Effects of Some Antibiotics on Paraoxonase from Human Serum *in Vitro* and from Mouse Serum and Liver *in Vivo*

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Paraoxonase (PON1, EC 3.1.8.1) is an esterase protein which plays multifunctional role in metabolism. Therefore, in this study the effects of commonly used antibiotics, namely sodium ampicillin, ciprofloxacin, rifamycin SV and clindamycin phosphate, on human PON1 were investigated *in vitro* and *in vivo*. Human serum paraoxonase (PON1) was separately purified by ammonium sulfate precipitation and hydrophobic interaction chromatography. The *in vitro* effects of the antibiotics in purifying human serum paraoxonase was determined using paraoxon as a substrate, and the IC$_{50}$ values of these drugs exhibiting inhibition effects were found from graphs of hydratase activity % by plotting the concentration of the drugs. It was determined that sodium ampicillin, ciprofloxacin, and clindamycin phosphate were effective inhibitors on human serum PON1, and the inhibition kinetics of interaction of sodium ampicillin, ciprofloxacin, and clindamycin phosphate with the human serum PON1 was also determined, with the K_i of sodium ampicillin, ciprofloxacin, and clindamycin phosphate being 0.00714±0.00068, $6.5 \times 10^{-6} \pm 4.59 \times 10^{-7}$, 0.0291±0.0077 mM, respectively. The *in vivo* effects of the antibiotics on paraoxonase enzyme activity in mouse serum and liver PON1 were also investigated. Mouse liver PON1 activity showed a statistically significant change at 2, 4 and 6 h of drug appliciation *in vivo*. Sodium ampicillin and clindamycin phosphate exhibited about 80% mouse liver PON1 at 2 or 4 h (p: 0.034, 0.003 and 0.021, respectively). In addition, ciprofloxacin and rifamycin SV only showed inhibition at 4 h incubation. Sodium ampicillin (17.12 mg/kg) lead to a significant decrease in mouse serum PON1 after 4 h drug administration. Ciprofloxacin (3.2 mg/kg), rifamycin SV (3.56 mg/kg) and clindamycin phosphate (2.143 mg/kg) did not exhibit any inhibition effect for the mouse serum PON1, *in vivo*.

Key words　paraoxonase; inhibition; antibiotics; IC$_{50}$; *in vivo*; *in vitro*

Serum paraoxonase (aryldialkilphosphatase, EC 3.1.8.1., PON1) is an esterase protein synthesized by the liver[1] and released into the serum, where it is associated with HDL (high density lipoprotein). The enzyme derives its name from its ability to hydrolyze paraoxon into *p*-nitrophenol and diethyl phosphate.[2] Paraoxon, a potent acethylcolinesterase inhibitor, is metabolically generated *in vivo* from the insecticide parathion by mitochondrial oxidation involving the cytochrome-P450 pathway.[3] The ability to hydrolyze paraoxon is routinely used for measuring PON1 activity *in vitro* in serum samples. The enzyme catalyses the hydrolysis of a broad range of substrates including arylesters[1] and carbamates[4] as well as cyclic carbonates and lactones. Also, it hydrolyzes OP (organophosphate compounds).[5] Furthermore, the enzyme inhibits atherogenesis by preventing the oxidation of HDL and low-density lipoprotein (LDL).[6] PON1 also hydrolyzes homocysteine thiolactone and prevents protein homocysteinylation, the process involved in atherogenesis.[7] PON1 and closely related proteins, PON2 and PON3, have been identified. PON3 is also contained in HDL particles,[8,9] whereas PON2 is not found in plasma but is expressed in many tissues.[10] PON2 and PON3 have antioxidant properties, but unlike PON1, lack paraoxon-hydrolyzing activity.

More recently, in addition to its role in lipid metabolism, and hence in treating cardiovascular disease and arteriosclerosis, PON1 has been shown to play a role in the metabolism of pharmaceutical drugs. Given the physiological importance of paraoxonase, its metabolic impact on medically important drugs should receive greater study. However, there are not many inhibition studies available on paraoxonase activity. The inhibitory effects of some diuretic and hypocholesterolemic drugs, such as spironolactone, mevastatin, lovastatin and simvastatin, pravastatin and prulifloxacin, have been investigated on terms of paraoxonase activity from human serum *in vitro* and *in vivo*. Differential effects of drugs on the PON enzyme activity was found. Some increased the activity and others decreased it.[11—13]

Many antibiotics are being used in therapies. There are few reports related to changes in enzyme activities. To our knowledge, the effects of any antibiotics on serum or liver paraoxonase have not been investigated. Therefore, the aim of this study is to determine the effect of some antibiotics, namely sodium ampicillin, ciprofloxacin, rifamycin SV and clindamiycin phosphate, on purified human serum paraoxonase *in vitro*, and mouse serum and liver paraoxonase *in vivo*.

MATERIALS AND METHODS

Materials　Sepharose 4B, ʟ-tyrosine, 1-napthylamine, protein assay reagents and chemicals for electrophoresis were obtained from Sigma Chem. Co. All other chemicals used were of analytical grade and obtained from either Sigma or Merck. 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.*[14] The reaction was monitored for 2 min at 37 °C by monitoring the appearance of *p*-nitrophenol at 412 nm in a Biotek automated recording spectrophotometer. The final substrate concentration during enzyme assay was 2 mM, and all rates were deter-

mined in duplicate and corrected for the non-enzymatic hydrolysis.

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,[15] with bovine serum albumin as a standard.

Purification of Paraoxonase from Human Serum by Hydrophobic Interaction Chromatography Human serum was isolated from 35 ml fresh human blood and put into a dry tube. The blood samples were centrifuged at 1500 rpm for 15 min and the serum was removed. Firstly, serum paraoxonase was isolated by ammonium sulfate precipitation (60—80%). The precipitate was collected by centrifugation at 15000 rpm for 20 min, and redissolved in 100 mM Tris–HCl buffer (pH 8.0). Next, we synthesized the hydrophobic gel, including Sepharose 4B, L-tyrosine and 1-napthylamine, for the purification of human serum paraoxonase.[16] The column was equilibrated with 0.1 M of a Na_2HPO_4 buffer (pH 8.00) including 1 M ammonium sulfate. The paraoxonase was eluted with an ammonium sulfate gradient using 0.1 M Na_2HPO_4 buffer with and without ammonium sulfate (pH 8.00). The purified PON1 enzyme was stored in the presence of 2 mM calcium chloride in order to maintain activity.

SDS Polyacrylamide Gel Electrophoresis SDS polyacrilamide gel electrophoresis was performed after purification of the enzyme. It was carried out in 10% and 3% acrylamide concentration for the running and stacking gel, respectively, containing 0.1% SDS according to Laemmli.[17] A 20 μg sample was applied to the elctrophoresis medium. Gel was stained overnight in 0.1% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid, then destained by frequently changing the same solvent, without dye. The electrophoretic pattern was photographed with the system of produce as an image of the gel.

In Vitro Inhibition Kinetic Studies and Determination of K_i Values For the inhibition studies of sodium ampicillin, ciprofloxacin, rifamycin SV and clindamiycin phosphate, different concentrations of medical drugs were added to the enzyme activity. Paraoxonase activity with medical drugs was assayed by following the hydration of paraoxon. Activity % values of paraoxonase for five different concentrations of each medical drug were determined by regression analysis using Microsoft Office 2000 Excel. Paraoxonase activity without a medical drug was accepted as 100% activity. For the drugs having an inhibition effect, the inhibitor concentration causing up to 50% inhibition (IC_{50} values) was determined from the graphs.

In addition, K_i values of sodium ampicillin, ciprofloxacin, rifamycin SV and clindamiycin phosphate were determined relative to paraoxonase activity. In order to obtain K_M values, K_M and V_{max} values of the enzyme for paraoxon at optimum pH (pH: 8.0) and temperature (37°C) was measured at seven different substrate concentrations (0.5, 1, 1.5, 2, 2.5, 3, 4). K_M and V_{max} values were determined by means of Lineweaver–Burke graphs. The final concentration of 0.0101 mg/ml and 0.0135 mg/ml for sodium ampicillin, 0.190 mg/ml and 0.381 mg/ml for ciprofloxacin, 0.0476 mg/ml and 0.0952 mg/ml for clindamycin phosphate were

added to the mixture, reaction resulting in two different fixed concentrations of the drug. K_i values were calculated from Lineweaver–Burke graphs.

In Vivo Inhibition Studies Mice (*Mus musculus diolecticus*, white type) (25±6) used for *in vivo* studies were kept under special conditions (in a windowless room, at a temperature of 22 °C, with light on for 12 h) for 1 month. Nine mice were selected for intramuscular administration of each drug, with three mice as a control group, not subjected to any drug administration. Drug dosage for the mice was calculated from the suggested dose for humans in mg/kg. 17.12 mg/kg sodium ampicillin, 3.2 mg/kg ciprofloxacin, 3.56 mg/kg rifamycin SV and 2.143 mg/kg clindamycin phosphate were injected intramuscularly into each mouse. For each drug, mice were sacrificed by cervical dislocation after 2 h, 4 h and 6 h following drug administration. Liver samples were taken and kept at −80°C until analysis. Blood was collected in dry tubes and plasma was separated by centrifugation and used immediately or stored at −80°C until analysis.

Preparation of the Microsomal Fraction Microsomal fractions were prepared by a modification of the method described by Gil *et al.*[18] Specifically, mouse liver was removed, which was placed in liquid nitrogen, rinsed with ice-cold homogenization buffer (5 mM Tris–HCl buffer pH 7.4, containing 0.25 M sucrose) and then placed in 4 vol. of ice-cold homogenization buffer. They were homogenized at 10500 rpm for 6 min. After homogenization, nuclei and mitochondria were removed by successive centrifugation at 1000 rpm for 10 min. The post-mitochondrial supernatant fraction was then centrifuged at 16000 rpm for 60 min. The microsomal pellet derived from 0.5 g liver tissue was suspended in 1 ml of 5 mM Tris–HCl buffer pH 7.4. Aliquots of microsomal fraction were used immediately or stored at −80 °C.

Solubilized Microsomal Membranes Paraoxonase was extracted by the addition of Triton X-100.[18] The microsomal fraction was adjusted to 0.75% Triton X-100, vortexed, stored at 4°C for 30 min and then centrifuged at 16000 rpm for 60 min. The resultant supernatant fraction was used for enzyme activity assay.

Statistical Analysis Statistical analysis was performed using a Minitab program for Windows, version 10.02. Analysis of variance, ANOVA, was used when more than two groups were compared. Data are presented as mean±S.D. Values of $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Paraoxonase (PON1) is a complex enzyme; its physiologic role has not yet been clarified. Interestingly, in addition to its role in lipid metabolism, cardiovascular disease and arteriosclerosis, PON1 has been shown to play a role in the metabolism of pharmaceutical drugs.[19] Billecke *et al.* found that PON enzyme hydrolyzes the diuretic spironolactone as well as hypocholestrolemic drugs.[11—13] Pravastatin was found to increase serum apolipoprotein A1, HDL cholesterol and PON activity.[11] 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.

In this study, the effects of penicillin, chinolon, antimi-

cobacterial and macrolid derived antibiotics on paraoxonase enzyme activity was studied in several aspects, including in vitro inhibition studies on purified human serum PON1 and in vivo studies on PON1 from mouse serum and liver.

In order to investigate the effect of these drugs on PON1 enzyme activity *in vitro*, human serum paraoxonase was purified by ammonium sulfate precipitation at 60—80% intervals, and subjected to hydrophobic interaction chromatography.[16] Different protocols are available for PON enzyme purification from serum and liver using three, four and seven steps.[14,20,21] We previously reported a purification strategy designed for the human PON1 enzyme consisting of two-step procedures resulting in a shorter and more straightforward approach in contrast to other purification procedures.[34,35] The gel for hydrophobic interaction chromatography was synthesized using Sepharose 4B, L-tyrosine and 1-napthylamine. The overall purification of human serum PON1 was obtained in a yield of 72.54, and specific activity of 1730.45 U/mg proteins, and this enzyme was purified 227-fold.[16] The purity of the enzyme was confirmed by SDS-PAGE. As seen in Fig. 1, a single band, 43 kDa, was obtained, which corresponds to the results of previous studies.[20—22]
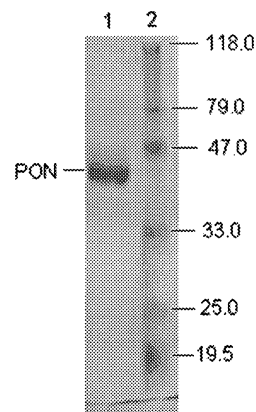

Fig. 1. SDS-PAGE Gel Electrophoresis of PON1 Purified by Ammonium Sulfate Precipitation and Hydrophobic Interaction Chromatography Gel

Serum hPON1 was purified with ammonium sulfate precipitation (60—80%) and hydrophobic interaction chromatography. Lane 1, a pooled sample obtained from a column showing paraoxonase enzyme activity. Lane 2 contains β-galactosidase (118 kDa), bovine serum albumin (79 kDa), ovalbumin (47 kDa), carbonic anhydrase (33 kDa), β-lactogloulin (25 kDa), lysozyme (19.5 kDa) protein marker. The molecular weight of PON1 was estimated to be approximately 43 kDa.
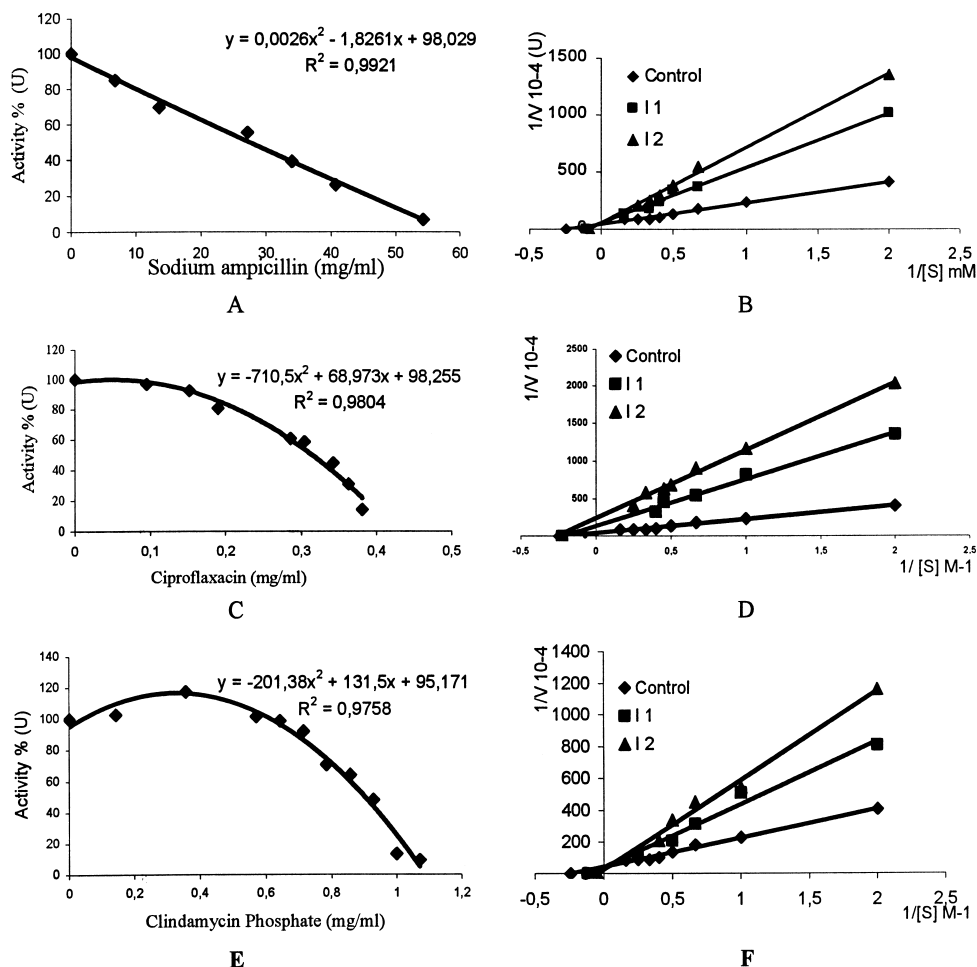

Fig. 2. Effects of Sodium Ampicillin (A), Ciprofloxacin (C) and Clindamycin Phosphate (E) Concentration on Purified Human Serum Paraoxonase and Lineweaver–Burk Plots for Sodium Ampicillin (B), Ciprofloxacin (D) and Clindamycin Phosphate (F) Induced the Inhibition of Human Serum Paraoxonase

Kinetic analysis of the inhibition of PON1 purified human serum by ammonium sulfate precipitation and hydrophobic interaction chromatography. The graph (B, D and F) shows a double-reciprocal plot of PON1 for paraoxon concentrations (0.5—4.0 mM) [S] in the absence (♦, controls) and in the presence of (■, 0.0101 mg/ml), (▲, 0.0135 mg/ml), (■, 0.190 mg/ml), (▲, 0.381 mg/ml) and (■, 0.0476 mg/ml), (▲, 0.0952 mg/ml) for sodium ampicillin ciprofloxacin and clindamycin phosphate, respectively. The inset shows the Dixon plot for K_i determination. All experiments were repeated at least three times and similar results were obtained.

Sodium ampicillin and rifamycin SV appear to prevent bacteria from making their cell walls; ciprofloxacin holds the chromosomes of bacteria; and clindamycin phosphate prevents the protein synthesis of bacteria, causing the cells to die. These drugs are used to treat many sensitive gram-negative and some gram-positive bacteria. As seen in Fig. 2, all of the selected drugs, except for rifamycin SV, *in vitro*, strongly inhibited the human serum PON1 activity. IC_{50} values were estimated as 27.51 mM, 0.313 mM and 0.902 mM for sodium ampicillin, ciprofloxacin and clindamycin phosphate, respectively (Table 1, Fig. 2). In addition, the kinetics of the interaction of drugs with the purified human serum PON1 was also studied. 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. K_M and V_{max} values were determined by means of these graphs. K_M: 4.16 mM and V_{max}: 227.27 were found using paraoxon at pH 8.0 and 37 °C. Reiner *et al.* reported that K_M of human serum PON1 enzyme was 2.5 mM using paraoxon as a substrate.[23] The K_i value (the dissociation of the enzyme-substrate-inhibitor complex) was calculated by the method of Dixon plots, which provides a simple way of determining the inhibition constant. The K_i values calculated for sodium ampicillin, ciprofloxacin and clindamycin phosphate were 0.00714±0.00068 mM 6.5×10⁻⁶±4.59×10⁻⁷ mM and 0.0291±0.0077 mM, respectively (*n*=3) (Table 1). The kinetic data indicate that the inhibition of paraoxonase activity by sodium ampicillin and clindamycin phosphate was of the competitive type, while ciprofloxacin showed non-competitive inhibition (Fig. 2).

Several studies report that, these antibiotics also inhibited other important metabolism enzymes. For example, sodium ampicillin showed inhibition on human carbonic anhydrase I, II and glucose 6-phosphate dehydrogenase.[24,25] It has also been reported that ciprofloxacin was a competitive inhibitor of the enzyme CYP1A2, and showed an inhibitory effect on topoisomerase IIα as well.[26—28] In addition, rifamycin SV has a strong inhibitory effect on polymerase enzymes.[29]

A few studies have investigated effects of some medical drugs on human serum PON1 enzyme *in vitro*. Most of these, on the modulation of PON1 by pharmaceutical compounds, have focused on lipid-lowering compounds. Others have

shown that the *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.[30] In the same cells, fenofibric acid caused a 50% and 30% increase in PON1 activity and mRNA, respectively.[30] 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.[31] A study in rats indicated that fluvastatin reduced both plasma and liver PON1 activity, while a lower dose was only effective on liver activity. Pravastatin, on the other hand, was devoid of significant inhibitory effects.[32]

For *in vivo* studies, mouse serum and liver PON1 were determined at three time points, namely 2 h, 4 h and 6 h after injection. The results of the *in vivo* effects of antibiotics are presented in Table 2. The PON1 activity of the control mouse which was not administered any drugs, was determined to be 31.929±8.053 EU and 15.145±0.938 EU in the serum and liver, respectively.

Ciprofloxacin (3.2 mg/kg), rifamycin SV (3.56 mg/kg) and clindamycin phosphate (2.143 mg/kg) did not exhibit any statistically significant inhibition effect for the mouse serum PON1 ($p>0.05$), while sodium ampicillin (17.12 mg/kg) showed a significant inhibition effect on mouse serum PON1. However, mouse liver PON1 activities, after drug administration, showed a statistically significant decrease or increase at 2, 4 and 6 h of drug application *in vivo*. For example, sodium ampicillin and clindamycin phosphate appearent at about 80% mouse liver PON1 at 2 or 4 h (*p*: 0.034, 0.003, 0.021, respectively). In addition, ciprofloxacin and rifamycin SV inhibits PON liver activity at 4 h. Similarly, a study in rats in-

Table 1. The Effects of Sodium Ampicillin, Ciprofloxacin, Rifamycin SV and Clindamycin Phosphate on Purification by Human Serum PON1 and Kinetic Analysis of the Inhibition

Antibiotic	IC_{50} (mg/ml)	K_i (mM)	Inhibition type
Sodium ampicillin	27.51	0.00714±0.00068	Competitive
Ciprofloxacin	0.313	6.5×10⁻⁶±4.59×10⁻⁷	Non-competitive
Rifamycin SV	Ineffective	Ineffective	Ineffective
Clindamycin phosphate	0.902	0.0291±0.0077	Competitive

Table 2. The *in Vivo* Effects of Sodium Ampicillin, Ciprofloxacin, Rifamycin SV and Clindamycin Phosphate on Mouse Serum and Liver PON1 Activity

Drugs (mg/kg)	Time (h)	Number (*n*)	Serum PON activity (U) (Mean±S.D.)	*p* value	Liver PON activity (U) (Mean±S.D.)	*p* value
	Control	3	31.929±8.05	—	15.145±0.938	—
Sodium ampicillin	2	3	48.93±11.90	>0.05	25.88±0.93	0.034
(17.12 mg/kg)	4	3	14.99±3.05	0.001	14.59±0.68	>0.05
	6	3	41.41±9.55	>0.05	13.07±1.075	>0.05
Ciprofloxacin	2	3	20.06±12.36	>0.05	15.964±3.250	>0.05
(3.2 mg/kg)	4	3	20.468±3.752	>0.05	11.871±1.545	0.035
	6	3	57.31±15.79	>0.05	19.444±3.900	>0.05
Rifamycin SV	2	3	41.75±17.06	>0.05	13.303±2.481	>0.05
(3.56 mg/kg)	4	3	28.25±21.31	>0.05	9.619±1.876	0.010
	6	3	58.941±	>0.05	15.145±1.545	>0.05
Clindamycin phosphate (2.143 mg/kg)	2	3	38.070±8.856	>0.05	27.836±3.382	0.003
	4	3	37.455±7.816	>0.05	20.468±2.325	0.021
	6	3	24.151±8.359	>0.05	13.097±0.356	0.024

Data represent the mean±S.D. *n*=3 denotes the number of mice used in each experiment. *p*<0.05 was considered statistically significant.

dicated that fluvastatin (20 mg/kg/d for 3 weeks) reduced both serum and liver PON1 activity, while a lower dose (2 mg/kg/d) was only effective toward liver activity. Pravastatin (4 or 40 mg/kg/d for 3 weeks), on the other hand, was devoid of significant inhibitory effects.[32] In addition, the anti-inflammatory glucocorticoid dexamethasone (1 mM) caused an eight-fold increase in PON1 mRNA in a mouse hepatoma cell line (Hepa cells), as well as in mice *in vivo*.[33] Furthermore, rifamycin SV showed statistically significant inhibitory effect on mouse liver *in vivo*, while it did not exhibit any inhibition of purified human serum PON1 *in vitro*.

In conclusion, sodium ampicillin, ciprofloxacin and clindamycin phosphate significantly inhibited purified human serum PON1 activity in a dose-dependent fashion. These antibiotics also showed different inhibition effects on mouse serum and liver. If it is required to give these antibiotics to the patient, their dosage should be carefully prescribed to decrease side effects, because these drugs may worsen the health of a patient, particularly patients who have arteriosclerosis and/or vascular disease.

Antibiotics activate PON1 activity in the liver *in vivo*. The reason for this could be that the other defense mechanisms may be involved in the activation of PON1 enzyme during drug metabolism in liver. Whereas, in *in vitro* studies, ciprofloxacin and rifamycin SV lead to the significant inhibition in liver. Antibiotics cause greater inhibition in *in vitro* studies. The reason for this could be the use of purified enzymes for *in vitro* study; it should be noted that another defense system may be involved *in vivo*.

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