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Goniothalamin Inhibits Growth of Human Lung Cancer Cells through DNA Damage, Apoptosis, and Reduced Migration Ability

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ABSTRACT: We evaluated the possible anticancer performance of a natural compound, goniothalamin (GTN), against human lung cancer using as a non-small cell lung cancer (NSCLC) cell line, H1299, as the model system. Cellular proliferation was significantly inhibited by GTN. Using an improved alkaline comet−nuclear extract (comet−NE) assay, GTN was found to induce a significant increase in the tail DNA. Wound healing and zymography assays showed that GTN attenuated cell migration and caused a reduction in the activity level of two major migration-associated matrix metalloproteinases, MMP-2 and MMP-9. It can be concluded that the DNA-damaging effect of GTN against lung cancer cells leads to growth inhibition as well as a depression in migration ability. Therefore, GTN has potential as a chemotherapeutic agent against lung cancer.

KEYWORDS: Goniothalamin, non-small cell lung cancer, comet assay, cell migration, matrix metalloproteinase

INTRODUCTION

Lung cancer is a leading cause of cancer-related mortality and has become a global health problem. Non-small cell lung cancer (NSCLC) causes more than 80% of all lung cancers, and the majority of NSCLC patients are unsuitable for surgery.[1] The development of chemotherapeutic drugs that target lung cancer seems to have reached a plateau.[2] Fortunately, many anticancer agents derived from natural products[3−5] have the possibility of improved therapeutic effects against lung cancer.

Goniothalamin (GTN) is one of the plant bioactive styryllactones found within the family Annonaceae, and it has been mainly isolated from the genus *Goniothalamus*.[6] GTN has been reported to induce cytotoxicity in a variety of cancers, including cervical,[7] gastric, kidney,[8,9] breast,[7,10] leukemia,[11−14] ovarian, melanoma, and colon cancers.[15] Some of these studies have demonstrated that the cytotoxicity induced by GTN occurs via apoptotic cell death.[11−14,16,17]

Accordingly, we proposed a hypothesis that GTN is also a potential anticancer drug against lung cancer. To test this hypothesis, we used *Goniothalamus amuyon*-derived GTN[18,19] to investigate the anticancer potential of the drug against a human NSCLC cell line, H1299, based on cell survival, an improved comet assay [comet−nuclear extract (comet−NE)],[20,21] changes induced in the cell cycle, apoptosis, and assays related to cell migration.

MATERIALS AND METHODS

Cell Cultures and Drug Information. A human NSCLC, H1299, was cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 μg/mL streptomycin, 0.03% glutamine, and 1 mM sodium pyruvate. The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. GTN from *G. amuyon*, prepared as described earlier,[18,19] was freshly dissolved in the dimethyl sulfoxide (DMSO) before being used to test the effects of the drug.

Assessment of Growth Proliferation. Cell growth was determined by the trypan blue dye exclusion assay as described earlier.[20,21] Briefly, cells were treated with the vehicle control (DMSO) or various doses of GTN for 24 and 48 h. Subsequently, the cells were exposed to 0.2% trypan blue and counted using a hemocytometer.

Assessment of DNA Damage. The comet−NE assay was performed as described previously,[20−22] except that the NE was derived from human gingival fibroblast cells. Briefly, 1×10^5 H1299 cells in suspension were added to 1.2% low-melting-point (LMP) agarose and subsequently transferred onto a precoated slide. A third layer (1.2% LMP gel) was added to the second gel and put on ice for solidification. The slides were soaked in freshly prepared ice-cold cell lysis solution

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at 4 °C for 2 h and then rinsed with deionized water 3 times. For NE digestion, a 20 μL excision mixture containing 0.6 μg of NE, 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-KOH (pH 7.9), 70 mM KCl, 5 mM $MgCl_2$, 0.4 mM ethylenediaminetetraacetic acid (EDTA), 2 mM ATP, 40 mM phosphocreatine, and 2.5 mM creatine phosphokinase was added to each slide. The slides were then incubated at 37 °C for 2 h in a humidified space with a coverslip. The slides were denatured and resolved by electrophoresis. Afterward, the DNA damage was analyzed.[22] Using a fluorescence microscope (TE2000-U; Nikon, Tokyo, Japan), the migration of DNA from the nucleus of each cell was measured with a computer program (http://tritekcorp.com) to calculate percent tail DNA,[20−22] which corresponds to the percentage of DNA in the comet tail (sum of intensities of pixels in the tail); i.e., head percent DNA = (head optimal intensity)/(head optimal intensity + tail optimal intensity) × 100. Tail percent DNA = 100 − head percent DNA.

Assessment of Cell Cycle Distribution and Sub-G1 Population. To determine the phase distribution of the DNA content of the cell, propidium iodide (PI) staining was performed as described earlier.[23] Briefly, cells were treated with solvent vehicle or 0.5, 1, 2, 5, and 10 μM GTN for 24 and 48 h. After treatment, the cells were collected, washed twice with phosphate-buffered saline (PBS), and fixed in 70% ethanol overnight. After centrifugation at 700 rpm for 5 min at 4 °C, the cell pellet was stained with 10 μg/mL PI (Sigma, St. Louis, MO) and 10 μg/mL RNase A in PBS buffer for 15 min at room temperature in the dark. The samples were assayed using a FACScan flow cytometer (Becton-Dickinson, Mansfield, MA) and CellQuest software (Becton-Dickinson).

Assessment of Apoptosis. To further confirm apoptosis in GTN-induced H1299 cells, annexin V staining (Pharmingen, San Diego, CA) was performed as described before.[23] A total of 1 × 10^6 cells per 100 mm Petri dish were treated with vehicle or GTN for 24 h. Subsequently, cells were labeled with 10 μg/mL annexin V−fluorescein isothiocyanate (FITC). The apoptotic cells were analyzed using flow cytometry.

Assessment of Cell Migration. In brief, a total of 5 × 10^5 H1299 cells were seeded onto 12-well plates, treated with GTN or DMSO, and then grown to complete confluence. A yellow 200 μL plastic pipet tip was used to create a clean 1 mm wide wound area in the H1299 confluent culture. After incubation for 24 and 48 h, the wound gaps were photographed. The wound areas were then examined and calculated using the software "TScratch".[24]

Gelatin Zymography. The gelatin zymography was performed using 10% polyacrylamide gels containing 0.1% gelatin. After electrophoresis, the sodium dodecyl sulfate (SDS) present was removed by treating with Triton X-100 (2.5%), followed by incubation in a Tris-based buffer containing NaCl, $CaCl_2$, and $ZnCl_2$ at 37 °C overnight. The gel was then stained with Coomassie Brilliant Blue, and gelatinase activity was detected as unstained gelatin-degradation zones within the gel. The signals were analyzed using Gel Pro, version 4.0, software (Media Cybernetics, Silver Spring, MD).

Statistical Analysis. All data are presented as the mean ± standard deviation (SD). Student's *t* test was used to test the significance of the mean difference between two groups.

RESULTS

GTN Induced Growth Inhibition in H1299 Lung Cancer Cells. As shown in Figure 1, the trypan blue assay showed that the relative cell proliferation at various concentrations of GTN (0, 1, 2, 5, and 10 μg/mL) after 24 and 48 h were 51.79 ± 5.92, 35.14 ± 5.09, 22.29 ± 6.04, and 9.30 ± 7.37 (n = 3) and 38.04 ± 3.82, 16.08 ± 1.14, 3.15 ± 0.17, and 0.45 ± 0.64 (n = 3), respectively. Treatments with GTN caused growth inhibition that was significant and dose-dependent (p < 0.05 at a dose of 1 μg/mL and p < 0.01 at doses of 2, 5, and 10 μg/mL for control versus GTN treatment). The IC_{50} values of the GTN-treated H1299 lung cancer cells at 24 and 48 h were 1.15 μg/mL (5.75 μM) and 0.83 μg/mL (4.15 μM), respectively.

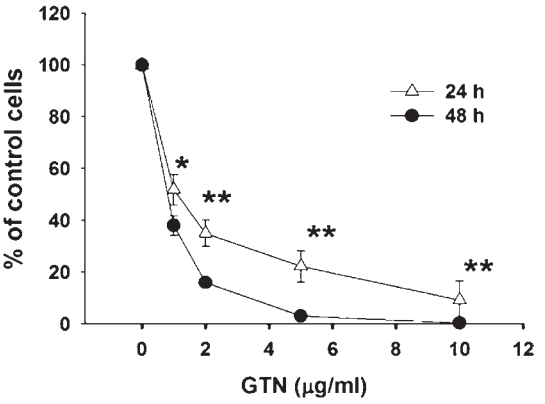


Figure 1. Proliferation of H1299 lung cancer cells is inhibited by GTN. Cells were incubated with various concentrations of GTN (0, 1, 2, 5, and 10 μg/mL) for 24 and 48 h. The proliferation was determined by the trypan blue assay. The data are expressed as the mean ± SD n = 3. (*) p < 0.005 and (**) p < 0.001 against the vehicle.

GTN Induced DNA Damage in H1299 Lung Cancer Cells When Measured by the Comet Assay. In the comet assay using H1299 cells (Figure 2A), no "tail" was found in the vehicle control (0.1% DMSO alone), whereas the "tail" grew following GTN treatment (2, 5, and 10 μg/mL for 2 h). Figure 2B shows that the values for the average percent of tail DNA of the GTN treatments (2, 5, and 10 μg/mL for 2 h) were 58.47 ± 18.47, 58.47 ± 18.47, and 90.69 ± 4.38 (n = 50), respectively (p < 0.001). Accordingly, GTN was able to induce DNA damage in H1299 cells in a significant and dose-dependent manner. These results suggested that DNA damage is involved in the antiproliferation effect of GTN on H1299 lung cancer cells.

GTN Induced Sub-G1 Accumulation in H1299 Lung Cancer Cells Using Flow Cytometry. As shown in Figure 3, the sub-G1 populations at concentrations of 0, 1, 2, 5, and 10 μg/mL GTN were 0.80 ± 0.07, 4.05 ± 0.38, 18.37 ± 0.67, 76.76 ± 0.04, and 98.76 ± 0.19 (n = 3), respectively. The sub-G1 population induced by GTN was slightly increased at a dose of 1 μg/mL (p < 0.05) and became significantly increased at the higher doses of 5 and 10 μg/mL (p < 0.001). It can be concluded that the GTN-induced sub-G1 population of H1299 lung cancer cells was significantly increased by GTN and that this occurred in a dose−responsive manner.

GTN Induces Apoptosis of H1299 Cells Measured by Flow-Cytometry-Based Annexin V Staining. To determine whether GTN inhibits cell survival by inducing apoptosis, flow-cytometry-based annexin V staining was performed. H1299 cells cultured with different concentrations of GTN for 24 h were stained with annexin V to detect the externalization of PS on the cell membrane. After treatments with vehicle control or 1, 2, 5, and 10 μM GTN for 24 h, the percentage of apoptotic cells was calculated and shown in Figure 4. These results showed that GTN significantly induced apoptosis of H1299 cells in a dose−response manner (p < 0.01 and 0.001 as indicated).

GTN Attenuated Migration of H1299 Lung Cancer Cells in the Wound Healing Assay. Figure 5A shows that the migration of H1299 lung cancer cells was dramatically inhibited by GTN. Figure 5B showed that the migration ability of H1299 cells treated with various GTN concentrations at 0, 1, 2, 5, and 10 μg/mL

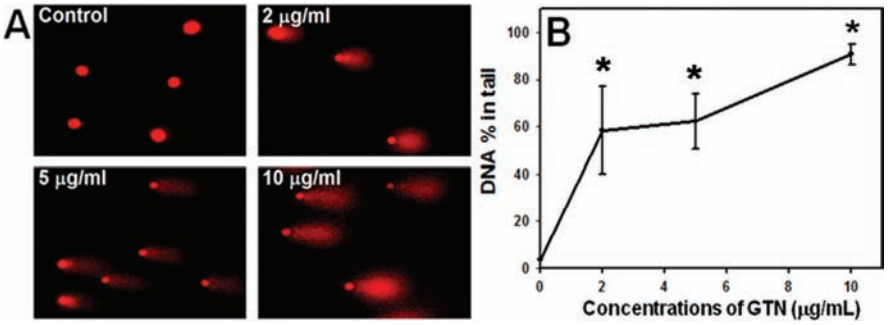
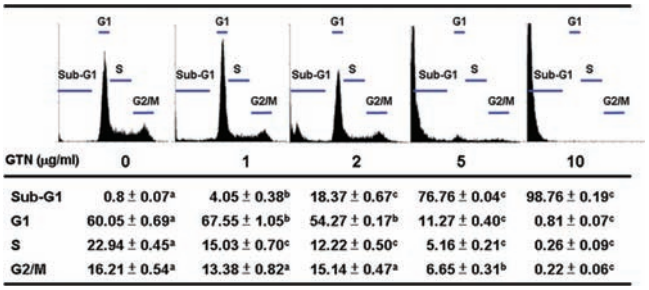


Figure 2. Comet−NE assays of H1299 lung cancer cells after treatment with GTN. (A) PI staining of the control (DMSO) and GTN-treated cells (1, 2, 5, and 10 μg/mL for 2 h). The circular spots in pink are the nuclei, and the tails indicated the DNA damage. (B) Average of tail percent DNA for the various GTN-treated cells. (∗) $p < 0.001$ against the vehicle.



GTN (μg/ml)	0	1	2	5	10
Sub-G1	0.8 ± 0.07[a]	4.05 ± 0.38[b]	18.37 ± 0.67[c]	76.76 ± 0.04[c]	98.76 ± 0.19[c]
G1	60.05 ± 0.69[a]	67.55 ± 1.05[b]	54.27 ± 0.17[b]	11.27 ± 0.40[c]	0.81 ± 0.07[c]
S	22.94 ± 0.45[a]	15.03 ± 0.70[c]	12.22 ± 0.50[c]	5.16 ± 0.21[c]	0.26 ± 0.09[c]
G2/M	16.21 ± 0.54[a]	13.38 ± 0.82[a]	15.14 ± 0.47[a]	6.65 ± 0.31[b]	0.22 ± 0.06[c]

Figure 3. GTN induces an accumulation in the sub-G1 population in H1299 lung cancer cells. Cells were treated with the indicated doses, 1, 2, 5, and 10 μg/mL, of GTN for 24 h, respectively. (A) Accumulation of the sub-G1 population in GTN-treated H1299 cells and vehicle controls at 24 h. (B) Quantification analysis. Data are presented as the mean ± SD ($n = 3$). Different letter notations indicate the statistical significance between the drug treatment and vehicle (a versus b and a versus c indicate $p < 0.005$ and 0.001, respectively).

was 100, 72.74 ± 1.28, 41.12 ± 0.99, 31.01 ± 2.43, and 7.30 ± 1.76% ($n = 3$), respectively. This indicates that cellular migration in H1299 lung cancer cells was inhibited by GTN in a dose−responsive manner.

GTN Inhibited MMP-2 and MMP-9 Activities in H1299 Lung Cancer Cells Based on Gelatin Zymography. The activity levels of MMP-2 and MMP-9 are shown in panels C and D of Figure 5, respectively. The fold changes in the MMP-2 activity at GTN concentrations of 0, 1, 2, 5, and 10 μg/mL were 1, 0.86 ± 0.07, 0.67 ± 0.04, 0.46 ± 0.02, and 0.40 ± 0.18 ($n = 3$), respectively. The fold changes in MMP-9 activity at GTN concentrations of 0, 1, 2, 5, and 10 μg/mL were 1, 0.68 ± 0.25, 0.51 ± 0.25, 0.35 ± 0.26, and 0.25 ± 0.17 ($n = 3$), respectively. These results show that GTN significantly attenuates MMP-2 and MMP-9 activities in H1299 cells in a dose−responsive manner.

DISCUSSION

The cytotoxic effect of GTN has been demonstrated in many types of cancer. For example, the IC_{50}, IC_{25}, and IC_{10} values for GTN-treated vascular smooth muscle cells (VSMCs)[16] after 72 h are 4.4 μg/mL (22 μM), 1.5 μg/mL (7.5 μM), and 0.5 μg/mL (2.5 μM), respectively. After 72 h of treatment with GTN, the IC_{50} values of HL-60 (a human promyelocytic leukemia cell line) and CEM-SS (a T-lymphoblastic cell line) are 4.5 and 2.4 μg/mL, respectively. In this study, we obtained a novel finding that GTN has a cytotoxic effect on H1299 lung cancer cells. In addition, the effect of GTN is time-dependent based on the 24 and 48 h results.

Because the normal human lung cells are not available in our lab, the test of cancerous lung cells was performed in the study. However, some studies have been discussed about the possible cytotoxicity of GTN upon the normal cells. For example, Tian et al. indicated that GTN and its derivative showed less toxicity on normal mice hepatocyte compared to human hepatocellular carcinoma (HCC) HepG2 and that the IC_{50} values of them on normal mice hepatocyte were about 3 times that on HepG2.[25] The study by Wattanapiromsakul et al. indicated that GTN exerted a promising cytotoxicity against colon cancer, breast cancer, and lung cancer cells but that the cytotoxic evaluation of GTN to normal cells revealed moderate toxicity against mouse skin fibroblast ST3 and human fibroblast HF cells.[26] Accordingly, these studies suggested that the cytotoxicity of GTN toward cancer cells and normal cells may be discrepant. However, we still cannot exclude the possibility that GTN may cause cell damage in normal lung cells.

Although GTN has been found to be cytotoxic against many types of cancer,[7,8,10−15,27] the detailed mechanisms have rarely been addressed, except in terms of apoptosis. Recently, DNA damage has been reported to be involved in the GTN-induced cytotoxic effect; this was based on the percentage of cells with DNA damage using VSMCs[16] and leukemia cell lines,[12,28] as measured by the comet assay.[29] Consistent with these findings,[12,28] in this study, we have demonstrated that GTN induces DNA damage in GTN-treated H1299 lung cancer cells based on the comet−NE assay (Figure 2). There are an about 60, 40, and 20% changes in tail length at IC_{50}, IC_{25}, and IC_{10}, respectively. Our results support the idea that GTN induces DNA damage in a dose-dependent manner in H1299 lung cancer cells prior to cytotoxicity.

According to the study by Inayat-Hussain et al., they demonstrated that GTN induced DNA damage and that reactive oxygen species (ROS) leads to apoptosis in human lymphocyte Jurkat T-cells. Additionally, the DNA damage induced by GTN is independent of topoisomerase IIα inhibition.[12] Furthermore, our preliminary result showed that GTN induced phosphorylation of γ-H2AX, a hallmark of DNA double-strand break (DSB) (data not shown). When these observations are taken together, they suggest that GTN seems to cause DSB of DNA damage via ROS generation but not inhibit topoisomerase IIα directly.

Our study has also shown that there is a dose-dependent accumulation in the sub-G1 population of cells (Figure 3) after

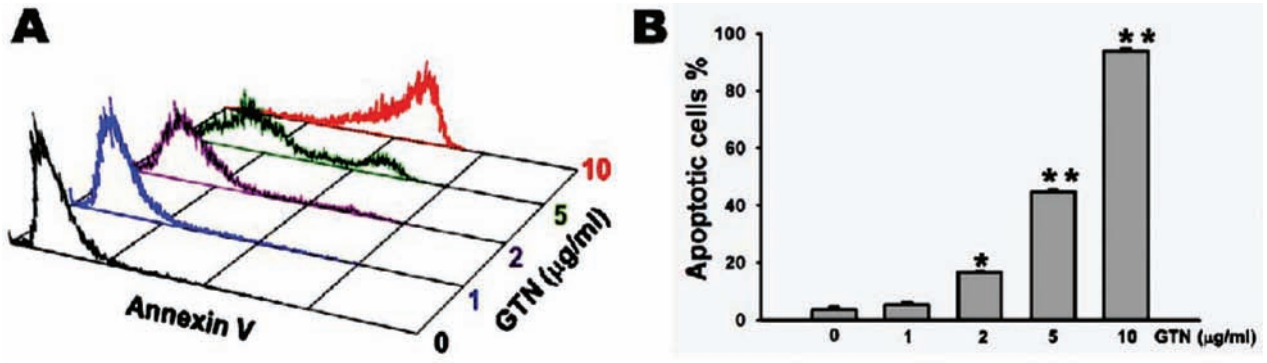


Figure 4. GTN induced apoptosis of H1299 cells. (A) Cells cultured with different concentrations of GTN for 24 h were stained with annexin V/PI to detect externalization of PS from the cell membrane. (B) Quantification analysis of annexin V staining. (∗) $p < 0.01$ and (∗∗) $p < 0.001$ for the control versus GTN-treated cells, respectively.

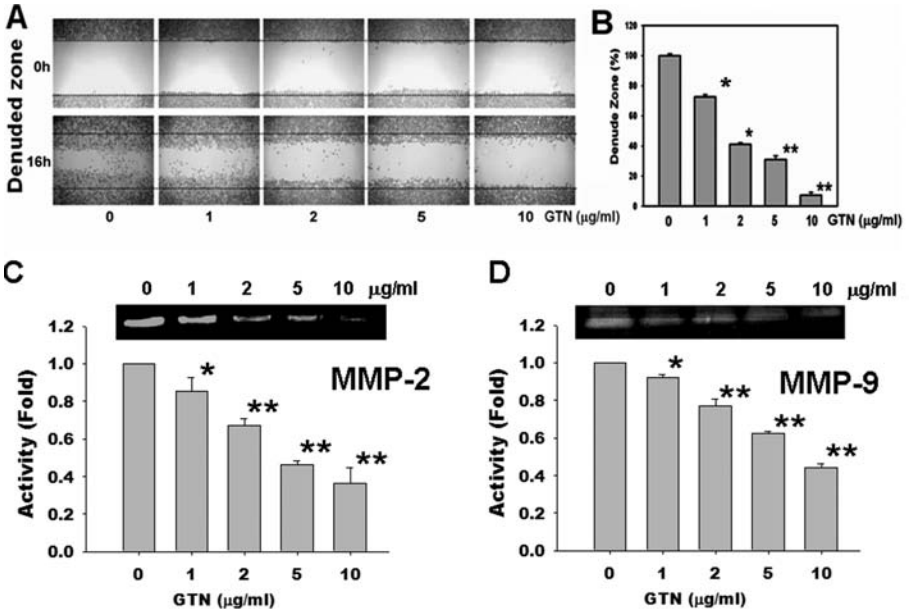


Figure 5. GTN inhibits cell migration in H1299 lung cancer cells. (A) A total of 5×10^5 cells were seeded onto a 12-well plate, and the cells were scraped to create a clean 1 mm wide wound area within the confluent culture. Cells were treated with the indicated doses of 0, 1, 2, 5, and 10 μg/mL GTN for 16 h. Afterward, the wound gaps were photographed using an inverted phase-contrast microscopy. (B) Quantification analysis. (C and D) Gelatin zymography assays of MMP-2 and MMP-9 activities. (∗) $p < 0.05$ and (∗∗) $p < 0.001$ against the vehicle.

GTN treatment of H1299 lung cancer cells but without clear cell cycle arrest occurring. These sub-G1 accumulations had been confirmed to be apoptosis based on the annexin V staining result (Figure 4). However, we cannot exclude the possibility for the involvement of necrosis and autophagy, and it needs to be further investigated in the future.

In breast cancer cells (MDA-MB-231), GTN has been reported to significantly induce cell cycle arrest at G2/M phase and then apoptosis occurred.[30] Although activation of the DNA damage response pathways is known to involve both cell cycle arrest and apoptosis, they are regulated by distinct cellular machinery. Moreover, apoptosis may happen independently of cell cycle arrest under certain conditions.[31,32] For instance, a low paclitaxel dose (10 nM) caused apoptosis without cell cycle arrest, but the same drug caused G2/M arrest at higher doses (>50 nM).[32] Similarly, we found that GTN induced a dose-dependent accumulation of the sub-G1 population and an apoptotic annexin V signal for H1299 cells. Therefore, the cell cycle arrested at a specific phase is not an essential event always associated with increased apoptosis.[33]

To further clarify the relationship between the higher dose GTN treatment and cell cycle arrest in H1299 lung cancer cells, we perform the annexin V staining assay and the result showed that a higher dose (5 and 10 μg/mL) of GTN triggered a significant apoptotic cell death of human lung cancer H1299 cells, whereas a lower dose (2 μg/mL) of GTN only induced a moderate accumulation of apoptotic population. Accordingly, these results suggest that the induction of apoptosis by GTN is a dose-dependent manner (Figure 4).

The extracellular matrix (ECM) plays an important role in tumor proliferation and migration.[34] Many anticancer therapies are based on killing cancer cells by generating high ROS.[35] A low level of intrinsic ROS may induce activation and releases of MMP-2 and MMP-9, which are responsible for cancer metastasis.[36]

Because GTN has been reported to be capable of modulating the redox status of cells and this is known to lead to apoptosis in breast cancer cells (MDA-MB-231),[30] we examined cell migration, MMP-2 activity, and MMP-9 activity (Figure 4) in H1299 cancer cells. In our study, the cell migration in wound healing (Figure 5) and transwell studies (data not shown) of H1299 lung cancer cells was markedly inhibited by GTN, even at the lowest (non-cytotoxic) dose. Therefore, these studies suggested that GTN has potential for inhibiting MMP-mediated lung cancer progression. To the best of our knowledge, this is the first demonstration that GTN dramatically attenuates the migration ability of cancer cells by inhibiting MMP-2 and MMP-9 activities.

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