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Impact of antibacterial drugs on human serum paraoxonase-1 (hPON1) activity: an *in vitro* study

Hakan Söyüt[1*], Elif Duygu Kaya[2], Şükrü Beydemir[3]

[1]*Department of Primary Education, Faculty of Education, Bayburt University, 69000 Bayburt, Turkey*

[2]*Department of Food Engineering, Faculty of Engineering, Iğdır University, 76000 Iğdır, Turkey*

[3]*Department of Chemistry, Faculty of Science, Atatürk University, 25240 Erzurum, Turkey*

PEER REVIEW

Peer reviewer

Dr. Chiara Pelillo, Callerio Foundation ONlus, Trieste, Italy.
Tel: 00390405588635
E-mail: chiara.pelillo@libero.it

Comments

The research proposed in this article has a meaningful potential presenting two key concepts: highlighting how antibiotics can be studied for their cardiovascular effects and anti-oxidant activity and developing a rapid assay to test anti-oxidant activity.
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ABSTRACT

Objective: To investigate the *in vitro* effects of the antibacterial drugs, meropenem trihydrate, piperacillin sodium, and cefoperazone sodium, on the activity of human serum paraoxonase (hPON1).

Methods: hPON1 was purified from human serum using simple chromatographic methods, including DEAE-Sephadex anion exchange and Sephadex G-200 gel filtration chromatography.

Results: The three antibacterial drugs decreased *in vitro* hPON1 activity. Inhibition mechanisms meropenem trihydrate was noncompetitive while piperacillin sodium and cefoperazone sodium were competitive.

Conclusions: Our results showed that antibacterial drugs significantly inhibit hPON1 activity, both *in vitro*, with rank order meropenem trihydrate piperacillin sodium cefoperazone sodium *in vitro*.

KEYWORDS

Paraoxonase, Inhibition, Meropenem trihydrate, Piperacillin sodium, Cefoperazone sodium

1. Introduction

Beta-lactams are a large family of antibiotics that include penicillin (*e.g.* Piperacillin), carbapenems (*e.g.* Meropenem) and cephalosporins (*e.g.* Cefoperazone). Meropenem is a carbapenem antibiotic that is active against common pathogens such as penicillin-sensitive, methicillin-sensitive and anaerobes[1]. Piperacillin/tazobactam is a β-lactam/β-lactamase inhibitor combination with a broad spectrum of antibacterial activity against many Gram-positive and Gram-negative bacteria, including *Pseudomonas aeruginosa*[2]. Cefoperazone is a third-generation cephalosporin antibiotic with a broad spectrum of activity against most Gram-positive and Gram-negative bacteria[3,4].

Regarding death from cardiovascular diseases worldwide, oxidative stress and/or weak antioxidant defence systems are considered to be major players in cardiovascular diseases. Oxidative stress occurs when free oxygen radicals are produced in excessive amounts. Oxidative stress is characterised by an imbalance between reactive oxygen species and antioxidant defences[5]. Oxidative stress is considered to be an important factor in the initiation and progression of atherosclerosis and to play a role in foam cell formation[6]. Atherosclerosis is the primary cause of cardiovascular disease and coronary heart disease. It is the

*Corresponding author: Dr. Hakan Söyüt, Ph.D., Assistant Professor, Department of Primary Education, Faculty of Education, Bayburt University, 69000, Bayburt, Turkey.
Tel: +90 458 2111175
E-mail: hsoyut@bayburt.edu.tr
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most significant cause of global morbidity and mortality in the modern world[7]. The defence system that protects free radical damage includes enzymatic antioxidant systems. The most important extracellular enzyme that is involved in this process is the high-density lipoprotein (HDL)-associated human serum paraoxonase-1 (hPON1)[8].

This enzyme catalyses the hydrolysis of organophosphates, aryl esters, and lactones[6,9,10]. hPON1 serves as an antioxidant enzyme by protecting low-density lipoproteins and HDL from oxidative stress, which is known to be associated with many vascular diseases, including atherosclerosis[11]. Actually, higher hPON1 activity plays a significant role in the prevention of atherosclerosis[12]. Epidemiological studies indicate that low hPON1 activity is correlated with increased risk of cardiovascular events and cardiovascular disease[13].

Serum hPON1 activity is influenced by environmental and genetic factors and varies among individuals in all populations. These variations in hPON1 activity are mainly related to the expression of two polymorphisms located in the coding regions of the hPON1 gene; Q192R (Q: glutamine, R: arginine)[14].

Due to the widespread prevalence of conditions it can be caused by reductions in hPON1 activity, its non-targeted inhibition is of vital importance. If any medication causes a reduction in hPON1 enzyme activity, many vascular diseases, including atherosclerosis, may occur due to increased oxidative stress. Indeed, further studies of the inhibitory effects of drugs should be performed because of the physiological role of hPON1. There are many studies regarding the effects of medications on the activity of hPON1. For example, Costa *et al*. reported that the cholinergic muscarinic antagonist atropine inhibited human serum hPON1[15]. Antibiotics, such as sodium ampicillin, ciprofloxacin and clindamycin sulphate have been reported to inhibit human serum hPON1[16].

As mentioned, these drugs are widely used for the treatment of serious bacterial infections. However, it is extremely important to adjust the dose of these drugs in patients. In this study, we used a simple and rapid procedure to purify hPON1 from human serum and investigated the *in vitro* effects of antibacterial drugs on its enzyme activity.

2. Materials and methods

2.1. Materials

The materials used in this study included DEAE-Sephadex A50, Sepharose 4B, 1-naphthylamine, paraoxon, protein assay reagents, and chemicals for electrophoresis and they were obtained from Sigma Chemical Co. All of the other chemicals used were of analytical grade and were obtained from either Sigma-Aldrich or Merck. Meropenem trihydrate, piperacillin sodium and cefoperazone sodium were obtained from local pharmaceutical manufacturing companies. We used a Chebios UV-vis spectrophotometer for the enzyme activity assays. The peristaltic pump used for enzyme purification was obtained from Ismatec (ISM833), the centrifuge machine was purchased from Hermle Labotechnic and the electrophoresis system was a BioRad Mini Protean system.

2.2. Paraoxonase activity assay

Human serum samples were supplied from the Research Hospital at Ataturk University. The activity of hPON1 was determined at 25 °C with paraoxon (diethyl p-nitrophenyl phosphate) (1 mmol/L) in 50 mmol/L glycine/NaOH (pH 10.5) containing 1 mmol/L $CaCl_2$. The hPON1 assay was based on the estimation of p-nitrophenol at 412 nm. The molar extinction coefficient of p-nitrophenol (ε=18.290 L/mol·cm at pH 10.5) was used to calculate hPON1 activity[17]. One enzyme unit was defined as the amount of enzyme that catalyses the hydrolysis of 1 μmol of substrate at 25 °C[18]. Assays were performed using a spectrophotometer.

2.3. Ammonium sulphate precipitation

Human serum precipitated with 60%-80% ammonium sulphate was carried out in our previous studies. The precipitate was obtained after centrifugation at 15 000 r/min for 20 min and redissolved in a 100 mmol/L Na-phosphate buffer (pH 7.0).

2.4. DEAE-Sephadex A50 anion exchange chromatography

At first, the anion exchange column was equilibrated with a 100 mmol/L Na-phosphate buffer (pH 7.0). Then, the enzyme solution, which had been dialyzed in the presence of 1 mmol/L Na-phosphate buffer (pH 7.0) for 2 h, was loaded onto the DEAE-Sephadex A50 anion exchange column (3 cm×30 cm). Later, the chromatography column was washed with a 100 mmol/L Na-phosphate buffer (pH 7.0), and then elution was carried out by an increasing linear gradient of (0-1.5) mol/L NaCl. The elution fractions which were collected were checked for enzyme activity at 412 nm. Tubes which displayed the same enzyme activity were combined. All these procedures were performed at 4 °C.

2.5. Sephadex G-200 gel filtration chromatography

In the first process, the sephadex G-200 column (60 cm×2 cm) was equilibrated with a 100 mmol/L Na-phosphate buffer (pH 7.0). The fractions obtained from the DEAE-Sephadex A50 anion exchange column were the mixed with glycerol and loaded onto the gel filtration column with the

same buffer. Finally, the enzyme solutions were eluated from the Sephadex G-200 column. The protein amount (280 nm) and enzyme activity (412 nm) for all tubes was recorded. The tubes showed enzyme activity were combined for other kinetic studies.

2.6. Protein determination

In previous studies that were also performed in our laboratory, it was found spectrophotometrically at 595 nm according to the Bradford method to quantitative protein assay during the purification steps[19].

2.7. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was applied to check the enzyme that was purified according to the Laemmli's procedure as in previous studies which were conducted in ours[20]. The obtained single band was photographed after electrophoresis.

2.8. In vitro studies for the drugs

We examined the inhibitory effects of the three antibacterial drugs: meropenem trihydrate, piperacillin sodium and cefoperazone sodium. All of the compounds were tested in triplicate for each used concentration. The hPON1 activities were measured in the presence of different drug concentrations. Control activity was assumed to be 100% in the absence of an inhibitor. A percentage of activity *vs.* drug concentration graph was drawn for each drug. For the determination of K_i values, three different inhibitor concentrations were tested for each drug. In these experiments, paraoxone was used as a substrate at five different concentrations (0.15, 0.30, 0.45, 0.60, and 0.75 mmol/L). Lineweaver-Burk curves were used for the determination of K_i and inhibitor type[21].

3. Results

We purified PON1 from human serum using only three procedures, which are ammonium sulphate fractionation (60%-80%), DEAE-Sephadex anion exchange chromatography and Sephadex G-200 gel filtration chromatography. The enzyme was obtained with a specific activity of 4060.00 EU/mg proteins and ~295-fold with a yield of 53.9% (Table 1).

Figure 1 shows the SDS-PAGE to determine the purity and molecular weight of hPON1. The molecular weight of the purified human serum paraoxonase was found to be 43 kDa, which is in agreement with other studies[22-29].

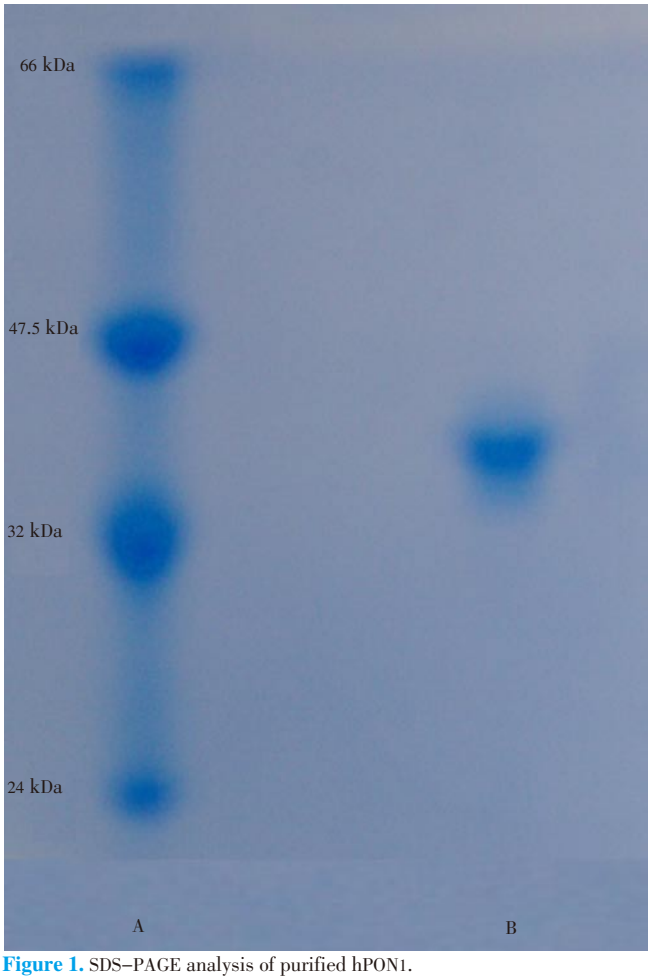

Figure 1. SDS-PAGE analysis of purified hPON1.
Lane (A) is standard proteins (kDa): Bovine serum albumin (66.000), aldolase (47.500), triosephosphate isomerase (32.000) and soy bean trypsin inhibitor (24.000). Lane (B) contains a human serum sample.

Antibacterial drugs showed inhibition effects on paraoxonase activity. IC_{50} values for meropenem trihydrate, piperacillin sodium, and cefoperazone sodium were determined to be 0.481 mmol/L, 23.105 mmol/L, and 25.342 mmol/L, respectively, via activity (%) *vs.* drug plots (Figure 2 and Table 2). K_i constants were calculated as (0.597±0.006) mmol/L, (1.285±0.624) mmol/L, and (1.414±0.639) mmol/L,

Table 1
Summary of the hPON1 purification procedure.

Purification step	Activity (EU/mL)	Total volume (mL)	Protein (mg/mL)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Yield (%)	Purification factor
Serum	110.70	10.00	8.00	80.00	1 100.7	13.76	100.00	1.00
Ammonium sulpate precipitation (60%-80%)	90.50	8.00	4.00	32.00	724.0	22.60	65.77	1.64
DEAE-Sephadex A50 anion exchange chromatography	50.20	6.00	0.10	0.60	301.2	502.00	41.60	36.48
Sephadex G-200 gel filtration chromatography	40.60	4.00	0.01	0.04	162.4	4 060.00	53.90	295.10

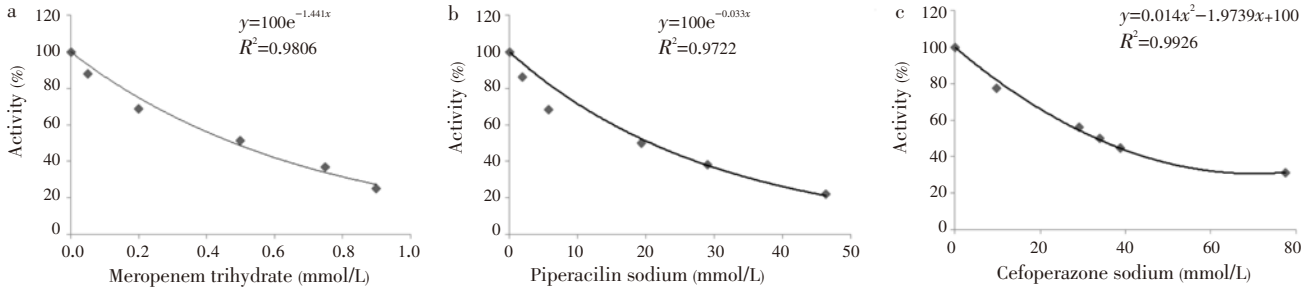

Figure 2. *In vitro* effect of antibacterial drugs at five different concentrations on hPON1 activity.
a: Meropenem trihydrate, b: Piperacillin sodium, c: Cefoperazone sodium.

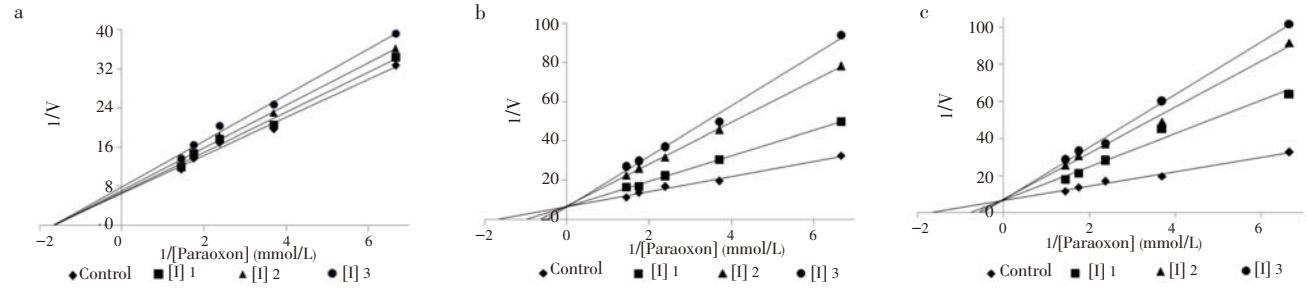

Figure 3. K_i graphs for paraoxonase from human serum. Lineweaver-Burk plots for 5 different substrate (paraoxon) concentrations and 3 different (a) meropenem trihydrate, (b) piperacillin sodium, and (c) cefoperazone sodium concentrations, which were used to determine the K_i values.

respectively using Lineweaver-Burk curves (Figure 3 and Table 2). Meropenem trihydrate showed noncompetitive inhibition, while others inhibited in a competitive manner. Figure 4 shows the structural and catalytic calcium ions of hPON1 interact with the nitrogen and oxygen electronegative atoms of the functional groups in piperacillin sodium and Lys 192.

Table 2
IC_{50}, K_i values and inhibition types.

Inhibitors	IC_{50} (mmol/L)	Average values of K_i (mmol/L)	Type of inhibition
Meropenem trihydrate	0.481	0.597±0.006	Noncompetitive
Piperacillin sodium	23.105	1.285±0.624	Competitive
Cefoperazone sodium	25.342	1.414±0.639	Competitive

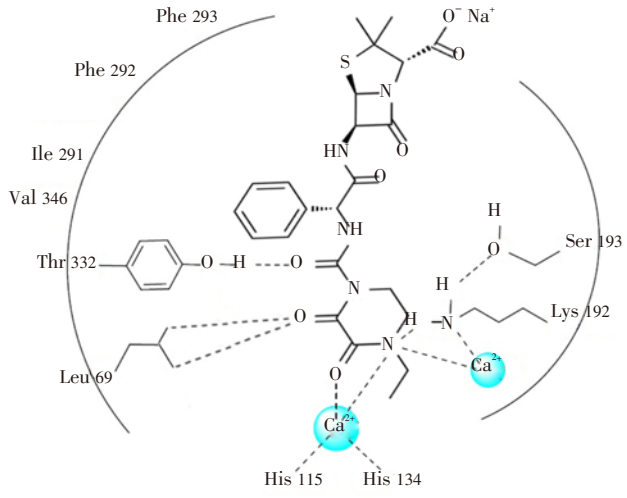

Figure 4. The default shape binding model for the interaction between piperacillin sodium and the hPON1 active site.

4. Discussion

Mammalian cells are protected from reactive oxygen species by antioxidant defense mechanisms, such as the activities of catalase, superoxide dismutase and glutathione peroxidase including PON. When the rate of the formation of reactive oxygen species exceeds the capacity of the antioxidant defense system, it has oxidative stress[30]. Oxidative stress is associated with cardiovascular diseases.

hPON1 is a calcium-dependent esterase that hydrolyses esters, such as organophosphate and lactone[17,31], a glycoprotein with a molecular weight of 43-45 kDa that is mainly synthesised by the liver[8] and one of the proteins involved in the antioxidant defense mechanisms in the human body. There are many cleaning systems for reactive oxygen species, including paraoxonases, in the human body[32]. hPON1 protects low-density lipoprotein, HDL and macrophages from oxidative stress by cleaning reactive oxygen species during living metabolism[33,34]. Therefore, hPON1 prevents vascular diseases and cardiovascular diseases[22-24].

According to the above information, it is clear that hPON1 plays important physiological roles in living metabolism. However, there is little information regarding the pharmacokinetic role of this enzyme. Recently, there are many researches performed different types of studies on hPON1.

There is a small number of studies on the interactions between hPON1 activity and drugs or certain chemicals[22-29]. It is known that hPON1 has two isoforms: Q and R. These isoforms play a crucial role in drug metabolism and decrease

the risk of atherosclerosis. hPON1 enzymatic activity exhibits observable differences based on this polymorphism. The effect of this polymorphism on hPON1 activity is dependent on the substrate and hPON1's activity may differ by ethnicity. Thus far, statins are the most widely studied pharmacological molecules that are used in hPON1 research. These studies show that various statins may have beneficial effects via lowering oxidative stress and increasing hPON1 activity. It is possible that hPON1 activity might be increased by statins as a result of oxidative stress limitations. Statins appear to be generally useful to determine the hPON1 status. Pravastatin, simvastatin and atorvastatin have positive effect on PON1 activity. These drugs prevent the inactivation of hPON1 via their anti-oxidative properties. In addition, the L55M and Q192R polymorphisms in hPON1 had no effect on the activity of hPON1 against paraoxon and phenyl acetate either before or after atorvastatin treatment in an atorvastatin polymorphisms study[7,35-39]. Aspirin is used extensively for the treatment and prevention of vascular disease and is also known for its antioxidant effects. The hypothesis that aspirin may have beneficial effects on hPON1 activity was tested. The use of aspirin significantly increased hPON1 activity in patients with coronary artery disease[40]. Other drugs with positive impact on cardiovascular health were examined for their action on hPON1. The effects of valsartan and barnidipine were investigated and it was found that they had no effect on hPON1 activity[41,42].

In an *in vitro* study, gentamycin sulphate and cefazolin sodium decreased hPON1 activity[16]. In the laboratory, various enzyme-drug interaction studies were conducted and have contributed a great deal to the literature[43,44]. In the laboratory studies, certain cardiovascular drugs, such as digoxin, metoprolol tartrate, verapamil, diltiazem, amiodarone, dobutamine and methylprednisolone were examined for their *in vitro* effects on hPON1. These drugs had a negative impact on hPON1 activity[24].

Pharmacological studies, including enzyme-drug interaction analyses, are becoming increasingly vital important[26,27,43-48].

The *in vitro* effects of antibacterial drugs meropenem trihydrate, piperacillin sodium and cefoperazone sodium on hPON1 activity were investigated in this study. It is crucially important that these antibacterial drugs are potent inhibitors of human serum hPON1. The compounds meropenem trihydrate, piperacillin sodium and cefoperazone sodium have inhibitory effects. For these drugs, the K_i values were determined by Lineweaver-Burk plots using different paraoxon concentrations. The drug meropenem trihydrate inhibited enzyme activity in a noncompetitive manner and both piperacillin sodium and cefoperazone sodium inhibited in a competitive manner.

The compounds piperacillin sodium and cefoperazone sodium behave in a similar way to paraoxon because they both have functional groups with the same number of electronegative atoms as paraoxon that interact with amino acids in the active site. Because piperacillin sodium and cefoperazone sodium have similar functional groups that they can compete with the substrate paraoxon by interacting with the active site of the enzyme. The compounds piperacillin sodium and cefoperazone sodium are close in K_i values due to similar functional groups and steric hindrance. It has formed a possible figure based on the studies by Harel *et al.* who have reported the crystal structure of the hPON1 enzyme and observed amino acid residues of the active site[49]. In pursuit of the intended model, it was found that the structural and catalytic calcium ions of hPON1 interact with the nitrogen and oxygen electronegative atoms of the functional groups in piperacillin sodium and Lys 192. It is known that an adult human has approximately 5 L of blood. Accordingly, the blood concentrations of piperacillin sodium and cefoperazone sodium were calculated as 0.740 and 0.298 mmol/L, respectively. These values were observed to be less than their respective IC_{50} values. However, the blood concentration of meropenem trihydrate was determined to be 0.228 mmol/L, which is similar with its IC_{50} value. The effect of this observation should be clarified by *in vivo* studies. As a result, the hPON1 was purified by using three simple purification steps and the *in vitro* effects of antibacterial drugs on hPON1 was investigated.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

Mammalian cells are protected from reactive oxygen species by antioxidant defense mechanisms, one of this is represented by the extracellular enzyme hPON1 mainly synthesised by the liver. hPON1 protects low-density lipoprotein, HDL, and macrophages from oxidative stress by cleaning reactive oxygen species during living metabolism.Therefore, hPON1 prevents vascular diseases and cardiovascular diseases.

Research frontiers

The cutting-edge of this research is to present a rapid tool to evaluate the anti-oxidant activity of antibiotics, in cardiovascular diseases acting on human serum hPON1, the most important extracellular enzyme that is involved in antioxidant activity acting in cardiovascular diseases.

Related reports

Few studies have shown interactions between drugs and hPON1. In an *in vitro* study, gentamycin sulphate and cefazolin sodium decreased hPON1 activity as certain cardiovascular drugs. In fact in their laboratory studies, certain cardiovascular drugs, such as digoxin, metoprolol tartrate, verapamil, diltiazem, amiodarone, dobutamine and methylprednisolone, were examined for their *in vitro* effects on hPON1. These drugs had a negative impact on hPON1 activity.

Innovations and breakthroughs

The innovation in this paper is the use of a rapid and easy tool to test drugs with anti-oxidant activity on hPON1 pxidative enzyme.

Applications

From literature, few studies showed that certain classes of antibiotics act on hPON1 reducing its activity as several cardiovascular drugs. In this paper they deepened the knowledge on the activity of antibiotics in cardiovascular diseases studying the effects of meropenem trihydrate, piperacillin sodium, and cefoperazone sodium.

Peer review

The research proposed in this article has a meaningful potential presenting two key concepts: highlighting how antibiotics can be studied for their cardiovascular effects and anti-oxidant activity and developing a rapid assay to test anti-oxidant activity.

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