The antithrombotic activity of corilagin purified from korean herb-Phyllanthus ussuriensis

Kum Chol Ri[1,*], Chol Ho Ju[1]

Abstract: Plasminogen activator inhibitor-1(PAI-1) is a main negative regulator of the fibrinolytic system. In animal studies, inhibition of PAI-1 activity prevents arterial and venous thrombosis, indicating that PAI-1 inhibitors may be used as a new class of antithrombotics. In this study, we revealed that corilagin which purified from Korean herb, Phyllanthus ussuriensis, inhibited plasma PAI-1 and increased the activity of t-PA in vitro and in vivo. We then observed that when injected more than 6mg/kg of corilain into the rat electrically stimulated carotid artery thrombosis rat, the rethrombosis ratio was lower than 28.6%, and it was much lower than when 20000IU/kg of urokinase was injected. At the end we found out that corilagin remarkably decreased the content of plasma fibrin in the thrombosis rat and the decrease at dose of more than 6mg/kg was about 4.4 fold than the control. Our data show that corilagin is a new class of small molecule PAI-1 inhibitors with anti-thrombotic potential and has great prospect as thrombosis therapeutic drug.

Key Words: *Plasminogen activator, Plasminogen activator inhibitor-1 (PAI-1), corilagin, thrombosis*

Introduction

Fibrinolysis is an important physiologic mechanism that removes intravascular thrombi to maintain vascular patency in the setting of thrombosis. Plasminogen activator inhibitor-1(PAI-1) is an immediate inhibitor of tissue-type plasminogen activator (t-PA) and urokinse-type plasminogen activator (u-PA), and therefore is a negative regulator of the fibrinolytic system.

Recent studies suggest that PAI-1 also contributes directly to the complications of obesity, including type 2 diabetes and coronary arterial thrombi, and may even influence the accumulation of visceral fat [1]. Other studies substantiate PAI-1 plays an important role in cellular motility and tumor angiogenesis, and an orally active PAI-1 inhibitor prevents angiogenesis in a Matrigel implant [2].

It was found out that high levels of PAI-1 reduce fibrinolytic potential and contribute to the development of thrombosis. In the clinic, elevated levels of PAI-1 antigen and activity have been reported in patients with a variety of thrombotic diseases including deep vein thrombosis, disseminated intravascular coagulation, unstable angina, myocardial infarction and coronary artery disease [4, 5]. In animal studies, transgenic mice expressing high levels of PAI-1 develop spontaneous thrombosis, whereas PAI-1-deficient mice are more resistant to venous or arterial thrombosis induced by either endotoxin or chemical and electric injury [6].

1 Life Science Department, Univercity of Science, Pyongyang 999093, Democratic People's Republic of Korea .* address for correspondence: 15124579835 @163.com..Kum Chol Ri

Inhibition of PAI-1 activity by monoclonal or polyclonal antibodies prevents thrombus formation in animal models, indicating that inhibition of PAI-1 is a potential strategy to prevent thrombosis. Mechanistically, the anti-thrombotic effects of PAI-1 inhibition are achieved by enhancing endogenous fibrinolytic potential without directly affecting blood coagulation or platelet function. In PAI-1-deficient mice, for example, no significant defects were found in coagulation assays. PAI-1-deficient mice showed no significant abnormalities in bleeding tests and exhibited no spontaneous bleeding [7, 17]. These data suggest that an antithrombotic agent based on PAI-1 inhibition may have a lower risk of bleeding than that of conventional antiplatelet and anticoagulant drugs, and therefore have a better therapeutic index.

Therefor, some studies to find out small molecule compounds which inhibit either PAI-1 production or activity have been performed [3]. Recently, we have identified a small molecule PAI-1 inhibitor, corilagin, among different compounds purified from korean herbs by a high-throughput screening. We now report antithrombotic characteristic of corilagin.

Materials and methods

The corilagin was prepared by filtering the extract of Korean herb Phyllanthus ussuriensis at the gel chromatography, and its chemical structure was verified by nuclear magnetic resonance spectro-scopy and mass spectrometry. To study the effects of corilagin on the activity of PAI-1 in vitro, we collected blood from a healthy rat, prevented coagulation by using 3.8% citric

sodium solution, and obtained a platelet-free plasma by centrifugating for 30 minutes in 4℃, 2500rpm. The plasma was mixed with different concentrations of corilagin and kept for 45 minutes. The activities of t-PA and PAI-1 were measured in the 96-well microtiter plate according to the chromogenic substrate analysis kits (Chromogenix) [3]. ThermoMax plate reader(Molecular Devices, Sunnyvale, CA) was used for the chromogenic analysis.

We also studied the effects of corilagin on the activities of PAI-1 and tPA of the rat plasma in vivo. We divided rats into two groups and injected 0.9% saline solution and corilagin respectively for 20 minutes at the rate of 0.5mg/kg/min. We gathered blood before injection and 10 minutes, 30 minutes, 60 minutes, 90 minutes, 120minutes after injection respectively, and measured the the activities of PAI-1 and tPA in the plasma.

To examine the thrombolysis effect of corilagin in the thrombosis animals, electrically stimulated carotid artery thrombosis rats [9] were used. In the electrically stimulated carotid artery thrombosis rats, we divided rats into 5 groups and 0.9% saline solution, 20000 IU/kg of urokinase(Sigma, St.Louis, MO), 3mg/kg of corilagin, 6 mg/kg of corilagin, 9 mg/kg of corilagin were injected respectively. At one hour after injection, the hematocele was measured with supersonic tachometer (DVM-4200, Hayashi Denki). We evaluated the vessels to be opened if the hematocele is over half of the original hematocele (the hematocele measured before the thrombus was formed) and to fail to be opened if the hematocele is less than half of the original hematocele. The vessels which was opened at first but decreased to less than 25% at 1 hour after successful reperfusion were evaluated as re-occlusion.

In order to study the effect of corilagin on the content of the plasma fibrin, we divided thrombosis rats into 4 groups and 0.9% saline solution, 3 mg/kg of corilagin, 6 mg/kg of corilagin and 9 mg/kg of corilagin were injected respectively. At 1 hour after that, we gathered blood from each rat, prevented coagulation by using citric sodium solution, and obtained a platelet-free plasma by centrifuging for 15 minutes in 2500rpm. 1ml of 0.025M $CaCl_2$ solution was added to each of the 1.9ml of plasma from test groups in order to form the fibrin. After centrifuging the fibrin fibers, the wet and dried weight was measured with an electronic balance.

The MDA content of the brain was measured using the TBA method [15].

Results and Discussion

- *The chemical structure of corilagin*

The chemical structure of corilagin is shown in Fig. 1.

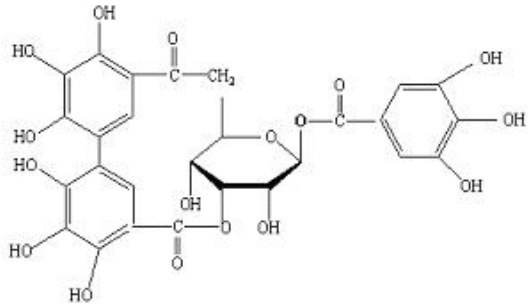

Fig. 1 The structure of corilagin

- *The effects of corilagin on the activities of PAI-1 and tPA of the rat plasma in vitro*

We examined the effects of corilagin on the activities of PAI-1 and tPA of the rat plasma, and the results were shown in Table 1.

Table 1. The effect of corilagin on rat plasma PAI-1 and tPA activities in vitro

corilagin mg/L	PAI-1 activity IU/mL	tPA activity IU/mL
0	19.2±2.5	3.3±1.4
10	16.1±2.4*	4.7±1.6*
20	11.5±2.2**	5.2±1.8*
40	7.4±3.5**	5.7±1.6**
80	4.5±1.6**	6.5±1.7**
160	3.7±1.4**	7.0±1.8**

(* P<0.05, ** P<0.01(comparison with saline solution)

As Table 1 shows, corilagin markedly inhibits the activity of PAI-1 of the rat plasma and raises that of tPA in vitro. The IC_{50} on the PAI-1 of the rat plasma calculated from Table 1 was 18mg /L.

- *The effects of corilagin on the activities of PAI-1 and tPA of the rat plasma in vivo*

Table 2 and Table 3 show the effects of corilagin on the activities of PAI-1 and tPA of the rat plasma in vivo, respectively .

As Table 2 and Table 3 show, corilagin decreased remarkably the activity of PAI-1 of the plama and increased that of tPA of the plasma. At 90 minutes after injection, the rat plasma PAI-1 activity was decreased to the minimum value of 16.1±3.7 IU/ml and the activity of the tPA of the plasma was mostly increased.

Table 2. The change in the activity of the rat plasma PAI-1 according to the time intervals since injection of corilagin

time, min / medicine	0	30	60	90	120
saline	26.4±4.8	25.9±5.0	27.4±4.3	26.9±5.7	27.4±4.9
corilagin	26.7±5.2	24.1±4.6#	20.3±5.4*#	16.1±3.7**#	18.8±3.5*#

The unit of PAI-1 activity is IU/ml.
*P<0.05, **P<0.01(comparison with the same time of saline solution), #P<0.01(comparison with 0 min)

Table 3. The change in the activity of the rat plasma tPA according to the time intervals since injection of corilagin

time, min / medicine	0	30	60	90	120
saline	2.4±0.6	1.9±0.3	2.0±0.5	1.9±0.2	2.1±0.4
corilagin	2.3±0.7	3.6±1.3**#	7.3±1.4**#	8.3±1.7**#	5.8±1.5**#

The unit of t-PA activity is IU/ml.
**P<0.01(comparison with the same time of saline solution), #P<0.01(comparison with 0 min)

- The thrombolysis effect of the corilagin on the thrombus in the carotid artery of electrically stimulated carotid artery thrombosis rats

We examined the thrombolysis effect of the corilagin on the thrombus in the carotid artery of electrically stimulated carotid artery thrombosis rat, and the results were shown in Table 4.

Table 4. The thrombolysis effect of the corilagin on the carotid artery thrombus in rats

Medicine	dose, mg/kg	reperfusion /total	reocclusion /reperfusion
saline	-	0/10	0/0
corilagin	3	2/10*	2/2*
	6	7/10*	2/7*
	9	7/10*	2/7*
urokinase	20000 IU/kg	7/10*	3/7*

* P<0.05(comparison with saline)

As Table 4 shows, when injected 6mg/kg of corilagin, reperfusion ratio was 70%, and reocclusion ratio was 28.6%, which is better than the injection of 20000IU/kg of urokinase (reocclusion ratio was 42.9% in this case).

- The change in the content of the plasma fibrin according to the variation in the corilagin content in the thrombosis rats

The change in the content of the plasma fibrin according to the variation in the corilagin dose in the thrombosis rats was shown in Table 5.

Table 5 shows that the plasma fibrin content was markedly reduced in thrombosis rats injected with corilagin in comparison with the control. When more than 6mg/kg of corilagin was injected, the fibrin content was decreased by 4.4 times in comparison with the control.

Table 5. The change in the content of the plasma fibrin according to the variation in the corilagin dose in the thrombosis rats

dose, mg/kg	the content of the plasma fibrin	
	wet weight, mg/ml	dried weight, mg/ml
saline	26.9±1.9	9.3±0.8
3	17.8±1.2*	5.1±0.5*
6	6.5±0.7**	2.1±0.3**
9	6.4±0.9**	2.0±0.2**

*P<0.05, **P<0.01(comparison with saline solution)

- The effect of corilagin on MDA content of the brain tissue in thrombosis rats

Table 6 shows the effect of corilagin on MDA content of the brain tissue in thrombosis rats.

Table 6. The effect of corilagin on MDA content of the brain tissue

corilagin dose, mg/kg	MDAcontent, nmol/g
saline	555.6±14.4
3	513.9±13.7*
6	395.7±15.8*
9	392.6±14.2*

*P<0.05(comparison with the same time of saline solution)

As Table 6 shows, the MDA content of the brain tissue in the rats was decreased according to the increase in the corilagin dose.

Conclusions

Through our experiments we revealed that corilagin inhibited plasma PAI-1 and increased the activity of t-PA in vitro and in vivo. We then observed that when injected more than 6mg/kg of corilagin into the electrically stimulated carotid artery thrombosis rats, the rethrombosis ratio was lower than 28.6%, and it was much lower than when 20000IU/kg of urokinase was injected. At the end we found out that corilagin remarkably decreased the content of plasma fibrin in the thrombosis rats and the decrease at dose of more than 6mg/kg was about 4.4 fold than the control.

Thrombosis is a major cardiovascular disease, afflicting millions people in the world. Currently used antithrombotic drugs are inhibitors of either platelets or blood clotting factors. More recently, several small molecule PAI-1 inhibitors have been identified [9]. Most of them have been reported that inhibit PAI-1 activity in vitro [8, 10–12]. One compound series among them, represented by XR5118, was shown to have antithrombotic efficacy in the electrically stimulated arterial thrombosis rats [13]. Another PAI-1 inhibitor, WAY-140312, was shown to enhance arterial blood flow and reduce venous thrombus weight when it was administered orally in the thrombosis rats [14]. These studies have demonstrated the feasibility of developing nonpeptic small molecule PAI-1 inhibitors as a new class of antithrombotic agents[16, 18, 19, 20]. Our data show that corilagin is a new class of small molecule PAI-1 inhibitors with anti-thrombotic potential and has great prospect as thrombosis therapeutic drug.

References

1. B De-Taeye, et al., Plasminogen activator inhibitor-1: a common denominator in obesity, diabetes and cardiovascular disease, Curr-Opin-Pharmacol, Vol 5(2005), 149-154pp
2. C. E. Leik, et al., Effect of pharmacologic plasminogen activator inhibitor-1 inhibition on cell motility and tumor angiogenesis, J-Thromb-Haemost, Vol 4(2006), 2710-2715pp
3. A. Liang, et al., Characterization of a small molecule PAI-1 inhibitor, ZK4044, Thromb-Res, Vol 115(2005), 341-350pp
4. B. Wiman, Plasminogen activator inhibitor-1(PAI-1) in plasma: its role in thrombotic disease, Thromb Haemost, Vol74(1995), 71-76pp
5. H. M. Hoffmeister, et al., Correlation between coronary morphology and molecular markers of fibrinolysis in unstable angina pectoris, Atherosclerosis, Vol 144(1999), 151-157pp
6. M. Eren, et al., Age-dependent spontaneous coronary arterial thrombosis in transgenic mice that express a stable form of human plasminogen activator inhibitor-1, Circulation, Vol 106(2002), 491-496pp
7. T. Abrahamsson, et al., Anti-thrombotic effect of a PAI-1 inhibitor in rats given endotoxin, Thromb Haemost, Vol 75(1996), 118-126pp
8. P. A. Chariton, et al., Evaluation of a low molecular weight modulator of human plasminogen activator inhibitor-1 activity, Thrombosis and Haemostasis, Vol 75(1996), 808-815pp
9. Q. Wu, Z. Zhao, Inhibition of PAI-1: a new anti-thrombotic approach, Curr Drug Targets Cardiovasc Haematol Disord, Vol 2(2002), 27–42pp
10. P. Bjorquist, et al., Identification of the binding site for a low-molecular-weight inhibitor of plasminogen activator inhibitor type 1 by site-directed mutagenesis, Biochemistry Vol 37(1998), 1227–1234pp
11. H. Elokdah, et al., Tiplaxtinin, a novel, orally efficacious inhibitor of plasminogen activator inhib-itor-1: design, synthesis, and preclinical characterization, J Med Chem Vol 47(2004), 3491–3494pp
12. B. Ye, et al., Synthesis and biological evaluation of piperazine-based derivatives as inhibitors of plasmino-gen activator inhibitor-1 (PAI-1), Bioorg Med Chem Lett Vol 14(2004), 761–765pp
13. P. A. Charlton, et al., XR5118, a novel modulator of plasminogen activator inhibitor-1 (PAI-1), increases endogenous tPA activity in the rat, Fibrinolysis Proteol-ysis Vol 11(1997) 51–56pp
14. Kumchol Ri, Yanqing Wang, Xiran Zhang. Innovator's Innovative Genetic Model: From Biological to Social Perspective, Science Journal of Business and Management, 2018; 6(2): 38-44
15. D. L., Crandall, et al., Characterization and comparative evaluation of a structurally unique PAI-1 inhibitor exhibiting oral in vivo efficacy, J Thromb Haemost Vol 2(2004) 1422–1428pp
16. Bing jing zheng et al., development and validation of an UPLC-PDA method for the determination of corilagin in rat plasma and its application to pharmacokinetic study, journal of chromatography B vol 1031(2016) 76-79pp
17. Kum Chol Ri, Jong Su Kim, Chol Kim,. Identification of*Klf6*-Related Super Enhancer in Human Hepatoma (HepG2) Cells by CRISPR Technique. (2017).Genetics and Molecular Research 16(4): gmr16039841.
18. F tong et al., corilagin attenuates radiation induced brain injury in mice, molecular neurobiology vol53(10),2016,6982-6996
19. Chen qq et al., determination of corilagin in rat plasma using a liquid chromatography-electrospray ionization tandem mass spectrometric method, biomed chromatogr, vol29(10),2015,1553-8
20. KumChol Ri, KumChol Kim, SunHyok Kong, JuHua Ri,. The Disruption of *Klf6*-Related Super- Enhancer Induces Growth Inhibition and Apoptosis in Human HepG2 Cells. (2018). Genetics and Molecular Research 17 (1): gmr16039888.