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CONTENTS

Volume 10, Number 3, 2016

Reviews

Original Articles

Brief Reports

Case Report

Guide for Authors

Copyright

Review

DOI: 10.5582/ddt.2016.01041

Menaquinone as a potential target of antibacterial agents

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Summary **The current trend of increasing infections by multidrug-resistant pathogens requires the discovery of novel antimicrobial agents with new target and selective toxicity towards pathogens. Menaquinone is a component of electron transport chains in a majority of anaerobic bacteria and Gram-positive bacteria. Due to its exclusivity in bacteria, menaquinone is thought to be a potential target for development of therapeutically effective antibacterial agents without side effects. In this review, we summarize inhibitors of menaquinone biosynthesis and antibiotics directly targeting menaquinone in bacteria.**

Keywords: Menaquinone, lysocin E, antibacterial agent

1. Menaquinone, its role and distribution

Bacteria use isoprenoid quinones such as ubiquinone (UQ) or menaquinone (MK) or demethylmenaquinone (DMK) (Figure 1) for their electron transport systems, which are found exclusively in cytoplasmic membranes (*1,2*). These quinones are important for the respiratory chain and play vital roles in cellular respiration, oxidative phosphorylation and formation of transmembrane potential in bacteria (*3*). Some bacteria have more than one of these quinones which they utilize according to growth conditions (*4*). MK, 2-methyl-3-polyprenyl-1,4-naphthoquinone, is the sole quinone in anaerobically growing bacteria, mycobacteria and most of the Gram-positive bacteria (*2*). MK exists in different forms according to the number of isoprene units which vary from 4 to 13 (*2*). In addition, some microbes require MK for virulence (*5*), regulation of certain gene expression such as nitrogen fixation (*6*), and during endospore and cytochrome formation (*7-9*).

Mammals utilize UQ as a sole quinone for respiration whereas MK is utilized for blood coagulation (*10*), bone metabolism (*11*), cell-cycle regulation (*12*) *etc*. The major source of MK in humans is either the diet or gut flora. Although UBIAD1, an enzyme that can catalyze the conversion of plant phylloquinone to MK-4, has been reported in humans, humans are not capable of *de novo* biosynthesis of MK (*13*). Therefore, it is expected that MK and its biosynthetic pathway serve as a platform for selectively targeting infections caused by pathogens that utilize MK. In this review, we summarize antimicrobial agents that either inhibit MK biosynthesis or directly interact with MK.

2. MK biosynthesis and its inhibition by small molecules

MK biosynthesis has been extensively studied. MK is synthesized from chorismate using either a classical or an alternative pathway. The recent understanding of MK biosynthesis and its critical roles for microbial growth has made it a potential target of antimicrobial agents and inhibitors of biosynthetic enzymes have been identified. Most of the inhibitors are analogues of either the substrate or cofactors of the enzymes.

The classical pathway involves enzymes, namely MenF, MenD, MenH, MenC, MenE, MenB, yfbB (MenI), MenA and MenG (Figure 2) (*3,14-17*). Targeting these enzymes that exist in bacteria and not in humans, can open up an avenue for novel antimicrobial agents with therapeutic potential. A number of inhibitors of these enzymes have already been identified (Figure 3). Some microorganisms such as *Helicobacter pylori*, *Wolinella succinogenes*, *Campylobacter jejuni*, *Geobacter sulfurreducens*, *Streptomyces coelicolor*, *Streptomyces avermitilis*, *Thermus thermophilus, Deinococcus radiodurans* and so on, synthesize MK using an alternative pathway

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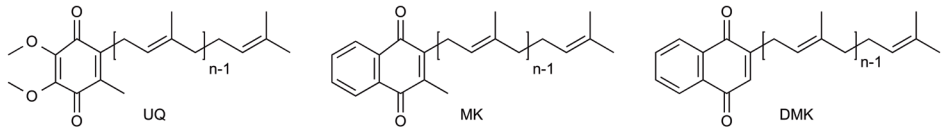


Figure 1. Quinones in bacterial electron transport systems

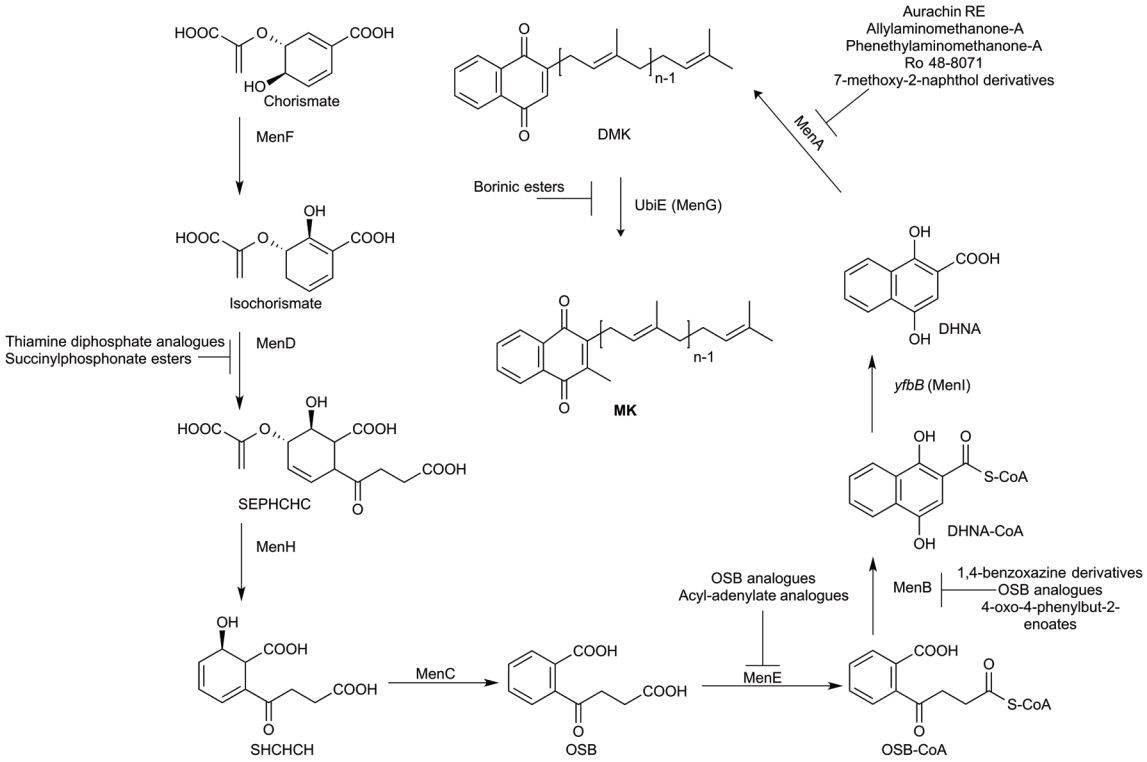


Figure 2. The classical MK biosynthesis pathway and inhibitors (SEPHCHC: 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate; SHCHC: 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate; OSB: o-succinylbenzoate; DHNA: 1,4-dihydroxy-2-naphthoyl).

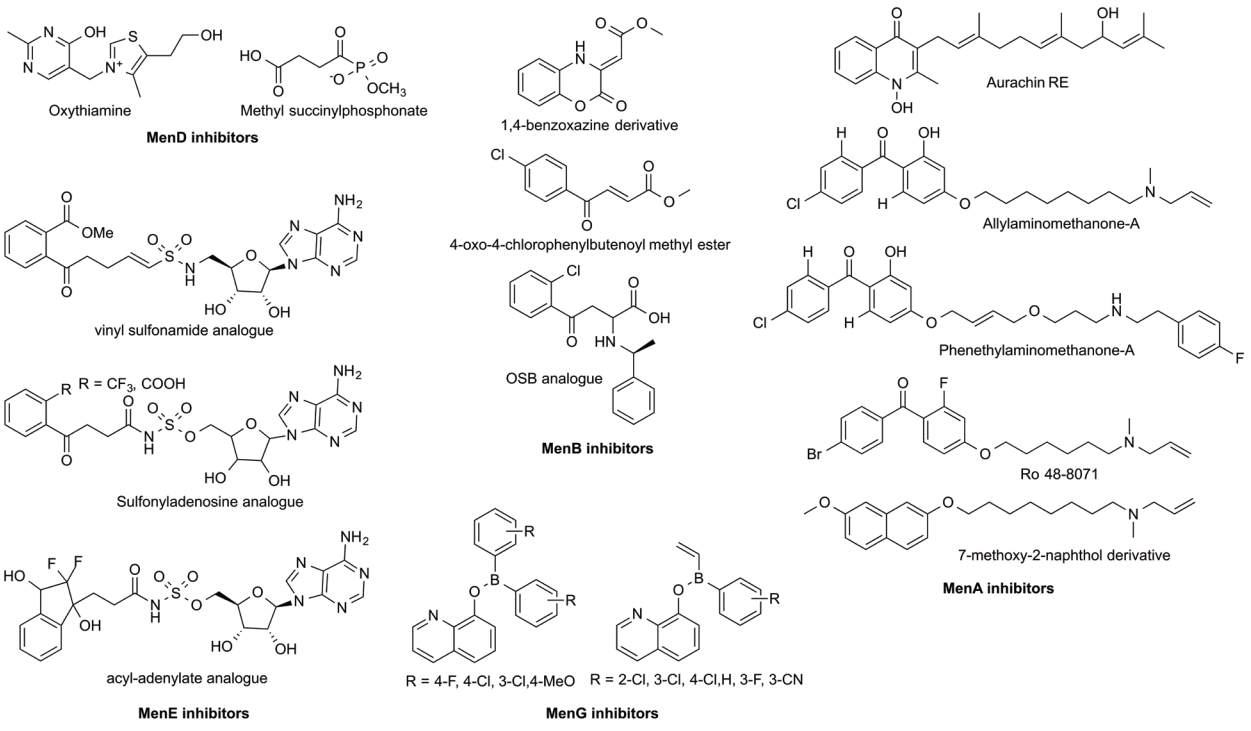


Figure 3. Inhibitors of classical MK biosynthetic pathway

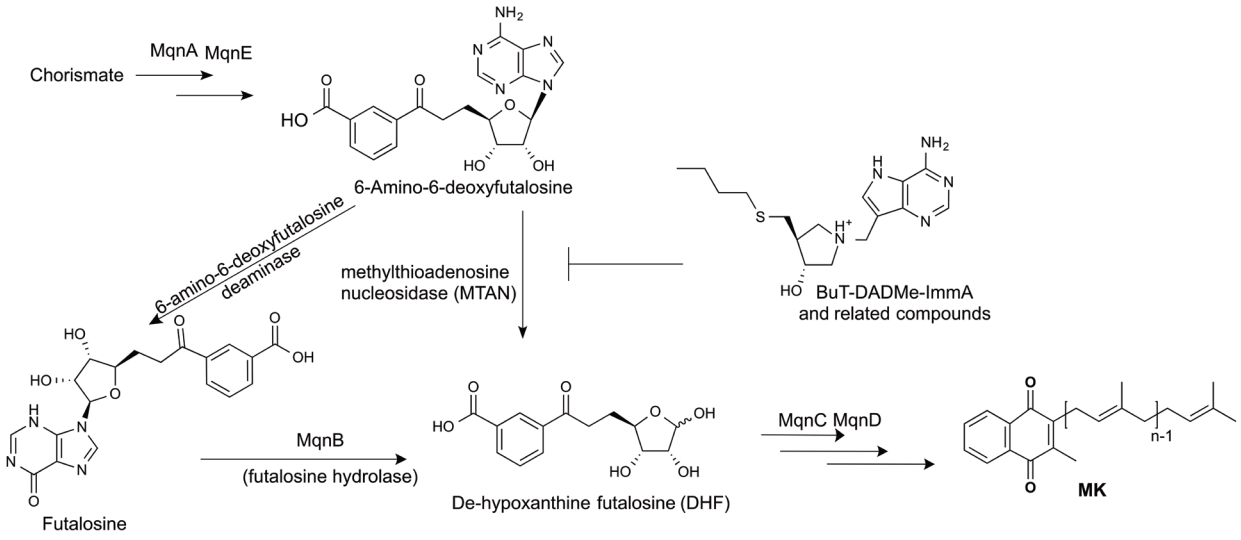


Figure 4. The alternative pathway for MK biosynthesis and inhibitors.

Table 1. MK biosynthesis inhibitors and their action

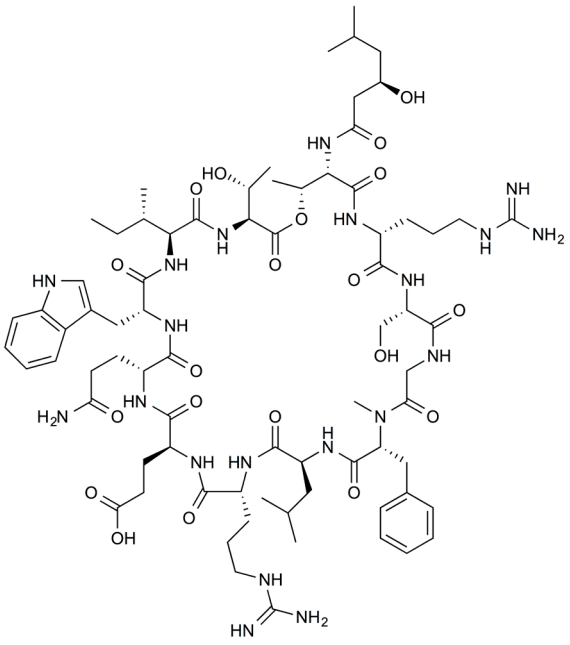
Target enzyme	Inhibitors (*Ref.*)	Growth inhibition
MenD	Thaimine diphosphate analogues (*24*)	+
	Succinyl phosphonate esters (*25*)	-
MenE	OSB analogues	
	Vinyl sulphonamide analogues (*26*)	-
	Sulfonyladenosine analogues (*27,28*)	ND
	Acyl-adenylate analogues (*29*)	+
MenB	OSB analogues (*24*)	+
	1,4-benzoxazine derivatives (*30*)	+
	4-oxo-4-phenylbut-2-enoates (*31*)	+
MenA	Aurachin RE (*17*)	ND
	Allylaminomethanone-A (*32*)	+
	Phenethylaminomethanone-A (*32*)	+
	Selective mycobacterial MenA inhibitor (*17*)	+
	Ro 48-8071 (*33*)	+
	7-methoxy-2-naphthol derivatives (*34*)	+
MenG	Borinic esters (*35*)	+
MTAN	BuT-DADMe-ImmA and analogues (*36,37*)	+

*ND: not determined

(*18,19*) that involves conversion of chorismate into 6-amino-6-deoxyfutalosine by MqnA and MqnE. Another important step of the alternative pathway, conversion of 6-amino-6-deoxyfutalosine to de-hypoxanthine futalosine (DHF), occurs by either a single step reaction catalyzed by methylthioadenosine nucleosidase (MTAN) as in *H. pylori* and *C. jejuni* or a two-step reaction catalyzed by 6-amino-6-deoxyfutalosine deaminase (*20,21*) and MqnB as in *S. coelicolor* and *T. thermophilus* (*18*) (Figure 4). The enzymatic reaction to convert DHF to MK involves MqnC, MqnD and possibly MenA and MenG (*22*). In the alternative pathway, an inhibitor of MTAN has been reported (Figure 4). Among the known inhibitors of MK biosynthesis, a MenB inhibitor 4-oxo-4-chlorophenylbutenoyl methyl ester showed therapeutic effects in a mouse model (*31*). Of note, not all the inhibitors of enzymes showed antimicrobial activity against microorganisms (Table 1).

3. Antibiotics interacting directly with MK

Lysocin E, a cyclic lipopeptide produced by Lysobacter sp. RH2180-5, directly interacts with MK and is the first antibiotic whose target is MK (*23*). It was found to directly bind to MK with a dissociation constant of 4.5 μM. The striking feature that makes lysocin E unique from other known antibiotics is its potent bactericidal



Microorganisms	MIC (μg/ml)	Quinone
Staphylococcus aureus	2-4	MK
Bacillus spp.	2	MK
Listeria monocytogenes	0.5	MK
Mycobacterium spp.	8	MK
Serratia marcescens	>100	UQ
Pseudomonas aeruginosa	>100	UQ
Candida spp.	>100	UQ
Cryptococcus neoformans	>100	UQ
Escherichia coli W3110	>100	UQ, MK, DMK
Streptococcus spp.	>128	None

Figure 5. Lysocin E, its MIC against various microorganisms (*23*), and quinones present in the microorganisms (*2*). MK: menaquinone, UQ: ubiquinone, DMK: demethylmenaquinone.

activity. *Staphylococcus aureus* showed rapid loss in absorbance at 600 nm in the presence of lysocin E indicating the lysis of bacteria. Besides bacteriolysis, potassium ion leakage from membranes and a rapid loss of bacterial membrane potential in *S. aureus* were observed in the presence of lysocin E. Spontaneous mutants resistant to lysocin E showed decreased production of MK and knockout mutants of the genes involved in the MK biosynthetic pathway, Δ*menA* and Δ*menB*, showed resistance to lysocin E. Moreover, antibacterial activity of lysocin E was decreased in the presence of MK, but not UQ, in the culture medium. This is probably due to the binding of lysocin E to the excessive amount of MK in the medium, leaving a small pool of lysocin E for binding with MK present in the bacterial membrane. The disruption of synthetic liposomes by lysocin E was dependent on the presence of MK. Further, Δ*menA* and Δ*menB* mutants of *S. aureus* showed repressed potassium leakage from their membranes. Thus, lysocin E directly targets MK, not the enzymes involved in MK biosynthesis. Lysocin E does not show antibacterial activity against *Escherichia coli* although the bacteria has MK in its cytoplasmic membrane. Membrane permeability might be the limiting factor for this Gram-negative bacteria (Figure 5). Lysocin E targets MK in the bacterial cytoplasmic membrane and causes membrane disruption, ultimately leading to cell death. Moreover, lysocin E was non-toxic to mice (acute toxic dose: > 400 mg/kg) and showed potent therapeutic activity in mice infected with MRSA (ED_{50}: 0.5 mg/kg). Little acute toxicity and potent therapeutic activity of lysocin E in animal infection models suggested that lysocin E has a potential for clinical application.

4. Conclusion

The respiration and electron transport chains are important for organisms. Since, most of the Gram-positive bacteria utilize MK and mammals utilize UQ as the sole cofactor in their electron transport system, inhibitors of MK are expected to show selective toxicity towards these bacteria. Many inhibitors of the enzymes of MK biosynthetic pathway have been developed and recent advances in the understanding of MK biosynthesis have attracted attention for MK as a target of antibacterial agents. Moreover, the discovery of MK targeting antibiotic, lysocin E, is a breakthrough in this field broadening the importance of MK as a potential target of antibacterial agents with therapeutic potential for the treatment of infectious diseases.

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Review

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Reevaluation of antithrombotic fruits and vegetables: great variation between varieties

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Summary **In the quest for prevention of atherothrombotic diseases, an antithrombotic diet may offer a promising approach. The major stumbling block in finding an effective diet is the lack of pathophysiological relevant techniques to detect potential antithrombotic effects of various diet components. Platelet function and coagulation/fibrinolysis tests currently in use do not allow assessment of global thrombotic status and their value in screening diet-components for antithrombotic effects. Recently, we combined the point-of-care shear-induced *ex vivo* thrombosis test (Global Thrombosis Test-GTT) with the Flow-mediated Vasodilation (FMV) *in vivo* test and found that the combination improved the assessment of thrombotic status in humans and could be used for screening diet-components for antithrombotic effects. In the present experiments, a combination of GTT, hemostatometry, laser-induced thrombosis tests and FMV were employed for screening. The results show that the overall antithrombotic effect is determined by the effect on thrombus formation and endogenous thrombolytic activities. This study showed a great variation in the observed antithrombotic effect between the tested varieties. Antithrombotic activities were independent from polyphenolic content or antioxidant activities. The presented experimental techniques seem to be suitable for establishing an antithrombotic diet, which may be effective in the prevention of atherothrombotic cardiovascular diseases in humans.**

Keywords: Thrombosis, thrombosis prevention, preventive cardiology, stroke, platelet function, nutrition, diet

1. Introduction

Prevention of arterial thrombotic diseases has higher priority than treatment of existing diseases. Compared to the ineffective Western-style diet, clinical trials provided evidence for reduced risk of arterial thrombosis and death from coronary heart disease in people on Mediterranean, Vegetarian and Japanese-style diets (*1-9*). As to the mechanism of such an antithrombotic effect, several nutrients and components of foods (omega-3 fatty acids; red wine; onion, garlic, kiwi; chocolate, *etc*.) were shown to inhibit platelet function *in vitro* (*10*).

In finding foods and dietary components with a potential antithrombotic effect, the use of pathologically relevant technique(s) is of crucial importance. Only those test(s) which have already proved to be useful in clinical practice in monitoring the overall thrombotic status and predicting major adverse thrombotic events should be used for screening dietary components and nutrients for the antithrombotic effect. Despite that platelets play a pivotal role in thrombosis, point-of-care platelet function tests failed to materialize clinical expectations. Tailoring antithrombotic medication based on monitoring platelet function by these tests

All authors belong to Antithrombotic Diet Discussion Group: (*http://www.nutr.kobegakuin.ac.jp/~seiri/english/*).

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did not improve clinical outcome (*11-13*). At present, prothrombotic status is assessed by measuring platelet aggregation to various soluble agonists (adenosine diphosphate, collagen, arachidonic acid, thrombin), and by extrapolating the results obtained using various biomarkers of coagulation and fibrinolysis. The major shortcoming of all these tests is the use of anticoagulated blood, in which activated platelets do not generate thrombin, the most significant contributor to arterial thrombogenesis. This could be the reason why most platelet function tests which measure platelet aggregation to various soluble agonists failed in guiding cardiac patients antithrombotic medication (*14-19*).

Evidence has been presented that only those tests, which take the arterial high shear and flow conditions as well as generation of thrombin by activated platelets into account, have relevance for the pathomechanism of occlusive arterial thrombosis *in vivo*. We compared results obtained from platelet function tests performed with anticoagulated blood and those obtained using shear-induced thrombosis and thrombolysis tests performed from non-anticoagulated blood. Our findings show that the commonly used platelet function tests performed at low shear conditions and from anticoagulated blood do not reflect the overall thrombotic status, while the innovative shear-induced thrombosis tests performed from non-anticoagulated blood do (*20,21*). We have shown in animal experiments that the combined use of high shear stress-induced thrombosis *in vitro* tests using non-anticoagulated blood (hemostatometry and global thrombosis test, GTT) in combination with the flow-mediated vasodilation *in vivo* test (FMV or FMD) provides reliable assessment of the global thrombotic status (*22*). In addition, GTT has been shown to be clinically useful for monitoring thrombotic status in patients on antithrombotic medication (*23,24*). It was therefore reasonable to employ these techniques to test fruits and vegetables and herbal drugs for antithrombotic effects.

2. *In vitro* tests

2.1. *Shear-induced platelet-rich thrombus formation in non-anticoagulated blood*

2.1.1. *Hemostatometry*

Details of hemostatometry have been described previously (*25,26*). Briefly, non-anticoagulated blood was withdrawn from the abdominal aorta of animals and tested with a hemostatometer built for this puspose in Kobe Gakuin University. Blood was forced to flow through a plastic tube by a paraffin oil replacement technique. While blood was flowing in it, the tubing was punched with a fine needle to induce "bleeding" from the holes into the surrounding warm saline. The perfusion pressure was monitored to assess the thrombotic reaction. Punching the tube caused a sharp drop in the perfusion pressure. Eventually "bleeding" stopped due to formation of platelet-rich hemostatic plugs in the holes and with this, the perfusion pressure returned to the pre-punching level. The recorded pressure changes reflect both the hemostatic and coagulation processes. In the recorded pressure curve, areas of 30% (H1) and 90% (H2) pressure recovery reflect the primary and completed hemostasis. Increase or decrease of H1 and H2 reflected inhibition or enhancement of hemostatic plug formation (platelet reactivity), respectively. In some occasions increased pressure was used to induce thrombolysis (Figure 1A).

2.1.2. *GTT*

GTT (Thromboquest Limited, London, UK) has been described in detail (*23,24,27,28*). Figure 1B shows the embodiment (a) and the principle of the technique (b) and a typical recording (c). There are flat segments along the inner wall of a conical plastic tube and when perfectly round ceramic ball bearings are placed into such a conical tube, the flat segments prevent the ball bearings from occluding the lumen. When non-anticoagulated blood is added to such a tube, it flows through the narrow gaps by the ball bearing and exits in droplets into an adjacent collecting tube. The latter is transilluminated by a light emitter and a sensor opposite the emitter generates a signal whenever a drop of blood interrupts the light path. In essence, the instrument detects the time interval (d; sec) between consecutive blood drops. Blood flows at 37°C by gravity through the narrow gaps formed between the upper ball bearing and the inner wall of the tube, where high shear stress activates and aggregates platelets. Platelet aggregates formed and then captured in the gaps by the lower ball bearing, arrest the blood flow. At the start, blood flow is rapid and hence (d) is small. Subsequently, the flow rate gradually decreases and hence (d) increases. When the actual (d) exceeds 15 seconds (occlusion-d), the instrument displays "Occlusion Time (OT)", which is the time elapsed from the detection of the first drop of blood until OT. Later, the blood flow is completely arrested. Eventually, due to thrombolysis, flow is restored as indicated by the detection of the first drop of blood after complete occlusion (Lysis Time- LT). Compared to controls, increased or decreased OT indicates inhibition or enhancement of platelet reactivity, respectively. Increase or decrease of LT indicates inhibition or enhancement of spontaneous thrombolysis, respectively. GTT can measure platelet reactivity and endogenous thrombolytic activity simultaneously.

3. *In vivo* tests

3.1. *Laser-induced thrombosis in the microcirculation and in the carotid artery of experimental animals*

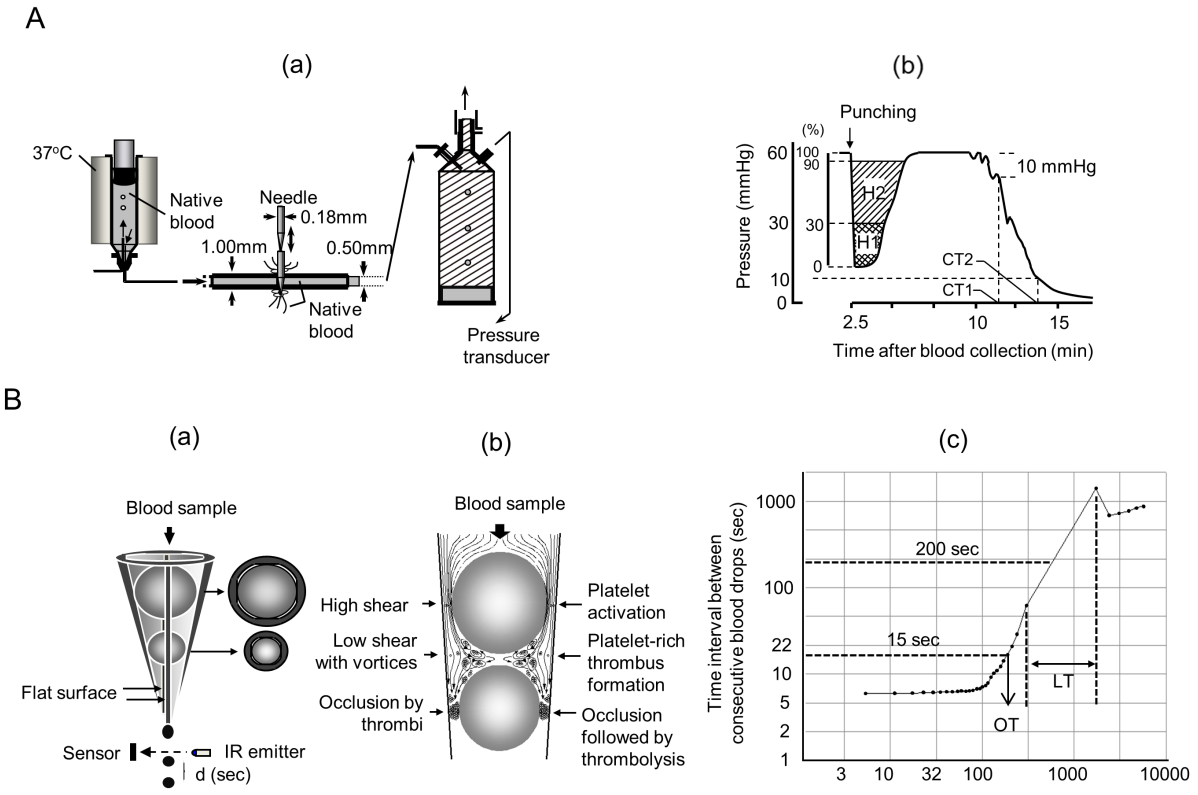


Figure 1. (A): (a) A principle of hemostatometry, (b) A typical pattern; (B): (a) Embodiment of GTT, (b) Principle of GTT, (c) A typical pattern.

Formation of platelet-rich thrombi and their embolization was initiated in the mouse carotid artery or the rat mesenteric or pial microvessels using the He-Ne laser-induced thrombosis technique. He-Ne laser-induced thrombosis method has been previously described in detail (*29-44*). In brief, the mesenteric or pial microvessels of anaesthetized rats or the left femoral artery of anaesthetized mice, was exposed and Evans blue dye was injected through the veins. The center of the mesenteric or pial microvessel or the carotid artery was irradiated with laser, and the formation of a thrombus at the site of irradiation was monitored and recorded on videotape. Thrombotic status of rats was expressed by the number of thrombosis events required to complete occlusion of blood flow and in mice expressed as the cumulative thrombus size. The latter was calculated by continuous observation of the thrombus mass every 10 seconds in the first 10 minutes after irradiation (Figure 2A).

3.2. *Flow-mediated Vasodilation test (FMV or FMD)*

We have adopted and modified the flow-mediated and nitroglycerin-mediated technique to anaesthetized mice, as shown in Figure 2B. Baseline images of the diameter of the femoral artery were taken before and after clamping for 180 sec at 30 sec intervals over 450 sec after restoration of blood flow. Nitroglycerin mediated vasodilation (endothelium-independent vasodilation) was induced by placing 70 microliters of 2.2 mM nitroglycerin/saline solution on the artery. A typical pattern of vasodilation after restoration of flow was transferred to a computer and the artery diameter changes were calculated. Changes in vessel diameter after restoration of flow were expressed as percentage of the baseline values (before clamping) and the peak vasodilation was calculated (*45-48*). A typical pattern of these is shown in Figure 3.

4. Screening antithrombotic fruits and vegetables by shear-induced thrombosis/thrombolysis *in vitro* tests, followed by He-Ne laser-induced *in vivo* test

Since overall antithrombotic and prothrombotic activities of fruits and vegetables were varied from varieties to varieties and determined by the balance between antithrombotic activity (platelet reactivity) and endogenous thrombolytic activity (fibrinolytic activity), special attention was paid to the sources of fruits and vegetables (*49*). Fruits and vegetables were ground using mortar and pestle. Juices obtained were prepared by filtration (test samples). One tenth volume of the test sample was mixed with nine tenths volume of non-anticoagulated rat blood collected from rat abdominal aorta immediately before the tests. Antithrombotic, prothrombotic and thrombolytic activities were

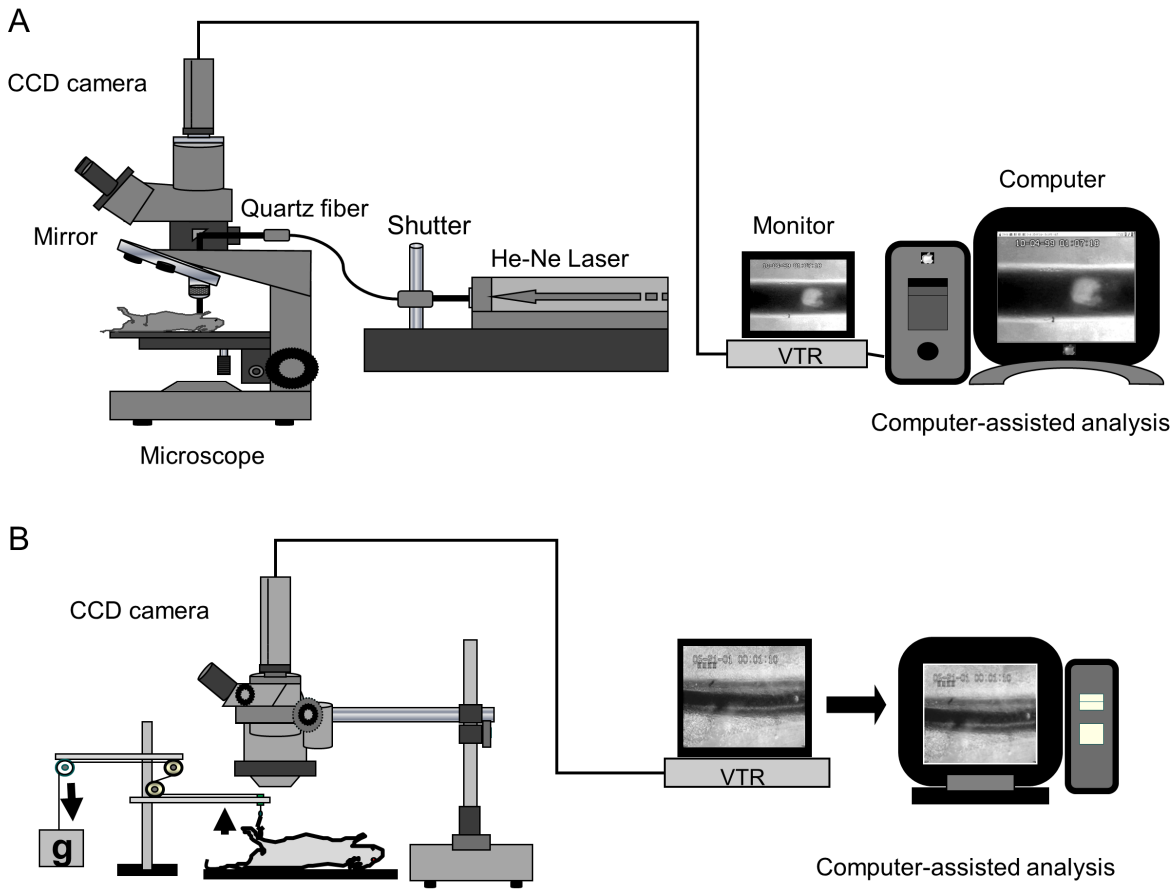


Figure 2. (A): He-Ne laser-induced thrombosis system; (B): A Flow-mediated vasodilation system.

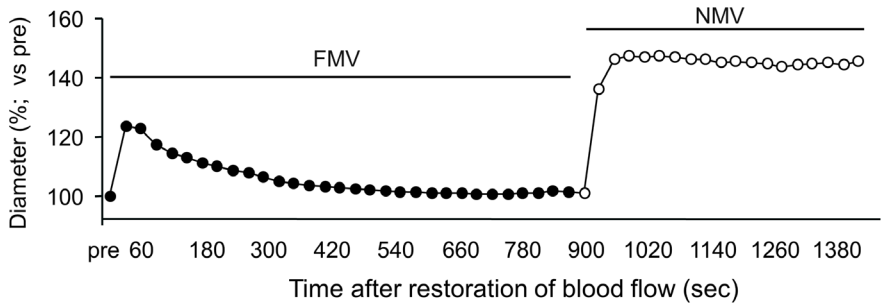


Figure 3. A typical pattern of flow-mediated vasodilation (FMV) and nitroglycerin-mediated vasodilation (NMV) in a mouse femoral artery.

measured by shear-induced thrombosis/thrombolysis *in vitro* test (hemostatometry or GTT) after quick mixing. The intensity of antithrombotic, prothrombotic and thrombolytic activities was expressed as the maximum dilution factor. At first these activities were screened using raw test samples, subsequently assessed using heat-treated (5-10 min) samples. Those samples which showed a significant antithrombotic or thrombolytic effect were administered to mice orally and tested by He-Ne laser-induced thrombosis *in vivo* test.

4.1. *Antithrombotic vegetables*

The overall effect of administered fruit or vegetable extracts on the *in vivo* thrombotic status is determined by the balance between thrombotic activity (effect on the growth of a platelet-rich thrombus) and thrombolytic activity (disintegration or fibrinolysis of the formed thrombus). For this reason, first we used relevant *in vitro* tests for screening and the active varieties were further tested *in vivo* to assess the overall antithrombotic effect.

4.1.1. *Tomatoes*

Ordinary size tomatoes Twenty-one varieties were measured by hemostatometry *in vitro*. The antithrombotic activity was dependent on the variety. The varieties were ranked into subgroups according to their activities, i.e. the group inhibiting platelet rich thrombus formation (antithrombotic group), the one enhancing the rate of thrombosis (prothrombotic group) and the group without effect (non-thrombotic group). Ten varieties were antithrombotic, out of them three had a highly significant antithrombotic effect. Two varieties showed a prothrombotic effect while in four varieties the effect was not clear. One variety with the strongest and heat stable antithrombotic effect was selected for further investigation. When tested *in vivo*, oral administration of this variety showed significant antithrombotic activity. This decreased during maturation (*49*).

Mini-type tomatoes Antithrombotic activity of four varieties was measured by the *in vitro* test GTT. Antithrombotic activities showed great variation between varieties, one was antithrombotic while the other three had no such effect. The antithrombotic activity decreased during maturation. Despite earlier suggestion of polyphenolic rich foods are antithrombotic, lycopene content was independent of antithrombotic activity. Thus, lycopene content cannot be used as an index of antithrombotic activity (*50*).

4.1.2. *Onions*

Onions from Hokkaido (Northern area in Japan) Antithrombotic activity of ten varieties was measured by the *in vitro* tests hemostatometry. Three varieties inhibited thrombus formation, one enhanced the rate of thrombus growth while six varieties had no effect on experimental thrombus formation. Five varieties enhanced endogenous thrombolytic activity while five varieties had no effect on it. Considering the balance between thrombotic and thrombolytic activities, one variety with the strongest antithrombotic effect was selected for further investigation. Subsequently, the antithrombotic activity of this variety was tested after oral administration in mice by the He-Ne laser-induced thrombosis *in vivo* test .This variety was heat stable (*51*).

Onions from Awaji Island, Hyogo (Middle area in Japan) Antithrombotic activity of five varieties was measured by the *in vitro* test GTT. One variety, which was antithrombotic by GTT test was further investigated by the laser-induced thrombosis test and the antithrombotic activity was confirmed after oral administration to mice. This activity was heat stable. Another variety inhibited endogenous thrombolytic activity, suggesting an overall prothrombotic activity (*22,52*).

4.1.3. *Strawberry*

Antithrombotic activity of seventeen varieties was measured by hemostatometry *in vitro*. Ten varieties inhibited platelet reactivity (antithrombotic), six varieties had no effect on thrombus formation and one could not be determined by this test. Varieties were ranked into subgroups on the basis of the intensity of their antiplatelet effect. Three varieties with the strongest antiplatelet activity were heat stable. Antithrombotic activity of these three varieties was demonstrated after oral administration in mice by the laser-induced thrombosis test *in vivo* (*53*).

4.1.4. *Potatoes*

Potatoes harvested in the spring Antithrombotic activity of twenty varieties was measured by the *in vitro* test GTT and ranked into subgroups. Three varieties, all heat-stable, were selected as antithrombotic varieties. Subsequently, antithrombotic activity was measured after oral administration in mice by the laser-induced thrombosis test. All three varieties showed antithrombotic activity *in vivo* (*54*).

Potatoes harvested in the autumn Antithrombotic activity of seven varieties was measured by the *in vitro* test GTT. Six varieties inhibited platelet reactivity and the antithrombotic activities were heat stable. One variety (heated) was further examined by the laser-induced thrombosis test *in vivo* and demonstrated to be prothrombotic under *in vivo* conditions (*55*).

4.1.5. *Carrots*

Antithrombotic activity of fifteen varieties and heat stability of selected varieties were measured by the *in vitro* test GTT. Effect on thrombus formation *in vivo* was measured by the laser-induced thrombosis test. Results of three varieties are shown in Figure 4.

As demonstrated by the laser *in vivo* test, the variety SAKATA-0421 inhibited platelet reactivity and enhanced endogenous thrombolysis, but after heat treatment, the inhibitory effect on platelets disappeared but the enhancing effect on endogenous thrombolytic activity remained (Figure 4A). SAKATA-0418 did not inhibit platelet reactivity but enhanced endogenous thrombolysis before heat treatment *in vitro*. After heat treatment, platelet reactivity was enhanced but effect on endogenous thrombolytic activity disappeared, suggesting prothrombotic activity *in vivo* (Figure 4B). The variety SAKATA-0420 enhanced platelet activity and endogenous thrombolytic activity before heat treatment and had no effect on the *in vivo* test. After heat treatment, it did not affect platelet reactivity but the effect on endogenous thrombolytic activity remained (Figure 4C). These findings showed that the *in vivo* effect on thrombosis variables can be predicted by the

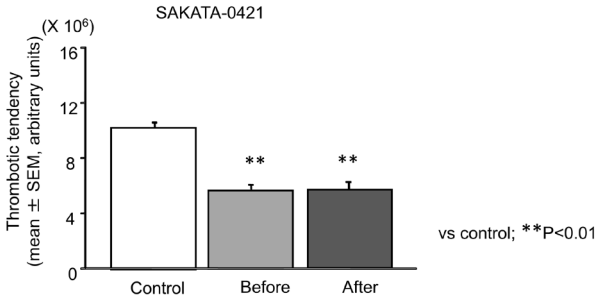
A

(a) GTT test, in vitro

Variety	Heat treatment	Occlusion time (OT)	Lysis time (LT)
SAKATA-0421	Before	135.3±12.3**	55.7±9.3*
	After	107.4±10.0	61.7±13.5*

vs control (%); *P<0.05; **P<0.01

(b) He-Ne laser-induced thrombosis test, in vivo



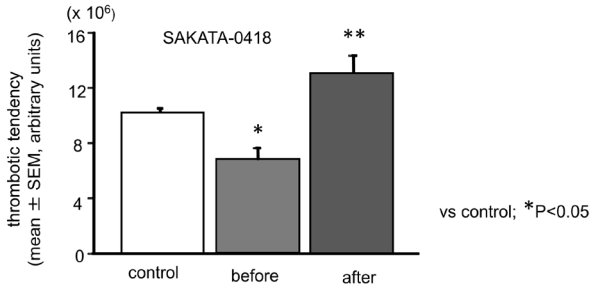
B

(a) GTT test, in vitro

variety	heat treatment	OT	LT
SAKATA-0418	before	90.6±8.3	83.5±4.2**
	after	79.9±8.5*	98.2±4.5

vs control (saline), (%); *P<0.05; **P<0.01

(b) He-Ne laser-induced thrombosis test, in vivo



C

(a) GTT test, in vitro

Variety	Heat treatment	Occlusion time (OT)	Lysis time (LT)
SAKATA-0420	Before	83.2±4.6*	67.6±4.7**
	After	105.4±4.6	71.1±6.7**

vs control (%); *P<0.05; **P<0.01

(b) He-Ne laser-induced thrombosis test, in vivo

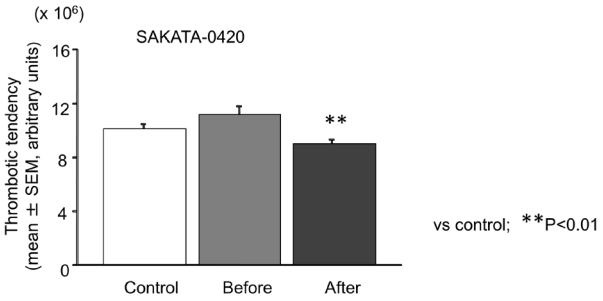


Figure 4. Effect of heat treatment of carrot variety (SAKATA-0421) (A), (SAKATA-0418) (B) and (SAKATA-0420) (C) on platelet reactivity (OT) and endogenous thrombolytic activity (LT) measured *in vitro* by GTT (a) and on thrombosis measured *in vivo* by He-Ne laser-induced thrombosis test (b). (Revised; Yamamoto *et al*: Blood Coagul Fibrinolysis 2008; 19:785-792.)

in vitro test GTT and that antithrombotic and the overall *in vivo* effect on thrombus formation and resolution is governed by the balance between the effect on platelets and endogenous fibrinolytic activities. These findings suggest that serving carrots as raw or heated dishes is beneficial as an antithrombotic diet (*56*).

4.1.6. *Herbs*

Antithrombotic activity and heat stability of twenty-five herb species were measured by the *in vitro* test hemostatometry. Herbs were classified into subgroups on the basis of their antithrombotic activity. Thirteen herbs were antithrombotic, five prothrombotic, six non-thrombotic and one undetermined. As to the mechanism of the heat stable antithrombotic effect of some herbs, we found that the antithrombotic effect was due to inhibition of platelet reactivity. Because at that time the GTT technique was not available, the quantitative effect on endogenous thrombolysis could not be measured. The antithrombotic effect was not related to a protection of endothelial function as measured by FMV (*57*).

4.1.7. *Sesame*

Whole grains of six accessions (varieties) were roasted at 110°C for 10 min and crushed. Diet containing whole grain flour was given to mice for 12 weeks and antithrombotic activity was measured by the laser-induced *in vivo* thrombosis test. Two accessions were antithrombotic and one variety showed prothrombotic effect (*58*).

4.1.8. *Rice*

The antithrombotic activity of five varieties (non-glutinous white rice) was measured. Diet containing non-glutinous white rice was given to mice for 3 months and antithrombotic activity was measured by the laser-induced *in vivo* thrombosis test. Four varieties had no effect on the overall thrombotic status while one variety had a prothrombotic effect (*59*).

4.2. *Antithrombotic fruits*

4.2.1. *Apples (Aomori Prefecture, northern area in Japan)*

Antithrombotic activity of sixteen varieties and heat stability of selected varieties were measured by the *in vitro* test GTT. Subsequently, antithrombotic activity *in vivo* was measured after oral administration to mice by the laser-induced *in vivo* thrombosis test. Sixteen apple varieties were classified into subgroups: antithrombotic, prothrombotic, and varieties having no effect on experimental thrombosis. It was demonstrated that antithrombotic activity in apple varieties was determined by the enhanced endogenous thrombolytic activity and not the effect on platelet reactivity. In apples the endogenous thrombolytic activity was caused by heat stable factors which increased the release of tissue plasminogen activator (t-PA), from endothelial cells and/or blood cells (*60*).

4.2.2. *Mulberries*

According to the GTT test results, eleven varieties were classified into three subgroups: antithrombotic, prothrombotic and without significant effect on experimental thrombosis. Subsequently, antithrombotic or prothrombotic effect was determined after oral administration to mice by the laser-induced *in vivo* thrombosis test. Combination of the effects on platelet reactivity and endogenous thrombolysis, as measured by GTT *in vitro* predicted the overall effects on thrombosis *in vivo* (*61*).

In contrast to vegetable varieties, much attention has to be paid to the area where these fruits were harvested. The antithrombotic activity of some fruits harvested in one area was different from those harvested in a different area (unpublished). Vegetable varieties but not fruit varieties are grown from the respective seeds and this may be the reason for the dependence of the measured antithrombotic effect on the harvest area.

4.2.3. *Grapes*

Antithrombotic activity of forty-six grape varieties (27 red grapes; 19 white grapes) donated from three institutes and heat stability of the selected varieties were measured by the *in vitro* test GTT. Effects of these varieties (raw) on platelet reactivity and endogenous thrombolytic activity are shown in Table 1. Three red varieties (Cabernet Sauvignon, Concord, Berry A) and one white variety (Honey Venus) were classified into an antithrombotic subgroup because of their effect on platelet reactivity and/or endogenous thrombolytic activity. The effect of Cabernet Sauvignon donated from two institutes (Cabernet Sauvignon A, Cabernet Sauvignon B) was different. Cabernet Sauvignon A inhibited platelet reactivity and enhanced endogenous thrombolytic activity, suggesting that Cabernet Sauvignon A could be considered as antithrombotic. Cabernet Sauvignon B enhanced platelet reactivity and inhibited endogenous thrombolytic activity, suggesting that Cabernet Sauvignon B was prothrombotic (Figure 5) (*62*). The results of mulberry and grape varieties showed that classification of fruit varieties according to their effect on experimental thrombosis should be re-defined together and the harvest areas should be considered.

The so-called French Paradox have prompted many epidemiological and laboratory studies on investigating antithrombotic grapes and wines (*63,64*).

Table 1. Effect of grape varieties (raw) on platelet reactivity and endogenous thrombolytic activity

Items	No. of red grape varieties	No. of white grape varieties
Inhibition of platelet reactivity	1	0
No effect	2	0
Enhancement of platelet reactivity	24	18
Not determined	0	0
Inhibition of endogenous thrombolytic activity	9	6
No effect	13	11
Enhancement of endogenous thrombolytic activity	3	1
Not determined	2	1

(Revised; Masahiro Iwasaki: Antithrombotic effect of grapes. Master's thesis, Kobe Gakuin University, 2006) (in Japanese).

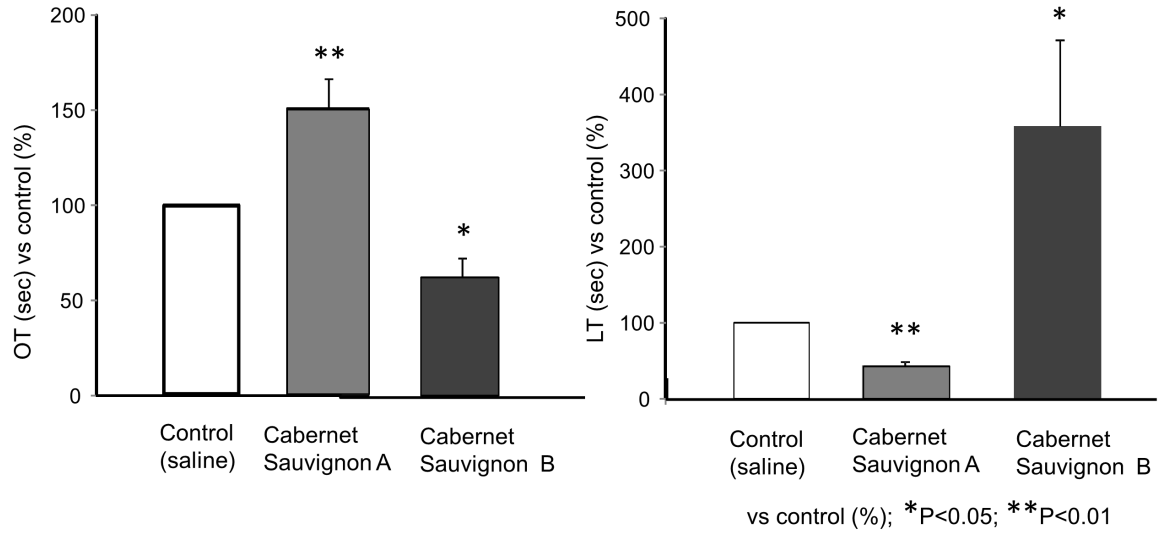


Figure 5. Effect of a red grape variety donated from two institutes (Cabernet Sauvignon A and B) on platelet reactivity (OT) and endogenous thrombolytic activity (LT) *in vitro* measured by GTT. (Revised; M. Iwasaki: Master's thesis of Kobe Gakuin University, 2006.)

Epidemiological studies have provided evidence that intake of fresh fruits and vegetables could help to prevent cardiovascular disease and stroke (*65-69*), while some studies have cast doubt on the red wine hypothesis (*70,71*).

Folts and his co-workers have investigated the mechanism of the French Paradox using the Folts animal model. This is the measurement of cyclic flow reductions (CFRs) in coronary blood flow after mechanical stenosis of the coronary artery and some damage to the vascular wall. The effect of grapes on platelet reactivity was measured by collagen-induced platelet aggregation *ex vivo* test in anticoagulated whole blood. The red wine (1987 Chateauneuf-du-Pape) and Welch's 100% natural purple grape juice inhibited thrombosis *in vivo* but the white wine (1990 Chateau Villotte Bordeaux) did not. The antithrombotic activity of the red wine was demonstrated *in vivo*. Whether the collagen-induced platelet aggregation test was suitable or not to screen and predict antithrombotic effect was not discussed at that time. Epidemiological verification of the antithrombotic effect of red wine consumption stimulated studies aimed to analyze red wine for certain chemical components like quercetin, rutin, resveratrol and antioxidants, which could be responsible for the antithrombotic effect (*72-76*).

Platelets play a pivotal role in arterial thrombotic diseases. Platelet function *in vitro*/*ex vivo* is widely assessed with platelet aggregometry using anticoagulated blood or platelet rich plasma (PRP), which measures platelet aggregation induced by various chemical agonists (*10*). Despite the recognition of the cardinal role of thrombin in thrombogenesis (*28,77-79*), a thrombin-induced platelet aggregation test could not be performed from (citrate) anticoagulated whole blood or PRP. Recently, high shear-induced thrombosis/thrombolysis tests using non-anticoagulated blood have become available for the measurement of thrombotic status or thrombotic and thrombolytic activities *ex vivo* and *in vitro*. In these tests, generation of thrombin from (shear) activated platelets plays the decisive role (*12,20,21,23-28*). Recent animal experiments show that the shear-induced thrombosis/thrombolysis *in vitro*/*ex vivo* tests using non-anticoagulated blood are useful for screening foods, dietary components and nutrients for antithrombotic effect (*22*).

5. Correlation between biologically active components

Table 2. Correlation between polyphenolics content/antioxidant activity and antithrombotic activity

Items	Polyphenolics content		Antioxidant activity		References
	Thrombotic effect	Thrombolytic effect	Thrombotic effect	Thrombolytic effect	
Strawberry	$p < 0.0001$ Antithrombotic$^{\clubsuit\clubsuit}$	ND	$p < 0.05$ Antithrombotic$^{\clubsuit\clubsuit}$	ND	Naemura *et al*. (*53*)
Grape (red)	ns	$p < 0.0001$ Prothrombotic$^{\clubsuit}$	ns	ns	Iwasaki (*62*)
Grape (white)	ns	$p < 0.0001$ Prothrombotic$^{\clubsuit}$	ns	ns	
Mulberry	ns	$p < 0.001$ Antithrombotic$^{\clubsuit\clubsuit}$	ns	$p < 0.001$ Antithrombotic$^{\clubsuit\clubsuit}$	Yamamoto *et al*. (*61*)
Carrot	$p < 0.01$ Antithrombotic$^{\clubsuit\clubsuit}$	ns	ns	ns	Yamamoto *et al*. (*56*)
Apple	ns	ns	ns	ns	Morishita *et al*. (*60*)

Antithrombotic and prothrombotic effects were measured by GTT *in vitro*. Significantly negative correlation between OT and polyphenolics content/ antioxidant activity suggests prothrombotic effect (Prothrombotic$^{\clubsuit}$). Significantly negative correlation between LT and polyphenolics content/ antioxidant activity suggests antithrombotic effect (Antithrombotic$^{\clubsuit\clubsuit}$). Significant positive correlation between OT and polyphenolics content/ antioxidant activity suggests antithrombotic effect (Antithrombotic$^{\clubsuit\clubsuit}$). Significantly positive correlation between LT and polyphenol content/ antioxidant activity suggests prothrombotic effect (Prothrombotic$^{\clubsuit}$). ns: not significant; ND: not determined. Prothrombotic$^{\clubsuit}$ and Antithrombotic$^{\clubsuit\clubsuit}$ activities measured by GTT are not conclusive but highly suggestive. Antithrombotic or prothrombotic activity *in vivo* has to be demonstrated by the laser-induced test *in vivo* (*22*).

of fruits and vegetables and the antithrombotic/ thrombolytic activities

Polyphenolics and antioxidant rich diets have been investigated for prevention of thrombotic diseases (*80-82*). We did not find a correlation between polyphenolics/ antioxidant contents of various fruits and vegetables and their experimental antithrombotic effect (Table 2). Although purified polyphenolics or antioxidants were shown to have antithrombotic activity (*83,84*), our results indicate that polyphenolics and/or antioxidants content of fruits and vegetables are not markers of the antithrombotic effect and cannot be used for screening such an effect

6. Effect of different cultivating fields and harvest times on antithrombotic activity

The antithrombotic strawberry variety, KYSt-4, was planted in the same field and harvested in December, January, February, March and April and antithrombotic activity was measured by GTT. In addition, KYSt-4 was planted in four different fields far from each other at the same time in Gifu Prefecture, Japan and harvested in April. We found that the antithrombotic activity of strawberry varieties grown in different environments (soil, fertilizer, temperature) were similar, thus this effect is probably governed by genes and it is resistant to environmental changes (*85*).

7. Effect of intake of strawberry varieties with and without antithrombotic activity in humans

Whole juice prepared from experimentally antithrombotic strawberry variety (KYSt-4) was given to healthy volunteers and the thrombotic status was measured by GTT two hours after intake. KYSt-4 juice significantly inhibited the shear-induced thrombosis test (GTT) *ex vivo* but whole juice from non-thrombotic variety (KYSt-10; Control 1) and water (Control 2) did not (*85*). This suggests that juices from experimentally antithrombotic fruit and vegetable varieties could prevent arterial thrombosis.

8. Conclusions

Vegetable and fruit varieties were screened for experimental antithrombotic effect by using shear-induced *in vitro* thrombosis tests (hemostatometry; GTT), followed by a laser-induced thrombosis *in vivo* test. The *in vivo* test of FMV was also used to detect any possible effect of the active varieties on endothelial function. Antithrombotic activities of fruits and vegetables were different from variety to variety even in the same species. Measurement of biologically active components in fruits and vegetables, which were suggested earlier to be responsible for the antithrombotic effect did not provide additional benefits in our screening. Further clinical studies are needed to prove the effectiveness of dietary components with experimental antithrombotic effect in humans and that daily intake of an antithrombotic diet is beneficial to prevent thrombotic disorders in humans.

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Original Article

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Complex secondary metabolites from *Ludwigia leptocarpa* with potent antibacterial and antioxidant activities

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Summary **Diarrhea continues to be one of the most common causes of morbidity and mortality among infants and children in developing countries. The aim of the present study was to evaluate the antibacterial and antioxidant activities of extracts and compounds from *Ludwigia leptocarpa*, a plant traditionally used for its vermifugal, anti-dysenteric, and antimicrobial properties. A methanol extract was prepared by maceration of the dried plant and this was successively extracted with ethyl acetate to obtain an EtOAc extract and with n-butanol to obtain an n-BuOH extract. Column chromatography of the EtOAc and n-BuOH extracts was followed by purification of different fractions, leading to the isolation of 10 known compounds. Structures of isolated compounds were assigned on the basis of spectral analysis and by comparison to structures of compounds described in the literature. Antioxidant activity was evaluated using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and gallic acid equivalent antioxidant capacity (GAEAC) assays. Antibacterial activity was assessed with the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) with respect to strains of a Gram-positive bacterium, *Staphylococcus aureus* (a major cause of community and hospital-associated infection), and Gram-negative multi-drug-resistant bacteria, *Vibrio cholerae* (a cause of cholera) and *Shigella flexneri* (a cause of shigellosis). All of the extracts showed different degrees of antioxidant and antibacterial activities. 2β-hydroxyoleanolic acid, (2R,3S,2''S)-3''',4',4''',5,5'',7,7''-heptahydroxy-3,8''-biflavanone, and luteolin-8-C-glucoside displayed the most potent antibacterial and antioxidant properties, and these properties were in some cases equal to or more potent than those of reference drugs. Overall, the present results show that *L. leptocarpa* has the potential to be a natural source of anti-diarrheal and antioxidant products, so further investigation is warranted.**

Keywords: *Ludwigia leptocarpa*, Onagraceae, triterpenoids, flavonoids, antibacterial, antioxidant

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1. Introduction

In developing countries, and particularly in Africa, poor sanitation exposes people to a wider array of microbial pathogens, increasing their susceptibility to bacterial infections (*1*). Each year, 3 million children are reported to die of diarrheal diseases. Cholera is a leading diarrheal disease in terms of its severity and outcomes. Several epidemics of cholera have been

reported in different parts of Cameroon and abroad (*2-5*). *Vibrio cholerae* strains belonging to the O1 and O139 serogroups cause epidemics and pandemics of cholera (*6,7*). Over the past few years, reported cases of cholera have increased steadily, numbering more than 300,000 cases and including more than 7,500 deaths in 2010 (*8*). As populations of poor countries continue to coalesce in mega-cities with low levels of sanitation and people move rapidly around the globe, new and more virulent strains of *V. cholerae* are expected to disseminate more rapidly (*9,10*). This makes cholera one of the most rapidly fatal infectious illnesses known.

The continuous emergence of multi-drug-resistant (MDR) *Vibrio cholerae* strains drastically reduces the efficacy of our antibiotic armory and, consequently, increases the frequency of therapeutic failure (*11,12*). In many regions affected by this pathogen, local and indigenous plants are often the only available means of treating such infections. Among the known plant species on Earth (estimated at 250,000-500,000), only a small fraction have been investigated for the presence of antimicrobial compounds and only 1-10% of plants are used by humans (*13,14*). Natural plant products also act as antioxidants. These include phenolic compounds, alkaloids, terpenoids, and essential oils. Plant-based antioxidant compounds (*15*) play a defensive role by preventing the generation of free radicals and hence are extremely beneficial to alleviating infectious diseases that generate free radicals as well as diseases caused by oxidative stress such as cardiovascular diseases, diabetes, inflammation, degenerative diseases, cancer, anemia, and ischemia (*16*).

Ludwigia leptocarpa (Nutt) Hara (Onagraceae or Oenotheraceae) is a herbaceous plant species that is also readily found in North America and in tropical Africa (*17*). In traditional medicine in Nigeria, an infusion of the plant is part of a mixture used to treat rheumatism (*18*). A leaf infusion has laxative, vermifugal, and anti-dysenteric properties. Previous studies of this genus have revealed the presence of flavonoids (*19,20*), cerebrosides, and triterpenoids (*20,21*). A study recently reported that alcoholic extracts of the leaves of *L. octovalvis*, *L. abyssinica*, and *L. decurrens* potentially have antioxidant, antibacterial, and antifungal activities (*22,23*). To the extent known, no study has reported on the antioxidant and antibacterial properties of *L. leptocarpa* with respect to bacterial strains causing diarrhea. Hence, the aim of this study was to investigate the antibacterial and antioxidant properties of extracts and compounds from *L. leptocarpa*.

2. Materials and Methods

2.1. *Experimental*

IR spectra were recorded with a Shimadzu FT-IR-8400S (Shimadzu, France) spectrophotometer. ^{1}H (500 MHz) and ^{13}C (125 MHz) nuclear magnetic resonance (NMR) spectra were recorded on a BRUKER Avance DRX-500 spectrometer (Bruker, Wissembourg, France) equipped with a BBFO + 5 mm probe. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR spectra were recorded on a BRUKER Avance III-600 spectrometer (Bruker, Wissembourg, France) equipped with a cryoplatform using CD_3OD, with tetramethylsilane (TMS) as the internal standard. Time-of-flight electrospray ionization mass spectrometry (TOF-ESIMS) and high-resolution time of flight electrospray ionization mass spectrometry (HR-TOFESIMS) experiments were performed using a Micromass Q-TOF micro instrument (Manchester, UK) with an electrospray source. The samples were introduced by direct infusion in a solution of methanol (MeOH) at a rate of 5 μL min^{-1}. Column chromatography was performed on Merck silica gel (VWR, France) 60 (70-230 mesh) and gel permeation chromatography was performed on Sephadex LH-20 (VWR, France), while thin layer chromatography (TLC) was carried out on silica gel GF254 pre-coated plates with detection accomplished by spraying with 50% H_2SO_4 followed by heating at 100°C or by visualization with an ultra-violet (UV) lamp at 254 and 365 nm.

2.2. *Plant material*

L. leptocarpa plants were collected in the village of Foto (Menoua Division, Western region of Cameroon), in April 2011. Authentication was performed by Victor Nana, a botanist at the Cameroon National Herbarium, Yaoundé, where a voucher specimen (N° 38782/HNC) was deposited.

2.3. *Extraction and isolation*

Dried *L. leptocarpa* (4 kg) was extracted with MeOH at room temperature for 3 days, and the extract was concentrated to dryness under reduced pressure to yield a dark crude extract (102 g). Part of the residue obtained (97 g) was suspended in water (200 mL) and successively extracted with ethyl acetate (EtOAc) and n-butanol (*n*-BuOH). The result was concentrated to dryness under reduced pressure to respectively yield EtOAc (20 g) and *n*-BuOH (40 g) extracts.

In accordance with antimicrobial and antioxidant assays, the EtOAc and *n*-BuOH extracts were submitted to further separation and purification. Part of the EtOAc extract (15 g) was purified over a silica gel column and eluted with hexane containing increasing concentrations of EtOAc (10%, 20%, 30%, 40%, 50%, 60%, 70%, and 80%). The purified extracted was also eluted with EtOAc containing increasing concentrations of MeOH (10% and 20%). Six fractions were obtained: A, B, C, D, E, and F. Fraction D (1.7 g) was purified over a silica gel column and eluted with a hexane-EtOAc mixture (7:3) to yield compounds **1** and **2** (17 mg and 22 mg,

respectively). Fraction E (3.1 g) was purified over a silica gel column and eluted with a hexane-EtOAc mixture (6:4) to yield compound **3** (17 mg). Part of the *n*-BuOH extract (30 g) was purified over a silica gel column and eluted with EtOAc containing increasing concentrations of MeOH (10%, 20%, 30%, 40%, and 50%). Five fractions (G_1-G_5) were obtained. Fraction G_1 (2.5 g) was purified over a silica gel column and eluted with EtOAc to yield the compounds **4** (19 mg) and **5** (16 mg). Fraction G_2 (3.1 g) was purified over a silica gel column and eluted with an EtOAc-MeOH mixture (8.5:1.5) to yield compounds **5** (25 mg) and **6** (13 mg). Fractions G_3 and G_4 (5.4 g) were combined and purified over a silica gel column; the fractions were then eluted with an EtOAc-MeOH-H2O mixture (8:1:1) to yield the compounds **7** (38 mg) and **8** (24 mg). Fraction G5 (2.5 g) was purified over a silica gel column and eluted with an EtOAc-MeOH-H_2O mixture (7:2:1) to yield the compounds **9** (66 mg) and **10** (40 mg).

Oleanolic acid (**1**): white amorphous powder from hexane-EtOAc; $C_{30}H_{48}O_3$.

2β-hydroxyoleanolic acid (**2**): white amorphous powder from hexane-EtOAc; $C_{30}H_{48}O_4$.

(2*R*,3*S*,2"*S*)-3'",4',4'",5,5",7,7"-heptahydroxy-3,8"-biflavanone (**3**): white amorphous powder from hexane-EtOAc; $C_{30}H_{22}O_{11}$; high resolution electron impact mass spectrometry (HRESIMS, positive-ion mode) m/z: 581.1057 $[M + Na]^+$(calcd. for $C_{30}H_{22}O_{11}Na$:581.1060).

Ellagic acid (**4**): yellow powder from EtOAc; $C_{14}H_6O_8$.

β-sitosterol-3-*O*-*β*-D-glucopyranoside (**5**): white amorphous powder from EtOAc$C_{35}H_{60}O_6$.

Luteolin-8-C-glucoside (**6**): yellow amorphous powder from EtOAc$C_{21}H_{20}O_{11}$.

28-*O*-*β*-D-xylopyranosyl-(1→4)-*α*-L-rhamnopyranosyl-(1→2)-[*α*-L-arabinopyranosyl-(1→3)]-4-*O*-(3'-hydroxybutanoyloxy-3-hydroxybutanoyloxy)-*β*-D-fucopyranosyl zanhic acid (**7**): white amorphous solid from EtOAc; $C_{60}H_{94}O_{27}$; HRESIMS (positive-ion mode) m/z: 1269.5870 $[M + Na]^+$(calcd. for $C_{60}H_{94}O_{27}Na$: 1269.5880).

3-*O*-*β*-D-glucopyranosyl-28-*O*-*β*-D-xylopyranosyl-(1→4)-*α*-L-rhamnopyranosyl-(1→2)-4-*O*-(3'-hydroxybutanoyloxy-3-hydroxybutanoyloxy)-*β*-D-fucopyranosyl medicagenic acid (**8**): white amorphous solid from EtOAc; $C_{61}H_{96}O_{27}$; HRESIMS (positive-ion mode) m/z: 1283.6044 $[M + Na]^+$ (calcd. for $C_{61}H_{96}O_{27}Na$: 1283.6037).

3-*O*-*β*-D-glucopyranosyl-28-*O*-*β*-D-xylopyranosyl-(1→4)-*α*-L-rhamnopyranosyl(1→2)-[*α*-L-arabinopyranosyl-(→3)]-4-*O*-(3'-hydroxybutanoyloxy-3-hydroxybutanoyloxy)-*β*-D-fucopyranosyl zanhic acid (**9**): white amorphous solid from EtOAc; $C_{66}H_{104}O_{32}$; HRESIMS (positive-ion mode) m/z: 1431.6395$[M+Na]^+$(calcd. for $C_{66}H_{104}O_{32}Na$: 1431.6408).

3-*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranosyl-28-*O*-*β*-D-xylopyranosyl-(1→4)-*α*-L-rhamnopyranosyl-(1→2)-[*α*-L- arabinopyranosyl-(1→3)]-4-*O*-(3'-hydroxybutanoyloxy-3-hydroxybutanoyloxy)-*β*-D-fucopyranosyl zanhic acid (**10**): white amorphous solid from EtOAc; $C_{72}H_{114}O_{37}$; HRESIMS (positive-ion mode) m/z: 1593.6927[M + Na]$^+$(calcd. for $C_{72}H_{114}O_{37}Na$: 1593.6937).

2.4. *Antibacterial assay*

2.4.1. *Microorganisms*

A total of six bacterial strains were tested for their susceptibility to compounds and these strains were from our laboratory collection (kindly provided by Dr. T. Ramamurthy, NICED, Kolkata). Among the clinical strains of *Vibrio cholerae* used in this study, strain NB2 belongs to the O1 serotype and strain SG24(1) belongs to the O139 serotype. These strains are able to produce cholera toxin and hemolysin (*24,25*). The other strains used in this study were non-O1 and non-O139 strains of *V. cholerae* (strains CO6 and PC2) (*24*) and strains of *Shigella flexneri* (*26*). The non-O1 and non-O139 strains of *V. cholerae* were positive for hemolysin production but negative for cholera toxin production (*24*). An American Type Culture Collection (ATCC) strain of *Staphylococcus aureus*, ATCC 25923, was used for quality control. The bacterial strains were maintained on an agar slant at 4°C and subcultured on appropriate fresh agar plates 24 h prior to any antibacterial testing. Mueller Hinton Agar (MHA) was used to activate bacteria. Mueller Hinton Broth (MHB) was used to determine minimum inhibitory concentrations (MICs) and nutrient agar (Hi-Media) was used to determine minimum bactericidal concentrations (MBCs).

2.4.2. *Determination of MICs and MBCs*

MICs and MBCs of extracts/compounds were assessed using the broth microdilution method recommended by the National Committee for Clinical Laboratory Standards (*27,28*) with slight modifications. Each test sample was dissolved in dimethylsulfoxide (DMSO, Fisher chemicals) to yield a stock solution. Ninety-six-well round-bottom sterile plates were prepared by dispensing 180 µL of the inoculated broth (1×10^6 CFU/mL) into each well. A 20 µL aliquot of a compound was added. The concentration of the tested samples varied from 0.125 to 1,024 µg/mL. The final concentration of DMSO in each well was <1% [preliminary analyses with 1% (v/v) DMSO did not inhibit the growth of the test organisms]. Dilutions of ampicillin (Sigma-Aldrich, Steinheim, Germany) and tetracycline (Sigma-Aldrich, Steinheim, Germany) served as positive controls, while broth with 20 µL of DMSO was used as a negative control. Plates were covered and incubated

for 24 h at 37°C. After incubation, MICs were read visually; bacteria were plated on nutrient agar (Conda, Madrid, Spain) and incubated at 37°C for 24 h. The lowest concentrations that yielded no growth after this subculturing served as the MBC.

2.5. *Antioxidant assay*

2.5.1. *DPPH free radical scavenging assay*

The free radical scavenging activity of extracts as well as their isolated compounds was assessed in accordance with the methods of Brand-Williams *et al*. (*29*) with slight modifications. Briefly, different concentrations (10 to 2,000 µg/mL) of extracts or compounds and vitamin C (Sigma-Aldrich, Steinheim, Germany) were thoroughly mixed with 3 mL of a methanolic DPPH solution (20 mg/L) in test tubes and the resulting solution was allowed to stand for 30 minutes at room temperature before the optical density (OD) was measured at 517 nm. The measurement was repeated 3 times and an average of those readings was determined. The percentage radical scavenging activity was calculated using the following formula: % scavenging [DPPH] = $[(A_0 - A_1)/A_0] \times 100$. Here, A_0 is the absorbance of the control and A_1 is the absorbance in the presence of the sample. The IC_{50} was determined from a graph obtained using standard vitamin C by using the formula "y = mx + c" for the slope of the graph.

2.5.2. *Gallic acid equivalent antioxidant capacity (GAEAC) assay*

A GAEAC assay was performed as previously described (*30*) with slight modifications. In a quartz cuvette, 20 µL of laccase (1 mM stock solution), 20 µL of a test sample, and 10 µL of ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (74 mM of stock solution) were added to 950 µL of an acetate buffer (pH = 5.0, 100 mM). The laccase was purified from *Sclerotinia sclerotiorum* according to a previously described protocol (*31*). The sample concentrations in the assay mixture were 800, 400, 200, 100, and 10 µg/mL for the extracts and 200, 100, 50, 25, and 125.5 µg/mL for the isolated compounds. The content of the generated $ABTS^{\bullet+}$ radical was measured at 420 nm after reaction for 240 s and this measurement was converted to the gallic acid equivalent using a calibration curve (Pearson's correlation coefficient: $r = 0.996$) created with 0, 4, 10, 14, 28, 56, and 84 µM of gallic acid rather than Trolox. Experiments were done in triplicate.

2.6. *Statistical analysis*

Data were analyzed using one-way analysis of variance followed by the Waller-Duncan post-hoc test. Results are expressed as the mean ± standard deviation (SD). $p < 0.05$ was considered to indicate a significant difference. All analyses were performed using the software Statistical Package for Social Sciences (SPSS, version 12.0).

3. Results and Discussion

3.1. *Chemical analysis*

In accordance with antibacterial assays of the MeOH, EtOAc, and *n*-BuOH extracts, the EtOAc and *n*-BuOH extracts were further separated and purified. This led to the isolation of 10 compounds. Structures (Figure 1) of these compounds have been assigned on the basis of spectroscopic data (^{1}H and ^{13}C NMR, ^{1}H-^{1}H COSY, HSQC, HMBC, ROESY, and NOESY), mass spectra, and by comparison of those compounds to compounds described in the literature. Hence, the isolated compounds were identified as oleanolic acid (**1**) (*32*); 2β-hydroxyoleanolic acid (**2**) (*32*); (2*R*,3*S*,2"*S*)-3"',4',4"',5,5",7,7"-heptahydroxy-3,8"-biflavanone (**3**) (*33*); ellagic acid (**4**) (*34*); 3-*O*-*β*-D-glucopyranosyl-*β*-sitosterol (**5**) (*35*); luteolin-8-*C*-glucoside (**6**) (*36*); 28-*O*-*β*-D-xylopyranosyl-(1→4)-*α*-L-rhamnopyranosyl-(1→2)-[*α*-L-arabinopyranosyl-(1→3)]-4-*O*-(3'-hydroxybutanoyloxy-3-hydroxybutanoyloxy)-*β*-D-fucopyranosyl zanhic acid (**7**) (*21*); 3-*O*-*β*-D-glucopyranosyl-28-*O*-*β*-D-xylopyranosyl-(1→4)-*α*-L-rhamnopyranosyl-(1→2)-4-*O*-(3'-hydroxybutanoyloxy-3-hydroxybutanoyloxy)-*β*-D-fucopyranosyl medicagenic acid (**8**) (*21*); 3-*O*-*β*-D-glucopyranosyl-28-*O*-*β*-D-xylopyranosyl-(1→4)-*α*-L-rhamnopyranosyl(1→2)-[α-L-arabinopyranosyl-(→3)]-4-*O*-(3'-hydroxybutanoyloxy-3-hydroxybutanoyloxy)-*β*-D-fucopyranosyl zanhic acid (**9**) (*20*); and 3-*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranosyl-28-*O*-*β*-D-xylopyranosyl-(1→4)-*α*-L-rhamnopyranosyl-(1→2)-[*α*-L-arabinopyranosyl-(1→3)]-4-*O*-(3'-hydroxybutanoyloxy-3-hydroxybutanoyloxy)-*β*-D-fucopyranosyl zanhic acid (**10**) (*21*).

3.2. *Antibacterial activity*

The susceptibility pattern and inhibition parameters of the tested organisms to the extracts and isolated compounds are indicated below (Table 1). Wells containing a concentration of 64-512 µg/mL of MeOH, EtOAc, and *n*-BuOH extracts inhibited the visible growth of all bacterial species. The most sensitive bacterial species were *S. aureus* and *S. flexneri*, while *V. cholerae* SG24(1) and *V. cholerae* NB2 were the species that were most resistant to the tested samples. All 3 plant extracts displayed less antibacterial activity than tetracycline. However, these extracts were active against *V. cholerae* NB2, *V. cholerae* PC2, and *S. flexneri* which were not sensitive to ampicillin. The antimicrobial activity of a plant extract was considered to be good if its MIC was less than 100.0 µg/mL,

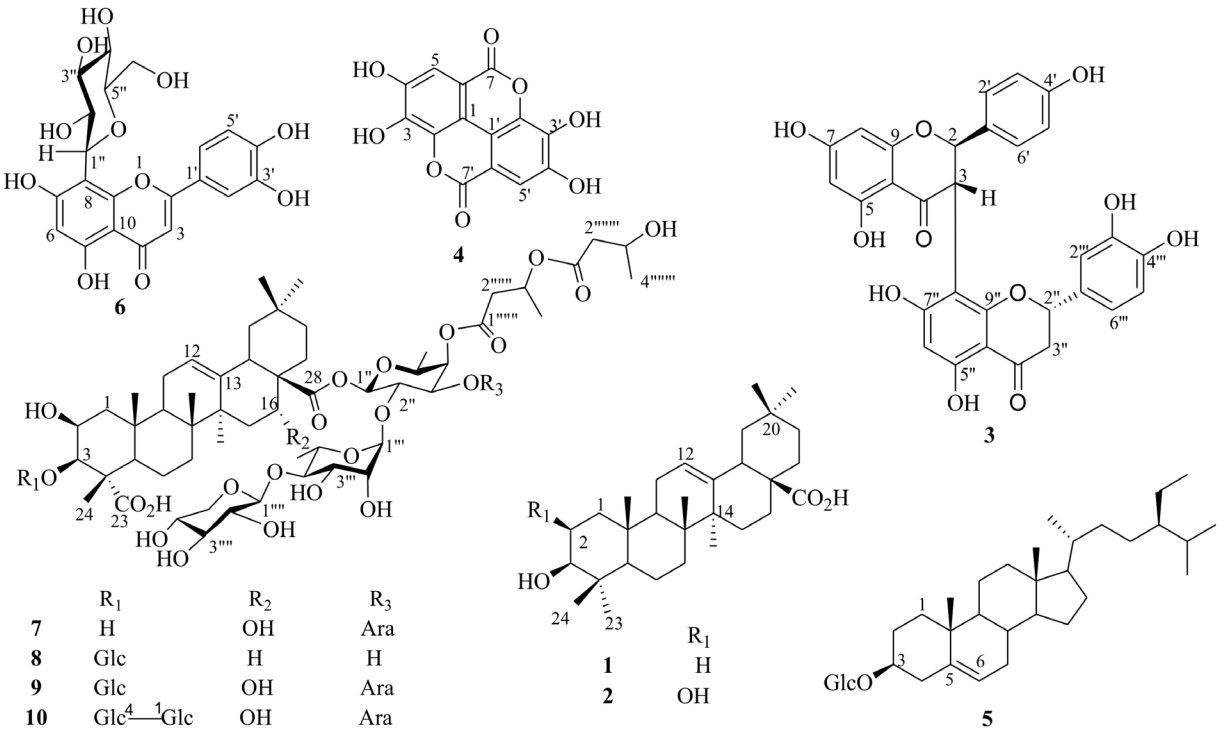

Figure 1. Structures of compounds isolated from the plant *L. leptocarpa*.

moderate if its MIC was from 100.0 to 500.0 µg/mL and poor if its MIC was over 500.0 µg/mL (*37*). Hence, the MeOH, EtOAc, and *n*-BuOH extracts of *L. leptocarpa* exhibited good activity against *S. aureus*, with an MIC of 64 µg/mL, whereas only the MeOH extract displayed poor activity against *V. cholerae* SG24(1). The present results for extracts of *L. leptocarpa* indicated that this plant species is a potential source of antibacterial agents. This *in vitro* study corroborated a previous study that found that alcoholic extracts of *L. octovalvis*, *L. abyssinica*, and *L. decurrens* leaves inhibited *Staphylococcus aureus* (*22,23,38*).

Compound **3** had the lowest MICs and MBCs, 2 µg/mL, for *S. aureus*; this compound has promise as an antibacterial since it was more potent at inhibiting S. *aureus* than the reference antibacterials ampicillin (MIC of 16 µg/mL and MBC of 16 µg/mL) and tetracycline (MIC of 16 µg/mL and MBC of 128 µg/mL) were. However, a MeOH extract had the highest MIC, 512 µg/mL, for *V. cholerae* SG24(1) while a MeOH extract had the highest MBC, 512 µg/mL, for *V. cholerae* SG24(1), *V. cholerae* CO6, and *V. cholerae* PC2. A lower MBC or MIC (≤ 4) means that a minimum amount of the plant extract or isolated compound was needed to kill the bacterial species while a higher value means that a comparatively higher concentration of the extract or compound was needed to control of the microorganism (*39*).

Ranked in order of antibacterial activity, compound **3** isolated from *L. leptocarpa* had the most potent antibacterial activity, followed by compound **6**, compound **2**, compound **4**, compounds **8** and **9**, compound **10**, compound **7**, and then compound **1**. Compounds **3**, **6**, **2**, **4**, **8**, **9**, and **10** were active against all of the tested pathogens whereas compound **1** was active only against *S. flexneri* and *S. aureus*. No activity was noted for compound **5** (results not shown). Antimicrobial cut-off points have been defined by several authors to enable an understanding of the antimicrobial potential of pure compounds. Activity of a compound is classified as: significant activity (MIC < 10 µg/mL), moderate activity (10 < MIC ≤ 100 µg/mL), and low activity (MIC> 100 µg/mL) (*40,41*). Accordingly, compound **3** had significant antibacterial activity against *V. cholerae* CO6, *V. cholerae* NB2, *V. cholerae* PC2, *S. flexneri*, and *S. aureus* while compound **6** had significant antibacterial activity against *Shigella flexneri* SDINT and *Staphylococcus aureus* ATCC 25923. The strains of *V. cholerae* NB2, PC2 (*24,25*) and *Shigella flexneri* (*26*) included in the present study were MDR clinical isolates and these were resistant to commonly used drugs such as ampicillin, streptomycin, tetracycline, nalidixic acid, furazolidone, and *co*-trimoxazole. However, most of the tested samples displayed antibacterial activity against these microbial strains, suggesting that their administration may represent an alternative treatment for *V. cholerae*, the cause of the dreadful disease cholera, and *S. flexneri*, the cause of shigellosis. Given the medical importance of the tested bacteria, the present results offer promise in terms of developing new antibacterials. The antibacterial activity of oleanolic acid, ellagic acid, and 2β-hydroxyoleanolic acid coincide with

Table 1. Antibacterial activity (MIC and MBC in µg/ml) of extracts, isolated compounds, and reference antibacterials

Extracts/ compounds	Inhibition parameters	*Vibrio cholerae* SG24(1)	*Vibrio cholerae* CO6	*Vibrio cholerae* NB2	*Vibrio cholerae* PC2	*Shigella flexneri* SDINT	*Staphylococcus aureus ATCC 25923*
MeOH extract	MIC	512	256	256	256	128	64
	MBC	512	512	256	512	128	128
	MBC/MIC	1	2	1	2	1	2
	MIC	128	256	128	128	128	64
EtOAc extract	MBC	256	256	>512	256	128	128
	MBC/MIC	2	1	/	2	1	2
	MIC	256	256	128	256	128	64
	MBC	256	>512	256	256	128	128
n-BuOH extract	MBC/MIC	1	/	2	1	1	2
	MIC	> 256	>256	>256	>256	256	256
	MBC	/	/	/	/	>256	>256
1	MBC/MIC	/	/	/	/	/	/
	MIC	128	64	64	64	16	16
	MBC	128	64	64	128	32	16
2	MBC/MIC	1	1	1	2	2	1
	MIC	16	8	8	8	4	2
	MBC	16	8	8	8	4	2
3	MBC/MIC	1	1	1	1	1	1
	MIC	128	64	64	128	64	32
	MBC	> 256	128	64	256	64	32
4	MBC/MIC	/	2	1	2	1	1
	MIC	16	32	32	16	4	4
	MBC	32	32	32	16	8	8
6	MBC/MIC	2	1	1	1	2	2
	MIC	> 256	256	256	256	128	128
	MBC	/	>256	>256	>256	>256	128
7	MBC/MIC	/	/	/	/	/	1
	MIC	128	256	128	128	128	64
	MBC	> 512	256	256	256	128	64
8	MBC/MIC	/	1	2	2	1	1
	MIC	256	128	128	128	64	64
	MBC	> 256	256	128	256	128	64
9	MBC/MIC	/	2	1	2	2	1
	MIC	256	256	256	256	128	128
	MBC	> 256	>256	>256	>256	>256	128
10	MBC/MIC	/	/	/	/	/	1
	MIC	16	16	>512	>512	>512	4
	MBC	16	16	>512	>512	>512	4
Ampicillin	MBC/MIC	1	1	/	/	/	1
	MIC	0.5	2	0.5	0.5	16	2
	MBC	4	16	4	4	128	8
Tetracycline	MBC/MIC	8	8	8	8	8	4

/: not determined; MIC: Minimum Inhibitory Concentration; MBC Minimum Bactericidal Concentration.

previous findings (*42,43*). All of the compounds that were found to be active in the present study are members of the triterpenoid, flavonoid, and phenolic acid groups. Although triterpenoid, flavonoid, and phenolic acid compounds have been reported to possess antibacterial activity (*39,44*), no study has reported the activity of compounds **3** and **6-10** on the types of MDR pathogenic bacterial strains used in the present study.

The mechanism of action of terpenoids (**1**, **2**, **5**, and **7-10**) is not fully understood, but it may involve membrane disruption by lipophilic compounds (*45*). Inhibition of the tested bacterial strains by phenolic acid (**4**) may be due to iron deprivation or hydrogen bounding with vital proteins such as microbial enzymes (*46*). The mechanism of action of flavonoids (3 and **6**) is still to be studied; nevertheless, their activity is probably due to their ability to form complexes with extracellular and soluble proteins and to form complexes with bacterial cell wall components. Moreover, lipophilic flavonoids may also disrupt microbial membranes (*47*).

3.3. *Antioxidant activity*

The MeOH, EtOAc, and *n*-BuOH extracts and their isolated compounds were evaluated for their antioxidant activity using two *in vitro* models. The results were expressed as the gallic acid equivalent antioxidant capacity of tested samples (Figure 2) and as equivalent concentrations of test samples scavenging 50% of the DPPH radical (Figure 3). $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radical scavenging activity were observed in all of the extracts. The MeOH and EtOAc extracts showed the most potent

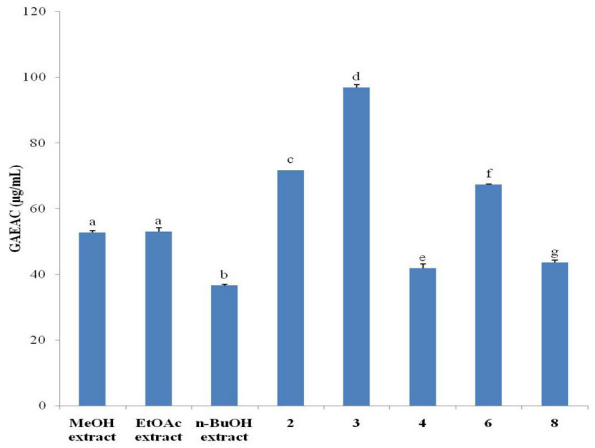

Figure 2. Gallic acid equivalent antioxidant capacity (GAEAC; µg/mL) of tested samples. Bars represent the mean ± S.D. of three independent experiments carried out in triplicate. Letters a-e indicate significant differences between samples according to one-way ANOVA and the Waller Duncan test; $p < 0.05$. Compounds **1**, **5**, **7**, **9**, and **10** were not active (results not shown).

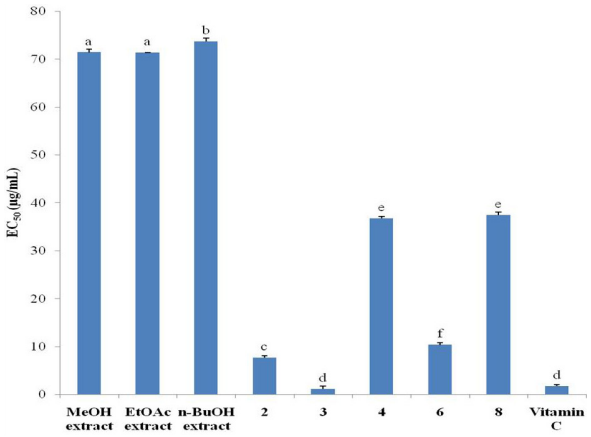

Figure 3. Equivalent concentrations of test samples scavenging 50% of the DPPH radical (EC_{50}). Bars represent the mean ± S.D. of three independent experiments carried out in triplicate. Letters a-f indicate significant differences between samples according to one-way ANOVA and the Waller Duncan test; $p < 0.05$. Compounds **1**, **5**, **7**, **9**, and **10** were not active (results not shown).

activity, followed by the *n*-BuOH extract (Figures 2 and 3). These results indicate the potential of the tested extracts to serve as a natural source of antioxidants with the potential to reduce oxidative stress and provide subsequent health benefits. The antioxidant capacity of the tested extracts may be due to the hydrogen-donating ability of phenols and flavonoids present in those extracts. Similarly, previous studies have reported that phenolic compounds contribute significantly to the antioxidant activity of medicinal plants (*39,48*). The compounds that had the most potent $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radical scavenging activity were compounds **2** (EC_{50} = 7.66 µg/mL; GAEAC= 71.64 µg/mL), **3** (EC_{50} = 1.09 µg/mL; GAEAC= 96.88 µg/mL), and **6** (EC_{50} = 10.34 µg/mL; GAEAC= 67.35 µg/mL), while the other compounds (compounds **4** and **8**) had moderate antioxidant properties. Compounds **1**, **5**, **7**, **9**, and **10** were found to be inactive in both models. Compound **3** was the most potent antioxidant compound and its $DPPH^{\bullet}$ radical scavenging activity was equal to that of vitamin C, which was used in the present study as reference antioxidant. This finding suggests that compound **3** is the best candidate to combat diseases associated with oxidative stress. This is very promising in terms of discovering antioxidants from plants. The antioxidant activity of compounds **2** and **4** agreed with previously reported findings (*42,49*). However, the present study is the first to document the antioxidant activity of the MeOH, EtOAc and *n*-BuOH extracts of *L. leptocarpa* as well as that of compounds **3**, **6**, and **8**.

4. Conclusion

Results indicated that MeOH and EtOAc extracts of *L. leptocarpa* as well as compounds **2**, **3**, and **6** possess the most potent antibacterial and antioxidant properties among the tested extracts and compounds. *L. leptocarpa* has the potential to be a natural source of products with health benefits, so it warrants further investigation.

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Original Article

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Development of chrysin loaded poloxamer micelles and toxicity evaluation in fish embryos

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Summary **Poloxamer micelles promise safety and efficacy for many water insoluble drugs. Chrysin has been reported to have anticancer, anti-inflammatory, antioxidant, and anti-aromatase activities but its water insoluble properties limit its pharmaceutical application. In the present study, chrysin loaded poloxamer micelles were developed. Two types of poloxamers, Pluronic F-68 and Pluronic F-127 were compared. It was found that chrysin loaded Pluronic F-68 micelles (CS-P68) and chrysin loaded Pluronic F-127 micelles (CS-P127) obviously increase the aqueous solubility of chrysin. The results also indicated that the type of polymer and ratio of drug to polymer affected size and desirable characteristics of the micelles. The micelle system of CS-P68 and CS-P127 formed at drug to polymer ratios of 1:4 and 1:2, respectively, was found to be the most suitable monodispersed system with a nanosize-range diameter. The *in vivo* study in zebrafish eggs indicates that the toxicity of CS-P68 and CS-P127 is a dose response. CS-P68 and CS-P127 at a drug dose of 10 ng/mL or less is safe for zebrafish embryo growth. The results of this study indicate enhanced water solubility of chrysin. Chrysin loaded poloxamer micelles are promising for further use in *in vivo* studies in mammalian animals and humans.**

Keywords: Chrysin, poloxamer, pluronic, polymeric micelles, solubility

1. Introduction

Chrysin, a natural flavonoid compound with the IUPAC name 5,7-dihydroxy-2- phenyl-4H-chromen-4-one, can be extracted from plants, honey, and propolis (*1*). It has been shown that chrysin is abundant in the fruit of *Oroxylum indicum* (*2*). The fruit of this plant is commonly used in Thailand and other East Asian countries as food and herbal medicine. Chrysin possesses several biological activities including, anti-cancer (*3,4*), anti-inflammation (*5,6*), and antioxidant properties (*7,8*) and is reported to cause an increase of testosterone production via suppression of aromatase, an enzyme that converts androgen to estrogen (*9*). However, biological actions are only facilitated given the stable structure and low molecular weights of the active compounds that can be soluble and pass through cell membranes (*10*). Unfortunately, chrysin has a major problem of solubility. Its insoluble aqueous property causes low absorption and low bioavailability. Therefore, it is essential to improve the solubility of chrysin in order to increase its pharmaceutical and medical applications.

Various techniques can be applied for enhancement of the solubility and dissolution rate of poorly water soluble drugs such as solubilization by cosolvents (*11*), salt formation (*12*), inclusion complex in cyclodextrins (*13*), solid dispersions (*14-16*), and micellization (*17,18*). For chrysin, enhancement of its water solubility by cosolvents has been reported (*19*). However, the reported cosolvents used were dimethylformamide and tetrahydrofuran which are harmful organic solvents. Therefore, a search for better techniques particularly with nontoxic carriers is still challenge.

Among several solubilizing techniques, micellization using polymeric micelles seems to be most promising for solubility enhancement of drugs, because it

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overcomes the limitations of the other techniques (*20,21*). Poloxamer is one of the most common polymers used to fabricate polymeric micelles (*22,23*). Poloxamer is an amphiphilic block copolymer. The non-covalent incorporation of many drugs into the hydrophobic core of poloxamer micelles resulted in an increase of solubility, stability, and bioavailability of the drugs. Therefore, this biocompatible polymer has been used for the encapsulation of various water insoluble drugs into nanoparticles in the form of polymeric micelles (*24*). Moreover, poloxamer is reported to be biocompatible, with low toxicity, and low degradation (*25,26*). In addition, poloxamer can minimize adsorption to surfaces due to hydrophilicity (*27*).

The aim of the present study is to develop chrysin loaded poloxamer micelles in order to enhance the water solubility of chrysin. The effect of polymer and solvent on the characteristics of the polymeric micelles obtained was investigated. Moreover, *in vivo* toxicity of the selected system of chrysin loading micelles on fish embryos was evaluated.

2. Materials and Methods

2.1. *Materials*

Chrysin was purchased from Sigma-Aldrich Co. (St. Louis, USA). Poloxamers (Pluronic F-68 and Pluronic F-127) were purchased from O-BASF Co. (Ludwigshafen, Germany). Tween 80 was obtained from Namsian Co. Ltd. (Bangkok, Thailand). Acetone was from RCI Labscan (Bangkok, Thailand). Ethanol was from Scharlau (Barcelona, Spain). All solvents were of analytical grade.

2.2. *Polymeric micelle preparation*

Two types of poloxamers; Pluronic F-68 and Pluronic F-127 were used in this study. Chrysin loaded polymeric micelles were prepared by dissolving chrysin separately in two different organic solvents; acetone or ethanol. The drug solution was added dropwise with Pluronic F-68 or Pluronic F-127 solution to obtain the mixture of chrysin-polymer at the weight ratios of 1:1, 1:2, 1:3, 1:4, 1:5, 1:10, and 1:15. After that, Tween 80 was added. Deionized water was added to volume and the mixture was frozen at -20°C and subsequently lyophilized under vacuum for 24 h. After lyophilization, the obtained dry chrysin loaded Pluronic F-68 and Pluronic F-127 micelles namely CS-P68 and CS-P127, respectively, were re-suspended in deionized water to the desired drug concentration for further studies.

2.3. *Determination of size, size distribution, and zeta potential of the micelles*

Size and size distribution (PDI) of CS-P68 and CS-P127 was measured using Zetasizer NanoZS (Malvern Instruments, UK) working on the principle of photon correlation spectroscopy (PCS). A portion of 1 mL of the micelle dispersion in water was transferred into a quartz cuvette and exposed to laser light diffraction at an angle of 173°. The intensity of the peak that showed the highest population of the micelles of that size was recorded. Zeta potential of the micelles was measured using the same instrument and determined five times for each sample. Results were automatically calculated by the analyzer.

2.4. *In vivo toxicity study*

The *in vivo* toxicity experiment was done using a method described previously (*28*) with some modification. Briefly, 4-h of age fresh zebrafish eggs were gently filled into a series of 10 mL aqueous clear mixtures containing CS-P68 or CS-P127 with chrysin concentrations of 1, 10, 100, 1,000 and 10,000 ng/mL. The incubation temperature was 28°C. The number of zebrafish eggs was 15 for each system. Water without any polymeric micelles was used as a control. The mortality of the embryos in each system was observed every 24 h for a period of 72 h under a stereo microscope (Nikon, Tokyo, Japan).

2.5. *Statistical analysis*

The preparation, size measurement, and toxicity study were done in triplicate and the results are expressed as mean ± S.D. Statistical analysis was done by using ANOVA and *P*-value at a level of 95% confidence limit.

3. Results and Discussion

3.1. *Preparation of chrysin loaded micelles*

In the process of chrysin loaded micelles, Tween 80 has been added for incorporation into the micelles. Tween 80 is a hydrophilic non-ionic surfactant widely used in emulsification and solubilizing of substances in medicinal, pharmaceutical, and food products. Moreover, it is used in conjunction with nanoparticles to improve specific delivery (*29*). Tween 80 is reported to be adsorbed on the surface by interacting with specific receptors on the blood brain barrier luminal face, and then transported into the brain (*30*). Therefore, incorporation of Tween 80 into the polymeric micelles of poloxamer in the present study was to obtain the most desirable carrier for the chrysin solubilization and delivery system. It was found that chrysin could be loaded into both Pluronic F-68 and Pluronic F-127. The systems obtained after preparation were transparent aqueous dispersions. After lyophilization, the products obtained were still transparent but the state of matter was changed to a semisolid form as a gel-

like product. After diluting with water, the semisolid products changed to transparent aqueous systems without any precipitation of chrysin. It was considered that all chrysin could be dissolved in the water. The result was in agreement with the previous results that the practically insoluble curcumin and xanthone could be solubilized by polymeric micelles and transparent aqueous mixtures obtained (*20,31*).

3.2. *Effects of polymer and solvent types on size and size distribution of the micelles*

Two types of solvents, ethanol and acetone, were compared in the preparation of the micelles of two types of poloxamer. It was found that using ethanol as a solvent for chrysin in the preparation of drug loaded micelles yielded micelles with different sizes depending on the polymer type and drug to polymer ratio. The size and PDI as well as % intensity of CS-P68 and CS-P127 are shown in Table 1 and Table 2, respectively. It was found that the size of drug entrapped micelles was slightly larger than that of empty micelles for both polymers. The size of CS-P68 was in the range of 12.6-17.8 nm whereas that of CS-P127 was in the range of 11.2-14.1 nm. The PDI was in the range of 0.1-0.2 for CS-P68 and 0.1-0.3 for CS-P127 indicating a good size distribution for both polymers. According to the peak intensity, the mixture at a weight ratio of 1:4 was considered to be the best formulation for CS-P68 whereas that of 1:2 was considered to be the best formulation for CS-P127 because it showed a peak intensity of 100% as shown in Figure 1.

Using acetone instead of ethanol as a solvent for chrysin to formulate chrysin loaded polymeric micelles of both polymers CP-68 and CP-127 could also be obtained. Similarly to those using ethanol as a solvent, it was found that the size of chrysin loaded micelles was slightly larger than that of empty micelles. As shown in Table 3 and Table 4 for CS-P68 and CS-P127, respectively, it was found that the size of CS-P68 and CS-P127 was in the range of 10.5-16.8 nm and 10.1-14.5 nm, respectively. It was observed that the size of chrysin loaded micelles prepared using acetone as a solvent for preparation of drug solution was slightly smaller but not significantly different than those using ethanol as a solvent. The PDI of the micelles of both polymers was in the same range as those prepared by using ethanol as a solvent. According to peak intensity,

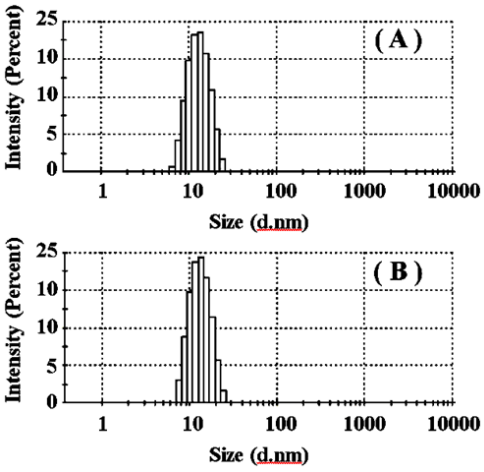

Figure 1. PCS analysis of CS-P68 (A) and CS-P127 (B) at drug to polymer ratios of 1:4 and 1:2, respectively obtained from the use of ethanol as a solvent.

Table 1. Characteristics of CS-P68 obtained from the use of ethanol as a solvent

Ratio of chrysin to polymer	Size (nm)	PDI	Intensity (%)
0:1	10.4 ± 3.4	0.132	100.0
1:1	17.8 ± 8.3	0.204	98.5
1:2	12.6 ± 4.2	0.156	98.6
1:3	12.5 ± 4.1	0.147	98.6
1:4	12.7 ± 4.1	0.139	100.0
1:5	13.5 ± 4.7	0.192	96.3
1:10	14.3 ± 5.6	0.197	97.0
1:15	15.1 ± 5.2	0.263	95.3

Table 2. Characteristics of CS-P127 obtained from the use of ethanol as a solvent

Ratio of chrysin to polymer	Size (nm)	PDI	Intensity (%)
0:1	9.1 ± 2.2	0.166	100.0
1:1	13.8 ± 5.2	0.179	96.8
1:2	11.7 ± 3.2	0.054	100.0
1:3	11.2 ± 2.7	0.195	97.6
1:4	13.3 ± 3.9	0.278	92.1
1:5	13.7 ± 2.9	0.247	79.7
1:10	14.1 ± 3.6	0.315	86.2
1:15	ND	ND	ND

ND = not detectable.

Table 3. Characteristics of CS-P68 obtained from the use of acetone as a solvent

Ratio of chrysin to polymer	Size (nm)	PDI	Intensity (%)
0:1	10.2 ± 2.9	0.076	100.0
1:1	11.4 ± 3.5	0.112	97.9
1:2	10.7 ± 3.6	0.181	97.6
1:3	10.5 ± 4.3	0.126	100.0
1:4	12.1 ± 4.3	0.259	93.2
1:5	10.8 ± 3.3	0.215	96.8
1:10	12.4 ± 5.2	0.193	97.3
1:15	16.8 ± 6.7	0.217	96.3

Table 4. Characteristics of CS-P127 obtained from the use of acetone as a solvent

Ratio of chrysin to polymer	Size (nm)	PDI	Intensity (%)
0:1	9.1 ± 2.3	0.081	100.0
1:1	10.9 ± 3.7	0.138	98.8
1:2	11.8 ± 3.9	0.288	88.6
1:3	10.6 ± 3.4	0.099	100.0
1:4	10.1 ± 2.5	0.331	95.7
1:5	14.5 ± 7.5	0.193	98.7
1:10	11.8 ± 4.3	0.145	100.0
1:15	11.1 ± 3.1	0.066	100.0

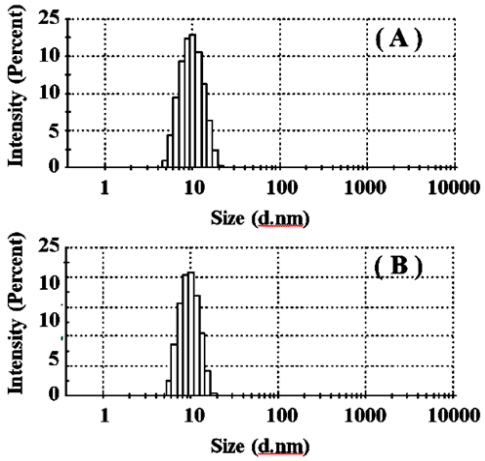

Figure 2. PCS analysis of CS-P68 (A) and CS-P127 (B) at drug to polymer ratio of 1:3 obtained from the use of acetone as a solvent.

the mixture at a weight ratio of 1:3 was considered to be the best formulation for CS-P68 because this system showed a peak intensity of 100% and showed a single size distribution peak as shown in Figure 2A. However, three systems of CS-P127 with drug to polymer ratios of 1:3, 1:10, and 1:15 showed a peak intensity of 100%. Considering the particle size of these systems, it was found that the micelles at a ratio of 1:3 showed the smallest size of 10.6 ± 3.4 nm. The single size distribution peak of this system was obtained as shown in Figure 2B.

These results indicate that chrysin can be successfully entrapped in micelles of both types of poloxamers, Pluronic F-68 and Pluronic F-127. The size of CS-P68 and CS-P127 obtained from all studied conditions are in the nanosize range. The results demonstrate that the types of polymers and the ratio of drug to polymer play an important role in the size of the developed drug loaded micelles whereas no significant difference between ethanol and acetone used as a solvent for drug dissolution was seen in the preparation process. It was found that the zeta potential of CS-P68 and CS-P127 from all conditions was approximately -12 to -14 mV (data not shown), indicating that the developed chrysin loaded polymeric micelles might have a possibility for aggregation. When comparing the developed chrysin loaded polymeric micelles to the intact chrysin added in water, it was found that clear aqueous systems of CS-P68 and CS-P127 were obtained whereas the intact chrysin at the same concentration precipitated in water. This result obviously indicates that water solubility of chrysin was increased dramatically when formed as CS-P68 and CS-P127. As poloxamer is composed of hydrophilic polyethylene oxide (PEO) and lipophilic polypropylene oxide (PPO) blocks, arranged in a PEOmPPOnPEOm structure (*32*), it can self-assemble into micelles in aqueous solution forming the hydrophobic PPO core surrounded by the hydrophilic PEO. The increased water solubility of chrysin using these polymeric micelles is

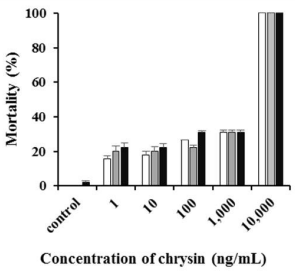

Figure 3. Mortality of zebrafish eggs at 24 h (white column), 48 h (gray column), and 72 h (black column) exposure to CS-P68.

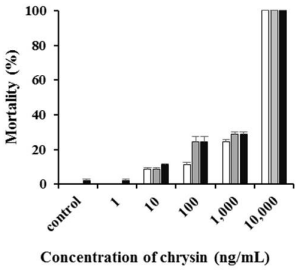

Figure 4. Mortality of zebrafish eggs at 24 h (white column), 48 h (gray column), and 72 h (black column) exposure to CS-P127.

considered to be due to the incorporation of chrysin into the hydrophobic portion of the micelles.

3.3. *In vivo toxicity of chrysin loaded micelles*

Regarding the solvent used, both ethanol and acetone could yield chrysin loaded micelles with a similar nano-size range but ethanol is considered to be a better solvent than acetone from the view point of environmental and human safety. Therefore, in the investigation of *in vivo* toxicity, only CS-P68 and CS-P127 with the proper ratio of drug to polymer of 1:4 and 1:2, respectively, and prepared using ethanol as a solvent were used. Chrysin has been reported to suppress an enzyme that converts androgen to estrogen resulting in an increase of testosterone (*9*). Therefore, it might be useful to know the safe dose or the maximum concentration of CS-P68 and CS-P127 which is considered as safe. In the present study, the embryo of zebra fish was used as a model for testing toxicity of the developed CS-P68 and CS-P127. The toxicity results expressed as mortality of zebrafish embryo are shown in Figure 3 for CS-P68 and Figure 4 for CS-P127. From these figures, it was noted that the mortality of the embryos with CS-P68 was higher than that with CS-P127 indicating that CS-P68 had higher toxicity than CS-P127. This effect was obviously seen particularly at the low dose range of 1-100 ng/mL. However, toxicity of both micelles was not significantly different at a concentration of 1000 ng/mL or more. It was found that the toxicity of all samples was seen in a dose dependent manner. A 10 ng/mL dose or less was

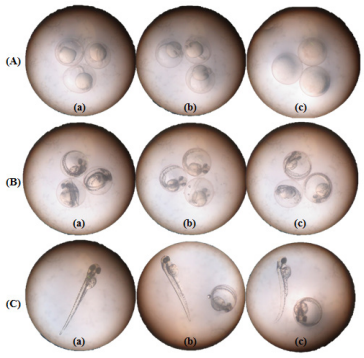

Figure 5. Morphology of surviving zebrafish embryos at 24 h (A), 48 h (B), and 72 h (C) exposure to water (a), CS-P68 at drug concentration of 1,000 ng/mL (b), and CP-127 at drug concentration of 1,000 ng/mL (c).

found to be safe for zebrafish embryos as less than 10% mortality was observed whereas doses of 100-1,000 ng/mL could be classified as a mild toxic dose as 10% to less than 30% mortality was observed. Higher than 1,000 ng/mL could be classified as moderate to severe toxicity to zebrafish embryos as 30-100% mortality was observed. The results also showed that the toxic effect of CS-P68 and CS-P127 was not time dependent. After incubating zebrafish eggs in the fixed drug dose systems but at different incubation times of 24, 48, and 72 h, the mortality of the fish was not significantly different at each dose. The results revealed that no zebrafish embryos were found after 24 h exposure to 10,000 ng/mL whereas more than 70% of the embryos developed normally (same as control) after exposure to 1-100 ng/mL. However, at a concentration of 1,000 ng/mL, some embryos (about 27 ± 1%) were dead after 24 h of exposure, the remaining embryos could develop but some showed delayed development. This effect could be seen clearly as shown in Figure 5. After 72 h, all embryos in the control group could hatch normally whereas only approx. 55 ± 5% of the eggs exposed to CS-P68 and CS-P127 (1,000 ng/mL) showed normal development like in the control group but the remaining embryos were still at an early stage. They were not dead but showed slow development as seen in Figure 5C. It was considered that polymer might be associated with the toxicity of CS-P68 and CS-P127. In CS-P68, the drug to polymer ratio was 1:4 whereas in CS-P127 it was 1:2. The amount of polymer in CS-P68 was 2 times higher than that in CS-P127. Therefore, high mortality particularly at low drug concentrations caused by CS-P68 was considered to be due to high concentration of polymer used in CS-P68.

4. Conclusion

Chrysin can be loaded in the polymeric micelles of Pluronic F-68 and Pluronic F-127. The obtained chrysin loaded micelles can increase its water solubility dramatically. The type of polymer and the ratio of drug to polymer play an important effect on size and desirable characteristics of the obtained micelles. The suitable chrysin loaded polymer micelles is composed of 1:4 and 1:2 of drug to Pluronic F-68 and to Pluronic F-127 ratios, respectively. The toxicity of these micelles is dose dependent but not time dependent. Chrysin at concentrations that do not exceed 10 ng/mL is considered safe for zebrafish embryos. The micelles with higher polymer ratios cause higher toxicity to the fish.

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Original Article

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Preparation of an oral acetaminophen film that is expected to improve medication administration: Effect of polyvinylpyrrolidone on physical properties of the film

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Summary **This study investigated the effect of polyvinylpyrrolidone (PVP) on a film containing carboxymethyl cellulose sodium (CMC) as a matrix to improve surface roughness caused by drug recrystallization. Acetaminophen (AA) was used as the model drug. Recrystallization is a problem encountered during the preparation of films that contain high drug doses, making them difficult to take. A film that does not disintegrate for clinical applications requires a smooth surface, moderate strength and elasticity, and a low level of adhesiveness to facilitate taking of the medication. Addition of PVP to the film formulation made the surface significantly smoother, and it was independent of the compounding method. Smooth films were obtained when the CMC concentration was kept constant and the amount of PVP was increased, but it also increased the adhesiveness and strength, and decreased the elasticity of the films. When high polymer concentration was kept constant and the ratio of CMC and PVP was varied, the films with smaller amounts of PVP tended to have a smoother surface and less adhesiveness. However, when the amount of PVP was reduced, the film strength increased and elasticity decreased. The amount of PVP had a negligible effect on drug dissolution behavior, making it useful for preparation of the AA film. However, it is necessary to determine the compounding method and the PVP load considering the adhesiveness, strength, and elasticity of the films.**

Keywords: Oral film, acetaminophen, carboxymethyl cellulose sodium, polyvinylpyrrolidone

1. Introduction

Oral preparations are widely used, and are an important type of formulation in pharmacotherapy. Tablets are most commonly used for their convenience, but there can be difficulties for infants and patients who have trouble swallowing. These problems lead to reduced patient compliance followed by reduction in the effectiveness of the drug. Liquids or powders can be used for such patients; however, there may be compliance issues, because these formulations do not have portability and they are also not easy to administer. To improve administration, jelly preparations (*1-3*), orally disintegrating tablets (*4-7*), and oral film preparations (*8-10*) have been developed. Although, almost all films can dissolve in the mouth, the films can only contain a small amount of drug. It makes the films usable for only those drugs that require a clinical dosage of no more than about 25 mg (*11*). Current drugs are administrated in a range of low to high dosage, such as acetaminophen (AA). In this study, the fundamentals of oral film preparations containing a high dose of AA are discussed. Even if it does not disintegrate, films with moderate strength and elasticity are easier to swallow with water, when they have a smooth surface. Current film preparations often contain hydroxypropyl methylcellulose (HPMC) as a matrix polymer. However we previously suggested that the possibility of using carboxymethyl cellulose sodium (CMC), instead of HPMC, was also evaluated (*12*). The main problem that

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results in rough surfaces is recrystallization of the drug at high doses during film preparation. To overcome this issue, polyvinylpyrrolidone (PVP), which does not crystallize and thereby reduces the effect of crystallization, was used for a solid dispersion (*13-15*). The effects of PVP addition on crystallization and physical properties of the films were investigated. As an alternative to tablets, the application of this concept for film formulations of other drugs could contribute to improved medication efficacy.

2. Materials and Methods

2.1. *Materials*

AA and CMC were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as a model drug and as a matrix, respectively. PVP K30 was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and used as an additive. Glycerin (GL) was obtained from Guaranteed Reagent Nacalai Tesque (Kyoto, Japan) and was used as a plasticizer.

2.2. *Film preparation*

Films were prepared using the solvent-casting method (Figure 1). AA was dissolved in purified water and mixed with various amounts of CMC and PVP. GL was added at a constant concentration. The mixture was stirred for 24 hours at room temperature using a magnetic stirrer. The resulting dispersed liquid was then dried by casting it on a flat tray at room temperature. The prepared films were cut to a size of 2 cm × 2 cm. Each sheet contained either 50 mg or 100 mg of AA. When the CMC concentration was constant, the amount of PVP was changed, and when concentration of polymer was constant, the mixing ratio of CMC and PVP was changed. The composition of each film is shown in Table 1.

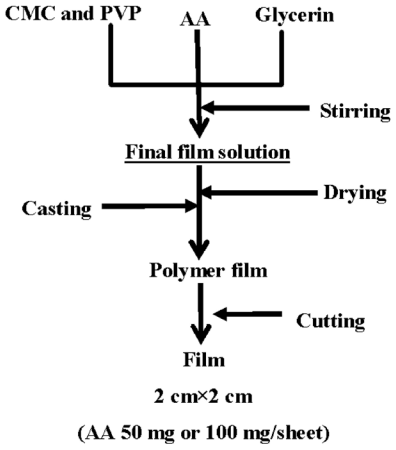

Figure 1. Preparation method for films containing AA.

2.3. *Film evaluation*

2.3.1. *Thickness measurement*

Film thickness was measured using a micrometer (Mitutoyo Co., Kanagawa, Japan) (n = 10).

2.3.2. *AA content measurement*

AA content per film sheet was determined using UV spectrophotometry (Shimadzu Co., Kyoto, Japan) at a wavelength of 244 nm (n = 10).

2.3.3. *Strength and elasticity measurement*

Film strength was measured using a rheometer (Sun Scientific Co. Ltd., Tokyo, Japan). The film was clipped 4 mm from the attachment, pulled down at a speed of 15 mm/min, and stretched until breakage occurred. A stress-displacement curve was obtained using these results. Film strength and extension were calculated from the stress displacement curve using formulae (1) and (2) (*16*) (n = 5):

$$\text{Tensile strength (N/mm}^2\text{)} = \frac{\text{Load at failure (N)}}{\text{Strip thickness (mm)} \times \text{Strip width (mm)}} \quad (1)$$

$$\text{Elongation at break (\%)} = \frac{\text{Increase in length (mm)}}{\text{Original length (mm)}} \times 100 \quad (2)$$

2.3.4. *Adhesive study*

Film adhesiveness was measured using a rheometer (Sun Scientific Co. Ltd.) as previously described by

Table 1. Composition of the film formulations prepared with carboxymethyl cellulose sodium and polyvinylpyrolidone

Material	A	B	C	D	E	F	G	H	I
Acetaminophen (mg)	50	50	50	100	100	100	50	50	50
Carboxymethyl cellulose sodium (mg)	30	30	30	30	30	30	20	15	10
Polyvinylpyrolidone (mg)	20	10	5	20	10	5	10	15	20
Glycerin (mg)	20	20	20	20	20	20	20	20	20
Total (mg)	120	110	105	170	160	155	100	100	100

Values indicate the amount per sheet.

Tamura *et al*. (*17*). The sample film was fixed on a table using double-sided tape and 100 µL of purified water was dropped onto it. Pressure was immediately applied using a circle attachment that had a 10-mm diameter, and the film was pulled down at a speed of 15 mm/min. A stress-displacement curve was obtained using these results. Adhesiveness was calculated based on isolated pressure ($n = 5$).

2.3.5. *Surface roughness study*

The surface roughness of the films was measured using a compact-sized roughness-measuring instrument, the Surf-test SJ210 (Mitutoyo Co. Ltd.). Six measurements were taken to obtain the arithmetic average surface roughness (Ra) for both sides of the film.

2.3.6. *Dissolution study*

The basket method, using the JP 16 dissolution apparatus (Toyama Sangyo Co. Ltd., Osaka, Japan), was used for this experiment. The dissolution medium was 900 mL purified water at 37°C, with a stirring rate of 100 rpm. At appropriate time intervals, after the film was put in dissolution medium, samples were withdrawn and replaced with the same volume of purified water. AA concentrations were determined using UV spectrophotometry (Shimadzu Co., Kyoto, Japan) at a wavelength of 244 nm ($n = 3$).

2.4. *Statistical analysis*

The results are presented as the mean ± standard deviation (SD) values. Statistical differences were analyzed using the Tukey-Kramer test for multiple comparisons, and the level of significance was set at $p < 0.05$.

3. Results

3.1. *Film characterization*

The AA content of prepared films was 90-110%. Thickness of the films containing 50 mg of AA was 0.35-0.47 mm, and the thickness of films containing 100 mg of AA was 0.57-0.67 mm (Tables 2-4).

3.2. *Film strength*

The CMC concentration was kept constant in the films that contained 50 mg of AA and the amount of PVP was changed as in formulations A, B, and C. The strength of these films was 3.98-7.12 N/mm^2. Formulation A, which contained the highest amount of PVP, was the strongest, whereas formulation B was the weakest ($p < 0.05$; Table 2). When the concentration of AA was kept constant and the amounts of CMC and PVP were changed as in formulations G, H, and I, these films had strengths of 0.20-3.13 N/mm^2. Formulation I,

Table 2. Physical characteristics of films containing acetaminophen (50 mg), carboxymethyl cellulose sodium, and polyvinylpyrolidone

Items	A	B	C	Comparison between groups
Tensile strength (N/mm^2)	7.12 ± 0.56	3.95 ± 0.67	5.43 ± 0.27	$p < 0.05$: B *vs*. A and C, C *vs*. A
Elongation at break (%)	31.32 ± 2.36	43.62 ± 3.34	42.68 ± 5.18	$p < 0.05$: A *vs*. B and C
Adherence (N/mm^2)	6.43 ± 0.52	7.27 ± 0.29	3.65 ± 0.23	$p < 0.05$: C *vs*. A and B
Thickness (mm)	0.45 ± 0.02	0.39 ± 0.01	0.41 ± 0.05	

Values are presented as mean ± SD ($n = 5$).

Table 3. Physical characteristics of films containing acetaminophen (50 mg), carboxymethyl cellulose sodium, and polyvinylpyrolidone

Items	G	H	I	Comparison between groups
Tensile strength (N/mm^2)	3.11 ± 0.42	3.13 ± 0.73	0.20 ± 0.09	$p < 0.05$: I *vs*. G and H
Elongation at break (%)	21.7 ± 2.78	29.3 ± 3.86	51.5 ± 6.94	$p < 0.05$: I *vs*. G and H
Adherence (N/mm^2)	3.62 ± 0.17	2.48 ± 0.23	5.99 ± 0.67	$p < 0.05$: H *vs*. G and I, G *vs*. I
Thickness (mm)	0.39 ± 0.05	0.47 ± 0.001	0.35 ± 0.03	

Values are presented as mean ± SD ($n = 5$).

Table 4. Physical characteristics of films containing acetaminophen (100 mg), carboxymethyl cellulose sodium, and polyvinylpyrolidone

Items	D	E	F	Comparison between groups
Tensile strength (N/mm^2)	3.61 ± 0.15	2.91 ± 0.36	0.96 ± 0.08	$p < 0.05$: F *vs*. A and D, E *vs*. D
Elongation at break (%)	33.3 ± 2.99	47.21 ± 4.24	59.1 ± 2.46	$p < 0.05$: F *vs*. A and D, E *vs*. D
Adherence (N/mm^2)	5.27 ± 1.09	4.69 ± 0.39	4.48 ± 0.63	
Thickness (mm)	0.67 ± 0.05	0.58 ± 0.05	0.57 ± 0.02	

Values are presented as mean ± SD ($n = 5$).

Table 5. Surface roughness (Ra) of films containing acetaminophen (50 mg), carboxymethyl cellulose sodium, and polyvinylpyrolidone

Items	Ra (μm)						
	no PVP	A	B	C	G	H	I
Top surface	20.53 ± 2.82	1.86 ± 0.19*†	1.99 ± 0.32*†	6.20 ± 0.75*	2.5 ± 0.70*#	4.6 ± 0.42*	3.7 ± 0.40*#
Bottom surface	3.06 ± 0.35	1.02 ± 0.07*	1.33 ± 0.49*	2.61 ± 0.40	1.8 ± 0.69*	3.0 ± 0.33	1.2 ± 0.31*

Values are presented as mean ± SD (n = 5). Top surface: *p < 0.001 *vs*. no PVP, †p < 0.001 *vs*. C, G, H, and I, #p < 0.01 *vs*. H. Bottom surface: *p < 0.001 *vs*. no PVP, C and H.

which included CMC and PVP at a ratio of 1:2, was the weakest (p < 0.05; Table 3). When both formulation types were compared, formulations G, H, and I were weaker than formulations A, B, and C.

For films with 100 mg of AA (formulations D, E and F), the strength was 0.96-3.61 N/mm^2. The strength decreased with decreasing PVP content (p < 0.05; Table 4).

3.3. *Film elasticity*

Formulations A, B and C, which included 50 mg of AA, a constant amount of CMC and variable amounts of PVP, had an extension of 31.32-43.62%, which indicated elasticity. Formulation A, which contained the highest amount of PVP, showed the least elasticity (31.32 ± 2.36%, p <0.05; Table 2) whereas formulations B and C showed similar elasticity. Formulations G, H and I, which included 50 mg of AA, variable CMC and PVP mixing ratios, had an extension of 21.7-51.5%. Extension increased with an increase in the PVP mixing ratio. Formulation I, which included CMC and PVP in a ratio of 1:2, had the maximum extension (p < 0.05; Table 3). When both formulation types were compared, formulation I had the maximum elasticity among all the film formulations (p < 0.05). Formulations D, E and F, which contained 100 mg of AA, had an extension of 33.3-59.1%. Film elasticity increased with a decreasing amount of PVP (p < 0.05; Table 4).

3.4. *Film adhesiveness*

CMC concentration in the films that contained 50 mg of AA was kept constant and the amount of PVP was changed as in formulations A, B, and C. These formulations had an adhesiveness of 3.65-7.27 N/mm^2. Formulation C, which had the lowest amount of PVP, had the lowest adhesiveness (3.65 ± 0.23, p < 0.05; Table 2). Formulations A and B also showed similar results. When high polymer concentration was kept constant and the amounts of CMC and PVP were changed (formulations G, H, and I), adhesiveness was 2.48-5.99 N/mm^2. Formulation H, which included CMC and PVP in a 1:1 ratio, was least adhesive (p < 0.05; Table 3). Formulation I, which included CMC and PVP in a 1:2 ratio, had maximum adhesiveness (p < 0.05). In films with 100 mg of AA (formulations D, E and F), adhesiveness was 4.48-5.27 N/mm^2. These films showed almost the same level of adhesiveness regardless of the amount of PVP (Table 4).

Table 6. Surface roughness (Ra) of films containing acetaminophen (100 mg), carboxymethyl cellulose sodium, and polyvinylpyrolidone

Items	Ra (μm)		
	D	E	F
Top surface	2.61 ± 0.31#	4.39 ± 0.77	5.29 ± 0.99
Bottom surface	1.99 ± 0.25	0.77 ± 0.09$	1.07 ± 0.11*

Values are presented as mean ± SD (n = 5). #p < 0.05 *vs*. E and F, $$p$ < 0.05 *vs*. D and F, *p < 0.05 *vs*. D.

3.5. *Film surface roughness*

The upper surface roughness (Ra), which was exposed during film preparation, was compared. The films containing 50 mg of AA had a smooth surface and their Ra was significantly less than the films that did not contain PVP (20.53 vs. 1.86-6.2 μm, p < 0.001; Table 5). For PVP films, formulations A and B, which had a constant CMC concentration and a variable amount of PVP, had a similar level of surface roughness. When formulations A, B, and C were compared with each other, formulation C, which had the lowest amount of PVP, showed the highest roughness. Among formulations G, H, and I, which had a constant high polymer concentration and variable amounts of CMC and PVP, formulations G and I showed less roughness, but it was not different from that of formulations A, B, and C. However, surface roughness (Ra) on the lower side of the film, was less in films that had no PVP. Formulations A, B, G and I showed significantly less roughness than the films that did not contain PVP (p < 0.001). Similar trends were observed in upper surface roughness in formulations A, B, and C and formulations G, H, and I.

The differences between upper and lower surface roughness were similar. Formulations A, B and G showed less roughness, whereas formulations C, H and I showed comparably higher roughness (Table 5).

For films containing 100 mg of AA, formulation D showed the least upper surface roughness, which increased with a decreasing amount of PVP (p < 0.05, D *vs*. E and F). On the other hand, formulation E

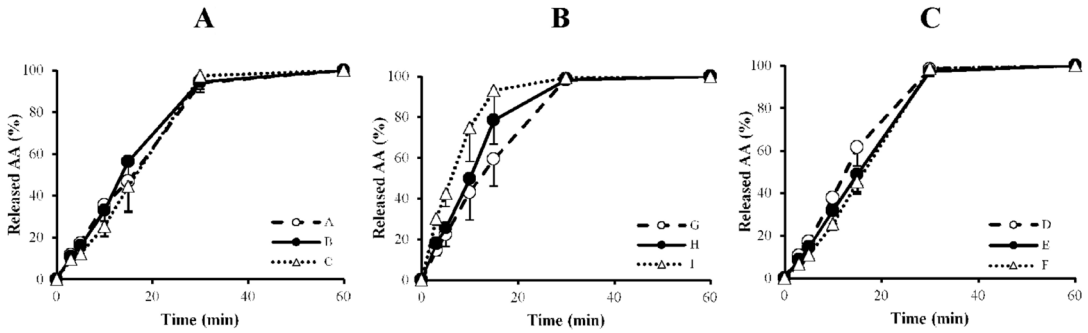

Figure 2. Dissolution profiles of acetaminophen from films prepared with carboxymethyl cellulose sodium and polyvinylpyrolidone. Values are presented as the mean ± SD (n = 3). **(A)** Formulation A, B, C (The film containing 50 mg AA and constant amount of CMC and variable amounts of PVP). **(B)** Formulation G, H, I (The film containing 50 mg AA and variable CMC and PVP mixing ratios). **(C)** Formulation D, E, F (The film containing 100 mg AA and constant amount of CMC and variable amounts of PVP).

showed the least (lower) surface roughness followed by formulation F and D ($p < 0.05$). Upper and lower surface roughness results were similar for films containing 100 mg of AA and those containing 50 mg of AA. Formulation D showed the smallest difference between upper and lower surface roughness (Table 6).

3.6. *Film dissolution behavior*

For films that contained 50 mg of AA, such as formulations A, B and C, which had a constant CMC concentration and variable amounts of PVP, there was no difference in the dissolution behavior based on the PVP content. These films showed approximately 100% dissolution at 30 minutes. Formulations G, H and I, which had a constant concentration of high polymer and a variable amount of CMC and PVP, also showed approximately 100% dissolution at 30 minutes. However, there were differences in the initial dissolution behavior when the CMC and PVP mixing ratio was changed. An increase in the PVP mixing ratio resulted in faster dissolution rate. Dissolution at 15 minutes was 59% for formulation G (CMC:PVP, 2:1) and 78% for formulation H (CMC:PVP, 1:1); it was the highest for formulation I (93%), which contained the most PVP (CMC:PVP, 1:2; $p < 0.05$). The dissolution at 15 minutes for all the films that contained 50 mg of AA (formulations A, B and C) was 47, 56, and 44%, respectively, and it was slower than that for formulations G, H, and I (Figure 2).

In the films that contained 100 mg of AA (formulations D, E, and F), which had a constant CMC concentration and variable PVP levels, dissolution behavior was similar to that of formulations A, B, and C. The dissolution at 30 minutes was 100%, and there were no differences in dissolution behavior in response to changes in the amount of PVP.

4. Discussion

This study investigated films that are easier to swallow, even if it is not a disintegrating film, have a smooth surface and that gel when taken with water. At high AA dose, there is drug recrystallization that results in films with reduced smoothness and a rough surface. In this study, the effect of PVP addition to reduce these problems was investigated.

A mixing method was used for this purpose where CMC was used as a matrix and amount of PVP was varied. The surface roughness of films containing PVP decreased and these films were smoother than the films that did not contain PVP. It has been shown that drug recrystallization is inhibited when a solid dispersion of polymer and PVP are used (*18,19*), making the surface smoother. This effect was dependent on the amount of PVP used. In both types of films containing either 50 mg or 100 mg of AA, when the CMC concentration was kept constant, surface roughness changed with change in PVP concentration. The film surfaces became smoother as the amount of PVP increased, and the surfaces became rougher as the amount of PVP decreased. For example, in films containing 50 mg of AA, when the high polymer concentration was kept constant and the mixing ratio was varied (formulations G, H, and I), the surfaces were smoother than those of formulation C, which contained 5 mg of PVP per sheet as compared to 10, 15, and 20 mg of PVP in formulations G, H, and I, respectively. PVP seemed to have a definite effect on surface smoothness, because these films contained 10-20 mg of PVP per sheet. These results suggest that the problem of drug recrystallization at high drug doses can be prevented by preparing CMC films that use PVP.

Sufficient strength and elasticity was required for the films prepared in this study (*20*). Films with 50 mg of AA, a constant amount of CMC, and a large amount of PVP (formulation A) showed higher strength and lesser elasticity than those shown by other films. Films that contained 100 mg of AA and a large amount of PVP showed similar trends: higher strength and lesser elasticity. Strength decreased while elasticity increased with a decreasing amount of PVP. These

results suggested that increasing the amount of PVP increases the film's strength and decreases the film's elasticity when the CMC concentration is kept constant. However, when the polymer concentration was kept constant and the mixing ratio was varied, elasticity increased and strength decreased with an increase in the PVP mixing ratio. Decreasing CMC concentration seems to significantly affect the mechanical properties of films. Thus, adhesiveness of the films was evaluated because it is a major factor for comfort when taking an experimental film formulation. When the CMC concentration was kept constant, formulation C, which included the least amount of PVP (PVP, 5 mg/sheet), had the least adhesiveness. In films with 100 mg of AA, the amount of PVP had little effect on adhesiveness. However, adhesiveness of these films was less than that of films with 50 mg of AA. On the other hand, among films with 50 mg of AA, which had a constant high polymer concentration and a variable CMC and PVP mixing ratio, films composed of a 1:1 mixing ratio showed the least adhesiveness. The adhesiveness of these films increased as the PVP mixing ratio increased. These results suggest that increasing the PVP mixing ratio increased adhesiveness of the films, due to the adhesiveness of PVP. At higher drug content (100 mg of AA), the influence of PVP on mechanical properties of the films may be smaller.

When the polymer concentration was kept constant and the CMC and PVP mixing ratio was varied, AA dissolution from films became faster with increase in the PVP mixing ratio in this condition. All films had 100% dissolution at 30 minutes and the PVP content did not seem to affect the dissolution behavior. In a clinical scene, it seems to be no problem.

These results suggested that films containing PVP have significantly smoother surfaces regardless of the compounding method used. When the CMC concentration was kept constant, an increase in the amount of PVP improved surface smoothness of the films. It increased adhesiveness and strength of the films; however, elasticity was decreased. When the high polymer concentration was kept constant, decreasing PVP mixing ratio improved film surface smoothness, and decreased the adhesiveness. When PVP mixing ratio was decreased, film strength increased and elasticity decreased. However, the amount of PVP did not affect the dissolution behavior of the drug from the films.

Compounding PVP to prepare and formulate AA films is a useful approach. However, it is necessary to consider adhesiveness, strength, and elasticity to determine the best mixing method and mixing loads.

AA was selected as a model drug because it is used widely in both children and the elderly, and it requires adjustments in dosage based on body weight and symptoms. Associated with increasing AA dosage are bulky tablet sizes or large amounts of powder, which can cause compliance problems. Films with high concentrations of drug could solve these issues. Application of this concept used in AA film formulation can be applied to prepare films of various other drugs. However, further studies are still required to mask the bitter taste of the drug.

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Brief Report

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Discovery of N-hydroxy-4-(1H-indol-3-yl)butanamide as a histone deacetylase inhibitor

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Summary **The indoles plant growth hormones have exhibited potentially antitumor activities. However, the targets of these indoles have not been clearly elucidated. By introduction of hydroxamic acid group to the structure of indolebutyric acid, the derived molecule (IBHA) exhibited potent HDAC2 (IC_{50} value of 0.32 ± 0.02 μM) and HDAC3 (IC_{50} value of 0.14 ± 0.01 μM) inhibitory activities compared with SAHA (IC_{50} value of 1.25 ± 0.06 μM and 0.97 ± 0.04 μM against HDAC2 and HDAC3). In the antiproliferative assays, the tested hematologic cell lines (U937 and K562) are more sensitive to IBHA than the solid tumor cell lines (MDA-MB-231 and PC-3). In the docking studies, the derived molecule (IBHA) could bind to the active site of human HDAC2 and HDAC3 by strong H-bond interactions and hydrophobic interactions. Pharmacophore mapping results revealed that properties of IBHA matches the receptor (HDAC3) based pharmacophore model.**

Keywords: HDACs inhibitor, indolebutyric acid, hydroxamic acid, docking, pharmacophore model

1. Introduction

Histone deacetylases (HDACs) are a family of enzymes responsible for the deacetylation of histone proteins by removing the acetyl moiety from the amino group of lysine residues on the N-terminal extension of core histones (*1-3*). Eighteen different HDAC isoforms which are divided into four classes have been identified in human. HDAC1, 2, 3 and 8 are classified as class I HDACs; class II HDACs are further subdivided into class IIa (HDAC4, 5, 7, and 9) and IIb (HDAC6 and 10); class III HDACs are a group of NAD+ dependent proteases known as sirtuins (sirt 1-7); Class IV HDACs (HDAC11), is an atypical category of its own.

Overexpression and aberrant recruitment of HDACs (especially class I and II HDACs) have significant roles in the genesis and development of tumor (*4*). Inhibition of HDACs has exhibited potent antitumor potential by induction of biological effects including apoptosis, cell cycle arrest, necrosis, autophagy, differentiation and migration (*5,6*). A number of structurally diverse HDAC inhibitors (HDACIs) have shown potent antitumor efficacy in various stage of clinical trials. Three HDACIs SAHA (*7*), FK228 (*8*) and PXD101 (*9*) have been approved by the US Food and Drug Administration (FDA) for the treatment of cancers.

The indoles plant growth hormones such as naphthaleneacetic acid, indolebutyric acid, indoleacetic acid and the widely studied indole-3-carbinol have showed antitumor potential in human (*10*). However, the targets of these molecules have not been detailed elucidated. Interestingly, the structure of indolebutyric acid is coincide with the pharmacophore of the classic histone deacetylase inhibitors (HDACIs) (Figure 1). The indol ring represents the cap of the HDACIs; the $(CH_2)_3$ of the butyric acid part is the linker; and the carboxylic acid group is the zinc binding group (ZBG). Therefore, hydroxamic acid group was introduced to indolebutyric acid, and the target compound (IBHA) was synthesized and evaluated by the enzymatic inhibition assay. The binding pattern of the designed molecule (IBHA) was predicted by the docking process. Pharmacophore modeling was also performed to evaluate the inhibitor-receptor binding.

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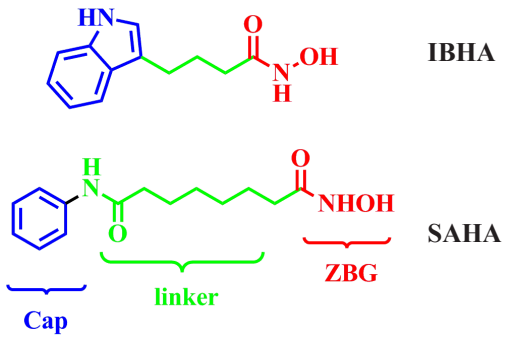

Figure 1. Structural comparison of IBHA with SAHA.

2. Materials and Methods

2.1. *Chemistry*

Target compound IBHA was derived by a single step reaction. The hydroxamic acid group was introduced by coupling of indolebutyric acid (Aladdin, Shanghai, China) and NH_2OH (Aladdin, Shanghai, China) using isobutyl chloroformate (Aladdin, Shanghai, China).

1H NMR spectra were recorded on a Bruker DRX spectrometer at 400 MHz, δ in parts per million and J in hertz, using TMS as an internal standard. High-resolution mass spectra were conducted by Shandong Analysis and Test Center in Ji'nan, China. ESI-MS were determined on an API 4000 spectrometer. Melting points were determined uncorrected on an electrothermal melting point apparatus.

N-hydroxy-4-(1H-indol-3-yl)butanamide (IBHA) To a solution of IBA (1.02 g, 5 mmol) in THF (50 mL), Et_3N (0.51g, 5 mmol) and IBCF (0.75 g, 5.5 mmol) were added in turn. After 10 min, NH_2OH (0.33 g, 10 mmol) was added. The reaction solution was stirred at room temperature for 8 h. Then, the solvent was evaporated with the residue being taken up in saturated citric acid (50 mL) and extracted with EtOAc (3 × 20 mL). The EtOAc solution was washed with brine (3 × 20 mL), dried over $MgSO_4$, and evaporated under vacuum. The desired compound IBHA (0.53 g, 49% yield) was derived by crystallization in EtOAc as white powder. Mp: 198-200 oC. ^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ 11.59 (s, 1H), 10.79 (s, 1H), 10.77 (s, 1H), 7.53-7.50 (m, 1H), 7.33 (d, J = 8.0 Hz, 1H), 7.13 (d, J = 5.6 Hz, 1H), 7.07-7.04 (m, 1H), 6.96 (t, J = 7.2 Hz, 1H), 2.77-2.69 (m, 2H), 2.18 (t, J = 7.2 Hz, 2H), 1.95-1.88 (m, 2H). ESI-MS: m/z: 219.3 $[M+H]^+$.

2.2. *Enzyme inhibition assay*

The method of enzymatic inhibition assay has been described in our previous work (*11*). Boc-Lys (acetyl)-AMC was used as the substrate of HDAC; and SAHA was used as a positive control. IBHA was diluted to six concentrations (25, 5, 1, 0.2, 0.04 and 0.008 uM/L) to investigate its HDAC inhibitory ability.

2.3. *In vitro antiproliferative assay*

Tumor cell inhibition was determined by the MTT method. Briefly, 2,000 cells were seeded into each well of 96-well plates, which were incubated at 37°C, 5% CO^2 overnight. The cells were then treated with compound sample at various concentrations for 48 h. After that, a 0.5% MTT solution was added to each well. After 4 h incubation, formazan was extracted by adding DMSO (200 mL) for 5 min. Optical density values were then detected at λ = 570 nm on a micro-plate reader.

2.4. *Molecular docking*

The molecular docking process was performed using Glide software (schrodinger Inc., supported by Shanghai Institute of Materia Medica Chinese Academy of Sciences). Crystal structure of HDAC2 (PDB Entry: 4LXZ), and HDAC3 (PDB Entry: 4A69) were obtained from the RCSB PDB data bank (*www.pdb.org*). Structural optimizations were performed to make the protein suitable for docking. The water molecules and the ligand crystallized in the protein structures were removed, and OPLS 2005 force field was assigned. The ligands used in the docking approach were sketched by maestro and refined by LigPrep. The active site was defined as a cubic box containing residues around Zn ion at a distance of 20 Å. Extra precision was applied in the docking process; other parameters were set as default.

2.5. *Pharmacophore modeling*

Discovery studio 2.5 software was used in the pharmacophore modeling process. The structure of IBHA-HDAC3 used in the present research was derived from the docking study. Structure of HDAC3 was defined as the receptor, and the binding site was defined as a sphere centered on IBHA with radius of 9 Å. Density of lipophilic sites and density of polar sites were set to be 25. The generated features were clustered and only the IBHA surrounding features were kept.

3. Results and Discussion

In order to evaluate the enzymatic inhibition activity and validate the assumption, the activity assay was performed. In this process, IBHA was tested against human HDAC2 and HDAC3 using SAHA as a positive control. The results showed that IBHA is a potent HDAC inhibitor with IC_{50} values of 0.32 ± 0.02 μM and 0.14 ± 0.01 μM against HDAC2 and HDAC3, respectively. Moreover, in the present test, molecule IBHA exhibited better performance than SAHA which showed IC_{50} value of 1.25 ± 0.06 μM and 0.97 ± 0.04 μM against HDAC2 and HDAC3, respectively.

To investigate the antiproliferative activity of IBHA,

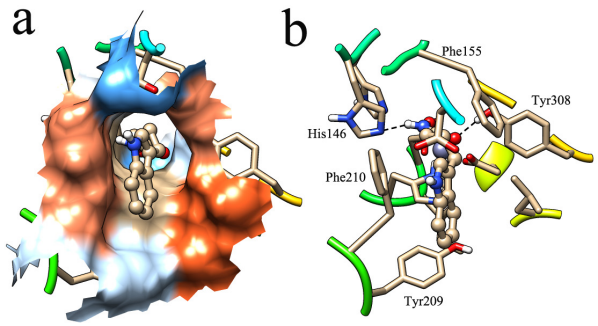

Figure 2. Results of binding IBHA to the active site of HDAC2. a: surface representation of the ligand-receptor binding; **b**: 3D representation of the interactions.

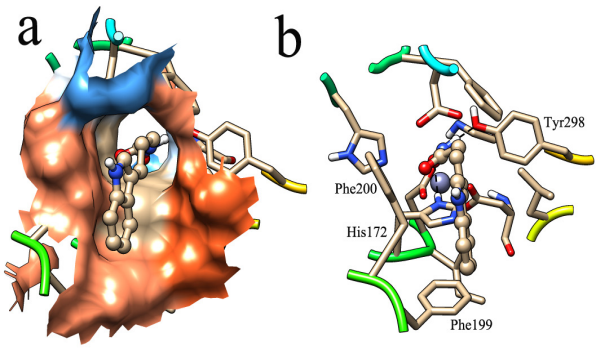

Figure 3. Results of binding IBHA to the active site of HDAC3. a: surface representation of the binding in the active site; **b**: 3D representation of the binding.

MTT assays were performed against 4 types of tumor cell lines. According to the results, IBHA exhibited inhibitory selectivity of hematologic cell lines (U937 and K562) compared with the tested solid tumor cell lines (MDA-MB-231 and PC-3). IBHA displayed IC_{50} values of 9.35 ± 0.12 μM and 11.76 ± 0.55 μM against U937 and K562 cell lines compared with SAHA (1.67 and 1.86 μM), respectively. While the IC_{50} values of IBHA against MDA-MB-231, PC-3 cell lines were 29.87 ± 1.44, 37.6 ± 2.18 μM compared with SAHA (2.91, 4.63 μM), respectively.

In order to predict the binding mode of IBHA in the active sites of HDAC2 and HDAC3, molecular docking approaches were performed using the Glide software. The docking results reveal that IBHA can access to the active site of both HDAC2 and HDAC3 (Figures 2 and 3). The surface plot revealed that the structure of IBHA has good spatial match in the sites, and hydrophobic interactions make significant contributions to the ligand-receptor bindings (Figure 2a and 3a). In the active site of HDAC2, there is significant π-π stacking interaction between the indole group of IBHA and phenyl ring of Phe210 (Figure 2b). Phe199 and Phe200 of HDAC3 play important roles in the hydrophobic interactions by Pi interactions (Figure 3b).

The hydroxamic acid group of IBHA not only chelates to the zinc ions in the active sites as expected, but also generates multiple H-bond interaction with surrounding residues. In the active site of HDAC2, CO of the hydroxamic acid group can form H-bond interactions with OH of Tyr308, and NH has H-bond interactions with NE2 of His146 (Figure 2b). In the catalytic site of HDAC3, the hydroxamic acid group of IBHA binds to His172 and Tyr298 with H-bond interactions (Figure 3b). All these involved interactions make IBHA bind tightly to the active sites of both HDAC2 and HDAC3.

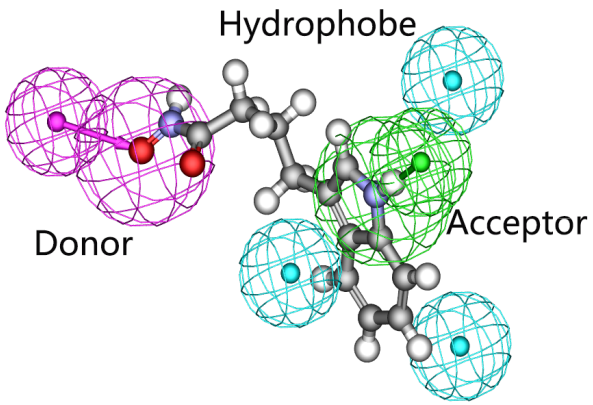

Figure 4. IBHA in the pharmacophore model of HDAC3.

Pharmacophore modeling was performed to further study the ligand-receptor interactions, and a receptor based pharmacophore model was generated on the active site of HDAC3 (Figure 4). The indole ring of IBHA located in the region that is rich in hydrophobic sites, and strong hydrophobic interaction can be formed. The superposition of the NH in the indole ring of IBHA and the H-bond receptor of the pharmacophore model reveals significant H-bond interactions. The NO of the hydroxamic acid group in the region of H-bond donor also make contributions to the H-bond interactions. The pharmacophore modeling results are consistent with that of the docking analysis.

In conclusion, structural modification was performed to make the indoles with antitumor potential (indolebutyric acid) bind to HDACs. Enzymatic inhibition assay results revealed that IBHA could potently inhibit the activity of both HDAC2 and HDAC3. Molecular docking studies showed that the designed molecule (IBHA) can bind to the active site of HDAC2 and HDAC3. Multiple H-bond interactions, hydrophobic interactions such as π-π conjugation and strong chelation, make significant contributions to the IBHA-HDACs bindings. The pharmacophore modeling results displayed good match between the structure of IBHA and the receptor based pharmacophore model. The present work revealed that IBHA could be used a lead compound in the development of novel HDACIs.

Acknowledgements

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Brief Report

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An ultra-low-molecular-weight heparin, fondaparinux, to treat retinal vein occlusion

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Summary **Retinal vein occlusions may decrease visual acuity. There is no known therapy to treat ocular thrombosis. The authors used fondaparinux, an ultra-low-molecular-weight heparin, to treat 13 consecutive cases of recent-onset retinal vein occlusions. Two patients with renal insufficiency were not included. Eight central retinal vein occlusions and 5 branch retinal vein occlusions in 13 patients were treated with subcutaneous fondaparinux 2.5 mg once a day. The patients were seen every 2 weeks. Macular edema was treated with intravitreal injections of anti-vascular endothelial growth factor or steroids. Two patients elected to discontinue treatment. Of the remaining 11, 9 occlusions resolved in 1.5 to 13.5 months with rapid resolution of retinal edema and hemorrhage as soon as the occlusions resolved. One patient had a retinal vein that was still occluded after 8 months of therapy and 1 had retinal vein occlusion that partially resolved after 15 months of treatment. Of the 9 eyes with occlusions that resolved, visual acuity improved in 7. In 2, visual acuity decreased due to macular ischemia. Occlusion recurred in 1 2.5 months after the suspension of initial treatment. This patient is again being treated with fondaparinux 2.5 mg. No hemorrhaging occurred. Fondaparinux 2.5 mg can be given subcutaneously once a day to patients with recent-onset retinal vein occlusions without renal insufficiency. An occlusion may take a number of months to resolve. Once the vein occlusion has resolved, retinal edema and hemorrhage rapidly resolve and vision improves. Macular edema should be treated while waiting for the vein occlusion to resolve.**

Keywords: Fondaparinux, ultra-low-molecular-weight heparin, low-molecular-weight heparin, central retinal vein occlusion, branch retinal vein occlusion

1. Introduction

Central retinal vein occlusion (CRVO) is the second most common vascular cause of the loss of visual acuity (VA) (*1*). The precise etiology is not known although different risk factors have been identified (*2*). Hayreh divided CRVOs into 2 categories: a nonischemic CRVO or "venous stasis retinopathy," and an ischemic CRVO or "hemorrhagic retinopathy" (*3*). A branch retinal vein occlusion (BRVO) involves one of the retinal vein branches and usually has less of an impact on vision. BRVOs have been divided into nonischemic, indeterminate, and ischemic forms depending on the amount of capillary non-perfusion (*4*). The current study used fluorescein angiography to assess capillary non-perfusion and the description by Magargal in order to categorize retinal vein occlusion (RVO) as ischemic or nonischemic. Minimal to moderate (less than 50%) capillary non-perfusion was considered nonischemic

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while more than 50% capillary non-perfusion was considered ischemic (*5*).

Ophthalmologists currently treat an RVO primarily with injections of intravitreal anti-vascular endothelial growth factor (anti-VEGF) agents or intravitreal steroids along with laser treatment of ischemic areas when needed. These injections can resolve severe macular edema and temporarily improve VA while waiting for the venous blockage to spontaneously resolve through recanalization or collateral vessel formation. These injections are not known to help remove or resolve a thrombus (*6,7*).

There are 3 types of heparins: unfractionated heparin (UFH), low-molecular-weight heparin (LMWH) such as enoxaparin (Clexane), and ultra-low-molecular-weight heparin (ULMWH) such as fondaparinux sodium (Arixtra). Due to their ability to bind to antithrombin III (AT-III), heparins have anticoagulant activity. AT-III undergoes a conformational change that allows it to interact with coagulant enzymes such as thrombin (factor IIa) and factor Xa. UFH functions well as an anticoagulant but adverse bleeding episodes can occur while UFH is used in antithrombotic therapy. This is due to its high antithrombin (anti-factor IIa) activity. UFH can also cause heparin-induced thrombocytopenia (HIT), a life-threatening complication, and allergic reactions. LMWHs, such as enoxaparin, also react with factor Xa but react much less with thrombin (factor IIa), which is why they are less apt to cause bleeding. Adverse bleeding, HIT, and interference with platelets can still occur with the use of enoxaparin. ULMWHs, such as fondaparinux sodium, have the highest anti-factor Xa activity and the lowest anti-factor IIa activity compared to UFH and LMWHs. This results in the lowest rate of bleeding among heparins and potent anticoagulant activity (Table 1) (*8*). Fondaparinux is a chemically synthesized pentasaccharide with highly potent anti-factor Xa activity. Since this pentasaccharide is too short to bridge AT-III to thrombin, fondaparinux has little anti-factor IIa activity and thus a markedly reduced risk of causing hemorrhaging (Table 1). As with enoxaparin, fondaparinux is administered subcutaneously; since it has a half-life of 17-21 hr, it is given once a day. Fondaparinux is metabolized principally in the kidneys and is contraindicated in patients with renal impairment. One advantage of fondaparinux is that the dosage does not need to be adjusted for weight or age because of its pharmacokinetics, *i.e*. its specific binding to anti-thrombin and its almost 100% bioavailability. Fondaparinux is administered subcutaneously in a dose of 2.5 mg once a day. Fondaparinux has several disadvantages. Unlike UFH, fondaparinux has no antidote. It has a long half-life and it accumulates in patients with renal insufficiency. Some immune mediators can affect the absorption, activity, and metabolism of fondaparinux (*8*).

As previously reported, enoxaparin was used in ophthalmology to treat 7 cases of RVO and 1 case of an orbital vein occlusion (*9*). In all 8 cases of venous thrombosis, the thrombus resolved and vision improved in 6 of 8 cases. The treatment lasted from weeks to months. There was one episode of spontaneous bleeding in an arm muscle requiring surgical drainage that did not affect the positive outcome of orbital thrombosis treatment.

Table 1. Comparison of the anti-Xa and anti-IIa activity of UF heparin, enoxaparin, and fondaparinux

Items	Anti-Xa (U mg-1)	Anti-IIa (U mg-1)
UF heparin	193	193
Enoxaparin	105	27
Fondaparinux	805 ± 27	< 0.1

IIa, factor IIa; Xa, factor Xa

Since LMWHs can resolve RVOs and have a minimal risk of causing HIT and bleeding, and also require constant platelet monitoring, the current study sought to evaluate an ULMWH, fondaparinux, with a markedly decreased risk of hemorrhaging and no need for platelet monitoring, in the treatment of RVOs. Presented here are the results of the use of fondaparinux, together with intravitreal anti-VEGF agents or steroids, in the treatment of RVOs.

2. Materials and Methods

Since March 2012, the current authors have seen 15 consecutive patients with recent-onset RVOs. All of the patients had complete eye examinations including the Snellen VA test (best-corrected), a slitlamp examination, Goldmann tonometry, and a dilated fundus examination. They also underwent fluorescein angiography to verify the diagnosis of an occlusion and to evaluate the extent of retinal ischemia. Ocular coherent tomography (OCT) was done to measure macular edema and, when necessary, peripheral retinal edema. At around the same time, all of the patients were seen by an internist for a complete medical evaluation, an electrocardiogram (EKG), and routine blood work including kidney function tests. Two patients had mild renal failure and were excluded from this study. Once RVO was diagnosed and the medical evaluation was complete, the remaining 13 patients received a through explanation of their condition and the risks involved in the experimental use of fondaparinux to resolve the RVO. Written consent was obtained before beginning treatment.

Thirteen patients began treatment with fondaparinux 2.5 mg a day. Two patients elected to discontinue the fondaparinux and seek treatment at other facilities, 1 after 5 months and 1 after 6 months of treatment. Dropouts were not due to complications. The remaining 11 patients continued treatment and have been followed to date. The patients were asked to avoid physical

Table 2. Clinical characteristics of patients and treatment outcomes

Pat	Age/sex	Type/I	Onset	Duration	VAb	VAa	OCTb	OCTa	Intravit	status
1	35M	CRVO	1d	4.5mo	2/10	10/10	473	205	dexam	open
2	78F	BRVO	10d	3.5mo	3/10	9/10	341	185	bevac	open
3	78M	I-CRVO	21d	4mo	1/30	1/30	601	153	aflib	open
4	45F	I-CRVO	2d	8mo	1/10	1/10	705	261	dexam	open
5	61M	CRVO	1d	5.5mo	10/10	10/10	260	223	none	open
6	73M	CRVO	1mo	1.5mo	5/10	7/10	524	194	aflib	open
7	82F	CRVO	1.5mo	2.5mo	1/30	4/10	510	236	dexam	open
8	74F	BRVO	35d	2mo	1/30	4/10	491	163	dexam	open
8a	74F	BRVO	14d		1/30		995		dexam	closed again
9	66F	BRVO	21d	15mo	8/10	10/10	310	213	none	partially open
10	76F	I-BRVO	NK	6mo	9/10	10/10	215	219	none	not open
11	64F	CRVO	1d	13.5mo	5/10	8/10	743	228	none	open

Aflib, aflibercept; bevac, bevacizumab; BRVO, branch retinal vein occlusion; CRVO, central retinal vein occlusion; dexam, dexamethasone; Duration, duration of treatment with fondaparinux; I, ischemic; NK, not known; OCTb, macular thickness in microns (um) according to OCT before treatment; OCTa, macular thickness in um according to OCT after treatment; Onset, time between onset of venous occlusion and the initiation of treatment; VAb, visual acuity before treatment; VAa, visual acuity after treatment.

activity or aggressive sports that could lead to bleeding.

3. Results and Discussion

Patients consisted of 4 males and 7 females with an average age of 66.5 yr and a range of 35-85 yr. There were 7 CRVOs and 4 BRVOs in these 11 patients. Of the 11 treated RVOs, 9 resolved. Of the remaining 2, 1 (Patient 9 in Table 2) resolved in the center of the macula but the periphery remained occluded with peripheral edema on OCT and clinical signs of an intraretinal hemorrhage and the other (Patient 10) did not change so peripheral blockage remained but there was no central edema. These 2 patients elected to continue treatment. Two of the CRVOs were ischemic and were treated with pan retinal argon laser photocoagulation, and 1 of the BRVOs was ischemic and was treated with sectoral retinal argon laser photocoagulation of the ischemic areas. The time from onset of the RVO to the beginning of therapy was 1-2 days in 4 patients, 10 days to 5 weeks in 6 patients, and unknown in 1 patient (Table 2)

Fondaparinux 2.5 mg was subcutaneously administered once a day to all 13 of the original patients. There were no episodes of systemic or ocular bleeding. The patients underwent eye examinations, including a VA test and fundus examination, every 2 weeks while being treated. Of the 11 patients who continued treatment, 7 received an intravitreal injection of steroids or other anti-VEGF agent to treat macular edema. One of the 7 required a second injection after 4 months because of recurrent macular edema. The drug that was intravitreally injected depended on its availability and the physician's medical judgement. Four received a dexamethasone intravitreal implant (Ozurdex), 2 received 0.05 cc of aflibercept (Eylea), and 1 received 0.05 cc of bevacizumab (Avastin). Three patients did not receive intravitreal injections because they did not have severe enough macular edema to justify an injection. One patient refused the injection (Patient 11). Arixtra was suspended 24 hr before each injection and restarted the same day 1-2 hr after the intravitreal injection. In all 11 patients, the fundus was examined every 2 weeks to evaluate whether the blockage resolved or not. Since the intravitreal injections resolved macular edema, the fundus was examined to ascertain a decrease in intraretinal bleeding and improvement in VA as an indication that the blockage had resolved. If clinical signs of a blockage were absent, Arixtra was reduced to every other day for 2 weeks and then suspended while waiting for a second OCT and fluorescein angiography. OCT and fluorescein angiography were performed, albeit not always immediately, to determine if the blockage had resolved or not. The rapid resolution of intraretinal bleeding, absence of new macular edema, and improvement in VA were evident clinically and served as indicators that the blockage had resolved. Of the 2 patients in whom a retinal vein was still occluded, one (Patient 9) had macular edema that resolved after 11 mo of therapy but peripheral edema persisted and was followed with OCT. This same patient with an occluded retinal vein (Patient 9 in Table 2) weighed 120 kilos at the start of treatment and 100 kilos at 12 months. Two patients developed collateral vessels at the optic nerve head before the occlusions resolved while being treated with fondaparinux (one dropped out at 5 months and the other was Patient 6). One patient (Patient 8) with a BRVO had an occlusion that resolved after 2 months with visual improvement, resolution of intraretinal bleeding, and resolution of macular edema. The therapy was gradually suspended and the patient was started on aspirin once a day. After 2.5 months on aspirin, the patient (Patient 8a) suddenly developed vision loss. A new BRVO occurred in the same area and the same therapy with fondaparinux 2.5 mg was started again. After 6 months of this treatment, the retinal vein remains occluded.

Nine of the RVOs resolved, 2 remained occluded, and 1 of the 9 that had resolved later recurred. Of the occlusions that resolved, 3 resolved in 1.5 to 2.5 months and the others took longer, with 1 resolving after 13.5 months. In the occlusions that recurred, the blockage remained after 6 months of therapy (Table 2). The patients were examined every 2 weeks while being treated. When clinical signs of an occlusion were absent, fondaparinux 2.5 mg was given every other day for 2 weeks and the patient was re-examined before stopping treatment. An OCT and fluorescein angiography were performed, albeit not always immediately, to verify that the occlusion resolved. The 2 patients who had occlusions that failed to resolve have BRVOs. One patient (Patient 9) had no macular edema but still had peripheral intraretinal bleeding and retinal edema after 15 months according to OCT and another (Patient 10) similarly had no macular edema but still had peripheral intraretinal bleeding and retinal edema after 7 months. Both patients elected to continue treatment. Macular edema improved in 1 patient (Patient 9) and, the patient elected to continue treatment because of amblyopia in the other eye.

Fondaparinux, an ULMWH, appears to be effective in resolving recent-onset RVOs and restoring VA without causing episodes of bleeding or requiring platelet monitoring. Nine patients out of the 11 treated had an occlusion that resolved, but the treatment took several months. One occulusion resolved after 13.5 mo. Of the 9 eyes with an occlusion, 7 had visual improvement and 2 with macular ischemia did not. Macular edema needs to be treated while waiting for an occlusion to resolve in order to restore VA. Macular edema can be treated with intravitreal steroids or injections of anti-VEGF agents. Anti-VEGF agents are not known to have anticoagulative effects in a vein occlusion but may have other benefits. Large amounts of VEGF are released in ischemic vein occlusions (*10*). This creates macular edema and anti-VEGF agents can be used to treat that edema (*6,7*). However, studies in primates have indicated that VEGF induces capillary endothelial cell proliferation within blood vessels, and particularly in veins, leading to intussusceptions and endothelial cell wall bridging within venules in some areas and vascular closure and non-perfusion in others (*11*). Inhibition of VEGF in the eye should reduce or prevent VEGF-induced intraluminal capillary endothelial cell proliferation and thus prevent a VEGF-induced reduction in venous flow. This would prevent the continued propagation of venous ischemia after the original vein occlusion and decrease the need for collateral vessel formation. Thus, the current study administered anti-VEGF agents while waiting for the occlusions to resolve. Treating edema hampers the clinical evaluation of when an RVO has resolved. Regular fundus examinations must be performed to follow an intraretinal hemorrhage or the recurrence of macular edema in order to determine when an occlusion has resolved. OCT and fluorescein angiography were used to help decide when the occlusion resolved. Once the RVO resolved, the edema and intraretinal hemorrhage rapidly resolved. Once the occlusion has resolved, fondaparinux should be stopped gradually every other day for 2 weeks before its suspension and the injections of anti-VEGF agents can be suspended. The authors' experience has been that suddenly stopping enoxaparin occasionally results in recurrence of an RVO within weeks in about 5% of patients (unpublished data). Therefore, once an RVO had resolved enoxaparin was administered every other day for 2 weeks in order to evaluate the risk of occlusion recurring before enoxaparin was completely suspended. The same approach was used for fondaparinux. Fondaparinux 2.5 mg was administered every other day for 2 weeks before it was completely stopped. When an RVO was treated with enoxaparin, occlusion recurred after its suspension. This occurred in Patient 8 2.5 months after fondaparinux was suspended and aspirin was given. In that patient, a blockage developed in the same area of the retina. Collateral vessels at the optic nerve head were noted in 2 patients. This is not surprising because the treatment to resolve an occlusion took several months in most of the current patients. Collateral vessel formation is a natural ocular response to blocked venous flow. The venous system takes a number of months to recover with fondaparinux and a natural ocular response can be expected in the interim.

Fondaparinux did not cause severe bleeding. UFH is an anticoagulant because of its effect on factor Xa but it has a high incidence of hemorrhaging because of its effect on factor IIa, in addition to its risk of HIT and platelet complications. Enoxaparin is an anticoagulant with a similar effect on factor Xa but a markedly reduced effect on factor IIa, with a much reduced incidence of hemorrhaging as well as HIT or platelet complications. Fondaparinux has a negligible effect on factor IIa, and therefore less of a risk of hemorrhaging, while having a potent effect on factor Xa. This means that fondaparinux has a potent anticoagulant effect in a venous occlusion (Table 1) (*8*). Platelet monitoring is not necessary, though it has been used in cases of HIT. Because of the bioavailability of fondaparinux, only 1 dose of 2.5 mg is administered subcutaneously per day. Fondaparinux should not be administered to patients with renal insufficiency (*8*).

The final VA may depend on the duration of macular edema and the presence of macular ischemia. It may also depend on the time from the onset of an occlusion to the beginning of treatment. In the current patients, the time from the onset of an occlusion to the beginning of treatment was 1 day to 5 weeks, though that period was not known in Patient 10. Improvement in VA and the extent to which an occlusion resolves may differ with a longer time before the initiation of treatment.

In conclusion, fondaparinux appears to be effective at resolving recent-onset RVOs without causing hemorrhaging. Macular edema and retinal ischemia also need to be treated while waiting for the occlusion to resolve. Patients need to be seen frequently in order to determine when to suspend treatment.

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Brief Report

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Cytokine expression profiles in the sera of cutaneous squamous cell carcinoma patients

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Summary **We focused on the interaction of cytokines in squamous cell carcinoma (SCC), and determined the expression profile of multiple cytokines in the serum of each patient with SCC in the present study. Serum samples were obtained from 12 SCC patients and 7 normal subjects. Four cytokines (IFN-γ, IL-6, GM-CSF, and TGF-β) were selected because they are reported to be involved in keratinocyte proliferation and SCC progression. Serum levels were measured using ELISA. We found a statistically significant increase of serum IFN-γ levels in SCC patients compared to those in normal subjects, and areas under the curve (AUC) of 0.82 for the serum levels of IFN-γ were higher than those for other cytokine levels according to ROC curve analysis. Patients with increased IFN-γ levels had a significantly more severe cancer stage. Furthermore, the combination of IFN-γ levels and TGF-β levels could improve the AUC to 0.84. We also found there was a significant correlation between IFN-γ levels and GM-CSF levels or between GM-CSF levels and TGF-β levels only in SCC patients. Our results suggest that the combination of IFN-γ levels and TGF-β levels is more effective to diagnose SCC, while serum levels of IFN-γ alone are useful to evaluate tumor progression. Furthermore, expression of these cytokines was not independent, but may be regulated by common upstream factors (*e.g.* cytokines or methylation) in SCC patients, and such factors may play some roles in the pathogenesis of SCC.**

Keywords: Squamous cell carcinoma, IFN-γ, IL-6, GM-CSF, TGF-β

1. Introduction

Squamous cell carcinoma (SCC) is one of the most frequent skin neoplasms. Compared to SCC seen in many other organs including esophagus, lungs and urinary bladder, cutaneous SCC is characterized by its strong correlation with cumulative ultraviolet exposure. Most cutaneous SCCs are usually low risk and treatable, but they have potential to recur and metastasize when they progress. To date, SCC antigen (SCC-Ag), tumor antigen that was originally purified from SCC, is the only reliable serum marker to diagnose SCC or to monitor the progress of the tumor. However, SCC-Ag has a drawback that the serum levels usually remain within normal limits at their early stage whereas they start to elevate only at the late stage (*1*). Therefore, it is mandatory to develop novel diagnostic methods for early detection and new therapeutic strategies. However, the pathogenesis of this malignant tumor is still to be clarified.

At present, several cytokines such as IFN-γ, IL-6, GM-CSF, and TGF-β have been reported to be involved in keratinocyte proliferation of the skin, and in the pathogenesis of SCC (*2-6*). In this study, we focused on the interaction of cytokines, and showed the expression profile of multiple cytokines in the serum of each patient with SCC.

2. Materials and Methods

2.1. *Clinical assessment and patient material*

Serum samples were obtained from 12 SCC patients (6

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Table 1. Clinical and laboratory features of 12 patients with SCC

Age	Gender	Location	Diameter (cm)	Stage	SCC antigen (ng/mL)	IFN-γ (pg/mL)	IL-6 (pg/mL)	GM-CSF (pg/mL)	TGF-β (ng/mL)	IFN-γ + TGF-β
70	M	face	0.5	0	1.0	20.5	59.0	19.6	1.6	22.1
91	F	face	0.7	0	1.0	16.6	59.0	21.0	2.1	18.7
83	F	lip	1.5	I	1.1	21.0	73.7	25.3	2.4	23.4
85	F	nose	1.5	I	0.8	24.9	125.1	26.8	2.5	27.4
89	M	lip	1.5	I	2.2	25.8	110.5	14.3	2.0	27.8
73	F	jaw	2.9	II	1.9	45.0	176.4	24.4	1.9	46.9
71	M	lip	4.5	II	1.6	52.5	165.4	27.2	2.6	55.1
83	M	lip	2.0	II	1.0	125.9	172.7	84.1	2.8	128.7
77	M	face	2.5	II	1.4	35.9	70.1	26.8	2.3	38.2
75	M	face	5.0	III	2.0	48.5	70.1	32.5	2.1	50.6
79	F	lip	2.0	III	3.2	8.3	95.8	29.2	2.3	10.6
64	F	head	8.0	III	0.5	8.7	143.5	24.4	2.4	11.1

M, male; F, female; SCC, squamous cell carcinoma; IFN-γ, interferon-γ; IL-6, interleukin-6; GM-CSF, granulocyte macrophage colony-stimulating factor; TGF-β, transforming growth factor-β.

males and 6 females; age range, 64-91 years) (Table 1). Control serum samples were obtained from 7 normal subjects with seborrheic keratosis. Institutional review board approval and written informed consent were obtained according to the Declaration of Helsinki.

2.2. *Statistical analysis*

Statistical analysis was carried out with Mann-Whitney's *U* test for the comparison of medians, and Fisher's exact probability test for the analysis of frequency. Correlations were assessed using Pearson's correlation coefficient. *p* values less than 0.05 were considered significant.

3. Results and Discussion

First, we measured the serum concentrations of multiple cytokines by ELISA to determine a cytokine profile for SCC sera. Four cytokines were selected because they are reported to be involved in keratinocyte proliferation of the skin, and in the pathogenesis of SCC (2-6). As a result, we found a statistically significant increase of the serum IFN-γ levels in SCC patients compared to normal subjects (Figure 1a). On the other hand, there was no significant difference in levels of the other cytokines between SCC patients and normal subjects (Figures 1b-1d).

Next, we performed receiver operating characteristic (ROC) curve analysis to evaluate the usefulness of the concentration of each cytokine for diagnosis of SCC. The areas under the curve (AUC) of 0.82 (95% CI, 0.61 to 1.03) for serum levels of IFN-γ (Figure 2a) was higher than those for other cytokine levels (Figures 2b-2d), indicating that serum IFN-γ might serve as a more useful biomarker for differentiating SCC patients and normal subjects than other cytokines. Furthermore, the combination of IFN-γ levels and TGF-β levels could improve the AUC to 0.84, suggesting that the combination is more effective to diagnose SCC.

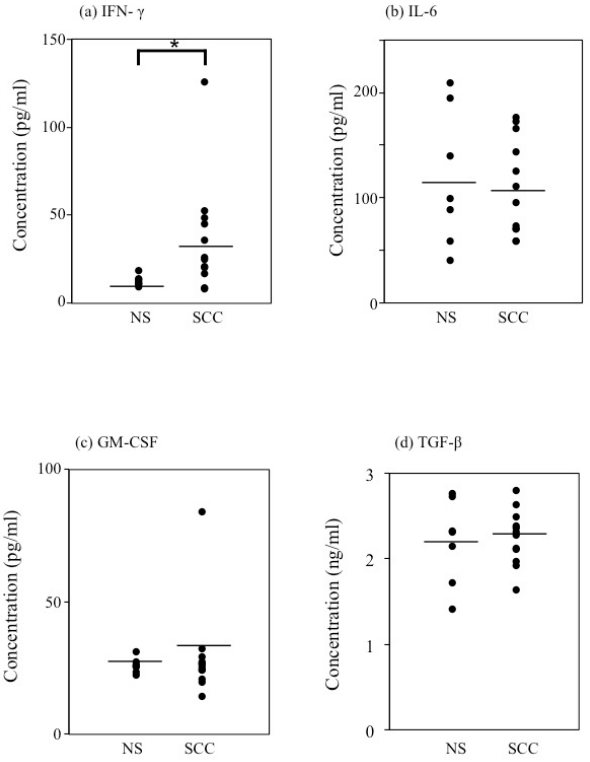

Figure 1. The concentrations of four cytokines (IFN-γ, IL-6, GM-CSF, and TGF-β) measured by ELISA using sera of normal subjects (NS) and SCC patients are shown on the ordinate. Bars show means. * $p < 0.05$.

Also, we examined the correlation among the levels of four cytokines. We could not find significant correlation among them in normal subjects, but there was mild and significant correlation between IFN-γ and GM-CSF ($r = 0.89$, $p < 0.01$) or between GM-CSF and TGF-β ($r = 0.64$, $p = 0.03$) in SCC patients (Figure 3). Therefore, expression of these cytokines was not independent in the sera of SCC patients.

When the cut-off value was set at mean + 6SD of normal subjects (= 30.4 pg/mL), serum IFN-γ levels were increased in 5 of 12 SCC patients. Patients with increased IFN-γ levels tended to have a significantly

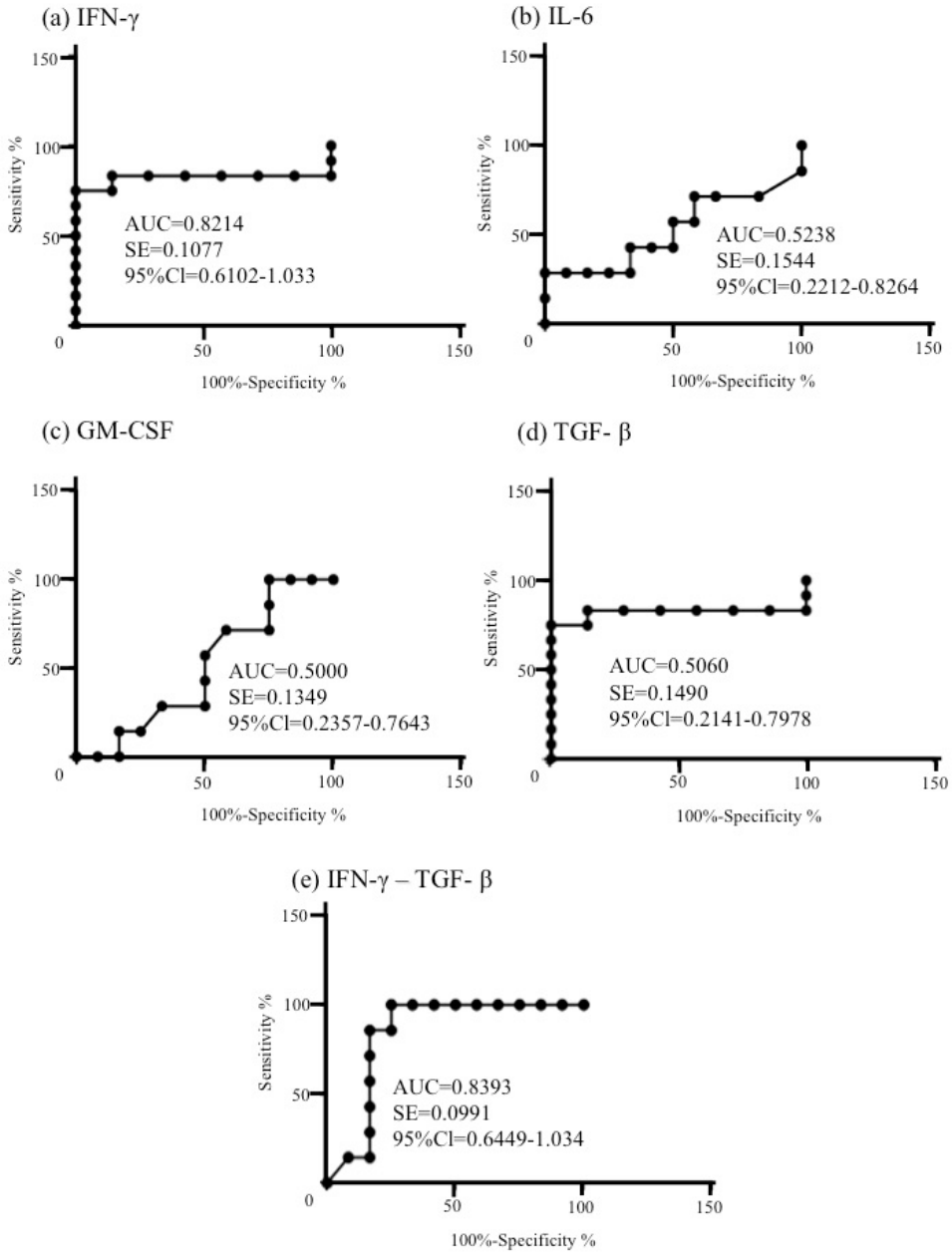

Figure 2. Receiver operating characteristic (ROC) curve for serum levels of indicated cytokines to distinguish SCC patients from normal subjects. AUC, areas under curves: SE, standard error: CI, confidence interval.

more severe cancer stage (II and III) than those with normal levels (100% vs. 28%, $p < 0.05$, Table 2).

Serum SCC-Ag was increased (> 1.5 ng/mL) in 1 of the 3 stage I patients, 2 of the 4 stage II patents, and 2 of the 3 stage IV patients (Table 1). Of note, increased serum IFN-γ levels were seen in all of the 4 stage II patients and 1 of the 3 stage IV patients. These results suggest that serum IFN-γ levels serve as a biomarker of tumor progression from early to middle stage, reflecting its contribution to tumor growth of SCC.

On the other hand, patients with increased IFN-γ levels also had increased levels of IFN-γ and TGF-β combination (> mean + 6SD, see Table 1), and analysis of correlation of the combination with clinical manifestations showed the same result as Table 2. Thus, the clinical significance of IFN-γ and TGF-β combination to evaluate tumor progression seems to be similar to that of IFN-γ alone in SCC patients.

In this study, we presented two novel findings: first, we found a statistically significant increase of serum IFN-γ levels in SCC patients compared to those in normal subjects. Patients with increased IFN-γ levels had a significantly more severe cancer stage. IFN-γ levels tended to be increased around stage II in SCC patients. Furthermore, the AUC of the combination of IFN-γ levels and TGF-β levels was higher than that of IFN-γ alone according to ROC curve analysis. Given that patients with increased levels of IFN-γ also showed increased levels of IFN-γ and TGF-β combination, our results suggest that the combination of IFN-γ levels and

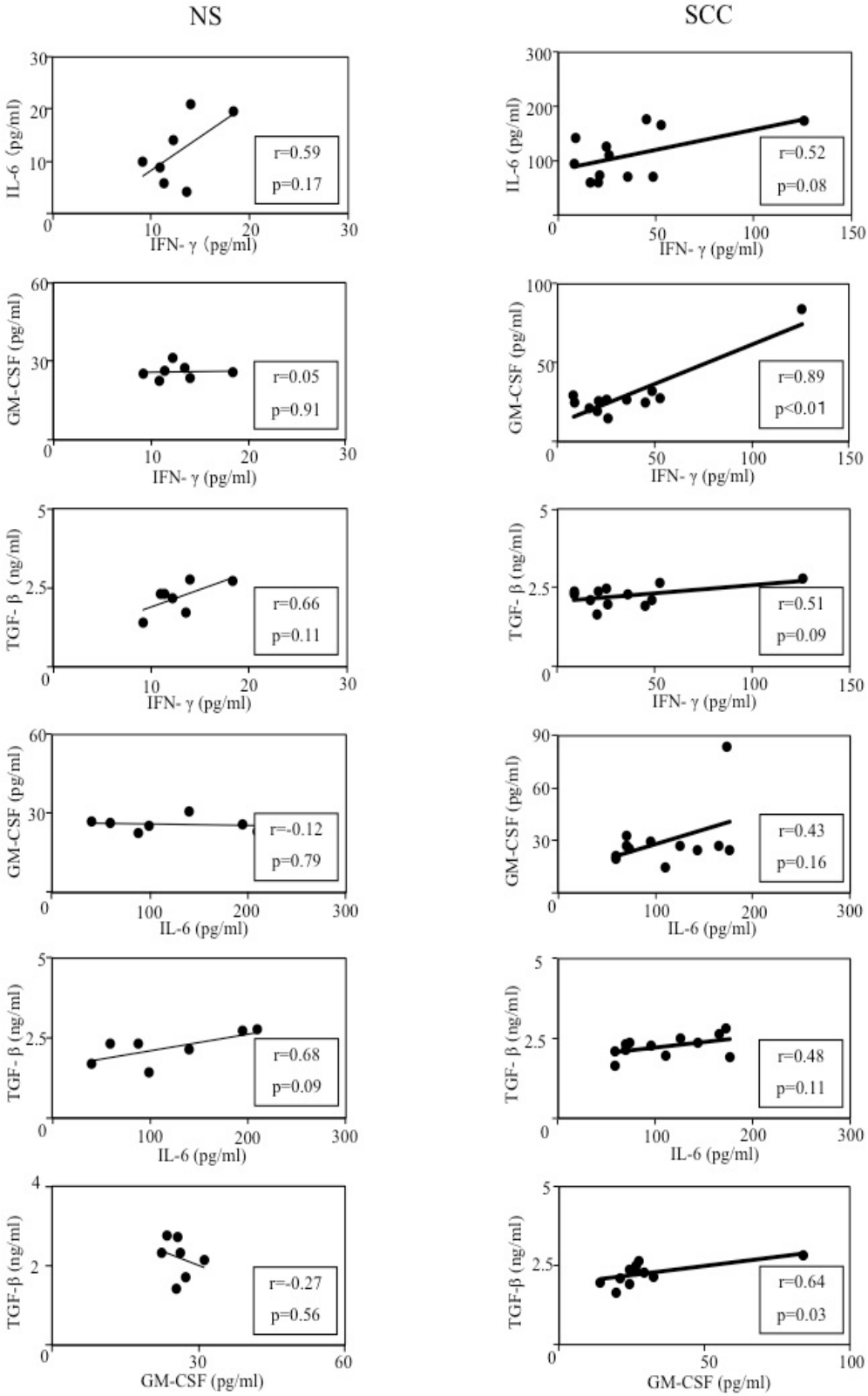

Figure 3. Correlation among the levels of four cytokines (IFN-γ, IL-6, GM-CSF and TGF-β) in each individual of normal subjects (NS) and SCC patients. Correlations were assessed by Pearson's correlation coefficient.

TGF-β levels is more effective to diagnose SCC, while serum levels of IFN-γ alone is sufficient to evaluate tumor progression from early to middle stage.

Second, we found there was significant correlation between IFN-γ levels and GM-CSF levels or between GM-CSF levels and TGF-β levels only in SCC patients. Therefore, expression of these cytokines was not independent, but regulated by common upstream factors (*e.g.* cytokines or methylation) in SCC patients, and such factors may play some roles in pathogenesis

Table 2. The association of serum IFN-γ levels with clinical and serological features of patients with SCC

Items	Patients with normal Interferon-γ levels (n = 7)	Patients with increased Interferon-γ levels (n = 5)
Age at the time of serum sampling (mean years)	80.14	75.80
Gender (M:F)	2:5	1:4
Clinical features		
Mean diameter (cm)	2.89	3.38
% of patients with stage II/III	28.57	100*
Laboratory features		
SCC antigen (ng/mL)	1.40	1.58

* $p < 0.05$ versus patients with normal IFN-γ levels using Fisher's exact probability test.

of SCC. Further studies are also needed to determine whether the upstream factors are the key molecules in SCC.

This is the first report focusing on the interaction of multiple cytokines, and demonstrating their expression profile in each patient with SCC. We suggest the possibility that the balance among multiple cytokines contribute to the pathogenesis of SCC, and indicate its clinical significance. This is a pilot study with a small number of patients. Although we could not find statistically significant correlation between cytokine levels and specific features of SCC (*e.g.* location or diameter), this may be because of the small patient number. Lager studies are needed in the future. Clarifying the involvement of the cytokine network in pathogenesis of SCC may lead to development of new diagnostic tools or new therapeutic strategies, and may contribute to the understanding of the mechanism of SCC.

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Case Report

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Three episodes of non-arteritic posterior ischemic optic neuropathy in the same patient treated with intravenous prostaglandin E1

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Summary **Non-arteritic posterior ischemic optic neuropathy (NA-PION) is a disorder involving reduced blood flow to the retrobulbar portion of the optic nerve. This disorder usually develops acutely, and research has suggested that high-dose steroid therapy soon after the onset of visual loss can result in significant visual improvement. This treatment, however, is not universally successful. The addition of a potent vasodilator could help to restore ocular blood flow. This case report describes the use of prostaglandin E1 (PGE1), a powerful vasodilator of the microcirculation, to treat three separate episodes of NA-PION over five years in the same patient. A 68-year-old white male was first seen in June 2009 with NA-PION in the left eye, and the condition was treated with steroids and PGE1. The patient had a subsequent episode in July 2010 that was treated with steroids and PGE1 and another in May 2014 that was treated with PGE1 alone. Visual acuity improved from 4/10 to 11/10 in 2009, from 4/10 to 11/10 in 2010, and from 5/10 to 10/10 in 2014. No complications due to the use of PGE1 were noted. PGE1 should be considered as a treatment for NA-PION to immediately restore blood flow and potentially improve vision.**

Keywords: Non-arteritic posterior ischemic optic neuropathy, prostaglandin E1, ophthalmic artery, central retinal artery, color Doppler imaging, hereditary hemochromatosis

1. Introduction

Posterior ischemic optic neuropathy (PION) is a disorder involving reduced blood flow to the retrobulbar portion of the optic nerve. PION usually develops acutely and can be classified as surgical, arteritic, or non-arteritic (*1,2*). The use of high-dose systemic steroids to treat non-arteritic PION (NA-PION) improves visual acuity (2). Steroid therapy is not universally successful.

Prostaglandin E1 (PGE1), a powerful vasodilator of the microcirculation, improves ocular blood flow in patients with peripheral vascular disease or diabetes (*3*). Intravenous PGE1 at a dose of 1 μg/kg and steroids have been used to treat acute non-arteritic anterior ischemic optic neuropathy (NA-AION) and acute arteritic AION (AAION) (*4,5*). Since NA-PION is a form of ischemia, PGE1 may help.

This case report describes 3 episodes of NA-PION in the same patient over 5 years that were successfully treated with IV PGE1 and steroids. The first episode and treatment in 2009 were previously reported (*6*). This case report includes blood flow measurements and color Doppler imaging of the ophthalmic artery and the central retinal artery before and after the first treatment. Over 5 years, the same patient had 2 more episodes of NA-PION; one was successfully treated with PGE1 and steroids and the other was treated with PGE1 alone. The results of color Doppler imaging and these 2 subsequent episodes are described here for the first time.

2. Case Report

A 68-year-old white male was first seen in June 2009

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eight hours after a loss of visual acuity in his left eye (*6*). The right eye was amblyopic and vision was 2/10 in the right eye and 4/10 in the left eye. The patient was being treated for hereditary hemochromatosis. The rest of the ocular examination, including an examination of the optic nerve heads, was normal. A medical examination was performed, initial blood work was done, and an electrocardiogram (EKG) was performed; results revealed no other problems like giant cell arteritis. NA-PION in the left eye was preliminarily diagnosed and 50 milligrams (mg) of prednisone was immediately given by mouth. The following morning, 50 mg of prednisone was again given by mouth and color Doppler imaging (CDI) was performed on the retina and optic nerve of both eyes. The blood flow velocity of the central retinal artery as it enters the eye, of the ophthalmic artery as its branches enter the sclera, and of the ophthalmic artery prior to branching was normal in the right eye and markedly reduced in the left eye (Table 1). Blood flow in the nasal and temporal posterior ciliary arteries was markedly reduced in the left eye but normal in the right eye. The patient weighed 70 kg. After a through explanation and written consent, the patient was given 60 μg of PGE1 intravenously over 2.5 h. After 1 h, the patient noted a gradual restoration of visual acuity in his left eye. CDI was performed immediately after the first intravenous treatment, revealing a marked increase in blood flow in the vessels of the left eye and a minimal increase in the blood flow in the vessels of the right eye. Blood flow in the left eye was still inferior to that in the right eye (Table 1). The next day, visual acuity remained the same in the right eye at 2/10 and improved to 11/10 in the left eye. The retina and optic nerve head were still normal and the patient was administered 25 mg of prednisone per day for 5 days. Three days after the first infusion of PGE1, CDI of the retrobulbar circulation indicated improvement from the original imaging, but a modest reduction in the flow velocity of the left eye was still present. The patient was intravenously administered 60 μg of PGE1. A Humphrey central 30-2 visual field test 1 week post-treatment indicated peripheral scotomas in both eyes. A thorough vascular and medical workup were done immediately after the second infusion. On follow-up visits over the course of a year, visual acuity in the left eye remained 11/10 with no further treatment (*6*).

In July 2010, 13 months after the first episode, the patient again awoke with decreased visual acuity in his left eye. He was seen 4 hours after onset and he had a Snellen visual acuity of 2/10 in his right amblyopic eye and a visual acuity of 4/10 in his left eye. The rest of the ophthalmic examination was normal, with no swelling of the optic nerve heads. Results of initial medical examinations were normal. The patient was immediately given 50 mg of prednisone by mouth and 60 μg of PGE1 intravenously over 2.5 h, and this treatment was repeated the following morning. Twenty-five mg of prednisone was administered for 5 days. The patient noted restored vision the afternoon immediately after the first treatment. After the second intravenous administration, vision was 2/10 in the right eye and 11/10 in the left eye. A medical workup revealed no giant cell arteritis. Follow-up ophthalmic visits were normal.

Table 1. Color Doppler imaging of the ocular vessels before and after PGE1

Items	Right Eye	Left Eye
CRA before PGE1	28/8 cm/sec	6/0 cm/sec
CRA after PGE1	30/16 cm/sec	18/8 cm/sec
OA anteriorly before PGE1	33/12 cm/sec	11/2 cm/sec
OA anteriorly after PGE1	36/16 cm/sec	19/8 cm/sec
OA retrobulbar before PGE1	22/10 cm/sec	7/0 cm/sec
OA retrobulbar after PGE1	24/14 cm/sec	16/6 cm/sec

CRA, central retinal artery; OA – anterior portion, branches of the ophthalmic artery entering the sclera; OA – posterior portion, the ophthalmic artery prior to branching.

On May 29, 2014, the patient (now 73 years of age) again awoke in the morning with a loss of visual acuity in the left eye. Five hours later, vision was 2/10 in the right eye and 5/10 in the left eye. He had mild cataracts in both eyes. The retina and optic nerve heads were normal. Within 1 hour of the eye examination, 60 μg of PGE1 was administered intravenously without steroids. That afternoon, the patient noticed visual improvement and the following morning 60 μg of PGE1 was administered intravenously without steroids. A week later, visual acuity was 2/10 in the right eye and 10/10 in the left eye. The level of vision in the left eye coincided with a mild cataract. A Humphrey central 24-2 visual field test 1 week post-treatment indicated peripheral scotomas in both eyes without central scotomas. Two years later, at the time of this report, the patient's vision has not changed.

3. Discussion

PION is a disorder involving reduced blood flow to the retrobulbar portion of the optic nerve (*1,2*). This condition was noted in the current patient because CDI revealed a reduced blood flow in the ophthalmic artery and central retinal artery of the left eye before treatment with PGE1 in 2009 (Table 1). There was no edema of the optic nerve head, a finding that corroborates the diagnosis of PION. As soon as ischemia was evident, *i.e*. 24 h after initial symptoms, intravenous PGE1 was immediately started. PGE1 is a potent vasodilator of the microcirculation (*3*). CDI immediately after the first administration of PGE1 revealed marked improvement in the ocular and retrobulbar blood flow (Table 1). Along with the increased blood flow, visual acuity also improved immediately after PGE1 in 2009. During the second and third episodes of visual loss over the next 5 years, visual acuity improved immediately after

treatment with intravenous PGE1. Since NA-PION is ischemia affecting the retrobulbar portion of the optic nerve, the use of a potent vasodilator, PGE1, could be crucial to immediately re-establishing blood flow. In all 3 episodes in the current patient, a through medical workup immediately following treatment was normal except for hereditary hemochromatosis and mild peripheral vascular disease. The optic nerve heads were never swollen and no other signs of giant cell arteritis were noted. Intravenous PGE1 was immediately begun after all 3 episodes without other examinations. Basic blood work and an EKG can be done immediately without delaying treatment. An extensive medical and ocular workup was not done before therapy with PGE1 since a delay in starting treatment could presumably lead to prolonged ischemia and possible permanent damage to the optic nerve. PGE1 has previously been reported to improve visual acuity in cases of NAION and AAION (*4,5*). In those cases, it was also given shortly after basic blood work and an EKG in order to immediately treat ischemia and avoid permanent damage to the optic nerve.

One intravenous infusion of PGE1 improves peripheral blood flow for up to 4 weeks in patients with peripheral vascular disease (*3*). Because of this prolonged effect of PGE1, PGE1 was given only two times in each of these 3 epi. Repeated administration could lead to systemic hypotension. To avoid this, 1 μg/kg of PGE1 was only administered intravenously twice after each episode. This patient weighed 70 kg and was treated with 60 μg of PGE1 after each episode.

PGE1 is a safe, potent vasodilator of the peripheral vascular system (the microcirculation or capillary system) that is used to treat patients with signs and symptoms of peripheral vascular disease such as intermittent claudication (*3,7*). Two days of intravenous administration of PGE1 causes vasodilatation of the capillary system that can last for 4 weeks or longer in patients with peripheral vascular disease. PGE1 is well tolerated with few side effects and can be used in patients who are hypotensive. The systemic blood pressure needs to be monitored frequently (every 15-20 min) during intravenous administration (*3,7*). The main mechanism of action of PGE1 is *via* vasodilatation of the microcirculation. PGE1 acts directly on the smooth muscle of the vascular wall, leading to vascular dilatation and increased blood flow. This vasodilatation varies depending on the anatomical location and is dose-dependent. PGE1 is also known to inhibit platelet aggregation (*8*). In addition to the known effects of PGE1 on platelet aggregation, fibrinolysis, blood flow, and viscosity, PGE1 also inhibits monocyte and neutrophil function, suggesting it has anti-inflammatory action as well. Research on gene expression has suggested that several genes in vascular smooth muscle cells and fibroblasts are modified by PGE1 at the transcriptional level. This may contribute to tissue protection in ischemic areas (*9*). These factors together promote an increase in the capillary flow. PGE1 is rapidly metabolized by oxidation during passage through the pulmonary circulation. It is excreted in the urine as metabolites within about 24 hours (*8*). This rapid elimination also contributes to its safety.

Steroids, in the form of prednisone, were immediately given to the current patient and were continued orally for a total of 7 days after the first 2 episodes but not after the third. The use of high-dose systemic steroids in a non-randomised retrospective study to treat NA-PION resulted in a significant improvement in visual acuity in comparison to untreated patients (*2*). Steroids in the same study did not significantly improve visual acuity in patients with arteritic or surgical PION. Another study has suggested the use of systemic corticosteroids in the form of oral prednisone to treat NAION (*10*). Steroid therapy is, however, not universally accepted. A more recent study using high-dose systemic steroids to treat acute NAION noted no visual or anatomic benefit and several serious complications from steroids (*11*). However, steroids were used in the current case for another reason, i.e. to try to reduce ischemia-reperfusion (I-R) injury. Ischemia leads to tissue hypoxia, depletion of energy-rich phosphates, accumulation of metabolic waste products including reactive oxygen species, and cellular edema, all of which may cause cellular injury (*12,13*). The immediate resumption of blood flow is needed to prevent further tissue damage but reperfusion itself may cause further tissue damage and reperfusion injury. Infiltrating leukocytes are thought to play a major role in I-R injury (*12,13*), and I-R injury was the reason why prednisone was used with PGE1 after the first 2 episodes in the current patient. Steroids were not used after the third episode and their non-use did not appear to have a negative effect. After treatment following the third episode, visual acuity in the left eye was 10/10, which coincides with a cataract in that eye, and the central visual field of the left eye did not appear to be compromised after treatment.

After the first and third episodes, a Humphrey central visual field test 1 week post-treatment indicated peripheral scotomas in both eyes. The visual field of the right eye was worse than that of the left eye, but the fact that the right eye was amblyopic could account for this. A central scotoma was not found in the visual field of the left eye, as is usually noted in NA-PION. However, at the time of the visual field test the patient had already been treated with PGE1, which improved visual acuity to 11/10. Optic disc pallor was not noted on follow-up visits. This also could be explained by the early treatment.

Since NA-PION is ischemia affecting the retrobulbar portion of the optic nerve, the use of a potent vasodilator could be crucial to immediately re-establishing blood flow. In current case, 3 episodes of NA-PION were promptly treated with PGE1, leading to immediate visual

improvement without causing any systemic or ocular side effects. Steroids may protect against I-R injury but their use needs to be evaluated further.

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