estimated Free Energy of Binding (kcal.mol$^{-1}$) | H-bond                  | Hydrophobic Interaction |
|----------------------|----------------------------------------------------|-------------------------|-------------------------|
| 4-ethylresorcinol    | -4.81                                              | Thr199, His200          | His119, Val143, Leu198, His200, Trp209 |
| 5-methylresorcinol   | -4.51                                              | Thr199, His200          | Leu198, His200          |
| AZA*                 | -5.75                                              | Tyr7, His64, Thr199     | Val143, Leu198, Val207, Trp209 |

* AZA was used as standard inhibitor for hCA-I

It was seen that AZA had three hydrogen bonds with the Tyr7, His64, Thr199 residues of enzyme and interacted hydrophobically at the residues of Val143, Leu198, Val207, Trp209. The estimated binding free energy of AZA was -5.75 kcal.mol$^{-1}$, which is lower than the other two compounds. It can be concluded that its higher potency of inhibition can be due to the H-bonds. 4-ethylresorcinol showed hydrogen bonds with the Thr199 (two hydrogen bonds), and His200 (two hydrogen bonds) residues of the hCA-I on its OH moiety. In addition to that, the molecule exhibited several hydrophobic interactions with the His119, Val143, Leu 198, His200, and Trp209 residues (see Figure 3A). Probably due to these interactions 4-ethylresorcinol showed higher potency than 5-methylresorcinol to inhibit hCA-I. The hydrophobic interaction of 4-ethyl resorcinol is similar to that of AZA. Lowest energy conformer of 5-methylresorcinol showed four hydrogen bonds at the active site of hCA-I. One of the oxygen atoms of hydroxyl moiety of the compound acted as hydrogen bond acceptor to form two H-bond with Thr199 with a distance of 3.01 Å and 2.91 Å, respectively. The oxygen atom of other hydroxyl moiety of it acted as hydrogen bond donor against His200 with a distance of 3.83 Å and 2.82 Å. Additionally, two hydrophobic interactions were observed between 5-methylresorcinol and the active site residues of hCA-I. The interactions were displayed in Figure 3B. On the basis, it could deduce from molecular docking study that both compounds showed inhibition potency through interacting with the active pocket of hCA-I.
**CONCLUSION**

In the present work, inhibition abilities of 4-ethylresorcinol and 5-methylresorcinol on pharmacologically significant human carbonic anhydrases hCA-I was evaluated. Both compounds showed good inhibition profiles against the enzyme and acted as competitive inhibitor. $K_i$ values were found to be 0.81±0.23 $\mu$M and 0.79±0.14 $\mu$M respectively. Hydrogen bonds and hydrophobic interactions were found to be dominant in inhibition mechanism of these molecules on hCA-I with estimated free energy of binding -4.81 and -4.51 kcal.mol$^{-1}$ respectively. Moving on these, it seems that 4-ethylresorcinol and 5-methylresorcinol can give an idea on the synthesis of novel inhibitors to be used in the drug design in the case of where hCA-I’s activity should be targeted.

**Conflict of Interest**

I declare that there is no conflict of interest during the planning, execution and writing of the article.

**Author’s Contributions**

I hereby declare that the planning, execution and writing of the article was done by me as the sole author of the article.

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