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Functionalized imidazolium and benzimidazolium salts as paraoxonase 1 inhibitors: Synthesis, characterization and molecular docking studies

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ABSTRACT

Paraoxonase (PON) is a key enzyme in metabolism of living organisms and decreased activity of PON1 was acknowledged as a risk for atherosclerosis and organophosphate toxicity. The present study describes the synthesis, characterization, PON1 inhibitory properties and molecular docking studies of functionalized imidazolium and benzimidazolium salts (**1a**–**5g**). The structures of all compounds were elucidated by IR, NMR, elemental analysis and structures of compounds **2b** and **2c** were characterized by single-crystal X-ray diffraction. Compound **1c**, a coumarin substituted imidazolium salt showed the best inhibitory effect on the activity of PON1 with good IC_{50} value (6.37 μM). Kinetic investigation was evaluated for this compound and results showed that this compound is competitive inhibitor of PON1 with K_i value of 2.39 μM. Molecular docking studies were also performed for most active compound **1c** and one of least active compound **2c** in order to determine the probable binding model into active site of PON1 and validation of the experimental results.

1. Introduction

Paraoxonase 1 (PON1) is the best studied member of the mammalian enzyme family including PON1, PON2 and PON3. PON1 is the calcium-dependent and high-density lipoprotein (HDL) associated enzyme that has 355 amino acids with a molecular mass of 43 kDA.[1–3] PON1 catalyses the hydrolysis of lactones,[4] thiolactones,[5] aromatic esters[6] and organophosphates,[7] but physiological substrate of this enzyme is still unknown. PON1 is a key enzyme in metabolism especially for two reasons; (i) it hydrolyses organophosphates such as serin and soman and protects the nervous system against the neurotoxicity of organophosphates[7] and (ii) it prevents the oxidation of low-density lipoproteins and reduced levels of oxidized lipids are involved in the initiation of atherosclerosis so decreased activity of PON 1 was acknowledged as a risk for atherosclerosis.[3] Therefore all factors affecting on PON1 activity have to be well understood. It is known that almost all chemical reactions in metabolism of living organisms are catalyzed by enzymes and they are important drug targets and effects of bioactive compounds on PON1 activity must also be examined.

Heterocyclic rings are widely found in the structures of large number of natural products and they are part of various biologically active compounds so synthesis of heterocyclic compounds is important target for organic chemists. Therefore, synthesis and biological evaluation of heterocyclic compounds are still the subject of intensive research area.[8–15] In addition, cyclic structures are more conformationally restricted than their acyclic analogues and this fact make them more selective and bioactive.[16,17]

Azoles and coumarins are well known and widely investigated class of compounds among the heterocyclic compounds. Imidazole, triazole and benzimidazole derived compounds have attracted great interest due to their wide range of pharmacological properties including anticancer, antitumor, antimicrobial, antiviral, anti-inflammatory and anticoagulant.[8–13] Coumarin derivatives have been widely investigated owing to their diverse pharmacological

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properties such as anticoagulant, anticancer, anti-HIV, antimicrobial[14,15] and carbonic anhydrase inhibitor.[18] As shown, these small heterocyclic compounds act as highly functional scaffolds and their vital role in drug design cannot be denied.

In our previous paper, we reported the inhibition of PON1 by some hydroxy coumarin-benzimidazole hybrid compounds (doi:10.3109/14756366.2015.1043297)[19] In the present study, we have synthesized nine imidazolium and eight benzimidazolium derivatives which containing coumarin, triazole, benzimidazole, benzoxazinone and some aryl or alkyl groups as substituent. The synthesized compounds were characterized by IR, NMR, elemental analysis and single-crystal X-ray diffraction. Among the various activities of these compounds, anti-coagulant effects of coumarin and benzimidazole derivatives make them more attractive especially for interactions with PON. Herein, we examined the in vitro effects of these twenty compounds which were functionalized with different substituents. Moreover, in order to determine the probable binding interactions, molecular docking studies were performed on PON1 active site.

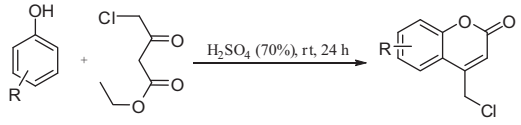
2. Results and discussion

2.1. Synthesis and spectral characterization of compounds (1a–5g)

Compounds **C1**–**3** were synthesized by the procedure described in literature by Frasinyuk[20] as shown in Scheme 1. Synthesis and structures of imidazolium salts (**1a**–**3c**) and benzimidazolium salts (**5a**–**g**) were outlined in Schemes 2 and 3, respectively. These compounds were synthesized by direct quaternization of 1-alkylimidazole and 1-alkylbenzimidazole derivatives with different alkyl chlorides in DMF. All compounds were obtained in good yields between 54% and 95%. In ^{1}H NMR spectra of imidazolium salts **1a**,**c**, **2a**,**c** and **3a**,**c**, signals of acidic NC*H*N protons were located in the range of 9.36–9.92 ppm while these signals were obtained in the range of 10.34–11.30 ppm for benzimidazolium salts (**5a**–**g**). These signals of acidic hydrogens are in good agreement with literature.[21] For compounds **3a**–**c**, signals of free —N*H* hydrogens were located in the range of 13.41–13.49 ppm. Signal of free —N*H* hydrogen of compound **5g** was located at 11.08 ppm. In IR spectra of all coumarin derived compounds (**C1**–**3**, **1a**–**c**, **5e**,**f**), sharp carbonyl peaks were observed in the range of 1707–1723 cm^{-1}. Two carbonyl peaks were observed at 1702 and 1685 cm^{-1} for compound **5g**. Elemental analysis of all compounds was also supportive for depicted structures. Hence, all of the synthesized compounds were characterized by ^{1}H NMR, ^{13}C NMR, IR and elemental analysis in satisfactory manner.

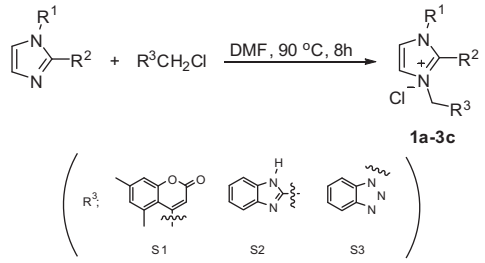
2.2. Structural description of the compounds 2b and 2c

The solid-state structures of compounds **2b** and **2c** have been determined by single crystal X-ray analysis. The perspective ORTEP-3 views of the compounds with the adopted atomic numbering scheme are depicted in Figures 1 and 2, while selected bond lengths and angles are given in Table 1. The crystallization charac-



C1, R: 5,7-$(CH_3)_2$; **C2**, R: 7,8-$(CH_3)_2$; **C3**, R: 6-$C(CH_3)_3$

Scheme 1. Synthesis of compounds **C1**–**3**.

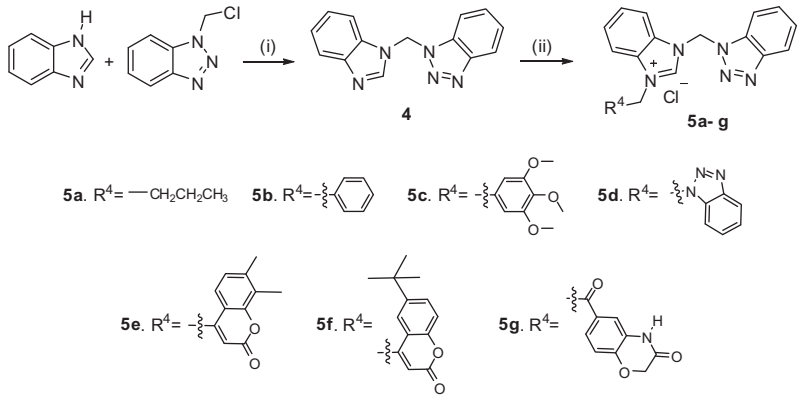


Compound no	R^1	R^2	R^3
1a	-CH_3	-H	S1
1b	-CH_3	-CH_3	S1
1c	-$CH_2CH_2CH_2CH_3$	-H	S1
2a	-CH_3	-H	S2
2b	-CH_3	-CH_3	S2
2c	-$CH_2CH_2CH_2CH_3$	-H	S2
3a	-CH_3	-H	S3
3b	-CH_3	-CH_3	S3
3c	-$CH_2CH_2CH_2CH_3$	-H	S3

Scheme 2. Synthesis and structures of imidazolium salts.

teristics of the two compounds are different, with compounds **2c** and **2b** crystallizing in the space groups $P2_1/c$ and $P\bar{1}$, respectively. In both compounds, the charge is balanced by a Cl^- anion. The asymmetric unit in the crystal structure of compound **2b** contains the two independent molecules, labelled A and B, and one water solvent molecule. In the following discussion, parameters for molecule B are quoted in square brackets.

The imidazole ring is planar with an rms deviation of 0.0012 Å for compound **2c**, and 0.0023 Å [0.0034 Å] for compound **2b**. The bonding within the imidazole ring indicates a pattern of delocalization that extends from atom N4 to atom N5 through atom C8, the N4—C8 {1.313(3) Å in compound **2c**, 1.346(2) Å [1.3388(19) Å] in compound **2b**} and N5—C8 {1.322(3) Å in compound **2c**, 1.323 (2) Å [1.333(2) Å] in compound **2b**} distances being significantly shorter than the N4—C10 {1.376(3) Å in compound **2c**, 1.380(2) Å [1.379(2) Å] in compound **2b**} and N5—C9 {1.373(3) Å in compound **2c**, 1.371(2) Å [1.380(2) Å] in compound **2b**} distances. The N1—C7 bond lengths of 1.446(3) and 1.440(2) Å [1.445(2) Å] in compounds **2c** and **2b**, respectively, show the C—N single bond character. The N1—C6 and N3—C1 bond lengths in the benzotriazole ring are 1.356(3) and 1.378(3) Å in compound **2c**, and 1.365 (2) and 1.379(3) Å [1.359(2) and 1.377(3) Å] in compound **2b**, and N1—N2 and N2—N3 bond lengths are 1.359(3) and 1.301 (3) Å in compound **2c**, and 1.356(2) and 1.291(2) Å [1.3615(19) and 1.298(3) Å] in compound **2b**, respectively. These shorter lengths suggest that the C—N and N—N bonds in benzotriazole ring have part double bond character. In these bond lengths, the N2—N3 bond is relatively shorter, which shows that the N2—N3 distance has relatively stronger double bond character. All nitrogen atoms in the benzotriazole ring (N1, N2 and N3) are sp^2 hybridized and occupy positions in the plane of the aromatic moiety. The rms deviation of the benzotriazole ring atoms from their mean plane is 0.0104 Å and 0.0124 Å [0.0089 Å] for compounds **2c** and **2b**, respectively. The triazole and benzene rings of the benzotriazole moiety are inclined to one another at an angle of 0.793(13)° in



Scheme 3. Synthesis and structures of benzimidazolium salts. Reagents and conditions: (i) KOH, EtOH, reflux, 24 h, (ii) R^4CH_2Cl, DMF, 90 °C, 8 h.

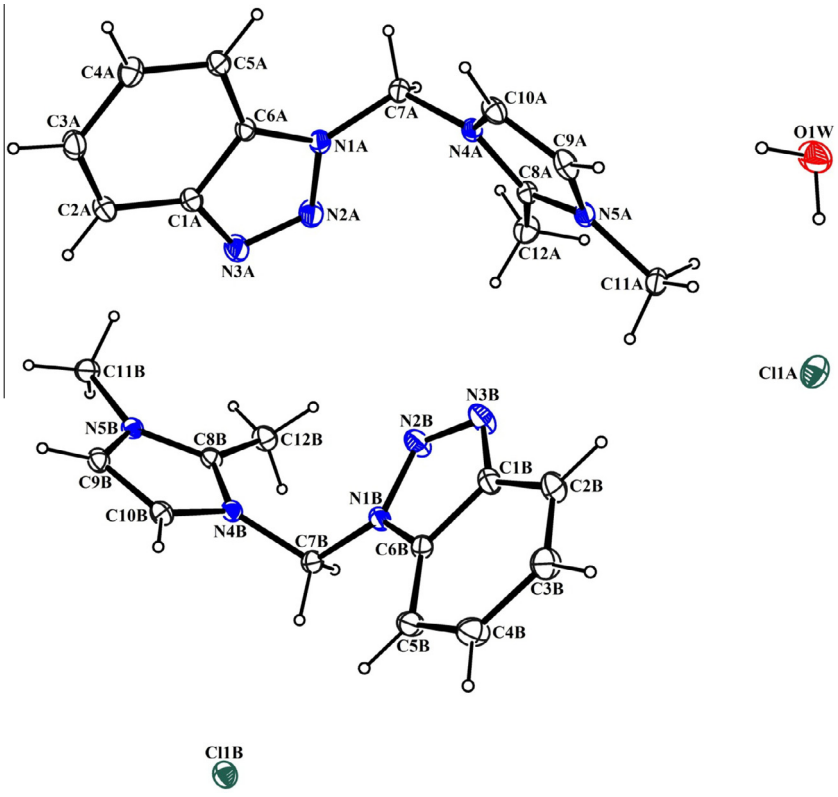


Figure 1. A view of compound **2b** showing the atom-numbering scheme. Displacement ellipsoids are drawn at the 10% probability level and H atoms are shown as small spheres of arbitrary radii.

compound **2c**, and 1.617(16)° [1.120(11)°] in compound **2b**. Furthermore, The mean plane of the benzotriazole system makes a dihedral angle of 71.536(82)° in compound **2c**, and 69.826(52)° [65.973(59)°] in compound **2b** with the imidazole ring. In both compounds, all the bond lengths and angles are within normal ranges[22] and comparable to those in the related compounds.[23–28]

2.3. Enzyme inhibition and structure activity relationship

As mentioned in Section 1, inhibition or activation properties of bioactive compounds on PON1 activity have to be well understood. According to our literature survey, inhibition effects of some drugs or metal ions on PON1 activity were reported[29–33] but studies about interactions of novel compounds and PON1 are scarce. Erzengin et al. reported the inhibition effects of some coumarin derivatives and they showed that 6,7-dihydroxy-3-(4-methylphenyl)-2*H*-chromen-2-one inhibits PON1 with IC_{50} value of 3 μM.[34] In addition, Akbaba et al. reported the interactions of some brominated compounds and bromophenols with PON1 and compounds inhibited PON1 with different sensitivities.[35]

In the present study, the effects of seven-teen imidazole and benzimidazole derivatives and three simple coumarin on purified PON1 were investigated. These compounds were synthesized first time in this study. For evaluating the PON1 activity, all compounds were subjected to PON1 inhibition assay with paraoxon as substrate. The IC_{50} values of compounds were given in Table 2. The results showed that twelve of seventeen imidazolium or benzimidazolium salts inhibit PON1 with different sensitivities while five compounds show no activity. IC_{50} values of active compounds were determined in the range of 6.37–3333.30 μM. Among the

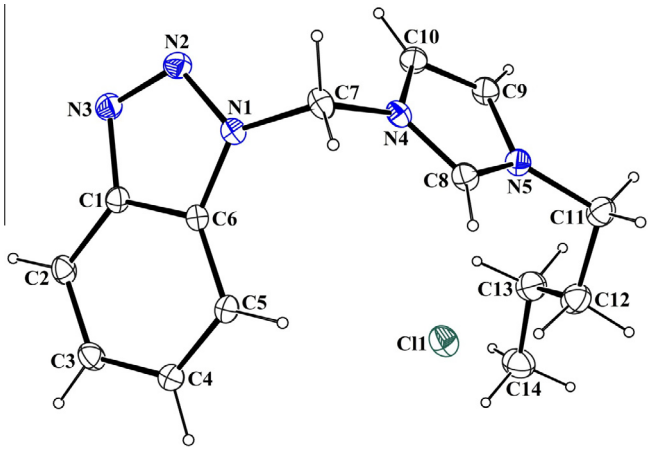


Figure 2. A view of compound **2c** showing the atom-numbering scheme. Displacement ellipsoids are drawn at the 10% probability level and H atoms are shown as small spheres of arbitrary radii. For clarity, only the major part of the disordered butyl moiety is shown.

Table 1
Selected geometric parameters for compounds **2b** and **2c**

Parameters	Compound **2c**	Compound **2b**	
		Molecule A	Molecule B
Bond lengths (Å)			
N1—N2	1.359(3)	1.356(2)	1.3615(19)
N1—C6	1.356(3)	1.365(2)	1.359(2)
N1—C7	1.446(3)	1.440(2)	1.445(2)
N2—N3	1.301(3)	1.291(2)	1.298(2)
N3—C1	1.378(3)	1.379(3)	1.377(3)
N4—C7	1.460(3)	1.458(2)	1.456(2)
N4—C8	1.313(3)	1.346(2)	1.3388(19)
N4—C10	1.376(3)	1.380(2)	1.379(2)
N5—C8	1.322(3)	1.323(2)	1.333(2)
N5—C9	1.373(3)	1.371(2)	1.380(2)
N5—C11A	1.483(4)	1.469(2)	1.465(2)
N5—C11B	1.483(4)	—	—
C9—C10	1.336(4)	1.335(3)	1.341(2)
C8—C12	—	1.472(2)	1.474(2)
Bond angles (°)			
N1—N2—N3	108.58(19)	108.43(15)	108.18(15)
N1—C7—N4	110.83(19)	111.50(13)	112.24(13)
N4—C8—N5	109.0(2)	107.28(14)	107.21(13)
N1—C6—C1	104.29(19)	103.58(14)	104.08(14)
C6—N1—N2	110.33(18)	110.67(13)	110.51(13)
C6—N1—C7	129.5(2)	129.62(14)	129.59(13)
N2—N1—C7	120.2(2)	119.71(14)	119.63(14)
N2—N3—C1	108.23(19)	108.80(15)	108.66(14)
N3—C1—C6	108.6(2)	108.52(15)	108.56(15)
C8—N4—C10	108.39(19)	108.73(14)	109.02(13)
C8—N4—C7	124.9(2)	126.19(14)	125.63(13)
C10—N4—C7	126.7(2)	125.02(15)	125.30(13)
C9—C10—N4	107.2(2)	106.95(16)	107.43(14)
C8—N5—C9	108.4(2)	109.55(15)	109.64(13)
C8—N5—C11A	125.7(4)	124.82(17)	125.68(15)
C9—N5—C11A	125.7(4)	125.63(16)	124.67(15)
C8—N5—C11B	125.0(7)	—	—
C9—N5—C11B	124.4(7)	—	—
C10—C9—N5	107.1(2)	107.49(15)	106.71(15)
N4—C8—C12	—	127.27(15)	126.72(15)
N5—C8—C12	—	125.45(16)	126.07(15)
N5—C11A—C12A	110.2(3)	—	—
N5—C11B—C12B	110.1(3)	—	—

tested compounds, **1c** was found out as most active compound with 6.37 μM value of IC_{50}. Kinetic investigation was performed for the compound **1c** and results showed that this compound inhibits PON1 activity in a competitive manner with K_i value of 2.39 μM (Table 3, Fig. 3).

In our previous study, we reported the inhibition of PON1 with benzimidazole-coumarin hybrid compounds. In the present study, we aimed to examine the effects of some different substituents to the inhibitory properties. Here we also examine whether the inhibitory properties of previously reported compounds are specific to combination of benzimidazole and coumarin or it arises from coumarin scaffold or some other substituents. For this purpose, firstly coumarin compounds **C1**–**3** were investigated. Results showed that these compounds have good inhibitory properties and IC_{50} values were ranged between 21.37 and 40.00 μM. Later, inhibitory properties of compounds **1a**–**c** were investigated and compounds **1a** and **1c** showed good inhibitory activities with IC_{50} values of 23.70 and 6.37 μM, respectively. In order to observe the effects of some different heterocyclic cores to the inhibitory activity, benzotriazole and benzimidazole moieties were attached to imidazole core. Interestingly, benzotriazole bearing imidazolium salts (**2a**–**c**) showed no activity. On the other hand, benzimidazole bearing imidazolium salts **3a** and **3c** showed good inhibitory activities with IC_{50} values of 19.05 and 28.98 μM, respectively. It was also observed that, for coumarin and benzimidazole substituted imidazolium salts, attaching a methyl group to the 2-position of imidazole core decreased activity (IC_{50} for **1b** is 317.31 μM and for **3b** 625.71 μM). When benzimidazole derived compounds were perused, compound **4** which containing benzimidazole and benzotriazole moieties inhibited PON1 activity with IC_{50} value of 564.13 μM. As seen from Table 2, quaternization of compound **4** with different substituent changed the inhibitory properties. Compounds **5a** and **5b** showed lower inhibitory activity than compound **4** with IC_{50} values of 3333.30 and 2064.20 μM, respectively. In addition, compounds **5c** and **5d** showed no activity. So it can be said that, attaching of butyl, benzyl, trimethoxybenzyl and benzotriazole moieties to compound **4** decreased inhibitory activities significantly. Attaching of 7,8-dimethyl substituted coumarin to the structure of compound **4** increased the inhibitory activity. Compound **5e** inhibited PON1 activity with IC_{50} value 237.50 μM. Among the benzimidazolium derivatives, compound **5g** which containing benzoxazinone moiety was found out as most active with IC_{50} value of 8.76 μM.

According to the results of enzyme inhibition assay the following generalizations can be made; (i) all coumarin derived compounds investigated in this study inhibited PON1, (ii) position of substituents on benzimidazole scaffold is important for inhibitory activity, benzimidazole derivatives which containing substituent on the 2-position were found out more active, (iii) benzotriazole

Table 2
IC_{50} values of the synthesized compounds

Compound no	IC_{50} (μM)
C1	31.41
C2	21.37
C3	40.00
1a	23.70
1b	317.31
1c	6.37
2a	NA
2b	NA
2c	NA
3a	19.05
3b	625.71
3c	28.98
4	564.13
5a	3333.30
5b	2064.20
5c	NA
5d	NA
5e	237.50
5f	627.00
5g	8.76

Table 3
Kinetic and molecular docking data of compounds **1c** and **2c**

Compound no	K_i (μM)	V_{max} (μmol/min)	K_m (μM)	Inhibiton type	Docking score (kcal/mole)	Close van der Waals contacts (bond length, (Å))	H-bonds (bond length, (Å))
1c	2.39	6.08	40.57	Competative	−5.4	ALA217 (4.39), GLU218 (3.47), LYS244 (5.47), TYR248 (5.17)	HIS246 (2.10)
2c	–	–	–	–	−3.6	PHE220 (5.10), HIS246 (4.87), VAl261 (3.70)	–

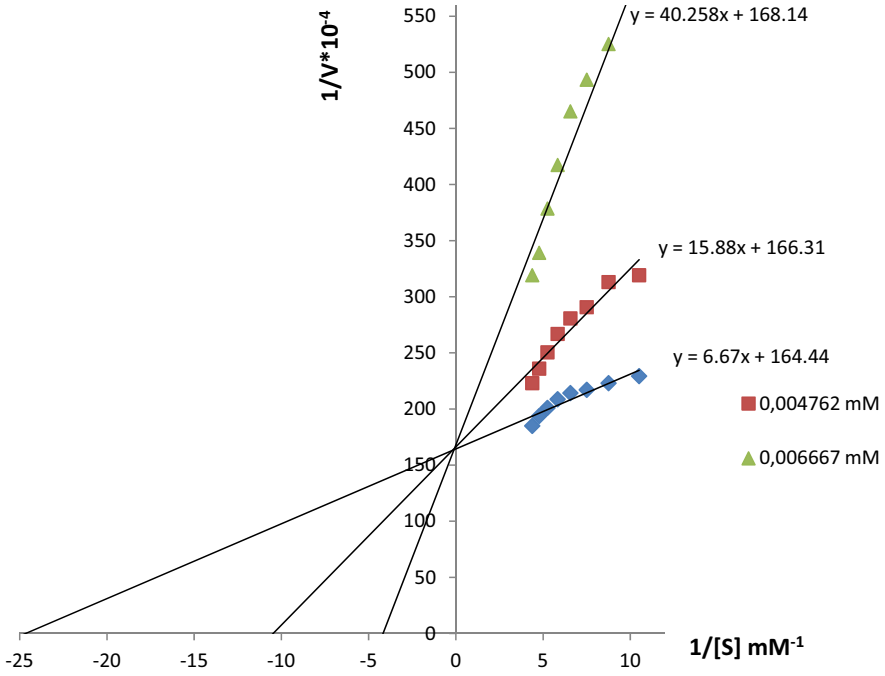


Figure 3. Inhibition of paraoxonase by compound **1c**. The slope of Lineweaver–Burk plots indicates competitive inhibition for paraoxon.

derivatives were found out as inactive against PON1, (iv) in this study only one benzoxazinone derived compound was investigated and attaching the benzoxazinone moiety to the structure of compound **4** increased inhibitory activity dramatically.

As mentioned in Section 1, PON1 catalyses the hydrolysis of lactones and in a study it was reported that, besides some lactone derivatives, dihydrocoumarin was hydrolyzed by PON1 but in the same study it was also shown that simple coumarin was not hydrolyzed by PON1.[4] As mentioned above, our results revealed that all coumarin compounds evaluated in this study inhibit PON1 activity and we suggest that coumarin derivatives are not suitable substrates for PON1 and they are likely possible inhibitors of PON1.

2.4. Molecular docking studies

Kinetic evaluation of compound **1c** showed that this compound inhibits PON1 activity in a competitive manner. Therefore molecular docking studies were performed in order to determine the probable binding model of compound **1c** into active site of PON1. Moreover, in order to validate the experimental results of compounds and for comparison with **1c**, molecular docking studies were also performed for compound **2c** which is one of the inactive compounds.

Docking scores were obtained using Lamarckian Genetic Algorithm and scoring function of AutoDock 4. After that, interactions were checked with the aid of ADT and Discovery Studio 4.0 Client. Docking scores and binding interactions of compounds **1c** and **2c** with PON1 (PDB code: 1V04)[1] are presented in Table 3. Final

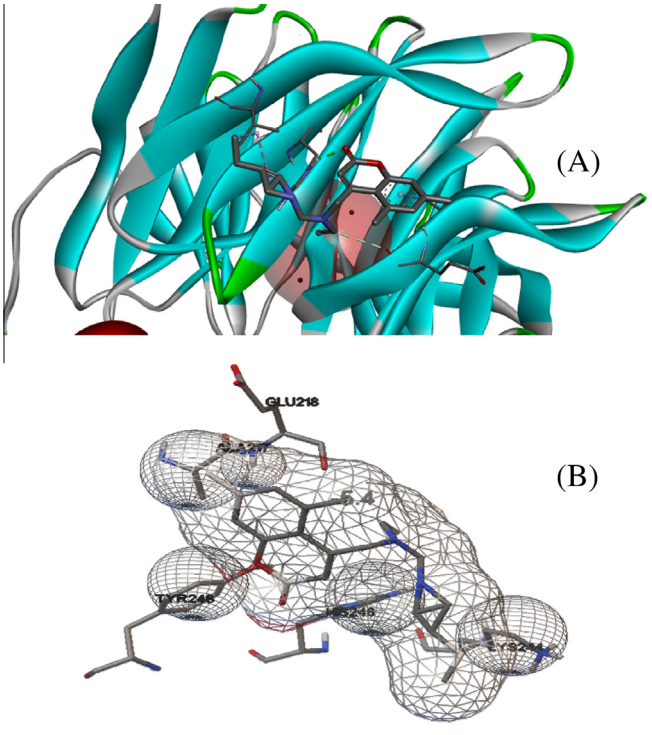


Figure 4. Docking of compound **1c** within the active site of PON1 (A) Discovery Studio 4.0 Client images and (B) ADT images.

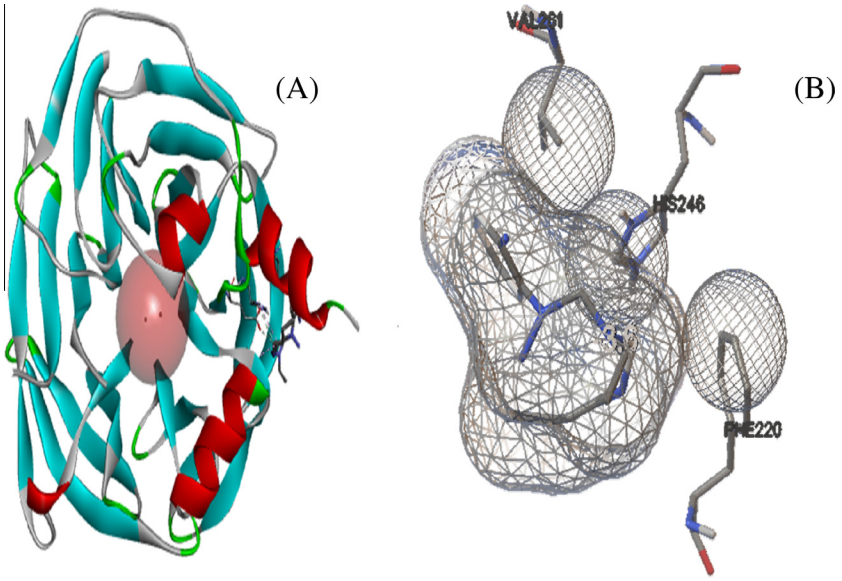


Figure 5. Docking of compound **2c** within the active site of PON1 (A) Discovery Studio 4.0 Client images and (B) ADT images.

images of compounds **1c** and **2c** for binding interactions are shown in Figures 4 and 5, respectively.

Molecular docking studies revealed that, compound **1c** which is the most active compound as PON1 inhibitory posses docking score of −5.4 kcal/mole and forms a hydrogen bond with His-246 (2.10 Å). Moreover **1c** in close van der Waals contacts with Ala-217 (4.39 Å), Glu-218 (3.47 Å), Lys-244 (5.47 Å) and Tyr-248 (5.17 Å). Compound **2c** which is an inactive compound exhibits weaker binding affinity with docking score of −3.6 kcal/mole. Compound **2c** forms no hydrogen bond and in close van der Waals contact with Phe-220 (5.10 Å), His-246 (4.87 Å) and Val-261 (3.70 Å). These results are in good agreement with experimental results. Compounds **1c** and **2c** are bearing same groups in their structures apart from coumarin or benzotriazole scaffold and stronger binding affinity of **1c** can attributable with coumarin scaffold. Harel et al. reported the crystal structure of recombinant PON1 which containing two calcium ions and these ions were shown essential for the catalytic activity and stability of PON1.[1] Molecular docking results also suggested that compound **1c** inhibits PON1 activity by not interaction with two calcium ions.

3. Conclusion

In summary, nine imidazolium salts and seven benzimidazolium salts were synthesized in good yields and well characterized. All compounds were subjected to PON1 inhibition assay with paraoxon as substrate. Results showed that twelve compounds inhibited PON1 and IC_{50} values were determined for active compounds. Among them, compound **1c** was found out as most active and kinetic evaluation revealed that PON1 is inhibited by **1c** in competitive manner. Molecular docking studies were performed for compounds **1c** and **2c** and results confirmed the data obtained experimentally. Moreover, according to molecular docking results **1c** inhibited PON1 by not interaction with two calcium ions.

Based on the finding in this study, we suggest that coumarins are potential inhibitory of PON1. In addition, some benzimidazole or benzoxazinone derived compounds inhibited PON1 effectively. The derivatives of coumarin, benzimidazole and benzoxazinones are being investigated as drug candidates for use in future. However inhibition of PON1 by these compounds reveals that more carefully and detailed investigations are required about dosage and some possible side effects of the derivatives of heterocyclic structures tested in this study.

4. Experimental

Chemicals and solvents were purchased from Sigma Aldrich, Merck (Istanbul-Turkey). 6-(chloroacetyl)-2*H*-1,4-benzoxazine-3(4*H*)-one, 1-(chloromethyl)-1*H*-benzo[*d*][1,2,3]triazole, 2-(chloromethyl)-1*H*-benzo[*d*]imidazole were supplied commercially and used without further purification. Melting points were determined by Electrothermal-9200 melting point apparatus. The FT-IR spectra were recorded on an ATR unit in the range of 400–4000 cm^{-1} with a Perkin Elmer Spectrum 100 Spectrophotometer. The ^{1}H NMR and ^{13}C NMR spectra were recorded using a Bruker FT spectrometer operating at 300.13 MHz (^{1}H), 75.47 MHz (^{13}C) and Bruker 600 Avance 3 HD spectrometer operating at 600.134 MHz (^{1}H) and 150.918 MHz (^{13}C). Chemical shifts are given in ppm relative to TMS. NMR multiplicities are abbreviated as follows: s = singlet, d = doublet, t = triplet, quin = quintet, sex = sextet, m = multiplet signal. Elemental analyses were performed by a LECO CHNS-932 elemental analyzer at IBTAM (Inonu University Scientific and Technological Research Center).

4.1. General procedure for the preparation of 4-chloromethylene substituted coumarin derivatives (C1–3)

4-Chloromethyl-5,7-dimethyl-2*H*-chromene-2-one (**C1**), 4-chloromethyl-7,8-dimethyl-2*H*-chromene-2-one (**C2**), 4-chloromethyl-6-tertiarybutyl-2*H*-chromene-2-one (**C3**) were synthesized by procedure described in the literature.[20] Crude products were recrystallized from ethanol.

4.1.1. 4-Chloromethyl-5,7-dimethyl-2*H*-chromene-2-one (C1)

White solid. Yield: 88%, mp: 180–181 °C, Elemental analysis: Calcd for: $C_{12}H_{11}ClO_2$; C, 64.73; H, 4.98; Found: C, 64.76; H, 4.94; IR(cm^{-1}): 3010, 2949, 1698(—C=O), 1617(—C=C—), 1603, 1550, 1453, 1423, 1389, 1272, 1231, 1161; ^{1}H NMR(300 MHz, DMSO-d_6): 7.11(s, 1H, Ar*H*), 7.04 (s, 1H, Ar*H*), 6.63(s, 1H, —C=C*H*—), 5.10(s, 2H, —CH_2Cl), 2.74(s, 3H, ArCH_3), 2.34(s, 3H, ArCH_3). ^{13}C NMR(75 MHz, DMSO-d_6): 159.4, 154.9, 152.3, 142.3, 136.1, 129.7, 115.7, 115.6, 110.1, 45.2, 22.3, 20.6.

4.1.2. 4-Chloromethyl-7,8-dimethyl-2*H*-chromene-2-one (C2)

White solid. Yield: 80%, mp: 176–177 °C, Elemental analysis: Calcd for: $C_{12}H_{11}ClO_2$; C, 64.73; H, 4.98; Found: C, 64.85; H, 4.87; IR(cm^{-1}): 2975, 2938, 1715(—C=O), 1631(—C=C—), 1602, 1563, 1419, 1371, 1281, 1260; ^{1}H NMR(300 MHz, DMSO-d_6): 7.57(d, 1H, Ar*H*, *J* = 8.1 Hz), 7.22(d, 1H, Ar*H*, *J* = 8.2 Hz), 6.60(s, 1H, —C=C*H*—), 4.99(s, 2H, —CH_2Cl), 2.36(s, 3H, ArCH_3), 2.27(s, 3H, ArCH_3). ^{13}C NMR(75 MHz, DMSO-d_6): 159.8, 151.3, 151.0, 141.8, 125.6, 123.9, 121.8, 114.7, 113.8, 41.3, 19.8, 11.2.

4.1.3. 4-Chloromethyl-6-*tert*-butyl-2*H*-chromene-2-one (C3)

White solid. Yield: 67%, mp: 134–135 °C, Elemental analysis: Calcd for: $C_{14}H_{15}ClO_2$; C, 67.07; H, 6.03; Found: C, 67.17; H, 6.12; IR(cm^{-1}): 3074, 2968, 1711(—C=O), 1606(—C=C—), 1569, 1426, 1376, 1261; ^{1}H NMR(300 MHz, DMSO-d_6): 7.78–7.36(m, 3H, Ar*H*), 6.67(s, 1H, —C=C*H*—), 5.10(s, 2H, —CH_2Cl), 1.34(s, 9H, —C(CH_3)$_3$). ^{13}C NMR(75 MHz, DMSO-d_6): 159.7, 151.3, 150.8, 146.8, 129.7, 121.4, 116.4, 116.3, 115.3, 41.4, 34.4, 31.0.

4.2. Synthesis of 1-((1*H*-benzo[*d*]1,2,3-triazol-1-yl)methyl)-1*H*-benzo[*d*]imidazole (4)

Compound **4** was synthesized by the procedure described in the literature with some differences in purification step,[36] crude product was recrystallized from ethanol/diethyl ether. White solid. Yield: 55%, mp: 191 °C, Elemental analysis: Calcd for: $C_{14}H_{11}N_5$; C, 67.46; H, 4.45; N, 28.10; Found: C, 67.54; H, 4.36; N, 28.17; IR (cm^{-1}): 3094, 1613, 1501, 1452, 1350, 1279, 1213; ^{1}H NMR (600 MHz, DMSO-d_6): 8.78(s, 1H, —NC*H*N—), 8.20–7.21(m, 8H, Ar*H*), 7.37(s, 2H, —NCH_2N—). ^{13}C NMR(150 MHz, DMSO-d_6): 145.7, 144.7, 143.9, 133.2, 132.5, 128.8, 125.1, 123.7, 123.0, 120.2, 113.3, 110.9, 55.4.

4.3. General procedure for the preparation of imidazolium (1a–3c) and benzimidazolium chlorides (5a–g)

1-Alkylimidazole or compound **4** (2 mmol) were dissolved in five mililiters of DMF. Two milimoles of corresponding alkyl chlorides was added to this solution and the resulting mixture was heated for eight hours at 90 °C. Later, the mixture was allowed to cool to ambient temperature. Ten mililiters of diethyl ether was added and precipitate was collected by filtration, The crude product was washed with hexane (2 × 10 mL) and diethyl ether (2 × 10 mL) the dried under reduced procedure. The suitable crystals of **2b** and **2c** for X-ray diffraction analysis was obtained from recrystallization of **2b** and **2c** from ethanol/diethyl ether.

4.3.1. 1-(Methyl)-3–((5,7-dimethyl-2*H*-chromen-2-one-4-yl) methyl)imidazolium chloride (1a)

White solid. Yield: 61%, mp: 279–281 °C, Elemental analysis: Calcd for: $C_{16}H_{17}ClN_2O_2$; C, 63.05; H, 5.62; N, 9.19; Found: C, 63.12; H, 5.72; N, 9.11; IR(cm^{-1}): 3070, 2955, 2942, 1707(—C=O), 1614(—C=C—), 1609, 1550, 1466, 1327, 1166; ^{1}H NMR(300 MHz, DMSO-d_6): 9.36(s, 1H, —NC*H*N—), 7.90–7.85(m, 2H, —NC*H*=C*H*N—), 7.16(s, 1H, Ar*H*), 7.10(s, 1H, Ar*H*), 6.18(s, 2H, —NCH_2coumarin), 5.49(s, 1H, —C=C*H*—), 3.95(s, 3H, —NCH_3), 2.72 (s, 3H, ArCH_3), 2.37(s, 3H, ArCH_3). ^{13}C NMR(75 MHz, DMSO-d_6): 159.1, 154.4, 152.4, 142.6, 137.8, 136.3, 129.9, 124.3, 123.0, 115.6, 114.3, 110.8, 51.3, 36.1, 23.6, 20.6.

4.3.2. 1-(Methyl)-2-(methyl)-3-((5,7-dimethyl-2*H*-chromen-2-one-4-yl)methyl)imidazolium chloride (1b)

White solid. Yield: 63%, mp: 330–332 °C, Elemental analysis: Calcd for: $C_{17}H_{19}ClN_2O_2$; C, 64.05; H, 6.01; N, 8.79; Found: C, 64.17; H, 6.12; N, 8.91; IR(cm^{-1}): 2944, 2935, 1711(—C=O), 1612 (—C=C—), 1606, 1554, 1541, 1460, 1328, 1170; ^{1}H NMR (300 MHz, DMSO-d_6): 7.71(d, 1H, —NC*H*=CHN—, *J* = 8.2 Hz), 7.57 (d, 1H, —NCH=C*H*N—, *J* = 8.1 Hz), 7.11(s, 1H, Ar*H*), 7.06(s, 1H, Ar*H*), 5.89(s, 2H, —NCH_2coumarin), 5.37(s, 1H, —C=C*H*—), 3.77(s, 3H, —NCH_3), 2.71(s, 3H, ArCH_3), 2.45(s, 3H, —NC(CH_3)N—), 2.32(s, 3H, ArCH_3). ^{13}C NMR(75 MHz, DMSO-d_6): 159.1, 154.5, 151.4, 145.9, 142.6, 136.6, 129.8, 123.2, 121.5, 115.5, 114.4, 110.0, 50.3, 35.1, 23.8, 20.6, 9.2.

4.3.3. 1-(Butyl)-3-((5,7-dimethyl-2*H*-chromen-2-one-4-yl) methyl)imidazolium chloride (1c)

White solid. Yield: 59%, mp: 266–268 °C, Elemental analysis: Calcd for: $C_{19}H_{23}ClN_2O_2$; C, 65.79; H, 6.68; N, 8.08; Found: C, 65.70; H, 6.59; N, 8.21; IR(cm^{-1}): 3071, 2960, 1709(—C=O), 1618 (—C=C—), 1605, 1559, 1456, 1316, 1157; ^{1}H NMR(300 MHz, DMSO-d_6): 9.49(s, 1H, —NC*H*N—), 8.01(m, 2H, —NC*H*=C*H*N—), 7.16(s, 1H, Ar*H*), 7.10(s, 1H, Ar*H*), 6.19(s, 2H, —NCH_2coumarin), 5.44(s, 1H, —C=C*H*—), 4.28(t, 2H, —NCH_2CH_2CH_2CH_3, *J* = 7.3 Hz), 2.71(s, 3H, ArCH_3), 2.37(s, 3H, ArCH_3), 1.32(quint, 2H, —NCH$_2$CH_2-CH_2CH_3, *J* = 7.4 Hz), 1.29(sex, 2H, —NCH_2CH_2CH_2CH_3, *J* = 7.6 Hz), 0.92(t, 3H, —NCH_2CH_2CH_2CH_3, *J* = 7.4 Hz). ^{13}C NMR(75 MHz, DMSO-d_6): 159.0, 154.4, 152.3, 142.7, 137.3, 136.3, 129.9, 123.3, 123.1, 115.6, 114.3, 110.9, 51.4, 48.9, 31.1, 23.6, 20.6, 18.8, 13.3

4.3.4. 1-(Methyl)-3-((1*H*-benzo[*d*]1,2,3-triazol-1-yl)methyl) imidazolium chloride (2a)

White solid, Yield: 89%, mp: 217–219 °C, Elemental analysis: Calcd for: $C_{11}H_{12}ClN_5$; C, 52.91; H, 4.84; N, 28.05; Found: C, 52.94; H, 4.87; N, 28.05; IR(cm^{-1}): 3091, 3031, 2947, 2879, 1615, 1593, 1560, 1458, 1308, 1301, 1229, 1220, 1166; ^{1}H NMR (300 MHz, DMSO-d_6): 9.82(s, 1H, —NC*H*N—), 8.45–7.49(m, 6H, Ar*H* and —NC*H*=C*H*N—), 3.87(s, 3H, —NCH_3), ^{13}C NMR(75 MHz, DMSO-d_6): 145.2, 137.6, 132.3, 128.6, 124.9, 124.3, 122.1, 119.5, 110.9, 57.6, 36.1.

4.3.5. 1-(Methyl)-2-(methyl)-3-((1*H*-benzo[*d*]1,2,3-triazol-1-yl) methyl)imidazolium chloride (2b)

White crystal. Yield: 95%, mp: 182–183 °C, Elemental analysis: Calcd for: $C_{12}H_{14}ClN_5$; C, 54.65; H, 5.35; N, 26.56; Found: C, 54.68; H, 5.37; N, 26.53; IR(cm^{-1}): 3086, 3037, 2953, 2944, 2881, 1559, 1466, 1312, 1307, 1231, 1224, 1178; ^{1}H NMR(600 MHz, DMSO-d_6): 8.46(d, 1H, —NC*H*=CHN—, *J* = 8.4 Hz), 8.29(d, 1H, Ar*H*, *J* = 2.2 Hz), 8.13(d, 1H, —NCH=C*H*N—, *J* = 8.4 Hz), 7.78(d, 1H, Ar*H*, *J* = 2.2 Hz), 7.69(t, 1H, Ar*H*, *J* = 6.2 Hz), 7.50(m, 3H, Ar*H* and —NCH_2-triazole), 3.77(s, 3H, —NCH_3), 2.87(s, 3H, —NC(CH_3)N—). ^{13}C NMR (150 MHz, DMSO-d_6): 146.8, 145.6, 132.9, 129.1, 125.4, 123.4, 121.8, 120.0, 111.4, 57.2, 35.5, 10.5.

4.3.6. 1-(Butyl)-3-((1*H*-benzo[*d*]1,2,3-triazol-1-yl)methyl) imidazolium chloride (2c)

White crystal. Yield: 94%, mp: 177–178 °C, Elemental analysis: Calcd for: $C_{14}H_{18}ClN_5$; C, 57.63; H, 6.22; N, 24.00; Found: C, 57.65; H, 6.25; N, 24.03; IR(cm^{-1}): 3165, 3085, 3023, 2950, 2872, 1614, 1592, 1557, 1457, 1434, 1306, 1223, 1165; ^{1}H NMR (300 MHz, DMSO-d_6): 9.92(s, 1H, —NC*H*N—), 8.41–7.47(m, 6H, Ar*H* and —NC*H*=C*H*N—), 7.43(s, 2H, —NCH_2triazole), 4.20(t, 2H, —NCH_2CH_2CH_2CH_3, *J* = 7.2 Hz), 1.76(quint, 2H, —NCH_2CH_2CH_2CH_3, *J* = 7.3 Hz), 1.19(sex, 2H, —NCH_2CH_2CH_2CH_3, *J* = 7.6 Hz), 0.86(t, 3H, —NCH_2CH_2CH_2CH_3, *J* = 7.3 Hz). ^{13}C NMR(75 MHz, DMSO-d_6): 145.2, 137.2, 132.3, 128.6, 124.9, 123.1, 122.3, 119.5, 110.9, 57.7, 48.9, 31.1, 18.7, 13.2).

4.3.7. 1-(Methyl)-3-((1*H*-benzo[*d*]imidazol-2-yl)methyl) imidazolium chloride (3a)

White solid. Yield: 56%, mp: 257–258 °C, Elemental analysis: Calcd for: $C_{12}H_{13}ClN_4$; C, 57.95; H, 5.27; N, 22.53; Found: C, 57.90; H, 5.20; N, 22.45; IR(cm^{-1}): 3055, 2952, 2940, 2856, 2768,

1564, 1533, 1440, 1327, 1270; ^{1}H NMR(300 MHz, DMSO-d_6): 13.41 (broad, 1H, —N*H*), 9.46(s, 1H, —NC*H*N—), 7.96–7.78(m, 2H, —NC*H*=C*H*N—), 7.57–7.18(m, 4H, Ar*H*), 5.82(s, 2H, —CH_2benzimidazole), 3.92(s, 3H, —NCH_3), ^{13}C NMR(75 MHz, DMSO-d_6): 148.0, 137.5, 123.6, 123.2, 122.2, 44.2, 35.9.

4.3.8. 1-(Methyl)-2-(methyl)-3-((1*H*-benzo[*d*]imidazol-2-yl) methyl)imidazolium chloride (3b)

White solid. Yield: 54%, mp: 253–255 °C, Elemental analysis: Calcd for: $C_{13}H_{15}ClN_4$; C, 59.43; H, 5.75; N, 21.32; Found: C, 59.36; H, 5.66; N, 21.38; IR(cm^{-1}): 2943, 2891, 2862, 2761, 1590, 1531, 1435, 1385, 1328, 1268; ^{1}H NMR(300 MHz, DMSO-d_6): 13.49(broad, 1H, —N*H*), 7.89(d, 1H, —NC*H*=CHN—, *J* = 2.1 Hz), 7.72(d, 1H, —NCH=C*H*N—, *J* = 2.1 Hz), 5.77(s, 2H, —NCH_2benzimidazole), 3.82(s, 3H, —NCH_3), 2.70(s, 3H, —NC(CH_3)N—), ^{13}C NMR (75 MHz, DMSO-d_6): 147.8, 145.4, 142.6, 134.2, 122.5, 121.8, 121.6, 118.8, 111.7, 45.1, 34.9, 9.7.

4.3.9. 1-(Butyl)-3-((1*H*-benzo[*d*]imidazol-2-yl)methyl)imidazolium chloride (3c)

White solid. Yield: 67%, mp: 238–239 °C, Elemental analysis: Calcd for: $C_{15}H_{19}ClN_4$; C, 61.96; H, 6.59; N, 19.27; Found: C, 62.09; H, 6.69; N, 19.24; IR(cm^{-1}): 3051, 2959, 2948, 1940, 2866, 2773, 1571, 1544, 1451, 1437, 1331, 1277; ^{1}H NMR(300 MHz, DMSO-d_6): 13.46(broad, 1H, —N*H*), 9.56(s, 1H, —NC*H*N—), 8.03–7.91(m, 2H, —NC*H*=C*H*N—), 7.78–7.46(m, 4H, Ar*H*), 6.08(s, 2H, —NCH_2benzimidazole), 4.23(t, 2H, —NCH_2CH$_2$CH$_2$CH$_3$, *J* = 7.2 Hz), 1.83(quint, 2H, —NCH$_2$CH_2CH$_2$CH$_3$, *J* = 7.5 Hz), 1.32(sex, 2H, —NCH$_2$CH$_2$CH_2CH$_3$, *J* = 7.6 Hz), 0.92(t, 3H, —NCH$_2$CH$_2$CH$_2$CH_3, *J* = 7.4 Hz), ^{13}C NMR(75 MHz, DMSO-d_6): 147.2, 137.6, 133.1, 125.0, 123.3, 122.8, 114.5, 48.8, 44.5, 31.1, 18.8, 13.3.

4.3.10. 1-((1*H*-Benzo[*d*]1,2,3-triazol-1-yl)methyl)-3-(butyl)-benzimidazolium chloride (5a)

White solid. Yield 92%, mp: 194–196 °C, Elemental analysis: Calcd for: $C_{18}H_{20}ClN_5$; C, 63.24; H, 5.90; N, 20.49; Found: C, 63.31; H, 5.95; N, 20.33; IR(cm^{-1}): 3097, 2962, 2943, 1617, 1505, 1457, 1442, 1366, 1352, 1288, 1217; ^{1}H NMR(300 MHz, DMSO-d_6): 10.93(s, 1H, —NC*H*N—), 8.57–7.45(m, 8H, Ar*H*), 7.80(s, 2H, —NCH_2triazole), 4.54(t, 2H, —NCH_2CH$_2$CH$_2$CH$_3$, *J* = 7.1 Hz), 1.90 (quint, 2H, —NCH$_2$CH_2CH$_2$CH$_3$, *J* = 7.3 Hz), 1.32(sex, 2H, —NCH$_2$-CH$_2$CH_2CH$_3$, *J* = 7.6 Hz), 0.90(t, 3H, —NCH$_2$CH$_2$CH$_2$CH_3, *J* = 7.3 Hz), ^{13}C NMR(75 MHz, DMSO-d_6): 145.1, 143.4, 132.4, 131.2, 130.3, 128.6, 127.2, 126.9, 124.9, 119.4, 114.1, 113.9, 111.2, 56.0, 46.8, 30.2, 19.0, 13.3.

4.3.11. 1-((1*H*-Benzo[*d*]1,2,3-triazol-1-yl)methyl)-3-(benzyl)-benzimidazolium chloride (5b)

White solid. Yield: 88%, mp: 193–195 °C, Elemental analysis: Calcd for: $C_{21}H_{18}ClN_5$; C, 67.11; H, 4.83; N, 18.63; Found: C, 67.20; H, 4.86; N, 18.56; IR(cm^{-1}): 3086, 3044, 2944, 1622, 1614, 1512, 1466, 1437, 1380, 1361, 1290, 1218; ^{1}H NMR(300 MHz, DMSO-d_6): 10.70(s, 1H, —NC*H*N—), 8.38–7.39(m, 13H, Ar*H*), 7.68 (s, 2H, —NCH_2triazole), 5.82(s, 2H, —NCH_2Ph), ^{13}C NMR(75 MHz, DMSO-d_6): 145.7, 144.1, 134.0, 132.9, 131.4, 131.0, 129.5, 129.3, 129.2, 128.9, 127.9, 127.6, 125.5, 120.1, 114.7, 114.5, 111.3, 56.7, 50.7.

4.3.12. 1-((1*H*-Benzo[*d*]1,2,3-triazol-1-yl)methyl)-3-(3,4,5-trimethoxybenzyl)-benzimidazolium chloride (5c)

Yellow solid. Yield: 91%, mp: 77–78 °C, Elemental analysis: Calcd for: $C_{24}H_{24}ClN_5O_3$; C, 61.87; H, 5.19; N, 15.03; Found: C, 61.91; H, 5.22; N, 14.91; IR(cm^{-1}): 3088, 3051, 3041, 2951, 1619, 1509, 1454, 1447, 1436, 1371, 1353; ^{1}H NMR(300 MHz, DMSO-d_6): 10.99(s, 1H, —NC*H*N—), 8.32–7.48(m, 8H, Ar*H*), 7.79 (s, 2H, —NCH_2triazole), 7.02(s, 2H, Ar*H*), 5.70(s, 2H, —NCH_2Ph), 3.76(s, 6H, ArOCH_3), 3.63(s, 3H, ArOCH_3), ^{13}C NMR(75 MHz, DMSO-d_6): 153.1, 145.2, 143.5, 137.7, 132.4, 130.9, 130.4, 128.8, 128.6, 127.3, 127.1, 124.9, 119.5, 114.3, 114.0, 111.0, 106.6, 59.9, 56.1, 50.4, 30.6.

4.3.13. 1,3-Di((1*H*-benzo[*d*]1,2,3-triazol-1-yl)methyl)benzimidazolium chloride (5d)

White solid. Yield: 73%, mp: 197–198 °C, Elemental analysis: Calcd for: $C_{21}H_{17}ClN_8$; C, 60.50; H, 4.11; N, 26.88; Found: C, 60.44; H, 4.16; N, 26.76; IR(cm^{-1}): 3087, 3061, 1623, 1540, 1509, 1466, 1372, 1344, 1288, 1222, 1209; ^{1}H NMR(600 MHz, DMSO-d_6): 11.30(s, 1H, —NC*H*N—), 8.47–7.46(m, 12H, Ar*H*), 7.76(s, 4H, —NCH_2triazole), ^{13}C NMR(150 MHz, DMSO-d_6): 145.6, 145.1, 132.9, 130.9, 129.2, 128.1, 125.5, 120.0, 114.7, 111.5, 56.7.

4.3.14. 1-((1*H*-Benzo[*d*]1,2,3-triazol-1-yl)methyl)-3-((7,8-dimethyl-2*H*-chromen-2-one-4-yl)methyl)benzimidazolium chloride (5e)

White solid. Yield: 78%, mp: 249–251 °C, Elemental analysis: Calcd for: $C_{26}H_{22}ClN_5O_2$; C, 66.17; H, 4.70; N, 14.84; Found: C, 66.26; H, 4.77; N, 14.77; IR(cm^{-1}): 2920, 1723, 1608, 1567, 1491, 1443, 1382, 1349, 1262, 1219; ^{1}H NMR(600 MHz, DMSO-d_6): 10.34(s, 1H, —NC*H*N—), 8.38–7.28(m, 10H, Ar*H*), 7.60(s, 2H, —NCH_2triazole), 6.18(s, 2H, —NCH_2coumarin), 6.06(s, 1H, —C=C*H*—), 2.41(3H, ArCH_3), 2.32(s, 3H, ArCH_3), ^{13}C NMR (150 MHz, DMSO-d_6): 160.1, 151.6, 149.0, 145.7, 145.0, 142.8, 133.0, 131.8, 131.2, 129.2, 127.9, 126.3, 125.5, 124.6, 122.2, 121.8, 120.0, 115.0, 114.6, 114.5, 111.3, 56.7, 20.4, 11.7.

4.3.15. 1-((1*H*-Benzo[*d*]1,2,3-triazol-1-yl)methyl)-3-((6-*tert*-butyl-2*H*-chromen-2-one-4-yl)methyl)benzimidazolium chloride (5f)

White solid. Yield: 70%, mp: 195–198 °C, Elemental analysis: Calcd for: $C_{28}H_{26}ClN_5O_2$; C, 67.26; H, 5.24; N, 14.01; Found: C, 67.36; H, 5.27; N, 13.90; IR(cm^{-1}): 3066, 2918, 1719, 1616, 1602, 1555, 1482, 1470, 1448, 1390, 1340, 1277, 1226; ^{1}H NMR (300 MHz, DMSO-d_6): 10.70(s, 1H, —NC*H*N—), 8.41–7.44(m, 11H, Ar*H*), 7.75(s, 2H, —NCH_2triazole), 6.36(s, 2H, —NCH_2coumarin), 6.09(s, 1H, —C=C*H*—), 1.27(s, 9H, —ArC(CH_3)$_3$), ^{13}C NMR (150 MHz, DMSO-d_6): 159.4, 151.0, 148.6, 147.0, 145.2, 144.6, 132.5, 131.4, 130.6, 128.6, 127.5, 127.4, 124.9, 120.6, 119.5, 116.4, 116.1, 114.2, 114.1, 112.8, 110.9, 56.3, 46.9, 34.5, 30.9.

4.3.16. 1-((1*H*-Benzo[*d*]1,2,3-triazol-1-yl)methyl)-3-(6-acetyl-2*H*-1,4-benzoxazine-3(4*H*)-one)benzimidazolium chloride (5g)

White solid. Yield: 54%, mp: 268–270 °C, Elemental analysis: Calcd for: $C_{24}H_{19}ClN_6O_3$; C, 60.70; H, 4.03; N, 17.70; Found: C, 60.61; H, 3.92; N, 17.82; IR(cm^{-1}): 3120, 3030, 1702, 1685, 1605, 1566, 1516, 1485, 1384, 1328, 1284; ^{1}H NMR(600 MHz, DMSO-d_6): 11.08(s, 1H, —N*H*), 10.38(s, 1H, —NC*H*N—), 8.36–7.19(m, 11H, Ar*H*), 7.86(s, 2H, —NCH_2triazole), 6.38(s, 2H, —NCH_2benzoxazinone), 4.75(s, 2H, —C(O)CH_2O—), ^{13}C NMR(150 MHz, DMSO-d_6): 189.6, 164.5, 148.8, 145.7, 144.9, 132.8, 132.4, 130.2, 129.3, 128.4, 128.1, 127.8, 127.7, 125.6, 125.3, 120.1, 116.8, 115.8, 114.9, 111.2, 67.2, 56.6, 53.7.

4.4. Single-crystal structure determination

The crystal data of the compounds **2b** and **2c** were collected on a STOE IPDS II diffractometer at room temperature (296 K) using graphite-monochromated Mo Kα radiation (λ = 0.71073 Å) by applying the ω-scan method. The structures were solved by direct methods using SHELXS-2013[37] and refined with full-matrix least-squares calculations on F^2 using SHELXL-2014[37] implemented in WinGX[38] program suit. All of the C-bound H atoms were placed at calculated positions and included in the refinement in the riding

model approximation, fixing the bond lengths at 0.93, 0.97 and 0.96 Å for aromatic CH, CH_2 and CH_3 atoms, respectively. The water H atoms in compound **2b** were located in a difference Fourier map and refined isotropically. The displacement parameters of the H atoms were fixed at $U_{iso}(H) = 1.2U_{eq}$ ($1.5U_{eq}$ for methyl) of their parent atoms. In compound **2c**, the butyl moiety shows positional disorder and the refined site-occupancy factors of the disordered parts, viz. C11A–C14A and C11B–C14B, are 0.644(4)/0.356(4)%. Data collection: X-AREA [39], cell refinement: X-AREA, data reduction: X-RED32.[39] Crystal data, data collection and structure refinement details are summarized in Table 4. The general-purpose crystallographic tool PLATON[40] was used for the structure analysis and presentation of the results. ORTEP-3 was used to generate the molecular graphics.[41]

CCDC 929882 and 1005944 contain the supplementary crystallographic data (excluding structure factors) for the compounds reported in this article. These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/cgi-bin/catreq.cgi [or from the Cambridge Crystallographic Data Centre (CCDC), 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44(0)1223-336033; e-mail: data_request@ccdc.cam.ac.uk].

4.5. Paraoxonase enzyme assay

Paraoxonase enzyme activity towards paraoxon was quantified spectrophotometrically by the method described by Gan et al.[42] The enzyme assay was based on the estimating of p-nitrophenol at 412 nm. The molar extinction coefficient of p-nitrophenol (ε = 17,100 M^{-1} cm^{-1}at pH 8) was used to calculate enzyme activity. The reaction was followed for 2 min at 37 °C by monitoring the appearance of p-nitrophenol at 412 nm in automated recording spectrophotometer (Biotek, Winooski, VT). 2 mM of final substrate concentration was used during enzyme assay, and all measurements were taken in duplicate and corrected for the non-enzymatic hydrolysis.

Table 4
Crystal data and structure refinement parameters for compounds **2b** and **2c**

Parameters	Compound **2b**	Compound **2c**
CCDC deposition no.	1005944	1003894
Color/shape	Colorless/prism	Colorless/plate
Chemical formula	$2(C_{12}H_{14}N_5)^+ \cdot 2Cl^- \cdot H_2O$	$C_{14}H_{18}N_5^+ \cdot Cl^-$
Formula weight	545.48	291.78
Temperature (K)	296	296
Wavelength (Å)	0.71073 Mo Kα	0.71073 Mo Kα
Crystal system	Triclinic	Monoclinic
Space group	$P\bar{1}$ (No. 2)	$P2_1/c$ (No. 14)
Unit cell parameters		
a, b, c (Å)	8.1818(6), 13.1643(9), 14.1659(10)	8.3066(5), 19.2956 (9), 9.7770(7)
α, β, γ (°)	102.865(5), 106.491(6), 104.493(5)	90, 99.164(5), 90
Volume (Å^3)	1343.70(17)	1547.06(16)
Z	2	4
D_{calc} (g/cm^3)	1.348	1.253
μ (mm^{-1})	0.280	0.245
Absorption correction	Integration	Integration
T_{min}, T_{max}	0.864, 0.962	0.889, 0.980
F_{000}	572	616
Crystal size (mm^3)	0.27 × 0.48 × 0.71	0.08 × 0.39 × 0.60
Diffractometer/measurement method	STOE IPDS II/ω scan	STOE IPDS II/ω scan
Index ranges	$-10 \leqslant h \leqslant 10$, $-17 \leqslant k \leqslant 17$, $-18 \leqslant l \leqslant 18$	$-10 \leqslant h \leqslant 10$, $-25 \leqslant k \leqslant 25$, $-12 \leqslant l \leqslant 9$
θ range for data collection (°)	$1.68 \leqslant \theta \leqslant 28.03$	$2.11 \leqslant \theta \leqslant 27.55$
Reflections collected	18208	10564
Independent/observed reflections	6480/4720	3559/2175
R_{int}	0.062	0.049
Refinement method	Full-matrix least-squares on F^2	Full-matrix least-squares on F^2
Data/restraints/parameters	6480/0/346	3559/113/218
Goodness-of-fit on F^2	1.061	1.006
Final R indices [$I > 2\sigma(I)$]	R_1 = 0.0426, wR_2 = 0.1117	R_1 = 0.0509, wR_2 = 0.1145
R indices (all data)	R_1 = 0.0613, wR_2 = 0.1186	R_1 = 0.0952, wR_2 = 0.1291
$\Delta\rho_{max}$, $\Delta\rho_{min}$ (e/Å^3)	0.23, −0.27	0.28, −0.23

4.5.1. Purification of paraoxonase from human serum by hydrophobic interaction chromatography

Human serum was isolated from 40 ml fresh human blood and put into a dry tube. The blood samples were centrifuged at 3000 rpm for 15 min and the serum was removed. Firstly, serum paraoxonase was isolated by ammonium sulphate precipitation (60–80%). The precipitate was collected by centrifugation at 15,000 rpm for 20 min, and re-dissolved in 100 mM Tris–HCl buffer (pH 8.0). Then, we synthesized the hydrophobic gel, including Sepharose 4B, L-tyrosine and 1-napthylamine, for the purification of human serum paraoxonase.[43] The purified enzyme had aspecific activity of 11.76 U/mg. The column was equilibrated with 0.1 M of a Na_2HPO_4 buffer (pH 8.00) including 1 M ammonium sulphate. The paraoxonase was eluted with an ammonium sulphate gradient using 0.1 M Na_2HPO_4 buffer with and without ammonium sulphate (pH 8.00). The purified PON1 enzyme was stored in the presence of 2 mM calcium chloride in order to maintain activity.

4.5.2. Total protein determination

The absorbance at 280 nm was used to monitor the protein in the column effluents and ammonium sulphate precipitation. Quantitative protein determination was achieved by absorbance measurements at 595 nm according to Bradford,[44] with bovine serum albumin as a standard.

4.5.3. SDS polyacrylamide gel electrophoresis

SDS polyacrylamide 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.[45] A 20 mg sample was applied to the electrophoresis 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.

4.5.4. In vitro kinetic studies and calculation of IC_{50} and K_i values

For the inhibition studies of synthesized compounds, different concentrations of compounds were added to the reaction medium. PON1 activity with compounds was assayed by following the hydration of paraoxon. Activity percentage values of PON for five different concentrations of each compounds were determined by regression analysis using the Microsoft Office 2000 Excel. PON1 enzyme activity without a synthesized compound was considered as 100% activity. The inhibitor concentration causing up to 50% inhibition (IC_{50} values) for compounds were determined from the graphs. So as to determinate K_i, K_m and V_{max} values of the PON1 enzyme using paraoxon as a substrate was measured at eight different substrate concentrations (0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1 and 1.2 mM) at pH 8.00 and 37 °C in presence of two different coumarin derivative concentrations of compound **1c** (6.7 μM and 4.7 μM) as knowing highest and lowest final concentrations. K_m and V_{max} values were calculated 40.57 μM, 6.08 μmol/min respectively through Lineweaver–Burke graphs. The coumarin compound numbered **1c** inhibited PON1 enzyme activity in a competitive inhibition type with K_i of 2.39 μM value. As a result of in vitro

experiments and by the means of Lineweaver–Burke graphs competitive inhibition type was controlled with docking for confirmation.

4.6. Molecular docking

4.6.1. Enzyme setup

Determination of the consistent receptor was based on previous study.[1] Macromolecule file (PDB code: 1V04) was modified using the ADT package version 1.5.6rc3 (Ankara, Turkey). All water molecules were deleted and polar hydrogens were added. Subsequently, Gasteiger charges were calculated and the generated pdbqt files were saved.

4.6.2. Ligand

Energy minimization of compounds **1c** and **2c** was carried out using GAMESS module for ChemOffice version Ultra 8.0.3 (Ankara, Turkey). All data were saved as pdb with the aid of Molegro Molecular Viewer version 2.5 (Ankara, Turkey). Further modification of these partial charges of pdb files was carried out through the ADT package so that the charges of the non-polar hydrogen atoms allocated to the atom to which the hydrogen is attached. These modified pdb files saved as pdbqt files. Appropriate grid box points were determined by centering on ligand separately for each compound.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2016.02.012.

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