Original Paper

A Blockade of IGF Signaling Sensitizes Human Ovarian Cancer Cells to the Anthelmintic Niclosamide-Induced Anti-Proliferative and Anticancer Activities

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Key Words

Ovarian cancer • Drug repurposing • Niclosamide • IGF signaling • IGF-1R • Cancer pathways • Cell signaling • Synergistic effect • Cancer therapy

Abstract

Background/Aims: Ovarian cancer is the most lethal gynecologic malignancy, and there is an unmet clinical need to develop new therapies. Although showing promising anticancer activity, Niclosamide may not be used as a monotherapy. We seek to investigate whether inhibiting IGF signaling potentiates Niclosamide's anticancer efficacy in human ovarian cancer cells. **Methods:** Cell proliferation and migration are assessed. Cell cycle progression and apoptosis are analyzed by flow cytometry. Inhibition of IGF signaling is accomplished by adenovirus-mediated expression of siRNAs targeting IGF-1R. Cancer-associated pathways are assessed using pathway-specific reporters. Subcutaneous xenograft model is used to determine anticancer activity. **Results:** We find that Niclosamide is highly effective on inhibiting cell proliferation, cell migration, and cell cycle progression, and inducing apoptosis in human ovarian cancer cells, possibly by targeting multiple signaling pathways involved in ELK1/SRF, AP-1, MYC/MAX and NFκB. Silencing IGF-1R exert a similar but weaker effect than that of Niclosamide's. However, silencing IGF-1R significantly sensitizes ovarian cancer cells to Niclosamide-induced anti-proliferative and anticancer activities both *in vitro* and *in vivo*. **Conclusion:** Niclosamide as a repurposed anticancer agent may be more efficacious when combined with agents that target other signaling pathways such as IGF signaling in the treatment of human cancers including ovarian cancer.

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Introduction

As the fifth most common cancer in women in the United States, ovarian cancer is the most deadly gynecologic malignancy [1, 2]. Due to the absence of an effective screening strategy, only approximately 20% of ovarian cancers are diagnosed while confined to the ovaries. Over the past two decades, the 5-year survival rate for ovarian cancer patients has substantially improved, largely due to improved surgical techniques and empirically optimized chemotherapy regimens of cytotoxic platinum-combination drugs. In spite of these improvements, the overall cure rate remains approximately 30% [1, 3]. Most patients experience recurrence within 12–24 months and die of progressively chemotherapy-resistant disease [1, 3]. Clinical management of ovarian cancer has met many challenges, which is in part because the origin and pathogenesis of epithelial ovarian cancer (EOC) are poorly understood [2]. Epithelial ovarian cancer (EOC) is the most common subtype. Increasing evidence indicates that EOC itself is composed of a diverse group of tumors that can be further classified on the basis of distinctive morphologic and genetic features [1, 2, 4–6]. Increasing evidence indicates that noncoding RNAs and cancer stem cells may contribute to the progression and metastasis of ovarian cancers [7–10]. Given the heterogeneity of human ovarian cancers, significant improvements in long-term survival will hinge on translating recent insights into the molecular and cellular characteristics of ovarian cancers into personalized treatment strategies, optimizing methods of screening or early detection, and developing novel therapeutics. While significant progress has recently been made in the development of novel targeted therapies for human cancers, including ovarian cancers [1, 4–6, 11], an effective alternative to drug development is repurposing drugs. Several examples of such drugs are currently in various stages of clinical trials [12, 13].

Repurposing clinically-used drugs represents a rapid and cost-effective approach to developing new anticancer agents. Niclosamide (trade name Niclocide) is a teniacide in the anthelmintic family and has been approved for use in humans for nearly 50 years. Niclosamide was thought to inhibit oxidative phosphorylation and stimulates adenosine triphosphatase activity in the mitochondria of cestodes (e.g. tapeworm), killing the scolex and proximal segments of the tapeworm both *in vitro* and/or *in vivo*, which is well tolerated in humans [14]. Niclosamide was identified as potential anticancer agent by various high-throughput screening campaigns [14]. It has been shown that Niclosamide exhibits effective anticancer activity and inhibits the growth of colon rectal cancer [15–17], osteosarcoma [18], lung cancer [19, 20], breast cancer [21–24], prostate cancer [21, 25], glioblastoma [26], head and neck cancer [27], leukemia [28, 29], human uterine leiomyoma [30], and ovarian cancer [31–33]. Nonetheless, Niclosamide may not be used as a single agent therapy for any human cancers including ovarian cancer. We have recently demonstrated that Niclosamide may exert its anticancer activity by targeting multiple signaling pathways in human osteosarcoma [18]. Thus, it is important to investigate whether blockades of other signaling pathways will potentiate or augment the Niclosamide's anticancer activity.

In this study, we investigate whether the anticancer activity of Niclosamide can be potentiated by inhibiting IGF signaling in human ovarian cancer cells. We find that Niclosamide is highly effective on inhibiting cell proliferation, cell migration, and cell cycle progression, and inducing apoptosis in human ovarian cancer cells. Silencing IGF1R exerts a similar but weaker effect than that of Niclosamide's. However, silencing IGF1R significantly sensitizes ovarian cancer cells to Niclosamide-induced anti-proliferative and anticancer activities both *in vitro* and *in vivo*. Therefore, our findings strongly suggest that Niclosamide as a repurposed anticancer agent may be more efficacious when combined with agents that target other signaling pathways such as IGF signaling in the treatment of human cancers including ovarian cancer.

Materials and Methods

Cell culture and chemicals

Human ovarian cancer cell lines SKOV3 and HeyA8 were provided by Dr. Ernest Lengyel. HEK-293 cells were from ATCC (Manassas, VA). HEK-293 derivative line 293pTP was previously reported [34]. These lines were maintained in complete Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA), 100 units of penicillin and 100 µg of streptomycin at 37°C in 5% CO₂ as described [35-37]. Niclosamide was purchased from Cayman Chemical (Ann Arbor, MI), and cisplatin was purchased from Sigma-Aldrich (St. Louis, MO). Unless indicated otherwise, all chemicals were purchased from Thermo-Fisher Scientific (Waltham, MA).

Viable cell counting assay

Viable cells were counted with Trypan blue exclusion staining assay as described [38]. Briefly, subconfluent SKOV3 and HeyA8 cells were treated with Niclosamide at the indicated concentrations or vehicle control. At 24h and 48h, cells were trypsinized, collected and stained with Trypan blue (0.1% Trypan blue). Unstained viable cells were counted under a bright field microscope. Each assay condition was done in triplicate.

Crystal violet cell viability assay

Crystal violet staining assay was conducted as described [39-41]. Briefly, subconfluent HeyA8 and/or SKOV3 cells were treated with varied conditions. At the indicated time after treatment, cells were gently washed with PBS and stained with 0.5% crystal violet/formalin solution at room temperature for 20-30min. The stained cells were washed with tape water and air dried for taking macrographic images [42, 43].

WST-1 cell proliferation assay

Cell proliferation was assessed by using Premixed WST-1 Reagent (Clontech, Mountain View, CA) as described [36, 44]. Briefly, subconfluent SKOV3 and HeyA8 cells seeded in 96-well plates were treated with varied conditions. At the indicated time points, the Premixed WST-1 Reagent was added to each well, followed by incubating at 37°C for 1-3h and reading at 440nm using the microplate reader (BioTek EL800, Winooski, VT). Each assay condition was done in triplicate.

Cell wounding/migration assay

Cell wounding/migration assay was performed as described [45, 46]. Briefly, exponentially growing ovarian cancer cells were seeded in 6-well cell culture plates and allowed to reach approximately 90% confluence. Then, the monolayer cells were wounded with sterile micro-pipette tips. At various time points, the wound healing status at the approximately same locations was recorded under bright field microscopy. Each assay condition was done in triplicate.

Apoptosis analysis (Hoechst 33258 staining)

As previously described [18, 42], exponentially growing HeyA8 and SKOV3 cells were treated with varied conditions. At 24h post treatment, cells were collected, fixed and stained with the Magic Solution (10x stock: 0.5% NP-40, 3.4% formaldehyde, 10 g/ml Hoechst 33258, in PBS). Apoptotic cells were examined and recorded under a fluorescence microscope. Each assay condition was done in triplicate. The results were repeated at least in three independent batches of experiments. The average numbers of apoptotic cells were calculated by counting apparent apoptotic cells in at least ten random fields at 100x magnification for each assay condition.

Cell cycle analysis

The exponentially growing HeyA8 and SKOV3 cells were seeded in 6-well plates at sub-confluence and treated with varied conditions. At 24h or 48h post treatment, cells were collected, fixed and stained with the Magic Solution for 30min. The stained cells were subjected to flow cytometry analysis using the BD FACSCalibur-HTS, as described [46]. The acquired flow cytometry data were analyzed with the FlowJo v10.0 software. Each assay condition was done in triplicate.

Construction and amplification of recombinant adenovirus expressing siIGF1R or RGFP

Recombinant adenovirus expressing siRNAs targeting human IGF-1R coding region was constructed by using the AdEasy system as described [47-50]. Briefly, the siRNA target sites were designed by using the Dharmacon's *siDESIGN* web-based program, as previously reported [51-53]. The selected three human IGF1R-targeting siRNA sites are 5'-GGC CAG AAA TGG AGA ATA A -3'; 5'-CCA AGG GTG TGG TGA AAG A-3'; and 5'-TCT CAA GGA TAT TGG GCT T-3'. The siRNA sites were assembled into our recently developed adenoviral shuttle vector pAdTrace-OK through Gibson DNA assembly [52, 53], resulting in pAdTrace-siIGF1R, which expresses the three siRNAs in one vector. The pAdTrace-siIGF1R shuttle vector was used to generate recombinant adenovirus in HEK-293 or 293pTP cells [34]. The resulting adenovirus was designated as AdR-siIGF1R, which also expresses RFP [54-57]. An analogous control adenovirus expressing a scrambled siRNA, as well as RFP and GFP (Ad-RGFP) was used as a control [58, 59]. For all adenoviral infections, polybrene (4-8 µg/ml) was added to enhance infection efficiency as previously reported [60].

Cell transfection and pathway-specific luciferase reporter assay

The Gaussia luciferase (GLuc) reporter assay was conducted as described [61, 62]. The 12 cancer-relevant signaling pathway GLuc reporters were homemade and previously described [61], including NFAT, HIF-1, TCF/LEF, E2F/DP1, Elk1/SRF, AP1, NFκB, Smad, STAT1/2, RBP-Jκ, CREB, Myc/Max reporters. A constitutively active reporter pG2Luc was used as a control [18, 63]. Experimentally, subconfluent SKOV3 cells were seeded in 25 cm² culture flasks and transfected with 3.0 µg per flask of the 13 reporter plasmids using Lipofectamine (Invitrogen). At 16h post transfection, cells were replated in 12-well plates and treated with various concentrations of Niclosamide or DMSO control. At 24h, 48h or 72h post treatment, culture media were taken and subjected to Gaussia luciferase assays using the BioLux Gaussia Luciferase Assay Kit (New England Biolabs). Each assay condition was done in triplicate. Luciferase activity was normalized by total cellular protein concentrations among the samples.

Total RNA isolation and touchdown-quantitative real-time PCR (TqPCR) analysis

Subconfluent ovarian cancer cells were infected with AdR-siIGF1R for 48h. Total RNA was isolated from the treated cells by using TRIZOL Reagents (Invitrogen) and subjected to reverse transcription reactions with hexamer and M-MuLV reverse transcriptase (New England Biolabs, Ipswich, MA). The cDNA products were used as PCR templates. The qPCR primers were designed by using Primer3 program [64] and used to amplify human IGF1R: 5'-ATG ACA TTC CTG GGC CAG TG-3' and 5'-TAG CTT GGC CCC TCC ATA CT-3'. TqPCR were carried out by using the SYBR Green-based qPCR analysis on a CFX-Connect unit (Bio-Rad Laboratories, Hercules, CA) [65]. The qPCR reactions were done in triplicate. *GAPDH* was used as a reference gene.

Xenograft tumors and Xenogen bioluminescence imaging

The use and care of animals were approved by the Institutional Animal Care and Use Committee. All experimental procedures were carried out in accordance with the approved guidelines. Briefly, HeyA8 stably labeled with firefly luciferase (HeyA8-FLuc) was constructed with *piggybac* system [37, 43, 66]. Exponentially growing HeyA8-FLuc cells were infected with AdR-siIGF1R or Ad-RGFP for 36h and collected, resuspended at 10⁷ cells/ml and injected subcutaneously into the flanks of athymic nude mice (Harlan Laboratories, 6-8 week old, male, 10⁶ cells per injection, and 4-6 sites per mouse). The mice were divided into four groups (n=5 per group). At three days post injection, the animals were treated with Niclosamide (10mg/kg body weight) or vehicle control intraperitoneally once every two days. Tumor growth was monitored by whole body bioluminescence imaging using Xenogen IVIS 200 Imaging System weekly after treatment. The average signal for each group at different time points was calculated using the Xenogen's Living Image analysis software as reported [39, 41, 46].

H & E staining

The mice were sacrificed at the end of week 4 and subcutaneous tumor masses were retrieved and fixed in 10% buffered formalin, and embedded in paraffin. Serial sections of the embedded specimens were stained with hematoxylin and eosin (H & E) as described [59, 67].

Statistical analysis

The quantitative assays were performed in triplicate and/or repeated three times. Statistical analysis was carried out using Microsoft Excel program. Data were expressed as mean $\pm$ SD. Statistical significances were determined by one-way analysis of variance and the student's *t* test. A value of $p < 0.05$ was considered statistically significant.

Results

Niclosamide effectively inhibits cell proliferation of human ovarian cancer cells

We sought to test the effect of the antibiotic Niclosamide on the proliferative activity of two commonly-used human ovarian cancer lines HeyA8 and SKOV3. We found cell proliferation was significantly inhibited by Niclosamide at as low as $1\mu\text{M}$ in both HeyA8 and SKOV3 cells in a time course and dose-dependent fashion (Fig. 1a). When treated with $1\mu\text{M}$ Niclosamide, the viable cell counts decreased to 65% and 30.1% of the control groups' at 24h and 48h, respectively, in HeyA8 cells (Fig. 1a-i). Similarly, for SKOV3 cells, treatment with $1\mu\text{M}$ Niclosamide led to a decrease in viable cells to 53.4% and 36% of the control group's at 24h and 48h, respectively (Fig. 1a-ii). These results were further confirmed by WST-1 proliferation assay (Fig. 1b). Specifically, when HeyA8 cells were treated with $1\mu\text{M}$ Niclosamide, cell proliferation rate reduced to 79.3%, 48.3%, and 39.3% of the control's at 24h, 48h, and 72h post treatment, whereas the two later time points are statistically significant (Fig. 1b-i). The cell proliferation of HeyA8 cells was effectively inhibited by Niclosamide in time and dose-dependent fashion and almost completely inhibited at $8\mu\text{M}$ (Fig. 1b-i). A

Fig. 1. Niclosamide effectively inhibits the proliferation of human ovarian cancer cells. (a) Viable cell counting assay. Subconfluent HeyA8 (i) and SKOV3 (ii) cells were seeded in 12-well plates and treated with Niclosamide at the indicated concentrations. At 24h and 48h post treatment, the viable cells were collected, stained with trypan blue and counted under a bright field microscope. (b) WST-1 cell proliferation assay. Subconfluent HeyA8 (i) and SKOV3 (ii) cells were seeded in 96-well plates and treated with Niclosamide at the indicated concentrations. At the indicated time points post treatment, the WST-1 reagent was added to plates and incubated for 1h and absorbance measurement was performed. All assay conditions were done in triplicate.

Fig. 2. Niclosamide induces apoptosis in human ovarian cancer cells. (a) Crystal violet staining assay. Subconfluent HeyA8 and SKOV3 cells were seeded in 12-well plates and treated with 2µM Niclosamide, 15µM cisplatin (CIS), or DMSO. At 24h, 48h and 72h post treatment, the cells were fixed and stained with crystal violet. (b) Hoechst 33258 staining assay. Subconfluent HeyA8 and SKOV3 cells were treated with 1 or 2µM Niclosamide or solvent control. At 24h post treatment, cells were collected, fixed and stained with Hoechst 33258 and examined under a fluorescence microscope (i). Apparent apoptotic cells (indicated by arrows) were counted in at least 10 random fields under 100x magnification (ii). “***” $p < 0.01$ (Niclosamide treated vs. control group).

similar trend was observed in SKOV3 cells as 1-2µM Niclosamide was shown to effectively inhibit cell proliferation at 48h and 72h post treatment (Fig.1b-ii). These results indicate that Niclosamide can effectively inhibit cell proliferation of human ovarian cancer cells.

Niclosamide is highly cytotoxic and induces apoptosis in human ovarian cancer cells

We conducted Crystal violet staining to compare the cytotoxicity between Niclosamide and the clinical cancer drug cisplatin. We found that 2µM Niclosamide exhibited similar cytotoxicity at 24h and 48h, but higher cytotoxicity at 72h than that of 15µM cisplatin in SKOV3 cells (Fig. 2a). HeyA8 cells were seemingly more resistant to cisplatin as significant portion of viable cells were observed at 72h post 15µM cisplatin treatment, while Niclosamide was shown to effectively inhibit cell proliferation at 72h (Fig. 2a).

We further investigated if Niclosamide can induce apoptosis in ovarian cancer cells. When HeyA8 and SKOV3 cells were treated with 0µM or 2µM Niclosamide for 24h and stained with Hoechst 33258, significant numbers of apoptotic cells were observed (Fig. 2b-i). Quantitative analysis indicated that the percentages of apoptotic cells were significantly increased in Niclosamide treated HeyA8 and SKOV3 cells ($p < 0.01$) (Fig. 2b-ii). These results indicate that Niclosamide exhibits higher cytotoxicity than cisplatin and effectively induces apoptosis in human ovarian cancer cells.

Fig. 3. Niclosamide inhibits cell migration and cell cycle progression in human ovarian cancer cells. (a) Cell wounding assay. Freshly seeded subconfluent HeyA8 (i) and SKOV3 (ii) cells were wounded with micro-pipette tips and treated with 2μM Niclosamide. The wounding gaps were recorded at the indicated time points after Niclosamide treatment. Each assay condition was done in triplicate. (b) Cell cycle analysis. Subconfluent HeyA8 cells were treated with Niclosamide or vehicle control for 24h. Cells were collected, fixed, stained with Hoechst 33258, and subjected to FACS analysis. Similar results were obtained in SKOV3 cells (data not included). Each assay condition was done in triplicate.

Niclosamide inhibits cell migration and cell cycle progression in human ovarian cancer cells

We next examined if Niclosamide exerts any effect on cell migration and wound healing in ovarian cancer cells. When freshly confluent HeyA8 monolayer cells were wounded, the gap was almost completely closed in DMSO control group, but the cells treated with 2μM Niclosamide failed to close the gap at 24h (Fig. 3a-i). Similar results were observed in SKOV3 monolayer cells, where control group closed the gap at around 18h, but approximately 75% of the gap remained open in the 2μM Niclosamide group Fig. 3a-ii). The rate of gap closures was further decreased when the cells were exposed to 4μM Niclosamide (data not shown). The above results suggest that Niclosamide may significantly inhibit cell proliferation and migration. We also performed cell cycle analysis on Niclosamide-treated cells and found a significant increase in the numbers of cells arrested in G1 phase, as well as decreased numbers of cells in S/M phase in Niclosamide-treated HeyA8 cells relative to the controls (Fig. 3b). Similar results were obtained in Niclosamide-treated SKOV3 cells (data not shown). These results suggest that Niclosamide's inhibition of ovarian cancer cell proliferation may be due in part to induction of apoptosis and inhibition of cell cycle progression.

Niclosamide inhibits multiple cancer-related signaling pathways, including the downstream effectors of IGF signaling

It has been reported that Niclosamide may exert anti-cancer activity in cancer cells by inhibiting Wnt/β-catenin activity [68]. Although Wnt/β-catenin signaling may play an important role in cancer development [69-71], our previous studies demonstrated low levels of endogenous Wnt/β-catenin activity in HeyA8 and SKOV3 cells, which can be activated

Fig. 4. Niclosamide inhibits multiple cancer-associated signaling pathways, including downstream mediators of IGF signaling. Subconfluent SKOV3 cells were transfected with the homemade GLuc reporters for the 12 cancer-associated pathways and a constitutively active reporter pG2Luc. At 16h post transfection the cells were treated with varied concentrations of Niclosamide. At 24h, 48h and 72h after treatment, 50μl of the culture medium were collected for GLuc activity assay using BioLux GLuc Assay Kit. (a) Niclosamide does not significantly affect 8 of the 12 cancer-associated pathway reporters (72h data point shown). (b) Niclosamide significantly inhibits the reporter activities of the 4 cancer-associated pathways. Each assay condition was done in triplicate. “*” $p < 0.05$, “**” $p < 0.001$.

by exogenous Wnt molecules [63]. Furthermore, it's conceivable that Niclosamide may target multiple signaling pathways to achieve its anticancer activity. Therefore, we sought to determine which, if any, cancer-associated pathways were modulated by Niclosamide. By introducing a panel GLuc reporters for 12 cancer-associated pathways as described [18, 42, 61, 72], along with a constitutively active reporter pG2Luc, we found that in SKOV3 cells seven pathways, including NFAT, HIF-1, TCH/LEF, E2F/DP1, Smad, STAT1/2, and RBP-JK, were not significantly affected by Niclosamide at up to 4μM for 72h treatment, while the CREB reporter was shown significantly inhibited at 72h ($p < 0.05$) but not at 24h and 48h ($p > 0.05$, data not shown) (Fig. 4a). However, four pathway reporters, including ELK1/SRF, AP-1, MYC/MAX and NFκB, were significantly inhibited by Niclosamide at all three time points (Fig. 4b). Similar reporter assay results were obtained in HeyA8 cells (data not shown). These reporter results suggest that Niclosamide may mainly target growth factor signaling pathways.

Fig. 5. Niclosamide synergizes with the silencing of IGF-1R expression in suppressing cell proliferation and migration of human ovarian cancer cells. (a) Effective knockdown of IGF-1R expression enhances Niclosamide-mediated inhibition of cell proliferation. The silencing efficacy of AdR-siIGF1R was assessed by TqPCR in human ovarian cancer lines (i). AdR-IGF1R-mediated silencing of IGF-1R expression in HeyA8 cells inhibits cell proliferation in a dose-dependent fashion. Crystal violet stain was carried out at 72h after infection. Lo MOI, lower multiplicity (10 infectious viral particles/cell); Hi MOI, higher multiplicity (20 infectious viral particles/cell). (iii) Silencing IGF1R expression potentiates Niclosamide-mediated inhibition of cell proliferation. HeyA8 cells were infected with AdR-IGF1R or Ad-RGFP for 16h, replated into 96-well plates, and treated with the indicated concentrations of Niclosamide. The WST-1 assay was conducted at 48h post treatment. “*” $p < 0.05$, “***” $p < 0.001$ (siIGF1R vs. RGFP groups at the given concentration). (b) Cell wounding assay. Subconfluent HeyA8 cells were infected with AdR-siIGF1R or Ad-RGFP for 16h and replated into 6-well plates. Freshly seeded near-confluent infected HeyA8 cells were wounded with micro-pipette tips and treated with 0, 2 and 4 μM Niclosamide. The wounding gaps were recorded at the indicated time points post Niclosamide treatment. Each assay condition was done in triplicate. (c) Niclosamide acts synergistically with silencing IGF-1R in suppressing cell cycle progression. Subconfluent SKOV3 cells were infected with AdR-siIGF1R or Ad-RGFP for 16h, replated into 6-well plates, and treated with Niclosamide at the indicated concentrations for 24h. Cells were collected, fixed, stained with Hoechst 33258, and subjected to FACS analysis. Each assay condition was done in triplicate. Representative results and selected combinations are shown.

Fig. 6. A combination of Niclosamide and IGF-1R knockdown effectively inhibits tumor growth in the xenograft model of human ovarian cancer cells. (a) Xenogen bioluminescence imaging of xenograft tumor growth. Firefly luciferase-labeled HeyA8 cells were injected into athymic nude mice subcutaneously. At three days post injection, the animals were treated with Niclosamide (10mg/kg) or vehicle control. The mice were imaged weakly after treatment, and sacrificed after 4 weeks. Representative images at week 2 are shown (i). The average signal for each group at different time points were calculated using the Xenogen's Living Image analysis software (ii). “***” $p < 0.001$ (treatment group vs. RGFP group). (b) Representative gross images of the retrieved tumor samples. (c) The retrieved tumor samples were paraffin-embedded, sectioned and subjected to H & E staining. Representative results are shown.

Niclosamide synergizes with the silencing of IGF-1R expression in suppressing cell proliferation and migration of human ovarian cancer cells

IGF signaling is one of the most commonly activated growth factor signaling pathways in human cancers [73-75]. Most of the Niclosamide-targeted cancer pathways are either direct downstream of and/or related to the IGF signaling pathway. Thus, we examined if silencing IGF1R in human ovarian cancer cells would potentiate Niclosamide's anti-proliferative activity. To accomplish efficient gene silencing of human IGF1R in ovarian cancers, we constructed the recombinant adenovirus AdR-silIGF1R, which expresses three siRNAs targeting IGF-1R coding region, based on our recently developed pSOK system [52]. AdR-silIGF1R was shown to effectively knock down IGF-1R expression in both HeyA8 and SKOV3 cells (Fig. 5a-i). Furthermore, AdR-IGF1R-mediated silencing of IGF-1R expression in HeyA8 cells inhibited cell proliferation in a dose-dependent fashion (Fig. 5a-ii), suggesting silencing IGF1R may exhibit modest anti-proliferation effect on ovarian cancer cells.

We further tested whether silencing IGF-1R would potentiate Niclosamide's growth inhibition activity. HeyA8 cells infected with AdR-silIGF1R were more sensitive to

Niclosamide as cell proliferation rate was significantly lower in AdR-silGF1R-infected cells than that in Ad-RGFP control virus-infected cell at 0.5 μ M Niclosamide ($p < 0.05$) (Fig. 5a-iii). More pronounced differences in terms of cell proliferation rates between AdR-silGF1R and Ad-RGFP-infected cells were observed in 1.0 μ M and 2.0 μ M Niclosamide treatment groups (Fig. 5a-iii). Similar results were obtained from SKOV3 cells (data not shown). These results suggest that silencing IGF-1R may sensitize ovarian cancer cells to Niclosamide.

We also analyzed the effect of silencing IGF-1R on Niclosamide inhibited cell migration. Wounded HeyA8 monolayer cells infected with AdR-silGF1R showed a significant delay in gap closure in a Niclosamide dose-dependent fashion (Fig. 5b). For example, at 12h and 28h, AdR-silGF1R-infected HeyA8 cells maintained much wider gaps, when treated with 2 μ M Niclosamide, than that of Ad-RGFP-infected cells' (Fig. 5b). Accordingly, we found that silencing IGF-1R in SKOV3 cells also enhanced the inhibitory effect of Niclosamide on cell cycle progression, as silencing IGF-R led to a significant increase in the numbers of cells in G1 phase and sub-G1 phase, as well as a decrease in the numbers of cells in S/G2 phases (Fig. 5c), which is consistent with the synergistic inhibitory effect between Niclosamide and silencing IGF-1R on cell proliferation in human ovarian cancer cells.

Niclosamide-inhibited xenograft tumor growth is potentiated by the inhibition of IGF signaling

We tested whether the *in vivo* anticancer activity of Niclosamide would be affected by silencing IGF-1R using the xenograft tumor model of human ovarian cancers. The firefly luciferase-tagged HeyA8 cells were first infected with AdR-silGF1R or Ad-RGFP and injected subcutaneously into the flanks of athymic nude mice. Each infected group was further divided into Niclosamide-treated or vehicle treated subgroups. At three days post-injection, the animals were treated with Niclosamide (10mg/kg body weight) or vehicle control. Tumor growth was monitored weekly by using Xenogen bioluminescence imaging (Fig. 6a-i). Quantitative analysis of Xenogen imaging data indicated that Niclosamide or silGF1R infection alone significantly inhibited tumor growth after two weeks when compared with the vehicle control group (Fig. 6a-ii). However, the AdR-silGF1R-infected HeyA8 cells and treated with Niclosamide exhibited the lowest signal among all four groups (Fig. 6a-ii). Xenogen imaging results were further confirmed by the retrieved tumor masses, indicating that the combination of silGF1R and Niclosamide yielded smallest tumor masses, while Niclosamide or silGF1R alone also showed apparent reduction of tumor sizes, compared with the RGFP alone group (Fig. 6b). H & E staining of the retrieved tumor samples revealed that while the RGFP control group exhibited high cellular proliferation, Niclosamide and/or silGF1R treated tumor samples exhibited varied extents of necrosis relative to samples of the control group (Fig. 6c). Thus, these *in vivo* studies further substantiate the synergistic anticancer effects between Niclosamide and silencing IGF-1R.

Discussion

Although the 5-year survival rate for ovarian cancer patients has improved over the past two decades, the overall cure rate remains approximately 30% [1, 3]. Thus, there is a critical need to develop more effective and novel therapies to treat ovarian cancers. Our results have demonstrated that Niclosamide acts synergistically with the inhibition of IGF signaling to suppress proliferation and growth of ovarian cancer cells, suggesting that the repurposed Niclosamide may be combined with the targeted inhibition of IGF signaling as a potential novel and efficacious chemotherapy strategy for the clinical management of ovarian cancer.

Through a high throughput screening of over 1,200 clinically approved drugs using stem-like ovarian tumor-initiating cells (OTIC), Niclosamide was identified as one of the hits that targeted OTICs, possibly by disrupting multiple metabolic pathways including biogenetics, biogenesis, and redox regulation [31]. A combination of Niclosamide and carboplatin produced increased cytotoxicity compared to single agent treatment in most of the tumor cells isolated from the ascites of 34 ovarian cancer patients, and Wnt/ β -catenin

pathway was inhibited by Niclosamide in ovarian cancer cells [33], as in several other types of human cancers [15, 16, 21, 30, 32, 33, 76], although our pathway reporter analysis failed to demonstrate a significant inhibition of the Wnt signal pathway by Niclosamide. Another study indicates that Niclosamide may also exert its anticancer activity through inhibition of Wnt/ β -catenin target gene FGF1 in ovarian cancer [32]. More recently, efforts have been devoted to developing Niclosamide analogs with higher bioavailability [77] and/or designing more efficacious formulations for preclinical and eventually clinical studies [78].

Nonetheless, the molecular underpinning of Niclosamide-mediated anticancer activity remains to be fully elucidated although several studies have been carried out to delineate its potential molecular mechanisms [14]. It was shown that niclosamide can inhibit Notch signaling in K562 cells [28]. Niclosamide was identified as a candidate compound capable of stimulating autophagy and inhibiting mTORC1 signaling [79, 80]. Consistent with our pathway reporter assay results, Niclosamide was shown to inactivate NF κ B pathway and generate reactive oxygen species in acute myelogenous leukemia stem cells [29]. Niclosamide was shown to inhibit STAT3 and consequently induced cell growth inhibition, apoptosis, and cell cycle arrest of the cancer cells with constitutively active STAT3 [81], which was shown to synergize with erlotinib against head and neck cancer [27], to overcome the acquired resistance to erlotinib in non-small cell lung cancer [19], and to reverse radioresistance of human lung cancer [20]. 100A4-induced metastasis formation in a mouse model of colon cancer was inhibited by niclosamide [17]. Furthermore, Niclosamide induced apoptosis, impaired metastasis and reduced immunosuppressive cells in breast cancer model [23].

Although our findings and other reports collectively demonstrate that Niclosamide has potent anticancer activity, it may not be used as a single agent therapy in human cancer. We found that Niclosamide can effectively target E2F, c-Myc and AP-1 pathways in human osteosarcoma cells [18]. In this study, we found that four pathway reporters including ELK1/SRF, AP-1, MYC/MAX and NF κ B, were significantly inhibited by Niclosamide in human ovarian cancer cells. The emerging evidence indicates that Niclosamide is a multiple-target agent. It's conceivable that Niclosamide may inhibit overlapping but distinct sets of cellular targets in different types of human cancers. Thus, identification and validation of cellular signaling pathways that may synergize or potentiate Niclosamide's anticancer activity will facilitate the translation of Niclosamide as an efficacious and attractive agent for the combination therapy of a broad range of human cancers including ovarian cancer.

In this study, we use the siRNA-mediated gene silencing strategy to specifically target IGF-1R signaling activity and explored a potential combination therapy with the repurposed Niclosamide in treating human ovarian cancers. The advantage of this approach is the high targeting specificity, while the disadvantage is the difficulty to effectively deliver such siRNA molecules into cancer cells. Targeting IGF signaling, especially IGF-1R activity, by either neutralizing/blocking antibodies or small molecule inhibitors has long been pursued as potential anticancer therapies [75, 82-84]. We have recently found that a naturally occurring IGF antagonist, IGFBP5, can inhibit tumor growth and metastasis [41, 75, 85, 86]. While the early use of IGF neutralizing antibodies has been less successful, several IGF-1R blocking antibodies have moved to various phases of clinical trials [83, 87, 88]. Meanwhile, numerous IGF-1R small molecule inhibitors have shown promising results in clinical trials [83, 87, 88]. Therefore, it's conceivable there is a great potential to develop efficacious anticancer therapies by combining Niclosamide and IGF-1R-targeting antibodies or small molecule inhibitors, which thus warrants further investigation.

In summary, we studied whether the anticancer activity of Niclosamide can be potentiated by inhibiting IGF signaling in human ovarian cancer cells. We found that Niclosamide alone was effective on inhibiting cell proliferation, cell migration, and cell cycle progression, and inducing apoptosis in human ovarian cancer cells, possibly by targeting multiple signaling pathways involved in ELK1/SRF, AP-1, MYC/MAX and NF κ B. Silencing IGF-1R exerted a similar but weaker anti-proliferation activity than that of Niclosamide's. However, silencing IGF-1R significantly sensitized ovarian cancer cells to Niclosamide-induced anti-proliferative and anticancer activities both *in vitro* and *in vivo*. Therefore, our

findings strongly suggest that Niclosamide as a repurposed anticancer agent may be more efficacious when combined with agents that target other signaling pathways such as IGF signaling in the treatment of human cancers including ovarian cancer. Future studies should be directed towards testing Niclosamide as a combination therapy agent in preclinical and clinical studies.

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Disclosure Statement

The authors declare no competing financial interests.

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