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RESEARCH ARTICLE

# Synthesis and evaluation of novel 2-[(1,2,4-triazol-3-yl)thio]acetamide derivatives as potential serum paraoxonase-1 (PON1) activators

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**ABSTRACT**

Coronary artery disease and low-density lipoprotein (LDL) levels in the blood have long been known to be associated with peripheral vascular diseases. Paraoxonase-1 (PON1) enzyme is related to serum levels of high-density lipoprotein (HDL) and protects LDL from oxidation which may result in development of microvascular disease in diabetes. The enzyme has a major role in the prevention of atherosclerosis besides antioxidant properties. Additionally, PON1 is important in the detoxification of organophosphate insecticides from the body. In this study, we aimed to synthesize highly active new compounds which can be a drug candidate and evaluate their effects on PON1 activity.

Nine novel triazole compounds bearing thioacetamide moiety (**5a-i**) were synthesized and their *in vitro* PON1 activity was investigated. The PON1 enzyme was purified from human serum using ammonium sulfate precipitation method. Also, it was further purified using Sepharose 4B-L-tyrosine-1-naphthylamine affinity chromatography. Among the synthesized triazole compounds, **5b, 5c, 5f** and **5h** have been determined to increase PON1 activity, remarkably. Compounds **5b, 5c, 5f** and **5h** bearing 5-nitrothiazole, benzothiazole, 6-ethoxybenzothiazole and 6-florobenzothiazole moieties could be considered to proceed in vivo investigations which is a further stage for a drug candidate.

**Key words:** Triazole; thioacetamide; paraoxonase (PON1); oxidative stress; antioxidant defence

## 1. Introduction

Human PON1 enzyme have been reported to comprise three enzymatic activity; phosphotriesterase, arylesterase and lactonase [1, 2]. PON1 enzyme plays an important role to prevent atherosclerosis and its antiatherogenic properties is associated with the protection of LDL, HDL and macrophages against oxidative stress [3-5]. It prevents accumulation of oxidized lipid, LDL and therefore the development of vascular disease is avoided [6]. Low levels of PON1 enzyme is a risk factor for vascular disease, due to these roles in lipid metabolism [7, 8]. PON1 name comes from paraoxon which is the toxic metabolite of organophosphate parathion, an insecticide. The enzyme hydrolyses various toxic oxon metabolite of the insecticide such as chlorpyrifosoxon, diazoxon as well as the nerve agents sarin, soman and VX etc. [9-13]. Phenyl acetate, thiophenyl acetate, 2-naphthyl acetate and other aromatic esters are the other PON1 substrates [14]. It is also determined to hydrolyze aromatic/aliphatic lactones and cyclic carbonates [15, 16]. PON1 enzyme is capable of hydrolysing at least 30 kinds of lactone consisting of 4-7 atoms including drugs and endogenous compounds. It

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has more affinity for aromatic lactones. However, coumarin compound bearing α and β-double bond on the lactone ring can not be hydrolyzed by the PON1, but dihydrocoumarin can be hydrolysed by the enzyme [17, 18].

The effects of anticholesterolemic drugs statins and fibrates on the expression and activation of PON enzyme were tested and these studies have given conflicting results [19-21]. Experimental animal studies support the clinical evidence of the stimulatory effects of lipid-lowering activity of drug therapy [22]. Fibrate group of drugs gemfibrozil, fenofibrate and ciprofibrate (Figure 1) were found to increase the serum PON activity [23-26]. In patients with coronary disease, low-dose aspirin increases ~ 13% of serum PON1 activity [27], but in healthy volunteers it was found not to increase the activity [28]. Nitro-aspirin and aspirin metabolite salicylic acid also determined to increase PON1 expression and activity [29]. Additionally, anticholesterolemic probucol and cholesterol, drug ezetimibe (Figure 1) reported to increase PON1 activity [30]. Moreover, a PPAR-γ agonist rosiglitazone, which is used in tip 2 diabetes theraphy, have caused increase of serum PON1 activity (9-13%) in diabetic patients [31], furthermore it increases the serum PON1 activity by increasing the synthesis of HDL particles in rabbit [32] (Figure 1).

Triazole ring is a stable, highly studied scaffold which exists in clinically used drugs and a lot of biactive molecules. Triazole derivatives have also capacity of hydrogen bonding which provides binding to biomolecular structures [33, 34]. On the other hand, thiazoles/benzothiazoles are another important class of heterocylic compounds found in many bioactive molecules and they have been reported in a large number of literature with a wide scale of biological activities [35, 36].

Nowadays, studies on the PON1 enzyme are more popular around the world. Despite this situation, there are a few studies in the literature on the interaction between drugs and PON enzyme activity. Therefore, we focused on novel 1,2,4-triazole and thiazole/benzothiazole bearing thioacetamide derivatives (**5a-i**) which possess high efficiency on various biological application area. The scope of this study includes synthesis of nine compounds and evaluation of their PON1 increasing effects which is a preliminary stage for a drug candidate.

## 2. Results

The synthesis of title compounds was carried out according to the previous study [37] of us as shown in Figure 2. At the beginning, starting material 3-cyclohexanepropionic acid was esterificated with ethanol to give ethyl 3-cyclohexylpropanoate (**1**). Then the obtained ester was reacted with hydrazine hydrate to give 3-cyclohexylpropanohydrazide (**2**). 1-(3-Cyclohexylpropionyl)-4-phenyl-3-thiosemicarbazide (**3**) was obtained with the reaction of hydrazide compound (**2**) and phenylisothiocyanate. Ring closuring product 4-phenyl-5-(3-cyclohexylpropionyl)-2,4-dihydro-3*H*-1,2,4-triazol-3-thione (**4**) was synthesized by refluxing compound **3** with aqueous KOH solution. Finally 4-phenyl-5-(3-cyclohexylpropionyl)-3-[*N*-(2-thiazolyl/

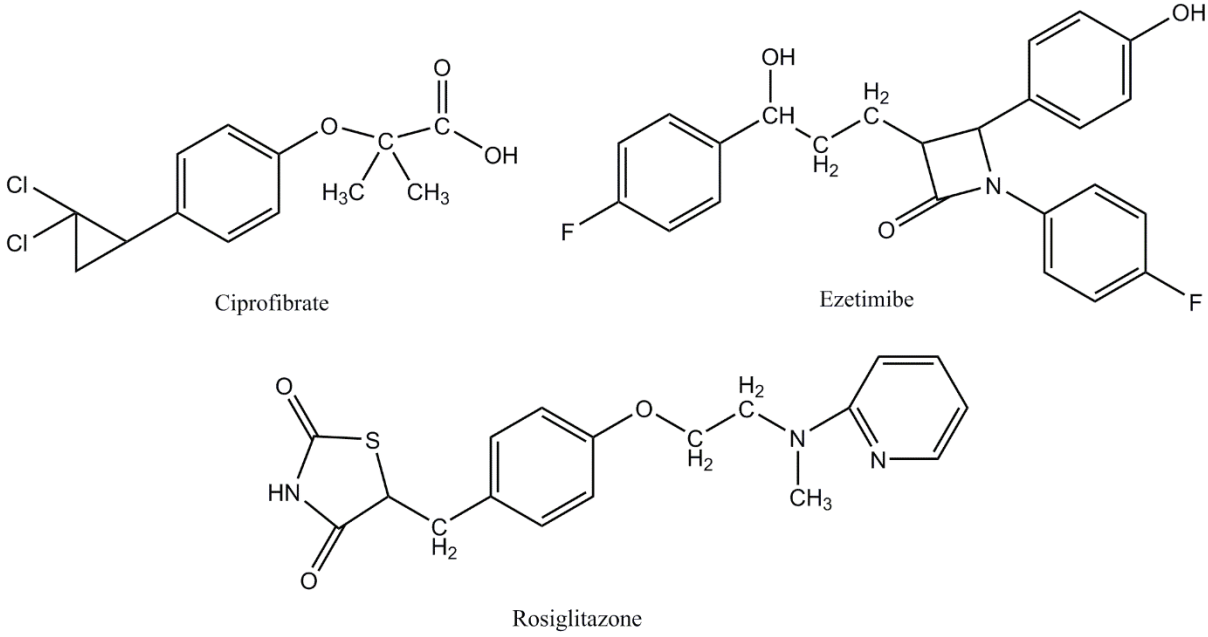


**Figure 1.** Some PON1 enzyme activator compounds

**Table 1.** Summary of Purification for PON1 from Human Serum

| Purification steps | Volume (mL) | Activity (U/mL) | Total Activity U | % | Protein (mg/mL) | Spesific Activity (U/mg) | Purification Fold |
|---|---|---|---|---|---|---|---|
| Serum | 25 | 122.8 | 3070 | 100 | 162.5 | 1.76 | - |
| Ammonium sulphate precipitation | 20 | 95.2 | 1904 | 62.0 | 42.1 | 2.26 | 1.3 |
| DEAE-Sephadex A 50 Ion Exchange | 20 | 85.6 | 1712 | 55.8 | 1.15 | 74.43 | 42.3 |
| **Purification steps** | **Volume (mL)** | **Activity (U/mL)** | **Total Activity U** | **%** | **Protein (mg/mL)** | **Spesific Activity (U/mg)** | **Purification Fold** |
| Enzyme fraction | 50 | 125.2 | 6260 | - | 145.2 | 0.86 | - |
| Sepharose 4B-L-tyrosine-1-naphthylamine affinity chromatograph | 20 | 61.6 | 1712 | 49.2 | 0.083 | 742.17 | 862.99 |

benzothiazolyl)acetamido]thio-4*H*-1,2,4-triazole derivatives (**5a-i**) were synthesized by the reaction of compound **4** and some appropriate 2-chloro-*N*-(substituted thiazolyl/benzothiazolyl)acetamide derivatives. The final compounds were yielded with a range of 75-88 %.

Structure elucidations of the final compounds were performed with IR, $^{1}$H-NMR, $^{13}$C-NMR and MS (ES$^{+}$) spectroscopic methods. Characteristic stretching absorption bands belonging to C=O groups and N-H groups were observed at 1679–1694 cm$^{-1}$ and at 3276-3293 cm$^{-1}$, respectively. In the $^{1}$H-NMR spectra of the compounds, cyclohexyl protons were observed at about 0.84-1.68 ppm as the furthest upfield resonances and methylene protons belonging to alkyl group were seen at about 2.46-2.56 ppm as multiplet peaks. The other methylene protons bonded to S atom were observed at 4.03-4.18 ppm and N-H protons belonging to amide moiety were observed at about 12.38-12.72 ppm as a broad singlet. In addition, aromatic protons were seen at expected regions in the spectrums. In the $^{13}$C-NMR spectra of the compounds, peaks at about 160-165 ppm were assigned corresponding to -C=O carbon atom. In the mass spectra of the compounds, M+1 peaks agreed well with the calculated molecular weight of the target compounds. All compounds gave satisfactory elemental analysis results.

PON 1 enzyme was purified from human serum using ammonium sulfate precipitation (60-80 %), DEAE-Sephadex anion exchange chromatography. PON1 enzyme was also purified from human serum using Sepharose 4B-L-tyrosine-1-naphthylamine affinity chromatography. Specific activities were calculated in both serum and purified PON 1 enzyme. Paraoxon was used as a substrate for determining PON1 enzyme activity.

The result of the purification of PON1 enzyme was presented in Table 1. The ammonium sulfate precipitation method was used as first step for the enzyme purification. PON1 enzyme was obtained from human serum with a yield of 62% by ammonium sulfate fractionation and it was purificated 1.3 fold. The sample which was applied ammonium sulfate fractionation which was then applied to DEAE-Sephadex anion exchange chromatography was obtained 42.3 fold, 55.8% yield and it was purificated 74.43 U/mg protein specific activity from human PON1 enzyme (Table 1). As summarized in Table 1, the purification of PON 1 was also carried out in simple one step using Sepharose 4B-L-tyrosine-1-naphthylamine affinity chromatography. The PON 1 enzyme was purified 862.99-fold with a specific activity of 742.2 (EU/mg) and 49.2% recovery. PON 1 enzyme purified by affinity chromatography was used for further kinetic studies. The mass and purity of the enzyme was assessed with SDS-PAGE electrophoresis and a single band at 45 kDa level was observed (Figure 3).

Compounds **5a-i** were tested to determine PON1 enzyme activity in *in vitro* conditions. The results were represented as graphics against to compound concentration (mM) (Figure 4). The effects of the triazole compounds **(5a-i)** on PON1 enzyme activity were studied at three concentrations (0.05, 0.1 ve 0.2 mM). In Figure 4, the relative activity obtained for the control sample was taken as 100% and the

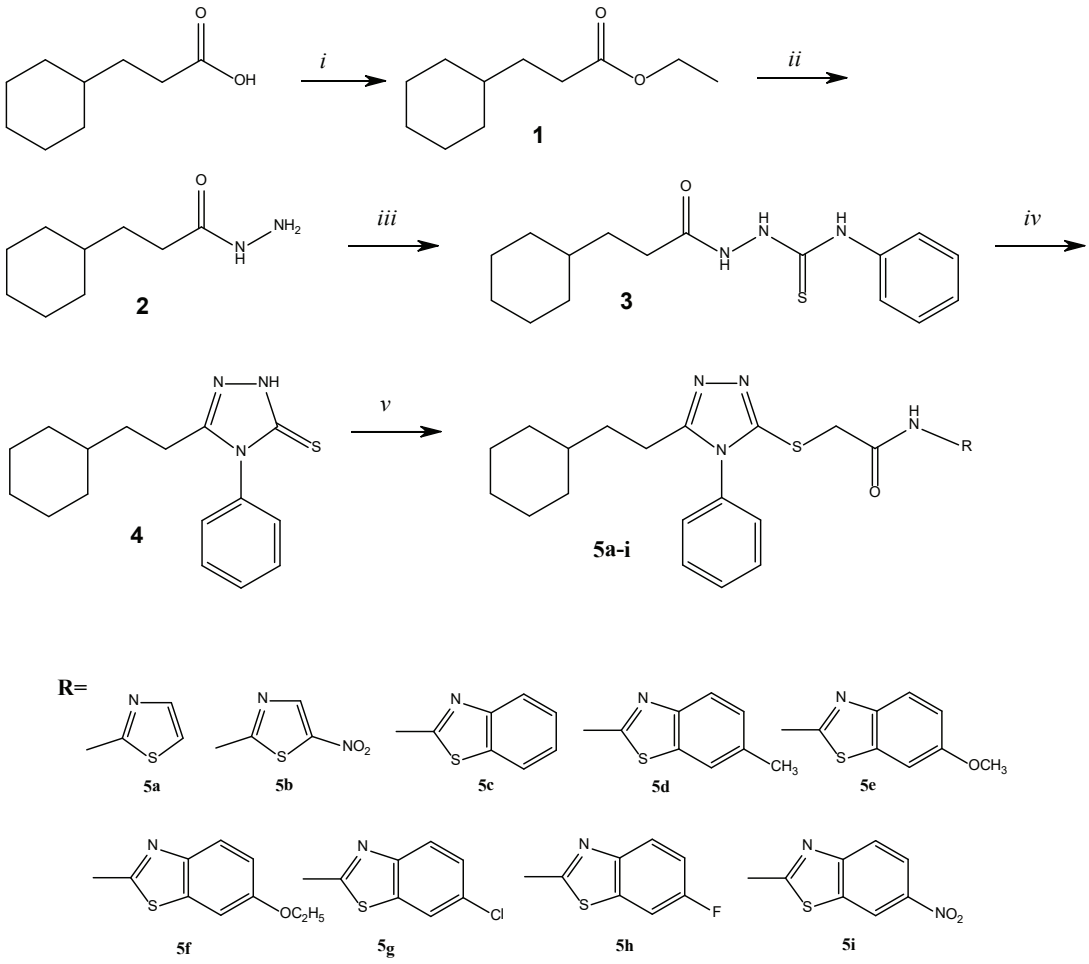


**Figure 2.** Synthesis of the compounds (**5a-i**). Reactants and conditions: *i*: EtOH, $H_2SO_4$, reflux, 12h; *ii*: $NH_2NH_2.H_2O$, EtOH, reflux, 6h; *iii*: Phenyl isothiocyanate, reflux, 2h; *iv*: 2N KOH, reflux, 3h; *v*: 2-Chloro-*N*-(substituted thiazolyl/benzothiazolyl) acetamide derivatives, $K_2CO_3$, acetone, r.t, 2h.

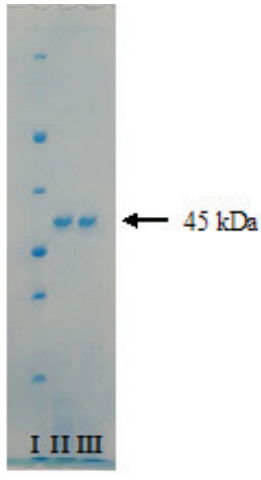


**Figure 3.** SDS-PAGE electrophoretic pattern of paraoxonase. [I: standart protein (myosin (200 kDa), β-galactosidase (125 kDa), bovine serum albumin (BSA) (66 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), lyzozme (14.3 kDa)); II : Purified PON 1 enzyme using ammonium sulfate precipitation (45 kDa) III: Purified PON1 enzyme using Sepharose 4B-L-tyrosine-1-naphthylamine affinity chromatography (45 kDa)].

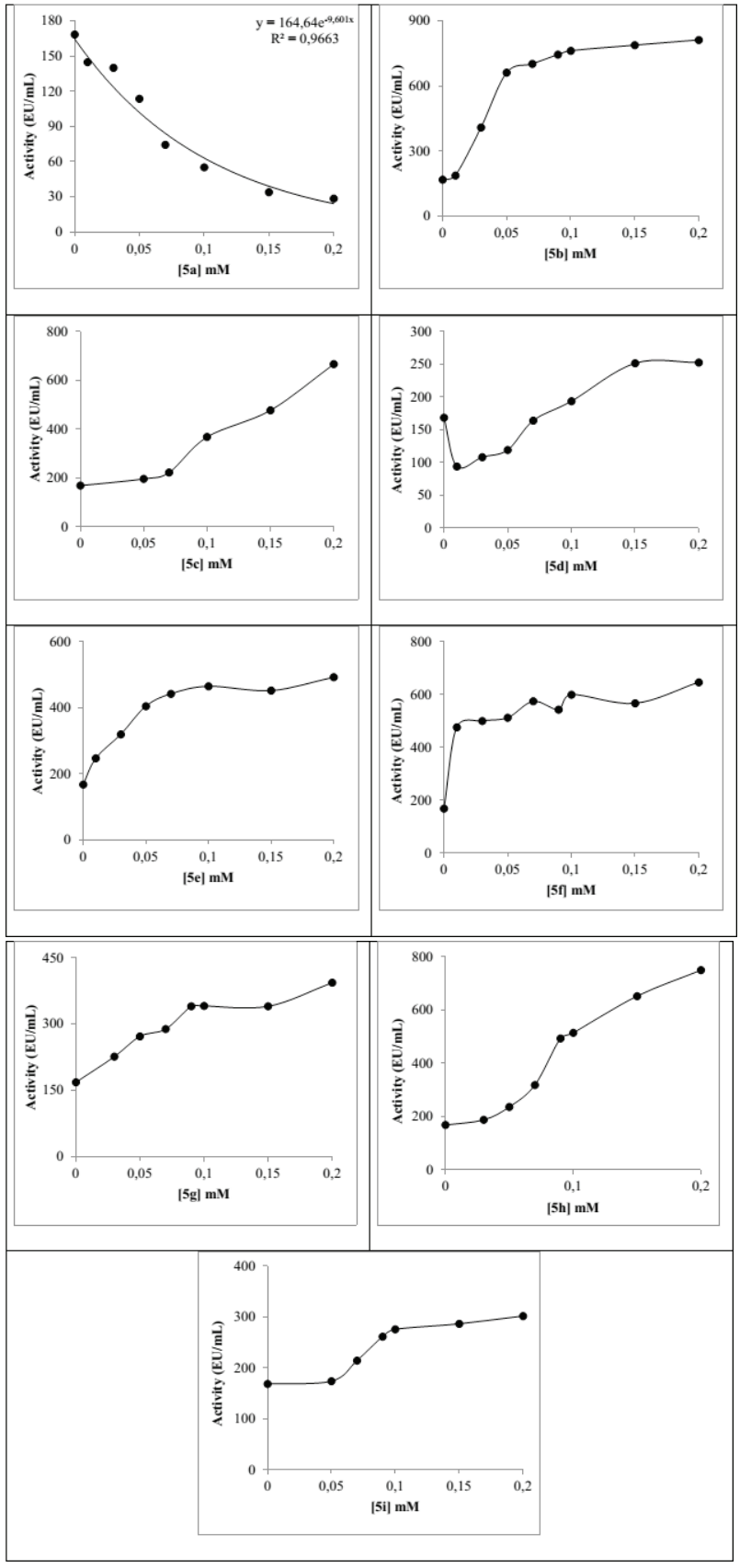


**Figure 4.** The *in vitro* PON1 enzyme activity of the compounds **5a-i.**

relative activities' values were calculated for the triazole compounds (**5a-i**) at indicated concentrations (0.05, 0.1 ve 0.2 mM). According to the obtained data, all compounds exhibited a dose dependent increase on PON1 activity. Only compound **5a** was an exception; the activator effect of this compound on PON1 decreased when the concentration increased (67.5% activity at 0.05 mM; 32.8% activity at 0.1 mM concentration; 16.9% activity at 0.2 mM concentration) (Table 2). The percentage of the relative activity of the final compounds ignored. When we compared relative percentage activity of the final compounds except **5a**, it was found 70.8%-392.9% at 0.05 mM, 114.9%-452.8% at 0.1 mM and 149.9%-482.5% at 0.2 mM concentration. Compound **5b** with 392.9% relative percentage and compound **5f** with 304.5% relative percentage exhibited the highest PON1 enzyme activator activity at 0.05 mM. When the concentration was increased to 0.1 mM, compound **5b** with 452.8% relative activity and **5f** with 356.5% relative activity were found as the most active compounds, again. Additionally, the relative activity of the compound **5h** was increased 2.18 fold (305.2% relative activity), when the concentration was increased from 0.05 mM to 0.1 mM. When the concentration was 0.2 mM, the activity was observed in sequence with **5b>5h>5c>5f** (Relative activity percentages were determined 482.5%, 444.8%, 395.5% and 384.4%, respectively). Herein, it is remarkable that the activatory effect of the compounds **5c** and **5h** were increased 3.4 and 3.17 fold, when the concentrations were changed from 0.05 mM to 0.2 mM. As can be seen from Table 2, another important point is the increase in the activity of the compound **5d**. The relative activity of the mentioned compound was determined as 70.8% at 0.05 mM, when the concentration was incerased to 0.2 mM the relative activity was increased to 149.9% with 2.12 fold. The % relative activity of the other derivatives was changed less than two fold, when the concentration was increased two fold.

## 3. Discussion

The activation of the paraoxonase enzyme maintains HDL reverse cholesterol transport function by protecting it oxidation protection. This situation prevents foam cell formation by the cholesterol accumulation in the cell wall and it also prevents the development of atherosclerosis [38]. Again, as a result of the activation of PON1 enzyme, the antioxidant power of the body increases; thus it provides to treat cancer, diabetes, connective tissue diseases, hepatic, and renal pathologies such as Parkinson's disease. When the obtained results are evaluated, the synthesized triazole compounds except **5a** have been activated the human PON1. Compounds **5b** with 5-nitrothiazole, **5c** with 2-benzothiazole, **5f** with 6-ethoxy-2-benzothiazole and **5h** with 6-fluoro-2-benzothiazole moieties may be considered to emerge on further studies as PON1 enzyme activator.

## 4. Conclusion

In this work, nine new triazole compounds including thiazole and benzothiazole rings were synthesized and evaluated for their increasing effects on paraoxonase-1 (PON1) activity. Compound **5b** bearing 5-nitrothiazole moiety provoked the highest relative PON1 enzymatic activity at 0.2 mM dose. Additionally, compounds **5c, 5f** and **5h** have also been determined to increase PON1 activity, remarkably. These four compounds have come into prominence and they are thought to investigate for further *in vivo* studies to be a drug candidate.

**Table 2.** The effect of **5a-i** series on purified PON1 activity from human serum

| Compounds | Concentration (mM) | Relative Activity (%) | Concentration (mM) | Relative Activity (%) | Concentration (mM) | Relative Activity (%) |
|---|---|---|---|---|---|---|
| **None** | - | 100 ± 0.0 (168.4±1.1 U/mL) | - | 100 ± 0.0 | - | 100 ± 0.0 (168.4±1.1 U/mL) |
| **5a** | 0.05 | 67.5 | 0.1 | 32.8 | 0.2 | 16.9 |
| **5b** | 0.05 | 392.9 | 0.1 | 452.8 | 0.2 | 482.5 |
| **5c** | 0.05 | 116.2 | 0.1 | 218.2 | 0.2 | 395.5 |
| **5d** | 0.05 | 70.8 | 0.1 | 114.9 | 0.2 | 149.9 |
| **5e** | 0.05 | 240.9 | 0.1 | 276.7 | 0.2 | 292.9 |
| **5f** | 0.05 | 304.5 | 0.1 | 356.5 | 0.2 | 384.4 |
| **5g** | 0.05 | 161.7 | 0.1 | 202.4 | 0.2 | 227.8 |
| **5h** | 0.05 | 140.3 | 0.1 | 305.2 | 0.2 | 444.8 |
| **5i** | 0.05 | 103.2 | 0.1 | 163.7 | 0.2 | 179.2 |

## 5. Materials and Methods

### 5.1. Chemistry

All chemicals and DEAE-cellulose, glycine, $(NH_4)_2SO_4$, protein assay reagents, and chemicals for electrophoresis were purchased from Sigma-Aldrich Co. (Sigma-Aldrich Corp., St. Louis, MO, USA) and Merck Chemicals (Merck KGaA, Darmstadt, Germany). Melting points were determined using an Electrothermal 9100 digital melting point apparatus (Electrothermal, Essex, UK) and were uncorrected. Spectroscopic data were recorded on the following instruments. IR: Shimadzu 8400 FTIR spectrophotometer (Shimadzu, Tokyo, Japan); $^1$H-NMR: Bruker DPX 400 NMR spectrometer (Bruker Bioscience, Billerica, MA, USA), in DMSO-$d_6$, TMS as internal standard; $^{13}$C-NMR: Bruker DPX 100 NMR spectrometer (Bruker Bioscience, Billerica, MA, USA), in DMSO-$d_6$, MS: AB SCIEX-3200 Q-TRAP LC/MS/MS MASS spectrometer (Fisons Instruments Vertriebs GmbH, Mainz, Germany). Elemental analyses were performed on a Leco TruSpec Micro CHN/CHNS elemental analyzer (Leco, Michigan, USA).

*5.1.1. Synthesis of ethyl 3-cyclohexylpropanoate* ***(1)****.* 3-Cyclohexanepropionic acid (0.20 mol) was refluxed in excess ethanol (0.25 mol) for 12 h. The obtained ester was treated with water and saline. Compound ethyl 3-cyclohexylpropanoate (**1**) was given with separation funnel. Boiling point was detected 105-113 °C for this compounds in a previous literature [39].

*5.1.2. Synthesis of 3-cyclohexylpropanohydrazide* ***(2).*** 0.15 mole of ethyl 3-cyclohexylpropanoate (**1**) and 0.3 mol of 85% hydrazine hydrate in 200 mL ethanol were stirred for six hours, then the precipitate was filtered and washed with water. The melting point was identified as 90 °C according to the reported literature [40].

*5.1.3. Synthesis of 1-(3-cyclohexylethyl)-4-phenyl-3-thiosemicarbazide* ***(3).*** Compound **2** (0.1 mol) was refluxed with phenylisothiocyanate (0.1 mol) in ethanol for 2h and the precipitate was filtered.

*5.1.4. 4-Phenyl-5-(3-cyclohexylethyl)-2,4-dihydro-3H-1,2,4-triazol-3-thione* ***(4).*** Compound **4** was given by refluxing compound **3** (0.05 mol) with aqueous KOH (200 mL) solution. After 3 h refluxing, the reaction mixture was cooled at room temperature and neutralized with acetic acid solution and then filtered.

*5.1.5. General procedure for the synthesis of 2-[[5-(2-cyclohexylethyl)-4-phenyl-4H-1,2,4-triazol-3-yl] thio]-N-(substitutedthiazol/benzothiazol-2-yl)acetamide derivatives* ***(5a-i).*** A mixture of compound **4** (0.002 mol), appropriate 2-chloro-*N*-(substituted thiazolyl/benzothiazolyl)acetamide derivative (0.002 mol) and potassium carbonate (0.002 mol) in acetone was stirred for 2 h. After evaporating the solvent, the precipitate was treated with water and then filtered. Afterwards, the raw products was recrstallised from ethanol.

*5.1.5.1. 2-[[5-(2-Cyclohexylethyl)-4-phenyl-4H-1,2,4-triazol-3-yl]thio]-N-(thiazol-2-yl)acetamide* ***(5a).*** Yield 83 %, m.p. 207 °C. IR (KBr, cm$^{-1}$) : $\nu_{max}$ 3291 (amide N-H), 1694 (C=O), 1543-1426 (C=C, C=N), 1268-1136 (C-O, C-N). $^1$H-NMR (400 MHz, DMSO-$d_6$, ppm) : δ 0.85-0.89 (m, 2H, cyclohexyl protons), 1.35-1.62 (m, 9H, cyclohexyl protons), 2.48-2.54 (m, 4H, -$CH_2$-$CH_2$-), 4.13 (s, 2H, -CO-$CH_2$), 7.22 (d, *J*=3.6 Hz, 1H, Ar-H), 7.43-7.47 (m, 3H, Ar-H), 7.58-7.56 (m, 3H, Ar-H), 12.38 (brs, 1H, N-H). For $C_{21}H_{25}N_5OS_2$ calculated: 58.99 % C, 5.89 % H, 16.38 % N, 15.00 % S; found: 58.94 % C, 5.87 % H, 16.33 % N, 15.03 % S. MS $[M+1]^+$: m/z 428.

*5.1.5.2. 2-[[5-(2-Cyclohexylethyl)-4-phenyl-4H-1,2,4-triazol-3-yl]thio]-N-(5-nitrothiazol-2-yl)acetamide* ***(5b).*** Yield 85 %, m.p. 116-117 °C. IR (KBr, cm$^{-1}$) : $\nu_{max}$ 3279 (amide N-H), 1687 (C=O), 1564-1456 (C=C, C=N, $NO_2$), 1238-1152 (C-O, C-N). $^1$H-NMR (400 MHz, DMSO-$d_6$, ppm) : δ 0.84-0.90 (m, 2H, cyclohexyl protons), 1.33-1.63 (m, 9H, cyclohexyl protons), 2.48-2.53 (m, 4H, -$CH_2$-$CH_2$-), 4.03 (s, 2H, -CO-$CH_2$), 7.44-7.46 (m, 2H, Ar-H), 7.56-7.59 (m, 3H, Ar-H), 8.41 (s, 1H, thiazole-H), 12.65 (brs, 1H, N-H). $^{13}$C-NMR (100 MHz, DMSO-$d_6$, ppm) : δ 24.48, 25.23, 32.44, 33.38, 39.43, 40.28, 127.99, 130.58, 133.95, 137.30, 146.45, 150.81, 156.08, 173.33, 174.86. For $C_{21}H_{24}N_6O_3S_2$ calculated: 53.37 % C, 5.12 % H, 17.78 % N, 13.57 % S; found: 53.34 % C, 5.13 % H, 17.74 % N, 13.60 % S. MS [M+1]+: m/z 473.

*5.1.5.3. 2-[[5-(2-Cyclohexylethyl)-4-phenyl-4H-1,2,4-triazol-3-yl]thio]-N-(benzothiazol-2-yl)acetamide* ***(5c).*** Yield 75 %, m.p. 262-264 °C. IR (KBr, cm$^{-1}$) : $\nu_{max}$ 3288 (amide N-H), 1679 (C=O), 1512-1478 (C=C, C=N), 1256-1110 (C-O, C-N). $^1$H-NMR (400 MHz, DMSO-$d_6$, ppm) : δ 0.85-0.93 (m, 2H, cyclohexyl protons), 1.34-1.68 (m, 9H, cyclohexyl protons), 2.49-2.56 (m, 4H, -$CH_2$-$CH_2$-), 4.22 (s, 2H, -CO-$CH_2$), 7.30-7.63 (m, 7H, Ar-H), 7.76 (d, *J*=7.6 Hz, 1H, Ar-H), 7.98 (d, *J*=7.6 Hz, 1H, Ar-H), 12.65 (brs, 1H, N-H). For $C_{25}H_{27}N_5OS_2$ calculated: 62.86 % C, 5.70 % H, 14.66 % N, 13.43 % S; found: 62.84 % C, 5.72 % H, 14.70 % N, 13.45 % S. MS [M+1]+: m/z 478.

*5.1.5.4. 2-[[5-(2-Cyclohexylethyl)-4-phenyl-4H-1,2,4-triazol-3-yl]thio]-N-(6-methylbenzothiazol-2-yl)acetamide* ***(5d).*** Yield 79 %, m.p. 285 °C. IR (KBr, cm$^{-1}$) : $\nu_{max}$ 3284 (amide

N-H), 1686 (C=O), 1546-1438 (C=C, C=N), 1230-1139 (C-O, C-N). $^1$H-NMR (400 MHz, DMSO-$d_6$, ppm) : δ 0.85-0.93 (m, 2H, cyclohexyl protons), 1.35-1.68 (m, 9H, cyclohexyl protons), 2.41 (s, 3H, -$CH_3$), 2.49-2.56 (m, 4H, -$CH_2$-$CH_2$-), 4.20 (s, 2H, -CO-$CH_2$), 7.47-7.49 (m, 1H, Ar-H), 7.58-7.65 (m, 6H, Ar-H), 7.76 (s, 1H, Ar-H), 12.59 (brs, 1H, N-H). For $C_{26}H_{29}N_5OS_2$ calculated: 63.51 % C, 5.95 % H, 14.24 % N, 13.04 % S; found: 63.55 % C, 5.93 % H, 14.21 % N, 13.06 % S. MS [M+1]+: m/z 492.

*5.1.5.5. 2-[[5-(2-Cyclohexylethyl)-4-phenyl-4H-1,2,4-triazol-3-yl]thio]-N-(6-methoxybenzothiazol-2-yl)acetamide* ***(5e).*** Yield 85 %, m.p. 264 °C. IR (KBr, $cm^{-1}$) : $\nu_{max}$ 3279 (amide N-H), 1679 (C=O), 1505-1439 (C=C, C=N), 1239-1051 (C-O, C-N). $^1$H-NMR (400 MHz, DMSO-$d_6$, ppm) : δ 0.85-0.93 (m, 2H, cyclohexyl protons), 1.34-1.68 (m, 9H, cyclohexyl protons), 2.49-2.56 (m, 4H, -$CH_2$-$CH_2$-), 3.81 (s, 3H, -$OCH_3$), 4.19 (s, 2H, -CO-$CH_2$), 7.03 (dd, *J*=8.4, 2.4 Hz, 1H, Ar-H), 7.47-7.66 (m, 7H, Ar-H), 12.52 (brs, 1H, N-H). For $C_{26}H_{29}N_5O_2S_2$ calculated: 61.51 % C, 5.76 % H, 13.80 % N, 12.63 % S; found: 61.56 % C, 5.78 % H, 13.87 % N, 12.66 % S. MS [M+1]+: m/z 508.

*5.1.5.6. 2-[[5-(2-Cyclohexylethyl)-4-phenyl-4H-1,2,4-triazol-3-yl]thio]-N-(6-ethoxybenzothiazol-2-yl)acetamide* ***(5f).*** Yield 76 %, m.p. 228-229 °C. IR (KBr, $cm^{-1}$) : $\nu_{max}$ 3281 (amide N-H), 1682 (C=O), 1538-1441 (C=C, C=N), 1284-1112 (C-O, C-N). $^1$H-NMR (400 MHz, DMSO-$d_6$, ppm) : δ 0.84-0.88 (m, 2H, cyclohexyl protons), 1.23-1.61 (m, 12H, cyclohexyl protons, $CH_3$), 2.46-2.53 (m, 4H, -$CH_2$-$CH_2$-), 4.04 (q, J=7 Hz, 2H, $CH_2$-$CH_3$), 4.16 (s, 2H, -CO-$CH_2$), 6.98 (dd, *J*=8.0, 2.8 Hz, 1H, Ar-H), 7.43-7.62 (m, 7H, Ar-H), 12.43 (brs, 1H, N-H). $^{13}$C-NMR (100 MHz, DMSO-$d_6$, ppm) : δ 15.35, 24.47, 25.22, 32.42, 33.31, 36.49, 39.41, 64.32, 106.13, 115.99, 121.88, 127.92, 130.63, 130.71, 133.48, 133.71, 143.23, 149.69, 156.11, 156.54, 167.52. For $C_{27}H_{31}N_5O_2S_2$ calculated: 62.16 % C, 5.99 % H, 13.42 % N, 12.29 % S; found: 61.20 % C, 5.98 % H, 13.46 % N, 12.31 % S. MS [M+1]+: m/z 522.

*5.1.5.7. 2-[[5-(2-Cyclohexylethyl)-4-phenyl-4H-1,2,4-triazol-3-yl]thio]-N-(6-chlorobenzothiazol-2-yl)acetamide* ***(5g).*** Yield 79 %, m.p. 272-273 °C. IR (KBr, $cm^{-1}$) : $\nu_{max}$ 3293 (amide N-H), 1683 (C=O), 1512-1463 (C=C, C=N), 1310-1128 (C-O, C-N). $^1$H-NMR (400 MHz, DMSO-$d_6$, ppm) : δ 0.81-0.89 (m, 2H, cyclohexyl protons), 1.30-1.64 (m, 9H, cyclohexyl protons), 2.46-2.53 (m, 4H, -$CH_2$-$CH_2$-), 4.18 (s, 2H, -CO-$CH_2$), 7.42-7.59 (m, 6H, Ar-H), 7.72 (d, *J*=8.8 Hz, 1H, Ar-H), 8.09 (s, 1H, Ar-H), 12.72 (brs, 1H, N-H). $^{13}$C-NMR (100 MHz, DMSO-$d_6$, ppm) : δ 24.46, 25.21, 32.42, 33.29, 36.43, 39.58, 122.15, 122.51, 127.19, 127.91, 128.36, 130.65, 130.74, 133.66, 133.85, 148.11, 149.66, 156.56, 159.38, 168.11. For $C_{25}H_{26}ClN_5OS_2$ calculated: 58.64 % C, 5.12 % H, 13.68 % N, 12.52 % S; found: 58.66 % C, 5.13 % H, 13.72 % N, 12.54 % S. MS [M+1]+: m/z 512.

*5.1.5.8. 2-[[5-(2-Cyclohexylethyl)-4-phenyl-4H-1,2,4-triazol-3-yl]thio]-N-(6-fluorobenzothiazol-2-yl)acetamide* ***(5h).*** Yield 88 %, m.p. 242-244 °C. IR(KBr, $cm^{-1}$) : $\nu_{max}$ 3287 (amide N-H), 1687 (C=O), 1553-1426 (C=C, C=N), 1285-1113 (C-O, C-N). $^1$H-NMR (400 MHz, DMSO-$d_6$, ppm) : δ 0.81-0.89 (m, 2H, cyclohexyl protons), 1.30-1.64 (m, 9H, cyclohexyl protons), 2.47-2.53 (m, 4H, -$CH_2$-$CH_2$-), 4.18 (s, 2H, -CO-$CH_2$), 7.26 (t, *J*=8.6 Hz, 1H, Ar-H), 7.44-7.60 (m, 5H, Ar-H), 7.72-7.76 (m, 1H, Ar-H), 7.86 (dd, J=8.0, 2.8 Hz, 1H, Ar-H), 12.65 (brs, 1H, N-H). $^{13}$C-NMR (100 MHz, DMSO-$d_6$, ppm) : δ 24.49, 25.22, 32.43, 33.31, 36.42, 39.41, 108.75, 109.02, 114.85, 115.09, 122.39, 122.48, 127.92, 130.66, 130.75, 133.34, 133.46, 133.68, 145.94, 149.68, 156.56, 158.18, 158.51, 160.56, 167.97. For $C_{25}H_{26}FN_5OS_2$ MS calculated: 60.58 % C, 5.29 % H, 14.13 % N, 12.94 % S; found: 60.63 % C, 5.30 % H, 14.17 % N, 12.97 % S. [M+1]+: m/z 496.

*5.1.5.9. 2-[[5-(2-Cyclohexylethyl)-4-phenyl-4H-1,2,4-triazol-3-yl]thio]-N-(6-nitrobenzothiazol-2-yl)acetamide* ***(5i).*** Yield 86 %, m.p. 235-237 °C. IR (KBr, $cm^{-1}$) : $\nu_{max}$ 3276 (amide N-H), 1684 (C=O), 1547-1445 (C=C, C=N, $NO_2$), 1281-1110 (C-O, C-N). $^1$H-NMR (400 MHz, DMSO-$d_6$, ppm) : δ 0.85-0.91 (m, 2H, cyclohexyl protons), 1.39-1.66 (m, 9H, cyclohexyl protons), 2.51-2.57 (m, 4H, -$CH_2$-$CH_2$-), 4.22 (s, 2H, -CO-$CH_2$), 7.49-7.51 (m, 2H, Ar-H), 7.60-7.63 (m, 3H, Ar-H), 7.83 (d, *J*=9.2 Hz, 1H, Ar-H), 8.24 (d, *J*=8.4 Hz, 1H, Ar-H), 8.96 (s, 1H, Ar-H), 12.72 (brs, 1H, N-H). $^{13}$C-NMR (100 MHz, DMSO-$d_6$, ppm) : δ 23.71, 24.45, 31.65, 32.54, 36.60, 38.64, 118.60, 119.97, 121.47, 127.16, 129.88, 129.93, 132.32, 132.96, 142.36, 149.19, 154.04, 155.67, 165.33, 169.08. For $C_{25}H_{26}N_6O_3S_2$ MS calculated: 57.45 % C, 5.01 % H, 16.08 % N, 12.27 % S; found: 57.43 % C, 5.03 % H, 16.11 % N, 12.29 % S. [M+1]+: m/z 523.

### 5.2. Activity Tests

*5.2.1. Purification of Paraoxonase-I (PON1) from human serum*

*5.2.1.1. Ammonium sulfate precipitation.* Firstly, the serum was precipitated at 60-80% saturated of ammonium sulfate. At the highest concentration, PON1 enzyme could be precipitated in the range of 60-80% ammonium sulphate saturation. For this reason, the proteins in human serum which were firstly collapsed by 60% saturation were removed.

Then, the supernatant was brought to 80% ammonium sulphate saturation. Lastly, it was centrifuged at 15.000 *xg* for 20 min and the PON1 enzyme was obtained in the precipitate. Then, precipitate was solved phosphate buffer (100 mM Na-phosphate, pH=7.0). The sample was placed in dialysis membrane and it was dialyzed against of dialysis buffer (25 mM Na-phosphate pH =7.0) during two hours.

*5.2.2. Human serum paraoxonase-I activity assay.* Paraoxonase activity of the enzyme was determined at 25°C in the presence of 1mM $CaCl_2$, 50mM glycine/NaOH buffer (pH = 10.5) by using paraoxon a substrate. Activity measurement is based on establishing the changes in absorption of formed *p*-nitrophenol as a result of the reaction of PON1 with paraoxon at 412 nm. *p*-Nitrophenol the molar extinction coefficient is e = 18.290$M^{-1}$ $cm^{-1}$ and an enzyme unit is number of micromole of hydrolysis paraoxon in per minute [41-43].

*5.2.3. Determination of effects of compounds of 5a-i derivatives on human serum PON1 enzyme activity in vitro.* Activity assays were done to determine the effect of compounds **5a-i** on the activity of human serum PON1 by joining compounds to the cuvette at different concentrations. All compounds were solved using DMSO. The stock solutions are diluted to create the 7 different concentrations of used **5a-i** derivatives. PON1 was measured by a spectrophotometric assay. The spectrophotometric assay based on the ability of PON1 to hydrolyse paraoxon substrate (diethyl *p*-nitrophenyl phosphate). Blank samples were prepared using DMSO. Results were given as Activity (U/mL)-Activator Concentration (mM) graphics. The relative activity obtained for the control sample was taken as 100% and the relative activities' values were calculated for the triazole compounds **(5a-i)** at indicated concentrations (0.05, 0.1 ve 0.2 mM) using Activity (U/mL)-Activator Concentration (mM) graphics.

*5.2.4. Sepharose 4B-L-tyrosine-1-naphthylamine affinity chromatograph*

The human serum was applied to the affinity column having a structure of Sepharose 4B-L-tyrosine-1-naphthylamine and equilibrated with 25 mM Tris/HCl (pH 8.0) / 10 mM $CaCl_2$. The affinity gel was washed with the solution 25 mM Tris/HCl (pH 8.0) / 10mM $CaCl_2$ / 3M NaCl. Human paraoxonase 1 was eluted with the solution 25 mM Tris/HCl (pH 8.0)/10 mM $CaCl_2$ at 0.5mL/min. Fractions (3 mL each) were collected as 3 mL, and those with the highest PON1 activity were combined [42, 43].

*5.2.5. Protein determination.* Protein assay was performed according to the method of Bradford for all purification steps of PON1 enzyme from human serum. This method is based on the principle which Coomassie brilliant blue G-250 is binding to proteins in medium of phosphoric acid. Formed complex shows a maximum absorbance at 595 nm. For this purpose, a standard was prepared using serum albumin. After the determination of protein content with Bradford method in the enzyme solutions, amount of protein was calculated using the standard graph. The sensitivity of this method is between 1-100 micrograms [44].

*5.2.6. SDS polyacrylamide gel electrophoresis.* After PON1 was purified, purity of enzyme was checked by using 3-10% discontinuous sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) electrophoresis according to Laemmli [45].

*5.2.7. Statistical analysis.* All of the tests were conducted in triplicate for determination of the PON1 activities of samples. Data were expressed as mean +/- standard deviation. Statistical analyses were performed using SPSS version 10.0 software (SPSS Inc., Chicago, IL, USA), and the significant differences were determined with a 95% confidence interval ($p < 0.001$ and $p < 0.05$) using Tukey's test.

## Authorship statement

Author contributions: Concept – L.Y; Design – Z.A.K.; Supervision – Z.A.K.; Resource – K.K.; Materials – H.N; Data Collection and/or Processing – L.Y.; Analysis and/or Interpretation – L.Y.; Literature Search – L.Y.; Writing – L.Y.; Critical Reviews – L.Y.

## Conflict of interest statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper. The authors report no conflicts of interest.

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