Antiproliferation and Apoptosis Induction in Colorectal Cancer Cells by Goniothalamin

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Objective: *To investigate the effect of goniothalamin on antiproliferation and apoptosis induction in three types of colorectal cancer cells.*

Background: *Colorectal cancer is the third of the twentieth most commonly diagnosed cancer. Different types of colorectal cancer cells differ in genotype and characteristics leading to different responses to anticancer drugs. Therefore, finding new anticancer compound for the colorectal cancer cells is necessary.*

Material and Method: *Antiproliferative response of goniothalamin on three colorectal cancer cell lines including Colo 205, SW480, and LoVo were determined by MTT assay. The antiproliferative response at different time and dose was also observed. Apoptosis induction by goniothalamin was observed in all three cell-lines via morphological changes and nuclear condensation by Hoechst33342 staining.*

Results: *Goniothalamin showed different antiproliferative response on Colo 205, SW480, and LoVo cells at the IC_{50} value is 9.86±0.38 μM, 22.00±4.40 μM, and 65.25±1.85 μM, respectively. In addition, the antiproliferative response of goniothalamin was a time- and dose- dependent manner. Apoptosis morphological changes and nuclear condensation were clearly observed in Colo 205, SW480 and LoVo cells treated with 10 μM, 25 μM, and 50 μM goniothalamin, respectively.*

Conclusion: *Goniothalamin showed antiproliferation and apoptosis induction in colorectal cancer cells with different sensitivity depending on cell type. Investigation of mechanisms underlying apoptosis and its potential use for colorectal cancer treatment should be further studied.*

Keywords: *Colorectal cancer cells, Goniothalamin, Apoptosis, Antiproliferative activity*

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Colorectal cancer (CRC) is the second most and the third most common cancer in women (represented in 9.2% of the total) and men (represented in 10.0% of the total) worldwide, respectively[1]. This study, three various CRC cell lines isolated from different tissues and stages were used for apoptosis induction including Colo 205 (derived from metastatic site: ascites; stage Dukes' D), SW480 (derived from primary tumor; stage Dukes'B), and LoVo (derived from left supraclavicular region; stage Dukes' C)[2]. In general, various cancer cell lines were differed by extensive genetic and epigenetic alteration causing different response to drug targets[3]. Thus, finding the new anticancer agents was necessary.

One of cancer therapy and prevention strategies is using the natural compound treatment. Goniothalamin (IUPAC name: 6-methylene-2-styryl-3, 6-dihydro-2H-pyran) is a major styryl-lactone compound extracted from plant genus *Goniothalamus*, indigenous plant in Southeast Asia region[4]. Many reports suggested that goniothalamin was an effective bioactive compound used for many medicinal treatment purposes, such as antimicrobial, anticandidal, anti-inflammatory and anticancer[5-8]. As mention above, different cell lines may also response differently to goniothalamin. Therefore, this study aims to investigate the effect of goniothalamin on various CRC cell lines on apoptosis-associated cell death induction.

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Material and Method

Cell culture

Three CRC cell lines, include Colo 205, SW480,

and LoVo, was obtained from the American Type Culture Collection (ATCC, Manassas, VA). They were maintained in RPMI 1640 medium (Invitrogen Life Science, USA) supplemented with 10% fetal bovine serum (GE Healthcare, UK), 100 U/ml penicillin and 100 µg/ml streptomycin (PAA Laboratories, Austria) at 37°C in a humidified 5% CO_2 atmosphere.

Natural compound and chemical reagent

Goniothalamin was obtained from Assoc. Prof. Wilawan Mahabusarakam, Faculty of Science, Prince of Songkla University, Thailand in purified powder form. It was extracted from the stems of *Goniothalamus macrophyllus*, which was collected from Songkhla province Thailand, in September 2007. Chemicals for cell viability assay including MTT (3- (4,5-dimethyl 0-2,5-diphenyl tetrazolium bromide) and dimethylsulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Chemicals for fluorescence microscope observation Hoechst33342 dye was obtained from Invitrogen (Carlsbad, CA, USA).

Cell proliferation and viability assay

All CRC cell lines were seeded at density of $5x10^3$ cells/well in 96-well plates and allowed to grow for 24 hours. Then each CRC cell line was treated with goniothalamin at various concentrations of 100, 50, 25, 12.5, 6.25, 3.125, and 1.562 µM, whereas the control group was treated with 0.5% DMSO. The antiproliferative effect of goniothalamin was evaluated by MTT assay for cell proliferation and viability as described by Denizot et al[9]. Briefly, the cells were incubated at 37°C with the indicated concentrations of goniothalamin for 24 hours to determine IC_{50} value. At the end of the stipulated time, 0.5 mg/ml of MTT solution dissolved in culture medium was replaced and the cells were further incubated for 2 hours in the incubator. After that, the MTT solution was aspirated from each well and 100 µl of DMSO was added to each well to dissolve the formazan crystals, a product of cell respiration as refer to viable cells, and the absorbance at 540 nm was quantified on Epoch™ Microplate Spectrophotometer and analyzed by Gen5™ Data Analysis Software (BioTek, CA, USA).

Time- and dose- dependent antiproliferative response assay

All CRC cell lines were seeded at density of $5x10^3$ cells/well in 96-well plates and allowed to grow for 24 hours. The cells were treated with various concentrations of goniothalamin based on the IC_{50} value of each CRC cell line (for Colo 205-5, 15, 25, and 50 µM; for SW480-15, 25, 50, and 75 µM; for LoVo-25, 50, 75, and 100 µM) for 3, 6, 9 and 12 hours, whereas the control group was treated with 0.5% DMSO. After treatment, time- and dose- dependent antiproliferative response of goniothalamin was evaluated by MTT assay as previously described.

Observation of cellular morphological changes and nuclear condensation

The fluorescent dye Hoechst33342 was commonly used to detect nuclear condensation, which is a characteristic of apoptotic cells. The protocol was modified from Oberhammer et al[10]. Each CRC cell lines were plated at a density of $2x10^5$ cells/well in 6-well plates and allowed to grow for 24 hours. Then, each CRC cell lines were treated with various concentrations of goniothalamin related to IC_{50} value (10 µM for Colo 205, 25 µM for SW480, and 50 µM for LoVo) for 12 hours and 24 hours at 37°C with 5% CO_2. After treatment, the treated cells were washed with PBS and fixed with 4% paraformaldehyde for 15 minutes at room temperature. The fixed cells were then washed with PBS and stained with 5 µg/ml of Hoechst 33342 solution in PBS for 15 minutes. Then, the cells were washed with PBS again, and the plates were observed using a fluorescence microscope IX73 model (Olympus, Tokyo, Japan). Using this microscope with the U-MWU2 mirror units for ultraviolet excitation was used for observation of nuclear condensation and the phase contrast mirror units were used for observation of cellular morphology.

Statistical analysis

Calculated data was obtained from at least three independent experiments and expressed as mean $\pm$ standard deviation (SD). Statistical analysis was performed with Student's t-test. A *p*-value of 0.05 was taken as minimum basis for assigning significance.

Results

Antiproliferative potential of goniothalamin on various colorectal cancer cell lines

To explore the antiproliferative effect of goniothalamin on various CRC cell lines, the IC_{50} value was assessed with the MTT assay using a panel of CRC cell lines, include Colo 205, SW480 and LoVo. Goniothalamin showed a different IC_{50} value in different CRC cell lines significantly. The IC_{50} value of goniothalamin is 9.86 ± 0.38 µM, 22.00 ± 4.40 µM, and 65.25 ± 1.85 µM for Colo 205, SW480, and LoVo cell lines, respectively (Fig. 1).

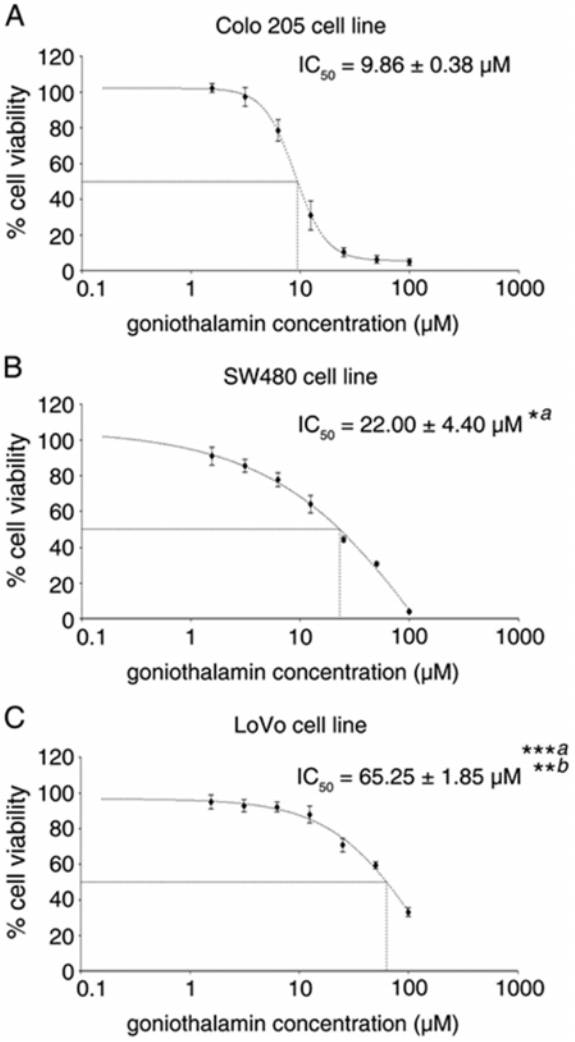

Fig. 1 The MTT cell viability assay for IC_{50} value of goniothalamin against Colo205 (A), SW480 (B) and LoVo (C) cell lines at 24 h exposure time. Values were expressed as mean ± SD from at least three independent experiments. *p<0.05, **p<0.01, ***p<0.001, were considered statistically significance. Statistical analysis, label *a* represents comparing with Colo205 as control and label *b* represents comparing with SW480 as control.

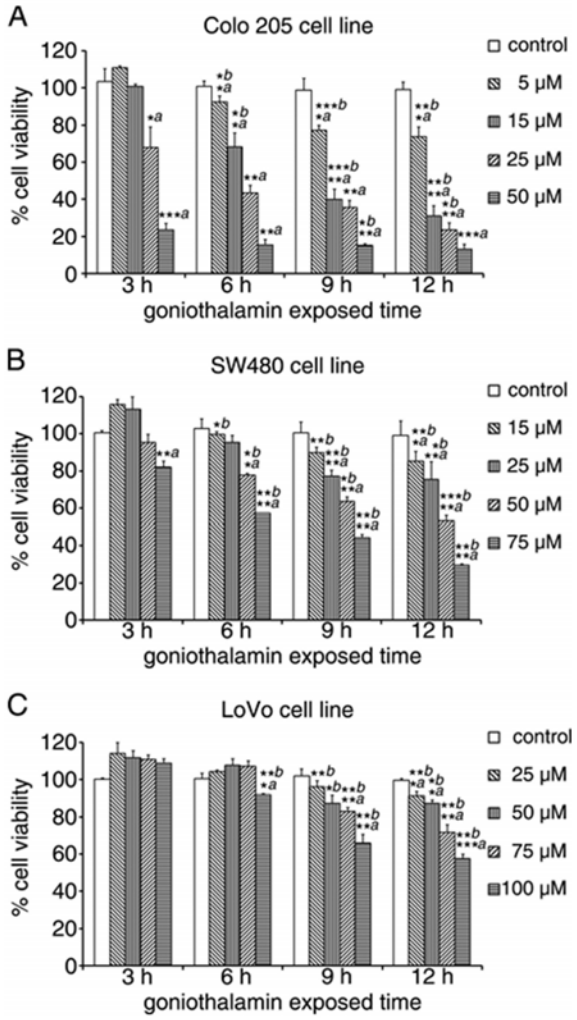

Fig. 2 The effect of goniothalamin on Colo205 (A), SW480 (B) and LoVo (C) cell viability at various concentrations and exposure time, the % cell viability was decreased in a time- and dose-response manner. Values were expressed as mean ± SD from at least three independent experiments. *p<0.05, **p<0.01, ***p<0.001, were considered statistically significance at each exposure time. Statistical analysis, label *a* represents comparison with 0.5% DMSO treated control of each exposure time and label *b* represents comparison with 3 hours exposure time of each concentration.

Antiproliferative response of goniothalamin on various colorectal cancer cell lines in time- and dose-dependent manner

The antiproliferative effect of various concentrations of goniothalamin response on each CRC cell lines after 3, 6, 9, and 12 hours treatment revealed that goniothalamin induced cell death in time- and dose-dependent manner (Fig. 2). The antiproliferation at each time and dose was displayed separately for each CRC cell line.

Apoptosis-associated cell death induction by goniothalamin in three colorectal cancer cell lines

For apoptosis, the morphology of the cells

was changed from normal to cell shrinkage and cell blebbing, also called "apoptotic bodies". After treatment, significant morphological changes in CRC cell lines were observed. The increased morphological changes evidently correlated with increased exposure time (Fig. 3).

Hoechst 33342 staining was based on fluorescent detection of nuclear condensation, which is typical feature of apoptotic cells of which a bright blue nucleus was observed. The result indicated that goniothalamin induced increased apoptotic cells in time dependent manner in CRC cell lines (Fig. 3).

Discussion

At present, many reports showed that cancer remains one of the most common causes of death in many countries. The GLOBALCAN project, which provides contemporary estimation of the incidence and prevalence of mortality from major types of cancer, at national level of 184 countries in the world, indicated that CRC is one of the most cancer problems worldwide. Almost 55% of the cases occur in more developed regions and 8.5% of the total case is mortality, with more deaths (52%) especially in the less developed regions of the world[1].

The current study investigated different response of goniothalamin on antiproliferative effect of each CRC cell line. An assay of cell proliferation and viability using MTT revealed that goniothalamin showed high antiproliferation with Colo 205 and SW480 cells but not LoVo cells. LoVo cells showed less antiproliferative response to goniothalamin which is correlated to previous report in normal fibroblast 3T3 cell line and normal liver Chang cell line, both shown IC_{50} value >50 µM[11,12]. The result suggested that Colo 205 cells, classified in stage of Dukes' D (widespread metastasis), and SW480 cells, classified in stage of Dukes' B (invasion through the bowel wall penetrating the muscle layer), were highly- and moderately-sensitive to goniothalamin, respectively while LoVo cells, classified in stage of Dukes' C (invasion involving lymph nodes), were less sensitive. It may be useful for anti-proliferation potential in CRC with Dukes' D and Dukes' B stage.

Bioactive compound that could induce apoptosis is beneficial to be used as an anticancer

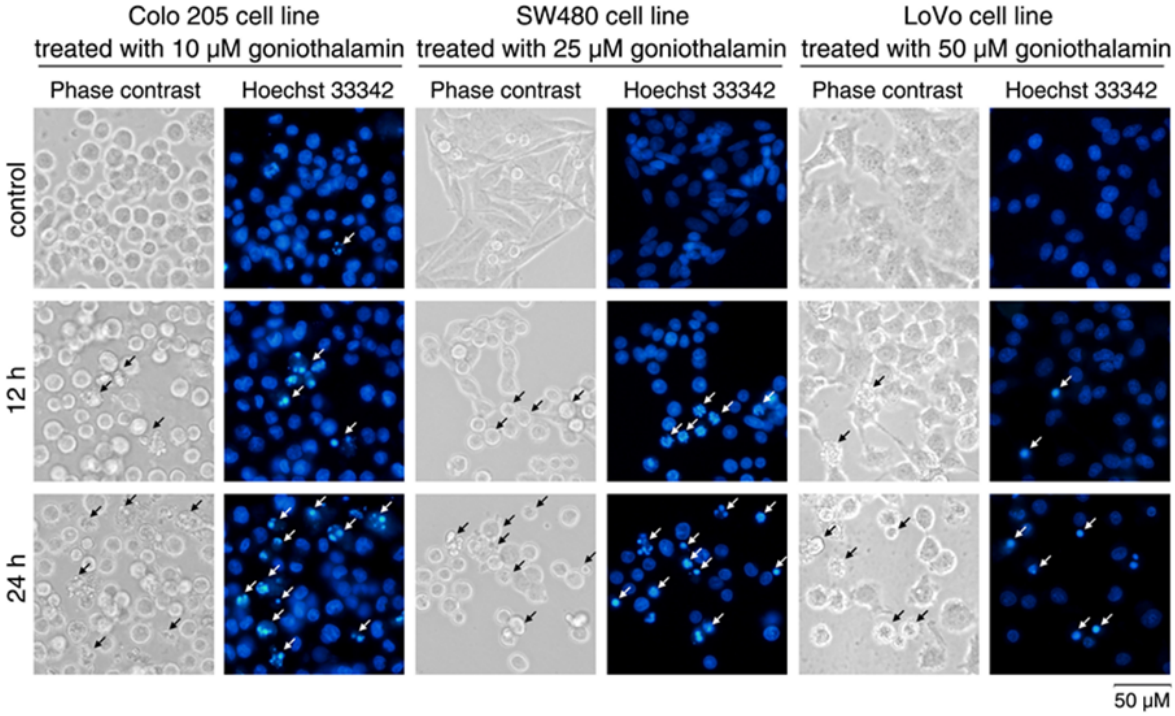

Fig. 3 Apoptosis induction in Colo 205, SW480 and LoVo cells treated with 10 µM, 25 µM and 50 µM goniothalamin, respectively. Cellular morphology and nuclear condensation was observed by phase contrast and Hoechst33342 staining under fluorescence microscope. Cellular morphological changes to apoptotic-like bodies were indicated by dark arrows. Nuclear condensation was indicated by white arrow.

agent. Apoptosis is generally characterized by distinct morphological characteristics; including cell shrinkage, nuclear condensation etc[13]. In this study, the apoptotic features were investigated by morphological changes and nuclear condensation observed under phase contrast mirror unit and ultraviolet excited mirror unit of fluorescence microscope, respectively. Antiproliferative effect of goniothalamin on each CRC cell line by MTT assay corresponded with morphological changes and nuclear condensation, which corresponded with other reports of apoptotic cell induction by goniothalamin[11,14] suggesting that goniothalamin induced cell death in CRC cell lines by apoptosis induction. However, the mechanism of apoptosis induction in CRC cell lines by goniothalamin is not yet determined, thus need to be further studied to reveal potential use as anticancer agent.

Conclusion

Goniothalamin showed selective antiproliferative effect towards Colo 205 and SW480 cell lines but not LoVo cell line. This compound showed the best antiproliferation with Colo 205 cell line, which presented in stage of widespread metastasis. However, SW480 cell line, which presented in stage of invasion, showed sensitivity with goniothalamin too. Thus, goniothalamin is potentially a good anticancer agent with non-toxic towards normal cells. Moreover, these results indicated that goniothalamin inhibited CRC proliferation via apoptosis-associated cell death induction determined by morphological changes and nuclear condensation.

What is already known on this topic ?

CRC is the second most and the third most common cancer in women and men worldwide, respectively.

Goniothalamin is an effective bioactive compound used for many medicinal treatment purposes including anticancer treatment.

Various cancer cell lines are differed by extensive genetic and epigenetic alteration causing different response to drug targets in general.

What this study adds ?

Different CRC cell lines response differently to goniothalamin on apoptosis-associated cell death induction.

Goniothalamin induce apoptosis-associated cell death induction that resulting the morphological changes and nuclear condensation in goniothalamin treated CRC cell lines.

Acknowledgement

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Potential conflicts of interest

None.

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การยับยั้งการเจริญเติบโตและเหนี่ยวนำการตายแบบอะพอพโทซิสในเซลล์มะเร็งลำไส้โดยสาร goniothalamin

ธเนศ โสภณนิธิประเสริฐ, วิลาวัลย์ มหาบุษราคัม, ยูคิโอ นาคามูระ, รมิดา วัฒนโภคาสิน

ภูมิหลัง: *มะเร็งลำไส้เป็นมะเร็งที่ถูกตรวจพบมากเป็นอันดับสามจากยี่สิบอันดับมะเร็งที่ถูกตรวจพบมากที่สุด เซลล์มะเร็งลำไส้พบมีหลากหลายชนิดโดยมีความแตกต่างกันของข้อบ่งชี้ทางพันธุกรรมและลักษณะที่แสดงออก ซึ่งเป็นสาเหตุให้เซลล์มะเร็งลำไส้และทวารหนักแต่ละชนิดเกิดการตอบสนองต่อยาต้านมะเร็งที่แตกต่างกัน ดังนั้นการค้นหาสารต้านมะเร็งชนิดใหม่สำหรับมะเร็งลำไส้และทวารหนักชนิดต่าง ๆ จึงมีความสำคัญ*

วัตถุประสงค์: *ศึกษาผลของสาร goniothalamin ในการยับยั้งการเจริญเติบโตและการเหนี่ยวนำการตาย แบบอะพอพโทสิสในเซลล์มะเร็งลำไส้สามชนิด*

วัสดุและวิธีการ: *ทดสอบฤทธิ์ยับยั้งการเจริญเติบโตต่อเซลล์ของสาร goniothalamin ในเซลล์มะเร็งลำไส้สามชนิด ได้แก่ Colo 205, SW480 และ LoVo ด้วยวิธี MTT assay ทดสอบฤทธิ์ยับยั้งการเจริญเติบโตต่อเซลล์ของสารที่ช่วงเวลาและความเข้มข้นต่าง ๆ ศึกษาการเหนี่ยวนำให้เกิดการตายแบบอะพอพโทซิสโดยสาร goniothalamin ในเซลล์ทั้งสามชนิดด้วยการวิเคราะห์การเปลี่ยนแปลงรูปร่างของเซลล์และการขดแน่นของนิวเคลียสโดยการย้อมด้วยสี Hoechst 33342*

ผลการศึกษา: *สาร goniothalamin แสดงฤทธิ์ยับยั้งการเจริญเติบโตต่อเซลล์ในระดับที่แตกต่างกันในเซลล์มะเร็งลำไส้ Colo 205, SW480 และ Lovo ที่ค่า IC_{50} เท่ากับ 9.86±0.38 μM, 22.00±4.40 μM และ 65.25±1.85 μM ตามลำดับ โดยฤทธิ์ยับยั้งการเจริญเติบโตต่อเซลล์มีรูปแบบเพิ่มขึ้นตามระยะเวลาที่ให้สารและความเข้มข้นของสารที่ใช้ ผลการศึกษาการเปลี่ยนแปลงรูปร่างของเซลล์และการขดแน่นของนิวเคลียสแสดงให้เห็นชัดเจนถึงการเหนี่ยวนำให้เกิดการตายแบบอะพอพโทซิสในเซลล์มะเร็งลำไส้ Colo 205, SW480 และ Lovo ที่ได้รับสาร goniothalamin ในความเข้มข้น 10 μM, 25 μM และ 50 μM ตามลำดับ*

สรุป: *สาร goniothalamin สามารถยับยั้งการเจริญเติบโตและเหนี่ยวนำการตายแบบอะพอพโทสิสในเซลล์มะเร็ง ลำไส้ที่ความไวในการตอบสนองต่อสารแตกต่างกันขึ้นกับชนิดของเซลล์ การศึกษากลไกในการเหนี่ยวนำการตายแบบอะพอพโทซิสและการนำไปใช้ในการรักษาโรคมะเร็งลำไส้ควรทำการศึกษาต่อไป*