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Research Article

Some Anticancer Agents Act on Human Serum Paraoxonase-1 to Reduce Its Activity

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Human serum paraoxonase (hPON1) is an important antioxidant enzyme. It protects low-density lipoproteins against oxidative stress and prevents atherosclerosis development. Anticancer agents have cardiotoxic effects, and this situation can lead to significant complications. Our aim was to evaluate the *in vitro* effects of some of the anticancer agents such as cetuximab, paclitaxel, etoposide, docetaxel, and ifosfamide on the activity of hPON1 in this study. For this reason, PON1 was purified from human serum with a specific activity of 3654.2 EU/mg and 16.84% yield using simple chromatographic methods. The five chemotherapeutic agents dose dependently decreased *in vitro* hPON1 activity. IC_{50} values for cetuximab, paclitaxel, etoposide, docetaxel, and ifosfamide were 0.0111, 0.042, 0.226, 0.665, and 23.3 mM, respectively. K_i constants were 0.0194, 0.0165, 0.131, 0.291, and 8.973 mM, respectively. The inhibition mechanisms of cetuximab, etoposide, docetaxel, and ifosfamide were non-competitive, and for paclitaxel was competitive. Consequently, inhibition of hPON1 by these anticancer agents may explain some of the cardiotoxic actions of these drugs.

Key words: anticancer agents, enzyme–drug interaction, high-density lipoprotein, inhibition, paraoxonase

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A free radical is an atom or molecule which has one or more unpaired electrons in its endmost orbitals. Free radicals are highly unstable, and they have extremely high chemical reactivity. Their electrons interact with other molecules within cells and damage various biological substances, including proteins, lipids, and DNA (1–4). Cells include some antioxidant defense mechanisms that protect these biological materials from the harmful effects of free oxygen radicals. For instance, lipid peroxidation, defined as the oxidative deterioration of lipid, is one of the harmful effects of free radicals on the metabolism. Lipid peroxidation is known to cause atherosclerosis in particular and some other vascular diseases (5). PON1 is an antioxidant enzyme which inhibits the lipid peroxidation. It has a crucial role in the lipid metabolism to prevent oxidative modifications from the low-density lipoproteins (6). PON1 also protects oxidation from the phospholipids in the high-density lipoprotein (HDL) structure. This situation slows the development of the foam cell formation and atherosclerosis by preventing the accumulation of cholesterol in the macrophages (7). Thus, PON1 is a protector against the development of atherosclerotic lesions in human and animal models (8–12). Low serum PON1 activity levels may be associated with increased levels of cardiovascular diseases. Namely decreased PON1 activity may be a prognostic factor for coronary heart diseases.

Firstly, Mackness *et al.* (13) showed that serum PON1 activity protects the oxidation of the LDL phospholipid at the beginning of the process of atherosclerosis (13). Also, many studies have been conducted that show the relationship between PON1 and atherosclerosis. Due to its antioxidant properties, PON1 activity is known as being important for cardiovascular physiology. Many studies show that PON1 activity reduces the risk of cardiovascular disease caused by oxidative stress (14). Besides, PON1 activity reduces inflammatory processes, hyperthyroidism, certain neuropathies, diabetes, cancer, and many other diseases, by preventing oxidative stress (15–20). For instance, Malik *et al.* (4) report that the role of oxidative imbalance in the oral squamous cell carcinoma may be associated with the antioxidant status. Thus, 'PON levels may act as an indicator of oxidative stress in cancer'. Also, some studies have shown that PON1 activity is lower in patients with various cancers than in the controls (21). On the other hand, recent studies emphasize the reduction of PON1 activity in mental diseases such as Alzheimer's disease (22), anxiety disorder (23), and Parkinson's disease (24). Therefore, inhibition of the PON1 enzyme is not desired and hazardous for living organisms (25).

Nowadays, novel drugs are still being developed for most diseases, including the above-mentioned ones. Thus, the determination of PON1 inhibitors and activators can be

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very important for new drug development, especially for diseases associated with oxidative stress. For this reason, in this study, we examined the effects on the purified hPON1 activity of some anticancer drugs commonly used in medical applications. These drugs were cetuximab, paclitaxel, etoposide, docetaxel, and ifosfamide (Figure 1). Cetuximab belongs to a group of cancer drugs known as monoclonal antibodies. It is especially used in the treatment of large bowel cancer and for head and neck cancer because it stops the division and growth of the cancer cells (26). Paclitaxel and docetaxel represent the taxane family of drugs. They are used for the treatments of various types of cancer. They work by preventing the division and growth of the cancer cells (27). Etoposide is an anticancer drug mainly used to treat lung and testicular cancers. It belongs to the topoisomerase inhibitor drug class. Etoposide works by blocking the topoisomerase 2 enzyme, which is necessary for the separation and the growth of the cancer cells (28). Ifosfamide is a chemotherapeutic agent. It is active as an alkylating agent and an immunosuppressive agent. This compound belongs to the isofamides. The action mechanism of the ifosfamide has not been determined, but appears to be similar to other alkylating agents as it also stops or slows down the growth of cancer cells. Ifosfamide is used to treat certain testicular cancers. Additionally, it is used for bone and soft tissue sarcomas or other types of cancers (29). These drugs are commonly used as chemotherapeutic agents, but their *in vitro* effects on purified hPON1 are not known. These anticancer agents also have cardiotoxic effects, which can lead to significant complications (30). Therefore, understanding these cardiotoxic effects is crucial for cancer survivors.

Materials and Methods

Materials

DEAE-Sephadex A-50, Sephadex G-100, paraoxon, protein assay reagents, and chemicals for electrophoresis were obtained from Sigma Chemical Co (Steinheim, Germany). All of the other chemicals used were of analytical grade and were obtained from either Sigma-Aldrich or Merck (Darmstadt, Germany). Anticancer agents were provided from the University Hospital Pharmacy (Atatürk University, Erzurum, Turkey).

Methods

Paraoxonase activity assay

Paraoxonase enzyme activity was determined at 25 °C with paraoxon (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. Assays were performed using a spectrophotometer (CHEBIOS UV–VIS, Fullerton, CA). The molar extinction coefficient of para-nitrophenol (ε = 18 290/M/cm at pH 10.5) is used for the calculation of enzyme activity. One enzyme unit was defined as the amount of enzyme that catalyzes the hydrolysis of 1 μmol of paraoxon at 25 °C (31).

Ammonium sulfate precipitation

Twenty five mililiters of Triton X-100-treated human serum was precipitated with ammonium sulfate (60-80%). The precipitate was collected by centrifugation at 15 000 × *g* for 20 min and redissolved in 100 mM Na-phosphate buf-

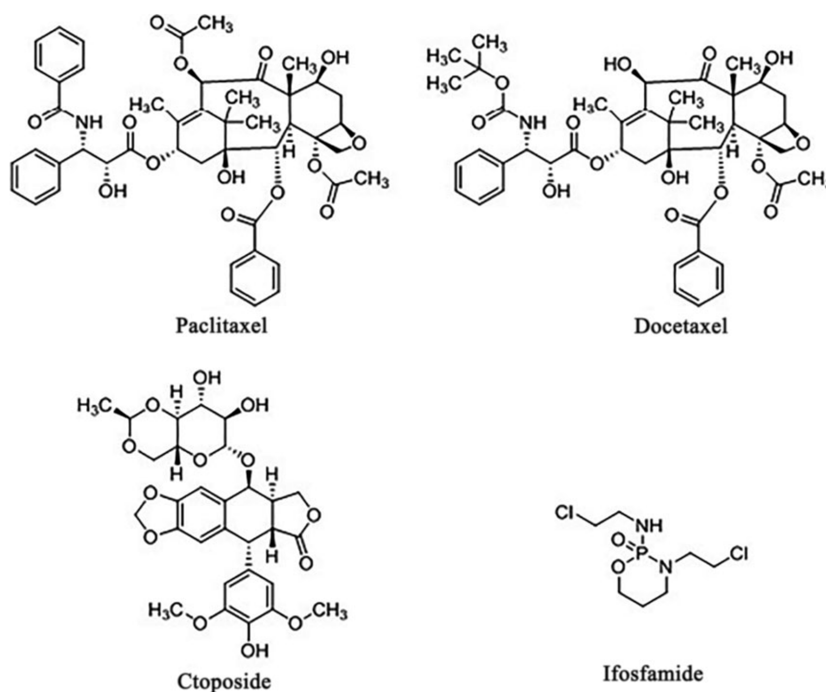

Figure 1: Chemical structures of anticancer agents such as paclitaxel, etoposide, docetaxel, and ifosfamide.

fer (pH 7.0). Then, it was dialyzed in the presence of 1 mM Na-phosphate buffer (pH 7.0) at 4 °C (25,32–35).

DEAE-Sephadex A-50 anion exchange chromatography

DEAE-Sephadex A-50 anion exchange column was prepared and equilibrated with 100 mM Na-phosphate buffer (pH 7.0). After dialysis in the presence of 1 mM Na-phosphate buffer (pH 7.0) at 4 °C, the enzyme solution was loaded onto the DEAE-Sephadex A-50 anion exchange column. 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. These processes were performed as in our previous studies (25,32–35). Enzyme activity of all elutions was checked at 412 nm. Active fractions were combined. All purification procedures were performed at 4 °C.

Sephadex G-100 gel filtration chromatography

The Sephadex G-100 gel was equilibrated with 100 mM Na-phosphate buffer (pH 7.0). After DEAE-Sephadex column, fractions were mixed with glycerol and loaded onto the Sephadex G-100 column for gel filtration process. Elution was performed with the same buffer. The measurement of the protein amount (280 nm) and enzyme activity (412 nm) was performed for each fraction. Elution tubes which observed the enzyme activity were combined for kinetic studies (25,32–35).

Protein determination

Quantitative protein determination was carried out by measuring the absorbance at 595 nm according to Bradford. Bovine serum albümin was used as a standard in this experiment (36).

SDS–polyacrylamide gel electrophoresis

Enzyme purity was controlled according to Laemmli's procedure. The procedure includes two different acrylamide concentrations as 3% and 8% for running and stacking gel, respectively (37). The experiment was carried out as our previous studies (38,39). SDS-PAGE was stained with silver reagent, and the electrophoretic pattern was photographed (Figure 2). Line 1 contains standard proteins. The standard protein marker was purchased from Thermo (USA). Line 2 contains purified human serum PON1 enzyme.

Drug–enzyme interaction studies

The inhibitory effects of cetuximab, paclitaxel, etoposide, docetaxel, and ifosfamide as antineoplastic drugs were examined. hPON1 activities were measured in the presence of different drug concentrations. Control activity was considered as 100% at the absence of any compound. Thus, Activity%–[Drug] curves were drawn, and drug concentrations that produced 50% inhibition (IC_{50}) were calculated from these curves for each drug. Paraoxon was used as a substrate in all kinetic studies. K_i values of the drugs were calculated by measuring enzyme activity at three different drug concentrations with five different substrate concentrations. Lineweaver–Burk graphs were used for the determination of K_i and inhibition type (40).

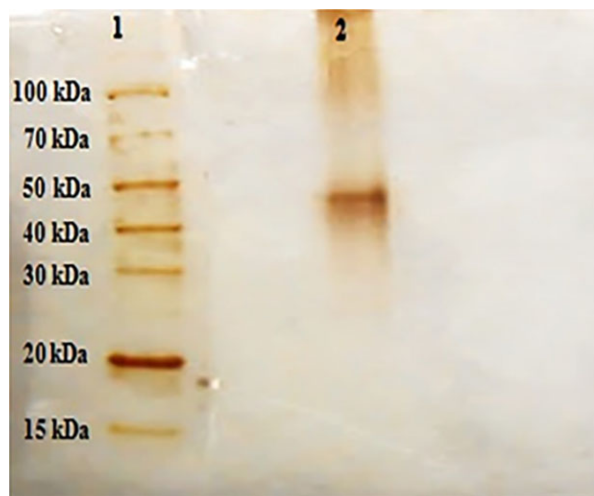

Figure 2: SDS-PAGE bands of human serum PON1. The procedure includes two different acrylamide concentrations as 3% and 8% for running and stacking gel, respectively. Line 1 contains standard proteins. The standard protein marker was purchased from Thermo (Carlsbad, CA, USA). PON1 enzyme is in line 2.

Results and Discussion

Oxidative stress is described as a disturbance in the balance between the production of reactive oxygen species and the antioxidant protection system. Many factors such as smoking, fast foods, lack of good nutrition, stress, air and water pollutants, alcohol, pesticides, exposure to toxins, and inadequate amounts of physical activity cause the increase in levels of free oxygen radicals. This situation leads to oxidative stress (1,3,41,42). Oxidative stress has been implicated in many diseases. Some of these include neurodegenerative and other diseases such as Parkinson's disease and Alzheimer's disease, atherosclerosis, hypertension, diabetes mellitus, ischemic diseases, and cancer (1). The cellular membrane damage is one of the best known toxic effects of free oxygen radicals. This process is initiated by lipid peroxidation. The oxidative modification of LDL is a crucial step in the pathogenesis of atherosclerosis (5). Atherosclerosis is a serious disorder caused by plaque that builds up inside the arteries (43) and is the biggest cause of death in the developed world.

The living metabolism is protected from free oxygen radicals in various ways. One way can be enzymatic.

Antioxidant enzymes and peptides such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione (GSH) including PON play a role in this metabolism. On the other hand, non-enzymatic compounds, particularly vitamins such as vitamins A, C, and E, are also forms of antioxidant defense mechanisms (41,44).

PON1 is one of the most important endogenous antioxidant enzymes in the human body. Human serum PON1 (EC 3.1.8.1) is associated with HDL and an antioxidant function. It can hydrolyze lactones and several non-physiological substrates, including aryl esters and organophosphates (OPs) (25,32,33). The paraoxonase gene family consists of three members: PON1, PON2, and PON3 (45). These isozymes show differences with regard to their subcellular localizations, catalytic activities, and substrate specificities. While PON1 and PON3 are expressed primarily in the liver, PON2 is widely expressed in various tissues including brain, liver, and kidney. These three human PON genes share approximately a 60% identity at the amino acid level and approximately 70% at the nucleotide level (45). In particular, PON1 is the best known member and the most studied in the paraoxonase family. The antioxidant properties of PON1 are known to be caused by free thiol groups in its three-dimensional structure. A free sulfhydryl at cysteine 284 plays a vital role in the antioxidant mechanism of PON1 such as the preventing of LDL oxidation (34). Therefore, PON1 is a preventive for vascular diseases and other cardiovascular diseases. These protector enzyme activity levels are not only vital for cardiovascular diseases, but also others associated with oxidative stress. Because of this, reduced PON1 activity may be linked to many diseases (25,32–35). Therefore, the protection of the PON1 activity in diseases associated with oxidative stress is crucial for the living metabolism. As in a number of enzymes, PON1 activity is also affected by a number of factors such as environmental chemicals, drugs, and smoking, alcohol, diet, age, and disease conditions (46). For example, in some studies, PON1 activity was found to be lower in smokers when compared to non-smokers. Cigarettes contain a substantial amount of free radicals which play a significant role in the impairment of the oxidative balance and cause cell damage. Chronic cigarette smoking is one of the major risk factors for the development of atherosclerosis and other diseases (47–49). It is known that cigarette smoking leads to a reduction of the quantity in the serum. The amount of HDL is vital in the serum, because PON1 is linked to the HDL structure. The reduction of HDL leads to the decreasing of the PON1 protein in the serum. Thus, atherosclerotic lesions are shown to increase inside the arteries.

Cancer is also one of the most serious health problems in the developing world. In recent years, some studies have been performed in regard to the relationship between the various cancers and PON1 activity. The end products of lipid peroxidation have an important role in oncogenesis. Thus, oxidized low-density lipoprotein is responsible for the development of oxidative stress-related cancer diseases (4,50–52). The results show that PON1 activity also has a vital role in cancer diseases. Due to the preventive effect of oxidative stress, scientists report the HDL-associated PON1 levels may act as a potential marker in cancer. For instance, several studies demonstrated that serum PON1 activity is lower in patients with lung (53), pancreatic (54), gastric (55), gastroesophageal (56), prostate (57), epithelial ovarian cancer (58), and oral squamous cell carcinoma (4) when compared to the control groups.

Many chemicals, including drugs, have a role to play in the increasing or decreasing of the various enzyme activities. Due to the balance of enzyme activities in the metabolism, extra-inhibition or activation may be a risk for living things. Many studies have been conducted on the modulation of PON1 activity by drugs. For example, Gouedard *et al.* (59) investigated the effects of pravastatin, simvastatin, and fluvastatin on human hepatoma cells PON1. They found that these drugs caused a 25–50% decrease in PON1 activity.

Table 2: IC_{50} and K_i values and inhibition types

Anticancer drugs	IC_{50} (mM)	K_i (mM)	Type of inhibition
Cetuximab	0.011	0.0194 ± 0.004	Non-competitive
Paclitaxel	0.042	0.0165 ± 0.011	Competitive
Etoposide	0.226	0.131 ± 0.071	Non-competitive
Docetaxel	0.665	0.291 ± 0.108	Non-competitive
Ifosfamide	23.30	8.973 ± 0.955	Non-competitive

Table 1: Summary of PON1 purification procedure from human serum

Purification steps	Activity (EU/mL)	Total volume (mL)	Protein (mg/mL)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Yield (%)	Purification fold
Serum sample	102.4	25	6.1	152.5	2560	16.78	100	1
Ammonium sulfate precipitation (60–80%)	100.2	15	5.191	77.865	1503	19.3	58.71	1.15
DEAE-Sephadex A-50 anion exchange chromatography	64.52	12	0.174	2.088	774.24	370.8	30.24	22.09
Sephadex G-100 gel filtration chromatography	53.9	8	0.0148	0.118	431.2	3654.2	16.84	217.7

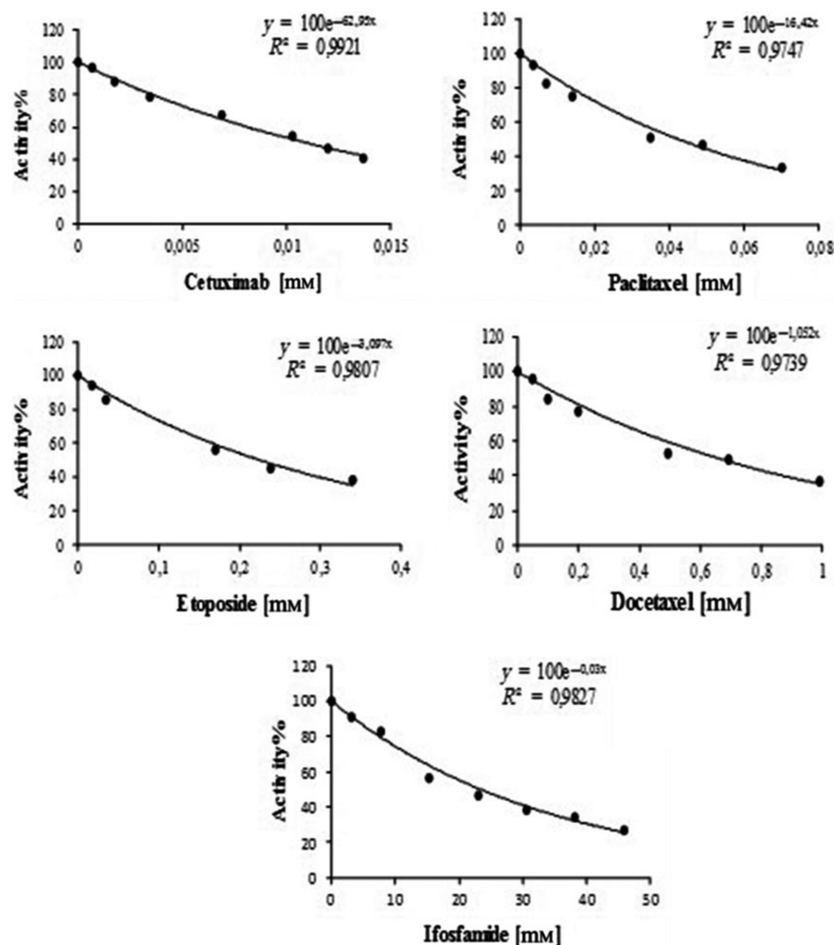

Figure 3: IC_{50} graphs for anticancer agents. Human serum paraoxonase (hPON1) activities were measured in the presence of different drug concentrations. Control activity was considered as 100% at the absence of any compound. Thus, Activity%–[Drug] curves were drawn, and drug concentrations that produced 50% inhibition (IC_{50}) were calculated from these curves for each drug.

There was a similar decrease in the mRNA of PON1 protein. In the same cells, fenofibric acid (250 mM) also caused a 50% and 30% increase in PON1 activity and mRNA, respectively (59). Other studies on PON1 activity investigated the effects of different hypocholesterolemic drugs such as spironolactone, mevastatin, lovastatin, pravastatin, and prulifloxacin (60–62). PON1–drug interaction studies are still being conducted in our laboratory. In our previous studies, the inhibitory effects of some cardiovascular therapeutics such as digoxin, metoprolol tartrate, verapamil, diltiazem, amiodarone, dobutamine, and methylprednisolone (34), and antibiotic drugs such as teicoplanin, rifamycin, tobramycin, ceftriaxone sodium, cefuroxime sodium, ceftazidime, ornidazole, and amikacin sulfate (25), anesthetics such as etomidate, propofol, and ketamine (32), calcium channel blockers such as nifedipine, nitrendipine, isradipine, and amlodipine besylate (35) were investigated. According to our findings, cardiovascular therapeutics inhibit the hPON1 enzyme, more strongly compared to the other drugs such as antibiotics, anesthetics, and calcium channel blockers. Other drugs show the inhibition effects in almost similar concentrations. In the present study, five chemotherapeutic agents were observed to have lower inhibition effects than antibiotics, anesthetics, and calcium channel blockers. It can be understood from the studies that PON1 is a drug-target enzyme. However, more extensive inhibition studies are necessary for a better understanding of the protective role of PON1 against the toxic effects of drugs, environmental chemicals, and oxidative stress. Thus, enzymatic toxicology studies are important in the development of new drugs.

The present study examined the *in vitro* inhibition effects of some anticancer drugs on the human serum PON1 enzyme. For this, PON1 was the purified human serum as mentioned in our previous studies (25,32–35). The enzyme was obtained with a specific activity of 3654.2 EU/mg protein, with a 217.7-fold purification and a yield of 16.84% (Table 1). SDS–polyacrylamide gel electrophoresis (PAGE) was performed after the purification of the enzyme, and the electrophoretic pattern is shown in Figure 2. After all the purification steps, the *in vitro* inhibition effects of cetuximab, paclitaxel, etoposide, docetaxel, and ifosfamide on the purified enzyme were investigated. The IC_{50} values were found by Activity%/[Inhibitor] graphs to be 0.011, 0.042, 0.226, 0.665, and 23.30 mM for cetuximab, paclitaxel, etoposide, docetaxel, and ifosfamide, respec-

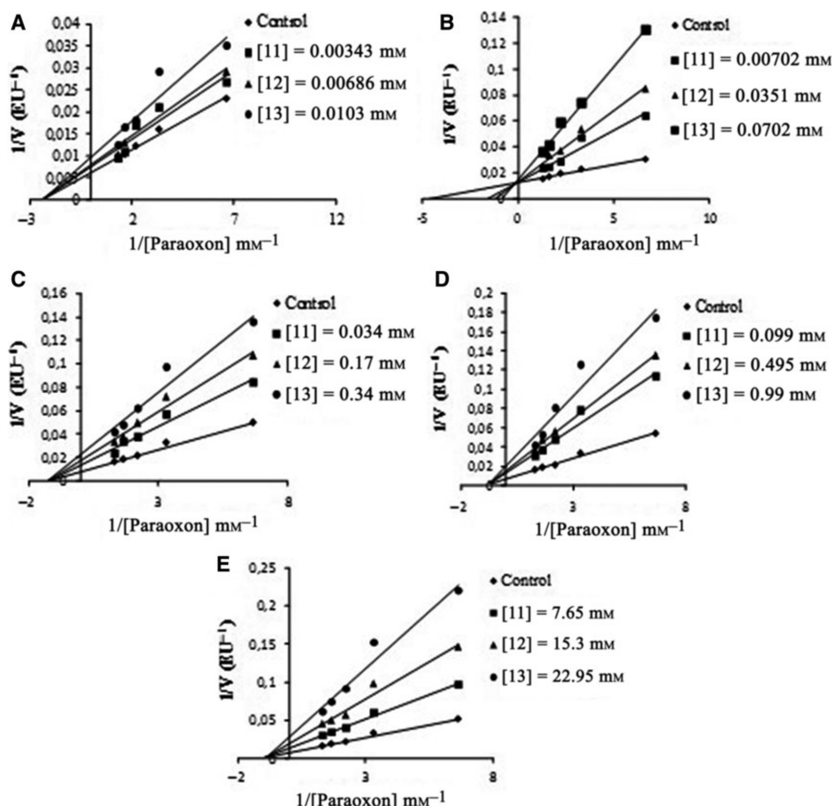

Figure 4: (A–E) Lineweaver–Burk graph of cetuximab, paclitaxel, etoposide, docetaxel, and ifosfamide, respectively. K_i values and inhibition types of the drugs were determined by measuring enzyme activity at three different drug concentrations with five different substrate concentrations.

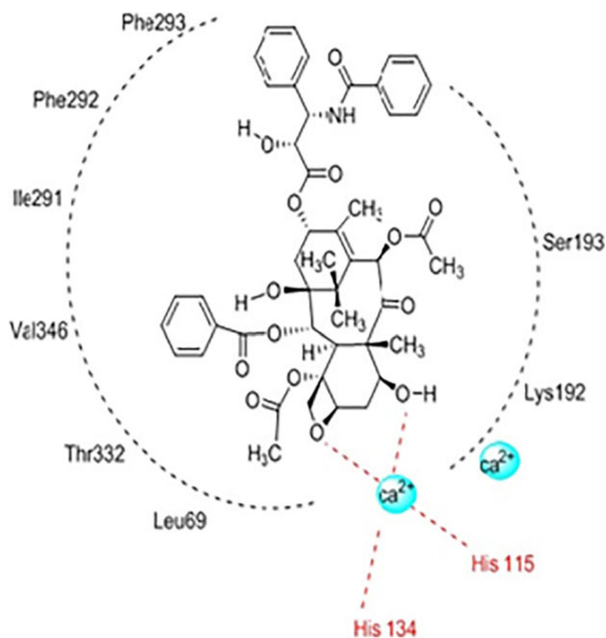

Figure 5: Schematic representation of the interaction of paclitaxel with the PON1 active site.

tively (Table 2; Fig. 3). According to the results, the order of the inhibitors is as follows: cetuximab > paclitaxel > etoposide > docetaxel > ifosfamide (Table 2; Fig. 3). The K_i values and inhibition types of these drugs were determined from the Lineweaver–Burk curves using different paraoxon concentrations (Table 2; Figure 4). We found that paclitaxel exhibited competitive inhibition, and the others exhibited non-competitive inhibition. Accordingly, paclitaxel may have a connection with the amino acids of the active site. According to this information, we designed a possible scheme for paclitaxel (Figure 5). Other drugs may interact with the enzyme structure outside of the catalytic site.

Conclusions

In conclusion, hPON1 was purified using three simple purification steps in a short time with high specific activity. We examined the *in vitro* effects of some anticancer agents on the purified hPON1 activity. We identified that cetuximab, paclitaxel, etoposide, docetaxel, and ifosfamide reduced the activity of hPON1 at different concentrations. These anticancer agents may have cardiotoxic effects which may lead to significant complications (30). Understanding these cardiotoxic effects may be crucial for cancer survivors. However, it may be necessary to carry out further studies such as *in vivo* studies to obtain a more concrete idea about the cardiotoxic actions of these drugs.

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