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RESEARCH ARTICLE

Effect of some analgesics on Paraoxonase-1 purified from human serum

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Abstract

The *in vitro* effects of the analgesic drugs, lornoxicam, indomethacin, tenoxicam, diclofenac sodium, ketoprofen and lincomycine, on the activity of purified human serum paraoxonase (hPON1) (EC 3.1.8.1.) were evaluated. hPON1 was purified from human serum with a final specific activity of 3840 U mg^{-1} and a purity of 25.3 % using simple chromatographic methods, including DEAE-Sephadex anion exchange and Sepharose 4B-L-tyrozine-1--napthylamine hydrophobic interaction chromatography. SDS-polyacrylamide gel electrophoresis indicated a single protein band corresponding to hPON1. The six analgesics dose-dependently decreased *in vitro* hPON1 activity, with IC_{50} values for lornoxicam, indomethacin, tenoxicam, diclofenac sodium, ketoprofen and lincomycine of 0.136, 0.195, 0.340, 1.639, 6.23 and 9.638 mM, respectively. K_i constants were 0.009, 0.097, 0.306, 0.805, 13.010 and 11.116 mM, respectively. Analgesics showed different inhibition mechanisms: lornoxicam, diclofenac sodium and lincomycine were uncompetitive, indomethacin and tenoxicam were competitive, ketoprofen was noncompetitive. According to the results, inhibition potency was lornoxicam>indomethacin>tenoxicam> diclofenac sodium>ketoprofen> lincomycine.

Keywords: *Paraoxonase inhibition; lornoxicam; indomethacin; tenoxicam; diclofenac sodium; ketoprofen*

Abbreviations: PON 1: Paraoxonase 1; OPs: Organophosphates.

Introduction

Human paraoxonase 1 (arylesterase, EC 3.1.8.1, hPON1) is a calcium dependent enzyme which is exclusively associated with high-density lipoprotein (HDL). The enzyme is synthesized in the liver and then secreted into the blood, and exhibits broad-substrate specificity. The serum enzyme protects low-density lipoproteins (LDL) from oxidative modification in metabolism [1–3]. Its gene family contains at least three members in mammals: PON1, PON2, and PON3, which are 65% and 70% similar at the nucleotide level [4,5]. PON1 and PON3 are expressed primarily in the liver while PON2 is widely expressed in various tissues, including brain, liver, and kidney [6–8]. The enzyme was firstly characterized as an organophosphate hydrolyzer including paraoxon from which it takes its name [9,10]. PON1 hydrolyses several organophosphates, for instance the nerve agents: sarin, soman and the pesticides: chlorpyrifos oxon, diazoxon [11–13]. It hydrolyses organophosphates (OPs), but physiological substrate and biological function of this enzyme is not clearly known yet. In addition to hydrolyzing OPs, PON1 also hydrolyses aromatic carboxyl esters, such as phenyl acetate, and is involved in drug and xenobiotic metabolism. Moreover, it hydrolyses various lactones, including naturally occurring lactone metabolites [14–16]. Recently, a decrease in PON-I activity has been determined in some oxidative stress-associated diseases [17]. PON1 acts as an antioxidant enzyme [18] by protecting low-density lipoproteins (LDL) from oxidative damage, which is known to be associated with many vascular diseases, including atherosclerosis [19]. PON1 activity reduces in cardiovascular diseases, such as diabetes mellitus, chronic renal failure, rheumatoid arthritis, hyperthyroidism and age-related macular degeneration [20–28]. PON1 is thought to play its anti-atherogenic role by inhibiting the oxidation of LDL, and hydrolyzing lipid peroxides [29]. HDL can influence different stages of the atherosclerotic process, and human paraoxonase-1 enzyme (PON1) contributes to the antiatherogenic effect of HDL.

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PON was first purified 13-fold from rabbit kidney by Mazur in 1946 [30]. Then, the enzyme has been purified using different methods from various sources. For instance, Mounter and colleagues purified the enzyme 65–100 fold in 1953 [30]. In addition, PON3 from rat liver microsomes was purified using hydroxyapatite, DEAE-Sepharose, Cibacron Blue (M_1), first DEAE (D_1), second DEAE, and Con A methods [1]. Sinan et al. purified hPON1 227-fold with ammonium sulfate fractionation and hydrophobic interaction chromatography [9].

Many drugs are known to activate or inhibit several body enzymes in vivo [31]. Should any drug inhibit human serum PON1, the increased oxidation of low-density lipoprotein (LDL) could cause many vascular diseases resulting in severe health problems, including atherosclerosis. Moreover, a decrease in the enzyme activity may cause vital effects in some oxidative stress-associated diseases. Many studies have been performed in our laboratory about interactions between drugs and different enzymes from a variety of sources [31–33]. Indeed, due to the physiological role of PON, more studies about the inhibitory effects of medical drugs should be performed. However, there are few studies about the effects of medical drugs on PON1 activity. For example, in vitro and in vivo effects of some diuretic and hypocholesterolemic drugs on paraoxonase activity have been investigated, such as spironolactone, mevastatin, lovastatin, simvastatin, pravastatin and prulifloxacin [34-36]. In addition, Sinan et al. reported the inhibition effects of cephalosporine and gentamycin sulfate on human serum PON1 activity. They found that gentamycin sulfate and cefazoline sodium were potential inhibitors [9].

Here, we purified human serum PON1 using a simple modified method. The simple three-step procedure consists of ammonium sulfate precipitation, DEAE-Sephadex anion exchange chromatography, and Sepharose 4B-L-tyrozine-1-napthylamine hydrophobic interaction chromatography. We also examined the effects of commonly used analgesics on hPON1 activity.

Materials and methods

Materials

Materials, including DEAE-Sephadex A50, Sepharose 4B, 1-Naphthylamine, Paraoxon, protein assay reagents, and chemicals for electrophoresis, were obtained from Sigma Chem. Co (Sigma-Aldrich Chemie GmbH Export Department Eschenstrasse 5, 82024 Taufkirchen, Germany). All other chemicals used were of analytical grade and were obtained from either Sigma-Aldrich or Merck (Merck KGaA Frankfurter strasse 250, D-64293 Darmstadt, Germany). Lornoxicam, indomethacin, tenoxicam, diclofenac sodium, ketoprofen and lincomycine were provided from the University Hospital Pharmacy (Atatürk University, Erzurum, Turkey).

Paraoxonase activity assay

Paraoxonase activity was determined at 25°C with paraoxon (diethyl p-nitrophenyl phosphate) (1 mM) in 50 mM glycine/NaOH (pH 10.5) containing 1 mM $CaCl_2$. The enzyme assay was based on the estimation of p-nitrophenol at 412 nm. The molar extinction coefficient of p-nitrophenol ($\varepsilon = 18.290$ $M^{-1}cm^{-1}$ at pH 10.5) was used to calculate enzyme activity [37]. One enzyme unit was defined as the amount of enzyme that catalyzes the hydrolysis of 1 μmol of substrate at 25°C. Assays were performed using a spectrophotometer (CHEBIOS UV-VIS).

Ammonium sulfate precipitation

Twelve milliliters of Triton X-100-treated human serum was precipitated with ammonium sulphate. The precipitation fractions for paraoxonase were 60%-80% [9]. The precipitate was collected by centrifugation at 15,000 rpm for 20 min and redissolved in 100 mM Na-phosphate buffer (pH 7.0).

DEAE-Sephadex A50 Anion Exchange Chromatography

The enzyme solution, which had been dialyzed in the presence of 1 mM Na-phosphate buffer (pH 7.0) at 4°C, was loaded onto the DEAE-Sephadex A50 anion exchange column (3 cm^2 x 30 cm), which had been equilibrated with 100 mM Na-phosphate buffer (pH 7.0). The column was washed with 100 mM Na-phosphate buffer (pH 7.0), and then elution was performed with a linear gradient of 0-1.5 M NaCl. Eluted fractions were collected and enzyme activity was checked at 412 nm. Tubes with enzyme activity were combined. All purification procedures were performed at 4°C.

Sepharose 4B-L-tyrozine-1-napthylamine hydrophobic interaction chromatography

Fractions from DEAE-Sephadex A50 column were mixed and prepared to apply to the Sepharose 4B-L-tyrozine-1-napthylamine hydrophobic interaction chromatography column which had been designed according to Sinan et al 2006 [9]. After equilibring the column with 100 mM Na-phosphate buffer including NaCl (pH=7.0), elution was performed with a linear gradient of decreasing NaCl concentration 0–1,5 M NaCl. Fractions were analyzed for both protein amount (280 nm) and enzyme activity (412 nm). Tubes with enzyme activity were combined for other kinetic studies.

Protein Determination

During the purification steps, protein quantity was determined spectrophotometrically at 595 nm according to the Bradford method using bovine serum albumin as the standard [38].

SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was performed after purification of the enzyme with 10% and 3% acrylamide concentrations for the separating and stacking gels, respectively, and 0.1% SDS [39]. Sample (20 μg) was applied to the electrophoresis medium. Gels were stained for 1.5 h in 0.1% Coomassie Brilliant Blue R-250, 50% methanol, and 10% acetic acid, then destained with several changes of the same solvent without dye.

In vitro studies for the drugs

We examined the inhibitory effects of six analgesic drugs: lornoxicam, indomethacin, tenoxicam, diclofenac sodium, ketoprofen and lincomycine. All compounds were tested in triplicate at each concentration used. PON activities were measured in the presence of different drug concentrations. Control activity was assumed to be 100% in the absence of inhibitor. For each drug, a percent activity versus drug concentration graph was drawn. For determination of K_i values, three different inhibitor concentrations were tested for each drug. In these experiments, paraoxone was used as substrate at five different concentrations (0.15, 0.3, 0.45, 0.6, and 0.75 mM). Lineweaver–Burk curves were used for determination of K_i and inhibitor type [40].

Results and discussion

Human serum paraoxonase (PON 1), a calcium dependent HDL-associated enzyme, is known for its detoxification and antiatherogenic properties [37]. The substrate specifity of PON is not well understood, so its physiologic function has not been clarified in vivo conditions. Yet, no natural substrates of this enzyme are known [9,30]. The enzyme catalyses the hydrolysis of organophosphates, aromatic carboxylic acid esters, lactones, and carbamates[41]. Organophosphates (OP) are pesticides that inhibit cholinesterase. They cause poisonings and deaths [42,43]. Bioscavengers are enzymes or antibodies neutralising highly toxic OPs before they reach biological targets [44]. Indeed, hPON1 also acts as an antioxidant enzyme, and is an in vivo bioscavencer [18]. In the recent years, PON1, PON2 and PON3 have been shown to be members of a multigene family in mammals. PON1 and PON3 are expressed primarily in the liver while PON2 is widespread throughout almost all tissues, including brain, liver, kidney and testis, [8].

Enzymes catalyze almost all chemical reactions in metabolism of the living organisms. Many chemicals influence metabolism at low concentrations by decreasing or increasing normal enzyme activity, especially by inhibiting specific enzymes [45] with critical function [46], and they are important drug targets [47]. A number of studies about interactions between drugs and different enzymes have been carried out by our group. For instance, Carbonic anhydrase, glucose 6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase were purified from different sources and investigated for inhibition and activation effects of commonly used drugs in medical applications [31,48,49]. However, there are few studies about effects of medical drugs on PON1 activity in literature. For instance, Sinan et al. investigated the in vitro effects of gentamycin sulfate and cefazolin sodium on the purified human serum PON1 enzyme and human liver PON1 activity in human hepatoma HepG2 cells [9]. In that study, they found that gentamycin sulfate and cefazolin sodium were potent inhibitors for human serum PON1, and IC_{50} values were 0.887 and 0.0084 mM, respectively. Metal ions, such as Co (II), Cu (II), Mn (II), Hg (II), and p-hydroxymercuribenzoate (pOHMB) change PON1 and PON3 activity in rat liver, indicating that their active sites may contain lysine, histidine, phenylalanine, cysteine, tryptophan, aspartic acid, glutamic acid, and asparagine residues, which can bind metals [50]. The rank order of inhibitors were different: for PON1, Hg^{2+} > pOHMB > Co^{2+} > Mn^{2+} > Cu^{2+} and for PON3, Hg^{2+} > Cu^{2+} > pOHMB > Mn^{2+} > Co^{2+}, suggesting that more work is necessary to determine the protective role of PONs against the toxic effects of xenobiotics, including environmental heavy metals and oxidative stress by-products.

In the present study, human serum PON1 (hPON1) was purified using only three procedures. The procedure consists of ammonium sulfate fractionation (60-80%), DEAE-Sephadex anion exchange and Sepharose 4B-L-tyrosine-1-napthylamine hydrophobic interaction chromatography. The purification procedure is relatively fast and takes only 7 to 8 h. The enzyme, with a specific activity of 3840 U/mg protein, was purified 314.9-fold with a yield of 25.3% (Table 1). PON has been purified using different purification procedures from a wide range of sources. In a study, human serum PON enzyme was purified approx. 62.1-fold using Agarose blue, Sephadex G 200, DEAE-Trisacryl M, Sepladex G 75 chromatography techniques. Pla and colleagues purified PON3 from rat liver with a final specific activity of 461 μmol min^{-1} mg^{-1} and a yield of 0.4% [50]. They obtained an overall purification factor of 177-fold using six steps, including hydroxyapatite adsorption, DEAE-Sepharose CL-6B chromatography, Cibacron Blue 3GA non-specific affinity chromatography, anion exchange on Mono Q HR 5/5, DEAE-cellulose, and a final affinity chromatography on Concovalin A-Sepharose. Thus, the present purification procedure both takes less time and has higher specific activity, yield, and purification. The final purified hPON1 had only one protein band on SDS-PAGE, with a molecular weight of 43 kDa, which is in agreement with other studies [9,11], although

Table 1. Summary of purification procedure of PON 1.

Purification step	Activity (EU/ml)	Total volume (ml)	Protein (mg/ml)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Yield (%)	Purification factor
Triton X-100 treated serum	114.6	12	9.4	112.8	1375.2	12.191	100	1
Ammonium sulpate precipitation	91.27	9	5.1	45.9	821.43	17.89	60	1.46
Ion exchance chromatography	64.6	7	0.103	0.721	452.2	627.18	32.98	51.44
Hydrophobic interaction	69.7	5	0.018	0.09	348.5	3840	25.3	314.9

Table 2. K_i values and inhibition types of 6 inhibitors for PON1.

Drug	IC_{50} (mM)	K_i (mM)	Avarage K_i (mM)	Inhibition Type
Lornoxicam	0.136	0.016	0.009	uncompetitive
		0.007		
		0.004		
İndomethacin	0.195	0.165	0.097	Competitive
		0.095		
		0.032		
Tenoxicam	0.34	0.490	0.306	Competitive
		0.262		
		0.168		
Diclofenac sodium	1.639	0.967	0.805	uncompetitive
		0.839		
		0.610		
Ketoprofen	6.23	15.781	13.010	noncompetitive
		13.7		
		9.551		
Lincomycine	9.638	14.304	11.116	uncompetitive
		10.560		
		8.486		

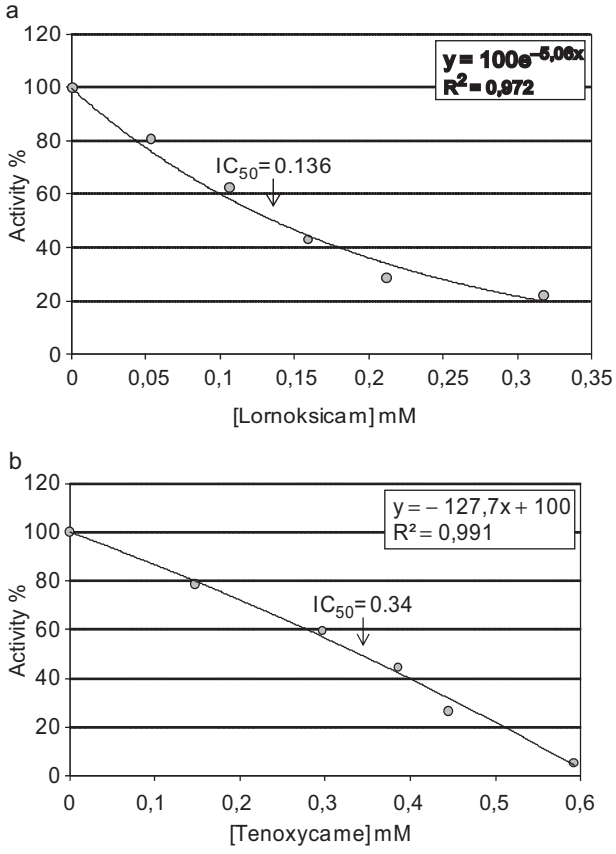

Figure 1. In vitro effect of the drugs – (a) lornoxicam, (b) tenoxicam at five different concentrations on paraoxonase-1 activity.

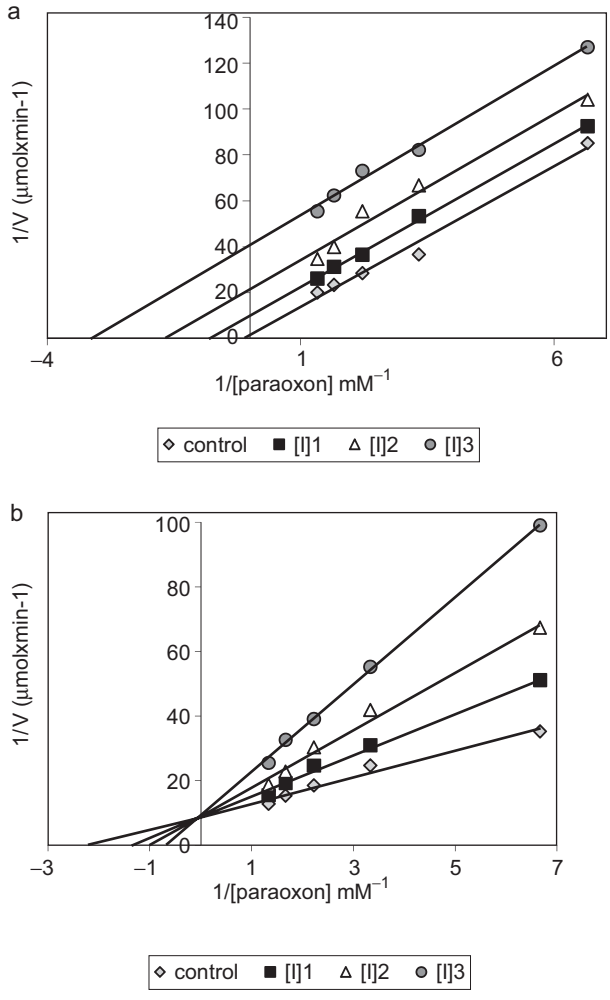

Figure 2. K_i graphs for paraoxonase from human serum. (a) and (b), Lineweaver-Burk graphs in 5 different substrate (paraoxon) concentrations and 3 different (a) lornoxicam, or (b) tenoxicam, concentrations for determination of K_i.

others show different migration patterns [51]. We also examined the in vitro effects of some analgesic drugs, such as lornoxicam, indomethacin, tenoxicam, diclofenac sodium, ketoprofen and lincomycine, on human serum PON1 activity. Both the IC_{50} and K_i parameters were determined. Inhibitory

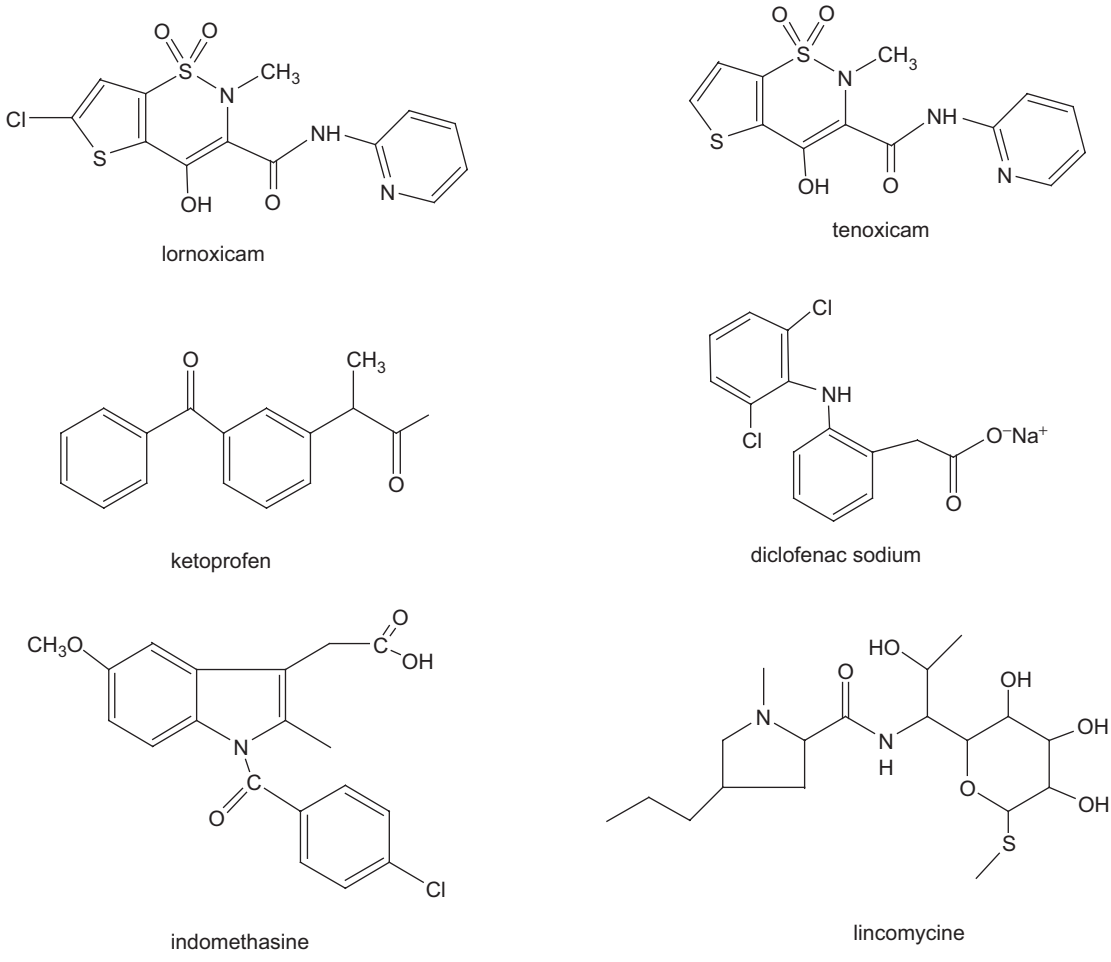

Figure 3. Structures of lornoxicam, indomethacin, tenoxicam, diclofenac sodium, ketoprofen and lincomycine.

effects of the drugs on enzyme activities were tested under in vitro conditions. Both the IC_{50} and K_i parameters were determined and are given in Table 2 and the representative graphs for lead are shown in Figures 1 and 2. It is important that the drugs inhibited the enzyme activity at low concentrations (Figure 1), and that they are potent inhibitors for human serum PON1. Especially, lornoxicam, indomethacin and tenoxicam have inhibitory effects at lower concentrations according to IC_{50} values, 0.136, 0.195 and 0.34 mM, respectively (Table 2 and Figure 1). According to these results, inhibition range can be given as; lornoxicam > indomethacin > tenoxicam > diclofenac sodium > ketoprofen > lincomycine. K_i graphs show that lornoxicam, diclofenac sodium and lincomycine inhibit the enzyme in an uncompetitive manner (Table 2 and Figure 2), ketoprofen inhibits in noncompetitive manner (Table 2), indomethacin and tenoxicam inhibit in competitive manner (Table 2 and Figure 2). Since lower K_i indicates greater inhibition, lornoxicam has a high inhibition rate for the enzyme in comparison with that of other drugs. When the chemical structures of the drugs are investigated, it can be clearly seen that the enzyme is susceptible to the molecules having electronegative atoms like chlor, sulphur, nitrogen and oxygen (Figure 3). It is so interesting that IC_{50} and K_i values of lornoxicam and tenoxicam are so different even though their chemical structures are the same except for an additional chlorine atom in lornoxicam. This may indicate that molecules containing chlor atoms are strong inhibitors for PON-I, but this is a little contradictory because PON activity is known to be stimulated in the presence of NaCl and $CaCl_2$ salts. So, these results should be supplemented with additional studies such as X-ray analysis and, etc.

In conclusion, it was aimed in this study to purify PON1 from human serum, and understand the inhibition effects of some drugs on this crucial enzyme.

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