Original Paper

Niclosamide Exhibits Potent Anticancer Activity and Synergizes with Sorafenib in Human Renal Cell Cancer Cells

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Key Words

Niclosamide • Renal cell carcinoma • Kidney cancer • Drug repurposing • Metastatic renal cancer • Targeted therapy

Abstract

Background/Aims: As the most lethal urological cancers, renal cell carcinoma (RCC) comprises a heterogeneous group of cancer with diverse genetic and molecular alterations. There is an unmet clinical need to develop efficacious therapeutics for advanced, metastatic and/or relapsed RCC. Here, we investigate whether anthelmintic drug Niclosamide exhibits anticancer activity and synergizes with targeted therapy Sorafenib in suppressing RCC cell proliferation.

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Methods: Cell proliferation and migration were assessed by Crystal violet staining, WST-1 assay, cell wounding and cell cycle analysis. Gene expression was assessed by qPCR. *In vivo* anticancer activity was assessed in xenograft tumor model. **Results:** We find that Niclosamide effectively inhibits cell proliferation, cell migration and cell cycle progression, and induces apoptosis in human renal cancer cells. Mechanistically, Niclosamide inhibits the expression of C-MYC and E2F1 while inducing the expression of PTEN in RCC cells. Niclosamide is further shown to synergize with Sorafenib in suppressing RCC cell proliferation and survival. In the xenograft tumor model, Niclosamide is shown to effectively inhibit tumor growth and suppress RCC cell proliferation. **Conclusions:** Niclosamide may be repurposed as a potent anticancer agent, which can potentiate the anticancer activity of the other agents targeting different signaling pathways in the treatment of human RCC.

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Introduction

Renal cell carcinoma (RCC) is the most lethal of the urological cancers and accounts for about 3% of all malignancies in adults, with about 300,000 new cases per year and about 120,000 deaths per year worldwide [1-3]. As it is most common in older men, active and passive cigarette smoking, obesity and hypertension are known risk factors although most patients do not have an identifiable risk factor; and the pathogenic mechanisms underlying the established risk factors remain unclear [1, 2]. Nonetheless, about 2-3% of RCC are associated with familial and several autosomal dominant syndromes, most notably von Hippel-Lindau (VHL) syndrome [4] and hereditary papillary renal carcinoma (HPRC) [3, 5, 6]. While Patients with RCC can present with local or systemic symptoms, many cases are symptomless until the stage is advanced, including flank pain, blood in the urine, or a lump in the abdomen [1, 2]. Pathologically, RCC comprises a heterogeneous group of cancers with diverse genetic and molecular alterations, each derived from the various parts of the nephron and possessing distinct genetic characteristics, histological features, and clinical phenotypes [1, 2, 6, 7]. Clear cell renal cell carcinoma accounts for about 70-80% of all RCC while papillary RCC represents about 10-15% of renal cancer [1, 2]. Other rare subtypes include papillary adenoma, multilocular cystic clear-cell carcinoma, hybrid oncocyctic chromophobe tumor, carcinoma of the collecting ducts of Bellini, renal medullary carcinoma, carcinoma associated with neuroblastoma, and mucinous tubular and spindle-cell carcinoma [1-3]. Although RCC can be sporadic or hereditary, and *VHL* mutations only occur in a small fraction of RCC, a majority is driven by dysfunction of the von Hippel-Lindau (VHL) gene function, which leads to aberrant activation of the hypoxic response and neoangiogenesis [2, 3, 7].

While nephrectomy remains an important intervention for localized RCC, systemic therapy is the mainstay of treatment for the patients with relapsed and/or metastatic RCC [1, 8, 9]. Despite recent advances in diagnostic imaging, surgical therapy and basic molecular understanding, many patients still experience metastatic disease, and their response rates to conventional therapies rarely exceed 25%, yet associated with serious adverse effects [1, 8, 10]. For the past decade, the therapeutic landscape of RCC has significantly expanded, mostly driven by targeting the dysregulated metabolic pathways involved in oxygen sensing (e.g., VHL/HIF pathway), energy sensing (e.g., HGF/MET pathway) and/or nutrient sensing cascade (e.g., AMPK-TSC1/2-mTOR and PI3K-Akt-mTOR pathways) [8-11]. For instance, small molecule inhibitors that target VEGF receptors (Sunitinib and Sorafenib) have a favorable toxicity profile and can prolong time to progression and preserve quality of life when used in newly diagnosed or previously treated patients [8, 9, 12]. Temsirolimus, an mTOR inhibitor, can prolong the survival of patients with poor-risk disease [8, 12]. Nonetheless, responses to these drugs are partial and of limited duration in most cases [8-10, 12]. Therefore, while new drugs, drug combinations and/or immunotherapies are undergoing clinical trials and may impact the treatment of RCC in future years, there is an unmet clinical need of alternative therapies for advanced RCC.

Even though small molecule inhibitor-based targeted therapies have significantly expanded cancer therapy landscape [13-15], it is conceivable that repurposing current FDA-approved drugs may represent a rapid and cost-effective approach to developing new anticancer agents. In fact, several such drugs are at various stages of clinical trials [16, 17]. Niclosamide (trade name Niclocide), a teniacide in the anthelmintic family, has been approved for human use for nearly 50 years. Niclosamide was thought to inhibit oxidative phosphorylation and stimulates ATPase activity in the mitochondria of cestodes of tapeworm, killing the scolex and proximal segments of the tapeworm, which is well tolerated in humans [18, 19]. Through various high-throughput screening campaigns, Niclosamide was identified as a potential anticancer agent [19], and showed effective anticancer activity in several types of human cancer [20-39]. Nonetheless, it is unlikely that Niclosamide may be used as a single agent therapy for human cancer.

In this study, we investigate whether Niclosamide exhibits anticancer activity and synergizes with the targeted therapy agent Sorafenib in suppressing cell proliferation of renal cancer cells. We find that Niclosamide is highly effective on inhibiting cell proliferation, cell migration and cell cycle progression, and inducing apoptosis in human renal cancer cells. Mechanistically, Niclosamide inhibits the expression of C-MYC and E2F1 while inducing the expression of PTEN, which may cause a feedback upregulation of PI3K/AKT/mTOR expression in RCC cells. Niclosamide is further shown to synergize with Sorafenib in suppressing RCC cell proliferation and survival. Niclosamide effectively inhibits tumor growth and suppresses RCC cell proliferation in the xenograft tumor model of human RCC. Collectively, our findings strongly suggest that Niclosamide may be repurposed as potent anti-renal cancer agent and may become more efficacious when combined with agents that target other signaling pathways in the treatment of human RCC.

Materials and Methods

Cell Culture and Chemicals

Human renal cell carcinoma (RCC) lines A498 and Caki-1 were purchased from ATCC (Manassas, VA) and 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 [40-42]. Niclosamide was purchased from Cayman Chemical (Ann Arbor, MI). Sorafenib was purchased from Selleckchem (Houston, TX). Unless indicated otherwise, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or ThermoFisher Scientific (Waltham, MA).

Crystal violet cell viability assay

Crystal violet staining assay was conducted as described [43, 44]. Briefly, subconfluent A498 and Caki-1 cells were treated with varied concentrations of Niclosamide or DMSO control. At 72h after treatment, cells were washed with PBS and stained with 0.5% crystal violet/formalin solution at room temperature for 20-30min. The stained cells were rinsed with tap water and air dried for taking macrographic images [18]. For quantitative measurement, the stained cells were dissolved in 10% acetic acid at room temperature for 20min, followed by measuring absorbance at 570–590nm [22].

WST-1 cell proliferation assay

Cell proliferation was assessed by using Premixed WST-1 Reagent (Clontech, Mountain View, CA) as described [18, 45]. Briefly, subconfluent A498 and Caki-1 cells were seeded in 96-well cell culture plates and treated with Niclosamide at the varied concentrations for 24h, 48h and 72h. The Premixed WST-1 Reagent was added to each well, followed by an incubation at 37°C for 60min and reading at 440nm using a microplate reader. Each assay condition was done in triplicate.

Cell wounding and migration assay

Cell wound healing migration assays were performed as previously described [45, 46]. Briefly, exponentially growing cells were seeded in 6-well cell culture plates at subconfluence. Once the cells

reached approximately 90% confluence, the monolayer cells were wounded with pipette tips and treated with various concentrations of Niclosamide. At various time points after wounding, the wound healing status at the approximately same fields was recorded under a bright field microscope. These assays were done in triplicate. The average gap widths were measured and determined from at least ten low-power field images for each assay condition using the Olympus Cellsens Digital Imaging software. The percentage of gap remaining open was calculated by dividing the average gap width at a given time point with the respective average gap width at 0h. Each assay condition was done in triplicate.

Apoptosis analysis (Hoechst 33258 staining)

As previously described [47, 48], exponentially growing A498 and Caki-1 cells were treated with 2 μ M Niclosamide or DMSO control. At 48h post treatment, cells were collected, fixed and stained with the Magic Solution (0.5% NP-40, 3.4% formaldehyde, 10 μ 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.

Cell cycle analysis

As previously described [18], the exponentially growing A498 and Caki-1 cells were seeded in 6-well plates at subconfluence and treated with varied concentrations of Niclosamide or DMSO control. 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. The acquired flow cytometry data were analyzed with the FlowJo v10.0 software. Each assay condition was done in triplicate.

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

Exponentially growing A498 and Caki-1 cells were treated 1 μ M and 4 μ M Niclosamide or DMSO. At 24h, 48h and 72h post treatment, 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 [49] and used to amplify the genes of interest. The primer sequences are as follows: *GAPDH*, ATGTTTTCCTGTGCCCTGAG and ATCTGTGGTGAGGGATGAGG; *E2F1*, ATGTTTTCCTGTGCCCTGAG and ATCTGTGGTGAGGGATGAGG; *c-FOS*, AGAATCCGAAGGGAAAGGAA and CTTCTCCTTCAGCAGGTTGG; *C-MYC*, CGTCTGGGAAGGGAGAT and CGCTGCTATGGGCAAAGT; *c-JUN*, CAGGTGGCACAGCTTAAACA and TTTTCTCTCCGTCGCAACT; *AKT1*, AGAAGCAGGAGGAGGAGGAG and TCTCCTTACCAGGATCACC; *PIK3CA*, CCCCTCCATCAACTTCTTCA and CGGTTGCCTACTGGTTCAAT; *MTOR*, CCAGGCGGAAGGAACTTGCA and TGCGCAGGAAAGGCATGA; and *PTEN*, GTTTACCGGCAGCATCAAAT and CCCCACCTTTAGTGCACAGT. TqPCR reactions were carried out by using the SYBR Green-based qPCR analysis using a CFX-Connect unit (Bio-Rad Laboratories, Hercules CA) [50]. All TqPCR reactions were done in triplicate. *GAPDH* was used as a reference gene.

Xenograft tumor formation 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, Caki-1 cells were stably labeled with firefly luciferase (Caki-FLuc) with the *piggyBac* transposon system as previously described [51, 52]. Exponentially growing Caki-FLuc cells were collected, resuspended at 10⁶ cells/ml and injected subcutaneously into the flanks of athymic nude mice (Harlan Laboratories, 6–8 week old male, 10⁶ cells per injection, and 6 sites per mouse). At three days post injection, the mice were randomly divided into three groups (n = 5 per group) and treated with various doses of Niclosamide (10mg or 20mg/kg body weight) or vehicle control (DMSO) intraperitoneally once every two days. Tumor growth was monitored by whole body bioluminescence imaging using Xenogen IVIS 200 Imaging System at 7, 14, 21, and 28 days post treatment. The average signal for each group at different time points was calculated using the Xenogen's Living Image analysis software as previously described [53–55].

H & E staining

The mice were sacrificed at the end of week 4, and subcutaneous tumor masses were retrieved, fixed in 10% buffered formalin and paraffin-embedded. Serial sections of the embedded specimens were stained with hematoxylin and eosin (H & E) as described [56-58].

Immunohistochemical (IHC) staining

The IHC staining was performed as described [59-61]. Briefly, sections of the paraffin-embedded tissue blocks were deparaffinized, rehydrated, and subjected to immunohistochemical staining with anti-PCNA (Santa Cruz Biotechnology) antibody. Control IgG and minus primary antibodies were used as negative controls.

Statistical analysis

The quantitative assays were performed in triplicate and/or repeated three times. Data were expressed as mean $\pm$ S.D. 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 renal cell carcinoma (RCC) cells

We first tested the effect of the antibiotic Niclosamide on the proliferative activity of two commonly-used human RCC lines A498 and Caki-1. Using the crystal violet staining assay, we found that cell proliferation was significantly inhibited by Niclosamide at as low as 2 μ M and 4 μ M in A498 and Caki-1, respectively (Fig. 1A-a). Quantitative analysis of the crystal violet staining studies indicates that Niclosamide significantly inhibited A498 and Caki-1 cell proliferation at as low as 1 μ M and 2 μ M, respectively ($p < 0.05$) (Fig. 1A-b). The growth inhibition activity of Niclosamide was further confirmed by the WST-1 proliferation assay (Fig. 1B). Specifically, the Niclosamide-exerted inhibitory effect on cell proliferation in both A498 and Caki-1 cells was shown in a dose-dependent fashion, as well as in a time-dependent manner (Fig. 1B). These results indicate that Niclosamide can effectively inhibit cell proliferation of human renal cancer cells.

Niclosamide inhibits cell migration and cell cycle progression while inducing apoptosis in human renal cancer cells

We next examined if Niclosamide exerts any effect on cell migration and wound healing in renal cancer cells. When the freshly confluent A498 monolayer was wounded, the gap was almost completely closed in DMSO control group at 36h, but the cells treated with 1 μ M or 4 μ M Niclosamide failed to close the gap at 24h (Fig. 2A-a). Quantitatively, the average percentages of the gap remaining open were significantly higher in the Niclosamide treatment groups ($p < 0.01$) (Fig. 2A-b). Similar results were obtained in Caki-1 cells, in which 1 μ M or 4 μ M Niclosamide was shown to effectively inhibit cell proliferation and gap closure (Fig. 2B-ab). These 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 decrease in numbers of cells in S/M/G2 phases in both 1 μ M and 4 μ M Niclosamide-treated A498 cells relative to the controls (Fig. 3A-a). Similar results were obtained in Niclosamide-treated Caki-1 cells (Fig. 3A-b). Most notably, a significant portion of the Niclosamide-treated cells in both lines, especially at 4 μ M Niclosamide, appeared in sub-G1 phase (Fig. 3A), suggesting Niclosamide may induce apoptosis. Thus, we investigated if Niclosamide can induce apoptosis in renal cancer cells. When A498 and Caki-1 cells were treated with 0 μ M or 2 μ M Niclosamide for 72h and stained with Hoechst 33258, significant numbers of apoptotic cells were observed (Fig. 3B-a). Quantitative analysis indicated that the percentages of apoptotic cells were significantly increased in Niclosamide treated A498 and Caki-1 cells ($p < 0.01$) (Fig. 3B-b). Collectively, these results suggest that Niclosamide's inhibitory effect

Fig. 1. Niclosamide inhibits the proliferation of human renal cell carcinoma (RCC) cells. (A) Exponentially growing A498 and Caki-1 cells were seeded in 12-well cell culture plates and treated with the indicated concentrations of niclosamide for 72h. The viable cells were fixed and stained with Crystal violet (a). For quantitative determination, the stained cells were dissolved in acetic acid and subjected to absorbance reading at 570nm (b). Each assay condition was done in triplicate and the results were repeated at least in three independent batches of experiments. Representative results are shown. “*” $p < 0.05$, “**” $p < 0.01$, compared with the DMSO control groups. (B) WST-1 cell proliferation assay. Subconfluent A498 and Caki-1 cells were seeded in 96-well plates and treated with Niclosamide (NA) at the indicated concentrations. At the indicated time points post treatment, the WST-1 reagent was added to plates and incubated for 3h and absorbance measurement was performed. All assay conditions were done in triplicate. “**” $p < 0.01$, compared with the DMSO control groups.

on renal cancer cell proliferation may be due in part to inhibition of cell cycle progression and induction of apoptosis.

Niclosamide inhibits multiple cancer-related signaling pathways and synergizes with Sorafenib in inhibiting the proliferation of human RCC cells

It has been reported that Niclosamide may exert anti-cancer activity in cancer cells by inhibiting Wnt/ β -catenin activity [62], as Wnt/ β -catenin signaling may play an important role in cancer development [63-65]. We previously reported that Niclosamide inhibits multiple cancer-related pathways in osteosarcoma cells and ovarian cancer cells [21, 22]. Here, we analyzed the effect of Niclosamide on several selected pathways. When A498 and Caki-1 cells were treated various concentrations of Niclosamide, the expression of both C-MYC and E2F1 was significantly inhibited in a dose-dependent fashion although the expression of FOS and JUN was not apparently affected (Fig. 4A).

We further examined the effect of Niclosamide on PI3K pathway. We found that the expression of PTEN was significantly up-regulated by Niclosamide in both A498 and Caki-1 cells (Fig. 4B-ab). Surprisingly, the expression of PIK3CA and MTOR, to a lesser extent AKT1, was also elevated in Niclosamide-treated A498 and Caki-1 cells (Fig. 4B-ab). While the exact mechanism underlying the up-regulation of PI3K pathway is not known, it is conceivable that Niclosamide-induced expression of PTEN may lead to up-regulation of PI3K pathway through a negative feedback fashion, or vice versa. Nonetheless, these results suggest that Niclosamide may significantly impact PI3K signaling pathway in renal cancer cells.

Fig. 2. Niclosamide inhibits the cell migration and wounding closure of human RCC cells. Exponentially growing A498 (A) and Caki-1 (B) cells were seeded in 6-well cell culture plates. Once cells reached confluence, cell wounding was created with pipet tips and then treated with 1.0 μ M, 4 μ M niclosamide or DMSO vehicle control. The wound closure status was monitored and recorded at the indicated time points (a). Each assay condition was done in triplicate. The results were repeated at least in three independent batches of experiments. Representative results are shown. Quantitatively, the average gap widths were measured and determined from at least ten low-power field images for each assay condition using Olympus Cellsens Digital Imaging software. The percentage of gap remaining open was calculated by dividing the average gap width at a given time point with the respective average gap width at 0h (b). “*” $p < 0.05$, “**” $p < 0.01$ (compared with the control group).

Fig. 3. Niclosamide effectively inhibits cell cycle progression and induces apoptosis of human RCC cells. (A) Cell cycle analysis. Subconfluent A498 (a) and Caki-1 (b) cells were treated with Niclosamide or vehicle control for 24h or 48h. Cells were collected, fixed, stained with Hoechst 33258, and subjected to FACS analysis. Percentages of cells in non-G1 phase were tabulated and graphed. Each assay condition was done in triplicate. (B) Exponentially growing A498 and Caki-1 cells were treated with 2 μ M Niclosamide or DMSO control (0 μ M). At 72h post treatment, cells were collected, fixed and stained with Hoechst 33258. Apoptotic cells (indicated by yellow arrows) were recorded under a fluorescence microscope (a). Each assay condition was done in triplicate. Apparent apoptotic cells were counted in at least 10 random fields under high-power magnification (b). “**” $p < 0.01$ (Niclosamide treated vs. control group).

Fig. 4. Niclosamide may target multiple cellular pathways in human renal cell carcinoma cells. Exponentially growing A498 and Caki-1 cells were treated with 1.0 μ M and 4.0 μ M niclosamide or DMSO. At 72h post treatment, total RNA was isolated and subjected to TqPCR analysis using primers specific for C-MYC, E2F1, C-FOS and C-JUN (A) or PTEN, PIK3CA, AKT1 and MTOR (B). All samples were normalized with endogenous GAPDH levels. Each assay condition was done in triplicate. “**”, $p < 0.01$ (compared with that of the DMSO or 0.0 μ M control groups).

Fig. 5. Niclosamide synergizes with sorafenib in inhibiting the proliferation of human RCC cells. Exponentially growing A498 (A) and Caki-1 (B) cells were seeded in 12-well cell culture plates and treated with the indicated concentrations of niclosamide or sorafenib (SOR), or with varied concentrations of niclosamide at the 10 μ M sorafenib. At 72h post treatment, the viable cells were fixed and stained with Crystal violet. Each assay condition was done in triplicate. Representative results are shown.

As several targeted therapies including Sorafenib have been approved as alternative therapies for renal cancer, we examined whether Niclosamide would synergize with these targeted therapies in renal cancer cells. In A498 cells, we found that Sorafenib exhibited significant cytotoxicity at 20 μ M, while no apparent cell death was observed at 10 μ M (Fig. 5A). However, in the presence of Niclosamide at as low as 0.25 μ M significant cytotoxicity was observed, and complete cell death was obtained at >0.5 μ M Niclosamide at the sub-lethal concentration of 10 μ M Sorafenib (Fig. 5A). Similar results were obtained in Caki-1 cells as Niclosamide was shown to significantly enhance the cytotoxicity of sub-lethal concentration of 10 μ M Sorafenib (Fig. 5B). These results strongly suggest that Niclosamide may synergize with Sorafenib to enhance its cytotoxicity against renal cancer cells.

Niclosamide inhibits RCC xenograft tumor growth and tumor cell proliferation in vivo

We tested the *in vivo* anticancer activity of Niclosamide using the xenograft tumor model of human renal cancer cells. Firefly luciferase-labeled Caki-1 cells were injected subcutaneously into the flanks of athymic nude mice, and treated with Niclosamide at three different doses (10mg/kg and 20mg/kg or DMSO vehicle control) intraperitoneally once every two days. Whole body optical imaging indicated that, while the xenograft tumors continued to grow over the 4-week testing period, the tumor growth was significantly inhibited after a 2-week treatment at 20mg/kg and a 4-week treatment at 10mg/kg, respectively (Fig. 6A-a), which were quantitatively analyzed and shown statistically significant ($p < 0.01$) (Fig. 6A-b). Consistent with the optical imaging results, the retrieved tumor volumes at the endpoint were significantly reduced in both Niclosamide treatment groups, compared with that of the control group ($p < 0.01$) (Fig. 6B-a), as shown in the gross images (Fig. 6B-b & c).

H & E staining of the retrieved tumor samples revealed that while the control group exhibited high cellular proliferation, Niclosamide treated tumor samples exhibited extensive necrosis (Fig. 7A). Immunohistochemical staining of the cell proliferation marker PCNA indicated that the Niclosamide-treated samples retrieved from the 10mg/kg group exhibited significantly decreased expression of PCNA, and almost no PCNA expression was detected in the 20mg/kg group, whereas the tumors retrieved from the control group exhibited a high level of PCNA expression (Fig. 7B). Quantitative analysis indicated that approximately >93% of the tumor cells were PCNA-negative non-proliferative cells in the 20mg/kg treatment group. Thus, these results further confirm that Niclosamide can effectively inhibit renal cancer cell proliferation *in vivo*, which may at least in part account for its anticancer activity.

Fig. 6. Niclosamide inhibits the growth of xenograft tumor of human renal cell carcinoma cells in athymic nude mice. (A) Xenogen bioluminescence imaging of xenograft tumor growth. Firefly luciferase-labeled Caki-1 cells were injected subcutaneously into athymic nude mice, and treated with niclosamide 10mg/kg and 20mg/kg or DMSO vehicle control at day 3 (indicated by an arrow), intraperitoneally once every two days. The mice were imaged at 3 (D3), 7 (D7), 14 (D14) and 28 (D28) days after drug treatment. Representative images are shown (a). The imaging data for day 21 are not shown. The average signal for each group at different time points were calculated using the Xenogen's Living Image analysis software (b). "****" $p < 0.01$ (treatment vs. control groups). (B) (a) the average tumor volume for each group. ** $p < 0.01$ (treatment vs. control groups). (b & c) Representative gross images of the retrieved tumor samples.

Fig. 7. Niclosamide inhibits the proliferative activity of human renal cell carcinoma cells in vivo. (A) H & E staining. The retrieved tumor samples from each group were paraffin-embedded, sectioned and subjected to H & E staining. (B) PCNA immunohistochemistry. Tumor sections were also subjected to immunohistochemical staining using anti-PCNA antibody. Control IgGs were used as negative controls (not shown). Representative images are shown.

Discussion

Even though considerable progress has been made in the treatment of RCC with the development of innovative surgical and systemic strategies [1, 8, 9], efficacious therapeutic options for metastatic renal cell carcinoma and/or relapsed RCC remain limited. Here, we demonstrated that the anthelmintic agent Niclosamide potently inhibits cell proliferation, cell migration and cell cycle progression, and induces apoptosis in human renal cancer cells. Mechanistically, Niclosamide was shown to inhibit the expression of C-MYC and E2F1 while inducing the expression of PTEN, which may lead to a feedback upregulation of PI3K/AKT/mTOR expression in RCC cells. Niclosamide is further shown to synergize with Sorafenib in suppressing RCC cell proliferation and survival. In the xenograft tumor model of human RCC, Niclosamide effectively inhibits tumor growth and suppresses RCC cell proliferation. Thus, our findings strongly suggest that Niclosamide may be repurposed as a potent anticancer agent and combine with inhibitors that target other signaling pathways in the treatment of human renal cell cancer.

Niclosamide has been shown to exhibit anticancer activity in several types of human cancer [19, 20, 23, 24], osteosarcoma [21], lung cancer [25, 26], breast cancer [27-30], prostate cancer [27, 31], glioblastoma [32], head and neck cancer [33], leukemia [34, 35], human uterine leiomyoma [36], and ovarian cancer [37-39]. We previously showed that Niclosamide exerts its anticancer activity by targeting multiple signaling pathways in human osteosarcoma [21]. More recently, we have demonstrated that the anticancer activity of Niclosamide can be potentiated by inhibiting IGF signaling in human ovarian cancer cells [22]. In this study, we demonstrate that Niclosamide exhibits anticancer activity and synergizes with the targeted therapy agent Sorafenib in suppressing cell proliferation of renal cancer cells. It was recently reported that autophagy may play an important role in Sorafenib resistance in liver cancer [66]. It would be interesting to investigate whether Niclosamide would exert any effects on autophagy in renal cancer. Overall, 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 [19].

It is conceivable that, in addition to Sorafenib, Niclosamide may potentiate or sensitize the anticancer activities of other currently-approved targeted therapies for RCC. The targeted drugs used to treat advanced RCC function by blocking angiogenesis or tyrosine kinases that help RCC cells grow and survive. As clear cell RCC usually harbors mutations or inactivation of VHL gene and resultant overexpression of VEGF, the first drug to validate VEGF

as a target in the treatment of clear cell RCC was the monoclonal antibody bevacizumab or Avastin [67]. Like Sorafenib, Sunitinib blocks several tyrosine kinases and attacks both blood vessel growth and other targets that help cancer cells grow. Other tyrosine kinase and/or neoangiogenesis inhibitors for RCC therapies include Pazopanib (Votrient), Axitinib (Inlyta), Cabozantinib (Cabometyx), and Lenvatinib (Lenvima). The mTOR is another validated therapeutic target as the mTOR inhibitors Temsirolimus and Everolimus have been shown to prolong progression-free survival in first-line treatment of poor prognosis RCC. Therefore, it is of translational significance to investigate whether Niclosamide can potentiate or augment the anticancer activity of these targeted therapies for RCC.

In summary, we demonstrate that Niclosamide effectively inhibit cell proliferation, cell migration and cell cycle progression, and induces apoptosis in human RCC cells. Niclosamide synergizes with Sorafenib in suppressing RCC cell proliferation and survival. Furthermore, Niclosamide effectively inhibits tumor growth and suppresses RCC cell proliferation in the xenograft tumor model of human RCC. Taken together, our findings strongly suggest that Niclosamide may be repurposed as potent anticancer agent and may potentiate the anticancer activity of other targeted therapies in the treatment of human renal cell cancer.

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Disclosure Statement

The authors declare to have no conflict of interests.

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