



Received on 30 May, 2017; received in revised form, 20 August, 2017; accepted, 29 August, 2017; published 01 March, 2018

TGF- β INCREASES CYTOTOXIC EFFECT AND SENESCENCE INDUCED BY PI3K/ AKT/ MTOR INHIBITOR IN HUMAN COLORECTAL CANCER CELLS

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Keywords:

NVP-BEZ235;
Colorectal cancer; XAV-939; TGF- β

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ABSTRACT: Although NVP-BEZ235 and XAV-939 have been shown a promising result in the treatment of colorectal cancer (CRC), our data is little with regard to the effect of environmental cytokine on this drugs. In order to explore the reaction of CRC to this novel therapy, the current study proposes to investigate the inhibitory effect of NVP-BEZ235 and/or XAV-939 in CRC cells in combination with TGF- β as a tumor environmental cytokine to analyze their effect on cancer cell proliferation, cell death and expression of cancer promoting genes. The cells were treated with single or combination of NVP-BEZ235, XAV-939 and TGF- β . Cell viability was evaluated by MTT assay. Flow cytometry method was used to determine cellular death. Real-time PCR was utilized to identify the effect of treatments on expression of snail, slug, C-myc and notch1 genes. The results revealed that NVP-BEZ235 inhibited CRC cells proliferation and induced senescence cells more than XAV-939. Although, XAV-939 increased cellular death and inhibited senescence induction of CRC cells better than NVP-BEZ235. TGF- β pretreatment sensitizes CRC cells to the cytotoxic effect of NVP-BEZ235 and XAV-939. Real-time PCR analysis showed that resistance cells to NVP-BEZ235 increased expression of slug. Expression of notch enhanced remarkably when cells treated with both NVP-BEZ235 and XAV-939. The results of the present study demonstrated that presence of anti-inflammatory cytokine like TGF- β augments the inhibitory effect of XAV-939 or especially NVP-BEZ235.

INTRODUCTION: Colorectal cancer (CRC) is the third most common cancer and the fourth-leading cause of cancer death worldwide¹. Although surgical resection and conventional therapy like chemotherapy are efficient at least in early, subsequent relapse and metastasis, as well as drug resistance frequently occurred². It is now apparent that tumor environmental cytokines such as TGF- β play a major role in colon tumor initiation as well as progression and metastasis^{3,4}.

Although several studies have reported that TGF- β inhibits tumor growth in early stages, whereas later it promotes invasion and metastasis⁵. As previously shown, TGF- β induces epithelial mesenchymal transition (EMT) Phenotype as well as EMT genes expression signature including snail and slug in CRC⁶⁻⁸.

Enhancement of mesenchymal markers leads to the cancer cells invasion, migration, drug resistance and immune system suppression⁹. Several studies have shown that TGF- β increases cancer cells migration and invasion through activation of PI3K/AKT/mTOR axis^{10,11}. The PI3K signaling is one of the crucial pathways in regulating cancer cells growth, motility, survival and metabolism. Activation of PI3K/AKT pathway frequently occurs in many different types of cancer¹².

<p>QUICK RESPONSE CODE</p> 	<p>DOI: 10.13040/IJPSR.0975-8232.9(3).924-31</p> <hr/> <p>Article can be accessed online on: www.ijpsr.com</p>
<p>DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.9(3).924-31</p>	

Furthermore, activation of the PI3K/Akt increases EMT phenotype through the up regulation of EMT-associated factors expression such as snail and slug¹³. In fact, Phosphorylation of beta-catenin (β -catenin) by AKT enhances β -catenin transcriptional activity and promotes expression of genes involved in EMT induction such as snail, slug and c-myc. Thus, activation of AKT increases EMT phenotype induction by up regulating the Wingless-Int/ β -catenin (Wnt/ β -catenin) signaling pathway^{14, 15}.

In addition, Notch1 and Wnt/ β -catenin have a crucial role in EMT induction¹⁶. Overactive Wnt/ β -catenin signaling pathway in CRC leads to the tumor progression and drug resistance to the PI3K/AKT inhibitors¹⁷. As regards, the PI3K is an attractive target for cancer therapy and inhibition of the PI3K/AKT can be important target for inhibition of cancer progression¹⁸.

Therefore, it is important to identify the effect of tumor environmental cytokine on responsiveness of CRC cells to new drugs treatment. As the molecular mechanisms of drug resistance to PI3K/AKT inhibitors and its relation with TGF- β and Wnt/ β -catenin in CRC remains unknown, this study investigated the combinatorial effects of Wnt/ β -catenin and PI3K /AKT inhibitors as well as TGF- β in CRC treatment.

MATERIAL AND METHODS:

Cell Culture: Caco2 cells were cultured in RPMI1640 (Gibco, UK) supplemented with 20% FBS (Gibco, UK) and 100 μ g/ml penicillin-streptomycin at 37°C in 5% CO₂ and 95% humidity. The cultured cells were incubated with/

without 5 ng/ml TGF- β (ebioscience) for 14 days. Then, the cells were treated with NVP-BE235 (cayman) at concentration of 240 nM for 48 hours or 220 nM for 72 hours and/or 10000 nM¹⁹ XAV-939 (Tocris) and incubated for 48 and 72 hours. The untreated cell line (caco2) was used as a control.

MTT Assay: MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) technique was used to find cell viability and IC₅₀ of the NVP-BE235. The MTT test is based on the enzymatic reduction of the tetrazolium salt. The reaction was carried out in 96-well plates. 30000 cells were cultured in each well and exposed to different concentration (100-500 nM) of the NVP-BE235 for 24 or 48h. Afterwards, the MTT substrate added to cells, usually at a final concentration of 0.2 - 0.5mg/ml, and incubated for 4 h. The quantity of formazan soluble in dimethylsulfoxide (presumably proportionate directly to the number of viable cells) was measured by recording changes in absorbance at 570 nm using a plate reading spectrophotometer.

RT-PCR: Expression of our interest mRNAs was measured by Real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was extracted from all cultures using TRIzol™ reagent (Invitrogen). Extracted RNA was reverse-transcribed using the reverse transcription kit (Thermo Fisher Scientific) according to manufacturer's instructions. The sequences of Snail, Slug, c-myc, notch1 and GAPDH primers were provided from published sources (**Table 1**).

TABLE 1: THE SEQUENCES OF SNAIL, SLUG, C-MYC, NOTCH1 AND GAPDH PRIMER

Gene	Forward primer sequences	Reverse primer sequences
C-myc	AATGAAAAGGCCCCCAAGGTAGTTATCC	GTCGTTTCCGCAACAAGTCCTCTTC
notch1	TCAGCGGGATCCACTGTGAG	ACACAGGCAGGTGAACGAGTTG
Snail	ACCACTATGCCGCGCTCTT	GGTCGTAGGGCTGCTGGAA
Snail	AACAGAGCATTTCGACAGGTC	GCTACACAGCAGCCAGATTCC
GAPDH	ACACCCACTCCTCCACCTTTG	GTCCACCACCCTGTTGCTGTA

Real-time RT-PCR was carried out with the SYBR Green Master Mix reagents (Takara, Japan) using the Corbett 6000 Rotor-Gene thermo cycler (Corbett research, Australia). Cycle parameters were as follow: enzyme activation for 5 min at 95°C, followed by 45 Cycles of denaturation at 95 °C for 15'sec and annealing at 60 °C for 20'sec and extension at 72 °C for 25 sec.

Melting curve analyses were performed in each run. Relative expression was determined using the 2^{- $\Delta\Delta$ CT} method.

Flow Cytometry: The ability of NVP-BE235 and/or XAV-939 to induce human colorectal cancer cell death was assessed. Cellular death was examined by flow cytometry (Partec system,

Germany) using the Annexin V-FITC/propidium iodide (PI) apoptosis detection kit (BD Biosciences) according to manufacturer's instruction. Briefly, after all indicted treatment were done, the cells were washed with cold PBS and resuspended in 1 ml of 1x binding buffer (provided with kit) at density of 5×10^5 . 100 μ l of cell suspension incubated with 5 μ l of Annexin V-FITC and PI for 15 minutes in a dark place at room temperature, then examined by flow cytometry. At least 10,000 events were recorded and the cellular death was determined.

Senescence: The identification of senescence cells is based on an increased level of lysosomal β -galactosidase activity. TGF- β treated/untreated cells were seeded in 12-well plate at a density of 2×10^5 cell per well and grown for 24 hours. Then, the cells were treated with indicated concentration of NVP-BEZ235 and/or XAV-939 for 48 hours and 72 hours. Afterward, aspirate the cell culture medium and wash the cells with PBS twice. Senescent cells was detected using a senescence detection kit (Abcam, UK) according to the manufacturer's instructions. The cells were observed under the microscope for development of blue color.

RESULTS:

Cytotoxicity Induced by NVP- BEZ-235 and XAV-939: In order to determine the IC_{50} value of NVP-BEZ235 the MTT assay was performed. NVP-BEZ235 has shown dose-dependent cytotoxicity on caco2 cells after 48 and 72 h incubation. IC_{50} value for the inhibitory effect of NVP-BEZ235 on caco2 cells was 240 nM and 220 nM for 48 hour and 72 hour, respectively. For the single or combination treatment, 10 μ g/ml of XAV-939 was used. Caco2 cells treated with 10 μ g/ml had no observable cytotoxicity. As expected, NVP-BEZ235 alone at 240 and 220 nM resulted in significant decreased cell survival (**Fig. 1**). However, XAV-939 increases cytotoxic effect of NVP-BEZ235 to 51% and 37% of the control group after 48 and 72h, respectively (**Fig. 1**).

Does TGF β Modulate Resistance to NVP-BEZ235 and XAV-939 in caco2 cells? To investigate the cell survival effect of TGF- β as a tumor environmental cytokine, the cells were exposure to TGF- β For 2 weeks and then were

treated with XAV-939 and/or NVP-BEZ235 for 48 and 72h. Results showed that, TGF- β treatment reduced cell survival, significantly (**Fig. 1**).

Although, TGF- β did not change proliferation inhibitory effect of NVP-BEZ235. But, combinatorial treatment of cells with TGF- β and XAV-939 reduced cell survival after 48 h ($p < 0.01$) Conversely, XAV-939 diminished the inhibitory effect of TGF- β after 72h (**Fig. 1**). However, the most reduction of cell survival (32%) was seen in cells pretreated with TGF- β and then were exposed to NVP-BEZ235 and XAV-939 for 72h (**Fig. 1**).

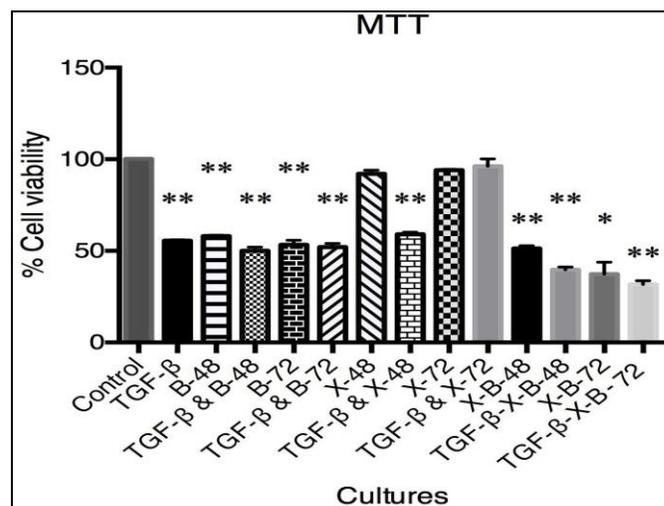


FIG. 1: TGF-B AND NVP-BEZ235 REDUCED CELL VIABILITY

Cell viability was assessed after TGF- β pretreated and untreated cells were exposed to with NVP-BEZ235 (240 nM or 220 nM for 48 or 72 h respectively) and/or 10000 nM XAV-939 for 48 and 72 h.

*= $P < 0.05$, **= $P < 0.01$ vs. untreated cells (control group). These results are presented as the mean \pm standard deviation of three independent experiments. B, NVP-BEZ235; X, XAV-939

Cellular Death Induction: In order to determine whether the inhibitory effects induced by proliferation inhibition or apoptosis induction, cellular death was determined by annexin-V/PI apoptosis detection kit. Our results, as depicted in **Fig. 2**, showed that NVP-BEZ235 (after 48 & 72h) and XAV-939 (after 48h) did not change cellular death significantly. Surprisingly, XAV-939 increased cellular death significantly, after 72h.

Is TGF β Able to Affect Cell Death? Exposure of cells to TGF- β for 14d increased cellular death significantly ($p < 0.05$; **Fig. 2**). Consistently, TGF- β pretreatment increased XAV-939 or NVP-BEZ235 induced cellular death significantly (**Fig. 2**).

In addition, combination of XAV-939 and NVP-BEZ235 was more effective in cellular death induction (after 72h), when the cells pretreated with

TGF- β (**Fig. 2**; $P < 0.05$), whereas the effect was not significant after 48h (**Fig. 2**; $P > 0.05$).

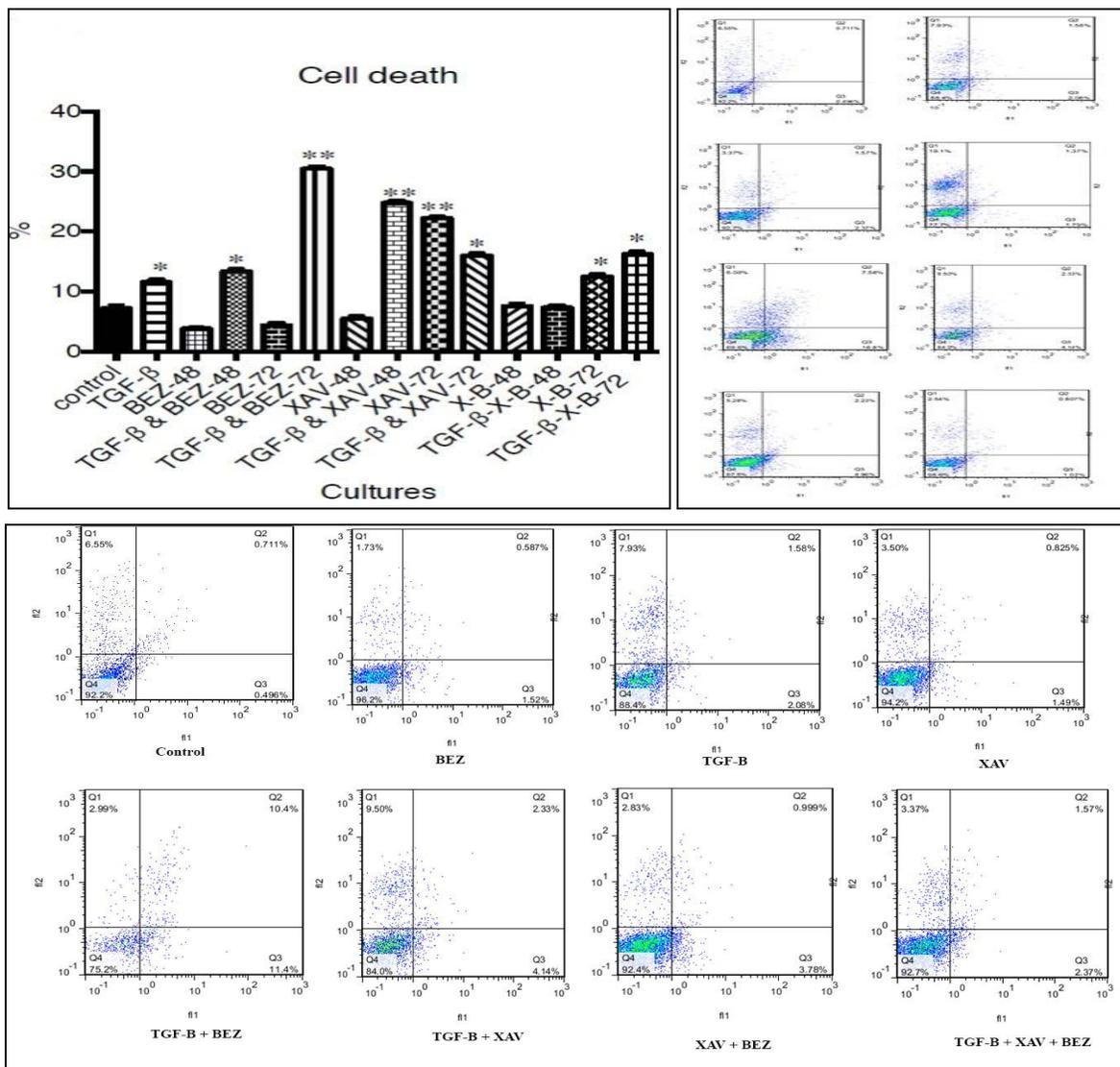


FIG. 2: TGF-B SENSITIZED NVP-BEZ235 INDUCED CELL DEATH

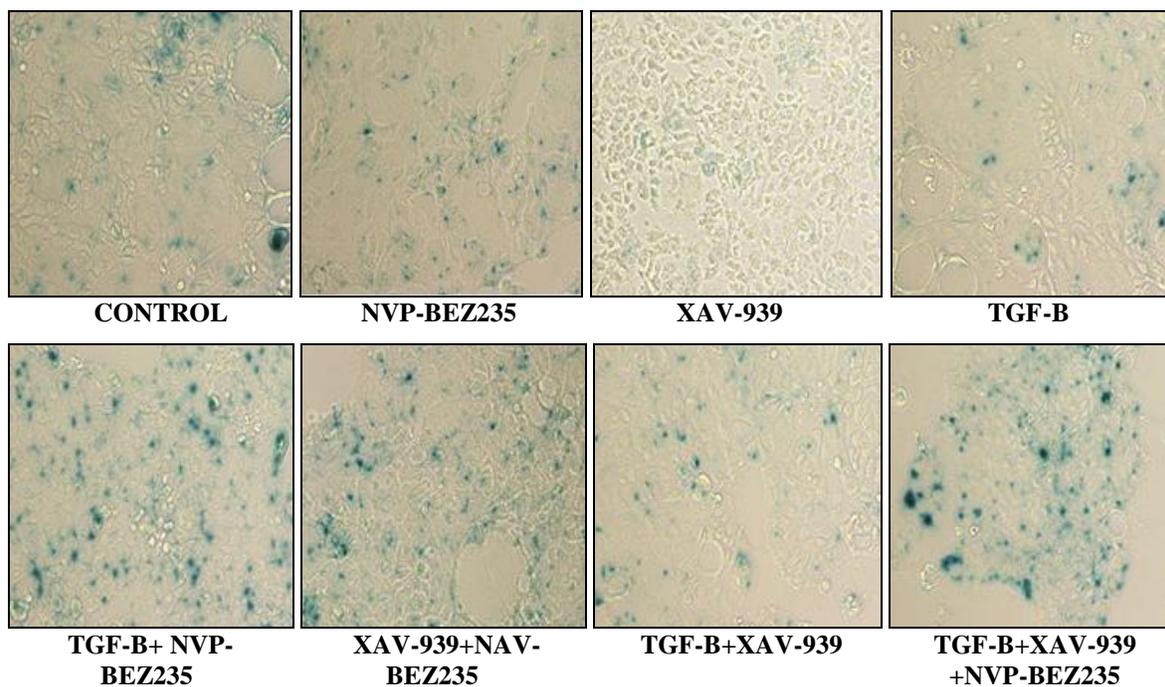
Flow cytometry analysis shows the apoptosis and necrosis (cellular death) following of single and combinatorial treatment with TGF- β , NVP-BEZ235 and XAV-939 after 48 and 72 h.

*= $P < 0.05$, **= $P < 0.01$ vs. untreated cells (control group). These results are presented as the mean \pm standard deviation of three independent experiments. B, NVP-BEZ235; X, XAV-939.

β -Gal in Human Caco2 Cell Lines: To assess and identify the cells with the senescence phenotype, the level of lysosomal β -galactosidase activity was investigated by formation local blue precipitant. In the control group, the level of lysosomal β -galactosidase activity was low (**Fig. 3**). The level of lysosomal β -galactosidase activity was increased in the cells treated with NVP-BEZ235 for 48 or 72 hour, compared to the control group (**Fig. 3**). In contrast, the cells treated with XAV-939, showed low level of enzymatic activity.

In combination treatment of XAV-939 and NVP-BEZ235 the senescence cells increased considerably (**Fig. 3**).

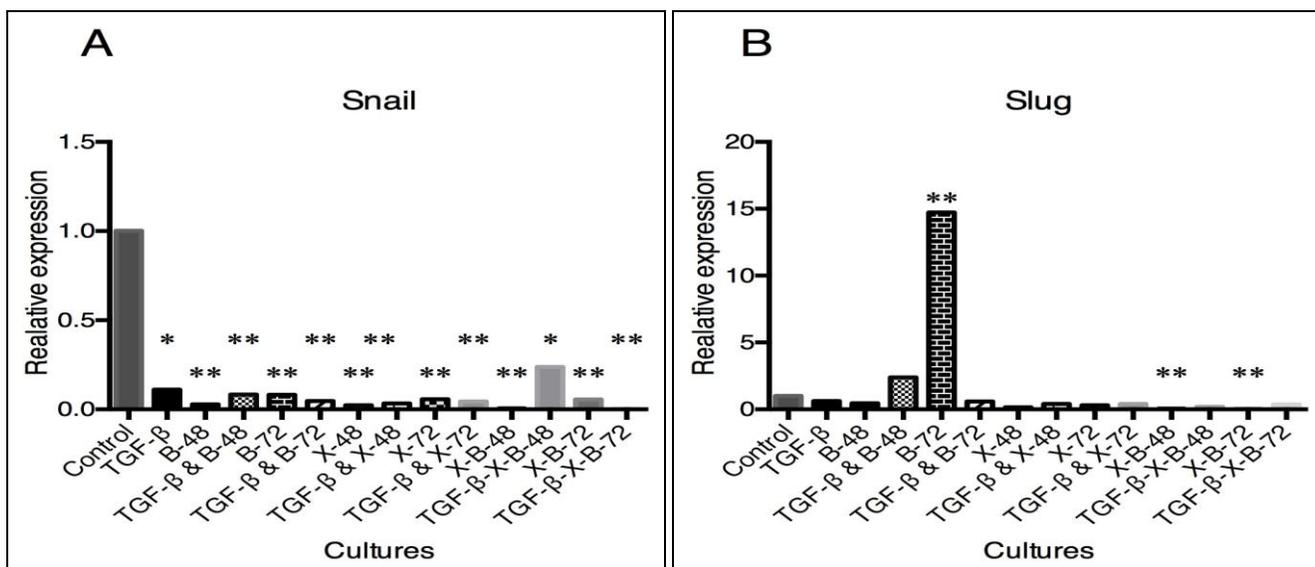
Is TGF β Able to Lead to an Increase in the Intensity of Blue Color? In the cells exposed to TGF β , an increase level of lysosomal β -galactosidase activity was seen, compared to the control group (**Fig. 3**). In the cells treated with TGF β , NVP-BEZ235 and XAV-939 combination, showed the most increase in senescence cells **Fig. 3**.



Expression of Snail, Slug, C-myc and Notch1 in Human Caco2 Cell Lines: We investigated Snail, Slug, C-myc and notch1 relative expression by Real-time PCR. Expression of Snail gene was distinctly lowered in the cells pre-treated with TGFβ compared to control group (Fig. 4A; p<0.01). In addition, a strong reduction of snail gene expression was found in the cells treated with NVP-BEZ235 or XAV-939 separately or in combination, (Fig. 4A; p<0.05). Expression of Slug gene in combinatorial treatment of XAV-939 and NVP-BEZ235 was reduced compared to the control group (Fig. 4B; P<0.05), but it did not reduce

significantly in the cells treated with NVP-BEZ235 or XAV-939 alone (Fig. 4B). In the cells treated with NVP-BEZ235, Slug expressed significantly at mRNA level (Fig. 4B; P<0.05). C-myc gene expression was decreased significantly in all the cultures compared to control group (Fig. 4C; P<0.05 or p<0.01).

A strong reduction of notch1 gene expression, in all of treated cells was found (p<0.01), except the cells exposure with NVP-BEZ235 and XAV-939 in combination for 72 hour (Fig. 4D; p<0.01).



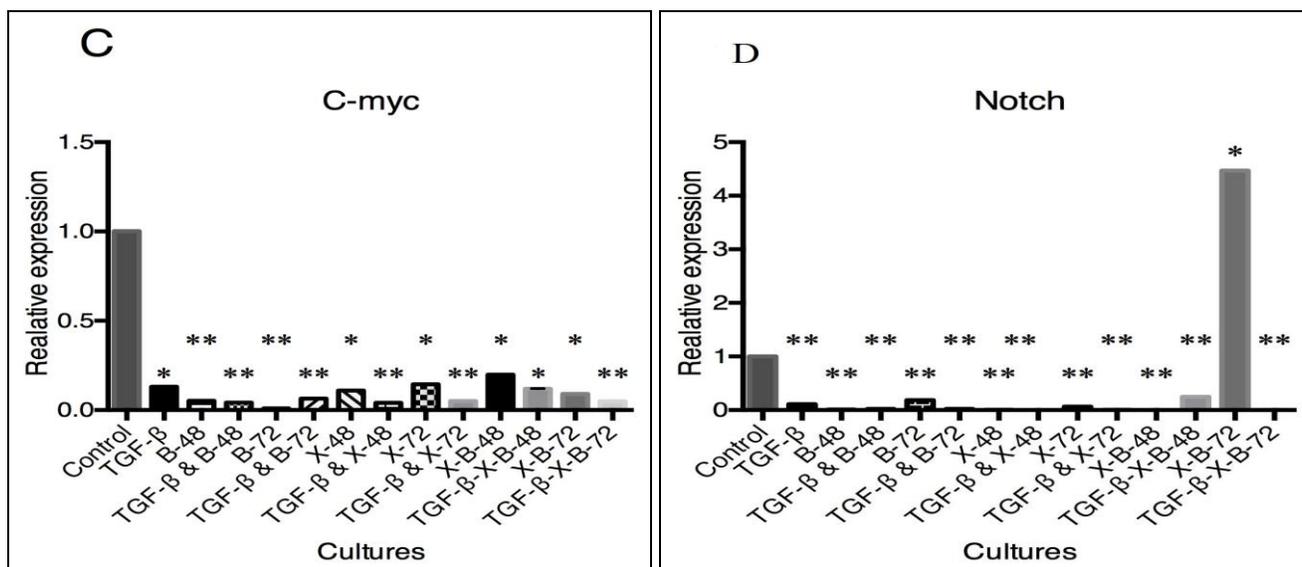


FIG. 3: EXPRESSION LEVELS OF SNAIL, SLUG, C-MYC AND NOTCH IN ALL CULTURES

Real-time PCR was used when TGF-β pretreated and untreated cells were exposed to with NVP-BE235 (240 nM or 220 nM for 48 or 72 h respectively) and/or 10000 nM XAV-939 for 48 and 72 h.

(A) Expression of snail. (B) Expression of slug. (C) Expression of C-myc. (D) Expression of notch. * = $P < 0.05$, ** = $P < 0.01$ vs. untreated cells (control group). These results are presented as the mean \pm standard deviation of three independent experiments. B, NVP-BE235; X, XAV-939.2

DISCUSSION: In this study, we found that TGF-β potentiates NVP-BE235 (PI3K/AKT/mTOR inhibitor) induced cytotoxicity in caco2 cancer cell line. NVPBEZ-235 or TGF-β showed anti proliferative effect in caco2 cells but only TGF-β induced cell toxicity to some extent. Surprisingly, TGF-β pretreatment vigorously promote NVP-BE235 induced cytotoxicity after 72h.

We also found that, blocking Wnt/βcatenin signaling via XAV-939 (tankyrase inhibitor) reduce antiproliferative effect of TGF-β but increase its cytotoxic effect. In addition XAV-939 increases NVPBEZ-235 induced cytotoxic effect after 72h. In Addition NVPBEZ-235 reduced the inhibitor effect of XAV-939 on TGF-β induce cell viability. Several Studies have been suggested that co treatment XAV-939 with everolimus (mTOR inhibitor) and erlotinib (tyrosine kinase inhibitor) resulted in synergistic cell proliferation inhibition²⁰. Also, similar to our study, XAV-939 was able to significantly increases the apoptosis induced by 5 floro uracil²¹.

In our study XAV-939 and NVP-BE235 together produced a higher cytotoxic effect in the presence of TGF-β. Thus, such a combinatorial treatment of colorectal cancer with XAV-939 and NVP-BE235 especially when TGF-β exist in tumor micro

environment may increase an additional benefit over conventional therapies.

Accumulating evidence suggests that T-box (TBX) gene play a major role in cancer cell proliferation and invasion. There is a direct relationship between TBX expression and cancer cell invasion, metastasis and proliferation²². TGF-β as a common environmental cytokine was shown to decreases cancer cell proliferation via Inhibition of TBX expression²³. In contrast to TGF-β, inhibition of Wnt signaling up regulates TBX expression. Thus, inhibition of Wnt/B-catenin pathway increase resistance to TBX repression, suggesting that how XAV-939 diminishes antiproliferative effect of TGF-B (**Fig. 1**). TBX was also found to inhibit p21 and repress senescence induction²².

Thus, in contrast to XAV-939, TGF-β decreases cell proliferation and increased senescence induction (**Fig. 3**) through the reduction of TBX, which in turn may increase sensitization of colorectal cancer cells to chemotherapy such as NVP-BE235. Senescent cells are a population that do not proliferate anymore but induction of senescence cells increase sensitization to chemotherapy^{24, 25}. However, senescence cells was shown as a main culprit in tumor recurrence^{26, 27}. So, XAV-939 may reduce disease relapses by decreasing senescence cancer cells (**Fig. 3**).

Moreover, our study suggested that combination of NVP-BEZ235 and XAV-939 for 72h increase NOTCH expression which, is one of the main factor in chemotherapy resistance²⁸. Surprisingly, all other groups decreased expression of notch significantly. In addition, we explored the expression of slug as a well-known metastatic factor that play a crucial role in drug resistance and CRC progression²⁹. Our data showed that expression of slug increased strongly when the cells were exposed to NVP-BEZ235 for 72 h. on the other hand, combinatorial treatment of cell with XAV-939 and NVP-BEZ235 decreased slug expression. Our study also showed that inhibition of Wnt/b-catenin pathway through XAV-939 prevents expression of slug in cells treated with NVP-BEZ235.

Furthermore, the expression of other tumor promoting factor such as C-myc and snail was assessed in all cultures. Our data interestingly indicated that the expression of C-myc and snail decreased in all cultures.

CONCLUSION: This study has shown for the first time that presence of anti-inflammatory cytokine such as TGF- β increases the efficacy of new chemotherapy drugs. NVP-BEZ235 was more potent in induction of cellular death when CRC cells were pretreated with TGF- β . TGF- β itself decreases the cell viability of CRC cells. Thus, induction of environmental anti-inflammatory cytokine like TGF- β improve CRC prognosis.

ACKNOWLEDGEMENT: This work was supported by the Shahrekord University of medical science [grant numbers 1980].

CONFLICT OF INTEREST: The authors declared no potential conflicts of interest.

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How to cite this article:

Karami R, Soltani A and Ghatrehsamani M: TGF- β increases cytotoxic effect and senescence induced by PI3K/Akt/mTOR inhibitor in human colorectal cancer cells. *Int J Pharm Sci & Res* 2018; 9(3): 924-31. doi: 10.13040/IJPSR.0975-8232.9(3).924-31.

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