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ORIGINAL ARTICLE

Cannabidiol Enhances Sunitinib Effect in Human Renal Cell Carcinoma by Inducing Apoptosis and Inhibiting Stat3 Signaling Pathway

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ABSTRACT

Background: Renal cell carcinoma (RCC) is considered to be the most frequent form of kidney cancer affecting both men and women worldwide. Monotherapy is not always effective and often results in resistance.
Aim: To evaluate cannabidiol and sunitinib combination in human RCC using 786-O cells as an *in vitro* model.
Study Design: Experimental study
Place and duration of study: Dow International Medical College, Karachi from 15th November 2021 to 14th May 2022.
Methodology: 786-O cells were culture and treated with sunitinib, cannabidiol and the combination of both and the growth inhibition was evaluated using MTT assay, while cell apoptosis was determine using flowcytometry. qPCR was used to analyze the expression of apoptotic genes and STAT3 in sunitinib and cannabidiol treated cells.
Results: Sunitinib, cannabidiol and the combination of both drugs showed a dose dependent inhibition on growth of 786-O cells ($p < 0.001$). The rate of apoptosis was 14.4% and 24.3% when treated with cannabidiol and sunitinib respectively. When the two drugs were combined, apoptosis was significantly increased to 50.3% ($p < 0.001$). qPCR showed that cannabidiol enhanced sunitinib induced apoptosis by downregulating anti-apoptotic gene (Bcl-2) and upregulating pro-apoptotic gene (caspase-3 and -9) ($p < 0.001$).
Practical Implication Using single drug for treating RCC is not always effective and often results in resistance. Our study suggests that cannabidiol in combination with sunitinib may be a preferable chemotherapeutic option for treatment of RCC.
Conclusion: Cannabidiol enhances sunitinib effect in inhibiting RCC and their combination achieved more strong inhibition than either drug alone.
Keywords: Cannabidiol, Renal cell carcinoma, Sunitinib, Apoptosis, STAT3, qPCR

INTRODUCTION

Kidney cancer is considered to be the commonest cancer worldwide affecting both men and women that accounts for 3.7% of all new cancer cases. The most common kidney cancer is renal cell carcinoma (RCC), accounting for 85% of all cases[1]. Due to its resistance to chemotherapy and radiotherapy, surgery is currently the sole treatment option for RCC. Therefore, it was urgently necessary to gain a deeper knowledge of the specific mechanisms underlying the etiology of RCC and develop more efficient treatment option[2].

Signal Transducer and Activator of Transcription 3 (STAT3) is a protein that has been recognized as a main factor for regulating cancer. Activation of STAT3 in cancer cells is mainly due to the tyrosine kinases such as platelet-derived growth factor receptor, epidermal growth factor receptor and vascular endothelial growth factor receptor. STAT3 is expressed in brain, breast, gastric, lung and renal tumors. It has a significant impact on tumor growth and metastasis. Therefore, targeting STAT3 in cancer treatment will provide a new therapeutic option[3,4].

Sunitinib is an antiangiogenic drug from the tyrosine kinase inhibitor family. It acts mainly by inhibiting VEGFR, PDGFR, macrophage colony-stimulating factor receptor (MCSFR) and stem cell growth factor receptor (KIT) [1]. It is extensively used as the first-line agent against RCC. However, treatment with sunitinib as a monotherapy is not always effective and often results in resistance and side effects. Therefore, establishing a novel drug combination might be an effective approach for treating RCC [5].

Cannabidiol is a member of the cannabinoid family that is derived from *cannabis sativa*. It acts as a CB1 receptor antagonist as well as a CB2 inverse agonist. Additionally, it acts as an agonist at TRP, PPAR, 5HT1A receptors [6] and have also been reported to inhibit vascular endothelial cell survival and migration. It is anxiolytic and has analgesic, anti-inflammatory, anti-angiogenic and anticancer properties [7, 8]. It is mainly used for treating anxiety and depression [9], but many studies have revealed that cannabidiol exhibits therapeutic effects in several tumors including glioma, breast cancer, prostate cancer and gastric cancer[6,7,10].

Monotherapy is not always effective and often results in resistance. Sunitinib and cannabidiol both possesses anticancer effect and have not been used in combination to date. Their combination will provide a new therapeutic option for treating RCC and will help in minimizing drug resistance.

Therefore, in this study we assessed the effect of sunitinib and cannabidiol combination on RCC and its molecular mechanism using 786-O cells as an *in vitro* model.

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MATERIAL AND METHODS

Ethical approval: This study was conducted in Dow University of Health Sciences after receiving Institutional Review Board permission (IRB-2206/DUHS/Approval/2021/575).
Cell culture: The human RCC cell line 786-O was purchased from the American Type Culture Collection. The cells were grown with 10% FBS in RPMI media. All cells were incubated at 37°C in the presence of 95% humidity and 5% CO_2. Cannabidiol and sunitinib stock solutions were made by dissolving them in dimethyl sulfoxide and diluting them to a final concentration of 5, 10 and 20 µg/ml cannabidiol and 1, 2 and 4 µM sunitinib in culture medium.
MTT assay: The effect of cannabidiol and sunitinib on the growth of 786-O cells was evaluated using MTT (3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyl tetrazolium bromide). A total of 8×10^3 cells were grown in a 96-well plate followed by 24 hours incubation until they reached 80% confluence. After 24 hours the media was replaced with fresh RPMI-1640 media and treated with various concentrations of cannabidiol (5, 10 and 20 µg/ml), sunitinib (1, 2 and 4 µM) and the combination of both drugs for 48 hours. Cells without addition of the drugs were used as control. Thereafter, each well was filled with a 0.5% MTT solution and incubated for an additional 4 hours. Finally, using a spectrophotometer, the absorbance was recorded at a wavelength of 570 nm.

Flowcytometry: Flowcytometry was used to determined cell apoptosis using an annexin apoptosis detection kit. Cells were treated with cannabidiol (5µg/ml), sunitinib (1µM) and the combination of both drugs for 48 hours. Thereafter the cells were collected by centrifugation, then at room temperature, 5 µl Annexin V-FITC and propidium iodide were added and incubated for 10-15 minutes. The data was analyzed by flowcytometry using FACSDiva Software 8.0.

Quantitative polymerase chain reaction (qPCR): RNA was isolated from the cells treated with cannabidiol, sunitinib and the combination of both by TRIzol reagent. The qPCR was performed using the SYBR Green Master Mix. The primer sequences are: Bcl-2: 5'-TCAGAGACAGCCAGGAGAAATCA-3' and 5'-CCTGTGGATGACTGAGTACCTGAA-3'; Caspase-3: 5'-GCAGCAAACCTCAGGGAAAC-3' and 5'-AACTGCTCCTTTTGCTGTGATCT-3'; Caspase-9: 5'-CTCCAACATCGACTGTGAGAAGTT-3' and 5'-GCGCCAGCTCCAGCAA-3'; STATE3: 5'-ACCTGCAGCAATACCATTGAC-3' and 5'-AAGGTGAGGGACTCAAACTGC-3'; GAPDH: 5'-TGACTTCAACAGCGACACCCA-3' and 5'-CACCCTGTTGCTGTAGCCAAA-3'. The mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis: All the procedures were carried out in triplicates. Data was entered using SPSS software 16.0. The control and experimental groups were compared using ANOVA and Tukey's post hoc test. The P value < 0.05 was set as the significance cutoff.

RESULTS

Effect of cannabidiol, sunitinib and its combination on growth of 786-O cells: Cannabidiol inhibited 786-O cells growth by 8%, 17.2% and 36.1% at 5, 10 and 20 µg/ml respectively. Sunitinib inhibited growth of 786-O cells by 11.1%, 23.3% and 48% at 1, 2 and 4 µM respectively. The combination of cannabidiol and sunitinib inhibited growth of 786-O cells by 21.4% at 5 µg/ml cannabidiol and 1 µM sunitinib, 43.1% at 10 µg/ml cannabidiol and 2 µM sunitinib and 88.2% at 20 µg/ml cannabidiol and 4 µM sunitinib respectively. These results indicated a dose dependent decrease in growth inhibition of 786-O renal cancer cells ($p < 0.001$) and at higher doses there was a marked reduction in cell growth, as shown in figure 1. Therefore, a dose of 5 µg/ml cannabidiol, 1 µM sunitinib and their combination, which produced mild to moderate effect was used for all subsequent experiments.

Effect of cannabidiol, sunitinib and its combination on apoptosis in 786-O cells: The flowcytometric examination showed that the rate of apoptosis was 14.4% and 24.3% when treated with cannabidiol and sunitinib respectively. When the two drugs were combined, apoptosis was significantly increased to 50.3% ($p < 0.001$) as shown in figure 2. These results suggest that cannabidiol and sunitinib promote apoptosis in 786-O renal cancer cells and thereby may have potential as a therapeutic option for treating RCC.

Effect of cannabidiol, sunitinib and its combination on apoptotic genes and STAT3 in 786-O cells: The results of the qPCR analysis disclosed that cannabidiol and sunitinib both downregulated anti-apoptotic gene (Bcl-2) and their combination displayed even higher level of reduction in gene expression. Meanwhile, cannabidiol, sunitinib and their combination upregulated pro-apoptotic genes (caspase-3 and -9). Both mechanisms led to the increased apoptosis of RCC with the co-treatment of cannabidiol and sunitinib ($p < 0.001$). Moreover, cannabidiol and sunitinib both reduces the expression of STAT3 and their combination displayed even higher level of reduction in the STAT3 expression ($p < 0.001$) as shown in figure 3.

Figure 1. Effect of drugs on growth inhibition of 786-O cells. The figure shows the effect of (A) cannabidiol, (B) sunitinib (C) and their combination on 786-O cells. The mean of three distinct experiments is represented by a bar graph. *p <0.05 in comparison to the control.

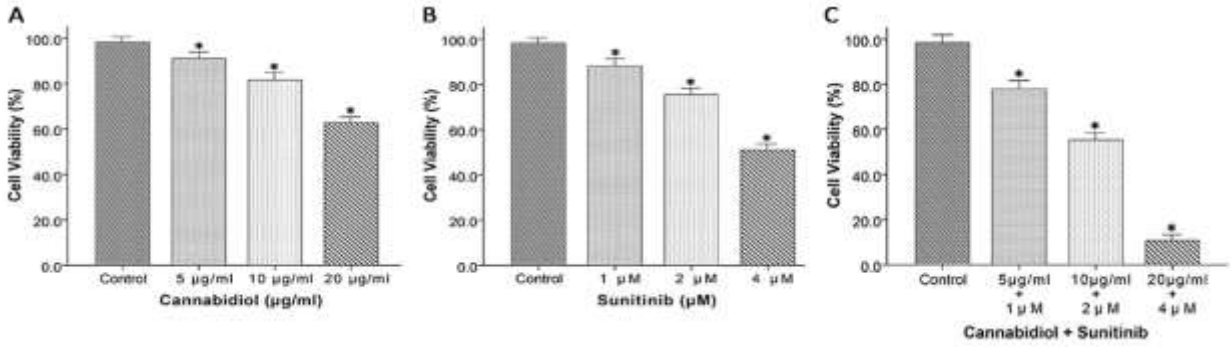


Figure 2. Effect of drugs on apoptosis in 786-O cells. The figure shows (A) the effect of cannabidiol, sunitinib and their combination on cell apoptosis in 786-O cells using flowcytometry (B) Showing the rate of apoptosis in 786-O cells. The mean of three distinct experiments is represented by a bar graph. *p <0.05 in comparison to the control.

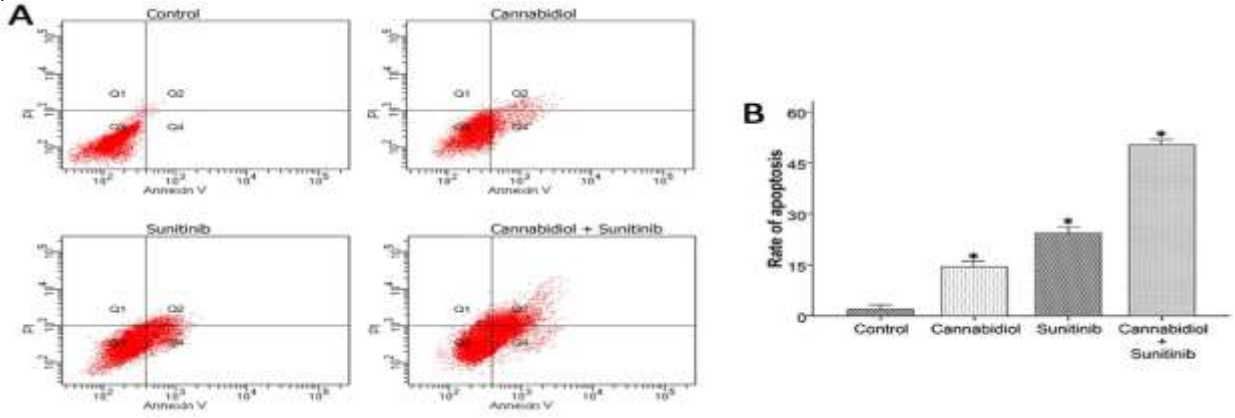
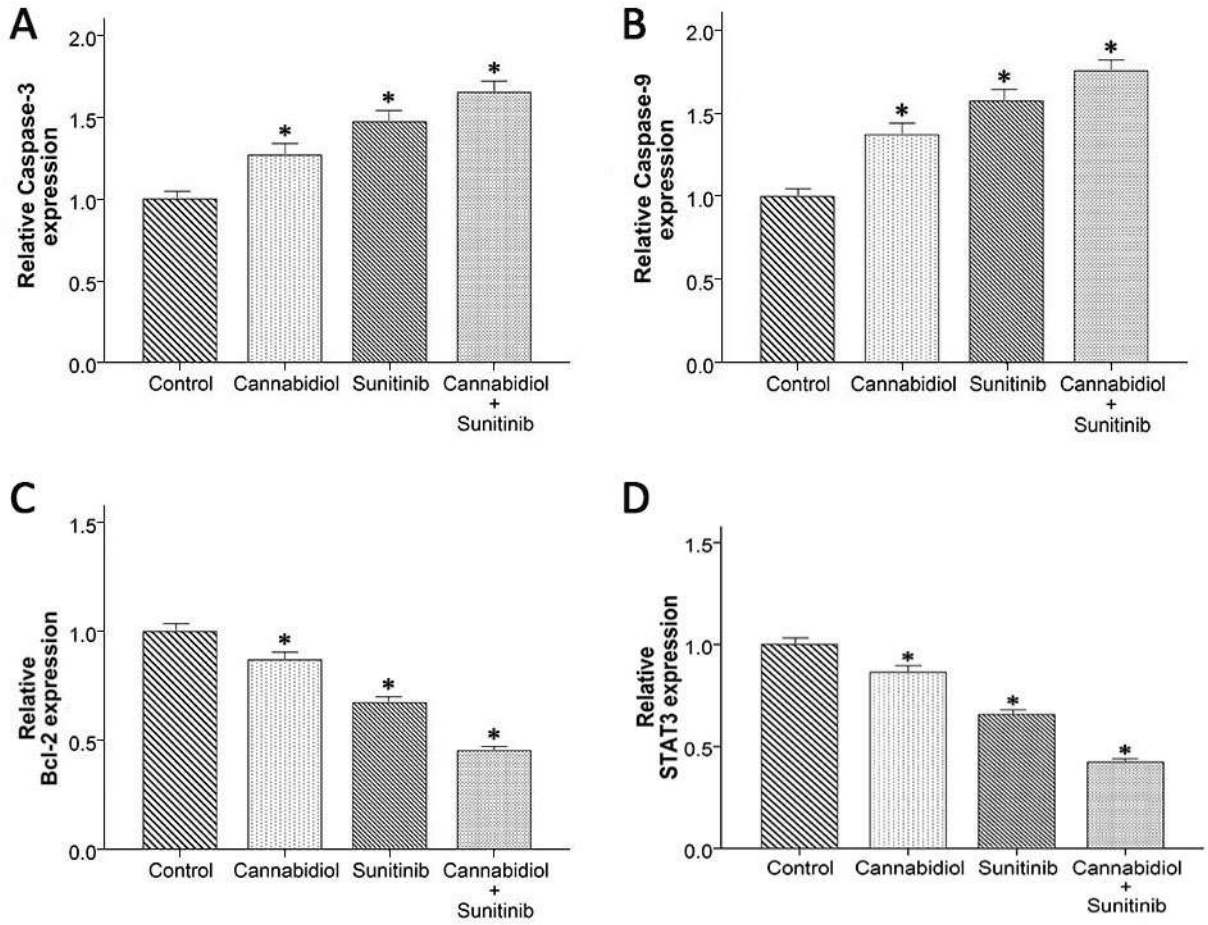


Figure 3. Effect of drugs on apoptotic genes and STAT3 in 786-O cells. The figure shows the effect of cannabidiol, sunitinib and their combination on the (A-B) pro-apoptotic genes (caspase-3 and -9), (C) anti-apoptotic gene (Bcl-2) and (D) STAT3 in 786-O cells using qPCR. The mean of three distinct experiments is represented by a bar graph. *p <0.05 in comparison to the control.



DISCUSSION

The RCC treatment has gone through a major change during the last decade. Drugs acting on VEGF receptors or mTOR, for example sunitinib and everolimus are usually used for treating RCC. However, most of the patients eventually go through cancer progression. So, new drugs for RCC treatment are highly required.

Despite the fact that both cannabidiol and sunitinib have a different mechanism of action, both drugs have the potential to inhibit cancer cells[11,12]. To the best of our knowledge, the combined effects of cannabidiol and sunitinib on human RCC have not been studied before. Therefore, we hypothesized that a combination therapy using both drugs would produce a stronger anticancer effect than using either drug alone.

In this study, the effect of cannabidiol with sunitinib on human RCC was evaluated using 786-O cells as an *in vitro* model. The current study's findings revealed that cannabidiol, sunitinib and their combination showed a dose-dependent inhibition on growth of 786-O cells consistent with the work reported in previous studies[3,10]. The results further confirmed that at initial dose both cannabidiol (5µg/ml), sunitinib (1µM) and their combination showed a mild to moderate effect on 786-O renal cancer cells and were considered safe.

Next, we evaluated apoptosis using flow cytometry. The results demonstrated that when both cannabidiol and sunitinib were combined the rate of apoptosis was increased significantly in comparison to the untreated control, which is in accordance with the work as reported in previous study[13].

To further confirm the mechanism behind the underlying apoptosis, we examined the effect of cannabidiol, sunitinib and their combination on apoptotic genes. Our results revealed that co-treatment with cannabidiol and sunitinib downregulated anti-apoptotic gene (Bcl-2) and upregulated pro-apoptotic genes (caspase-3 and -9), which is in accordance with the work as reported in previous study[12,13].

Finally, we evaluated the effect of cannabidiol and sunitinib on STAT3, which is expressed in various human cancers and plays a significant role in tumor growth and metastasis. Our results revealed that cannabidiol and sunitinib both inhibited the expression of STAT3, and this finding is completely in agreement with previously conducted studies[3]. When both cannabidiol and sunitinib were combined there was a stronger inhibition in the expression of STAT3 as compared to either drug alone.

CONCLUSION

The present study showed that cannabidiol enhances the effect of sunitinib in inhibiting RCC, and their combination accomplished a stronger inhibition than either drug alone. The strong efficacy exhibited by the drug combination supports their use as a new therapeutic option. However, the combined effects of both these drugs requires an extensive clinical trial for further affirmation.

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