Inhibitory Effect of Diarylheptanoids on Nitric Oxide Production in Activated Murine Macrophages

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Thirteen novel diarylheptanoids bearing a chalcone or a flavanone moiety (1–13), a new curcumin derivative, 1,2-dihydrobis(de-O-methyl)curcumin (14), and two known flavonoids (15 and 16) isolated from the seeds of *Alpinia blepharocalyx* K. Schum. were tested for their inhibitory effects on nitric oxide (NO) production in lipopolysaccharide (LPS)-activated murine macrophages J774.1 *in vitro*. All the tested compounds inhibited NO production in a concentration-dependent manner (IC_{50} =36–568 μ M). Among the compounds examined, blepharocalyxin B (13) was the most potent inhibitor of NO production (IC_{50} =36 μ M). Analysis of the structure activity relationship among these novel diarylheptanoids led to the conclusion that the position of attachment of a chalcone or a flavanone to a diarylheptanoid does not affect their inhibitory potency although their presence in association causes a substantial enhancement of the inhibitory activity. Moreover, a conjugated double bond in a chalcone moiety potentiated the inhibitory activity. On the other hand, hexamethoxydeoxycalyxin A (17) and pentamethoxycalyxin B (18), a methylated product of calyxin A (1) and an epimeric mixture of calyxin B, showed greatly reduced activity suggesting that phenolic hydroxyl groups are involved in the inhibitory activity.

Key words Diarylheptanoid; nitric oxide (NO) production; macrophage; calyxin; blepharocalyxin

In inflammatory diseases, macrophages are activated to produce a variety of mediators such as cytokines, oxyradicals and nitric oxide (NO) radicals. The NO radicals act as a mediator, a messenger, or a regulator of cell function in a number of physiological systems¹⁾ and pathophysiological states.²⁾ However, excessive production of NO can also destroy functional normal tissues during acute and chronic inflammation.³⁾ This, therefore, suggests that inhibition of chronic NO production in macrophages could lead to potential anti-inflammatory drugs.

In our search for biologically active substances from traditional Chinese medicines, we recently reported several novel diarylheptanoids, named calyxins A–H, epicalyxins B–D and blepharocalyxins A, B, from the seeds of *Alpinia blepharocalyx*.⁴⁾ In this series of compounds, calyxins A (1), B (2), F (9), H (11) together with epicalyxin B (3) and 6-hydroxycalyxin F (10) are diarylheptanoids with a chalcone moiety, while calyxins C (4), D (6), E (8) and epicalyxins C (5), D (7) have a flavanone moiety. On the other hand, blepharocalyxins A (12) and B (13) possess two diarylheptanoids joined by a chalcone moiety.

Diarylheptanoids have interesting biological and pharmacological properties such as anti-inflammatory,⁵⁾ antitumorigenic⁶⁾ and antihepatotoxic activity.⁷⁾ Curcumin, a well investigated typical diarylheptanoid, is capable of scavenging a variety of reactive oxygen species,⁸⁾ including NO.⁹⁾ Similarly, flavonoids have also been shown to be good scavengers of free radicals and to act as natural anti-oxidants.¹⁰⁾ In this context, Kröl *et al.* have shown that flavones inhibit production of NO in murine macrophages.¹¹⁾ On the other hand, polyphenols have been reported to inhibit the formation of 3-nitrotyrosine in peroxynitrite-modified collagen.¹²⁾

The compounds (1–13) possess both diarylheptanoid and flavonoid structural units. In the belief that the novelty of the structure found in these diarylheptanoids could have a good biological profile, we carried out further investigation to

evaluate the role of these novel diarylheptanoids in the regulation of NO release in activated murine macrophage J774.1 cells.

MATERIALS AND METHODS

Materials The compounds (1–16) were isolated from the seeds of *A. blepharocalyx* and 17 and 18 were obtained by methylating calyxin A (1) and an epimeric mixture (1 : 1) of calyxin B.⁴⁾ The murine macrophage cell line, J774.1, was obtained from the Riken Cell Bank, Japan and lipopolysaccharide (LPS) WE. Coli 055:B5 (Difco Laboratories, Detroit, U.S.A.) was used throughout this study. Phosphate-buffered saline (PBS), RPMI 1640 medium, and heat-inactivated foetal calf serum (FCS) were purchased from Nissui Pharmaceutical Co., Ltd., Tokyo, Japan. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma, St. Louis, U.S.A.

Cell Culture The cells were maintained continuously in a 75 cm² plastic culture flask (Falcone, Becton Dickinson, NJ, U.S.A.) in RPMI-1640 supplemented with penicillin (100 units/ml), streptomycin (100 μ g/ml) and 10% FCS. Cells were harvested by gentle scraping and passed every 3–4 d by dilution of a suspension of the cells in fresh medium.¹³⁾

Macrophage populations were enriched by adherence to plastic in 24-well plates (Falcone, Becton Dickinson, NJ, U.S.A.) with a calculated 10⁶ macrophages per well and allowed to adhere for 4 h. Thereafter, the medium was replaced with fresh medium or medium containing LPS (10 μ g/ml) and/or the test compounds, and the cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂ for 48 h. In all experiments, each concentration of test sample was examined in triplicate.

Nitrite Assay Nitrite concentrations in the culture medium were measured as an indicator of NO production by

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Chart 1.

a microplate assay method based on the Griess reaction.¹⁴⁾ Equal volumes of culture medium supernatant and Griess reagent (0.5% sulfanilamide, 0.05% naphthylene-diamide dihydrochloride in 2.5% H₃PO₄) were added to Eppendorf tubes and left for 10 min. Absorbance was read at 560 nm and the nitrite concentration in the medium was determined with sodium nitrite as a standard. Data are expressed as the mean $\pm$ S.E. of triplicates. Statistical significance was evaluated using Student's *t*-test.

Cell Viability Cell viability was assessed by the MTT method. Cells (5×10^3 cells/well) in 96-well plates were exposed to test samples at 37 °C in a humidified atmosphere containing 5% CO₂ for 48 h. After completion of the drug treatment, 10 μ l MTT (5 mg/ml) and fresh medium were added to each well. After incubation for 4 h at 37 °C, medium was removed and the crystals of viable cells were solubilized with 100 μ l dimethylsulfoxide. The absorbance of each well was then read at 540 nm using an ELISA plate reader.

RESULTS AND DISCUSSION

Stimulation of cells with LPS (0.1–10 $\mu\text{g/ml}$) led to NO production, which resulted in the accumulation of nitrite ion in the medium (Fig.1). This NO production from LPS-activated macrophages was inhibited by diarylheptanoids and flavonoids in a concentration-dependent manner (Table 1). Their inhibitory activities were much higher than those of dexamethasone, a known inhibitor of inducible NO synthase.¹⁾ Furthermore, compound **14** showed a comparatively stronger inhibitory effect than that of curcumin. Among the compounds examined, blepharocalyxins A (**12**), B (**13**) and calyxin B (**2**) showed stronger inhibitory activity (IC_{50} =39.0, 36.0, 39.0 μM , respectively) than their analogues without any cytotoxicity. In particular, blepharocalyxin B (**13**) was the strongest inhibitor of NO production, showing significant activity even at 11 μM .

The differences in inhibitory potency between calyxin A type and calyxin B type compounds (**1** vs. **2**, **3**, **11**; **8** vs. **4**–**7**) were less clear cut, suggesting that the position of attachment of a chalcone or a flavanone to a diarylheptanoid does not affect their inhibitory potency. However, the presence of both diarylheptanoid and flavonoid moieties is important for inhibitory activity, because the diarylheptanoid **14** and the flavonoids **15** and **16** exhibited much weaker inhibitory activity.

Another conclusion drawn from Table 1 is that helichrysetin (**15**, a chalcone) is a better inhibitor of NO production than 5-*O*-methylnaringenin (**16**, a flavanone) and accordingly, the diarylheptanoids with a chalcone moiety (**1**–**3**, **9**–**11**) exhibited stronger inhibitory activity than the corre-

sponding compounds with a flavanone moiety (**4**–**8**). This indicates that the conjugated double bond in the chalcone moiety contributes to the inhibitory activity. The role of phenolic hydroxyl groups in NO inhibition was found to be very substantial due to the fact that hexamethoxydeoxycalyxin A (**17**) and pentamethoxycalyxin B (**18**) exhibited very weak NO inhibitory activity under similar experimental conditions. Since there is a paucity of data, no relationship between the stereostructure of these compounds and their inhibitory activity could be deduced from the present study.

The present report shows that a series of diarylheptanoids (**1**–**13**) and, to a lesser extent, other phenolic compounds significantly inhibit LPS-induced NO production in macrophages. Thus, diarylheptanoids may have a role to play in modulating inflammatory and immune responses by affecting

Fig. 1. Dose-Dependent Effect of LPS on Nitrite Production in Macrophage J774.1 Cells

Cells were incubated for 48 h with increasing concentrations of LPS. Each value is the mean $\pm$ S.E. of triplicate measurements.

Table 1. Inhibitory Activity of **1**–**18** on Nitric Oxide Formation in Murine Macrophage J774.1 Cells Stimulated by LPS

Compound	Dose ($\mu\text{g/ml}$)	Inhibition %	IC_{50} (μM)	Compound	Dose ($\mu\text{g/ml}$)	Inhibition %	IC_{50} (μM)
1	100	97.23 $\pm$ 0.8	49	10	100	90.80 $\pm$ 6.80	51
	10	50.56 $\pm$ 2.44			10	36.70 $\pm$ 1.5	
	1	17.60 $\pm$ 4.0			1	ND	
2	100	96.60 $\pm$ 0.46	39	11	100	99.05 $\pm$ 0.55	62
	10	40.03 $\pm$ 1.50			10	33.33 $\pm$ 1.15	
	1	6.00 $\pm$ 0.5			1	18.83 $\pm$ 0.80	
3	100	78.30 $\pm$ 1.20	84	12	100	93.38 $\pm$ 2.80	39
	10	32.06 $\pm$ 3.11			10	26.54 $\pm$ 1.60	
	1	7.70 $\pm$ 0.10			1	ND	
4	100	96.40 $\pm$ 2.5	84	13	100	93.58 $\pm$ 1.15	36
	10	16.30 $\pm$ 2.1			10	26.65 $\pm$ 5.58	
	1	2.20 $\pm$ 0.7			1	ND	
5	100	80.73 $\pm$ 3.8	95	14	100	93.35 $\pm$ 3.00	181
	10	11.80 $\pm$ 1.60			10	8.73 $\pm$ 1.50	
	1	2.10 $\pm$ 0.60			1	ND	
6	100	82.35 $\pm$ 6.7	103	15	100	57.05 $\pm$ 1.50	159
	10	20.05 $\pm$ 2.5			10	30.02 $\pm$ 3.00	
	1	ND			1	ND	
7	100	93.80 $\pm$ 2.0	83	16	100	35.45 $\pm$ 1.40	568
	10	21.00 $\pm$ 1.7			10	9.05 $\pm$ 0.5	
	1	ND			1	ND	
8	100	97.50 $\pm$ 1.5	76	17	100	2.20 $\pm$ 1.4	
	10	27.55 $\pm$ 5.05			100	7.50 $\pm$ 3.0	
	1	6.00 $\pm$ 0.5			100	34.02 $\pm$ 3.5	
9	100	97.50 $\pm$ 1.65	51	Dexametha-	100		
	10	31.76 $\pm$ 0.08		sone			
	1	17.03 $\pm$ 2.50		Curcumin ^{a)}	100	77.70 $\pm$ 1.5	190
					50	31.80 $\pm$ 3.0	

Values are mean $\pm$ S.E. of triplicate measurements. Cells were incubated with a combination of endotoxin (10 $\mu\text{g/ml}$) and compounds for 48 h. Nitrite accumulation in the culture medium was determined using the Griess reaction. ND=not detected. ^{a)} No inhibition was detected with 1 and 10 $\mu\text{g/ml}$.

NO production. Studies to investigate whether these polyphenolic compounds act as direct inhibitors of NO synthase or act upon a control system are underway.

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