Antihelminth Compound Niclosamide Downregulates Wnt Signaling and Elicits Antitumor Responses in Tumors with Activating APC Mutations

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Abstract

Wnt/β-catenin pathway activation caused by adenomatous polyposis coli (APC) mutations occurs in approximately 80% of sporadic colorectal cancers (CRC). The antihelminth compound niclosamide downregulates components of the Wnt pathway, specifically Dishevelled-2 (Dvl2) expression, resulting in diminished downstream β-catenin signaling. In this study, we determined whether niclosamide could inhibit the Wnt/β-catenin pathway in human CRCs and whether its inhibition might elicit antitumor effects in the presence of APC mutations. We found that niclosamide inhibited Wnt/β-catenin pathway activation, downregulated Dvl2, decreased downstream β-catenin signaling, and exerted antiproliferative effects in human colon cancer cell lines and CRC cells isolated by surgical resection of metastatic disease, regardless of mutations in APC. In contrast, inhibition of NF-κB or mTOR did not exert similar antiproliferative effects in these CRC model systems. In mice implanted with human CRC xenografts, orally administered niclosamide was well tolerated, achieved plasma and tumor levels associated with biologic activity, and led to tumor control. Our findings support clinical explorations to reposition niclosamide for the treatment of CRC. Cancer Res; 71(12); 4172–82. ©2011 AACR.

Introduction

Currently available systemic therapies increase survival but do not cure advanced colorectal cancer (CRC). One explanation for the failure of these therapies to address the subset of malignant cells with stem cell–like characteristics. The Wnt signaling pathway, fundamental to embryonic tissue patterning, is also activated in stem-like cells (1–3). Binding of Wnt ligands, of which there are 19 subtypes in humans, to Frizzled (Fzd) receptors, of which there are at least 10 (4, 5), results in the recruitment of intracellular Dishevelled (Dvl), leading to Fzd receptor internalization (6). Downstream signaling events include both the stabilization of cytosolic β-catenin and the translocation of the stabilized β-catenin to the nucleus that result in the activation of the transcription factor T-cell factor/lymphoid enhancer factor (TCF/LEF; refs. 3, 7). In the absence of pathway stimulation, a complex consisting of axin, GSK-3, and adenomatous polyposis coli (APC) promotes the proteolytic degradation of β-catenin.

The canonical Wnt pathway is activated in approximately 80% of sporadic CRC primarily because of mutations in the APC gene (8, 9) and in a small proportion of cases, activating mutations of the β-catenin gene (CTNNB1; ref. 9). Although one would expect that inhibition of the Wnt pathway would require drugs that inhibit β-catenin or its downstream intermediaries, recent observations reveal that Wnt ligands or inhibitors may affect the growth and survival of colon cancer cells in spite of the presence of APC or CTNNB1 mutations (10–12). This suggests that inhibition of upstream receptors, feasible drug targets compared with downstream protein–protein interactions, may provide therapeutic benefit in CRC (5, 13–16). We instituted a translational small molecule screening program to identify agents that inhibit Wnt/Fzd signaling. Using libraries containing Food and Drug Administration (FDA)-approved drugs, we discovered that the antihelminthic niclosamide promotes Fzd1 endocytosis, downregulates Dvl2 protein, and inhibits Wnt3A-stimulated β-catenin stabilization and downstream β-catenin signaling (TCF/LEF reporter activity). We noted that Fzd1 colocalizes in vesicles containing transferrin and agonist-activated β(2)-adrenergic receptor following niclosamide-mediated internalization and could serve as a negative modulator of Wnt/Fzd1 signaling by depleting Fzd and Dvl (17).

We now extend our previous studies by showing that niclosamide inhibits Fzd1 signaling in human colon cancer cell lines and in human colorectal metastasectomy specimens. We found that niclosamide was well tolerated in experimental
animal models and had antitumor effects in vitro and in vivo, despite mutations in APC.

Materials and Methods

Reagents

The following reagents were purchased for this study: niclosamide, protease inhibitors (P8340), phenylmethanesulfonylfluoride (P7626), orthovanadate (220590), and the 1x kinase (IKK) inhibitor PS-1145 (P6624; Sigma-Aldrich); 7-aminoactinomycin D and Annexin V–biotin kits (Immunotech); TNF-α (R&D Systems); mTOR inhibitor everolimus (Novartis); and oxaliplatin (Sanofi Aventis).

Mice

NOD.CB17-Prkdcscid/J [nonobese diabetic (NOD)/severe combined immunodeficient (SCID)] mice were purchased from Jackson Labs and bred in the Duke Comprehensive Cancer Center Isolation Facility. All work was conducted under a Duke University IACUC–approved protocol.

Cell lines

CRC cell lines, HT29 (ATCC HTB-38), HCT116 (ATCC CCL-247), CaCO2 (ATCC HTB-37), and MCF-10A (ATCC CRL-10317) were purchased from the American Type Culture Collection (ATCC). ATCC characterizes these cells by morphology, immunology, DNA fingerprint, and cytogenetics. HT29 and CaCO2 cells were cultured in Dulbecco’s modified Eagle’s medium, and HCT116 cells were cultured in McCoy’s 5A medium supplemented with 10% FBS. MCF-10A cells were cultured in mammary epithelial basal medium with 5% horse serum and mammary epithelial growth medium growth factors (Lonza). All cultures were monitored routinely and found to be free of contamination by mycoplasma or fungi. All cell lines were discarded after 4 months and new lines were propagated from frozen stocks.

Tumor cell isolation from patients’ CRC specimens and establishment of explants in NOD/SCID mice

Patients undergoing resection of CRC metastatic to the liver provided signed informed consent approved by the Duke University Medical Center Institutional Review Board to allow harvest of tumor remaining viable despite prior systemic chemotherapy. CRC cells were isolated from metastatic specimens as previously described (18), and CRC cells growing in vitro were used as target cells in subsequent assays.

APC and β-catenin mutation analyses

Genomic DNA was prepared from CRC explants growing in NOD/SCID mice by using standard methods. Hematoxylin and eosin staining was initially carried out on the CRC explants and areas of malignant epithelial cells were macrodissected. Genomic DNA was isolated and used to PCR amplify the DNA fragments of mutation hotspot of APC (19) or β-catenin gene (20). PCR fragments were agarose gel purified and subjected to DNA sequencing. The sequences obtained were compared with the published DNA sequence of APC (NCBI accession: NG_008481) or β-catenin gene (NCBI accession: NG_013302).

MTT assay

CRC cell lines (HT29, HCT116, and CaCO2) and CRC explant cells growing in vitro (CRC007, CRC010, CRC020, CRC025, CRC028, CRC039, CRC057, and CRC119) were put into 96-well flat-bottomed plates at 5,000 and 10,000 cells/well, respectively. Niclosamide (0.4, 2, and 10 μmol/L) was added and further incubated for 3 days. Oxaliplatin (10 μmol/L) was added to control wells for comparison purposes. MTT assay was conducted as described elsewhere (18). To examine the overtime effect of niclosamide, cells were incubated with niclosamide (0.4, 2, and 10 μmol/L) for 24, 48, 72, and 96 hours, and MTT assays were conducted at each time point. To examine the involvement of mTOR and NF-κB signaling in the cytotoxic effect of niclosamide on CRC cells, everolimus (0.1, 0.3, 1, 3, and 9 μmol/L; ref. 21) or PS-1145 (0.4, 2, 10, and 50 μmol/L; ref. 22) was added to the medium and MTT assays were conducted after 72-hours of incubation.

Flow-based apoptosis assay

Colon cancer cell lines and explants (1 × 10⁵ tumor cells), normal mammary epithelial cells (MCF-10A), fibroblasts, and peripheral blood mononuclear cells (PBMC) derived from normal donors were cultured in 12-well flat-bottomed plates with niclosamide (0.2–20 μmol/L) for 3 days. All cells were harvested with 0.05% trypsin/EDTA, washed, labeled with biotin-conjugated Annexin V, and then stained with 7-AAD and streptavidin-APC. Samples were acquired on a FACSCalibur machine (BD Bioscience) and analyzed with CellQuest software for expression of Annexin V as a marker of apoptosis.

Luciferase reporter assay

Cancer cells (CaCO2 and HCT116) were seeded in a 96-well plate and cotransfected with 30 ng/well TOPflash or FOPflash plasmid DNA with 3 ng/well pRL-TK Renilla luciferase plasmid DNA (Promega) by using Lipofectamine LTX plus (Invitrogen). TOPflash and FOPflash were obtained from Dr. Randy Moon at University of Washington. Sixteen hours after transfection, cells were treated with DMSO or niclosamide at several concentrations and further incubated for 24 hours. Luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega). The firefly luciferase activity was normalized to Renilla luciferase activity.

Western blot analysis

Cytosolic fractions of lysates were isolated as described elsewhere (23). Anti-De2 (clone 1085), anti-β-catenin (clone 7D11), and anti-β-actin (clone C-11) monoclonal antibodies (Santa Cruz Biotechnology) were used to probe the blots. To examine the NF-κB activation status of niclosamide-treated cancer cells, HT29, HCT116, and CaCO2 cell lines were treated with/without niclosamide (1 μmol/L) or PS-1145 (10 μmol/L) for 24 hours and then with/without TNF-α (10 ng/mL) for 1 hour. Whole-cell lysates were analyzed by Western blotting with anti-phospho-NF-κB p65 (clone 93H1; Cell Signaling...
Technology) and anti-NF-κB p65 (clone F-6, Santa Cruz Biotechnology) antibodies.

**Immunohistochemistry and fluorescent immunostaining**

Tumors grown in the flank of NOD/SCID mice, treated with/without niclosamide, were analyzed by immunohistochemistry (24). Anti-β-catenin (clone 7D11; Santa Cruz Biotechnology) and anti-Dvl2 (clone 10B5; Santa Cruz Biotechnology) monoclonal antibodies were used at 1:100 and 1:50 dilution, respectively. An Olympus Vanox AHBS3 microscope (Olympus) was used for the analyses and photographs were taken with an Olympus DP-70 camera. To analyze the effect of niclosamide on NF-κB signaling, cancer cell lines (HT29, HCT116, and CaCO2) were incubated with/without niclosamide (1 μmol/L) or PS-1145 (10 μmol/L) for 24 hours and then with/without TNF-α (10 ng/mL) for 1 hour. Cells were fixed with 3% paraformaldehyde (Sigma), permeabilized with 0.01% Triton X-100 (Sigma) in PBS, and then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-NF-κB p65 antibody (clone F-6, 1:100 dilution, Santa Cruz Biotechnology) for 1 hour at room temperature. Nuclei were labeled with DAPI (4 ′,6-diamidino-2-phenylindole). An Axio Observer fluorescence microscope (Carl Zeiss) was used, and images were captured on a Hamamatsu ORCA ER CCD camera (Hamamatsu Photonics) and analyzed with MetaMorph Premier 7.6.5 software (Molecular Devices). Additional methodologies are provided in the Supplementary sections.

**Pharmacokinetics of niclosamide in plasma and tumor**

NOD/SCID mice (weighting 23–25 g) received oral administration of niclosamide (200 mg/kg of body weight). Blood samples were obtained at predose and at 0.25, 0.5, 0.75, 1, 1.5, 4, 8, 12, and 24 hours after drug administration. Plasma was isolated by centrifugation and stored at −20 °C until liquid chromatography/tandem mass spectrometric (LC/MS-MS) analysis.

To study tumor uptake of niclosamide, NOD/SCID mice were inoculated with HCT116 cells (5 × 10⁶ cells), and on day 4, oral gavage of niclosamide (200 mg/kg body weight) or control solvent was initiated. After 3 weeks of treatment, mice were sacriﬁced 24 hours after the last drug administration, and blood and tumor tissue were collected simultaneously. Tumor tissue was cryocrushed in liquid nitrogen and homogenized with deionized water in the FastPrep apparatus (4-mm ceramic bead, 20 seconds, speed 4). Quantification of niclosamide in mouse plasma and tumor tissue was guided by a published LC/MS-MS method (25). A Shimadzu 20A series LC system and an Applied Biosystems API 4000 QTrap tandem mass spectrometer were used.

**In vivo antitumor effect of niclosamide**

HCT116 cells were harvested from ﬂasks with 0.05% trypsin/EDTA and resuspended with Hank’s buffered solution (5 × 10⁶ cells/100 μL). CRC explants (CRC039) cultured in vitro were harvested with the same procedure and mixed with equal volume of Matrigel to make 1 × 10⁶ cells/100 μL concentration. The cell suspensions (100 μL) were inoculated into the flanks of NOD/SCID mice. Four days later, niclosamide administration by gavage 6 d/wk for 2 (HCT116) or 3 weeks (CRC039) began. Tumor size was measured 3 times a week until mice were euthanized.

**Statistical analysis**

Student’s t test was used to analyze differences in tumor volumes for each niclosamide concentration compared to vehicle control. Differences at P < 0.05 were considered statistically significant.

**Results**

**Niclosamide inhibits proliferation of CRC cell lines and explants**

The CRC cell lines used in this study, HT29 and CaCO2 (APCmut and β-cateninwt) and HCT116 (APCmut and β-cateninmut), all express Fzd1 and Fzd2. These cell lines were incubated for 3 days with various concentrations of niclosamide (0.4–10 μmol/L) or 10 μmol/L of oxaliplatin, prior to analysis in an MTT assay. Niclosamide inhibited proliferation of all 3 cell lines with HCT116, most sensitive, and HT29, least sensitive (Fig. 1A). The antiproliferative effect of niclosamide, as low as 2 μmol/L concentration, was greater than 10 μmol/L oxaliplatin against HCT116 and CaCO2 cells. The antiproliferative effect of niclosamide against cultured CRC explants derived from resection specimens (CRC007, CRC010, CRC020, CRC025, CRC028, CRC039, CRC057, and CRC119) was similarly studied in MTT assays after 3-day incubation with various concentrations of niclosamide. The mutational status of the APC and β-catenin genes for these CRC explants is reported in Table 1. Niclosamide inhibited proliferation of all CRC explants tested in a dose-dependent fashion regardless of whether there were APC/β-catenin mutations. Although there was a trend for greater inhibition of proliferation of explants without mutations at low concentrations of niclosamide (0.4 and 2.0 μmol/L), there was equivalent inhibition of proliferation (of explants with and without APC/β-catenin mutations) at higher doses (Table 1; representative cases shown in Figure IB). These data suggest that niclosamide has anticaner activity despite mutations in the downstream tumor suppressor APC.

To assess the effect of niclosamide on CRCs over time, the 3 cell lines were incubated with various concentrations of niclosamide or oxaliplatin for 24, 48, 72, or 96 hours. As shown in Figure 1C, in general, the antiproliferative effect increased over time.

**Niclosamide is nontoxic to normal fibroblasts and PBMCs**

To assess the toxicity of niclosamide on nonmalignant tissues, PBMCs from a normal donor, fibroblasts isolated from CRC patient’s tumor tissue, and mammary epithelial cells (MCF-10A) were compared with the colorectal explants for their sensitivity to niclosamide by using an Annexin V–based flow cytometric assay (Fig. 1D). CRC explants and HCT116 cells showed steep increases in Annexin V–positive cells at 1 or 0.2 μmol/L of niclosamide, respectively, reaching 60% to
Figure 1. Niclosamide inhibits the proliferation of CRC cell lines and has minimal toxicity on normal fibroblasts, PBMCs, or immortalized mammary epithelial cells. CRC cell lines HT29, HCT116, and CaCO2 (A) or CRC explant cells growing in vitro CRC039, CRC057, and CRC119 (B) were cultured in 96-well flat-bottomed plates and treated with several doses (0.4, 2, and 10 µmol/L) of niclosamide for 3 days. Oxaliplatin (10 µmol/L) was used as a positive control. MTT assay was conducted and OD562 was measured after cell lysis with DMSO. C, HT29, HCT116, and CaCO2 cells were incubated with niclosamide (Niclo; 0.4, 2, and 10 µmol/L) for 24, 48, 72, and 96 hours. Oxaliplatin (Oxa; 10 and 20 µmol/L) was used as a positive control. Percentages of proliferations [each OD562 value divided by OD562 value of the control (Cont.; medium alone) of the same time point] are shown. D, HCT116 and CRC119 tumor cells, fibroblasts, MCF-10A cells, or PBMCs derived from normal donors were incubated for 3 days with niclosamide at concentrations ranging from 0.2 to 20 µmol/L. All cells were harvested and labeled with Annexin V. The percentage increase in Annexin V–positive population (compared with untreated control) is shown.

Table 1. APC and β-catenin mutation status and effect of niclosamide on CRC explant cells in vitro

<table>
<thead>
<tr>
<th>APC mutation</th>
<th>β-Catenin mutation</th>
<th>% Inhibition of proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.4 µmol/L</td>
</tr>
<tr>
<td>CRC007</td>
<td>Ser&lt;sup&gt;1356&lt;/sup&gt; to a stop codon</td>
<td>No</td>
</tr>
<tr>
<td>CRC010</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>CRC020</td>
<td>Glu&lt;sup&gt;1317&lt;/sup&gt; to Gln</td>
<td>No</td>
</tr>
<tr>
<td>CRC025</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>CRC028</td>
<td>Ser&lt;sup&gt;1356&lt;/sup&gt; to a stop codon</td>
<td>No</td>
</tr>
<tr>
<td>CRC039</td>
<td>Ser&lt;sup&gt;1315&lt;/sup&gt; to a stop codon</td>
<td>No</td>
</tr>
<tr>
<td>CRC057</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>CRC119</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

NOTE: APC and β-catenin mutations were analyzed as described in Materials and Methods. The antiproliferative effect of niclosamide against each CRC explant was analyzed by MTT assay, and the percentage inhibition compared with medium alone is reported. Those conditions with 50% or greater inhibition are presented in bold.
80%, suggesting a significant induction of apoptotic cell death. However, fibroblasts, MCF-10A cells, or PBMCs from a normal donor did not show a significant increase in apoptotic cell death. Thus, niclosamide does not have significant toxicity against nontumor cells.

Niclosamide inhibits Wnt/β-catenin pathway activation and decreases the cytosolic expression of endogenous Dvl2 and β-catenin in CRC cells

To analyze the effect of niclosamide on Wnt/β-catenin signaling, we conducted TOPflash assay with CRC cell lines (CaCO2 and HCT116) and could show that niclosamide inhibits the activation of this pathway in a dose-dependent manner (Fig. 2A). In our previous study, we reported the downregulation of Dvl2 and β-catenin expression in U2OS cells by niclosamide. In the present study, we also analyzed Dvl2 and β-catenin expression in CRC explants and cell lines and observed that these cancer cells show similar downregulation of these molecules in response to niclosamide treatment (Fig. 2B). Interestingly, HT29 cells, assessed as less sensitive to niclosamide on the basis of MTT assay (Fig. 1A), showed only moderate changes in cytosolic expression of Dvl2/β-catenin (data not shown), whereas sensitive CRC explants and cell lines (CRC057, CRC119, HCT116, and CaCO2) showed more evident downregulation, suggesting a correlation of the cytotoxic effect and Wnt/Fzd1 signaling inhibition.

CRC cells not affected by NF-κB or mTOR inhibitors are inhibited by niclosamide under conditions in which it inhibits Wnt pathway signaling

Others have reported that niclosamide inhibits the NF-κB pathway in leukemia cell lines (26) or mTOR signaling in MCF-7 breast cancer cells (27). Therefore, we wished to examine this effect in our model of CRC cells. First, we examined whether specific inhibitors of the NF-κB or mTOR pathways would affect the viability of CRC cells sensitive to the effects of niclosamide. The pan-IKK inhibitor PS-1145 was used to block NF-κB signaling (22) and everolimus (21) was used to block mTOR signaling. As shown in Figure 3A, the 3 cell lines, under conditions in which niclosamide causes inhibition of cell proliferation, were not sensitive to PS-1145 or everolimus, suggesting that neither the NF-κB nor mTOR pathway inhibition was a mechanism of action for niclosamide in our models. We also examined whether the NF-κB pathway could be perturbed by niclosamide. When exposed to TNF-α, these cells showed activation in the NF-κB pathway [elevated phospho-p65 (p-p65) and translocation of NF-κB p65 to the nucleus; Fig. 3B and C]. In the same cell lines, TNF-α-induced activation of NF-κB could not be blocked by niclosamide whereas they were blocked by the IKK inhibitor PS-1145 at least in HCT116 and CaCO2 cells (Fig. 3B and C). Furthermore, to determine whether niclosamide-induced killing of CRCs could be prevented by blocking the NF-κB pathway, we

![Figure 2. Niclosamide inhibits Wnt/β-catenin signaling and downregulates Dvl2 and β-catenin expression by CRC cells in vitro. A, CRC cell lines (CaCO2 and HCT116) were transiently cotransfected with TOPflash or FOPflash plasmid DNA with pRL-TK Renilla Luciferase plasmid DNA. Cells were treated with different concentrations of niclosamide (0, 1, 5, and 10 μmol/L) for 24 hours. Activity of Wnt/β-catenin signaling pathway was quantified by measuring relative firefly luciferase activity units (RLU) normalized to Renilla luciferase. B, CRC explants and CRC cell lines were treated with different concentrations (0, 1, 5, and 10 μmol/L) of niclosamide overnight (18 hours). After washing cells with PBS, cell lysates were prepared with hypotonic lysis buffer. Cytosolic fractions of lysates were isolated and analyzed by Western blotting with anti-Dvl2 (clone 10B5), anti-β-catenin (clone 7D11), and anti-β-actin (clone C-11) monoclonal antibodies.](cancerres.aacrjournals.org)
Niclosamide Inhibits Wnt Signaling and Colorectal Cancer Growth

Exposed these cell lines to PS-1145 during niclosamide treatment. PS-1145 could not inhibit the niclosamide-induced killing of these cancer cells (Fig. 3D). Therefore, we concluded that neither NF-kB nor mTOR pathway inhibition was a dominant mechanism of action in our CRC model system and niclosamide exerts its effect primarily through the Wnt pathway.

Additive antiproliferative activity of niclosamide combined with oxaliplatin

We sought to assess the combination of niclosamide with oxaliplatin, commonly used to treat CRC. The addition of oxaliplatin, even at lower concentration (1-2 μmol/L), induced more killing of CaCO2 cells than niclosamide alone (Fig. 4A). Percent proliferation value (right panel) was also calculated by adjusting optical density (OD) at 562 nm (OD562) values of niclosamide 0 μmol/L at each oxaliplatin concentration to 100%. The effect of the 2 drugs was at least additive (right panel). Similar additive effects were observed with CRC explant cells at low niclosamide concentrations (Fig. 4B). These data suggest that niclosamide enhances the antitumor effect of oxaliplatin.

Pharmacokinetic analysis of niclosamide following oral administration in NOD/SCID mice

Niclosamide is administered orally for the treatment of intestinal helminthic infections and is reported to have little systemic absorption. However, we wished to determine whether niclosamide was sufficiently absorbed to attain the inhibitory concentrations required in vitro. Plasma
concentrations of niclosamide were measured in mice following oral administration of 200 mg/kg mixed into polyethylene glycol (Fig. 3A). Win-Nonlin software was used for the noncompartmental pharmacokinetic analysis of the rather complex concentration–time profile observed. Within the first hour, the concentration–time data showed a sharp increase ($t_{max} = 0.15$ minutes, $C_{max} = 893.7$ ng/mL) and decrease (30–45 minutes, $C = 50–40$ ng/mL), followed by a small but definite “rebound” (1.5 hours, $C = 78$ ng/mL) after which plasma concentration decreased gradually through at least 2 exponential decay processes. Elimination rate ($\lambda_Z = 0.217$ h$^{-1}$) and the half-life ($t_{1/2} = 3.2$ hours) were calculated from the slope of the 2-point line (12 and 24 hours) and the area under the curve (AUC) between $t = 0$ and $t = 24$ hours was calculated as AUC$_{last} = 1,011$ mg h/L. The simplest explanation for the complex profile observed is that the initial “burst” in niclosamide plasma concentration is caused by a smaller portion of the drug in more soluble form (AUC$_{0-1h} = 206$ mg h/L) and that the “rebound” is the final portion of the absorption profile of the main and less soluble fraction of the drug (AUC$_{1-24h} = 805$ mg h/L). The 2-phase profile at later times cannot be explained by saturation of liver enzymes, as in mammals niclosamide is not metabolized by the liver. The behavior can be explained either by differing absorption profiles at different regions of the gastrointestinal tract or, less likely, by enterohepatic recycling (reabsorption from gall bladder). The overall complex behavior is most probably the consequence of high doses administered or by the specificity of the solvent system (simple pharmacokinetics observed in rats at 5 mg/kg DMSO/cremophor EL; ref. 25). It is important that from 0.5 to 12 hours after oral intake, plasma concentrations were relatively stable at the range of 39.5 to 77.6 ng/mL (~0.1–0.2 µmol/L).

To study the niclosamide uptake by solid tumors, we measured the niclosamide concentrations in tumor tissue (ng/g tissue) obtained from mice implanted with HCT116 tumor cells and treated with niclosamide (200 mg/kg body weight per day) or control solvent for 3 weeks. At 24 hours after the final administration, niclosamide concentrations in tumor tissue (37 ng/g tissue) were similar to those in plasma (38 ng/mL; Fig. 5B), suggesting the efficient and reliable distribution of niclosamide from blood to tumor tissue. Considering that the vascular volume accounts for only a small fraction of the tissue volume, our data indicate that niclosamide is retained in the tissue and that its concentration at the site of action may be several-fold higher than that in plasma (i.e., in the µmol/L range).

**Niclosamide has antitumor activity in vivo**

To study the in vivo antitumor effect of niclosamide, HCT116 and CRC039 were inoculated subcutaneously into...
Niclosamide inhibits Wnt signaling and colorectal cancer growth

mice and 4 days later, oral gavage of niclosamide was initiated 6 times a week. During the course of treatment with niclosamide, no obvious side effects were observed in the mice. As shown in Figure 6A, niclosamide significantly inhibited the growth of both HCT116 and CRC039 tumors. In the more rapidly growing tumor (HCT116), a dose of 200 mg/kg of body weight was needed to suppress the tumor growth.

However, 100 mg/kg of niclosamide could suppress the growth of the relatively slow-growing tumor (CRC039) to the same level. We analyzed another CRC explant (CRC028) and observed the similar inhibition of tumor growth with niclosamide (25 mg/kg per day) compared with control-treated mice. Thus, niclosamide was confirmed to inhibit the growth of human CRCs in NOD/SCID mice.

Dvl2 and β-catenin expression declines in niclosamide-treated CRC tumors in vivo

We wished to document that Wnt pathway intermediaries would decline after in vivo treatment with niclosamide. CRC masses (HCT116, CRC028, and CRC039) from mice treated with niclosamide (25 or 100 mg/kg per day) for 2 or 3 weeks were excised, formalin-fixed, and analyzed by immunohistochemistry for the expression of Dvl2 and β-catenin. Niclosamide-treated tumors showed decreased levels of cytoplasmic expression of Dvl2 and β-catenin compared with control-treated tumors (Fig. 6B). This result indicates the prolonged inhibitory effect of niclosamide in vivo for Wnt/β-catenin signaling.

Discussion

The importance of inappropriate Wnt signaling to the development and progression of advanced cancers such as CRC has been well documented. Therefore, components of the Wnt pathway are prime drug development targets, particularly the accessible plasma membrane receptors such as Fzd (5, 13–16). However, there are currently no FDA-approved drugs that regulate Wnt signaling at the level of the Fzd receptor. We had previously found that niclosamide promotes Fzd1 internalization and inhibits Wnt/Fzd function, suggesting that it may have antitumor effects. Indeed, in the current study, we showed that niclosamide has antitumor effects in vitro against CRC cell lines and CRC cells obtained from patient resection specimens at low micromolar concentrations, but it has no significant toxicity against nontumor cells, including PBMCs.

In the current study, we could show by TOPFlash assay that niclosamide can inhibit Wnt pathway activation in CRC. The mechanism of action of the niclosamide in our studies is thought to be through internalization of Fzd1 and downregulation of Wnt pathway intermediaries as we reported previously (17). In its common usage as an antihelminthic (28, 29) is believed to uncouple oxidative phosphorylation (30).

It has recently been found to be effective at low micromolar concentrations in preventing the synthesis of corona virus proteins in a tissue culture model of severe acute respiratory syndrome (31). Its mechanism of action in this instance has not been well defined, but niclosamide can interact with DNA (31). We do not believe that either of these mechanisms are the operative mechanism in the tumor models, because we would have expected much greater toxicity to normal tissue if there were uncoupling of oxidative phosphorylation or DNA-interacting effects in mammalian tissues. Recently, Jin and colleagues (26) reported that niclosamide inhibited the NF-κB pathway and increased reactive oxygen species levels.
to induce apoptosis in acute myelogenous leukemia cells. In contrast, we did not observe any inhibitory effect of niclosamide on NF-κB signaling in our CRC model (Fig. 3). Balgi and colleagues (27) reported mTORC1 inhibition and autophagosome accumulation leading to autophagy in response to niclosamide exposure. Although we did not formally study the mTOR pathway in our models, it should be noted that the cell lines tested in our studies did not have mTOR pathway dysregulation and were not sensitive to the potent mTOR inhibitor everolimus (Fig. 3A).

We were encouraged to find that niclosamide had activity despite the presence of APC mutations in some of the cell lines and CRC explants. Because APC mutations that impair the process of targeting β-catenin for elimination are a common finding in CRCs, we were concerned that niclosamide, exerting its effect through upstream molecules, would not have activity in APC-mutated tumors. Nonetheless, we saw similar activity regardless of the APC mutational status, suggesting that inhibition of upstream molecules could impact downstream molecules as reported by He and colleagues (11). Because functions of both Dvl2 and β-catenin are shown to be controlled by ubiquitination and degradation by the proteasome (32), another possible mechanism of niclosamide function might be its effect on either the ubiquitination or a proteolytic pathway to cause degradation of Dvl2 and β-catenin, independent of APC and β-catenin mutation status.

One potential concern for the use of niclosamide as an anticancer therapeutic agent is the poor absorption of this drug. Indeed, we required higher doses (100–200 mg/kg body weight) to show significant inhibition of tumor growth in NOD/SCID mice. Nonetheless, we confirmed relatively stable concentrations of niclosamide in the plasma at the range of 39.5 to 77.6 ng/mL (~0.1–0.2 μmol/L) from 0.5 to 12 hours after oral intake (200 mg/kg). Importantly, niclosamide concentrations in tumor tissue showed good local accumulation (Fig. 3B).

Figure 6. Niclosamide inhibits the growth of CRCs in NOD/SCID mice and downregulates Dvl2 and β-catenin expression. A, HCT116 colon cancer cells were harvested from flasks with 0.05% trypsin/EDTA and resuspended with Hank’s buffered solution at 1 × 10⁶ cells/100 μL concentration. CRC explants (CRC039) cultured in vitro were harvested with the same procedure and mixed with equal volume of Matrigel to make a concentration of 1 × 10⁶ cells/100 μL. The cell suspension (100 μL) was inoculated into the flank of NOD/SCID mice 4 days before the start of treatment. Niclosamide (10, 100, and 200 mg/kg body weight) was administered by gavage for 6 d/wk for 2 (HCT116) or 3 weeks (CRC039). Tumor size was measured 3 times a week until mice were euthanized. *, P < 0.05; **, P < 0.01. Cont., control. B, HCT116 and CRC039 tumors grown in the flank of NOD/SCID mice were treated with/without niclosamide (200 mg/kg body weight) for 2 or 3 weeks. β-Catenin and Dvl2 immunohistochemistry was conducted for tumor sections as described in Materials and Methods. Scale bars, 100 μm. Insets show higher magnification (scale bars, 20 μm).
correlation with those in plasma, suggesting the efficient distribution of niclosamide from blood to tumor tissue. Furthermore, we observed downregulation of Dvl2 and β-catenin cytosolic expression in niclosamide-treated tumor cells in vitro. Thus, oral administration of niclosamide does result in significant distribution of the drug into tumor tissue to prove a prolonged inhibitory effect on Wnt/β-catenin signaling, resulting in tumor growth inhibition.

In summary, we showed downregulation of Wnt/β-catenin signaling and antitumor effects of niclosamide in vitro and in vivo. No significant toxicity was shown against nontumor cells in vitro and no obvious side effects were observed in niclosamide-treated mice. We now propose to study niclosamide in phase I human studies for patients with CRC. Also, because niclosamide is minimally absorbable from gastrointestinal tract, we are studying modifications that might permit greater absorption of the drug. Finally, high exposure of niclosamide occurs in the intestinal lumen, which supports the use of niclosamide to prevent induction, progression, or recurrence of premalignant lesions and/or adenocarcinomas of the gastrointestinal tract. The relatively poor absorption and low systemic levels of niclosamide may make this an attractive agent for cancer prevention.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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