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In Vitro Inhibition Effect of Some Dihydroxy Coumarin Compounds on Purified Human Serum Paraoxonase 1 (PON1)

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Abstract Human serum paraoxonase 1 (PON1; EC 3.1.8.1) is a high-density lipoprotein associated, calcium-dependent enzyme that hydrolyses aromatic esters, organophosphates and lactones and can protect the low-density lipoprotein against oxidation. In this study, in vitro inhibition effect of some dihydroxy coumarin compounds namely 6,7-dihydroxy-3-(2-methylphenyl)-2H-chromen-2-one (A), 6,7-dihydroxy-3-(3-methylphenyl)-2H-chromen-2-one (B) and 6,7-dihydroxy-3-(4-methylphenyl)-2H-chromen-2-one (C) on purified PON1 were investigated by using paraoxon as a substrate. PON1 was purified using two-step procedures, namely ammonium sulphate precipitation and Sepharose-4B-L-tyrosine-1-naphthylamine hydrophobic interaction chromatography. The purified enzyme had a specific activity of 11.76 U/mg. The dihydroxy coumarin derivatives of A and B compounds inhibited PON1 enzyme activity in a noncompetitive inhibition manner with K_i of 0.0080±0.256 and 0.0003±0.018 mM values, respectively. C compound exerted an uncompetitive inhibition of PON1 enzyme activity with K_i of 0.0010± 0.173 mM. Moreover, dihydroxy coumarin derivatives of A, B and C compounds were effective inhibitors on purified human serum PON1 activity with IC_{50} of 0.012, 0.022 and 0.003 mM values, respectively. IC_{50} value of unsubstituted 6,7 dihydroxy coumarin was found as 0.178 mM. The present study has demonstrated that PON1 activity is very highly sensitive to studied coumarin derivatives.

Keywords Paraoxonase 1 · In vitro inhibition · Coumarin derivatives

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Introduction

In humans, the paraoxonase (PON) gene family has three members; PON1, PON2 and PON3 aligned next to each other on the long arms of chromosome 7(q21.22) [1, 2]. Human serum paraoxonase 1 (PON1; EC 3.1.8.1) is the best studied member of the family of mammalian enzymes. PON1 is synthesised primarily in the liver and a portion is secreted into the blood, where it is associated exclusively with high-density lipoproteins (HDLs) through its retained hydrophobic leader sequence [2–4].

PON1 is a calcium dependent serum enzyme that has 354-amino acids with a molecular mass of 43 kDa. PON1 received its name from paraoxon, the toxic metabolite of the organophosphate insecticide parathion, which is one of its most studied substrate [3, 5]. PON1 also catalyses the hydrolysis of multiple substrates; lactones, thiolactones, cyclic carbonate esters, aromatic esters and phosphotriesters [5–9]. PON1 can bind reversibly to organophosphate substrates, which it hydrolyses [1]. Therefore, PON1 plays a major role in the detoxification of these compounds and other artificial substrates, so that it may alter significantly an individual's susceptibility to the toxicity of these chemicals. One natural physiological function of PON1 is the metabolism of toxic oxidised lipids of both low density lipoprotein (LDL) and HDL particles [3]. In vitro studies show that PON1 both prevents the formation of oxidised LDL and inactivates LDL-derived oxidised phospholipids once they are formed. PON1 also protects phospholipids in HDL from oxidation [3, 10, 11].

Serum PON1 was found to possess important antioxidant and antiatherogenic properties [12–15]. PON1 levels and its arylesterase/PON activities were shown to be inversely related to the risk of coronary heart diseases, hypercholesterolemia and diabetes [16–19]. Atherosclerotic patients, who are under oxidative stress, are characterised by decreased serum PON1 levels and activities [14]. Therefore, due to the relationship of PON1 with atherosclerosis, which is a disease underlying cause of 50 % mortality in Western society and organophosphates poisoning, this enzyme is subject to intensive research [20]. The physiological substrates and mechanisms of antiatherogenic activity of PON1 still remain unknown. Despite its traditional assignment as PON/arylesterase, pieces of evidence have been accumulated to indicate PON1 as lactonase [21]. Although the endogenous substrates of PONs are still uncertain, they are likely to include lactones consumed as drug metabolites, food ingredients and derivatives of fatty acid oxidation processes such as 5-HETEs lactone that resides in HDL [21]. PON1 exhibits a substrate-dependent polymorphism. This polymorphism is related to two polymorphic sites at amino acid positions 55 and 192. The Q isozyme of PON1 (formerly called A-type, glutamine at position 192) is several times less efficient than the R isozyme (formerly called B-type, arginine at position 192) in hydrolysing paraoxon [22]. On the other hand, soman, sarin and diazoxon are hydrolysed much better by the Q than the R-isozyme [23].

Natural products have been important sources of new pharmacological active agents. Plant-derived products have led to discovery of many clinically useful drugs for the treatment of human diseases such as antitumor and anti-infective drugs. Coumarins are well-known natural products displaying a broad range of biological activities [24]. Coumarins comprise a very large class of compounds found throughout the plant kingdom [25, 26]. Coumarins owe their class name to 'Coumarou', the vernacular name of the tonka bean (*Dipteryx odorata* Willd., Fabaceae), from which coumarin itself was isolated in 1820 [27].

Coumarin is classified as a member of the benzopyrone family of compounds, all of which consist of a benzene ring joined to a pyrone ring [28]. The benzopyrones can be subdivided into the benzo-α-pyrones to which the coumarins belong and the benzo-γ-pyrones, of which the flavonoids are principal members [28]. The biological activity of

coumarin and more complex-related derivatives appears to be based on the coumarin nucleus [29]. The diverse biological activities of natural and synthetic coumarin derivatives as anticoagulants and antithrombotics are well known [30]. Biological effects observed include antibacterial, antithrombotic and vasodilatory, antimutagenic, lipoxygenase and cycloxygenase inhibition, scavenging of reactive oxygen species and antitumourigenic effects [29].

In view of the biological interference of coumarin compounds with coagulation and thrombotic events and the reported antiatherogenic properties of PONs, we tried to examine the in vitro effects of 6,7-dihydroxy coumarin derivatives on the purified human serum PON1.

Materials and Methods

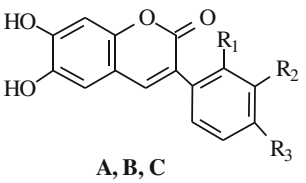
Materials

The materials used include Sepharose 4B, L-tyrosine, 1-napthylamine, paraoxon, 6,7-dihydroxy coumarin and protein assay reagents were obtained from Sigma Chem. Co. 6,7-dihydroxy-3-(2-methylphenyl)-2H-chromen-2-one (A), 6,7-dihydroxy-3-(3-methylphenyl)-2H-chromen-2-one (B) and 6,7-dihydroxy-3-(4-methylphenyl)-2H-chromen-2-one (C) (Scheme 1) were prepared by previously described method [31]. All other chemicals used were of analytical grade and purchased from either Sigma-Aldrich or Merck.

Purification of PON1 Enzyme from Human Serum by Hydrophobic Interaction Chromatography

Human serum was isolated from 175 ml fresh human blood and put into a dry tube. The blood samples were centrifuged at 1,500 rpm for 15 min, and the 50 ml serum was removed. Firstly, serum PON1 enzyme was isolated by ammonium sulphate precipitation. The precipitation intervals for PON1 were 60–80 % [20]. The precipitate was collected by centrifugation at 15,000 rpm for 20 min and redissolved in 100 mM Tris–HCl buffer at pH 8.00. The pooled precipitate obtained from human serum by using ammonium sulphate precipitation was subjected to hydrophobic interaction chromatography. The final saline concentration of precipitate was adjusted to 1 M ammonium sulphate, prior to that it was loaded onto the hydrophobic column prepared from Sepharose 4B-L-tyrosine-1-napthylamine [20]. The column was equilibrated with 0.1 M Na_2HPO_4 buffer at pH 8.00 including 1 M ammonium sulphate. The PON1 enzyme was eluted with ammonium sulphate gradient using 0.1 M Na_2HPO_4 buffer with and without ammonium sulphate at pH 8.00. The purified PON1 enzyme was stored in the presence of 2 mM $CaCl_2$ at +4 °C, in order to maintain activity.

Scheme 1 Chemical structures of dihydroxy coumarin derivatives tested for PON1 inhibition and antimicrobial activity



A, B, C

	R_1	R_2	R_3
A	CH_3	H	H
B	H	CH_3	H
C	H	H	CH_3

PON Enzyme Assay

PON activity towards paraoxon was quantified spectrophotometrically by the method described by Gan et al. [32]. The enzyme assay was based on the estimation of *p*-nitrophenol at 412 nm. The molar extinction coefficient of *p*-nitrophenol (ε=17,100 $M^{-1}cm^{-1}$ at pH 10.5) 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). Two millimolars of final substrate concentration was used during enzyme assay, and all measurements were taken in duplicate and corrected for the non-enzymatic hydrolysis.

In Vitro Inhibition Kinetic Studies and Determination of IC_{50} and K_i Values

For the inhibition studies of coumarin derivatives, different concentrations of coumarin derivatives were added to the reaction medium. PON activity with coumarin derivatives was assayed by following the hydration of paraoxon. Activity percentage values of PON for five different concentrations of each coumarin derivatives were determined by regression analysis using the Microsoft Office 2000 Excel. PON1 enzyme activity without a coumarin derivative was accepted as 100 % activity. The inhibitor concentration causing up to 50 % inhibition (IC_{50} values) for coumarin derivatives were determined from the graphs. In addition, K_i values of coumarin derivatives were determined on PON activity. In order to obtain K_i values, K_m and V_{max} values of the PON1 enzyme using paraoxon as a substrate was measured at seven different substrate concentrations at pH 8.00 and 37 °C. K_m and V_{max} values were determined by means of Lineweaver–Burk graphs. Seven different concentrations of substrate, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 4.0 mM, were added to the reaction medium in the presence and the absence of coumarin derivative. The lowest and the highest final concentrations of 0.0019 and 0.0343 mM for A and 0.0021 and 0.0463 mM for B and 0.00103 and 0.0206 mM for C were obtained in the reaction medium at two different fixed concentrations of coumarin derivatives. K_i values were calculated from Lineweaver–Burk graphs. IC_{50} and K_i values of unsubstituted 6,7-dihydroxy coumarin were also determined to elaborate on structure activity relationship (SAR) of tested substituted 6,7-dihydroxy coumarin derivative inhibitors of PON1.

Statistical Analysis

Statistical analysis was performed using Minitab program for Windows, version 10.02. Analysis of variance was used when more than two groups were compared. Data are presented as mean±SD.

Results and Discussion

In this study, human serum PON1 was purified by two-step procedures using ammonium sulphate precipitation and Sepharose-4B-L-tyrosine-1-napthylamine hydrophobic interaction chromatography which was specifically designed to the retained N-terminal hydrophobic signal peptide for PON1 enzyme. Ammonium sulphate precipitation has been chosen for the initial purification step of PON1 enzyme. The PON enzyme activity determined from each step, shows that the highest enzyme activity were at the interval of 60–80 %. Subsequently, prior to loading onto hydrophobic interaction column, the precipitate was saturated with 1 M

ammonium sulphate in order to improve its efficiency for binding to hydrophobic gel of the column. A hydrophobic gel has been used in order to reduce the number of the purification steps of PON enzyme. The hydrophobic gel was designated based on the retained N-terminal hydrophobic signal peptide for PON1 enzyme. 1-napthylamine, which is a hydrophobic group, was added to Sepharose-4B gel matrix with the extension of L-tyrosine arm [20]. A 150.19-fold purification with a yield of 11.89 % was achieved (Table 1).

Many chemicals at relatively low doses affect metabolism by altering normal enzyme activity, particularly through inhibition of a specific enzyme [33]. Therefore, we investigated the effect of dihydroxy coumarin compounds namely A, B and C on purified human serum PON1 using paraoxon as a substrate.

Coumarins are active components of herbs used for the treatment of various diseases [34]. A wide spectrum of biochemical and pharmacological activities is displayed by coumarins and their derivatives. Coumarin derivatives are used widely as anticoagulants (such as warfarin, –OH group is attached at four positions) for the treatment of disorders in which there is excessive or undesirable clotting, such as thrombophlebitis, pulmonary embolism and certain cardiac conditions. In the literature, two coumarin-based sulfamate drugs (667COUMATE and STX 118) have been reported to have IC_{50} values of 25–59 nM for the inhibition of hCA-II activity. Synthesis of coumarin derivatives has been accomplished either by Knoevenagel condensation of anilinosulfonylacetic acids with suitable salicylaldehydes or by the reaction of methyl anilinosulfonylacetates with substituted salicylaldehydes in the presence of a catalytic amount of a base [31]. Recently, new synthetic routes to this class of compounds have been reported, also detailing their variable and interesting biological and pharmacological activities [34–37]. There have also been very interesting studies of the synthesis and selective biological properties of 7-hydroxy-4-methyl-2H-chromen-2-one, 7-hydroxy-4,5-dimethyl-2H-chromen-2-one and some of their derivatives [34].

The ability to hydrolyse paraoxon and phenyl acetate is routinely used for measuring PON1 activity in vitro. In this study, V_{max}, K_m and the specific activity of the purified enzyme for paraoxon were determined 1.5×10^{-3} U, 0.666 mM and 11.76 U/ml, respectively. The IC_{50} values are presented in Table 2. All the three dihydroxy coumarin derivatives of A, B and C compounds demonstrated higher inhibitory activities against PON1 enzyme than unsubstituted 6,7-dihydroxy coumarin (Fig. 1). Comparing them, it is clear that the most potent inhibitor of PON1 enzyme was C with an IC_{50} value of 0.003 mM (Fig. 1; Table 2).

We determined the inhibition constant values (K_i) and inhibition types using the Lineweaver–Burk method with different paraoxon concentrations (Table 2). The kinetics of interaction of unsubstituted 6,7-dihydroxy coumarin and coumarin derivative compounds with the purified human serum PON1 indicated a different inhibition pattern. The dihydroxy

Table 1 Summary of the steps involved in the purification of human serum PON1

Steps	Volume (ml)	Activity (U/ml)	Total activity (U)	Protein amount (mg/ml)	Total protein (mg)	Specific activity (U/mg)	Overall yield (%)	Overall purification (fold)
Serum	50	66.316	3,315.8	1,482.88	74,147	0.0448	100	1
Ammonium sulphate fractionation	18	83.588	1,504.58	1,067.5	19,215	0.0783	45.37	1.74
Hydrophobic interaction chromatography	3	131.404	394.21	11.17	33.51	11.76	11.89	150.19

Table 2 The IC_{50} and K_i values and inhibition types of unsubstituted 6,7-dihydroxy coumarin, A, B and C compounds on purified PON1 activity

Dihdydroxy coumarin compounds	IC_{50} (mM)	K_i (mM)	Inhibition type
A	0.012	0.0080±0.256	Noncompetitive
B	0.022	0.0003±0.018	Noncompetitive
C	0.003	0.0010±0.173	Uncompetitive
Unsubstituted 6,7-dihydroxy coumarin	0.178	0.0930±0.096	Uncompetitive

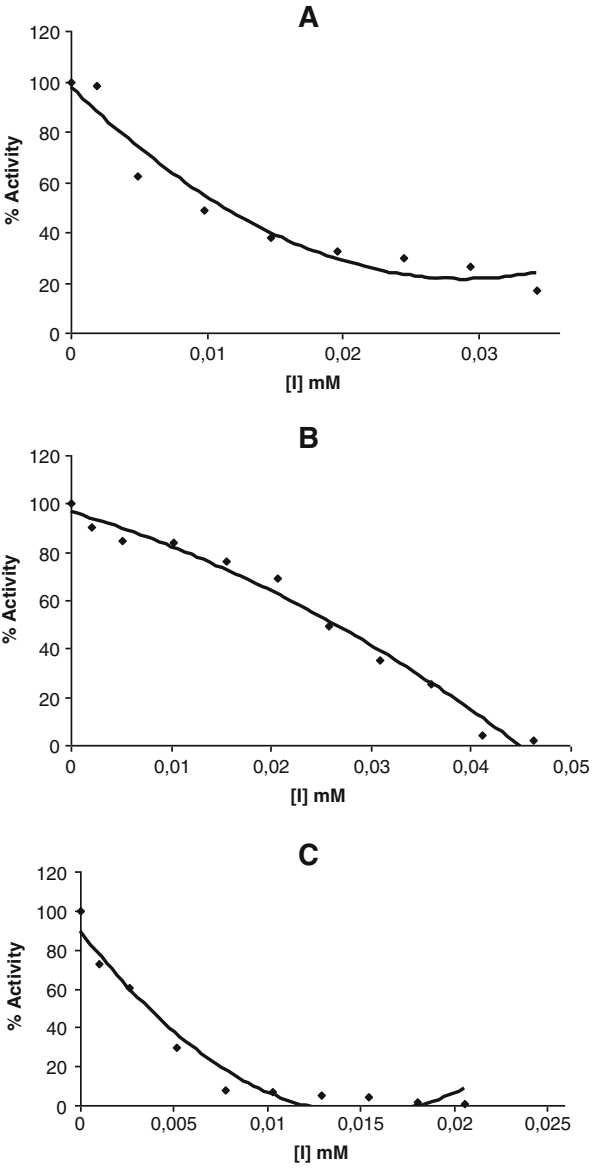


Fig. 1 In vitro effect of A, B and C compounds at several different concentrations on purified PON1 activity

coumarin derivatives of A and B compounds inhibited PON1 enzyme activity in a noncompetitive manner inhibition with K_i of 0.0080±0.256 and 0.0003±0.018 mM values, respectively. However, C compound exerted an uncompetitive inhibition of PON1 enzyme activity with K_i of 0.0010±0.173 mM (Fig. 2; Table 2). On the other hand, unsubstituted 6,7-dihydroxy coumarin compound weakly inhibited (with IC_{50} of 0.178 mM) PON1 enzyme activity in an uncompetitive inhibition manner with K_i of 0.0930±0.096 mM.

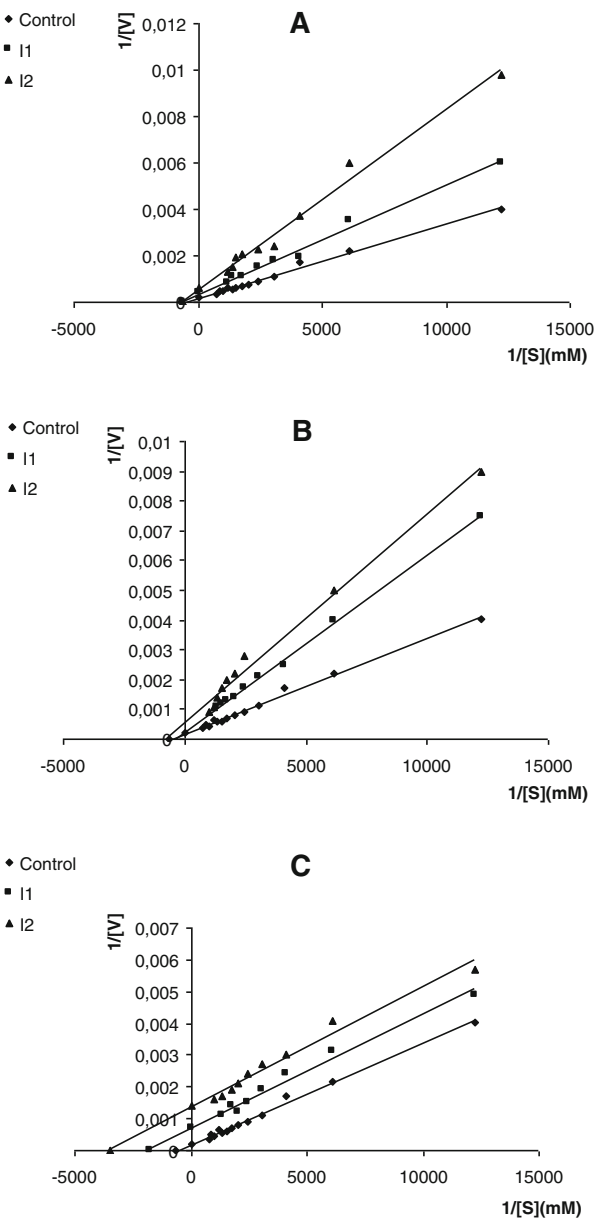


Fig. 2 K_i graphs for purified PON1 from human serum: **A**–**C** Lineweaver–Burk plots at five different substrate (paraoxon) concentrations and at two different A, B and C concentrations

In the literature, there are no previous reports about inhibition studies on PON1 activity from different sources with these dihydroxy coumarin derivatives. Only, Basaran et al. studied their in vitro inhibitory effects on hCA-I and hCA-II [31]. They have reported that among the compounds tested, B was the least effective inhibitor of hCA-I, exhibiting IC_{50} values of 0.837 and 0.320 mM against esterase and hydratase activities, respectively. For hCA-II, B was the weakest inhibitor of esterase activity (IC_{50} of 0.201 mM), but C was the weakest hydratase inhibitor (IC_{50} of 0.561 mM), closely followed by B (IC_{50} of 0.504 mM). The most effective inhibitor of hCA-I esterase activity was A (IC_{50} of 0.153 mM) and that of hCA-II esterase activity was C (IC_{50} of 0.127 mM).

In this study, a group of some dihydroxy coumarin compounds namely A, B and C were designed, synthesised and evaluated as potential inhibitors of human serum PON1 enzyme. The present study has demonstrated that PON1 activity is very highly sensitive to studied coumarin derivatives. All the tested compounds exhibited good concentration-dependent inhibitory activities against PON1 enzyme. Among the compounds, **C** showed the best PON1 inhibition activity with IC_{50} (0.003 mM) and K_i (0.0010±0.173 mM) values.

The SAR studies suggested that methyl substituent on the side ring of 6,7-dihydroxy coumarin would be a major factor in inhibitory potency of PON1 activity. The impact of these compounds on the PON1 activity is fairly clear. So, further studies are needed to elaborate the effects of various substituents on the inhibitory activity of these compounds.

In conclusion, due to the lack of inhibition studies on PON from different sources with the dihydroxy coumarin derivatives, there is not enough data with which to compare our results and consequently it is not possible to establish definite differences or similarities among PON. In spite of the contribution of our study to increase in the knowledge of the biochemical properties of PON, more study is required to analyse the inhibitory effects of coumarin derivatives on PON activity.

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