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Celecoxib and bevacizumab synergistically inhibit non-small cell lung cancer by inducing apoptosis and modulating VEGF and MMP-9 expression

Abdul Qadir[1*], Zurqa Khalid[2], Fareha Kashan Theba[3], Muhammad Mujtaba Ali[1], Mahayrookh Asif[4] and Fatima Rizvi[4]

[1]Department of Pharmacology, United Medical and Dental College, Karachi, Pakistan.
[2]Department of Physiology, Dow Medical College, Dow University of Health Sciences, Karachi, Pakistan.
[3]Department of Physiology, Dow Dental College, Dow University of Health Sciences, Karachi, Pakistan.
[4]Department of Pharmacology, Dow International Medical College, Dow University of Health Sciences, Karachi, Pakistan.

Abstract: Lung cancer is the most typical form of cancer that results in death worldwide. Patients with non-small cell lung cancer (NSCLC) have an around 15% survival rate despite of advancement in cancer treatment. This study aimed to evaluate the combined effect of celecoxib and bevacizumab on NSCLC using A549 cells as an *in vitro* model. The A549 cells were culture and treated with celecoxib, bevacizumab and their combination and the cell proliferation was assessed using MTT assay, whereas cell apoptosis was analyzed using flowcytometry. The effects on the apoptotic genes were examined using western blotting, while qPCR was used for analyzing the VEGF and MMP-9 expression. Celecoxib, bevacizumab and their combination exhibited a dose dependent inhibition ($p<0.001$). The rate of apoptosis was 14.1% and 26.5% but when the two drugs were combined, the rate of apoptosis was significantly increased due to synergism by 52.2% ($p<0.001$). Western blotting displayed that co-treatment significantly upregulated proapoptotic genes (caspase-3 and -9) and downregulated anti-apoptotic gene (Bcl-2) ($p<0.001$). Additionally, VEGF and MMP-9 expression were both significantly reduced with co-treatment compared to the control ($p<0.001$). Celecoxib combined with bevacizumab synergistically inhibited NSCLC by inducing apoptosis and modulating VEGF and MMP-9 expression.

Keywords: Bevacizumab, celecoxib, non-small cell lung cancer.

INTRODUCTION

Lung cancer is considered to be the most prevalent cause of cancer-related death and it continues to remained as one of the most serious health issue despite advancement in the field of medicine (Shen and Su, 2021). The deaths due to lung cancer is higher than breast, prostate and colon cancers. Lung cancer is of two types: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). SCLC comprises about 15%, whereas NSCLC comprises about 85% of all types of lung cancers (RelliTrerotola, 2019). The survival rate of patients with NSCLC is about 15% despite advancements in cancer treatment. Therefore, new therapeutic options are needed for treating the patients with NSCLC (Shen *et al.*, 2021).

There are various factors involved in regulating tumor growth and metastasis and among them vascular endothelial growth factor (VEGF) and matrix metalloproteinases-9 (MMP-9) are considered important molecular target for treating lung cancer (LiHuang, 2018). VEGF and MMP-9 are expressed in breast, lung, colon, uterus and ovarian cancer (DangZhang, 2017, RamachandranSørensen, 2017). VEGF is a glycoprotein that is responsible for tumor angiogenesis (ChenYang, 2018), whereas MMP-9 is an enzyme that is responsible for the breakdown of extracellular matrix and stimulates tumor invasion and metastasis (HaoDeng, 2020). They are regarded as key mediators of tumor growth and metastasis, therefore targeting VEGF and MMP-9 is an optimal strategy for treating cancer (Li *et al.*, 2018).

Bevacizumab is a humanized monoclonal antibody, discovered by Genentech and marketed by Roche. It primarily targets the VEGF to prevent tumor growth and metastasis. It is approved by the FDA for treating various human cancers (HeydarMansouri, 2018, Pentheroudakis Mavroeidis, 2019, KimKim, 2019). It is extensively used as a first line treatment in the mid and advanced stage of cancers, including NSCLC, renal cell carcinoma, colorectal cancer and glioblastoma (JinWei, 2018, McDermottHuseni, 2018, Pentheroudakis *et al.*, 2019, NayakMolinaro, 2021). Monoclonal antibodies are selective, have least side effects and can exert synergistic effects when used in combination with other drugs (YuScherwitzl, 2019).

Celecoxib is a non-steroidal anti-inflammatory drug (NSAIDs), which inhibits the inflammatory process by predominantly targeting COX-2 enzyme. It works incredibly well and has a low systemic toxicity (Chen *et al.*, 2018). It is primarily used for treating arthritis but in recent years, its use in cancer treatment has drawn more attention. Studies have disclosed that celecoxib besides being an anti-inflammatory drug, can inhibit tumor

*Corresponding author: e-mail: abdul.qadir@umdc.edu.pk

formation via inducing apoptosis in cancer cells, whereas it has no side effects on normal cells. Currently, various studies are being conducted to examine its therapeutic impact when combined with other drugs (Chen *et al.*, 2018, Khafaga Shamma, 2021).

In this study for the first time, the therapeutic effects of celecoxib and bevacizumab combination on NSCLC and its underlying molecular mechanism were evaluated using A549 cells as an *in vitro* model.

MATERIALS AND METHODS

Ethical approval

This study was carried out at Dow University of Health Sciences after getting approval from the Institutional Review Board (IRB-2483/DUHS/Exemption/2022/805).

Cell culture

The human NSCLC A549 cell line was bought from the American Type Culture Collection (ATCC). The cells were cultured in RPMI-1640 medium with 10% FBS. All cells were kept at 37°C in cell culture incubator containing 95% humidified air and containing 5% CO_2 (Abd-Rabou and Ahmed, 2019). Celecoxib (Celbexx) was purchased from Getz Pharma (PVT) limited and bevacizumab (Avastin) was purchased from Roche Pakistan Limited. The stock solution of celecoxib and bevacizumab was prepared by dissolving it in dimethyl sulfoxide (DMSO) and was diluted to a final concentration of 30, 60 and 120 µM celecoxib and 5, 10 and 20 µM bevacizumab in culture medium. The concentration of DMSO in the culture media was kept at 0.1%.

Cell proliferation assay

The effect of celecoxib, bevacizumab and their combination on the growth of A549 cancer cells was investigated using the MTT assay (Methylthiazolyldiphenyl-tetrazolium bromide) (Sigma-Aldrich) (Heydar *et al.*, 2018). Around 1 x 10^4 cells were added into a 96-well plate and then incubated for 24 hours until they reached 80% confluence. After the desired time, the media was exchanged with fresh RPMI-1640 media and treated with different doses of celecoxib (30, 60 and 120µM), bevacizumab (5, 10 and 20µM) and their combination for 48 hours. Then, MTT solution (5mg/mL) was added and the absorbance at a 560 nm wavelength were recorded using a spectrophotometer. The untreated cells (vehicle alone) were used as the negative control. The cell viability was calculated after performing three independent experiments.

Cell apoptosis analysis by flowcytometry

Cell apoptosis was analyzed by flowcytometry (BD FACS Celesta) using an annexin apoptosis detection kit (BD Pharmingen) (Chen *et al.*, 2018). Cells grown to 80% confluence were treated with celecoxib (30µM), bevacizumab (5µM) and their combination for 48 hours. Thereafter, centrifugation was used to collect the cells, which were then resuspended in 500µl binding buffer followed by 15-20 minutes incubation at room temperature. Then, in accordance with the manufacturer's instructions, propidium iodide (PI) and annexin V-FITC were added and the data was analyzed by flowcytometry using FACSDiva Software 8.0.

Western blot analysis

Western blotting was used to analyze the impact on the apoptotic genes (Huang Song, 2018). The A549 cancer cells were treated with celecoxib (30µM), bevacizumab (5 µM) and their combination for 48 hours. The cells were then collected using RIPA buffer and BCA Protein assay Kit (Pierce Biotechnology) was used to calculate the total protein concentration. Around 50µg proteins were subjected to 10% SDS-PAGE for electrophoresis. Primary antibodies, including GAPDH, Bcl-2, caspase-3, caspase-9 and secondary antibodies IgG-horseradish peroxidase were used for western blot. Data were gathered using Elecsys-2010 (Roche Professional Diagnostics) and ImageJ software 1.52 was used to determine each band's optical density.

Quantitative real-time polymerase chain reaction (qPCR):

The RNA was extracted after treating the cells with celecoxib, bevacizumab and their combination by AllPrep DNA/RNA Mini kit (Qiagen). Using a Reverse Transcription System kit (Promega), approximately 1 µg of RNA was reverse transcribed and SYBR Green PCR Master Mix (Qiagen) was used to perform the qPCR (Heydar *et al.*, 2018). The primer sequences were as follows: VEGF: 5'- GGCTGGCAACATAACAGAGAA-3' and 5'-CCCCACATCTATACACACCTCC-3'; MMP-9: 5'-TGGGCTACGTGACCTATGACAT-3' and 5'-GC CCAGCCCACCTCCACTCCTC-3'; GAPDH: 5'-TGAC TTCAACAGCGACACCCA-3' and 5'-CACCCTGTTGC TGTAGCCAAA-3'. Using the $2^{-\Delta\Delta CT}$ method, the mRNA expression levels were determined and normalized to GAPDH.

STATISTICAL ANALYSIS

All the experiments were conducted in triplicate. The statistical analysis was executed using SPSS software, version 21.0 and the comparison of control and treatment groups was done using ANOVA and Tukey's post hoc test. The p-value <0.05 was set as the significance cutoff.

RESULTS

Effect of celecoxib, bevacizumab and their combination on proliferation of A549 lung cancer cells

Celecoxib inhibited the growth of A549 lung cancer cells by 9% at 30µM, 16.1% at 60µM and 30.7% at 120µM. Bevacizumab inhibited the growth of A549 lung cancer cells by 12% at 5µM, 20% at 10µM and 35.5% at 20µM

respectively. The combination of celecoxib and bevacizumab inhibited growth of A549 lung cancer cells by 23.1% at 30µM celecoxib and µM bevacizumab, 39% at 60µM celecoxib and 10µM bevacizumab and 71.2% at 120µM celecoxib and 20µM bevacizumab respectively. These results indicated a concentration dependent decrease in proliferation of A549 lung cancer cells ($p<0.001$) and at higher concentrations there was a marked reduction in cell proliferation, as shown in fig. 1.

Effect of celecoxib, bevacizumab and their combination on apoptosis in A549 lung cancer cells

The Flowcytometric analysis showed that the rate of apoptosis was 14.1% when treated with celecoxib (30µM) and 26.5% when treated with bevacizumab (5µM). When both drugs were combined the rate of apoptosis was significantly increased to 52.2% as compared with the untreated control ($p<0.001$) as shown in fig. 2. These findings imply that celecoxib and bevacizumab combination might be effective in treating lung cancer.

Effect of celecoxib, bevacizumab and their combination on pro-apoptotic genes and anti-apoptotic genes in A549 lung cancer cells

The results disclosed that pro-apoptotic genes (caspase-3 and -9) expression in celecoxib + bevacizumab group were more significantly increased than the celecoxib and bevacizumab groups ($p<0.001$). Moreover, the anti-apoptotic gene (Bcl-2) expression in celecoxib+ bevacizumab group was significantly lower compared to the celecoxib and bevacizumab groups respectively ($p <0.001$). These findings imply that celecoxib and bevacizumab co-treatment resulted in an increase in apoptosis of A549 lung cancer cells through both pathways as shown in fig. 3.

Effect of celecoxib, bevacizumab and their combination on VEGF and MMP-9 in A549 lung cancer cells

The qPCR analysis showed that celecoxib and bevacizumab both reduces VEGF and MMP-9 expression in A549 lung cancer cells and their combination displayed more significant reduction ($p<0.001$) as compared to the single drug treatment as shown in fig. 4.

DISCUSSION

The NSCLC treatment has gone through a major change during the last decade. Bevacizumab monotherapy is approved for treating NSCLC patients with mid and advanced stages (ZhouHe, 2021), while celecoxib monotherapy is not standard for treating NSCLC patients but studies have reported that celecoxib beside being an NSAIDs possesses anticancer effect can induce apoptosis in cancer cells (Khafaga *et al.*, 2021, KimKim, 2017). Therefore, the current study's objective was to determine whether the addition of celecoxib would improve the anticancer effect of bevacizumab against NSCLC using A549 lung cancer cells as an *in vitro* model.

First, we examined the effect of celecoxib, bevacizumab and its combination on growth of A549 lung cancer cells using MTT assay. It is one of the most rapid, reliable and quantifiable method used for displaying the viability of the cells. Our results exhibited that celecoxib, bevacizumab and their combination suppressed the A549 lung cancer cells growth in a concentration-dependent manner, which is in line with the findings of earlier studies (Kim *et al.*, 2017). The results further demonstrated that celecoxib (30µM), bevacizumab (5µM) and their combination had a mild to moderate effect on A549 lung cancer cells at initial doses and were considered safe.

The next step was to evaluate downstream apoptosis signaling. Results of the flowcytometry showed that the rate of apoptosis was considerably higher in the celecoxib+ bevacizumab group compared to the other groups respectively, which is consistent with the findings of a prior study (Kim *et al.*, 2017).

The mechanism behind the underlying apoptosis was further elaborated by examining the effect of celecoxib, bevacizumab and their combination on anti-apoptotic (Bcl-2) and pro-apoptotic genes (caspase-3 and -9). Our findings demonstrated that Bcl-2 expression was considerably lower in the celecoxib+ bevacizumab group than in the other groups ($p<0.001$), while caspase-3 and -9 expression were both considerably higher in the celecoxib+ bevacizumab group than in the other groups, respectively ($p<0.001$) (Huang *et al.*, 2018, Kim *et al.*, 2017). This shows that celecoxib and bevacizumab combination regulate the expression of genes linked to apoptosis.

Finally, we examined how celecoxib and bevacizumab affected VEGF and MMP-9, which are expressed in various human malignancies and plays a crucial role in tumor growth and metastasis (Hao *et al.*, 2020). Our results shows that as a single agent both celecoxib and bevacizumab inhibited the expression level of VEGF and MMP-9 and this finding is in accordance with the work as reported in previously conducted studies (Heydar *et al.*, 2018, Li *et al.*, 2018). When both celecoxib and bevacizumab were combined there was a more significant inhibition in the expression level of VEGF and MMP-9 than in the other groups.

CONCLUSION

Compared with single drug, celecoxib combined with bevacizumab synergistically inhibited NSCLC by inducing apoptosis and modulating VEGF and MMP-9 expression. The strong inhibition displayed by celecoxib and bevacizumab combination favors their use as a new therapeutic option.

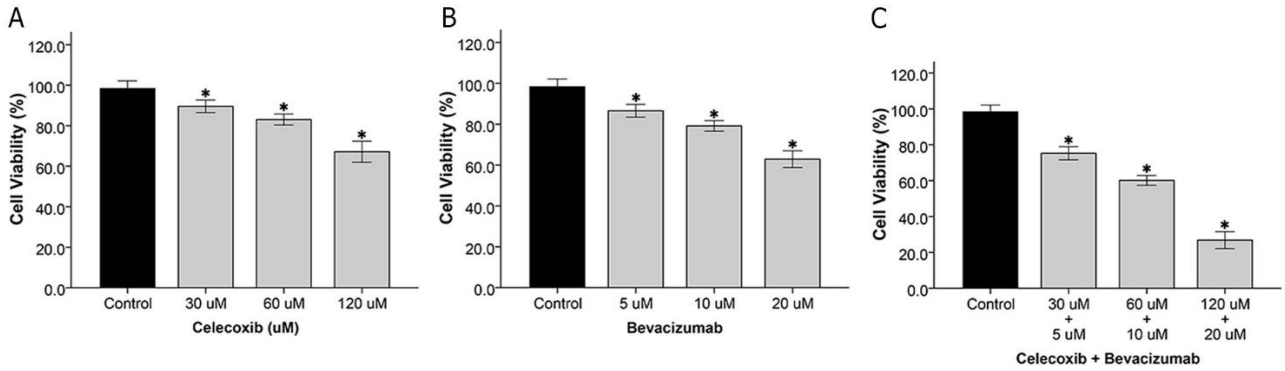

Fig. 1: Effect of (A) celecoxib (B) bevacizumab (C) and their combination on growth inhibition of A549 cancer cells. Each bar represents the mean ± SD of three independent experiments. *p <0.05 as compared to control (n=3).

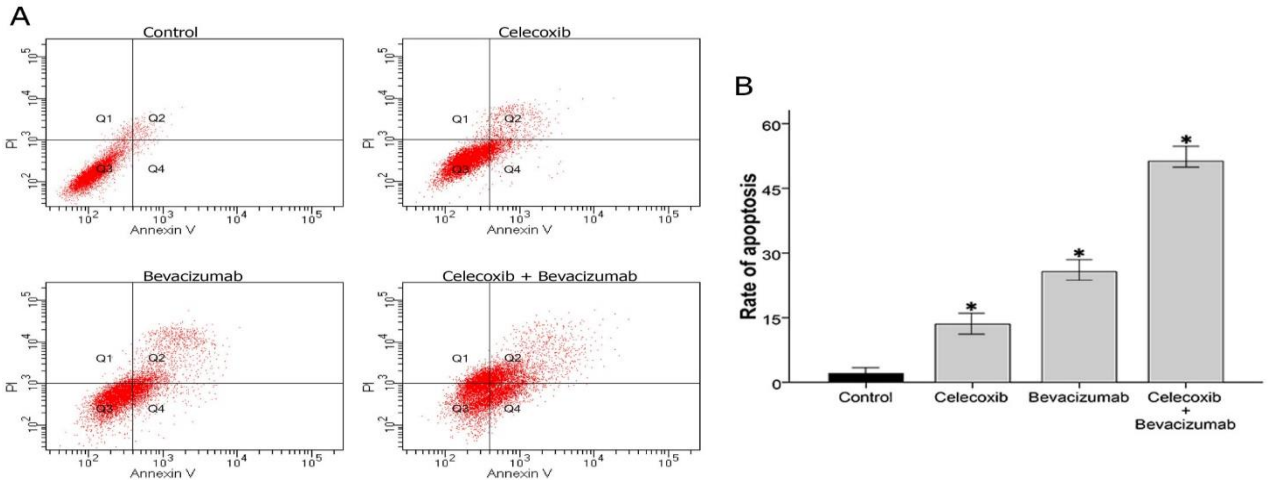

Fig. 2: The Flowcytometric analysis showing (A) the effect of celecoxib, bevacizumab and their combination on cell apoptosis in A549 lung cancer cells. (B) Showing the rate of apoptosis in A549 lung cancer cells. Each bar represents the mean ± SD of three independent experiments. *p<0.05 as compared to control (n=3).

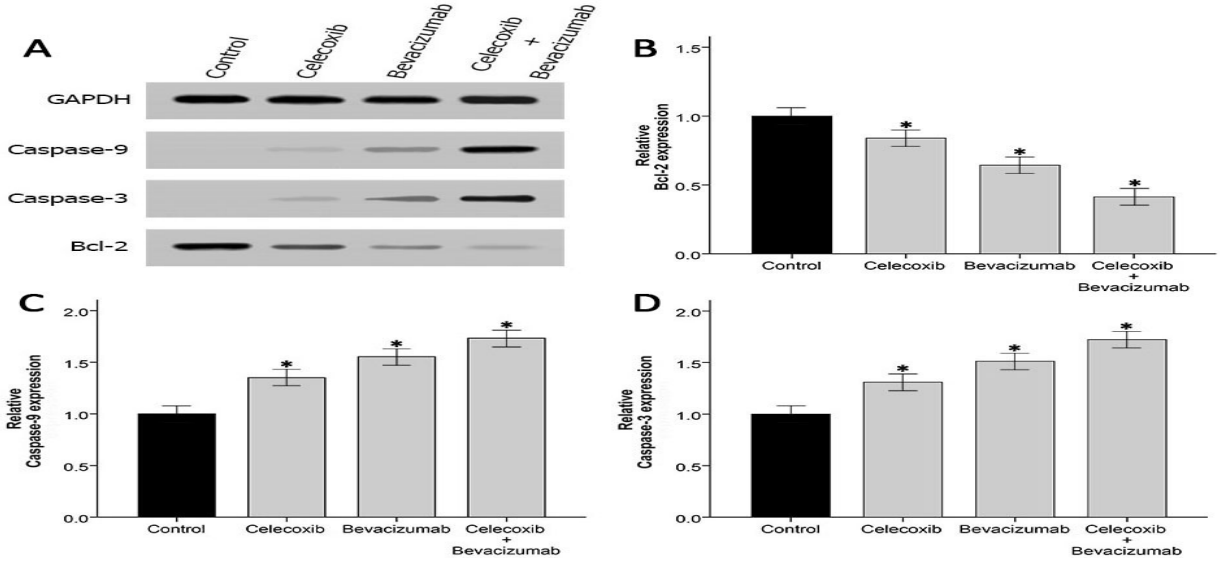

Fig. 3: The western blot analysis showing (A) the effect of celecoxib, bevacizumab and their combination on expression of pro-apoptotic genes (Caspase-3 & -9) and anti-apoptotic gene (Bcl-2) in A549 lung cancer cells. (B-D) showing the quantification of caspase-3, caspase-9 and Bcl-2. Each bar represents the mean ± SD of three independent experiments. *p<0.05 as compared to control (n=3).

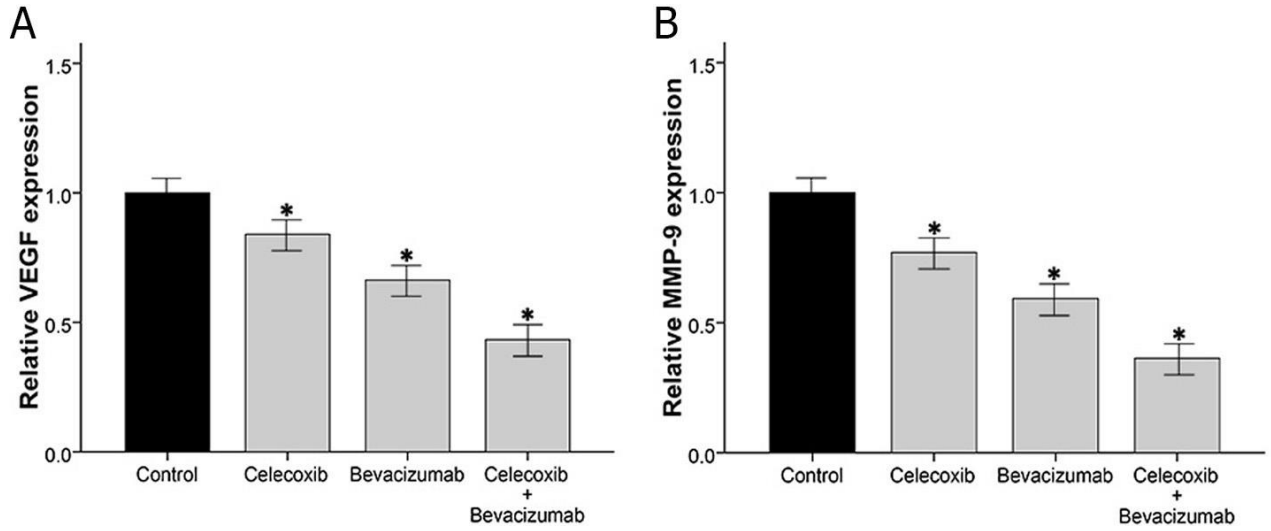

Fig. 4: Effect of celecoxib, bevacizumab and their combination on (A) VEGF and (B) MMP-9 in A549 lung cancer cells using qPCR. Each bar represents the mean±SD of three independent experiments. *p<0.05 as compared to control (n=3).

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