Am J Transl Res 2017;9(8):3776-3786
www.ajtr.org /ISSN:1943-8141/AJTR0053660

Original Article

Inhibition of Wnt/β-catenin by anthelmintic drug niclosamide effectively targets growth, survival, and angiogenesis of retinoblastoma

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Received March 24, 2017; Accepted July 26, 2017; Epub August 15, 2017; Published August 30, 2017

Abstract: Retinoblastoma is an angiogenesis-dependent ocular tumor, the clinical management of which remains a challenge. Agents that can target tumor cells and angiogenesis, as well as augment current chemotherapy efficacy, present a promising therapeutic strategy for retinoblastoma. We demonstrated that niclosamide, an FDA-approved anthelmintic drug, is effective against multiple aspects of retinoblastoma. Niclosamide inhibited proliferation via causing cell cycle arrest at the G2/M phase and induced caspase-dependent apoptosis in a panel of retinoblastoma cell lines, including Y79, RB116, and WERI-Rb-1. In addition, niclosamide inhibited retinoblastoma angiogenesis by disrupting capillary network formation, decreasing migration and proliferation, and inducing apoptosis of human primary retinal microvascular endothelial cells. We also demonstrated that niclosamide specifically suppresses the levels of p-LRP6, Dvl2, and β-catenin, but not p-STAT3, in Y79 cells. It decreased β-catenin activity and the mRNA expression levels of Wnt/β-catenin target genes. Stabilization of β-catenin with the Wnt activator lithium or overexpression of β-catenin reversed the inhibitory effects of niclosamide in Y79 cells, confirming Wnt/β-catenin as the molecular target of niclosamide in retinoblastoma cells. Importantly, niclosamide significantly enhanced the *in vitro* and *in vivo* efficacy of carboplatin and inhibited Wnt/β-catenin signaling in a retinoblastoma xenograft mouse model. Our data suggest that niclosamide is a promising candidate for the treatment armamentarium for retinoblastoma. Our work also highlights that targeting Wnt/β-catenin is a potential therapeutic strategy in retinoblastoma.

Keywords: Niclosamide, retinoblastoma, angiogenesis, Wnt/β-catenin, chemotherapy

Introduction

Retinoblastoma, the most common ocular cancer in children, is initiated by the mutation of the tumor suppressor gene retinoblastoma 1 (*RB1*) [1, 2]. The treatments for retinoblastoma include enucleation, chemotherapy, and radiation, along with ophthalmic therapies; however, the prognosis for retinoblastoma is still poor in developing countries [3, 4]. In addition to *RB1* gene mutation, other genetic and biological factors can lead to the development of malignancy [5, 6]. Tumor angiogenesis also plays an essential role in the progression and chemoresistance of retinoblastoma and serves as a prognostic factor for disease dissemination [7, 8]. The development of novel strategies that target both tumor cells and tumor angiogenesis is a promising approach for improving the clinical outcome of retinoblastoma.

Niclosamide is an FDA-approved anthelmintic drug that has been used for the treatment of tapeworms in the last several years. However, independent high-throughput screenings have identified niclosamide as a potent anti-cancer compound [9, 10]. It has been demonstrated to inhibit the Wnt/β-catenin pathway and mitochondrial functions, which leads to growth inhibition and apoptosis in various types of cancer [11-14]. Niclosamide also acts on cancer cells via suppression of STAT3 and NF-κB, and the modulation of metabolic signaling pathways. Its inhibitory effects on tumor-initiating cells and synergistic effects when combined with chemotherapeutic agents make niclosamide an attractive candidate for cancer treatment [10, 11, 15-17].

In the present study, we explored the multiple targets affected by niclosamide in retinoblasto-

ma. We also investigated the combinatory effects of niclosamide with carboplatin (a common chemotherapeutic agent for retinoblastoma) and its underlying molecular mechanisms. We demonstrated that niclosamide effectively prevents the growth retinoblastoma cells and inhibits angiogenesis. In addition, niclosamide significantly enhanced the inhibitory effects of carboplatin against retinoblastoma *in vitro* and *in vivo*. Finally, we demonstrated that the molecular mechanism of the action of niclosamide in retinoblastoma is inhibition of the Wnt/β-catenin pathway.

Materials and methods

Cell culture and drugs

Three human retinoblastoma cell lines were used in this study. Y79 and WERI-Rb-1, and RB116 were purchased from American Type Culture Collection and Kerafast, respectively. All retinoblastoma cell lines were subcultured in RPMI medium supplemented with 1% HEPES (Life Technologies, USA) and 10% fetal bovine serum (FBS; Hyclone™, UK). Human primary retinal microvascular endothelial cells (HRECs) (Cell Systems, USA) were grown on gelatin-coated surfaces in M131 medium containing microvascular growth supplements (Invitrogen, USA). Z-VAD-FMK (CalBiochem, USA), lithium chloride (LiCl), and niclosamide (Sigma-Aldrich, USA) were reconstituted in dimethyl sulfoxide (DMSO), and carboplatin (Sigma-Aldrich, USA) was reconstituted in water. The drugs were stored in aliquots at -20°C.

Measurement of proliferation, cell cycle status, and apoptosis

Cell proliferation activity was measured using the MTS Cell Proliferation Assay Kit (Abcam, USA) according to the manufacturer's instructions. Cell cycle status was measured by labeling cells with propidium iodide (PI; Abcam, USA) to stain DNA, according to the manufacturer's protocol, followed by flow cytometric analysis on a Beckman Coulter FC 500. Cell cycle status was analyzed by assessing PI staining (presented as a histogram plot). Apoptosis was measured by labeling cells with an annexin V and 7-aminoactinomycin D (7-AAD) kit (BD Pharmingen, USA) according to the manufacturer's protocol. Apoptotic cells were quantified by flow cytometry on a Beckman Coulter FC 500.

Assessment of in vitro capillary tube formation

Liquid complete Matrigel® matrix (150 µl, BD Biosciences, USA) was added to a 96-well plate and incubated for 30 minutes at 37°C to allow the Matrigel® to solidify. M131 medium (50 µl) containing 2×10^4 HRECs and the drug were then gently plated onto the complete Matrigel® matrix. The capillary network formed after 6-8 h of incubation. Photos were taken under phase-contrast microscopy. We quantified the capillary network formation using ImageJ software.

Boyden chamber migration assay

Migration assays were performed using a Boyden chamber consisting of a cylindrical cell culture insert (containing a 6.5-mm diameter polycarbonate membrane) nested inside the well of a cell culture plate. HRECs, together with drugs, were seeded in the upper chambers of the plates in serum-free media and serum was placed in the wells below. After 6-8 h of incubation, migratory cells that had moved through the pores towards the lower surface of the filter were fixed and stained with 0.4% Giemsa. The cells on the lower surface were counted under a light microscope.

Western blot analyses

Cells (1×10^7, after drug treatment) were homogenized in RIPA lysis buffer (Thermo Scientific, USA) supplemented with 1% Halt Protease Inhibitor Cocktail (Thermo Scientific, USA) and phosphatase inhibitors (Sigma-Aldrich, USA). Total protein concentrations were determined using a Pierce™ BCA Protein Assay Kit (Thermo Scientific, USA). Equal quantities of protein were denatured and size-fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, then transferred to nitrocellulose membranes for western blot analyses. The antibodies used were anti-p-LRP6, anti-LRP6, anti-Dvl2, anti-β-catenin, anti-β-actin, anti-p-STAT3, and anti-STAT3 (Santa Cruz Biotechnology, USA). The blots were visualized with an Amersham ECL Western Blotting Detection Kit (GE Healthcare, UK). Protein density was determined using ImageJ software.

Plasmid transfection

Plasmid (1.5 µg) transfection was conducted using Lipofectamine® 2000 (Invitrogen, USA)

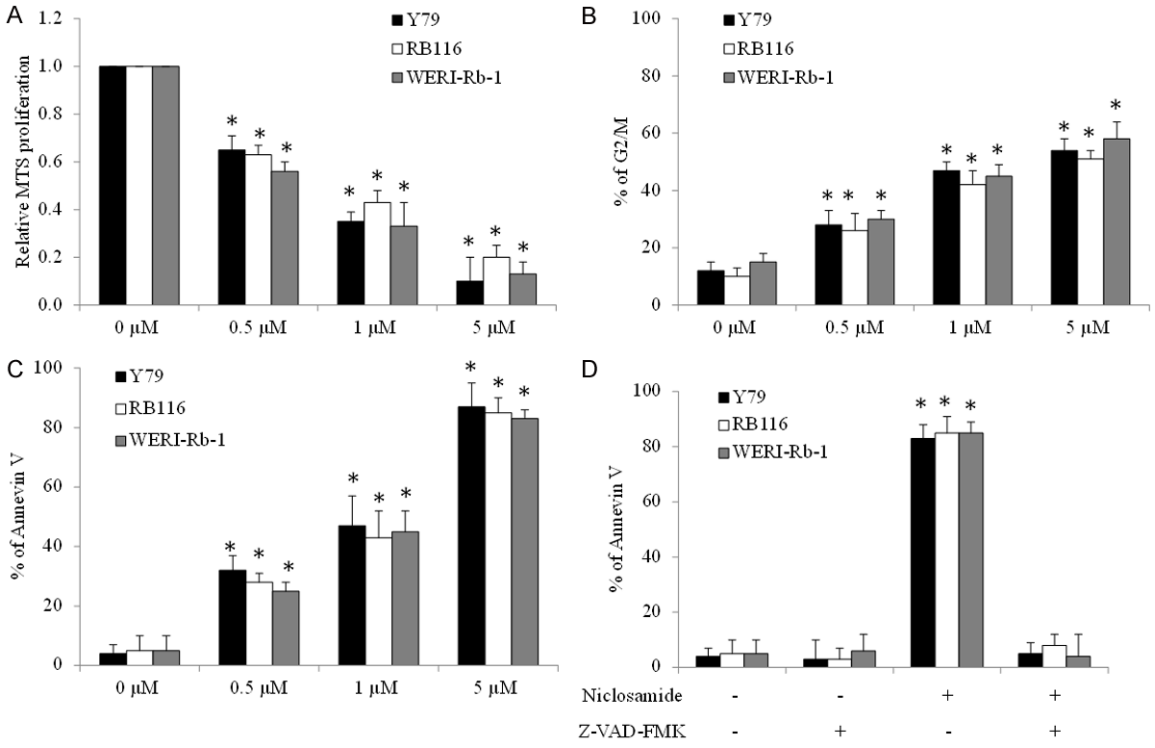

Figure 1. Niclosamide effectively targeted retinoblastoma cells. A: Niclosamide (0.5 μM, 1 μM, and 5 μM) significantly inhibited the proliferation of the retinoblastoma cell lines Y79, RB116, and WERI-Rb-1. Proliferation was measured using an MTS kit after drug treatment for 3 days. B: Niclosamide significantly increased the percentage of retinoblastoma cells in G2/M phase. Cell cycle analysis was performed by flow cytometry after 24 hours of drug treatment. C: Niclosamide induced significant levels of apoptosis in retinoblastoma cells in a dose-dependent manner. D: The pan-caspase inhibitor Z-VAD-FMK completely abolished the apoptosis induced by 5 μM niclosamide. Cells were treated with niclosamide or Z-VAD-FMK alone, or in combination, for 3 days prior to flow cytometric analysis of Annexin V and 7-AAD staining. Niclosamide (5 μM) and Z-VAD-FMK (80 μM) were used in the rescue experiment. *P<0.05, compared to control.

in serum-free RPMI medium in 6-well plates, according to the manufacturer's instructions. The pcDNA-β-catenin construct (a kind gift from Dr. Eric Fearon) [18] was used to overexpress β-catenin, and a TCF/LEF reporter kit (Invitrogen, USA) was used to determine β-catenin activity. Luciferase reporter activity was measured in a dual-luciferase assay, then normalized to Renilla luciferase relative light unit values.

Reverse transcription PCR

Total RNA was isolated from cells using the Absolutely RNA Miniprep Kit (Stratagene, USA). First-strand complementary DNA (cDNA) was synthesized by using an iScript cDNA Synthesis Kit (Bio-Rad, USA). We amplified the cDNA by incubating it with a SsoFast EvaGreen Supermix (Bio-Rad, USA). Reverse transcription (RT)-PCR was conducted on a CFX96™ RT-PCR system. The primer sequences have been previously reported [19].

Retinoblastoma tumor xenograft in immunodeficient mice

All procedures were conducted in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice (4-6 weeks old) were purchased from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences. Phosphate-buffered saline (100 μl) containing 10×10^6 Y79 cells were subcutaneously injected into the flanks of mice. After the mice developed palpable tumors (~100 mm^3), they received drug treatment. The mice were randomly divided into 4 groups. The drug treatments included 20% DMSO in saline (vehicle

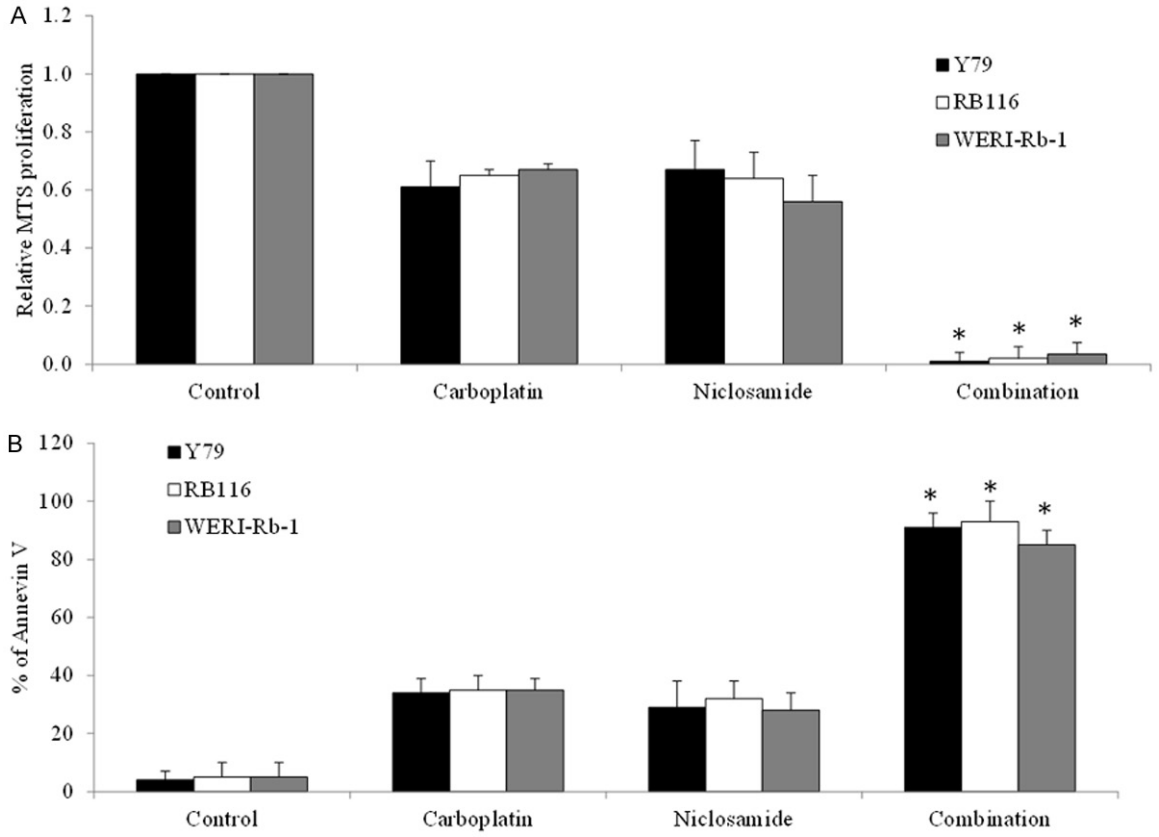

Figure 2. Niclosamide significantly enhanced the inhibitory effects of a chemotherapy agent on retinoblastoma cells. The combination of carboplatin with niclosamide inhibited proliferation to a significantly greater degree (A) and induced significantly more apoptosis (B) than carboplatin or niclosamide alone in Y79, RB116, and WERI-Rb-1 cells. We used 0.5 μM niclosamide and 0.5 μM carboplatin for the combination studies. The cells received drug treatment for 72 hours prior to MTS and apoptosis analysis. *P<0.05, compared to carboplatin or niclosamide alone.

control), 0.5 mg/kg niclosamide, 1 mg/kg carboplatin, and a combination of niclosamide and carboplatin. The drugs were given daily by intraperitoneal (i.p.) injection for 3 weeks. The tumor length and width were measured every 3 days and the tumor size was calculated using the formula: (length^2 × width)/2. The mice were then euthanized, and the tumors were isolated and weighed.

Statistical analyses

Each experiment was performed at least thrice, and data were expressed as the mean ± standard deviation. For the *in vitro* cell assay results, the error bars indicate the values of the standard deviations among 3 independent experiments. For the *in vivo* results, the error bars indicate the values of the standard deviations among the mice in each group. Statistical comparisons were performed with the unpaired Student's t test. The values were considered statistically significant at P<0.05.

Results

Niclosamide effectively targeted retinoblastoma cells and synergistically enhanced the inhibitory effects of a chemotherapeutic agent

We first examined the possible biological effects of niclosamide alone in retinoblastoma, using 3 retinoblastoma cell lines: Y79, WERI-Rb-1, and RB116. These cell lines are genetically related and contain subpopulations expressing stem cell markers; Y79 and WERI-Rb-1, but not RB116, have *RB1* mutations [20-22]. We found that niclosamide significantly inhibited the proliferation of the retinoblastoma cell lines, as assessed by MTS assay (**Figure 1A**). In addition, the IC_{50} of niclosamide was similar (~0.65 μM) for Y79, WERI-Rb-1, and

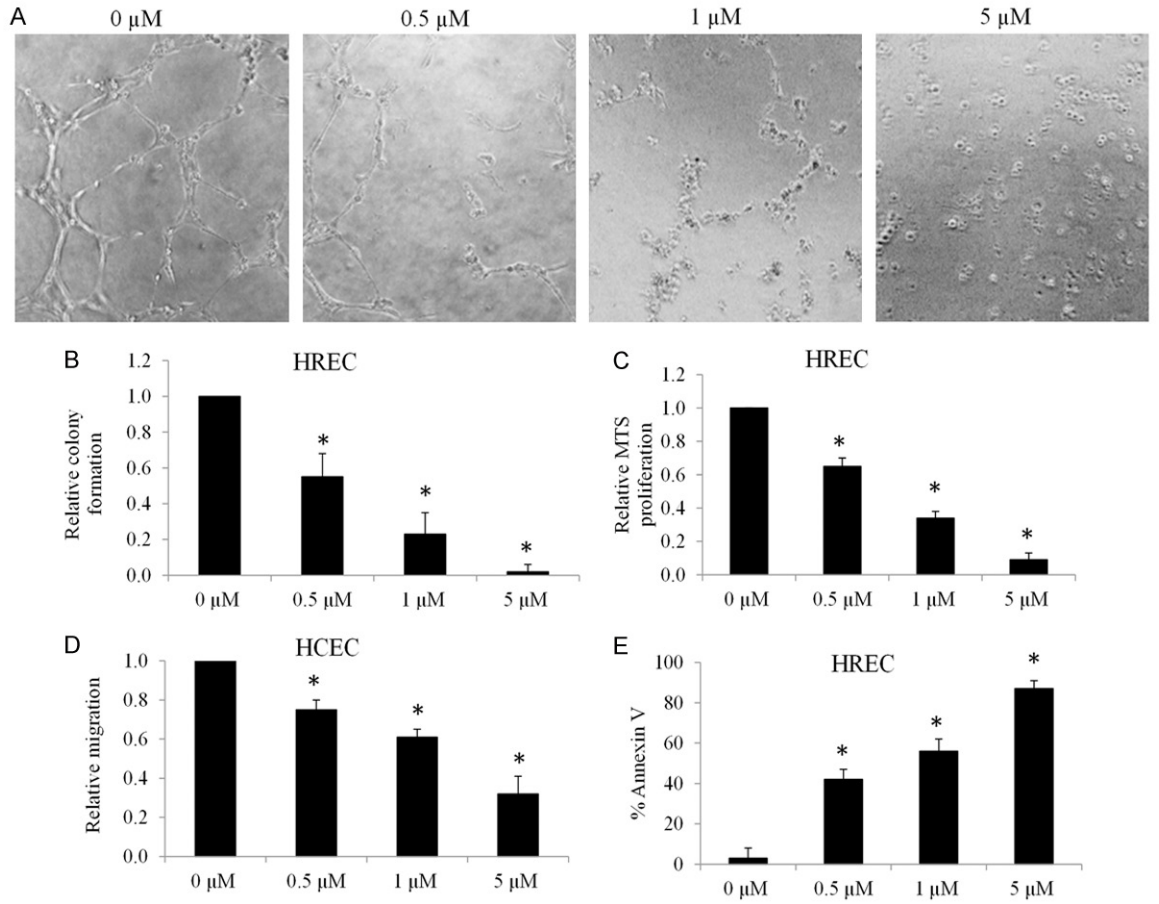

Figure 3. Niclosamide significantly inhibited retinal angiogenesis. Representative images (A) and the quantification (B) of capillary network structures revealed the potent inhibitory effect of niclosamide on retinal angiogenesis. The photos were taken after 6 hours of *in vitro* capillary tube formation by HRECs. Quantification was performed with ImageJ software. Niclosamide significantly decreased the migration (C) and proliferation (D), and increased the apoptosis (E), of HRECs. The cells received drug treatment for 72 hours prior to MTS and apoptosis assays, and for 6 hours prior to capillary tube formation and migration assays. *P<0.05, compared to control.

RB116 cells. Cell cycle analysis using PI staining demonstrated that niclosamide inhibited cell proliferation by causing cell cycle arrest at the G2/M phase (**Figure 1B**). Niclosamide (0.5, 1, and 5 μM) also significantly induced apoptosis of retinoblastoma cells, as assessed by quantification of annexin V staining (**Figure 1C**). A pan-caspase inhibitor, Z-VAD-FMK, completely abolished the apoptosis-inducing effect of niclosamide (**Figure 1D**), demonstrating that niclosamide induces apoptosis of retinoblastoma cells in a caspase-dependent manner.

We then investigated the combinatory effects of niclosamide and the standard chemotherapy agent carboplatin in retinoblastoma cells. The concentrations of niclosamide or carboplatin used for the combination studies were those that individually inhibited proliferation by ~30% and induced apoptosis in ~30% of cells in Y79, WERI-Rb-1, and RB116 cultures (**Figure 2**). However, when niclosamide and carboplatin were combined, they inhibited ~100% of proliferation and induced apoptosis in ~100% of cells (**Figure 2**), suggesting that niclosamide synergistically augments the effects of carboplatin in retinoblastoma cells.

Niclosamide significantly inhibited multiple aspects of retinoblastoma angiogenesis

Retinoblastoma is an angiogenesis-dependent tumor, and anti-angiogenesis therapy has been shown to inhibit retinoblastoma growth [7, 8, 23, 24]. In order to test if niclosamide affected retinoblastoma angiogenesis, we performed *in vitro* angiogenesis assays on gelled basement membrane extracts (Matrigel®) using HRECs.

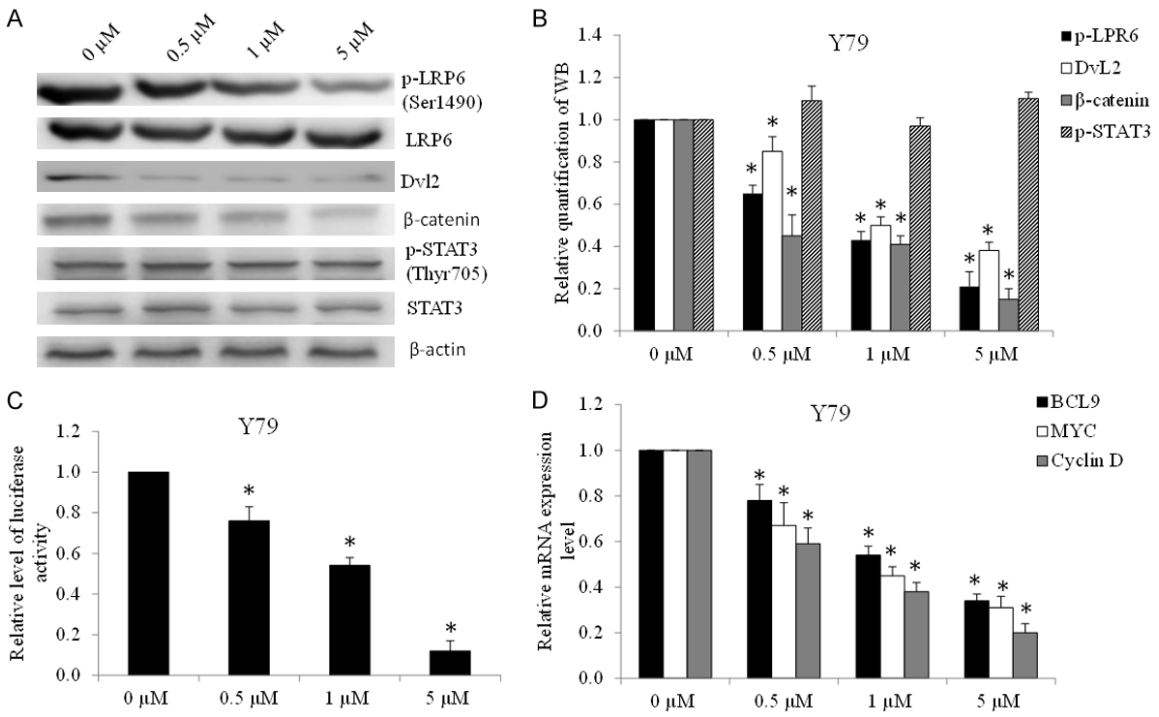

Figure 4. Niclosamide inhibited the Wnt/β-catenin pathway in retinoblastoma cells. Representative images (A) and quantification (B) of western blots showing decreased protein levels of Dvl2 and β-catenin, and decreased levels of phosphorylated LRP6, but not STAT3, in niclosamide-treated Y79 cells. Protein levels were normalized to β-actin. Niclosamide significantly decreased β-catenin activity (C) and the transcript levels of Wnt/β-catenin target genes, including *BCL9*, *MYC*, and *CCND1* (D) in Y79 cells. Cells were treated with niclosamide at different concentrations for 24 hours prior to western blotting, and luciferase activity and gene expression analyses. The results shown are relative to the control. **P*<0.05, compared to control.

HRECs formed capillary tubes within 6 h of culture on Matrigel® [25] (**Figure 3A**). However, HRECs failed to form proper capillary tubes in the presence of niclosamide. Importantly, cells exposed to 5 μM niclosamide formed minimal tubular networks (**Figure 3A** and **3B**), suggesting that niclosamide is a potent angiogenesis inhibitor. In addition, niclosamide significantly inhibited other biological functions of HRECs, such as migration, proliferation, and survival (**Figure 3C-E**).

Niclosamide targeted retinoblastoma cells through inhibition of the Wnt/β-catenin pathway

The anti-tumor activities of niclosamide have been attributed to its inhibition of the Wnt/β-catenin, mTOR, and STAT3 signaling pathways [12, 13, 26, 27]. Given the importance of the Wnt/β-catenin and STAT3 pathways in cancer development and the maintenance of cancer stem cells [28-30], we examined if niclosamide acts on retinoblastoma via targeting Wnt/β-catenin and/or STAT3.

We found that niclosamide decreased the levels of essential molecules involved in Wnt/β-catenin signaling, including p-LRP6, β-catenin, and Dvl2, whereas it did not affect the level of p-STAT3, in Y79 cells (**Figure 4A** and **4B**, Supplementary Data), suggesting that niclosamide specifically inhibits Wnt/β-catenin signaling in retinoblastoma cells. We consistently observed decreased β-catenin activity and Wnt/β-catenin target gene (*MYC*, *CCND1*, and *BCL9*) transcript levels in Y79 cells exposed to niclosamide (**Figure 4C** and **4D**). We next rescued β-catenin levels using the Wnt activator LiCl and found that the anti-proliferative and pro-apoptotic effects of niclosamide were abolished by LiCl (**Figure 5A** and **5B**). Similarly, β-catenin overexpression reversed the inhibitory effects of niclosamide in retinoblastoma cells (**Figure 5C** and **5D**). These findings demonstrated that the inhibitory effects of niclosamide in retinoblastoma cells can be

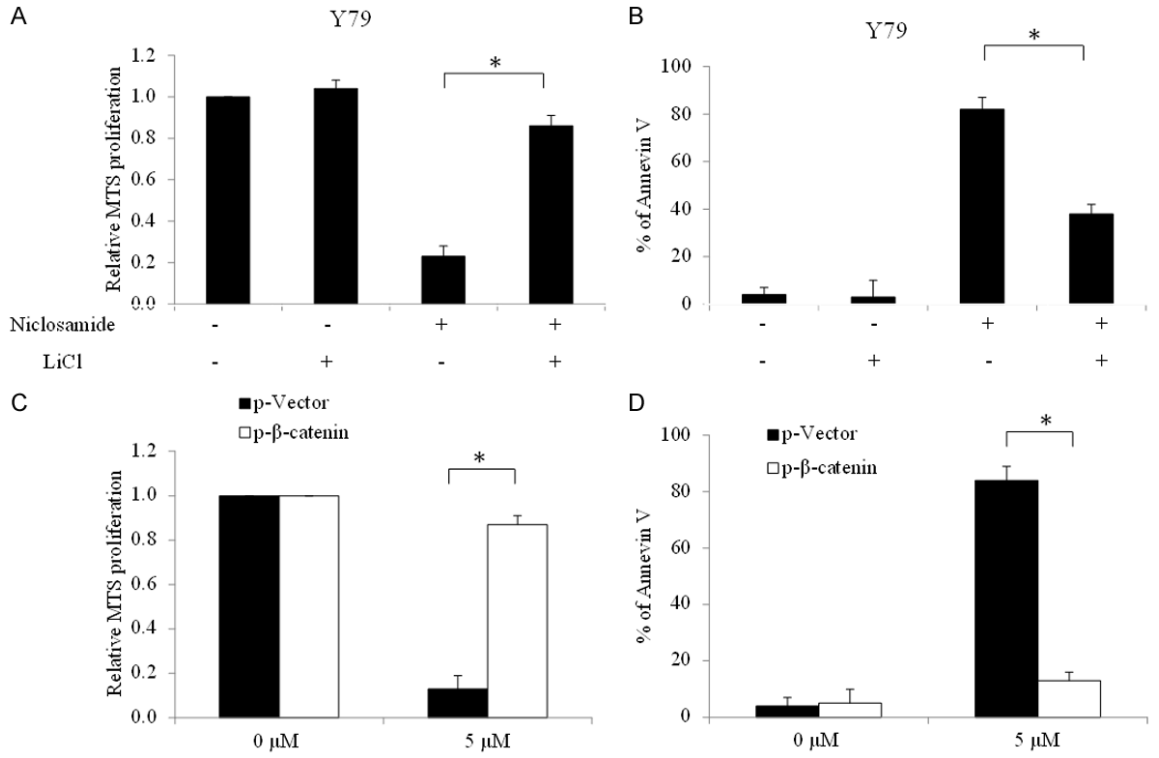

Figure 5. β-catenin overexpression via pharmacological and genetic approaches reversed the inhibitory effects of niclosamide on retinoblastoma cells. Niclosamide was ineffective at inhibiting proliferation (A) and inducing apoptosis (B) in Y79 cells in the presence of the Wnt activator LiCl. We used 5 μM niclosamide and 10 μM LiCl in the rescue experiment. The overexpression of β-catenin abolished the anti-proliferative (C) and pro-apoptotic (D) effects of niclosamide in Y79 cells. Niclosamide was added to the cells 24 hours post-transfection. **P*<0.05, compared to niclosamide or p-Vector.

attributed to its ability to suppress the Wnt/β-catenin signaling pathway.

Niclosamide significantly augmented the in vivo efficacy of a chemotherapeutic agent in retinoblastoma cells

We further investigated the translational potential of niclosamide for retinoblastoma treatment. We established a retinoblastoma xenograft mouse model by subcutaneously implanting Y79 cells into the flanks of NOD/SCID mice, then tested the *in vivo* efficacy of carboplatin and niclosamide combination treatment. After the development of palpable tumors, the mice were treated daily with vehicle, each drug alone, or the drug combination for 3 weeks. We did not observe significant toxicity in the mice treated with niclosamide (0.5 mg/kg) or carboplatin (1 mg/kg) alone, as shown by their body weights, appearance, and behavior. Administered singly, niclosamide or carboplatin moderately inhibited tumor growth (**Figure 6A** and **6B**). However, when the 2 drugs were combined, we detected a significantly greater inhibition of tumor growth throughout the treatment (**Figure 6A-C**). In addition, the decreased levels of p-LRP6, Dvl2, and β-catenin observed in the tumors of the mice treated with niclosamide and the drug combination demonstrated that niclosamide inhibits Wnt/β-catenin signaling *in vivo* (**Figure 6D** and Supplementary Data).

Discussion

More effective chemotherapy strategies are needed for the clinical management of retinoblastoma. Development of a combination therapy consisting of 2 agents with different molecular targets is a compelling approach to maximize potency and minimize toxicity [31]. Drug repurposing offers a good opportunity to identify potent combinations of FDA-approved drugs with potentially new indications, and has been hotly investigated in major drug discovery programs [32]. In line with these efforts, we

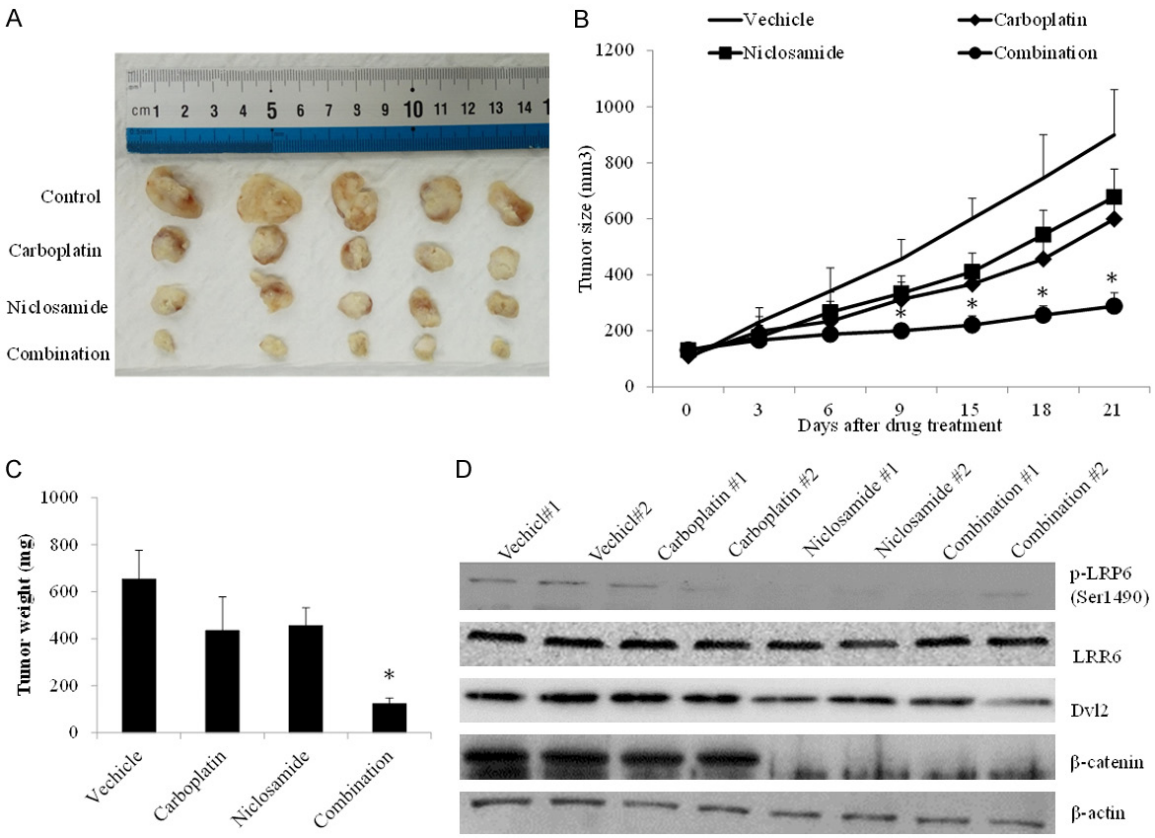

Figure 6. Niclosamide enhanced the inhibitory effects of carboplatin on Wnt/β-catenin signaling in retinoblastoma cells *in vivo*. (A) Representative images of tumors isolated from mice treated with vehicle (20% DMSO in saline), carboplatin (i.p. 1 mg/kg) or niclosamide (i.p. 0.5 mg/kg) alone, or a combination of carboplatin and niclosamide. Tumor growth curves (B) and tumor weights (C) demonstrated the potent inhibitory effects of the combination of carboplatin and niclosamide on tumor growth throughout the treatment. (D) Representative western blot images showing the decreased p-LRP6, Dvl2, and β-catenin levels in tumors from niclosamide- and combination-treated mice. The retinoblastoma xenograft mouse model was established by subcutaneously injecting Y79 cells into NOD/SCID mice. *P<0.05, compared to niclosamide or carboplatin alone.

screened a few FDA-approved drugs and identified niclosamide as a potential addition to the treatment armamentarium for retinoblastoma. In this study, we demonstrated that niclosamide effectively targets multiple aspects of retinoblastoma via inhibition of the Wnt/β-catenin pathway. Importantly, niclosamide acted synergistically with carboplatin against retinoblastoma *in vitro* and *in vivo*.

We demonstrated the inhibitory effects of niclosamide on 3 retinoblastoma cell lines of different cellular origins, morphology, and karyotypes [20-22]. Niclosamide arrested cell growth at the G2/M phase and induced caspase-dependent apoptosis with a similar IC_{50} in all tested cell lines (**Figure 1**), suggesting that the inhibitory effects of niclosamide are universal in retinoblastoma. We also tested the *in vivo* efficacy of niclosamide using a retinoblastoma xenograft mouse model. Niclosamide, at doses that were not toxic to mice, effectively inhibited retinoblastoma tumor growth (**Figure 6**). Niclosamide has been demonstrated to inhibit the growth and survival of breast, ovarian, and prostate cancers, as well as glioblastoma and leukemia [11, 14, 17, 33-35]. Our results are consistent with, and extend to retinoblastoma, the previous findings on the anti-cancer activities of niclosamide.

In addition to targeting retinoblastoma cells, we are the first demonstrate that niclosamide effectively inhibits retinal angiogenesis via suppression of the capillary network formation, migration, growth, and survival of HRECs (**Figure 3**). Retinoblastoma is a well-vascularized solid tumor that depends on angiogenesis

for growth, survival, and metastasis [36]. Quantification of angiogenesis can help to identify patients at high risk of retinoblastoma dissemination after enucleation [8]. Targeting angiogenesis has been shown to inhibit retinoblastoma growth in a xenograft mouse model without producing substantial systemic toxicity [23, 24]. Despite a strong initial response to treatment, some patients still develop chemoresistance and relapse [37]. Our findings showed that niclosamide significantly sensitizes retinoblastoma cells to carboplatin (**Figures 2** and **6**), suggesting that the combination of niclosamide with a chemotherapeutic agent is likely to overcome the chemoresistance of retinoblastoma. This is consistent with previous reports that niclosamide enhances the effects of cisplatin or dasatinib in breast cancer and chronic myeloid leukemia [11, 17]. The abilities of niclosamide to inhibit retinal angiogenesis and synergize with a chemotherapeutic agent may confer greater success than other anticancer compounds.

The molecular mechanisms of the anti-cancer activities of niclosamide vary in different types of cancer, but mainly include the induction of mitochondrial dysfunction, or the inhibition of the Wnt/β-catenin, STAT3, and NF-κB pathways [11, 26, 38, 39]. A significant finding of this work is the identification of Wnt/β-catenin inhibition as the mechanism of niclosamide action in retinoblastoma. We observed decreased levels of essential molecules involved in Wnt/β-catenin, but not p-STAT3, as well as reduced β-catenin activity and expression of Wnt/β-catenin target gene mRNA in retinoblastoma cells exposed to niclosamide (**Figure 4**). Stabilization of β-catenin using both pharmacological and genetic approaches completely reversed the effects of niclosamide on retinoblastoma, further confirming that niclosamide acts on retinoblastoma via inhibition of Wnt/β-catenin signaling (**Figure 5**). The roles of Wnt/β-catenin in promoting cancer development and facilitating cancer stem cell maintenance have been well-documented in various cancers [29]. However, the roles of Wnt/β-catenin in retinoblastoma are controversial. Tell et al. showed that Wnt signaling promoted tumor suppression in retinoblastoma, whereas Silva et al. and Gao et al. demonstrated that Wnt/β-catenin signaling positively regulated the growth of retinoblastoma cells and stem-like cells [40-42]. Our findings suggest that Wnt/β-catenin activation is critical to retinoblastoma proliferation and survival.

In conclusion, our work shows that niclosamide is a potential candidate for retinoblastoma treatment, given its individual potency and its synergism with a chemotherapeutic agent *in vitro* and *in vivo*. Our work also demonstrates that targeting the Wnt/β-catenin pathway is a promising alternative strategy for treating retinoblastoma.

Acknowledgements

This work was supported by a research grant provided by the Natural Science Foundation of Hubei (2014CFB214).

Disclosure of conflict of interest

None.

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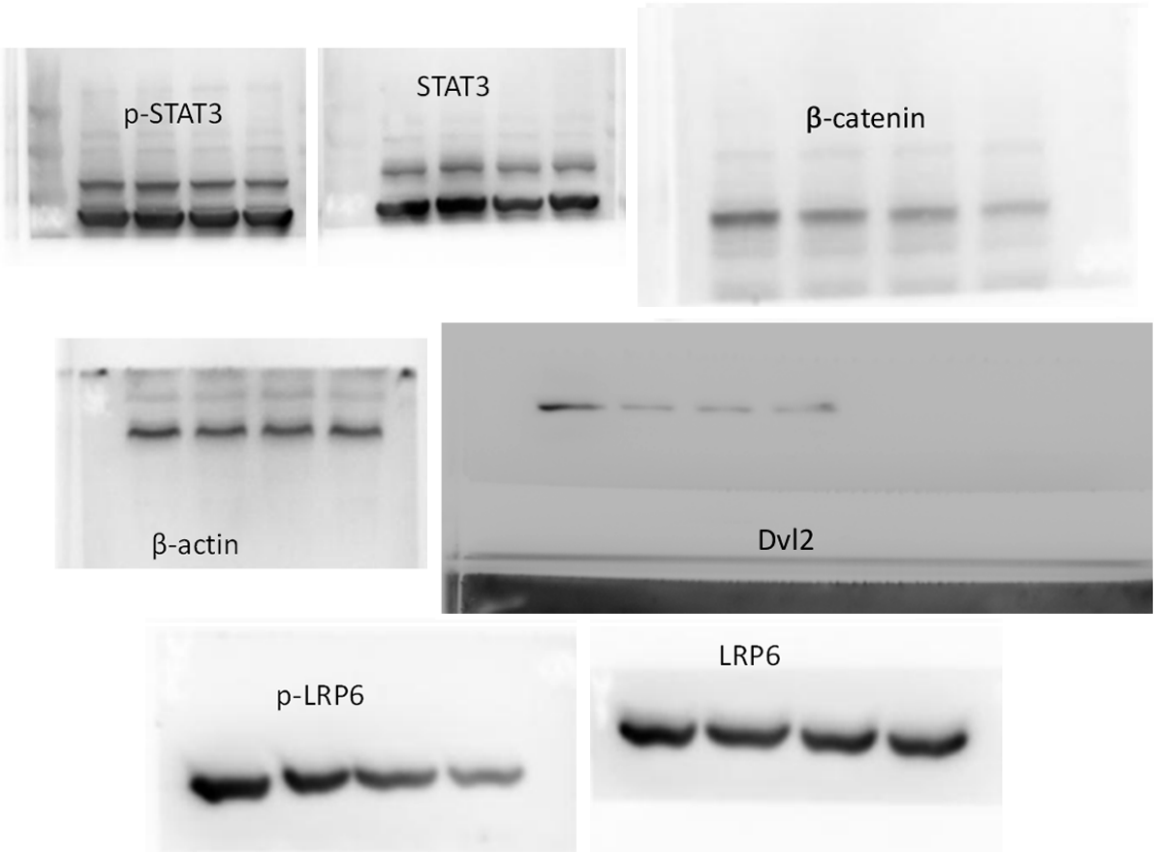
Full unedited gels for Figure 4A



Full unedited gels for Figure 6D