BE-18591 as a new H^+/Cl^- symport ionophore that inhibits immunoproliferation and gastritis

Keiji Tanigaki[a], Tomohiko Sato[a,1], Yasufumi Tanaka[a,2], Takahiro Ochi[a,3], Asako Nishikawa[a,4], Kazuo Nagai[b,5], Hiroyuki Kawashima[c], Shoji Ohkuma[a,*]

[a] *Laboratory of Biochemistry and Molecular Cell Biology, Department of Pharmaceutical Biology, Faculty of Pharmaceutical Sciences, Kanazawa University, Takara-machi 13-1, Kanazawa, Ishikawa 920-0934, Japan*
[b] *Department of Bioengineering, Tokyo Institute of Technology, 4259 Nagatsuta-chou, Midori-ku, Yokohama, Kanagawa 226, Japan*
[c] *Department of Pharmacology, Niigata University School of Dentistry, 2-Bancho 5274, Gakkocho-dori, Niigata 951-8514, Japan*

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Abstract **In our previous papers [e.g. Sato et al., J. Biol. Chem. 273 (1998) 21455–21462], we have shown that prodigiosins can uncouple various H^+-ATPases through their H^+/Cl^- symport activity. BE-18591 is an enamine of 4-methoxy-2,2′-bipyrrole-5-carboxyaldehyde (tambjamine group antibiotics) which resembles the prodigiosins. We found that BE-18591 was a new group of antibiotics that uncouples various H^+-ATPases: it inhibited proton pump activities with IC_{50}s of about 1–2 nM (about 20 pmol/mg protein) for submitochondrial particles as well as gastric vesicles and of 230 nM (about 230 pmol/mg protein) for lysosomes, but it had little effect on their ATP hydrolyses (up to 10 μM), a property of H^+/Cl^- symport activity. At low concentrations (< 1 μM), BE-18591 inhibited immunoproliferation, the IC_{50} of lipopolysaccharide-stimulated mouse splenocytes was 38 nM, that of Concanavalin A-stimulated cells was 230 nM. Gastritis of rabbits was also inhibited. At higher concentrations (> 1 μM), BE-18591 induced neurite outgrowth (15% induction in 48 h at 4 μM), inhibited bone resorption (~35% in 48 h at 10 μM) and caused cell death (~30% in 48 h at 4 μM) but with little apoptosis.**

Key words: BE-18591; Proton pump; H^+/Cl^- symport; Immunoproliferation; Gastritis

1. Introduction

We have been studying the activity of vacuolar-type H^+-ATPase (V-ATPase) inhibitors on cell differentiation (neurite outgrowth (NOG) of PC12 cells [1] and macrophage production from M1 cells [2]), the inhibition of cell growth [3] and induction of apoptosis in PC12 cells [4]. We have also shown previously that the prodigiosin group of antibiotics, red pigments produced by microorganisms *Streptomyces* and *Serratia*, are a group of H^+/Cl^- symporters that uncouple various proton transporters [5–8]. Cycloprodigiosin was able to induce differentiation and apoptosis in HL60 cells [9]. Recently, BE-18591 (Fig. 1) was produced by streptomycete strain BA18591 as a yellowish green pigment in the methanol extract [10,11]. It inhibited the growth of some Gram-positive bacteria but had little effect on those of Gram-negative bacteria and *Saccharomyces cerevisiae*. It also inhibited the growth of P388 cells and MNK human stomach cancer cells in culture, as well as transplanted Ehrlich ascites tumor cells in BDF_1 mice [10]. BE-18591 is regarded as a member of the tambjamine group of antibiotics (including tambjamine E and tambjamine aldehyde) as well as the prodigiosin group [11].

In this paper we report that BE-18591 reversibly uncoupled various H^+-ATPases through its H^+/Cl^- symport activity and inhibited gastric acid secretion as well as the immune responses. Furthermore, at higher concentrations, it showed some degree of inhibition of bone resorption (pit formation), induction of NOG (in PC12 cells) and cell deaths (apparently necrosis) in various cells.

2. Materials and methods

2.1. Materials

Rats (Wistar, male) were obtained from Sankyo Labo Service (Tokyo, Japan). Fluorescein-labeled dextran (FD) was either synthesized according to [12] or supplied in the form of FITC (fluorescein isothiocyanate)-dextran (MW 70 000) from Sigma (St. Louis, MO, USA). Stomachs of freshly slaughtered hogs were provided by the Meat

*Corresponding author. Fax: (81)-76-234 4462.
E-mail address: ohkuma@kenroku.kanazawa-u.ac.jp (S. Ohkuma).

[1] Present address: Santen Seiyaku Co., Ltd., Nagoya Office, Nagoya, Aichi 461-0025, Japan.
[2] Present address: Lemon Dispensing Pharmacy, Tsu, Mie 514-0001, Japan.
[3] Present address: Menikon Research and Development, Kasugai, Aichi 487-0032, Japan.
[4] Present address: Fuji Chemical Industries, Ltd., Takaoka, Toyama 933-0951, Japan.
[5] Present address: Department of Biological Chemistry, College of Bioscience and Biotechnology, Chubu University, Kasugai, Aichi 487-8501, Japan.

Abbreviations: Con A, concanavalin A; ΔpH, transmembrane pH gradient; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; FDA, fluorescein-diacetate; F-ATPase, F-type H^+-ATPase; FBS, fetal bovine serum; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; FD, fluorescein-labeled dextran; HEPES, *N*-[2-hydroxy-ethylpiperazine-*N*′-[2-ethanesulfonic acid]; LPS, lipopolysaccharide; MES, 2-[*N*-morpholino]-ethanesulfonic acid; NOG, neurite outgrowth; PIPES, piperazine-*N*,*N*′-(bis-2-ethanesulfonic acid); P-ATPase P-type H^+-ATPase; Pyranine, 8-hydroxy-1,3,6-pyrenetrisulfonic acid trisodium salt; SMP, submitochondrial particle; TMAH, tetramethylammonium hydroxide; V-ATPase, vacuolar-type H^+-ATPase

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Inspection Center of Kanazawa City (Kanazawa, Japan). Rabbits (Japan white rabbits, male, 2–2.5 kg in body weight) were obtained from Shiraishi Laboratory Animal (Tokyo, Japan). BE-18591 was extracted in methanol from streptomycete strain BA18591and provided from Tsukuba Research Institute, Banyu Pharmaceutical (Tsukuba, Japan). Prodigiosin 25-C and metacycloprodigiosin were prepared from the culture broth of *Streptomyces hiroshimensis* as described elsewhere [13]. Omeprazole was obtained from Fujisawa-Astra Japan (Osaka). Protease inhibitors of microbial origin were obtained from the Peptide Research Institute (Osaka). Bafilomycin A_1 was kindly provided by Professor K. Altendorf (University of Osnabrück, Osnabrück, Germany). 8-Hydroxy-1,3,6-pyrenetrisulfonic acid, trisodium salt (pyranine), laser grade was from Eastman Kodak (Rochester, NY, USA). Acridine orange, carbonylcyanide *p*-trifluoromethoxy-phenylhydrazone (FCCP), 2-[*N*-morpholino]-ethanesulfonic acid (MES), *N*-(2-hydroxy-ethylpiperazine-*N*′-[2-ethanesulfonic acid) (HEPES), tetramethylammonium hydroxide (TMAH), sodium gluconate, and glucono-D-lactone were all supplied from Wako (Tokyo, Japan). Phospholipids were obtained from Sigma (Sigma type II-S phosphatidylcholine from soybean phospholipids), and used without purification. Other reagents were purchased as commercial products mostly from Sigma (St. Louis, MO, USA) and used without further purification.

2.2. Cell culture

Rat pheochromocytoma PC12 cells (RCB 009) were obtained from the RIKEN Cell Bank (Tsukuba, Japan) and cultured in Dulbecco's modified Eagle's medium (DMEM, Flow Laboratories) supplemented with 10% fetal bovine serum (FBS, M.A. Laboratory) 5% horse serum (COSMO Bio, Japan) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37°C in 5% CO_2 and 95% air. CHO-K1 cells were cultured in F-12 medium (HAM, Nissui, Tokyo, Japan) supplemented with 0.1% Bactopeptone (Difco Laboratories), 10% FBS and antibiotics (as above).

2.3. Preparation of organelles

FD-loaded lysosomes (FD-dextranosomes) were isolated from the liver of rats injected i.p. with FD 16–20 h earlier according to the method described elsewhere [14], with the exception that the Percoll washout procedure was omitted and the lysosomal layer in the Percoll gradient was used for the experiments. Submitochondrial particles (SMPs) were prepared from rat liver according to the published procedure [15,16] before being suspended in the isotonic buffer and stored at −80°C until use. Membrane vesicles containing (H^++K^+)-ATPase were mostly purified from the gastric mucosa of freshly slaughtered hogs according to [17] with slight modifications [8]. The resulting pellet was resuspended in 2 ml of 5 mM piperazine-*N*,*N*′-(bis-2-ethanesulfonic acid) (PIPES)-Tricine (pH 6.8) and stored at −80°C until use. The specific activity for the vanadate-sensitive K^+-ATPase in the final preparation was about 0.72–075 U/mg protein with little detectable ouabain-sensitive ATPase activity.

2.4. Measurement of proton pump activity

The formation of a transmembrane pH gradient (ΔpH, inside acid) in lysosomes was measured by the fluorescence quenching of FD incorporated into lysosomes [5,18–20]. The formation of ΔpH in the vesicles (SMPs and gastric vesicles) was measured by the fluorescence quenching of a permeant basic dye, acridine orange [6,8]. The assay buffer for lysosomal acidification contained 0.15 M KCl, 20 mM HEPES–TMAH (pH 7.5), and 2.5 mM $MgCl_2$ in a final volume of 2.0 ml. The reaction was started by the addition of 1.0 mM adenosine triphosphate (ATP)–Na_2 [5]. The assay buffer for SMPs was 0.15 M KCl, 20 mM HEPES–TMAH (pH 7.5), 2.5 mM $MgCl_2$ (with or without 1 mM valinomycin) and 1 μM acridine orange in a final volume of 2.0 ml. The reaction was started by the addition of 2.5 mM ATP–Na_2 [6]. The assay buffer for gastric vesicles contained 150 mM KCl, 8 mM PIPES–TMAH (pH 6.8), 2 mM $MgCl_2$, 5 mM valinomycin and 2 μM acridine orange in a final volume of 2.0 ml. The reaction was started by the addition of 1.0 mM ATP–Na_2 [8]. Fluorescence was measured at 37°C with a Hitachi 850 or F-4500 spectrofluorometer, with combinations of excitation at 495 nm and emission at 520 nm for FD and at 480 nm and 520 nm for acridine orange. The slit widths were 5 nm for both monochrometers. The initial velocity of acidification was defined by the rate of ATP-dependent quenching (ΔF) of the fluorophores. For the reversibility of BE-18591, the membrane vesicles of hog gastric mucosa (0.38 mg protein) were preincubated in a total volume of 100 μl containing valinomycin with or without the inhibitors (10 nM BE-18591) for 3 min at 37°C. After the preincubation, 1.9 ml of assay medium containing acridine orange, with or without inhibitors, was added, and the acidification of these membrane vesicles was started by the addition of 1.0 mM ATP. The change in fluorescence was traced with the excitation at 493 nm and emission at 530 nm.

2.5. Measurement of ATPase activity

The assay buffer for lysosomal V-ATPase (bafilomycin A_1-sensitive ATPase) contained 0.15 M KCl, 20 mM HEPES–TMAH (pH 7.5), 2.5 mM $MgCl_2$, and 2.5 mM ATP–Na_2 in a final volume of 1.0 ml. The samples were incubated at 37°C for 40 min [21]. The assay buffer for SMP contained 0.15 M KCl, 20 mM HEPES–TMAH (pH 7.5), 2.5 mM $MgCl_2$, and 2.5 mM ATP–Na_2 in a final volume of 1.0 ml. The incubation temperature and period were 37°C and 40 min, respectively [6]. The assay buffer for gastric mucosa membranes contained 150 mM KCl, 8 mM PIPES–TMAH (pH 6.8), 2 mM $MgCl_2$, 5 mM valinomycin, 1.0 mM ATP–Na_2, and 5 μg/ml of protease inhibitors (leupeptin, antipain, chymostatin, and pepstatin), in a final volume of 1.0 ml. The negative control buffer contained 150 mM choline chloride instead of KCl. Assays were started by adding membrane vesicles (about 2.38 mg gastric mucosa protein/tube) and the samples incubated at 37°C for 10 min [8,17]. The liberated phosphate was estimated by the malachite green method [22].

2.6. Estimation of H^+/Cl^- symport activity using liposomes

H^+/Cl^- symport activity was measured by the fluorescence quenching of pH-sensitive pyranine incorporated into liposomes and osmotic swelling of liposomes in ammonium chloride-containing medium [7]. Liposomes were prepared from phosphatidylcholine (Sigma type II-S phospholipids, so-called asolectin) essentially as described elsewhere [7]. Briefly, phosphatidylcholine was suspended at 50 mg/ml in a 20 mM HEPES–TMAH (pH 7.5) buffer containing 25 mM of gluconate (usually 2 ml). The mixture was vortexed vigorously and sonified to clarity at room temperature for 5 min. It was then applied onto a spin column equilibrated with the assay buffer and centrifuged at 2000 rpm for 30 s just before use. The swelling of liposomes in hypertonic ammonium salts was monitored by the decrease in absorbance at 550 nm [23]. Liposomes prepared in 2 mM HEPES–TMAH (pH 7.5), and 0.25 M sucrose were loaded in 0.2 M ammonium salt of the anion (e.g. NH_4Cl, NH_4-gluconate), and then after 30 s the BE-18591 was added while the OD_{550} was monitored.

2.7. Immunosuppression

Immunosuppressive activity was assessed by the growth inhibition of stimulated mouse spleen lymphocytes [24]. Briefly, in the mitogenic response, mice splenocyte (5×10^5 cells/well, 200 μl) were plated in a flat-bottom microtiter plate in triplicate cultures. Cells were cultured for 48 h either with 2 μg/ml lipopolysaccharide (LPS) or with 1 μg/ml concanavalin A (Con A). [^{3}H]Thymidine (9.23 kBq/well) was added to each well 4 h before harvesting. The cells were harvested on a glass filter and the radioactivity was measured by a liquid scintillation counter.

2.8. Fluorescent measurement of cell growth, cell viability and chromosome condensation

Cell growth was estimated essentially according to [3]. The fluorescence was measured using a fluorescence spectrophotometer (Hitachi model F-4500, λ_{Ex} = 356 nm, λ_{Em} = 458 nm). Cell viability was accessed by dual fluorescence staining with propidium iodide and fluorescein-diacetate (FDA) [25]. Cells plated in 24-well culture dishes were incubated in a medium containing propidium iodide (50 nM) and FDA (0.15 nM) at 37°C for 10 min, before being washed and observed by epifluorescence microscopy (Olympus, BH2-RFK or IMT-2-21CA3 with B-excitation). The viable cells fluoresced green due to the fluorescein accumulated through the cytoplasmic hydrolysis of FDA, while the nuclei of dead cells fluoresced red because of the propidium iodide taken up due to loss of the permeability barrier. Apoptosis was accessed morphologically as chromatin condensation by staining mostly with Hoechst 33258 (or 33342) after the cells had been fixed with 2% glutaraldehyde and washed once with DMEM by centrifugation ($3000\times g$ for 5 min) [4].

2.9. Gastric secretion

Parietal cells of rabbit gastric glands were isolated essentially as described previously [26]. Parietal cells were isolated from the gastric gland by combined collagenase digestion [27] and Percoll density gradient cell fractionation methods [28], with slight modifications [8]. A primary culture of isolated parietal cells was performed according to the method of Chew et al. [29] with slight modifications [8]. The isolated parietal cells were cultured on glass coverslips coated with collagen type IV in a medium consisting of DMEM plus 10% FBS at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The acid secretion was essentially as described elsewhere [30] using the accumulation of the fluorescent weak base, acridine orange, in apical vacuoles. Cells were preincubated in a medium containing 5 μM acridine orange, before being stimulated with histamine (10^{-4} M). They were then further incubated for 40 min and viewed under a phase-contrast fluorescent microscope (Axiovert+35M, Zeiss) using a B-type dichroic mirror (excitation filter, BP450–490; dichroic mirror, FT510; barrier filter, LP520) [8].

2.10. NOG of PC12 cells

NOG of PC12 cells was estimated as described previously [1]. A neurite was defined as a process whose length was more than 1.5 times longer than that of the cell body as judged from the photographs taken under phase contrast microscopy (Olympus, Tokyo). About 150 cells were counted per well, and the percentage of cells with neurites was calculated.

2.11. Pit assay for bone-resorbing activity

To determine the bone-resorbing activity, we measured the area and number of resorption pits formed on dentine slices by isolated mature osteoclasts [31,32]. The concentrations of parathyroid hormone and vitamin D_3 were both 10 μM.

2.12. Other analytical methods

Protein was determined by the amido black/solid phase method [33], or with the Coomassie brilliant blue/liquid phase method [34] using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA), with BSA as the standard.

3. Results

3.1. Effect of BE-18591 on the proton pump and ATPase activities of V-, F-, and P-ATPases

Proton pump activities of V-, F- and P-ATPase were inhibited by prodigiosins (prodigiosin 25-C, metacycloprodigiosin and prodigiosin) at relatively low concentrations [5–8]. The effect of BE-18591 on the proton pump activity was first investigated in this study. As shown in Fig. 2, it inhibited the proton pump activity of the lysosomal proton pump (V-ATPase) with an IC_{50} of 80 nM (about 230 pmol/mg protein) (350 μg protein/ml). It also inhibited the mitochondrial ATP-dependent proton pump activity (F-ATPase) with an

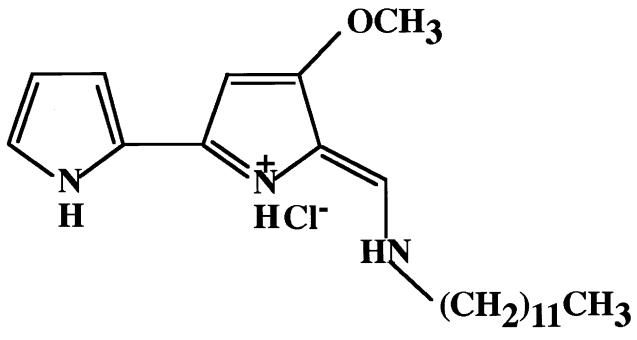

Fig. 1. Chemical structure of BE-18591. BE-18591 used was the hydrochloric acid salt ($C_{22}H_{35}N_3O$·HCl) and its molecular weight was approximately 393.5.

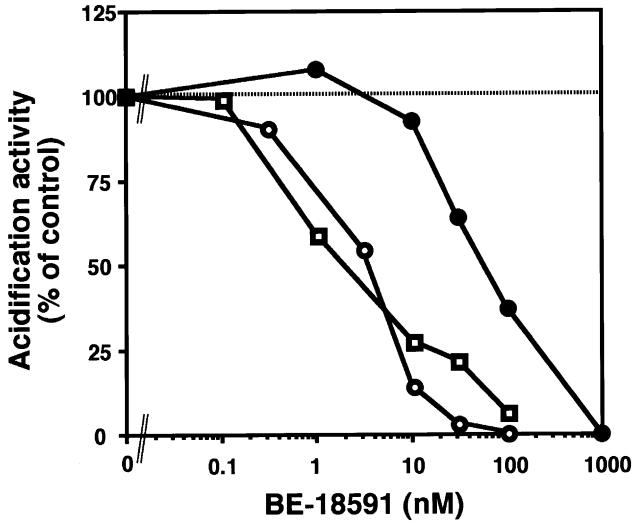

Fig. 2. Effect of BE-18591 on the acidification activity of various H^+-ATPases. Preparations of subcellular organelles and assay methods of the proton pump activity were performed according to Section 2. ○, rat liver submitochondrial H^+-ATPase; ●, rat liver lysosomal H^+-ATPase; □, hog gastric membrane vesicle (H^+/K^+)-ATPase; Control, 1% dimethyl sulfoxide (DMSO, solvent of BE-18591).

IC_{50} of 1 nM (about 10 pmol/mg protein) (100 μg protein), as well as the ATP-dependent proton pump activity (P-ATPase) of rabbit gastric mucosa with an IC_{50} of 2 nM (100 μg protein) (about 20 pmol/mg protein). Furthermore, the proton pump inhibiting activity was reversible for P-type proton pumps as shown in Fig. 3. The proton pump activity of gas-

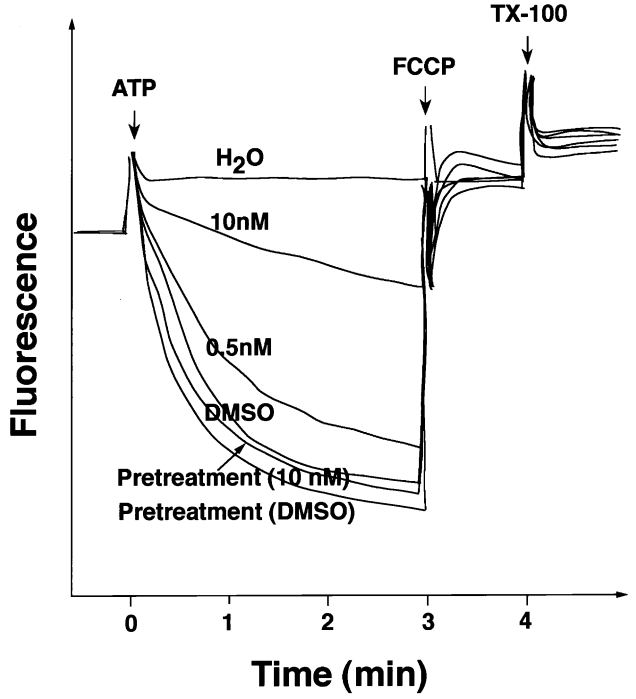

Fig. 3. Reversibility of BE-18591 on the acidification of hog gastric membrane vesicles. Preparations of hog gastric membrane vesicles and the reversibility of the proton pump activity were performed according to Section 2. The final concentration of BE-18591 pre-incubated at 10 nM was 0.5 nM in the proton pump assay medium. H_2O, distilled water instead of ATP; DMSO, 1%; FCCP, 1 mM (ethanol was less than 1%); TX-100, 0.1% Triton X-100.

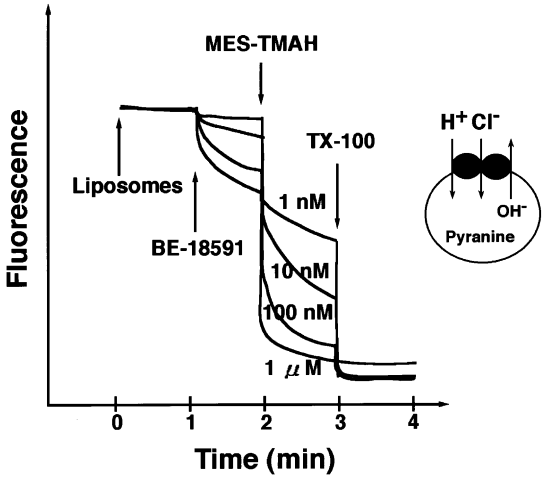

Fig. 4. H^+/Cl^- symport activity of BE-18591 as revealed by the use of liposomes. Liposomes containing pyranine, prepared in a buffer (20 mM HEPES–TMAH, pH 7.5) containing either K-gluconate (25 mM K-gluconate, 2.5 mM $MgSO_4$) were diluted 40-fold in external buffer (20 mM HEPES–TMAH, pH 7.5) containing 25 mM NaCl. At the times indicated, BE-18591 (1 nM–1 μM) was added to decrease intraliposomal pH and this was followed by a further rapid and significant decrease in the intraliposomal pH by 50 mM MES–TMAH (pH 6.0). MES–TMAH, 50 mM MES–TMAH (pH 6.0); TX-100, 0.2% Triton X-100; BE-18591, 1 nM–1 μM.

tric membrane vesicle inhibited by 10 nM BE-18591 was reversed after a 20-fold dilution (final BE-18591 was 0.5 nM) with medium. Essentially similar results were obtained with other types of ATPases. However, BE-18591 inhibited ATP hydrolysis only at higher concentrations with an IC_{50} of about 400 nM (about 160 nmol/mg protein) (2.5 μg protein/ml) for ATP hydrolysis by hog gastric mucosa membrane vesicle P-ATPase. A similar uncoupling tendency has been observed with other types of ATPases. These results indicate that BE-18591 is a non-specific inhibitor against various types of H^+ pump.

3.2. H^+/Cl^- symport activity

The inhibitions of the proton pump activities by BE-18591 were all prevented by a large amount of liposomes (data not shown), suggesting that they were related to the non-specific lipid solubility of BE-18591. Therefore, its effect on the liposomes themselves was observed. BE-18591 induced acidification of the intraliposomal pH by the addition of chloride, and this was further accelerated by the addition of acidic buffer, both in a chloride concentration dependent manner (Fig. 4). On the other hand, BE-18591 did not produce intraliposomal acidification upon the addition of an anion gradient or by the addition of acidic buffer when the anion was gluconate (membrane-impermeant) (figure not shown). However, BE-18591 did cause swelling of liposomes in the presence of chloride anion (ammonium chloride, figure not shown). These results suggest that BE-18591 inhibited the ATP-dependent H^+-pump activities through its H^+/Cl^- symport (functionally equal to H^+/OH^- antiport) activity.

3.3. Immunosuppression

Prodigiosins have been shown to inhibit lymphocyte proliferations [29]. BE-18591 also inhibited the proliferation of mouse spleen lymphocytes as shown in Fig. 5. However, the concentration of BE-18591 required for the inhibition of the growth of mouse splenocytes was one order higher than that of prodigiosin 25-C. Furthermore, the specificity of BE-18591 for LPS (specific B-cell mitogen) rather than Con A (specific T-cell mitogen) was less than that of prodigiosin 25-C. Thus, the IC_{50} of BE-18591 against LPS-stimulated splenocytes was 38 nM and against Con A-stimulated cells 230 nM, while the IC_{50} of prodigiosin 25-C against LPS-stimulated cells was 0.95 nM and against Con A-stimulated cells 21.45 nM. These results suggest that BE-18591 was weaker and less specific to lymphocyte mitogens than prodigiosin 25-C.

3.4. Growth inhibition

BE-18591 inhibited the growth of mouse spleen cells (see above) and CHO cells (IC_{50} = 200–300 nM, figure not shown). However, BE-18591 had little affect on the growth of PC12 cells (IC_{50} > 10 μM). This suggests that PC12 cells were relatively resistant to BE-18591. BE-18591 seemed to inhibit the growth of mouse splenocytes and CHO cells through its cytotoxic rather than cytostatic effect, because the dose-responses were similar. However, BE-18591 did not cause apoptosis as the cells died from necrosis (figure not shown). This is in contrast to the apoptotic effects of bafilomycins and prodigiosins [4,24].

3.5. Inhibition of gastric acid secretion

Prodigiosins have been shown to inhibit rabbit gastritis by inhibiting apical vacuole acidification of parietal cells from rabbit gastric mucosa [8]. BE-18591 also inhibited rabbit gastritis by inhibiting apical vacuole acidification of parietal cells from rabbit gastric mucosa as observed by quinacrine fluorescence (Fig. 6). BE-18591 inhibited the orange fluorescence of quinacrine more strongly than omeprazole or prodigiosin 25-C, suggesting that BE-18591 was the most potent among these substances at inhibiting gastritis.

3.6. Induction of NOG

Prodigiosins as well as bafilomycins have been shown to induce cell differentiation in a dose-dependent manner [1,9]. BE-18591 also induced NOG of PC12 cells but only at higher concentrations (> 100 nM) and weakly (< 15%).

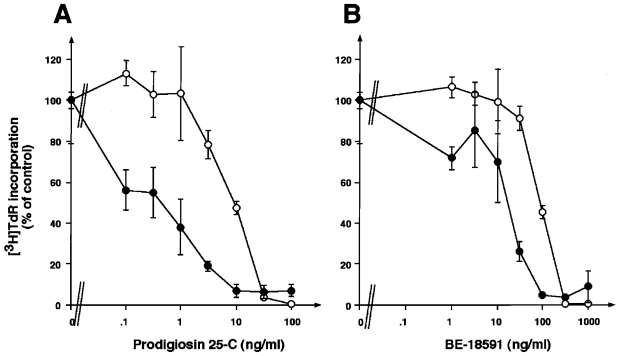

Fig. 5. Immunosuppressive activities of BE-18591. Blastogeneses of mouse splenocytes were performed according to Section 2. Concentrations of LPS and Con A were 2 μg/ml and 1 μg/ml, respectively. IC_{50}s of prodigiosin 25-C were 0.4 ng/ml for Con A-stimulated and 9 ng/ml for LPS-stimulated mouse splenocytes, and those of BE-18591 were 15 ng/ml and 90 ng/ml, respectively. All the data shown are the averaged results (mean ± S.D.). [^{3}H]TdR, [^{3}H]thymidine; A: prodigiosin 25-C; B: BE-18591; ○, LPS-stimulated mouse splenocytes; z.cirf;, Con A-stimulated mouse splenocytes.

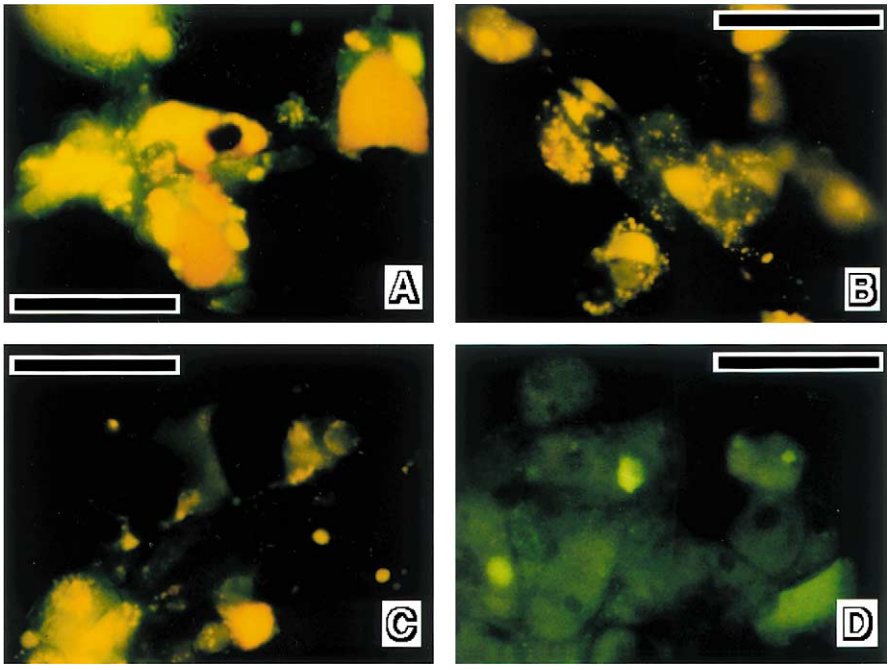

Fig. 6. Inhibition of acid secretions. Parietal cells isolated from rabbit gastric glands were preincubated in medium containing 5 μM acridine orange, and then stimulated with histamine (10^{-4} M) before being further incubated for 40 min in the presence or absence of inhibitors (omeprazole, prodigiosin 25-C or BE-18591 with DMSO as the solvent control). The cells were viewed under a phase contrast fluorescent microscope (B-type dichroic mirror). A: control (1% DMSO). B: 100 μM omeprazole. C: 2.5 μM prodigiosin 25-C. D: 1 μM BE-18591. Bar represents 50 μm. Accumulation of orange fluorescence in the large vacuoles disappeared after treatment of parietal cells with prodigiosin 25-C, and BE-18591 as well as with omeprazole.

3.7. Inhibition of bone resorption

Prodigiosin 25-C and metacycloprodigiosin have been reported to suppress the bone resorption by rat osteoclasts [35]. BE-18591 also inhibited bone resorption in a dose-dependent manner in the presence of 10 μM parathyroid hormone and 1,25-vitamin D_3. However, its bone resorbing activity (70% at 10 μM BE-18591) was evidently less than that of calcitonin (≦20% at 10^{-6} M) (figure not shown). This suggests that the bone-resorbing activity of BE-18591 is limited.

4. Discussion

BE-18591 is regarded as a member of both the tambjamine group of antibiotics as well as the prodigiosin group [11]. The tambjamine group of antibiotics were first isolated from the ascidian *Atapozoa* sp. and its nudibranch predators [36]. In this paper, we have shown that BE-18591 (1) inhibited various ATP-dependent proton pumps, (2) showed H^+/Cl^- symport activity on liposomes, (3) inhibited acid secretion by rabbit gastric parietal cells, and (4) inhibited the mitogenic response of mouse splenocytes (and growth of CHO cells). Therefore, BE-18591 is similar to prodigiosins in both structure and activity [8,11]. In fact, we also observed the inhibition of *Helicobacter pylori* [NCTC 11637] growth by BE-18591 (MIC [minimum inhibitory concentration] = 0.5 μg/ml [37]). Cycloprodigiosin induced both cell differentiation and apoptosis in HL60 cells [9]. BE-18591 also induced NOG in PC12 cells in a dose dependent manner, but its effect was weaker than that of prodigiosins (data not shown). BE-18591 also induced growth inhibition and cell death in a dose dependent manner in mouse splenocytes, CHO cells, P388 cells (IC_{50} = 0.258 μg/ml [∼724 nM]) and MNK human stomach cancer cells (IC_{50} = 0.52 μg/ml [∼1.320 μM]) [9]. However, the growth inhibiting effect was weaker than the prodigiosins and it had little affect on the growth of PC12 cells. Furthermore, BE-18591 did not cause apoptosis, as the cells probably died through necrosis. These results suggest that BE-18591 inhibited cell growth because it induced cell death, and it is somehow specific to cells. In vivo, BE-18591 has been reported to have anti-tumor effects on Ehrlich tumor cells but not on P388 leukemia cells (both ascites types) in BDF_1 mice [10]. The NOG-inducing activity of BE-18591 is probably be caused by a mechanism other than apoptosis. In fact, tetrapyrrole [38,39], an analogue of 4-methoxy-2,2′-bipyrrole-5-pyrromethene, strongly induced apoptosis but induced little NOG (data not shown).

The mechanism of growth inhibition by BE-18591 remains unclear. There is a paper insisting that its copper-nuclease activity correlated well with its death inducing effect [40,41]. However, its H^+/Cl^- activity also showed a good correlation with death-inducing activity. 4-Methoxy-2,2′-bipyrrole-5-carboxyaldehyde (also called tambjamine aldehyde), a precursor of BE-18591, neither inhibited the proton pump nor ATP hydrolysis activities of the P-ATPase in hog gastric mucosa membrane vesicles (data not shown). These results suggest that at least three nitrogens, among which two should be in pyrrole rings, are required for the H^+/Cl^- symport activity in this type of compound. These results also indicate that the correlation itself does not explain the mechanisms. However, H^+/Cl^- symport activity does not seem to be restricted to this type of compound; other types of H^+/Cl^- symporters ($TlCl_3$ [42], Bepridil [43], Hg^{2+} and Cu^+ [44]) have completely different structures. Furthermore, pamamycin [45] and cryptate [46] (both completely different from tambjamines) might be candi-

dates for other types of H^+/Cl^- symporters. These compounds did not show a correlation between the inhibition of cell growth and the H^+/Cl^- symport activity. At present we do not have any data on the copper-nuclease activity of these compounds or BE-18591. PNU156804, a prodigiosin 25-C analogue, has been shown to affect cellular JAK kinase 3 [47]. A similar effect might be related to the growth inhibition by BE-18591. Further studies are necessary to clarify the precise mechanism through which BE-18591 acts.

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References

[1] Tamura, H. and Ohkuma, S. (1991) FEBS Lett. 294, 51–55.
[2] Kinoshita, K., Hidaka, H. and Ohkuma, S. (1994) FEBS Lett. 337, 221–225.
[3] Ohkuma, S., Shimizu, S., Noto, M., Sai, Y., Kinoshita, K. and Tanura, H. (1993) In Vitro Cell. Dev. Biol. 29A, 862–866.
[4] Kinoshita, K., Waritani, T., Noto, M., Takizawa, K., Minemoto, Y., Nishikawa, A. and Ohkuma, S. (1996) FEBS Lett. 398, 61–66.
[5] Ohkuma, S., Sato, T., Okamoto, M., Matsuya, H., Arai, K., Kataoka, T., Nagai, K. and Wasserman, H.H. (1998) Biochem. J. 334, 731–741.
[6] Konno, H., Matsuya, H., Okamoto, M., Sato, T., Tanaka, Y., Yokoyama, K., Kataoka, T., Nagai, K., Wasserman, H.H. and Ohkuma, S. (1998) J. Biochem. 124, 547–556.
[7] Sato, T., Konno, H., Tanaka, Y., Kataoka, T., Nagai, K., Wasserman, H.H. and Ohkuma, S. (1998) J. Biol. Chem. 273, 21455–21462.
[8] Matsuya, H., Okamoto, M., Ochi, T., Nishikawa, A., Shimizu, S., Kataoka, T., Nagai, K., Wasserman, H.H. and Ohkuma, S. (2000) Biochem. Pharmacol. 60, 1855–1863.
[9] Yamamoto, D., Uemura, Y., Tanaka, K., Nakai, K., Yamamoto, C., Takemoto, H., Kamata, K., Hirata, H. and Hioki, K. (2000) Int. J. Cancer 88, 121–128.
[10] Kojiri, K., Nakajima, S., Suzuki, H., Okura, A. and Suda, H. (1993) J. Antibiot. 46, 1799–1803.
[11] Nakajima, S., Kojiri, K. and Suda, H. (1993) J. Antibiot. 46, 1894–1896.
[12] Grabe, C.G., Harty, P.K. and Rosen, S.D. (1983) Anal. Biochem. 130, 287–294.
[13] Harashima, K., Tsuchida, N., Tanaka, T. and Nagatsu, J. (1967) Agric. Biol. Chem. 31, 481–489.
[14] Arai, K., Kanaseki, T. and Ohkuma, S. (1991) J. Biochem. 110, 541–547.
[15] Bustamante, E., Soper, J.W. and Pedersen, P. (1977) Anal. Chem. 80, 401–408.
[16] Wehrle, J.P., Cintrón, N.M. and Pedersen, P.L. (1978) J. Biol. Chem. 253, 8598–8603.
[17] Maeda, M., Tagaya, M. and Futai, M. (1988) J. Biol. Chem. 263, 3652–3656.
[18] Ohkuma, S., Moriyama, Y. and Takano, T. (1982) Proc. Natl. Acad. Sci. USA 79, 2758–2762.
[19] Ohkuma, S. (1989) Methods Enzymol. 174, 131–154.
[20] Ohkuma, S. and Takano, T. (1997) Cell Struct. Funct. 22, 253–268.
[21] Arai, K., Shimaya, A., Hiratani, N. and Ohkuma, S. (1993) J. Biol. Chem. 268, 5649–5660.
[22] Chan, K.-M., Delfert, D. and Junger, K.D. (1986) Anal. Biochem. 157, 375–380.
[23] Selwyn, M.J., Dawson, A.P., Stockdale, M. and Gains, N. (1970) Eur. J. Biochem. 14, 120–126.
[24] Lee, H.-M., Kataoka, T., Magae, J. and Nagai, K. (1995) Biosci. Biotech. Biochem. 59, 1417–1421.
[25] Ormerod, M.G. (1990) in: Flow Cytometry: A Practical Approach (Ormerod, M.G., Ed.), pp. 265–273, IRL Press, Oxford.
[26] Urushidani, T. and Nagao, T. (1996) Am. J. Physiol. 270, G103–G112.
[27] Brown, M.R. and Chew, C.S. (1987) Can. J. Physiol. Pharmacol. 65, 1840–1847.
[28] Payne, N. and Gerber, J.G. (1990) Methods Enzymol. 187, 505–513.
[29] Chew, C.S., Ljungström, M., Smolka, A. and Brown, M.R. (1989) Am. J. Physiol. 256, G254–G263.
[30] Mangeat, P., Gusdinar, T., Sahuquet, A., Hanzel, D.K., Forte, J.G. and Magous, R. (1990) Biol. Cell 69, 223–232.
[31] Kameda, T., Mano, H., Yuasa, T., Mori, Y., Miyazawa, K., Shiokawa, M., Nakamaru, Y., Hiroi, E., Hiura, K., Kameda, A., Yang, N.N., Hakeda, Y. and Kumegawa, M. (1997) J. Exp. Med. 186, 489–495.
[32] Takahashi, K., Liu, Y.-C., Hayashi, N., Goto, F., Kato, M., Kawashima, H. and Takeuchi, T. (1997) Peptide 18, 439–444.
[33] Schaffner, W. and Weissmann, C. (1973) Anal. Biochem. 56, 502–514.
[34] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[35] Woo, J.-T., Ohba, Y., Tagami, K., Sumitani, K., Kataoka, T. and Nagai, K. (1997) Biosci. Biotech. Biochem. 61, 400–402.
[36] Lindquist, N. and Fenical, W. (1991) Experientia 47, 504–506.
[37] Tsukamoto, M., Kojiri, K., Suda, H. and Ohkuma, S. (2001) JAPAN KOKAI H09-315968.
[38] Wasserman, H.H. and Friendland, D.J. (1968) Tetrahedron Lett. 1968, 641–644.
[39] Matsunaga, S., Fusetani, N. and Hashimoto, K. (1986) Experientia 42, 85.
[40] Melvin, M.S., Ferguson, D.C., Lindquist, N. and Manderville, R.A. (1999) J. Org. Chem. 64, 6861–6869.
[41] Melvin, M.S., Woolton, K.E., Rich, C.C., Saluta, G.R., Kucera, G.L. and Manderville, R.A. (2001) J. Inorg. Biochem. 87, 129–135.
[42] Diaz, R.S. and Monreal, J. (1994) Mol. Pharmacol. 46, 1210–1216.
[43] Kovacic, H., Gallice, P., Sarrazin, M. and Crevat, A. (1993) J. Pharmacol. Exp. Ther. 267, 1509–1514.
[44] Karniski, L.P. (1992) J. Biol. Chem. 267, 19218–19225.
[45] Kondo, S., Yasui, K., Natsume, M., Katayama, M. and Marumo, S. (1988) J. Antibiot. 41, 1196–1204.
[46] Dietrich, B., Fyles, T.M., Hosseini, M.W., Lehn, J.-M. and Kaye, K.C. (1988) J. Chem. Soc. Commun. 1988, 691–692.
[47] Stepkowski, S.M., Erwin-Cohen, R.A., Behbod, F., Wang, M.-E., Qu, X., Tejpal, N., Nagy, Z.S., Kahan, B.D. and Kirken, R.A. (2002) Blood 99, 680–689.