Original article **P73**

Vasorelaxant activity and inhibitory effects on leukocyte function of a flavanoid compound from *Kaempferia Parviflora*

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Abstract

The rhizomes of *Kaempferia parviflora (K. parviflora)* (Zingiberaceae) have been used in Thai traditional medicine for gastrointestinal disorders, pain, allergy, and inflammation. The alcoholic infusion of its rhizome has also been used as a tonic for rectifying male impotence. Ethanolic extract of *K. parviflora* was reported to exhibit acute anti-inflammatory activities in the rat paw edema model (unpublished data) and vasorelaxant activity in isolated rat aortic rings. The purposes of this study were to investigate the effects of a flavanoid compound from *K. Parviflora*, (VR-F6/1) on vasorelaxant activity, the expression of ICAM-1 on human umbilical vein endothelial cells (HUVEC), CD62-L and Mac-1 on neutrophils, human neutrophil functional responsiveness and T-lymphocyte proliferation. VR-F6/1 was primarily investigated for its cytotoxic effects on HUVECs, neutrophils and T-lymphocytes using XTT cytotoxicity assay. The vasorelaxant activity of VR-F6/1 was assessed using myography technique. The effects of VR-F6/1 on fMLP-induced L-selectin shedding and Mac-1 expression on neutrophils were assessed using flow cytometry. Human neutrophil functional responsiveness was determined by measuring fMLP-induced chemotaxis, superoxide anion generation (SAG), and MPO release. PHA-induced-T-lymphocyte proliferation was quantified by [H^3] thymidine incorporation. Viability of HUVECs, neutrophils, and T-lymphocytes were not significantly affected by VR- F6/1 (1-100 μg/ml) (IC_{50}>1000 μM). VR-F6/1 concentration-dependently caused acute relaxation of isolated mouse mesenteric arteries. VR-F6/1 (10 μM) inhibited ICAM-1 expression on HUVECs, and inhibited fMLP-induced L-selectin shedding and Mac-1 surface expression on neutrophils. VR-F6/1 concentration-dependently inhibited fMLP-induced chemotaxis, SAG, and MPO production in neutrophils. VR-F6/1 also significantly inhibited PHA-induced T-lymphocyte proliferation in a concentration-dependent manner. These findings suggest that anti-inflammatory activity of VR-F6/1 might be, in part, attributable to inhibition of ICAM-1 expression on HUVEC, inhibition of CD62L shedding and Mac-1 surface expression on neutrophils, neutrophil chemotaxis and SAG as well as T-lymphocyte proliferation. This study also demonstrates an acute vasorelaxant activity of VR-F6/1 where nitric oxide may mediate the effect.

Keywords: *K.Parviflora*, vasorelaxation, ICAM-1, neutrophil chemotaxis, superoxide anion generation, MPO, T-lymphocyte proliferation.

Introduction

The rhizomes of *Kaempferia parviflora* (Zingiberaceae) have been used in Thai traditional medicine for gastrointestinal disorders, pain, allergy, and inflammation. The alcoholic infusion of its rhizome has also been used as a tonic for rectifying male impotence. Ethanolic extract of *K. parviflora* was reported to exhibit acute anti-inflammatory activities in the rat paw edema model (unpublished data) and vasorelaxant activity in isolated rat aortic rings. *K. parviflora* contains many flavonoids that possess anti-inflammatory, anti-allergic and anti-oxidant activities. In this study, therefore, the effects of VR-F6/1 on TNFα-induced ICAM-1 expression on human umbilical vein endothelial cells (HUVEC), neutrophil

functional responsiveness including, fMLP-induced L-selectin shedding and Mac-1 expression, chemotaxis, SAG, and MPO production as well as PHA-induced T-lymphocyte proliferation were investigated in order to elucidate the cellular mechanisms of its anti-inflammatory activity. In addition, the effect of VR-F6/1 on vasorelaxant activity was also investigated.

Materials and Methods

Plant

The isolation of VR-F6/1 from ethyl acetate extract of *K. Parviflora* was performed by silicagel column chromatography using ethyl acetate and hexane as eluents. The derived fractions isolated from column chromatography were re-chromatographed and followed by crystallization in order to obtain the pure compounds.

Isolation of human neutrophils

Human neutrophils (PMNs) were isolated by Percoll density gradient centrifugation. Venous blood obtained from healthy donors, was mixed with an equal volume of Percoll, and the mixture centrifuged at 280*g* for 20 min. After centrifugation, PMN were harvested and washed. Any contaminating red cells were removed. The cells were >99% viable as determined by trypan blue exclusion and were resuspended as required.

Isolation of human T-lymphocytes

Human PBMC were isolated by Ficoll-Hypaque centrifugation. T-lymphocytes were isolated from PBMC by Rosetting with neuraminidase-treated sheep red blood cells (SRBC). PBMC were mixed with neuraminidase-treated SRBC and FCS, incubated then centrifuged. PBMC/FCS/SRBC mixture were layered over Ficoll-Hypaque and centrifuged. The E-rosette-positive population (T-cells) was separated from SRBC by hypotonic lysis. The cells were > 95% viable as determined by trypan blue exclusion.

Cytotoxicity assay (1)

VR-6/1 was primarily investigated for its cytotoxic effects on neutrophils, T-lymphocytes and HUVECs. Human neutrophils, T-lymphocytes and HUVECs were incubated with VR-F6/1 (0.1-10 μM) for 4 h, 3 days and 12h, respectively in 96-well plates, then XTT was added and incubated at 37°C for an additional 4 h. The XTT formazan product was measured spectrophotometrically at 450 nm.

Determination of Neutrophil Chemotaxis (2)

Human neutrophil chemotaxis can be assessed by measuring of neutrophil migration across the polyvinylcarbonate filter using chemotaxis chamber. The bottom wells of the chamber were filled with fMLP. The top plate with the installed filter was placed onto the filled bottom plate, and upper wells were filled with neutrophils treated with VR-F6/1 (0.1-10 μM). After incubation, the filter removed, washed, fixed and stained with DiffQuik TM. Chemotaxis was quantified spectrophotometrically by measuring absorbance at 550 nm. Indomethacin was used as a reference compound.

Determination of Superoxide Anion Generation (SAG) (3)

Neutrophil SAG is determined by spectrophotometric evaluation of the reduction of ferricytochrome C to ferrocytochrome C in the presence of cytochalasin B**.** Neutrophils resuspended in PBS containing cytochrome C/cytochalasin B were preincubated with VR-F6/1 (0.1-10 μM) before stimulating with fMLP. The reaction was terminated followed by centrifugation. The absorbance of the supernatant from each tube was measured at 550 nm. Indomethacin was used as a standard reference compound.

Determination of Myeloperoxidase (MPO) production (4)

Neutrophils were preincubated with VR-F6/1 (0.1-10 μM) before stimulating with fMLP. After centrifugation, supernatants were incubated with the reaction mixture of

3,3',5,5'-Tetramethylbenzidine (TMB) supplemented with H_2O_2. The reaction was then stopped by adding H_2SO_4 and absorbance was measured spectrophotometrically at 450 nm. Indomethacin was used as a reference compound.

Determination of adhesion molecule expression on human neutrophils (5)

Neutrophils were preincubated with VR-F6/1 (0.1-10 μM) in incubator before stimulating with fMLP. After incubation, neutrophils were stained with CD62L-PE antibody and CD11b/Mac-1-APC antibody. The analysis of immunofluorescence as a measure of L-selectin (CD62L) and Mac-1 (CD11b/CD18) surface expression was performed on a FACS Calibur flow cytometer.

In vitro culture of human umbilical vein endothelial cells (HUVECs) and determination of ICAM-1 expression

HUVECs were cultures in 6-well plate in endothelial cell growth medium at 37ºC with 35% CO_2 in a humidified incubator. HUVECs incubated with VR-F6/1 (0.1-10 μM) for 10 h, then activated with TNF-α (50 ng/ml). Cells were trypsinized and counted, and then incubated with 2μl of antibody CD54-PE. The reaction was stopped by adding PBS. Cells were washed and the expression of ICAM-1 was measured using flow cytometry.

T-lymphocyte proliferation assay

T-lymphocytes were incubated with VR F6/1(0.1-100 μM) in the presence of 0.001% (w/v) PHA for 3 days at 37ºC with 5% CO_2. After incubation, the cells were labeled with [^{3}H] thymidine (0.5mCi/well). After a further incubation, the cells were harvested, washed, dried using Cell Harvester and radioactivity thymidine incorporation was determined by liquid scintillation counting.

Vasorelaxant activity

The vasorelaxant activity of VR-F6/1 was investigated using myography technique. Male mice were killed by cervical dislocation. The segments of third order branches of the mouse superior mesenteric arteries were removed, cut into rings (1.5 mm long) and mounted in a myograph chamber for measuring the isometric wall tension. The chamber was filled with physiological salt solution maintained at 37ºC and continuously bubbled with a 95% O_2 and 5% CO_2 mixture. After equilibration, the arteries were set to a circumference 90% of that obtained when the arteries were stretched. The rings were precontracted with phenylephrine and tested for the prescence of functional endothelium. The vasorelaxant effect of VR-F6/1 was tested by precontracting arteries with phenylephrine and then cumulatively adding VR-F6/1 ($3x10^{-8}$–$3x10^{-5}$M) to induce a concentration-dependent response. Relaxation is expressed as the percentage of decrease in the maximal tension induced by phenylephrine.

Results

Cytotoxic effects

The viability of neutrophils, T-lymphocytes and HUVECs was not affected by VR-F6/1 (0.1-10 μM) (IC_{50} > 1000μM, n=6).

Neutrophil Superoxide Anion Generation (SAG)

VR-F6/1 exerted strong inhibitory effects on fMLP-induced human neutrophil SAG (IC_{50} of 4.1±1.3 μM) compare with that of indomethacin (0.1-10μM), IC_{50} of 33.8± 6.2 μM (Fig.1).

Neutrophil chemotaxis

VR-F6/1 significantly suppressed fMLP-induced human neutrophil chemotaxis in a concentration-dependent manner with an IC_{50} of 3.23±1.1 μM. Indomethacin (0.1-100μM) exerted strong inhibition of fMLP-induced human neutrophil chemotaxis, with IC_{50} of 1.1 ± 0.3 μM (Fig.2)

Neutrophil MPO production

VR-F6/1 exerted very weak inhibitory effect on MPO production in human neutrophils induced by fMLP (IC_{50}>1000μM). Indomethacin (0.1-10μM) caused strong inhibition of fMLP-induced MPO production in human neutrophil, with IC_{50} of 42.9± 5.2 μM.

Neutrophil adhesion molecules expression

Activation of neutrophil by fMLP caused L-selectin shedding and increase in Mac-1 surface expression. VR-F6/1 at 10 μM statistically significant inhibited shedding of L-selectin and Mac-1 surface expression (Table 1).

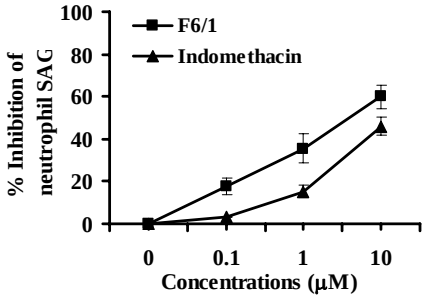

Figure 1. Inhibitory effects of VR-F6/1 (0.1-10μM) on fMLP- induced-SAG in human neutrophils Results are mean±S.E.M of 5 experiments using cells from different donors.

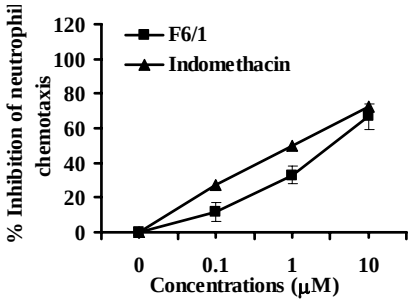

Figure 2 Inhibitory effects of VR-F6/1(0.1-10μM) on fMLP-induced chemotaxis in human neutrophils.Results are mean± S.E.M of 5 experiments using cells from different donors.

Intercellular adhesion molecule-1 (ICAM-1)

Activation of HUVECs by TNF-α (50ng/ml) caused an increase in ICAM-1 expression and this increase was suppressed by VR-F6/1 (1-10 μM) in a concentration–dependent manner (Table 2).

Table 1. Inhibitory effects of VR-F6/1 on fMLP-stimulated shedding of CD-62L (A) and Mac-1 surface expression on neutrophils (B). Results are expressed as means±S.E.M. of 3 independent experiments. *p<0.05 indicates a significant difference from fMLP- stimulated cells.

Concentration (μM)	A. Relative intensity (CD62L)			B. Relative intensity (Mac-1)		
	Negative	Positive	VR-F6/1	Negative	Positive	VR-F6/1
-	30.3±2.1	5.3±0.8	-	4.3±0.9	51.1± 3.4	-
1	-	-	6.8±1.6	-	-	47.1±2.4
10	-	-	12.0±3.2*	-	-	40.9± 3.4*

Table 2. Inhibitory effects of VR-F6/1 on TNF-α stimulated ICAM-1 expression in HUVECs. Results are expressed as means±S.E.M. of 3 independent experiments. *p<0.05 indicates a significant difference from fMLP- stimulated cells.

Concentration (μM)	Relative intensity (ICAM-1)		
	Negative	Positive	VR-F6/1
-	2.1±0.4	51.2±4.6	-
1	-		40.2± 4.4*
10	-		25.2± 3.8*

T-lymphocyte proliferation

VR-F6/1(0.1-100 μM) caused a concentration-dependent inhibition of PHA-induced T-lymphocyte proliferation with IC50 of 6.3 ± 0.4 μM (Fig. 3).

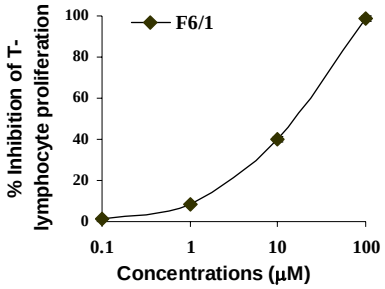

Figure 3. Log concentration-effect curves for inhibition of PHA-induced T-lymphocyte proliferation by VR-F6/1. Results are the mean ± S.E. mean of 5 different donors.

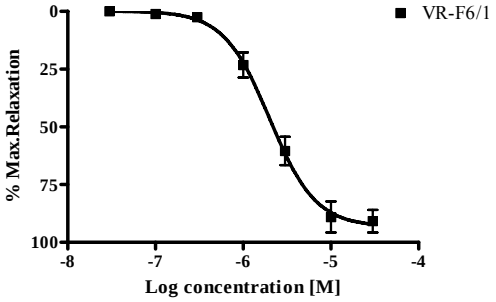

Figure 4. The concentration–dependent relaxant effect of VR-F6/1 on mesenteric artery precontracted by phenylephrine (10^{-5}M). Relaxation is expressed as the percentage of decrease in the maximal tension induced by phenylephrine. Results are the mean ± S.E. mean (N=4).

Vasorelaxant activity

VR-F6/1 ($3x10^{-8}$–$3x10^{-5}$M) produced a concentration-dependent relaxation of the phenylephrine-preconstricted mouse mesenteric arterial rings with endothelium-intact. The maximal relaxation and EC_{50} of VR-F6/1 were 90.7±3.8 % and 2.06±0.3μM, respectively, n=4, (Fig.4).

Discussion and Conclusion

In acute inflammation, neutrophils rapidly migrate to the site of injury and die through apoptosis during the resolution of inflammation. Chronic inflammation is characterized by infiltration of mononuclear cells. The activation of lymphocytes causes cytokine production and produces persistent inflammatory response. Neutrophil recruitment, into inflamed tissue proceeds via multi-step processes. Neutrophil emigration through endothelial cells involves several adhesion processes including cell rolling, arrest, and transmigration. Rolling is mediated by selectins, while arrest and transmigration both require activated integrins (LFA-1 (CD11 a/CD18) and Mac-1 (CD11 b/CD18). During neutrophil activation, L-selectin, which initially has a high basal expression, is rapidly down-regulated and causes up-regulated Mac-1 surface expression. Integrins and selectin ligands are also able to signal into the cell, where they initiate neutrophil extravasation, promote cytoskeletal rearrangement and ultimately induce superoxide production and degranulation.

VR-F6/1 (10μM) significantly inhibited fMLP-induced L-selectin shedding and an increase in Mac-1 surface expression. TNF-α –induced ICAM-1 expression on HUVECs was also inhibited by VR-F6/1. Pre-treatment of neutrophils with VR-F6/1 (0.1-10μM) significantly inhibited fMLP-induced chemotaxis and SAG. VR-F6/1 (0.1-100μM) also inhibited PHA-induced T-lymphocyte proliferation in a concentration-dependent manner. These findings suggest that the anti-inflammatory activity of VR-F6/1 from *K. Parviflora* extract may be attributed to its inhibitory effects on neutrophil functions and T-lymphocyte proliferation. However, further investigation is required to define the underlying molecular mechanisms of the inhibitory effects of this compound on neutrophil and T-lymphocyte function. Penile erection involves relaxation of smooth muscle of the corpus cavernosum and its associated arterioles. The alcoholic infusion of its rhizome has been used as a tonic for rectifying male impotence. This study demonstrated an acute, significant vasorelaxant activity of VR-F6/1 where nitric oxide may mediate the effect. Interestingly, its vasorelaxant activity may be one of the contributing factors for improving impotence problem in men.

However, further investigation to elucidate molecular mechanisms for its vasorelaxant activity is required.

Acknowledgements

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