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APIGENIN INCREASES CISPLATIN INHIBITORY EFFECTS ON THE TELOMERASE ACTIVITY OF TRIPLE NEGATIVE BREAST CANCER CELLS

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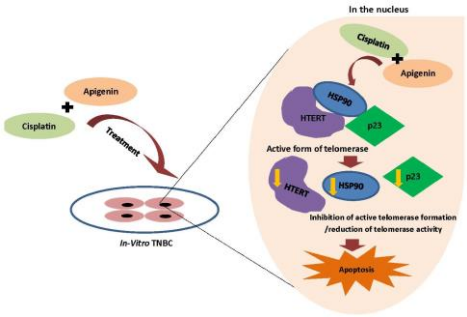
Graphical abstract



Abstract

Inhibition of telomerase activity has emerged as a promising strategy to combat cancer cells, especially ones with no specific molecular targets such as triple negative breast cancer (TNBC). Cisplatin, a chemotherapeutic drug, is causing DNA damage while apigenin, a plant-derived antioxidant, induces apoptosis in various cancer cell types. Little is known about their combined ability to inhibit telomerase activity in TNBC cells. In the current study, the effect of cisplatin in combination with apigenin was investigated with regards to telomerase activity and expression of the telomerase catalytic subunit *hTERT* as well as Heat Shock Protein 90 (Hsp90) and p23 in two types of TNBC (MDA-MB-231; HCC1806) and one non-tumorigenic (MCF10A) epithelial cell line. The results showed that the combined treatment of cisplatin and apigenin significantly down-regulated telomerase activity. The inhibition of telomerase activity was accompanied by a down-regulation of *hTERT*, Hsp90 and p23 at transcriptional and translational level in both TNBC cells, as compared to control cells. The results of the current study suggest that apigenin and cisplatin synergistically inhibit telomerase activity by downregulating the enzyme's catalytic subunit. However, the exact roles of Hsp90 and p23 in the regulation of telomerase activity requires further investigation as they seem to be TNBC subtype-specific.

Keywords: Telomerase activity, *hTERT*, Hsp90, p23, cisplatin, apigenin

Abstrak

Strategi perencatan aktiviti telomerase untuk menghalang pertumbuhan sel kanser telah menjanjikan kesan positif terutamanya bagi rawatan sel kanser yang tiada sasaran molekul khusus seperti rawatan kanser payudara-tiga negatif (TNBC). Cisplatin, sejenis ubat kemoterapi yang bertindak pada kanser sel dengan menyebabkan kerosakan pada DNA sel manakala apigenin adalah sebatian antioksidan yang diperolehi daripada tumbuhan, dilaporkan membunuh kanser sel melalui pengaktifkan program sel mati secara terancang dalam pelbagai jenis sel kanser. Setakat ini, tidak banyak maklumat yang diketahui tentang kesan gabungan rawatan kedua-kedua sebatian ini untuk menghalang aktiviti telomerase di dalam sel kanser TNBC. Dalam kajian ini, kesan gabungan rawatan cisplatin dan apigenin ke atas aktiviti telomerase

dan subunit pemangkin telomerase seperti *hTERT*, 'Heat shock protein 90' (Hsp90) dan p23 dilakukan ke atas dua jenis sel subunit TNBC (MDA- MB-231; HCC1806) dan sel epithelium normal (MCF10A). Hasil kajian menunjukkan bahawa rawatan gabungan cisplatin dan apigenin telah menurunkan aktiviti telomerase dan pada masa yang sama berlakunya penurunan aktiviti transkripsi dan translasi *hTERT*, Hsp90 dan p23 di dalam kedua-dua sel TNBC berbanding sel epithelium normal. Hasil kajian ini menunjukkan bahawa gabungan cisplatin dan apigenin dapat menghalang aktiviti telomerase dengan mengurangkan aktiviti subunit pemangkin telomerase. Walau bagaimanapun, peranan sebenar Hsp90 dan p23 dalam mengawal aktiviti telomerase tidak diketahui dan memerlukan kajian lanjutan yang lebih terperinci kerana kesan rawatan bergantung kepada jenis sel kanser yang digunakan.

Kata kunci: Telomerase aktiviti, *hTERT*, Hsp90, p23, cisplatin, apigenin

1.0 INTRODUCTION

Triple negative breast cancer (TNBC) is an aggressive breast cancer subtype with a poor 5-year prognosis that mainly affects young women (<50 years). Treatment of this type of cancer poses a great challenge to the clinicians as there is a lack of specific therapeutic molecular targets associated with this cancer and a high incidence of drug resistance after the initial treatment [1, 2]. In the search of new therapeutic targets in the treatment of TNBC, the telomerase reverse transcriptase represents a promising molecular candidate for cancer therapy [3].

Almost non-detectable in healthy cells, about 85-90% of cancer cells not only express telomerase but also have an increased telomerase activity [4, 5, 6, 7]. The primary functions of telomerase are to stabilize and maintain the telomere length of chromosomes during cell replication, thus preventing the fusion of chromosome ends and potential rearrangement of DNA segments [8]. Inhibiting telomerase activity in cancer cells will lead to the shortening of telomeres, destabilizing of the chromosomes and eventually leads to cell death via apoptosis [9, 10]. Activation of telomerase activity in cancer cells requires the interaction of its structural protein subunits such as *hTERT*, Hsp90 and p23 [11, 12, 13]. Blocking of telomerase structural subunits was shown to inhibit telomerase activity in cancer cells [13, 14]. Therefore, targeting telomerase activity and its structural subunits would be a good strategy in inhibiting cancer progression.

Cisplatin (*cis*-diammine-dichloro-platinum) a platinum-based drug, is a chemotherapeutic drug used to treat TNBC [15, 16, 17] and various types of malignant tumours such as ovarian [18], prostate and colon cancers [19] has shown various disadvantages like not being cancer specific, causing multiple side effects and drug resistance [19, 20]. The side effects of cisplatin include nephrotoxicity, neurotoxocity and ototoxicity [21] to healthy cells [22].

Various natural products have shown potential in preventing several diseases including cancer. Numerous anticancer agents have successfully been isolated from various sources of natural products such as plants, animals and microorganisms [23, 24]. Apigenin, a bioactive flavonoid derived from *Tinospora crispa*, has been shown to inhibit human cancer cell growth through cell cycle arrest and apoptosis without causing any side effects to normal cells [25, 26, 27, 28, 29]. An alternative approach by combining a current drug therapy with potential natural active compound with lower side effects has been showed to increase the toxicity effect of cisplatin in TNBC cells and overcome the toxicity problem of cisplatin in normal cells. Recently, Al-Rashidi *et al*. demonstrated that the combination of *Tinospora crispa* and cisplatin significantly increased the apoptosis in TNBC while sparing the normal MCF10A breast epithelial cells [30]. As apigenin is a major active compound in *Tinospora crispa*, it is postulated that apigenin could increase the cisplatin-induced apoptotic activity in TNBC while reducing the cytotoxic effect of cisplatin in non-cancerous cells. Furthermore, the potential effect of apigenin in inhibiting cell growth and attenuating telomerase activity in human leukemic cells has been previously reported [31]. However, the single and combined effects of apigenin on telomerase in TNBC cells remain unclear.

Therefore, the present study was aimed to determine the effects of apigenin and cisplatin on telomerase activity and the expression of various telomerase subunits in TNBC. We hypothesized that a combination of cisplatin and apigenin would be able to specifically target telomerase activity and its structural subunits in TNBC cells without harming normal cells as well as increasing the potency of cisplatin and apigenin as a single drug treatment in TNBC via telomerase inhibition.

2.0 METHODOLOGY

2.1 Cell Culture

The normal epithelial breast cell line (MCF10A) and the two triple negative breast cancer (TNBC) cell lines, MDA-MB-231 (mesenchymal stem-like subtype) and HCC1806 (basal-like 2 subtype) were obtained from the American Type Culture Collection (ATCC, USA). MDA-MB-231 and HCC1806 with a passage number less than 30 were propagated in Roswell Park Memorial Institute (RPMI) 1640 (GIBCO) medium supplemented with 10% heat-inactivated FBS (GIBCO Life Technologies, USA) and 1% 10 U/ml penicillin, and 100µg/ml streptomycin (GIBCO Life Technologies, USA) in 5% CO_2 at 37°C. The MCF10A cells (passage number <8) were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (GIBCO Life Technologies, USA) medium supplemented with 10% heat-inactivated fetal bovine serum (GIBCO Life Technologies, USA), 20ng/ml epidermal growth factor (Sigma-Aldrich, USA), 100µg/ml hydrocortisone (Sigma-Aldrich, USA), insulin and 1% penicillin-streptomycin (GIBCO Life Technologies, USA) in 5% CO_2 at 37°C. Cells were harvested for treatment once they have reached 70-80 % confluences.

2.2 Cell Cytotoxicity Assay

Before the treatment, 100mg/ml of stock solution of cisplatin (purity ≥ 99.9%, molecular weight: 300.05, CAS Number: 1566-27-1), apigenin (purity ≥ 97%, molecular weight: 270.24, CAS Number: 520-36-5) obtained from Sigma Aldrich (USA) were prepared in 100% DMSO (Sigma Aldrich, USA). The concentration of the diluents in treated cells did not exceed 0.1% (v/v) for all the experiment. The cells were seeded at 2 x 10^5 cells/ml per well in 96 well plates and incubated overnight before treated with 5, 10, 20, 40, 60, 80 and 100µg/ml of cisplatin or apigenin diluted with media for 72 hours in 5% CO_2 at 37°C. Cell viability for each treatment was measured by adding 20 µl of CellTiter 96® Aqueous One Solution (Promega, USA) to each well followed by 2 hours of incubation. The absorbance was measured at 490nm using a microplate reader (SpectroMax 190, USA). Percentage of cell viability was calculated based on the following formula: (A-B)/A x 100%, where A was the absorbance value for untreated cells while B was the absorbance value for treated cells. The IC_{50} values for each treatment were identified from the graph "percentage of cell viability vs. concentration".

2.3 Telomerase Activity of MDA-MB-231 and HCC1806

Telomerase activity was measured by using the TeloTAGGG PCR ELISA^{Plus} kit (Roche, USA) according to the manufacturer's instructions. Briefly, the cell extracts were prepared from MDA-MB-231 and HCC1806 cells treated with the IC_{50} values of cisplatin, apigenin and the combination of both for 72 hours. The total protein concentration of the cell extracts were measured using Nanodrop (ND-1000 spectrophotometer from Agilent, USA) and standardized to 2µg of total protein. The PCR reactions were performed as follows; elongation at 25°C for 30 minutes, telomerase inactivation at 94°C for 5 minutes and product amplification for 30 cycles with the following temperature settings: 94°C for 30s, 50°C for 30s, and 72°C for 30s. The PCR products and the internal standard were quantitated by reading the absorbance at 450nm using a SpectraMax 190 microplate reader. Heat inactivated extracts and lysed buffer were used as negative control and blank respectively.

2.4 RNA Extraction and Reverse Transcriptase Reaction

Total RNA was extracted from 2x10^5 MDA-MB-231 cells after 72 hours incubation with 12µg/ml cisplatin (IC_{50}) and 8µg/ml apigenin (IC_{50}) and the combination while HCC1806 cells with 12 µg/ml cisplatin (IC_{50}) and 14µg/ml apigenin (IC_{50}) and the combination of cisplatin and apigenin. Total RNA including the one from MCF10A cells treated with all the above concentration was extracted using RNeasy Mini Kit (Qiagen, Germany). RNA concentration was measured by Nanodrop (ND-1000 spectrophotometer from Agilent, USA) and total RNA was standardized to 10ng/µl followed by reverse transcription reaction using iScript reverse transcription supermix from Bio-Rad (USA). Briefly, 15 µl of the master mix containing 4µl of iScript supermix and nuclease-free water were added to the tube containing 5µl of RNA (concentration 10ng/µl) for each reverse transcription reaction. The samples were mixed by pipetting gently up and down and then incubated in a thermal cycler using the following protocol: priming (25°C for 5 min), reverse transcription (42°C for 30 min) and RT inactivation (85°C for 5 min).

2.5 Quantitative-Real-Time Polymerase Chain Reaction (qRT-PCR) of *hTERT*, p23 and Hsp90

The PCR master mix was prepared according to the manufacturer's instruction. Forward and reverse primers are listed in Table 1. The components of master mix used in qRT-PCR are listed in Table 2. The qRT-PCR was completed under the following conditions: activation at 95°C for 15 seconds, annealing and melting at 60°C for 45 seconds (48 cycles) and extension at 72°C for 10 seconds. The samples were run in a Bio-Rad CFX 96 Real-Time PCR system and analyzed with CFX Manager™ software, version 1.5 (Bio-Rad Laboratories, USA). A standard curve was generated from a dilution series of untreated TNBC cells. qRT-PCR results were presented as fold change compared to controls calculated using the comparative C_T method ($2^{-\Delta\Delta C_T}$ method) and normalized using the housekeeping gene (Actin-

S and GAPDH). Each biological sample was run in triplicates of three independent experiments (n=9).

Table 1 List of forward and reverse primers used for qRT-PCR

Gene	Forward primer	Reverse primer	Accession No.
hTERT	CTTGGGAACCAGGACAAAGG	TAAAATTATCCACATGGCTCACGT	XM011514106.1
Hsp90	TCCTTCGGGAGTTGATCTCTAATGC	GAATTTTGAGCTCTTTACCACTGTCCAA	NM00127972.1
P23	ACCAGTTCGCCCGTCCC	CCTTCGATCGTACCACTTTGCAGA	NM006601.6
Actin S	GTGGGGCGCCCCAGGCACC	CTCCTTAATGTCACGCACGATTTC	NM001101.3
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG	NG007004.4

Table 2 Component of SYBR Green Master Mix for qRT-PCR

Reagents	Volume per reaction (µl)	Final Concentration
MyIQ SYBR Green	5	1x
Primer (Forward)	1	200 nM
Primer (Reverse)	1	200 nM
Deionised Water	2	-
cDNA template	1	-
Total volume	10	-

2.6 Protein Expression of Heat Shock Protein 90

The samples treated with cisplatin and apigenin were analysed for Hsp90 protein content by using Heat Shock Protein 90 kDa Alpha A1 ELISA kit (USCN Life Science Inc., USA). In brief, 100µl each, of standards, blank and samples were added to the appropriate wells and incubated at 37°C for 2 hours. 100µl of Reagent A was added to each well and incubated at 37°C for 1 hour. Then, the solution was aspirated, and the wells were washed with wash buffer for three times. 100µl of Reagent B was added to each well and incubated at 37°C for 30 minutes. The solutions were aspirated and the wells were washed five times. Then 90µl of substrate solution was added to each well, and the plate was incubated at 37°C for 15 to 20 minutes. After the incubation, 50µl of stop solution was added to each well and the absorbance was read at A450nm using a SpectraMax 190 microplate reader. The standard curve was plotted as absorbance versus concentration (ng/ml). The intensity of Hsp90 expression for each sample was calculated using the standard curve.

2.7 Protein Expression of p23

The effect of cisplatin and apigenin treatments on p23 protein expression in TNBC cells and MCF10A cells was measured using ELISA (Cusabio Biotech Co, Ltd, China). The assay was performed according to the manufacturer's instructions. Briefly, 100µl of standard or sample solutions were added to each well and incubated at 37°C for 2 hours. Then, the solution was removed from each well, and 100µl of biotin-labelled antibody was added, and the plate was incubated at 37°C for 1 hour. The liquid was aspirated, and the wells were washed five times with washing buffer. Then 90 µl of the Tetramethyl-benzidine substrate solution was added to each well, and the plate was incubated at 37°C in the dark for 15 to 30 minutes. Finally, 50 µl of stop solution was added to each well of the plate, and the absorbance was read at 450nm within 5 minutes of adding the stop solution.

2.8 Statistical Analysis

Statistical analyses were performed using SPSS software version 16. An initial descriptive analysis was carried out using mean ± SD. The differences between the treated and untreated groups were tested using one-way ANOVA followed by Bonferonni post-hoc analysis. Differences were considered as statistically significant at value $*p<0.05$.

3.0 RESULTS AND DISCUSSION

3.1 Cell Viability Assay

The combination of a chemotherapeutic drug, cisplatin, with a natural product, apigenin, was investigated with regards to their effects on cancer and healthy cells. The cytotoxicity of apigenin and cisplatin were evaluated on two human TNBC cell lines (MDA-MB-231 and HCC1806) and the non-tumorigenic MCF10A using MTS assays. The untreated cells served as negative control. The results are listed in Table 3. Cisplatin at a concentration of 12µg/ml and apigenin at a concentration of 8µg/ml decreased 50% of the cell viability in MDA-MB-231. In HCC1806 cells, 6µg/ml cisplatin and 14 µg/ml apigenin inhibited 50% of the cell viability. In MCF10A cells, a higher dose of cisplatin (30µg/ml) and apigenin (94µg/ml) were required to reach the IC_{50} dose compared to those in the TNBC cell lines.

The antioxidant effect of apigenin and many other flavonoid compounds are well documented.

For example, apigenin showed potential anti-cancer activity against several breast cancer cells such as MCF-7 [32], MDA-MB-453 [33], as well as MDA-MB-231 cells [34]. In the present study, cisplatin and apigenin exhibited growth inhibition against MDA-MB-231 and HCC1806 cells in a dose-dependent manner.

Table 3 The IC_{50} values of cisplatin and apigenin on MDA-MB-231, HCC1806, and MCF10A after 72 hours of treatment

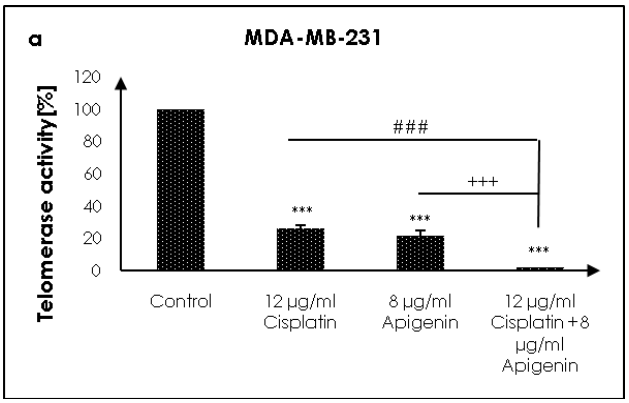
Cell line	Cisplatin IC_{50}	Apigenin IC_{50}
MDA-MB-231	12 ± 2 µg/ml (***)	8 ± 4 µg/ml (***)
HCC1806	6 ± 5 µg/ml (***)	14 ± 6 µg/ml (***)
MCF10A	30 ± 2µg/ml (***)	94 ± 3 µg/ml (***)

Note: The concentration of cisplatin and apigenin inhibits 50% of cell viability of MDA-MB-231, HCC1806, and MCF10A cells. The values shown are means ± SD of three independents experiments. Statistically different from control (***, $p < 0.005$).

3.2 Effect of Cisplatin and Apigenin on Telomerase Activity in MDA-MB-231 and HCC1806 Cells

Cancer cells have multiple strategies to escape cell death, especially apoptosis. One of the strategies is the continuous expression of telomerase activity that allows cancer cells not only to survive but also to proliferate and metastasize [32, 35]. Several studies could show that cancer cells exhibit not only telomerase activity but also that the activity is comparatively high while normal cells have no or very little telomerase activity [6, 7]. Due to this selective activity, any chemopreventive agent that can inhibit telomerase activity in cancer could play an important role in future cancer treatment.

In this present study, cisplatin or apigenin alone significantly reduced the telomerase activity in MDA-MB-231 and HCC1806 cells as shown in Figure 1(a and b). In MDA-MB-231 cells, the effect was greater when cisplatin was combined with apigenin, indicating that cisplatin and apigenin acted synergistically (Figure 1a). However, there was no significant difference in the effect on telomerase activity between the combined treatment of cisplatin and apigenin and cisplatin or apigenin alone in HCC1806 cells (Figure 1b).



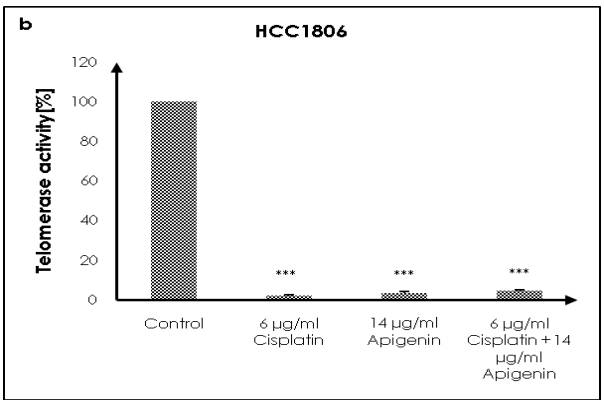


Figure 1 The Effects of Cisplatin, Apigenin and Combined Treatment on Telomerase Activity in MDA-MB-231 and HCC1806 Cells. a) Percentage of telomerase activity in MDA-MB-231 cells after cisplatin or/and apigenin treatment. b) Percentage of telomerase activity in HCC1806 cells after cisplatin or/and apigenin treatment. Data are expressed as mean ± S.D. of three independent experiments, ***p<0.005 compared to the control cells. +++ p<0.005 compared to the apigenin treated cells. ### p<0.005 compared to the cisplatin treated cells

3.2 Inhibition of *hTERT* Gene Expression in TNBC by Cisplatin or Apigenin Alone, and the Combination of Both

hTERT, is reported to be the rate-limiting factor of telomerase activity in several cancer cells [36, 37, 38]. So, inhibition of this subunit could contribute to suppression of telomerase activity in cancer cells. In this present study, combined treatment of apigenin and cisplatin showed positive effects in reducing the level of *hTERT* expression and telomerase activity in both TNBC cells lines. The basal expression levels of *hTERT* in the two TNBC cell lines and the control cell line (MCF10A) confirmed that there is only minimal telomerase activity in the control cells while both TNBC cell lines exhibited increased expression levels. However, the level of *hTERT* expression is significantly lower in HCC1806 cells compared to those in MDA-MB-231 cells. After the treatment with cisplatin or apigenin alone, and the combination of both, the gene expression of *hTERT* in MDA-MB-231 (Figure 2a) and HCC1806 (Figure 2b) cells were significantly reduced in comparison to that in the untreated cells. When compared to the effects of cisplatin or apigenin alone, the combined treatment of cisplatin and apigenin showed no further reduction in the gene expression of *hTERT* in MDA-MB-231 and HCC1806 cells.

The result in agreement with previous data demonstrated a reduction of *hTERT* expression and telomerase activity in head and neck squamous cell carcinoma cell lines (PNUH-12 and SNU-899) after 48 hours of treatment with cisplatin [39]. Moreover, apigenin has been reported to decrease telomerase activity via down-regulation of *hTERT* in leukaemic cells [31]. Our data also suggest that apigenin has a

latent effect in inhibiting telomerase activity in TNBC cells. To our knowledge, there are no data that have been reported previously regarding the special effect of apigenin alone or in combination with cisplatin on telomerase activity in TNBC cells.

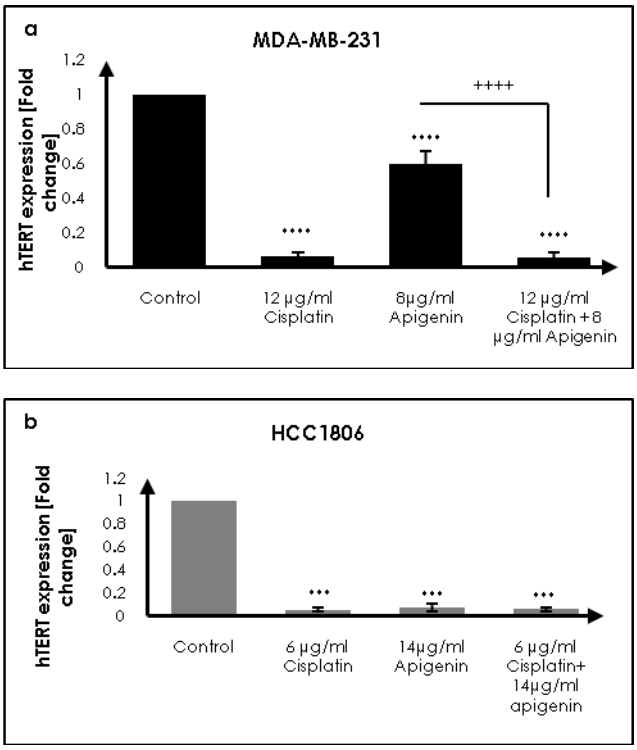


Figure 2 The Effect of Cisplatin and/or Apigenin on the Expression of *hTERT* in TNBC cell lines: Shown are the data for MDA-MB-231 (a) and HCC1806 (b). MDA-MB-231 was treated with cisplatin (12µg/ml), apigenin (8µg/ml) and the combination for 72 hours. HCC1806 was treated with cisplatin (6µg/ml), apigenin (14µg/ml), and the combination for 72 hours. Analysis was performed by qRT-PCR using specific primer sequences. Control cells were normalized to 1. Fold changes of gene expression were measured based on the ratio of *hTERT* gene in treated versus control cells. All data were normalized with two reference genes; GAPDH and Actin S. Data are expressed as mean ± S.D. of three independent experiments, *** $p<0.005$, **** $p<0.001$ compared to control cells. ++++ $p<0.001$ compared to apigenin treated group

3.4 Cisplatin/apigenin Combined Treatment Altered Hsp90 Expression Levels in TNBC and MCF10A Cells.

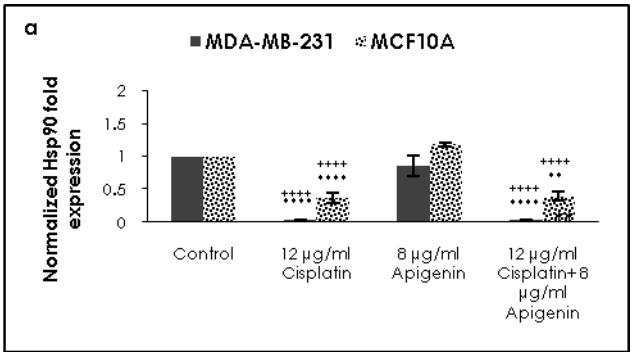
The telomerase protein is a complex structure containing *hTERT*, Hsp90, p23, and other proteins which are critical for telomerase activity in cancer cells [13, 40, 41]. Hsp90 is found in all cells and plays an important role in facilitating cellular protein folding. Hsp90 maintains the specific structure of proteins in order to perform their role in the cells.

The incubation of the two TNBC and the control cell lines with cisplatin or apigenin alone, and the combined treatment of cisplatin and apigenin, resulted in partially contradictory results. In MDA-MB-231 cells, cisplatin alone and the combined treatment showed reduced Hsp90 mRNA expression (0.03 ± 0.01, $p<0.001$) compared to that in the control cells while no change was observed in the apigenin-treated group (Figure 3a). Cisplatin or apigenin alone resulted in higher mRNA expression of Hsp90 in HCC1806 cells (1.3 ± 0.04, $p<0.05$; 1.6 ± 0.15, $p<0.001$), respectively compared to that in the control cells. Interestingly, an inhibitory effect was seen in the combined treatment group where Hsp90 mRNA was suppressed to 0.35 ± 0.05 ($p<0.001$) (Figure 3b).

The Hsp90 protein levels in both TNBC cells were reduced following cisplatin and apigenin single treatment groups to 38 ± 7.2% ($p<0.001$) and 69 ± 16% ($p<0.05$). The Hsp90 protein levels in MDA-MB-231 and HCC1806 cells were greatly reduced after the combined treatment to 70 ± 10%, p<0.05 and 41 ± 7.2 % ($p<0.001$), respectively (Figure 4).

In MCF10A cells, high concentration of apigenin (14µg/ml) resulted in an elevation of Hsp90 mRNA (1.5 ± 0.2, $p<0.01$) and protein expression (129 ± 3.05 %, $p<0.001$). Meanwhile, low concentration of cisplatin (6µg/ml) caused a decreased Hsp90 protein expression in MCF10A to 113 ± 8.7 %, $p<0.05$). Interestingly, the combined treatment caused a down-regulation of Hsp90 mRNA and protein in MCF10A (Figure 4 & 5).

Inhibition of Hsp90 function by cisplatin and apigenin were also reported in another study [42, 43]. Ishida *et al*., [44] reported that the direct binding of cisplatin to the amino and carboxyl terminals of the human Hsp90 also cause the inhibition of Hsp90 activity. Huang and his co-researchers reported that oxidative stress caused up-regulation of Hsp90 expression in atrazine-treated MCF10A cells, resulting in increased cytotoxicity of atrazine in human cells [45]. However, no data has been published on the effect of down-regulation of Hsp90 in MCF10A or other normal epithelium cells.



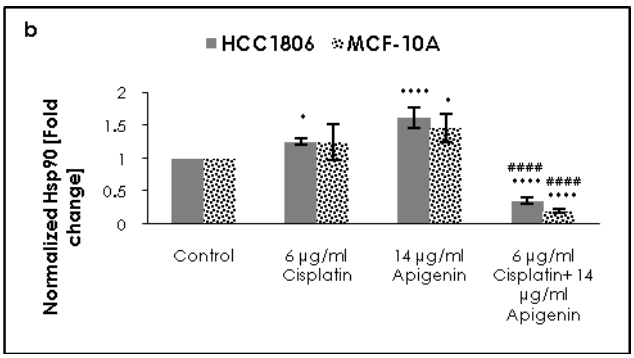


Figure 3 The Effects of Cisplatin and/orApigenin on the Gene Expression of Hsp90 in TNBC and MCF10A. a) The Hsp90 mRNA level in MDA-MB-231 & MCF10A cells treated with IC_{50} concentration of cisplatin and/or apigenin for 72 hours. b) The Hsp90 mRNA level in HCC1806 & MCF10A cells treated with IC_{50} concentration of cisplatin and/or apigenin for 72 hours. Analysis was performed by qRT-PCR using specific primer sequence. All data were normalized with GAPDH and Actin S as reference genes. Data are expressed as mean ± S.D. of three independent experiments, * p<0.05, ** p<0.01, *** p<0.005 compared to control cells. ++++ p<0.001 compared to apigenin in treated cells. #### p<0.001 compared to both single treatment

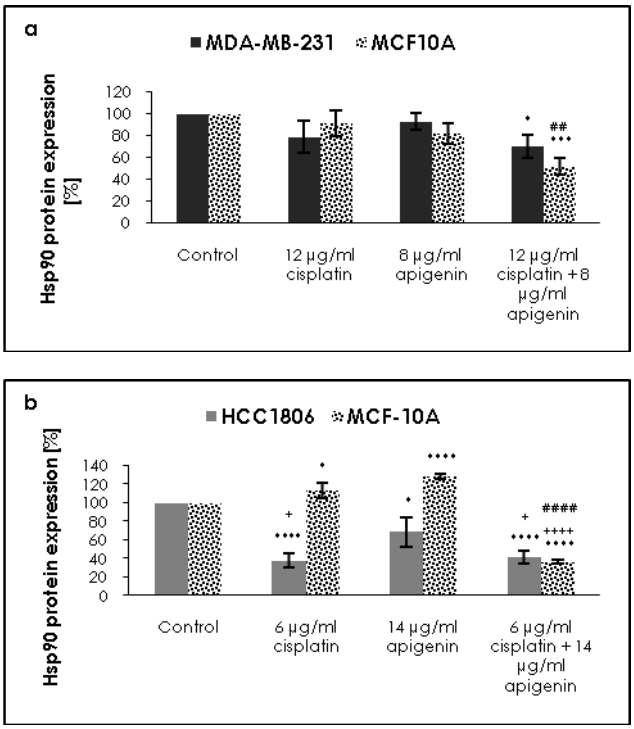


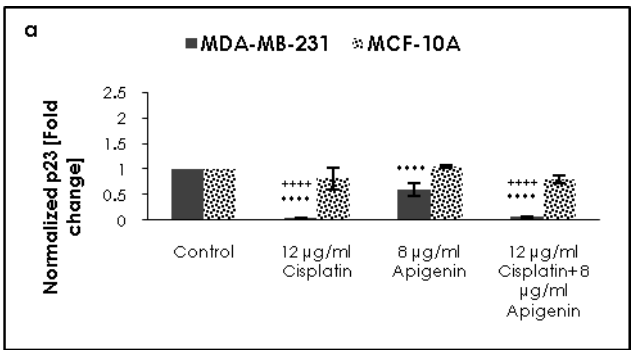
Figure 4 The Effect of Cisplatin or/and Apigenin Treatment on Hsp90 Protein Expression Level in MDA-MB-231, HCC1806 and MCF10A Cell Lines. a) Percentage of Hsp90 level in MDA-MB-231 and MCF10A cells after cisplatin or/and apigenin treatments. b) Percentage of Hsp90 levels in HCC1806 and MCF10A cells after cisplatin or/and apigenin treatments. Combined treatment significantly inhibits Hsp90 expression in both TNBC cell lines. Data are expressed as mean ± S.D. of three independent experiments, *p<0.05, **P<0.01, ***p<0.005, **** p<0.001 compared to the control cells. + p<0.05, ++++ p<0.001 compared to the apigenin treated cells. ## p<0.01, #### p<0.001 compared to the cisplatin treated cells

3.5 Apigenin with Cisplatin Inhibits p23 Levels in TNBC

P23 is a co-chaperone of Hsp90 and functionally important to stabilize the protein complex [46]. Several studies reported that the specific binding of Hsp90 and p23 to *hTERT* are required for the assembly of the active telomerase *in-vitro* and *in-vivo* [12, 47]. Blocking the telomerase activity through reduction of its subunits is considered as an effective strategy for the development of novel cancer therapies [13, 41, 48].

This present study demonstrated the effects of apigenin and cisplatin treatments on Hsp90 co-chaperone, p23, which is reported to be involved in the activation of telomerase activity. The qRT-PCR results showed a significant reduction of p23 mRNA expression after single and combination treatments in MDA-MB-231 and HCC1806 cells (Figure 5). The inhibitory effect on p23 after apigenin and cisplatin treatment was also observed at the protein level in both TNBC cell lines (Figure 6). Although, combined treatment showed potential effect in reducing p23 level in MDA-MB-231 cells, however, still no statistical difference was seen when compared to individual treatment. In MCF10A cells, apigenin alone increased p23 expression level and only the combined treatment showed a significant inhibition of p23 expressions at both transcriptional and translational levels. This study proposes the concentration of cisplatin and apigenin may play an important role in inducing the toxicity in MCF10A cells. Nevertheless, the data on p23 expressions in TNBC and MCF10A cells are scarce. Several studies found that up-regulation of p23 were crucial for normal mouse development [49]. However, the importance of p23 for cell survival *in-vitro* may vary between different types of tissues and cells [40, 50, 51]. Furthermore, Hsp90 and p23 may also have other functions and future studies are required to determine the specific function of these proteins in both cancer and normal cells. In addition, we also cannot deny that other mechanisms also influence the level of telomerase and its subunit in TNBC cells.

The inhibition of Hsp90 and p23 in MDA-MB-231 cells, further explains the inhibition of telomerase activity in this cell line. This is similar to previous data which reported that inhibition of p23 causes disruption of Hsp90 phosphorylation and activates proteasomal degradation of *hTERT* with down-regulation of telomerase activity [41]. Moreover, Lee and Chung (2010) demonstrated that the disruption of *hTERT* and p23 binding resulted in inhibition of telomerase activity in a time and dose-dependent manner [13].



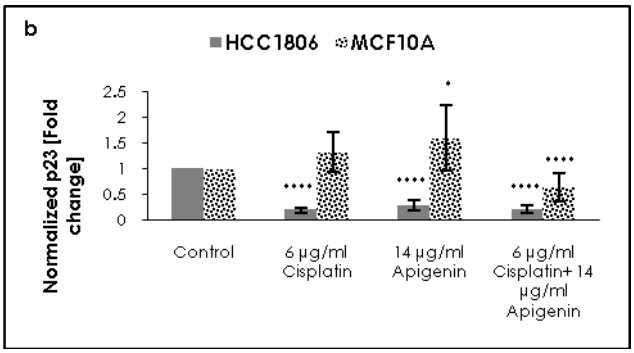
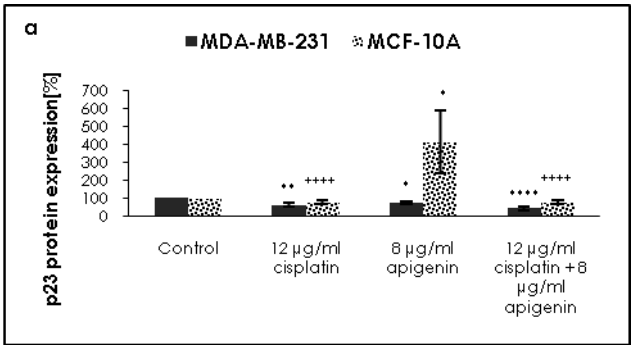


Figure 5 The Effect of Cisplatin and/or Apigenin on the Gene Expression of P23 in TNBC and MCF10A Cells. a) The p23 mRNA expression level in MDA-MB-231 and MCF10A cells treated with IC_{50} concentration of cisplatin and/or apigenin for 72 hours. b) The p23 mRNA expression level in HCC1806 and MCF10A cells treated with IC_{50} concentration of cisplatin and/or apigenin for 72 hours. Analysis was performed by qRT-PCR using specific primer sequences. All data were normalized with GAPDH and Actin S as reference genes. Data are expressed as mean ± S.D. of three independent experiments, * p<0.05, **** p<0.001 compared to control cells. ++++ p<0.001 compared to apigenin treated cells



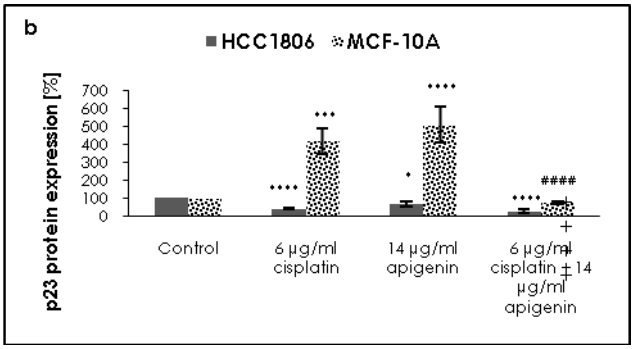


Figure 6 Protein Expression of P23 after Cisplatin, Apigenin and Combined Treatment in MDA-MB-231, HCC1806 and MCF10A Cells. a) Percentage of p23 protein expression after cisplatin or/and apigenin treatment in MDA-MB-231 and MCF10A cells. b) Percentage of p23 protein expression after cisplatin or/and apigenin treatment in HCC1806 and MCF10A cells. Data are expressed as mean ± S.D. of three independent experiments, *p<0.05, **p<0.01, ***p<0.005, ****p<0.001 compared to the control cells. ++++p<0.001 compared to the apigenin-treated cells. ####p<0.001 compared to the cisplatin-treated cells

4.0 CONCLUSION

To our knowledge, this is the first study that shows the effect of apigenin on telomerase activity and its subunits in TNBC cells. The study showed that the additive effect of apigenin is highly dependent on the TNBC subtype. Further studies by using various concentrations of apigenin and cisplatin could be done to achieve a more positive effect. A general inhibition of telomerase activity will affect all telomerase positive cells i.e. stem cells, cancer cells and cancer stem cells. In order to target specifically cancer stem cells and telomerase positive cancer cells, additional markers or delivery methods will have to be developed. Respectively the side effects of a global telomerase inhibition will have to be carefully investigated in clinical trials before these drugs can be used in patients.

In summary, the present study showed that the additive effect of apigenin is different across the cell lines. Based on this study, telomerase and its subunits may be excellent therapeutic targets for treating TNBC cells however, additional marker are required to confirmed the mechanism of action of combined treatment in both TNBC and normal cell lines.

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