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ORIGINAL ARTICLE

Activation of Two Different Drugs Used in Alzheimer's Disease Treatment on Human Carbonic Anhydrase Isozymes I and II Activity: an *In Vitro* Study

Alzheimer Hastalığının Tedavisinde Kullanılan İki Farklı İlacın İnsan Karbonik Anhidraz I ve II İzoenzim Aktiviteleri Üzerindeki Aktivasyonu: *İn Vitro* Çalışma

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ABSTRACT

Objectives: Human carbonic anhydrase I and II (hCAI, II) isoenzymes were purified from human erythrocyte. Kinetic interactions between the enzymes and memantine and donepezil, two different drugs used in Alzheimer's disease (AD) treatment, were investigated.

Materials and Methods: The purification procedure was composed of preparation of homogenate (or hemolysate) and affinity chromatography on Sepharose 4B-L-tyrosine-sulfanilamide.

Results: Both drug exhibited *in vitro* activator effects on hCAI and II enzymes activity. Strong activations were found for these compounds: The CA values of memantine and donepezil against hCAI were 0.013 µM and 1.8 µM, respectively. The K_A values of memantine and donepezil against hCAII were 0.045 µM and 3.7 µM, respectively.

Conclusion: Since the levels of CA isoenzymes are low in patients with AD or in the older population, increasing activities of these isoenzymes are important for these patients. The effect of these drugs used in AD treatment was thought to be caused by positive changes in the levels of carbonic anhydrase isoenzymes.

Key words: Human CAI, human CAII, enzyme activation, memantine, donepezil

ÖZ

Amaç: İnsan karbonik anhidraz I ve II (hCAI ve II) izoenzimleri insan eritrositlerinden saflaştırıldı. Alzheimer hastalığının (AH) tedavisinde kullanılan memantin ve donepezil ilaçlarının bu enzimlerle olan kinetik etkileşimleri incelendi.

Gereç ve Yöntemler: Saflaştırma prosedürü homojenat (ya da hemolizat) hazırlama ve Sefaroz-4B-L-tirozin-sülfonamid afinite kromotografisi yönteminden oluşmaktadır.

Bulgular: Her iki ilaç da CAI ve II izoenzim aktiviteleri üzerinde *in vitro* aktivatör etkisi gösterdi. Bu bileşikler için güçlü aktivasyon değerleri elde edildi: hCAI izoenzimine karşı memantin ve donepezil için CA değerleri sırasıyla 0.013 µM ve 1.8 µM. hCAII izoenzimine karşı memantin ve donepezil için K_A değerleri sırasıyla 0.045 µM and 3.7 µM.

Sonuç: AH ve yaşlı nüfusta CA izoenzim seviyeleri düşük olduğu için, bu hastalarda bu izoenzimlerin aktivitelerinin artması önem arz etmektedir. Bahsi geçen bu iki ilacın AH tedavisindeki etkisini CA izoenzimleri seviyesinde yapmış olduğu pozitif artış ile göstermiş olduğu düşünülmüştür.

Anahtar kelimeler: İnsan CAI, insan CAII, enzim aktivasyonu, memantin, donepezil

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INTRODUCTION

Carbonic anhydrases (CA) (CA, EC 4.2.1.1) are belong to family of metalloenzyme and have 16 isoforms in mammals. They catalyze from the reversible hydration of CO_2 to the bicarbonate ion and protons and are expressed as pH regulatory enzyme in most tissues especially in erythrocytes.[1-6] Many such CA isozymes which make these processes are important therapeutic targets with the potential to be inhibited/activated for the treatment of diseases such as glaucoma, edema, obesity, osteoporosis, epilepsy and cancer.[2-8] Activation of several these isoenzymes was reported to be a possible therapy for increasing of synaptic efficacy. This increase might represent the new approach for the treatment of Alzheimer's disease (AD). At the same time, it may ensure to improvement aging, spatial learning and memory therapy.[9]

AD is characterized clinically as a progressive dementia. The neurobiological mechanisms influencing the progressive impairments in memory and intellectual performance that are the hallmarks of AD are not well understood. In addition, the levels of several CA isozymes, including human carbonic anhydrase (hCAI), are diminished in patients affected by AD or in the older population.[10]

Several classes of CA activators are known. One of them is histamine. Histamine is an organic compound including nitrogen and both mediates local immune responses and acts as a neurotransmitter. It was reported to increase the activity of CA and to attend the proton shuttling process.[11] Function of CA activators is to bind at the entrance of the enzyme active site, at the same time to ease the proton transfer processes between active site and solvent system. Histidine, phenylalanine, sildenafil citrate have been shown to be potential activators of different CA isozymes. D-3,4-dihydroxyphenylalanine; dextrodopa (D-DOPA), L-Tyr, and 4-amino-L-Phe act as perfect activators for CAs like the histamine. But LHis, L-Trp, L-Adrenaline, and dopamine have been demonstrated weak activating effects for different CAs.[2,12-15]

Generally it is known that activators bind to different site from the inhibitors within the enzyme active cavity.[11,16] Also, they participate in facilitated the proton transfer processes between active site and solvent system, shuttling protons with groups which have an appropriate pKa such as the carboxylate groups.[17]

Memantine is an antagonist of N-methyl-D-aspartate glutamate receptors as uncompetitively. It is proposed to treat of patients with moderate to severe AD. Additionally, benefits of memantine in AD are reported.[18,19] Memantine was chosen because of its similarity to histamine which is activator of CA isoenzymes (Figure 1). Both compounds have -NH_2 group. Donepezil is a drug used in the palliative treatment of AD. It is approved for treatment in patients with mild to moderate AD.[20,21]

In light of the above information, we thought that these drugs could activate hCAI and II isoenzymes. We have purified hCAI and hCAII from human erythrocytes and analyzed the *in vitro* effects of these drugs memantine (1) and donepezil (2) on these isoenzymes. We used the esterase activity of hCAI and hCAII and 4-nitrophenyl acetate (NPA) as substrate. We are justified in our opinion. Because we found that memantine (1) and donepezil (2) are a potent activator of hCAI and hCAII.

RESULTS AND DISCUSSION

CA purification, assay and activation

We used a simple one step method which is the Sepharose-4B-L-tyrosine-sulfanilamide affinity chromatography for the purification of the two CA isozymes.[22,23] These isozymes have important roles in different tissues.[24-29] In many studies, they have been purified from different tissues. Theirs activity have been investigated with various chemicals, pesticides and drugs.[22,23,30-36] In this study, activities of purified hCAI and hCAII isoenzymes from human erythrocytes were determined by using the esterase activity method. And we used NPA as substrate as previous study.[36]

Activator effects of these drugs memantine (1) and donepezil (2) on enzyme activities were tested under *in vitro* conditions. %Activity / (drug concentration) curves was drawn (Figure 2, 3) and they was used at determination of activation constant (K_A) values of the drugs for CAI and II isoenzymes. The K_A values of memantine against hCAI was found to be 0.013 µM which whereas that of donepezil was of 1.8 µM. The K_A values of memantine against hCAII were found to be 0.045 µM whereas that of donepezil was of 3.7 µM (Table 1).

Histamine (3) which taken as the reference compound have

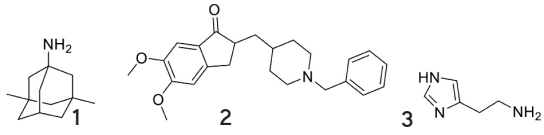

Figure 1. Chemical structures of memantine (1), donepezil (2) and histamine (3)

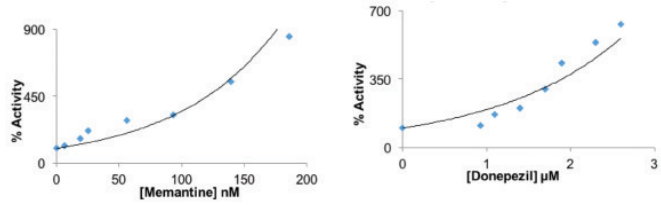

Figure 2. %Activity / (drug concentration) curves was used at determination of K_A values of the drugs for CAI isoenzyme

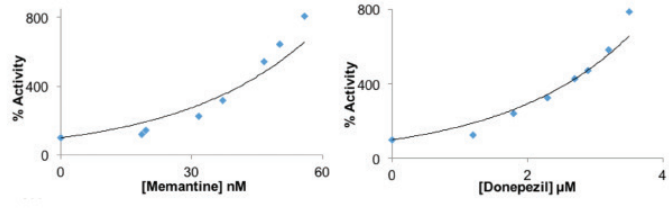

Figure 3. %Activity / (drug concentration) curves was used at determination of K_A values of the drugs for CAII isoenzyme

the K_A values against hCAI and hCAII of 2 µM, and 125 µM, respectively, being a highly potent activator against both the isoforms (Table 1).[37] The best activator of hCAI is memantine with respective K_A of 0.013 µM. Likewise, the best activator of hCAII is memantine with respective K_A of 0.045 µM. Donepezil activated hCAI almost the same rate compared with histamine. But it activated hCAII more activated than histamine (Table 1). As shown in Figure 1, memantine has bicyclic structure. Other structures are planar. Memantine has been easily interacted with the amino acids in active site of CA isoenzymes and these isoenzymes have been more active.

Memantine and donepezil acted as perfect activators for CAI and II isoenzymes like LHis, L-Adrenaline, D-DOPA, L-Tyr, and 4-amino-L-Phe. But these two isoenzymes more activated than L-Trp and dopamine have been demonstrated weak activating effects for different CAs.[2,12-15]

We reported here the first study on the activator effects of these drugs memantine (1) and donepezil (2) on the hCA esterase activity. The structures of active substances were shown in Figure 1. Consequently, memantine and donepezil are much more potent compared with histamine. These compounds may be used as leads for developing novel activators. This study will contribute to understand the relationship between CA isoenzymes and AD. Also, it will provide important information for the diagnosis of AD and its treatment.

EXPERIMENTAL

Chemicals

Sepharose-4B, protein assay reagents, 4-nitrophenylacetate and chemicals for electrophoresis were purchased from Sigma-Aldrich Co. All other chemicals were analytical grade and obtained from Merck.

Purification of carbonic anhydrase

Erythrocytes suspension was obtained from the Blood Center of the Research Hospital at Erzincan University. The red cells were washed twice with 0.9% NaCl, and hemolyzed with 1.5 volumes of ice-cold water. The ghost and intact cells were removed by centrifugation at 3100 g for 30 min at 4°C. The pH of the hemolysate was adjusted to 8.7 with a solid Tris base, and applied to the prepared Sepharose 4B-L-tyrosine-sulfonamide affinity column equilibrated with 25 mM Tris-HCl/22 mM Na_2SO_4 (pH 8.7).[22-25] The hCAI and hCAII isozymes were eluted with 1 M NaCl/25 mM Na_2HPO_4 (pH 6.3) and 0.1 M CH_3COONa/0.5 M $NaClO_4$ (pH 5.6), respectively. The absorbance of the protein in the column effluents was determined spectrophotometrically at 280 nm.[22,23,36]

Table 1. Activation constants of hCA isozymes I, and II with memantine, donepezil and histamine

Compound	K_A (µM)	
	hCAI	hCAII
Memantine	0.013	0.045
Donepezil	1.8	3.7
Histamine	2	125

K_A: Activation constant, hCA: Human carbonic anhydrase

CA activation assay

CA activity was assayed by following the change in absorbance at 348 nm of NPA to 4-nitrophenylate ion over a period of 3 min at 25°C using a spectrophotometer (Shimadzu UV-VIS) according to the method described by Verpoorte et al.[38] A reference measurement was obtained by preparing the same cuvette without enzyme solution.[39] The activation effects of memantine and donepezil were examined. Different activator concentrations were used. Stock solutions of activators (10 mM) were prepared in distilled-deionized water and dilutions up to 0.1-0.9 µM were done thereafter with the assay buffer. Then, %Activity / (drug concentration) curves was drawn (Figure 2, 3) and they was used at determination of K_A values of the drugs for CA I and II isoenzymes.

The K_A is defined similarly like the inhibition constant (K_i). It is obtained with the help of the classical Michaelis-Menten equation as shown below:

$$\upsilon = \upsilon_{max} / \{ 1 + \frac{K_M}{[S]\left(1 + \frac{[A]_f}{K_A}\right)} \}$$

$[A]_f$ is the free concentration of activator and can be represented in the form of the total concentration of the enzyme ($[E]_t$) and activator ($[A]_t$). Because we work at substrate concentrations considerably lower than K_M ($[S] \ll K_M$), the obtained competitive steady-state equation for determining the activation constant is given by the following equation:

$$\upsilon = \upsilon_o.K_A / \{K_A + [A]_t\}$$

$$-0.5\{([A]_t + [E]_t + K_A) - ([A]_t + [E]_t + K_A)2 - 4[A]_t.[E]_t)1/2\}$$

υ_o represents the initial velocity of the enzyme-catalyzed reaction without activator.[12,25,27]

Protein determination

We determined amount of protein during the purification steps according to the Bradford method. We measure it spectrophotometrically at 595 nm, using bovine serum albumin as the standard.[36,40-43] We have used ten tubes with different concentrations of albumin as shown in Figure 2. Then we mixed them with the Bradford reagent (Coomassie Brilliant Blue G-250) and measured the absorbance at 595 nm. Our unknown sample concentration was defined as µg/µL according to standard curve in Figure 4.

CONCLUSION

The K_A values of memantine against hCAI was found to be 0.013 µM which whereas that of donepezil was of 1.8 µM. The K_A

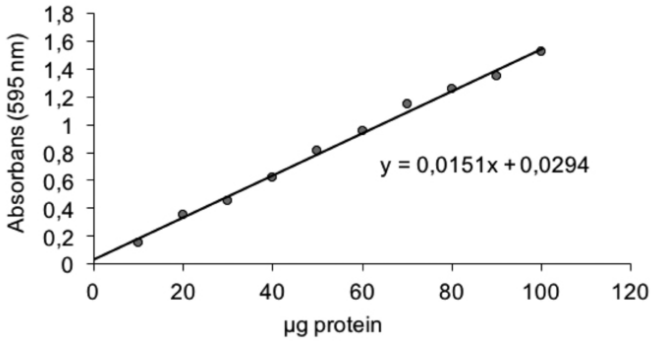

Figure 4. Protein standard curve displaying with just µg of protein at 595 nm

values of memantine against hCAII were found to be 0.045 µM whereas that of donepezil was of 3.7 µM (Table 1).

We used the histamine as the reference compound. It has the K_A values against hCAI and hCAII of 2 µM, and 125 µM, respectively. For two isoenzymes were reported that it is a highly potent activator.[37] It was reported that histamine attends the proton shuttling process and increases the activity of CA.[12]

Activation of these isoenzymes can be a potential target for drug development because of the physiological relevance of CAs.[2] CA activators may be designed as a derivative for increasing of synaptic efficacy.[37] The pharmacological effects of memantine and donepezil not yet been developed clinically for hCA I and hCA II isoenzymes. Thus, in the near future, the novel therapeutic applications will make for enzyme activators.

Conflict of Interest: No conflict of interest was declared by the author.

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