

RESEARCH ARTICLE

Phytochemicals from *Goniothalamus griffithii* Induce Human Cancer Cell Apoptosis

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Abstract

Bioactive compounds extracted from leaves and twigs of *Goniothalamus griffithii* include pinocembrin (PCN) and goniothalamine (GTN). The objectives of this study were to investigate the cytotoxic activities of PCN and GTN and their influence on molecular signaling for cell death in several human cancer cell lines compared to normal murine fibroblast NIH3T3 cells. GTN exhibited the most potent cytotoxicity against MCF-7 > HeLa > HepG2 > NIH3T3 cells with IC₅₀ values of 7.33, 14.8, 37.1 and 65.4 μM, respectively, whereas PCN was cytotoxic only to HepG2 cells with IC₅₀ values of ~80 μM. Apoptotic cell death was confirmed by staining the cells with annexin V-FITC and propidium iodide (PI) employing flow cytometry. Apoptosis was shown by externalization of phosphatidylserine in goniothalamine-treated MCF-7 cells in a dose response manner. Positive PI-stained cells with the typical morphology of apoptotic cells were increased dose-dependently. Furthermore, reduction of mitochondrial transmembrane potential was found in goniothalamine-treated MCF-7, HepG2 and HeLa cells. GTN treatment in MCF-7 increased caspase-3, -8 and -9 activities while GTN-induced HeLa cells showed an increase of both caspase-3 and -9 activities. But an increased caspase-8 activity was demonstrated in GTN- and PCN-treated MCF-7 and HepG2 cells, respectively. Taken together, GTN- and PCN-induced human cancer cell apoptosis was through different molecular mechanisms or signaling pathways, which might be due to different machineries in different types of cancer cells, as evidenced by the compound-modulated caspase activities in both intrinsic and/or extrinsic pathways.

Keywords: Pinocembrin – goniothalamine - *Goniothalamus griffithii* – cancer cells - apoptosis

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Introduction

Goniothalamine (GTN) is a styryllactone constituent or 5, 6, dihydro-6-styryl-2-pyrone (C₁₃H₁₂O₂), isolated from the stems of *Goniothalamus griffithii*, which is in the family of Annonaceae {Mu, 2003 #936}. GTN shows antibacterial and antifungal activities against a wide range of gram positive and gram negative bacteria and fungi (Mosaddik and Haque, 2003).

GTN shows different antiproliferative response on human colon adenocarcinoma Colo 205, SW480, and LoVo cells at the IC₅₀ value is 9.86 ± 0.38 μM, 22.00 ± 4.40 μM, and 65.25 ± 1.85 μM, respectively. It induces apoptosis in these three colorectal cancer cell lines with different sensitivity depending on cell type (Sophonnithprasert et al., 2015b). GTN also has anticancer effect on human oral squamous cell carcinoma (H400 cells) via mitochondria and caspase-9, -3/7 in dose-dependent manners. GTN-induced apoptosis is closely associated with the regulation on Bcl-2 family proteins, cell cycle arrest at S phase and inhibition of NF-κβ translocation in H400 cells (Li et al., 2016).

GTN enhances TRAIL-induced apoptosis in colorectal

cancer cells through DR5 upregulation and cFLIP downregulation (Sophonnithprasert et al., 2015a). A combination of GTN and TRAIL enhances caspase-dependent apoptosis induction in colorectal cancer LoVo cells via both death receptor- and mitochondria-mediated apoptosis pathways. GTN increases the translocation of DR5 to cell surface and consequently contributes to the enhancement of TRAIL-induced apoptosis (Sophonnithprasert et al., 2015a).

Pinocembrin (5,7-dihydroxyflavone) or PCN is a bioflavonoid compound extracted from honey, propolis (*Sonoran propolis*), ginger roots, wild marjoram, pine needles and *Alpinia galanga* and *Goniothalamus griffithii*. Its pharmacological and biological activities include antimicrobial, anti-inflammatory, antioxidant, protect blood brain barrier, modulate mitochondrial function, and induce apoptosis as its anticancer effect as well as neuroprotective effects against cerebral ischemic injury (Lan et al., 2015)

In the characters of pharmaceuticals, PCN has a high potential to be used as a drug for ischemic stroke treatment and for other clinical diseases. It is highly safe proved by the experiments in rats and normal human volunteers. The

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pharmacokinetics of PCN in multiple-dose is similar to those in the single dose administration, with no evidence of acute toxicity except skin urticaria (Cao et al., 2015). It consists of the anti-inflammatory and anti-stroke effects for neuroprotection. It is tolerated well up to 150 mg/day (Lan et al., 2015).

PCN reduces hippocampal inflammation, oxidative perturbations and apoptosis in a rat model of global cerebral ischemia reperfusion (Saad et al., 2015). PCN ameliorates global cerebral damage induced by ischemia-reperfusion (I/R) through suppressing oxidative stress, inflammatory and apoptotic markers as well as mitigating glutamate and lactate dehydrogenase activity. PCN attenuates endoplasmic reticulum stress-mediated apoptosis (Wu et al., 2013). PCN-treatment significantly attenuates neurological deficit scores, infarct volume, cerebral edema and improves pathological lesion in the I/R injury rats. It reduces the number of TUNEL-positive and caspase-3-positive neurons, and upregulates the expression of LC3B-II and Beclin1 in penumbra area. Hence, PCN also protects the brain against focal ischemia-reperfusion injury by inhibiting apoptosis and reverses the autophagy dysfunction in the penumbra area (Zhao et al., 2014).

PCN reduces the expressions of tumor necrosis factor- α , interleukin-1 β , intercellular adhesion molecule-1, vascular cell adhesion molecule-1, inducible NO synthase and aquaporin-4; inhibits the activation of microglia and astrocytes; and downregulates the expression of matrix metalloproteinases (MMPs) in the ischemic brain (Gao et al., 2010).

PCN triggers Bax-dependent mitochondrial apoptosis in colon cancer HCT116 cells. It shows cytotoxicity against colon cancer cells including normal lung fibroblasts compared to relative no toxicity to human umbilical cord endothelial cells. It induces loss of mitochondrial membrane potential with subsequent release of cytochrome c and processing of caspase-9 and -3 in HCT 116 cell line (Kumar et al., 2007).

The aims of this study were to compare the cytotoxic effects of these two active compounds purified from *G. griffithii*, which are pinocembrin (PCN) and goniotalamin (GTN), on various human cancer cell lines, viz., MCF-7, HeLa and HepG2 cells, and normal murine fibroblast cells. Their mechanisms of growth inhibition were also elucidated.

Materials and Methods

Materials

Active compounds, goniotalamin, was prepared and isolated from the stem of *Goniothalamus griffithii* as previously reported and the structure is shown in Figure 1A (Mu et al., 2003). Purification of pinocembrin from *G. griffithii* follows the protocol of Trakoontivakorn, et al. (Trakoontivakorn et al., 2001) and its structure is shown in Figure 1B. RPMI1640 medium and caspase-3, -8 and -9 determination kits were obtained from Invitrogen, USA. MTT (3-(4,5-dimethyl)-2,5-diphenyl tetrazolium bromide), propidium iodide (PI) and 3,3'-dihexyloxycarbocyanine iodide (DiOC₆) were

obtained from Sigma-Aldrich (St. Louis, MO, USA). Annexin V-fluorescence staining kit was purchased from Roche, Indianapolis, IN, USA.

Cells and Cell Culture

Human non-invasive breast cancer MCF-7 cell line was a gift from Dr. Preeyanart Vongchan at Faculty of Associated Medical Science, Chiang Mai University. Whereas human hepatocellular carcinoma HepG2 cell line was obtained from Dr. Prachya Kongtawelert and Human cervical cancer HeLa cell line was a gift from Dr. Ariyapong Wongnoppawich and murine normal fibroblasts cell line was obtained from Dr. Usanee Vinitketkumneun at Faculty of Medicine, Chiang Mai University, respectively. The cells were cultured in 10% fetal bovine serum in RPMI-1640 medium supplemented with penicillin G (100 units/ml) and streptomycin (100 μ g/ml) at 37°C in a humidified atmosphere containing 5% CO₂. The 80% confluent cells were applied for further experiments.

Cytotoxicity assay

MCF-7, HeLa, HepG2 and NIH3T3 cells were plated at concentrations of 2x10⁴ cells per well in 96-well plate. The cells were grown in the presence of GTN or PCN at concentrations ranging from 10 to 80 μ M for 24 h. Following phytochemical treatment, cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay. Briefly, MTT dye solution was added and incubated in carbon dioxide gas incubator for 4 h. Then, 100 μ L of dimethyl sulfoxide were added to dissolve the violet dye crystals. Absorbance was measured by employing a microplate reader (Biotek, Winooski, VT, USA) at 570 nm. The percentage of cell viability was calculated and 20% and 50% inhibitory concentrations (IC₂₀ and IC₅₀) were determined (Banjerdpongchai et al., 2011). In each experiment, determinations were carried out in triplicate.

Determination of Apoptosis

After treatment with GTN or PCN at the concentrations of IC₀, IC₂₀ and IC₅₀ for 24 h the cancer cells were washed twice with PBS (centrifuged at 200 x g for 5 minutes) and re-suspended in 100 μ L of PBS. The cell suspension was then stained with 100 μ L of binding buffer containing the reagent annexin V-FITC and PI for 15 min. The samples were processed by flow cytometer, and results were analyzed and reported as the percentage of selected cells that are positive for either annexin V-FITC or PI or both (Banjerdpongchai et al., 2013).

Assay for the Reduction of Mitochondrial Transmembrane Potential ($\Delta\Psi_m$)

Loss of mitochondrial transmembrane potential or MTP ($\Delta\Psi_m$) during the induction of apoptosis was examined by using 3,3'-dihexyloxycarbocyanine iodide (DiOC₆). Each type of human cancer cells (5x10⁵ cells/mL in 24-well plate) were treated with GTN or PCN at the concentrations of IC₀, IC₂₀ and IC₅₀ and incubated at 37°C under 5% CO₂ atmosphere for 24 h. Then the cells were added with 3,3'-dihexyloxycarbocyanine iodide

(DiOC₆) solution to the final concentration of 40 nM and incubated at 37°C for 15 minutes. The cells were washed twice with PBS and analyzed by flow cytometry technique (Banjerdpongchai et al., 2015b).

Determination of Caspases-3, -8 and -9 Activities

Human cancer cells (1x10⁶ cells/well in 24-well plate) were treated with IC₀, IC₂₀ and IC₅₀ for 24 h. Then the treated cells were washed with ice-cold PBS and centrifuged at 200 x g at room temperature for 5 min. The cell pellets were lysed with lysis buffer for 15 minutes on ice. The cell suspension was then centrifuged at 10,000 x g for 10 minutes and 50 μL supernatant was transferred to 96-well plate. The reaction buffer was prepared by adding 10 μL of DTT to 1 mL of 2x reaction buffer. The reaction buffer, 50 μL, was added to each well. Caspase-8 colorimetric substrate (IETD-*p*-nitroaniline, or IETD-*p*-NA) or caspase-3 colorimetric substrate (DEVD-*p*-NA) or caspase-9 colorimetric substrate (LEHD-*p*-NA), 5 μL, was added and incubated at 37°C for 1 h. Finally, the absorbance was measured at 412 nm. (Banjerdpongchai et al., 2014).

Statistical analysis

Results were expressed as mean ± S.D. (standard deviation). Statistical difference between control and treated group was determined by a nonparametric one-way ANOVA (Kruskal Wallis test) with a limit of $p < 0.05$ in three independent experiments. For comparison between two groups, data were analyzed using Student's *t*-test.

Results

Cytotoxicity of GTN and PCN against various human cancer cell lines

As tested by MTT assay, GTN was toxic to human cancer cells with the most to the least potential as followings: MCF-7 > HeLa > HepG2, whereas normal murine fibroblast NIH3T3 cells were least sensitive as evidenced by the highest value of IC₅₀ concentration (65.36 μM) (Table 1). For PCN, it was toxic to human hepatocellular carcinoma HepG2 cells only, whereas other tested cancer cell lines and normal fibroblasts were less sensitive, with the IC₅₀ values more than 80 μM. When the effects of these two phytochemicals from *G. griffithii* on the same cell type, viz., HepG2 cell line, were compared,

GTN (with IC₅₀ = 44.58 μM) was more toxic than PCN (with IC₅₀ ~ 80 μM).

Apoptosis in human cancer cells induced by both GTN and PCN

The mode of cell death was apoptosis as proven by the number of MCF-7 cells treated with GTN in the right lower quadrants of the dot plot analysis with annexin V-FITC- and PI-positive cells (early apoptosis). The number of MCF-7 cells underwent early apoptosis (at IC₂₀) and late apoptosis (at IC₂₀ and IC₅₀ concentrations) (as in Figure 2). Whereas HepG2 cells treated with GTN or PCN also underwent apoptotic cell death as shown in Figure 3 and 4. The dot plots of each type of cancer cells stained with annexin V-fluorescein isothiocyanate and propidium iodide are shown and compared with their corresponding histograms. The number of cells stained with both fluorescent dyes increased dose dependently (as shown by the peak height of each fluorescence dye). Since cytotoxic doses of PCN at IC₂₀ and IC₅₀ against MCF-7 and HeLa cells could not be obtained, hence, the experiments of annexin V-FITC/PI staining employing flow cytometer are not performed.

Disruption of mitochondrial transmembrane potential in GTN- and PCN-treated cancer cells

Both GTN and PCN induced reduction of the mitochondrial transmembrane potential as evidenced by the decrease of DiOC₆ fluorochrome, which is usually uptaken by normal healthy cells into their mitochondria. When the cancer cells undergo apoptosis via intrinsic pathway, mitochondrial transmembrane potential decreases (Banjerdpongchai et al., 2015a). When MCF-7, HeLa and HepG2 cells treated with GTN and HepG2 cells treated with PCN, their mitochondrial transmembrane potential was disrupted as shown in the P area (green

Table 1. IC₂₀ and IC₅₀ Concentrations of Pinocembrin (PCN) and Goniothalamine (GTN) in Various Human Cancer Cell Lines and Normal Murine Fibroblast Cells

Cell Types	Inhibitory concentrations			
	Pinocembrin (μM)		Goniothalamine (μM)	
	IC ₂₀	IC ₅₀	IC ₂₀	IC ₅₀
MCF-7	> 100	> 100	2.56	7.33
HeLa	> 100	> 100	5	14.77
HepG2	19.86	80	15.02	44.58
NIH3T3	> 100	> 100	43.97	65.36
PBMCs	N.D.	N.D.	68.32	> 80

N.D. = not determined

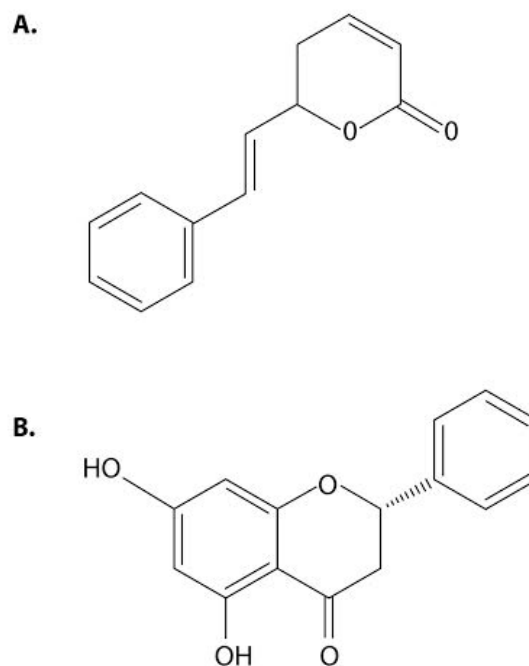


Figure 1. Structures of Goniothalamine (A) and Pinocembrin (B)

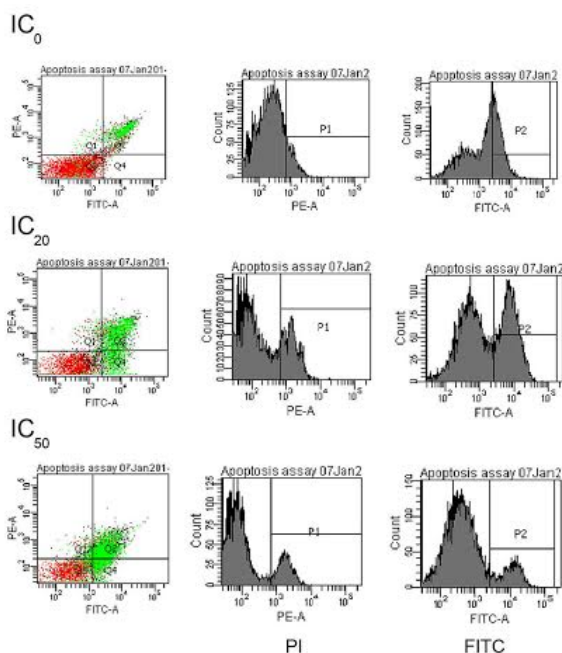


Figure 2. Apoptotic MCF-7 Cell Death Induced by GTN. Dot plots and histograms of MCF-7 cells treated with GTN for 24 h and stained with annexin V-FITC and PI employing flow cytometry technique

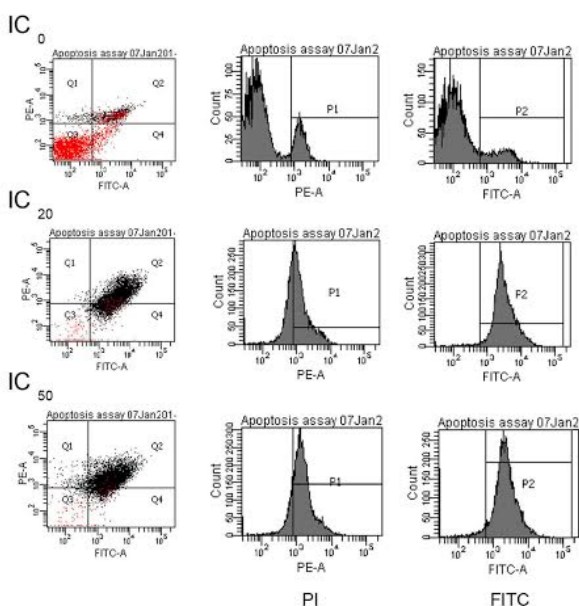


Figure 4. Apoptotic HepG2 Cell Death Induced by PCN. Dot plots and histograms of HepG2 cells treated with PCN for 24 h and stained with annexin V-FITC and PI employing flow cytometry technique

peak) in Figure 5.

Activation of Caspases-9, -8 and -3 activities in GTN- and PCN-treated cancer cells

When the cancer cells treated with natural products, such as zearalenone, the cells underwent apoptosis with the activation of caspase-9 (initiator caspase in intrinsic or mitochondrial pathway), caspase-8 (initiator caspase in extrinsic or death receptor pathway) and

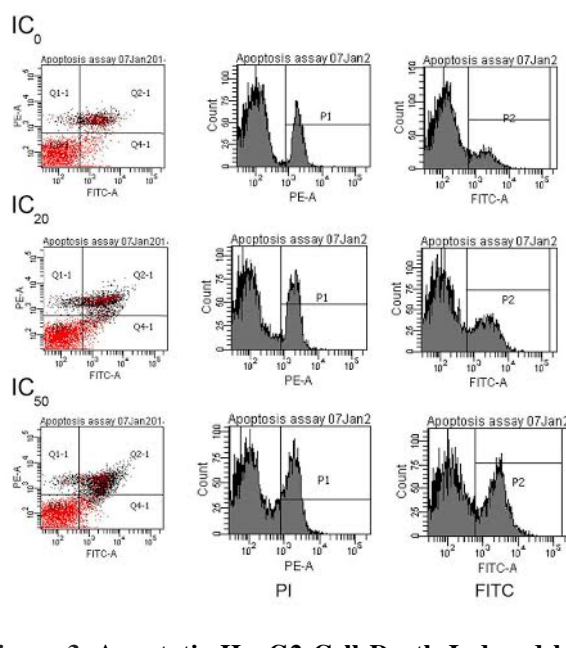


Figure 3. Apoptotic HepG2 Cell Death Induced by GTN. Dot plots and histograms of HepG2 cells treated with GTN for 24 h and stained with annexin V-FITC and PI employing flow cytometer

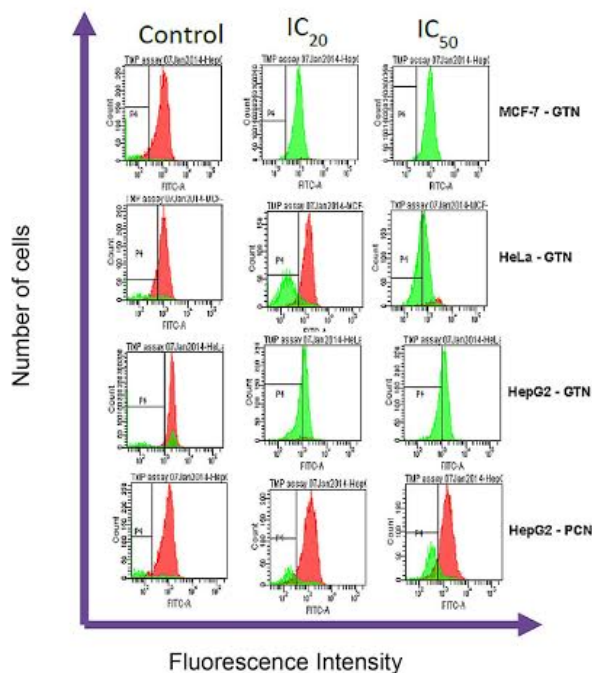


Figure 5. Reduction of Mitochondrial Transmembrane Potential of Various Cancer Cells. Histograms of MCF-7 or HeLa or HepG2 cells treated with GTN or PCN for 24 h and stained with DiOC₆. The relative fluorescence intensity was measured by flow cytometry technique

caspase-3 (executioner or effector caspase of apoptosis) (Banjerdpongchai et al., 2010). In the three cancer cell lines treated with GTN or PCN the caspase-9 (mitochondria-mediated) and/or caspase-8 (death receptor-mediated) and caspase-3 were activated due to the pathways involved as shown in Figure 6A-C. GTN induced MCF-7 cell apoptosis via caspase-9 and 8 whereas it induced HeLa cell apoptosis via caspase-9 only, but not caspase-8. GTN

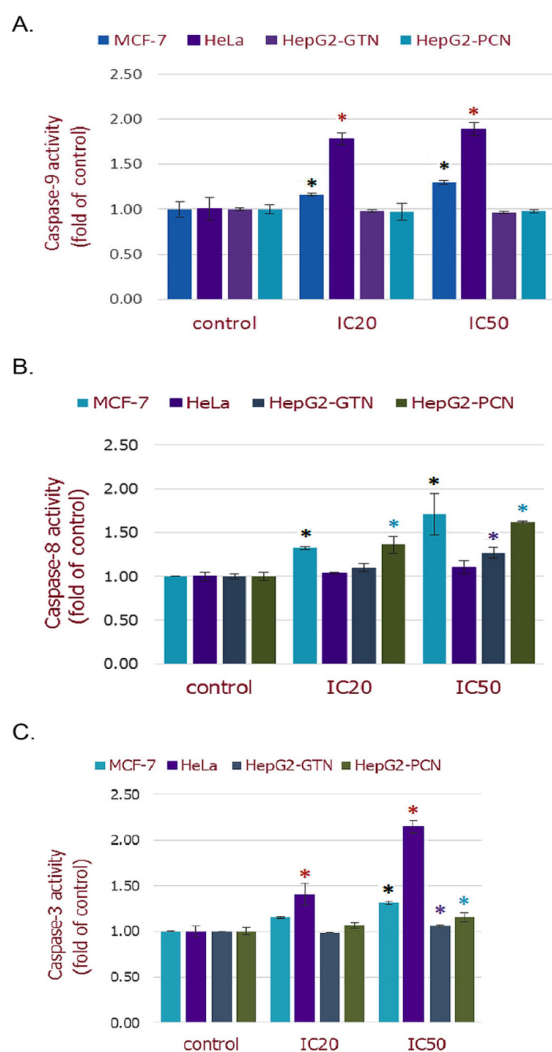


Figure 6. Activities of Caspases -9, -8 and -3 in Human Cancer Cells Treated with GTN and PCN.

The caspase-9 (A), -8 (B) and -3 (C) activities of various human cancer cell lines after treatment with GTN or PCN for 24 h were measured by using specific tetrapeptide substrates tagged with para-nitroaniline and the absorbance of cleaved *p*-NA was determined by spectrophotometer. The significance of statistical value compared to control (without treatment) is marked with asterisk, $p < 0.05$

or PCN induced caspase-8-mediated apoptosis in HepG2 cells. Whereas both GTN and PCN induced caspase-3 activity as their effector and executioner caspase in the cascade followed. The response of each cancer cells was dependent on the stimuli (whether it was GTN or PCN) and types of cancer cells, which determined the outcome as percentage of apoptotic cells and the intensity of MTP reduction together with types of caspase activation.

Discussion

Goniothalamine is potentially a genotoxic or clastogenic substance without any anti-genotoxic properties (Umar-Tsafe et al., 2004). GTN induces apoptosis in hepatoblastoma cancer cells via induction of caspase-3 with less sensitivity on the normal liver Chang cells (Al-Qubaisi et al., 2011; Al-Qubaisi et al., 2013).

GTN inhibits growth of HL-60 and U937 leukemic cell lines by apoptosis induction. Reduction of mitochondrial transmembrane potential, expressions of caspase-8, -9 and Smac/Diablo are increased in HL-60 cells. GTN-treated human leukemic cells undergo apoptosis via intrinsic and extrinsic pathways (Petsophonsakul et al., 2013). However, GTN induces early apoptosis in HeLa cells without the mechanistic revelation (Alabsi et al., 2012).

Antiproliferative effects of goniothalamine on Ca9-22 oral squamous cancer cells (OSCC) through apoptosis, ROS induction, mitochondrial depolarization and DNA double strand breaks, as evidenced by the higher level of γ H2AX (Yen et al., 2012).

Phorbol-12-myristate-13-acetate-induced protein 1 (NOXA) induces p53-dependent and -independent apoptosis in hepatocellular carcinoma-derived cells (Kuo et al., 2011). The proliferation of non-small cell lung cancer (NSCLC) cell line, H1299 is inhibited by GTN via DNA damage measured by comet assay. It attenuates cell migration and a reduction in the activities of two major migration-associated matrix metalloproteinases, MMP-2 and MMP-9 (Chiu et al., 2011).

GTN also plays pivotal roles on normal coronary artery smooth muscle cells (CASMCs). GTN induces DNA damage with concomitant elevation of p53 level demonstrating an upstream signal for apoptosis. The p53 elevation in GTN-treated CASMCs is independent of NAD(P)H: quinone oxidoreductase 1 and Mdm-2 expression. An increase in hydrogen peroxide and reduction in free thiols confirm the role of oxidative stress in GTN treatment. The sequential activation of caspase-2 and -9 but not caspase-8 leading to downstream caspase-3 cleavage is observed in GTN-treated CASMCs. Reduction of ATP level and decrease in oxygen consumption further confirm the role of mitochondria in GTN-induced apoptosis in CASMCs. The mitochondrial release of cytochrome c is found and is independent of cardiolipin (Chan et al., 2010). Restenosis represents a major impediment to the success of coronary angioplasty. Abnormal proliferation of vascular smooth muscle cells (VSMCs) has been shown to be an important process in the pathogenesis of restenosis. GTN may be a drug candidate for anti-restenosis (Chan et al., 2006).

GTN induces oxidative stress, DNA damage and apoptosis via caspase-2-independent and Bcl-2-independent pathways in Jurkat T-cells (Inayat-Hussain et al., 2010). GTN causes a decrease in GSH with an elevation of reactive oxygen species. Analysis using topoisomerase II processing of supercoiled pBR 322 DNA shows that GTN causes DNA damage via a topoisomerase II-independent pathway, suggesting that cellular oxidative stress may contribute to genotoxicity. Caspase-2 activity is activated and cytochrome c releases in GTN-treated Jurkat cells. Apoptosis is completely abrogated by N-acetylcysteine further confirming the role of oxidative stress (Inayat et al., 2010).

GTN from *Goniothalamus andersonii* also induces apoptosis in Jurkat T-cells as assessed by the processing of caspases-3 and -7 with the appearance of their catalytically active subunits of 17 and 19 kDa, respectively. Activation of these caspases is further evidenced by detection of

poly(ADP-ribose) polymerase cleavage (PARP). Pre-treatment with the caspase inhibitor benzoyloxycarbonyl-Val-Ala-Asp fluoromethyl ketone (Z-VAD.FMK) blocks apoptosis and the resultant cleavage of these caspases and PARP (Inayat et al., 1999).

GTN shows an inhibitory effect against Leukemic HL-60 cells and stomach cancer SGC-7901 cells (Zhou et al., 2005). MDA-MB-231-treated cells significantly induce cell cycle arrest at G(2)/M phase and apoptosis with an increase in intracellular reactive oxygen species, a decrease in intracellular free thiol contents and enhancement of cdc25C degradation (Chen et al., 2005). The metabolism of GTN by *Streptomyces aurofaciens* and *Nocardia* species produces two metabolites, i.e., 3,4-dihydrogoniothalamine and 3,4,7,8-tetrahydrogoniothalamine, which are also detected and identified in the urine and blood samples of the GTN-treated Sprague-Dawley rats (El-Sharkawy et al., 1996).

However, the reports of the pinocembrin inhibitory effect on cancer cytotoxicity are less than those of GTN. Most of the cytotoxic effects of PCN are neuroprotective against ischemic injury of the neurons. However, there is a report of PCN reversing autophagy induction and inhibiting apoptosis in prenumbra neurons (Zhao et al., 2014).

Even though there were previous reports of the effect of GTN or PCN on human cancer cells, the mechanisms involved are not clearly demonstrated. The caspase-mediated apoptosis via both intrinsic (caspase-9) and/or extrinsic (caspase-8) are shown in these three cell types with various extents. Both compounds were isolated from *G. griffithii* showing different degree of apoptotic cell induction.

In conclusion, the cytotoxic and apoptotic response of MCF-7, HeLa and HepG2 to GTN and HepG2 to PCN were different in the extent showing by their IC₅₀, percentage of cell positive for annexin V-FITC and negative/positive for PI, percentage of cells with reduced MTP and percentage of cells with the activation of caspases. These were due to the types of stimuli (GTN or PCN) and the types of cancer cells with different machineries, such as their expression of genes, whether oncogenic or tumor suppressor genes, their genomic instability and DNA damage and gene mutation.

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