GONIOTHALAMIN INHIBITS CELL GROWTH, PERTURBS CELL CYCLE AND INDUCES APOPTOSIS IN HUMAN OSTEOSARCOMA SAOS-2 CELLS

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Abstract

Goniothalamin (GTN), a naturally occurring styryl-lactone extracted from *Goniothalamus sp.* has been reported to possess a potent antitumor effect against several types of cancer cells. Nevertheless, the anticancer effect of GTN has not been explored in bone cancer. The present study was designed to evaluate GTN potential anticancer effects in human osteosarcoma Saos-2 cells and to determine the possible mechanisms with respect to apoptosis induction, reactive oxygen species (ROS) release, activation of executioner caspase 3/7 and effects on the cell cycle. The current data demonstrated that cell proliferation was significantly inhibited by GTN in a concentration- and time-dependent manner. This was achieved primarily *via* apoptosis where the flow cytometric analysis showed a significant increment of apoptotic cells, from 7.23±0.75 to 39.86±0.54 and 1.38±0.15% to 3.27±0.27% for early and late apoptotic cells, respectively. Moreover, GTN induced a significant increase in intracellular ROS levels and activated caspase 3/7. Cell cycle analysis of GTN-treated cells showed the population of G2/M phase was significantly arrested with 53.12±0.84% compared to the untreated cells with 28.64±0.73%. Taken together, these results suggest that the suppression of GTN-treated Saos-2 cells was associated with apoptosis and G2/M cell cycle arrest. Due to its antiproliferative and proapoptotic effects, GTN has a potential to be used as a chemotherapeutic agent against bone cancer.

***Keywords*:** Apoptosis; Caspase; Cell Cycle; Goniothalamin; Saos-2

Introduction

Osteosarcoma, which usually affects young adults, is the most common histological subtype of primary bone cancer (1). This aggressive malignant neoplasm originates from primitive transformed cells of mesenchymal origin, displays osteoblastic differentiation, and generates malignant osteoid (1, 2). Osteosarcoma is always treated with a combination of chemotherapy and surgical removal of the tumour. The most common chemotherapeutic

drugs used in clinical include doxorubicin, methotrexate, cisplatin and ifosfamide. However, new strategies and innovative approaches are in demand to tackle the complexity of osteosarcoma since only up to 65% probability of survival has been documented in non-metastatic osteosarcoma patients. The successful treatment is usually associated with high toxicity and non-selective effect, suggesting the unwanted adverse effects on the normal cells counterpart (3). Therefore, it is necessary to identify better compounds with selective inhibitory effects against cancer cells.

The development of numerous important classes of drugs has historically relied on natural products, particularly those derived from plants, which have continued to serve as templates even to this day (4). Paclitaxel, epipodophyllotoxin, vinblastine, vincristine, combretastatin and betulinic acid are some examples of plant-derived drugs that have been used clinically and there are many more that have been studied for further improvement and development including those with anticancer properties (5).

Goniothalamus is a genus of palaeotropical Annonaceae that is widely distributed throughout tropical Southeast Asia, consisting of more than 160 species. The region of Indochina and Western Malaysia is the primary location of its diversity (6). One of its bioactive compounds, goniothalamin (GTN) has been reported to possess anticancer activities in many cancer cell lines including leukaemic, breast, lung, oral, cervical, colon, ovarian, pancreatic and prostate cancer cells (7-10). Our previous study also demonstrated that GTN is an effective and selective therapeutic agent with a very strong range of cytotoxicity against some cancer cell lines such as Saos-2, MCF-7, UACC, A549, HT29 and less toxicity towards the normal cells, human mesenchymal stem cell HMSC (11). The selective inhibitory effects suggest that GTN has unique molecular properties that can cause cytotoxic effects on specific cells and thus signify the importance of further exploring this compound for the development of a new cancer-specific anticancer agent. The purpose of this study was to examine the possible mechanisms of GTN in Saos-2 cells related to release of ROS, cell cycle effects, induction of apoptosis, and activation of executioner caspase 3/7.

Materials and methods

Goniothalamin (GTN)

The white crystal compound isolated from the roots of *Goniothalamus macrophyllus* was kindly provided by Prof. Dr. Abdul Manaf Ali from Universiti Sultan Zainal Abidin (UniSZA). It was previously extracted as described by Jusoh et al. 2015 (12). ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) spectra were measured in deuterated dimethyl sulfoxide (dimethyl sulfoxide-d_6) using Bruker Spectrometer at Institute of Marine Biotechnology, Universiti Malaysia Terengganu (400 MHz for proton and 100 MHz for ^{13}C) to validate the compound. Prior to any experimental activities, the stock and working solution were prepared as described in our previous study (11). Briefly, the working solution was prepared at a concentration of 60 µg/mL by diluting the stock solution with Phosphate Buffered Saline (PBS).

Cell culture and treatment

The osteosarcoma cell line Saos-2 (HTB-85, ATCC), was grown in complete DMEM medium (Sigma, USA), containing 10% foetal bovine serum (Nacalai Tesque, Japan), penicillin (100 IU/mL) and streptomycin (100 µg/mL) and incubated in 5% CO_2 incubator at 37°C with 90% humidity. The cells were subcultured when attained 80 % confluence, which was observable using an inverted microscope.

The cells were seeded into a 96-well plate at a concentration of 5×10^4 cells/mL prior to the cell treatment. The treatments of Saos-2 cells were performed based on the strong activity of GTN as obtained in our previous study, with IC_{50} values below 5 µg/mL at 24, 48 and 72 hours of incubation times (11). To further examine the effect of GTN on the proliferation of Saos-2 cells, different GTN concentrations ranging from 0.31 to 5.00 µg/mL were applied. GTN at a concentration of 5 µg/mL was used in a scratch wound assay to treat the confluent cells. For other experiments such as the ROS release, cell cycle, Annexin V FITC/PI and caspase assay, the cells were treated with the IC_{50} concentration of GTN, 1.2 µg/mL (11). Doxorubicin (DOX) at 0.5 µg/mL concentration was used as a positive control in this study.

Real-time assessment of cell proliferation

Saos-2 cells were plated in a 96-well plate and left to incubate overnight at a temperature of 37°C in a 5% CO_2 incubator (Shellab, USA). The following day, the cells were treated and placed in IncuCyte™ ZOOM System (Essen BioScience, USA) to measure the real-time proliferation of live Saos-2 cells quantitatively for up to 70 hours.

Scratch assay

The Saos-2 cells were seeded in IncuCyte® ImageLock 96-well microplates and incubated in 5% CO_2 incubator at 37°C temperature (Shellab, USA) until the cells formed a confluent monolayer. Then, they were scratched using the WoundMaker™ (Essen BioScience) which is a mechanical tool with 96 pins that can create 700-800 µm homogeneous wounds in cell monolayers on the plate. The plate was then washed twice with PBS to remove cell debris and followed by treatment with GTN. The time-

lapse images were taken hourly and the proliferation of cells covering the scratch-gap was monitored using the IncuCyte ZOOM® system (Essen BioScience) at 10× magnification.

Intracellular ROS release

The OxiSelect™ Intracellular ROS Assay Kit (Cell Biolabs, USA) was used to detect the intracellular ROS generation. Briefly, the seeded Saos-2 cells were treated and incubated for 2 and 24 hours. The cells were washed using PBS. Then, 1 µg/mL of 2, 7-dichlorodihydrofluorescein diacetate (DCFH-DA) dye was added and incubated for 30 minutes at 37°C. The cells were then washed twice with fresh culture media. DCF fluorescence was measured using a fluorescence microplate reader (BMG Labtech, Germany), at 485 nm excitation and 520 nm emission.

Cell cycle analysis

The Saos-2 cells were seeded in 6-well microplates under serum deprivation conditions to synchronise the cell phase and incubated overnight in 5% CO_2 incubator at 37°C (Shellab, USA). The treatment was carried out the next day in a complete growth medium and then incubated for a further 24 hours. After incubation time, the cells were collected and fixed in 70% ethanol and incubated for 1 hour at 4°C. The fixed cells were spun down at 300 × *g* and a volume of 500 µL of FxCycle™ PI/RNase staining solution was added to the pellet followed by incubation for another 15 to 30 minutes in the dark and at room temperature. The cell cycle progression of the stained cells for 15,000 events were analysed using FACSCalibur flow cytometer (BD Biosciences, USA) with CellQuest Pro software. The data obtained were further analysed using ModFit software (Verity Software House Inc).

Annexin V FITC assay

The apoptotic event was detected using FITC Annexin V Apoptosis Detection Kit II (BD Biosciences, USA) according to the kit's manual. The Saos-2 cells were seeded at a concentration of 5×10^4 cells/mL in 6-well microplates and left to incubate overnight in a 5% CO2 incubator (Shellab, USA) at 37°C. After 24 hours of treatment, the culture media was transferred to a new tube while the cells were detached and mixed back to their respective culture media followed by centrifugation at 300 × *g*. A volume of 100 µL of 1× binding buffer was added to the cell pellet and transferred to a 5 mL round bottom culture tube (BD Biosciences, USA). The cell was then stained with 5 µL Annexin V-FITC-Propidium Iodide (PI) (1:1). The mixture was incubated for 15 minutes in the dark at room temperature. A volume of 400 µL of 1× binding buffer was added to stop the reaction and the stained cells were analysed by FACSCalibur flow cytometry (BD Biosciences, USA) for at least 5,000 events.

Caspase 3/7 assay

The activation of caspase 3 and 7 was examined using Cell Meter™ Caspase 3/7 Activity Assay Kit (AAT Bioquest®, USA) accordance to the instructions in the manufacturer's manual. Briefly, after treating the cells for 24 hours, a volume of 100 µL of caspase solution (caspase 3/7 substrate DEVD-ProRed™) was added and further incubated at room temperature for 1 hour, protected from light. The fluorescence intensity was measured by a fluorometer plate reader (BMG Labtech, Germany) at 535 nm excitation and 620 nm emission.

Statistical analysis

All experiments were conducted in three independent experiment (n=3) and each data represents mean±standard deviation (SD). The comparison between untreated and the GTN-treated Saos-2 cells was done using one-way ANOVA, followed by the Dunnett's post-test to detect any significant differences (* $p<0.05$; ** $p<0.01$; *** $p<0.001$; ns = not significant).

Results

GTN inhibits the proliferation of Saos-2 cells

The real-time proliferation of Saos-2 cells was assessed quantitatively using the IncuCyte™ ZOOM System (Essen BioScience, USA). As shown in Figure 1, GTN was found to inhibit Saos-2 cells proliferation in a dose- and time-dependent manner. The untreated Saos-2 cells exhibited normal proliferation and achieved 95% confluence after 70 hours. In contrast, the GTN-treated cells at 0.31, 0.63 and 1.25 µg/mL only reached confluence at 60, 55 and 45%, respectively. GTN treatment at the highest concentration (5 µg/mL) gave the lowest percentage of confluence of Saos-2 cells with 30%, indicating approximately three-fold decrease compared to the untreated cells. As depicted in Figure 2, the confluent monolayer untreated cells took less than 21 hours to close the cell-free area, whereas the addition of GTN to the culture of confluent cells has clearly affected cell proliferation by delaying the closure of the scratch. Figure 3 shows that the percentage of wound confluence had significantly ($p<0.05$) dropped from 15.99±1.13% to 8.21±2.26% after 6 hours of treatment with GTN. The percentage of wound confluence was significantly ($p<0.001$) reduced by the GTN treatment at 9 and 12 hours, from 21.42±2.85 and 24.41±3.38% to 10.12±2.25 and 11.67±2.52%, respectively.

GTN induces the production of intracellular ROS

Figure 4 shows a relatively low level of ROS observed in control cells, whereas a marked (p<0.05) increase in intracellular ROS levels was observed after adding GTN for 2 hours, however, it remains constant after 24 hours. ROS levels were also increased significantly (p<0.05) in cells treated with DOX, although not significantly until 24 hours after treatment.

GTN perturbs the progression of cell cycle

The effects of GTN on cell cycle progression of Saos-2 cells after 24 hours were evaluated by flow cytometry, as illustrated in Figure 5. Noticeably, the highest peak of G2/M phase was observed in cells treated with GTN (b) while the highest peak of S phase was observed in cells treated with DOX (c). As shown in Figure 6, treatment with GTN had caused a significant (p<0.001) cell cycle arrest at the G2/M phase, with 53.12±0.84% detected compared to 28.64±0.73% in the untreated cells. The increased percentage of G2/M phase accumulation had caused a significant (p<0.001) reduction of S phase with 4.95±1.15%. Meanwhile, treatment with DOX caused a significant (p<0.001) accumulation of S phase with 63.01±1.36% increment detected compared to only 36.42±0.45% in untreated cells. Table 1 summarises the percentage events according to the respective cell cycle stages; G0/G1, S and G2/M.

GTN induces apoptosis and activates caspase 3/7

As shown in Figure 7, After 24 hours of treatment with GTN and DOX, there was a significant (p<0.001) reduction in the number of viable cells and an increase in apoptotic cells, both for early apoptotic cells (FITC+PI-) and late apoptotic cells (FITC+PI+). In comparison to the untreated cells, GTN decreased the percentage of viable cells from 90.45±0.86 to 56.77±0.64% (Table 2). The percentage of early and late apoptotic cells in the untreated cells were 7.23±0.75 and 1.38±0.15% respectively and were found to be increased to 39.86±0.54 and 3.27±0.27%, respectively in the GTN-treated Saos-2 cells. The percentage of viable, early apoptotic, late apoptotic and necrotic cells in DOX-treated Saos-2 cells were 67.11±1.45, 22.18±0.39, 10.65±1.40 and 0.04±0.01%, respectively. The involvement of effector caspase, caspase 3/7 in response to the treatment of GTN was also evaluated. As shown in Figure 8, both GTN and DOX activated caspase 3/7 after 24 hours of exposure time.

Discussion

GTN has been reported to possess anticancer activities in many cancer cell lines including leukaemia cells Jurkat T, K562, CEMSS and HL60, liver cancer cells HepG2 and HCC, breast cancer cells MCF-7, T47D and MDA-MB-231, lung cancer cells COR-L23, NCI-H460 and NSCLC, oral cancer cells Ca9-22, cervical cancer cells HeLa, colon cancer cells HT29 and LS174T, ovarian cancer cells Caov-3, pancreatic cancer cells PANC-1 and prostate cancer cells PC-3 (13–22). Besides, GTN has also been reported to have less toxicity to normal liver Chang cell, normal kidney cell (MDBK) and normal mouse fibroblast (7, 20, 22).

Our previous study also demonstrated GTN as an effective and selective therapeutic agent with a very strong range of cytotoxicity against some cancer cell lines such as Saos-2, MCF-7, UACC, A549, HT29 and less toxicity towards normal cells, human mesenchymal stem cell HMSC. GTN demonstrated very selective and potent cytotoxic activities against Saos-2 cells with low IC_{50} value; 1.29±0.08 µg/mL and high selectivity index; 5.04±0.64 as compared to normal HMSC cells (11). The real-time analysis conducted in this study revealed that GTN inhibited the proliferation of Saos-2 cells in a manner that was dependent on both the dose and duration of treatment. A higher dose of GTN resulted in a lower confluency of Saos-2 cells. In addition, the cell progression on the scratch was found to be significantly delayed by GTN, leaving the gap open even after 21 hours. The underlying mechanism associated with the inhibitory effects of GTN on Saos-2 cells was further explored by evaluating the release of ROS, the effects on the cell cycle, induction of apoptosis and activation of executioner caspase 3/7.

Emerging evidence has shown that the formation of ROS and the resulting oxidative stress play a crucial role in mechanism of cell death, either by nonphysiological (necrotic) or regulated pathways (apoptotic) (23, 24). In this study, the control cells had a comparatively minimal level of ROS, while the addition of GTN for 2 hours resulted in a noticeable increase in the formation of intracellular ROS. However, it remains constant after 24 hours. Cells treated with DOX also showed an increase in ROS levels, although this increase was not statistically significant until 24 hours post-treatment.

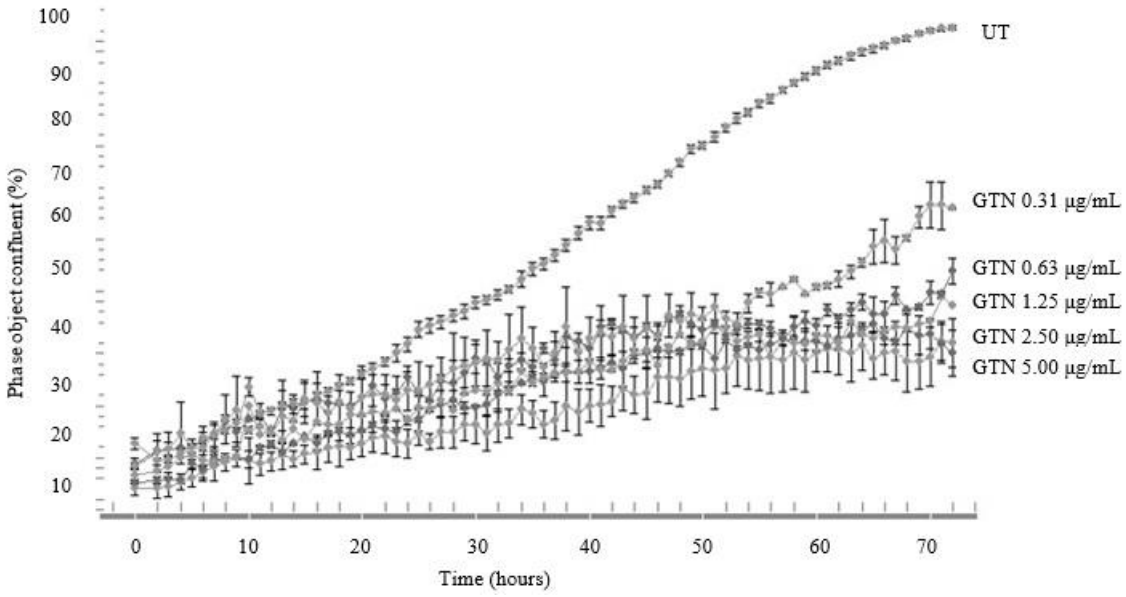

Figure 1: The real-time proliferation of Saos-2 cells measured by IncuCyte™ ZOOM Live Cell System for up to 70 hours. The cells were treated with GTN at different concentrations ranging from 0.31 to 5.00 µg/mL.

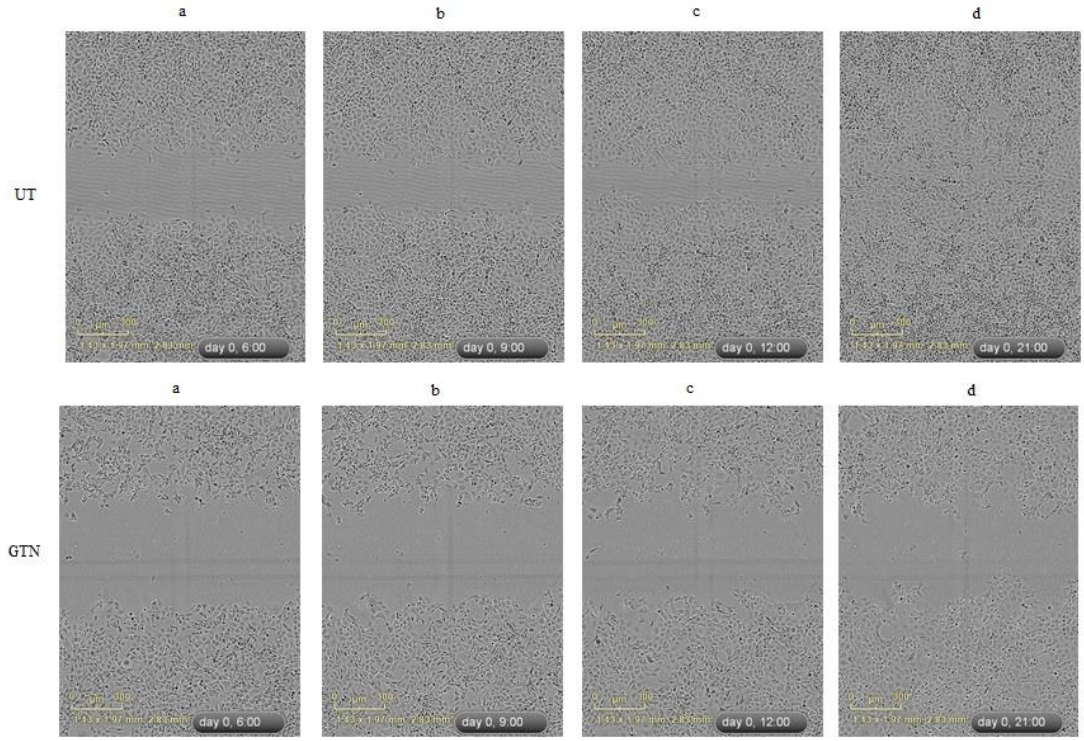

Figure 2: A confluent monolayer of Saos-2 cells was mechanically wounded (scratch assay), leaving two wound edges separated by a 700-800 µm wide void (cell-free area). The proliferation of untreated and GTN-treated Saos-2 cells towards covering the void were monitored using the IncuCyte ZOOM® system at 10× magnification. The time-lapse images were taken at 6, 9, 12, and 21 hours represented as a, b, c and d, respectively.

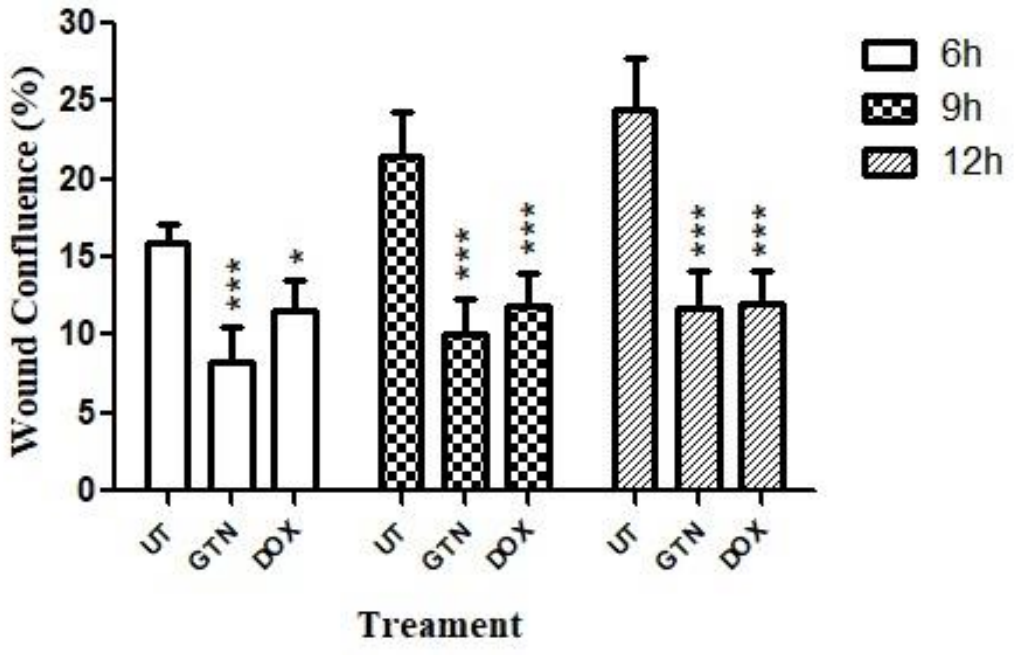

Figure 3: The proliferation of untreated and treated Saos-2 cells on the void (cell-free area) after 6, 9 and 12 hours were measured as the percentage of wound confluence. The data is reported as the mean ± standard deviation, with a sample size of 3. One-way ANOVA was employed for group comparisons, followed by the Dunnett's post-test to identify any significant differences between the treated and untreated cells (* p<0.05; *** p< 0.001).

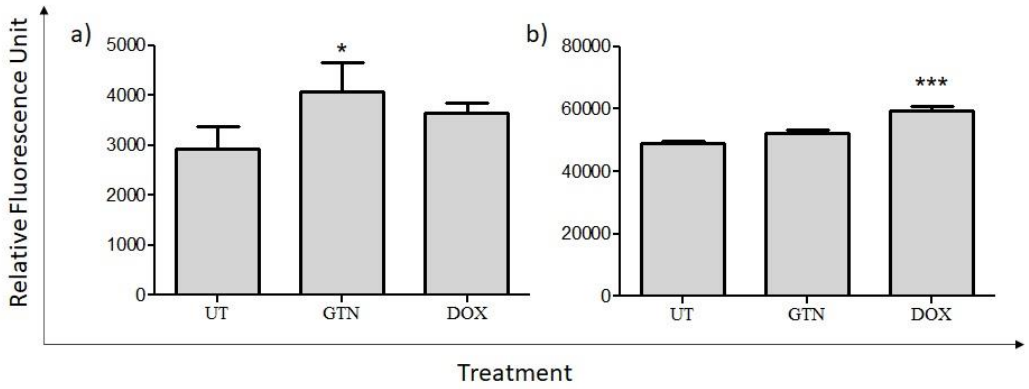

Figure 4: The assessment of intracellular ROS production in untreated, GTN-treated and DOX-treated Saos-2 cells after a) 2 hours and b) 24 hours of exposure time. The data is reported as the mean ± standard deviation, with a sample size of 3. One-way ANOVA was employed for group comparisons, followed by the Dunnett's post-test to identify any significant differences between the treated and untreated cells (* p<0.05).

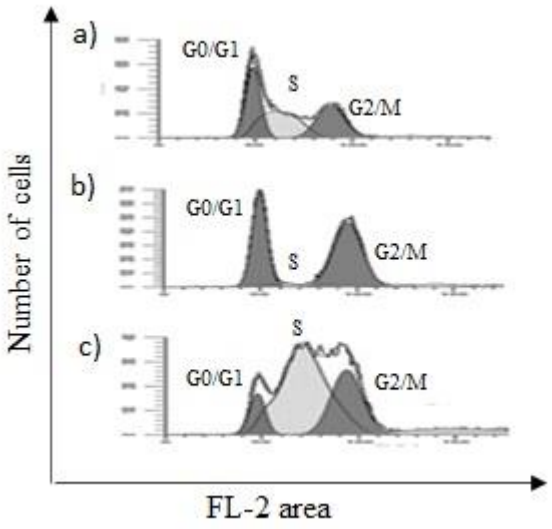

Figure 5: DNA histogram of Saos-2 cells with a) untreated, b) GTN-treated, c) DOX-treated at 24 hours exposure time.

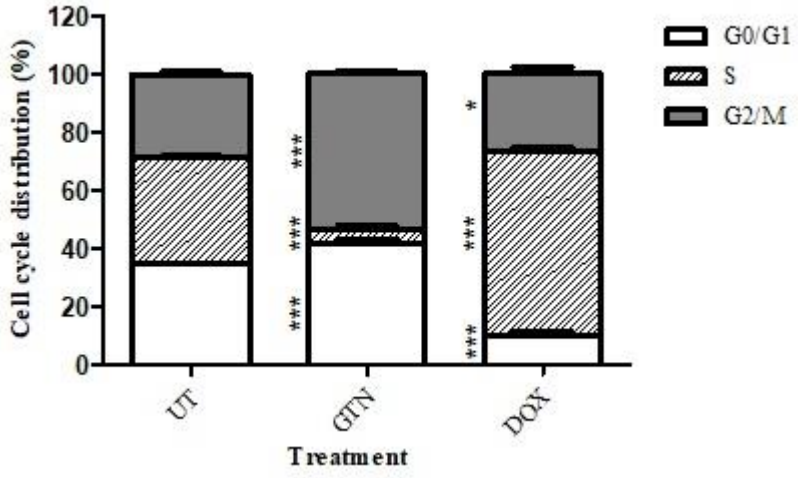

Figure 6: Quantification of cell cycle analysis of Saos-2 cells treated with either GTN or DOX for 24 hours. The data is reported as the mean ± standard deviation, with a sample size of 3. One-way ANOVA was employed for group comparisons, followed by the Dunnett's post-test to identify any significant differences between the treated and untreated cells (* p<0.05, *** p<0.001).

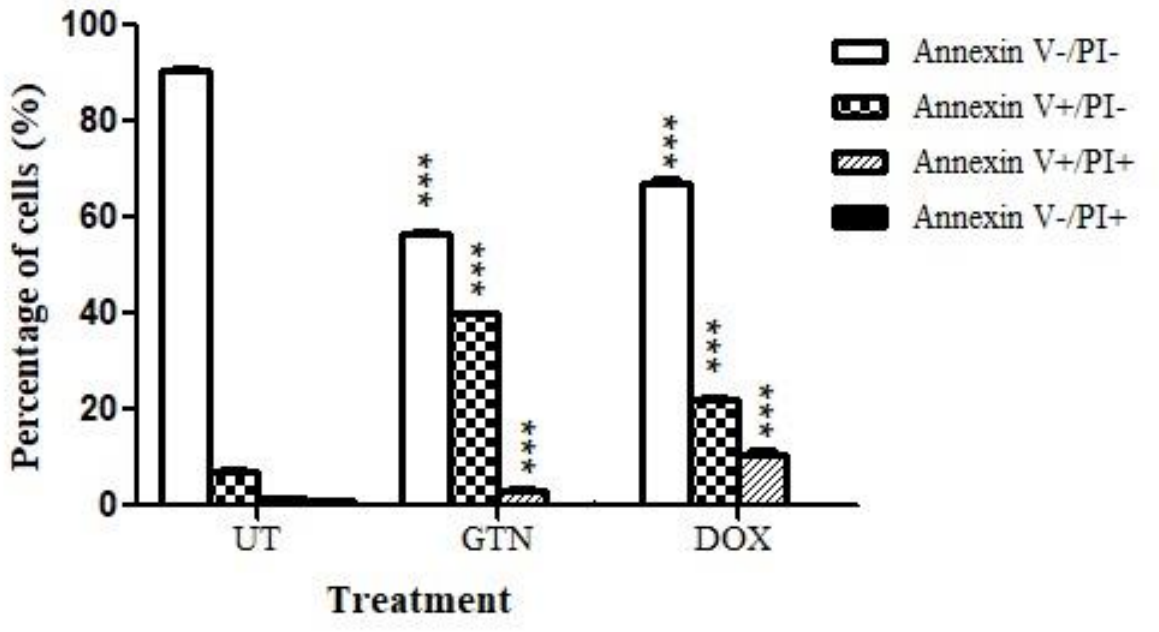

Figure 7: Analysis of apoptosis in Saos-2 cells treated for 24 hours with GTN or DOX. The percentages of stained cells; viable cells (Annexin V-/PI-), early apoptotic (Annexin V+/PI-) and late apoptotic (Annexin V+/PI+) and necrotic cells (Annexin V-/PI+) were represented as bar histograms. The data is reported as the mean ± standard deviation, with a sample size of 3. One-way ANOVA was employed for group comparisons, followed by the Dunnett's post-test to identify any significant differences between the treated and untreated cells (*** p< 0.001).

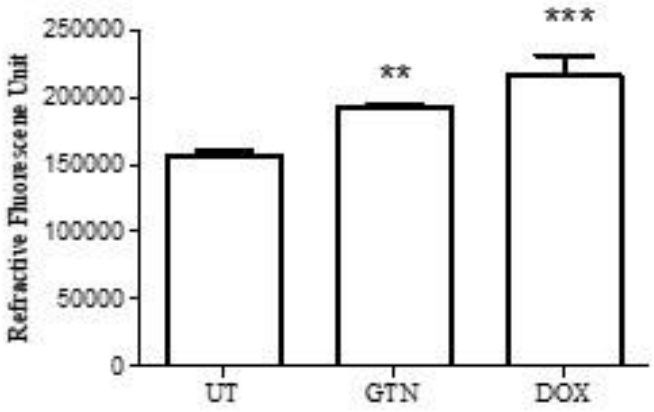

Figure 8: The activation of caspase 3/7 in untreated, GTN-treated and DOX-treated Saos-2 cells at 24 hours exposure time. The data is reported as the mean ± standard deviation, with a sample size of 3. One-way ANOVA was employed for group comparisons, followed by the Dunnett's post-test to identify any significant differences between the treated and untreated cells (*** p<0.001).

Table 1: Cell cycle distribution (G0/G1, S and G2/M) of untreated, GTN-treated and DOX-treated Saos-2 cells after 24 hours incubation. The data is reported as the mean ± standard deviation, with a sample size of 3.

Treatments	Cell Cycle stages		
	G0/G1	*S*	*G2/M*
Untreated	34.94±0.32	36.42±0.45	28.64±0.73
GTN	41.93±1.45	4.95±1.15	53.12±0.84
DOX	10.42±0.97	63.01±1.36	26.58±2.25

Table 2: The percentage of viable, early apoptosis, late apoptosis and necrosis of Saos-2 cells following treatment with GTN or DOX after 24 hours incubation. The data is reported as the mean ± standard deviation, with a sample size of 3.

Treatments	Events			
	Viable	*Early apoptosis*	*Late apoptosis*	*Necrosis*
Untreated	90.45±0.86	7.23±0.75	1.38±0.15	0.93±0.08
GTN	56.77±0.64	39.86±0.54	3.27±0.27	0.10±0.02
DOX	67.11±1.45	22.18±0.39	10.65±1.40	0.04±0.01

In another study, ROS levels increased significantly in Saos-2 cells treated with DOX only after 14 hours, and the increase was proportional to the length of DOX incubation (25). Previous studies have also shown that GTN induced oxidative stress in MDA-MB-231 breast cancer cells, leading to cdc25C degradation and subsequent G2/M phase arrest (14).

A cell cycle is a series of events in which cells grow and duplicate. It consists of cell cycle checkpoints that are responsible in preventing genetic errors during cell division. Two probable consequences might occur at this checkpoint, either delaying the cell cycle progression for DNA repair mechanism or inducing cell cycle exit or cell death in the case of irreparable DNA damage. Therefore, insights into which cell cycle checkpoint arrested by the GTN will reveal how the therapeutic approach can be best exploited (26). In the present study, cell cycle analysis by measuring the quantitative cellular DNA content, followed by sample acquisition using flow cytometry was applied. Our data indicates that the cell cycle was arrested at the G2/M phase in the GTN-treated Saos-2 cells with a concomitant decrease in the population of cells in the S phase. We found that the S phase was accumulated in cells treated with positive control DOX. This is concurred with the previous report on its established mechanism by blocking an enzyme called topoisomerase II and subsequently inhibits the DNA synthesis (27).

The decrease in cell viability observed after treatment with GTN may serve as a reliable indication of apoptosis. However, it should be noted that these cells could also undergo necrosis as a result of external factors and environmental conditions that can impede their ability to proliferate and divide. Thus, additional experiments were conducted to ascertain the mode(s) of cell death triggered by GTN. One of the characteristics of apoptosis involves alterations in the plasma membrane, where phosphatidylserine (PS) is relocated from the inner layer to the outer layer of the membrane, thereby making PS accessible to external cellular processes. The annexin V has a high affinity towards PS and thus provide the basis for apoptosis detection. Flow cytometry can detect and measure apoptotic cells at the single-cell level through the use of FITC-conjugated Annexin V. Simultaneously staining cells with FITC-Annexin V (green fluorescence) and the non-vital dye propidium iodide (red fluorescence) enables the differentiation of intact cells (Annexin V-/PI-), early apoptotic cells (Annexin V+/PI-), late apoptotic cells (Annexin V+/PI+), and necrotic cells (Annexin V -/PI+) (28). As expectedly, we found that the GTN has significantly decreased the percentage of viable cells compared to the untreated cells. The treatment of GTN led to a rise in the proportion of cells undergoing early and late stages of apoptosis. This outcome may be attributed to the expression of phosphatidylserine on the outer membrane layer, which is activated during apoptosis. Our findings provide additional support to previous research indicating that apoptosis is the primary mechanism of cell death in cells treated with GTN (8-10, 13-22).

Caspases activation is the key event of apoptosis as they initiate irreversible steps of the cell demise (29). Our results demonstrated that caspase 3/7 was activated after 24 hours of exposure time in Saos-2 cells. This result indicates that GTN-induced apoptosis in Saos-2 cells

could involve a caspase-mediated mechanism. The activation of caspase 3/7 may be resulted from the cleavage of the initiator caspases, caspase 8 and 9. This cascade of caspase cleavage and activation leads to the direct or indirect degradation of various crucial cellular proteins and ultimately results in the initiation of cell death (29).

Conclusion

These findings revealed that GTN is a promising anticancer agent against Saos-2 cells due to its cytotoxic activities by inhibiting the cell proliferation in a dose- and time-dependent manner, inducing the production of intracellular ROS and arresting the cell cycle at G2/M phase. The mode of cell death in GTN-treated Saos-2 cells was mainly apoptosis which may occur through a caspase-mediated mechanism. Further studies of the molecular signalling pathways are necessary to understand the detailed targeted mechanisms.

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Competing interests

The authors have no conflict of interest associated with this study.

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