The Extract from *Nandina domestica* THUNBERG Inhibits Histamine- and Serotonin-Induced Contraction in Isolated Guinea Pig Trachea

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Although the fruit of *Nandina domestica* THUNBERG (ND) has been used to treat respiratory disorders such as coughing and breathing difficulty in Japan for many years, very little is known about mechanisms underlying its action. In the present study, we investigated effects of the crude extract from ND (NDE) and one of its constituents, nantenine, on contractile responses in isolated guinea pig tracheal ring preparations. In normal experimental condition, guinea pig trachea remained tonically contracted during the resting state, and addition of NDE (1 mg/ml) caused a relaxation of tracheal smooth muscles, but had little effect on the responsiveness of trachea to acetylcholine. The basal, tonic contraction was abolished by the presence of atropine and indomethacin. In this condition, NDE at 0.1–1 mg/ml inhibited histamine-induced contraction in both competitive and non-competitive manners. NDE at 0.01–1 mg/ml inhibited serotonin-induced contraction in a competitive manner. Nantenine (2–20 μ M) did not affect histamine-induced contraction, and slightly inhibited serotonin-induced contraction. These results suggest that NDE has inhibitory effects on tracheal smooth muscle contraction, and nantenine cannot account solely for this effect of NDE.

Key words *Nandina domestica*; tracheal smooth muscle; histamine; serotonin; nantenine

The fruits of *Nandina domestica* THUNBERG (ND), called in “nantenjitsu” in Japan, have been used for the treatment of respiratory diseases such as asthma, whooping cough and pharynx tumor for many years in Japan.¹⁾ However, little is known about mechanisms underlying its pharmacological action. Since the trachea is a lower respiratory tract that controls respiratory air flow, it is possible that ND improves breathing difficulty by relaxing tracheal smooth muscles. To examine this possibility, in the present study, we investigated effects of the crude extract from ND (NDE) on contractile responses of isolated trachea *in vitro*.

Although chemical studies on the constituents of NDE have revealed that it contains numerous alkaloids, it remains unclear which constituent is responsible for beneficial effects of NDE in treating respiratory disorders. Nantenine (9,10-methylenedioxy-1,2 dimethoxyaporphine or *O*-methyldomes-ticine) is a major alkaloid isolated from NDE,¹⁾ and has been reported to show several pharmacological activities. For example, nantenine (3 μ M) competitively inhibited serotonin-induced contractile responses in isolated rabbit aorta and isolated rat stomach,²⁾ suggesting that it is a serotonergic receptor antagonist. Nantenine (0.3–3 μ M) competitively inhibited phenylephrine-induced contraction in isolated rat aorta,³⁾ suggesting that it is also an α_1 -adrenoceptor antagonist. Nantenine at higher concentrations ($2.35, 4.7 \times 10^{-4}$ M) inhibited KCl-induced increase in intracellular Ca^{2+} concentration and contraction in isolated rat vas deferens,⁴⁾ suggesting that it works like Ca^{2+} antagonists. Intravenous administration of nantenine (3–6 mg/kg) produced dose-dependent decreases in mean arterial blood pressure and heart rate in anesthetized rats.⁵⁾ Intraperitoneal administration of nantenine (20–50 mg/kg) inhibited pentylenetetrazol- and electroshock-induced seizures in mice.⁶⁾ The chemical structure of nantenine is similar to that of 3,4-methylenedioxymethamphetamine (MDMA), which is a “club drug” often sold under the name of ecstasy, and nantenine blocked MDMA-induced hyperthermia in mice.⁷⁾ However, to the best of our knowledge,

there has been no report describing pharmacological effects of nantenine on the respiratory system. Therefore, we compared the effects of NDE and nantenine in the present study.

Contractile responses of tracheal smooth muscles are regulated by various neurotransmitters and autacoids including acetylcholine, histamine, serotonin, *etc.*^{8–11)} As mentioned above, nantenine has been reported to inhibit serotonin-induced contractile responses in isolated rabbit aorta and isolated rat stomach.²⁾ It is possible that NDE and nantenine affect serotonin-induced contraction of tracheal smooth muscles. Therefore, in the present study, we used acetylcholine, histamine and serotonin as stimulants to induce tracheal contraction. Furthermore, we chose the guinea pig as an experimental animal, because it is well known that the guinea pig trachea responds more sensitively to histamine and serotonin than the rat trachea.

MATERIALS AND METHODS

Drugs and Chemicals NDE was manufactured through a conventional process by Tokiwa Pharmaceutical Co., Ltd., Noevir Group (Osaka, Japan). Briefly, the dried fruits of ND were extracted with 10 parts of hot water (90–100 °C) for 1 h, and the solution was spray-dried to give NDE. The yield of NDE was 25.0%. To verify the quality of NDE, the content of nantenine in NDE was checked with gas chromatography. NDE used in the present study contained 0.67% nantenine. The NDE was suspended in distilled water and sonicated at room temperature for 10 min before use. Nantenine was obtained from Matsuura Yakugyo Co., Ltd. (Nagoya, Japan). Nantenine was first dissolved at the concentration of 2 mM in 0.01 N HCl, and then pH was adjusted to 7.0 by adding appropriate volume of 0.1 N NaOH. The nantenine solution was diluted to desired concentrations by distilled water immediately before use. Acetylcholine chloride, histamine, indomethacin, ketanserin tartrate and serotonin hydrochloride were purchased from Sigma Chemical (St. Louis, MO,

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U.S.A.). Atropine sulfate monohydrate, diphenhydramine hydrochloride and papaverine hydrochloride were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals were of reagent grade from Wako.

Animal and Tracheal Tissue Preparation All experiments were performed in accordance with the "Guiding Principles for the Care and Use of Laboratory Animals" approved by the Japanese Pharmacological Society. The trachea was isolated from 6–9-week-old male Hartley guinea pigs (Tokyo Laboratory Animals Science, Tokyo, Japan), weighing 350–550 g, and immersed in Krebs–Henseleit (K–H) solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl_2 , 1.2 mM MgSO_4 , 1.2 mM KH_2PO_4 , 25 mM NaHCO_3 , 10 mM glucose) gassed with 95% O_2 /5% CO_2 (pH 7.4). The trachea was carefully cleaned of unnecessary adipose and connective tissues, and cut into transverse strips of two adjacent cartilage rings (2–3 mm wide).

Measurement of Contractile Responses Each tracheal ring preparation was mounted in a 5-ml organ bath (UC-5TD; UFER Medical Instrument, Kyoto, Japan) at a resting tension of 0.5 g weight (approximately 5 mN). The bath contained K–H solution maintained at 32°C and gassed with 95% O_2 /5% CO_2 . The isometric tension was measured with a force displacement transducer. Before testing effects of drugs, the tracheal ring preparations were allowed to equilibrate for 60 min, during which time the bathing solution was removed and replaced with fresh K–H solution every 20 min and changes in resting tension were adjusted back to 0.5 g weight. At the end of the equilibration, the ring preparation was contracted with 10 mM acetylcholine to ensure stabilization of the smooth muscles. After the contraction reached a steady state, the tissue was rinsed several times with fresh K–H solution until tension returned to the resting level. Concentration–response curves were then constructed by cumulative addition of acetylcholine, histamine or serotonin. In case histamine or serotonin was used as a contractile agent, atropine (1 μM) and indomethacin (10 μM) were added to the bathing solution in order to abolish the basal, tonic contraction possibly produced by endogenous acetylcholine or arachidonate metabolites.^{12,13} Since guinea pig tracheal smooth muscles contracted very slowly and it usually took 1–2 h to obtain one cumulative concentration–response curve, only one concentration of NDE, nantenine or other antagonists was tested on each tissue. The first concentration–response curve for an agonist was constructed in the absence of NDE, nantenine or other antagonists. The same tissue was pre-incubated with one concentration of NDE, nantenine or other antagonists for 10 min, and the second concentration–response curve for the same agonist was constructed in the presence of NDE, nantenine or other antagonists.

Data Analysis The concentration–response curves were analyzed for each experiment. The magnitude of contraction induced by each agonist in each condition was expressed as the percentage of the maximal contraction induced by the agonist in the first concentration–response curve (control). Median effective concentrations (EC_{50} values) for agonists were calculated as described previously.¹⁴ Data are presented as the means $\pm$ S.E.M. of n independent observations. Significance of the differences was determined with Student's t -test.

RESULTS

As shown in Fig. 1A, cumulative addition of acetylcholine at concentrations of 10^{-9} to 10^{-2} M produced a concentration-dependent contraction in guinea pig tracheal ring preparations used in the present study. In normal condition, addition of 1 mg/ml NDE alone caused a decrease in basal tone of the preparation. Cumulative addition of acetylcholine in the presence of 1 mg/ml NDE produced a concentration-dependent contraction. The effects of acetylcholine in the absence and presence of 1 mg/ml NDE were summarized as in Fig. 1B. The basal tone was decreased by the presence of NDE, but maximal contraction induced by acetylcholine was not different in the absence and presence of NDE. The concentration–response curve for acetylcholine was slightly shifted to the right by the presence of 1 mg/ml NDE. The EC_{50} values of acetylcholine were increased by the presence of 1 mg/ml NDE, from $2.48 \pm 0.56 \mu\text{M}$ ($n=5$) to $8.29 \pm 1.70 \mu\text{M}$ ($n=5$; $p<0.01$ by Student's t -test). As a positive control, the effect of the muscarinic receptor antagonist atropine was also checked. The EC_{50} values of acetylcholine were increased by the presence of 10^{-7} M atropine, from $1.94 \pm 0.70 \mu\text{M}$ ($n=5$) to $352.37 \pm 46.62 \mu\text{M}$ ($n=5$; $p<0.01$ by Student's t -test).

The guinea pig tracheal ring preparations used in the present study appeared to tonically contract during the resting state in normal experimental conditions. In fact, addition of

Fig. 1. Effects of NDE on the Basal Tone and Acetylcholine-Induced Contraction in Isolated Guinea Pig Tracheal Ring Preparations

All experiments were done in normal K–H solution without atropine and indomethacin. (A) Typical recordings of contractile responses induced by cumulative addition of acetylcholine in the absence (left) and presence of 1 mg/ml NDE (right). (B) Effect of NDE on the concentration–response curve for acetylcholine. The first concentration–response curve was constructed in the absence of NDE (○). The same tissue was pre-incubated with 1 mg/ml NDE for 10 min, and then the second concentration–response curve was constructed in the presence of 1 mg/ml NDE (●). The ordinate indicates the amplitude of acetylcholine-induced contraction expressed as the percentage by taking the basal tone as 0% and acetylcholine-induced maximal contraction in the absence of NDE as 100%. As seen in the graph, addition of 1 mg/ml NDE alone caused a decrease in the basal tone. Data are the means $\pm$ S.E.M. ($n=5$).

Fig. 2. Typical Examples Showing Effects of NDE and Papaverine on the Basal Tone of Isolated Guinea Pig Tracheal Ring Preparations

(A) In normal K-H solution, the trachea was relaxed by addition of papaverine ($3 \mu\text{M}$). (B) In normal K-H solution, the trachea was relaxed by addition of 1 mg/ml NDE, and the subsequent addition of papaverine caused no further relaxation. (C) In a K-H solution supplemented with atropine ($1 \mu\text{M}$) and indomethacin ($10 \mu\text{M}$), addition of NDE and papaverine caused no change in the baseline.

papaverine ($3 \mu\text{M}$) caused a relaxation of the preparation (Fig. 2A). After the preparation was relaxed by addition of 1 mg/ml NDE, papaverine caused no further relaxation, indicating that NDE at 1 mg/ml produces maximal relaxation of the trachea (Fig. 2B). The presence of the basal tone should make it difficult to quantitatively analyze the effect of NDE on tracheal responses to drugs. Since the basal tone is likely to be produced by endogenous acetylcholine or arachidonate metabolites,^{12,13} the following experiments were done under the presence of atropine, a muscarinic acetylcholine receptor antagonist, and indomethacin, a cyclooxygenase inhibitor. In a K-H solution supplemented with atropine ($1 \mu\text{M}$) and indomethacin ($10 \mu\text{M}$), the basal tone was abolished, and addition of NDE or papaverine caused no change in the baseline (Fig. 2C).

In the presence of atropine and indomethacin, concentration-response curves for histamine and serotonin were constructed. As shown in Fig. 3, cumulative addition of histamine produced a concentration-dependent contraction in guinea pig tracheal ring preparations. The tracheal response to histamine was not affected by the presence of 0.01 mg/ml NDE (Fig. 3A), but significantly inhibited by the presence of 0.1 mg/ml NDE (Fig. 3B). The EC_{50} values of histamine were increased by the presence of 0.1 mg/ml NDE, from $1.12 \pm 0.10 \mu\text{M}$ ($n=5$) to $2.24 \pm 0.29 \mu\text{M}$ ($n=5$; $p<0.01$). The histamine-induced maximal contraction was decreased to $90.0 \pm 3.1\%$ ($n=5$) by 0.1 mg/ml NDE. The tracheal response to histamine was further inhibited by the presence of 1 mg/ml NDE (Fig. 3C). The EC_{50} values of histamine were increased

Fig. 3. Effect of NDE on Histamine-Induced Contraction in Isolated Guinea Pig Tracheal Ring Preparations

All experiments were done in a K-H solution supplemented with $1 \mu\text{M}$ atropine and $10 \mu\text{M}$ indomethacin. Only one concentration of NDE was tested on each tissue. The first concentration-response curve was constructed in the absence of NDE ($\circ$; A–C). The same tissue was pre-incubated with NDE for 10 min, and then the second concentration-response curve was constructed in the presence of NDE ($\bullet$; A, 0.01 mg/ml; B, 0.1 mg/ml; C, 1 mg/ml). The ordinate indicates the amplitude of histamine-induced contraction expressed as the percentage by taking the basal tone as 0% and histamine-induced maximal contraction in the absence of NDE as 100%. Data are the means $\pm$ S.E.M. ($n=5$).

by the presence of 1 mg/ml NDE, from $0.76 \pm 0.10 \mu\text{M}$ ($n=5$) to $5.94 \pm 0.67 \mu\text{M}$ ($n=5$; $p<0.01$). The histamine-induced maximal contraction was decreased to $55.4 \pm 2.5\%$ ($n=5$) by 1 mg/ml NDE. As a positive control, the effect of the histamine H_1 receptor antagonist diphenhydramine was also checked. The EC_{50} values of histamine were increased by the presence of 10^{-6}M diphenhydramine, from $0.74 \pm 0.13 \mu\text{M}$ ($n=5$) to $15.9 \pm 2.4 \mu\text{M}$ ($n=5$; $p<0.01$).

As shown in Fig. 4, cumulative addition of serotonin produced a concentration-dependent contraction in guinea pig tracheal ring preparations. The concentration-response curve for serotonin was slightly shifted to the right by the presence of 0.01 mg/ml NDE (Fig. 4A). The EC_{50} values of serotonin were increased by the presence of 0.01 mg/ml NDE, from $0.20 \pm 0.03 \mu\text{M}$ ($n=5$) to $0.33 \pm 0.04 \mu\text{M}$ ($n=5$; $p<0.05$). The concentration-response curve for serotonin was further shifted to the right by the presence of 0.1 mg/ml NDE (Fig. 4B) and 1 mg/ml NDE (Fig. 4C). Since it was hard to obtain serotonin-induced maximal contraction in the presence of 0.1 and 1 mg/ml NDE (Figs. 4B,C), we were not able to quantitatively analyze the effects of 0.1 and 1 mg/ml NDE on the potency and efficacy of serotonin. As a positive control, the effect of the serotonin 5-HT_{2A} receptor antagonist ketanserin was also checked. The EC_{50} values of serotonin were increased by the presence of 10^{-8}M ketanserin, from $0.08 \pm 0.01 \mu\text{M}$ ($n=5$) to $0.74 \pm 0.15 \mu\text{M}$ ($n=5$; $p<0.01$).

Fig. 4. Effect of NDE on Serotonin-Induced Contraction in Isolated Guinea Pig Tracheal Ring Preparations

All experiments were done in a K-H solution supplemented with $1 \mu\text{M}$ atropine and $10 \mu\text{M}$ indomethacin. The concentration-response curves for serotonin were constructed in the absence ($\circ$; A–C) or presence of NDE ($\bullet$; A, 0.01 mg/ml ; B, 0.1 mg/ml ; C, 1 mg/ml). The experimental protocol and representation of the graphs are as in Fig. 3. Data are the means $\pm$ S.E.M. ($n=5$).

Since chemical analysis with gas chromatography revealed that NDE contained 0.67% nantenine (molecular weight; 339.385), nantenine was estimated to be present at approximately $20 \mu\text{M}$ in 1 mg/ml NDE. Therefore, we investigated the effects of nantenine at 0.2 , 2 and $20 \mu\text{M}$ on histamine- and serotonin-induced contraction. As shown in Fig. 5, histamine-induced contraction was not at all affected by the presence of 0.2 – $20 \mu\text{M}$ nantenine. As shown in Fig. 6A, serotonin-induced contraction seemed to be augmented by $0.2 \mu\text{M}$ nantenine, but the effect of nantenine was not statistically significant. The concentration-response curve for serotonin was slightly shifted to the right by the presence of $2 \mu\text{M}$ nantenine (Fig. 6B). The EC_{50} values of serotonin were increased by the presence of $2 \mu\text{M}$ nantenine, from $0.19 \pm 0.05 \mu\text{M}$ ($n=5$) to $0.50 \pm 0.05 \mu\text{M}$ ($n=5$; $p<0.01$). The concentration-response curve for serotonin was further shifted to the right by the presence of $20 \mu\text{M}$ nantenine (Fig. 6C). The EC_{50} values of serotonin were increased by the presence of $20 \mu\text{M}$ nantenine, from $0.28 \pm 0.05 \mu\text{M}$ ($n=5$) to $4.24 \pm 0.52 \mu\text{M}$ ($n=5$; $p<0.01$). The inhibitory effect of nantenine on serotonin-induced contraction (Fig. 6) was weaker than that of NDE (Fig. 4).

DISCUSSION

NDE at 1 mg/ml caused a relaxation in tonically contracted tracheal preparations (Fig. 1). This is the first report demonstrating that NDE relaxes tracheal smooth muscles. The basal, tonic contraction of isolated guinea pig tracheal

Fig. 5. Effect of Nantenine on Histamine-Induced Contraction in Isolated Guinea Pig Tracheal Ring Preparations

All experiments were done in a K-H solution supplemented with $1 \mu\text{M}$ atropine and $10 \mu\text{M}$ indomethacin. The concentration-response curves for histamine were constructed in the absence ($\circ$; A–C) or presence of nantenine ($\bullet$; A, $0.2 \mu\text{M}$; B, $2 \mu\text{M}$; C, $20 \mu\text{M}$). The experimental protocol and representation of the graphs are as in Fig. 3. Data are the means $\pm$ S.E.M. ($n=5$).

preparations was abolished by atropine and indomethacin (Fig. 2), indicating that it is produced by endogenous acetylcholine and arachidonate metabolites, as previously reported.^{12,13} The concentration-response curve for acetylcholine was shifted to the right by 3.3-fold in the presence of 1 mg/ml NDE (Fig. 1B), suggesting that NDE has a weak anti-cholinergic action. However, the inhibitory effect of NDE on acetylcholine-induced contraction was very slight, and cannot account for NDE-induced decrease in the basal tone. It is possible that NDE relaxes tracheal smooth muscles through non-specific mechanisms like papaverine or Ca^{2+} antagonists. This possibility is under investigation in our laboratory.

The main finding in the present study was that NDE inhibited histamine- and serotonin-induced tracheal contraction. The concentration-response curve for histamine was shifted to the right by 2.0- and 7.8-fold in the presence of 0.1 and 1 mg/ml NDE, respectively (Figs. 3B, C). The maximal contraction induced by histamine was decreased to 90 and 55% in the presence of 0.1 and 1 mg/ml NDE, respectively. These results suggest that NDE at 0.1 – 1 mg/ml inhibits histamine-induced tracheal contraction through both competitive antagonism at histamine receptors and non-competitive smooth muscle relaxant mechanism. It has previously been reported that histamine-induced contraction of isolated guinea pig trachea is mediated by histamine H_1 receptors,^{13,15} and we have confirmed in the present study that histamine-induced tracheal contraction is competitively blocked by the H_1 receptor antagonist diphenhydramine. Therefore, NDE at 0.1 mg/ml

Fig. 6. Effect of Nantenine on Serotonin-Induced Contraction in Isolated Guinea Pig Tracheal Ring Preparations

All experiments were done in a K-H solution supplemented with $1 \mu\text{M}$ atropine and $10 \mu\text{M}$ indomethacin. The concentration-response curves for serotonin were constructed in the absence (○; A–C) or presence of nantenine (●; A, $0.2 \mu\text{M}$; B, $2 \mu\text{M}$; C, $20 \mu\text{M}$). The experimental protocol and representation of the graphs are as in Fig. 3. Data are the means $\pm$ S.E.M. ($n=5$).

or more is likely to bind to H_1 receptors and antagonize H_1 receptor-mediated responses in tracheal smooth muscles.

NDE at 1 mg/ml did not affect acetylcholine-induced maximal contraction (Fig. 1B), but significantly decreased histamine-induced maximal contraction (Fig. 3C). This may be attributed to differences in the efficacy of acetylcholine and histamine in inducing tracheal contraction. It has been reported that acetylcholine-induced tracheal contraction is less sensitive to Ca^{2+} antagonists than histamine-induced contraction.^{16,17} Therefore, it is conceivable that NDE has smooth muscle relaxant properties like Ca^{2+} antagonists.

NDE also inhibited serotonin-induced tracheal contraction. The concentration-response curve for serotonin was shifted to the right by 1.7-fold in the presence of 0.01 mg/ml NDE (Fig. 4A). Although we were not able to exactly calculate the EC_{50} values of serotonin in the presence of 0.1 and 1 mg/ml NDE in Figs. 4B and C, the concentration-response curve for serotonin appeared to be shifted to the right by approximately 100- and 1000-fold in the presence of 0.1 and 1 mg/ml NDE, respectively. These results suggest that NDE antagonizes approximately 10 times more potently against serotonin than against histamine. It has previously been reported that serotonin-induced contraction of isolated guinea pig trachea is mediated by serotonin 5-HT_{2A} receptors,^{12,15,18–20} and we have confirmed in the present study that serotonin-induced tracheal contraction is competitively blocked by the 5-HT_{2A} receptor antagonist ketanserin. Therefore, NDE at 0.01 mg/ml or more is likely to bind to 5-HT_{2A}

receptors and antagonize 5-HT_{2A} receptor-mediated responses in tracheal smooth muscles.

Chemical analysis has revealed that NDE at 0.01 – 1 mg/ml contains nantenine at 0.2 – $20 \mu\text{M}$. NDE at 0.1 – 1 mg/ml inhibited histamine-induced tracheal contraction (Fig. 3), while nantenine at 0.2 – $20 \mu\text{M}$ had no effect (Fig. 5). These results suggest that nantenine is not responsible for the inhibitory effect of NDE on histamine-induced tracheal contraction.

Nantenine at 2 – $20 \mu\text{M}$ inhibited serotonin-induced tracheal contraction (Fig. 6). The concentration-response curve for serotonin was shifted to the right in the presence of nantenine. It has previously been reported that nantenine at $3 \mu\text{M}$ competitively inhibited serotonin-induced contractile responses in isolated rabbit aorta and isolated rat stomach.² These results suggest that nantenine competitively antagonizes 5-HT_{2A} receptor-mediated responses in the trachea as well as in the aorta or stomach. Therefore, nantenine may be partly responsible for the anti-serotonergic effect of NDE. However, the inhibitory effect of nantenine on serotonin-induced tracheal contraction (Fig. 6) was much less potent than that of NDE (Fig. 4). NDE at 1 mg/ml shifted the concentration-response curve for serotonin by approximately 1000-fold (Fig. 4C), while nantenine at $20 \mu\text{M}$ shifted by 15.1-fold (Fig. 6C). It is possible that NDE contains more potent serotonin receptor antagonist than nantenine.

In conclusion, we have found for the first time that NDE acts as a non-competitive smooth muscle relaxant and competitive antagonists at H_1 receptors and 5-HT_{2A} receptors in tracheal smooth muscles. The antagonistic potency of NDE is approximately 10 times higher against serotonin than against histamine. Nantenine is not responsible for the anti-histaminergic effect of NDE, but may partly contribute to the anti-serotonergic effect of NDE. Since nantenine cannot account solely for the inhibitory effects of NDE on tracheal smooth muscle contraction, we are exploring other bioactive constituent(s) in NDE than nantenine.

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