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PHARMACOLOGICAL PROPERTIES OF N-(3',4'-DIMETHOXYCINNAMOYL) ANTHRANILIC ACID (N-5'), A NEW ANTI-ATOPIC AGENT

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1 N-(3',4'-dimethoxycinnamoyl) anthranilic acid (N-5') exhibited a dose-dependent, potent inhibition of the passive cutaneous anaphylaxis (PCA) mediated by homocytotropic antibodies (HTA), which was hardly affected by anti-inflammatory agents such as phenylbutazone, indomethacin and prednisolone at any dose used. The HTA-induced PCA was significantly inhibited by combined treatment with diphenhydramine and cyproheptadine.

2 Doses of N-5' which potently inhibited HTA-induced PCA inhibited only slightly the heterologous PCA produced by anti-bovine serum albumin (BSA) rabbit serum. This heterologous PCA was clearly inhibited by phenylbutazone, indomethacin and prednisolone. Diphenhydramine and cyproheptadine, singly or combined inhibited the heterologous PCA only slightly.

3 The increased vascular permeability caused by histamine and 5-hydroxytryptamine was significantly inhibited by diphenhydramine or cyproheptadine, but not by N-5' and the anti-inflammatory agents used.

4 N-5' 150 mg/kg orally inhibited rat paw oedema induced by carrageenin by about 26% while phenylbutazone, indomethacin and prednisolone produced significant inhibition.

5 N-5' at concentrations of 100 and 1000 μM significantly inhibited (by about 52% and 95%, respectively) the histamine release from rat peritoneal cells induced by HTA; 10 μM N-5' had little effect. Histamine release was inhibited by phenylbutazone or indomethacin at 1000 μM but not at 100 μM. Prednisolone had no effect on histamine release at any of the concentrations used.

6 These findings suggest that the inhibition of the HTA-induced PCA by N-5' may be due to inhibition of histamine release and is clearly different from the actions of anti-inflammatory agents such as phenylbutazone, indomethacin and prednisolone.

Introduction

N-(3',4'-dimethoxycinnamoyl) anthranilic acid (N-5') is a newly synthesized, anthranilic acid derivative. Given orally, N-5' potently inhibits the passive cutaneous anaphylaxis (PCA) mediated by homocytotropic antibodies (HTA).

Koda, Nagai, Watanabe, Yanagihara & Sakamoto (1976) reported that N-5' inhibited HTA-induced PCA and mast cell degranulation in rats and guinea-pigs. They suggested that the anti-allergic properties of N-5' may be clinically useful in the treatment of allergy-related diseases, especially asthma.

In view of the anti-inflammatory actions of anthranilic acid derivatives, N-5' has been compared with several established anti-inflammatory agents.

Methods

Passive cutaneous anaphylaxis in rats

Preparation of antisera. Antiserum containing homocytotropic antibody (HTA, reagin antibody) was prepared in rats as described by Tada & Okumura (1971). Male Wistar rats weighing 180 to 200 g were splenectomized and 5 days later immunized by injecting into all four footpads a total of 1 mg of 2,4-dinitrophenyl-coupled ascaris extract (DNP-As) mixed with 10^{10} *Bordetella pertussis*; 5 days later, 0.5 mg of DNP-As alone was injected subcutaneously into the back. Eight days after the first immunization, blood was collected by aortic puncture, under ether anaesthesia. The antiserum obtained by these procedures remained, when injected intradermally, in the skin for at least 1 week and was inactivated by heating at 56°C for 30 minutes. The titre of the antiserum was determined in rats by homologous PCA 48 h after injecting it intradermally. The dilution of antiserum producing a diameter of approximately 5 mm was usually 1:500. Anti-bovine serum albumin (BSA) rabbit serum (anti-BSARS) was prepared by 4 weekly intramuscular injections of BSA (10 mg/kg) emulsified with an equal volume of complete Freund's adjuvant. Anti-BSARS injected intradermally

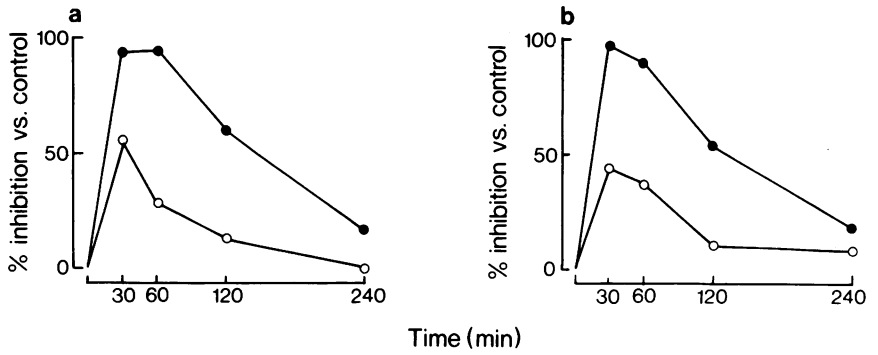

Figure 1 Time course of inhibitory action of N-5′ on (a) the amount of leakage of dye and (b) the area resulting from passive cutaneous anaphylaxis produced by homocytotropic antibodies. N-5′ 100 (O) or 200 (●) mg/kg was administered orally at 30, 60, 120 or 240 min before challenge with antigen (DNP-As). Each point represents the mean value of 6 observations.

disappeared within 24 h and the titre determined by 4 h-PCA was 1 : 32. All antisera obtained were stored at −20°C until used.

Assessment of passive cutaneous anaphylaxis in rats. Normal male Wistar rats weighing 140 to 160 g were sensitized passively by injection into the shaved skin of the diluted antisera (1 : 200 in the case of HTA, 1 : 4 in the case of anti-BSARS). After 48 h in the case of HTA and 4 h in the case of anti-BSARS, 0.5 mg of antigen (DNP-As or BSA, respectively) and 0.5 ml of 0.5% Evans blue were injected intravenously. The animals were exsanguinated 30 min after challenge with the antigens. The area blued as a result of PCA was excised and measured. The amount of dye leaked was extracted with acetone containing sodium sulphate and measured photometrically (620 nm). The drugs to be tested were suspended in a 0.5% aqueous solution of sodium carboxymethylcellulose (CMC) and administered orally except where stated otherwise. Animals were fasted for 16 h before experiments and water was provided *ad libitum.*

The increase in vascular permeability caused by intradermal injections of histamine (10 μg/0.1 ml) or 5-hydroxytryptamine (0.1 μg/0.1 ml) was also assessed by the leakage of Evans blue.

Oedema of rat hind paw

Oedema was induced in male Wistar rats (weighing 120 to 140 g and fasted for 16 h) by subcutaneous injection of 1.0% carrageenin (0.1 ml) into the hind paw. Drugs to be tested were administered orally 30 min before the injection of carrageenin. The oedema was measured volumetrically every hour for 5 h and expressed as $(Et-En/En)\times 100$, where En = volume of hind paw before injection of carrageenin, and Et = volume after injection.

Anaphylactic histamine release from rat peritoneal cells in vitro

Isolation and sensitization of peritoneal cells. Male Wistar rats weighing 180 to 220 g were exsanguinated and injected intraperitoneally with 10 ml of a solution containing (mmol/l): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2.6H_2O$ 1.0 and glucose 5.6; 5 units/ml of heparin was added and the pH adjusted to 7.2 with 5% (v/v) of 0.1 M phosphate buffer. The abdominal region was massaged for several minutes, and the peritoneal exudate then collected into siliconized glassware.

The cells were washed with the buffer solution and centrifuged (200 *g* for 3 min at 4°C) several times, and then separated. Cells from 10 animals were pooled. Peritoneal cells obtained in this way contained 34 to 35% mast cells.

The cells were sensitized by incubation, with occasional stirring, for 1 h at 37°C in 4 ml of homocytotropic antiserum diluted with 0.9% w/v NaCl solution (saline) and containing 10 units of heparin. They were then washed twice with the buffer solution. Finally a suspension of about 6×10^4 mast cells/ml was prepared.

Histamine release and its assay. After pre-warming to 37°C for 5 min, 2.5 ml of the cell suspension was incubated for 20 min with 0.3 ml of test solution and 0.2 ml of 300 μg/ml DNP-As as antigen. The reaction mixture was then centrifuged at 500 *g* for 10 min at 4°C.

The supernatant was assayed for released histamine and the precipitate for the residual cellular histamine according to the method of Shore, Burkhalter & Cohn (1959).

Percentage release of histamine was calculated as $(A/A+B)\times 100$, where A = μg of released histamine, and B = μg of residual cellular histamine.

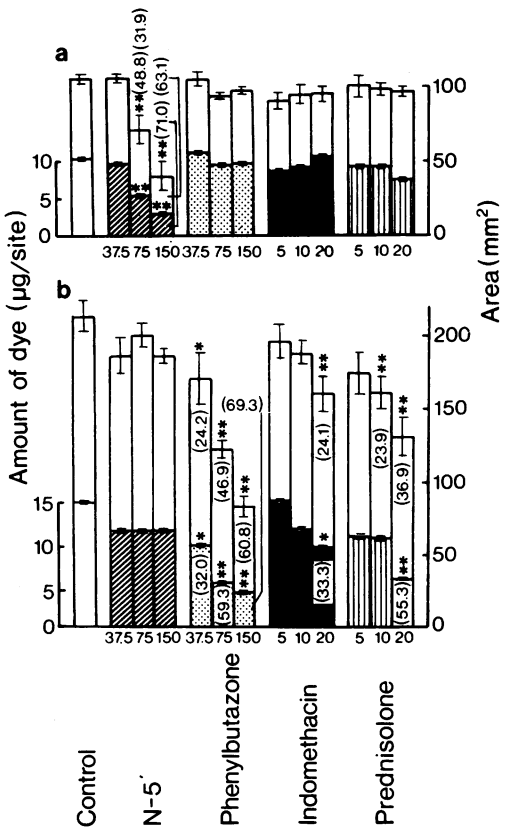

Figure 2 Effect of drugs on (a) HTA-induced passive cutaneous anaphylaxis (PCA) and (b) anti-BSARS-induced PCA in rats. Each drug was administered orally 30 min before challenge with antigen (DNP-As in (a) and BSA in (b)), see text. Doses of drugs (mg/kg) are given at the base of the columns. Numbers in parentheses indicate inhibition as a percentage of the control value. Mean values from 5 (a) or 6 (b) observations are given. Vertical lines indicate s.e. mean. $^{*}P<0.05$, $^{**}P<0.01$. The amount of dye leaked (lower parts of columns) as a result of PCA-induced by HTA or by anti-BSARS in controls was 10.4 ± 0.90 and 15.0 ± 0.90 μg/site respectively; the area was 105.1 ± 2.90 and 211.2 ± 10.30 mm², respectively.

The inhibition of histamine release by test drugs was calculated as $(1-(D-S/C-S))\times 100$, where C, D, S = percentage release of histamine in controls, in the presence of test drugs, and in the absence of antigen (spontaneous), respectively. Drugs to be tested were dissolved in 1% aqueous solution of $NaHCO_3$.

Drugs

The following drugs were used: phenylbutazone, indomethacin, prednisolone, histamine dihydrochloride, 5-hydroxytryptamine creatinine sulphate, diphenhydramine hydrochloride, cyproheptadine hydrochloride, carrageenin, Evans blue (Merck), heparin (Novo), bovine serum albumin (Sigma) and Freund's complete adjuvant (Difco).

Results

Passive cutaneous anaphylaxis induced with homocytotropic antibodies

To examine the effect of N-5′ at doses of 100 and 200 mg/kg orally on PCA induced by HTA, N-5′ was administered at 30, 60, 120 or 240 min before challenge with antigen (DNP-As). Inhibition was most potent at 30 or 60 min pretreatment with N-5′, and negligible at 240 min pretreatment (Figure 1). Thirty minutes pretreatment with drugs was therefore used when N-5′ was compared with phenylbutazone, indomethacin and prednisolone. The amount of dye leaked as a result of PCA in controls was 10.4 ± 0.90 μg/site and the area 105.1 ± 2.90 mm². N-5′ produced a dose-dependent, potent inhibition of the leakage, whereas phenylbutazone, indomethacin or prednisolone had little or no effect (Figure 2a). The PCA was significantly inhibited by the combined pretreatment with 50 mg/kg of diphenhydramine and 10 mg/kg of cyproheptadine (not shown in Figure 2a).

Passive cutaneous anaphylaxis induced with anti-bovine serum albumin rabbit serum

The mean amount of dye leaked as a result of PCA induced by anti-BSARS in controls was 15.0 ± 0.90 μg/site and the area 211.2 ± 10.30 mm² (Figure 2b). Doses of N-5′ which potently inhibited homologous PCA mediated by HTA, inhibited only slightly PCA induced by anti-BSARS. On the other hand, heterologous PCA was clearly inhibited by phenylbutazone, indomethacin and prednisolone in a dose-dependent manner. Diphenhydramine (50 mg/kg) and cyproheptadine (10 mg/kg), singly or combined, produced only a slight inhibition (not shown in Figure 2b).

Increased vascular permeability caused by histamine or 5-hydroxytryptamine in rats

N-5′, phenylbutazone, indomethacin or prednisolone inhibited only slightly at the doses used the increased vascular permeability caused by histamine and 5-hydroxytryptamine (Figure 3). Diphenhydramine (50 mg/kg p.o.) and cyproheptadine (10 mg/kg p.o.), inhibited increased vascular permeability by 76% and 83%, respectively.

Oedema of rat hind paw

The rat paw oedema induced by carrageenin was inhibited by all tested doses of N-5′, phenylbutazone,

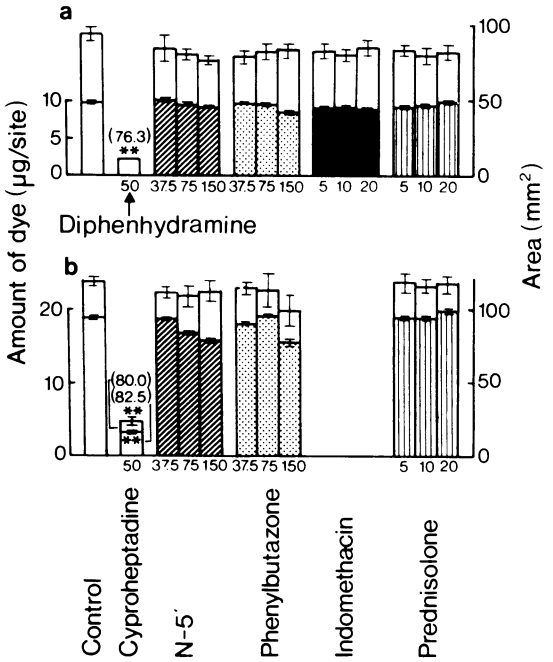

Figure 3 Influence of drugs on the increased vascular permeability caused by (a) histamine or (b) 5-hydroxytryptamine. Each drug was administered orally 30 min before injection of histamine (10 μg per site in 0.1 ml) or 5-hydroxytryptamine (0.1 μg per site in 0.1 ml). Doses of drugs (mg/kg), percentage inhibition, mean ± s.e. mean (5 observations) and *P* values are given as in Figure 2. The amount of dye leaked as a result of intradermal injection of histamine and 5-hydroxytryptamine in controls was 9.7 ± 0.43 and 18.9 ± 1.25 μg/site, and the area 94.1 ± 4.63 and 118.9 ± 2.68 mm², respectively. The influence of indomethacin on the increased vascular permeability caused by 5-hydroxytryptamine was not tested.

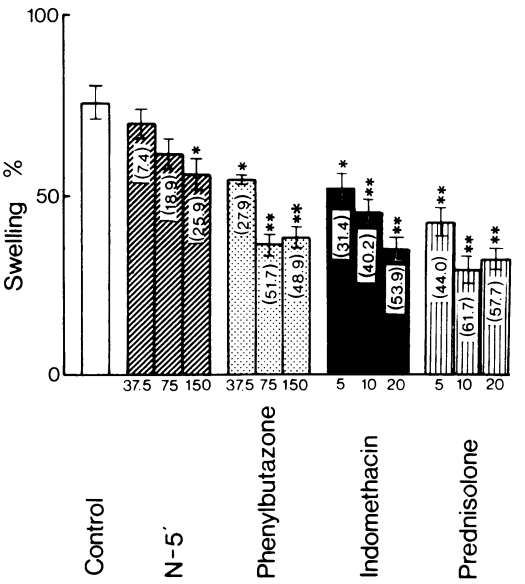

Figure 4 Influence of drugs on oedema of rat hind paw induced by carrageenin. Each drug was administered orally 30 min before injection of carrageenin (1.0%, 0.1 ml/paw s.c.). Doses of drugs, percentage inhibition, mean ± s.e. mean (5 observations) and *P* values are given as in Figure 2. Swelling was measured 3 h after carrageenin injection (see text).

indomethacin and prednisolone (Figure 4). N-5′ produced only about 26% inhibition at 150 mg/kg orally, while phenylbutazone, indomethacin and prednisolone were more potent. Inhibition was determined 1, 2, 3, 4 and 5 h after carrageenin injection but the values at 3 h were used, because both the second phase of carrageenin oedema and the inhibitory action of the drugs were most pronounced at that time.

Release of histamine from rat peritoneal cells induced by homocytotropic antibodies

Cells sensitized with HTA were incubated with test drugs 1, 5, 10 or 20 min before challenge with antigen (DNP-As). Inhibition of the allergic histamine release by N-5′ was most potent after 1 min and gradually decreased with longer preincubation times (not shown in Figure). One minute pretreatment with drugs was therefore used (Figure 5).

Figure 5 shows the effect of 1, 10, 100 and 1000 μM N-5′, phenylbutazone, indomethacin and prednisolone on the allergic histamine release from peritoneal cells. Mean total amount of histamine released as a result of antigen-antibody reaction from about 1.5×10^5 of rat peritoneal mast cells was 2.77 ± 0.130 μg, corresponding to 38.1 ± 1.56% of the total histamine content (7.3 μg/1.5×10^5 cells). The spontaneous release without antigen was 11.1 ± 1.87%.

The histamine release was significantly inhibited by 100 and 1000 μM N-5′ but was hardly affected by the drug at a concentration of 10 μM or 1 μM. Phenylbutazone and indomethacin inhibited histamine release by 50% at a concentration of 1000 μM but had no effect at 100, 10 or 1 μM. Prednisolone had no effect at any concentration used.

Discussion

Homocytotropic antibodies (HTA) resembling human reaginic antibodies in their physicochemical and

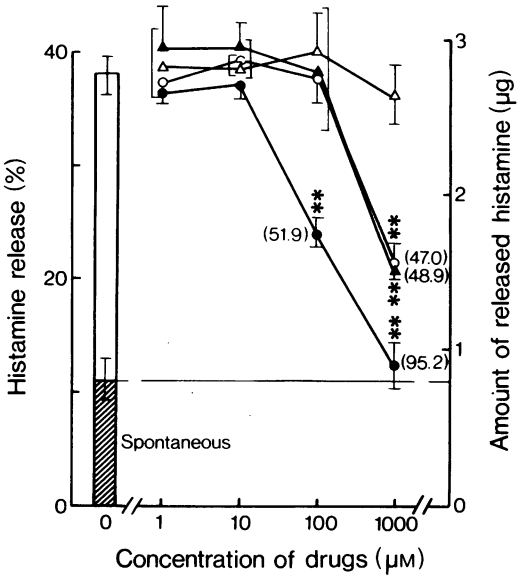

Figure 5 Influence of N-5′ (●), phenylbutazone (○), indomethacin (△) and prednisolone (▲) on histamine release from rat peritoneal cells induced by homocytotropic antibodies (HTA) and antigen. Each drug was applied 1 min before challenge with antigen (DNP-As) (see text). Each experiment included 6 observations. Vertical lines indicate s.e. mean. ** $P<0.01$. The antigen-antibody reaction released from about 1.5×10^5 rat peritoneal mast cells 2.77 ± 0.130 μg of histamine or $38.1\pm1.56\%$ of the total histamine. Spontaneous release of histamine from sensitized cells without antigen was $11.1\pm1.87\%$. Numbers in parentheses indicate percentage inhibition (see text).

immunological properties (Mota, 1964; Bianaghi, Benacerraf, Block & Kourilsky, 1964) are induced in rats when antigen is injected with killed *B. pertussis* organisms as adjuvant. The rat antisera used in the present experiments was inactivated by a temperature of 56°C and persisted in the skin for at least 1 week. This supports the view that the homologous PCA involved reagin type antibodies only, and no attempts were made to obtain reagin fractions from rat sera. It is well established that reagin type antibodies induce histamine release by sensitizing mast cells.

N-5′ significantly inhibited the HTA-induced PCA in rats in a dose-dependent manner, while inhibition of anti-BSARS-induced PCA was only slight. Phenylbutazone, indomethacin and prednisolone produced the reverse pattern. Combined treatment with diphenhydramine and cyproheptadine significantly inhibited HTA-induced PCA but not anti-BSARS-induced PCA. This suggests that histamine and 5-hydroxytryptamine may mediate HTA-induced PCA but not anti-BSARS-induced PCA. Our data are supported by the findings of Goose & Blair (1969).

N-5′ and the anti-inflammatory agents inhibited only slightly the increased vascular permeability caused by histamine or 5-hydroxytryptamine. This suggested that the inhibitory action of N-5′ on HTA-induced PCA was not due to antagonism to histamine and 5-hydroxytryptamine but to inhibition of the release of these mediators. The experiments with rat peritoneal cells suggested that N-5′ acts mainly on histamine release. Phenylbutazone and indomethacin inhibited this release only in 1 mM concentrations. Prednisolone was inactive. According to Mota & Ishii (1960), phenylbutazone (1540 μg/ml, about 5 mM) reduced anaphylactic histamine release only by 45%. The report that cortisone did not decrease the anaphylactic histamine release (Trethewie, 1958) agrees with our data. The inhibition by 1 mM phenylbutazone and indomethacin may depend on uncoupling of oxidative phosphorylation and/or membrane stabilization since Miller & Smith (1966), Yamasaki & Saeki (1967) and Ignarro (1971) have shown that phenylbutazone and indomethacin inhibit the mast cell degranulation induced by compound 48/80, by depressing the processes generating ATP and their anti-inflammatory effects are associated with stabilization of the lysosomal membrane.

Rat paw oedema induced by carrageenin was effectively inhibited by the anti-inflammatory agents, but only slightly by N-5′. Yamasaki & Saeki (1967), reported that phenylbutazone, indomethacin, flufenamic acid and mefenamic acid inhibited the inflammatory oedema produced in rats by rabbit anti-rat serum. This oedema was not inhibited by N-5′ (Koda *et al.*, 1976). The pharmacological properties of the anthranilic acid derivative, N-5′ appear to differ from those of anti-inflammatory agents. As proposed by Koda *et al.* (1976), N-5′ could be an anti-allergic clinical drug, effective by oral application. Other pharmacological properties of N-5′ are now under investigation.

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