The effects of cardiac drugs on human erythrocyte carbonic anhydrase I and II isozymes

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ABSTRACT
Cardiovascular diseases are the leading cause of mortality worldwide. In recent years, the relationship between cardiac anhydrase inhibitors and atherosclerosis has attracted attention. In this study, we aimed to determine the in vitro effects of 35 frequently used cardiac drugs on human carbonic anhydrase I (hCA I) and II (hCA II). The inhibitory effects of the drugs on hCA I and hCA II were determined with both the hydratase and esterase methods. The most potent inhibitors observed were propafenone (hCA I: 2.8 \( \mu \)M and hCA II: 3.02 \( \mu \)M) and captopril (hCA I: 1.58 \( \mu \)M and hCA II: 6.25 \( \mu \)M). Isosorbide mononitrate, propanolol, furosemide, and atorvastatin were also potent inhibitors. The inhibitor constant, \( K_i \), value from the Lineweaver-Burk plot for propafenone was 2.38 \( \mu \)M for hCA I and 2.97 \( \mu \)M for hCA II. The tested cardiac drugs showed potent in vitro inhibition of the hCA I and II isozymes. Especially, in patients with atherosclerotic heart disease, these drugs may be preferred primarily due to the beneficial effects of carbonic anhydrase inhibition on atherosclerosis.

1. Introduction
Carbonic anhydrases (CAs, EC 4.2.1.1) are a group of enzymes that catalyse the transformation of carbon dioxide into bicarbonate. There are 15 human carbonic anhydrase (hCA) isoforms, and they all differ in their cellular/tissue localisation and enzymatic features. Investigation of the properties of this family of enzymes is crucial for human health. Analyses of the inhibitory effects of different drugs on enzymes in the CA family are crucial for life. The cytosolic forms of these enzymes are CA-I and CA-II. The isozymes hCA I and hCA II are involved in respiration and acid-base homeostasis. CAs’ catalysis of carbon dioxide hydration is of paramount importance for many physiological processes, which include pH and bicarbonate homeostasis, respiration, bone metabolism, and tumorigenesis. In recent years, the relationship between CA and atherosclerosis has attracted attention. Atherosclerotic cardiovascular diseases are the leading cause of death worldwide. Atherosclerotic lesions cause coronary artery disease, stroke, and peripheral artery disease. Coronary atherosclerotic plaque-related thrombotic occlusion results in acute myocardial infarction. Calcium accumulation is a crucial step in atherosclerosis and is associated with a higher risk for cardiovascular mortality due to coronary artery disease. CAs have an important role in vascular calcification in humans. However, the potential role of CA inhibition related to cardiac drugs in atherosclerosis has not been sufficiently researched. Additionally, drug–enzyme interaction studies have recently gained great interest. Many adverse drug events may result from CA isozyme inhibition. Thus, CA has been increasingly studied by several scientists worldwide. Our laboratory has also specialised in this subject. However, no studies have investigated the effects of some cardiac drugs on hCA activity. Therefore, the current study aimed to determine and compare possible alterations in the activity of hCA I and II caused by cardiac drugs.

2. Experimental part
2.1. Materials
Sepharose 4B, L-tyrosine, protein assay reagents, phenol red, and chemicals for electrophoresis were obtained from Sigma-Aldrich Co. All other chemicals were of analytical grade and obtained from either Sigma-Aldrich Co. or Merck. Medications were provided by a local pharmacy. The study was approved by the local ethics committee (Balikesir University Faculty of Medicine Clinical Research Ethical Committee, Balikesir, Turkey, Decision No. 2019/138 and Date: 09.10.2019).

2.2. Preparation of hemolysate and purification of enzyme
Blood samples (25 ml) from healthy human volunteers were collected. They were centrifuged at 1000g for 20 min at 4°C, and the supernatant was removed. The packed erythrocytes were washed three times with 0.9% NaCl and then were hemolyzed in cold water. The pH of the hemolysate was adjusted to 8.5 with the solid Tris base. The 25-ml hemolysate was applied to an affinity column containing y Sepharose 4B-ethylene diamine-4-isothiocyano-benzensulfonamide. CA isozymes were then eluted with 0.1 M NaCl/25 mM Na2HPO4 (pH 6.3) and 0.1 M CH3COONa/0.5 M NaClO4 (pH 5.6), which recovered hCA I and II, respectively.
The IC₅₀ values for cardiac drugs of human carbonic anhydrase I and II are presented in Table 1. The IC₅₀ values were calculated from activity %–[I] graphs and are shown in Table 1. CA activity in the absence of a drug was set as 100% activity.

The most potent inhibitors were propafenone (hCA I: 2.8 μM and hCA II: 3.02 μM) and captopril (hCA I: 1.58 μM and hCA II: 6.25 μM). Isosorbide mononitrate (hCA I: 6.08 μM and hCA II: 5.5 μM), propranolol (hCA I: 1.25 μM and hCA II: 6.25 μM), furosemide (hCA I: 6.23 μM and hCA II: 4.95 μM), and atorvastatin (hCA I: 7.75 μM and hCA II: 9.85 μM) were also potent inhibitors. The Kᵢ values from the Lineweaver–Burk plot for propafenone were 2.38 μM (non-competitive) for hCA I and 2.97 μM (non-competitive) for hCA II (Table 2).

CAs are drug-target enzymes. The inhibitors of these enzymes are important compounds for discovering new therapeutic agents and understanding enzyme–drug interactions in detail at the molecular level. Propafenone is a sodium channel-blocking antiarrhythmic drug. It works by blocking the activity of particular electrical signals in the heart that can cause arrhythmias. It is used to restore a normal heart rhythm and maintain regular beats in supraventricular arrhythmias. The effect of these cardiovascular drugs on CA has not been studied; however, the effects of some of these agents on aldehyde oxidase were studied. Propafenone (2.5 μM), amlodipine (5.5 μM), and nifedipine (79% inhibition at 50 μM) have been shown to inhibit aldehyde oxidase to a certain extent.

Atherosclerosis is a dynamic process that influences the aorta and its branches. Atherosclerotic plaques are a local thickening of the intima layer caused by cholesterol, hydroxyapatite and fibrous connective tissue accumulation, and proliferation of smooth muscle cells. The determinant step of atherosclerosis is calcium precipitation, which traps cholesterol in the plaque precursor matrix, which includes calcium carbonate, triglycerides, lipoproteins, hydroxyapatite, and calmodulin. In addition, calcification is a marker of atherosclerosis and is used to detect atherosclerosis severity.

Studies have shown that both CA-I and CA-II play an important role in the aetiology of vascular calcification as a component of atherosclerosis. In a study by Oksala et al., CA-II was highly expressed in human atherosclerotic plaques in patients with advanced atherosclerosis.

Ayari et al. detected overexpression of CA-II in human atheroma plaques compared with healthy arterial tissue from the same patient in a comparative genome-wide microarray expression profiling. The inhibitors of these enzymes are important compounds for discovering new therapeutic agents and understanding enzyme–drug interactions in detail at the molecular level. Propafenone is a sodium channel-blocking antiarrhythmic drug. It works by blocking the activity of particular electrical signals in the heart that can cause arrhythmias. It is used to restore a normal heart rhythm and maintain regular beats in supraventricular arrhythmias. The effect of these cardiovascular drugs on CA has not been studied; however, the effects of some of these agents on aldehyde oxidase were studied. Propafenone (2.5 μM), amlodipine (5.5 μM), and nifedipine (79% inhibition at 50 μM) have been shown to inhibit aldehyde oxidase to a certain extent.

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analysis. When patients were compared with each other, CA-II was overexpressed more than 1.7 fold in the atherosclerotic plaques.

Yuan et al. studied the effects of CA on atherosclerosis in an atherosclerotic rat model and human aortic dissection and aneurysm caused by atherosclerosis. They demonstrated significantly higher CA-I levels in the animal atherosclerotic tissues, and CA-I levels increased in human atherosclerotic tissues. Moreover, methazolamide, a CA inhibitor, decreased CA-I levels in the animal model. These results suggest that the higher levels of CA-I are related to vascular calcification, and CA-I plays an important role in atherosclerosis and its progression.

Atorvastatin belongs to a group of drugs known as statins, or HMG-CoA reductase inhibitors. Statins inhibit de novo cholesterol synthesis and decrease low-density lipoprotein (LDL). Their hypolipidemic effects result in stabilisation of atherosclerotic plaques, hence they are used for coronary and peripheral artery diseases.

Another study revealed that atorvastatin inhibited CYP3A4 enzyme activity in a concentration-dependent manner with an IC_{50} value of 48 μM. Guidelines and studies have demonstrated that early use of statin therapy correlates with evident clinical benefits and reduced mortality in patients with atherosclerotic coronary artery disease. Another study revealed that atorvastatin showed submicromolar–low nanomolar inhibition of the 15 hCA isoforms (hCA I–XIV).

A recent study by Yuan et al. demonstrated that CA-I expression and CA-I-mediated calcification are significantly associated with atherosclerosis progression, and methazolamide significantly reduces atherosclerosis and suppresses CA-I expression. According to the results of our study, CA may be responsible for the atherosclerosis-reducing effects of statins as a secondary pathway in addition to the LDL-lowering effect.

Captopril is a competitive inhibitor of angiotensin converting enzyme (ACE). This enzyme is responsible for the conversion of angiotensin I to angiotensin II. Angiotensin II regulates blood pressure and is a key element of the renin–angiotensin–aldosterone system. Leppala et al. reported that captopril is an angiotensin I converting enzyme inhibitor with an IC_{50} value of 0.007 μM. ACE inhibitors improve endothelial function, retard the progression of atherosclerosis, and reduce the risk of cardiovascular death, myocardial infarction, and stroke via ventricular remodelling and neurohumoral regulation. Therefore, these agents are recommended in the treatment of a wide range of diseases, including coronary artery disease, peripheral artery disease, heart failure, stroke, diabetes, and hypertension. CA inhibition by captopril may be an additional pathway to prevent atherosclerosis.

Beta-blockers inhibit the sympathetic activity of beta-adrenergic receptors. Propranolol, a non-selective beta blocker, inhibits all beta receptors. This activity decreases cardiac contractility and heart rate. Propranolol is frequently used in the treatment of patients with ischaemic heart disease and hypertension. Sozzani et al. reported that propranolol is also an inhibitor of protein kinase C. The IC_{50} value of propranolol was approximately 150 μM. In addition, propranolol has been reported to inhibit ATPase activity with an IC_{50} value of 4.4 μM. Beta-adrenergic inhibitors significantly decrease the activity of CA.

Furosemide is a loop diuretic that acts on the kidney. Furosemide inhibits the Na⁺–K⁺–2Cl⁻ cotransporter on the membrane of the epithelial cells of the thick ascending limb of the loop of Henle. The decreased sodium and chloride reabsorption results in diuresis and natriuresis. Furosemide is used to treat edema in patients with heart failure. Temel et al. reported that furosemide inhibits the activity of glucose-6-phosphate dehydrogenase with an IC_{50} of 0.526 mM. Furosemide has been reported to contain primary sulfamoyl moieties and inhibit CA isoforms in the kidneys and other organs.

Isosorbid mononitrate is a drug mainly used to treat angina pectoris. It relaxes the coronary arteries, thereby increasing the circulation in the ischaemic zone. Isosorbid mononitrate relaxes vascular smooth muscles through the formation of nitric oxide (NO). NO activates guanylyl cyclase, resulting in decreased blood pressure and relaxation of the veins and arteries. In our study, this drug inhibited CA isoenzymes at the micromolar level.

Acetazolamide is a classic CA inhibitor. It has been reported to show notable inhibitory effects on hCA I with an IC_{50} value of 5.8 nM. The IC_{50} values of cardiac drugs used in our study were higher than the IC_{50} value for acetazolamide in a previous study.

4. Conclusions

In this study, the in vitro effects of 35 frequently used cardiac drugs were investigated on human erythrocyte CA-I and CA-II. In conclusion, the drugs showed potent inhibitory effects on hCA I and hCA II in vitro. Our results underline the potential of cardiac drugs to target atherosclerosis through hCAI-II inhibition. Especially, in patients with atherosclerotic heart disease, cardiac drugs that inhibit hCAs may be preferred primarily due to the beneficial effects of CA inhibition.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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References

1. Supuran CT, Altamimi ASA, Carta F. Carbonic anhydrase inhibition and the management of glaucoma: a literature and patent review 2013–2019. Expert Opin Ther Pat 2019; 29:781–92.
2. Supuran CT. Carbonic anhydrase inhibitors. Bioorg Med Chem Lett 2010;20:3467–74.
3. Nowbar AN, Gitto M, Howard JP, et al. Mortality from ischaemic heart disease. Circ Cardiovasc Qual Outcomes 2019;12: e003573.
4. Gamble W. Atherosclerosis: the carbonic anhydrase, carbon dioxide, calcium concerted theory. J Theor Biol 2006;239:16–21.
5. Goodman WG, London G, Amann K, et al. Vascular calcification in chronic kidney disease. Am J Kidney Dis 2000;43:572e579.
6. Ramanan R, Kannan K, Sivanesan S, et al. Bio-sequestration of carbon dioxide using carbonic anhydrase enzyme purified from Citrobacter freundii. World J Microbiol Biotechn 2009;25:981e987.
7. Ramanan R, Kannan K, Deshkar A, et al. Enhanced algal CO(2) sequestration through calcite deposition by Chlorella sp. and Spirulina platensis in a mini-raceway pond. Bioreour Technol 2010;101:2616e2622.
8. Mirjafari P, Asghari K, Mahinpey N. Investigating the application of enzyme carbonic anhydrase for CO₂ sequestration purposes. Industrial Eng Chem Res 2007;46:921e926.

9. Adeva-Andany MM, Fernández-Fernández C, Sánchez-Bello R, et al. The role of carbonic anhydrase in the pathogenesis of vascular calcification in humans. Atherosclerosis 2015;241:183–91.

10. Taskin M, Bilen C, Ergun A, et al. In vitro effects of estrogen and progesterone containing drugs on human erythrocyte carbonic anhydrase I and II isozymes in women smokers and nonsmokers. J Chin Med Assoc 2015;78:513–9.

11. Sanoglu N, Bilen C, Sackes Z, et al. The effects of bronchodilator drugs and antibiotics used for respiratory infection on human erythrocyte carbonic anhydrase I and II isozymes. Arch Physiol Biochem 2015;121:56–61.

12. Koc ER, Erken G, Bilen C, et al. The effects of anti-epileptic drugs on human erythrocyte carbonic anhydrase I and II isozymes. Arch Physiol Biochem 2014;120:131–5.

13. Erzengin M, Bilen C, Ergun A, et al. Antipsychotic agents screened as human carbonic anhydrase I and II inhibitors. Arch Physiol Biochem 2014;120:29–33.

14. Bozdag M, Isik S, Beyaztas S, et al. Synthesis of a novel affinity gel for the purification of carbonic anhydrases. J Enzyme Inhib Med Chem 2015;30:240–4.

15. Maren TH. A simplified micromethod for the determination of carbonic anhydrase and its inhibitors. J Pharm Exp Ther 1960;130:2629–34.

16. Verpoorte JA, Mehta S, Edsall JT. Esterase activities of human carbonic anhydrases B and C. J Biol Chem 1967;242:4221–9.

17. Lineweaver H, Burk D. The determination of enzyme dissociation constants. J Am Chem Soc 1934;56:658–66.

18. Mintz GS, Wiviott SD, Diaz R, et al. Early intensive vs a delayed conservative simvastatin strategy in patients with acute coronary syndromes: phase Z of the A to Z trial. JAMA 2004;292:1307–16.

19. Navarese EP, Kowalewski M, Andreotti F, et al. Meta-analysis of time-related benefits of statin therapy in patients with acute coronary syndrome undergoing percutaneous coronary intervention. Am J Cardiol 2014;113:1753–64.

20. Almotrefi A, Dzimiri N. Effects of beta-adrenoceptor blockers on mitochondrial ATpase activity in guinea-pig heart preparations. Eur J Phar 1992;215:231.

21. Adeva-Andany MM, Fernández-Fernández C, Sánchez-Bello R, et al. The role of carbonic anhydrase in the pathogenesis of vascular calcification in humans. Atherosclerosis 2015;241:183–91.