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Original Research Paper

Investigating the Cytotoxic and Apoptotic Effects of Telmisartan, both independently and in combination with Doxorubicin, on a Lung Cancer Cell Line

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Abstract: Telmisartan (Tel), an angiotensin receptor blocker, has shown potential in enhancing the anticancer efficacy of Doxorubicin (Dox) against lung cancer, a leading cause of global mortality. This study investigated the cytotoxic and apoptotic effects of Tel alone and in combination with Dox on A549 lung cancer cells. Cells were divided into four groups (control, Tel-treated, Dox-treated, and Tel + Dox-treated) and exposed to six drug concentrations. After 72 hours, cytotoxicity and IC50 values were assessed, followed by BCL-2 expression analysis and apoptosis quantification. Results showed that Tel alone had modest cytotoxicity, but its combination with Dox significantly enhanced anticancer activity, achieving 86.78% cytotoxicity at 800 µg/ml. The combination therapy reduced Dox's IC50 from 292.46 µg/ml to 72.21 µg/ml (CI = 0.45), indicating strong synergy. The dose reduction index revealed a significant decrease in the required Dox dosage when combined with Tel. Furthermore, Tel treatment significantly reduced BCL-2 expression ($P < 0.0458$) compared to the control, similar to the reduction observed in Dox-treated cells. Apoptosis analysis demonstrated a higher percentage of apoptotic cells in Tel-treated groups compared to controls, with Dox inducing slightly greater apoptosis than Tel, though not statistically significant. These findings suggest that Telmisartan enhances Doxorubicin's anticancer effects by inducing apoptosis through a BCL-2-dependent mechanism, highlighting its potential as a combinational therapeutic strategy for lung cancer treatment.

Keywords: Telmisartan, Anticancer, Lung cancer, BCL-2, Apoptosis

1. Introduction

Lung cancer is one of the most common cancers and the leading cause of cancer-related deaths. Globally, lung cancer claimed 1.8 million lives, making it the cancer with the greatest mortality rate [1]. It represent the second in term of the occurrence in Iraq [2], and it account for approximately 8% of all cancer cases [3]. Lung cancer's pathophysiology is complex and poorly understood. Lung cancer development typically begins with genetic mutations in lung epithelial cells. Oncogene's Mutations, such as EGFR (epidermal growth factor receptor), result in continuous activation of signaling pathways which promote division and cell growth, additionally, deletions or mutations in tumor suppressor genes, like P53, which normally control cell cycle checkpoints and DNA repair, allow the cells having damaged DNA to proliferate and survive [4].

The therapeutic approaches to lung cancer are stage-specific and must practically consider histology, molecular pathology, age, co-morbidities, and the patient's desires [5]. The treatment regimen for lung cancer is contingent upon several aspects, including the disease kind (NSCLC or SCLC), its stage, genetic

alterations, and the patient's overall health and preferences. More than 70% of non-small cell lung cancer cases are diagnosed at an advanced stage, precisely stage III or IV; therefore, Cytotoxic chemotherapy remains the backbone of treatment and is crucial for individuals with advanced and metastatic stages of NSCLC [6].

Resistance to chemotherapeutic drugs is a substantial impediment in the treatment of lung cancer patients. It signifies a significant problem that contributes to treatment failure, tumor growth, and relapse. [7]. another challenge of chemotherapeutic agents is their side effects, for example, Doxorubicin (Anthracyclines),which used to treat many type of cancer like Breast and lung cancers [8],develop cardiotoxicity and Exceeding a dose of 400 mg/m2 increases the likelihood of toxicity occurrence [9].Therefore, creating a novel formulations or combination therapies have been evaluated to improve Doxorubicin efficacy and reducing its side effects. Numerous in vivo and in vitro investigations demonstrated that ARBs (angiotensin receptor blockers) like Telmisartan, prevented endometrial cells from proliferating [10], in breast [11], and gastric cancer cells [12]. Angiogenesis, invasion, proliferation, and pro-survival signaling are among the many functions that the Renin-Angiotensin System (RAS) controls.

A large number of these processes are collaborative and interconnected. There is strong evidence from preclinical research that almost every aspect of cancer is regulated by the Angiotensin II/Angiotensin I Receptor axis [13]. Telmisartan also has the capacity to reverse myocardial remodeling and enhance left ventricular hypertrophy in cases of hypertension [14]. Furthermore, Telmisartan lowers the levels of apoptosis-related proteins, thereby reducing cell death in cardiac tissues [15]. It lowers oxidative stress indicators such as malondialdehyde (MDA) [16].

Although Epidemiological surveys indicate that ARB (angiotensin receptor blockers) treatment in hypertensive patients is associated with reduced cancer incidence and mortality rates [17], [18]. Nonetheless, the impact of ARB on the many effects induced by doxorubicin as a combinatorial treatment for cancer, as well as its antitumor efficacy against lung cancer, need further elucidation. Examining this gap can improve the comprehension of the potential role of ARB in lung cancer therapy. It may facilitate the development of innovative therapeutic strategies for lung cancer. This study aims to evaluate the anticancer and apoptotic effects of Telmisartan on the A549 lung cancer cell line, both independently and in conjunction with Doxorubicin.

2.Methodology

Cell line and Chemicals

A549 cancers were acquired in Basra, Iraq, from Biotech Cell Bank. more than 1,000 cell generations and have been extensively researched as models of lung cancer [19]. MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) powder and RIPA lysis solution was purchased from sigma, USA. Phosphate -buffered saline (PBS) and Roswell Park Memorial Institute -1640 were purchased from Gibco, USA. Trypsin-EDTA was purchased from Capricorn, USA. The Trypan blue dye was obtained from Flow Laboratories, United Kingdom. Acridine orange (AO) dye was purchased from Sigma-Aldrich, USA. Benzylpenicillin and streptomycin were procured from Troge, Germany.

Doxorubicin hydrochloride (lyophilized Powder) obtained from Zydus, India. Telmisartan powder obtained from suzhou nuopal new material technology co., Ltd, China. Human Bcl-2(B-cell/Lymphoma2) is obtained from Elabscience Technology Laboratory, USA.

Cellular Cultivation and MTT Assay

Lung cancer cell line (A549) was separated utilizing trypsin-EDTA as a proteolytic agent, phosphate-buffered saline (PBS) for media cleansing, and fetal bovine serum (FBS) to inactivate trypsin. The cells were then grown on a 96-well plate utilizing RPMI-1640 liquid media augmented with 100 Units per ml of penicillin and 100 µg/mL of streptomycin, then the sample incubated for 24 hour at 37 ° C, with 5 % CO2 and 95% humidity to promote the development of a cell monolayer (80% growth phase). The cells which are viable were enumerated utilizing trypan blue dye. Four primary groups were employed: control (untreated cells), Doxtreated cells, Tel-treated cells, and cells subjected to both Dox and Tel (ratio 0.5:0.5), Each experimental group comprised of six concentrations, each with four replicates: 800, 400, 200, 100, 50, and 25 µg/ml. A blank composed just of the medium was utilized to assess the non-specific transformation of Formazan and the pharmaceuticals under investigation. Following 72 hours of incubation, the MTT assay was employed to assess the viability of the cells, and dose-response curves were constructed via non-linear regression employing a four-parameter logistic model Hill equation. IC50 for cell viability, indicating 50% inhibition, was determined for each group using GraphPad Prism 10. The percentage of cell viability was assessed using the subsequent formula:

$$Cell\ viability\% = \frac{As - Ab}{Ac - Ab} \times 100\%$$

Whereas as signifies the absorbance of the sample, Ab denotes the absorbance of the blank, and Ac represents

the absorbance of the control. All assessments were performed in quadruplicate

Measurement of BCL-2 level

After seeding, A549 cells were subjected to the resultant IC50 treatment for 36 hours in four flasks, each including three replicates (control, Dox-treated cells, Tel-treated cells, Dox plus Tel). Subsequent to cell extraction and centrifugation, the supernatants were eliminated. The pellets were gathered, and lysis buffer was utilized to lyse. The cells for protein extraction, followed by the measurement of BCL-2 using ELISA test kits according to the provided instructions.

Apoptosis assay utilizing double staining with acridine orange (AO) and ethidium bromide (EB)

Lung cancer cells were cultured at a density of 4 × 10^4 cells per well in a culture plate and incubated for 24 hrs. at 37°C to achieve optimal confluence; thereafter, the media was discarded, and fresh medium containing the IC50 concentration of the investigated compounds was introduced. The cells were cultivated for a further 24 hours, whereas the control group remained untreated. Subsequently, the cells were washed twice with PBS and stained with fluorescent dye containing AO and EEtBr. Subsequently, an instant investigation and imaging were conducted using a fluorescent microscope [20]. The photos were examined using ImageJ software version 1.43 to determine the total cell count, apoptotic cells, and viable cells [21]. The proportion of apoptosis was determined as follows:

$$\text{Apoptosis\%}=\frac{number\ of\ apoptotic\ cells}{total\ cells\ count}*100$$

3. Results and discussion

MTT Cell Viability Assay

This study aimed to evaluate the anti-cancer effects of Telmisartan on A549 cancer cells, both alone and in combination with Doxorubicin. The viability of the cancer cells was assessed using the MTT test.

concentration (mg/ml)	Cytotoxicity % Doxorubicin alone	Cytotoxicity % Telmisartan alone	Dox:Tel conc.	Cytotoxicity % Doxorubicin plus Telmisartan
Control	0_+0	0	0	0
25	10.11+_0.47	9.62+_5.84	12.5+12.5	51.21 +_ 5.09 a, b,c
50	11.22+_1.27	11.01+_8.21	25+25	52.86 +_9.69a, b,c
100	18.75 +_6.44b	13.76+_4.54	50+50	60.34 +_10.19a,b.c
200	27.98+_ 7.03b	22.54+_5.199b	100+100	76.086 +_6.57a,b ,c
400	50.99+_ 5.64b	51.56+_8.22b	200+200	81.913 +_3.90a,b,c
800	63.72+_7.35b	71.95+_2.73b	400+400	86.78 +_2.02a,b

Table 1 Comparison between cytotoxic effect of doxorubicin alone and the combination of doxorubicin and telmisartan against A495 cell line
a: Significant from Doxorubicin alone p value< 0.05
b: Significant from Control p value < 0.05
c: Significant from telmisartan p value < 0.05

Cytotoxicity escalates significantly with the concentration of Doxorubicin, however not in a totally linear fashion. The increase is notably more significant at elevated dosages, especially between 400 mg/ml and 800 mg/ml, where the rise in cytotoxicity is 12.73% (from 50.99% to 63.72%).

The greater consistency of data at elevated doses for Telmisartan (reduced standard deviation at 800 mg/ml) suggests that higher concentrations of Telmisartan exhibit more predictable cytotoxic effects than Doxorubicin.

The combination therapy at the maximum dose of 800 mg/ml yields 86.78% cytotoxicity, the highest value recorded in this dataset. This indicates that the combined therapy produces more significant cytotoxic effects than either drug individually, as evidenced by figure 1.

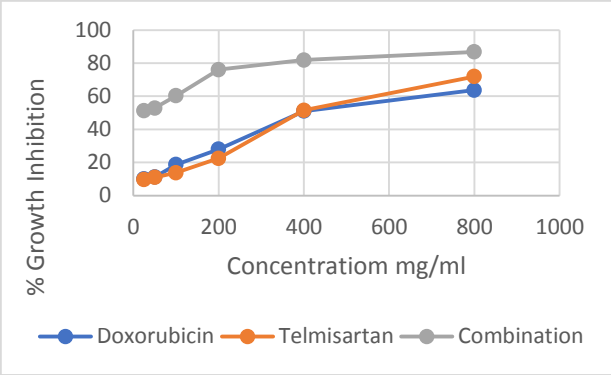


Fig 1. Effect of doxorubicin and telmisartan combination on A549 cancer cell line at various concentrations.

There was a significant difference on growth inhibition % between doxorubicin alone and doxorubicin plus telmisartan combination. P value < 0.021.

The Effect of Doxorubicin plus Telmisartan combination on IC50, CI,DRI on the A495 Cell line

The IC50 of Doxorubicin plus Telmisartan was 72.21 µg/ml compared to 292.46 and 341.97 for Doxorubicin and Telmisartan alone, respectively. This was adjusted through calculating of DRI for Dox equal to 8.11 and CI equal to 0.45. These findings are displayed in Table (2).

Table 2: The Effect of Doxorubicin plus Telmisartan combination on IC50 of A549 cell line.

Parameter/Drug	Doxorubicin	Telmisartan	Combination
IC50 µg/ml	292.46	341.97	72.21
CI	_	_	0.45
DRI	8.11	9.47	__

CI: combination Index, where CI less than 1 refers to synergism. DRI :dose reduction index .

Doxorubicin effect on BCL-2 Concentration

The study's findings indicated a substantial reduction in BCL-2 concentration (P< 0.0229) following the treatment of A549 cells with the IC50 of Doxorubicin, in comparison to the control group, as illustrated in figure (2).

Telmisartan effect on BCL-2 Concentration

The study results indicate a substantial reduction in BCL-2 concentration (P < 0.0458) after treating A549 cells with the IC50 of Telmisartan, in comparison to the control group, as illustrated in Figure 2.

Comparison between the effect of Doxorubicin alone and against Doxorubicin plus Telmisartan combination on the BCL-2 Concentration

Subjecting A549 to Doxorubicin plus Telmisartan combination IC50 showed no significant decrease in BCL-2 level (P= 0.1290) compared to cells treated to Doxorubicin alone, as shown in figure (3).

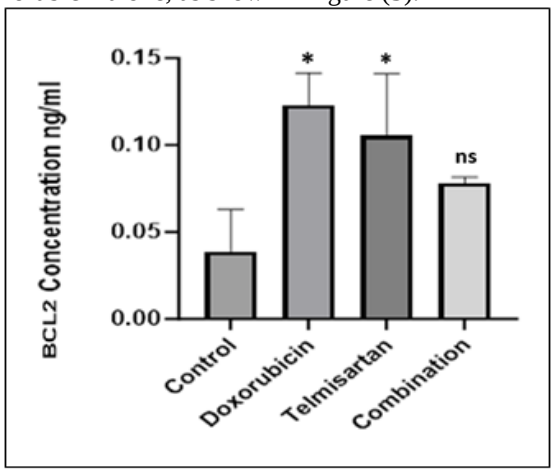


Fig 3. Statistical analysis (One-Way ANOVA), Effects of Doxorubicin, Telmisartan and their combination on BCL-2 concentration in the A549 cell line. * P<0.05, ns P>0.05 compared to control.

The IC50 values of Doxorubicin, Telmisartan, and their combinations were utilized to treat the A549 cell line 24 hours prior to evaluating the existence of apoptotic cells.

A549 cells were discovered to be undergoing apoptosis (cells with orange-red fluorescent color and nuclear shrinkage) following AO-EtBr labelling, while the control group (untreated cells) always had a green, fluorescent color. Figure (3.4).

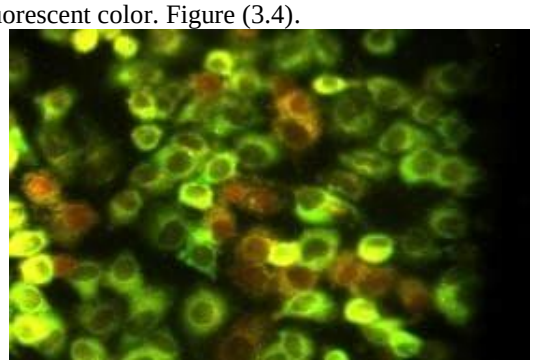


Fig 4. Detection of apoptosis by (AO/EB) in A549 cells (Doxorubicin-treated cells).

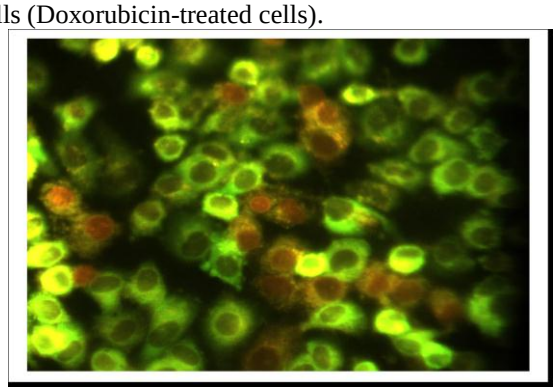


Fig 5. Apoptosis Detection by (AO/EB) in A549 cells (Telmisartan -treated cells).

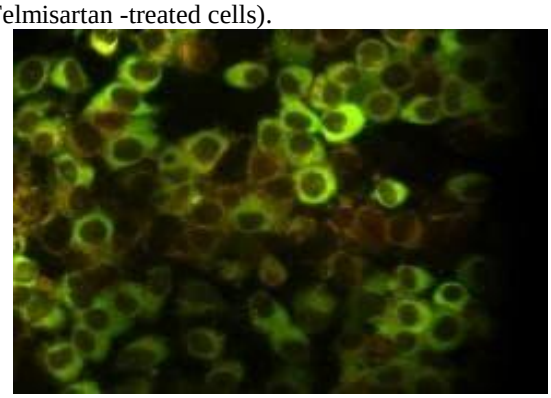
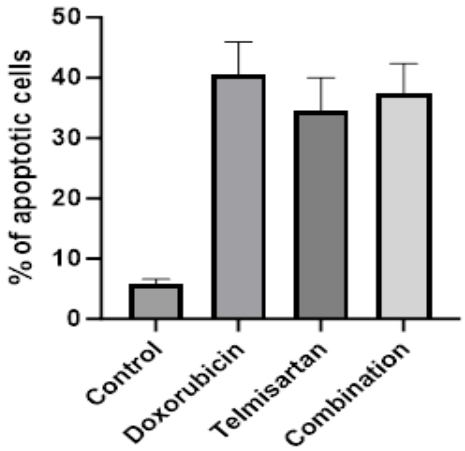


Fig 6. Apoptosis Detection by (AO/EB) in A549 cells, (Doxorubicin plus Telmisartan treated cells)



a: significant from control

Fig 7. Analysis of Apoptosis by ImageJ (Doxorubicn,Telmisartan and their combination) vs Control.

Lung cancer remains one of the most prevalent cancers globally, exhibiting substantial mortality rates in both genders. Despite several treatment modalities, including chemotherapy, surgery, immunotherapy, radiation, and targeted therapy, the processes contributing to treatment resistance in certain lung cancer cell types remain inadequately comprehended [22]. This study was initiated due to the growing demand for innovative therapeutic strategies to improve cancer treatment efficacy and reduce toxicities, focusing on the synergistic effects of Telmisartan (Tel), an angiotensin receptor blocker (ARB), and Doxorubicin (Dox), a widely utilized chemotherapeutic drug, on A549 lung cancer cells.

This study found that Telmisartan exhibited minor cytotoxic effects when used independently, aligning with prior reports indicating that ARBs may have certain anticancer capabilities. Nonetheless, its combination with Doxorubicin produced substantial synergistic effects, leading to a notable enhancement in cell mortality. The combination therapy attained a cytotoxicity rate of 86.78% at the maximum concentration, indicating a significant improvement compared to Doxorubicin treatment alone. The findings indicate that Telmisartan may enhance the susceptibility of A549 cells to the cytotoxic effects of Doxorubicin, potentially addressing certain issues related to Doxorubicin resistance and toxicity.

This study demonstrated a notable reduction in the IC50 of Doxorubicin from 292.46 µg/ml to 72.21 µg/ml when co-administered with Telmisartan., demonstrating a potent synergistic interaction between the two agents. The combination index (CI) value of 0.45 reinforces this synergy, as values below 1 signify a synergistic interaction. This indicates that Telmisartan may improve the effectiveness of Doxorubicin at reduced doses, potentially mitigating the side effects linked to greater chemotherapeutic doses.

The notable decrease in BCL-2 expression after Telmisartan medication corresponds with the suggested mechanism of action. BCL-2 is an anti-apoptotic protein that is essential for the regulation of cell viability. The downregulation signifies the initiation of apoptosis, a crucial mechanism for the eradication of cancer cells. The combination treatment led to a more pronounced decrease in BCL-2 levels, and this impact was statistically significant when compared to the control group with Telmisartan alone. This suggests that Telmisartan may not only improve cellular sensitivity to chemotherapy but also directly promote cell death by affecting pro-apoptotic and anti-apoptotic proteins, such as BCL-2.

Both Telmisartan and Doxorubicin significantly elevated the fraction of apoptotic cells relative to the control; however, the combination therapy yielded the highest rate of apoptosis.

Nonetheless, the disparities between Telmisartan and Doxorubicin treatments were not statistically significant, suggesting that both medications independently facilitate the activation of apoptosis. The BCL-2 expression data further substantiates a possible convergence in the mechanisms of action of both medicines.

The Dose Reduction Index (DRI) further substantiates the prospective clinical efficacy of co-administering Telmisartan with Doxorubicin. The reduction of the required dose of Doxorubicin in combination therapy may mitigate the side effects typically linked to high-dose chemotherapy, including cardiotoxicity and myelosuppression, thus enhancing the overall safety profile of cancer treatment.

The present study offers significant insights into the synergistic effects of Telmisartan and Doxorubicin on lung cancer cells; nonetheless, numerous limitations merit attention. This inquiry was initially performed in vitro utilizing a singular cell line (A549). While A549 cells serve as a prevalent model for lung adenocarcinoma, additional investigations utilizing diverse cell lines and in vivo models are essential to validate our results and evaluate the therapeutic efficacy of the Telmisartan-Doxorubicin combination within a more intricate biological framework. Furthermore, while the study emphasizes apoptosis through BCL-2 regulation, it is imperative to investigate additional molecular processes, including as autophagy, DNA damage response, and p53 activation, to achieve a more thorough comprehension of the synergistic effects of the medications.

This study demonstrates Telmisartan's ability to augment the anticancer efficacy of Doxorubicin in lung cancer cells. The combined therapy exhibited synergistic cytotoxic effects and triggered apoptosis via a BCL-2 dependent mechanism, proposing a feasible strategy to improve the effectiveness of conventional chemotherapeutic agents while reducing their associated toxicities. Additional study is required to corroborate these findings in vivo and investigate the wider therapeutic ramifications of combining Telmisartan with other chemotherapy drugs.

4. Conclusion

Telmisartan (ARABs) have shown anticancer efficacy against A549 cells, as indicated by MTT assay findings.

Moreover, in conjunction with Dox, they collaboratively diminished the survival of A549 cells, potentially lowering the required Dox dosage and alleviating its harmful effects on humans during cancer treatment protocols.

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Author's Declaration

The authors hereby assert that the work provided in this article is original, that they accept full responsibility for any claims regarding its content, and that the study was conducted under the supervision of the Faculty of Medicine at the University of Basra.

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