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Note

Immunomodulating Activity of DW-116, A New Quinolone Antibiotic

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DW-116, {1-(5-fluoro-2-pyridyl)-6-fluoro-7-(4-methyl-1-piperazinyl)-1,4-dihydro-4-oxoquinoline-3-carboxylic acid hydrochloride}, is a new quinolone antibiotic with a broad antibacterial spectrum against G(+) and G(-) bacteria. DW-116 was evaluated for the immunomodulating activities, which is one of the efforts to investigate the mechanism of action related to the good *in vivo* antibacterial efficacy. The results of *in vitro* studies revealed there was no statistically significant increase in B and T lymphocyte proliferation. But the results of *in vivo* studies showed that the number of plaque forming cells (PFC), the amount of polyclonal antibodies and delayed-type hypersensitivity (DTH) were significantly increased after the repeat administration with 12 and 60 mg/kg of DW-116. Taken together, these results proposed that immunostimulating effect of DW-116 could be one of the action mechanisms for demonstrating *in vivo* antibacterial activities under these experimental conditions.

Key words : DW-116, PFC, DTH, Immunoglobulin

INTRODUCTION

Quinolone antibiotics have been developed since nalidixic acid was discovered. But quinolone antibiotics have side effects on central nervous system, phototoxicity, arthropathy and other problems. Now, it is required to search for the drugs with improved drug efficacy and decreased side effects. Therefore, it is important to develop new drug of quinolone antibiotics of which drug efficacy is stronger, the absorption rate is higher, half-life is longer and toxic side effect is lower than before. Developed antibiotics should be once-a-day and oral dosing therapeutics.

DW-116, a new quinolone antibiotic, has a broad antibacterial spectrum against gram-positives and gram-negatives, and its activity is a little lower than that of ciprofloxacin (Han *et al.*, 1995). But when DW-116 was orally administered, pharmacokinetic profile was better than those of other quinolone antibiotics, its oral absorption was good, half-life was long, tissue distribution was better and drug concentration of tissue was higher than those of other quinolones (Lee, 1995; Lee *et al.*, 1995). As a result of *in vivo* tests, DW-116 reflected comparable activity as good as ciprofloxacin and ofloxacin and showed better activity than rufloxacin (Han *et al.*, 1995a). Especially, in the case of thigh infection test, DW-116 showed stronger cure rate than ciprofloxacin (Han *et al.*, 1995b). Also, DW-116 had the lower toxicity and side effect than other quinolone antibiotics (Kwon *et al.*, 1995). With these results, it was expected DW-116 could be a new quinolone antibiotic as a once-a-day treatment therapeutic agent.

In this study, we investigated the immunomodulating activities of DW-116 in order to illustrate its exceptional *in vivo* stronger efficacies than *in vitro* antibacterial activities, besides its good pharmacokinetic profile.

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MATERIALS AND METHODS

Animals and chemicals

SPF male CD-1 and BALB/c mice of 7 weeks old were purchased from Charles River Laboratories (Japan). Animals were randomized to 6 heads and acclimated in polycarbonate cages of animal facilities which were regulated at 23±1°C of temperature, 55±5% of humidity, 10~18 circulation/hour of ventilation, 12 hour cycle of light/dark and 300~500 Lux of illumination before use. Body weights of animals used in experiment

ranged at mean body weight (g) ±20%. Rodent diet 20 as food from Pico-Lab (U.S.A.) was freely provided and water *ad libitum*.

Unless otherwise indicated, chemicals used were purchased from Sigma Co. Ltd. (U.S.A.). Guinea pig complement, RPMI 1640 media and fetal bovine serum (FBS) were purchased from GIBCO (U.S.A.).

Lymphocyte proliferation

Mice were sacrificed by cervical dislocation and spleens were aseptically obtained to RPMI 1640 media containing 10% FBS (RPMI-FBS). Single cell population of splenocytes were prepared by teasing spleens with plunger of syringe. After removal of debris of fat and tissue, cell suspension was centrifuged at 1000 rpm for 15 min. Supernatant was aspirated and cell pellet was repeatedly resuspended in RPMI-FBS. 2×10^5 cells were plated on each well of 96-well plate and incubated for 48 hours in the presence of LPS (mitogen for B lymphocytes) or ConA (mitogen for T lymphocytes). MTT was added to the cultures and incubated for additional 4 hours. After formazan formed was dissolved in DMSO (Sigma, St. Louis, MO), absorbance as cell density was measured at 540 nm with ELISA reader (Bio-Tek, Winooski, VT).

PFC assay for IgM production

PFC assay was performed by the method of Cunningham and Szenberg (1968) and briefly described as follows. SRBC for sensitization was washed with saline three times, diluted to 5×10^9 SRBCs/ml and intravenously injected to tail vein. SRBC was stored in Alservier's solution until use. The number of SRBCs was counted with hematocytometer. 60 mg/kg and 12 mg/kg of DW-116 was orally administered to mice from the day of sensitization of SRBC (day 0) for consecutive 4 days (day 3). On day 4, mice were sacrificed by cervical dislocation and spleens were obtained. Spleens were washed with PBSS (glucose 250 mg/l, KH_2PO_4, 15 mg/l, $Na_2HPO_4\cdot12H_2O$ 190 mg/l, phenol red 2.5 mg/ml, $CaCl_2\cdot2H_2O$, 46.5 mg/l, KCl 100 mg/ml, $NaCl_2$ 2 g/l, $MgCl_2\cdot6H_2O$ 50 mg/ml, $MgSO_4\cdot7H_2O$ 50 mg/l, pH 7.2) and splenocytes were prepared by homogenization. Debris of fat and tissues were removed through sieve and cell pellets were obtained by centrifugation at 1500 rpm for 6 min. Supernatant was removed and red blood cells were hemolysed by the addition of ACK buffer (NH_4Cl 747 mg/ml, Tris-base 206 mg/ml, pH 7.2). Splenocytes were washed with PBSS and the number of splenocytes was counted with trypan blue exclusion method and adjusted to 5×10^6 cells/ml. 50 μl of splenocyte suspension, 100 μl of 1/4 diluted guinea pig complement (GIBCO, Grand Island, NY), 100 μl of 12.5% SRBC and 250 μl of PBSS were added to 1.5 ml microcentrifuge tube and mixed by vortex. Dual microchamber was filled with 50 μl of the mixture. Dual microchamber was made by sticking the center of slide, which the side faces of two microscope slide (76×26 mm) were overlaped with double-sided tapes, completely. Dual microchamber containing the mixture was slightly sealed by dipping in paraffin wax-vaselin (1 : 1) which was dissolved in petri dish by warming and incubated at 37°C for 1 hour. The formation of PFC was counted and expressed as PFCs/10^6 cells.

Measurement of immunoglobulin amount

The amount of polyclonal immunoglobulin in serum was measured by ELISA method of Vunakis and Langone (1980) and briefly described as follows. Schedule for the treatment of animals with DW-116 was the same as that of PFC assay. On day 4, after repeat dosing of DW-116 into mice, blood was collected from mice for the determination of immunoglobulin amount. 100 μl of anti-polyclonal antibodies (Sigma, St. Louis, MO) was diluted in carbonate-bicarbonate buffer (pH 9.6) to 1 : 160 and coated on 96-well plate at 4°C overnight and then, wells were washed three times with PBS containing 0.1% Tween 20 and 1% bovine serum albumin (PBS-Tween, pH 7.4). Sera from animals were appropriately diluted and 100 μl of diluted serum was added to 96-well plate coated polyclonal antibodies and incubated for 3 hours. Wells were washed again three times with PBS-Tween and then 100 μl of polyclonal antibodies conjugated to alkaline phosphatase were added. After incubation for 2 hours, wells were washed three times with PBS-Tween and then 100 μl of 4-nitrophenyl phosphate (pNPP, 1 mg/ml) dissolved in diethanolamine buffer (pH 9.8) was added and incubated at room temperature for 30 min. Reaction was measured with ELISA reader (Bio-Tek, Winooski, VT) as OD 405 nm.

Delayed-type hypersensitivity (DTH)

DTH was performed according to the method of Titus and Chiller (1981) and briefly described as follows. 0.2 ml of SRBC solution (5×10^6 cells/ml) to be sensitized was injected into tail vein of BALB/c mice (day 0). 60 mg/kg and 12 mg/kg of DW-116 were orally administered into mice for 5 days from the day after sensitization with SRBC. On the fifth day (day 4), footpad thickness was measured as control of swelling, in advance and left footpads of mice were subcutaneously challenged with 40 μl of SRBC. SRBC was stored in Alservier's solution until use. SRBC was washed with saline three times, the number of SRBCs was counted with hematocytometer and then adjusted to 5×10^6 SRBCs/ml for sensitization and to 2.5×10^9

SRBCs/ml for challenge. The thickness of footpad swelled was measured with vernier calipers (Mitutoyo, Japan) after 24 hours (day 5).

Statistical analyses

All data were represented as mean±SD. The comparison between the results of treated groups and that of control was performed by Student's t-test.

RESULTS

To study the effect of DW-116 on lymphocyte proliferation, splenocytes were incubated with LPS, mitogen of B lymphocytes, or ConA, mitogen of T lymphocytes, in the presence of specific doses of DW-116. The concentratioins of DW-116 were 4 and 20 μg/ml which was the highest concentration causing no effect on cell viability of splenocytes for experimental period. The results showed 4 and 20 μg/ml of DW-116 have no significant effect on *in vitro* lymphocyte proliferation by LPS (Fig. 1) or ConA (Fig. 2).

To show whether DW-116 has the effect on the number of plaque forming cells (PFCs) after the repeated *in vivo* exposure, DW-116 was orally administered to mice for 4 days from the day after immunization with SRBC. After animals were sacrificed by cervical dislocation, spleens were excised and splenocytes were prepared for the measurement of PFCs. In addition, blood was collected and serum was separated by centrifugation for the measurement of immunoglobulin amount. Administration doses of DW-116 were 12 and 60 mg/kg because those doses of DW-116 showed good and significant *in vivo* antibacterial efficacies (Hwang *et al.*, 1997). The results showed the numbers of PFCs of 12 and 60 mg/kg of DW-116 administered groups was significantly elevated about 40% and 50% compare to that of control group, respectively, in a

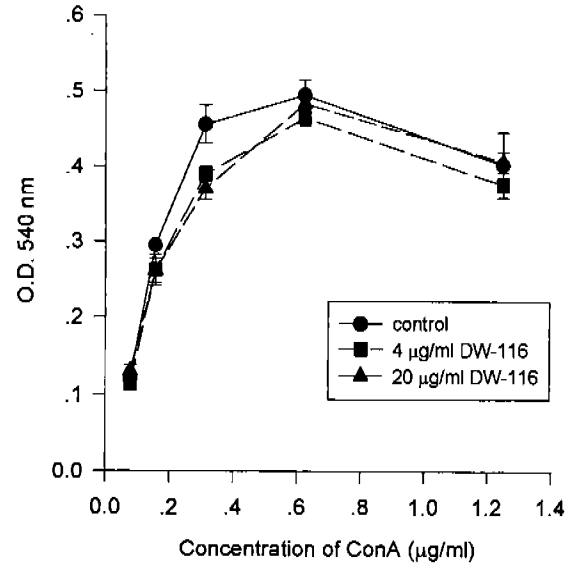

Fig. 2. The effect of DW-116 on blastogenesis of splenocytes by ConA stimulation. Splenocytes were prepared from CD-1 mice, stimulated with ConA and cultured in the presence of specific concentrations of DW-116 for 48 hours. Cell proliferation was measured by MTT assay. All data represented here are mean±SD.

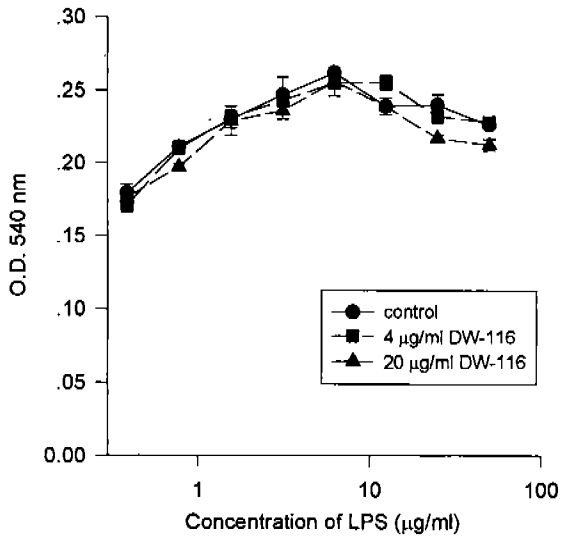

Fig. 1. The effect of DW-116 on blastogenesis of splenocytes by LPS stimulation. Splenocytes were prepared from CD-1 mice, stimulated with LPS and cultured in the presence of specific concentrations of DW-116 for 48 hours. Cell proliferation was measured by MTT assay. All data represented here are mean±SD.

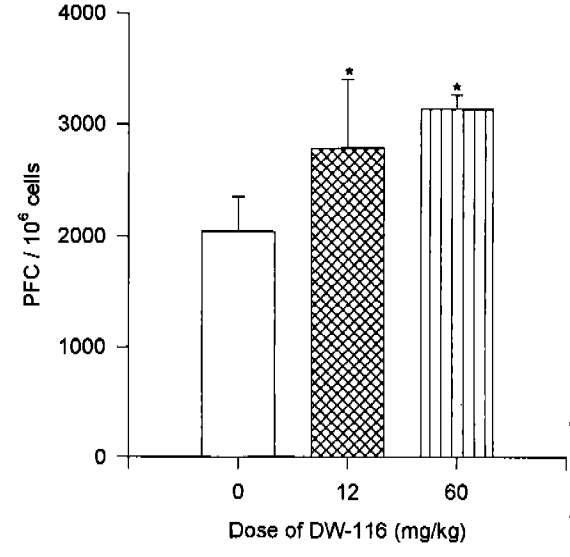

Fig. 3. The effect of DW-116 on the number of plaque forming cells. DW-116 was orally administered to mice sensitized with SRBC for 4 days, daily. Mice were sacrificed, splenocytes were prepared and PFC assay was performed as described in materials and method. All data represented here are mean±SD. *$P<0.05$, Significantly different from control group.

dose-dependent manner (Fig. 3). The amount of immunoglobulins in serum was about 10% and 16% more than control, repectively (Fig. 4). From these data, it is suggested that DW-116 increases the function of B lymphocytes related to humoral mediated immunity after *in vivo* exposure.

To determine the effect of DW-116 on delayed-type hypersensitivity, DW-116 was orally administered to mice for 5 days from the day after sensitization with SRBC. After mice were challenged with SRBC on the 5th day, footpad thickness on 6th day was increased by DW-116 at the dosages of 12 and 60 mg/kg about 18% and 32%, respectively (Fig. 5). These data showed DW-116 affects T lymphocytes related to cellular mediated immunity.

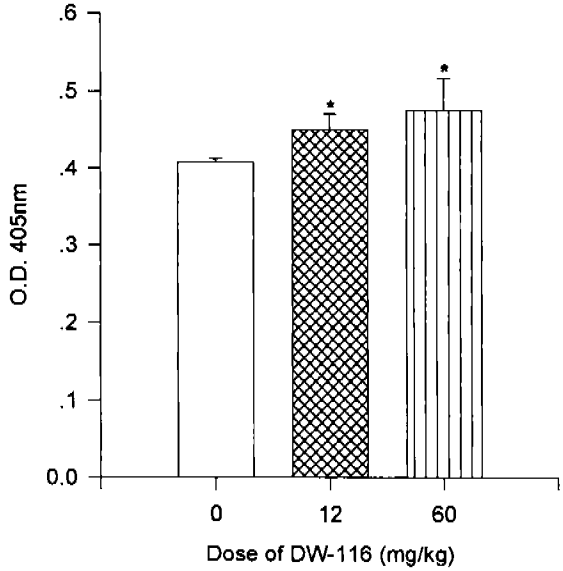

Fig. 4. The effect of DW-116 on the production of immunoglobulins. DW-116 was orally administered to mice sensitized with SRBC for 4 days, daily. Blood were collected and serum was separated by centrifugation for the measurement of immunoglobulins as described in materials and method. All data represented here are mean±SD. *$P<0.05$, Significantly different from control group.

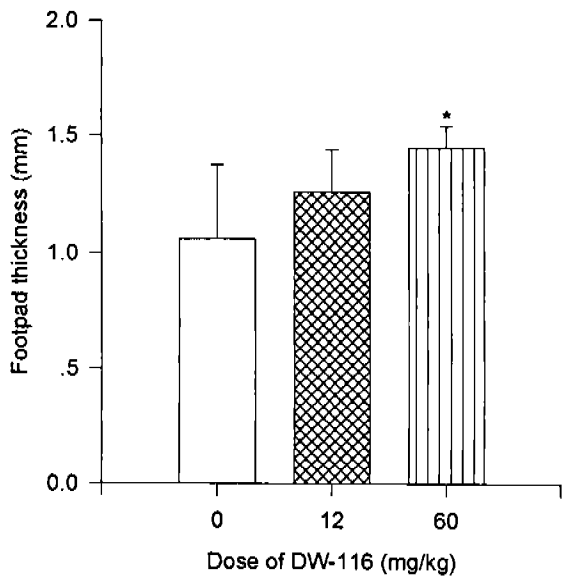

Fig. 5. The effect of DW-116 on delayed-type hypersensitivity. DW-116 was orally administered to mice sensitized with SRBC for 5 days, daily. Mice were challenged with SRBC on the last administration day and keep up for 24 hours. Footpad thickness was measured with vernier calipers as described in materials and method. All data represented here are mean±SD. *$P<0.05$, Significantly different from control group.

DISCUSSION

DW-116, a new quinolone antibiotic, has better *in vivo* antibacterial activity than expected from *in vitro* experiment. Against respiratory tract infection (RTI) caused by *Pseudomonas aeruginosa* in rats and *Klebsiella pneumoniae* in mice, DW-116 showed significant activity in both experiments. In urinary tract infection (UTI) caused by *E. coli*, DW-116 also showed good efficacy for the treatment of model disease (Hwang *et al.*, 1997). In addition, DW-116 showed strong activity against thigh infections in mice challenged with *Staphylococcus aureus* Smith (Han *et al.*, 1995b).

It has been well known that immune responses play an important role to show antibacterial effect (Morgan and Weigle, 1987). Healthy individuals protect themselves against microbes by means of many different immunological mechanisms. These include physical barriers, phagocytic cells in the blood and tissues, a class of lymphocytes called natural killer (NK) cells, and various blood-borne molecules, all of which particulate in defending individuals from a potentially hostile environment. With these reports, the purpose of this study was to investigate the mechanism of action on *in vivo* antibacterial activity of DW-116 through the measurement of immunomodulating effect of DW-116 on immune cells.

For years, scientists have searched for the ways to trigger the body's own defenses against external invasion by microorgarnisms or internally arising diseases. Scientists have recognized certain biologic interactions between microorgarnisms and host, to indicate that agents which can enhance host immunity may play a significant role in controlling infectious diseases and autochthonous diseases. Several of the cellular mechanisms which are regulated by immunomodulators are the same in combating infections by microorgarnisms.

In *in vitro* experiments, it appeared that DW-116 had no immunomodulating effect on immune cell proliferation by LPS or ConA. But DW-116 had a good *in vivo* immunomodulating activities such as

increase of the number of PFCs and delayed-type hypersensitivity after the administration of DW-116 for 5 consecutive days. It means that DW-116 affects the lymphocyte functions. These data propose that *in vivo* immunomodulating effect of DW-116 was induced by several mechanisms as follows; (1) *In vivo* immunomodulating effect of DW-116 can be induced by its metabolites rather than DW-116 itself. (2) The immunomodulating effect of DW-116 was not by a direct action of DW-116 against lymphocytes but by an indirect action through various mediators such as cytokines from macrophages to lymphocytes. The effect of DW-116 on the various functions of macrophages will be further investigated.

Taken together, it is suggested that the immunostimulation of DW-116 is one of the mechanism of action to explain *in vivo* antibacterial activities of DW-116. Then, we can say that *in vivo* antibacterial activities of DW-116 may not only be resulted from direct killing microorganisms of DW-116, itself but also from the immunomodulation of DW-116 and its metabolites.

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