Inhibitory Effect of Liposomal Rhinacanthin-N Isolated from Rhinacanthus nasutus on Pulmonary Metastasis in Mice

Pongpun Siripong, a,b Jantana Yahuafai, a,b Suratsawadee Piyaviriyaakul, a Kwanjai Kanokmedhakul, c Hiroyuki Koide, b Takayuki Ishii, b Kosuke Shimizu, b Somsak Ruchirawat, d and Naoto Oku* a,b

a Natural Products Research Section, Research Division, National Cancer Institute; Bangkok 10400, Thailand; b Department of Medical Biochemistry, School of Pharmaceutical Sciences, University of Shizuoka; Shizuoka 422–8526, Japan; c Faculty of Science, Khon Kaen University; Khon Kaen 40002, Thailand; and d Laboratory of Medicinal Chemistry, Chulabhorn Research Institute; Bangkok 10900, Thailand.

Received March 12, 2012; accepted May 1, 2012

We previously observed that rhinacanthins, which are the main naphthoquinone esters isolated from the roots of a Thai medicinal plant, Rhinacanthus nasutus Kurz. (family Acanthaceae), suppress the growth of Meth-A sarcoma in the tumor-bearing mice and that rhinacanthin-N has the strongest antitumor activity among these naphthoquinone esters tested. In the present study, we investigated the effect of rhinacanthin-N on pulmonary metastasis induced by B16F10 melanoma cells in mice. C57BL/6 male mice were injected intravenously with B16F10 melanoma cells, and liposomal rhinacanthin-N was administered intraperitoneally from day 1 to 7 after tumor implantation. Liposomes were used to formulate an injectable form of the hydrophobic agent. Treatment of the mice with 5 or 10 mg/kg/d of liposomal rhinacanthin-N significantly inhibited the pulmonary metastatic colonization of the melanoma cells. Based on these data, our findings demonstrate that rhinacanthin-N possesses antimetastatic efficacy, which may make it a lead compound for the development of a new anticancer drug for use in cancer chemotherapy.

Key words rhinacanthin; metastasis; liposome; naphthoquinone ester; Rhinacanthus nasutus

Rhinacanthins, the naphthoquinone esters derived from the roots of Rhinacanthus nasutus Kurz. (family Acanthaceae) have been traditionally used for treatment of various cancers in Thailand,11 and they exhibit antiproliferative activity against various cancer cells.2) Rhinacanthin-C, -N and -Q, which are the 3 main bioactive compounds, display an apoptosis-inducing effect on human cervical carcinoma (HeLaS3) cells.3) Since these isolated compounds are hydrophobic, the activity is variable because of their limited solubility; and thus they cannot be injected into the bloodstream in a soluble form. Therefore, rhinacanthin was entrapped in liposomes formulated in a previous study, in which we observed that these liposomal formulations show strong antiproliferative activity against human cervical carcinoma (HeLaS3) cells in a dose and time-dependent manner.5,6) Moreover, they suppress the tumor growth and enhance the survival of Meth-A sarcoma-bearing mice when given at the dose of 5 mg/kg/day. Among them, liposomal rhinacanthin-N significantly suppresses the growth of the solid tumor (p<0.05): The tumor growth suppression by liposomal rhinacanthin-C, -N and -Q was about 54, 69 and 58%, respectively. However, the antimetastatic effects of these natural naphthoquinone compounds and their liposomal formulations have not yet been determined. Therefore, in the present study, we evaluated the anti-metastatic effect of liposomal rhinacanthin-N.

Liposomal formulations have been frequently used to improve drug efficacy and bioavailability, and to reduce undesirable side effects. In addition, liposomes with the characteristic of long circulation are known to accumulate in the interstitial spaces of tumor tissues, which have highly permeable blood vessels. On the other hand, liposomes have also been used for the injection of hydrophobic drugs, since hydrophobic materials can be encapsulated into the lipid layer of the liposomes. Moreover, liposomal formulation does not cause any unfavorable effect such as hemolysis, which would be accompanied with solubilizing agent such as Cremophore EL.5) In such a case, liposomes having a rather fluid bilayer are used; and when the drug-bearing liposomes are into the bloodstream, the drug is transferred to lipoproteins, which, in turn, deliver the drug to the whole body.5,6) In this context, to extend our study, we evaluated the antimitotic activity of liposomal rhinacanthin-N by injecting such liposomes via the peritoneal route into C57BL/c mice previously injected intravenously with B16F10 melanoma cells.

MATERIALS AND METHODS

Materials Egg yolk phosphatidylcholine (EPC) and egg yolk phosphatidylglycerol (EPG) were kindly provided by Nippon Fine Chemicals Co., Ltd. (Takasago, Hyogo, Japan). A Tetracolor ONE cell proliferation assay kit was purchased from the Seikagaku Co., Ltd., Tokyo, Japan.

Preparation of Rhinacanthin-N from Rhinacanthus nasutus Rhinacanthin-N (Fig. 1) was prepared as described previously.5) Briefly, dried roots of R. nasutus were ground and extracted with methanol. After removal of the solvent, the extract was further partitioned with n-hexane followed by chloroform and methanol. A portion of the active chloroform extract was chromatographed on a silica gel column and eluted with chloroform and methanol. The fraction containing rhinacanthin-N was rechromatographed on silica gel, and recrystallized from n-hexane. The identity of rhinacanthin-N was confirmed by spectroscopic data (UV, IR, 1H and 13C NMR and MS).

Preparation of Liposomal Rhinacanthin-N Rhinacanthin-N-entrapped liposomes were prepared with EPC, EPG, and rhinacanthin-N (6:3:1 as a molar ratio) as described previously.5) In brief, lipids and rhinacanthin-N dissolved in

* To whom correspondence should be addressed. e-mail: siripong_nci@yahoo.com; oku@u-shizuoka-ken.ac.jp

© 2012 The Pharmaceutical Society of Japan
chloroform were evaporated under reduced pressure. After the addition of t-butyl alcohol the mixture was lyophilized and then hydrated with 0.3 M trehalose solution. Next the liposomal solution was freeze-thawed for 3 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-diameter pores (Nucleopore, Maistone, U.K.). Particle sizes and ζ-potential of the liposomes bearing a given rhinacanthin were determined by use of an ELS 800 electroforetic light-scattering spectrophotometer. The average size and ζ-potential of rhinacanthin-N-encapsulating liposomes were 106nm and −45.4 mV, respectively. For the determination of encapsulation efficiency of rhinacanthin-N into liposomes, an aliquot of liposomal solution was solubilized by the addition of 10% reduced Triton X-100; and the amount of the drug was determined at 253 nm, after the pH of the solutions had been adjusted to 1.0. The trapping efficiency was determined to be about 100%.

**Cell Growth Assay** Highly metastatic B16F10 mouse melanoma cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM)/F12, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% kanamycin, and 0.1% sodium bicarbonate (Wako Pure Chemical Ind., Ltd., Japan) and incubated at 37°C in a humidified atmosphere of 5% CO₂. The cells were suspended in 200 μL of DMEM/F12 supplemented with 10% FBS and seeded into the wells of a 96-well culture plate (5 × 10³ cells/well). After a 24-h pre-incubation at 37°C, each of various concentrations of liposomal or free rhinacanthin-N was added; and incubation was subsequently carried out for 24h. In the case of free rhinacanthin-N, 0.5% dimethyl sulfoxide (DMSO) was included in the incubation medium. At the end of incubation, the cells were washed with phosphate-buffered saline (PBS) and incubated at 37°C for 3 h in a solution comprising 10 μL of TetraColor ONE and 190 μL of serum-free medium. The amount of formazan dye formed in the solution had been adjusted to 1.0. The trapping efficiency of rhinacanthin-N at the dose of 5 mg/kg/d or 10 mg/kg/d in 0.3 M trehalose solution was administered intraperitoneally (i.p.) to the tumor-bearing mice (n=6) at days 1 to 7 after injection of the tumor cells. The control group also was injected i.p. with 0.3 M trehalose solution. Mice from each group were sacrificed 14 d after injection of the tumor cells. The lungs were flushed with saline and dissected out. The numbers of metastatic colonies in the lungs were macroscopically counted. To evaluate the severity of possible side effects of the liposomal drug, we measured the body weight of these animals from day 0 to day 9.

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

**RESULTS AND DISCUSSION**

Our previous data showed that rhinacanthin-C, -N, and -Q, naphthoquinone esters derived from *Rhinacanthus nasutus* roots, as well as synthetic compounds such as 1,2-naphthoquinones and 1,4-naphthoquinones, selectively suppress the growth of KB, HeLa, and HepG2 cells (all human cancer cell lines) and Vero (monkey) cells.⁷⁻⁸ The effect has been suggested to be related the inhibition of DNA topoisomerase II.²⁻⁵ We also earlier observed that these rhinacanthins induce apoptosis of HeLaS3 cells.⁹ In the present study, we firstly examined the anti-proliferative activity of liposomal rhinacanthin-N (Fig. 1), against B16F10 melanoma cells. As shown in Fig. 2, the proliferation of B16F10 melanoma cells *in vitro* was significantly inhibited by these liposomes after a 24-h exposure. The IC₅₀ value of liposomal rhinacanthin-N was 19 μM, and that of the free rhinacanthin-N was 35 μM.

We next investigated the effect of liposomal rhinacanthin-N at the dose of 5 or 10 mg/kg/d for 7d' administration on lung metastasis of intravenously injected B16F10 melanoma cells (5 × 10⁷ cells/0.2 mL) into C57BL/6 mice. The liposomal rhinacanthin-N significantly reduced the number of pulmonary metastatic colonies of B16F10 melanoma cells in C57BL/6 mice (p<0.001; Fig. 3A). The percent inhibition of metastatic...
To evaluate the severity of possible side effects of liposomal rhinacanthin-N, we measured the body weight each day for 9 days after the injection. None of the animals in either of the liposomal rhinacanthin-N treatment groups or in the control group showed any abnormalities in general condition after injection of the drugs. Figure 3B shows the body weight changes in C57BL/6 mice after the treatment with liposomal rhinacanthin-N. The body weight did not differ significantly between the control group and the treatment groups at any time after injection of the liposomes.

Our findings demonstrate that liposomal rhinacanthin-N, a naphthoquinone ester isolated from the roots of *Rhinacanthus nasutus* Kurz., possessed anti-metastatic efficacy against B16F10 melanoma cells injected into C57BL/6 mice. Recently, it was reported that a rhinacanthin-rich extract showed bacterial activity against *Streptococcus mutans*, *Propionibacterium acnes*, *S. epidermidis*, and *S. aureus*. Moreover, Kongkathip and co-workers developed anti-cancer agents and anti-malaria agents with rhinacanthins used as a lead compound. Therefore, rhinacanthins have potential as lead compounds for the treatment of various diseases including cancer.

**Acknowledgements** This work was supported by the JSPS-NRCT Fellowship Program, Japan and a Thai Government Grant from the National Cancer Institute, Department of Medical Service, Ministry of Public Health, Bangkok, Thailand.

**REFERENCES**


9) Pradidphol N, Kongkathip N, Sittikul P, Boonyalai N, Kongkathip...
