Antitumor Activity of Liposomal Naphthoquinone Esters Isolated from Thai Medicinal Plant: *Rhinacanthus nasutus* Kurz.

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We previously observed that rhinacanthins-C, -N and -Q, three main naphthoquinone esters isolated from the roots of Thai medicinal plant; *Rhinacanthus nasutus* Kurz. (Acanthaceae) induced apoptosis of human cervical carcinoma HeLaS3 cells. Since these rhinacanthins showed limited solubility in aqueous medium, we attempted to entrap them into liposomal membrane: Liposomalization enabled injection of the drugs and the drugs were expected to transfer to lipoproteins in the bloodstream. Liposomal formulations of rhinacanthins-C, -N and -Q showed strong antiproliferative activity against HeLaS3 cells with the IC50 values of 32, 17, 70 μM; 19, 17, 52 μM and 2.7, 2.0 and 5.0 μM for the exposure time of 24, 48, and 72 h, respectively. These liposomes suppressed the tumor growth in Meth-A sarcoma-bearing BALB/c mice at the dose of 5.0 mg/kg/d for 10 d. Among rhinacanthins, liposomal rhinacanthin-N significantly suppressed solid tumor growth. Based on these results, our findings demonstrated that rhinacanthin-N suppressed tumor growth *in vivo*, and suggested that liposomes are useful for preparing injectable formulation of hydrophobic drugs.

Key words  *Rhinacanthus nasutus*; naphthoquinone ester; rhinacanthin; liposome; antitumor activity

*Rhinacanthus nasutus* Kurz. (family Acanthaceae), has been used in Thai traditional medicine for the treatment of various diseases such as eczema, pulmonary tuberculosis, herpes, hepatitis, diabetes, hypertension and various skin diseases, and its active components were widely investigated.1—7) The main bioactive compounds were revealed to be naphthoquinone; rhinacanthone and lignan groups.1—8,10,11) Antitumor activity of *R. nasutus* Kurz. extracts,5) and that of rhinacanthin in the *in vivo* study were reported.15) We previously reported that rhinacanthins-M, -N and -Q and related naphthoquinone esters as well as synthetic compounds, 1,2-naphthoquinones and 1,4-naphthoquinones, selectively suppressed the growth of KB, HeLa and HepG2 human cancer cells, and normal Vero cells.12,13) We also performed mechanistic study and observed that synthetic rhinacanthins-N and -Q inhibited DNA topoisomerase II.13) A naphthoquinone compound β-lapachone, that was also derived from natural products and has a similar structure to rhinacanthone, was reported to be a potent inhibitor of DNA topoisomerase I and II,14—16) and to induce apoptosis of various cancer cells.17—21) Recently, we observed that rhinacanthins-C, -N and -Q suppressed human cervical carcinoma HeLaS3 cells through induction of apoptosis.22) Therefore, in the present study, we attempted to determine actual antitumor activity of rhinacanthins in tumor-bearing mice.

Since rhinacanthins-C, -N and -Q are hydrophobic compounds, they are not able to inject into bloodstream without solubilization. For *in vivo* use of the drugs, we attempted to solubilize them in liposomes. By the way, liposomes have been widely used in the field of drugs delivery systems (DDS): Liposomalization reduces side effects and toxicities of drugs encapsulated, and improves their bioavailability. Therefore, liposomes tended to be used for cancer treatment, since most of anticancer drugs accompany severe side effects.23—25) Furthermore, liposomes with long circulating characteristics are known to accumulate in interstitial spaces of tumor tissues of which blood vessels are highly permeable.26,27) For the purpose of DDS, liposomes having rather rigid lipid bilayer are favorable, and they are usually composed of phospholipids with saturated fatty acyl chains and cholesterol. On the other hand, liposomes have been used for the injection of hydrophobic drugs, since hydrophobic materials can be encapsulated into the lipid bilayer of the liposomes. For example, Visudyne, a liposomal formulation of benzoporphyrin derivative monoacid ring A (BPD-MA), is being used clinically in photodynamic therapy for age-related macular degeneration. In Visudyne, liposomes are used as a “solvent” of BPD-MA for the purpose of injection of the drug into bloodstream.28) In such case, liposomes having rather fluid lipid bilayer are used, and the injected drug in the bloodstream is transferred to lipoproteins, which, in turn, deliver the drug to the whole body. In this experiment, we used liposomes for the formulation of injectable rhinacanthins, which have limited solubility in water, and evaluated *in vivo* therapeutic efficacy in Meth-A sarcoma-bearing BALB/c mice.

MATERIALS AND METHODS

Materials Egg yolk phosphatidylcholine (EPC) and egg yolk phosphatidylglycerol (EPG) were gifts of Nippon Fine Chemical Co., Ltd. (Takasago, Hyogo, Japan). Tetracolor ONE cell proliferation assay kit was purchased from the Seikagaku Co., Ltd., Tokyo, Japan. All other reagents and chemicals were of analytical grade. Rhinacanthins-C, -N and -Q (Fig. 1) were prepared as described previously.22) In brief, dried roots of *R. nasutus* Kurz. (1.5 kg) were grounded and extracted with methanol. After removal of the solvent,
methanolic extract was further partitioned with the n-hexane and following with chloroform and methanol. A portion of the chloroform extract (7.6 g) was chromatographed on a silica gel column and eluted with chloroform and methanol in the order of increasing polarity. Fractions of 100 ml were collected and combined to provide 5 fractions. Second fraction was chromatographed on silica gel and eluted with n-hexane and chloroform by increasing polarity, affording rhinacanthin-C as red oil (1.3 g). Third fraction was chromatographed on silica gel, eluted with chloroform: methanol (19:1) and two compounds were isolated. Recrystallization of these compounds from n-hexane afforded rhinacanthin-C (690 mg) and orange needles of rhinacanthin-N (112 mg). Forth fraction was also chromatographed on silica gel column, eluted with ethyl acetate: methanol (4:1) and two compounds were obtained; rhinacanthin-N (620 mg) and rhinacanthin-Q as yellow powder (580 mg), respectively. The identity of these active compounds was confirmed by spectroscopic data (UV, IR, 1H- and 13C-NMR and MS).13,22)

**Preparation of Rhinacanthins-C, -N and -Q Entrapped Liposomes** Rhinacanthin-entrapped liposomes were constructed of EPC and EPG with rhinacanthins-C, -N or -Q (6:3:1 as a molar ratio). Lipids and one of three rhinacanthins dissolved in chloroform were evaporated under reduced pressure after the addition of t-butyl alcohol. The residual mixture was then further lyophilized and hydrated with 0.3 M trehalose solution (Hayashibara, Biochemical Laboratory, Japan). The liposomal preparations were freeze-thawed for three cycles by using liquid nitrogen and sonicated for 15 min at 60 °C. Finally, the liposomes were sized by extrusion thrice through a polycarbonate membrane filter with 100-nm pores (Nucleopore, Maidstone, U.K.). Particle sizes were housed in cages with a 12-h light–12-h dark cycle. Food and water were available ad libitum. Animals were cared for according to the Guidelines for the Care and Use of Laboratory Animals of the University of Shizuoka.

Five week-old BALB/c male mice (body weight, 18.3 ± 0.9 g) were implanted subcutaneously (s.c.) into the left posterior flank with 1×10⁶ cells/0.2 ml of Meth-A sarcoma cells (day 0) and they were randomly divided into 4 groups (1 control and 3 treatment groups, each n = 6). Liposomes containing rhinacanthins-C, -N or -Q at the dose of 5.0 mg/kg/d as rhinacanthins were administered i.p. into Meth-A sarcoma-bearing mice daily from day 1 to day 10. The control group was injected i.p. with 0.3 ml trehalose solution. The size of the tumor and body weight of each mouse were monitored daily thereafter. Two bisecting diameters of each tumor were measured with slide calipers to determine the tumor volume and calculation was performed using the formula 0.4 (a×b²), where a is the largest diameter and b is the smallest one. The tumor volume thus calculated correlated well with the actual

**Spin Column Assay** Spin column assay was performed as described previously with a slight modification.38 Sepharose™ 4 Fast Flow (Amersham Pharmacia Biotech AB) was loaded into a 1-ml syringe with saline by centrifugation at 800 rpm for 30 s. Liposomes containing rhinacanthins-C or -N were mixed with an equal volume of HEPES-buffered saline (HBS, pH 7.4) or fetal bovine serum (FBS, Sigma Chemical Co., St. Louis, MO, U.S.A.), and incubated for 30 min at 37 °C. Then, 100 μl of the liposomal solution was applied on the spin column and centrifuged at 500 rpm for 30 s, and the eluent was collected as the first fraction. Then, 100 μl of saline was applied to the column, and centrifugation was done at 500 rpm for 30 s to obtain the second fraction. The procedure was continued until the bedded volume fraction had been eluted. Each fraction obtained was mixed with 10% reduced Triton X-100 and the amount of rhinacanthins was determined with a photometer.

**Growth Assay of HeLaS3 Cells** Human cervical carcinoma (HeLaS3) cells were maintained in Dulbecco’s modified Eagle medium (DMEM), supplemented with 10% heat-inactivated FBS, 1% kanamycin and 0.1% sodium bicarbonate. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂. For the growth assay, HeLaS3 cells were suspended in 200 μl of DMEM supplemented with 10% heat-inactivated FBS and seeded on a 96-well culture plate (5×10⁴ cells/well). After a 24-h incubation at 37 °C, various concentrations of liposomal rhinacanthins-C, -N and -Q were added to each well to the final concentrations of 0, 3, 10, 30, 100, and 300 μM, and incubated for another 24, 48 or 72 h. At the end of each time point, the medium containing the drugs was discarded and the cells were washed with PBS (–). Subsequently, 10 μl of Tetracolor ONE solution and 190 μl of serum free DMEM medium were added to each well and then incubated at 37 °C for 3 h. The amount of formazan formed was measured on a microplate reader at a test wavelength of 492 nm and a reference wavelength of 630 nm. Each assay was performed in quadruplicate. The IC₅₀ values were calculated from the mean of absorbance.

**Therapeutic Experiment** Meth-A murine sarcoma ascites cells (1×10⁶ cells/0.2 ml) were intraperitoneally (i.p.) inoculated and were grown in the abdomens of male BALB/c mice (SLC, Shizuoka, Japan). At 8 to 10 d later, the ascites was collected and centrifuged at 510 rpm in 4 °C for 10 min. The supernatant was removed and washed 3 to 6 times with saline and kept the cells on ice before implantation.26,29 Mice were housed in cages with a 12-h light–12-h dark cycle. Food and water were available ad libitum. Animals were cared for according to the Guidelines for the Care and Used of Laboratory Animals of the University of Shizuoka.
tumor weight ($r=0.980$). The mean survival days from tumor implantation were determined for evaluating the life-prolonging effects of rhinacanthins.

**Statistical Analysis** All data were expressed as the mean ± standard deviation (S.D.). Differences between groups were described by the Student’s unpaired $t$-test. $p$ value less than 0.05 was considered statistically significant.

**RESULTS AND DISCUSSION**

**Liposomal Formulation of Rhinacanthins** Since rhinacanthins showed limited solubility in water, we had dissolved them in DMSO in the previous in vitro experiment. Concerning the in vivo use, however, appropriate solvent is needed for dissolving the drugs instead of DMSO. By the way, Visudyne, clinically available liposomal BPD-MA, is formulated as liposomes because of limited solubility of BPD-MA in an aqueous media. Therefore, we attempted to prepare liposomal rhinacanthins for in vivo use of the drugs. Rhinacanthins-C, -N and -Q were liposomalized with EPC and EPG. These drugs were well dissolved in the liposomes: Encapsulation efficiencies were about 100% for all three rhinacanthins. The average sizes of rhinacanthins-C, -N and -Q liposomes were, 98.9 nm, 106.2 nm, and 100.3 nm, and the z-potentials for them were $-36.4 \text{ mV}$, $-45.4 \text{ mV}$, and $-37.4 \text{ mV}$, respectively.

When liposomes are used as a solvent for hydrophobic materials, it is well known that the materials easily transfer from liposomal membrane to serum lipoproteins. Therefore, we determined the transfer of rhinacanthins-C and -N from liposomes to serum lipoproteins by use of spin column procedures. Figure 2 shows the typical elution pattern of rhinacanthin-N. Liposomes are known to be eluted at void volume (fractions 3, 4). As a result, rhinacanthins were eluted at the lipoprotein fractions (fractions 5—10 in the figure) when the liposomal rhinacanthins were incubated for 30 min in the presence of 50% FBS: The transfer % calculated from the elution profiles was $71.1 \pm 0.7\%$ and $63.6 \pm 1.8\%$ for rhinacanthins-C and -N, respectively. These data suggested that rhinacanthins injected into bloodstream would be delivered to various tissues including tumor tissue via the lipoproteins.

**Antiproliferative Effects of Liposomal Rhinacanthins-C, -N and -Q on HeLaS3 Cells** Next the antiproliferative effects of liposomal rhinacanthins-C, -N and -Q on HeLaS3 cells were measured by the Tetracolor ONE cell proliferation assay. As shown in Fig. 3, the proliferation of HeLaS3 cells were significantly inhibited by rhinacanthins when the concentration of these drugs was increased (3—300 $\mu\text{M}$) as well as the exposure time was prolonged (24—72 h). These profiles of the antiproliferative effect was quite similar to that of DMSO-dissolved rhinacanthins as previously reported. The IC$_{50}$ values of rhinacanthins-C, -N and -Q were 32, 17 and 70 $\mu\text{M}$ for 24 h; 19, 17 and 52 $\mu\text{M}$ for 48 h; and 2.7, 2.0 and 5.0 $\mu\text{M}$ for 72 h, respectively. These data indicated that liposomal rhinacanthins showed a little bit stronger cytotoxicity compared with DMSO-dissolved ones. Furthermore, since the toxic effects of rhinacanthins appeared time-dependently and the strongest cytotoxicity was observed after 72 h incubation, the antiproliferative activities would be not due to direct toxicity against tumor cells but due to blocking of some proliferative steps. We previously observed that rhinacanthins-C, -N and -Q induced apoptosis of HeLaS3 cells through the activation of caspase 3. The cell cycle analysis by FACScan flow cytometry revealed that rhinacanthins induced accumulation of sub G1 hypodiploid cells in a dose-dependent manner: apoptotic death was observed after 48 and 72 h whereas no significant change was observed after a 24-h incubation. Interestingly, rhinacanthin-treated cells were partly arrested at G2/M phase after 24 h exposure. This result suggested the growth inhibitory effect of rhinacanthins might be due to cycle arrest during the G2/M phase that helps to prevent further damage and give the cell time to repair the defect, or undergo apoptosis. Liposomal rhinacanthins also damaged cells time-dependently, and strong cytotoxic action was observed after the 72-h incubation, suggesting that the mode of action of the liposomal rhinacan-
Encapsulation of chemotherapeutic agents in liposomal formulations has shown decreased side effects and increased bioavailability of the drug to the target tissue, thereby leading to an enhanced therapeutic efficacy in patients.\(^\text{31,32}\) However, the purpose of liposomal formulation in this study was not for reducing side effects, but for making injectable form of hydrophobic drugs.\(^\text{33}\) Furthermore, it is possible that rhinacanthins transferred to serum lipoprotein effectively accumulated in tumor tissues, since lipoproteins, especially low density lipoproteins, are known to deliver hydrophobic materials to tumor cells.\(^\text{35—36}\)

In the present study, the results demonstrated for the first time that rhinacanthins-N, -Q and -C entrapped in liposomes showed antiproliferative activities against HeLaS3 cells: The activities were slightly higher than that of free drugs after 24- and 48-exposure. Corresponding to the \textit{in vitro} data, these liposomal drugs effectively suppressed tumor growth and increased the survival of Meth-A sarcoma-bearing BALB/c mice. Among them, liposomal rhinacanthin-N was most potent compound (\(p<0.05\)). We previously observed that a C-3 hydroxy group on the naphthoquinone ring (\(R_3=\text{OH}\)), two methyl substituents on the C-2’ of propyl chain (\(R_1\) and \(R_2\)), and naphthoate ester containing a hydroxyl group at C-1 and a methoxy group at C-4 on the naphthalene ring related with antiproliferative effects.\(^\text{13}\) In the present study, we found that rhinacanthin-N was the most potent for tumor growth suppression in agreement with previous observation. Moreover, the naphthoate ester moiety in 1, 4 naphthoquinone structure might contribute antitumor activity in comparison with aliphatic ester. Finally, we conclude that liposomes are useful for injectable formulation of hydrophobic drugs.

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