Effects of NIP-502 on Antigen-Induced Bronchial Responses and Allergic Reactions in Animal Models

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ABSTRACT—We examined the effect of a newly synthesized pyridazinone derivative, NIP-502 [4-chloro-5-(3-ethoxy)-4-phenoxybenzamine)-3(2H)-pyridazinone], on antigen-induced bronchial responses and allergic reactions in several animal models. NIP-502 (10 mg/kg, p.o.) inhibited the antigen-induced immediate asthmatic response in passively sensitized guinea pigs. The inhibitory effect was also observed in metyrapone (an inhibitor of 11(3-hydroxylase)-pretreated guinea pigs. NIP-502 improved ovalbumin (OA)-induced airway hyperresponsiveness to acetylcholine and inhibited the OA-induced increase in the number of inflammatory leukocytes in the bronchoalveolar lavage fluid. These inhibitory effects on OA-induced responses were similar to those of prednisolone. NIP-502 also showed an inhibitory effect on the passive cutaneous anaphylactic reaction in rats but did not inhibit the reversed cutaneous anaphylactic reaction, reversed Arthus reaction or delayed type hypersensitivity reaction. On the other hand, prednisolone showed broad inhibitory effects except for the reversed cutaneous anaphylactic reaction. In the in vitro study, NIP-502 (30 µM) significantly inhibited Formyl-Met-Leu-Phe-induced superoxide anion production by the guinea pig alveolar macrophages. These results indicate that the inhibitory effects of NIP-502 on bronchial responses are similar to those of prednisolone, but this compound seemed to act more selectively on the respiratory tract than prednisolone. Because of its effectiveness against a variety of bronchial responses, NIP-502 may be useful in the treatment of bronchial asthma.

Keywords: NIP-502, Antigen-induced bronchial response, Prednisolone
Materials and Methods

Materials

NIP-502 was synthesized by the Central Research Laboratory, Nissan Chemical Ind. (Funabashi). Sources of the other compounds were as follows: Egg albumin (OA; Seikagaku Co., Tokyo), prednisolone acetate (Takeda Chemical Ind., Osaka), sheep red blood cell (Toyo Serum Co., Tokyo), metyrapone (Aldrich Chemicals, Milwaukee, WI, USA), acetylcholine chloride (ACh; Nacalai Tesque, Kyoto), cytochalasin B, cytochrome C, Formyl-Met-Leu-Phe (FMLP) (Sigma, St. Louis, MO, USA).

Animals

Male or female Hartley guinea pigs (250–500 g), male or female Wistar rats (150–250 g), Balb/c mice (approximately 20 g), male albino rabbits (2.5–3 kg) were purchased from Japan SLC, Inc. (Shizuoka).

Antigens and antisera

Benzylpenicillole bovine γ-globulin (BPO-BGG), bovine serum albumin (BPO-BSA) or OA were used as antigens in the guinea pigs. Anti-BPO-BGG guinea pig serum was prepared according to the methods described by Levine and Redmond (11). The IgE antibody titer was 1:2^11 estimated by the 7-day homologous passive cutaneous anaphylactic reaction (PCA). The IgG antibody titer in the same serum was 1:2^1 estimated by 7-day homologous PCA. These results indicate that this anti-serum contains mainly IgE antibody.

Dinitrophenylated Ascaris suum extract (DNP-As) and bovine serum albumin (DNP-BSA) were used as antigens in the rats. Anti-DNP-As rat serum was prepared according to the method described by Tada and Okumura (12), with some modification. The IgE antibody titer of this antisera preparation, estimated by 48-hr homologous PCA, was 1:2^9. Anti-ovalbumin (OA) rabbit serum was prepared according to the method described by Nagai et al. (13). The antibody titer of this antisera preparation was 1:2^9.

Antigen-induced bronchoconstriction in guinea pigs

Guinea pigs were passively sensitized with anti BPO-BGG guinea pig serum (0.5 ml/kg, i.v.). Forty-eight hours after sensitization, the guinea pigs were anesthetized with pentobarbital sodium (50 mg/kg, i.p.). The trachea of each guinea pig was cannulated, and the cannula was connected to a transducer (MFP-1100, TV-142, TV-241, TP602-T; Nihon Kohden, Tokyo) and a respirometer (RM-25, RPM-6018; Nihon Kohden). Then the guinea pigs were challenged with BPO-BSA (40 μg/kg, i.v.). Changes in respiration were measured by counting changes in the respiratory rate and volume and determining the ratio of expiration time to inspiration time. NIP-502 (3, 10 mg/kg, p.o.) was administered 1 hr before the antigen challenge. In a separate experiment, we examined the effect of NIP-502 on metyrapone-pretreated guinea pigs. We have previously reported that pretreatment with metyrapone, which inhibits 11β-hydroxylase in glucocorticoid biosynthesis (14, 15), tended to reduce plasma cortisol (16) and dramatically increases the ratio between the time required for expiration and inspiration (8). Metyrapone (10 mg/kg, i.v.) was given 24 hr and 2 hr before the BPO-BSA (30 μg/kg, i.v.) challenge. Other experimental conditions and measurement procedures were similar to those used with non-metyrapone treated animals.

Antigen-induced airway hyperresponsiveness in guinea pigs

The guinea pigs were sensitized i.p. with 5 mg OA/animal on day 0 and with 10 mg OA/animal on day 2. From day 14 to day 34, 0.1%, 0.2%, 0.4%, 0.5%, 1% and 1% OA aerosol were inhaled at 4-day intervals. The guinea pigs were placed in the transparent chambers connected to an ultrasonic nebulizer TUR-3200 (Nihon Kohden) and were exposed to OA aerosol for 1 min. On day 35 (24 hr after the final 1% OA inhalation), the guinea pigs were anesthetized with pentobarbital sodium and changes in the rate and the volume of respiration were recorded by the transducers. The degree of bronchial responses was determined by measuring changes in the rate and volume of respiration and the ratio of expiration to inspiration time induced by increasing doses of ACh (15.6–2000 μg/kg, i.v.) at 5-min intervals. Measurements were taken for 30 sec immediately following the ACh injection. NIP-502 (p.o.) or prednisolone (i.p.) was administered 2 hr before every OA aerosol, and aminophylline (p.o.) was administered 1 hr before the aerosol.

Bronchoalveolar lavage fluid study in guinea pigs

Guinea pigs were sensitized with 0.5 mg OA/animal, i.p. on day 0 and 1 mg OA/animal, i.p. on day 2. These animals were repeatedly exposed to OA aerosol (0.1% on
day 14, 0.2% on day 17, 0.4% on day 20, 0.5% on day 25 and 1% on day 28 and day 31). On day 32, guinea pigs were killed by urethane (i.p.). Bronchoalveolar lavage fluid (BALF) was obtained by the previously reported method (9). Briefly, BALF was obtained by the injection of 10 ml of saline containing 0.1% BSA into the trachea, and then fluid was withdrawn. The total number of nucleated cells in the lavage fluid was counted after addition of Turk’s solution to the resuspended cell pellet. In order to count the differentiated leukocytes, the smear was prepared using a cytocentrifuge and stained with May-Grünwald and Giemsa stains. NIP-502 (30 mg/kg, p.o.) and aminophylline (50 mg/kg, p.o.) were administered 1 hr before and prednisolone (20 mg/kg, i.p.) was administered 3 hr before every OA aerosol.

Forty-eight hour homologous PCA reaction in rats

The rat 48-hr homologous PCA reaction was induced according to a previously described method (17). Briefly, two reaction sites were prepared on the shaved dorsal skin of a rat. Rat anti-DNP-As 48-fold diluted antiserum (0.1 ml) was injected intradermally into one reaction site.

Fig. 2. Effects of NIP-502 on BPO-BSA-induced bronchoconstriction in saline- (a) and in metyrapone- (b) treated guinea pigs that were passively sensitized with anti-BPO-BGG. NIP-502 was administered p.o. 2 hr before the BPO-BSA challenge. Each value represents mean±S.E. of percentage of initial value before antigen challenge for respiratory rate, respiratory volume and the ratio of expiration time to inspiration time (Ex/In). *P<0.05, significantly different from the time matched control response. Control (○); NIP-502: 3 mg/kg (▲), 10 mg/kg (●); n = 6–8.
Fig. 3. Effects of NIP-502 on ovalbumin (OA)-induced airway hyperresponsiveness in guinea pigs. Animals were sensitized with OA at 5 mg/kg, i.p. on day 0 and OA at 10 mg/kg, i.p. on day 2. From day 14, 0.1%, 0.2%, 0.4%, 0.5% and 1% OA aerosols were inhaled at 4-day intervals. Airway reactivity was examined by i.v. administration of acetylcholine (15.6-2000 pg/kg) 24 hr after the 1% OA challenge on day 34. NIP-502 was administered orally 2 hr before each OA aerosol. Each value represents mean±S.E. of percentage of initial value before acetylcholine administration for respiratory rate, respiratory volume and the ratio of expiration time to inspiration time (Ex/In). *P<0.05, significantly different from the control by Student's t-test. Normal (+); Control (○); NIP-502: 10 mg/kg (●), 30 mg/kg (■); n=5–9.

for sensitization. As a control response, an equivalent volume of saline was injected into another site at the same time. Forty-eight hours after sensitization, 1 mg of DNP-BSA and 5 mg of Evans blue dye in saline were injected intravenously. After 30 min, the rats were sacrificed, and the two reaction sites were excised for the determination of extravasated dye and determined colorimetrically, according to the method of Katayama et al. (18). NIP-502 (30–50 mg, p.o.) was administered 1 hr before and prednisolone (20 mg/kg, i.p.) was given 2 hr before the antigen challenge.

**Reversed cutaneous anaphylactic reaction in rats**

Rabbit anti-rat serum (15%) and saline were injected intradermally into separate sites (0.1 ml/site) on the shaved back surface of each rat. Two hours later, the rats were sacrificed and the reaction sites were excised for the determination of weight, as an indicator of the increase in cutaneous edema. NIP-502 (200 mg/kg, p.o.) was administered 1 hr before and prednisolone was give 2 hr before antiserum challenge.

**Reversed Arthus reaction in rats**

Anti-OA rabbit serum was given i.v. immediately after the injection of OA into the right hind footpads of male Wistar rats. The volume of the footpad was measured both before the antiserum challenge and 3 hr after. NIP-502 (100, 200 mg/kg, p.o.) was administered 1 hr before and prednisolone (20 mg/kg, i.p.) was given 3 hr before the antiserum challenge.

**Delayed type hypersensitivity (DTH) reaction in mice**

Female Balb/c mice were sensitized with sheep red blood cells (SRBC) (1 x 10^7/0.2 ml/animal/i.v.). Four days later, DTH was induced by the injection of SRBC (1 x 10^8/20 μl) into the right hind footpad, and then the increase in footpad volume was measured. The volume was measured 24 hr later using a plethysmometer. NIP-502 (200 mg/kg, p.o.) was administered 1 hr before and 16 hr after the SRBC challenge. Prednisolone (20 mg/kg, i.p.) was given 1 hr before the SRBC challenge.

**Superoxide anion (O_2^-) production**

The generation of superoxide anion (O_2^-) was measured according to the method of Yamashita et al. (19). Guinea pig alveolar macrophages were prepared according to the previously described method (9). Briefly, the animals were killed by pentobarbital (i.p.). The trachea was cannulated, and the airway lumen was washed with 3 x 10 ml of saline containing 0.1% BSA warmed at 37°C. The lavage fluid from each animal was centrifuged (150 x g at 4°C for 10 min). The pellets were washed with Ca^2+-, Mg^2+-free Hanks' balanced salt solution (HBSS, pH 7.3) and resuspended at 2 x 10^6 cells/ml in HBSS containing 0.1% BSA (HBSS-BSA). These cells contained more than 92% alveolar macrophages as identified by May-Grunwald and Giemsa staining, and the viability of these cells were more than 98% measured by trypan blue
staining. Cells in 1 ml HBSS-BSA were pre-incubated with cytochrome C (1.24 mg/ml) and cytochalasin B (5 μg/ml). After 10 min, the cells were stimulated by 1 μM FMLP for 10 min. NIP-502 (10–300 μM) or aminophylline (100, 300 μM) was added 5 min before the stimulation. To stop the reaction, the test tubes were placed on ice, and then centrifuged at 1500 × g for 10 min at 4°C. An aliquot of the supernatant was measured spectrophotometrically at 550 nm. The amount of reduced cytochrome C was calculated from the molar extinction coefficient of 21.1 mM⁻¹ cm⁻¹. Results are expressed as the number of nanomoles of cytochrome C reduced by superoxide dismutase.

Fig. 4. Effects of prednisolone (a) and aminophylline (b) on ovalbumin (OA)-induced airway hyperresponsiveness in guinea pigs. Animals were sensitized with OA at 5 mg/kg, i.p. on day 0 and OA at 10 mg/kg, i.p. on day 2. From day 14, 0.1%, 0.2%, 0.4%, 0.5% and 1% OA aerosols were inhaled at 4-day intervals. Airway reactivity was examined by i.v. administration of acetylcholine (15.6–2000 μg/kg) 24 hr after the 1% OA challenge on day 34. Prednisolone was administered intraperitoneally 2 hr and aminophylline was administered orally 1 hr before each OA aerosol, respectively. Each value represents mean ± S.E. of percentage of initial value before acetylcholine administration for respiratory rate, respiratory volume and the ratio of expiration time to inspiration time (Ex/In). *P < 0.05, significantly different from the control by Student’s t-test. Normal (+); Control (○); prednisolone, 20 mg/kg (●); aminophylline, 50 mg/kg (■); n = 6–8.
Statistics
Statistical analysis was performed using Dunnett's, paired or Student's t-test as appropriate for the data.

RESULTS

Antigen-induced bronchoconstriction in guinea pigs

In passively sensitized guinea pigs, an antigen induced decrease in respiratory rate, prolongation of the ratio between expiration/inspiration and respiratory volume were observed. Figure 2 shows the change of respiratory parameters and the influence of NIP-502 on non-metyrapone-treated (a) and metyrapone-treated (b) guinea pigs following antigen-induced bronchoconstriction. Oral administration of NIP-502 (10 mg/kg) showed effective inhibition irrespective of metyrapone treatment.

Antigen-induced hyperresponsiveness in guinea pigs

Repeated inhalation of OA induced airway hyperresponsiveness to intravenous administration of acetylcholine. NIP-502 administered orally at a dose of 10 mg/kg 1 hr before each OA challenge showed a tendency to reduce the enhanced bronchial reactivity (Fig. 3). At a dose of 30 mg/kg, NIP-502 significantly inhibited the OA-induced airway hyperresponsiveness. Prednisolone, a potent glucocorticoid (Fig. 4a), which was administered intraperitoneally (20 mg/kg) 2 hr before each OA inhalation, was also effective under similar conditions, but the bronchodilator aminophylline (50 mg/kg, p.o.) had no effect (Fig. 4b).

Bronchoalveolar lavage fluid study in guinea pigs

Repeated inhalation of OA induced an increase in the number of macrophages, eosinophils, neutrophils and lymphocytes in the BALF of guinea pigs obtained 24 hr after the final OA aerosol (Fig. 5, solid column). NIP-502 significantly inhibited the OA-induced increase of all inflammatory leukocytes except lymphocytes. Prednisolone, given at a dose of 30 mg/kg, i.p. 3 hr before every OA aerosol, showed non-selective inhibition of the inflammatory response.

Fig. 5. Effects of NIP-502 and aminophylline on ovalbumin (OA)-induced increase in the numbers of total cells, macrophages, eosinophils, neutrophils and lymphocytes in the bronchoalveolar lavage fluid (BALF) of guinea pigs. Animals were sensitized with 0.5 mg/kg, i.p. on day 0 and 1 mg/kg, i.p. on day 2, and they were repeatedly exposed to aerosolized OA (0.1% on day 14, 0.2% on day 17, 0.4% on day 20, 0.5% on day 25 and 1% on days 28 and 31). BALF were obtained 24 hr after the last inhalation of OA on day 31. NIP-502 (30 mg/kg, p.o., *) and aminophylline (50 mg/kg, p.o., ) were administered 1 hr and prednisolone (20 mg/kg, i.p., ) was administered 3 hr before each OA aerosol. Other columns represent the following: normal (no sensitization) and , control (sensitization and no test compound treatment). Each value represents mean ± S.E., *p < 0.05, significantly different from the control response by Dunnett's multiple range test; n = 3 – 8.

Fig. 6. Effects of NIP-502 and prednisolone on 48-hr homologous passive cutaneous anaphylactic reactions in rats. Male Wistar rats were sensitized with anti-DNP-As serum (0.1 ml/site, i.d.). After 48 hr, PCA was induced by i.v. injection of DNP-BSA solution (1 mg/animal) containing 0.5% Evans blue. NIP-502 was administered p.o. 1 hr and prednisolone was given 3 hr after the injection of DNP-As solution. Each value represents mean ± S.E., *p < 0.05, significantly different from the control response by Dunnett's multiple range test; n = 6 – 7.
leukocyte cell accumulation in BALF. Aminophylline inhibited the increase of macrophages and neutrophils, but did not inhibit those of eosinophils and lymphocytes. Though there was a slight inhibition of lymphocyte accumulation following treatment with NIP-502, it was not statistically significant.

Forty-eight hour homologous PCA reaction in rats
As shown in Fig. 6, NIP-502 at a dose of 30 mg/kg, p.o. administered 1 hr before the antigen challenge, showed a tendency to inhibit passive cutaneous reactions in rats. At the dose of 50 mg/kg, NIP-502 showed a significant inhibition. Prednisolone, which was administered 3 hr before the antigen challenge, clearly inhibited the PCA reaction.

Reversed cutaneous anaphylaxis in rats
NIP-502 (300 mg/kg, p.o., 1 hr before antiserum challenge) or prednisolone (20 mg/kg, i.p., 2 hr before antiserum challenge) did not inhibit reversed cutaneous anaphylaxis in rats (control group skin swelling 0.20±0.01 g, NIP-502-group 0.24±0.01 g and prednisolone-group 0.24±0.01 g, ns).

Reversed Arthus reaction in rats
NIP-502 (200 mg/kg, p.o.) did not affect the Arthus reaction in rats (control group footpad increased volume 0.69±0.12 ml, NIP-502-group 0.71±0.12 ml, ns). Prednisolone (20 mg/kg, i.p.) significantly inhibited the increase in footpad volume (0.36±0.10 ml, P<0.05).

SRBC-induced delayed type hypersensitivity in mice
At the dose of 200 mg/kg (1 hr before and 16 hr after SRBC challenge), NIP-502 had no effect on the increase in footpad volume (control 45±5 μl, NIP-502 treated group 43±5 μl, ns). In contrast, pretreatment with prednisolone (20 mg/kg, i.p., 1 hr before the challenge) clearly inhibited that response (20±3 μl, P<0.05).

Superoxide anion (O$_2^-$) production
FMLP (1 μM) generated superoxide anion from guinea pig alveolar macrophages and the concentration of cytochrome C reduced by superoxide dismutase in the control responses were from 4 to 13 nmols/2×10⁶ cells. NIP-502 inhibited the production of O$_2^-$ in a concentration-dependent manner (30–300 μM). Aminophylline slightly but significantly inhibited O$_2^-$ production only at a dose of 300 μM (Fig. 7).

DISCUSSION
In this study, we have demonstrated that a newly synthesized pyridazinone derivative, NIP-502, inhibited not only antigen-induced bronchoconstriction but also antigen-induced airway hyperresponsiveness and accumulation of inflammatory leukocytes in the bronchoalveolar fluid of guinea pigs. This ability to inhibit a wide variety of allergic symptoms suggests that NIP-502 is a promising treatment for bronchial asthma.

NIP-502 inhibited the antigen-induced bronchoconstriction, one of the main symptoms of asthma, even in the presence of metyrapone. Metyrapone treatment causes a decrease in the concentration of cortisol (16, 20), and an increase in the level of antigen-induced bronchoconstriction in guinea pigs (8). Furthermore, the enhanced bronchoconstriction and the deteriorated respiratory parameters were observed within 30 min after the antigen challenge in metyrapone treated guinea pigs and they were not influenced by the β-stimulant salbutamol and were effectively inhibited by prednisolone or hydrocortisone (8). Nakazawa (21) reported that in asthmatic patients who show late asthmatic responses (LAR), the plasma cortisol level was decreased when LAR appeared. Moreover, Sasaki et al. (20) showed that a late asthmatic response to the antigen challenge was observed in metyrapone-treated dogs. It is reported that a β-adrenoceptor agonist inhibited only the immediate-phase airway response, while glucocorticoid inhibited the late-phase airway response (10, 22). Although the antigen-induced bronchoconstriction in metyrapone treated guinea pigs
NIP-502 effectively prevented the ovalbumin-induced increase in the number of macrophages, eosinophils and neutrophils. NIP-502 inhibited lymphocyte accumulation in BALF, but not to a significant degree. The bronchodilator aminophylline also inhibited the increase of macrophages and neutrophils. The inhibitory effect on eosinophil accumulation, which seems to have a major role in airway inflammation and airway hyperresponsiveness (6), was clearly seen after treatment with NIP-502, but no significant inhibition was observed in aminophylline-treated animals. Furthermore, NIP-502 inhibited OA-induced airway hyperresponsiveness, but aminophylline had no effect. This is the most significant difference between NIP-502 and aminophylline. These results suggest that the change in the total number of cells is not a completely satisfactory measure of the inhibitory efficacy of the drug. Eosinophils may be involved in the change of airway responsiveness induced by antigen (23, 24). This does not contradict findings that prednisolone showed non-selective inhibition of the increase in the number of inflammatory leukocytes and the increase in airway reactivity induced by OA. As shown in the results from the superoxide production experiment, NIP-502 also inhibited the function of alveolar macrophages in a dose-related manner. This inhibitory effect was also seen by the treatment of aminophylline, but it was weak. Kaneko et al. (25) reported that theophylline (below 100 μM) enhanced O₂⁻ production by human neutrophils, but at a higher concentration of 300 μM, it had an inhibitory effect. The concentration of aminophylline used in the present study seems to be high enough to inhibit the O₂⁻ production by guinea pig macrophages.

The results of the present study show that NIP-502 may affect not only on the immediate asthmatic response, but also various pathological conditions observed in bronchial asthma. The inhibitory mechanisms of NIP-502 on antigen-induced bronchial reactions have not been clarified yet, but the ability of NIP-502 to inhibit O₂⁻ generation (present study) and inhibition of the LTD₄-induced contraction in isolated guinea pig trachea at a concentration of 3–10 μM (A. Yamamoto, unpublished observation) may contribute to the inhibitory mechanism of this compound. Further experiments are needed, however.

NIP-502 inhibited PCA reactions in rats but did not have any significant effect on other allergic responses even at extremely high doses (200 mg/kg, p.o.). The PCA reaction is one of the most common of the immediate cutaneous responses that define the Type I allergic reaction. There is some evidence that Type II–IV allergic reactions, which were not effectively inhibited by NIP-502 in the present study, are related to asthmatic responses. On the other hand, because of the selective inhibition of NIP-502 on the PCA reaction, it is likely that the process of the PCA reaction is related to the asthmatic response to a certain degree. Furthermore, NIP-502 and prednisolone have similar effects on bronchial reactivity and leukocyte accumulation, but the inhibitory effects of NIP-502 seem to act more selectively on the respiratory tract than corticosteroids do.

In conclusion, NIP-502 shows an inhibitory effect on antigen-induced bronchoconstriction, airway hyperresponsiveness and accumulation of inflammatory leukocytes into guinea pig BALF, and the mode of action of this compound may be different from that of prednisolone or aminophylline. These results suggest that NIP-502 possesses a highly beneficial profile for the treatment of bronchial asthma.

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