Anticancer activities of genistein-topotecan combination in prostate cancer cells

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Abstract

Prostate cancer is one of the leading causes of death in men aged 40 to 55. Genistein isoflavone (4′, 5′, 7-trihydroxyisoflavone) is a dietary phytochemical with demonstrated anti-tumour activities in a variety of cancers. Topotecan Hydrochloride (Hycamtin) is an FDA-approved chemotherapy drug, primarily used for secondary treatment of ovarian, cervical and small cell lung cancers. This study was to demonstrate the potential anticancer efficacy of genistein-topotecan combination in LNCaP prostate cancer cells and the mechanism of the combination treatment. The LNCaP cells were grown in complete RPMI medium, and cultured at 37°C, 5% CO_2 for 24–48 hrs to achieve 70–90% confluency. The cells were treated with varying concentrations of genistein, topotecan and genistein-topotecan combination and incubated for 24 hrs. The treated cells were assayed for (i) post-treatment sensitivity using MTT assay and DNA fragmentation, (ii) treatment-induced apoptosis using caspase-3 and -9 binding assays and (iii) treatment-induced ROS generation levels. The overall data indicated that (i) both genistein and topotecan induce cellular death in LNCaP cells, (ii) genistein-topotecan combination was significantly more efficacious in reducing LNCaP cell viability compared with either genistein or topotecan alone, (iii) in all cases, cell death was primarily through apoptosis, *via* the activation of caspase-3 and -9, which are involved in the intrinsic pathway, (iv) ROS generation levels increased significantly with the genistein-topotecan combination treatment. Treatments involving genistein-topotecan combination may prove to be an attractive alternative phytotherapy or adjuvant therapy for prostate cancer.

Keywords: Topotecan ● genistein ● prostate cancer

Introduction

An estimated 217,730 men were diagnosed with prostate cancer within the United States in 2010, while over 32,000 died as a result of this disease [1]. American men have a 1 in 6 chance of developing prostate cancer during their life time, making it the second most common carcinoma, next to skin cancer. Risk factors for prostate cancer include age, specifically men over the age of 60, and previous family history with the carcinoma.

Genistein is the major dietary flavonoid found in soy (derived from soybean). Soy naturally contains genistin: a beta-glucoside, which is broken down in the gastrointestinal tract, into genistein through fermentation by microbes [2, 3]. Several studies have shown that the incidence of carcinoma is significantly lower in populations where soy constitutes a large part of the diet. Japanese and Asians average a daily genistein ingestion rate of 1.5–4.1 mg/person, which is significantly higher than the average consumption in Western European countries and the United States [2]. Asian immigrants relocating into higher risk countries loose their low-risk advantage by the second generation, and run a higher risk of developing carcinomas [4, 5].

Exposure of malignant cells to genistein has shown genistein's ability to inhibit cell growth and proliferation. Potential mechanism of actions has been identified in several studies and include: alternation of signal transduction pathways, caspase protease activation and regulation of the cell cycle. Research has also shown that genistein modulates epidermal growth factor activity and signal transduction in both breast and prostate cancer cells [5–8].

In addition, genistein inhibits topoisomerase II enzyme, and angiogenesis through the blockage of VEGF signalling. Furthermore, genistein possesses oestrogenic and anti-oestrogenic capabilities due to its structural similarity to oestrogen [8–10].

Genistein-induced apoptosis in carcinoma cells has been shown to be due by genistein's ability to control expression of apoptosis-related genes; such as up-regulation of Bax, and utilization of an independent

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p-53 pathway. Genistein also induces apoptosis *via* other signal pathways including: increase in caspase-3 protease activity, initiation of DNA damage and halting of the cell cycle at the G2/M phase [6, 11]. Existing research data indicate that genistein has significantly less cytotoxicity compared with standard chemo and radiation therapy.

Another recently known anti-tumour phytochemical is topotecan, which also induces apoptosis in cancer cells. Topotecan Hydrochloride (under the trade name Hycamtin® by SmithKline Beecham Pharmaceutical) is an FDA approved chemotherapeutic agent for the treatment of ovarian, cervical and small cell lung cancer. The drug is a semi-synthetic derivative of camptothecin, the active ingredient found in the bark and stem of the Chinese tree *Camptotheca accuminata* [12, 13]. Camptothecin, an alkaloid phytochemical, is able to arrest cell growth and proliferation in several carcinoma cell lines. However, it has an extremely low solubility and early clinical trials reported adverse side effects, including bone marrow suppression [13–15].

Administration of topotecan is limited to patients with recurring carcinomas, and/or those who do not respond to standard chemotherapeutic and/or radiation treatments. Side effects include: myelosuppression, low blood counts and suppression of the immune system, leading to an increase in susceptibility to infection [16, 17]. Topotecan's mechanism of action is through inhibition of Topoisomerase I enzyme. To relieve DNA strain during replication and repair, the topoisomerase I enzyme makes single stranded cut in the phosphate backbone of the genetic code. Topotecan interposes itself between the topoisomerase and the unwound DNA strand, thus preventing the annealing of DNA sister strands. The topotecan-DNA complex destabilizes the genetic material, leading to double strand cuts and eventually apoptosis in the cells [16–18].

The induction of apoptosis through the formation of the topotecan-DNA complex induces cell cycle arrest in various stages, depending on the drug dosage utilized. Topotecan treatment groups exposed to 0.05 μM of the drug, arrested the cell cycle at S/G2/M, while concentrations higher than 0.1 μM halted the cell cycle at the G1 phase [19]. In addition, Topotecan can also induce oxidative stress by increasing the levels of reactive oxygen species (ROS) and nitrite. Elevation of ROS can cause irreversible damage and modification to proteins by inducing the formation of protein carbonyl derivatives [20, 21]. Increases in ROS combined with reduced production of antioxidant, elevates DNA stress and damage; ultimately leading to the induction of intrinsic apoptotic cell death [20].

The aim of this study was to investigate the potential efficacy of Gn-TPT combination on the viability of LNCaP prostate cancer cells. The hypothesis was that Gn-TPT combination would maintain the therapeutic efficiency of topotecan, with significantly less cytotoxicity; implying potentially less side effects in patients.

Materials and methods

Cell line

The LNCaP (ATCC, Washington, DC, USA) and PNT2: normal prostate epithelium (Sigma-Aldrich, St. Louis, MO, USA) cells were cultured in complete RPMI 1640 media with 10% Foetal Bovine Serum, 1% penicil-

lin/streptomycin and L-glutamine. All cells were grown in a humidified atmosphere at 37°C with 5% CO_2, until 70–90% confluency levels were reached. Genistein isoflavone (Gn) (Sigma-Aldrich) was constituted with dimethylsulfoxide (DMSO) and diluted with RPMI-media to produce aliquots ranging from 10 to 200 μM (Gn_{10-200}). Topotecan Hydrochloride (TPT) (Drummond Scientific Co., Broomall, PA, USA) was diluted into a stock solution with dimethylsulfoxide (DMSO). Stock solutions of TPT were further diluted with RPMI-media to produce aliquots ranging in concentration from 0.1 to 10 μM ($TPT_{0.1-10}$). Final concentration of DMSO for both genistein and topotecan did not exceed 0.05%.

Treatment

Cell cultures were split into treatment groups, with single ($Gn_{10-200\ \mu M}$ and $TPT_{0.1-10\ \mu M}$) and TPT-Gn combination ($TPT_{0.1-10\ \mu M} + Gn_{EC50}$) dosages. Treatment groups were incubated for 24 hrs at 37°C with 5% CO_2. Experiments had control groups consisting of LNCaP cells cultured in RPMI 1640 media (with 10% FBS, 1% penicillin/streptomycin and L-glutamine) without treatment.

MTT assay

The MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide],a tetrazolium dye, is used to determine cell viability. As the colour intensity generated, correlates directly with the amount of metabolically active cells in each well, cell viability can be quantitatively determined by measuring the optical density (OD) in individual wells.

Briefly, cells were plated at a density of 5×10^4 cells per well in a 96-well plate and incubated for 24–48 hrs to achieve >80% confluency. The cells were then treated with varying concentrations of Gn, TPT, Gn-TPT combination and incubated for 24 hrs; after which 10 μl of MTT reagent was added to each well. The plates were further incubated at 37°C and 5% CO_2 for 4 hrs in the dark, after which 100 μl of DMSO was added to the cells and incubated overnight under the same conditions. Absorbance (OD) was read at 490 nm using a Multiskan microplate reader (Molecular Devices Inc., Sunnyvale, CA, USA). The OD obtained was graphed against the concentrations.

DNA fragmentation assay

The DNA fragmentation assay distinguishes between necrotic and apoptotic cell death. Cells undergoing apoptosis will cleave their nuclear DNA into 180–200 bp DNA breaks, which can be detected through electrophoresis. Necrotic-induced cell death results in irregular DNA breaks, which will appear as smears in the gel. Briefly, after treating the LNCaP cells as previously described, cells were collected and washed in PBS. A maximum of 5×10^6 cells were isolated from the Gn, TPT and Gn-TPT combination treatment groups. Following the protocol from the Qiagen-DNeasy® Blood and Tissue Kit, DNA was extracted from the pellet and quantified through spectrophotometry. Ten nanograms of DNA extract (+2 μl of loading dye) was loaded into a 1.5% agarose gel with 5 μl EtBr and ran at 80 mV for 2 hrs. A 1 kb DNA ladder was also loaded into the gel as a marker to aide quantification and sizing of the DNA fragments.

Caspase protease analysis

The involvement of caspases-3 and -9 in treatment-induced apoptosis was determined by using a Caspase colorimetric activity assay kits.

Caspase-9 (initiator) activity correlates with the intrinsic apoptotic pathway, while caspase-3 (effector) is activated by both the intrinsic and extrinsic pathways. The assay involves the caspase-mediated cleavage of a labelled peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) for caspase-3.

$$Ac\text{-}DEVD\text{-}pNA \xrightarrow{caspase-3} Ac\text{-}DEVD + pNA$$
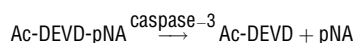
The *p*-nitroaniline (*p*NA) product can be quantified using a spectrophotometer or plate reader at 405 nm. This gives a direct indication of caspase-3 activity. Similar techniques were utilized for caspase-9 detection.

After cells were treated and incubated as previously described, cell pellets were isolated and washed in PBS. Following Qiagen-Protein Purification protocol, protein was extracted and quantified. Forty micrograms of protein lysate of each sample was exposed to the substrate of the individual caspase. Inhibitors for caspase-3 and -9 were also utilized as negative controls. Fold-increase in caspase activity was calculated by comparing the absorbance (OD) values of the treated samples with that of the control sample.

Nitroblue tetrazolium-reactive oxygen species assay

The ROS species such as superoxide and hydrogen peroxide are natural products of metabolic processes. During periods of stress, the level of ROS in cells will increase exponentially leading to DNA and cellular damage and death. In the presence of superoxide ions, nitroblue tetrazolium (NBT) is converted to the insoluble NBT-diformazan.

Briefly, cells (1×10^5 cells/well) were grown in a six-well plate, and then treated as discussed previously. The NBT (1 mg/ml) in HBSS medium was added to the wells after treatment, and incubated for 4 hrs at 37°C in the dark. After incubation, cells were trypsinized and counted. Finally, samples were washed and diluted to equal amounts in PBS buffer and finally lysed with DMSO to release the cellular content. The ROS production levels from released cellular content was read at 560 nm and calculated by comparing the absorbance values of the treated samples to that of the control samples.

Statistical analyses

Experiments were performed in triplicates, and repeated twice to confirm similar results. Significance of the differences in mean values was determined using ANOVA and the Student's *t*-test. Statistical significance was defined as $P \leq 0.05$.

Results

Growth inhibition

The MTT assay was utilized to test the effects of genistein and topotecan on cell viability of the LNCaP prostate carcinoma cell line. Data collected were analysed based on the knowledge that the absorbance is directly correlated with the amount of viable cells. Results showed that the number of viable cells decreased, as the concentration of genistein increased; demonstrating a dose-dependent relationship.

Based on the MTT results, the EC50 dose of genistein was calculated as 30 μM (Fig. 1A). Exposures of genistein to normal prostate epithelium did not show a significant decrease in cell viability up to 80 μM concentration (Fig. 1B).

Similar dose-dependent decreases in viability were observed for topotecan treatments. Combination treatments of topotecan (0.5–10 μM) with the EC dose of genistein showed a more significant dose-dependent decrease in cell viability than the single treatments ($P < 0.05$) (Fig. 1C).

Apoptosis induction and caspase activation

Treatment of LNCaP cells with genistein and topotecan, in singular and combination dosages, induced apoptotic cell death as shown by the presence of uniform DNA fragments in electrophoresis (Fig. 2, Lanes 2–5). Control samples did not demonstrate evidence of cell death shown by the single band (Lane 1).

The data obtained revealed that genistein and topotecan in single treatments, caused an increase in both caspase-9 (initiator) and caspase-3 (effector) activities. The TPT-Gn combination induced a significantly higher increase in caspase-9 and -3 levels compared to the Gn and TPT single treatments ($P < 0.05$) (Fig. 3A and B).

ROS generation

The ROS species are natural products of metabolic processes. During periods of stress, ROS levels will increase exponentially in response to stress, leading to plasma membrane damage, DNA and cellular damage and eventual induction of the intrinsic apoptosis pathway. Treatment regimens which increase cell's ROS levels can therefore negatively impact cellular viability. The NBT dye allows for a colorimetric observation of cellular ROS levels. The data obtained indicates elevated levels of ROS in all the treatment groups in a dose-dependent manner. The TPT-Gn combination induced significantly higher levels of ROS ($P < 0.05$) relative to the single, Gn and TPT treatments (Fig. 4).

Discussion

The aim of this project was to investigate the anticancer activities of genistein-topotecan combination in prostate cancer cells. Existing research data indicate that development of alternative preventative and/or treatment options utilizing a combination of phytochemicals and chemotherapeutic drugs could be an attractive alternative compared to conventional carcinoma treatments. The potential combined activity of both compounds may allow for a more targeted treatment option, with lower drug dosages and less cytotoxicity; implying potentially less side effects in patients.

Previous studies completed on both androgen-resistant and androgen-sensitive cell lines have shown the ability of genistein to induce apoptosis [8, 22–28]. Data obtained showed similar trends with the exposure of genistein to LNCaP cells. Genistein-induced dose-dependent apoptosis in the LNCaP cell lines, this observation is consistent
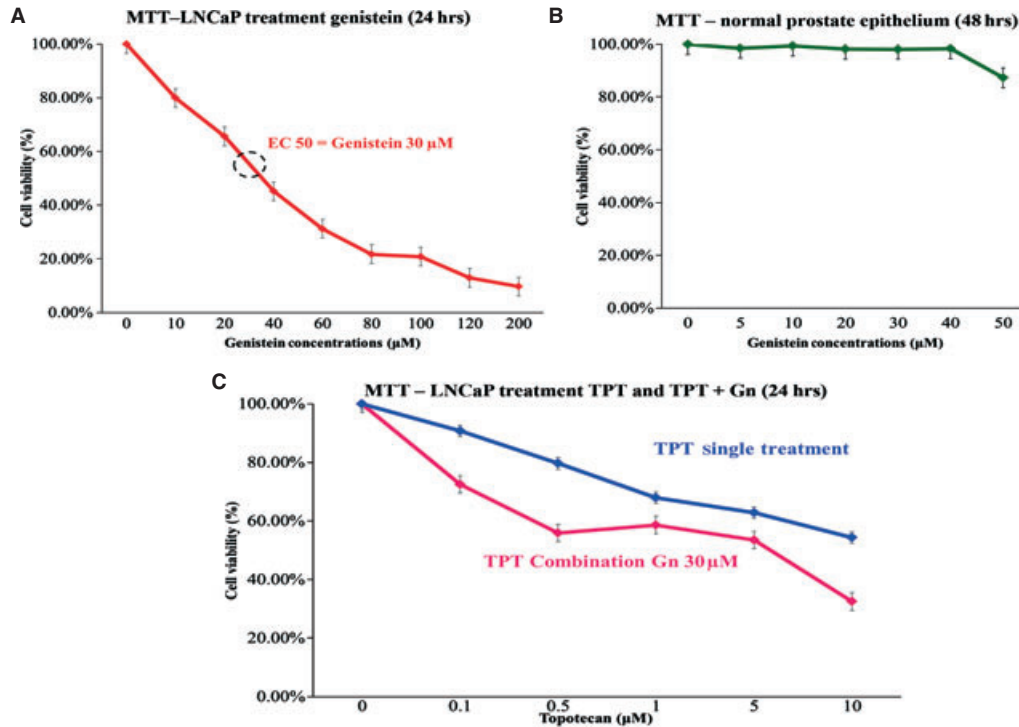
Fig. 1 The growth and viability of LNCaP was investigated using the MTT assay. Genistein is an attractive treatment option as it does not significantly ($P > 0.05$) affect the viability of normal epithelium cells (**B**); however, it reduces cell viability in a dose-dependent manner when exposed to carcinoma cells (**A**). The EC 50 dose of genistein for LNCaP cells was measured at 30 μM (**A**). The TPT as a single treatment also decreased LNCaP cell viability in a dose-dependent manner; the combination treatments of TPT + Gn 30 μM killed the carcinoma cells more efficiently than the individual TPT concentrations (**C**).
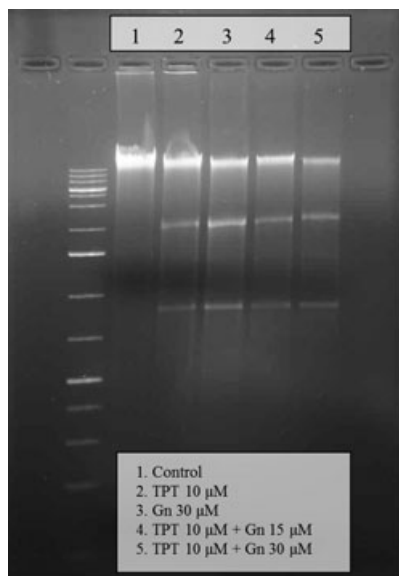

Fig. 2 DNA Fragmentation and laddering through electrophoresis confirmed the presence of apoptotic cell death in treatment groups. No fragments were detected in the control sample.

with previous studies which demonstrate genistein's ability to inhibit growth and proliferation of prostate carcinoma cells, while being non-toxic to normal prostate epithelial cells [5, 6, 29, 30] Topotecan as a single treatment also decreased LNCaP cell viability in a dose-dependent manner. However, topotecan (10 μM) – genistein (30 μM) combination was significantly more effective in killing the cells than either of the topotecan and genistein treatments. This observation correlated significantly with the combination-induced apoptosis.

The caspase experiment showed an involvement of caspase-9 and -3 in the signalling pathway of genistein/topotecan-induced apoptosis. There was significantly more increase in both caspase-9 and -3 with the combination treatments, when compared with the single treatments. The activation of these caspases indicates the utilization of the intrinsic apoptotic pathway [31–33].

The NBT–ROS assay was carried out to determine the amount of ROS species in the LNCaP cells. The ROS species are natural products of metabolic processes. During periods of stress, ROS levels will increase exponentially leading to plasma membrane damage, DNA and cellular damage and eventual induction of the intrinsic apoptotic pathway [20, 21]. Treatment regimens which increase ROS levels in cells can therefore inhibit cellular viability. In this study, significantly higher levels of ROS were observed in the combination-treated LNCaP cells compared with the single treatments. Treatment-induced elevated
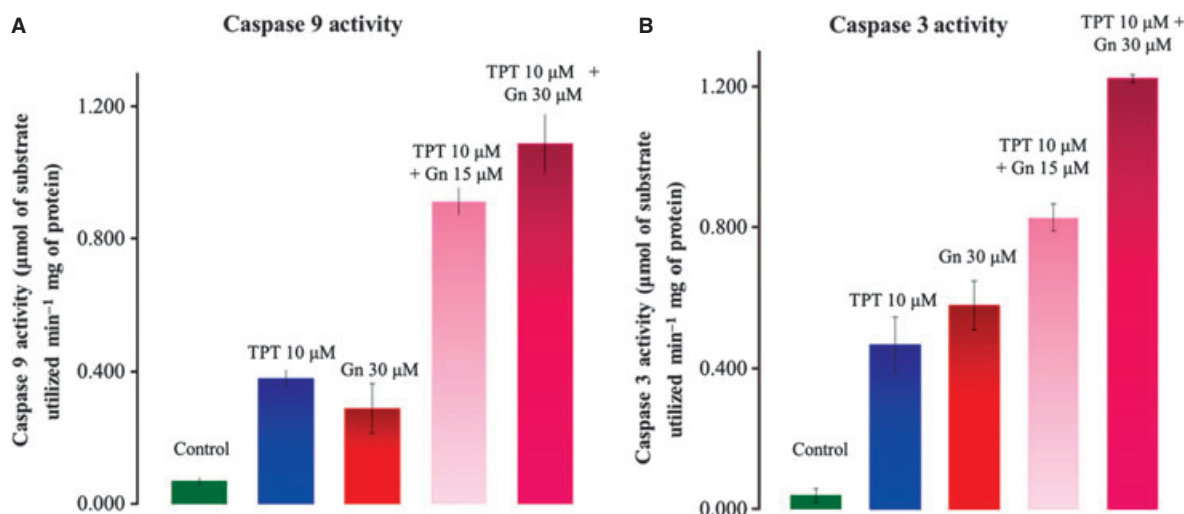
Fig. 3 Caspase-9 (initiator) and caspase-3 (effector) activity in LNCaP cells after 24 hrs exposure to Gn and TPT single and combination treatment. Compared with the control groups, both Gn and TPT in single and combination treatments showed increased caspase activation in both caspase-9 and -3, with a more heightened response in the combination groups.
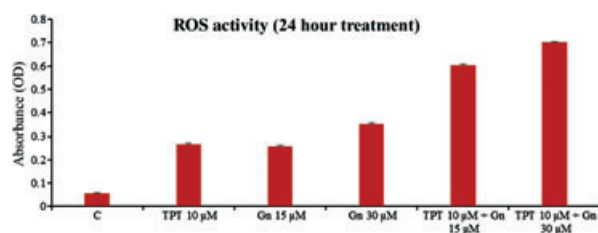

Fig. 4 ROS levels were observed through the NBT-ROS assay. Increase levels of ROS often correlate with induction of apoptotic cell death. When compared with the control groups, all treatment regimens had elevated ROS levels. Combination groups with genistein and topotecan had the highest absorbance rates of ROS species.

levels of ROS could therefore be of potential clinical significance; implying the potential significance of the combination treatment.

The overall data consistently indicated the effectiveness of all the treatment regimens on apoptosis induction. However, Gn-TPT combination was more efficacious than either of the single treatments in inhibiting the growth of LNCaP cells.

Conclusion

The overall data highlights the potential significance of (i) genistein as adjuvant in chemotherapy and (ii) Gn-TPT combination treatment in LNCaP apoptosis induction *via* elevation of caspase-9 and -3 levels and ROS expression in the cells.

Acknowledgements

Conflict of interest

The authors confirm that there are no conflicts of interest.

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