Induction of Apoptosis in Tumor Cells by Three Naphthoquinone Esters Isolated from Thai Medicinal Plant: *Rhinacanthus nasutus Kurz*.

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*Rhinacanthus nasutus* Kurz. (Acanthaceae) has been used as Thai traditional medicine for the treatment of various cancers. Recently, we reported that rhinacanthins, active components of the plant, had antiproliferative activity against human cancer line cells. In the present study, we investigated the growth inhibitory mechanism of rhinacanthins-C, -N and -Q, three main naphthoquinone esters isolated from the roots of *R. nasutus* Kurz. in human cervical carcinoma (HeLaS3) cells by means of TUNEL staining, DNA fragmentation assay, flow cytometry, and cleavage assay of Asp-Glu-Val-Asp-peptide-nitroanilide, a caspase-3 substrate. After the HeLaS3 cells were exposed with different concentrations of the drugs, rhinacanthins-C, -N and -Q exhibited antiproliferative effects on HeLaS3 cells with the IC_{50} values of 80, 65, 73 \mu M; 55, 45, 55 \mu M; and 1.5, 1.5 and 5.0 \mu M for 24, 48 and 72 h time points, respectively. Morphological changes showing nuclear fragmentation of rhinacanthins-treated cells were clearly observed after 48 h exposure. Consistent with this observation, the appearance of a ladder formation was also evident with an agarose gel electrophoresis of the extracted DNA. Flow cytometric analysis revealed that rhinacanthin-N caused G2/M arrest of HeLaS3 cells after 24 h incubation, and increased the proportion of sub-G1 hypodiploid cells, apoptotic cells, in the population of HeLaS3 cells after 48 and 72 h incubation. Moreover, the drug treatment markedly elevated the activity of caspase-3. Based on these results, our findings demonstrated for the first time that the inhibitory effects of three main naphthoquinone esters isolated from the roots of *R. nasutus* Kurz. on the growth of HeLaS3 cells appear to arise from the induction of apoptosis, that might be associated with the activation of caspase-3 pathway.

Key words *Rhinacanthus nasutus*; naphthoquinone ester; rhinacanthin; antitumor activity; apoptosis; caspase-3 activation

Cancer causes significant morbidity and mortality and is a major public problem world widely. Cancer chemotherapy, a routine method for the treatment in cancer patients, faces on the problems of unstable efficiency, severe side effects, and cost. Therefore, new drugs with high efficacy without severe side effects, along with understanding the molecular basis in cancer treatment, are awaited.

Many attentions have been focused on natural products as potential sources of novel anticancer drugs over the decades. 1) *Rhinacanthus nasutus* Kurz. (family Acanthaceae) has been used as Thai traditional medicine for the treatment of various cancers, e.g. cervical and liver cancers. 2) Various parts of this plant have been also used for the treatment in various other diseases such as eczema, pulmonary tuberculosis, herpes, hepatitis, diabetes, hypertension, and various skin diseases, and the active components of this plant have been widely investigated. 2–12) The main bioactive components of the plant are known to be naphthoquinones such as rhinacanthins (A—D, G—Q), rhinacanthone and lignan groups. 3–12) Naphthoquinone compounds have been reported to possess antiproliferative activity against a panel of cancer cells. 3,9–12) Recently, our group reported that synthesized compounds of 1,2-naphthoquinones and 1,4-naphthoquinones as well as rhinacanthins-M, -N and -Q, and related naphthoquinone esters showed selective antiproliferative activity against human cancer cells (KB, HeLa and HepG2) with moderate cytotoxicity against non-tumorigenic Vero cells. 13,14) Regarding to the antitumor effects, it was reported that ethanolic and aqueous extracts of the roots and leaves of *R. nasutus* Kurz. suppressed the growth of ascites sarcoma 180 in Swiss mice, and that rhinacanthin-C was expected as the bioactive compound. 11) Moreover, the antitumor activity of rhinacanthone against Dalton’s ascites lymphoma was also observed. 12) However, there was no report elucidating the action targets and anticancer mechanism of these potential natural compounds, except one report that both synthesized rhinacanthins-N and -Q could inhibit DNA topoisomerase II, which is known to play a key role in the regulation of cells growth. 13)

By the way, it is well recognized that many of available chemotherapeutic drugs including topoisomerase inhibitors mediate their anticancer effects *via* apoptosis induction in cancer cells. 15) Moreover, many drugs derived from natural products such as etoposide, camptothecin, \(\beta\)-lapachone, and so on are known to induce apoptosis. 16–17) Especially, \(\beta\)-lapachone, a naturally produced from naphthoquinone compound having similar basic structure to rhinacanthone, showed potent inhibition of DNA topoisomerase I and II, and induced apoptosis in various cancer cells. 25–31) Therefore, it is possible that rhinacanthins would damage tumor cells through the induction of apoptosis. The present study, we investigated the growth inhibitory effect of rhinacanthins-C, -N and -Q, three main naphthoquinone esters isolated from the roots of Thai medicinal plant, *R. nasutus* Kurz., against human cervical carcinoma HeLaS3 cells, and observed that these compounds actually induced apoptosis of the cells.
MATERIALS AND METHODS

Materials 4,6-Diamidino-2-phenylindole (DAPI), propidium iodide (PI), ribonuclease-A and proteinase-K were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). TetraColor ONE cell proliferation assay kit was purchased from the Seikagaku Co. Ltd. (Tokyo, Japan). Apop Tag® Fluorescein In Situ Cell Death Detection and Caspase-3 Colorimetric Assay Kits were obtained from Chemicon (Temecula, CA, U.S.A.). BSA Protein Assay Kit was from Bio-Rad Laboratories, Inc. (Hercules, CA, U.S.A.). All other reagents and chemicals were of the highest purity grade available.

Isolation of Bioactive Naphthoquinone Compounds from R. nasutus Kurz. Roots Three main naphthoquinone esters were isolated from the roots of R. nasutus Kurz. Briefly, dried roots of R. nasutus Kurz. (1.5 kg) were ground and extracted with methanol using a Soxhlet apparatus. The solvents were filtered and evaporated in vacuo by a rotary evaporator. The methanol extract was further partitioned with the n-hexane and following with chloroform and methanol, affording the residues of n-hexane (5.9 g), chloroform (14.6 g) and methanol (36.7 g) extracts, respectively.

A portion of the chloroform extract (7.6 g) was chromatographed on a silica gel column (200 g) and eluted with chloroform and methanol in the order of increasing polarity. Fractions of 100 ml were collected to provide 5 fractions (A—E) and 3 middle fractions were used for further purification. Fraction B was rechromatographed on silica gel and eluted with n-hexane and chloroform by increasing polarity, affording rhinacanthin-C as red oil (1.3 g). Fraction C was then 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 (0.69 g) and orange needles of rhinacanthin-N (0.112 g). Fraction D was also rechromatographed on silica gel column, eluted with ethyl acetate: methanol (4:1) and two compounds were obtained; rhinacanthin-N (0.62 g) and rhinacanthin-Q as yellow powder (0.58 g), respectively. The identity of three active compounds was confirmed by spectroscopic data (UV, IR, 1H- and 13C-NMR, MS).

Cell Cultures Human cervical carcinoma (HeLaS3) cells were maintained in Dulbecco’s modified Eagle medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS, Wako Pure Chemical Ind., Ltd., Japan), 1% kanamycin and 0.1% sodium bicarbonate. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO2.

Cell Proliferation Assay Inhibition of cell proliferation by rhinacanthins was determined by TetraColor ONE cell proliferation assay kit. In briefly, HeLaS3 cells (5×104 cells/well) were suspended in 200 μl of DMEM containing 10% FBS and seeded on a 96-well culture plate (Costar, Cambridge, MA, U.S.A.). After 24 h of preincubation at 37 °C, various concentrations of rhinacanthins-C, -N and -Q dissolved in 10% DMSO were added to each well at the final concentrations of 0, 3, 10, 30, 100, and 300 μM, and incubated for a further indicated times (24, 48 or 72 h). At the end of each time point, the medium containing the drugs were discarded and the cells were washed with PBS. Subsequently, 10 μl of TetraColor ONE solution and 190 μl of 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. DMSO (0.1%) had no cytotoxic effect on the cells. The IC50 values were calculated from the mean of absorbance.

TUNEL Assay After treatment of HeLaS3 cells with rhinacanthins (C, N and Q), apoptotic cells were determined with terminal deoxynucleotidyl transferase (TdT)-mediated deoxoyuridine triphosphate (dUTP) nick-end labeling (TUNEL) assay using an Apop Tag® Fluorescein In Situ Cell Death Detection Kit according to the manufacturer’s instructions. The test is based on the ability of TdT to catalyze polymerization of nucleotides to free 3′-OH DNA end generated during apoptosis. Adriamycin and 0.1% DMSO-treated cells were used as positive and negative controls. For this assay, HeLaS3 cells (1×104 cells/well) were grown on a 8-well chamber slide (Nalge Nunc Int., U.S.A.) and treated with 40, 60, 90, and 120 μM of rhinacanthins-C, -N and -Q for 48 h. The cells were then fixed with 1% paraformaldehyde for 10 min, washed three times with PBS. Nuclear DNA was denatured with a cooled ethanol/acetate acid (2:1) at −20 °C for 5 min. The cells were rehydrated with PBS and incubated in TdT buffer at 37 °C for 1 h. At the end of incubation, the reaction was blocked by adding the stop/wash buffer and incubated with antidigoxigenin-fluorescein for 30 min in dark humidified chamber at room temperature. After washing with PBS, the staining cells were counterstained with 10 μg/ml of DAPI and incubated for 10 min at room temperature. Finally, the slides were mounted with Perma Fluor aqueous mounting medium under a glass coverslip and were then observed under a Carl Zeiss LSM 510 confocal laser scanning microscope (Thornwood, NY, U.S.A.). Fluorescein was excited by using an argon laser at 458 nm.

DNA Fragmentation Analysis DNA fragmentation served as a late apoptosis marker was detected by agarose gel electrophoresis. HeLaS3 cells (1×106 cells/ml) at exponentially growing phase were treated with or without different concentrations of rhinacanthins (40, 60, 90 μM) for 24, 48 or 72 h. The cells were washed twice with cold PBS, pelleted by centrifugation and lysed in 20 μl lysis buffer (50 mM Tris–HCl, pH 7.4; 10 mM EDTA and 0.5% sodium N-lauroylsarcosinate). Lysate was incubated sequentially with 500 μg/ml ribonuclease A at 50 °C for 30 min and 500 μg/ml proteinase K at 50 °C for 60 min in a shaking water bath. Equivalent amounts of DNA (2–3 μg) were then analyzed by 2% agarose gel electrophoresis at 50 V in TBE buffer (2 mM EDTA, pH 8; 89 mM Tris–HCl and 89 mM boric acid). The DNA fragmentation pattern was visualized and photographed under transmission UV light after ethidium bromide staining. Adriamycin- and 0.1% DMSO-treated cells were used as positive and negative controls.

Cell Cycle Analysis To estimate the proportion of cells in different phases of cell cycle, cellular DNA was analyzed by FACSscan after treatment with or without the rhinacanthin drugs in various dose and time (40, 60, 90 μM for 24, 48, 72 h). Briefly, HeLaS3 cells (5×105) were harvested, pelleted by centrifugation at 500×g for 10 min and fixed overnight in ice-cold 70% ethanol at 4 °C. The cells were washed with PBS and suspended in PBS containing 100 μg/ml ribonuclease A at 37 °C for 20 min. Cellular DNA was labeled with...
250 μl PI (100 μg/ml) in PBS and stored in the dark at 4 °C at least 30 min. At the end of incubation, the cell suspensions were filtered through a 41 μm-pore size nylon mesh filter and analyzed by an EPICS flow cytometer (Coulter Electronics). The percentage of cell populations in different phases of the cell cycle was determined by Multicycle software (Phoenix Flow Systems, San Diego, CA, U.S.A.).

**Caspase-3 Assay** The activity of caspase-3 was determined by a Caspase-3/CPP32 colorimetric assay kit according to the manufacturer’s instruction. In brief, 0.1% DMSO and different concentrations of rhinacanthin-N-treated HeLaS3 cells (1×10^6 cell/dish) were harvested, washed twice with cold PBS and lysed in lysis buffer. After standing on ice for 30 min and centrifuged at 12000×g for 10 min, the supernatant was collected. The protein concentration in the cell lysate was determined by Bradford assay (Bio-Rad). An equal amount protein was placed in 96-well plate containing 20 μl of assay buffer and 10 μl of caspase-3 substrate (DEVD-p-nitroanilide). Following incubation at 37 °C for 3 h, the release of p-nitroanilide was measured at 405 nm using a microplate reader (Benchmark 550, Bio-Rad, U.S.A.). Quantification of caspase-3 activity was calculated as fold-increase over vesicle control.

**RESULTS**

**Antiproliferative Effects of Rhinacanthins-C, -N and -Q on HeLaS3 Cells** Figure 1 shows the structures of three major isolated compounds: rhinacanthins-C, -N and -Q purified from chloroform extract of the roots of *R. nasutus* KURZ. These chemical structures are confirmed by UV, IR, 1H- and 13C-NMR and MS spectroscopy. The basic structure of these compounds is a naphthoquinone ester.

The antiproliferative effects of rhinacanthins-C, -N and -Q on HeLaS3 cells were measured by the TetraColor ONE cell proliferation assay. As shown in Fig. 2, the proliferation of HeLaS3 cells was significantly inhibited by rhinacanthins when the concentration of these drugs was increased (3—100 μM) as well as the exposure time was prolonged (24—72 h). The IC_{50} values of free rhinacanthin-C, -N and -Q were 80.0, 65.0 and 73.0 μM of 24 h; 55.0, 45.0 and 58.0 μM for 48 h as well as 1.5, 1.5 and 5.0 μM for 72 h. These results suggested that three main naphthoquinone esters; rhinacanthins-C, -N and -Q could inhibited the growth of HeLaS3 cells in a dose- and time-dependent manners. Among of them, rhinacanthin-N was the most effective. Interestingly, it took long time exposure of rhinacanthins to obtain remarkable cytotoxicity.

**Induction of Apoptosis by Rhinacanthins-C, -N and -Q in HeLaS3 Cells** To determine whether cells death induced by rhinacanthin drugs is associated with apoptosis, we confirmed the apoptotic characterizations in HeLaS3 cells by several approaches e.g. morphological changes, DNA fragmentation and cell cycle arrest, detecting by TUNEL staining assay, agarose gel electrophoresis and FACSscan.

At first, the morphological changes induced by rhinacanthins-C, -N and -Q in HeLaS3 cells (40, 60, 90 μM for 48 h) were examined by using confocal laser scanning microscopy.
after staining the cells with *in situ* TUNEL assay. As shown in Fig. 3, TUNEL-positive cells were observed over a period of 48 h exposure of rhinacanthin-N. The level of apoptosis gradually increased in a dose-dependent manner, especially highest concentration (as shown in Fig. 4). Similar results were obtained with rhinacanthins-C and -Q (data not shown). The results suggested that rhinacanthins-C, -N and -Q were able to induce marked apoptotic morphology in HeLaS3 cells. Subsequently, genomic DNA fragmentation as a hallmark of apoptotic cell death was confirmed using an agarose gel electrophoresis. After treatment HeLaS3 cells with rhinacanthin-N, which is the most potent compound, at different concentrations (40, 60, 90 μM) for various time periods (24, 48 or 72 h), a typical ladder pattern of internucleosomal DNA fragmentation was observed. With the time delay of 48 h, the formation of DNA ladder pattern was much more obvious in high concentration of rhinacanthin-N (as shown in Fig. 5). Furthermore, to understand the mode of action of the drugs, the effect of rhinacanthin-N on the cell cycle distribution in HeLaS3 cells was also explored by FACScan. After the cells exposed to different concentrations of rhinacanthin-N (40, 60, 90 μM) for various time periods (24, 48 or 72 h), the proportion of sub G₁ hypodiploid cells were significantly accumulated from 16.9 to 39.2% for 48 h and to 42.2% for 72 h (Fig. 6), that correlated with the enhanced DNA fragmentation observed under these conditions. On the other hand, as early as a 24 h treatment of this drug, the cell cycle arrested at G2/M phase appeared (37.2%) and subsequent apoptotic cell death occurred as shown in Fig. 5.

Based on these findings, it is confirmed that rhinacanthins, especially the most potent rhinacanthin-N naphthoquinone ester, can induce apoptosis in HeLaS3 cells in a dose- and time-dependent manner. In addition, it maybe induces cell cycle arrest in G₂/M phase of cell cycle before the cells undergo apoptosis.

**Activation of Caspase-3 by Rhinacanthins-C, -N and -Q on HeLaS3 Cells** Caspases, the cytoplasmic aspartate-specific cysteine protease, have been shown to play a central role in the apoptotic signaling pathway. Activation of caspase-3 appears to be directly responsible for many of the molecular and structural changes in apoptosis. Among the identified caspases, the activation of caspase-3 is a crucial event in numerous types of cells leading the execution of apoptosis. In this experiment, we examined the role of caspase-3 activation in rhinacanthins-mediated apoptosis in HeLaS3 cells using *in vitro* colorimetric substrate, DEVD-pNA, which is specific for caspase-3 activity. As shown in Fig. 7, rhinacanthins-C and -N treatment caused the activation of caspase-3 in a dose-dependent manner after 48 h exposure, whereas the activity was decreased at a highest concentration of rhinacanthin-Q. Rhinacanthin-N (90 μM), the most effective drug, induced a marked increase in caspase-3 activity to 2.5 fold over the control cells. The pattern of the increases in caspase-3 activity well correlated with that of rhinacanthin-N induced fragmentation of DNA. Therefore, these findings demonstrated that a caspase-3 dependent pathway might be involved in rhinacanthins-induced apoptosis.

**DISCUSSION**

Currently, DNA topoisomerases have been recognized as potential chemotherapeutic targets; etoposide, camptothecin and several naphthoquinonoid natural products with anticancer activities e.g. shikonin, lapachol, β-lapachone etc. function to topoisomerases, albeit with wide variety of mechanisms controlled by their respective chemical structures. Again, all these compounds are reported as inducers of apoptosis in human cancer cells. A recent study on the mode of action of rhinacanthin drugs demonstrated that synthetic rhinacanthins-N and -Q relaxed supercoiled DNA possibly through the inhibition of topoisomerase II. Moreover, natural naphthoquinonoid β-lapachone having the chemical structure related with rhinacanthone which was also isolated from *R. nasutus* Kurz., is revealed to induce apoptosis through p53 independent pathway, cell cycle arrest at S phase, and activation of caspase-3. Based on this knowledge, it would be interesting to examine the apoptosis-inducing activity of three rhinacanthin drugs, a main of naphthoquinone esters isolated from the roots of *R. nasutus* Kurz., in human cancer cells, since precise mechanisms of these naphthoquinone drugs remain unclear.

In the present study, we examined the antiproliferative ac-
tivity of three naturally rhinacanthins-C, -N and -Q against HeLaS3 cells. Interestingly, their antiproliferative effect was rather weak after 24 h exposure (IC50 values of 80.0, 65.0, 55.0 μM) and it gradually augmented with the prolonged exposure time (IC50 values of 55.0, 45.0, 55.0 μM for 48 h and 1.5, 1.5, 5.0 μM for 72 h). These results suggested that the activity is not due to the direct toxicity against tumor cells but due to the blocking of some steps in the proliferative cascade. In fact, long exposure of rhinacanthins is needed for obtaining strong cytotoxic effect: HeLaS3 cells treated with rhinacanthins-C, -N and -Q for 24 h, washed, and incubated with fresh medium until 72 h, were far less damaged by the drugs compared with the treatment of the cells for 72 h throughout the presence of the same concentrations of the drugs (Fig. 2).

Next, whether the apoptotic pathway is involved in the cell death caused by rhinacanthin drugs was investigated. Several lines of evidence are presented here for the first indication that apoptosis is the mode of cells death caused by these rhinacanthin drugs. Morphological characterizations such as nuclear fragmentation of the HeLaS3 cells were clearly observed after 48 h exposure of the drugs by the TUNEL staining. Consistent with this observation, the appearance of such fragments resulting in a ladder formation was also evident and was much more obvious after prolonged incubation time. Cleavage of DNA at the internucleosomal linker sites yielding DNA fragments in multiples of 180 base pairs is regarded as a biochemical hallmark of apoptosis.41) Further study on the cell cycle analysis by FACScan revealed that sub-G1 hypodiploid cells, a marker of cell death by apoptosis, accumulated in response to rhinacanthins treatment in a dose dependent: Again, G1 hypodiploid cells were only ob-

![Fig. 6. Cell Cycle Analysis of HeLaS3 Cells Treated with Rhinacanthin-N by Using Flow Cytometry](image)

HeLaS3 cells (1×10^6 cells/ml) were treated with rhinacanthin-N (40 μM; B, F, J, 60 μM; C, G, K and 90 μM; D, H, L) for indicated time periods (24, 48, 72 h). 0.1% DMSO-treated cells were used as negative controls (A, E, I). The cells were fixed with ice-cold 70% ethanol, treated with RNase A and stained with propidium iodide in the dark. The percentage of non-apoptotic and apoptotic cells with in each cell cycle was observed by using flow cytometry.
caspase-3 is thought to be the key enzyme that induces apoptosis in driving apoptosis. Activation of caspase appears to be needed to understand the relationship between cell cycle arrest and naphthoate ester containing a hydroxyl group at C-1 and a methoxy group at C-4 on the naphthalene ring (R5). It has been shown that the growth inhibitory effects of rhinacanthin drugs result from multiple actions, such as interfering with cell cycle progression, inducing apoptosis mediated by the activation of caspase-3 activity as well as targeting DNA topoisomerase II of tumor cells. These observations also support that R. nasutus Kurz., a Thai medicinal plant, may possibility served to use as a naturally therapeutic potential drug for treating in cancer patients.

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