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Research Article
Investigation of Inhibition of Busulfan (Chemotherapeutic Drug) on Human Serum Paraoxonase-1 (PON1)

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Abstract

Background and Objective: Paraoxonase 1 (PON1) is an antiatherogenic lactonase. It plays the role of an antioxidant enzyme by protecting HDLs from oxidative stress and also has important roles such as protection of Low-Density Lipoprotein (LDL) against oxidation and detoxification of highly toxic substances. Lowering the levels of this enzyme is a major risk in patients with cardiovascular disease. This study, it was aimed to investigate the *in vitro* effect of busulfan which is widely used in chemotherapy, on the human paraoxonase-1 enzyme. **Materials and Methods:** This study identifies interactions between the enzyme and busulfan, which is widely used in cancer chemotherapy. Paraoxonase activity measurement was used to determine the chemotherapeutic drug constants. **Results:** This study found that busulfan exhibited very potent inhibitory properties for human serum PON1 with an IC_{50} value of 77 μM and an average K_i value of 42.83 μM. Busulfan showed competitive inhibition. **Conclusion:** The use of busulfan, which has a very strong inhibition, in chemotherapeutic treatment can be very dangerous.

Key words: Paraoxonase, busulfan, inhibition, chemotherapeutic drug, PON1

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Paraoxonase-I (EC 3.1.8.1, PON1) is a member of the lactonase enzyme family. This enzyme has three enzymatic activities: lactonase, arylesterase and paraoxonase activity. However, the physiological activity of the enzyme is still unclear[1]. Because of the enzyme's ability to hydrolyze paraoxon, it is called paraoxonase. Organophosphate compounds (OPs) are widely used in agricultural and rural areas. For example, paraoxon, chlorpyrifos-oxon and diazoxide are frequently used in agricultural applications and these compounds are substrates for PON1. These compounds are very toxic to other organisms, including humans, insects and plants[2]. PON1 is an HDL-dependent anti-oxidative enzyme. Therefore, some of the antioxidant activity of HDL is provided by PON1. The enzyme also has an important role in preventing lipid peroxidation with its antioxidant properties. PON1 protects Low-Density Lipoprotein (LDL) and other lipoproteins from oxidative stress caused by some types of free radicals. This property of paraoxonase-I protects HDL and LDL against oxidation. It prevents atherosclerotic lesions that may occur due to oxidation[3]. PON1 not only prevents atherosclerosis with its antioxidant role but also prevents the development of diabetes, hypercholesterolemia and many diseases[4]. In many diseases, drugs show their effects by increasing or decreasing the activities of enzymes[5-11]. Determining the *in vitro* and *in vivo* effects of drugs on these enzymes can be an important step for new drug development studies against many diseases. Many studies have been done on drug inhibition of the PON1 enzyme before. For example, in a study conducted by Türkeş, they determined that pantoprazole, omeprazole and esomeprazole drugs effectively inhibited the PON1 enzyme. These drugs competitively inhibited the paraoxonase enzyme[12]. In another study, Ekinci and Beydemir[13] investigated the effects of various analgesic drugs on human PON1 activity. They found IC_{50} values for lornoxicam, indomethacin, tenoxicam, diclofenac sodium, ketoprofen and lincomycin as 0.13, 0.19, 0.34, 1.63, 6.23 and 9.63 mM, respectively. In addition, the authors stated that the inhibition type of indomethacin and tenoxicam was competitive, while ketoprofen, lornoxicam, diclofenac sodium and lincomycin were noncompetitive. However, more research is needed on chemotherapeutic drugs. Because chemotherapy drugs are powerful inhibitors.

Busulfan is a chemotherapeutic drug widely used to treat pediatric and adult chronic myelogenous leukaemia. Busulfan causes male infertility by damaging germ cells. In this study, it was aimed to investigate the *in vitro* effect of busulfan on the human paraoxonase-1 enzyme.

MATERIALS AND METHODS

Study area: This study carried out in 2021. This work took about three months.

Chemicals: All chemicals were obtained from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). Busulfan was obtained from Bursa Uludağ University, Faculty of Medicine Oncology Department.

Paraoxonase activity measurement: Human serum samples were obtained from Erzincan Mengücekgazi Research Hospital. PON1 activity was determined with paraoxonase (diethyl p-nitrophenyl phosphate) (1 mM) in 50 mM glycine/NaOH (pH 10.5) buffer solution containing 1 mM $CaCl_2$ at 25°C. The activity measurement is based on the absorption at 412 nm of p-nitrophenol formed as a result of the reaction of paraoxon and PON1 enzyme in Table 1. The molar extinction coefficient of p-nitrophenol ($\varepsilon = 18.290\ M^{-1}\ cm^{-1}$ at pH 10.5) was used to calculate PON1 activity. The enzyme unit of paraoxonase is the number of micromoles of paraoxon hydrolyzed in 1 min. Activity measurement was performed using a spectrophotometer (CHEBIOS UV-VIS). The activity calculation formula for the PON1 enzyme is as follows[14]:

$$EU\ (mL) = \frac{\Delta OD}{18,290} \times \frac{V_T}{V_E} \times 1000$$

The symbols in this formula are explained below:

EU (Ml) : Enzyme unit in 1 mL
ΔOD : Change in absorbance in one min

Table 1: Activity measurement cuvette content of human serum PON1 enzyme

	Control cuvette		Sample cuvette	
Stock activity solutions	Volume (µL)	Concentration (mM)	Volume (µL)	Concentration (mM)
Glycine/NaOH (pH:10.5)	500	25	500	25
Substrate solution	330	1	330	1
Distilled water	120	-	Variable	-
Medication solution	0	-	Variable	Variable
Enzyme sample	50	-	50	-

18.290 : Molar extinction coefficient of para nitrophenol at pH: 10.5 (M^{-1} cm^{-1})
VT : Total cuvette volume measured (mL)
VE : The volume of the enzyme sample added to the cuvette where the measurement was made (mL)

Determination of IC_{50} and K_i constants for a chemotherapeutic drug: The inhibitory effect of busulfan, which is widely used in cancer chemotherapy, was investigated. This chemotherapeutic drug was tested three times for each concentration. Paraoxonase activities of the enzyme were analyzed at different drug concentrations. Graphs were plotted showing percent activity for busulfan as a function of drug concentration. Control activity in the absence of inhibitor was accepted as 100%. The 50% inhibition (IC_{50} value) of busulfan was obtained from the graphs using different inhibitor concentration values To calculate K_i values, three different inhibitory concentrations of busulfan were tested Lineweaver-Burk curves were used to determine the values of K_i and the type of inhibition.

RESULTS

In this study, the inhibition effect of busulfan on paraoxonase enzyme activity was studied. The *in vitro* inhibition impact of busulfan on the enzyme was determined. The IC_{50} value is the inhibitor concentration inhibiting 50% of enzyme activity. The IC_{50} value was calculated as 77 μM from the curve equation in the Activity (%)-[Busulfan] graph in Fig. 1. In the present study, activity measurements were made by taking 5 different concentrations of paraoxon (1.33, 1.66, 2.22, 3.33 and 6.66 μM) with three different concentrations of fixed busulfan ([I1]:20, [I2]:40 and [I3]:80 μM). Lineweaver-Burk graph was drawn for busulfan with the help of the obtained activity values in Table 2.

K_i is the equilibrium constant expressing the inhibitor relation of the enzyme. K_i constants were determined from the expression:

$$K_M^I = \frac{K_M}{1+\frac{I}{K_i}}$$

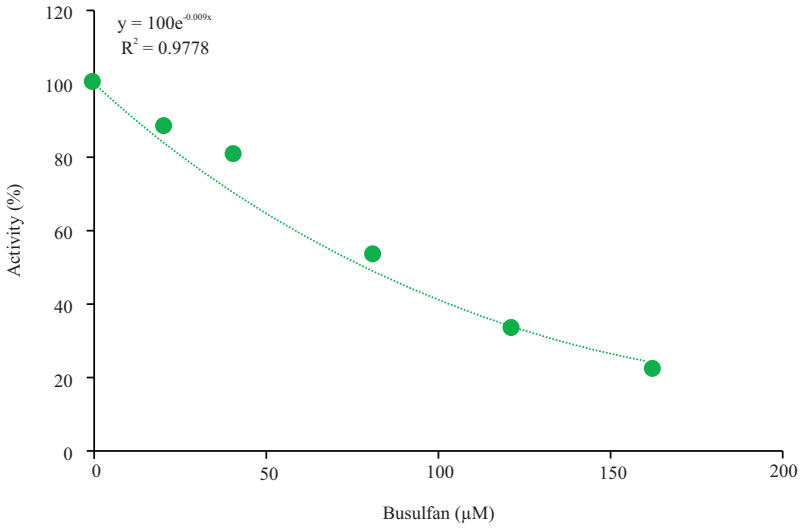

Fig. 1: Activity (%)-(concentration) graph used to determine the IC_{50} value

Table 2: $1/V \times 10^3$-1/Paraoxon (μM^{-1}) values for Lineweaver-Burk graph

	1/Paraoxon (μM^{-1})				
	6.66	3.33	2.22	1.66	1.33
$1/V \times 10^3$					
Control	0.103	0.074	0.052	0.044	0.035
I1	0.166	0.119	0.08	0.058	0.041
I2	0.274	0.182	0.119	0.085	0.057
I3	0.46	0.249	0.195	0.13	0.094

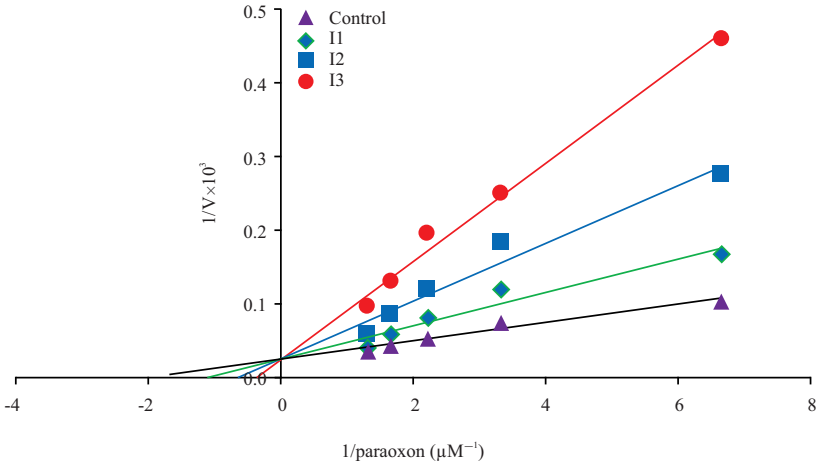

Fig. 2: Lineweaver-Burk graph used to determine K_i constant

which equals the slope in the graph equation for competitive inhibition. The average K_i value was found to be 42.83 μM in Fig. 2.

DISCUSSION

PON1 enzyme is found in many tissues such as the brain, heart, kidney, liver, lung and small intestine. However, PON1 is mostly synthesized by the liver in metabolism and bound to HDL and then released into the bloodstream[15]. PON1 is directly responsible for several antiatherogenic functions of HDL. These tasks include protecting lipoproteins and cells against oxidative stress, as well as preventing atherogenesis and lipoprotein peroxidation[16]. These protective effects of PON1 are related to the enzyme level in serum. In recent studies, it has been stated that PON1 levels are low in patients with atherosclerosis, diabetes and hypercholesterolemia. Overexpression of PON1 has been found to inhibit the development of cardiovascular diseases in particular. It has also been determined that PON1 stimulates HDL efflux in macrophages and inhibits LDL biosynthesis[17]. In another study, PON1 was shown to be transported from HDL to the outer surface of the cell membrane to protect metabolism against oxidative stress-related conditions such as coronary artery disease, diabetes, obesity[3].

Enzymes are suitable targets for chemotherapeutic application in patient metabolism. Drug molecules bind to enzyme active sites and other allosteric binding sites. They exert their biological effects by competitive, non-competitive and uncompetitive inhibition on the active site of the enzyme. There are few studies on PON1-chemotherapeutic drug interaction.

In a previous study, Türkeş *et al.*[18] were investigated the inhibition effects of palonosetron hydrochloride, bevacizumab and cyclophosphamide on PON1. They found that IC_{50} values were in a range of 00.25-2.462 mM against PON1. The *in vitro* impact of vincristine sulfate, epirubicin hydrochloride and doxorubicin hydrochloride (chemotherapy drugs) on hPON1. They found that chemotherapy drugs inhibited the enzyme at low concentrations[19]. In a study by Gökçe *et al.*[20], they determined that salbutamol sulfate, tiotropium, varenicline tartrate, ciprofloxacin hydrochloride, fluticasone propionate, theophylline sodium glycinate, moxifloxacin hydrochloride, ampicillin trihydrate and montelukast sodium drugs strongly inhibited the PON1 enzyme. Salbutamol sulfate showed the strongest inhibitory effect, while montelukast sodium showed the weakest inhibition effect.

This study showed that the IC_{50} value of busulfan was 77 μM against purified PON1. Busulfan inhibited the PON1 enzyme in very small concentrations. The fact that busulfan inhibits the enzyme at very small concentrations indicates that it is a very potent inhibitor. When these study findings were compared with previous drug inhibition studies in the literature, busulfan inhibited PON1 enzyme at concentrations similar to the inhibition concentrations of those drugs.

CONCLUSION

In conclusion, this study focused on investigating the *in vitro* effects of busulfan, which is frequently used in leukaemia cancer, on PON1 activity, which has a role in serious cases such as cardiovascular diseases. A wide variety of risks may arise in patients taking these drugs due to PON1 inhibition. Decreased activity of PON1 is an important risk factor for atherosclerosis. This study should be supported by

in vivo studies. Further studies are needed as there is no universal conclusion about whether PON1 can be used as a biomarker for cardiovascular disease.

SIGNIFICANCE STATEMENT

This study discovered the inhibition effect of busulfan on PON1 that can be beneficial for understanding the adverse effects of busulfan on cardiovascular diseases, that many researchers were not able to explore. Thus a new theory on the usage of busulfan in cancer treatment may be arrived at.

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