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Journal of Enzyme Inhibition and Medicinal Chemistry

ISSN: 1475-6366 (Print) 1475-6374 (Online) Journal homepage: https://www.tandfonline.com/loi/ienz20

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To cite this article: Basak Gokce, Nahit Gencer, Oktay Arslan, Mert Olgun Karatas & Bulent Alici (2016) *In vitro* inhibition effect of some coumarin compounds on purified human serum paraoxonase 1 (PON1), Journal of Enzyme Inhibition and Medicinal Chemistry, 31:4, 534-537, DOI: 10.3109/14756366.2015.1043297

To link to this article: https://doi.org/10.3109/14756366.2015.1043297

Journal of Enzyme Inhibition and Medicinal Chemistry

www.tandfonline.com/ienz
ISSN: 1475-6366 (print), 1475-6374 (electronic)

J Enzyme Inhib Med Chem, 2016; 31(4): 534–537
 DOI: 10.3109/14756366.2015.1043297

informa healthcare

RESEARCH ARTICLE

In vitro inhibition effect of some 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* effect of some hydroxy and dihydroxy ionic coumarin derivatives (**1**–**20**) on purified PON1 activity was investigated. Among these compounds, derivatives **11**–**20** are water soluble. In investigated compounds, compounds **6** and **13** were found the most active ($IC_{50} = 35$ and 34 μM) for PON1, respectively. The present study has demonstrated that PON1 activity is very highly sensitive to studied coumarin derivatives.

Keywords

Coumarin derivatives, *in vitro* inhibition, paraoxonase

History

Received 9 February 2015
Revised 14 April 2015
Accepted 15 April 2015
Published online 18 May 2015

Introduction

Paraoxonase, the calcium-dependent enzyme, (arylesterase, EC 3.1.8.1, hPON1) has an important role in living metabolism. It is an organophosphate hydrolyser. It also hydrolyses aromatic carboxyl esters such as phenyl acetate, various lactones, including naturally occurring lactone metabolites and it is involved in drug and xenobiotics metabolism[1–4]. ''PON'' name derives from one of its most commonly used *in vitro* substrates, paraoxon. hPON1 also acts as an antioxidant enzyme that is an *in vivo* bioscavenger[5].

Coumarin is a member of a class of compounds known as benzopyrones. Numerous biological activities of natural and synthetic coumarin derivatives are well known. Anticancer[6], anticoagulant[7], anti-HIV[8], lipid lowering[9], anti-inflammatory[10], antimicrobial[11], antibacterial[12], antifungal[12], anticonvulsant[13] activities of coumarin derivatives were reported. By the reason of their fluorescence ability they are widely used on fluorescent probes in biology and medicine[14].

The diverse biological activities of natural and synthetic coumarin derivatives as anticoagulants and antithrombotics are well known[15]. The biological effects observed include antibacterial, antithrombotic and vasodilatory, antimutagenic, lipoxygenase and cyclooxygenase inhibition, scavenging of reactive oxygen species and antitumourigenic effects[16].

In recent years, coumarin derivatives were reported as inhibitor of metalloenzyme carbonic anhydrase (CA)[17]. However, there are a few inhibition studies on PON1 activity in the literature. Only Erzengin et al. reported coumarin derivatives (three derivatives) as PON1 inhibitors[18].

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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 20 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. (Izmir, Turkey). Twenty ionic coumarins were prepared by previously described methods[19,20].

Paraoxonase enzyme assay

Paraoxonase enzyme activity towards paraoxon was quantified spectrophotometrically by the method described by Gan et al.[21]. The enzyme assay was based on the estimation of *p*-nitrophenol at 412 nm. The molar extinction coefficient of *p*-nitrophenol ($\varepsilon = 17\,100\ M^{-1}\,cm^{-1}$ 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). Two millimolar of final substrate concentration was used during enzyme assay, and all measurements were taken in duplicate and corrected for the non-enzymatic hydrolysis.

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. First, serum paraoxonase

was isolated by ammonium sulphate precipitation (60–80%). The precipitate was collected by centrifugation at 15 000 rpm for 20 min, and redissolved in 100 mM Tris–HCl buffer (pH 8). Next, we synthesized the hydrophobic gel, including Sepharose 4B, L-tyrosine and 1-napthylamine, for the purification of human serum paraoxonase[22]. The column was equilibrated with 0.1 M of a Na_2HPO_4 buffer (pH 8) 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). The purified PON1 enzyme was stored in the presence of 2 mM calcium chloride in order to maintain activity.

In vitro kinetic studies

For the inhibition studies of coumarin derivatives, the different concentrations of coumarin derivatives were added to the reaction medium. PON1 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 considered as 100% activity. The inhibitor concentration causing up to 50% inhibition (IC_{50} values) for coumarin derivatives was determined from the graphs.

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[23], with bovine serum albumin as a standard.

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[24]. 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.

Results and discussion

In this study, the effects of 20 (Figure 1) ionic coumarin derivatives on PON1 activity were investigated. These compounds had been synthesized and CA inhibitory properties of these compound had been reported previously[19,20]. Compounds **1–10** are 7-hydroxy coumarin derivatives and compounds **11–20** are 7,8-dihydroxy coumarin derivatives. 7,8-Dihydroxy coumarin derivatives are water soluble compounds. In this study, the result showed that water soluble coumarin inhibited PON1 activity effectively. The IC_{50} values are presented in Table 1. The results showed that all compounds inhibited the PON1 enzyme activities. Among all the compounds, **13** was found to be most active one for PON1 activity ($IC_{50} = 34\ \mu M$). In the literature, there are no previous reports about inhibition studies on PON1 activity from different sources with these dihydroxy coumarin derivatives. Only, Erzengin et al. studied their *in vitro* inhibitory effects on PON1[18]. They have reported that among the compounds tested, C (6,7-dihydroxy-3-(4-methylphenyl)-2H-chromen-2-one) was the most effective inhibitor of PON1 (IC_{50} value of 0.003 mM).

In the content of this study, PON1 inhibitory activities of sixteen benzimidazolium, two imidazolium and two quaternary ammonium salts of coumarin derivatives were investigated. When we peruse the inhibitory activities of these compounds; it can be said that benzimidazolium derivatives are much more active than non-benzimidazolium derivatives. According to these results, the addition of a benzene ring to structure increased lipophilicity of compounds and making them more active than other coumarin derivatives. For the comparison of benzimidazolium salts, we can classify them in two parts; (i) 7-hydroxy coumarin (**3–8**) and (ii) 7,8-dihydroxy coumarin-bearing compounds (**11–20**). Some compounds are bearing same groups in their structures apart from coumarin scaffold. We may use these compounds for comparison. Comparison of compounds **3** and **11** revealed that each of these compounds are bearing methyl group as substituent, and 7,8-dihydroxy coumarin-bearing compound **11** is more active than compound **3** which includes 7-hydroxy coumarin. Comparison of compounds **4** and **12** which bear butyl group apart from coumarin revealed that 7-hydroxy coumarin derivative compound **4** is more active than compound **12**. This misfit can be seen in the comparisons of compound **5** with **16** and **6** with **20**. So, these results suggest that there is no significant difference between 7-hydroxy and 7,8-dihydroxy coumarin scaffolds.

Pharmacological studies, including enzyme–drug interaction analyses, are becoming increasingly vital important[25–29]. In a study, it was shown that a lactam derivative namely 2-hydroxyquinoline inhibited PON1 effectively[30]. Coumarin derivatives contain unsaturated lactone (namely pyron) ring and lactones are isosteric form of lactams in which the ring nitrogen replaced by a oxygen. As main distinction between them, coumarin derivatives are aromatic, whereas lactam has a saturated ring. Therefore, it had been reported that some 6,7-dihydroxy-3-aryl coumarin derivatives inhibited PON 1 in another paper[18]. We had reported CA inhibitory properties of compounds **1–20** in our previous studies[19,20] and these compounds have good IC_{50} values for CA which are in the micromolar range. In this article, compounds **1–20** are ionic compounds but they have apolar character and dissolve in water partly. In view of that fact, the inhibition mechanisms of CA and PON may contain some similarities.

In a study, it was shown that simple lactone derivatives were hydrolyzed by PON1[31]. In the same study authors reported that it decreases the rate of hydrolysis when the hydroxy group is on the lactone ring. So lactam derivatives and a coumarin were not hydrolyzed by PON1. In our study, synthesized compounds are bearing both hydroxy and azolium substituent so according to the results of our and previous studies, compounds **1–20** are not suitable substrate for PON1 and they inhibited PON1 effectively.

Conclusions

There is great interest in coumarins out of their physiological roles. They have many derivatives that are natural and synthetic. Coumarins could be found of pharmacological agents, consisting of a wide range of biological properties such as bacteriostatic, anticancer, anticoagulant, anti-inflammation, antioxidant and analgesic. Besides their important activities in drugs and their intense flavour in foods, they are also toxic in certain levels. Synthetic coumarin was used as a good flavouring in food industry during years after its first synthesis in 1868[32].

Recently, in the literature exceeding reports and experiments dealing with the question of hazard factor for coumarin have focused on animal and its estimation based on scientific information[32–34]. Nonetheless, significant human assays are available now on the hepatoxycity of using coumarin as a pharmaceutical cure[35–37]. These implementations in the regulations related with the use of coumarin on European level are

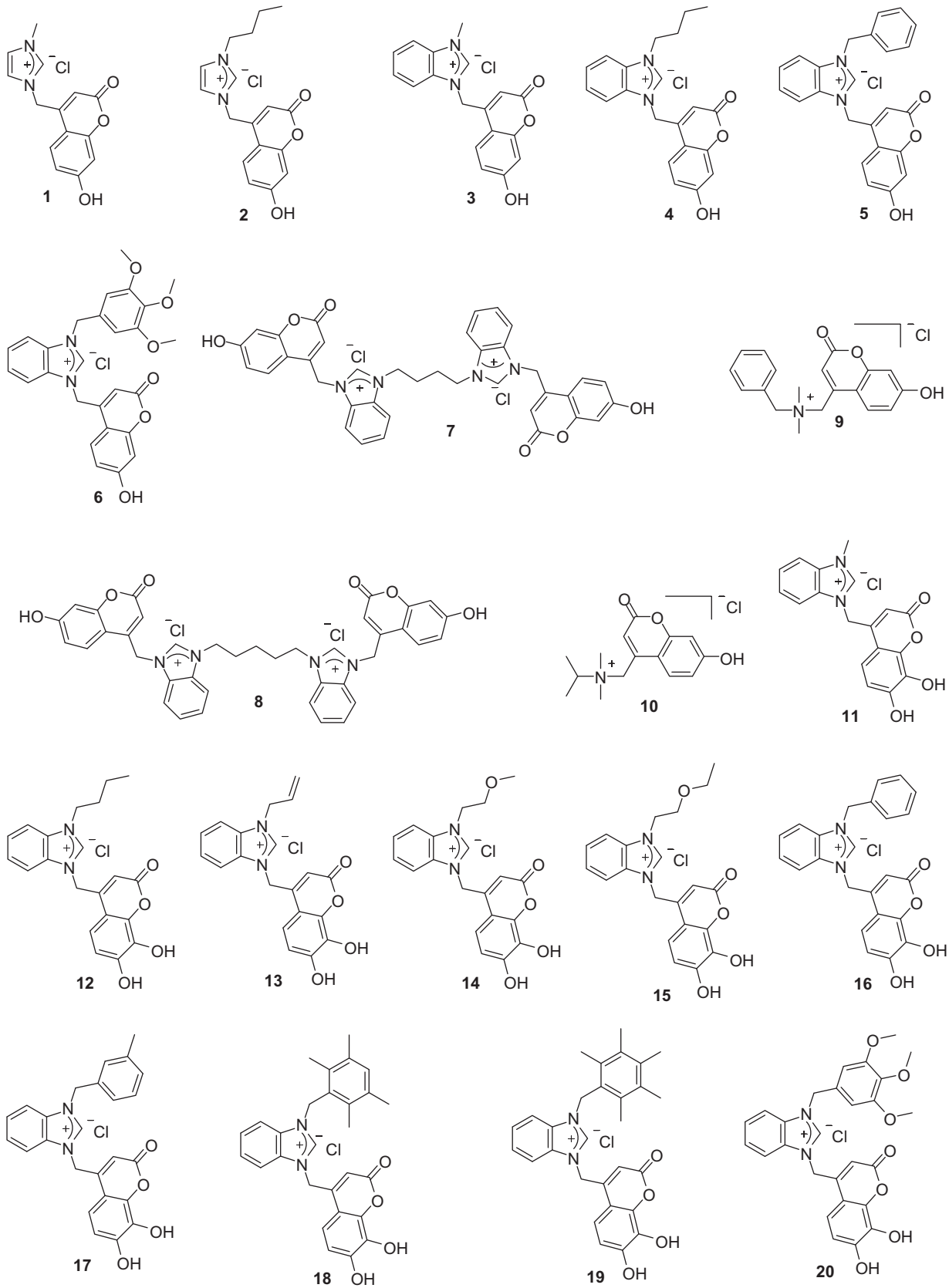

Figure 1. Prepared compounds by literature[19,20].

evidence for understanding better that it is toxic. Both their many rewarding features in health and numerous experiments on hepatoxic effect of coumarin in laboratory animals make these compounds attractive for future comprehensive opinions.

In conclusion, we present here a hPON1 inhibition study of several coumarins as CA I–II inhibitors[19,20]. From these results that can be confirmed with coumarin compounds, toxicological experiments *in vivo*, inhibited effectively paraoxonase which has

Table 1. The IC_{50} values of coumarin derivatives on purified PON1 activity.

Compound No.	IC_{50} (μM)	Compound No.	IC_{50} (μM)
1	287	**11**	104
2	88	**12**	100
3	133	**13**	34
4	94	**14**	84
5	95	**15**	90
6	35	**16**	42
7	87	**17**	42
8	68	**18**	84
9	340	**19**	88
10	192	**20**	143

important detoxification role in metabolism. Our findings provide a substructure to support further consideration of limitation dosage of coumarin as a drug and as a flavour cause of risk assessment.

Acknowledgements

The authors would like to thank Fehim İlhan for his advice on English grammar and expression.

Declaration of interest

This work has been supported by Balikesir University Research project (2014/54).

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