Original Article

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Cytotoxic activity of marcanine C and some other aminoquinone derivatives against MCF-7 cells

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Abstract

Alkaloids from *Goniothalamus* have been isolated and renowned for their cytotoxicity. Recently, there has been an increment of interest to study on their chemical structures and the molecular mechanisms of action. The study herein, reported the cytotoxic effects of some *Goniothalamus* alkaloids in MCF-7 human breast adenocarcinoma cells as well as NB4 acute promyelocytic leukemia cells. The effects of marcanine B, C, and other structural-related compounds namely liriodenine and 5-hydroxy-3-amino-2-aceto-1,4-naphthoquinone (HAANQ) on apoptosis, caspase-3 activation and cell cycle arrest were evaluated by flow cytometry. We found that, marcanine C showed the most prominent apoptosis inducing effect on both MCF-7 and NB4 cells. As proven by the result of caspase-3 activation, marcanine C 7.5 μM for 24 hr induced more than 23% the activation of caspase-3 on MCF-7 cells. Furthermore, marcanine C caused cell cycle arrest in the G1 phase along with low viability of the cells in S and G2/M phases suggesting the prevention of DNA synthesis and the consequent apoptosis. In summary, marcanine C exerted the most prominent cytotoxic effect among all test compounds. This study is the first to report the effects of marcanine C on apoptosis induction in breast cancer cells via caspase-3 activation and the cell cycle arrest in G1 phase.

Keyword: marcanine C, 1-azaanthraquinones, apoptosis, cell cycle, caspase-3, *Goniothalamus*

1. INTRODUCTION

Cancer, a group of diseases defined by the uncontrolled growth and spread of abnormal cells, is irrefutably a worldwide major public health issue. With a high mortality, as of 2012, more than 8.2 million people died of cancer, making it the second major cause of death, inferior to cardiovascular disease.[1]

Cancer treatment can be performed with many procedures as monotherapy or combination, including surgery, radiotherapy and systemic therapy. Regardless of severe adverse reactions, anticancer drugs still play an important role as adjuvant or palliative chemotherapy for treatment of cancer, particularly in a metastatic disease. For this reason, many efforts have been made to develop novel cytotoxic compounds since 1940s, up to present.[2-4]

The genus *Goniothalamus*, belonging to the family Annonaceae, has been reported with at least 160 species of trees or erect shrubs which spontaneously distribute throughout Asian tropical countries. The genus is renowned for being a source of naturally occurring cytotoxic compounds which can be categorized into three main classes of secondary metabolites: acetogenins, styryllactones, and alkaloids.[5-11] Studies on various cancer cell lines have shown that acetogenins exert cytotoxicity by either inhibition of

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mitochondrial complex I, enhancing caspase-3 activity, and arresting cells in G1 phase, whereas styryllactones act through apoptosis induction either by caspase-3 or caspase-7 activation or loss of mitochondrial transmembrane potential, inducing reactive oxygen species leading to DNA damage, and arresting cells in G2/M phase.[12,13] Although alkaloids, especially 1-azaanthraquinones, have been discovered with good cytotoxic potential, the mechanism of action remains unclear.

In this study, apoptotic inducing activity of some alkaloids (Fig. 1) including marcanine B, marcanine C, liriodenine, and 5-hydroxy-3-amino-2-aceto-1,4-naphthoquinone (HAANQ), was evaluated and compared in MCF-7 human breast adenocarcinoma cells as well as in NB4 acute promyelocytic leukemia cells. Furthermore, effects of marcanine C on caspase-3 activation and cell cycle arrest were analyzed.

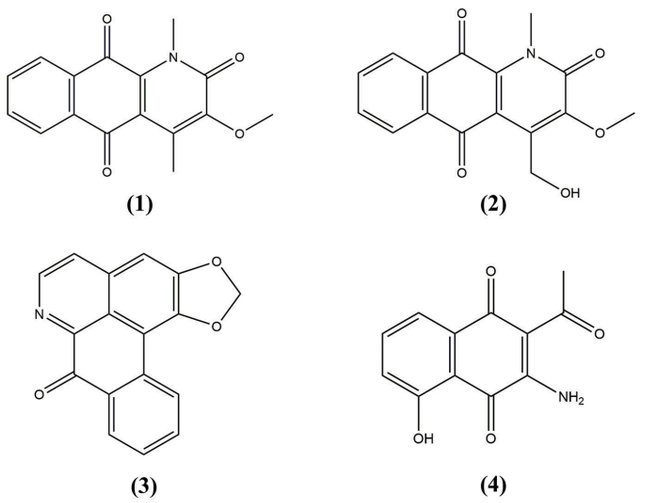

Figure 1. Chemical structures of test compounds: marcanine B (1); marcanine C (2); liriodenine (3); 5-hydroxy-3-amino-2-aceto-1,4-naphthoquinone (4).

2. MATERIALS AND METHODS

Chemicals and Reagents

Marcanine B, marcanine C, and HAANQ, were obtained from *Goniothalamus marcanii* Craib by the methods as described previously.[7] Liriodenine was obtained from *Anomianthus dulcis*.[14] Methanol (MeOH), ethanol (EtOH), dichloromethane (CH_2Cl_2), ethylacetate (EtOAc), acetonitrile (ACN) and n-hexane were purchased from Sigma-Aldrich. RPMI-1640 medium, DMEM-high glucose medium, penicillin-streptomycin, trypsin-EDTA 0.25%, trypan blue 0.4%, L-glutamine and fetal bovine serum (FBS) were from GIBCO (Grand Island, NY). Camptothecin was purchased from Sigma-Aldrich. Media used in the study was a mixture of either RPMI-1640 for NB4 cells or DMEM for MCF-7 cells, 1% penicillin-streptomycin, 1% L-glutamine and 10% FBS and this will be hence referred to as ''complete medium''.

Cell culture

The human acute promyelocytic leukemia-M3 (NB4) and human breast carcinoma (MCF-7) cell lines were kindly provided by Mrs. Yaowalak U-pratya (Division of Hematology, Department of Medicine, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand) and Dr. Chanitra Thuwajit (Department of Immunology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand), respectively. NB4 and PBMC were maintained in RPMI complete medium, whereas MCF-7 was maintained in DMEM-HG complete medium at 37°C in a humidified atmosphere of 5% CO_2.

Test compounds and control

All compounds were prepared as stock solutions in dimethyl sulfoxide (DMSO) at the concentration of 4 mM on the day of the experiment. Working solutions of test compounds were achieved by dilution in the complete medium prior to addition to the cells. Camptothecin as a positive control was also prepared in the complete medium with the same manner aforementioned.

Cell number and viability

The trypan blue exclusion assay was performed to determine cell viability. Briefly, after being incubated in a humidified 5% CO_2 atmosphere, an aliquot of the previously seeded cells (1 x 10^5) was resuspended in an equal volume of 0.4% trypan blue solution. After incubation at room temperature for 10 min, viable cells were counted on a hemocytometer (Fisher Scientific, Itasca, IL).

Apoptosis assay and cell cycle analysis by flow cytometry

Previously described standard techniques with a slight modification were utilized for the assessment of cell cycle inhibition and apoptosis.[15,16] Briefly, after being seeded in 24-well culture plate, cells (1 x 10^5) were incubated with or without the test compounds at concentrations ranging from 0.5 to 10 μM for 48 hr in a humidified 5% CO_2 atmosphere at 37ºC. Only MCF-7 cells were harvested with trypsin-EDTA 0.25% by centrifugation at 500g for 10 min. Cell pellets were rinsed with PBS before a staining process. Cells undergoing apoptosis were analyzed using flow cytometric method using annexin V-FITC apoptosis detection kit [Becton Dickinson Biosciences (BDB)] under the principle of measurement of membrane redistribution of phosphatidylserine. After the acquisition of 5,000 events/sample, percentage of apoptotic cells was analyzed by a FACSCanto™II flow cytometer (BDB, San Jose, CA). Spontaneous apoptosis in untreated cells was used as a control. For cell cycle analysis, BD Cycletest™ Plus DNA Reagent Kit was utilized. Staining process was performed according to the manufacturer's instruction. The amounts of cells at G1, S, and G2/M phases were analyzed by a FACSCalibur flow cytometer (BDB, San Jose, CA) equipped with CellQuest software (BDB).

Active caspase-3 assay

As guided by standard techniques,[15,16] caspase-3 activity was analyzed using the PE-conjugated monoclonal active caspase-3 antibody apoptosis kit (BDB). The manufacturer's instruction was followed to perform the analysis. The percentage of caspase-3 activity was determined by a FACSCalibur flow cytometer and CellQuest software.

Statistical analysis

The statistical analysis was performed by PASW Statistics 18 (SPSS Inc., Chicago, IL). Experiments were compared using Independent-Samples T-test, and One-Way ANOVA. Results were displayed as mean ± standard deviation (S.D.). *P*-value of less than 0.05 was used to determine as statistically significant.

3. RESULTS

Apoptosis inducing activity

Herein, we evaluated the apoptosis inducing activity of some 1-azaanthraquinones, namely marcanine B, and marcanine C, in comparison to some other compounds called liriodenine and HAANQ using camptothecin as a positive control. MCF-7 cells were treated with the aforementioned compounds in various concentrations ranging from 0.5-10 μM for 48 hr at 37ºC. The result showed a significantly ($p \leq 0.024$) increasing percentage of apoptotic cells in an increasing concentration manner of marcanine C whereas camptothecin resulted in a significantly ($p = 0.00$) high percentage of apoptotic cells even at a very low concentration of 0.5 μM (Fig. 2A).

The apoptotic effect of marcanine C was further evaluated after an incubation period of 72 hr. When treated with marcanine C from 1-10 μM, the percentage of cells undergoing apoptosis was significantly ($p \leq 0.020$) greater than that observed in an incubation period of

48 hr. A maximum effect of both compounds was displayed at the highest concentration of 10 µM (Fig. 2B). To this point, it can be concluded that treatment with marcanine C induces cell apoptosis in both dose- and time-dependent fashion.

We further studied the selectivity of marcanine C in comparison to the apoptosis inducing activity on nonproliferating normal cells. Regarding this, PBMCs and MCF-7 were treated with marcanine C at various concentrations from 0.5-10 µM for 48 hr at 37ºC. As a result, percentage of apoptotic PBMC cells was not different from a negative control when treated with marcanine C from 0.5-5 µM. However, a significant (p = 0.00) increase of apoptotic cells was found in marcanine C-treated PBMCs at the highest concentration of 10 µM. In contrast, a slightly significant (p = 0.044) increase of apoptotic MCF-7 cells was encountered after being treated with 0.5 µM marcanine C which reached a maximum effect at concentration of 10 µM (Fig. 2C). In summary, the effect of marcanine C on MCF-7 was selectively more notable than that displayed on normal PBMCs.

To determine the cancer type specificity, the apoptosis inducing activity of marcanine C was evaluated using NB4, a type of promyelocytic leukemic cells. Consequently, marcanine C at a concentration from 0.5-3 µM had no significant effect on increasing of NB4 cells undergoing apoptosis compared to the control. But, a significant (p = 0.00) effect on increasing of apoptotic cells was detected when treated with marcanine C at 5 and 10 µM. A similar result, as mentioned earlier, regarding the effect of marcanine C on MCF-7 was found in dose-dependent manner with a maximum effect at the highest concentration of 10 µM (Fig. 2D). In conclusion, the apoptosis inducing effect of marcanine C was more obvious on MCF-7 than that encountered on NB4 cells.

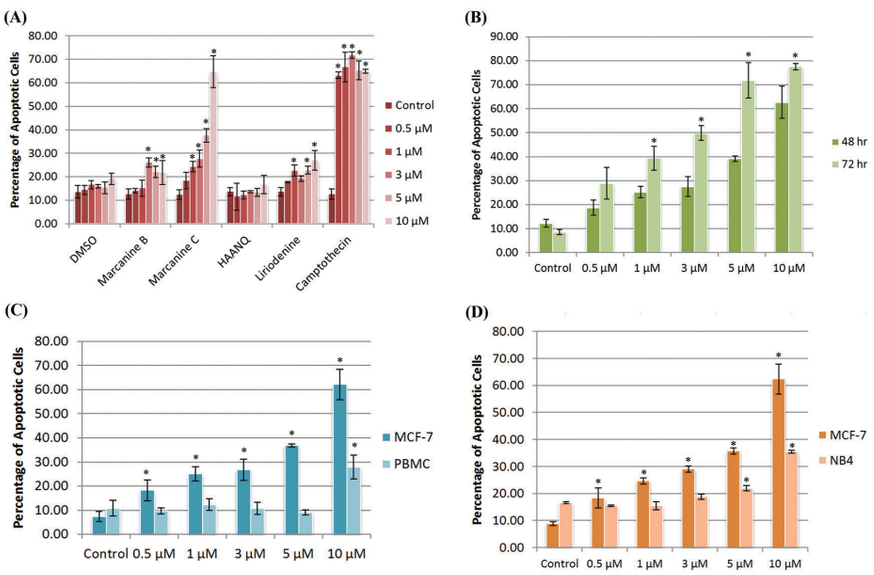

Figure 2. Analysis of apoptosis inducing activity: (A) percentage of apoptotic MCF-7 cells after incubation with marcanine B, marcanine C, HAANQ, liriodenine, camptothecin, or the solvent DMSO in range of 0.5-10 µM for 48 hr; (B) percentage of apoptotic MCF-7 cells after incubation with marcanine C 0.5-10 µM for 48 or 72 hr; (C) percentage of apoptotic MCF-7 cells or normal peripheral blood mononuclear cells (PBMCs) after treatment with marcanine C 0.5-10 µM for 48 hr; (D) percentage of apoptotic MCF-7 cells or NB4 promyelocytic leukemia cells after treatment with marcanine C 0.5-10 µM for 48 hr. Results were displayed with mean ± S.D. of three experiments.

Analysis of caspase-3 activation

For the assessment of caspase-3 activation, MCF-7 cells were treated with marcanine C at concentrations of 5 and 7.5 µM for 24 hr at 37ºC. The untreated cells resulted in a very low percentage of cells with active caspase-3 with a baseline of 2.7%. When treated with camptothecin (0.5 µM) as a positive control, more than 38% of cells were detected with active caspase-3. Cells treated with 5 µM and 7.5 µM marcanine C also resulted in a similar trend (Fig. 3) with the percentage of cells with active caspase-3 of 4.4% and 23.37%, respectively. Therefore, marcanine C was able to activate caspase-3 leading to cell apoptosis.

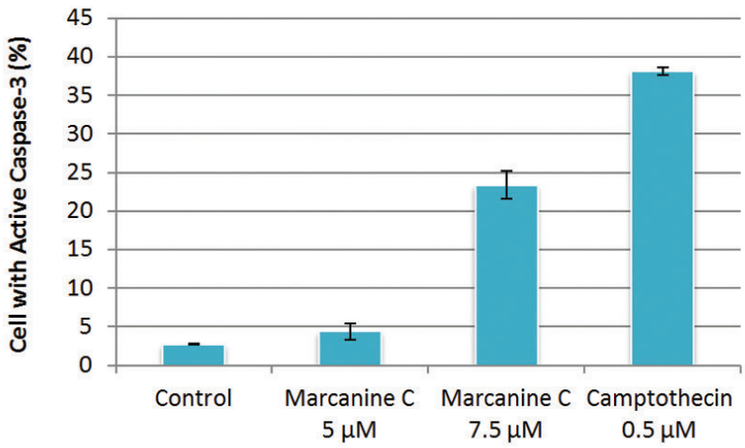

Figure 3. Analysis of caspase-3 activation. The percentage of MCF-7 cells with active caspase-3 after treatment with 5, 7.5 µM marcanine C, or 0.5 µM camptothecin for 24 hr. Results were displayed with mean ± S.D. of three experiments.

Cell cycle analysis

With an obvious result from the apoptosis inducing assessment, we had taken marcanine C for evaluating its ability for cell cycle inhibition. Next, MCF-7 cells were treated with different concentrations of marcanine C (1-10 µM) for 24 hr at 37ºC. The untreated portion of MCF-7 cells had come across with the percentage of cells in G1, S, and G2/M phases as 62%, 20%, and 18%, respectively. Incubation with higher concentrations of marcanine C had given a tendency showing greater proportions of the cells in the G1 phase in a dose-dependent style, with implicitly decreasing proportions of cells in the S and G2/M phases (Fig. 4A).

The cell cycle inhibiting pattern was not due to the presence of very low concentrations of DMSO as a solubilizing vehicle. This had been proved by incubation of cells with different concentrations of DMSO as same as present when treated with marcanine C. Apparently, the result showed no significant ($p > 0.86$) alteration on the proportion of cells in G1 and S phases (Fig. 4B). However, a significant ($p = 0.016$) depletion in G2/M phase was found on after treating with the highest concentration of 0.25% of DMSO.

Moreover, marcanine C was evaluated for cell cycle inhibition on PBMCs to assess the effect of this agent on normal cells (Fig. 4C). As a result, no significant change on the proportion of cells in each phase was noticed, implying that marcanine C had no effect on the cell cycle of PBMCs. In summary, marcanine C had a potent effect on cell cycle arrest in the G1 phase. The effect was more obviously sensitive on MCF-7 cells compared to normal PBMCs.

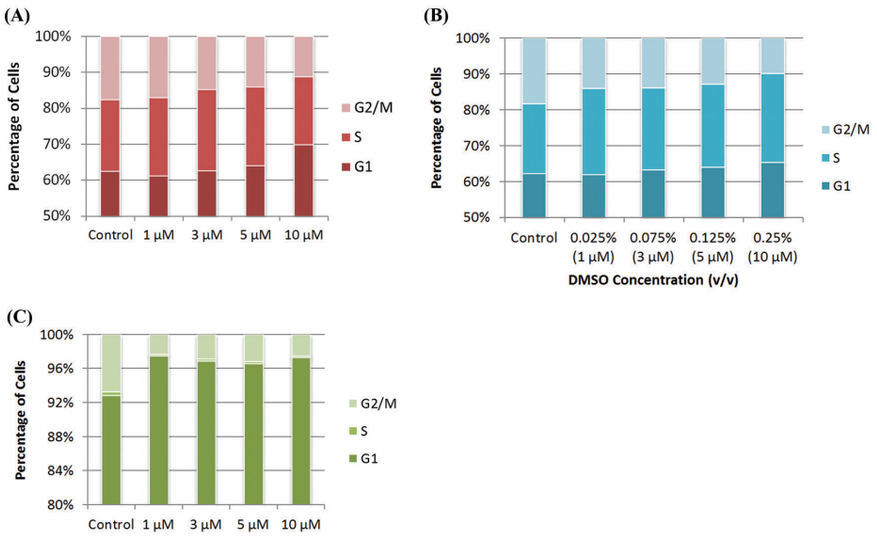

Figure 4. Cell cycle analysis. The percentage of MCF-7 cells in the G1, S and G2/M phases after a treatment for 24 hr with: (A) marcanine C in the range of 1-10 µM; (B) DMSO. The percentage of normal peripheral blood mononuclear cells (PBMCs) in the G1, S and G2/M phases after a treatment for 24 hr with marcanine C from 1-10 µM (C) was also shown. Results were displayed with mean ± S.D. of three experiments.

3. DISCUSSION

A few studies have previously reported the comprehensive mechanism of action of some cytotoxic compounds isolated from the genus *Goniothalamus*.[12,17,18] It has been revealed that some of those compounds exert cytotoxic activity via apoptosis induction, generating reactive oxygen species and triggering a cell signaling pathway resulting in DNA damage and mitochondrial membrane depolarization. However, there are only a few studies regarding the mechanism of action of 1-azaanthraquinones. Recently, an increasing number of reports have revealed that some aminoquinone derivatives from *Goniothalamus species* possess their cytotoxic effect related to apoptosis which is a fundamental biological process playing an important role in both physiological and pathological conditions.[17,18] In the present study, four alkaloids namely, marcanine B, marcanine C, liriodenine, and HAANQ, were assessed on their ability to induce apoptosis in MCF-7 cells with the Annexin V-FITC assay. Among those compounds, marcanine C was considered the most active compound resulting in the highest percentage of apoptotic cells. When compared to marcanine C, marcanine B was considered less active, this might be due to a more hydrophobic methyl group at C4 position. This result was consistent with finding from a previous study showing that more hydrophobic substitution at C4 position resulted in less cytotoxic activity of 1-azaanthraquinones.[17] Moreover, HAANQ was considered less active in terms of apoptosis inducing activity compared to both marcanine B and marcanine C. This poor activity can be explained by lacking a lactam scaffold which was previously described as a pharmacophore.

Further experiment on assessment of caspase-3 activation ability of marcanine C resulted in higher percentage of cells with active caspase-3 compared to the untreated control cells. This indicates the stimulation of caspase-3 at the point of execution phase. Caspase-3 is activated by initiator caspases. Caspase-3 is considered to be the most important executioner caspase cleaving many substrates that consequently lead to morphological changes seen

in apoptotic cells.[19-21] Our result on caspase-3 stimulating activity was similar with another study reported that cleistopholine, a compound bearing 1-azaanthraquinones moiety, also activated caspase-3 function.[18]

Cell cycle analysis revealed that marcanine C inhibited cell proliferation and arrested cell cycle in the G1 phase. An increase in marcanine C concentration resulted in higher percentage of cells in the G1 phase together with corresponding lower percentage of cells in both S and G2/M phases. Disruption in the G1 phase prevents DNA synthesis and replication, consequently leads to DNA damage and apoptosis.

In summary, our study demonstrated the apoptosis inducing activity of some nitrogen bearing compounds including marcanine B, marcanine C, liriodenine, and HAANQ against MCF-7 cells. Among these compounds, marcanine C demonstrated the most prominent effect. Further studies in this work firstly revealed that the cytotoxic effect of marcanine C was related to caspase-3 activation pathway as well as induction of cell cycle arrest in the G1 phase on MCF-7 cells.

4. Acknowledgement

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5. Conflict of Interest

The authors declare no conflict of interest.

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