Inhibitory Effects of Bis(2-aminohexyl)disulfide and Its Analogues on Polymorphonuclear Leukocyte Functions *in Vitro*

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Water soluble analogues of the anti-inflammatory compound, bis(2-aminopropyl)disulfide dihydrochloride (compd. I) with a butyl (II), phenyl (III), benzyl (IV) or pyrrolidinyl group (V) instead of the methyl group were synthesized, and their effects on the functions of cells related to inflammation were studied *in vitro*. Compounds II, III and IV showed much higher inhibitory activity than compd. I on formyl Met–Leu–Phe (FMLP)-induced O_2^--generation of polymorphonuclear leukocytes (PMNs) and platelet aggregation. Compound II showed the strongest activity among the compounds (IC_{50} values: 2.6 μM). The inhibition of O_2^--generation of PMNs by compd. II was the most effective when FMLP was used as a stimulant rather than when phorbol myristate acetate, A-23187 and opsonized zymosan were used. However, compd. II was not an O_2^--scavenger. Compounds II, III and IV significantly inhibited a series of activation processes in PMNs, chemotaxis, phagocytosis and lysosomal enzyme release at doses ranging from 10 to 100 μM. Under these doses, compds II, III and IV did not affect the histamine release from mast cells or the hemolysis of erythrocytes. These results strongly suggest that the anti-inflammatory action caused by compd. II and its analogues was at least partly due to inhibition of several functions of PMNs and platelets.

Keywords bis(2-aminohexyl)disulfide; polymorphonuclear leukocyte; inflammation; O_2^--generation; chemotaxis; phagocytosis; lysosome enzyme release; platelet; platelet aggregation

During our search for pharmacological activities of marine products we focused on D-cysteinolic acid, isolated from sardines, which possesses platelet anti-aggregant activity.[1] Previously, we found that the platelet anti-aggregant activity of D-cysteinolic acid analogues with an S–C–C–N skeleton became more potent when the hydrophobicity was increased at the 2-C-position of the skeleton. Thus, the bis(2-aminopropyl)disulfide dihydrochloride (compd. I) was obtained.[2] Furthermore, we reported that compd. I inhibited carrageenin- and serotonin-induced paw edema formation.[3] Also, we reported that several bis[2-(*E*-2-alkenoylamino)ethyl]-disulfides (compds. A series), synthesized from cystamine and 2-*trans* fatty acids, showed potent anti-inflammatory activity.[4] The effects of compd. I and the compd. A series were suggested to be due at least partly to the inhibition of prostaglandin biosynthesis.[2,5] Because of their high insolubility in water, the series of compd. A could not be investigated for biological characteristics on a cellular level *in vitro*. The findings that compounds of such a simple chemical structure demonstrated useful pharmacological activity prompted us to investigate the activities of water soluble compd. I analogues with more hydrophobic substituents than the methyl group at the 2-C-position. In this paper, we describe the *in vitro* effects of the analogues of bis(2-aminohexyl)disulfide dihydrochloride (compd. II) on several functions of polymorphonuclear leukocytes (PMNs), platelets and mast cells related to inflammation.

Experimental

Materials Formyl Met–Leu–Phe (FMLP), phorbol 12-myristate-13-acetate (PMA), cytochrome c (type IV), zymosan, latex beads, indomethacin, superoxide dismutase (SOD), xanthine oxidase, phenolphtalein glucuronic acid, Micrococcus lysodeikticus, lysozyme, bovine serum albumin, disodium cromoglycate (DSCG), propidium iodide and fluorecein acetate were purchased from Sigma Co. (St. Louis, Mo, U.S.A.). Calcium ionophore A-23187 was obtained from CalBiochem Behring Diagnostic (San Diego, Ca, U.S.A.). Luminol, casein (from milk) and lactic acid dehydrogenase (LDH) UV Test Wako were obtained from Wako Pure Chemical Co. (Osaka, Japan). Histamine dihydrochloride and *o*-phthalaldehyde were obtained from Nacalai Tesque (Kyoto, Japan). Hanks solution (HBSS, Nissui 2) was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Epinephrine and collagen were purchased from Kyoto Daiichi Kagaku (Kyoto, Japan). Arachidonic acid was obtained from P-L Biochemicals Inc. (Milwaukee, WI, U.S.A.). Nucleopore (5μm) was purchased from Nomura Micro Science Co., Ltd. (Osaka, Japan). All other reagents and solvents used were of an analytical grade.

Syntheses of Compd. I and Its Analogues Melting points are uncorrected. Infrared (IR) spectra were taken in Nujol mulls with a JASCO IR-810 instrument. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a Hitachi R-90H with D_2O as the solvent and tetramethylsilane as an internal standard. Compound I and its analogues listed in Fig. 1, compd. II, (*S*)-bis-2-amino-3-phenylpropyl)disulfide dihydrochloride (compd. III), (*S*)-bis(2-amino-4-phenylbutyl)disulfide dihydrochloride (compd. IV), and (*S*)-bis[2-(2-pyrrolidinyl)-ethyl]disulfide dihydrochloride (compd. V), were synthesized from their corresponding alcoholic compounds according to the method of Kondo *et al.*[6] Briefly, DL-2-amino-1-propanol, DL-2-amino-1-hexanol, (*S*)-2-phenyl glycinol, (*S*)-2-amino-3-phenyl-1-propanol or (*S*)-2-pyrrolidinemethanol was protected by *tert*-butoxycarbonyl (Boc) group for its NH_2-group, converted to the tosyl derivative and treated with thioacetic acid to afford thioester derivative. Alkaline hydrolysis of this thioester, air oxidation, and the following treatment with HCl/dioxane gave the corresponding disulfide compound. Reduction of Boc-compound II afforded 2-aminohexanethiol hydrochloride (compd. II-1). Compound I: mp 229 °C (dec.). *Anal.* Calcd for $C_6H_{18}Cl_2N_2S_2$: C, 28.45; H, 7.16; N, 11.06. Found: C, 28.50; H. 7.20; N, 11.10. IR: 1580, 1560, 1505, 1190, 1105, 455 m^{-1}. ^{1}H-NMR: 1.54 (3H, d, J=7 Hz, CH_3), 2.9—3.4 (2H, m, $-CH_2-$), 3.7—4.0 (1H, m, $-CH-$).

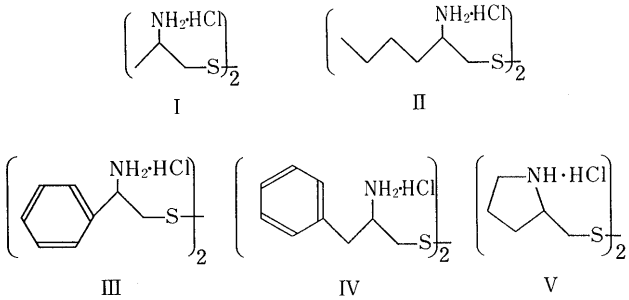

Fig. 1. Bis(2-aminopropyl)disulfide and Its Analogues

Compound II: mp177—179 °C. *Anal.* Calcd for $C_{12}H_{30}Cl_2N_2S_2$: C, 42.71; H, 8.96; N, 8.30. Found: C, 42.50; H, 9.00; N, 8.20. IR: 1590, 1240, 1095, 720 cm^{-1}. ^{1}H-NMR δ: 0.8—1.2 (3H, m, CH_3), 1.3—1.7 (4H, m, CH_2CH_2), 1.7—2.1 (2H, m, CH_2CH–), 3.01 (1H, dd, $J_1=15$ Hz, $J_2=8$ Hz, CH_2S–), 3.30 (1H, dd, $J_1=15$ Hz, $J_2=5$ Hz, CH_2S) 3.5—4.0 (1H, m, CH). Compound III: mp 225 °C (dec.). *Anal.* Calcd for $C_{16}H_{22}Cl_2N_2S_2$: C, 50.92; H, 5.88; N, 7.42. Found: C, 50.9; H, 5.80; N, 7.40. IR: 1600, 1515, 1505, 770, 740, 695 cm^{-1}. ^{1}H-NMR δ: 3.32 (2H, d, $J=7$ Hz, CH_2S), 4.70 (1H, t, $J=7$ Hz, CH–), 7.2—7.7 (5H, m, Ph). Compoound IV: mp 250 °C (dec.). *Anal.* Calcd for $C_{18}H_{26}Cl_2N_2S_2$: C, 53.32; H, 6.46; N, 6.91. Found: C, 53.40; H, 6.60; N, 6.80. IR: 1600, 1560, 1515, 1085, 740, 700 cm^{-1}. ^{1}H-NMR δ: 2.83 (2H, d, $J=7$ Hz, CH_2 Ph), 3.02 (1H, dd, $J_1=14$ Hz, $J_2=8$ Hz, CH_2S), 3.22 (1H, dd, $J_1=14$ Hz, $J_2=6$ Hz, CH_2S), 3.7—4.2 (1H, m, Ph). Compound V: mp 208 °C (dec.). *Anal.* Calcd for $C_{10}H_{12}Cl_2N_2S_2$: C, 39.33; H, 7.26; N, 9.18. Found: C, 39.10; H, 7.10; N, 9.00. IR: 1585, 1410, 1240, 1080, 560, 450 cm^{-1}. ^{1}H-NMR δ: 1.7—2.6 (4H, m, CH_2CH_2–), 2.8—3.6 (4H, m, CH_2NH–, CH_2S–), 3.9—4.4 (1H, m, CH–).

Assay for PMN Function Male Hartley guinea pigs (400—600 g body weight) purchased from Japan Keari Co. (Osaka, Japan) were used. PMNs were isolated from the peritoneal cavity 16 h after peritoneal injection of 40 ml of 2% sterilized casein dissolved in saline. PMNs were collected by centrifugation at $250\times g$, 4 °C for 5 min after decomposition of contaminating erythrocytes using a hypotonic NaCl solution, and resuspended in HBSS.[7] Purity and viability of the isolated PMNs was more than 96% and 98%, respectively. Generation of a superoxide anion was assayed spectrophotometrically by reduction of cytochrome c,[8] and the continuous assay was performed using an immunoreader (InterMed. NJ2001, Osaka, Japan) as described by Pick and Muzel.[9] The reaction mixture, composed of 2×10^6 PMNs, 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 the stimulator was added to the reaction mixture. The reduction of the cytochrome c was measured at 550 nm with a reference at 540 nm. The 50% inhibitory concentration (IC_{50}) values were calculated for the concentration–response curve of the inhibition (maximum rate of cytochrome c reduction) of O_2^--generation by test sample. The result was expressed as an average of 2 experiments. Active oxygen was also measured using a chemiluminescence probe (Labo-science, TD-4000, Tokyo, Japan). The PMNs (2.5×10^5 cells) were incubated with 2 nmol of luminol and the sample in 100 μl of HBSS for 2 min at 37 °C, followed by stimulation with 10 μl of 1 μM FMLP. The superoxide anion generated from the reaction of xanthine with xanthine oxidase was measured by the cytochrome c reduction assay method.[10] The reaction mixture was composed of 500 nmol of xanthine, 50 nmol of cytochrome c and the sample in 0.975 ml of 50 mM phosphate buffer (pH 7.4)—0.1 mM ethylenediamine tetraacetic acid. After pre-incubation for 5 min at 25 °C, 25 μl of xanthine oxidase (1 U/ml) was added.

Chemotaxis was assayed by employing a Boyden chamber with a nitrocellulose filter.[11] In the lower compartment, 200 μl of chemotactic factor (20 nM) was placed, and an equal volume of PMNs suspension (10^6 cells) preincubated for 7 min at 37 °C with the sample was placed in the upper compartment. After incubation for 20 min at 37 °C, the filter was taken out and washed with several drops of 1% egg albumin. Next, the filter was fixed with 10% formalin, washed in water and stained with 10% Giemsa solution for 90 min. After the filter was washed in water, the number of PMNs which had emigrated through the filter was counted under a microscope. The result was expressed as the number of emigrated PMNs per 5 random fields.

Phagocytosis by PMNs was determined by ingestion of polystylene latex beads according to Takehiro *et al.*[12] PMNs (5×10^6 cells) were incubated with the sample in 0.25 ml of HBSS for 10 min at 37 °C. To start the reaction, 0.1 ml of the polystylene latex bead suspension (4×10^9 beads/ml) was added to the mixture. The reaction was stopped by immersing the incubation tubes into an ice bath. After washing the PMNs three times with 10 ml of HBSS (centrifuged at $100\times g$, 10 min), precipitated PMNs were heated for 10 min at 100 °C with 2 ml of dioxane and maintained for 2 h at room temperature. The resulting supernatant by centrifugation for 20 min at $1870\times g$ was measured on an absorbance at 253 nm. The number of particles ingested by PMNs was calculated on a calibration curve, with data obtained by the same dioxane extraction procedure. The result was expressed as the number of particles ingested per 100 cells.

Lysosome enzyme secretion was measured by the method of Kobayashi *et al.*[13] Cell suspension (5×10^6 cells) and the sample in 10 mM *N*-hydroxyethylpiperazine-*N'*-2-ethansulfonate (Hepes)–HBSS was preincubated for 5 min at 37 °C. Then the stimulant, latex beads (2.5×10^8 beads), was added to the mixture and incubated for 15 min at 37 °C. The reaction was stopped by immersion in an ice bath for 5 min. After centrifugation (10000 rpm, 15 min), an aliquot of the supernatant was used for enzyme assay (experimental release lysozyme, β-glucuronidase and LDH) according to the standard methods.[14] Enzyme activity was determined after the disruption of whole cell freeze-thawing. Enzyme release activity was expressed in terms of (experimental release per maximum release) $\times$ 100 (%).

A cytotoxity test[15] was done by incubating samples with 2×10^5 cells in a final volume of 0.22 ml for 30 min at 37 °C. Cells were counted by using an Epics profile II (Coulter Co., Fl, U.S.A.) with fluorescein diacetate-propidumiodide.

Assay for Platelet Aggregation Blood was taken after informed consent from healthy laboratory volunteers who did not ingest aspirin or other drugs. Blood was mixed with one-tenth volume of 3.8% sodium citrate in 10 ml-centrifuged tubes. Siliconized or plastic equipment was used throughout the experiments. After centrifugation at $400\times g$ for 10 min, platelet-rich-plasma (PRP) was removed and used for the platelet aggregation study. PRP contained $0.15—0.35\times10^9$ platelets per ml. Platelet aggregation studies were performed using an Aggrecoder PA-3210 (Kyoto Daiichi Kagaku, Kyoto, Japan) according to the turbidimetric method of Born and Cross.[16] To 240 μl of PRP was added 10 μl of test sample 3 min prior to the induction of platelet activation by epinphrine (final concentration: 8.3 μM), collagen (1.67 μg/ml) or arachidonate (0.42 mM), and (IC_{50} values were calculated from the concentration–response curve of the inhibition (maximum ampliuted of light transmittance) of platelet aggregation by samples. The result was expressed as an average of 2 experiments.

Assay of Histamine Release from Mast Cells Rat (Male Wister strain) peritoneal mast cells were isolated by the method of Üvanas and Thon.[17] Isolated mast cells were adjusted to 2.8×10^4 cells/ml by phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin. The cell suspension (1.8 ml) was incubated for 10 min at 37 °C. Then, with or without 0.1 ml of compd. II analogues dissolved in PBS, 0.1 ml of A-23187 as a stimulator was added to the cell suspension, which was then incubated for 10 min at 37 °C. The reaction was stopped by cooling in iced water to 0 °C and subsequent centrifugation at 1200 rpm for 5 min. The resulting supernatant and precipitate were assayed for histamine concentration using the method of Shore *et al.*[18] Results were expressed in terms of percentages of histamine release in supernatant per total histamine.

Assay for Hypotonic Hemolysis of Erythrocytes[19] Blood was taken from healthy laboratory workers with sodium heparin as an anticoagulant. To reduce the resistance of membranes and to obtain more distinct effect, heparinized blood was allowed to stand for 24 h at 37 °C. Then the heparinized blood was incubated with compd. II analogues for 10 min at 37 °C. A part of the mixture was mixed with PBS (pH 7.4, 0.10—0.85% of sodium chloride) and stood for 30 min at room temperature.

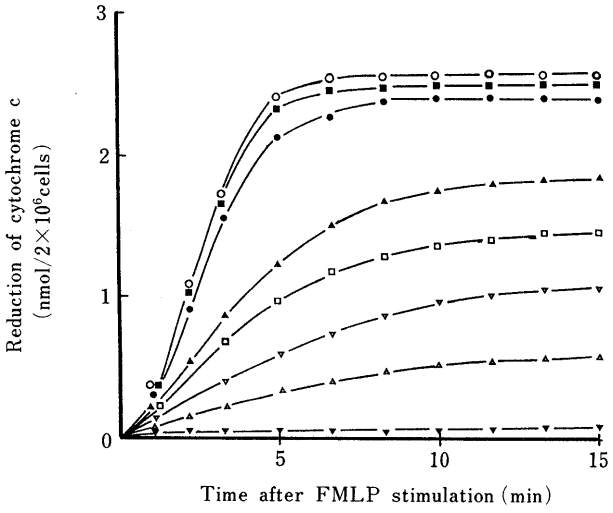

Fig. 2. Effect of Compd. II and Its Analogues on FMLP-Induced O_2^--Generation in PMNs

After preincubation with the compounds (5 μM) or SOD (10 μg/ml) for 2 min at 37 °C, PMNs (2×10^6 cells) were stimulated by FMLP (0.1 μM). ○, control; ●, compd. I; △, compd. II; ▲, compd. III; □, compd. IV; ■, compd. V; ▽, compd. II-1 (5 μM as a dimer); ▼, SOD.

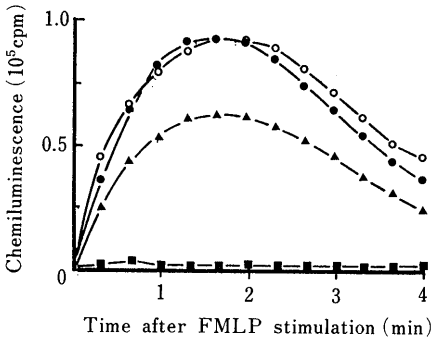

Fig. 3. Effect of Compd. II on FMLP-Induced Chemiluminescence in PMNs

After preincubation with luminol (20 μM) and compd. II at 37 °C for 2 min, PMNs (2.5×10^5 cells) were stimulated by FMLP (0.1 μM). ○, control. compd. II; ●, 0.1 μM; ▲, 1 μM; ■, 10 μM.

Table I. Effect of Compd. II on Several Stimulators-Induced O_2^--Generation in PMNs

Sample	Concentration (μM)	% inhibition of O_2^--generation			
		FMLP (0.1 μM)	PMA (0.1 μg/ml)	A-23187 (10 μM)	Opsonized zymosan (1 mg/ml)
Compd. II	1	19	10	10	11
	10	89	12	26	17
	25	92	31	46	24
Indomethacin	100	41	0	0	—
SOD	10 μg/ml	85	82	77	79

Table II. Effect of Compd. II and Its Analogues on FMLP-Induced Chemotaxis of PMNs

Sample	Concentration (μM)	No. of PMNs[a] emigrated	Inhibition (%)
Control	—	362.4±34.4	—
Compd. I	1	337.4±28.9	6.9
	10	311.2±12.4	14.1
	25	298.4±24.6	17.7
Compd. II	1	273.8±24.7	24.4
	10	203.6±34.3[b]	43.8
	25	91.6±13.9[c]	74.4
Compd. III	1	269.4±33.3	25.7
	10	227.2±17.4[b]	37.3
	25	168.6±22.0[c]	53.5
Compd. IV	1	341.4±24.0	5.8
	10	178.6±38.3[b]	50.7
	25	157.2±24.3[c]	56.6

a) Mean±S.E. (n=5—10). b) $p<0.01$, c) $p<0.001$: *versus* control.

Each supernatant obtained by centrifugation at $700 \times g$ for 5 min was determined spectrophotometrically on the degree of hemolysis at 540 nm.

Results

Generation of Superoxide Anion in PMNs Figure 2 illustrated the effect of compd. II and its analogues on FMLP-induced O_2^--generation in PMNs by the cytochrome c method. Compound II showed a higher inhibitory effect than other analogues at 5 μM. The inhibitory effect of this compound was in a dose–dependent manner (see Table I) and its inhibitory potency (IC_{50}) was about 2.6 μM. The rank order of potency was compd. II > compd. II-1 (IC_{50}: 3.0 μM) > compd. IV (4.2) > compd. III (6.1) > compd. I (32) > compd. V (>100). In addition, we tested the effect of compd. II on FMLP-induced chemiluminescence in PMNs (Fig. 3). The chemiluminescence was completely inhibited in the presence of 10 μM compd. II and in this assay an IC_{50} was 2.0 μM. On the other hand, this compound did not scavenge O_2^--generated by the xanthine–xanthine oxidase reaction, unlike SOD (data not shown). Furthermore, compd. II was tested on the inhibitory effect on O_2^--generation induced by several stimulators (Table I). This compound was clearly a stronger inhibitor against FMLP than against PMA, Ca ionophore A-23187 or opsonized zymosan. Indomethacin, a strong inhibitor (IC_{50}: 10^{-5} M) of O_2^--generation of non-stimulated PMNs or oil-induced peritoneal macrophages,[20] inhibited only FMLP-induced O_2^--generation at 100 μM. SOD, which promotes the dismutation of superoxide to H_2O_2, scavenged the reduction of cytochrome c induced by all tested stimulators ranging from 77 to 85%.

Table III. Effect of Compd. II and Its Analogues on Latex Bead-Induced Enzyme Release in PMNs

Sample	Concentration (μM)	Enzyme release (%)[a]		
		β-Glucuronidase	Lysozyme	LDH
Control	—	12.2±0.2	50.5±0.6	2.32±0.55
Compd. I	1	12.4±1.6	50.9±2.5	0.25±0.06[b]
	10	13.2±0.5	51.5±1.0	0.23±0.16[b]
	100	12.1±0.5	52.9±3.0	0.08±0.04[b]
Compd. II	1	12.2±0.8	49.0±0	1.87±0.69
	10	9.7±1.0	51.8±0.8	0.35±0.07[b]
	100	4.8±0.2[d]	32.4±0.8[d]	0.06±0.06[b]
Compd. III	1	11.7±0.3	48.6±0.4	0.67±0.23
	10	9.4±0.2[d]	43.0±3.4	0.52±0.16[b]
	100	3.7±0.2[d]	22.1±0.8[d]	0.32±0.12[b]
Compd. IV	1	12.7±0.6	50.9±3.2	0.34±0.20[b]
	10	8.5±0.2[d]	44.2±0.6[c]	0.57±0.08[b]
	100	4.3±0.3[d]	22.8±1.2[d]	0.41±0.36[b]

a) Mean±S.E. (n=3). b) $p<0.05$, c) $p<0.01$, d) $p<0.001$: *versus* control.

Table IV. Effect of Compd. II and Its Analogues on Phagocytosis of Latex Beads by PMNs

Sample	Concentration (μM)	No. of latex[a] (/100 cells)	Inhibition (%)
Control	—	916± 32	—
Compd. I	1	852± 92	6.9
	10	682± 51[b]	25.5
	100	561± 25[d]	38.8
Compd. II	1	930±121	−1.5
	10	436± 30[d]	52.4
	100	228± 5[d]	75.1
Compd. III	1	606± 62[c]	33.8
	10	286± 67[d]	68.8
	100	130± 56[d]	85.8
Compd. IV	1	788±123	14.0
	10	306± 55[d]	66.6
	100	185± 22[d]	79.8

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

Chemotaxis of PMNs As shown in Table II, compds. II, III and IV inhibited significantly FMLP-induced chemotaxis. Inhibition percentages of compds. II, III and IV against FMLP at 25 μM were 74.4%, 53.5% and 56.6%,

Table V. Platelet Anti-aggregant Activity of Compd. II and Its Analogues in Human PRP

Compound	IC_{50} (μM)		
	Epinephrine (8.3 μM)	Collagen (1.67 μg/ml)	Arachidonate (0.42 mM)
I	105	75	70
II	11	21	39
III	9	19	78
IV	13	9	15
V	19	66	77
II-1[a]	15	38	52

a) Calculated as a dimer.

respectively. These compounds were much stronger in inhibiting FMLP-induced chemotaxis than compd. I.

Enzyme Release in PMNs The inhibitory effect of compds. II, III and IV on latex bead-induced enzyme release is shown in Table III. Compounds II, III and IV significantly inhibited the release of lysosomal enzymes, lysozyme and β-glucuronidase. These compounds also suppressed the leakage of the cytoplasmic enzyme, LDH.

Phagocytosis of PMNs As shown in Table IV, compds. I—IV inhibited dose-dependently the phagocytosis of latex beads by PMNs. Compound III showed a higher inhibitory effect (inhibition: 85.8%) than other analogues at 100 μM. The rank order of potency was compd. III > compd. IV (inhibition: 79.8%) > compd. II (75.1%) > compd. I (38.9%).

Cytotoxicity of PMNs None of the compounds (compds. I, II, III and IV) tested affected cell viability when they were incubated with PMNs (2×10^5 cells/ml) at 37 °C for 30 min at concentrations of 25, 50 and 100 μM (data not shown).

Platelet Aggregation Table V showed the inhibitory effect of compd. II and its analogues on epinephrine, collagen or arachidonate-induced platelet aggregation. While compd. I represented IC_{50} values of approximately 100 μM, other newly synthesized compounds had lower IC_{50} values. Thiol compound, II-1, demonstrated a slightly weaker activity than did its disulfide.

Histamine Release from Mast Cells None of the tested compounds affected non-stimulated or A-23187-stimulated histamine release from mast cells (data not shown). An anti-inflammatory drug, DSCG, inhibited the release by 59.3 and 79.1% at the concentrations of 200 and 1000 μM, respectively.

Hypotonic Hemolysis of Erythrocytes None of the disulfides affected the hypotonic hemolysis of erythrocytes (data not shown).

Discussion

In our previous reports, we found that compd. I possessed platelet anti-aggregant activity[2] and anti-inflammatory activity.[3] Inflammation has been considered a protective reaction system in animals. Leukocytes, platelets, macrophages and mast cells are related to the primary processes of the protective reactions.

One of the major protective reactions in inflammation is the emigration of leukocytes. Leukocytes, especially PMNs, migrate to the inflammatory site, phagocytose pathogenic bacteria and release lysosomal enzyme. Simultaneously, generated active oxgens sterilize the invading microorganisms. As shown in Fig. 2, compds. II, III, IV and II-1 strongly inhibited O_2^--generation induced by FMLP in PMNs. These compounds, with more hydrophobic substituents than the methyl group at the 2-C-position, were more effective in inhibiting O_2^--generation and also platelet aggregation than compd. I. Compound II-1 (thiol) was a slightly weaker inhibitor than compd. II (corresponding disulfide). The thiol compound might undergo rapid atmospheric oxidation under the present experimental conditions.[21] Therefore, the inhibitory activity observed might have been caused by a disulfide form rather than thiol form. Compound V, with the pyrrolidinyl group, was the least potent inhibitor. The most potent inhibitors of O_2^--generation, compds. II, III and IV, were tested for their effect on several functions of PMNs. Compounds II, III and IV, ranging from 10 to 100 μM, significantly inhibited a series of activation processes, chemotaxis, phagocytosis and lysosomal enzyme release in guinea pig peritoneal PMNs, without cytotoxicity. To look for clues of the action mechanism of compd. II and its analogues, its effect on O_2^--generation induced by several stimulations in PMNs was tested. It was found that unlike SOD, compd. II was not an O_2^--scavenger. As shown in Table I, compd. II was a stronger antagonist of FMLP than any of the other stimulators. It has been recognized that the FMLP signal is mediated to reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation *via* receptor binding, guanosine triphosphate (GTP) binding protein activation, phospholipase c activation, phosphatidylinositol response, intracellular [Ca^{2+}] mobilization and protein kinase c activation.[22] PMA directly activates protein kinase c, and calcium ionophore A-23187 activates phospholipase c by elevating intracellular [Ca^{2+}].[22] It is likely that the effect of compd. II and its analogues involves at least partly a certain process of signal tranduction from the FMLP membrane receptor to phospholipase c.

Platelets are rapidly activated by various stimulators such as epinephrine, collagen, and arachidonate. In activated platelets, chemical mediators such as serotonin and histamine are released, resulting in elevated vascular permeability. As shown in Table IV, newly synthesized compd. I analogues, compds. II, III, IV, V and II-1, were much stronger inhibitors of epinephrine, collagen and arachidonate-induced platelet aggregation than compd. I

On the other hand, we tested whether the hypotonic hemolysis of erythrocytes and histamine release of mast cells were influenced by these disulfide compounds. But none of the compounds tested showed any effect on the hemolytic reactions. And they did not affect non-stimulated or A-23187-stimulated histamine release from mast cells. Compound II was a weak antogonist of O_2^--generation induced by A-23187. It is likely that compd. II and its analogues do not mainly relate to the influx of [Ca^{2+}] in either PMNs or mast cells.

It was strongly suggested that compd. II and its analogues possessed an anti-inflammatory action through the inhibition of emigration, O_2^--generation, lysosomal enzyme release and phagocytosis of PMNs, and by inhibition of platelet aggregation. Althouth their biochemical action mechanism is not obscure, it is noteworthy that compd. I

has the ability to inhibit serotonin-induced paw edema formation,[3] differing from nonsteroidal anti-inflammatory drugs, which inhibit O_2^--generation.[23]

Glutathione (GSH) shows significant protective effects from scavenging, such as a free radical and peroxide. GSH and several cystamine derivatives are known to inactivate γ-glutamylcysteine synthetase, the rate-limiting enzyme for GSH synthesis.[24] Disulfides may be metabolized by either chemically or enzymatically exchanging thiol–disulfide between disulfides and GSH.[25] In general, it is believed that disulfide compounds modify receptor proteins and/or regulatory proteins physicochemically. Their oxidation-reduction potential, including thiol–disulfide exchange, and electrophilic reaction are thought to be a driving force. Catalytic activities of certain enzymes are reported to be affected by the formation of enzyme–GSH mixed disulfides or by modifying intramolecular disulfide bonds.[26] Further studies are required to clarify in detail the effects of compd. II and its analogues on functions of PMNs and platelets.

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