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Research Article

Effects of mono and dinuclear copper(II) complexes derived from non-steroidal anti-inflammatory drug naproxen on human serum paraoxanase1 (PON1) activity

Esra Dilek[a*] and Sema Caglar[b]

[a]*Department of Biochemistry, Division of Pharmaceutical Basic Sciences, Faculty of Pharmacy, Erzincan University, 24030, Erzincan-Turkey*
[b]*Department of Chemistry, Faculty of Sciences and Arts , Erzincan University, 24030, Erzincan-Turkey*

Abstract

The inhibition of human paraoxonase1 (PON1, EC 3.1.8.1) enzyme, with two Cu (II) complexes derived from naproxen was investigated by using the paraoxonase activity method with diethyl 4-nitrophenyl phosphate as substrate. The complexes $[Cu_2(\mu\text{-nap})_4(3\text{-pic})_2]$ **(1)** and $[Cu(nap)_2(H_2O)(4\text{-pic})_2]$ **(2)** decreased the in vitro PON1 activity with different inhibition mechanisms. The inhibition mechanism of complex 1 was uncompetitive whereas complex 2was noncompetitive inhibitors. In this study, complexes mentioned above showed effective inhibitory activity on PON1. IC_{50} values for $[Cu_2(\mu\text{-nap})_4(3\text{-pic})_2]$ (1) and $[Cu(nap)_2(H_2O)(4\text{-pic})_2]$ (2) were of 0.109 mM and 0.103 mM for PON1, respectively. K_i values for complex 1 and complex 2 were of 0.116 mM and 0.121 mM for PON1, respectively.

Keywords: paraoxonase1; inhibition; copper(II)complexes; enzyme; inhibitor.

1. Introduction

Paraoxonase (PON) (arylesterase, [EC 3.1.8.1]) is a enzyme of 354 amino acids, having a molecular weight of 43 kDa. It was first discovered in 1946 by Abraham Mazur, in later years, it is defined as the human serum PON. It is Ca-linked serum esterase and synthesized in liver. It is released to blood stream and found as linked with high-density lipoprotein (HDL) [1]. There are Ca^{2+} ions in the structure of PON, required for not only the enzyme activity but also the enzyme stability. One Ca^{2+} ion plays role in catalytic reactions and the second ensures proper conformation of active site on the enzyme [2].

PON has two main functions. First PON hydrolysis paraoxon, the active metabolite of parathion which is an insecticide in organophosphate [1,3].Second, it protects low-density lipoprotein (LDL) from oxidation by hydrolyzing of lipid peroxides[4].

Sulfhydryl group has antioxidant properties and PON has free sulfhydryl group (cysteine 284) which make it an antioxidant enzyme [5]. Therefore is it is thought to play a protective role against development of many diseases such as diabetes, cardiovascular disease, sepsis, Alzheimer and Parkinson [6].

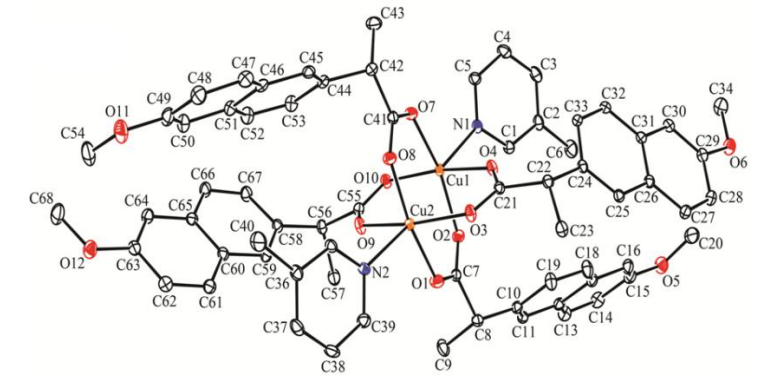
The activity of PON changes depending on some parameters such as age, sex, diet, life style and drug usage. Serum PON1 activity is nearly half of the adult levels in newborns and premature babies. After one year of birth, it reaches to normal adult level in babies. In addition, studies showed that PON1 activity decreases by age [7]. When analyzed in terms of sex, women have higher activity than men [8]. Moreover, the replacement of saturated fat with trans-fat causes a reduction in PON activity in contrast olive oil consumption increases the PON1 activity by acting contrary. This effect is particularly pronounced in women[9,10].The factors that reduce the enzyme activity are cigarette, alcohol, whereas some drugs (statins, aspirin, fenofibrate, such as dexamethasone) is known to cause an increase in the PON1 level and its activity[11].

* Correspondence Info

Mrs. Esra Dilek
Assistant Professor,
Department of Biochemistry,
Faculty of Pharmacy, Erzincan University,
Erzincan-Turkey,24030,
E-mail: edilek@erzincan.edu.tr;esrabayram25@gmail.com

It is clearly shown that significant effects of PON1on living organisms. Therefore, further study on interactions of PON with drugs is needed. We conducted studies related to purification of some protein and the investigation of protein-chemical or protein-drug interactions [12,13]. For example, our previous research indicated that PON1 enzyme is affected by some antibiotic drugs which are commonly used in medicine at low concentrations [13]. There are aslo other studies showing *in vitro* and *in vivo* effect of some drugs such as simvastatin, lovastatin, mevastatin, spironolactone, prulifloxacin and pravastatin on PON activity [14-16]. Nevertheless, there are still few studies related to protein-chemical or protein-drug interactions. In this study, we studied the *in vitro* effects of two different Cu(II) well-defined complexes with on human serum PON1 enzyme activity. The structures of these complexes were previously determined as shown in Figure 1[17].

Figure 1: Structures of the $[Cu_2(\mu\text{-nap})_4(3\text{-pic})_2]$ (1) and $[Cu(nap)_2(H_2O)(4\text{-pic})_2]$ (2), respectively.[23]



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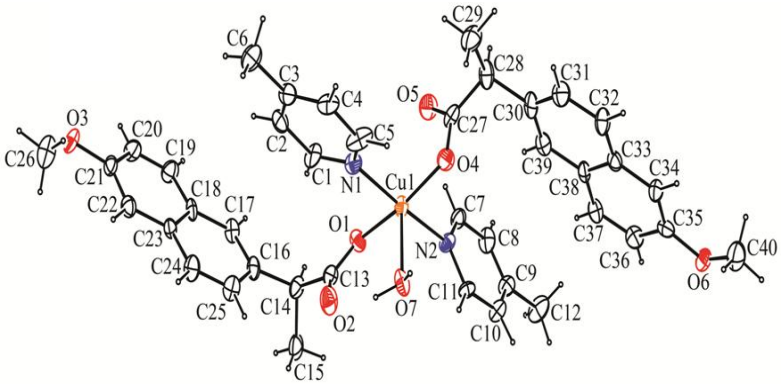

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2. Materials and methods

2.1 Materials

DEAE-Sephadex A50, Paraoxon (diethyl 4-nitrophenyl phosphate), and Sephadex G-200 were purchased from Sigma Chem. Co. All other chemicals were analytical grade and obtained from Merck. Fresh human serum was taken from the Blood Center of the Mengücek Gazi Education and Research Hospital at Erzincan University.

2.2 Paraoxonase activity assay

Paraoxonase activity of PON was determined with paraoxon (1 mM) in 50 mM glycine-NaOH (pH 10.5) buffer including 1 mM $CaCl_2$ at 25 ºC. Enzyme activity assay is based on spectrophotometrical measurement of p-nitrophenol at 412 nm. SHIMADZU UV-VIS spectrophotometer was used analysis. The molar extinction coefficient of paranitrophenol ($\varepsilon = 18{,}290 M^{-1}\ cm^{-1}$ at pH 10.5) is used for calculation of activity.

2.3 Ammonium sulfate precipitation and dialyzed

Precipitation intervals for PON1 were 60-80% [13].The precipitate obtained was redissolved in 100 mM Na-phosphate buffer (pH 7.0). And then, the mixture is dialyzed in 1 mM Na-phosphate buffer for two hours (pH 7.0).

2.4 DEAE-Sephadex A50 anion exchange chromatography

First, the mixture dialyzed was charged onto the DEAE-Sephadex A50 anion exchange column (3 cm × 30 cm) equilibrated with 100 mM Na-phosphate buffer (pH 7.0). Then, elution was initiated. For each elution tube, the enzyme activity was examined at 412 nm. Tubes which have activity were combined [13].

2.5 Sephadex G-200 gel filtration chromatography

Fractions obtained from anion exchange column were mixed with glycerol and charged onto the Sephadex G-200 column (2 cm × 60 cm) equilibrated with 100 mM Na-phosphate buffer (pH 7.0). Then, elution was initiated. For each elution tube, amount of protein at 280 nm and the enzyme activity at 412 nm were recorded. Tubes which have activity were combined for other kinetic studies [13].

2.6 Quantitative protein assay

Quantitative protein assay was carried out according to Bradford method. Bovine serum is used as the standard.. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the Laemmli's procedure as described previously [13,18]. The single band obtained was photographed after electrophoresis (Figure 2).

2.7 *In vitro* inhibition of purified human Paraoxnase 1

The purified enzyme from the last column was kept for kinetics studies and paraoxon was used as the substrate. The stock solutions at a certain concentration were prepared for each complex. Different concentrations were created by dilution with water.PON activities were measured separately for each concentration. Control activity was considered to be 100% in the absence of complexes. A graph was drawn for each complexes concentration against activity as a percent for each complex.

At the inhibition study, five different substrate (paraoxon) concentrations (0.15 - 0.75 mM) were used. K_i values of each complex were determined by selecting three different complex concentrations showing inhibition. Line weaver-Burk curves were drawn for identifying of K_i values and inhibition type [19].

3. Results and Discussion

PON1 was obtained with a yield of 40.1% a specific activity of 5857.1 U/mg proteins, and purified approximately 207-fold (Table 1). Figure 1 shows the SDS-PAGE results used to determine the purity of PON1. The figure 2 shows also that there is one single band for human serum, which is an indication of a successful purification. Figure 3 shows the *in vitro* effects of $[Cu_2(\mu\text{-nap})_4(3\text{-pic})_2]$ (1), and $[Cu(nap)_2(H_2O)(4\text{-pic})_2]$ (2) on paraoxonase activities of PON1. When the concentrations of complexes increase, the activity of PON1 was reduced up to approximately 20%. The *in vitro* effects of complex 1 and 2 on paraoxonase activity of PON1 were shown Table 2. IC_{50} values were determined as 0.109 mM and 0.103 mM, respectively (Table 2). K_i values and inhibition types of the drugs were given in Figure 4 and Table 2.

When comparing the $IC_{50}$and K_i values of the complexes, they were found to have similar values. Mono and dinuclearcopper(II) complexes derived from non-steroidal anti-inflammatory drug naproxen containing 3-picoline and 4-picolinedecreased the *in vitro* PON1 activity (Figure 4). The inhibition mechanism of complex 1 was uncompetitive whereas complex 2was noncompetitive inhibitors (Figure 4).

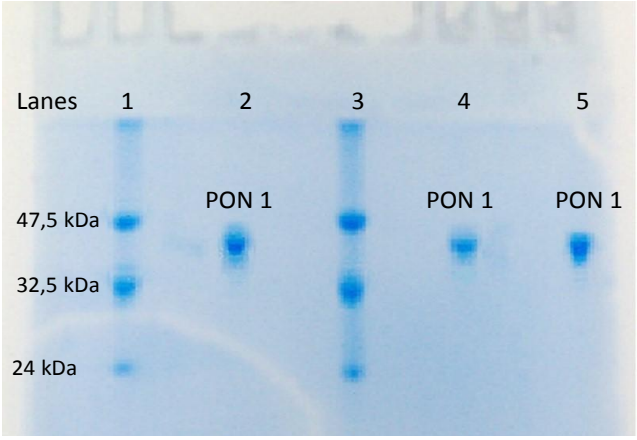
Important functions of PON1in living systems are clearly indicated in the scientific studies[5-11]. In addition, the role of the enzyme in pharmacokinetics will obviously be important. Lately, many scientists in the world have performed a variety of studies on PON1. But there are few studies on the PON activity and its interactions with some chemicals or drugs. For example, Leviev and James looked at the effects of simvastatin on biosynthesis and plasma levels of PON and found that simvastatin increased the plasma PON activity. Other studies on PON1 activity also looked at the effects of different hypocholesterolemic drugs such as spironolactone, mevastatin, lovastatin, pravastatin, and prulifloxacin[14-16]. Isgor and Beydemir evaluated the effects of certain cardiovascular drugs such as metoprolol tartrate, digoxin, amiodarone, diltiazem, verapamil, methylprednisolone, and dobutamine on human serum PON1 enzyme activity [20]. The *in vitro* effects of some calcium channel blocker such as isradipine, nifedipine, amlodipine, and nitrendipinbesylate on PON 1 activity was also investigated [21]. In our previous study, we evaluated the impacts of some antibiotics such as netilmicin sulfate, oxytetracyclinehidrochloride, clindamycin phosphate, streptomycin sulfate, and lincomycin hydrochloride on PON1 from human serum [13].

PON was initially purified from human serum by Gan *et al*. Then, studies on the purification procedures developed and began to rise[22-24]. In our previous study, we used three simple procedures: ammonium sulfate precipitations (60-80%), DEAE-Sephadex anion exchange, and Sephadex G-200 gel filtration chromatography [13]. We chose the same procedures in this study, because of these procedures are inexpensive, simple and can be used in a large scale of purification.

In this study, we investigated the *in vitro* effects of two different Cu(II) complexes with well-defined chemical structures on human serum PON1 enzyme activity. For this reason, human serum PON1 was purified using ammonium sulfate fractionation (60–80%), DEAE–Sephadex-A50 anion exchange, and Sephadex G-200 gel filtration chromatography. According to table 1, these results are consistent with our previous report and other studies[13,15,20,25]. We confirmed the purity of the enzyme with SDS-PAGE.

The enzyme / drug or enzyme / chemical interactions have become one of the major studies on pharmacokinetics and may scientists are publishing papers on this issue[12,26,27]. Drugs taken into the body and toxic substances exhibit generally their biological effects by interacting with enzymes. We thought that mono- and dinuclear copper (II) complexes derived from non-steroidal anti-inflammatory drug naproxen containing 3-picoline and 4-picolinedefinedby Caglar and co-workers might interact with PON1 enzyme. Therefore, we investigated the *in vitro* effects of these complex **1** and complex **2** on PON1 activity. We identified that complex **1** uncompetitively, and complex **2** inhibited noncompetitively paraoxonase activity of this enzyme. It can be concluded from the table 2 complex 2 exhibited the most effective inhibition. This could be due to the fact that complex **2** can be attached to a region outside the active site of the enzyme. Complex1, on the other hand, can be attached only to the enzyme-substrate complex.

Figure 2: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of purified paraoxonase 1



Lane 1 and 3: Standard proteins: 47.5, 32.5 and 24 kDa, Lane 2 and 5: Human serum (12µg protein) and Lane 4: Human serum (4µg protein)

Figure 3: *In vitro* effect of (A) complex 1, and (B)complex 2 at different seven concentrations on paraoxonase1 activity.

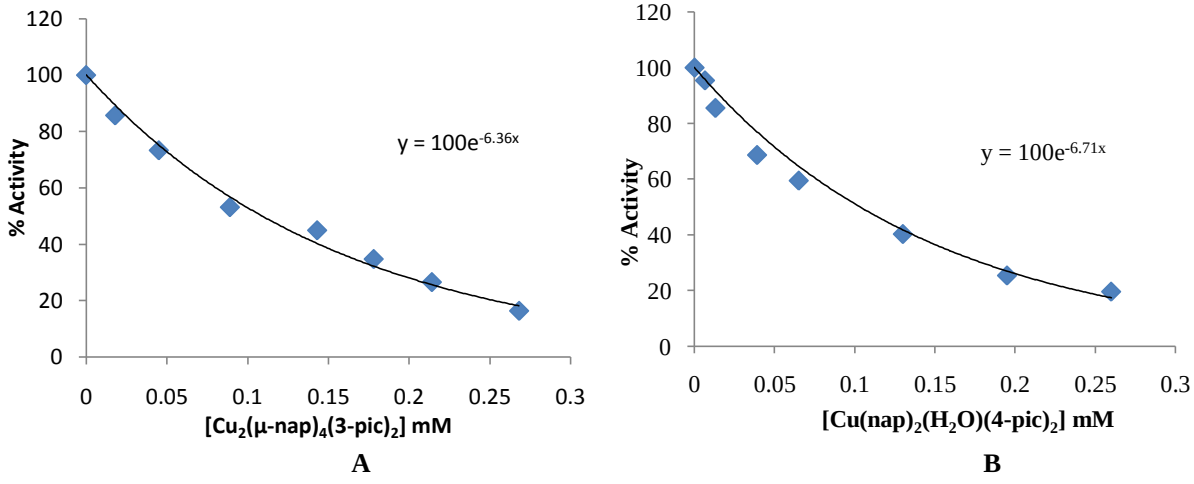
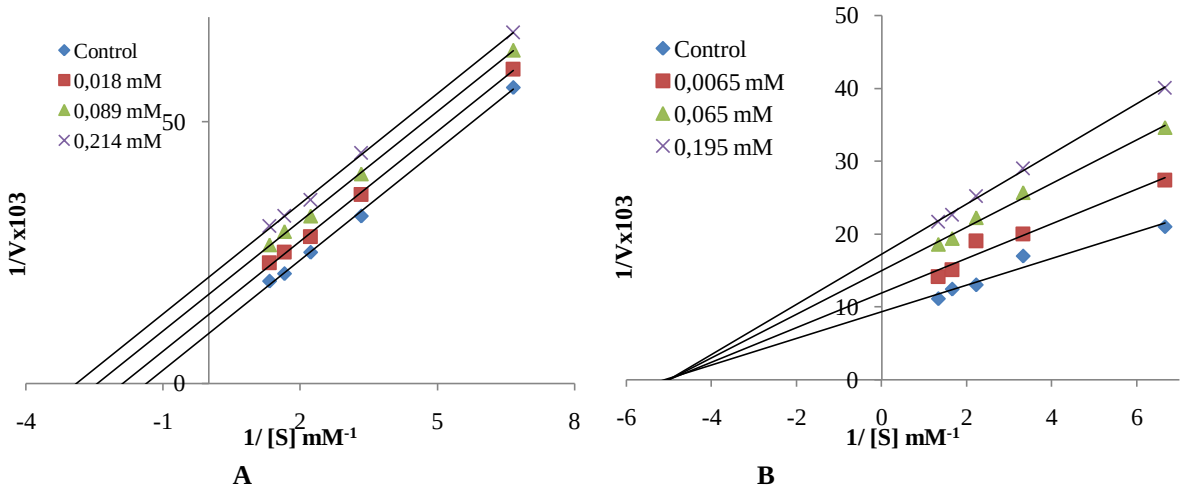


Figure 4: Determination of inhibition types and K_i values of (A) complex 1, and (B) complex 2 by using Lineweaver-Burk curves.



K_i values of each complex were determined by selecting three different complex concentrations showed inhibition. Five different substrate (paraoxon) concentrations (0.15 - 0.75 mM) were used. Control activity was considered to be 100% complex absence. Activity assays were performed as described in Materials and Methods.

Table 1: Summary of the paraoxonase1 purification procedure from human serum sample

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
Serum	122	2406	19,72	100	-
Ammonium sulfate precipitation (60-80%)	74,2	1836,8	24,75	76,3	1,26
DEAE-Sephadex A50 anion-exchange chromatography	1,89	1426,5	754,8	59,3	38,3
Sephadex G-200 gel filtration chromatography	0,168	984,0	5857,1	40,1	297,0

DEAE: diethyl aminoethyl cellulose.

Table 2: IC_{50}, K_i values and inhibition types for two complexes

Inhibitor	IC_{50} (mM)	K_i (mM)	Inhibition Type
$[Cu_2(\mu\text{-nap})_4(3\text{-pic})_2]$	0.109	0.116	Uncompetitive
$[Cu(nap)_2(H_2O)(4\text{-pic})_2]$	0.103	0.121	Noncompetitive

4. Conclusion

PON1 synthesized in liver and released into blood in humans, is physically linked with high-density lipoprotein (HDL). Therefore, it is interacting with all kinds of substances which can pass into blood. Consequently, the effect of the substances entering the body is important in terms of health. An increasing disease has led scientists to discover new drugs. Chemicals synthesized by chemists should be investigated in terms of biological activities to be able to use in medicine. In this study we aimed the effect of two Cu complexes on PON1 enzyme activity. In the future, drugs containing these complexes could be mediated to animals to see the effect on serum.

Conflict of interest

The authors have no conflict of interest to disclose.

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