American Journal of Applied Sciences 6 (12): 2018-2023, 2009
ISSN 1546-9239

Anticancer Activities of Styrylpyrone from the Leaves and Twigs of *Goniothalamus maewongensis via* Cell Cycle Arrest

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Abstract: Problem statement: *Goniothalamus maewongensis* is one of three new species which are found in Thailand recently. The genus goniothalamus is not only well known for the rich of styrylpyrones but also famous for the potential of biological activities, especially the cytotoxic activity against a number of human cancer cell lines. The phytochemical and biological investigations of this plant are interesting to bioassay-guided fractionation, particularly cell cycle arrest. **Approach:** The investigation was carried out to extract, isolate, purify and elucidate structure of the active compound from the leaves and twigs of *Goniothalamus maewongensis*. Both of the solvent extracts and isolated compound were evaluated with kinds of mammalian cancer cell lines, i.e., A549, GLC4, GLC4/Adr, K562 and K562/Adr for antiproliferation assay and cell cycle analysis. **Results:** Styrylpyrone from the leaves and twigs of *Goniothalamus maewongensis* was isolated from the active hexane extract. The spectroscopic techniques were provided for success in structure elucidation. In addition, a styrylpyrone compound was the most powerful to biological activities, which this molecular is significantly more toxic to small cell lung cancer than non small cell lung cancer cell ($p<0.05$). On the other hand, the goniodiol was not recognized by both multidrug resistance protein (ABCC1 and ABCB1). The study of cell cycle arrest explained antiproliferation effect by goniodiol at G2/M arrest in both lung cancer type (A549 and GLC4) and erythroleukemia cell (K562), while cell cycle arrest by goniodiol on both resistant cell lines are positioned on G0/G1 or S-phase. **Conclusion:** Goniodiol exhibits anti-proliferative on cancer cell line and un-recognized by multidrug resistant protein (ABCB1 and ABCC1).

Key words: *Goniothalamus maewongensis*, styrylpyrone, cell cycle arrest

INTRODUCTION

The genus *Goniothalamus* (Annonaceae) consists of 115 species, distributed throughout the tropics and subtropics of the world[1] with some used widely as traditional medicines. Extensive survey of the genus Goniothalamus by Smittinand[2] indicated the presence of ten species in Thailand. Furthermore, 3 new species, i.e., *Goniothalamus aurantiacus*, *Goniothalamus maewongensis* and *Goniothalamus rongklanus* were determinated by Saunders and Piya[3]. Several acetogenins, styrylpyrones and alkaloids have been isolated from the plants in this genus and their cytotoxic activity against a number of human cancer cell lines has been reported[4]. Styrylpyrones are a series of natural products which exhibit moderated to significant biological activity including antitumor and antifungal properties, as well as antibiotic potential[5]. Phytochemical studies on leaves and twigs of *Goniothalamus maewongensis* has led to the isolation and characterization of a styrylpyrone, which was found to posse significant cytotoxic activities of this compound. The styrylpyrone

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compound has been determinated from IR, ^{1}H NMR, ^{13}C NMR, 2D NMR and MS Spectroscopic data.

MATERIALS AND METHODS

General procedures: Melting point was measured on a BÜchi 322 micromelting point apparatus and has to be uncorrected. IR spectrum was recorded as KBr pellets using a Shimadzu 8900 FTIR spectrophotometer. The ^{1}H and ^{13}C NMR (1D and 2D) spectra were recorded at 400 MHz and 100 MHz respectively using a DPX on a BrÜker DPX 400 spectrometer in $CDCl_3$ as an internal standard. ESIMS (positive mode) were obtained by using a Finnigan LC-Q Advantage Thermoquest spectrometer equipped with Xcalibur software. Silica gel 60 (Merck, 70-230 mesh) was used for column chromatography; while TLC analysis was carried out on Si gel GF_{254} precoated plates with detection using UV detector.

Plant material: Fresh leaves and twigs of *Goniothalamus maewongensis* were collected in Kampangphet Province, Thailand, in April 2009. They were identified by Saunders and Piya[3] voucher specimen (BKF no. 152891) has been deposited at the Forest Herbarium, Department of National Park, Wildlife and Plant Conservation, Ministry of Natural Resources and Environment, Bangkok, Thailand.

Extraction and isolation: Air-dried powdered leaves and twigs of *Goniothalamus maewongensis* (1.2 kg) were successively defatted with hexane, WP0639 (3.5 L×3 days×5 times) and then sequential extracted consecutively at room temperature with ethyl acetate, WP0640 (3.5 L×3 days×5 times), acetone, WP0641 (3.5 L×3 days×5 times) and methanol, WP0642 (3.5 L×3 days×5 times), followed by filtration. The filtrates were combined and evaporated to dryness under reduced pressure to afford 20.84, 63.55, 19.91 and 84.69 g of residues, respectively. The extracts were submitted for bioassays. Preliminary biological investigation for cytotoxic of the four extracts (WP0639-0642) were carried out by our collaborators from Chiang Mai University of Thailand. Effect of *Goniothalamus maewongensis* extracts on cell viability of A549 cells. A549 cells (1.0×10^4 cell well^{-1}) were grown in the presence of 0.1% DMSO or various concentrations of the *Goniothalamus maewongensis* extract for 72 h. The number of viable cells was determined by SRB assay. Each point represents the mean values ± standard deviation of three independent experiments performed in triplicate. It was also found that the hexane extract displayed strong activity in the A549 assay with % inhibition at 16 μg mL^{-1}. From the bioassay-guided fractionation, the hexane fraction was firstly separated by column chromatography. The hexane extract (20.84 g) was subjected to a coarse separation on a silica gel column (300 g), eluting with various proportions of ethyl acetate-n-hexane, followed by the increasing amount of methanol in ethyl acetate and finally with methanol. Fractions (200 mL each) were collected and combined on the basis of TLC behavior. The solvents were evaporated to dryness to afford six fractions (F_1-F_6). Fraction F_5 (4.95 g), eluted by 5% ethyl acetate-n-hexane, was obtained as a semisolid. Further separation by column chromatography over silica gel (150 g), eluting with n-hexane, then with various proportions of ethyl acetate: n-hexane, followed by the increasing amount of methanol in ethyl acetate and finally with methanol. Fractions were collected and combined then the solvents were removed under reducing pressure to afford subfractions A_1-A_2. The subfraction A_2 (1.15 g), was separated by column chromatography over silica gel to yield subfraction B_1. Further, purification by preparative thin-layer chromatography on silica plates (EtOAc: Hexane, 35:65) afforded white solid 0.28 g. The solid was recrystallized from ethanol to give purified colourless needle 0.2 g which identified as styrylpyrone, goniodiol (Fig. 3).

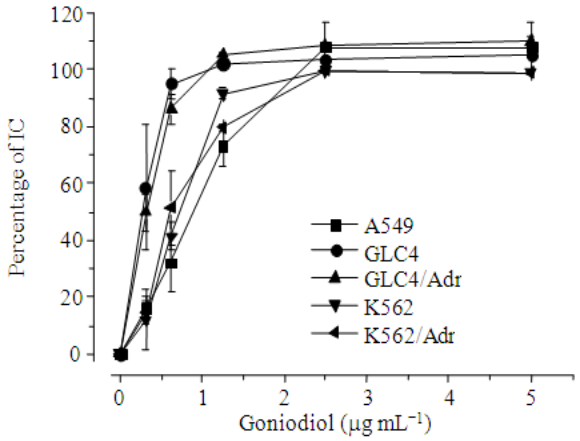

Fig. 1: The effect of goniodiol on cell proliferation of A549 (■), GLC4 (●), GLC4/Adr (▲), K562 (▼) and K562/Adr (◄) cells. The percentage of IC represented the percentage of inhibited cell proliferation at the concentration of goniodiol up to 5 μg mL^{-1}. The IC_{50} value was recalculated to molarity unit and printed in Table 2. Data shown are mean ± SD obtained from 4 independent experiments

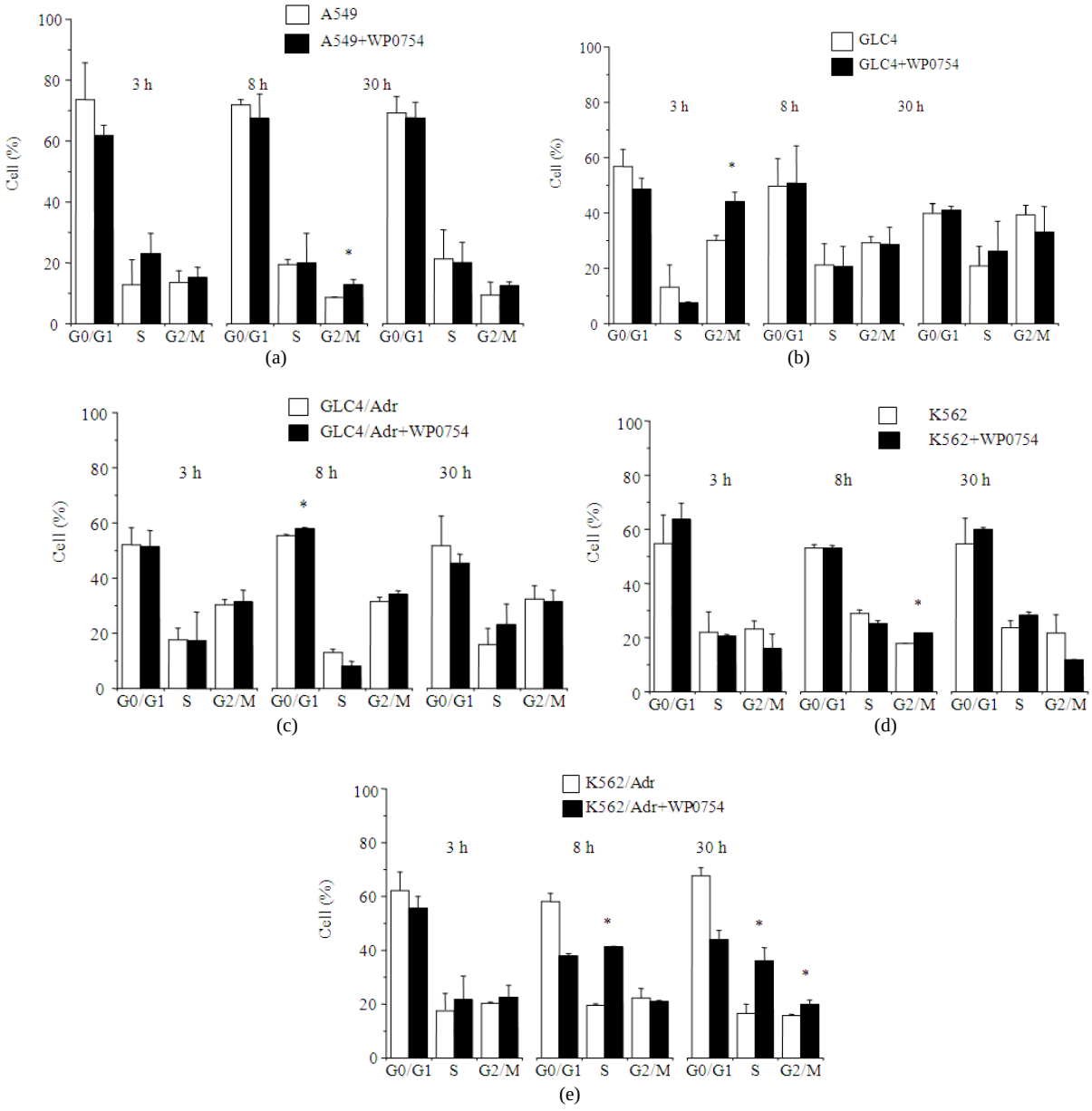

Fig. 2: (a) Cell cycle analysis of A549; (b) GLC4; (c) GLC4/Adr; (d) K562 and (e) K562/Adr cell, cells were incubated with goniodiol(WP0754) for 3, 8 and 30 h at IC_{50} concentration (■) and control cell (□). (*p<0.05)

Cell lines and culture conditions: The bioactivity of extracted compound was investigated on 5 cancer cell lines, that were non small cell lung cancer (A549), adriamycin-sensitive small cell lung cancer (GLC4), adriamycin-resistant small cell lung cancer (GLC4/Adr) with the expressing of ABCC1/MRP1, adriamycin-sensitive erythroleukemia cell (K562) and adriamycin-resistant erythroleukemia cell (K562/Adr) with the expressing of ABCB1/MDR1 (P-glycoprotein)[6,7]. All cell lines were cultivated in RPMI-1640 medium supplemented with 1% fetal bovin serum 1% penicillin/streptomycin in a 37°C incubator under a 5% CO_2.

The anti-proliferation assay: The effect of compound on cell proliferation was observed in those five cell lines.

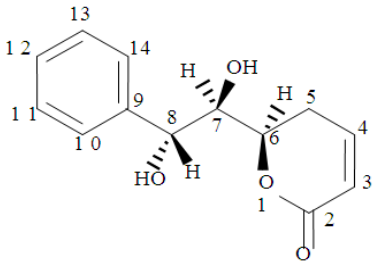

Fig. 3: The chemical structure of goniodiol

Cells were incubated at cell culture condition with a various concentrations of compound up to 5 μg mL^{-1} for 72 h. The amount of living cells were counted by using COULTER EPICS CL-MCLTM (Coultronics France SA).

Cell cycle distribution analysis: Cells (5×10^5) were seeded in mulitidish 24 wells and treated at the concentration of IC_{50} value of each cell line for 3, 8 and 30 h (Fig. 2). Cells were harvested parallel with non treated cells and washed with Phosphate Buffer Solution pH 7.3 (PBS) twice before fixed with 70% ethanol overnight at 4°C. After fixation, cells were collected and resuspended in PBS and consecutively added then mixed with final concentration of 1% triton-X100, 20 μg mL^{-1} RNase and 10 μg mL^{-1} propidium iodide. Then, cells were dark incubated at room temperature for 30 min. before cell cycle distribution analysis by using flow cytometer.

RESULTS

Chemical structure determination: In this investigation, some of biological activity of *Goniothalamus maewongensis* has been tested, whereby; hexane, ethyl acetate, acetone and methanol extract were assayed for their anticancer activities. The active hexane extract was carried out for purification, separation, crystallization and structure elucidation of the isolated goniodiol. The structure has been established on the basis of spectral and chemical evidence. It is worth nothing that the data from spectroscopic techniques, especially the ^1H, ^{13}C-1D and 2D NMR were performed on the accurate molecular structure (Table 1).

The anti-proliferation assay: The antproliferation of gonidiol on cancer cell lines was presented in Table 2 as the IC_{50} value which is defined as the concentration in which inhibited cell proliferation by 50%. The results showed that this compound potentially inhibited all cell lines proliferation in micromolar level.

Cell cycle arrest: The quantitative analysis of histogram to estimate the percentage of G0/G1, S and G2/M phases of control cells and treated cells with IC_{50} value of goniodiol for 3, 8 and 30 h. clearly showed that this compound holdup cell cycle progression of GLC4 and moderately of A549 and K562 cell at G2/M phase. At 8 h. incubation time, G0/G1 phase of GLC4/Adr cell was slightly increased while K562/Adr cell S-phase arrest was dramatically discovered in either 8 or 30 h.

Goniodiol was isolated as a colourless needle. In the IR spectrum, absorption bands attributable to the hydroxyl (3410 cm^{-1}) and carbonyl of the unsaturated δ-lactone (1720 cm^{-1}) groups were presented. Additional, the C-O stretching vibration occurs at 1245 cm^{-1} indicated the secondary alcohol moiety. The UV spectrum of this compound had maxima at 187 and 266 nm that exhibited the $\pi\rightarrow\pi^*$ of aromatic together with double bond and $n\rightarrow\pi^*$ which belong to carbonyl chromosphore. In the ESIMS, the molecular weight was indicated by the peak at m/z 234 $(M)^+$. Thus, the molecular formula was established as $C_{13}H_{14}O_3$. The presence of two hydroxyl groups were suggested in the mass spectrum by a fragment ion base peak at m/z 91(100) due to the direct lose of 46 a.m.u. ethanol unit from the $C_8H_9O_2^+$, $(M\text{-phenyl})^+$ to $(M\text{-phenyl-EtOH})^+$. In addition, the fragment ion at m/z 89 (99.29) revealed to go down the formaldehyde from the $C_8H_9O_2^+$, followed by misplacing the water. The fragmentation patterns are as shown in Fig. 4. The five proton multiplet at δ 7.25-7.39 in the ^1H NMR spectrum indicated a monosubstituted phenyl group. Three deshielded one-proton signal at δ 4.46 (d (t), J = 15.14, 5.41), 3.27 (dd, J = 5.41, 1.96), 3.89 (d, J = 1.96) were revealed of oxygen bearing methine protons. The olefinic functional group was inferred from two methine doublet at δ 6.07 (d, J = 9.82) and methane double of doublet of doublet at δ 6.94 (ddd, J = 9.82, 5.40, 3.72). In the ^{13}C NMR spectrum, the presence of a δ-lactone unit was primarily assigned on the basis of a lactone carbonyl carbon shift at δ 162.73 and oxymethine carbon at δ 76.67. In order to obtain more information about the location of carbonyl group in the structure, a 2D NMR HMBC was carried out. The correlations observed at δ 6.07 (H-3), 6.94 (H-4), 2.58 (H-5) and 4.46 (H-6) were exhibited the carbon signal at 162.73 (C-2). The carbon signals at δ 61.40 and 57.15 were obviously due to the carbinol methine carbons. The HMBC spectrum of this compound, which provided correlations from δ 3.89 (H-8) to 125.87 was clearly shown that the C-10 and C-14 located on an aromatic ring.

Table 1: ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectral data (δ val-use; $CDCl_3$), ^{1}H-^{13}C long-range and ^{1}H-^{1}H correlations of goniodiol obtained by DEPT, HMBC and COSY

Position	δ ^{13}C (DEPT)	δ ^{1}H (J Hz)	HMBC correlation	COSY correlation
1	-	-	-	-
2	162.73 (C)	-	-	-
3	121.07 (CH)	6.07 (d,J = 9.82)	C-2, C-5	H-4 (6.94)
4	144.29 (CH)	6.94 (ddd, J = 9.82, 5.40, 3.72)	C-2, C-5, C-6	H-3 (6.07)
5	25.78 (CH_2)	2.58 m	C-2, C-3, C-4, C-6, C-7	H-6 (4.46)
6	76.67 (CH)	4.46 (d(t), J = 15.14, 5.41)	C-2, C-4, C-5, C-7, C-8	H-5 (2.58),H-7 (3.27)
7	61.40 (CH)	3.27 (dd, J = 5.41, 1.96)	C-5, C-6, C-8, C-9	H-6 (4.46),H-8 (3.89)
8	57.15 (CH)	3.89 (d, J = 1.96)	C-6, C-7, C-9, C-10, 14	H-7 (3.27)
9	135.61 (C)	-	-	-
10	125.87 (CH)	7.25-7.39 m	-	-
11	128.59 (CH)	7.25-7.39 m	-	-
12	128.59 (CH)	7.25-7.39 m	-	-
13	128.59 (CH)	7.25-7.39 m	-	-
14	125.87 (CH)	7.25-7.39 m	-	-

Table 2: The effect of goniodiol on cell viability of A549, GLC4, GLC4/Adr, K562 and K562/Adr

	IC_{50} (μM) n = 4	
Cell line	Mean	SD
A549	3.89	0.56
GLC4	1.15	0.51
GLC4/Adr	1.32	0.17
K562	2.95	0.13
K562/Adr	2.82	0.17

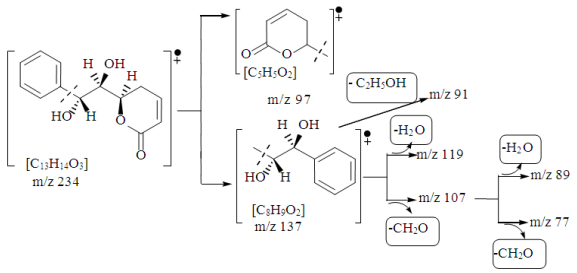

Fig. 4: The mass spectral fragmentation patterns of goniodiol

Therefore, three carbon signals which were the same at δ 128.59 were assignable for the C-11, C-12 and C-13 in that aromatic ring together. The entire sequence of ^{1}H-^{1}H spin-spin coupling connectivities starting from C-3 to C-8 were observed in the COSY spectrum which allowed to place on the functional groups as shown in Table 1 and the HMQC data led to the assignment of all carbon shifts in the molecule. The relationship between the dihedral angle and vicinal coupling constant ^{3}J is given theoretically by the Karplus equation:

$$^{3}Jab = J^{0}\cos^{2}f\text{-}0.28(0°<f<90°)$$

and

$$^{3}Jab = J^{180}\cos^{2}f\text{-}0.28(90°<f<180°)$$

So, the relative configuration at H-7 and H-8 could be determined by the $^{3}J_{7,8}$, H-C-C-H (1.96 Hz) coupling constant which indicated the two protons were located opposite side with dihedral angle 119.05°[2]. The bioactivity of goniodiol showed the cytotoxic efficacy on five cancer cell lines (Table 2, Fig. 1). By the result, this molecular is significantly more toxic to small cell lung cancer than non small cell lung cancer cell ($p<0.05$) the same as goniotriol which was extracted from *Goniothalamus laoticus* (Annonaceae)[8]. To compare the IC_{50} value of both GLC4/Adr and K562/Adr drug resistant and its parental drug sensitive cell, the difference of the population means is not significantly different ($p<0.05$). These mean that goniodiol is not recognized by both multidrug resistance protein (ABCC1 and ABCB1). The study of cell cycle arrest explain antiproliferation effect by goniodiol at G2/M arrest in both lung cancer type (A549 and GLC4) and erythroleukemia cell (K562) corresponding to the finding in hepatocellular carcinoma HepG2 and HepG2-R (doxorubicin resistance) by Tian[9] and a styryl dihydropyron (goniothalamin) in human breast cancer MDA-MB-231[9,10]. It should be noted that the cell cycle arrest by goniodiol on both resistant cell lines are positioned on G0/G1 or S-phase.

CONCLUSION

Phytochemical investigation of the hexane extract of *Goniothalamus maewongensis* had led to the isolation of goniodiol. This compound was showed the power of anti-proliferative on cancer cell line and un-recognize by multidrug resistant protein (ABCB1 and ABCC1).

ACKNOWEDGEMENT

We gratefully acknowledge Center of Innovation in Chemistry (PERCH-CIC) for financial support and Center of Excellent for Molecular Imaging (CEMI), Department of radiologic technology, Faculty of Associated Medical Science, Chiang Mai University for convenient use on flow cytometer, COULTER EPICS CL-MCLTM (Coultronics France SA). The authors are thankful to Dr. Kruawon Wongpaiboon for English editing.

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