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ORIGINAL ARTICLE

Some indazoles reduced the activity of human serum paraoxonase 1, an antioxidant enzyme: *in vitro* inhibition and molecular modeling studies

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ABSTRACT

Background: Paraoxonase 1 (PON1: EC 3.1.8.1) is a vital antioxidant enzyme against mainly atherosclerosis and many other diseases associated with oxidative stress. Thus, studies related to PON1 have an important place in the pharmacology. In this study, we aimed to evaluate the *in vitro* inhibition effects of some indazoles on the activity of human PON1.

Methods: PON1 was purified from human serum with a specific activity of 5000 U/mg and 13.50% yield by using simple chromatographic methods.

Results: The indazoles showed K_i values in a range of 26.0 ± 3.00–111 ± 31.0 μM against hPON1. All these indazoles exhibited competitive inhibition. In addition, molecular docking studies were performed in order to assess the probable binding mechanisms into the active site of hPON1. Molecular modeling studies confirmed our results.

Conclusions: Inhibition of PON1 by indazoles supplies a verification to further consideration of limitation dosage of indazole molecule groups as drug.

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Enzyme inhibition; molecular modeling; paraoxonase 1; purification; indazole

Introduction

The paraoxonase enzyme family (PON1, PON2, PON3) is derived from the toxic metabolite "paraoxon" of parathion, an insecticide known as paraoxonase (Furlong *et al.* 2016). In humans, PON1 and PON3 genes expressed predominantly in the kidney and liver. PON1 and PON3 are synthesized from the liver and then secreted into the bloodstream by binding to HDL (Mackness and Mackness 2015). Conversely, the PON2 is synthesized from many different tissues and is not found in circulation (Ng *et al.* 2001, Levy *et al.* 2007). The PON1 enzyme is the most studied and well-known member of the paraoxonase family (Litvinov *et al.* 2012). Unlike other PON subtypes, the PON1 enzyme, which has paraoxonase activity as well as aryl esterase activity, metabolizes many organophosphates and drugs. At the same time PON1 hydrolyzes aryl esters thiolactones, cyclic carbonates, nerve gases, glucuronides, and estrogen esters, which means it has a wide variety of substrates (Rajkovic *et al.* 2011). PON1 enzyme creates a defense system and eliminates the harmful effects of organophosphates (Androutsopoulos *et al.* 2011). Thus, PON1 is known as a detoxifier protein.

On the other hand, PON1 plays a role in inhibiting lipoprotein oxidation by hydrolyzing lipid peroxides in the structure of oxLDL, which is involved in HDL cholesterol structure (Mackness *et al.* 1991). Because of this feature, PON1 shows protective properties against atherosclerosis and the place of PON1 in cardiovascular physiology is quite important. PON1, an antioxidant enzyme, is also thought to be important in the development of hyperthyroidism, Alzheimer's disease, anxiety disorder and Parkinson's disease, diabetes, and cancer, which are caused by lipid peroxidation and oxidative damage (Alim and Beydemir 2016). Therefore, the conservation of PON1 activity is very important and inhibition of the PON1 is an undesirable situation for cardiovascular diseases, some other diseases related to oxidative stress and organophosphate toxicity (Isgor and Beydemir 2010). The investigation of the effects of bioactive molecules used in pharmacological applications on the activity of this cardioprotective enzyme is very important for the development of new drugs. A number of studies investigating the relationship between PON1 activity and commonly used drugs are available in the literature (Isgor and Beydemir 2010, Alim and Beydemir 2016). Further, PON1 plays a significant role in drug metabolism studies due to PON1 participating in both biological activation and inactivation of specific drugs (Tougou *et al.* 1998, Furlong *et al.* 2016).

In view of the pharmacological aspect of PON1, we examined the *in vitro* inhibition effects of some indazoles on human serum PON1 (hPON1) activity in this study. Indazole is an important class of heterocyclic compounds formed by the fusion of benzene and pyrazole rings (Gaikwad *et al.* 2015). Indazole derivatives display a wide range of biological activities such as anti-inflammatory (Cheekavolu and Muniappan 2016), anti-tumor (Abbasi *et al.* 2014), anti-HIV (Kim *et al.* 2013), antimicrobial, antiparasitic, antiplatelet (Cerecetto *et al.* 2005), and serotonin 5-HT3 receptor antagonist activities (Bermudez *et al.* 1990). For this reason, indazole derivatives have a very important role in

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pharmacological applications for the synthesis of new drugs. There are three tautomers, 1H-indazole, 2H-indazole, and 3H-indazole, according to position of the heteroatoms in the ring of the indazole molecule (Shrivastava *et al.* 2016). Among these, 1H-indazole exhibits a wide range of biological and pharmaceutical activities (Markina *et al.* 2012).

We examined the *in vitro* inhibition effects of some indazoles [1H-indazole (A), 4-bromo-1H-indazole (B), 6-bromo-1H-indazole (C), 7-bromo-1H-indazole (D), 4-chloro-1H-indazole (E), 6-chloro-1H-indazole (F), 7-chloro-1H-indazole (G), 4-fluoro-1H-indazole (H), 6-fluoro-1H-indazole (I), 7-fluoro-1H-indazole (K)] on the activity of hPON1 in this study (Figure 1). The indazole compounds dose-dependently significantly decreased *in vitro* hPON1 activity. IC_{50}, K_i values and inhibition types were determined for each inhibitory molecule. In addition, molecular docking studies were performed for A, D, G, and H compounds which have low K_i values in order to assess the probable binding mechanisms into the active site of hPON1.

Materials and methods

Materials

All the chemicals including indazoles, column packing materials for chromatographic processes and paraoxon, we used in the experimental procedure were obtained from Sigma-Aldrich Co. (Sigma-Aldrich Chemie GmbH Export Department Eschenstrasse 5, 82024 Taufkirchen, Germany).

Paraoxonase activity assay

The paraoxonase activity assay was based on the estimation of *p*-nitrophenol at 412 nm. hPON1 activity was measured using paraoxon (diethyl p-nitrophenyl phosphate) as substrate (1 mM) in 50 mM glycine/NaOH (pH 10.5) including 1 mM $CaCl_2$. The molar extinction coefficient of *p*-nitrophenol ($\varepsilon = 18{,}290 M^{-1} cm^{-1}$ at pH 10.5) was used for calculation of activity. One enzyme unit was described as the amount of enzyme catalyzing the hydrolysis of 1 mmol of substrate at 25 °C. The assays were performed spectrophotometrically using the method described by Renault *et al.* (2006).

Purification of paraoxonase 1 from human serum

Triton X-100 treated human serum (20 ml) was subjected to ammonium sulfate precipitation in the range of 60–80%. After centrifugation (at 1500 g for 20 min), precipitate was collected and redissolved in 100 mM Na-phosphate buffer (pH 7.0). The enzyme solution was dialyzed against 1 mM sodium phosphate buffer (pH 7.0) for about 2 h. Then, the enzyme solution

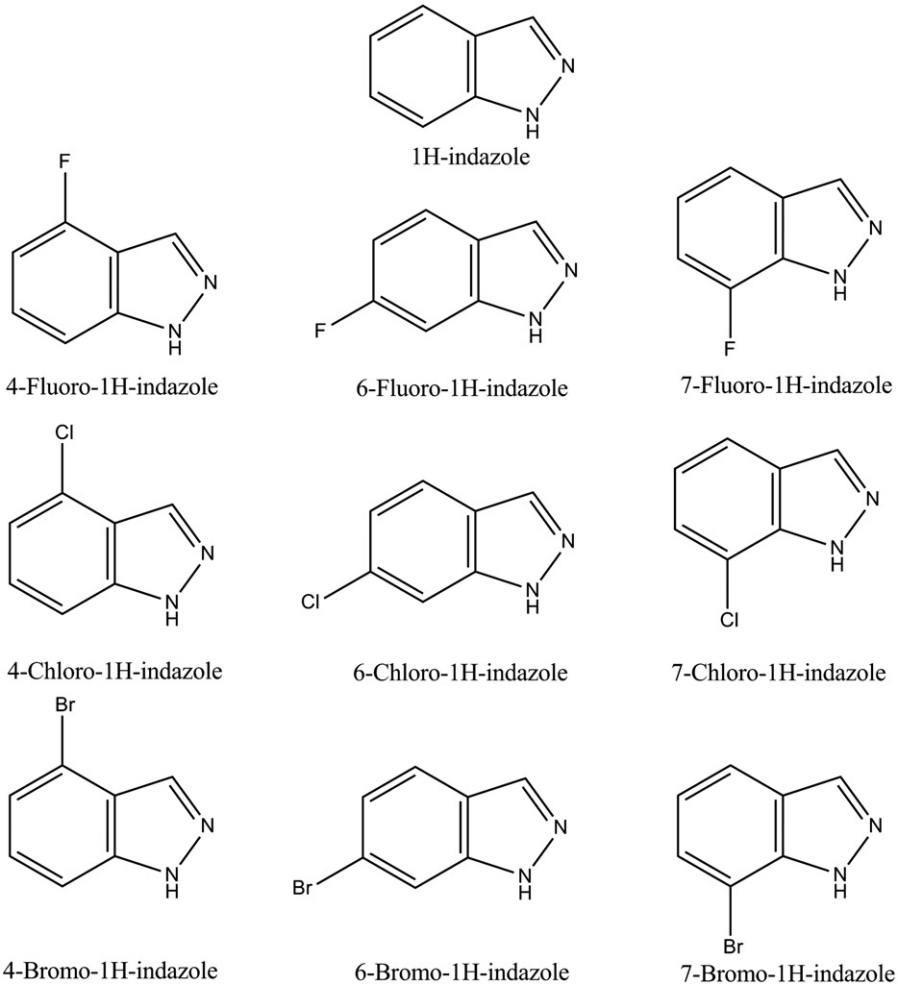

Figure 1. The molecular structures of indazoles used in this study.

Table 1. Summary of the paraoxonase-1 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	124	20	7.2	144	2480	17.22	100	1
Ammonium sulfate precipitation (60–80%)	132	18	5.6	100.8	2376	23.57	95.8	1.369
DEAE-Sephadex A-50 anion exchange chromatography	71	10	0.152	1.52	710	467.11	28.63	27.13
Sephadex G-100 gel filtration chromatography	56	6	0.0112	0.0672	336	5000	13.5	290.36

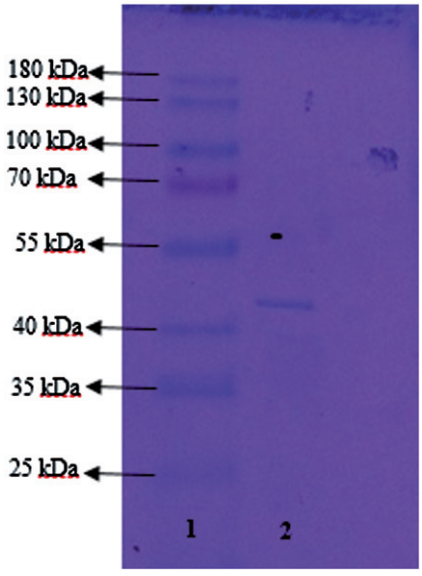

Figure 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of purified hPON1. Lane 1: Standard proteins (kDa), lane 2: Purified hPON1.

was loaded onto the DEAE-Sephadex A50 anion exchange column previously equilibrated with the 100 mM Na-phosphate buffer (pH 7.0). The column was washed with a 100 mM Na-phosphate buffer to remove any other impurities. A linear gradient of 0–1.5 M NaCl (100 ml) was used to elute the enzyme. The activity measurements were made at 412 nm in all the elutions, and the active fractions were pooled. After anion exchange chromatography, active enzyme solution was loaded onto the gel filtration column (Sephadex G-100) equilibrated with a 100 mM Na-phosphate buffer (pH 7.0). Both the qualitative protein identification (280 nm) and enzyme activity (412 nm) were monitored in the eluates. The tubes observed in the enzyme activity were combined for other kinetic studies. All purification procedures were made at 4 °C. These processes were performed as in our previous studies (Isgor and Beydemir 2010, Alim and Beydemir 2016). Quantitative protein assay was performed using the Bradford method (Bradford 1976) and the enzyme purity was controlled according to Laemmli's procedure (Laemmli 1970, Demir and Beydemir 2015).

In vitro *inhibition studies of some indazoles with hPON1*

The *in vitro* effects of A–K molecules on purified hPON1 were examined. About 10% DMSO was used to dissolve the indazole molecules and DMSO was added as much as the amount of inhibitor added to enzyme medium in the blind medium in each activity measurement. Thus, the possible effect of DMSO on hPON1 activity was eliminated. Final concentration for A was 6.54 mM, for B, C, and D were 6.55 mM, for E, F, and G were 5.07 mM, for H and I were 0.734 mM, for K molecule 7.34 mM, respectively. hPON1 enzyme activity without an indazole compound was considered as 100% activity. Then hPON1 enzyme activity was measured at five different indazole concentration. This process was performed for all indazole molecules. Activity percentage values of hPON1 for five different concentrations of each compounds were determined and Activity%-[Indazole] graphs were drawn. IC_{50} values for each indazole molecules were calculated from these graphs. To determine the K_m and V_{max} values for the hPON1 enzyme, activity measurements were made at five different concentrations of paraoxon (0.15, 0.3, 0.45, 0.60, 0.75 mM) and Lineweaver–Burk plot was drawn to determine K_m and V_{max} values. So as to determine K_i values and inhibition types of the indazoles using paraoxon as substrate was measurement at five different substrate concentration (0.15, 0.3, 0.45, 0.60, 0.75 mM) and three different indazole concentration. The values obtained from these measurements were plotted with Lineweaver–Burk graphs. K_i values and inhibition types were determined from these graphs (Lineweaver and Burk 1934).

Molecular docking studies

The crystal structure of hPON1 in complex with 2-hydroxyquinoline (PDB ID: 3SRG) (Ben-David *et al.* 2012) was prepared by the Protein Preparation Wizard of Schrödinger (Sastry *et al.* 2013). Three-dimensional structures of A, D, G, and H were obtained using the ligand preparation tool of Maestro. The co-crystallized 2-hydroxyquinoline was selected as the center of the docking box, and the grid files were created in the receptor grid generation section. The docking scores were obtained using the Glide Extra Precision (Glide XP) module of the Schrödinger to determine the binding energy of the compounds to PON1 (Halgren *et al.* 2004, Friesner *et al.* 2006).

Results

In this study, PON1 was purified from human serum using DEAE-Sephadex anion exchange and Sephadex G-100 gel filtration chromatography. The overall purification, hPON1 was

obtained with a yield of 13.50% a specific activity of 5000 U/mg proteins, and this enzyme was purified ~290.36-fold (Table 1). The purity of the enzyme was checked by SDS-PAGE and the hPON1 with a molecular mass of about 43 kDa was displayed as a single band in the gel (Figure 2). K_m and V_{max} values were determined 2.196 mM and 94.34 U/ml, respectively. These results are consisted with the literature (Reiner *et al.* 1989, Sinan *et al.* 2006). After the purification steps, the *in vitro* inhibition effects of A–K molecules on the purified enzyme were investigated. Activity%-[Indazole] graphs were drawn to determine the IC_{50} values and IC_{50} values were found as to be 358, 133, 154, 166, 331, 86.40, 72.90, 282, 249, and 226 μM for A, H, I, K, E, F, G, B, C, and D, respectively. Lineweaver–Burk graphs were drawn by studying at five different paraoxonase concentrations and three different inhibitor concentrations to determine K_i values and inhibition types for the each indazole molecule. K_i values were found as to be 111 ± 31.0, 37.0 ± 10.0, 55.0 ± 3.00, 68.0 ± 12.0, 69.0 ± 6.00, 41.0 ± 2.00, 26.0 ± 3.00, 81.0 ± 24.0, 49.0 ± 16.0, and 45.0 ± 15.0 μM for A, H, I, K, E, F, G, B, C, and D, respectively (Table 2). All indazole molecules exhibited competitive inhibition. In addition, molecular docking studies

Table 2. IC_{50}, K_i values and inhibition types of indazole compounds for hPON1.

Indazoles	IC_{50} (μM)	K_i (μM)	Inhibition type
A	358	111 ± 31.0	Competitive
B	282	81.0 ± 24.0	Competitive
C	249	49.0 ± 16.0	Competitive
D	226	45.0 ± 15.0	Competitive
E	331	69.0 ± 6.00	Competitive
F	86.40	41.0 ± 2.00	Competitive
G	72.90	26.0 ± 3.00	Competitive
H	133	37.0 ± 10.0	Competitive
I	154	55.0 ± 3.00	Competitive
K	166	68.0 ± 12.00	Competitive

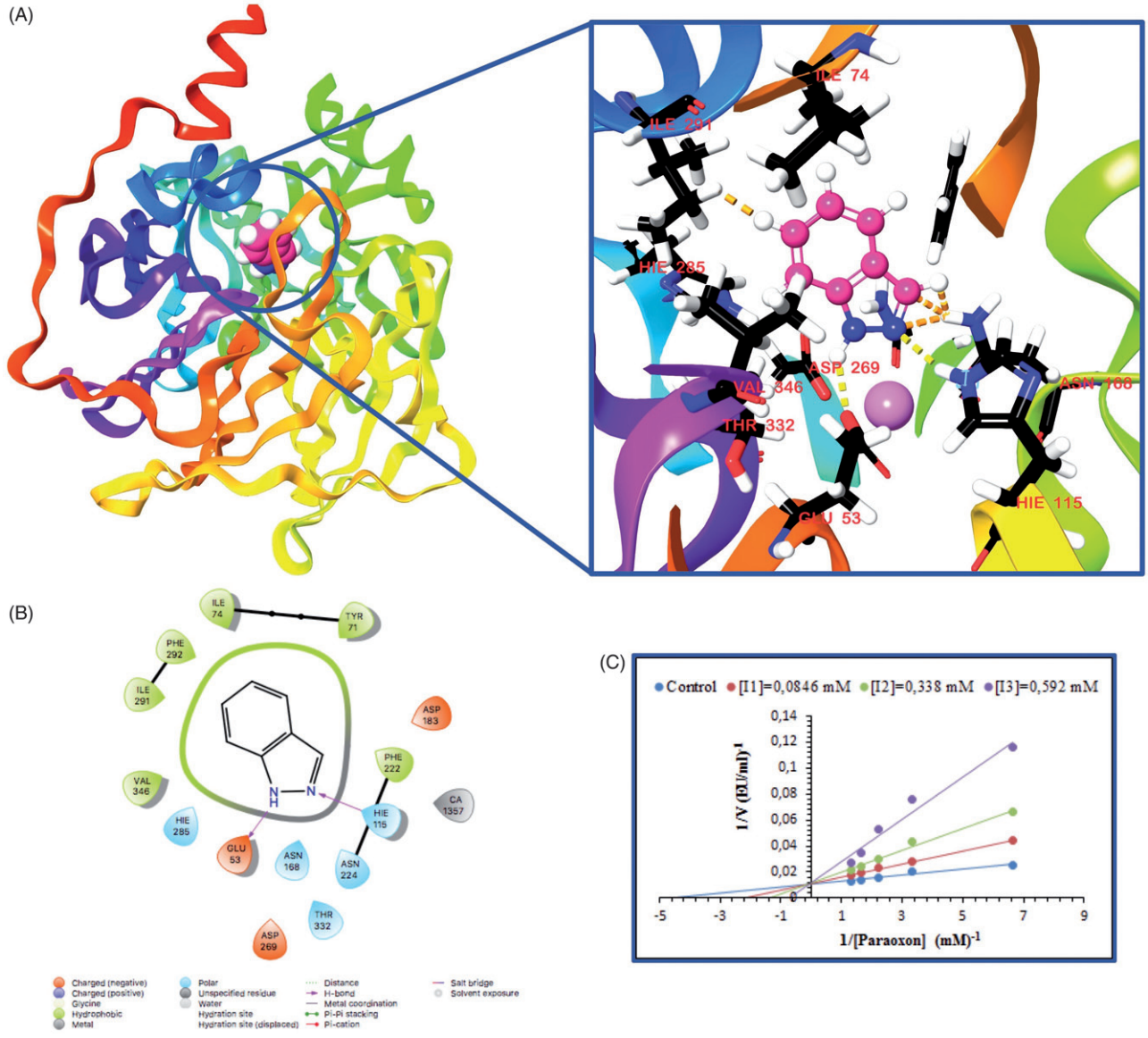

Figure 3. Docking pose of compound 1H-indazole in ligand binding site of hPON1 (PDB ID: 3SRG). In the image, the key residues in ligand binding site are represented in ball and stick model with black carbon atoms, white hydrogen atoms, blue nitrogen atoms and red oxygen atoms. Ca atom is represented as pink ball. Compound 1H-indazole is represented in ball and stick model with salmon pink carbon atoms and white hydrogen atoms, blue nitrogen atoms (A). Molecular interactions along with nearby residues of compound 1H-indazole are represented in 2D diagram. Pink arrows symbolize a hydrogen bond interaction with a protein (B). Lineweaver–Burk graph of 1H-indazole (C).

were performed for A, D, G, and H compounds in order to assess the probable binding mechanisms into the active site of hPON1 (Figures 3–6). The docking scores of these molecules were found to be −6.27, −6.62, −6.84, and −7.50 kcal/mol for A, D, G, and H, respectively by using the Glide/XP. The poses of A, D, G, and H in the ligand-binding site of the hPON1 are represented in (Table 3 and Figures 3(A)–6(A)).

Discussion

Atherosclerosis is the formation of fat and cholesterol plaques that inhibit or slow the flow of blood on the inner surface of the arteries (Galkina and Ley 2009). Each of the endogenous and exogenous factors such as age, gender, smoking, family history of ischemic heart disease, hypercholesterolemia, diabetes mellitus and hypertension significantly increases atherosclerosis (Simon and Vijayakumar 2013). The most important step in the development of atherosclerosis is the exchange of low-density lipoprotein (LDL) with oxidation. Thus, inhibition of oxidative modification of LDL is an important step towards atherosclerosis (Alberti *et al.* 2002). Thus, the PON1 enzyme plays a vital role in the prevention of atherosclerosis (Alici *et al.* 2008). PON1 also plays an important role in the development of many diseases such as hyperthyroidism (Azizi *et al.* 2003), diabetes (Abbott *et al.* 1995), cancer, aging (Mackness and Mackness 2015), obesity (Ferre *et al.* 2013), Alzheimer's disease (Pi *et al.* 2012), anxiety disorder (Bulut *et al.* 2013), Parkinson's disease (Kirbas *et al.* 2014), and many other diseases associated with oxidative damage. For this reason, this enzyme has become a focus for many researchers (Ekinci and Beydemir 2009a, Isgor and Beydemir 2010, Alim and Beydemir 2016).

Recently, there has been a growing interest in studies to determine the role of PON1 in drug metabolism (Furlong *et al.* 2016). There are many reports on the interactions of PON1 with various drugs or bioactive molecules. Generally,

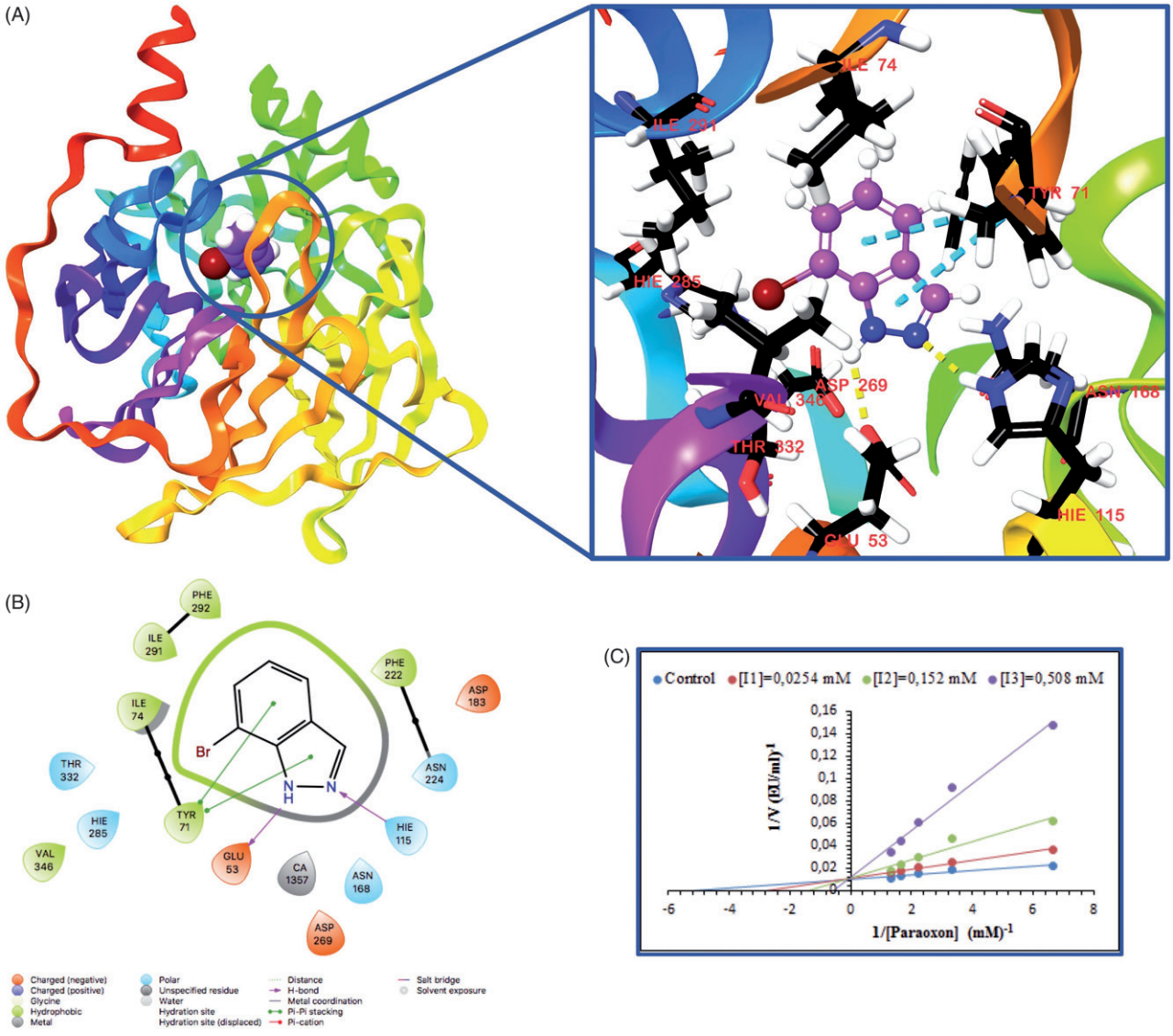

Figure 4. Docking pose of compound 7-bromo-1H-indazole in ligand binding site of hPON1 (PDB ID: 3SRG). In the image, the key residues in ligand binding site are represented in ball and stick model with black carbon atoms, white hydrogen atoms, blue nitrogen atoms and red oxygen atoms. Ca atom is represented as pink ball. Compound 7-bromo-1H-indazole is represented in ball and stick model with purple carbon atoms and white hydrogen atoms, blue nitrogen atoms, dark red bromine atom (A). Molecular interactions along with nearby residues of compound 7-bromo-1H-indazole are represented in 2D diagram. Pink arrows symbolize a hydrogen bond interaction with a protein and two-point green line represents π–π stacking (B). Lineweaver–Burk graph of 7-bromo-1H-indazole (C).

these studies focus on the pharmacological aspects of PON1 and the interaction between the enzyme with various drugs due to the association of PON1 with various diseases. All these studies are significant to better understand of the role of PON1 in drug metabolism. In addition, it is necessary to know which drugs and nutrients reduce or increase PON1 activity in order to prevent atherosclerosis and other diseases associated with oxidative stress. Many studies have been conducted on the modulation of hPON1 activity by drugs. For example, recent studies have shown that some drugs, such as analgesics (Ekinci and Beydemir 2009b), anesthetics (Senturk *et al.* 2011), calcium channel blockers (Turkes *et al.* 2014), antibacterial agents (Turkes *et al.* 2015), chemotherapeutics (Alim and Beydemir 2016), and antiepileptics (Beydemir and Demir 2016) reduce human serum PON1 activity. In addition, the effects of chemicals with prominent biological activity such as sulfonamides (Ekinci *et al.* 2010, Alim *et al.* 2017) and dihydroxycoumarin derivatives (Erzengin *et al.* 2012) on hPON1 activity were examined and it was determined that these molecules exhibit an inhibitory effect on hPON1 activity. In this study, we aimed to investigate the effects of some indazole molecules on hPON1 activity. The indazoles are important heterocyclic compounds having a wide range of biological and pharmacological applications (Gaikwad *et al.* 2015).

In the direction of our purpose, firstly the PON1 enzyme was purified from human serum. Purification process is important for a better understanding of the catalytic and kinetic properties of the enzyme. In this study, we purified PON1 from human serum by using DEAE–Sephadex anion exchange and Sephadex G-100 gel filtration chromatography. After purification process, the *in vitro* inhibition effects of the

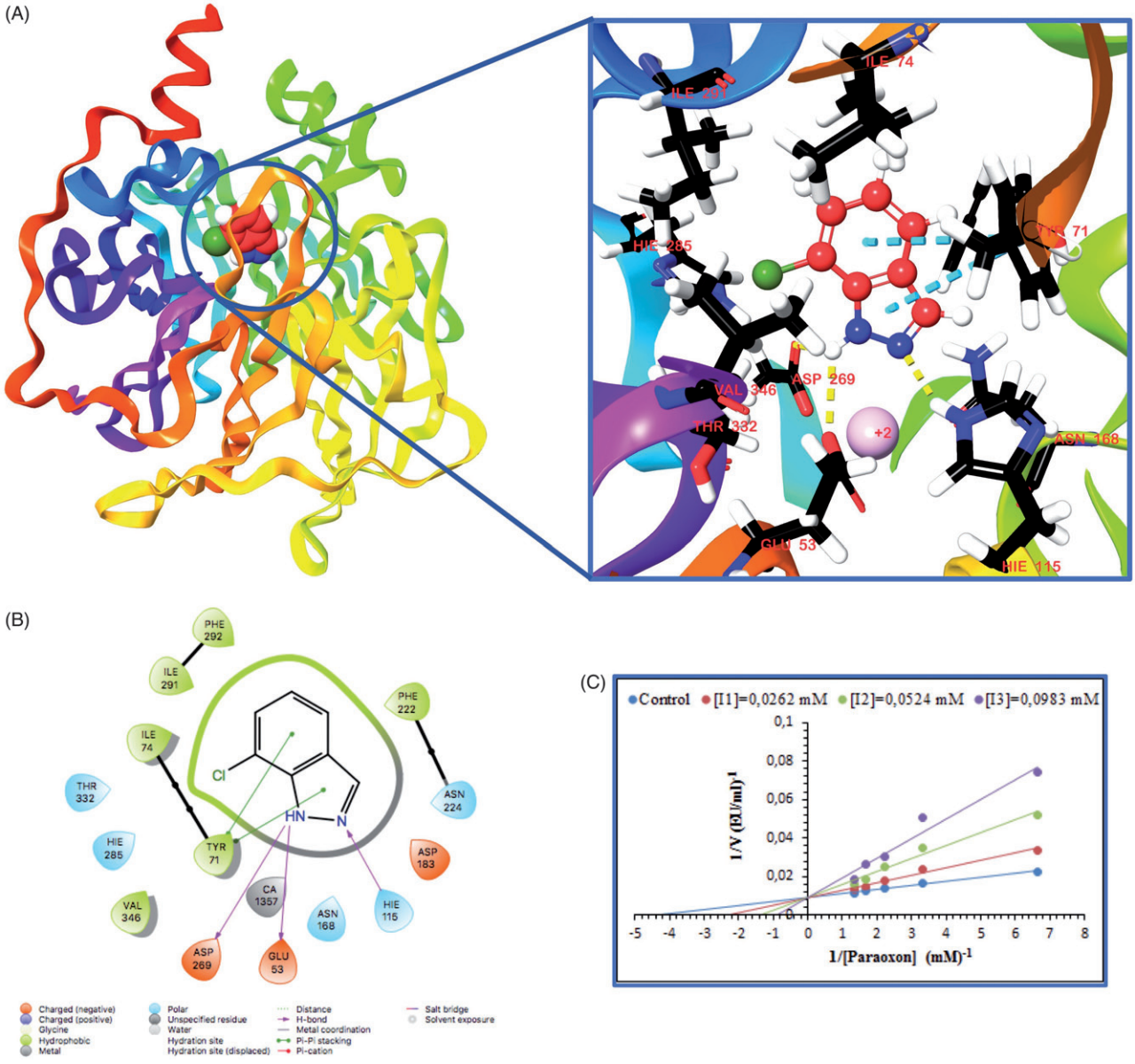

Figure 5. Docking pose of compound 7-chloro-1H-indazole in ligand binding site of hPON1 (PDB ID: 3SRG). In the image, the key residues in ligand binding site are represented in ball and stick model with black carbon atoms, white hydrogen atoms, blue nitrogen atoms and red oxygen atoms. Ca atom is represented as pink ball. Compound 7-chloro-1H-indazole is represented in ball and stick model with red carbon atoms and white hydrogen atoms, blue nitrogen atoms, dark green chlorine atom (A). Molecular interactions along with nearby residues of compound 7-chloro-1H-indazole are represented in 2D diagram. Pink arrows symbolize a hydrogen bond interaction with a protein and two-point green line represents π–π stacking (B). Lineweaver–Burk graph of 7-chloro-1H-indazole (C).

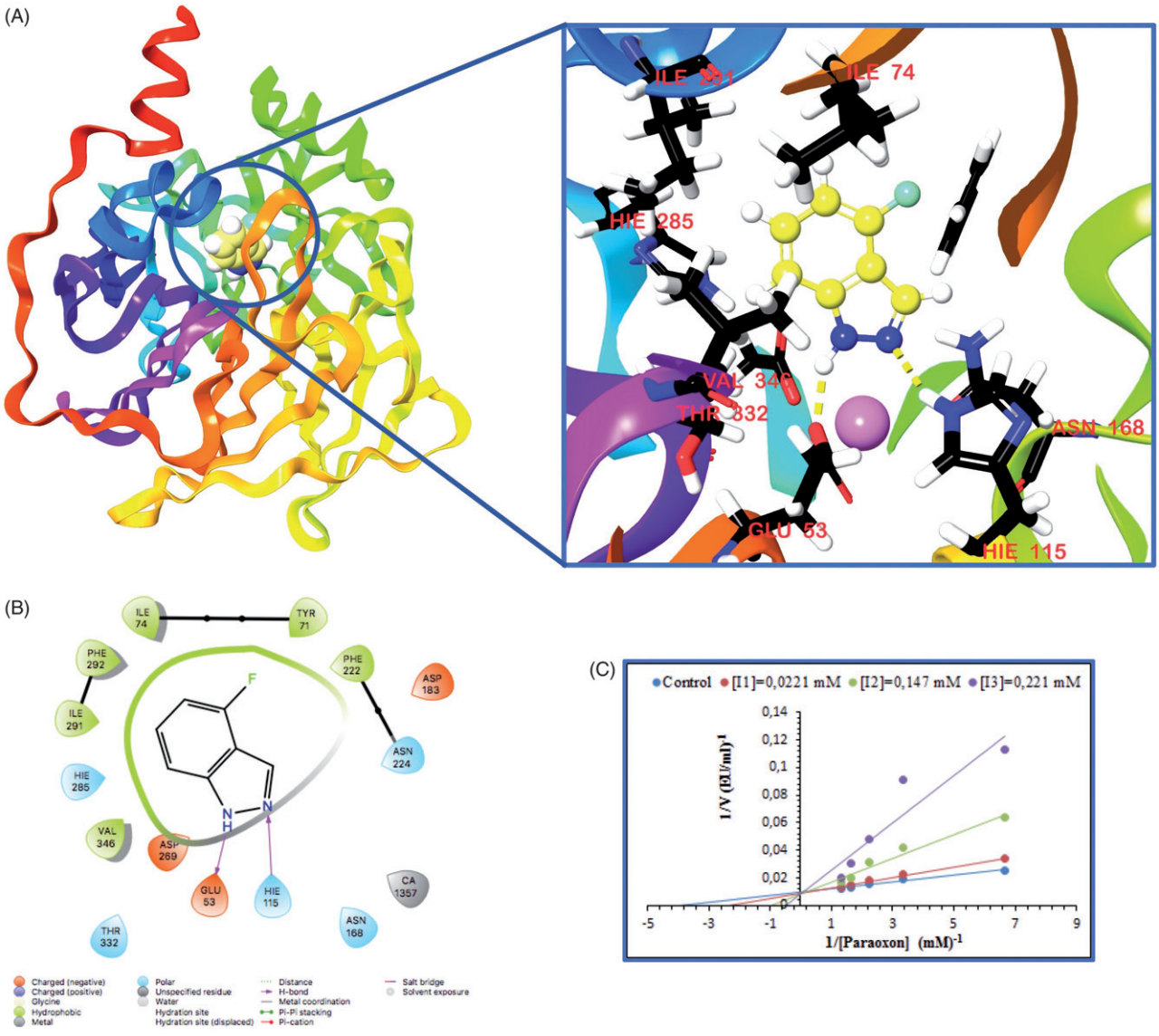

Figure 6. Docking pose of compound 4-fluoro-1H-indazole in ligand binding site of hPON1 (PDB ID: 3SRG). In the image, the key residues in ligand binding site are represented in ball and stick model with black carbon atoms, white hydrogen atoms, blue nitrogen atoms and red oxygen atoms. Ca atom is represented as pink ball. Compound 4-fluoro-1H-indazole is represented in ball and stick model with yellow atoms and white hydrogen atoms, blue nitrogen atoms, green fluorine atom (A). Molecular interactions along with nearby residues of compound 4-fluoro-1H-indazole are represented in 2D diagram. Pink arrows symbolize a hydrogen bond interaction with a protein (B). Lineweaver–Burk graph of 4-fluoro-1H-indazole (C).

Table 3. Molecular docking scores and the interactions of A, D, G and H molecules with the ligand binding site of hPON1.

Indazole compounds	Hydrogen bonds	Pi-pi interaction	Polar interactions	Hydrophobic interactions	Negative charge interactions	Docking score (kcal/mol)
A	GLU53, HIE115	–	HIE115, HIE285, ASN168, ASN224, THR332	PHE222, TYR71, ILE74, ILE291, VAL346	GLU63, ASP269, ASP183	−6.27
D	GLU53, HIE115	TYR71	THR332, HIE285, ASN168, ASN224, HIE115	TYR71, VAL346, ILE291, PHE292, ILE74, PHE222	GLU63, ASP269, ASP183	−6.62
G	GLU53, HIE115, ASP269	TYR71	THR332, HIE285, HIE115, ASN224, ASN168	TYR71, PHE222, ILE74, PHE292, ILE291, VAL346	GLU63, ASP269, ASP183	−6.84
H	GLU53, HIE115	—	ASN168, HIE115, ASN224, THR332, ASN270, HIE285	LEU69, VAL346, LEU240, PHE347, ILE74, ILE291, PHE222, PHE292, TYR71	GLU63, ASP269, ASP183	−7.50

some indazoles on the purified enzyme were investigated. Both the IC_{50} and K_i parameters of the each indazole compounds were determined in this study from Activity%-[Inhibitor] graphs and Lineweaver–Burk graphs (1/V-1/[S]), respectively. IC_{50} values were found by Activity%/[Inhibitor] to be 358, 133, 154, 166, 331, 86.4, 72.9, 282, 249, and 226 μM for A, H, I, K, E, F, G, B, C, and D, respectively. As is known, the inhibitor with the smaller IC_{50} value has a higher inhibitory effect. According to this, the order of the inhibitors is as follows: G > F> H > I > K > D> C > B> E > A. As can be seen, the different order of binding of fluoro, chloro, and bromo to the 1H-indazole molecule caused different inhibitory effects of these molecules on PON1 activity.

The K_i values and inhibition types of the indazole molecules were determined from the Lineweaver–Burk curves using five different paraoxon and three different inhibitor concentrations. K_i values were found to be 111 ± 31.0, 37.0 ± 10.0, 55.0 ± 3.00, 68.0 ± 12.0, 69.0 ± 6.00, 41.0 ± 2.00, 26.0 ± 3.00, 81.0 ± 24.0, 49.0 ± 16.0, and 45.0 ± 15.0 μM for A, H, I, K, E, F, G, B, C, and D, respectively. The inhibitor with a small K_i constant has a greater affinity for the enzyme and its inhibitory effect on the catalytic activity of the enzyme is known to be great. According to our results, the G molecule has a higher affinity for hPON1 than other molecules, and the inhibitory effect of this molecule on hPON1 catalytic activity is greater. This result is also supported by the IC_{50} value we determined for the G molecule. In addition to this, we also found that these indazoles exhibited competitive inhibition. Accordingly, all these indazole molecules may have a connection with the amino acids of the hPON1 active site. Molecular docking studies are important to show the interaction between a small molecule and a protein at the atomic level. Nowadays, molecular docking has become an increasingly useful tool in drug design studies by finding a wide range of research fields (Xuan-Yu *et al.* 2011). Thus, we performed the molecular docking studies in order to determine the probable binding mechanism of A, D, G, and H into the active site of the hPON1. Docking scores of these molecules were found to be −6.27, −6.62, −6.84, and −7.50 kcal/mol for A, D, G, and H, respectively by using the Glide/XP docking protocols (Table 3).

In this study, we showed 3D images and 2D diagrams of the docking poses of A, D, G, and H in the ligand binding site of the hPON1 receptor. 2D structure diagrams are widely used in the scientific literature to represent interactions between ligands and biomacromolecules (Caboche 2013). The interactions of A, D, G, and H molecules with the ligand binding site of hPON1 are given in Table 3 and Figures 3(B)–6(B). Docking results and K_i values, we obtained are in conformity with each other (Figures 3(C)–6(C)).

Conclusions

In conclusion, we observed that the indazoles reduced the hPON1 activity at low concentrations by binding to the active site of the hPON1 enzyme. In general, the results of this study suggest that caution should be exercised in anticipation of PON1 inhibition in the use of drugs containing indazole molecule groups with significant biological activity. In addition to, the results of this study will help us to identify new therapeutic strategies for the indazoles.

Disclosure statement

No potential conflict of interest was reported by the authors.

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