Evaluation on Pharmacological Activities of 2,4-Dihydroxybenzaldehyde

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Abstract – 4-Hydroxybenzaldehyde, a phenolic compound found in a variety of natural sources, was previously shown to contain anti-inflammatory and related anti-angiogenic and anti-nociceptive activities. The present work was designed to assess some pharmacological activities of 2,4-dihydroxybenzaldehyde (DHD), an analogue of 4-hydroxybenzaldehyde. DHD exhibited a significant inhibition in the chick chorioallantoic membrane (CAM) angiogenesis, and its IC₅₀ value was 2.4 µg/egg. DHD also contained *in vivo* anti-inflammatory activity using acetic acid-induced permeability and carrageenan-induced air pouch models in mice. In the air pouch model, DHD showed significant suppression in exudate volume, number of polymorphonuclear leukocytes and nitrite content. DHD showed an anti-nociceptive activity in the acetic acid-induced writhing test in mice. It also suppressed enhanced production of nitric oxide (NO) and elevated expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophage cells. It was able to slightly decrease the level of reactive oxygen species in the stimulated macrophages. DHD at the used concentrations couldn't modulate the viabilities of RAW264.7 cells. Taken together, like 4-hydroxybenzaldehyde, DHD contains anti-angiogenic, anti-inflammatory and anti-nociceptive activities.

Keywords: Anti-angiogenic, Anti-inflammatory, Anti-nociceptive, Cyclooxygenase-2, 2,4-dihydroxybenzaldehyde, Inducible nitric oxide synthase, Nitric oxide, RAW264.7, Reactive oxygen species

INTRODUCTION

2,4-Dihydroxybenzaldehyde (DHD, β -resorcyaldehyde, Fig. 1) is one of the phenolic components identified in various natural products, including *Cassia grandis* (González *et al.*, 1996) and acacia wood (de Simón *et al.*, 2009). It can also be synthesized and used to be a starting material for organic synthesis of a variety of useful substances (Pattekhon and Divakar, 2001; Yang and Meng, 2008). DHD was shown to contain antibacterial activity against the four pathogenic bacteria, such as *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica* (Friedman *et al.*, 2003). Its phosphate exhibited slow-binding inhibition on fructose biphosphate aldolase, which involved active-site Schiff-base formation

and required a phenol group ortho to the aldehyde moiety (Blonski *et al.*, 1997). DHD showed weak scavenging activities towards peroxynitrite and hydroxy radicals, potent oxidants that have a potentially causative effect on cancer and other deleterious pathogenesis of various diseases

2,4-Dihydroxybenzaldehyde (DHD).

Fig. 1. Chemical Structure of 2,4-dihydroxybenzaldehyde (DHD), a phenolic component of plant origin.

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(Yang *et al.*, 2001). Until recently, other biological activities of DHD have not been identified. 4-Hydroxybenzaldehyde, a DHD analogue and a major constituent of *Gastrodia elata* Blume, was shown to contain anti-angiogenic, anti-inflammatory and anti-nociceptive activities via down-regulating inducible nitric oxide synthase and cyclooxygenase-2 (Lim *et al.*, 2008a). In this following work, it is demonstrated that DHD also possesses considerable anti-angiogenic, anti-inflammatory and anti-nociceptive activities in *in vivo* and *in vitro* experimental models.

MATERIALS AND METHODS

Chemicals, fertilized eggs and experimental animals

2,4-Dihydroxybenzaldehyde (DHD), 3,4-dihydroxybenzaldehyde, 2,4,6-trihydroxybenzaldehyde, 3-bromo-4-hydroxybenzaldehyde, 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde, Evans blue, *E. coli* lipopolysaccharide (LPS), indomethacin and dexamethasone were obtained from Sigma Chemical Co. (St. Louis, MI, USA). 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) was from Invitrogen (Carlsbad, CA, USA). All other chemicals used were of reagent grade or better. Fertilized brown Leghorn eggs were purchased from Pulmuone Food Co., Seoul, Korea. Male ICR mice (male, 4-week-old, about 25 g) were obtained from Samtaco Animal Farm, Osan, Korea, and acclimated for one week prior to treatment. The animal room was maintained at $23 \pm 2^\circ\text{C}$ with a 12 h light/dark cycle. Food and tap water were supplied *ad libitum*. The ethical guidelines, described in the NIH Guide for Care and Use of Laboratory Animals, were followed throughout the experiments.

Cell culture

The RAW264.7 cells, a murine macrophage cell line, were from American Type Culture Collection (Manassas, VA, USA). The macrophage cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum (FBS), 25 mM HEPES (pH 7.5), 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. The mammalian cells were maintained in a humidified atmosphere containing 5% CO_2 , plated at a density of 1×10^6 and preincubated for 24 h at 37°C . For all experiments, the macrophage cells were grown to 80-90% confluence, and subjected to no more than 20 cell passages.

Chorioallantoic membrane (CAM) assay

Anti-angiogenic activity of DHD was determined using CAM assay (Song *et al.*, 2003) as follows. The fertilized eggs were kept at 37°C in a humidified egg incubator. After

3.5-day incubation, about 2 ml of albumen was aspirated from the eggs through the small hole drilled at the narrow end, permitting the small CAM and yolk sac to drop away from the shell membrane. The shell covering the air sac was punched out and removed by forceps, and the shell membrane on the floor of the air sac was peeled away. In the 4.5-day-old chick embryo, a DHD-loaded Thermanox coverslip was applied onto the CAM surface. Two days after returning the chick embryo to the incubator, an appropriate volume of 10% fat emulsion (Intralipose, 10%) was injected into a 6.5-day-old embryo chorioallantois. Using a score system under a microscope, the branching pattern of each egg was graded as 0, 1+ or 2+. Convergence of a few vessels toward the CAM surface was denoted as 1+, and 2+ reflected an increased density and length of vessels toward the CAM surface.

Acetic acid-induced vascular permeability test

According to a modification of the method of Whittle (1964), acetic acid-induced vascular permeability test was used to assess anti-inflammatory activity of DHD. One hour after oral administration of DHD (10, 30 or 100 mg/kg) or indomethacin (IND, 10 mg/kg), 0.1 ml/10 g body weight of 2% Evans blue solution was injected intravenously in each mouse. Ten min later, 0.1 ml/10 g body weight of 0.7% acetic acid in saline was intraperitoneally injected. Twenty min after the administration of acetic acid, the mice were sacrificed by cervical dislocation. After 10 ml of saline was injected into the peritoneal cavity, the washing solutions were collected in test tubes. The concentration of Evans blue in the washing solution was spectrophotometrically determined by the absorbance at 590 nm. The vascular permeability was represented in terms of the absorbance (A_{590}) which leaked into the peritoneal cavity.

Carrageenan-induced air pouch formation

According to a modification of the procedure of Ghosh *et al.* (2000), λ -carrageenan-induced air pouch formation was performed. Six days prior to the drug treatment, the air pouch was formed in the intrascapular region of rats by initial subcutaneous injection of 4 ml sterile air and successive injections of 2 ml sterile air every 3 days to sustain its patency (Sedgwick and Lees, 1986). On day 0, vehicle (2% Tween 80 in saline), DHD (0.03, 0.1 and 0.3 mg/pouch) or dexamethasone (DEXA, 0.01 mg/pouch) was administered into the pouch just prior to the λ -carrageenan injection (0.1 ml of 1.0% solution). After 16 hour, the pouch cavity was opened and the exudates were collected. The exudate volumes were measured using a graduate tube. Aliquots were diluted with Turk solution, and the poly-

morphonuclear leukocytes were counted in a standard hemocytometer chamber. Experiments were performed in triplicate.

Acetic acid-induced writhing response

An anti-nociceptive activity of DHD was assessed as previously described (Olajide *et al.*, 2000). The response to an intraperitoneal injection of acetic acid solution into mice, manifesting as a contraction of the abdominal muscles and stretching of hind limbs, was evaluated. Nociception was induced by intraperitoneal injection of 0.7% acetic acid solution at the dose of 0.1 ml/10g body weight. Each experimental group was orally treated with vehicle (1% CMC in saline), DHD (10, 30 or 100 mg/kg) or indomethacin (IND, 10 mg/kg) as a positive control. From 10 min later, the number of writhes during the following 10 min was counted.

Nitrite analysis

Accumulated nitrite (NO_2^-) in the culture media or the exudates obtained from air-pouch experiment was determined according to a colorimetric assay based on the Griess reaction (Sherman *et al.*, 1993; Kim *et al.*, 2009). The samples (100 μl), obtained after centrifugation, were mixed with 100 μl Griess reagent (6 mg/ml) at room temperature for 10 min, and then NO_2^- concentration was determined by the absorbance at 570 nm. The calibration curve was constructed using the known concentrations of sodium nitrite.

Determination of intracellular ROS

For determination of intracellular ROS level, the redox-sensitive fluorescent probe DCFH-DA was used as previously described (Royall and Ischiropoulos, 1993). After preincubation with DHD (0, 0.02, 0.05 mM) for 1 h, the RAW264.7 cells were treated with LPS for 24 h. Afterwards they were incubated with 5 μM DCFH-DA for 30 min at 37°C. The harvested cells were immediately analyzed by a flow cytometry.

Western analysis

The RAW264.7 macrophage cells were treated with LPS (1 $\mu\text{g/ml}$) in the presence or absence of DHD for 24 h and then washed twice with ice-cold phosphate-buffered saline (PBS). The mammalian cells were lysed in a buffer containing 20 mM HEPES (pH 7.9), 0.1 M KCl, 0.3 M NaCl, 10 mM EDTA, 1% SDS, 1 mM PMSF, 1 $\mu\text{g/ml}$ leupeptin and 1 $\mu\text{g/ml}$ pepstatin. For immunoblotting, anti-inducible nitric oxide synthase (anti-iNOS; Transduction Laboratories, Lexington, KY, USA), anti-cyclooxygenase-2 (anti-

COX-2; Transduction Laboratories, Lexington, KY, USA) and anti- β -actin (Sigma-Aldrich, St. Louis, MO, USA) antibodies were used.

MTT reduction assay

The cellular viability was quantified by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Freshney, 1994). Briefly, cells incubated with various concentrations of DHD were treated with 5 μl of MTT (Sigma-Aldrich Co., St. Louis, MI, USA) solution (5 mg/ml) for 2 h. The cells were then lysed with isopropyl alcohol, and the absorbance was read at the wavelength of 540 nm.

Statistical analysis

The results were expressed as mean $\pm$ S.E. Comparison between experimental groups was performed by ANOVA followed by the Tukey's multiple range tests. *p* values less than 0.05 were considered to be significant.

RESULTS AND DISCUSSION

Down-regulation of angiogenesis prevents neoplastic growth and inflammation. Some anti-angiogenic components are effective in animal models of inflammation models, while several anti-inflammatory drugs, such as indomethacin, methotrexate and corticosteroids, contain anti-angiogenic activities (Tong *et al.*, 2004). The CAM assay is *in vivo* used to measure both angiogenesis and anti-angiogenesis. Since 4-hydroxybenzaldehyde was previously found to contain an anti-angiogenic activity (Lim *et al.*, 2008a), anti-angiogenic activities of its five analogues, such as 2,4-dihydroxybenzaldehyde (DHD), 3,4-dihydroxybenzaldehyde, 2,4,6-trihydroxybenzaldehyde, 3-bromo-4-hydroxybenzaldehyde, 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde, were preliminarily examined using the CAM assay (Table I). As shown in Table I, DHD appeared to be most

Table I. Comparison of anti-angiogenic activities of 4-hydroxybenzaldehyde analogues

4-Hydroxybenzaldehyde analogues	IC ₅₀ ($\mu\text{g/egg}$)
2,4-Dihydroxybenzaldehyde	2.4
3,4-Dihydroxybenzaldehyde	13.9
2,4,6-Trihydroxybenzaldehyde	7.2
3-Bromo-4-hydroxybenzaldehyde	> 50
3,5-Di- <i>tert</i> -butyl-4-hydroxybenzaldehyde	30.6

Anti-angiogenic activities of five 4-hydroxybenzaldehyde analogues were determined using chick chorioallantoic membrane (CAM) assay.

effective among them. Accordingly, its anti-angiogenesis-related activities were elucidated.

After the 2-day treatment, retinoic acid, used as a positive control, at 1 $\mu\text{g}/\text{egg}$ exhibited an inhibition of 89.5% in the branching patterns of blood vessels (Fig. 2). When 0.1, 0.3 and 3.0 $\mu\text{g}/\text{egg}$ of DHD was applied onto the CAMs, the inhibition percentages in the CAM angiogenesis were 35.3%, 42.9% and 60.0%, respectively (Fig. 2). This clearly indicates that DHD contains an anti-angiogenic activity in a dose-dependent manner. The IC_{50} value of DHD was 2.4 $\mu\text{g}/\text{egg}$. Taken together, DHD contains an anti-angiogenic activity.

Though an anti-inflammatory activity of DHD has not been suggested so far, its *in vivo* anti-inflammatory activity has been assessed using the two *in vivo* models, vascular permeability and air pouch models. In a vascular permeability test as a typical model of the first stage inflammatory reactions, inflammatory mediators, released following sti-

Fig. 2. Dose-dependent anti-angiogenic activity of 2,4-dihydroxybenzaldehyde (DHD) in the chick embryo chorioallantoic membrane (CAM) assay. Retinoic acid (RA, 1 $\mu\text{g}/\text{egg}$) was used as a positive control. Each group contained at least 20 eggs. Each column represents mean $\pm$ S.E. of the three independent experiments. ** $p < 0.01$, *** $p < 0.001$. Compared with the control group.

mulation, lead to dilation of blood vessels and result in enhanced vascular permeability (Vogel and Vogel, 1997). DHD at the oral doses of 10, 30 and 100 mg/kg showed an inhibition of 16.7%, 27.9% and 45.2% on vascular permeability, respectively (Table II). An inhibitory effect of DHD at 100 mg/kg is comparable to that of indomethacin (10 mg/kg) used as a positive control (Table II). In the carrageenan-induced air pouch model, an excellent model of acute inflammation, fluid extravasation, leukocyte migration and biochemical parameters in the exudate can be easily detected. Injection of carrageenan into an air pouch on the dorsal surface of mice triggers an inflammatory process. In the carrageenan-induced air pouches, dexamethasone (DEXA, 0.01 mg/pouch), a nonselective cyclooxygenase inhibitor, reduced the exudate volume by 66.4% (Table III). Treatment with DHD at 0.03, 0.1 and 0.3 mg/pouch displayed an inhibition of 47.2%, 68.9% and 85.9%, respectively, as compared to the control exudate volume (Table III). Total numbers of the polymorphonuclear leukocytes in the air pouches were also decreased by DHD at 0.03, 0.1 and 0.3 mg/pouch, the inhibitory percentages of which were 26.3%, 58.6% and 73.8%, respectively (Table III). DHD contains an acute anti-inflammatory

Table II. Inhibitory effects of DHD in acetic acid-induced vascular permeability and writhing response in mice

Materials	Dose (mg/kg)	A ₅₉₀	Number of writhings
Control	-	1.06 $\pm$ 0.18	26.2 $\pm$ 1.30
DHD10	10	0.88 $\pm$ 0.11 (16.7)	13.50 $\pm$ 0.99 (48.4)
DHD30	30	0.76 $\pm$ 0.09 ^b (27.9)	9.33 $\pm$ 0.71 ^a (64.3)
DHD100	100	0.58 $\pm$ 0.07 ^b (45.2)	3.50 $\pm$ 0.67 ^a (86.6)
IND	10	0.65 $\pm$ 0.09 ^b (39.0)	4.50 $\pm$ 0.85 ^b (82.8)

Indomethacin (IND, 10 mg/kg body weight) was used as a positive control. DHD (10, 30 and 100 mg/kg body weight) was orally administered. Vascular permeability was represented by the absorbance at 590 nm. The results are expressed as mean $\pm$ S.E. Each group contained 8 mice. This experiment was performed in triplicate. Figures in parentheses indicate inhibitory percentage with respect to the control group treated only with 1% CMC in saline. ^a $p < 0.05$, ^b $p < 0.01$, compared with the control group.

Table III. Inhibitory effects of DHD on carrageenan-induced inflammation in the air pouch model

Group	Dose (mg/pouch)	Exudate (ml)	Total leukocytes ($\times 10^7$ cells)	Nitrite content (μM)
Control	-	2.12 $\pm$ 0.06	4.51 $\pm$ 0.32	6.24 $\pm$ 0.62
DHD0.03	0.03	1.12 $\pm$ 0.11 ^b (47.2)	3.32 $\pm$ 0.09 ^b (26.3)	5.82 $\pm$ 0.43 (6.7)
DHD0.1	0.1	0.66 $\pm$ 0.12 ^b (68.9)	1.87 $\pm$ 0.24 ^b (58.6)	4.88 $\pm$ 0.39 (21.7)
DHD0.3	0.3	0.30 $\pm$ 0.06 ^b (85.9)	1.18 $\pm$ 0.08 ^b (73.8)	2.59 $\pm$ 0.27 ^a (58.4)
DEXA	0.01	0.72 $\pm$ 0.07 ^b (66.04)	1.45 $\pm$ 0.04 ^b (67.7)	2.30 $\pm$ 0.19 ^b (63.1)

The results are expressed as mean $\pm$ S.E. of $n=8$. Figures in parentheses indicate inhibitory percentages with respect to the corresponding control group. Dose was expressed in mg/pouch. Dexamethasone (DEXA, 0.01 mg/pouch) was used as a positive control. The control group was treated only with 2% Tween 80 in saline. This experiment was repeated in triplicate. ^a $p < 0.05$, ^b $p < 0.01$, compared with the control group.

activity. In brief, it is assumed that an acute inflammatory activity of DHD results from its prevention on the release of inflammatory mediators at the first stage.

In the acetic acid-induced writhing response, an inhibitory mechanism of abdominal writhing induced by acetic acid involves the release of arachidonic acid metabolites via cyclooxygenase, and prostaglandin biosynthesis (Franzotti *et al.*, 2002). As shown in Table II, DHD at 10, 30 and 100 mg/kg, p.o., exhibited an inhibition by 48.4%, 64.3% and 86.6%, respectively, on the writhing response induced by acetic acid. Collectively, DHD contains an anti-nociceptive activity in addition to an anti-inflammatory activity, suggesting that prostaglandin biosynthesis might be commonly involved in the pharmacological activities of DHD.

Nitric oxide (NO), an important proinflammatory mediator in the pathogenesis of inflammatory process, is produced from L-arginine by the three isoforms of nitric oxide synthase (NOS), such as neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). For expression of iNOS, the mammalian cells should be experimentally triggered by specific stimulants, including pro-inflammatory cytokines and bacterial LPS (Chesrown *et al.*, 1994). Since iNOS-derived NO plays an important role in a variety of pathological conditions, such as inflammation and autoimmune diseases, and gives rise to cellular injury, suppression of iNOS is closely linked with anti-inflammatory action (Singh *et al.*, 2000). Inhibitory effects of DHD was examined on LPS-induced NO production in the

RAW264.7 macrophages (Fig. 5). Level of the accumulated nitrite, determined by the Griess method, in the culture supernatants was used as an index for NO. After treatment with LPS, nitrite level markedly increased. When the RAW264.7 macrophage cells were treated with 0.02, 0.05 and 0.1 mM DHD, NO production induced by LPS was suppressed in a dose-dependent manner (Fig. 3). The *in vitro* suppression by DHD in the induction of NO was supported by the result obtained with the air pouch model. As shown Table III, DHD was able to significantly decrease the nitrite content in the air pouches in a dose-dependent manner. With the assumption that suppression by DHD of NO induction might be caused by a decrease in the iNOS level, the effect of DHD on the iNOS expression was examined in the RAW264.7 macrophages cells activated with LPS. As shown in Fig. 4, DHD dose-dependently suppressed iNOS induction without changes in the levels of β -actin, an internal control. This result suggests that DHD specifically inhibits iNOS induction in the LPS-stimulated macrophage cells. Non-steroidal anti-inflammatory drugs (NSAIDs), known to be very beneficial in the treatment of various inflammatory disorders, target cyclooxygenase (COX), a rate-limiting enzyme involved in the conversion of arachidonic acid into inflammatory prostaglandins. In contrast to constitutive expression of cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2) is induced by tissue damage during inflammatory process. Various natural products of plant origin contain anti-inflammatory activities via suppression of COX-2 (Jachak, 2006). DHD was able to suppress COX-2 induction in LPS-stimulated RAW264.7 macrophage cells (Fig. 4). Although vanillin, an analogue

Fig. 3. Inhibitory effect of 2,4-dihydroxybenzaldehyde (DHD) on LPS-induced NO production in RAW264.7 macrophage cells. The mammalian cells were incubated for 24 h with LPS (1 μ g/ml) in the presence or absence of indicated concentrations of DHD. Accumulated nitrite in the culture medium was determined by the Griess reaction. The values are mean $\pm$ S.E. of the three independent experiments. * p < 0.05, ** p < 0.01, compared with the LPS only.

Fig. 4. Inhibitory effects of 2,4-dihydroxybenzaldehyde (DHD) on LPS-induced expression of iNOS and COX-2 in RAW264.7 macrophage cells. The mammalian cells were incubated for 24 h with LPS (1 μ g/ml) in the presence or absence of indicated concentrations of DHD. After 24 h incubation, the cell lysates (30 μ g protein) were separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane and blotted with appropriate antibodies. β -Actin was used an internal control. This blot is a representative of the three independent experiments.

Fig. 5. Reduction of reactive oxygen species (ROS) level by 2,4-dihydroxybenzaldehyde (DHD) in RAW264.7 macrophage cells activated with LPS. The mammalian cells were incubated for 24 h with LPS (1 μ g/ml) in the presence or absence of indicated concentrations of DHD. After 24 h incubation, the intracellular ROS levels were determined. ROS level was represented as DCF fluorescence. The values are mean $\pm$ S.E. of the three independent experiments. * $p < 0.05$, compared with the LPS only.

of DHD, contains anti-angiogenic, anti-inflammatory and anti-nociceptive activities, it was unable to suppress COX-2 induction in the LPS-activated macrophages (Lim *et al.*, 2008b). However, vanillin suppressed NO production and iNOS induction in the LPS-activated macrophages (Lim *et al.*, 2008b). Taken together, DHD is estimated to exhibit its anti-inflammatory and anti-nociceptive activities via the suppression of iNOS and COX-2 induction.

Although ROS at low concentrations act as important intracellular messengers of various physiological events, including cell proliferation and apoptosis, the increased production of ROS is cytotoxic and related with various diseases. As shown in Fig. 5, DHD was able to reduce ROS level in the macrophages, which was elevated by LPS. Previously, 4-hydroxybenzyl alcohol was identified to reduce the ROS level in the LPS-activated macrophages (Lim *et al.*, 2007). No cytotoxic effects of DHD on the RAW264.7 macrophages were observed at the used concentrations, which was determined by the MTT assay (Fig. 6). DHD at 0.02, 0.05 and 0.1 mM showed cellular survival similar to that of the untreated control (Fig. 6)

In conclusion, it has been clearly demonstrated that DHD possesses some novel pharmacological activities, such as anti-inflammatory, anti-angiogenic and anti-nociceptive activities via suppression of iNOS and COX-2 induction. This is the first finding on the pharmacological

Fig. 6. Effect of 2,4-dihydroxybenzaldehyde (DHD) on the viability of RAW264.7 macrophage cells. Cell viability was measured by the MTT assay. The mammalian cells were treated with indicated concentrations of DHD or 1 μ g/ml LPS for 24 h. Data indicate mean $\pm$ S.E. from three independent experiments.

activities of DHD.

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