Effects of some drugs on human cord blood erythrocyte carbonic anhydrases I and II: an \textit{in vitro} study

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Cord blood remains in the placenta and in the attached umbilical cord after childbirth. Cord blood is obtained from the umbilical cord when the child is born. It is collected because its stem cells content, including hematopoietic cells, which can be used to treat hematopoietic and genetic disorders. The placenta is a much better source of stem cells since it contains up to ten times more stem cells than cord blood.\(^1\)\(^-\)\(^4\) On the other hand, enzymes are very important for the life attendance. Carbonic anhydrases is a crucial family in enzymes because they catalyze the reversible reactions of CO\(_2\) and water.\(^5\)\(^-\)\(^7\) CA is of broad interest because it is one of the fastest enzymes known; the turnover number or kcat of some CA isoforms exceeds \(1 \times 10^6\) s\(^{-1}\). The reactions of CA izoenzymes are essential to several of physiological processes such as calcification, photosynthesis, respiration, ionic, acid-base and fluid balance, metabolism and cell growth.\(^8\) Therefore, CA appears to be almost ubiquitously expressed in living organisms.\(^9\)\(^-\)\(^10\) Investigation of property of this family is very important. Analyses of inhibition effects of different drugs on CA family are crucial for life.

Antibiotics are widely used to deal with various disorders, but there are few reports of their effects on enzyme activities. Some studies found either increases or decreases in mammalian enzyme activities, and the inhibitor or activator effects of some antibiotics have been investigated. Several investigations are reported, different type of drugs and chemicals have inhibition effects on CAs.\(^11\)\(^-\)\(^14\) Ampicillin is a beta-lactam antibiotic that has been used extensively to treat bacterial infections. Ceftriaxone is a third-generation cephalosporin antibiotic. It has broad spectrum activity against gram-positive and gram-negative bacteria. Ceftizoxime is a parenteral third-generation cephalosporin. Ranitidine is a histamine H\(_2\)-receptor antagonist that inhibits stomach acid production.\(^15\)\(^-\)\(^18\) All of these drugs are used during pregnancy.

Most of investigations focus on the property of umbilical cord. Such as the immunologic properties of cord blood differ from mature bone marrow or peripheral

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**Abstract**

In the present study, we purified hcbCA I and II from human cord blood erythrocytes using by Sepharose-4B-tyrosine-sulfanilamide affinity gel chromatography. Also, it was checked the inhibition effects of ampicillin sulfate, ceftriaxone, ceftizoxime and ranitidine on hcbCA I and hcbCA II. IC\(_{50}\) values for ceftriaxone, ceftizoxime and ranitidine were found to be 27.1, 79.4 and 55.5 \(\mu\)M for hcbCA I and of 21.0, 79.1 and 66.1 \(\mu\)M for hcbCA II, respectively. According to these results, Ampicillin sulfate inhibited only hcbCA II and IC\(_{50}\) values of this antibiotic was found to be 56.8 \(\mu\)M. All these substances were found non-competitive inhibitors. It is important to study the inhibition effects of these drugs on hcbCA I and II izoenzymes. Because, pregnant woman is take all of these substance. For this reason, these drugs should be carefully used and the dosage should be very well ordered to minimize side effects.

**Keywords:** Carbonic anhydrase, cord blood, erythrocytes
blood stem cells. Also, cord blood cells produce increased amounts of anti-inflammatory cytokine interleukin-10, which may downmodulate graft-versushost disease1,19. The effects of many of the known antibiotics have not been analyzed on human cord blood CA isozymes yet, which are contained at the highest molar amounts in blood. Usage of these drugs may worsen hcbCA I and II activity in cord blood. So, they may be used in pregnant patients; it is important to explore the effect of these on CA I and II activity during pregnancy. The present study was realized on the in vitro effect of ampicillin sulfate, ceftriaxone, cefotizoxime, ranitidine on hbcCA I and II isozymes purified from human cord blood erythrocytes by Sepharose-4B-l tyrosine-sulfanilamide affinity gel chromatography.

Materials and methods

Chemicals

Sepharose 4B, protein assay reagents and 4-nitrophenylacetate were obtained from Sigma-Aldrich Co. (Sigma-Aldrich Chemie GmbH Export Department Eschenstrasse 5, 82024 Taufkirchen, Germany). All other chemicals were analytical grade and obtained from Merck (Merck KGaA Frankfurter strasse 250, D 64293 Darmstadt Germany).

Purification of hbcCA isozymes from human cord blood by affinity chromatography

The purification of the two hcbCA isozymes from human cord blood was performed in a simple single-step method by means of Sepharose-4B-l tyrosine-sulfanilamide affinity gel chromatography. Erythrocytes were purified from fresh human cord blood obtained from the Blood Centre of Erzincan Hospital. From normal pregnant women ad term, following the birth by caesarian section or normal delivery, 2 mL of umbilical cord blood was withdrawn by syringe just before the separation placenta and transferred into K3EDTA anticoagulated blood tubes and mixed. The blood samples were stored at −40°C until analysis. The blood samples were centrifuged at 2250 g for 15 min and the plasma and buffy coat were removed. The red cells were isolated and washed twice with 0.9% of NaCl and hemolyzed with 1.5 g of sodium nitrate 5 mL ice-cold water). After 10 minutes of reaction, the diazotized sulfanilamide was poured to 40 mL of the Sepharose 4B-l tyrosine suspension. The pH was adjusted to 9.5 with 1 M NaOH. After gentle stirring for 3 h at room temperature, the coupled red Sepharose derivative was washed with 1 L of water and then 200 mL of Tris-sulfate (0.05 M, pH 7.5). The hemolysate was applied to the prepared Sepharose-4B-l tyrosine-sulfanilamide affinity column equilibrated with 25 mM Tris- HCl / 0.1M Na2SO4 (pH 8.7). The affinity gel was washed with 25 mM Tris-HCl/22mM Na2SO4 (pH 8.7). The human cord blood carbonic anhydrase (hbcCA I and hcbCA II) isozymes were eluted with 1 M NaCl / 25 mM NaH2PO4 (pH 6.3) and 0.1 M CH3COONa / 0.5 M NaClO4 (pH 5.6), respectively. All procedures were performed at 4°C20-22.

Hydratase activity assay

CA activity was assayed by following the hydration of CO2 according to the method described by Wilbur and Anderson23 described previously by Ozturk Sarikaya (2010)24. CO2-hydratase activity as an enzyme unit (EU) was calculated by using the equation (t o - t c/tc) where t o and t c are the times for pH change of the non-enzymatic and the enzymatic reactions, respectively.

Esterase activity assay

CA activity was assayed according to method of Verpoorte et al.25 described previously by Innocenti et al.26,27 CA activity was determined by following the change in absorbance at 348 nm of 4-nitropheny lacetate (NPA) to 4-nitrophenylate ion over a period of 3 min at 25°C using a spectrophotometer (CHEBIOS UV-VIS). The enzymatic reaction, in a total volume of 3.0 mL, contained 1.4 mL 0.05 M Tris–SO4 buffer (pH 7.4), 1 mM, 3 mM 4-nitropheny lacetate, 0.5 mM H2O and 0.1 mL enzyme solution. A reference measurement was obtained by preparing the same cuvette without enzyme solution. The inhibitory effects of ampicillin sulfate, ceftriaxone, cefotizoxime, ranitidine were examined. All compounds were tested in triplicate at each concentration used. Different inhibitor concentrations were used. HCA-I enzyme activities were measured for ceftriaxone (0.003–0.033 mM), cefotizoxime (0.017–0.096 mM), ranitidine (0.026–0.053 mM) at cuvette concentrations and HCAII enzyme activities were measured for ampicillin sulfate (0.038–0.095 mM), ceftriaxone (0.003–0.021 mM), cefotizoxime (0.026–0.061 mM) and ranitidine (0.032–0.058 mM) at cuvette concentrations. Control cuvette activity in the absence of inhibitor was taken as 100%. For each inhibitor an Activity (%)-[Inhibitor] graphs were drawn. To determine K i values, three different inhibitor concentrations were tested. In these experiments, 4-nitropheny lactate was used as substrate at five different concentrations (0.15–0.75 mM). The Lineweaver-Burk curves were drawn28.
Protein determination
Protein during the purification steps was determined spectrophotometrically at 595 nm according to the Bradford method using bovine serum albumin as the standard.

SDS polyacrylamide gel electrophoresis
SDS polyacrylamide gel electrophoresis was performed after purification of the cord blood iso enzymes. It was carried out according to Laemmli procedure. The electrophoretic pattern was photographed (Figure 1).

Results and discussion
Cord blood is alternative to stem cell source to treat cancer and genetic diseases. Therefore, analyzing enzyme property of cord blood is crucial for organism. Umbilical cord blood contains different enzymes such as glucose 6-phosphate dehydrogenase, glutathione reductase, carbonic anhydrases. So far, 16 different CA isozymes have been identified in mammals and several novel isoforms have also been identified in non-mammalian vertebrates. Much of the research on the evolution of the structure and function of CA has been focused on the widely distributed CA I and II isozymes. The purification of the two CA isozymes used here was performed with a simple step method by a Sepharose 4B L-tyrosine sulfanilamide affinity column (Table 1). Inhibition effects of drugs derivatives on enzyme activities were tested under in vitro conditions; IC₅₀ values were calculated by Activity (%)-[Inhibitor] graphs and are given in Table 2. Kᵢ values were calculated from Lineweaver–Burk graphs and are given in Table 2.

CA catalyzes the conversion of CO₂ to H₂CO₃ at cells and intracellular fluid. Inhibitors of carbonic anhydrases are very important for the treatment of different diseases such as antiglaucoma, diuretics, antiepileptics, in the management of mountain sickness, gastric and duodenal ulcers, neurological disorders or osteoporosis. CA I and II have been purified many times from different organisms and the effects of various chemicals, pesticides and drugs on its activity have been investigated.

In this study, CA I and II were purified from human cord blood erythrocytes by a simple one step procedure using Sepharose 4B L-tyrosine sulfanilamide affinity column. The activity of the effluents was determined by the hydratase method, with CO₂ as substrate and further kinetic studies were performed using the esterase activity method, with 4-nitrophenyl acetate (NPA) as substrate. hcbCA I was purified 142.79-fold with specific activity (1115.22 EU mg/µL) and yield (73%).

Similarly, hcbCA II was purified 730.69-fold with specific activity (5706.67 EU mg/µL) and yield (67%). SDS-PAGE of the enzymes showed a single polypeptide band (Figure 1). Four inhibitors were prepared and evaluated for the inhibitory effects on hcbCA I and II. The inhibitory effects of drugs were tested in range of 0.001-1000 mM. Among the four inhibitor, three of them (Ceftriaxone, Ceftizoxime, Ranitidine) showed inhibition effects on hcbCA I and all of them on hcbCA II.

Some researchers reported that Ampicillin sulfate showed inhibition on human carbonic anhydrase. IC₅₀ value of this work is 385 µm for HCA I and 774 µm for HCA II. Other study on rainbow trout erythrocytes pointed out 2160 µM Kᵢ value and 1870 µM IC₅₀ value for carbonic anhydrase. If we check our result, this antibiotic did not prove any inhibition on hcbCA I but showed high inhibition effect on hcbCA II with IC₅₀ value of 56.8 µm and Kᵢ of 83 µm. Ranitidine is H₂ blocker and available in prescription strength and over the counter. This drug provides short-term relief as well as over doses of H₂ blockers. The studies showed that Ranitidine inhibited cytosolic carbonic anhydrases. IC₅₀ and Kᵢ values determined by that study were 2004 µM and 1900 µM for hCA-I and 2542 µM and 29.7 µM for hCA-II. The obtained IC₅₀ values were 1900 µM for hCA-I and 2542 µM for hCA-II for Ceftizoxine. We found IC₅₀ and Kᵢ values lower than those of Beydemir et al. For example, IC₅₀’s were 27.1 µM for hcbCAI and 21.0 µM for hcbCA II. Kᵢ’s were 28.5 µM for hcbCAI and 15.8 µM for hcbCA II. Results of Ceftizoxime were found similar to those of Ranitidine.

It is well-known that many chemicals and drugs affect the metabolism by altering enzyme activities and the results may be very dangerous. Thus, the drug-enzyme interactions should be well-characterized. Although there is a lack of studies regarding drug-enzyme
interactions, the investigations on this issue is increasing day by day. Here, we purified the CA enzymes with simple chromatographic methods and determined inhibitory potentials of commonly used drugs. As seen in Table 2, all drugs showed inhibitory action on the enzymes except for ampicillin which had no effect on hcbCA I. Ceftriaxone was the most powerful inhibitor for both enzymes. The reason could be the bulk of the molecule as well as the electronegative groups. The second most potent inhibitor was ranitidine for both enzymes. The result could be the bulk of the molecule as well as the electronegative groups. The second most potent inhibitor was ranitidine with the Ki values of 54.7 and 29.7 µM for hcbCA I and hcbCA II, respectively. Ceftrizoxime and ampicillin showed rather low inhibitory effect as compared with others. Overall results indicate that these drugs inhibit the enzymes in non-competitive manner. This result is not surprising because molecular structures of the drugs are not similar to physiological substrate of CA enzymes. Also, non-physiological substrates of CA e.g, paranitrophenyl acetate, have quite different molecular structures compared to these drugs investigated here. The drugs are probably interacting with the regions other than the active site, probably with the sulphhydryl groups as they are quite bulky. As mentioned above, drug-enzyme interaction studies have gained a great interest over the recent years. Our study is thus a contribution to the literature data on the inhibitors or carbonic anhydrase enzymes. Results of our study provide some useful information. First, depending on our data, it is understood that usage of these drugs must be well-determined as they might have serious side effects on CA enzymes which may result in the disruption of acid-base balance and salt transport. Second, our data is very important for design and exploration of novel carbonic anhydrase inhibitors. As known, carbonic anhydrase inhibitors are very important for the treatment of different diseases such as antiglaucoma, diuretics, antiepileptics, mountain sickness, gastric and duodenal ulcers, neurological disorders or osteoporosis, and thus discovery of novel CA inhibitors is very important for medicinal chemists.

**Conclusion**

HcbCA-I and II isozymes were purified in one-step with high specific activity by the purification method used in this study. Ampicillin sulfate sulfate, Ceftriaxone, Ceftrizoxime and Ranitidine at low concentrations showed *in vitro* inhibitory effects on hcbCA-I (without

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**Table 1. Purification steps of hcbCA I and II from human cord blood erythrocytes by Sepharose-4B-l tyrosine-sulfanilamide affinity gel chromatography.**

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Activity (EU/mL)</th>
<th>Volume (mL)</th>
<th>Protein (mg/mL)</th>
<th>Total Protein (mg)</th>
<th>Total Activity (EU)</th>
<th>Specific Activity (EU/mg)</th>
<th>Recovery (%)</th>
<th>Purification (Fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemolysate</td>
<td>151.00</td>
<td>51.00</td>
<td>19.33</td>
<td>985.83</td>
<td>7701.00</td>
<td>7.81</td>
<td>100</td>
<td>1.00</td>
</tr>
<tr>
<td>hcbCA I</td>
<td>513.00</td>
<td>11.00</td>
<td>0.46</td>
<td>5.06</td>
<td>5643.00</td>
<td>1115.22</td>
<td>73</td>
<td>142.79</td>
</tr>
<tr>
<td>hcbCAII</td>
<td>856.00</td>
<td>6.00</td>
<td>0.15</td>
<td>0.90</td>
<td>5136.00</td>
<td>5706.67</td>
<td>67</td>
<td>730.69</td>
</tr>
</tbody>
</table>

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**Table 2. Inhibitory activities of drugs.**

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Compound Structure</th>
<th>IC50 hcbCA I (µM)</th>
<th>IC50 hcbCA II (µM)</th>
<th>IC50 hcbCA I (µM)</th>
<th>IC50 hcbCA II (µM)</th>
<th>Inhibition type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin sulfate</td>
<td><img src="ampicillin.png" alt="Image" /></td>
<td>* 57.8</td>
<td>83.0</td>
<td>* 56.8</td>
<td>83.0</td>
<td>Non-competitive</td>
</tr>
<tr>
<td>Ceftrizoxime</td>
<td><img src="ceftrizoxime.png" alt="Image" /></td>
<td>79.4</td>
<td>86.1</td>
<td>Non-competitive</td>
<td>79.1</td>
<td>51.8</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td><img src="ceftriaxone.png" alt="Image" /></td>
<td>27.1</td>
<td>28.5</td>
<td>Non-competitive</td>
<td>21.0</td>
<td>15.8</td>
</tr>
<tr>
<td>Ranitidine</td>
<td><img src="ranitidine.png" alt="Image" /></td>
<td>55.5</td>
<td>54.7</td>
<td>Non-competitive</td>
<td>66.1</td>
<td>29.7</td>
</tr>
</tbody>
</table>

*The inhibition was not determined.*
Ampicillin sulfate) and hcbCA-II activity. Ceftriaxone had the strongest inhibitory effects on hcbCA-I and II, when compared to the Ceftriaxone and others. However, Ceftriaxone and Ranitidine inhibited hCA-I and II at low concentrations. For that reason, uncontrolled usage all of these drugs can cause serious side effects and can be deleterious to health. For this reason, these drugs must be used carefully and the dosage should be closely monitored to decrease side effects.

**Declaration of interest**
The authors report no conflicts of interest.

**References**
2. www.gentlebirth.org