Niclosamide Suppresses Proliferation, Induces Apoptosis and Inhibits Wnt/β-catenin Signaling Pathway in Human Ovarian Cancer Cells

Niklozamid İnsan Over Kanseri Hücrelerinde Proliferasyonu Baskılar, Apoptozisi İndükler ve Wnt/β-katenin Sinyal Yolağını İnhibe Eder

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ABSTRACT

Objective: The aim of this study is to investigate in vitro effects of an antihelminthic drug niclosamide on human ovarian carcinoma cell line OVCAR-3.
Methods: MTT assay was applied to investigate the cytotoxic effects of niclosamide on the cells. β-catenin levels in the cells were analyzed by immunocytochemistry, in order to assess the potency of niclosamide on Wnt/β-catenin signaling pathway that function in cell proliferation. The effects of the drug on apoptosis were detected by TUNEL method. All the assays were also performed for chemotherapy agent 5-fluorouracil (5-FU) and anticancer effects of these two drugs were compared.
Results: It was found that niclosamide at 1 μM and 2 μM concentrations reduced cell viability, whereas 5-FU showed its significant proliferation inhibitory effect at higher concentrations. Niclosamide led to an increase in apoptosis while this effect was weaker compared with 5-FU. Niclosamide treatment decreased β-catenin staining in the cells significantly but 5-FU did not affect β-catenin levels.
Conclusion: The results indicate that niclosamide induces apoptosis and suppresses cell proliferation by inhibiting Wnt/β-catenin signaling pathway in OVCAR-3 cells. In conclusion, these findings warrant further evaluation of niclosamide as a promising therapy for ovarian cancer.

Key Words: OVCAR-3, niclosamide, 5-fluorouracil, Wnt/β-catenin, proliferation, apoptosis

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ÖZET

Amaç: Bu çalışmanın amacı, antihelmintik bir ilaç olan niklozamidin insan over kanseri hücre hattı OVCAR-3 üzerindeki in vitro etkilerini araştırmaktır.
Yöntemler: Niklozamidin hücreler üzerindeki sitotoksik etkilerini incelemek için MTT testi uygulanmıştır. Niklozamidin hücre proliferasyonunda görev alan Wnt/β-katenin sinyal yolağına potansiyel etkisini değerlendirmek üzere immünositokimya yöntemi kullanılarak hücrelerdeki β-katenin seviyeleri analiz edilmiştir. İlacın apoptozise etkileri TUNEL yöntemi ile değerlendirilmiştir. Tüm deneyler ayrıca kemoterapi ajanı olan 5-florourasil (5-FU) için de yapılarak iki ilacın antikanser etkileri karşılaştırılmıştır.
Bulgular: 1 μM ve 2 μM konsantrasyonlardaki niklozamidin hücre canlılığını azalttığı, 5-FU'nun ise ancak daha yüksek konsantrasyonlarda anlamlı bir proliferasyon inhibisyonu sağladığı bulunmuştur. Niklozamidin apoptoziste artışa neden olduğu ancak 5-FU ile karşılaştırıldığında bu etkinin daha zayıf olduğu belirlenmiştir. Niklozamid uygulaması hücrelerdeki β-katenin miktarının anlamlı şekilde azalmasına neden olmuş, 5-FU ise β-katenin seviyelerini etkilememiştir.
Sonuç: Sonuç olarak, niklozamid OVCAR-3 hücrelerinde apoptozisi indüklemekte ve Wnt/β-katenin sinyal yolağını inhibe ederek hücre proliferasyonunu baskılamaktadır. Bu bulgular, niklozamidin over kanseri için ümit verici bir tedavi olarak daha fazla incelenmeye değer olduğunu göstermektedir.

Anahtar Sözcükler: OVCAR-3, niklozamid, 5-florourasil, Wnt/β-katenin, proliferasyon, apoptozis

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INTRODUCTION

Ovarian cancer is the most lethal gynecological malignancy although it is relatively uncommon among the female cancers (1). This high mortality rate is caused by the diagnosis of ovarian cancer usually at an advanced stage. The standard treatment for advanced ovarian cancer is based on the combination of surgical tumor debulking and chemotherapy with platinum and taxanes. Although the treatment increases survival rates, the overall survival remains poor because most patients eventually develop tumour recurrence (2). Therefore, it is critical to search for novel therapeutic targets and explore new agents for the treatment of ovarian cancer.

Niclosamide (trade name Niclocide) is an oral salicylanilide in the antihelmintic family that is especially effective against cestodes which infects humans (3). It has been approved by FDA for the treatment of various tapeworm infections and has been used in humans for approximately 50 years (4). It is believed that niclosamide exhibits its anthelmintic effects by inhibiting oxidative phosphorylation in the mitochondria of the tapeworm, but its mechanism of the action has not been well defined (5). Niclosamide has been identified as a potential cancer therapeutic agent and has antiproliferative activity in many cancer cells (e.g. head and neck cancer, colon cancer, breast cancer, prostate cancer, non-small cell lung cancer, ovarian cancer and acute myeloid leukemia) (6-12). In the previous studies it has been demonstrated that niclosamide targets multiple signaling pathways including Stat3, NF-kB, ROS, Notch and mTORc1 (10, 12-14). Recently, it has been reported that niclosamide can promote downregulation of cytosolic β-catenin expression to inhibit Wnt/β-catenin signaling (15, 16).

Wnt/β-catenin signaling activation requires the stabilization of cytosolic β-catenin, which enters the nucleus for the activation of Wnt target genes by binding transcription factors of the T-cell factor and lymphoid enhancing factor (TCF/LEF) family (17, 18). If the Wnt ligand does not bind to the receptors on the cell membrane, the pathway is “off” and β-catenin is degraded by a destruction complex containing Axin, adenomatous polyposis coli (APC), and glycogen synthetase kinase 3β (GSK3β). β-catenin is phosphorylated by the kinases casein kinase 1 (Ck1) and GSK3β followed by ubiquitination and proteasomal degradation by the 26S proteasome. The Wnt ligand binds to the transmembrane receptors causing the Wnt pathway to turn "on," which in turn causes the inhibition of the destruction complex leading to the accumulation of cytosolic β-catenin. β-catenin levels increase and it accumulates in the cytoplasm, translocates to the nucleus and regulates expression of specific target genes (17-19). Over 100 target genes regulated by Wnt pathway have been identified and 23 of them are found to be upregulated in ovarian cancer (20).

The Wnt/β-catenin signaling pathway plays important roles in the various processes including cell fate specification, differentiation, proliferation, survival, migration, polarity and apoptosis in the great majority of cell types. Abnormalities in the levels and activities of Wnt signaling components may cause defects in embryonic development. Aberrations in the Wnt/β-catenin signaling pathway are also observed in variety of types of human cancer (21-23). Inhibitors of the Wnt/b-catenin pathway have become a focus of research in trying to find targeted therapeutic agents for the treatment of various cancers.

In the present study we sought to investigate the potential effects of niclosamide in human ovarian cancer cells. We analyzed cytotoxic and apoptotic effects of the drug by MTT and TUNEL assays. In addition we explored the inhibitory action of niclosamide against β-catenin levels in the cells by using immunocytochemistry. We compared the results to the findings obtained from the anti-cancer agent 5-fluorouracil (5-FU).

METHODS

Reagents and cell culture

Niclosamide (2’,5-dichloro-4’-nitrosalicylanilide) (N3510) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and was dissolved in DMSO (dimethyl sulfoxide) at a 10 mM concentration. 5-FU (03738) was obtained from Fluka (Italy) and dissolved in DMSO at a 100 mM concentration. Stock drug solutions were stored at 4°C before use and diluted in the relevant assay media, and 0.1% DMSO served as a vehicle control. Human ovarian epithelial adenocarcinoma cell line OVCAR-3 was purchased from American Type Culture Collection (ATCC) (Rockville, MD, USA) and was cultured in RPMI-1640 medium (Lonza, Walkersville, MD) supplemented with 20% heat inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin, 0,01 mg/mL bovine insulin and 1 mM sodium pyruvate (Sigma-Aldrich, St. Louis, MO, USA) at 37°C in a humidified incubator with a mixture of 95% air and 5% CO_2.

Cell proliferation assay

The effects of niclosamide and 5-FU on cell proliferation were determined by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay as previously described, with some modification (24). Briefly, the exponentially growing cells ($5x10^3$ cells/well) were seeded in 96-well plates. After 48 h incubation, the cells were treated with various concentrations of niclosamide (0.2 μM, 0.5 μM, 1 μM, 2 μM, 4 μM, 8 μM) and 5-FU (1 μM, 2 μM, 5 μM, 10 μM, 20 μM, 40 μM). After incubation for 24, 48 and 72 h, the incubation medium was replaced with 25 μL 5 mg/ml MTT solution for incubation for 3 h at 37°C. The MTT solution was subsequently discarded, and 100 μL DMSO was added to dissolve the precipitate completely at room temperature. The optical density (OD) of each well was then measured at 570 nm using a BioTek EL808 microplate spectrophotometer (Winooski, VT, USA). The cell viability was expressed as relative viable cells (%) to control OVCAR-3 cells and median inhibitory concentration (IC_{50}) values were calculated. Each experiment was performed as quadruplicate and replicated at least 3 times.

TUNEL staining

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was used to detect DNA fragmentation and apoptotic cell death in OVCAR-3 cells. The TUNEL assay was performed using the ApopTag® Plus Peroxidase *In Situ* Apoptosis Detection Kit (Cat no. S7101) (Chemicon, Temecula, CA, USA). OVCAR-3 cells were plated in adherent conditions in round coverslips inserted in 24-well plates at $3x10^5$ cells per well. After 48 h incubation, the cells were treated with 1 μM and 2 μM niclosamide or 10 μM and 20 μM 5-FU for 48h. Cells were fixed in 1% paraformaldehyde and permeabilized with 0.1% Triton X-100 solution in PBS for 5 min at 4°C. After endogenous peroxidase was inactivated in 3% hydrogen peroxide, cells were equilibrated and applied with TdT enzyme in a humidified chamber at 37°C for 1 h. Reactions were stopped by incubating cells in stop/wash buffer for 10 min before proceeding anti-digoxignenin conjugate in a humidified chamber for 30 min. After the cells were applied with peroxidase substrate 3,3’-diaminobenzidine (DAB) (00-2020) (Invitrogen, Carlsbad, CA) to detect TUNEL positive cells counterstained in methyl green for 10 min. Stained cells were washed in 100% N-butanol and dehydrated in xylene before the coverslips were mounted using mounting medium and examined under computerized photolight microscope (Leica DM4000 B, Germany).

Cells were scanned in the 6 areas (1 central and 5 peripheral) of each coverslip. TUNEL index was calculated as percentage of TUNEL-positive nuclei among total number of 100 counted nuclei.

Immunocytochemical staining

Immunocytochemical staining (ICC) was performed on the cell-bearing coverslips of each experimental group. Rabbit polyclonal anti-β-catenin (71-2700) was purchased from Invitrogen (Carlsbad, CA) and used according to the manufacturer’s instruction. Briefly, the coverslips were washed with phosphate-buffered solution (PBS, pH 7.4), incubated for 5 minutes in 3% H_2O_2 and epitopes were stabilized by application of serum blocking solution for 5 min. Cells were incubated with diluted primary antibody (1:100) overnight at 4°C in a humidity chamber followed by treatments with the biotinylated secondary antibody (859043) (Invitrogen, Carlsbad, CA) for 20 min and streptavidin peroxidase (859043) (Invitrogen, Carlsbad, CA) enzyme for 20 min. Color reaction was developed using DAB and cells were counterstained with Harris's hematoxylin (008011) (Invitrogen, Carlsbad, CA). Cells were examined under a computerized photolight microscope (Leica DM4000 B, Germany).

Cells with cytoplasmic β-catenin staining were scanned in the 6 areas (1 central and 5 peripheral) of each coverslip. According to the staining intensity, the staining results were evaluated by two independent researchers and scored as negative (0), faint (1), weak to moderate (2), moderate to strong (4) and strong (5).

Statistical analysis

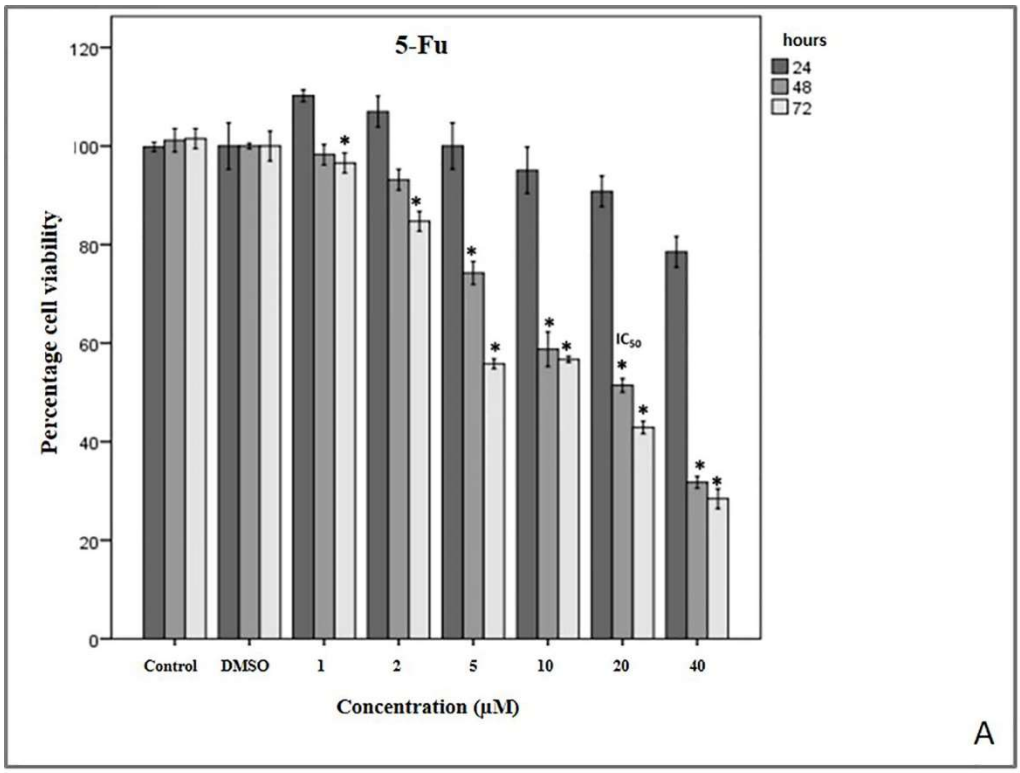
Data values of MTT assays were given as mean ± standard deviation (SD). Statistical differences were analyzed by one-way analysis of variance (ANOVA) carried out using SPSS 11.5 for windows (SPSS Inc, Chicago, USA). Scheffe and Tamhane tests were used as a post-hoc method to determine differences among groups and were considered statistically significant when $p<0.001$. SPSS 17.0 for windows (SPSS Inc, Chicago, USA) was used to analyze the data obtained from TUNEL assay and immunocytochemical staining. To determine whether TUNEL-positive cell count in each experimental group is statistically significant, z-test was applied. Proportion of positive cells to total cell number in one group compared with the other groups and differences at $p<0.05$ were considered statistically significant. The nonparametric Kruskal–Wallis test was conducted for comparing β-catenin staining intensity between groups. Since the statistical significance ($p<0.001$) was determined, Mann–Whitney *U* test was used to analyze differential immunostaining in the groups and p values less than 0.05 were considered to be statistically significant.

RESULTS

Niclosamide inhibits proliferation of OVCAR-3 cells

Cytotoxic effect of niclosamide in OVCAR-3 cells was evaluated by MTT assay. OVCAR-3 cells were incubated for 3 days with various concentrations (1~40 µM) of 5-FU to assess the antiproliferative action of the drug. 24 h after the treatment, there was no significant effect on cell viability. Concentrations 20 µM and above of 5-FU significantly inhibited OVCAR-3 cell growth and 20 µM concentration was determined as the IC_{50} value of the drug (Figure 1A).

OVCAR-3 cells were exposed to increasing concentrations of niclosamide (0.2~8 µM) for 24, 48 and 72 h. Niclosamide exhibited weak antiproliferative activity against cancer cells after 24 h incubation. It inhibited cell growth after 48h of treatment at 1 µM, 2 µM, 4 µM and 8 µM concentrations with 50% inhibition concentration (IC_{50}) of 2 µM. The viability of cells was decreased dramatically after 72 h exposure of niclosamide (Figure 1B)



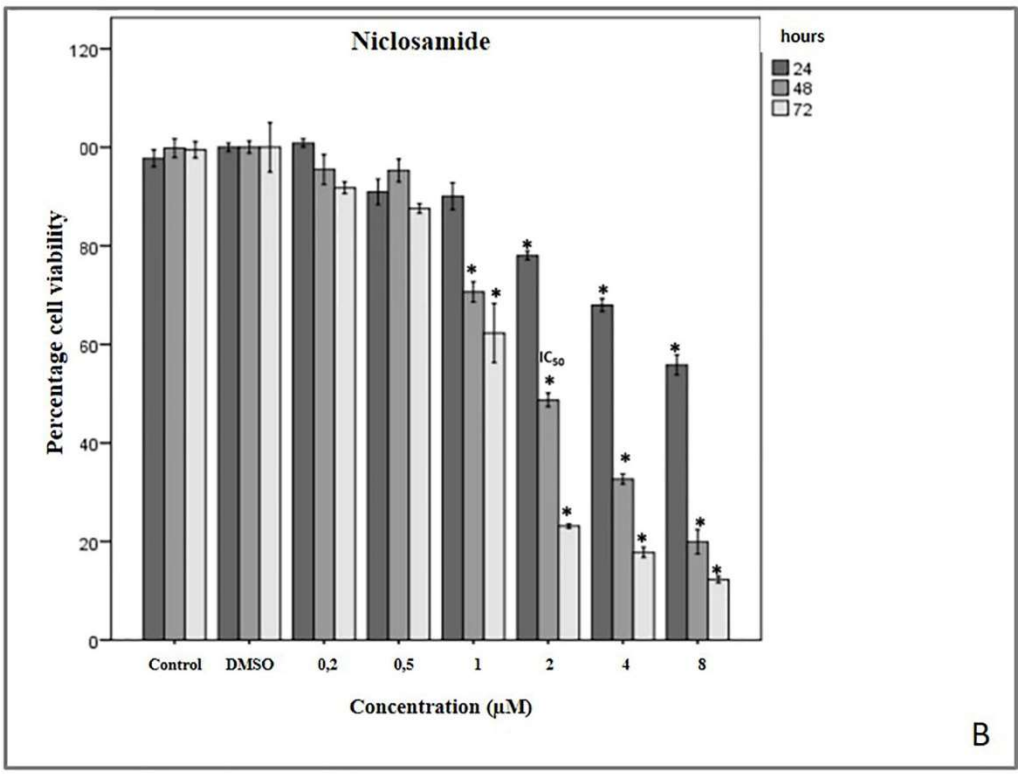
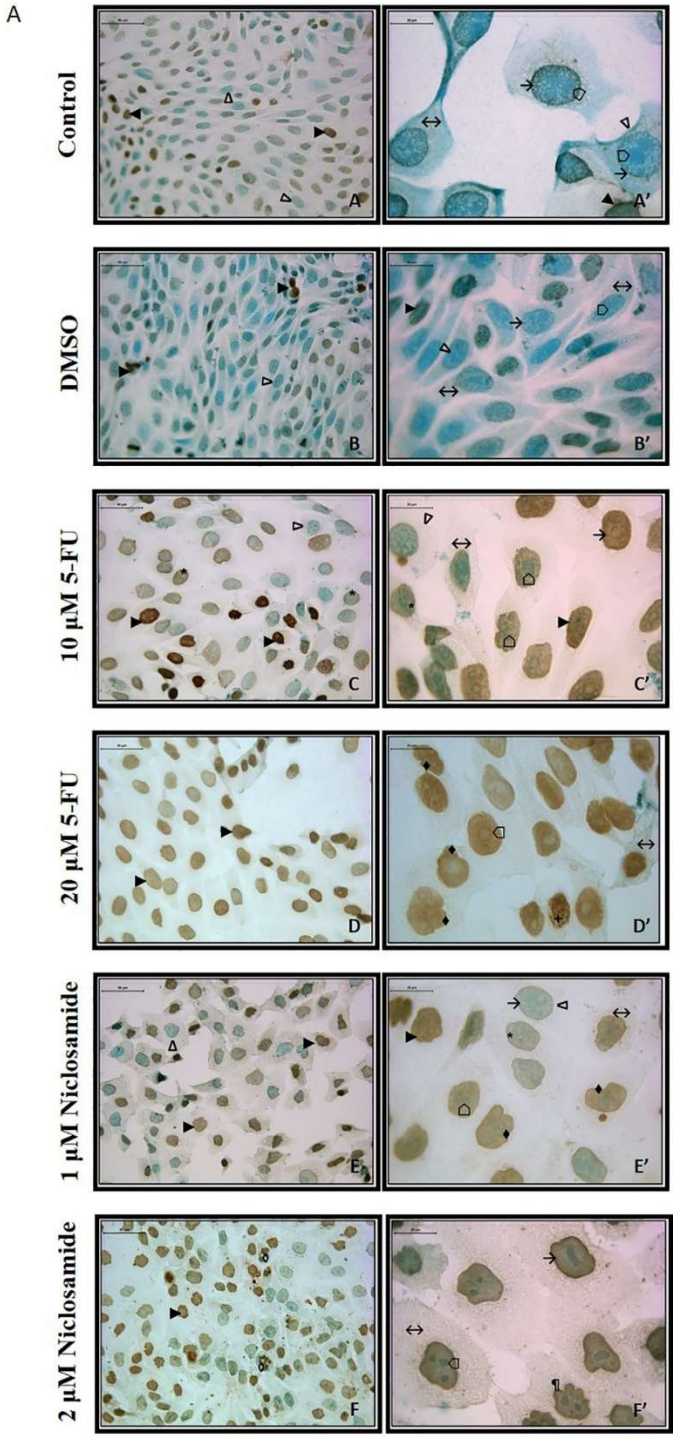


Figure 1. Viability of OVCAR-3 cells treated with drugs for 24, 48 and 72 hours, performing MTT test (* p≤ 0.001). DMSO: 0.1% (v/v). 5-FU treatment **(a)**. Niclosamide treatment **(b)**

Niclosamide induces apoptosis in OVCAR-3 cells

Data obtained from TUNEL assay showed that niclosamide significantly induced apoptosis in a dose-dependent manner in OVCAR-3 cells compared with the control and the DMSO control.



However, percentage of TUNEL-positive cells indicated that 5-FU was more competent to induce apoptosis (Figure 2A). When 10 µM and 20 µM 5-FU triggered cell death in 63% and 90% of OVCAR-3 cells, apoptotic cell rates were 45% and 67% at the 1 µM and 2 µM niclosamide concentration respectively. Significant differences between groups were analyzed statistically (Figure 2B).

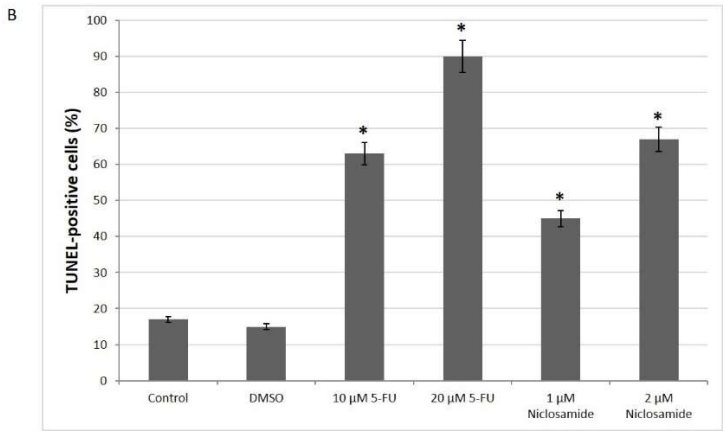
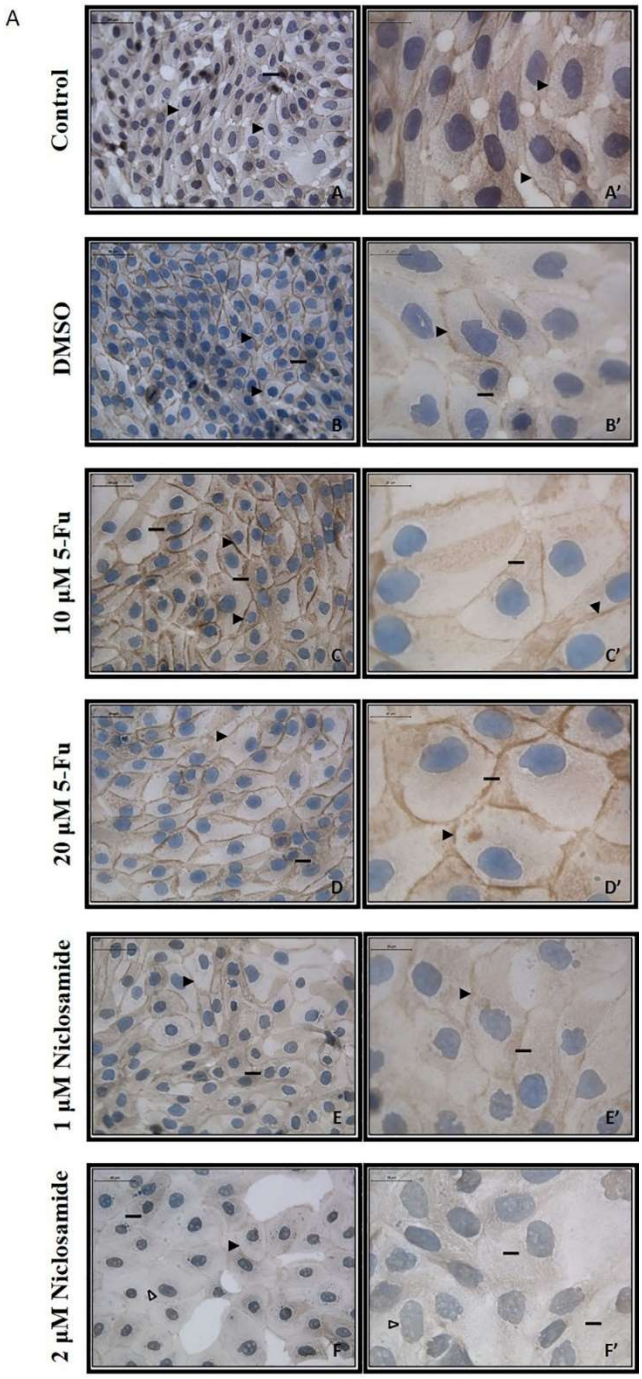


Figure 2. TUNEL staining of OVCAR-3 cells after 5-FU and niclosamide exposure for 48 h. TUNEL-positive cells (▶), TUNEL-negative cells (Δ), nucleus (→), nucleolus (⌂), cytoplasm (↔), weakly stained cells (*), ondulation on cell membrane (♦), nucleus with damaged chromatin structure (+), nuclear fragments as apoptotic bodies (◊) and lobulated nucleus (¶). Scale bars: 50µm for A, B, C, D, E, F; 20µm for A', B', C', D', E', F' **(A)**. TUNEL-positive cell percentages compared between groups (*p < 0.001) **(B)**

Wnt/ß-catenin pathway inhibition in OVCAR-3 cells by niclosamide

To elucidate the effects of niclosamide on β-catenin levels immunocytochemical staining was performed and location and relative abundance of the protein were evaluated. β-catenin was distributed in cell membrane and cytoplasm of OVCAR-3 cells under normal culture conditions. Strong membranous and moderate cytoplasmic staining was observed in the control and DMSO control cells. After 1 µM niclosamide treatment for 48 h, staining intensity became weaker. At the 2 µM concentration of niclosamide, β-catenin staining was significantly decreased. Most of the cells showed negative immunoreactivity whereas weak cytoplasmic staining was detected in a small number of cells (Figure 3A). Intensity comparisons between groups showed that reducing effect of niclosamide on β-catenin levels in the cells was statistically significant (Figure 3B). Staining protocol was also performed for the 10 µM and 20 µM concentrations of the 5-FU and reactivity intensity was found quite similar to control and DMSO control at both concentrations (Figure 3A). There were no statistically significant differences among control, DMSO control and 5-FU groups (Figure 3B).



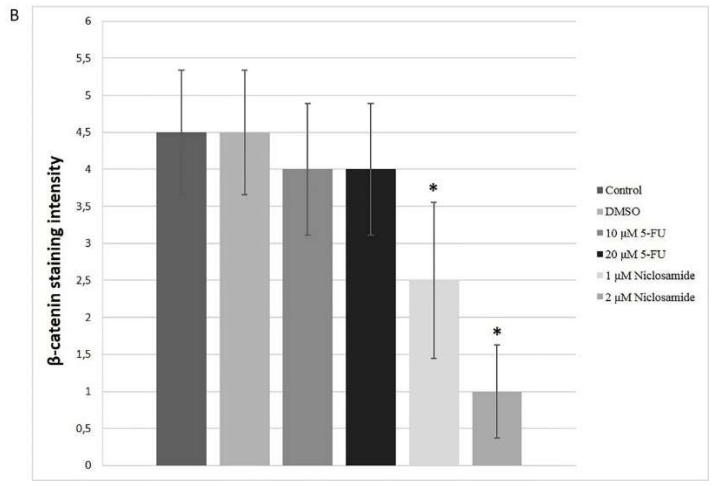


Figure 3. Immunocytochemistry analysis of OVCAR-3 cells stained with β-catenin antibody after 5-FU and niclosamide exposure for 48 h. Membranous staining (►), cytoplasmic staining (▬), and negative staining (Δ). Scale bars: 50µm for A, B, C, D, E, F; 20µm for A', B', C', D', E', F' **(A)**. β-catenin staining intensity of OVCAR-3 cells compared between groups (*p < 0.001) **(B)**

DISCUSSION

Development of novel anticancer drugs that show less toxicity and are able to be administrated orally is important to improve the ovarian cancer treatment. Repurposing existing drugs that have been used for other indications to find new uses is an attractive strategy for the drug development process. Niclosamide is a salicylic acid derivative anti-helminthic drug that is effective against human tapeworms. It has low toxicity in mammals (oral LD_{50} in rats, >5,000 mg/kg) and is safe (25, 26). Niclosamide has been recently investigated for use in cancer therapy because of its convenient properties.

It has been demonstrated that niclosamide is able to inhibit cell proliferation and induce apoptosis at very low concentrations in many human cancer cells. IC_{50} value was found to be less than 1 µM and niclosamide induced apoptosis at 1.2 and 2.4 µM concentrations in prostate and breast cancer cell lines (14). Significant anti-proliferative actions were detected at niclosamide concentrations 0.5–1.0 µM and the apoptotic induction was established in multiple myeloma cells (27). In the present study, we demonstrated the potential therapeutic activity of niclosamide against human ovarian cancer cells. Niclosamide inhibited cell proliferation in a dose- and time-dependent fashion. We showed that niclosamide displayed a significant anti-proliferative effect at a concentration as low as 2 µM after 48 h incubation of OVCAR-3 cells. This action was greater than 5-FU, the anti-cancer drug which is already used for treatment of several cancers, whose IC_{50} value has been found to be 20 µM against OVCAR-3 cells in dose-response experiments. We also determined that niclosamide was able to induce ovarian cancer cell apoptosis significantly at 2 µM concentration compared to the control ($p<0.05$). However, 20 µM of 5-FU was more potent than niclosamide in apoptosis induction after 48 h incubation and this finding was validated with statistical comparison ($p<0.001$). Our data revealed that niclosamide is a potential therapeutic agent for ovarian cancer.

The Wnt/β-catenin signaling pathway is involved in tissue development and homeostasis. The target genes of the pathway regulate cell proliferation and apoptosis, and evidences indicated that aberrant up-regulation of this pathway promotes tumorigenesis of a variety of cancers (3-5). Dysregulation of Wnt/β-catenin signaling on the cell membrane, in the cytoplasm, and in the nucleus leads to aberrant activation of pathway and is involved in carcinogenesis of ovarian cancer (8). Thus, targeting the Wnt/β-catenin pathway is a promising new approach for the ovarian cancer therapy. Several studies demonstrated that niclosamide could inhibit Wnt/β-catenin signaling by down-regulating cytosolic β-catenin expression (22, 23).

Yo *et al.* used a drug screening method with more than 1200 clinically approved drugs and demonstrated that niclosamide selectively inhibits the growth of stem-like ovarian cancer-initiating cells by modulating metabolic signaling pathways including Wnt pathway (18). A significant reduction of Wnt/β-catenin signaling was observed in tumor cells isolated from patients' ascites with primary ovarian cancer treated with niclosamide and carboplatin combination (28).

It has been reported that more soluble niclosamide analogs are able to produce cytotoxicity and inhibit Wnt signaling in ovarian cancer patient samples (29). *King et al.* reported that niclosamide inhibits WNT7A levels and TCF/LEF activity stimulated by a constitutively active β-catenin in human ovarian cancer cells (30). In this study, we investigated the status of Wnt/β-catenin signaling in OVCAR-3 cells and effects of niclosamide on the pathway. This was the first study which uses immunocytochemistry to investigate the Wnt/β-catenin signaling pathway mediated cell proliferation inhibitory effect of the drug on OVCAR-3 cells. We showed that cytosolic β-catenin levels were increased in the cancer cells and after 2 μM niclosamide treatment for 48 h, cytoplasmic distribution of β-catenin was significantly reduced compared to the control ($p<0.05$). There was no statistically significant alteration of β-catenin labeling following 5-FU treatment of cells ($p>0.05$). These data suggest that niclosamide is a potent Wnt/β-catenin signaling inhibitor by inhibiting cytosolic β-catenin accumulation in ovarian cancer cells.

In conclusion, our data indicate that niclosamide inhibits ovarian cancer cell growth and induces apoptosis *in vitro* at low concentrations. Its antiproliferative activity might be caused by inhibiting Wnt/β-catenin signaling pathway in ovarian cancer cells. Niclosamide is a candidate monotherapy or in combination with current chemotherapeutics for the treatment ovarian cancer and worthy of further investigations.

Conflict of interest

No conflict of interest was declared by the authors.

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