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Amphenicol and Macrolide Derived Antibiotics Inhibit Paraoxonase Enzyme Activity in Human Serum and Human Hepatoma Cells (HepG2) *in vitro*

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Abstract—Human serum paraoxonase (PON1) was separately purified by ammonium sulfate precipitation and hydrophobic interaction chromatography. The *in vitro* effects of commonly used antibiotics, namely clarithromycin and chloramphenicol, on purified human serum paraoxonase enzyme activity (serum hPON1) and human hepatoma (HepG2) cell paraoxonase enzyme activity (liver hPON1) were determined. Serum hPON1 and liver hPON1 were determined using paraoxon as a substrate and IC_{50} values of these drugs exhibiting inhibition effects were found from graphs of hydratase activity (%) by plotting concentration of the drugs. We determined that chloramphenicol and clarithromycin were effective inhibitors of serum hPON1.

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Key words: paraoxonase, inhibition, clarithromycin, chloramphenicol, *in vitro*, HepG2

Human serum paraoxonase (hPON1) (EC 3.1.8.1) is an antioxidant enzyme physically related to high density lipoprotein (HDL) and has roles in blocking oxidation of low density lipoprotein (LDL) and in detoxification of organophosphates [1-3]. PON1 is a member of a multi gene family that includes at least two other genes in humans and mice [4]. Previous studies showed that PON1 possesses hydrolytic activity with organophosphates such as paraoxon, soman, sarin, and tabun [5-7]. PON activity has been found in a variety of mammalian tissues, with liver and serum having the highest levels [8], and the source of serum PON is believed to be primarily the liver [9]. The serum HDL concentration is inversely correlated with atherosclerosis risk [10]. The initial focus of attention was on the role of HDL in reverse-cholesterol transport. However, recent studies have suggested more diverse mechanisms. HDL protects against oxidative modification of LDL [11-13]. This is believed to be central to the initiation and progression of atherosclerosis [14]. The antioxidant activity of HDL relates to its enzymes, primarily PON1, but also lecithin cholesterol acyl transferase [15], and these can prevent lipid peroxide accumulation on LDL both *in vitro* and *in vivo* [13, 16-18].

Most studies on the modulation of PON1 by pharmaceutical compounds have focused on lipid-lowering compounds. *In vitro* exposure of HuH7 human hepatoma cells to provastatin, simvastatin, and fluvastatin caused a 25-50% decrease in PON1 activity in the culture medium and a similar decrease in PON1 mRNA; both effects were reversed by mevalonate [19]. In the same cells, fenofibric acid caused a 50 and 30% increase in PON1 activity and PON1 mRNA content, respectively [19]. In another *in vitro* study on isolated lipoproteins, two oxidized metabolites of atorvastatin and a metabolite of gemfibrozil were found to increase HDL-associated PON1 activity [20]. A study in rats indicated that fluvastatin reduced both plasma and liver PON1 activity, while a lower dose was only effective toward liver activity. Provastatin, on the other hand, was devoid of significant inhibition effects [21]. Studies in humans have provided similar contrasting results. An increase in serum PON1 activity was found in patients treated with simvastatin and other statins, gemfi-

Abbreviations: PON1) paraoxonase enzyme; serum hPON1) human serum paraoxonase enzyme; liver hPON1) human liver paraoxonase enzyme in HepG2 cell line; HDL) high density lipoproteins; LDL) low density lipoproteins; HepG2) human hepatoma cell.

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brozil and fenofbirate [22-25]. On the other hand, no changes in serum PON1 activity were reported by other studies in patients treated with ciprofibrate [26], bezofibrate, and gemfibrozil [27]. The cholinergic muscarinic antagonist, atropine, was shown to inhibit human plasma and pig liver PON1 *in vitro* [28]. In a cohort of aspirin users, a significant increase of plasma PON1 activity and concentration was reported [29]. In addition, the anti-inflammatory glucocorticoid dexamethasone caused an eightfold increase in PON1 mRNA in a mouse hepatoma cell line (Hepa cells), as well as in mice *in vivo* [30].

Clarithromycin is a macrolide antibiotic that is widely used for the treatment of a myriad of infections such as those caused by *Hemophilus influenzae*, *Mycobacterium avium*, and *Helicobacter pylori*. Clarithromycin is oxidatively metabolized to 14-(*R*)-hydroxyclarithromycin or *N*-demethylated to *N*-desmethylclarithromycin and members of the CYP3A subfamily mediate these reactions [31]. Like erythromycin, clarithromycin is a potent mechanism-based inhibitor of CYP3A [32]. Chloramphenicol is an amphenicol antibiotic, which is effective on *Rickotsiae*, *Lymphogranuloma-Psittaccosis* group and *Vibrio cholera*, and it is especially effective on *Salmonella typhi* and *Hemophilus*. The antibiotic chloramphenicol is an irreversible inhibitor, which forms a covalent amide bond with a lysine amino acid residue at the active site of the enzyme cytochrome P-450 [33].

Many antibiotics are used to deal with some tissues disorders but there are few studies of their effects on enzyme activities. To our knowledge the effects of some widely used antibiotics on serum and liver paraoxonase have not been investigated. The present study therefore investigated *in vitro* the effects of chloramphenicol and clarithromycin on serum hPON1 and on liver hPON1 in HepG2 cell line.

MATERIALS AND METHODS

Materials. Sepharose 4B, L-tyrosine, 1-naphthylamine, protein assay reagents, and chemicals for electrophoresis were obtained from Sigma (USA). All other chemicals used were analytical grade and obtained from either Sigma or Merck (Germany). Medical drugs were provided by the local pharmacy.

Paraoxonase enzyme assay. Paraoxonase enzyme activity towards paraoxon was quantified spectrophotometrically by the method described by Gan et al. [3]. The reaction was followed for 2 min at 37°C by monitoring the appearance of *p*-nitrophenol at 412 nm in a Biotek (Russia) automated recording spectrophotometer. The final substrate concentration during enzyme assay was 2 mM and all rates were determined in duplicate and corrected for the non-enzymatic hydrolysis. PON1 activity (1 U/liter) was defined as 1 μmol of *p*-nitrophenol formed per minute.

Total protein determination. The absorbance at 280 nm was used to monitor the protein in the column effluents and ammonium sulfate precipitation. Quantitative protein determination was achieved by absorbance measurements at 595 nm according to Bradford [34], with bovine serum albumin (BSA) standard.

Purification of paraoxonase from human serum by hydrophobic interaction chromatography. Human serum was isolated from 35 ml fresh human blood taken to dry tube. The blood samples were centrifuged at 1500*g* for 15 min and the serum was removed. First, serum paraoxonase was isolated with ammonium sulfate precipitation (60-80%). The precipitate was collected by centrifugation at 15,000*g* for 20 min and redissolved in 100 mM Tris-HCl buffer (pH 8.0). Then, we synthesized hydrophobic gel including Sepharose 4B-L-tyrosine and 1-naphthylamine for the purification of human serum paraoxonase in our laboratory [35]. The synthesized gel was placed in a 10 cm column. Subsequently, the column was equilibrated with 0.1 M Na_2HPO_4 buffer (pH 8.0) containing 1 M ammonium sulfate. The A_{280} absorbance of the collected fractions was measured throughout equilibration. The equilibration was carried out to reach 0.009 absorbance value. The 15 ml sample was loaded onto the column. First, a salt gradient was carried out with 0.1 M Na_2HPO_4 buffer (pH 8.0) with and without 1 M ammonium sulfate buffer in a gradient mixer. After the completion of salt gradient, elution was performed with 15 ml 0.1 M Na_2HPO_4 buffer (pH 8.0) containing 2 mM $CaCl_2$. Fractions were collected into 1.5 ml tubes and the PON activity and protein concentrations were determined for each tube. The purified PON enzyme was stored in the presence of 2 mM $CaCl_2$ in order to maintain activity.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed after the purification of the enzyme. It was carried out in 10 and 3% acrylamide concentration for the running and the stacking gel, respectively, containing 0.1% SDS according to Laemmli [36]. A 20-μg sample was applied to the electrophoresis medium. Gels were stained for 1.5 h in 0.1% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid, then destained with several changes of the same solvent without the dye. The electrophoretic pattern was photographed to produce an image.

***In vitro* inhibition kinetic studies.** Amphenicol and macrolide derived antibiotics chloramphenicol and clarithromycin were selected as medical drugs. For the inhibition studies of chloramphenicol and clarithromycin, different volumes of the medical drugs at constant concentration were added to the reaction mixture. Paraoxonase activities with the medical drugs were assayed by following the hydration of paraoxon.

Activity (%) values of paraoxonase for five different concentrations of each medical drug were determined. Paraoxonase activity without a medical drug was taken as

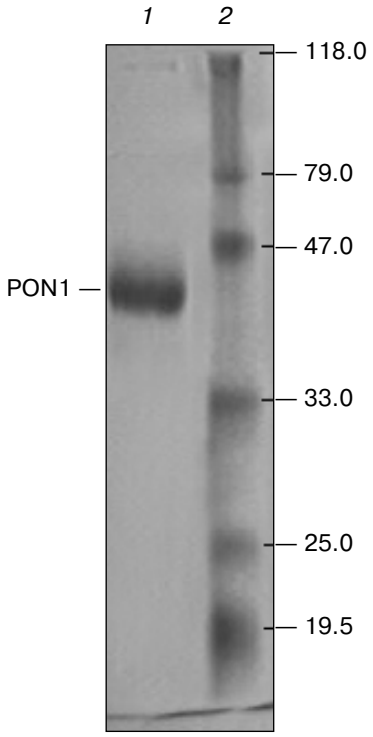

Fig. 1. SDS-PAGE gel electrophoresis of PON1 purified by ammonium sulfate precipitation and hydrophobic interaction chromatography gel. Lanes: *1*) a pooled sample obtained from column showing paraoxonase enzyme activity; *2*) molecular mass standards: β-galactosidase (118 kD), BSA (79 kD), ovalbumin (47 kD), carbonic anhydrase (33 kD), β-lactoglobulin (25 kD), lysozyme (19.5 kD). The molecular weight of PON1 was estimated to be approximately 45 kD.

100% activity. For the drugs having an inhibiting effect, the inhibitor concentration causing 50% inhibition (IC_{50} values) was determined from the graphs.

In addition, K_i, K_m, and V_{max} values of chloramphenicol and clarithromycin were determined on serum hPON1. These values of the enzyme for paraoxon were measured at optimum pH (8.0) and temperature (37°C) at seven different substrate concentrations.

Cell culture of HepG2 cells. Human hepatoma cell line (HepG2) was used in this study. For experiment, cells were seeded at 250,000/well into 12-well plates containing DMEM medium supplemented with glutamine (0.2 mM), penicillin and streptomycin (100 U/ml and 100 μg/ml, respectively), and bovine fetal calf serum (10% (v/v)), and cells were incubated at 37°C under 5% (v/v) CO_2. After cells were grown for 16 h, six different concentrations of chloramphenicol and clarithromycin antibiotics were added into the medium (1, 25, 50, 75, 100, 110 μg and 1, 15, 30, 50, 75, 100 μg, respectively) For each drug, cells were lysed with a lysis buffer (10% Triton X-100 and 500 mM potassium phosphate buffer, pH 8.0) according to Foka et al. after 2, 4, and 6 h time points of the drug application [37]. Subsequently, enzyme activity of the supernatant was determined according to Gan et al. [3]. For each drug, an activity (%)–[drug] graph was plotted at six different inhibitor concentrations, and the drug concentrations causing 50% inhibition (IC_{50}) were calculated.

RESULTS AND DISCUSSION

Paraoxonase hydrolyzes several organophosphorus compounds used as insecticides as well as nerve agents; it metabolizes toxic oxidized lipids associated with both LDL and HDL; and it can hydrolyze a number of lactone-containing pharmaceutical compounds, inactivating some, while activating others. Therefore, the determination of the effect of different pharmaceutical drugs on paraoxonase enzyme activity is required in order to clarify PON1 status in the metabolism.

The effect of amphenicol and macrolide derived antibiotics on paraoxonase enzyme activity was studied in several aspects including *in vitro* inhibition studies on serum hPON1 and *in vitro* studies on liver hPON1 from HepG2 cell line.

In order to investigate the effect of these drugs on serum hPON1 *in vitro*, human serum paraoxonase was purified by ammonium sulfate precipitation at 60-80% interval and hydrophobic interaction chromatography [35]. Different protocols are available for PON enzyme purification from serum and liver using three, four, and seven steps [3, 38, 39]. A purification strategy designed for the human PON1 enzyme consists of two-step procedures resulting in shorter and more straightforward approach in contrast to other purification procedures. The gel for hydrophobic interaction chromatography was synthesized with Sepharose 4B, L-tyrosine, 1-naphthylamine. The enzyme was purified 227-fold with a final specific activity of 51.1 U/mg. The purity of enzyme was confirmed by SDS-PAGE. As seen in Fig. 1, a single band of 43 kD was obtained, which corresponds to the previous studies [38-40].

The IC_{50} value of chloramphenicol and clarithromycin antibiotics on paraoxonase activity in HepG2 cells (2, 4, and 6 h represent the time points after the drug application)

Antibiotic	IC_{50}, μg/ml		
	2 h	4 h	6 h
Chloramphenicol	43.89	29.98	23.98
Clarithromycin	30.116	52.152	15.77

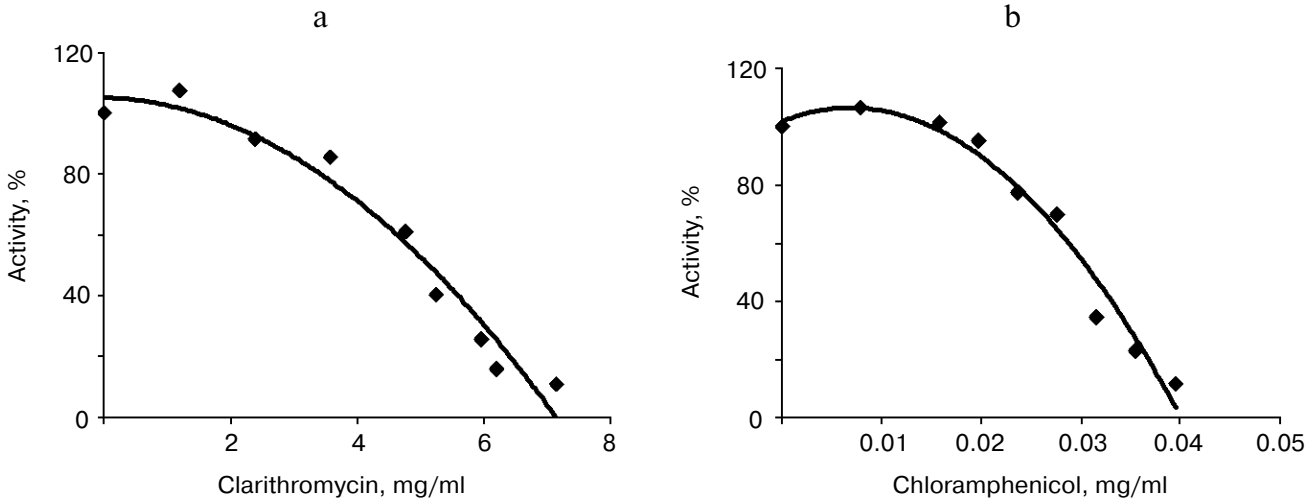

Fig. 2. Effects of clarithromycin (a) and chloramphenicol (b) concentration on purified human serum paraoxonase.

As seen in Fig. 2, both of the selected drugs *in vitro* strongly inhibited the serum hPON1 activity. IC_{50} values were estimated as 0.0309 and 5.121 mg/ml for chloramphenicol and clarithromycin, respectively. The next step was to study the kinetics of the interaction of drugs with the purified serum hPON1. The Lineweaver–Burk double-reciprocal graph was plotted using a range of paraoxon concentrations (0.5-4 mM) in the absence or presence of each drug. Moreover, K_m and V_{max} values were determined by means of these graphs (data not provided). K_m (4.16 mM) and V_{max} (227.27 μmol/min) were found using paraoxon at pH 8.0 and 37°C. Reiner et al. reported that K_m of serum hPON1 enzyme was 2.5 mM using paraoxon as substrate [41]. In a different study, K_m and V_{max} values of liver hPON1 were 1.83 ± 0.25 mM and 47.64 ± 2.95 μmol/min, respectively [42]. In fact, a study reported that chloramphenicol was an irreversible inhibitor of the enzyme cytochrome P-450 and clarithromycin significantly inhibits CYP3A activity *in vivo* [43].

There are a few studies investigating the effects of some medical drugs on serum hPON1 enzyme *in vitro*. Most of these on the modulation of PON1 by pharmaceutical compounds have focused on lipid-lowering compounds. One of these, provastatin, was found to increase serum apolipoprotein A1, HDL cholesterol, and PON activity [41, 42]. Similarly, in a cohort of aspirin users, a significant increase in plasma PON1 activity and concentration was reported [29]. On the contrary, the cholinergic muscarinic antagonist atropine was shown to inhibit human plasma and pig liver PON1 *in vitro* [28].

In order to investigate the effect of antibiotics on liver hPON1, HepG2 cells were used as a model. Six different concentrations of the related drugs were applied on the HepG2 cells at 2, 4, and 6 h time points. IC_{50} values were determined by using a graph of activity (%)–[drug] (data not shown). Chloramphenicol and clarithromycin caused a decrease in paraoxonase activity in the Hep2G cells (table). This decrease was in dose-dependent and time-dependent manner for chloramphenicol (data not shown). On the other hand, clarithromycin exhibited the most potent inhibitory effect at 6 h time point. Other studied medical drugs, provastatin, simvastatin, and fluvastatin (10-100 μM), caused a 25-50% decrease in PON1 activity in the culture medium of HuH7 human hepatoma cells [19]. However, in the same cells, fenofibric acid (250 μM) caused a 50 and 30% increase in PON1 activity and PON1 mRNA content, respectively [19]. In another *in vitro* study on isolated lipoproteins, two oxidized metabolites of atorvastatin (5-50 μM) and a metabolite of gemfibrozil (2-80 μM) were found to increase HDL-associated PON1 activity [20].

In conclusion, due to the lack of inhibition studies on paraoxonases from different sources it is not possible to establish definite differences or similarities among paraoxonases. In spite of the contribution of our study to increase in the knowledge of the biochemical properties of paraoxonases, more extensive inhibition studies are necessary before the identity of paraoxonases can be stated.

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