Effects of Alkaloids from *Aconitum yezoense* var. *macroyezoense* on Cutaneous Blood Flow in Mice

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Received December 24, 1996; accepted May 29, 1997

Nine alkaloid constituents in the root of *Aconitum yezoense* var. *macroyezoense*, as well as three acetylated derivatives, were examined for their peripheral vaso-activities by measuring laser-flowmetrically the cutaneous blood flow in the hind foot of mice after intravenous administration. The major constitutive delosine (1), 14-acetyldelosine (2) and luciduscline (3), respectively, had little or very mild vaso-activity. Kobusine (4) and pseudokobusine (5) and three minor constituents, lucucleine (6), 1-acetyllucucleine (7) and dehydrolucucleine (8), together exhibited a rapid increase in blood flow reaching a peak with a magnitude almost equal to that produced by hydralazine, when administered intravenously at the same dosage level of 20 mg/kg. Among them, 4 was characterized by successive reversal of the increase to a decrease in blood flow, while 7 produced a flow with a more delayed peak time. Dehydrolucucleine (9) exhibited a transient decrease in blood flow prior to occurrence of the increase, as did papaverine. Consequently, it is assumed that the alkaloids, especially those of the C19-diterpenoid type, in the root of this *Aconitum* plant have peripherally vaso-dilating activities to varying degrees in mice, probably due to their direct action on the cutaneous microvasculature in a similar fashion to that shown by hydralazine. The laser blood flowmetric method would be useful as an *in vivo* means of qualitative as well as quantitative screening of chemically modified derivatives of peripherally vasoactive agents in mice.

Key words *Aconitum* alkaloid; cutaneous blood flow; laser blood flowmeter

The roots of *Aconitum* plants have long been used as “bushi,” an important herbal drug in some prescriptions of traditional Chinese medicine for the treatment of hypometabolism, dysuria, cardiac weakness, chill, neuralgia, gout and certain rheumatic diseases. *Aconitum* alkaloids are generally assigned to the two chemical structures; the former includes aconitine, mesaconitine, hyaconitine and jesaconitine, all having extremely high toxicity, whereas the latter, such as luciduscline, kobusine, pseudokobusine and atisine, are commonly known far less toxic.¹

Pharmacologically, *Aconitum* alkaloids of the C19-norditerpenoid type have been extensively studied and reviewed.² Aconitine is a representative toxin that exerts its action both centrally and peripherally, with predominant effects on the cardiovascular and respiratory systems by preventing the normal closing of sodium channels.³ In contrast, there is little information about the pharmacological properties of the C20-diterpenoid alkaloids and their chemically transforming products.

*Aconitum yezoense* var. *macroyezoense* (NAKAI) TAMURA is a plant naturally grown in the Jozankei district of Hokkaido in Japan and the extracts from the root contain delosine (1) and 14-acetyldelosine (2) of the C19-norditerpenoid type and kobusine (4), pseudokobusine (5) and luciduscline (3) of the C20-diterpenoid type as major constitutive alkaloids, together with twenty-seven minor alkaloids, in the absence of aconitine-type alkaloids.³–⁷ Although there is a paucity of data on the pharmacological properties of these *Aconitum* alkaloids, 3 in particular has been studied and is known to have some activities such as peripheral and coronary vasodilation⁸ and reduction of blood pressure.⁹ An antagonistic effect of lucucleine (6) on aconitine-induced arrhythmia has also been shown.¹⁰

Our preceding paper reported that laser blood flowmetry provides the means of quantification of cutaneous blood flow changes after intravenous administration of various already-known vaso-active drugs in anesthetized mice.¹⁰

In the present study, the *Aconitum* alkaloids from the root of *A. yezoense* var. *macroyezoense* were examined for peripheral vaso-activity by continuously measuring blood flow in the hind foot of anesthetized mice with the Doppler-type laser blood flowmeter.

MATERIALS AND METHODS

**Chemicals** The following *Aconitum* alkaloids were used after extraction from the root of *A. yezoense* var. *macroyezoense*, followed by purification and identification by the methods described previously⁴,⁶: delosine (1), 14-acetyldelosine (2), kobusine (4), pseudokobusine (5), luciduscline (3), lucucleine (6), 1-acetyllucucleine (7), dehydrolucucleine (8) and dehydroluciduscline (9). Three acetyl derivatives were obtained as amorphous powder by the procedures described below and were also used as test material. The reference drugs, prazosin hydrochloride, hydralazine hydrochloride, papaverine hydrochloride and nifedipine were obtained from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). These test compounds were dissolved in 50% ethanol and injected intravenously to mice in a constant volume of 0.02 ml per 10 g of body weight. The vehicle served as the negative control. All other chemical used were of analytical grade.

**Preparation of Acetyl Derivatives** Alkaloids 1, 6 or 8 was mixed with acetic anhydride in pyridine and stirred

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for a suitable time in the cold. The acetylate formed was extracted with chloroform after addition of water and 28% ammonia water to the reaction mixture, washed with 5% aqueous NaHCO₃ and NaCl-saturated water, and purified by silica gel column chromatography. Identification was performed as follows. Melting points were determined on a Yanagimoto micro melting point apparatus and were uncorrected. IR spectra were recorded on a JASCO IRA-2 or a FT-7000 spectrometer. NMR spectra in CDCl₃ were recorded on a JEOL GX-270 spectrometer using tetramethylsilane as an internal standard. Chemical shifts are given in ppm. The following abbreviations are used: s = singlet, d = doublet, t = triplet, q = quartet. MS spectra were measured on a Hitachi M-2000 spectrometer by electron impact (EI).

1-Acetyldelcosine (10) From 1 (200 mg), 10 (189.4 mg) was obtained as amorphous powder (yield 87%). ¹H-NMR (CDCl₃) δ: 1.08 (t, 3H, J = 7.2 Hz, N-CH₂-CH₃), 2.02 (s, 3H, OCOCH₃), 3.30, 3.37 and 3.42 (each s, 3H, OCH₃), 3.89 (s, 1H, 6α-H), 3.93 (m, 1H; changed into t after addition of D₂O, J = 4.6 Hz, 14β-H), 4.72 (dd, 1H, J = 7.5, 10.5 Hz, 1β-H). IR (KBr) cm⁻¹: 3482 (OH), 1734 (C=O), 1249. HRMS m/z: 495.2852 (Calcd for C₂₃H₄₁NO₄: 495.2830). MS m/z: 495 (M⁺), 480 (M⁺ – CH₃), 464 (M⁺ – OCH₃), 436 (M⁺ – OCOCH₃, base peak).

12-Acetyldelcosine (11) Acetylation of 6 (200 mg) produced amorphous 11 (59.6 mg, 27%), accompanying 12,15-diacetyldelcosine ⁴ (36.6 mg, 15%) and 1,12,15-triacetyldelcosine ⁴ (11.1 mg, 4%).

11: ¹H-NMR (CDCl₃) δ: 0.76 (s, 3H, 18-CH₃), 1.06 (t, 3H, J = 7.0 Hz, N-CH₂-CH₃), 2.02 (s, 3H, OCOCH₃), 3.89 (dd, 1H, J = 5.9, 7.9 Hz, 1β-H), 4.17 (s, 1H, 15-H), 4.50 (t, 1H, J = 8.7 Hz, 12β-H), 5.19 and 5.29 (each s, 1H, 17-H₂). IR (KBr) cm⁻¹: 3426 (OH), 1736 (C=O), 1636 (C=O), 1247, 895. HRMS m/z: 401.2593 (Calcd for C₂₂H₃₅NO₄: 401.2564). MS m/z: 401 (M⁺), 384 (M⁺ – OH), 342 (M⁺ – OCOCH₃), 340 (base peak).

12-Acetyldelcosine (12) Amorphous powder of 12 (15.4 mg, 45%), as well as of 12,15-diacetyldelcosine ³ (3.3 mg, 9%) was obtained from the acetylation of 8 (31 mg).

12: ¹H-NMR (CDCl₃) δ: 0.81 (s, 3H, 18-CH₃), 1.02 (t, 3H, J = 7.1 Hz, N-CH₂-CH₃), 2.04 (s, 3H, OCOCH₃), 3.69 (s, 1H, 19-H), 4.04 (d, 1H, J = 4.9 Hz, 1β-H), 4.24 (s, 1H, 15-H), 4.59 (dd, 1H, J = 8.0, 6.0 Hz, 12β-H), 5.23 and 5.34 (each s, 1H, 17-H₂). IR (KBr) cm⁻¹: 3340 (OH), 1730 (C=O), 1640 (C=O), 1230, 1080, 895. HRMS m/z: 399.2400 (Calcd for C₂₄H₄₅NO₄: 399.2410). MS m/z: 399 (M⁺), 343 (M⁺ – C₃H₆O), 340 (M⁺ – OCOCH₃, base peak).

Animals Male ICR SPF mice at 5 weeks of age were purchased from Japan SLC Inc. (Shizuoka, Japan) and used after acclimatization for 7d. They were housed 5 mice in a polycarbonate cage, with free access to food and water. The animal room was maintained at a constant temperature of 23 ± 1°C, in a relative humidity of 50 ± 5%, and with a 12-h light and 12-h dark cycle.

Measurement of Cutaneous Blood Flow A Doppler-type laser blood flowmeter (Type ALF21R, Advence Co., Ltd., Tokyo, Japan) was used for measurement of cutaneous blood flow in the hind foot of mice under anesthesia with an intraperitoneal dose of 70 mg/kg of sodium pentobarbital (Nembutal®, Abbott Labs., North Chicago, IL, U.S.A.) followed by intravenous administration of each test compound. The probe was fixed in place on the center of the dorsal skin surface of the left hind
foot by wrapping it with elastic adhesive tape and the flow signals generated in the cutaneous region of skin 2 mm in diameter and 1 mm in depth were detected continuously for 20 min. Blood flow was digitally recorded as ml/min/100 g of tissue with a three channel pen recorder (EB 22005, Chino Co., Tokyo) and the differences before and after administration were determined.

**Statistical Analysis** The data obtained were expressed as the mean ± S.E. of 3 to 5 mice of a group and the differences from the values determined before administration were analyzed statistically by Student’s paired t-test at the significant level of 0.05 or less.

**RESULTS**

**Effects of Reference Drugs** Figure 1 shows the time-courses of cutaneous blood flow in the hind foot of anesthetized mice after intravenous administration of four well-known vasodilators. Prazosin caused a sustained rise in blood flow with time from 5 min after the administration of 0.2 mg/kg. A biphasic pattern of increase in blood flow was obtained after the administration of 0.05 mg/kg of nifedipine; the first peak was reached early at 1 min and the second at 5 min, which then slowly declined. Hydralazine increased blood flow reaching a peak at 3 min after the administration of 20 mg/kg which was nearly equal to the second peak of nifedipine. However, the increase tended to be replaced by a decrease in blood flow after return to the basal level. When papaverine was administered at 10 mg/kg, temporary, but not significant, fall followed by moderate rise in blood flow was observed.

**Effects of Alkaloids and Synthetic Compounds** All the test compounds were administrated at a constant dose of 20 mg/kg. As illustrated in Fig. 2, 1 and two acetates were virtually ineffective, but 2 was produced an early, slight increase followed by decrease after return to the basal level significantly. Alkaloids 4 and 5 both increased blood flow rapidly to a similar extent, reaching peaks that were fairly comparable in magnitude to that with hydralazine, or the second peak with nifedipine. However, the increase in blood flow with the former alkaloid was then replaced by a decrease lasting until the end of observation, whereas the decline of the peaked flow with the latter was as slow as observed with nifedipine. The results from the experiments with 3 and related compounds are summarized in Fig. 3. Alkaloid 3 increased blood flow only temporarily to a similar degree as did 2. Alkaloids 6 and 12 both exhibited an apparently biphasic pattern of the increase in blood flow; the first was very transient with a sharp peak that was 2 times larger in magnitude than that with 3 and the second occurred to a milder extent. Either 8 or 11 also produced a temporarily increased blood flow with a peak that was of almost the same magnitude as the first peaks obtained with the above two alkaloids, but was devoid of secondary rise. In contrast to these related compounds, 7 demonstrated gradual increase and decrease in blood flow with a single peak of magnitude corresponding nearly to that of the maximum with hy-

![Fig. 1. Time Courses of Cutaneous Blood Flow in Anesthetized Mice after Intravenous Administration of Reference Drugs with Vaso-activity](image-url1)

Blood flow was measured by laser blood flowmeter. Each point is presented as a mean of 3–5 experiments. ■, control; □, prazosine 0.2 mg/kg; ●, hydralazine 20 mg/kg; △, papaverine 10 mg/kg; ○, nifedipine 0.05 mg/kg. *p < 0.05, **p < 0.01: significantly different from the basal value determined before administration (Student's paired t-test).

![Fig. 2. Time Courses of Cutaneous Blood Flow in Anesthetized Mice after Intravenous Administration of 1, 4 and Their Related Alkaloids](image-url2)

Blood flow was measured by laser blood flowmeter. Each point is presented as a mean of 3–5 experiments. ■, 1 20 mg/kg; ●, 2 20 mg/kg; △, 10 20 mg/kg; □, 4 20 mg/kg; ○, 5 20 mg/kg. *p < 0.05, **p < 0.01: significantly different from the basal value determined before administration (Student's paired t-test).

![Fig. 3. Time Courses of Cutaneous Blood Flow in Anesthetized Mice after Intravenous Administration of Lucidusculine-Related Alkaloids](image-url3)

Blood flow was measured by laser blood flowmeter. Each point is presented as a mean of 3–5 experiments. ■, 6 20 mg/kg; ●, 7 20 mg/kg; △, 11 20 mg/kg; □, 3 20 mg/kg; ○, 8 20 mg/kg; △, 12 20 mg/kg; ○, 9 20 mg/kg. *p < 0.05, **p < 0.01: significantly different from the basal value determined before administration (Student's paired t-test).
DISCUSSION

Laser flowmetric monitoring of cutaneous blood flow revealed the characteristics of mode of action of the reference vasodilators to the peripheral microcirculation in mice. Prazosin did not cause an immediate increase in blood flow, as occurred when nifedipine was administered, but produced only a continual rise during the observation time of 20 min. Nifedipine showed a biphasic pattern of blood flow increase, in which the second rise was larger than the initial rise. Prazosin is known as a selective \(\alpha_1\)-adrenergic antagonist accompanying a relatively potent activity of inhibiting cyclic nucleotide phosphodiesterases, and thus is capable of reducing peripheral vascular resistance without secondary reflex tachycardia. In contrast, nifedipine relaxes rather selectively arterial resistance vessels by blocking the entry of Ca\(^{2+}\) into the smooth muscle cell with little effect on venous pooling, resulting in the reduction in afterload and compensatory increases in heart rate and ejection fraction. Likewise, hydralazine causes direct relaxation predominantly of the arteriolar smooth muscle, and does not relax venous smooth muscle; the action is believed to be associated with powerful stimulation of the sympathetic nerve endings to release catecholamines. Papaverine also dilates directly vascular smooth muscle in combination with the inhibition of cellular cyclic nucleotide phosphodiesterases. In the present study, the increase in blood flow with hydralazine was replaced by the decrease in the basal flow level, whereas a transitory fall followed by a rise in blood flow occurred when papaverine was administered.

Among the five major alkaloids from the root of Aconitum yessoense var. macroyessoense (Nakai) Tamura, 4 and 5 both were fairly comparable to hydralazine regarding the intensity of the activity to increase cutaneous blood flow in the hind foot of mice and the peak-time of the increase. However, the former alkaloid was characterized by rapid reversal of the increased blood flow to a decrease which lasted for the period after recovery of the basal flow. As compared to these alkaloids, 2, 3 and 1, although most abundantly present in this order in the root, had little to mild effect on blood flow. Acetylization of 1 at C-1 failed to enhance the activity of the parent alkaloid significantly. Nevertheless, it is interesting to note that 2 and 3 both have peripherally analgesic activities in mice, whereas 4 and 5 are virtually inactive. Of the luciduscine-like related compounds, 6 and 8, as well as their 12-acetyl derivatives (11, 12), together were approximately two times more active in increasing blood flow immediately after their administration than 3, but this early effect was transitory and they differed as to whether blood flow was thereafter increased or decreased. On the other hand, 7 was unique in its slow producing of a single peak on the increase in blood flow, the magnitude of which was nearly equal to those of the peaks attained earlier by the four compounds mentioned above, while 9 showed a papaverine-like action producing a transient fall of blood flow before a gradual increase. To sum up, alkaloids, especially of the C\(_{20}\)-diterpenoid type, in the root of this Aconitum plant are believed to have a peripherally vaso dilating activity to varying degrees in mice, probably due to their direct action on the cutaneous microvasculature, as that shown by hydralazine. It is also assumed that chemical modification of these alkaloids contributes to production of compounds more specifically acting in the peripheral microcirculation system.

The laser blood flowmeter used in this study was designed on the basis of the finding that there is a linear relationship between the integrated intensity of the power spectrum of scattered light from a given tissue and the volume of red blood cells moving at a low density in the microcirculation; it has also been used as a tool for estimating the microcirculation in the tips of human fingers. Our preceding paper reported that this method, when applied to the quantitative measurement of cutaneous blood flow in mice, provided data on several well-known vasodilators that were individually dose-related and closely comparable to those reported in the literature. It is further emphasized in the present study that the method is applicable to \textit{in vivo} experiments using small animals such as mice for the purpose of qualitative, as well as quantitative, screening of vaso-active agents.

Acknowledgment The authors gratefully acknowledge Prof. E. Fujihira, Hokkaido College of Pharmacy, for his helpful advice and support throughout this work.

REFERENCES
