PYRROLOMYCIN GROUP ANTIBIOTICS INHIBIT SUBSTANCE P-INDUCED RELEASE OF MYELOPEROXIDASE FROM HUMAN POLYMORPHONUCLEAR LEUKOCYTES

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In order to search for microbial modulators of the activity of neuropeptide, we established a screen based on substance P (SP)-induced myeloperoxidase (MPO) release from human polymorphonuclear leukocytes (PMN). SP induced MPO release in a dose-dependent manner at concentrations ranging from $1 \sim 10 \times 10^{-4}$ M. In comparison at 1×10^{-4} M, induction was also observed with SP derivatives but not with other neuropeptides such as neurokinin and enkephalin. Based on this, we searched for microbial inhibitors against SP-induced MPO release. An actinomycete metabolite designated HS3, which turned out to be identical with dioxapyrrolomycin or A1-R2081, and structurally related pyrrolomycins were found to inhibit SP-induced MPO release. In addition, these compounds inhibited the f-Met-Leu-Phe (FMLP)-induced MPO release from PMN. Pyrrolomycin derivatives with an *N*-methylated pyrrole ring showed, however, a selective inhibition of the SP-induced MPO release. This was in contrast to results with aseanostatin P5 which selectively inhibited FMLP-induced MPO release.

We have been studying human polymorphonuclear leukocytes (PMN) functions. One of our major concerns is the PMN response to stimuli such as lung carcinoma-derived chemotaxin (LUCT/interleukin-8)[1~3], inflammatory mediators and neurosubstances. We have thought that such PMN response would be useful as a screen to search for novel biologically active substances or to reveal unknown activity of known compounds including antibiotics. Eventually we were successful to discover aseanostatins that inhibit the f-Met-Leu-Phe (FMLP)-induced myeloperoxidase (MPO) release from PMN[4].

Substance P (SP) has been known as a neuropeptide involved in inflammatory reaction[5,6]. Recently, it has been reported that SP induced the chemotaxis of PMN as well as the release of lysosomal β-glucuronidase (BGL)[7] and the respiratory burst[8,9]. In addition, the SP receptor is distributed widely among tissues[10]. Based on this, we thought that SP-induced MPO release from human PMN could be established as a screen to search for inhibitors of SP function. If such inhibitors are available, we may improve our understanding how SP is involved in physiological events such as inflammation or on the mechanism of SP binding to its receptor.

As a result of screening, an actinomycete metabolite designated HS3, which turned out to be identical with Al-2081[11], LL-F4228α[12] or dioxapyrrolomycin[13], was found to exhibit an inhibitory effect on MPO release. In this paper, the effect of HS3 and related antibiotics (pyrrolomycins) on SP-induced MPO release is described.

Materials and Methods

Chemicals

SP, other neuropeptides (Table 1) and f-Met-Leu-Phe (FMLP) were purchased from Protein Research Foundation (Osaka, Japan). Cytochalasin B (CB) and 3,3',5,5'-tetramethylbenzidine (TMB) were obtained from Sigma Chemical Company (St. Louis, U.S.A.). TMB was dissolved in *N*,*N*-methylformamide (Katayama Chemical, Osaka, Japan). Pyrrolomycin and its derivatives were obtained from Meiji Seika Kaisha, Ltd. (Japan)[14~18].

Preparation of PMN

PMN were isolated from venous blood of healthy human adult volunteers on a Lymphoprep density as described previously[19].

Assay for MPO Release

For induction of MPO release from PMN, the prewarmed (37°C, 10 minutes) PMN suspension (37.5 μl; 2×10^6 cells/ml of Hank's balanced salt solution (HBSS)) was mixed with SP (7.5 μl), phosphate buffered saline (PBS; 7.5 μl) and HBSS (22.5 μl). The mixture (75 μl) was then incubated at 37°C for 10 minutes in a plastic plate with 96 wells (Nunc, Denmark). Microbial culture extracts or purified chemicals (7.5 μl) were substituted for PBS when their effect on MPO release was examined. After incubation, the plate was chilled on ice for 1 minute and centrifuged at $330 \times g$ at 4°C for 5 minutes to separate supernatants from the cell. The cell pellets were then disrupted by suspending and mixing well with 150 μl of cold HBSS containing 0.1% Triton X-100 to obtain cell-lysates. Both the cell-lysates and the supernatants were assayed for their MPO activity according to the method described previously[20,21]. MPO activity in the supernatants was regarded as the MPO release from the PMN and total MPO activity referred to the supernatant MPO activity + the cell lysate MPO activity. Percent MPO release was therefore calculated as 100 × supernatant MPO activity/(supernatant + cell lysate) MPO activity. One unit of MPO activity was defined as the 1.0 increase in absorbance at 655 nm ($10^3 \times \Delta A_{655}$/minute/ml of sample) due to formation of the oxidation product from tetramethylbenzidine as an appropriate substrate.

SP was dissolved in HBSS right before the assay. PBS (pH 7.3) consisted of NaCl 8.0 g, KCl 0.2 g, Na_2HPO_4 (anhydrous) 1.15 g and KH_2PO_4 (anhydrous) 0.2 g/liter of double distilled water (DDW).

Table 1. Effect of neuropeptides on the release of MPO from human PMN.

Compounds tested (1×10^{-4} M)	% Release of MPO[a]	Compounds tested (1×10^{-4} M)	% Release of MPO[a]
HBSS	6.6±0.6	HBSS	4.3±0.5
FMLP (1×10^{-6} M)+CB (5 μg/ml)	20.4	FMLP (1×10^{-6} M)+CB (5 μg/ml)	39.9±12.7
SP	24.4	SP	5.1
[Tyr[8]]-SP	8.9	Neurotensin	3.5
[D-Pro[2], D-Trp[7,9]]-SP	10.2	α-Endorphin	2.3
[D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]]-SP	13.5	Bradikinin	2.6
[D-Arg[1], D-Trp[7,9], Leu[11]]-SP	19.2	Neuropeptide Y (1×10^{-5} M)	1.9
HBSS	2.7	HBSS	4.2±0.3
FMLP (1×10^{-6} M)+CB (5 μg/ml)	6.2	0.1% DMSO	4.9
Neurokinin A	1.5	FMLP (1×10^{-6} M)+CB (5 μg/ml)	22.3±2.4
Neuromedin B	1.7	SP	30.1
Neoromedin C	2.2	Bradikinin-potentiator B	7.1
Leucine-enkephalin	1.9	Leucine-enkephalin (Sulfate)	3.9
Methionine-enkephalin	2.2	[D-Ala[2], D-Leu[5]]-enkephalin	5.8
[D-Ala[2], Met[5]]-enkephalin	1.5	β-Endorphin	1.8
[D-Ala[2], Met[5]]-enkephalinamide	2.0	γ-Endorphin	3.7
PHM-27 (1×10^{-5} M)	2.3	Des-Thr[1]-γ-endorphin	3.9
Bombesin (1×10^{-5} M)	2.2		

[a] 100 × supernatant/(cell lysate + supernatant).

HBSS consisted of NaCl 8.0 g, KCl 0.4 g, Na_2HPO_4 (anhydrous) 47.9 mg, KH_2PO_4 60 mg, $MgSO_4$ (anhydrous) 48.8 mg, $MgCl_2$ (anhydrous) 46.8 mg, $CaCl_2$ (anhydrous) 140 mg and glucose 1.0 g/liter of DDW.

Preparation of Microbial Culture Extracts

Soil isolates of actinomycetes, bacteria and fungi were cultivated on a rotary shaker at 26~28°C for 3~4 days in 500-ml Erlenmeyer flasks each containing 80~100 ml of the following media: Medium for actinomycetes consisted of malt syrup 3.0%, soy bean oil 0.15%, soy bean meal 1.5%, soluble vegetable protein (SVP) 0.5%, Pharmamedia 1.0%, $NaNO_3$ 0.3% and $CaCO_3$ 0.2%, pH 7.0. Medium for bacteria and fungi consisted of glucose 2.0%, starch 1.0%, soy bean meal 1.25%, wheat germ 0.8%, NaCl 0.125% and $CaCO_3$ 0.15%, pH 7.0.

After incubation, each broth was added to an equal volume of EtOAc and mixed vigorously. The EtOAc layers were taken out and evaporated. The resultant residues were dissolved in an equal volume of PBS and diluted to 3 and 10% with PBS for the MPO release assay.

Purification and Characterization of HS3

A slant culture of strain HS3 was inoculated into 100 ml of a seed medium in a 500-ml Erlenmeyer flask and incubated at 28°C for 2 days on a rotary shaker at 220 rpm. The seed medium consisted of starch 1.0%, glucose 1.0%, peptone 0.5%, meat extract 0.2%, yeast extract 0.3%, soy bean meal 0.2% and $CaCO_3$ 0.2%, pH 7.0. The seed culture (1.6 ml) was then transferred into twenty 500-ml Erlenmeyer flasks each containing 80 ml of a production medium and incubated for 65 hours under the same conditions as those for the seed culture. The production medium consisted of starch 2.0%, wheat germ 2.0%, SVP 0.5%, NaCl 0.25%, $CaCO_3$ 0.3% and $CoCl_2 \cdot 6H_2O$ 0.001%. The cultured broth was mixed with EtOAc (1.0 liter) and filtered at pH 7.5 with the aid of Celite. The EtOAc layer was separated, washed with water and concentrated to dryness. The resultant residue (0.25 g) was chromatographed on a silica gel column (50 g) developed with $CHCl_3$-MeOH (50:1). The active fractions were pooled and concentrated and the resulting powder (75 mg) was purified on a Sephadex LH-20 column developed with MeOH. Finally, recrystallization of the crude powder from hot benzene was carried out. The recrystallization yielded 38 mg of pale yellow crystals designated HS3.

Physico-chemical characterization of HS3 was carried out by NMR, EI-MS, IR, TLC.

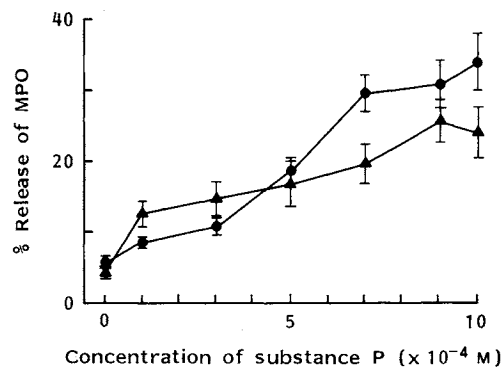
Results

Effective Concentration of SP for Stimulation of MPO Release from Human PMN

In order to establish the basis for a novel screen, we first examined the effective dose range of SP on MPO release from human PMN. As shown in Fig. 1, a dose-dependent induction of MPO release was observed at concentrations ranging from $1 \sim 10 \times 10^{-4}$ M. The highest effect was obtained at 10×10^{-4} M. No significant induction was detected at concentrations lower than 1×10^{-4} M (data not shown). It has been known that FMLP causes MPO release from human PMN in the presence of CB[22]. Therefore, CB (5 μg/ml) was examined for its effect on the SP-induced MPO release (Fig. 1). It turned out that a stimulative effect of CB was observed in the lower range of SP concentrations ($1 \sim 3 \times 10^{-4}$ M), whereas a suppressive effect of

Fig. 1. SP-induced MPO release from human PMN.

● MPO release with SP, ▲ MPO release with SP in the presence of CB (5 μg/ml). Mean ± S.E. ($n=9$) values were plotted. Total MPO activity was about 17 units.



CB was observed at higher SP concentrations over 5×10^{-4} M.

Extracellular levels of lactate dehydrogenase (LDH) as a cytosol enzyme of PMN were also examined according to the method of PESCE *et al.*[22]. The LDH levels remained at the basal level in the presence of $5 \sim 10 \times 10^{-4}$ M SP (data not shown), indicating that SP did not cause the leakage of PMN cytosol.

SP derivatives and several other neuropeptides such as neurokinin and enkephalin were also examined for their capability of inducing MPO release at the concentration of 1×10^{-4} M (Table 1). None of the latter substances showed obvious induction, while SP derivatives clearly induced MPO release. The effect of SP derivatives was, however, weaker than that of SP.

Screening of Microbial Metabolites Inhibiting the SP Effect

Based on the above results, we employed 1×10^{-4} M of SP for the induction of MPO release to screen for microbial inhibitors against the SP-dependent MPO release. We have screened EtOAc extracts of 100 actinomycete, 100 fungal and 50 bacterial cultured broths. Inhibitory activity was reproducibly observed with samples from 12 actinomycete, 3 fungal and 5 bacterial cultured broths. Total MPO activity of PMN that referred to the combined activity of supernatant MPO and cell lysate MPO remained at a constant level (data not shown). This indicated that a low level of MPO activity in the supernatants should not be due to the inhibition of the enzyme activity of MPO but to the inhibition of MPO release from PMN. Among the active samples, the sample derived from an actinomycete strain designated HS3 with the strongest activity was chosen for further characterization because a nematoda, *Caenorhabditis elegans*, was also inhibited in a manner suggesting activity on the nervous system (data not shown).

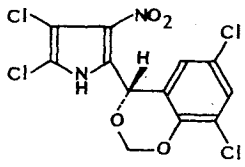
Inhibition of SP-induced MPO Release by HS3 and Pyrrolomycins

An active principle designated HS3 was isolated and purified from the EtOAc extract of the cultured broth of the actinomycete strain HS3. Chemical characterization revealed that HS3 was identical with Al-R2081[10] or dioxapyrrolomycin[12] (Fig. 2). In brief, HS3 showed the following physico-chemical properties: UV λ_{max}^{MeOH} nm 276, 324; IR (KBr) cm^{-1} 1570, 1360; ^1H NMR ($CDCl_3$-CD_3OD) δ 3.42 (2H, m), 5.38 (1H, s), 5.55 (1H, s), 6.85 (1H, s), 7.32 (1H, s); EI-MS 382 (M^+), 354 ($M^+ - 28$). These data indicated that the chemical structure of HS3 was closely related to that of the pyrrolomycin group antibiotics. Finally, HS3 was identified as dioxapyrrolomycin (Al-R2081) on the basis of TLC, mp and IR data. Taxonomic properties of the HS3 producer fell into those of *Streptomyces* (data not shown). Therefore the strain was designated as *Streptomyces* sp. HS3.

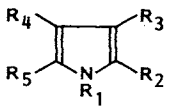
Fig. 3 shows the inhibitory effect of HS3 on MPO release induced by 7×10^{-4} M SP and by a mixture of FMLP (1×10^{-6} M) and CB (5 μg/ml). While approximately 2- and 3-fold increases in MPO release were observed with SP and a mixture of FMLP and CB, respectively, without addition of HS3, these increases were inhibited by the addition of HS3 in a dose-dependent manner. The MPO release induced by both SP and the mixture of FMLP and CB was inhibited almost completely by 1 μg/ml of HS3. IC_{50} was estimated at 0.1 μg/ml for the MPO release induced by SP and 0.3 μg/ml for that induced by the mixture of FMLP and CB. HS3 itself showed no effect on the MPO release from human PMN.

Since HS3 turned out to be one of the pyrrolomycin group of antibiotics (Fig. 2), we examined the inhibitory effect of these compounds at the concentrations of 0.1 μg/ml and 10 μg/ml. As shown in Table 2, HS3, pyrrolomycins (A, B, C, D and F1) and their methylated derivatives strongly inhibited SP-induced MPO release; $45 \sim 80\%$ at 0.1 μg/ml and almost completely at 10 μg/ml, indicating a dosage effect. The FMLP-induced release in the presence of CB was also inhibited by these compounds except for the

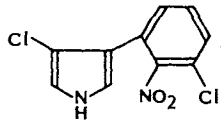
Fig. 2. Structures of HS3 and its related compounds.



HS3 (Dioxapyrrolomycin)



Pyrrolomycins



Pyrrolnitrin

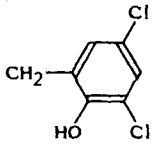
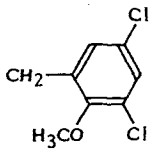
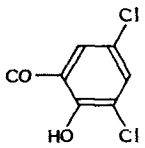
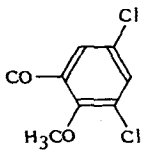
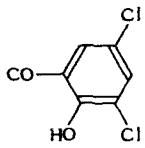
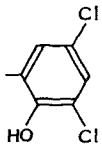
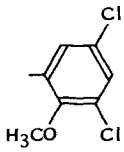
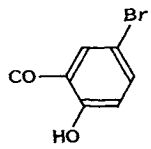
Pyrrolomycins (PM)	R_1	R_2	R_3	R_4	R_5
PM-A	H	H	NO_2	Cl	Cl
N-Methyl-PM-A	CH_3	H	NO_2	Cl	Cl
PM-B	H		NO_2	Cl	Cl
N,O-Dimethyl-PM-B	CH_3		NO_2	Cl	Cl
PM-C	H		H	Cl	Cl
N,O-Dimethyl-PM-C	CH_3		H	Cl	Cl
PM-D	H		Cl	Cl	Cl
PM-E	H		NO_2	Cl	Cl
N,O-Dimethyl-PM-E	CH_3		NO_2	Cl	Cl
PM-F1	H		Br	Br	Br

Fig. 3. Effect of HS3 on the MPO release induced by either SP or a mixture of FMLP and CB.

■ SP-induced MPO release, ▲ FMLP-induced MPO release in the presence of CB, ● MPO release in the presence of HS3 alone.

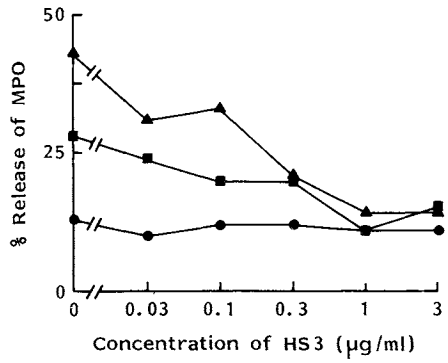


Table 3. Effect of aseanostatin P5 (P5) on the SP-induced or FMLP-induced MPO release from human PMN.

Compounds	MPO release			
	SP-induced		FMLP-induced[a]	
	% Release	% Control	% Release	% Control
None (control)	16.7	100.0	41.2	100.0
P5 (1.0 μg/ml)	15.9	92.7	27.1	60.2

[a] In the presence of CB (5 μg/ml).

Table 2. Inhibitory effect of HS3 and its related compounds on the MPO release induced by SP and FMLP.

Compounds	MPO release (% Control)[a]			
	0.1 μg/ml[b]		10 μg/ml[b]	
	SP	FMLP	SP	FMLP
None (control)	100.0	100.0	100.0	100.0
HS3	56.1	67.9	1.3	36.8
PM-A	54.1	104.1	−15.3	62.9
N-Methyl-PM-A	51.6	101.6	59.2	104.7
PM-B	43.3	77.0	−31.2	22.6
N,O-Dimethyl-PM-B	31.8	114.2	−7.0	103.5
PM-C	42.0	97.8	−28.7	62.6
N,O-Dimethyl-PM-C	43.9	94.0	35.0	104.1
PM-D	49.7	80.8	−29.9	−12.6
PM-E	18.5	67.9	−9.6	66.7
N,O-Dimethyl-PM-E	40.9	91.5	−2.5	89.6
PM-F1	40.1	90.9	−30.6	59.4
Pyrrolnitrin	33.1	83.6	70.7	95.3

[a] 100 × (compound − basal)/(SP or FMLP − basal). In this equation, 1) compound, 2) SP (or FMLP) and 3) basal refer to MPO release in the presence of the following: 1) compound (0.1 or 10 μg/ml) + SP (7×10^{-4} M) or FMLP (1×10^{-6} M) + CB (5 μg/ml) in 0.1% DMSO. 2) SP or FMLP alone at the same concentrations as 1). 3) 0.1% DMSO. The MPO release under the conditions 2) reached 27.5% with SP and 43.6% with FMLP, respectively. The basal MPO release with 0.1% DMSO was 11.8%. Total MPO activity was approximately 30~40 units.

[b] Concentrations of compounds added.

methylated pyrrolomycin derivatives. Their inhibition was, however, weaker than that against the SP stimulation. Furthermore, the dosage effect of the methylated pyrrolomycins was not parallel with that of the nonmethylated pyrrolomycins.

Mono- or di-methyl pyrrolomycins (10 μg/ml) showed little or no inhibition against the FMLP-induced MPO release in the presence of CB selectively, while they showed high inhibition (comparable to nonmethylated ones) against the SP-induced MPO release.

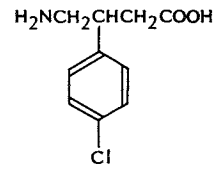
Pyrrolnitrin, another antibiotic containing the pyrrole ring (Fig. 2), also showed a relatively high activity against the MPO release induction by SP. The FMLP-induced MPO release was not significantly inhibited by the antibiotic.

Aseanostatin P5[4] (12-methyltetradecanoic acid) that was recently discovered by us as an inhibitor against the FMLP-induced MPO release was also examined for its capability of inhibiting SP-induced MPO release (Table 3). Aseanostatin P5 did not inhibit SP-induced MPO release at the concentration that exhibited a clear inhibition of the FMLP-induced MPO release. It seems likely that aseanostatin P5 is reciprocal to the pyrrolomycin derivatives with *N*-methylated pyrrole rings in terms of inhibition against the MPO release inductions by SP and FMLP.

Discussion

In the present study, we established conditions for the induction of MPO release from PMN by SP. The effect of SP was detected at 1×10^{-4} M and increased with increasing the concentration. In comparison at 1×10^{-4} M, SP derivatives also induced MPO release, but other neuropeptides including neurokinin A did not. Thus the induction of MPO release by the neuropeptides we examined was remarkable with only SP. Since the extracellular activity of LDH (a cytosol enzyme) of PMN induced with SP remained at the basal level, the increase in the extracellular activity of MPO caused by SP would not be due to leakage by membrane damage but to release by exocytosis. SP and neurokinin A are tachykinin family neuropeptides that share Phe-X-Gly-Leu-Met-NH_2 as the common carboxyl terminal sequence[23,24]. It is therefore suggested that the *N*-terminal side sequence of SP is effective for the induction of the MPO release.

Fig. 4. Structure of baclofen [β-(4-chlorophenyl)-GABA].



It will be noted that the SP derivatives we used are known as antagonists of SP[25] and induced the MPO release from PMN. This suggests that SP antagonists can be detected by testing the increase in MPO release from PMN. Since the MPO release from PMN can be easily and rapidly assayed in a small scale, the PMN response to exogeneous substances will be useful not only to search for novel compounds with novel activity but also to reveal activities of known antibiotics.

Pyrrolomycin group antibiotics have so far been noted only for their antibiotic activity[11~14]. It was revealed in the present study that they showed a high inhibitory activity against the MPO release induced by either SP or FMLP. Thus unknown activities of pyrrolomycin group antibiotics was first visualized. For high activity against the FMLP-induced MPO release, the NH of the pyrrole ring seems to be critical, since *N*-methylation of the pyrrole ring resulted in the selective reduction of their activity against FMLP-induced MPO release. This was paralleled with the loss of antibiotic activity of pyrrolomycins (unpublished data) and was in contrast to the result of aseanostatin P5 that did not inhibit the SP-induced MPO release from human PMN but rather the FMLP-induced MPO release. One possibility for this differential activity between pyrrolomycins and their *N*-methylated derivatives will be differential binding to the receptor(s) for SP and FMLP. It has been known that SP binds to the FMLP chemotaxis receptor on the rabbit neutrophil[7]. The same thing might be true for the human neutrophil or PMN. Based on this postulation, pyrrolomycins may block binding sites for both SP and FMLP on the receptor and the *N*-methylated derivatives only the SP site. However, we can not rule out that PMN has a different receptor for SP from that for FMLP. Another possibility will be the differential activity to initiate signal transduction or exocytosis for the release of lysosomal enzymes. Comparative studies on the mode of action of these compounds including aseanostatin[4] are now in progress.

SP has been known as a neurotransmitter of pain[23] and recently as a likely mediator or regulator for inflammatory reactions or immune responses[5,26]. In relation to SP function in the nervous system, baclofen[23] (a GABA derivative) has been known as an SP antagonist whose structure is not similar to SP (Fig. 4). We recently noticed that this antagonist has a 4-chlorophenyl moiety that has similarity with the pyrrolomycin group antibiotics. Since GABA is not an antagonist of SP, the 4-chlorophenyl moiety will be critical for the antagonistic activity to SP of baclofen. It is therefore of interest whether pyrrolomycin group antibiotics act as antagonists of SP in the nervous system.

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