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: Ersin Hopa, Ismet Basaran, Selma Sinan, Yusuf Turan & Umit Cakir (2014) *In vitro* inhibition effects of some coumarin derivatives on human erythrocytes glucose-6-phosphate dehydrogenase activities, Journal of Enzyme Inhibition and Medicinal Chemistry, 29:5, 728-732, DOI: 10.3109/14756366.2013.845819

To link to this article: https://doi.org/10.3109/14756366.2013.845819

Journal of Enzyme Inhibition and Medicinal Chemistry

http://informahealthcare.com/enz
ISSN: 1475-6366 (print), 1475-6374 (electronic)

J Enzyme Inhib Med Chem, 2014; 29(5): 728–732
 DOI: 10.3109/14756366.2013.845819

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ORIGINAL ARTICLE

In vitro inhibition effects of some coumarin derivatives on human erythrocytes glucose-6-phosphate dehydrogenase activities

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Abstract

Inhibitory effects of some synthesized dihydroxycoumarin compounds on purified G6PD were investigated. For this purpose, initially human erythrocyte G6PD was purified 7069-fold in a yield of 33.6% by using ammonium sulfate precipitation and affinity chromatography which includes 2′,5′-ADP Sepharose 4B. The purified enzyme showed a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Enzyme activity was determined spectrophotometrically according to Beutler method at 340 nm. 6,7-Dihydroxy-3-(2-methylphenyl)-2H-chromen-2-one (**OPC**), 6,7-dihydroxy-3-(3-methylphenyl)-2H-chromen-2-one (**MPC**) and 6,7-dihydroxy-3-(4-methylphenyl)-2H-chromen-2-one (**PPC**) were used as dihydroxycoumarin compounds. This study has demonstrated that G6PD activity is very highly sensitive to study coumarin derivatives.

Keywords

Coumarin derivatives, glucose-6-phosphate dehydrogenase, *in vitro* inhibition

History

Received 19 June 2013
Revised 15 September 2013
Accepted 15 September 2013
Published online 5 February 2014

Introduction

Glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) is the first enzyme of the pentose phosphate pathway (PPP) that converts β-D-glucose-6-phosphate (G6P) to 6-phosphoglucono-δ-lactone with the reduction of $NADP^+$ to NADPH[1]. PPP is the only source of NADPH in the erythrocytes. The formation of NADPH in erythrocytes has vital importance. The major role of NADPH in erythrocytes is regeneration of reduced glutathione (GSH), which prevents hemoglobin denaturation, preserves the integrity of red blood cell membrane sulfydryl groups and detoxifies hydrogen peroxide and oxygen radicals in and on the red blood cells. G6PD deficiency significantly reduces the production of NADPH and this situation blocks the GSH formation. With the GSH deficiency, free radicals cannot be reduced and causes hemolysis of erythrocytes.

Natural products have been important sources of new pharmacological active agents. Coumarins (2*H*-chromen-2-ones, 2*H*-1-benzopyran-2-ones) are among the best known oxygen heterocyclics with an δ-lactone ring and comprise a very large class of compounds found throughout the plant kingdom[2]. A wide spectrum of biochemical and pharmacological activities are displayed by coumarins and their derivatives[3,4]. Coumarin derivatives are used widely as anticoagulants (such as warfarin, –OH group is attached at the 4th position) for the treatment of disorders in which there is excessive or undesirable clotting, such as thrombophlebitis, pulmonary embolism and certain cardiac conditions. They are also used as rodenticides due to their ability to cause fatal hemorrhaging[5]. In recent years, increasing human population leads to the development and variation of a lot of industry such as cosmetics, drugs and food. It is extremely important to investigate the effects of coumarin type compounds on the activity of enzymes which are important for physiological functions, especially in metabolism.

The effect of coumarins on different enzyme activities such as carbonic anhydrase (CA) and paraoxonase (PON) has been investigated[6–9]. However, no reports could be found in the literature on the effects of coumarin derivatives on human G6PD enzyme.

Thus, the aim of this study was mainly to design, synthesize and investigate *in vitro* inhibitory effects of these compounds (OPC, MPC and PPC) that may be in interaction with human body throughout medicinal treatment on purified enzyme.

Materials and methods

2′,5′-ADP-Sepharose 4B was purchased from Pharmacia. NADP, glucose-6-phosphate, protein assay reagents and chemicals for electrophoresis and synthesis were obtained from Sigma (St. Louis, MO) and Aldrich (Germany). The structure of all synthesized dihydroxy coumarin compounds were identified from their IR (Perkin Elmer Spectrum BX II) and ^{1}H-NMR spectra (Bruker GmbH DPX-400). Melting points were measured on an Electrotermal 9200 instrument. 6,7-dihydroxy coumarin compounds, 6,7-dihydroxy-3-(methylphenyl)chromenones, were synthesized according to literature[10].

General procedure for the synthesis of 6,7-dihydroxy-3-(methylphenyl)chromenones

A solution of 2,3,4-trimethoxybenzaldehyde (25.0 mmol) and methylphenylacetonitrile (37.5 mmol) in ethanol (100 mL) was heated to 70 °C. Twenty percent NaOH solution was then added

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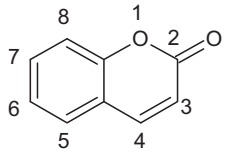


Coumarin
(2*H*-chromen-2-one)

Scheme 1. Structure of coumarin.

dropwise to the stirred solution until the onset of turbidity. The acrylonitrile was precipitated by cooling the solution to room temperature. The precipitate was filtered, washed with water and dried. Pyridine hydrochloride (100.0 mmol) was added to the crude product and it was heated for 2 h at 180 °C. After cooling, the product was crystallized from water. The precipitated coumarin was washed with water until neutral, dried and recrystallized from ethanol. The yield, melting point and NMR spectra of dried product were determined (Scheme 1).

*6,7-Dihydroxy-3-(2-methylphenyl)-2H-chromen-2-one (**OPC**)*

Yield: 76.5%; mp 242 °C; IR (KBr), ν (cm^{-1}): 3464(OH), 1655(C=O), 1297(C–H), 1151(C–O). ^{1}H-NMR ($CDCl_3$ + DMSO), δ (ppm): 2.20 (s, 3H, CH_3), 6.83 (s, H, ArH), 6.98 (s, H, ArH), 7.32 (m, 4H, ArH), 7.50 (s, H, cumH), 7.86 (s, 2H, OH).

*6,7-Dihydroxy-3-(3-methylphenyl)-2H-chromen-2-one (**MPC**)*

Yield: 76.8%; mp 211 °C; IR (KBr), ν (cm^{-1}): 3172(OH), 1669(C=O), 1258(C–H), 1168(C–O). ^{1}H-NMR ($CDCl_3$ + DMSO), δ (ppm): 2.32 (s, 3H, CH_3), 6.75 (s, H, ArH), 6.91 (s, H, ArH), 7.08 (d, H, ArH), 7.21 (d, H, ArH), 7.37 (d, 2H, ArH), 7.70 (s, H, cumH), 7.75 (s, 2H, OH).

*6,7-Dihydroxy-3-(4-methylphenyl)-2H-chromen-2-one (**PPC**)*

Yield: 80.5%; mp 257–258 °C; IR (KBr), ν (cm^{-1}): 3151(OH), 1660(C=O), 1273(C–H), 1188(C–O). ^{1}H-NMR ($CDCl_3$ + DMSO), δ (ppm): 2.30 (s, 3H, CH_3), 6.75 (s, H, ArH), 6.90 (s, H, ArH), 7.13 (d, 2H, ArH), 7.48 (d, 2H, ArH), 7.67 (s, H, cumH), 7.73 (s, 2H, OH).

Purification of G6PD enzyme from human erythrocytes by affinity chromatography

Appropriate amount of fresh blood samples from human volunteers were collected in the tubes containing EDTA and sample tubes were centrifuged at $3000 \times g$ for 10 min. The plasma and leukocyte coat were removed. The packed erythrocytes were washed with isotonic KCl solution three times, and hemolysate with five volumes of ice-cold water and then centrifuged at 15 000 *g* for 20 min at 4 °C to remove the ghost and intact cells[11]. The hemolysate was subjected to precipitation with ammonium sulfate (30–70%). Ammonium sulfate was slowly added to hemolysate for completely dissolution. This mixture was centrifuged at $15\,000 \times g$ for 30 min. The precipitate was dissolved in 50 mM potassium phosphate buffer (pH: 7.0) and then dialyzed at 4 °C in 50 mM potassium acetate/50 mM potassium phosphate buffer (pH: 7.0) at overnight. All of the purification procedures were performed at 4 °C. Two grams of dry 2′,5′-ADP-Sepharose 4B was used for a 10-mL column volume. The gel was washed with 400 mL distilled water to remove foreign bodies and air bubbles of swollen gel were eliminated. The gel was resuspended in 0.1 M potassium acetate/0.1 M potassium phosphate buffer (pH: 6.0) and packed in a column (1 x 10 cm) and equilibrated with the same buffer. The dialyzed enzyme solution was loaded onto the 2′,5′-ADP-Sepharose 4B column was then sequentially washed with 25 mL of 0.1 M potassium acetate/0.1 M potassium phosphate buffer (pH: 6.0) and 25 mL of 0.1 M potassium acetate/0.1 M potassium phosphate buffer (pH: 7.85). The final washing with 0.1 M potassium chloride/0.1 M potassium phosphate buffer (pH: 7.85) was continued until the final absorbance difference became about 0.05 at 280 nm. The enzyme was eluted with a solution of 80 mM potassium phosphate + 80 mM potassium chloride + 0.5 mM $NADP^+$ + 10 mM EDTA (pH: 7.85) and 1 mL fractions were collected. The enzyme activity was measured in the final fractions and the activity-containing tubes were pooled[12,13].

G6PD enzyme assay and protein determination

G6PD enzyme activities of synthesized dihydroxy coumarin compounds were measured spectrophotometrically at 340 nm by Beutler's method[6]. One enzyme unit was defined as the enzyme amount that reduces 1 µmole of $NADP^+$ per min under the assay conditions. This method depends on the reduction of $NADP^+$ by G6PD in the presence of G6P. The activity measurements were made by monitoring the increase in absorbance at 340 nm due to the reduction of $NADP^+$ at 25 °C. Quantitative protein determination was performed at 600 nm according to Lowry's method[14] using bovine serum albumin (BSA) as a standard. Protein samples were fractionated on 12% SDS-PAGE gel[15] using a Minigel system. Gel was stained with Coomassie Brilliant Blue R-250, and destained using standard methods to detect protein bands.

Determination of kinetic parameters and *in vitro* inhibition studies

To obtain K_m and V_{max} values, various final concentration of G6P (0.06–1.2 mM) and NADP + (0.02–0.3 mM) were used. All kinetic studies were performed at 25 °C and optimum pH (1 M Tris-HCl, pH:8.0). Inhibition experiments were done using G6P as substrate and a group of dihydroxy coumarin derivatives (**OPC**, **MPC** and **PPC**) with different final concentration as possible inhibitors. Kinetic parameters were calculated from Lineweaver–Burk graphs. G6PD activity without a coumarin derivative was accepted as 100% activity. For the coumarin compounds having an inhibition effect, the inhibitor concentration causing up to 50% inhibition (I_{50} values) were determined by regression analysis graphs drawn by using a Microsoft Excel 2000 computer program. In addition, K_i values of coumarins were calculated on G6PD activity.

Results and discussion

Pentose phosphate metabolic pathway involves the conversion of glucose into pentose sugars, necessary for various biosynthetic reactions. Produced pentose sugars are required as precursors in the biosynthesis of a number of important molecules, such as ATP, CoA, NAD, FAD, RNA and DNA. Pentose phosphate metabolic pathway also provides a form of NADPH which is a reducing power in various biosynthesis reactions.

G6PD is an enzyme which catalyzes the first reaction in the PPP[16,17]. The activity of G6PD changes depending on nutrition, hormones and especially the concentration of NADPH[18]. Although there are a lot of research reports about enzyme deficiency in erythrocyte cells[19,20], G6PD deficiency which is concerning to all human biology is yet to be investigated[21].

For the solution to the growing number of health problems, various drugs from plants are produced. But to meet to needs of drugs, a large number of chemical substances synthesized in

the laboratories are used as pharmaceutical active ingredient. In this way, the use of drugs obtained from various sources causes the problem of side effects. Many chemicals at relatively low dosages affect the metabolism of biota by altering normal enzyme activity, particularly inhibition of a specific enzyme[22]. The effects can be dramatic and systematic[23].

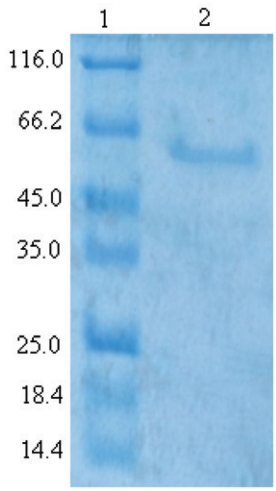


Figure 1. SDS-PAGE of purified human erythrocyte G6PD. The enzyme was electrophoresed at pH: 8.3 on a 12% polyacrylamide gel and stained with Coomassie Brilliant Blue R-250. Lanes: 1, molecular weight standards (β-galactosidase, 116 kDa; bovine serum albumin, 66.2 kDa; egg albumin, 45 kDa; lactate dehydrogenase, 35 kDa; Rease Bsp981 (*Escherichia coli*), 25 kDa; β-lactoglobulin, 18.4 kDa; Lysozyme, 14.4 kDa; 2, purified human erythrocyte G6PD).

Table 1. Kinetic parameters of human erythrocyte glucose-6-phosphate dehydrogenase.

Substrate	K_m (mM)	V_{max} (U/mg)
G6P	0.22	1.94
NADP	0.14	2.76

Coumarins are active components of herbs used for the treatment of various diseases[24]. The diverse biological activities of natural and synthetic coumarin derivatives as anticoagulants and antithrombotics are well known[25]. Some of the coumarin derivatives are also reported anti-HIV agents[26], lipid lowering agents[27] and antioxidants[28]. They have also been shown to inhibit lipid peroxidation and to possess vasorelaxant[29], anti-inflammatory[30], and anticarcigonenic activities[31].

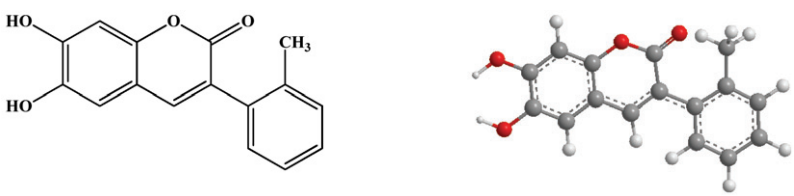
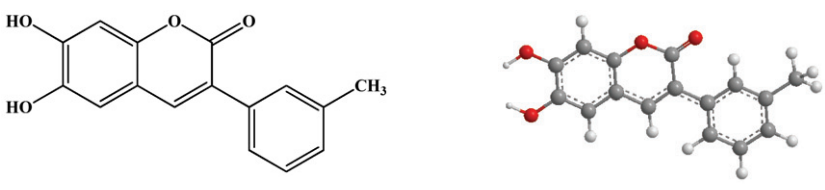
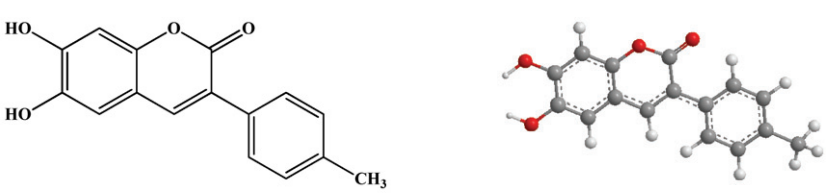
In the literature, inhibition effects of some coumarin derivatives on the various enzymes were investigated but there are not any study related to G6PD enzyme. In a study, two coumarin-based sulfamate drugs (667 COUMATE and STX 118) have been reported to have IC_{50} values of 25–59 nM for the inhibition of hCA-II activity[6]. Inhibition of some important enzymes, which play a key role in a metabolic pathway, may lead to pathologic conditions or disorders. These stimulated us to investigate inhibitory effects of a number of dihydroxy coumarin compounds on human erythrocyte G6PD enzyme.

In this study, G6PD was purified from human erythrocytes by ammonium sulfate precipitation and 2′,5′-ADP-Sepharose 4B affinity chromatography, respectively. Purification factor for ammonium sulfate precipitation and affinity chromatography were 1.2- and 7068.9-fold in a yield of 68.18% and 33.65%, respectively. Figure 1 shows the SDS-PAGE gel for the determination of purity of the enzyme in the study. A high purity was obtained for the enzymes.

The reaction kinetics of the purified G6PD was determined from Lineweaver–Burk plots using glucose-6-phosphate and $NADP^+$ as substrates with K_m values of 0.22 and 0.14 mM and V_{max} values of 1.94 and 2.76 U/mg, respectively (Table 1). Affinity of the enzyme for $NADP^+$ was considerably higher than for glucose-6-phosphate. The higher G6PD affinity for $NADP^+$ has also been reported[32].

In the literature, effects of various drugs and chemical substances on the catalytic activity of the G6PD enzyme were investigated. K_i values of these substances are higher than the

Table 2. Chemical structures of the synthesized coumarins.

Structure	Abbreviation
	**OPC**
	**MPC**
	**PPC**

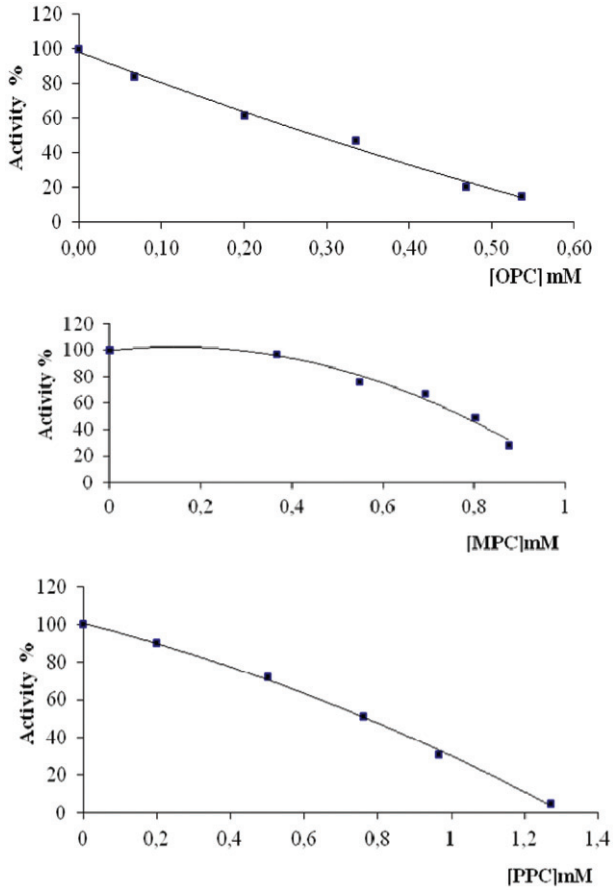


Figure 2. Activity % curve of G6PD in different **OPC**, **MPC** and **PPC** concentrations.

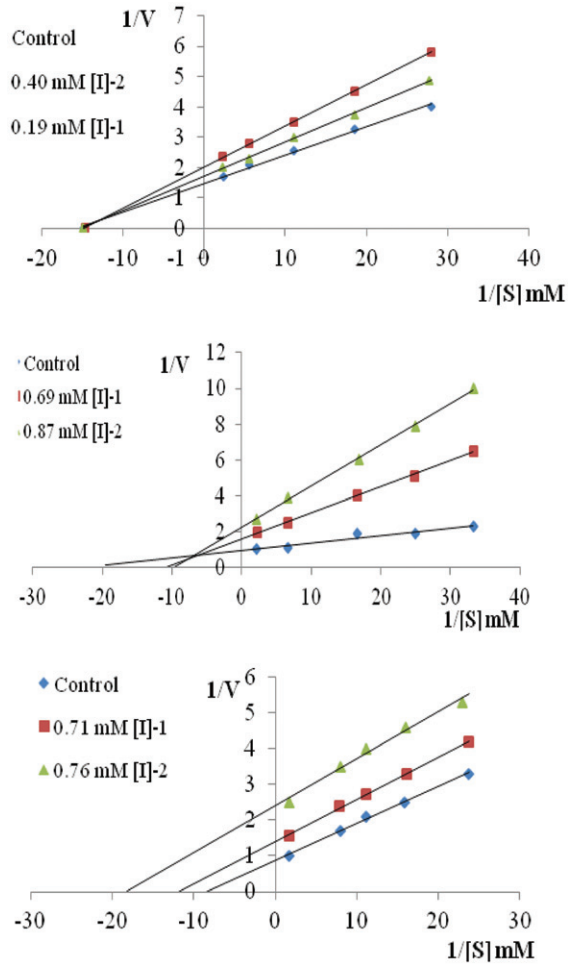


Figure 3. Lineweaver–Burk graphs in five different substrate (G6P) concentrations sand in two different **OPC**, **MPC** and **PPC** concentrations for determination of K_i.

Table 3. Inhibition type, K_i and I_{50} values obtained from regression analysis graphs for G6PD in the presence of coumarin derivatives.

Coumarin	Inhibition type	K_i (mM)	IC_{50} (mM)
OPC	Noncompetitive	1.37	0.305
MPC	Mixed	0.734	0.769
		0.269	
PPC	Uncompetitive	0.835	0.790

values calculated for the coumarin derivatives. K_i values of isepamicin sulfate, omepirazol, morphine sulfate, vankomycine, magnesium sulfate, metamizol and granisetron hydrochloride were reported as 1.7 mM, 8.2 mM, 25.9 mM, 2.71 mM, 13.2 mM, 6.3 mM, 4.5 mM, respectively[33–36].

In this study, for investigation of inhibition effects, IC_{50} and K_i parameters of coumarin derivatives (Table 2) for G6PD were determined. IC_{50} values of **OPC**, **MPC** and **PPC** were 0.305 mM, 0.769 mM and 0.790 mM (Figure 2, Table 3), and the K_i constants were 1.37 mM, 0.734 mM–0.269 mM, 0.835 mM (Figure 3, Table 3), respectively.

I_{50} values showed the same trend for **MPC** and **PPC** and higher than **OPC**. These results show that **MPC** and **PPC** are weak inhibitors on G6PD enzyme than **OPC**. **OPC**, **MPC** and **PPC** compounds inhibited G6PD enzyme activity in a non-competitive, mixed and uncompetitive manner respectively (Table 3). Differences in chemical structures of coumarins also lead to different types of inhibition. As shown in the Table 2, the chemical structures of coumarin derivates are similar to each other. The only difference is due to the different positions of methyl groups on phenyl ring. Methyl group is located in the 2nd position in **OPC**, 3rd position in **MPC** and 4th position in **PPC**. This situation may be because **OPC** connects to the enzyme from a different region than the active region. The presence of methyl group in position 3 instead of 2 influences the mechanism of enzyme action. Therefore, **MPC** can be connected to the enzyme in both active and inactive region. Methyl group is located in 4th position in the structure of **PPC** because it only binds to the enzyme when the enzyme–substrate complex occurs. Our results indicate that studied coumarin derivatives have more potent inhibitory effect on the G6PD enzyme.

Declaration of interest

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

The financial support of Scientific Research Council of Balikesir University (Project No: 2009/20) is gratefully acknowledgement.

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