

Investigation of the Effects of Favipiravir and Oseltamivir Active Substances Used in the Treatment of Covid-19 on Carbonic Anhydrase I-II Isoenzymes and Acetylcholine Enzyme Activities in Vitro

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ABSTRACT

Covid-19, originating from Wuhan, China, is a worldwide health problem. Immune system abnormalities caused by covid-19 lead to infections, septic shock, and severe multi-organ dysfunction. The drugs used for treatment are palliative pharmacological alternatives and help manage symptoms or complications that occur during the course of the disease. Both carbonic anhydrases and cholinesterases can be target enzymes for drugs. The goal of this study is to determine how the drugs used in covid-19 affect patients being treated for Alzheimer's disease, myasthenia gravis, glaucoma, or epilepsy, and to determine if there are drug-drug interactions. In case of possible interactions, it is crucial for these patients to consider alternative treatments and to recheck the dosage of the drugs used. To this end, the effects of the drugs favipiravir and oseltamivir, which are used in the covid-19 clinic and whose relationship with these enzymes has not been previously studied, on the isoenzymes of carbonic anhydrase I- II and the enzyme acetylcholinesterase were studied in vitro. No inhibition or activation was observed on the enzyme acetylcholinesterase, while inhibition was observed for the isoenzyme carbonic anhydrase I - II.

Keywords: Alzheimer's disease, Carbonic anhydrase, Favipiravir, Oseltamivir, Covid-19arikueda@gmail.com<https://orcid.org/0000-0003-3275-7035>ukocyigit@cumhuriyet.edu.tr<https://orcid.org/0000-0001-8710-2912>

Introduction

Investigations into a group of patients who experienced respiratory symptoms (fever, cough, and shortness of breath) in Wuhan Province, China led to the discovery of covid-19 on January 13, 2020. After that, the pathogen spread very quickly and became a global threat, affecting millions of people around the world and causing the death of thousands.

In the treatment of covid-19 during the pandemic: Lopinavir/ritonavir, danoprevir/ritonavir, oseltamivir, favipiravir, remdesivir, umifenovir, molnupiravir, antivirals, camostat mesylate/nafamostat mesylate, ivermectin, chloroquine/hydroxychlorozone, anticoagulant drugs, drugs such as dexamethasone, treatment trials, such as dexamethasone, treatment. However, none of the proposed treatments have proven effective in completely eradicating covid-19. The drugs used are palliative pharmacological alternatives and have helped to treat the symptoms or complications that occur during the course of the disease [1].

Acid-base balance is one of the most important requirements for the survival of an organism and is achieved by the combined efforts of the kidneys and lungs [2]. CA Enzymes, on the other hand, maintain acid-base balance by catalyzing the reversible hydration of carbon dioxide to bicarbonate and H⁺ ions. It has been characterized as a pH-regulating enzyme [3].

One of the most prevalent neurological conditions in clinical medicine is seizures. Although the exact causes of

seizures are still unknown, it is believed that variations in brain pH and intracellular potassium concentration as well as ion variability are involved. The pH buffering of the extracellular and intracellular spaces is mainly carried out by the CO₂/HCO₃ buffer, and the balance of the two species is maintained by the zinc enzyme carbonic anhydrase (CA). Some carbonic anhydrase inhibitors (CAIs) are prescribed to treat epilepsy as anticonvulsants[4].

In glaucoma, one of the eye diseases that lead to blindness in 15-20%, the enzyme carbonic anhydrase has a triggering effect. For this reason, carbonic anhydrase inhibitors such as acetazolamide and dorzolamide have been used to treat glaucoma for years [5].

Recently, it has been suggested that activators of carbonic anhydrase may be one of the key factors in pathologies related to pharmacological development of synaptic activity, learning, and memory.

It has been demonstrated that administering amino acid-type CAAs enhances spatial learning, which is counteracted by concurrently administering a sulfonamide inhibitor such as acetazolamide. These trials also revealed impairment in the consolidation of fear memories, which may open the door to pharmacological uses in new therapies for phobias and post-traumatic shock. Extracellular signal-regulated kinase (ERK) pathways, which are engaged in a crucial stage of memory formation in both the cortex and hippocampus, were

found to be quickly activated by CAA administration. The use of CAAs for memory treatment in aging or neurodegenerative illnesses like Alzheimer's disease may result from these intriguing findings [6]. As a result, it has been proposed that CA activators may be helpful in the management of phobias and cognitive impairment.

The fact that acid-base balance dysregulation observed in covid patients has not been adequately defined has attracted the attention of researchers; in the study by Gaetano Alfano et al. ABG analyzes of 211 covid patients were examined, and acid-base balance irregularity was observed in 79.7% of patients [7]. It is suggested that CA enzymes, which are characterized as pH-regulating enzymes and provide acid-base balance in many tissues, may be a crucial factor in the pathogenesis of the disease; CA, ACE2 and MMA biomolecules contribute to the RAS system in the pathogenesis of covid, and dysregulation of these biomolecules triggers respiratory acidosis, pulmonary edema, cardiac and renal failure [8]. In the study of Seçil Deniz et al, it was hypothesized that patients with covid-19 problems have an acid-base state affected by the activity of CA, and the blood levels of CA were measured in acute covid patients, noncovid patients, and post-covid patients. It was found that the activity of CA in blood was significantly increased in covid-19 patients and was higher in post-covid patients than in acute covid patients. In the same study, it was suggested that CA inhibitors could be used as pharmacological treatment in the treatment of covid [9].

Our study involves investigating the effects of favipiravir- and oseltamivir-based drugs used in the treatment of covid-19 on acid-base balance through the enzymes carbonic anhydrase isoenzyme I and II (hCA I and hCA II). This study, which examined the use of drugs in specific patient groups based on acid-base balance and the drug-enzyme relationship, is a contribution to the literature.

For the brain to function properly, acetylcholine (ACh), norepinephrine, dopamine, gamma-aminobutyric acid, serotonin, and glutamate all need to be in balance [10]. Alzheimer's disease (AD) is characterized by behavioral signs as well as a steady decline in cognitive ability. Acetylcholinesterase/cholinesterase inhibitors are the principal type of medications now used to treat AD. A crucial aspect of AD is cholinergic neurotransmission [11].

Targeted antibodies to the muscle's acetylcholine receptors (AChRs) are the main cause of the rare autoimmune disease myasthenia gravis, which affects the neuromuscular junction. Muscle weakness and exhaustion are brought on by the loss of AChRs, which affects neuromuscular transmission. Myasthenia gravis can today be effectively controlled with reasonably safe and effective medications, despite the fact that the condition used to typically be deadly. Acetylcholinesterase inhibitors (AChEI), which enhance neuromuscular transmission, are the first step in treatment and are crucial for early diagnosis [12].

The relationship between vascular deficits and retinal ganglion cell (RGC) loss in glaucoma was investigated.

They wanted to know if the acetylcholinesterase inhibitor galantamine, which supports RGC survival, might shield the retinal microvasculature and enhance blood flow in an experimental glaucoma model. The outcomes demonstrated that systemic administration of galantamine improved retinal blood flow, preserved the density of microvessels in glaucomatous retinas, and mediated the vasoactive effect of galantamine on retinal microvessels through activation of muscarinic acetylcholine receptors both in vitro and in vivo [13].

As shown in the above studies, acetylcholinesterase inhibitors are used in the treatment of serious diseases such as Alzheimer's disease, myasthenia gravis, and glaucoma. It is very important to know how acetylcholinesterase is affected when these patients need to take these two antiviral drugs used at covid-19 clinic and to determine if there is a drug interaction. In the event of an interaction, it is imperative that these patients consider alternative treatments and recheck the dosage of the medications used.

Materials and Methods

Chemicals

p-nitrophenylacetate, Tris-SO₄ (0.5 M pH 7.4), Tris-HCl buffer (1M, pH 8), acetylcholine iodate solution (10 mM), DTNB solution (10 mM), enzyme solution (carbonic anhydrase I-II isoenzymes and acetylcholinesterase), purified water, favipiravir (favicovir film-coated tablet 200 mg 40 tablets) and oseltamivir phosphate (oseflu 30 mg 10 capsules) active ingredient drug samples

Tools and Devices Used

Spectrophotometer (SOIOptical instruments/China), Magnetic stirrer (Elektro-MAG/Turkey), Precision balance (Weightlab Instrument/Turkey), pH meter (HANNA/United States), Vortex instrument (Velp Scientifica/Italy), Micropipette types (Weightlab Instrument/Turkey, Nichipet EXII/Japan, ISOLAB/Turkey, A.B.T. Laboratory Industry/Turkey)

Activity Assignments

Esterase activity method investigation of the effects of drugs on carbonic anhydrase (Ca) isoenzymes

Carbonic anhydrase is an enzyme with esterase activity, so this method was studied. The principle of the esterase activity method is that the enzyme carbonic anhydrase hydrolyzes the compound *p*-nitrophenyl acetate as a substrate to *p*-nitrophenolate, which absorbs at a wavelength of 348 nm. In this method, *p*-nitrophenol and *p*-nitrophenolate in both compounds have the same absorbance values at a wavelength of 348 nm. Therefore, the formation of phenol or phenolate during the reaction affects the measured values [83-86].

In this experiment, *p*-nitrophenyl acetate, which has very low absorbance at a wavelength of 348 nm, was used

as a blank sample. 1-ml quartz cuvettes were also used in the measurements. In the method, the procedure for activity determination was applied in the following order.

Table 1. Contents of 1ml cuvette used in esterase activity studies for carbonic anhydrase isoenzymes

Substances	Control (Blind) (μ l)	Sample (μ l)
Tris-SO ₄ (0,5 M) pH 7,4	400	400
p-Nitrophenol acetate	360	360
H ₂ O	240	230
Enzyme solution	-	10
Total final volume	1000	1000

The absorbance values of the reaction mixture prepared according to Table 1 were measured every 15 seconds, and the absorbance value at a wavelength of 348 nm at 25°C was read at the end of 3 minutes, and the difference between the absorbance value at zero second and the absorbance value was taken. [14-17]. In the kinetic studies applied in the study, the procedures for activity determination were applied according to the procedure for esterase activity of the enzyme. In this study, the effects of favipiravir and oseltamivir phosphate-based drugs on the isoenzymes of carbonic anhydrase I- II were investigated. IC₅₀ and K_i values were calculated using data obtained by this method.

Determination of the effects of drugs on Acetylcholinesterase enzyme

The effects of the drugs under study on the enzyme acetylcholinesterase were investigated. The acetylcholinesterase method was used for this purpose.

The principle of the method: AChE catalyzes the hydrolysis of acetylcholine and the formation of its degradation products thiocholine and acetate.

5-Thio-2-nitrobenzoic acid, a yellow compound, is formed by the interaction of the DTNB chemical used in the research with thiocholine, one of the degradation products. The absorbance value of this colored compound is measured at 412 nm [18]. In this method, the measurements of samples and blanks are performed at a wavelength of 412 nm. It should be noted that the absorbance values are measured and recorded at the beginning and after the fifth minute.

Table 2. The contents of the cuvette used during the kinetic studies of the acetylcholinesterase enzyme

Substances	Control (Blind) (μ l)	Sample (μ l)
Tris-HCl	100	100
H ₂ O	790	780
Inhibitor	-	10
DTNB	50	50
Enzyme solution	10	10
Acetylcholinethiodide	50	50

Results

The inhibition potentials of drugs against two physiologically relevant CA isoforms, the slower cytosolic isoform (hCA I), the more rapid cytosolic isoenzyme (hCA II) were investigated by using an esterase assay method. The inhibition data of compounds against CA I, and II isoforms were summarized in Table 3 (IC₅₀ and K_i values expressed as micromolar (μ M)).

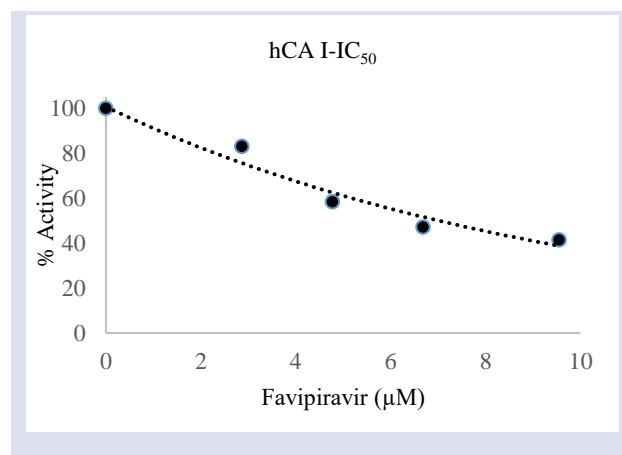
Table 3. The enzyme inhibition results of the drugs against carbonic anhydrase I and II isoenzymes

Compounds	IC ₅₀ (μ M)		K _i (μ M)			
	hCA I	r ²	hCA II	r ²	hCA I	hCA II
Favipiravir	6.9810	0.9531	5.3950	0,9526	6.2507 ±1.0564	5.9018±0.2938
Oseltamivir phosphate	1.2459	0.9461	1.0279	0.9783	2.2773±0.4405	1.5910±0.6036
AZA*	12.6200	0.9712	19.810	0.9706	12.08±2.00	18.2200±4.900

*AZA: acetazolamide, standard.

The drug concentrations (IC₅₀) at which 50% of CA enzymes activities were inhibited were calculated. The drugs were remarkably inhibited both the cytosolic isoforms hCA I (IC₅₀ 6.9810 and 1.2459 μ M) and hCA II (IC₅₀ ranging between 5.3950 and 1.0279 μ M). Figure 1 show IC₅₀ plots.

Finally, inhibition constants (K_i) were determined for t CA enzymes from Lineweaver-Burk plots. K_i values were calculated as 6.2507 ±1.0564 μ M and 2.2773±0.4405 μ M for hCA I and 5.9018±0.2938 μ M and 1.5910±0.6036 μ M for hCA II, respectively. The K_i value for the standard item AZA was found to be 18.2200±4.900 μ M. Figure 2 show K_i plots.



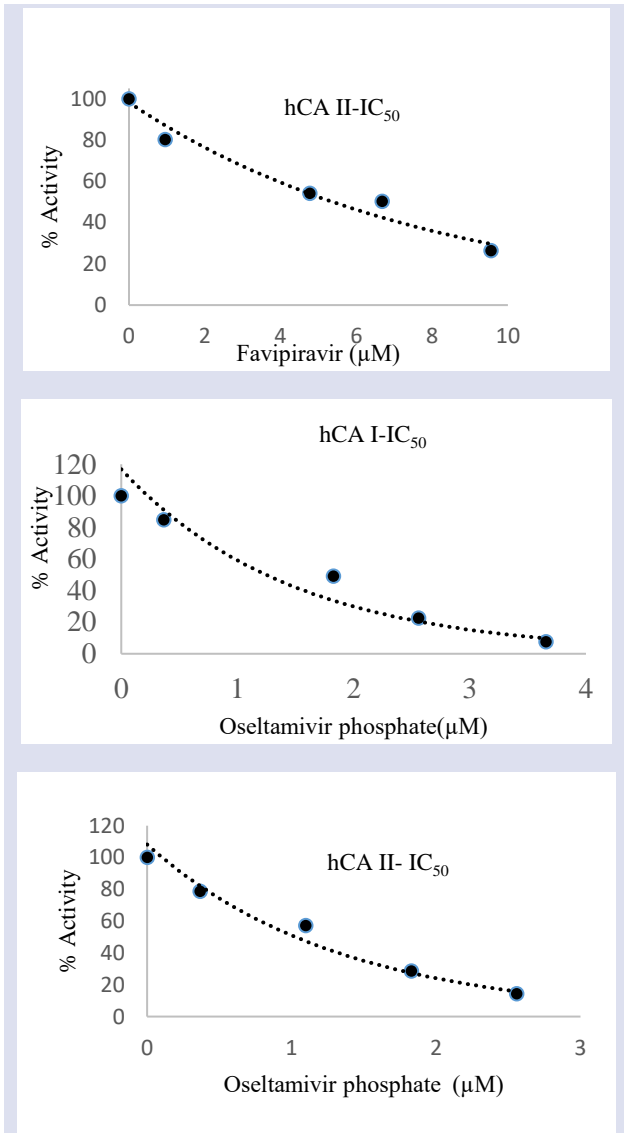


Figure 1. IC₅₀ graphics of the drugs against carbonic anhydrase I and II isoenzymes

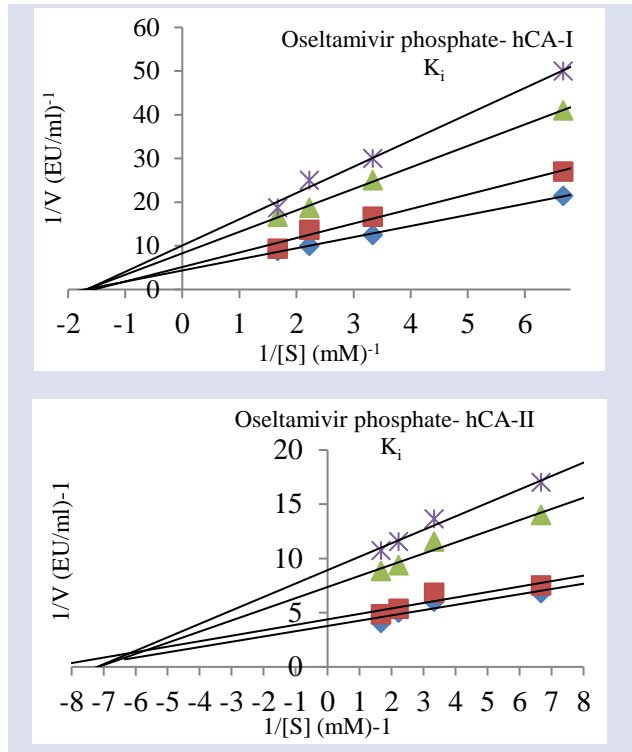
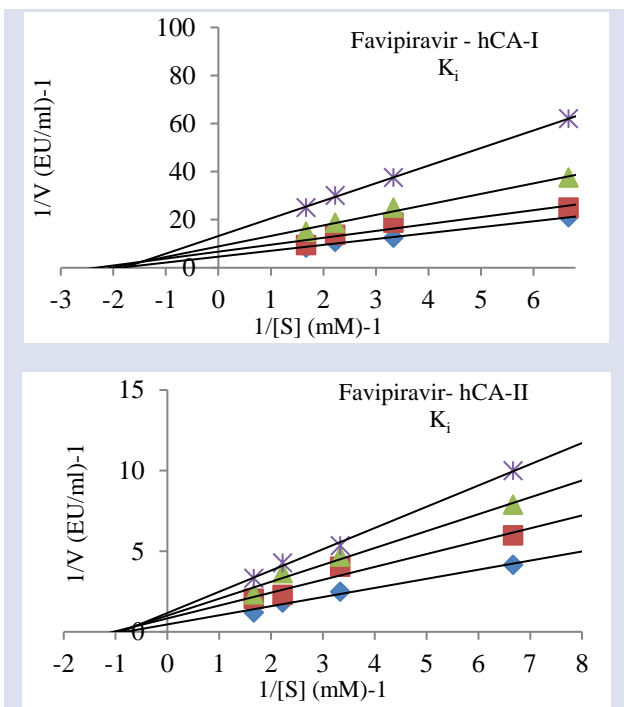


Figure 2. K_i graphics of the drugs against carbonic anhydrase I and II isoenzymes



Finally, the effects of these drugs on acetylcholinesterase enzyme activity were investigated in vitro using the acetylcholiniodate method. No effect of drugs on enzyme activity was found.

Discussion and Conclusion

In the study of Gaetano Alfano et al., it was mentioned that 79.7% of 211 covid-19 patients had acid-base disorders. Based on this study, it was thought that favipiravir and osetamivir phosphate active agents used in the treatment of covid-19 may have inhibition or activation effects on carbonic anhydrase I-II isoenzymes. IC₅₀ and K_i results were calculated for both drugs, and it was observed that the absorbance difference value decreased as the amount of inhibitor substance studied increased. The results showed that favipiravir and osetamivir phosphate-based drugs have inhibitory effects on carbonic anhydrase I-II isoenzymes. No inhibition or activation was observed on the acetylcholinesterase enzyme.

Carbonic anhydrase enzymes, which are characterized as pH-regulating enzymes and provide acid-base balance in tissues, are a critical factor in the pathogenesis of covid-19 disease. In this context, carbonic anhydrase inhibition by favipiravir and osetamivir phosphate active substances used in treatment is very important.

Carbonic anhydrase inhibitors are used clinically as diuretics, anti-glaucoma agents, and anti-epileptics, but new applications have recently been reported in the treatment of cancer, neuropathic pain, sleep apnea, migraine, lowering intracranial pressure, and cerebral ischemia. The carbonic anhydrase inhibition observed for

favipiravir and oseltamivir phosphate active substances used in covid-19 may contribute to the development of new treatment strategies.

When special patient groups with conditions such as epilepsy and glaucoma have to receive treatment with these antivirals, the drug doses used by the experts in the field should be reviewed and the appropriate dose determined, taking into account drug-drug interactions.

As a result, enzyme inhibitors are very important in the discovery of new therapeutic agents and in a detailed understanding of protein-drug interactions at the molecular level. This study contributed to the literature by investigating the effects of favipiravir and oseltamivir phosphate-based drugs on acetylcholinesterase and carbonic anhydrase I-II isoenzymes. Drug research is generally a long and laborious process. Our results will provide insight to researchers and may be a preliminary step for many new projects.

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Conflicts of interest

There are no conflicts of interest in this work.

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