Inhibitory Effect of Bis(2-aminohexyl) Disulfide and Bis(2-amino-3-phenylpropyl) Disulfide on Several Mouse Inflammations

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The anti-inflammatory profile of the analogues of bis(2-aminopropyl) disulfide dihydrochloride with butyl (compd. II) and phenyl (compd. III) instead of the methyl group was studied in several mouse models related to phagocyte functions. The test samples were administered 2—3 h before the inflammatory stimulation or the peak of inflammation. Subcutaneously administered, compds. II and III significantly inhibited serotonin-induced paw edema in a dose–dependent manner (50% inhibitory dose values: 10 and 5 mg/kg, respectively), when orally administered at 25 mg/kg, these compounds were significantly effective, but their potencies were weaker. Neither compound had any irritant activity when administered at a dose of 12.5 μg/5 μl/paw into the paw. In a sheep red blood cells (SRBC)-induced delayed-type hypersensitivity (DTH) reaction model, compd. II (25 mg/kg, s.c.) significantly inhibited the DTH responses when administered at two different times in relation to the time of challenge. However, there was only slight inhibition by compd. III (25 mg/kg, s.c.) on paw edema formation when administered 14 h after secondary immune response. In a model of experimental acute hepatic failure induced by successive injections of *Propionibacterium acnes* and lipopolysaccharide, both compounds increased mouse survived, compared with the control mice, and kept the serum levels of components involved in hepatic failure to nearly normal levels. These results demonstrate that compds. II and III possess an inhibitory effect on inflammation related to phagocytes.

Keywords bis(2-aminohexyl) disulfide; bis(2-amino-3-phenylpropyl) disulfide; anti-inflammatory compound; phagocyte; paw edema; peritonitis; delayed-type hypersensitivity; acute hepatic failure

In the acute inflammatory process, leukocytes, especially phagocytes, play an important role in the host defense against foreign noxious substances invading the body.[1] During our research on pharmacological activities of marine products, we previously reported that the analogues of bis(2-aminopropyl) disulfide dihydrochloride (compd. I) with butyl (compd. II), phenyl (compd. III) and benzyl (compd. IV) in place of the methyl group had potent suppressive effects *in vitro* on several functions of polymorphonuclear leukocytes (PMNs) among cells related to inflammation.[2] In this report, an inhibitory profile of these analogues *in vivo* has been demonstrated by investigating the inhibitory effects of compds. II and III on several mouse inflammations related to phagocytes.

Experimental

Animals Male Hartley guinea pigs (400—600 g body weight), male and female ICR mice (6 weeks old) and male BALB/c mice (8 weeks old) were purchased from Shimizu Jikken Zairyou, Ltd. (Kyoto, Japan). Sheep red blood cells (SRBC) were obtained from the Research Institute of Microbial Diseases, Osaka University.

Materials Bis(2-aminohexyl) disulfide (compd. II) and bis(2-amino-3-phenylpropyl) disulfide (compd. III) were previously[2] synthesized by the method of Kondo *et al.*[3] Hank's balance salt solution (HBSS) and RPMI 1640 medium were obtained from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan), fetal calf serum (FCS) from Gibco (Grand Island, NY, U.S.A.), *N*-formyl-Met-Leu-Phe (FMLP), heparin, superoxide dismutase (SOD), cytochrome c (type IV), lipopolysaccharide (LPS, *Escherichia coli*) from Sigma Co. (St. Louis, Mo, U.S.A.) and yeast (dried beer yeast), serotonin creatinine sulfate, giemsa solution and casein (from milk) from Wako Pure Chemical Co., Ltd. (Osaka, Japan). All other reagents were of analytical grade.

Superoxide Anion Generation and Chemotaxis of Peritoneal Macrophages Guinea pigs were injected intraperitoneally with 50 ml of 2% casein sterilized in an autoclave dissolved in saline. Four days later peritoneal exudate cells (PEC) were removed by washing with HBSS containing 10 units/ml heparin. The PEC were washed 3 times with HBSS and suspended in RPMI 1640 medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin and 10% FCS (complete RPMI 1640 medium). The cell suspension was plated in tissue culture dishes and allowed to adhere for 2 h at 37 °C in a humidified atmosphere of 5% CO_2 in air. Non-adherent cells were removed by shaking and by washing the dishes with HBSS. The adherent cells (macrophages) were collected with a soft, wide-tipped rubber policeman and suspended in HBSS.[4]

Generation of superoxide anions (O_2^-) was assayed spectrophotometrically by reduction of cytochrome c, as previously described for PMNs.[2,5] Briefly, the reaction mixture, composed of 2×10^6 macrophages, 150 nmol of cytochrome c and the sample, was suspended in 0.15 ml of HBSS. After preincubation for 2 min at 37 °C, 50 μl of 0.4 μM FMLP was added to the reaction mixture. The reduction of the cytochrome c was measured by the absorbance change at 550 nm.

Chemotaxis of macrophages was assayed by employing a Boyden chamber[6] with a nitrocellulose filter, as previously described.[2] Briefly, 200 μl of 20 nM FMLP was placed in the lower compartment, and an equal volume of suspension of 1.3×10^6 macrophages preincubated for 20 min at 37 °C with the sample was placed in the upper compartment. After incubation for 20 min at 37 °C, the emigrated macrophages in the filter were counted.

Yeast-Induced Peritonitis According to the method of Hisadome *et al.*,[7] the sample was subcutaneously administered to male ICR mice. After 2 h, the animals were injected intraperitoneally with 0.5 ml of 5% (w/v) yeast suspended in HBSS. After 3 h, the animals were sacrificed by decapitation and intraperitoneally administered 5 ml of HBSS containing 10 units/ml heparin. A small incision was made into the abdominal cavity and the PEC were harvested. The total number of PEC and cells phagocytized with yeast were counted with a hemocytometer.

Serotonin-Induced Paw Edema According to the method of Oyanagui,[8] serotonin paw edema was induced by injecting 5 μl of a serotonin solution (containing 1.2 μg of serotonin) in the right hind paw of male ICR mice. The same volume of saline was injected into the left hind paw. The sample was subcutaneously or orally administered before injection of serotonin. The difference in thickness between the two paws was measured with a dial thickness gauge (Mitsutoyo MGF., Co., Ltd.) 15 min after the injection of serotonin.

Irritant Activity Five μl of both the sample and saline was injected in the right and left, hind paws of male ICR mice, and the difference in swelling rate between the two paws was measured.

Delayed-Type Hypersensitivity (DTH) DTH specific for SRBC was assessed by the paw reaction as described by Harada *et al.*[9] Briefly, female mice were sensitized by intravenous injection of 10^8 SRBC in 200 μl of saline and 5 d later they were challenged with 10^8 SRBC in 20 μl of saline in the right hind paw. Paw swelling was measured under a dissecting

microscope at 24 h after the eliciting injection. The effect of sample on the expression of DTH to SRBC when administered, respectively, 2 h before and 14 h after the challenge injection was measured.

Experimental Acute Hepatic Failure According to the method of Tsutsui *et al.*,[10] acute hepatic failure was induced by an intravenous injection of heat-treatment *Propionibacterium acnes* (1 mg/mouse) into BALB/c mice followed by an intravenous injection of LPS (10 μg/mouse) 7 d later. Mortality was measured by counting the number of mice that died of massive hepatic necrosis within 24 h of the LPS injection. Sample was subcutaneously administered 2 h before the LPS injection. Mice surviving 24 h after the LPS injection were bled from the heart, and the serum enzyme activities and levels of TP and Alb were determined.[11]

Results

O_2^- Production and Chemotaxis *in Vitro* of Peritoneal Macrophages The *in vitro* effect of compds. II and III on O_2^- generation and chemotaxis of the elicited macrophages brought into the guinea pig peritoneum in response to an injection of sterile casein was examined. Figure 1 illustrates the representative effect of these compounds on FMLP-induced O_2^- generation in macrophages by the cytochrome c method. Both compounds inhibited FMLP-induced O_2^- generation in a dose-dependent manner. The 50% inhibitory concentration (IC_{50}) values calculated from the concentration–response curve of the inhibition (maximum rate of cytochrome c reduction) of O_2^- generation by compds. II and III were 2.4 and 3.4 μM, respectively. An O_2^- scavenger, SOD (10 μg/ml), used as a positive control compound completely inhibited O_2^- generation (data not shown). Also, both compounds significantly inhibited FMLP-induced chemotaxis, as shown in Table I. The IC_{50}

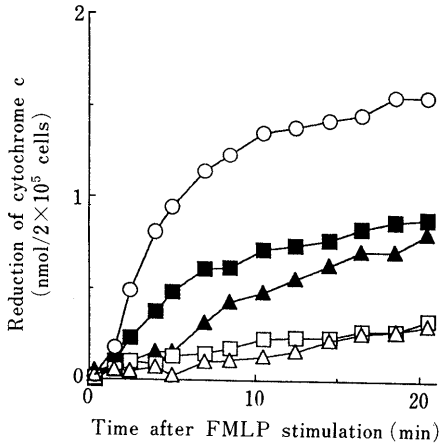

Fig. 1. Representative Effect of Compds. II and III on FMLP-Induced O_2^- Generation in Guinea Pig Peritoneal Macrophages

After preincubation with the samples for 2 min at 37 °C, macrophages (2×10^6 cells) were stimulated by FMLP (0.1 μM). ○, control; ▲, compd. II (2.5 μM); △, (10 μM); ■, compd. III (2.5 μM); □, (10 μM).

Table I. Effect of Compds. II and III on FMLP-Induced Chemotaxis of Macrophages

Sample	Concentration (μM)	No. of macrophages emigrating[a]	Inhibition (%)
Control	—	330±11	—
Compd. II	1	229±12[c]	31
	10	121± 6[c]	63
	100	50±22[c]	85
Compd. III	1	203±44[b]	38
	10	136±18[c]	59
	100	17± 3[c]	95

a) Mean±S.E. ($n=3$). *b*) $p<0.01$, *c*) $p<0.001$: *versus* control.

Table II. Effect of Compds. II and III on Yeast-Induced Peritonitis in Mice

Sample[a]	Dose (mg/kg)	No. of leukocytes migrating[b] ($\times 10^6$)	Inhibition (%)	No. of yeasts[b] ($\times 10^6$)	Inhibition (%)
Control	—	7.6±0.8	—	2.2±0.6	—
Compd. II	5	5.8±0.9	23	2.0±0.5	10
	25	3.7±0.2[c]	51	1.2±0.1	45
Compd. III	5	6.1±1.6	20	1.7±0.6	22
	25	3.5±0.4[c]	53	1.4±0.2	38

a) Sample was subcutaneously administered 2 h before yeast injection. *b*) Mean± S.E. ($n=5$). *c*) $p<0.01$: *versus* control.

Table III. Effect of Compds. II and III on Serotonin-Induced Paw Edema Formation in Mice

Sample[a,b]	Dose (mg/kg)	Swelling[c] (mm)	Inhibition (%)
Subcutaneous administration[a]			
Control	—	0.766±0.047	—
Compd. II	1	0.635±0.074	17
	5	0.454±0.036[f]	41
	25	0.300±0.016[f]	61
Compd. III	1	0.635±0.013[d]	17
	5	0.372±0.104[e]	51
	25	0.194±0.056[f]	75
Dexamethasone	1	0.394±0.086[e]	49
SOD	5	0.400±0.078[e]	48
Oral administration[b]			
Control	—	0.643±0.040	—
Compd. II	25	0.451±0.030[e]	30
Compd. III	25	0.417±0.044[e]	35

a) Sample was administered 2 h (Compds. and SOD) or 4 h (dexamethasone) before serotonin injection (1.2 μg/paw). *b*) Sample was administered 3 h before serotonin injection. *c*) Mean±S.E. ($n=5$). Edema was measured 15 min after serotonin injection. *d*) $p<0.05$, *e*) $p<0.01$, *f*) $p<0.001$: *versus* control.

Table IV. Irritant Activity of Compds. II and III in Mice

Sample	Dose (μg/5 μl/paw)	Swelling (mm)[a] Time after sample injection (min)				
		15	30	60	120	180
Control	—	0.031±0.008	0.036±0.011	0.030±0.007	0.025±0.010	0.023±0.006
Compd. II	12.5	0.044±0.004	0.035±0.009	0.043±0.010	0.034±0.006	0.020±0.012
Compd. III	12.5	0.051±0.014	0.074±0.009[b]	0.055±0.008	0.029±0.007	0.015±0.009
Serotonin	1.2	0.732±0.118[c]	0.575±0.009[c]	0.428±0.375[c]	0.171±0.038[c]	0.118±0.011[d]

a) Mean±S.E. ($n=4$). *b*) $p<0.05$, *c*) $p<0.01$, *d*) $p<0.001$: *versus* control.

values of compds. II and III were 3.8 and 4.4 μM, respectively.

Yeast-Induced Peritonitis In the experiment on yeast-induced peritonitis in mice, compds. II and III (25 mg/kg, s.c.) significantly inhibited the emigration of leukocytes into the peritoneum and tended to inhibit the phagocytosis of leukocytes (Table II).

Serotonin-Induced Paw Edema As shown in Table III, subcutaneously administered compds. II and III significantly inhibited serotonin-induced paw edema in mice in a dose–dependent manner at a dosage of 1 to 25 mg/kg. Their 50% inhibitory dose (ED_{50}) values calculated from the dose–response curve of the inhibition of edema were approximately 10 and 5 mg/kg, respectively. Both compounds showed a stronger inhibitory effect than did compd. I (ED_{50}: >25 mg/kg). Dexamethasone[12] and SOD used as positive control compounds showed a stronger inhibition. Also, when orally administered at 25 mg/kg, both compounds were significantly effective, but their potencies were weaker than when subcutaneously administered compounds.

TABLE V. Effect of Compds. II and III on SRBC-Induced Delayed-Type Hypersensitivity in Mice

Sample[a,b]	Dose (mg/kg)	Swelling[c] (mm)	Inhibition (%)
Experiment 1[a]			
Control	—	0.363±0.072	—
Compd. II	5	0.252±0.070	30
	25	0.162±0.024[d]	55
Compd. III	5	0.294±0.037	19
	25	0.122±0.050[d]	66
Experiment 2[b]			
Control	—	0.414±0.026	—
Compd. II	5	0.274±0.025[e]	34
	25	0.196±0.033[f]	53
Compd. III	5	0.380±0.047	8
	25	0.312±0.052	25

a, b) Sample was subcutaneously administered *a*) 2 h before or *b*) 14 h after challenge of SRBC. *c*) Mean±S.E. (n=5—8). *d*) $p<0.05$, *e*) $p<0.01$, *f*) $p<0.001$: *versus* control.

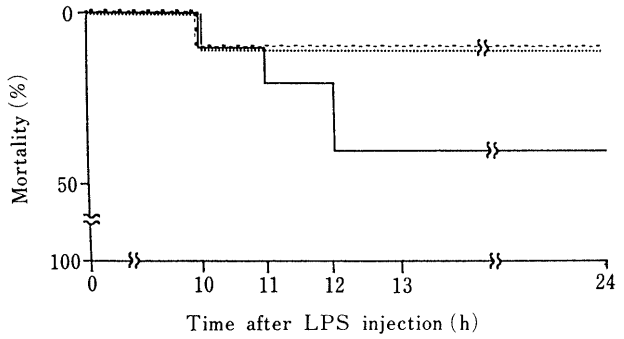

Fig. 2. Effect of Compds. II and III on Acute Hepatic Failure Induced by *P. acnes* and LPS in Mice

The samples (25 mg/kg, s.c.) were administered 2 h before the LPS injection (10 μg/kg, i.v.) in mice treated with heat-treated *P. acnes* (1 mg/kg, i.v.) at 1 week intervals. ——, control; ------. compd. II; – – –, compd. III.

Irritant Activity To confirm whether the inhibitory effect on the edema formation derived from a counter irritant effect[13] of the test compounds, their irritant activity was investigated. When administered at a dose of 12.5 μg/5 μl/paw into mouse paw, both compounds formed a small little edema during the 180 min after the injection, except that formed by compd. III was only slight edema but significant at 30 min, as shown in Table IV. Judging from the result of serotonin which was used as a positive control compound, compds. II and III are considered to have no irritant activity.

SRBC-Induced DTH Table V shows the effect of compds. II and III in relation to the time of SRBC-induced DTH in mice when subcutaneously administered 2 h before or 14 h after secondary immunization by challenge injection of SRBC. At both administration times compd. II (25 mg/kg, s.c.) significantly inhibited the edema formation induced by DTH.

Compd. III (5 and 25 mg/kg) significantly inhibited edema formation upon SRBC challenge when administered 2 h before immunization but showed only a tendency to inhibit when administered 14 h afterwards.

Experimental Acute Hepatic Failure As shown in Fig. 2, the control animals with acute hepatic failure which was induced by successive injections of *P. acnes* and LPS, started to die about 10 h after the LPS injection. In the control groups the rate of 24-h survival mice was 60%. In the groups given compds. II or III 2 h before the LPS injection, the survival rate was 90%.

In this acute hepatic failure model, several serum parameters, glutamate pyruvate transaminase (GPT), glutamate oxaloacetate transaminase (GOT) and lactate dehydrogenase (LDH) increased, total protein (TP) and albumin (Alb) decreased, and alkaline phosphatase (ALP) changed slightly from the untreated control group (Table VI). Both compounds kept the serum levels of parameters related to hepatic functions near those of the untreated control group. The potency of compd. II seemed to be stronger than that of compd. III, especially in GPT and GOT levels.

Discussion

Leukocytes, particularly PMNs and macrophages, act as a host defense mechanism against foreign stimulatory substances invading into the body.[1] PMNs and macrophages migrate to the inflammatory site through the

TABLE VI. Effect of Compds. II and III on Several Serum Parameters

Sample	GOT (U/l)	GPT (U/l)	ALP (U/l)	LDH (U/l)	TP (g/dl)	Alb (g/dl)
Normal	21± 2	49± 3	142±5	410± 40	5.6±0.1	4.0±0.1
Control	322±12	1345±275	91±3	4263±987	4.0±0.2	2.7±0.0
Compd. II	107±41[a]	135± 21[a]	94±4	452± 86[a]	5.2±0.2[a]	3.9±0.1[a]
Compd. III	244±51[a]	244± 86[a]	95±2	456± 63[a]	4.8±0.1[a]	3.8±0.1[b]

Data (mean±S.E.) are expressed as the assay values on sera obtained from 5 mice arbitrarily selected from surviving animals 24 h after the LPS injection, except normal control group (n=5). *a*) $p<0.01$, *b*) $p<0.001$: *versus* control.

mechanism of chemotaxis, phagocytose pathogenic bacteria and release lysosomal enzymes. Simultaneously, generated active oxygen species sterilize the invading microorganisms.[1,14] It is also well known, however, that excessive responses of leukocytes to foreign noxious stimulators cause inflammatory reactions, that is, they harm the body. In the previous[2] and present experiments, compds. II and III were demonstrated to be potent inhibitors of several functions *in vitro* involving O_2^- generation, chemotaxis, phagocytosis and enzyme release in peritoneal PMNs and macrophages induced by sterilized casein injection. Further, the efficacy of these compounds against inflammation *in vivo* was evaluated in the present study using several mouse models.

In the yeast-induced peritonitis model it is possible to examine the emigration and phagocytosis of leukocytes which are the primary steps of the inflammatory process. Because both compounds inhibited the yeast-induced peritonitis, their inhibitory effect on the emigration and phagocytosis of leukocytes *in vitro* was also confirmed in the *in vivo* model. In the experiment on serotonin-induced paw edema accompanied by the increased vascular permeability, it was found that both compounds had the inhibitory effect (Table III). The swelling of this model was rapid and no participation of arachidonate cascade products was noted.[15] Based on the fact that the edema was inhibited by steroidal anti-inflammatory drugs and SOD which is an O_2^- scavenger, it is suggested that the action of steroidal anti-inflammatory drugs is due to the induction of the vascular permeability inhibitory protein "vasoregulin," and that the action of SOD is due to the scavenging of O_2^- which inactivates vasoregulin.[16] In general pharmacologic aspects, part of the drugs which act to inhibit serotonin action may possibly blockade receptors. Compds. II and III, however, do not seem to be antagonists of serotonin (*i.e.* indolealkylamines, lysergic acid derivatives, beta halo ethylamines and phenothiazines)[17] because of their structural difference. Furthermore, Ludány *et al.*[18] and Northover[19] reported that serotonin enhanced phagocytosis by both rat and rabbit leukocytes. But it was found that serotonin (up to 1 mM) did not stimulate O_2^--generation in guinea pig peritoneal PMNs (data not shown). Although compds. II and III have no scavenging activity of O_2^-, they inhibit O_2^--generation.[2] The inhibitory effect on serotonin-induced edema may thus be due, at least partly, to the inhibition of phagocytosis and/or of O_2^--generation in phagocytes followed by enhanced phagocytosis. We also investigated the effect of compds. II and III on the inflammation involving cell-mediated immunity, employing the paw reaction to assess DTH to SRBC. The DTH responses are composed of the induction phase in which lymphokine-responsive monocytes, migrate and accumulate in the SRBC-injected sites, and the effector phase in which the cells are activated by the challenge injection and form an inflammatory site.[9] Compd. II inhibited the DTH responses in two different administrations in relation to the time of challenge. However, slight inhibition of paw edema formation was shown only by compd. III when administered 14 h after the secondary immune response. This result indicates that the compounds inhibit both chemotaxis and activation of monocytes.

Recently, heat-treated *P. acnes* plus LPS-induced hepatitis has been used as an immunological liver injury model in mice. As mechanism of this acute hepatic failure, adherent cells are thought to undergo priming and accumulate in liver by intravenous injection of *P. acnes*, and then are further activated by LPS to produce a cell injury factor.[10,20] In this model compds. II and III reduced mortality and inhibited the leak of enzymes from the injured liver cells to serum. This suggests that both compound may inhibit the functions of monocytes and macrophages in which the immune system is involved.

Consequently, it was confirmed that compds. II and III had an inhibitory effect on inflammation related to phagocyte functions.

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