Evaluation of Anticancer Activity of Olmesartan and Ramipril On A549 Cell Line

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Angiotensin Converting Enzyme Inhibitor (ACEI) and Angiotensin II type 1 receptor antagonist (ARBs) are the most efficient cardiovascular drugs and exhibited efficient cytostatic activity in vitro in many malignant and normal cells[1].OBJECTIVE: This study aims to assess the anticancer activity of these two drugs in a dose dependant manner using A549 cell line through MTT assay and Cell cycle analysis.. MATERIALS AND METHODS: Ramipril and Olmesartan were added to A549 at various concentrations ranging from 10{ v to 10mM.The dot plot of the cytotoxicity results were used to extrapolate the IC50 values. The dot plot of flow cytometry results were used to extrapolate the DNA percentage in phases of cell cycle. The plates were read at 570 nm by using a PERCLIN ELMER (multimode reader). Measurements for concentration required for 50% inhibition was noted. RESULTS: Ramipril and Olmesartan were added to A549 at various concentrations ranging from 10{ v to 10mM.The dot plot of the cytotoxicity results were used to extrapolate the IC50 values. The dot plot of flow cytometry results were used to extrapolate the DNA percentage in phases of cell cycle

Angiotensin converting enzyme inhibitor (ACEI) and Angiotensin II type 1 receptor antagonist (ARB) are the most efficient hypertensive drugs. They are the first line drugs in cardiac failure and preferred drugs in hypertension with diabetes mellitus [2]. It was established in various studies that ACEI and ARBs inhibits Angiogenesis, proliferation of cancer cells, migration of cells, and Tissue development[3, 6]. The drugs in current use are Ramipril and Olmesartan both are available orally with less adverse effects.

In the Renin Angiotensin system cascade the multifunctional potent endogenous chemical is angiotensin II which is an efficient vasoconstrictor[7]

(suganuma et al). Angiotensin II plays an important role in enhancing tumor angiogenesis and growth.[8] Studies have shown that angiotensin receptor antagonist Candesartan inhibits tumor angiogenesis by preventing the lung metastasis of renal murine cells[8.]

Various studies have shown that anticancer agents produce deleterious side effects by damaging normal cells [9, 11]. So therefore use of ACE Inhibitors and ARBs can be efficient adjuvants in anticancer therapy with a propensity for less toxicity. A few studies report that ACEI and ARBs induce cancer[12] and some studies have reported they exhibit anticancer property[3]. The present study aims to

demonstrate the anticancer activity of the two drugs Ramipril and Olmesartan through MTT assay and cell cycle analysis on human A549 lung cancer cell line. The prevalence of new lung cancer accounts for 13% [13] in the world and In India it accounts for 6.9%[14].

The study drug Ramipril is a selective oral Inhibitor of ACE which is currently used to treat blood pressure and cardiovascular disease[15]. Latest studies shown that Ramipril inhibits the proliferation of human mesangial cell and regulates cell growth and division[16]. This drug cause adverse reaction like cough, dizziness, nausea, vomiting and light headedness. Olmesartan is a selective oral inhibitor of Angiotensin 1 receptor which is also currently used to treat blood pressure and cardiovascular disease[17]. It also has shown strong apoptotic activity against HeLa human cell lines[18.] This drug causes adverse reactions like dizziness, weakness and light headedness. Owing to the association of ACEI and ARBs with lung cancer this study was carried to evaluate the anticancer potential of Ramipril and Olmesartan through MTT assay and cell cycle analysis on human A549 lung cancer cell line.

Objective

This study aims to assess the anticancer potency of these two drugs through MTT assay and flow cytometry analysis on human A549 lung cancer cell lines.

MATERIALS AND METHODS

Chemicals and reagents

A549 cells were procured from National Centre for Cell Sciences, Pune. Ramipril, Olmesartan, 3- (4, 5-Dimethylthiazol-2-Yl), 2, 5-diphenyl tetrazolium (MTT), Dimethyl sulfoxide (DMSO) and propidium iodide were purchased from sigma aldrich. Fetal bovine serum (FBS), Phosphate-Buffered saline (PBS) was purchased from Gibco, Invitrogen Life Technologies. This study was done in Imrahl labs and VClin Bio lab at central research facility of Sri Ramachandra deemed to be university

MTT Assay

The cytotoxicity of the two drugs was performed according to the method of Mossmann[19.] Briefly, cells (1×10^5/well) were seeded in 24-well plates following which cells were incubated at

37^0C with 5% CO_2 atmosphere. Upon reaching confluence the cells were washed in Phosphate Buffered Saline (PBS) and the medium was changed. Increasing concentrations of both the drugs obtained through serial dilution were added to the different wells and were marked respectively. One of the wells was treated only with the diluent which served as the control. The culture plates were incubated for 24 hrs at 37^0C with 5% CO_2 atmosphere. After incubation, the treatment medium were collected from all the wells and washed with PBS (pH 7.4). $100\mu l$/ well (5mg/ml) of 0.5% 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl—tetrazolium bromide (MTT) was added to each well and the cell plate was incubated for 4 hours following which 1ml of DMSO was added in all the wells. This helps in dissolving the insoluble crystalline formazan product for effective absorbance measurement. The absorbance at 570 nm was measured with UV-visible Spectrophotometer using DMSO as the blank and the results were tabulated. Measurements for concentration required for 50% inhibition was noted.

Cell Cycle Analysis Flow Cytometry

Flow cytometry is a quantitative assay was done to measure the DNA content of cells. Cell cycle analysis was performed by method of Del Bino *et al*[20]. This assay was done in VClin Bio lab. Cells were seeded into a 6 well plate 0.5×10v cells/well. Upon reaching 80% confluence the cells were washed with PBS and drugs at different concentrations i.e. 5ìM and 10ìM and 15μM were added to the A549 cell lines. The treatment medium were collected after Trypsinisation then fixed with 70% ethanol. Then these cells were centrifuged at 1500 RPM then stored at 4R"c overnight and incubated for one hour. Then stained with propidium iodide in presence of 1% RNASE at 37R"c and analyzed the sample in a flow cytometer. A gate (R_1) was drawn to show the total events. The dot plot of flow cytometry was drawn to extrapolate the DNA percentage in phases of cell cycle by setting a gate in the single population (G0/G1/S/G2/M). The emitted fluorescent light of the DNA dye (PI emits fluorescence in FL2 channel) generates an electronic signal that can be recorded as high (FL2H) for the intensity of the staining as well as measured as pulse-area (FL2A) and pulse-width (FL2W) of the samples. In the FL2-width *vs*

FL2-area dot plot, a gate (G0/G1/S/G2/M) was set around the single population. Ten thousand events were acquired and the percentage of DNA content in each cell cycle phase were analyzed using Cell Quest pro software (Becton Dickinson,USA).

RESULTS

MTT Assay

The concentrations ranged from 10{ᵛ to 10mM, IC50 value of Ramipril was 0.012 mM respectively. The cell viability of Ramipril at different concentrations on lung cancer cell line were shown in Table- 1.The IC50 value of Olmesartan was 0.35mM.The cell viability of Olmesartan at different concentrations on lung cancer cell line were shown in fig 1.

$$\% \text{ of cell viability} = \frac{A549 \text{ of treated cells}}{A549 \text{ of control cells}} \times 100$$

Flow cytometry

We performed cell cycle analysis for determining the inhibitory effect of Ramipril and Olmesartan on lung cancer cell lines. Cells were treated with control, Ramipril 5µM, 10 µM, 15 µM and Olmesartan 300 µM. The percentage of cells in the G0/G1, S, G2/M phases was counted by flow cytometry shown in table-2. Ramipril when treated with different concentrations 5µM, 10 µM, 15 µM there was increase in the percentage of G2/M cells to 10%, 11%, 13% shown in fig.2.Similarly Olmesartan at 300 µM percentage of G2/M cells increased to 15% shown in fig.3 when compared with the control. Our results showed that Ramipril and Olmesartan arrested G2/M phase.

DISCUSSION

Our study shows the potential antitumor activity of Ramipril and Olmesartan on human

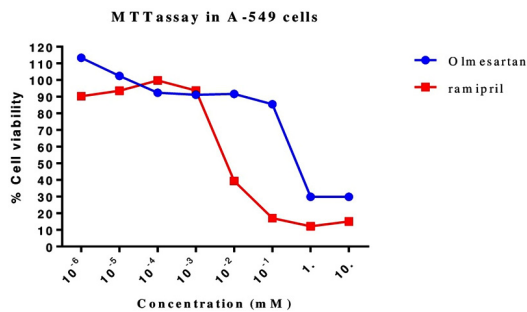**Table 1.** Effect of cell viability of Ramipril and Olmesartan on lung Cancer cell line

MTT ASSAY

CONCENTRATION	%OF CELL VIABILITY	
mM	RAMIPRIL	OLMESARTAN
10^{-6}	90.30±2.20	113.31±2.87
10^{-5}	93.53±2.86	102.42±2.19
10^{-4}	99.73±1.47	92.78±2.80
10^{-3}	93.62±2.79	91.13±3.82
10^{-2}	39.35±2.84	91.67±3.42
10^{-1}	17.07±1.27	85.85±2.81
1	12.13±0.22	29.84±0.38
10	15.09±0.49	29.84±1.65

Table 2. Shows the percentage of Ramipril and Olmesartan in different phases of cell cycle at various concentrations 5 µM, 10 µM and 15 µM and 300 µM

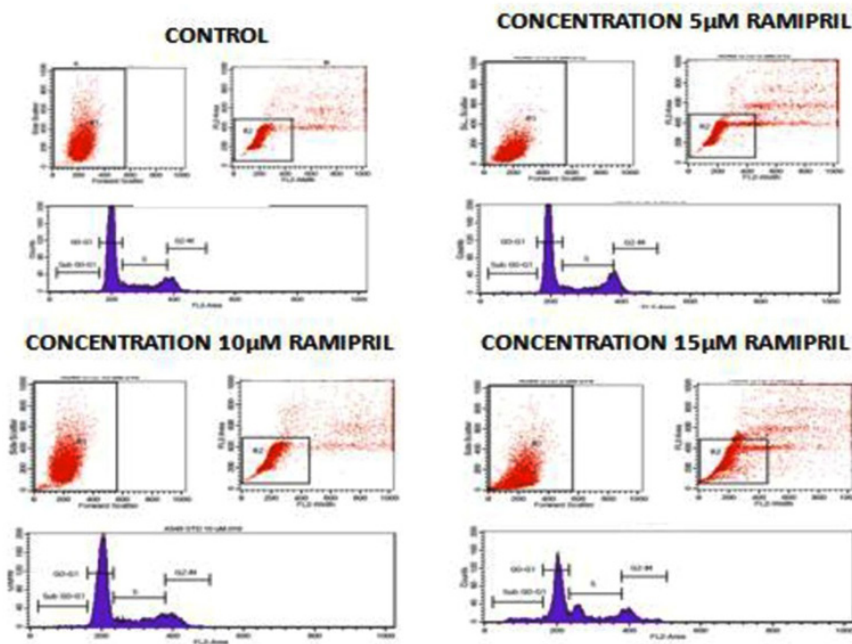| Concentration(µM)	Gated Cells Percentage			
	SUB GO-G1 Phase	G0-G1 Phase	S Phase	G2-M Phase
Control	0.26	71.21	20.28	9.90
Ramipril 5 µM	0.28	68.21	21.68	10.52
Ramipril 10 µM	0.70	66.99	21.46	11.28
Ramipril 15 µM	6.51	60.91	19.97	13.24
Olmesartan 300µM	1.36	68.16	15.67	15.21

A549 lung cancer cell lines using MTT and flow cytometry analysis. From the graph half maximal inhibitory concentration (IC50) was calculated and it proves that Ramipril was more effective than Olmesartan. We found that Ramipril and Olmesartan exhibited half maximum inhibitory concentration (IC50) using MTT at 0.012& 0.35mM. Based on the MTT result we further preceded to flow cytometry analysis. In this assay Ramipril and Olmesartan induced G2/M phase
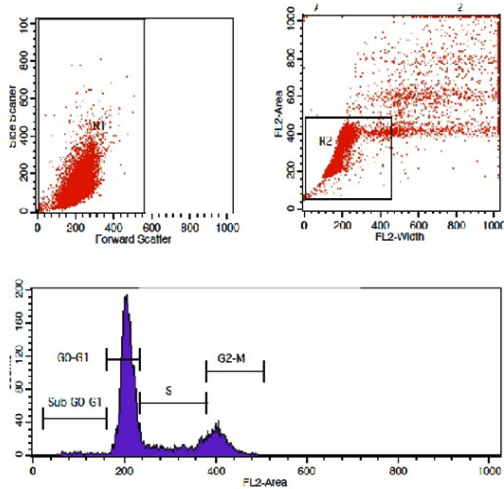

Fig. 1. Dot plot of the cell viability percentage versus increasing concentration of Ramipril and Olmesartan. Graphs were plotted using the % of cell viability at Y-axis and concentration of the drug on X-axis

arrest. Cell cycle progression in A549 when exposed to Ramipril at different concentrations i.e. 5, 10 and 15 µM, (figure2) shifted the population of cells into the G2/M phase from G1 phase. In the control only 6% cells were in G2/M phase when Ramipril exposed to 5µM increased to 10% cells and at 10 and 15 µM increased to 11% and 13% in G2/M phase. There was a reduction in the G1 Phase when compared with the control. The Cell cycle progression in A549 cells, when exposed to Olmesartan at concentration (300µM) a similar increase in G2-M fraction was observed i.e. 15.22% when compared with the control i.e. 9.30% and induced G2/M phase arrest.G2/M cell cycle inhibits the cell entry into the mitotic stage. Ramipril and Olmesartan induced a dose dependent arrest of lung cancer cell lines at G2/M phase with reduced number of cells in the G0/G1 phase. From this study we could say that Ramipril and Olmesartan induced G2/M phase arrest. Further investigations are needed for the role of various proteins involved in the G2/M phase arrest.The antitumor effects were first described in a study by *Lever et al*[3] in which breast cancer incidence decreased in patients receiving ACE inhibitors. Previously Attoub *et*

Fig. 2. Showing the effect of cell cycle activity of control and Ramipril at on various concentrations of 5 µM, 10 µM and 15 µM

CONCENTRATION 300µM OLMESARTAN

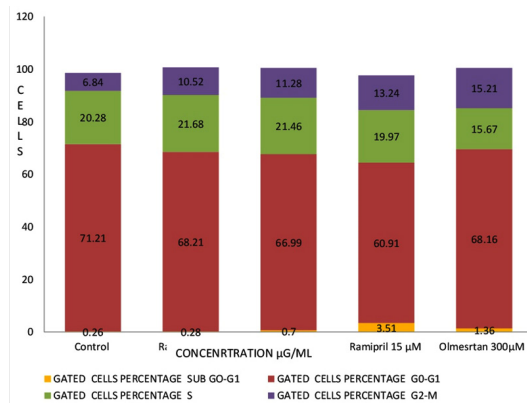**Fig. 3.** Showing the effect of cell cycle activity of Olamesartan at 300 µM

Fig. 4. The graph shows the percentage of Ramipril and Olmesartan in different phases of cell cycle at various concentrations of 5 µM, 10 µM and 15 µM and 300µM, % of cells at Y-axis and concentration of the drug on X-axis

al[21] using MTT and cell cycle analysis showed Captopril inhibited the viability of LNM35 (human lung adenocarcinoma) and induced apoptosis in flow cytometry. Whereas Perindopril [13]another ACE inhibitor, showed no direct cytotoxicity (invitro) against tumor cells but inhibited tumor growth in murine hepatocellular cancer cell lines by suppressing VEGF induced angiogenesis.

However kiyoaki *et al* showed that Telmisartan at 300 µM Angiotensin receptor antagonist inhibited cell viability and induced apoptotic cell death in prostate cells[22, 25.] Elham *et al* showed that Olmesartan inhibited cell viability (MTT) and also induced apoptotic cell death in flow cytometry on HeLa and MCF-7 cancer cell lines[18]. However there are only limited studies for Ramipril and Olmesartan in lung cancer cell lines. This study demonstrates the antineoplastic activity of Ramipril and Olmesartan, invitro.

Previous studies demonstrated that Olmesartan block the RAS and NF-kappaB pathway[18] whereas Ramipril inhibited platelet derived growth factor expression[15].NF-kappaB pathway and platelet derived growth factor is significant in the proliferation of cells. This could probably be the reason for the anticancer property in our study. Nowadays surgery, chemotherapy, radiation, hormones and immunotherapy are the main treatment approaches for the cancer. In the existing treatment methods resistance and toxicity

is the serious problems encountered. This work is the first of its kind in demonstrating antineoplastic activity of Ramipril and Olmesartan, invitro. These drugs inhibit the angiogenesis, NF-kappaB pathway, stand as an anticancer drug. Ramipril and Olmesartan inhibited cell viability and also induced cell cycle arrest in A549 cell lines. In our study Ramipril was found to be more effective at lower concentrations than Olmesartan. This will pave way for generating newer drugs with the drugs existing in the market which are safe and effective.

Limitations

Further confirmatory studies in this line are required to demonstrate the anticancer effect of Ramipril and Olmesartan on A549 lung cancer cell line.

CONCLUSION

The present study concludes that Ramipril is more efficient than Olmesartan in lung cancer (A549) cell line.

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