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Activation of the intrinsic-apoptotic pathway in LNCaP prostate cancer cells by genistein- topotecan combination treatments

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ABSTRACT

Background: Prostate cancer is the second most common cancer in American men. The 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. Genistein isoflavone is the primary dietary phytochemical found in soy and has demonstrated anti-tumor activities in LNCaP prostate cancer cells. Topotecan Hydrochloride (Hycamtin) is an FDA-approved chemotherapy for secondary treatment of lung, ovarian and cervical cancers. The purpose of this study was to detail the potential activation of the intrinsic apoptotic pathway in LNCaP prostate cancer cells through genistein-topotecan combination treatments.

Methods: LNCaP cells were cultured in complete RPMI medium in a monolayer (70-80% confluency) at 37ºC and 5% CO_2. Treatment consisted of single and combination groups of genistein and topotecan for 24 hours. The treated cells were assayed for i) growth inhibition through trypan blue exclusion assay and microphotography , ii) classification of cellular death through acridine/ ethidium bromide fluorescent staining, and iii) activation of the intrinsic apoptotic pathway through Jc-1: mitochondrial membrane potential assay, cytochrome c release and Bcl-2 protein expression.

Results: The overall data indicated that genistein-topotecan combination was significantly more efficacious in reducing the prostate carcinoma's viability compared to the single treatment options. In all treatment groups, cell death occurred primarily through the activation of the intrinsic apoptotic pathway.

Conclusion: The combination of topotecan and genistein has the potential to lead to treatment options with equal therapeutic efficiency as traditional chemo- and radiation therapies, but lower cell cytotoxicity and fewer side effects in patients.

Key words: topotecan; genistein; intrinsic apoptotic cell death

BACKGROUND:

Prostate cancer is the leading diagnosed carcinoma in American men and has a mortality rate of 1 out of 36, according to the American Cancer Society. Men with a family history, especially a first degree relative such as a father and brother diagnosed with prostate cancer are in a high risk category, as well as men of African-American descent. The majority of new carcinoma patients are 65 years or older, often with elevated Prostate Specific Antigen (PSA) levels [1, 2].

Conventional treatment options such as chemo- and radiation therapy have not been able to completely eradicate prostate cancer. Besides, both treatments induce necrotic and apoptotic cell death in malignant and healthy epithelia [3, 4]. Alternative treatment methods utilizing phytochemicals or bioactive plant components have shown great potential as preventative and/or adjuvant therapies in a wide range of carcinomas, including breast and prostate cancers. Published studies show that phytochemicals such as genistein, pomegranate extract and curcumin are able to inhibit carcinoma proliferation and induce mainly apoptotic cell death [5–8]. Carcinoma treatment options involving a combination of chemotherapeutic agents and phytochemicals have shown an increase in sensitivity to drug toxicity, elevated reactive oxygen species (ROS), DNA damage and ultimately acceleration of induced- apoptotic cell death [9–12].

Recently, our lab reported that combination treatments of genistein and the chemo therapeutic drug, topotecan in LNCaP prostate cancer cell lines, were able to reduce cell viability more effectively compared to singe treatments [10]. Results of the *in- vitro* experiments indicated that by combining genistein and topotecan, the carcinoma cells had higher activation of caspase 9 (initiator) and caspase 3 (effector) proteases, as well as elevated ROS species and DNA fragmentation; all characteristics of apoptotic cell death [10]. Our current research aims are focused on mapping the activation of the apoptotic pathway, regarding the induction of intrinsic and/or extrinsic initiating factors.

Genistein, a soy extract, is able to limit carcinoma proliferation and induce apoptotic cellular death by inhibition of the topoisomerase II enzyme, increasing of ROS levels, lowering the levels of anti-oxidants and induction of DNA damage [13, 14]. *In-vitro* studies in various cancer cell lines showed cell arrest in the G2/M cell cycle phase, up-regulation of the pro-apoptotic factor Bax and decrease of vascular epithelium growth factor (VEGF) correlating with a reduction in angiogenesis [5, 6, 15, 16].

The proliferation of LNCaP prostate cancer is dependent on androgen secretions, especially in the early stages of development. The chemical structure of genistein is similar to estrogen which aids the phytochemical in blocking and down-regulating androgen receptors found on the cell surface and stroma of testosterone dependent prostate carcinoma, results were similar in both *in-vitro* and *in-vivo* experiments [17, 18]. Genistein treatments have shown a dose and time-

dependent induction of carcinoma death in androgen sensitive and insensitive cell lines, but did not significantly affect the viability of normal, healthy epithelia [5, 8, 19].

Topotecan, under the trade name Hycamtin® by SmithKline Beecham Pharmaceutical, is a chemo therapeutic agent used for the treatment of cervical, ovarian and lung cancers [20–22]. The effectiveness of the drug is partly due to blockage of the topoisomerase I enzyme, which is essential for DNA replication. Topotecan inserts itself between the unwound DNA strands and the topoisomerase I enzyme; preventing the re-annealing of the genetic strand and the induction of DNA fragment, resulting in apoptotic cell death [22, 23].

Cellular viability is further decreased by induction of oxidative stress by elevation of ROS and nitrite levels, as well as activation of the apoptotic pathway through caspase-3 proteases and p53-independent pathways. *In –vitro* carcinomas exposed to topotecan showed a dose-dependent response to the induction of cellular death. At lower doses the cell cycle was halted at S/G2/M phase, while dosages exceeding 0.1μM halted in the G1 phase [10, 24–28].

Side effects from the topotecan treatment include suppression of the bone marrow, anemia and leukopenia. The low blood count can severely reduce the patient's immune system and correlates with higher bacterial and viral infection rates. The severity of the side effects limits topotecan treatment regimens to patients with relapse carcinomas or who did not respond to conventional radiation and chemo-therapeutic regimens[21, 22, 29, 30].

In- vitro combination treatments involving topotecan and genistein have shown increased carcinoma cell sensitivity to drug toxicity. Lowering the chemo-therapeutic dosages and combining a phytochemical such as genistein can lower the cell cytotoxicity yet maintain the therapeutic efficiency noted at higher concentrations of topotecan [10]. Combination treatments would results in overall lower chemo-therapeutic exposure and lessened side effects.

The aim of this investigation was to further detail the activation of the apoptotic pathway in LNCaP prostate cancer cells, when treated with topotecan and genistein, in single and combination treatments.

MATERIALS AND METHODS:

Cell Line:

LNCaP cells (ATCC, Washington, D.C, U.S.A) were cultured in complete RPMI 1640 media with 10% Fetal Bovine Serum, 1% penicillin/streptomycin and L-glutamine. Cells were grown in a monolayer (70-80% confluency) at 37ºC and 5% CO_2. Stock volume of genistein isoflavone (Gn) (Sigma-Aldrich, St. Louis, Missouri, U.S.A) and Topotecan Hydrochloride (TPT) (Drummond Scientific Co., Broomall, Pennsylvania, U.S.A) were diluted with dimethylsulfoxide (DMSO) and RPMI-media to produce aliquots of Gn 15 and 30 μM (Gn_{15} and Gn_{30}) and TPT 10 μM (TPT_{10}). Final concentration of DMSO did not exceed 0.05% for both stock solutions of genistein and topotecan.

Treatment:

Cells were split into 5 treatment groups: single treatments of Gn_{15}, Gn_{30} and TPT_{10} and TPT-Gn combinations of (TPT_{10} + Gn_{15}) and (TPT_{10} + Gn_{30}). Treatment values were determined by

preliminary data indicating the EC 25 dose of Gn (Gn_{15}) and EC 50 dosage of TPT (TPT_{10}) and Gn (Gn_{30})[10]. Each experiment completed had control groups of LNCaP cells cultured in complete RPMI media, as previously described. Incubation time for all control and treatment groups was 24 hours, at 37ºC with 5 % CO_2.

Trypan Blue Exclusion assay:

The trypan blue exclusion assay was utilized to assess pre- and post-treatment viability of the cells. Viable cells were unable to uptake the dye, while dead (non-viable), cells took up the dye and stained blue. After the cells were exposed to the trypan blue dye (1:1 ratio), 10 µL of the suspension was loaded onto a hematocytometer, and inserted into an automated cell counter to quantitatively distinguish between viable (non-stained) and non-viable (colored) dead cells. Percentage cell viability was calculated by the formula:

$$\textbf{Cell viability (\%)} = \frac{\textbf{No. of viable cells (unstained cells)}}{\textbf{Total no. of cells}} \times 100\%$$

Control and treatment groups were also viewed and photographed under a compound microscope, total magnification 100X.

Acridine Orange/ Ethidium Bromide fluorescent assay:

The fluorescence-based acridine orange/ethidium bromide assay was utilized to differentiate between viable, apoptotic and necrotic cells. The two dyes have different emission spectra, and a cocktail solution is particularly useful. Briefly, Ethidium bromide (10µl) and acridine orange (75µl) were combined in a cocktail, of which 1µl was added to 25µl cell suspension. This is incubated at room temperature in the dark for 2 minutes, after which 10 µl of each suspension is transferred onto a microscope slide, covered with a cover slip and analyzed under a fluorescent microscope with a band-pass filter. Detection of apoptosis was based on morphological and fluorescent characteristics of the stained cells. Ethidium bromide is dependent on disrupted cell membranes to enter cells, thus can only stain dead (non-viable) cells; producing red nuclei. Viable cells were indicated by bright green, apoptotic cells by orange/brown, and necrotic cells by red. Cell death was quantified by counting a total of 250 cells in various fields per slide and recording percentage values of viable, apoptotic and necrotic cells.

Mitochondrial Membrane Potential Assay (JC-1 Staining)

Jc-1 is a lipophilic, cationic dye (5, 5^1, 6, 6^1 –tetrachloto-1, 1^1, 3, 3^1 – tetraethyl benzimedazolyl carbocyanine iodine) which can selectively enter the mitochondria and illuminate green to red flourescence correlating to the level of mitochondrial membrane potential. Initiation of the intrinsic apoptotic pathway is reliant on the loss of mitochondrial membrane potential ($\Delta\Psi m$), causing a release of cytochrome c from the mitochondria into the cytoplasm. The Jc-1 dye formed aggregates in the mitochondria of healthy cells resulting in an intense red fluorescence. Apoptotic cells with a reduction in mitochondrial membrane potential ($\Delta\Psi m$), displayed green fluorescence since the Jc-1 dye remained in its' monomeric form.

Briefly, LNCaP cells were cultured as a monolayer in a 24 well MTP and grown to 70-80% confluency. Treatment and control groups were set up as described previously. After incubation, 25µL of Jc-1 dye was added to each well and incubated for 15 minutes. Cells were analyzed with the fluorescent microscope; excitation/emission= 540/570nm for red fluorescence (viable cells) and 485/535nm for green fluorescence (apoptotic cells).

Western Blotting: Cytochrome C and Bcl-2 Detection:

The induction of the intrinsic apoptotic pathway is regulated by several factors, including the mitochondrial release of cytochrome c into the cytoplasm, and a family of (pro- and anti-apoptotic) Bcl-2 proteins. Anti-apoptotic proteins such as Bcl-2 and Bcl-X_L reduce the permeability of the mitochondrial membrane, preventing cytochrome c release and therefore inhibit cells from undergoing apoptosis. The release of cytochrome c and down-regulation of the Bcl-2 protein correlating with the activation of the intrinsic-apoptotic pathway can be detected through western blotting.

Briefly, after treatment of LNCaP cells as described, cells were pelleted and washed in PBS. Using the Qiagen- Protein Purification kit, protein was extracted and quantified per manufacturer's protocol. Utilizing standard western blotting techniques, 30µg of protein lysate was separated on a 12.5% SDS-polyacrylamide gel and electroblotted onto polyvinylidene diflouride membranes overnight at 4^0C in transfer buffer. The blots were washed with a 5g/100mL nonfat milk TBST washing buffer for 1 hour and incubated with the primary antibody: cytochrome c (Cell Signaling) or Bcl-2 (Cell Signaling) overnight at 4^0C. After washing the membrane with standard washing buffer containing Tween, an anti-rabbit secondary antibody (cytochrome c) or anti-mouse secondary antibody (bcl-2) were utilized. Final western analysis was dependent on band intensity correlating with heightened cytochrome c release and diminished bcl-2 protein expression. Samples were also blotted with GAPDH antibody, as a protein loading control guide.

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

Trypan Blue Exclusion assay was utilized to quantify the amount of viable and dead cells in control and treatment groups of LNCaP prostate carcinoma cells. Results showed a higher degree of stained cells in all treatment groups. The TPT + Gn combination treatment groups had the highest amount of non-viable cells in a dose-dependent response. % of viable cells was graphed with a significant decrease noted in TPT_{10} + Gn_{30} treatment groups ($P \leq 0.05$) (Figure 1B). Analysis of the Trypan Blue Exclusion assay and MTT data (data not shown) through the Bliss independence model suggested an additive effect when combining genistein and topotecan treatments.

Cellular morphology was photographed with a compound light microscope at 100X total magnification (Figure 1A). Control groups showed a high degree of healthy, actively proliferating LNCaP cells with 70-80% confluency. All treatment groups had a decrease in cell viability, as revealed by the lowered confluency rate. Combination treatment groups (TPT + Gn) showed lower cell viability; the presence of apoptotic bodies and a smaller cellular morphology all of which were indicative of apoptotic cell death.

1A

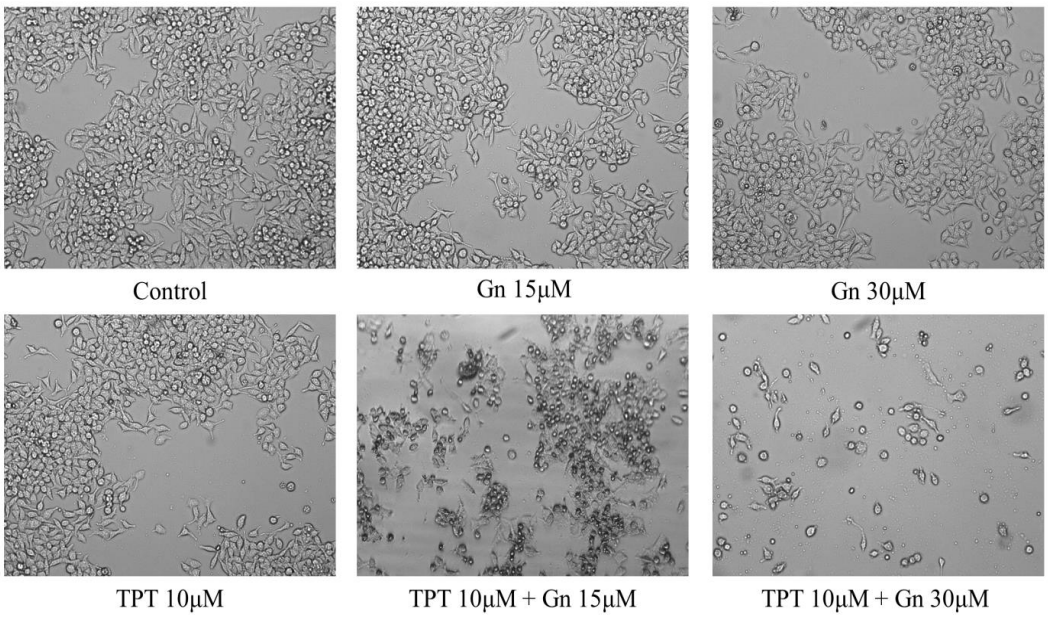

Fig 1A. LNCaP cellular morphology was photographed through a compound microscope (100X TM). Control cells had 70-80% confluency, with a fibroblastic appearance. All treatment groups had a decline in cell viability as noted by a decrease in cell number. The most drastic change was noted in the combination treatment groups (TPT + Gn) with shrinkage of cell size and formation of apoptotic bodies.

1B

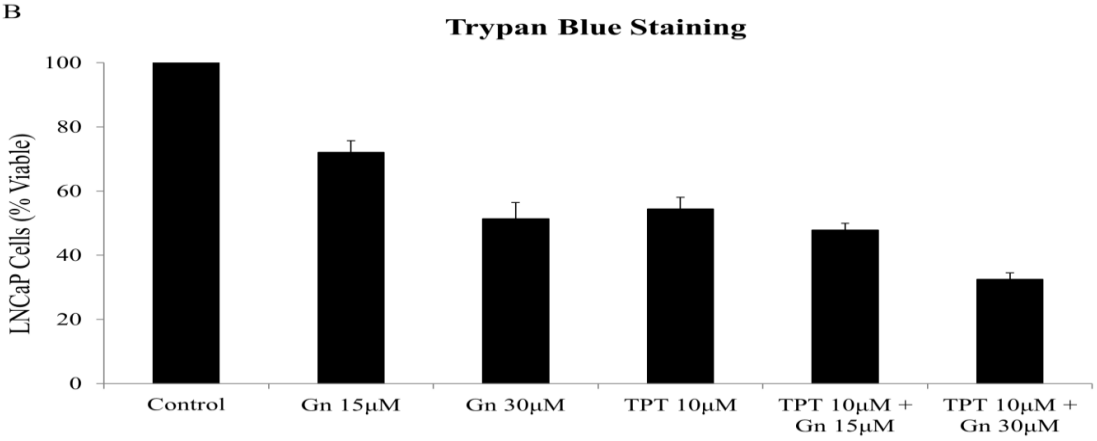

Fig 1B. The viability of the LNCaP cells was tested through Trypan Blue Exclusion Assay. Viable cells were unable to uptake the dye, while dead (non-viable), cells took up the dye and stained blue. % of viable cells decreased in all treatment groups, with a significant decrease noted in TPT_{10} + Gn_{30} treatment groups ($P \leq 0.05$)

Classification of Cellular Death:

Treatment of LNCaP cells with Gn and TPT induced cell death in both singular and combination dosages. Morphological changes correlating to necrotic and apoptotic cell death are also observed during this staining technique. Apoptotic cells will show cell blebbing, formation of apoptotic bodies and overall cell shrinkage. Cells undergoing necrotic cell death will swell in size and ultimately release their cellular components into the extracellular environment.

In all treatment groups, both necrotic and apoptotic cell death was noted. Treatment-induced cell death was mostly apoptosis, which was dose-dependent (Figure 2). Comparatively, genistein-induced apoptosis was significantly higher than topotecan-induced apoptosis ($P \leq 0.05$). However, TPT-Gn combination induced the highest apoptotic cell death in the LNCaP cells, relative to the single treatments (Figure 2). In all treatment groups, there was evidence of necrosis with increasing concentrations.

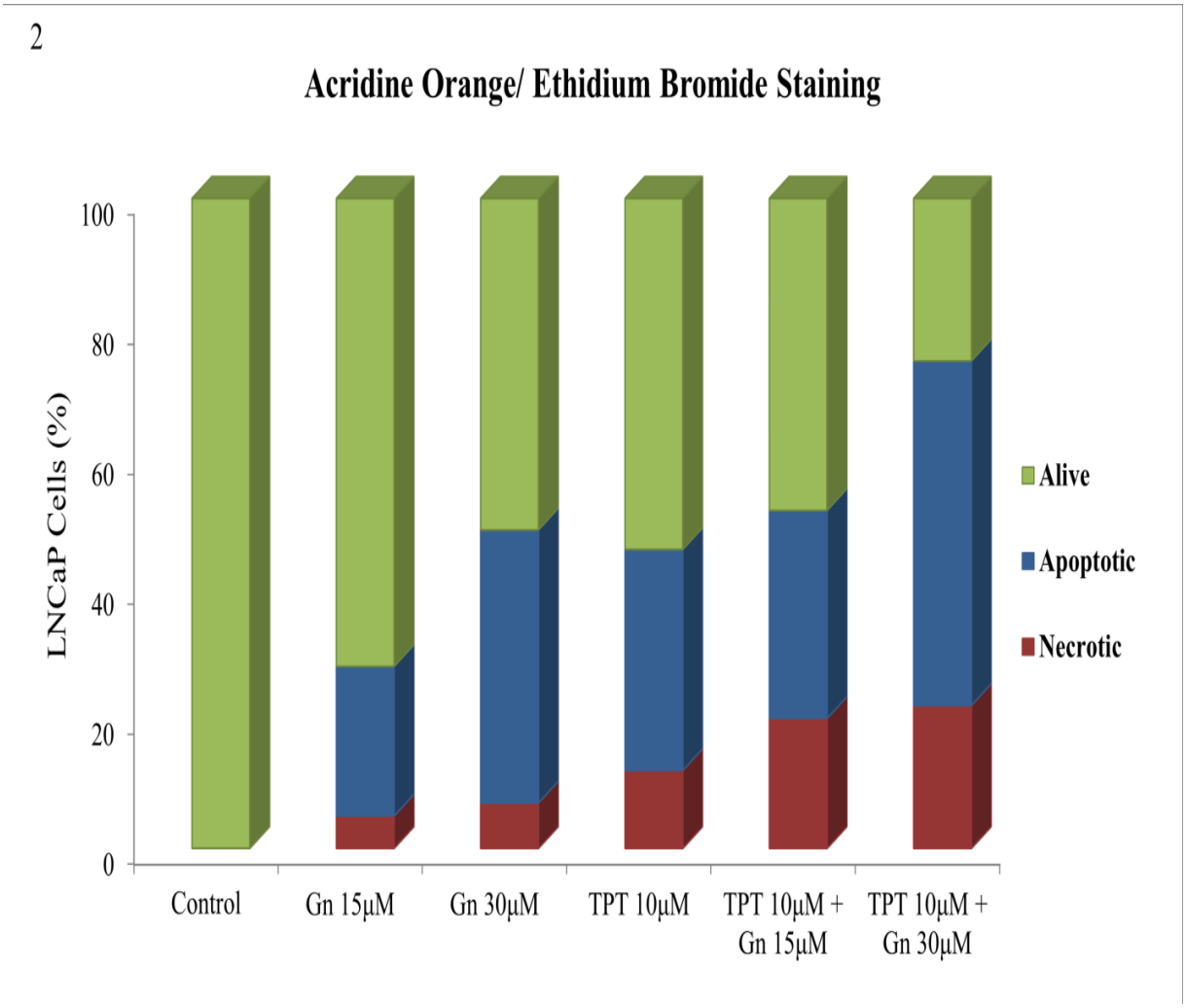

Fig 2. Acridine Orange/ Ethidium Bromide staining distinguish viable, apoptotic and necrotic cells based on green or red fluorescence staining and cell morphology. Genistein-induced apoptosis was significantly higher than topotecan-induced apoptosis ($P \leq 0.05$). The primary mode of cellular death in all treatment groups was apoptosis, with the highest percentage observed in TPT+ Gn combination treatments.

Activation of the Intrinsic-Apoptotic Pathway:

Apoptotic cell death is initiated through the intrinsic and/or extrinsic apoptotic pathway. The intrinsic pathway is reliant on DNA damage, suppression of anti-apoptotic factors and release of cytochrome c due to loss of mitochondrial membrane potential. Mitochondrial membrane potential was observed in all control and treatment groups through the Mitochondrial Membrane Potential Assay (Jc-1 Staining). Viable cells with intact mitochondrial membrane potential will cause the formation of Jc-1 aggregates; thus fluorescing red. Treatments involving the activation of the intrinsic pathway will lower mitochondrial membrane potential; leading to cytochrome c release in to the cytoplasmic region. LNCaP cells with lowered mitochondrial membrane potential will have a green fluorescence, as the Jc-1 monomers remain in the cytoplasm.

In the present study, the highest percentage of red fluorescence (indicating viable cells) was seen in the control, untreated groups. Individual and combination treatments of TPT and Gn all showed a minimal amount of red fluorescence (viable cells) with a high degree of green fluorescence; indicating loss of mitochondrial membrane potential and initiation of the intrinsic apoptotic pathway (Figure 3).

To further trace the activation of the intrinsic apoptotic pathway, western blotting was done to detect levels of cytosolic cytochrome c and bcl-2 expression. Protein lysate collected from all treatment groups were utilized to detect cytochrome c levels. The loss of mitochondrial potential (Figure 3) allowed cytochrome c release from the mitochondria into the cytoplasmic region of the LNCaP cells. Single and combination treatments of TPT and Gn showed varying band intensities correlating with cytochrome c release (Figure 4A). Gn single treatment showed a darker band formation in higher dosages (Gn_{30}). Combination treatments were also positive for cytochrome c release, with the darkest band intensity observed in TPT_{10} + Gn_{30} samples (Figure 4A). Control samples did not show cytochrome c release.

The expression of the anti-apoptotic protein bcl-2 was also detected. The highest level of bcl-2 was seen in the control LNCaP samples, while all treatment groups had diminished expression bands (Figure 4A). The single Gn treatments had a lower bcl-2 expression levels at higher genistein concentration (Gn_{30}). TPT_{10} protein extracts also had low bcl-2 expression, but the TPT+ Gn combinations showed the highest amount of bcl-2 down-regulation with the faintest band patterns (Figure 4A), corresponding to lowest bcl-2 expression. Optical density values were calculated for all samples to allow for a quantitative analysis of band intensity (Figure 4B and 4C)

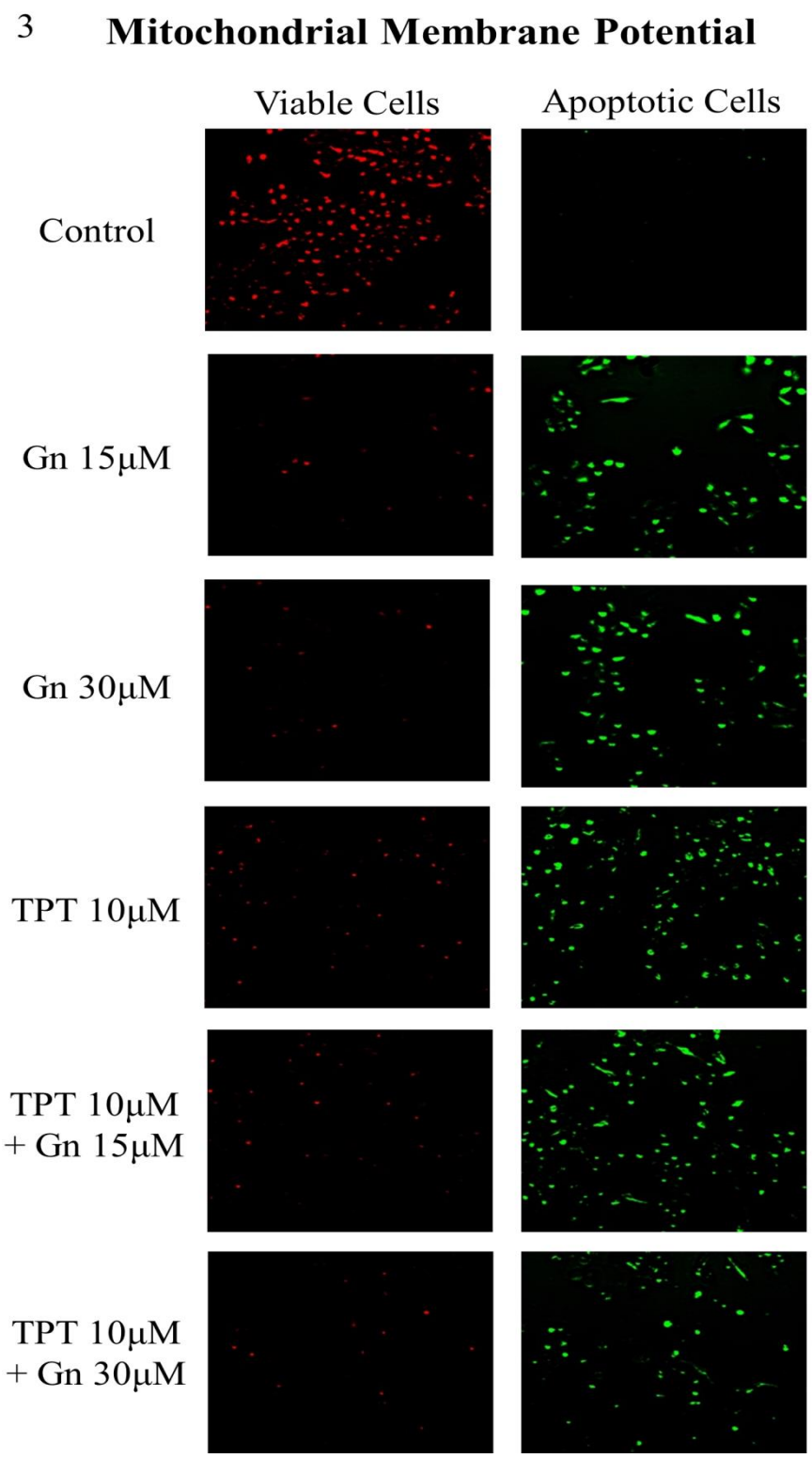

Fig 3. Mitochondrial Membrane Potential was tested through fluorescent staining. Healthy cells, with intact mitochondria, stained red. Unhealthy cells undergoing apoptosis will have a diminished mitochondrial membrane potential; therefore staining green. Control samples stained red, with a minimal amount of green fluorescence. Single and combination treatment groups all displayed a high amount of apoptotic cells through green fluorescent staining. Data is representative of two independent experiments

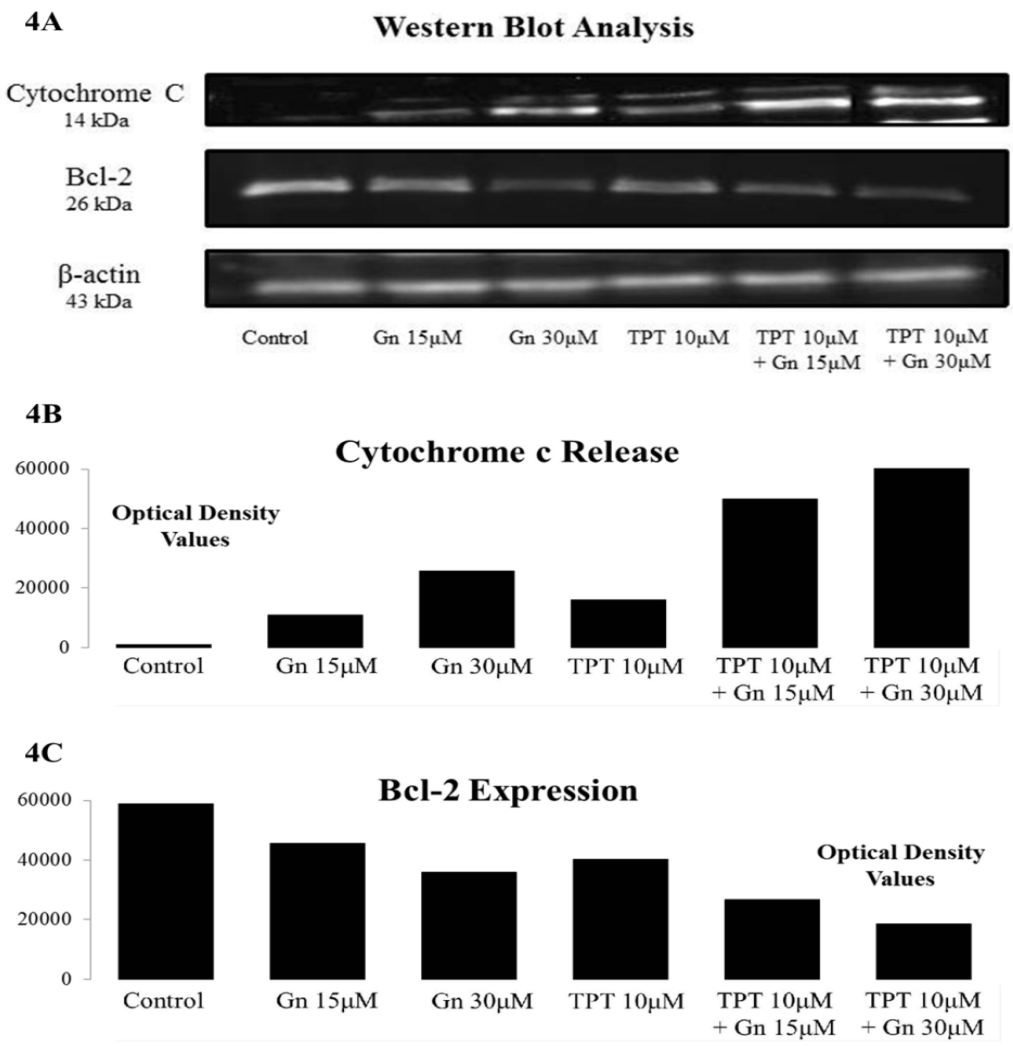

Fig 4A. Western blotting technique was completed to detect the release of cytochrome c (14kDa) and Bcl-2 (26kDa) expression. Mitochondrial cytochrome c release into the cytoplasm was detected in all treatment groups with the highest band intensity noted in the combination treatment groups (TPT + Gn). The expression of the anti-apoptotic Bcl-2 protein was diminished in all treatment groups, with the lowest band intensity noted in combination treatment groups (TPT + Gn). B-actin (43kDa) was loaded as a protein loading control. **Fig 4B- 4C.** Optical density values were calculated to allow for a quantitative analysis of band expression. Western signal and background coloration was reversed to allow for better analysis of band patterns.

DISCUSSION:

The aim of this project was to further investigate the activation of the apoptotic pathway of TPT and Gn combination treatments in LNCaP prostate cancer cells. Our lab has previously shown that TPT+ Gn combination more effectively reduced the number of viable carcinoma cells when compared to TPT and Gn single treatment. Anti-cancer activities of TPT + Gn combination include an increase in caspase 9 and 3 protease activity, elevated ROS levels and formation of

DNA fragmentation [10]. Further assays and experiments were completed to investigate the method of cell death and the potential activation of the intrinsic apoptotic pathway.

LNCaP cells exposed to single and combination treatments of TPT and Gn in a 24 hour incubation period showed a decrease in cell viability. This data is consistent with previous studies showing that TPT and Gn, individually can limit cellular growth and proliferation [5, 14, 26–28, 31]. Combination of TPT + Gn was significantly more effective in reducing cellular viability than single treatments and TPT_{10} + Gn_{30} had the lowest cell viability after the 24 hour incubation. Micro-photography images of the treated cells were indicative of treatment-induced apoptosis.

Apoptotic cell death is an organized method of cellular suicide; the cell will decrease in size, slice its DNA in consistent strand length and exhibit membrane blebbing. Apoptosis does not induce significant inflammation and does not inhibit the immune system; the exact opposite of the more traumatic form of cell death; necrosis [32, 33]. Therapeutic regimens which induce apoptotic cell death would induce far less cytotoxicity and potentially fewer side effects in patients. Most of the cellular death observed in all the treatment groups was apoptosis, as evidence by the Acridine Orange/ Ethidium bromide fluorescence assay, consistent with previous studies[6, 15–17]. The highest percentage of apoptotic cell death in LNCaP cells was observed in the TPT_{10} + Gn_{30} treatment groups; implying the potential clinical benefits of combining the chemo-therapeutic drug TPT, with Gn.

Gn and TPT have been shown to hinder the topoisomerase II and I enzyme, respectively. These enzymes are essential for DNA replication and repair. Inactivation leads to induction of DNA damage which can initiate the intrinsic apoptotic pathway [14, 22, 23]. Our lab has previously shown that TPT + Gn combination treatments cause increased activation of the caspase 9 and 3 proteases, both utilized by the intrinsic pathway [10]. The intrinsic apoptotic pathway is regulated by mitochondrial release of cytochrome c into the cytoplasm due to decrease in mitochondrial membrane potential. [4, 6]. The loss of mitochondrial membrane potential should correlate with the release of cytochrome c into the cytoplasmic region of the cells. Western blot results showed dose-dependent increase release of cytochrome c in all treatment groups with a more heightened band intensity for the TPT_{10} + Gn_{30} dosage group. This is consistent with previous studies [4, 6].

The intrinsic apoptotic pathway is also regulated by a family of pro- and anti-apoptotic proteins. Anti-apoptotic proteins such as bcl-X_L and bcl-2 have to be down-regulated to allow for reduced mitochondrial membrane potential, cytochrome c release and activation of caspase 9 and 3 proteases [3, 4, 34]. Both Gn and TPT expressed lower levels of bcl-2 protein in the western blot assays when compared to the dark band formation in the control LNCaP cells. The TPT_{10} + Gn_{30} combination had the least expression of the bcl-2 protein when compared to all treatment and control groups. Diminished bcl-2 expressions, as well as loss of mitochondrial membrane potential and heightened cytochrome c release in TPT_{10} + Gn_{30} treatments all indicate the activation of the intrinsic apoptotic pathway. The greatest response was observed in the TPT + Gn combination therapies relative to the single treatments implying potential therapeutic significance of the combination treatments.

CONCLUSION:

The results from this study demonstrate the activation of the intrinsic apoptotic pathway in LNCaP prostate carcinoma cells when exposed to TPT and Gn in single and combination treatments. The TPT_{10} + Gn_{30} dosage was able to reduce a greater quantity of cancer cells, mostly through programmed cell death, then either TPT or Gn individually. The combination of TPT with the soy extract genistein has the potential to lead to treatment options with equal therapeutic efficiency as traditional chemo- and radiation therapies, but lower cell cytotoxicity and fewer side effects in patients.

Competing Interests: The authors have no financial interests or conflicts of interest.

Author's Contribution: All authors contributed to this study.

Abbreviations: GN, genistein; ROS, reactive oxygen species; TPT, topotecan.

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