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Influence of phosphorohydrazone derivatives of benzopyrones on polymerization and viscosity of fibrin

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The synthesis and antitumour and antibacterial activity of coumarin and chromone phosphorohydrazones have been reported. This study describes influence of phosphorohydrazones derivatives of coumarin and chromone on the polymerization and viscosity of fibrin. The fibrin polymerization assay was performed by the Shen and Lorand method and the clot viscosity was measured on the basis of Shen and Lorand and Marchi and coworkers methods. Among the eight compounds tested, one coumarin derivative and two chromone derivatives showed significant activity.

Keywords: phosphorohydrazone, coumarin, chromone, polymerization, viscosity, fibrin

INTRODUCTION

Natural and synthetic compounds containing benzo-γ-pyrone (chromone) or benzo-α-pyrone (coumarin) moieties are known for their broad spectrum of biological activities.

For example, warfarin (Rettie & Tai, 2006), dicumarol and other anticoagulants have shown antiangiogenic properties and demonstrated activity against various types of cancer (Kostova, 2005). In recent years numerous studies have been carried out on the synthesis of new benzo-γ-pyrone (Di Braccio *et al.*, 2003) and benzo-α-pyrone derivatives (Kawase *et al.*, 2001; Kostova, 2005) and their metal complexes (Zyner & Ochocki, 1999; Kośmider *et al.*, 2004; Kostova *et al.*, 2007) showing biological activity.

In our earlier papers we presented preliminary results of research on antitumour (Nawrot-Modranka *et al.*, 2004; 2006) and antibacterial (Nawrot-Modranka *et al.*, 2006) activity of newly synthesized phosphorohydrazone derivatives of chromone and coumarin. Among the tested compounds one (**1**) exhibited significant cytotoxic activity *in vivo* against mouse leukemia L1210 and P388, which was injected parerentally (Nawrot-Modranka *et al.*, 2006). The synthesis and antibacterial properties of Pd(II) chelates with some phosphorohydrazone derivatives of chromone and coumarin were also described (Nawrot-Modranka & Nawrot, 2007).

The aim of this study was to test the effect of coumarin phosphorohydrazones **1–3** and five chromone derivatives **4–8** on the polymerization and viscosity of fibrin *in vitro*.

Physical and chemical properties of fibrin play an important role in thrombo-embolic and tumour changes (Richard *et al.*, 2001; Fernandez *et al.*, 2004; Wojtukiewicz *et al.*, 2004). The structure of fibrin and the rate of its polymerization may be studied by viscosity tests (Mosesson, 1990; Gron *et al.*, 1992; Ryan *et al.*, 1999; Oenick, 2004). The evalu-

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Abbreviations: bFGF, basic fibroblast growth factor; DMSO, dimethylsulfoxide; L1210, P388, tumour cell lines; PBS, phosphate-buffered saline; VEGF, vascular endothelial growth factor.

ation of the influence of external factors upon this process has been carried out by numerous authors. They studied the effect of metal ions (Michalska & Wierzbicki, 1990; 1993), pharmaceuticals (Standeven *et al.*, 2002) and some pathological changes or fibrin properties (Blombäck *et al.*, 1990; Libionka *et al.*, 2005). Incorrect course of fibrin polymerization leads to serious disturbances of hemostasis (Hayen *et al.*, 1999; Bromberg & Capello, 1999). Fibrin makes a hook for neoplastic cells and influences biosynthesis of interleukins and growth factors. Fibrin formation and angiogenesis control are important issues in the therapy, especially in cancer treatment. The therapy methods may be based on angiogenesis inhibition (thrombospondin-1, antiangiogenic antithrombin III) or thrombin inhibition and fibrin structure alteration. Fibrin belongs to angiogenic factors (Richard *et al.*, 2001; Wojtukiewicz *et al.*, 2004; Fernandez *et al.*, 2004). It increases the expression of pro-angiogenic interleukin 8 (IL 8), bFGF and VEGF.

Studies of the fibrin structure and polymerization make it possible to evaluate the influence of known pharmaceuticals or newly synthesized compounds on angiogenesis which is linked directly to the cancerogenesis of tumour and metastasis.

MATERIALS AND METHODS

Studies of the polymerization and viscosity of fibrin were carried out *in vitro* in the presence of one of eight phosphorohydrazone derivatives of 4-hydroxycoumarin and chromone (Fig. 1). The compounds **1-8** were obtained according to methods published earlier (Nawrot-Modranka *et al.*, 2006).

Studies on fibrin polymerization. The sample used for fibrin polymerization tests following the method of Shen and Lorand (1983) contained:

1 ml of human fibrinogen (Kabi) at 3.2 mg/ml, 980 μl of PBS buffer, 0.01 mol/l, pH=7.3, 20 μl of the compound in DMSO (Sigma Aldrich) at concentrations 10^{-5} or 10^{-6} mol/l, 1 ml of thrombin (EC 3.4.21.5) (Biomed Lublin, Poland) at 8 NIH u/ml.

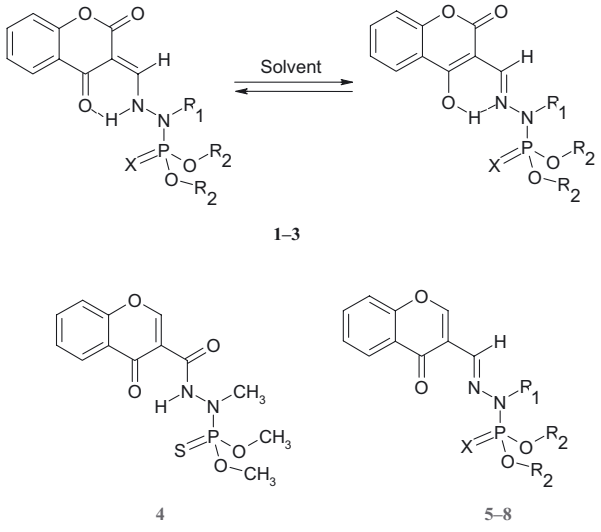
Measurements were taken at 350 nm on a Specol-11 spectrophotometer (Carl Zeiss, Jena, Germany). Absorbance (A) changes were measured with the accuracy ±0.0001 at time t±1 s. It was assumed that the reaction rate could be expressed as the rate of changes in the apparent absorbance of the solution (a turbidimetric measurement – attenuation of radiation caused by its scattering on the fibrin network). A so-called incrementary ratio $\Delta A/\Delta t$ being the measure of the reaction rate was calculated. The results were collected at Δt=20 s intervals, so ΔA in the equation given above is the absorbance change for this period of time. Calculations were carried out for subsequent time intervals. The total measurement lasted 3 min, so it was possible to determine nine values of the instantaneous rate. The calculated $\Delta A/\Delta t$ values were assigned to the midpoint of the time interval, i.e. the value $\Delta A/\Delta t$ calculated for the interval between t=20 s and t=40 s was assigned to the time t=30 s.

Measurements of fibrin viscosity. The clot viscosity was measured on the basis of the methods of Shen and Lorand (1983) and Marchi *et al.* (2004). The sample under investigation contained:

200 μl of human fibrinogen, fraction I, type I (Sigma Aldrich) at 3.2 mg/ml

200 μl of sodium chloride solution (0.15 mol/l) containing 1 NIH u/ml of thrombin and calcium chloride at 0.18 mol/l, 20 μl of a solution of factor XIII (transglutaminase, EC 2.3.13), 2 units/ml, (Sigma Aldrich) in 0.01 mol/l Tris buffer, pH 7.6, 20 μl of DMSO solution of the tested compound at 10^{-5} or 10^{-6} mol/l. The control sample contained 20 μl DMSO without the compound. The samples were incubated for 15 min at room temperature. Viscosity was measured with a Brookfield cone and digital plate rheometer DV-III, version 3.0 connected with a thermostatic bath PGW-E1 (Medingen). The measurement of viscosity is based on the determination of the relationship between the angular velocity of a rotating measuring element and the torque connected with these rotations. It is therefore possible to find the relationship between the shear rate and the shear stress to determine the flow curves of the material under investigation.

Statistical analysis of the results was performed using *t*-Student and Cochran-Cox tests. The



1: $R_1 = R_2 = CH_3$ **5**: $R_1 = R_2 = CH_3$; X = S
2: $R_1 = CH_3$, $R_2 = C_2H_5$ **6**: $R_1 = CH_3$; $R_2 = C_2H_5$; X=S
3: R_1 = H; $R_2 = C_2H_5$ **7**: R_1 = H; $R_2 = C_6H_5$; X=S
8: $R_1 = CH_3$; $R_2 = C_6H_5$; X=O

Figure 1.

normality of distribution of the measured values was confirmed with Kolmogorov-Smirnov test. Variance homogeneity was tested with Fisher test.

RESULTS

Fibrin polymerization

In our research we evaluated the changes of absorbance (ΔA) during fibrin polymerization in the presence of tested compounds, derivatives of benzo-α-pyrone **(1–3)** and benzo-γ-pyrone **(4–8)**. The studied compounds were applied as DMSO solutions at concentrations of 10^{-5} and 10^{-6} mol/l.

In the group of coumarin derivatives, compound **1** at 10^{-5} mol/l caused a statistically significant decrease in absorbance compared to the control sample. This compound caused the reduction of fibrin polymerization by 79.1±12.2% at 10^{-5} mol/l and by 38.4±18.9% at 10^{-6} mol/l (Table 1).

Among the chromone derivatives, **6** and **8** significantly reduced fibrin polymerization. At 10^{-5} mol/l compound **6** caused a reduction by 74.3±6.0% and compound **8** by 78.9±11.3% at 10^{-6} mol/l the polymerization was reduced by 72.5±8.1% and 76.6 ±11.6% compared to the control. Compounds **2, 3** (coumarin derivatives) and **4, 5, 7** (chromone derivatives) also caused absorbance reduction compared to the control but these changes were not statistically significant. A sample diagram presenting the kinetics of fibrin polymerization in the presence of compounds **1, 3** and **4** at 10^{-5} mol/l is shown in Fig. 2.

Coagulation of fibrinogen under the influence of thrombin in the presence of chromone or coumarin derivatives at 10^{-6}–10^{-5} mol/l leads to increased optical density of the samples, suggesting their higher turbidity (Fig. 2). The increased turbidity does not arise from non-specific precipitation of the protein under the influence of the studied compounds. Samples containing fibrinogen alone or thrombin in the presence of chromone or coumarin derivatives at the studied concentrations did not show changes in absorbance (not shown). It may, therefore, be concluded that the reduced polymerization of fibrin monomers is caused by structural changes in the resulting system.

Table 1. Changes in absorbance (ΔA) over 20 s during of fibrin polymerization in the presence of coumarin and chromone derivatives

Compound No.	Reduction of absorbance compared to control (%)	
	Concentration 10^{-5} (mol/l)	Concentration 10^{-6} (mol/l)
Coumarin derivatives		
1	79.07±12.21*	38.44±18.91
2	23.34±12.34	28.42±11.29
3	33.41±24.36	26.42±11.02
Chromone derivatives		
4	32.76±19.67	15.68±11.80
5	40.39±12.17	40.79±17.25
6	74.34±5.97*	72.52±8.09*
7	49.01±16.58	58.79±18.82
8	78.94±11.27*	76.58±11.63*

*statistically significant difference $P<0.05$, n=10

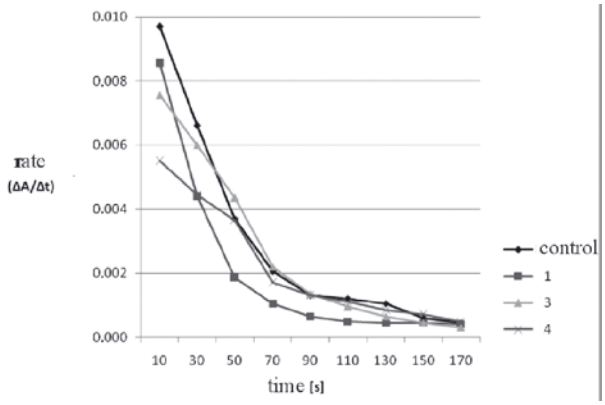


Figure 2. Kinetics of fibrin polymerization in the presence of compounds 1, 3 and 4 (the final concentraction in the probe is 10^{-5} mol/l).
The sample under investigation contained 1 ml of human fibrinogen (Sigma-Aldrich) at 3.2 mg/ml, 980 μl of PBS buffer, 0.01 mol/l, pH 7.3, and 20 μl of the compound in DMSO.

Fibrin viscosity

Measurements of fibrin viscosity in the presence of phosphorohydrazone derivatives of chromone and coumarine were taken with a digital Brookfield rheometer DVIII at a shear rate of 675 1/s. Both the derivatives of coumarin and chromone caused a reduction of cross-linked fibrin viscosity (Table 2).

Among the coumarin derivatives, compound **1** at 10^{-5} mol/l caused the most significant viscosity reduction (η=1.82±0.02 mPas) compared to the control group (3.93±0.08 mPas). No such effect was observed for the concentration of 10^{-6} mol/l. Compound **3** reduced the viscosity at a statistically significant level while applied at only 10^{-6} mol/l (1.74±0.04 mPas).

In the group of chromone derivatives, compound **4** at 10^{-5} and 10^{-6} mol/l caused a statistically significant reduction of viscosity to 1.56±0.01 and 1.34±0.06 mPas, respectively, compared to the control group. Compound **5** significantly reduced the viscosity of fibrin to 1.16±0.05 mPas at 10^{-5} mol/l whereas the viscosity of samples for the concentration of 10^{-6} mol/l was only slightly reduced to 2.07±0.08 mPas. In this group, compound **8** at 10^{-5} mol/l caused a statistically significant reduction of

Table 2. Changes in viscosity of cross-linked fibrin compared to control in the presence of coumarin and chromone derivatives (shear rate 675 1/s).

Compound No.	Concentration 10^{-6} (mol/l)			
	τ (N/m^2)	η (mPa·s)	τ (N/m^2)	η (mPa·s)
C	2.65	3.93±0.08	2.65	3.93±0.08
Coumarin derivatives				
1	1.23	1.82±0.02*	9.93	4.71±0.12
3	2.28	3.38±0.03	1.18	1.74±0.04*
Chromone derivatives				
4	1.05	1.56±0.01*	0.91	1.34±0.06*
5	0.78	1.16±0.05*	1.40	2.07±0.08
7	1.91	2.83±0.07	5.62	8.33±0.03
8	1.42	2.11±0.21*	1.18	1.74±0.07*

*Statistically significant difference $P<0.05$, n=10; C-control; τ – tangent stress (N/m^2); η, viscosity (mPa·s).

fibrin viscosity to 2.11±0.02 mPas and at 10^{-6} to 1.74 mPas. Compound **7** was of a weaker activity and reduced the viscosity only at 10^{-5} mol/l. Compounds **2** and **6** did not reduce the sample viscosity at either of the applied concentrations.

DISCUSSION

Earlier crystallographic studies have shown that compound **1** in the solid state has the structure of a phosphorohydrazone 2,4-dione (Rybarczyk-Pirek *et al.*, 1999) and compounds **2** and **3,** of 4-hydroxycoumarin (Rybarczyk-Pirek *et al.*, 2003; 2006) (Fig. 1).

For compounds **1–3** in the studied DMSO solutions, equilibrium exists between both tautomers (Nawrot-Modranka *et al.*, 2006). Compound **1** studied *in vivo* manifested a significant antitumour activity against mouse leukemia L1210 and P388 (Nawrot-Modranka *et al.*, 2004; 2006).

The results of our research show that phosphorohydrazone derivatives of coumarin and chromone have varying influence on the fibrin polymerization and viscosity. Compound **1**, whose antitumour activity was shown earlier, influences both fibrin polymerization and its physical properties. This compound significantly reduces the fibrin polymerization rate and its viscosity at 10^{-5} mol/l and 10^{-6} mol/l (Tables 1 and 2). Compounds **2** and **3**, derivatives of 4-hydroxycoumarin applied at both concentrations caused slight absorbance reduction compared to the control sample (Table 1).

Among the chromone phosphorohydrazones, a statistically significant reduction of absorbance compared to the control was noted for both concentrations, 10^{-5} and 10^{-6} mol/l, for compounds **6** and **8** (Table 1). In this group of derivatives compounds **4** and **8** statistically decreased the viscosity of fibrin. Compound **5** reduced it at the higher concentration (10^{-5} mol/l) and slightly at 10^{-6} mol/l.

To summarize, on the basis of the data presented above it may be concluded that among the three coumarin derivatives under investigation, compound **1** influences both fibrin polymerization and viscosity. This compound contains a methylated hydrazine nitrogen atom and is a methyl ester of thiophosphoric acid. Among the chromone derivatives such an activity is exhibited by compound **8**, also containing a *N*-methylhydrazone moiety. On the other hand, derivative **6** acts only on fibrin polymerization. The common structural feature of these compounds (**1**, **6**, **8**) is the moiety mentioned above. The least active of the tested compounds were **2** (a coumarin derivative) and **7** (a chromone derivative).

The derivatives of coumarin and chromone studied earlier with respect to their antitumour and antibacterial activity act also upon the polymerization and viscosity of fibrin. The mechanism of the activity of these compounds on the processes under investigation is difficult to explain due to the possibility of their interactions with fibrinogen, fibrin monomers, the course of the polymerization reaction itself, and cross-linking (Mills *et al.*, 2002; Marchi *et al.*, 2004). Formation of an appropriate structure of fibrin of physiological conditions is a complex process comprising a reaction of proteolysis (liberation of fibrinopeptides A and B, γ-multimers and α-polymers) and the stabilization process catalyzed by factor XIII. A detailed knowledge of the course of fibrin polymerization as well as factors influencing its viscosity may promote new directions in treatment of cardiovascular diseases and cancer (Bromberg & Capello, 1999; von Tempelhoff *et al.*, 1998; 2002). According to the literature, it may be concluded that both polymerization and viscosity of fibrin may be influenced by calcium and magnesium ions, heavy metal ions, some pharmaceuticals, and diseases. The structure of fibrin fibers is a factor that may affect the migration of tumour cells (Furlan *et al.*, 1966; Carr & Gabriel, 1980; Hayen *et al.*, 1999; Bromberg & Capello, 1999; Standeven *et al.*, 2002) and metastasis.

Results presented in this paper facilitate a preliminary evaluation of the relationship between the structure of phosphorohydrazone derivatives of coumarin and chromone and their activity. Antiangiogenic properties of the most active compounds will be investigated soon.

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