

Niclosamide suppresses hepatoma cell proliferation via the Wnt pathway

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Background: The Wnt pathway plays an important role in hepatocarcinogenesis. We analyzed the association of the Wnt pathway with the proliferation of hepatoma cells using Wnt3a and niclosamide, a drug used to treat tapeworm infection.

Methods: We performed an MTS assay to determine whether Wnt3a stimulated proliferation of Huh-6 and Hep3B human hepatoma cell lines after 72 hours of incubation with Wnt3a in serum-free medium. The cells were subjected to hematoxylin and eosin staining and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) after 48 hours of incubation. RNA was isolated 48 hours after addition of Wnt3a or niclosamide, and *cyclin D1* expression levels were analyzed by real-time quantitative polymerase chain reaction. The promoter activity of *T-cell factor* was analyzed by luciferase assay 48 hours after transfection of TOPflash. Western blot analysis was performed with antibodies against β -catenin, dishevelled 2, and cyclin D1.

Results: Cell proliferation increased with Wnt3a. Niclosamide suppressed proliferation with or without Wnt3a. Hematoxylin and eosin and TUNEL staining suggested that apoptosis occurred in cells with niclosamide. *Cyclin D1* was upregulated in the presence of Wnt3a and downregulated with addition of niclosamide. The promoter activity of *T-cell factor* increased with Wnt3a, whereas *T-cell factor* promoter activity decreased with niclosamide. Western blot analysis showed that Wnt3a upregulated β -catenin, dishevelled 2, and cyclin D1, while niclosamide downregulated them.

Conclusion: Niclosamide is a potential candidate for the treatment of hepatoma.

Keywords: Wnt3a, niclosamide, luciferase assay, *cyclin D1*, *T-cell factor*

Introduction

Molecular therapy is a promising treatment for hepatoma. Currently, molecular therapy targets signaling pathways that are aberrantly activated specifically in cancer cells. The Wnt signaling pathway has been found to be dysregulated in hepatoma, as revealed by comparison of expression levels of Frizzled (Fz), the receptor for Wnt protein, between cancerous and noncancerous tissues.¹ Based on these results, the Wnt signaling pathway is expected to be a target of molecular therapy for hepatoma.

The Wnt signaling pathway plays a crucial role in regulating cell growth and differentiation. Cytoplasmic β -catenin is maintained at low levels in the absence of Wnt signals through destruction by the glycogen synthase kinase-3 β complex.² Wnt proteins bind to their receptor, Fz, and coreceptor, low-density lipoprotein receptor-related protein 5 and 6, to form a complex.³ The complex phosphorylates low-density lipoprotein receptor-related protein 5 and 6 and sequesters the axin-adenomatous polyposis coli-dishevelled

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(DVL) destruction complex to the receptor domain, inhibiting glycogen synthase kinase-3 β . β -catenin then accumulates in the cytosol and translocates to the nucleus. In the nucleus, β -catenin acts as a cofactor of T-cell factor (TCF)/lymphoid enhancer factor and activates target genes. Constitutive activation of the Wnt pathway leads to abnormal cell growth and development of cancer.⁴ The Fz family of genes, which consists of 10 members, function as cell surface receptors.⁵ Fz9 is widely expressed in hepatoma cell lines, and its inhibition suppresses hepatoma cell proliferation.⁶

Nicosamide was originally developed for the treatment of tapeworm infections, and is still being used for this purpose worldwide;^{7,8} however, its pharmacologic mechanism is not well understood. Among the number of chemicals screened, nicosamide was found to cause autophagy and reversibly inhibit the mammalian target of rapamycin complex 1.^{9,10} Sack et al showed that nicosamide exerts a suppressive effect on the metastasis-promoting activity of S100-A4, a calcium-binding protein that promotes colon cancer metastasis.¹¹ In addition, they demonstrated that nicosamide suppresses cell proliferation and metastasis, and induces apoptosis of colorectal cancer cells.¹¹ Chen et al proposed that nicosamide induces internalization of Fz1.¹² Using an established cell line, Fzd1GFP-U2OS, which stably expresses Fz1 labeled with green fluorescent protein, they found the green fluorescent protein signal to be internalized in cells incubated with nicosamide. Downregulation of DVL2 is another mechanism by which nicosamide inhibits the Wnt pathway.¹³ Further, Yo et al screened 1,200 clinically approved drugs for drug-resistant ovarian cancer cells to identify nicosamide-disrupting metabolic pathways.¹⁴ These reports suggest that nicosamide is a potential anticancer agent.

The Wnt pathway is activated in human hepatoma. Wnt3a, Wnt4a, and Wnt5a, as well as Fz3, Fz6, and Fz7, are upregulated in human hepatoma tissues¹ when compared with surrounding normal tissues. Fz1 is also upregulated, although not as frequently as Fz3, Fz6, and Fz7. Fujimoto et al analyzed the expression of Fz in the human fetal and adult liver and in seven hepatoma cell lines with reverse transcriptase polymerase chain reaction (PCR).⁶ They found that Fz1 is expressed in three cell lines (HLF, PLC/PRL/5, and Huh-6) and in the fetal liver, but not in the adult liver. These reports indicate that Wnt ligands act as growth factors and are inhibited by nicosamide in hepatoma.

Materials and methods

Cell culture

In this study, Huh-6 cells and Hep3B cells were purchased from RIKEN Cell Bank (Tsukuba, Japan) because the former

expresses Fz1 and the latter does not.⁶ Both cell lines were cultured in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum (Life Technologies, Grand Island, NY, USA). The cells were cultured with 5% carbon dioxide at 37°C in a humidified chamber. Cells were spread onto four-well chamber slides (Becton Dickinson, Franklin Lakes, NJ, USA) and subjected to hematoxylin and eosin staining and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL). The stained slides were observed under an AX80 microscope (Olympus, Tokyo, Japan).

Cell proliferation analysis

Huh-6 cells were trypsinized, harvested, and spread onto 96-well flat-bottom plates (Asahi Techno Glass, Tokyo, Japan) at a density of 1,000 cells per well, and then incubated for 24 hours in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum. After culture, the cells were treated with Wnt3a (R&D Systems, Minneapolis, MN, USA) or nicosamide (Sigma-Aldrich) at the indicated concentrations for 72 hours. The cell cultures were then examined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) according to the manufacturer's instructions (Promega Corporation, Tokyo, Japan). MTS is bio-reduced by cells into a colored formazan product that reduces absorbance at 490 nm. Absorbance was analyzed at a wavelength of 490 nm with an iMark Microplate Absorbance Reader (Bio-Rad, Hercules, CA, USA).

TUNEL staining

Apoptotic cells were detected using an apoptosis in situ detection kit (Wako Pure Chemicals, Osaka, Japan). Analysis of apoptotic cells was based on the TUNEL procedure, which consists of the addition of apoptotically fragmented DNA to the 3' termini by terminal deoxynucleotidyl transferase followed by immunochemical detection using an anti-fluorescein antibody conjugated with horseradish peroxidase and diaminobenzidine as a substrate.

Real-time quantitative PCR

Cells were spread onto 6-well plates (Asahi Techno Glass) and cultured. After 48 hours of treatment with Wnt3a and nicosamide, total RNA (5 μ g) was isolated with Isogen (Nippon Gene, Tokyo, Japan) and used to generate cDNA with SuperScript III and oligo(dT) primers according to the manufacturer's instructions (Life Technologies). The PCR primers and product sizes were as follows: *cyclin D1*

(NM_053056, 5'-AGAGGCGGAGGAGAACAAACAG, 5'-AGGCGGTAGTAGGACAGGAAGTTG; 180 bp) and *RPL19* (BC095445, 5'-CGAATGCCAGAGAAGGTCAC, 5'-CCATGAGAATCCGCTTGTTT; 157 bp). Real-time quantitative PCR was performed with 40 cycles of 5 seconds of denaturation and 5 seconds of annealing extension. *RPL19* was used as an internal control.

Luciferase assay

Huh-6 cells were spread onto 24-well plates (Asahi Techno Glass) and cultured for 24 hours. When cells reached 70% confluence, they were transfected with Lipofectamine LTX (Life Technologies), 0.5 µg of TOPflash reporter plasmid (Millipore, Temecula, CA, USA), and 0.05 µg of pRL-TK (Promega, Madison, WI, USA) in the medium. Transcriptional activity was measured with a dual luciferase reporter assay system (Promega) using Gene Light (GL-200A) (Microtech Co Ltd, Funabashi, Japan). Plasmid in medium without transfection was used as a negative control.

Western blot analysis

Protein was isolated from cells after 48 hours of culture. A 10 µg sample of protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, and transferred to a nylon filter. After 30 minutes of incubation with 5% skim milk, the filters were incubated with primary antibodies for one hour at room temperature (1:2,500). After one hour of incubation with secondary antibodies at room temperature (1:2,500), the specific antigen-antibody complexes were visualized by enhanced chemiluminescence (GE Healthcare, Pittsburg, PA, USA). Rabbit monoclonal anti-β-catenin antibody and anti-DVL2 antibody were purchased from Cell Signaling Technologies (Danvers, MA, USA). Rabbit polyclonal cyclin D1 antibody and mouse monoclonal anti-tubulin-α antibody were purchased from Epitomics Inc (Burlingame, CA, USA) and Lab Vision (Fremont, CA, USA), respectively. Horseradish peroxidase-linked anti-rabbit antibody and horseradish peroxidase-linked anti-mouse antibody were purchased from GE Healthcare. The filter was reprobed with anti-tubulin-α antibody. Expression levels of β-catenin, DVL2, and cyclin D1 were normalized with tubulin-α and analyzed using ImageJ64 imaging software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Cell proliferation and real-time quantitative PCR data were analyzed by one-factor analysis of variance. Statistical analysis was performed using JMP5.0 J software (SAS Institute

Japan, Tokyo, Japan). $P < 0.05$ was accepted as being statistically significant.

Results

We performed the MTS assay to determine whether niclosamide suppressed proliferation of Huh-6 cells and Hep3B cells. Both cell lines were incubated with niclosamide for 72 hours. Serum was not added to the medium to quench its effects and focus on the effects of Wnt3a or niclosamide.¹⁵ Cell proliferation was suppressed to $29\% \pm 9.0\%$ in Huh-6 cells ($P < 0.05$, Figure 1A) and $41\% \pm 11\%$ in Hep3B cells ($P < 0.05$, Figure 1B) at 1 µM of niclosamide. We performed the MTS assay to analyze whether Wnt3a stimulated proliferation of Huh-6 cells and Hep3B cells. Cell proliferation increased to $175\% \pm 33\%$ in Huh-6 cells ($P < 0.05$, Figure 1C) and $170\% \pm 20\%$ in Hep3B cells ($P < 0.05$, Figure 1D) with 20 ng/mL Wnt3a, respectively. Niclosamide was added to medium containing 20 ng/mL Wnt3a to determine if this suppressed the proliferative effect of Wnt3a. The MTS assay was performed 72 hours after addition of niclosamide at the indicated concentration. Niclosamide at 1.0 µM suppressed proliferation of Huh-6 cells to $17.0\% \pm 6\%$ ($P < 0.05$, Figure 1E) and Hep3B cells to $62\% \pm 23\%$ ($P < 0.05$, Figure 1F). These data indicate that niclosamide inhibits the Wnt3a-mediated proliferative effect.

The cells were stained with hematoxylin and eosin for morphologic analysis (Figure 2). We observed no changes in cells cultured in serum-free medium containing 20 ng/mL Wnt3a. On the other hand, Huh-6 cells and Hep3B cells showed morphologic changes after addition of 0.1 µM niclosamide, some with pyknotic nuclei suggesting apoptotic cell death (arrows). TUNEL staining was performed to confirm apoptosis. Positive results were observed in Huh-6 cells and Hep3B cells treated with niclosamide alone or with Wnt3a and niclosamide.

Cyclin D1 is a downstream target of the Wnt pathway and is involved in regulation of cell cycle progression. Reverse transcriptase PCR analysis showed decreased levels of *cyclin D1*. RNA was isolated after 48 hours of treatment with Wnt3a or Wnt3a with niclosamide. *Cyclin D1* expression levels were downregulated by the increased concentration of niclosamide. *Cyclin D1* was downregulated to $30\% \pm 15\%$ in Huh-6 cells ($P < 0.05$, Figure 3A) and to $44\% \pm 14\%$ in Hep3B cells ($P < 0.05$, Figure 3B) at 1.0 µM. *Cyclin D1* expression levels were upregulated by increased Wnt3a concentrations to $236\% \pm 76\%$ with 6 ng/mL Wnt3a in Huh-6 cells ($P < 0.05$, Figure 3C) and $180\% \pm 20\%$ with 20 ng/mL Wnt3a

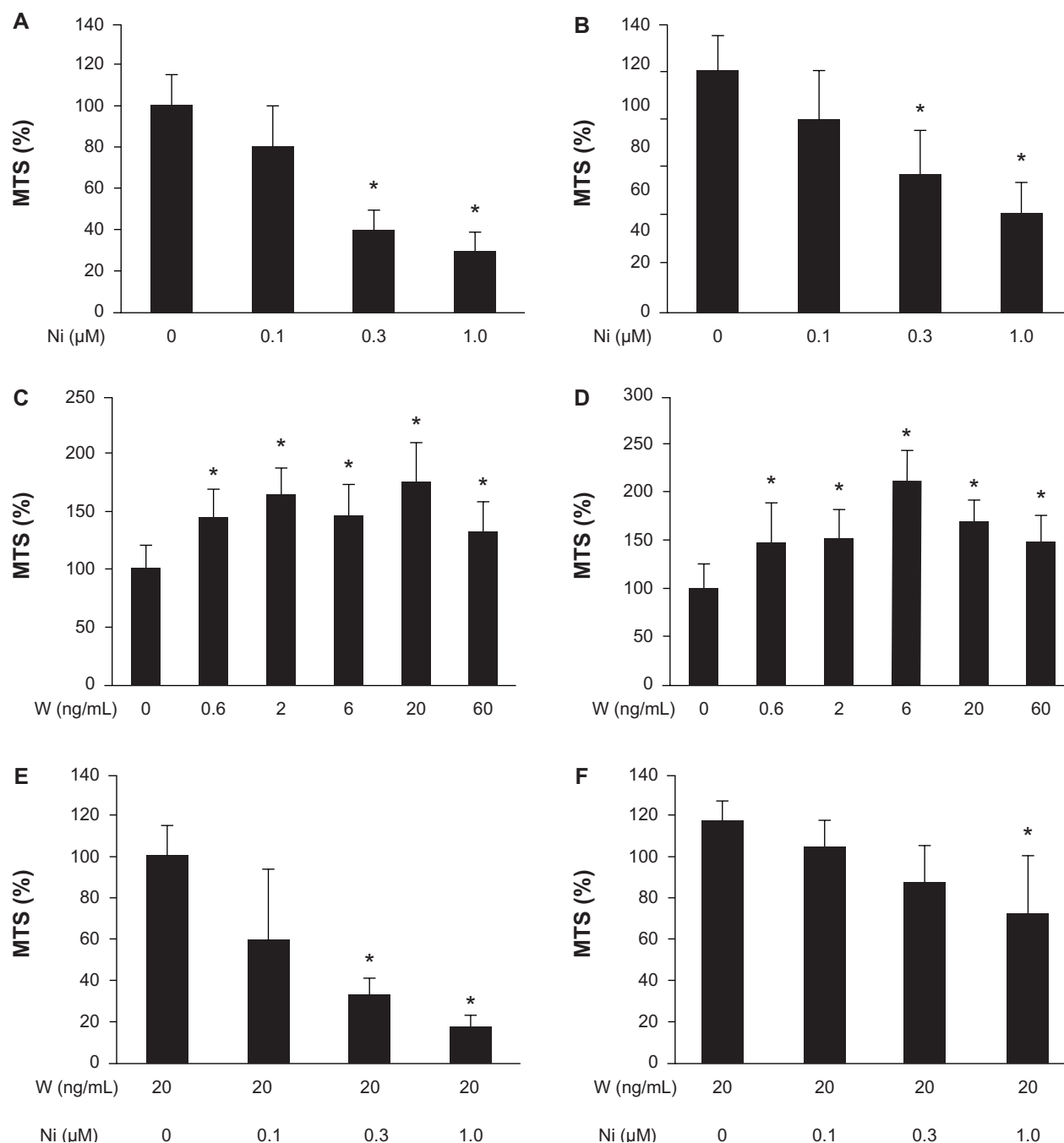


Figure 1 Cell proliferation assay. A cell proliferation assay was performed after addition of Wnt3a or niclosamide to Huh-6 cells (**A**, **C**, and **E**) and Hep3B cells (**B**, **D**, and **F**). Niclosamide suppressed proliferation without Wnt3a (**A** and **B**) or with Wnt3a (**E** and **F**). Wnt 3a increased proliferation (**C** and **D**).

Notes: * $P < 0.05$; $n = 3$.

Abbreviations: W, Wnt3a; Ni, niclosamide; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt.

in Hep3B cells ($P < 0.05$, Figure 3D). Niclosamide was added to medium containing 20 ng/mL Wnt3a and cultured for 48 hours. *Cyclin D1* expression levels were downregulated to $35\% \pm 10\%$ in Huh-6 ($P < 0.05$, Figure 3E) and $50\% \pm 15\%$ in Hep3B (Figure 3F) with 1 μM niclosamide treatment. These data clearly demonstrate that Wnt3a upregulates *cyclin D1* expression levels and that niclosamide inhibits Wnt3a activity.

The TOPflash reporter plasmid contains three copies of the T-cell factor binding site, which is one of the targets of the Wnt pathway.¹⁶ The promoter activity of *TCF* decreased to $8\% \pm 3\%$ in Huh-6 cells ($P < 0.05$, Figure 4A) and $13\% \pm 8\%$ in Hep3B cells ($P < 0.05$, Figure 4B) with 1 μM niclosamide. The promoter activity of *TCF* increased to $260\% \pm 30\%$ in Huh-6 cells ($P < 0.05$, Figure 4C) and $200\% \pm 35\%$ in Hep3B cells ($P < 0.05$, Figure 4D) with 6 ng/mL Wnt3a. The *TCF*

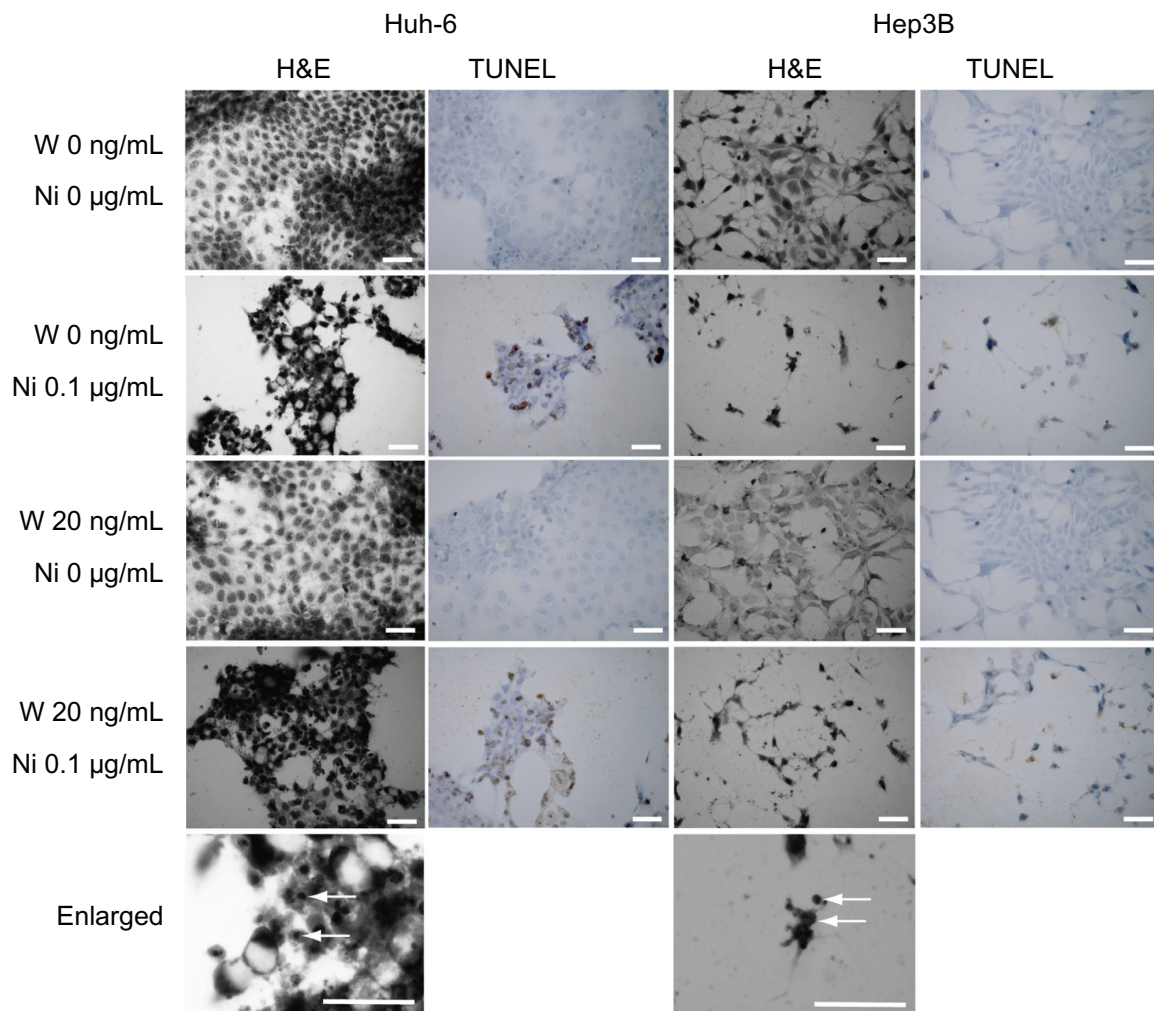


Figure 2 H&E staining and TUNEL staining. Huh-6 and Hep3B cells were subjected to H&E and TUNEL staining. While no change was seen in cells cultured without niclosamide cells, those treated with 0.1 μ M niclosamide were damaged and had pyknotic nuclei (enlarged, white arrows).

Notes: Original magnification $\times 400$. Scale bar 50 μ m.

Abbreviations: W, Wnt3a; Ni, niclosamide; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling; H&E, hematoxylin and eosin.

promoter activity decreased to 0 in Huh-6 cells ($P < 0.05$, Figure 4E) and to $20\% \pm 10\%$ in Hep3B cells ($P < 0.05$, Figure 4F) with 1 μ M niclosamide treatment. Expression levels of β -catenin, DVL2, and cyclin D1 were analyzed using Western blot analysis (Figure 5). One μ M niclosamide suppressed expression levels with or without 20 ng/mL Wnt3a.

Discussion

Niclosamide suppressed cell proliferation to 70% at 1 μ M in the human colon cancer cell line HCT116, and at 1.5 μ M in the human ovarian cancer cell line CP70.¹⁴ In our experiments, 1 μ M niclosamide suppressed cell proliferation to 29% in Huh-6 cells and 41% in Hep3B cells without Wnt3a, and to 17% in Huh-6 cells and 62% in Hep3B cells with Wnt3a. These results are comparable with those obtained in previous reports. We added niclosamide directly to hepatoma

cells without serum or Wnt3a and found that niclosamide suppressed cell proliferation. Huh-6 cells expressed all *Fz* genes except *Fz8*, while Hep3B expressed all but *Fz1* and *Fz8*.⁶ This suggests that the Wnt pathway is constitutively active and inhibited by niclosamide. Sack et al suggested that niclosamide inhibits formation of the β -catenin/TCF complex.¹¹ Previous reports and our present data indicate that niclosamide may suppress cell proliferation with or without Wnt ligands. This hypothesis is supported by the fact that hepatoma cell proliferation is suppressed by sorafenib in serum-free culture.¹⁷

Dysregulation of the Wnt pathway has been implicated in various types of cancer, including hepatoma.¹⁸ Wnt3a is upregulated in human hepatoma tissues.¹ It is speculated that Wnt3a is involved in the growth of hepatoma cells and increases the promoter activity of *TCF*.¹⁹ With this

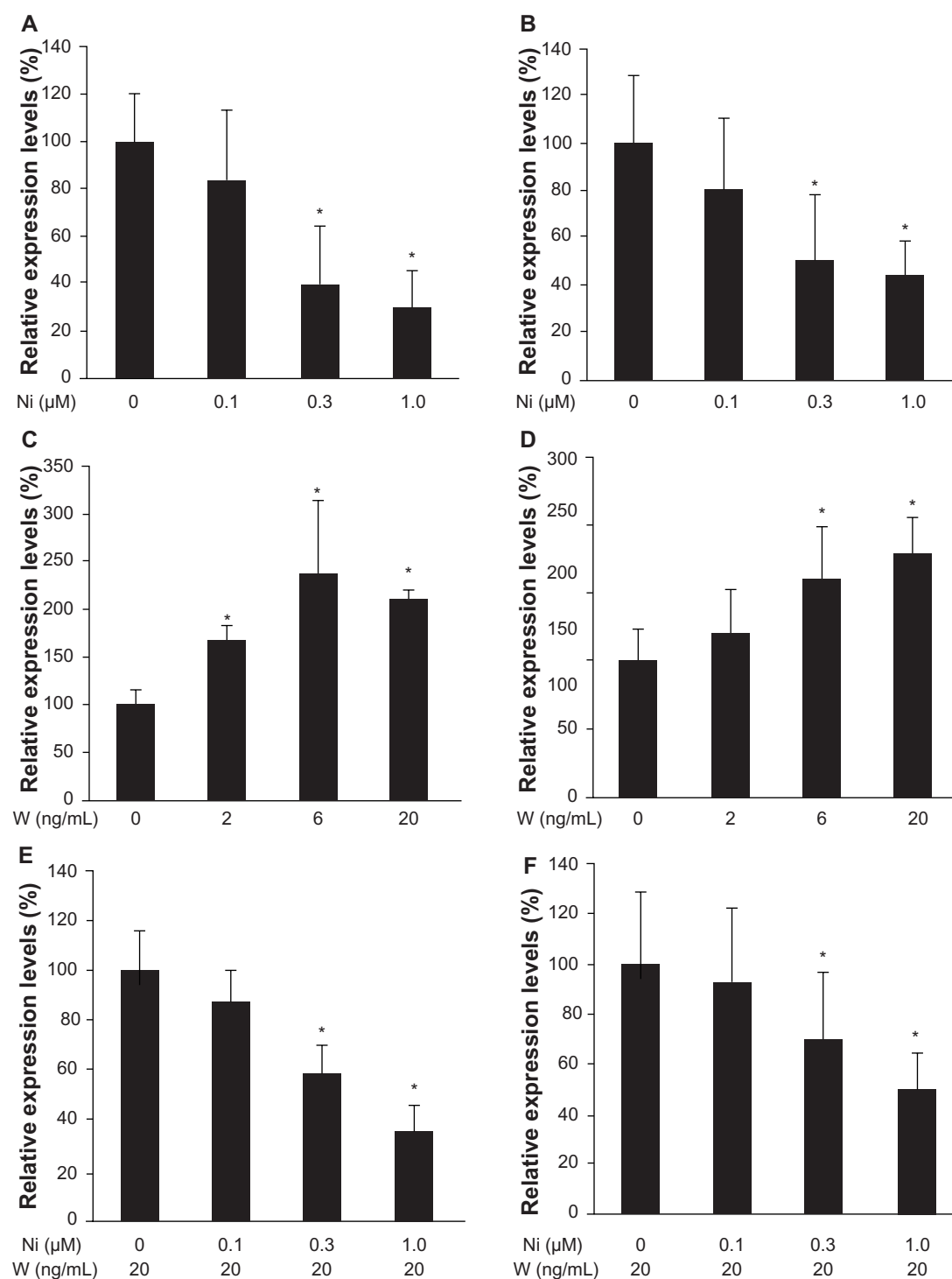


Figure 3 Real-time quantitative polymerase chain reaction. The expression level of *cyclin D1* was analyzed by real-time quantitative polymerase chain reaction with Huh-6 cells (A, C, and E) and Hep3B cells (B, D, and F). Wnt3a upregulated *cyclin D1* expression (C and D). Niclosamide downregulated *cyclin D1* expression with (E and F) or without (A and B) Wnt3a (2 ng/mL).

Notes: * $P < 0.05$; $n = 3$.

Abbreviations: W, Wnt3a; Ni, niclosamide.

upregulation, TCF binds to the promoter of *cyclin D1* and increases its expression.²⁰ Our study clearly demonstrates that Wnt3a increased the proliferation of Huh-6. Moreover, Wnt3a increased expression of *cyclin D1* and stimulated the promoter activity of TCF with TOPflash. These data suggest

that Wnt3a activates the Wnt pathway, upregulates *cyclin D1* expression, and promotes proliferation of Huh-6 cells, consistent with previous reports.

The role of the Wnt pathway in hepatocarcinogenesis is intriguing. Hepatitis C core proteins are involved in

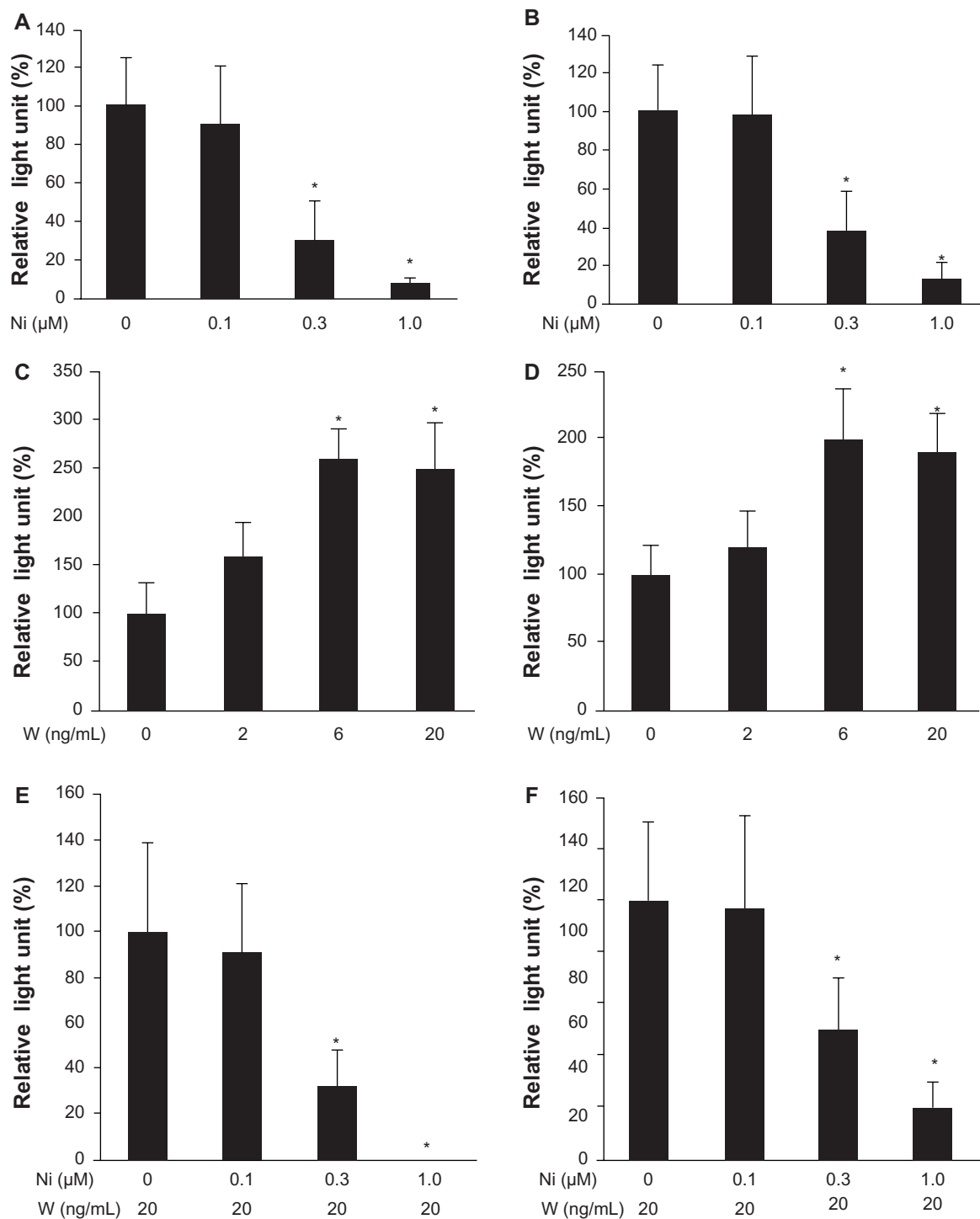


Figure 4 Luciferase assay. A luciferase assay was performed with TOPflash after the addition of Wnt3a or niclosamide to Huh-6 cells (A, C, and E) and Hep3B cells (B, D, and F). Luciferase activity of TOPflash was calculated as relative light units divided by that of pRL-TK. Wnt3a increased the luciferase activity of TOPflash (C and D). Luciferase activity was suppressed by niclosamide with (E and F) or without (A and B) Wnt3a (2 ng/mL).

Notes: * $P < 0.05$; $n = 3$.

Abbreviations: W, Wnt3a; Ni, niclosamide.

hepatocarcinogenesis²¹ and upregulation of Wnt3a and Fz1 in SMMC-7721 cells.²² Interestingly, the promoter activity of *TCF* is stronger with the HCV core protein and Wnt3a than it is with Wnt3a alone.¹⁹ Yang et al cultured HepG2 cells with Wnt3a²³ and found an upregulation of

Kruppel-like factor 8, an oncogenic transcription factor leading to increased β -catenin accumulation. For the treatment of hepatoma, inhibition of the activated Wnt pathway is essential. In our present study, niclosamide successfully inhibited the Wnt pathway and suppressed proliferation of

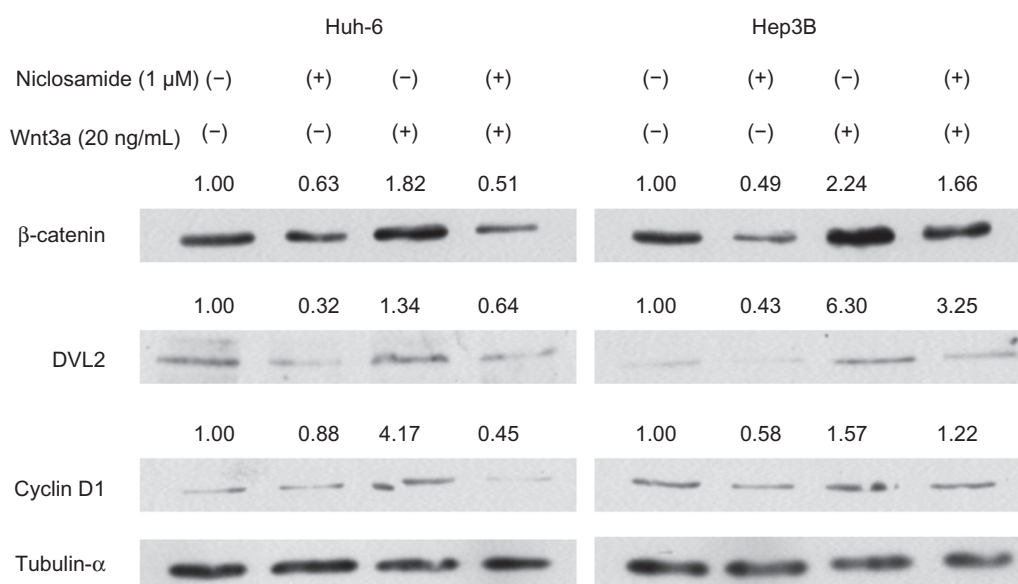


Figure 5 Western blot analysis. Protein was subjected to Western blot analysis 24 hours after addition of nicosamide and/or Wnt3a. The relative expression levels of each antibody were calculated against tubulin- α .

Abbreviation: DVL, dishevelled.

Huh-6 cells. Our data are in accordance with the previous report by Sack et al.¹¹

In this study, nicosamide suppressed the promoter activity of *TCF*. Our present data corroborate our previous report and suggest that nicosamide inhibits the Wnt pathway. Our data clearly show that nicosamide successfully suppresses hepatoma cell proliferation. Figure 2 shows that nicosamide induces apoptosis as evidenced by the presence of pyknotic nuclei.²⁴

Human hepatoma tissues harbor TUNEL-positive cells and proliferation cell nuclear antigen-positive cells.²⁵ Thus, the results of the cell proliferation assay should reflect the accumulative results of both effects. Nicosamide antagonized Wnt3a-enhanced cell proliferation (Figure 1) while inducing cell apoptosis. The accumulative result was, therefore, indicative of hepatoma, and our results showed a decrease in total cell number.

Nicosamide inhibits the Wnt signaling pathway by internalizing Fz1. However, Fz1 is not upregulated in cancerous tissues.¹ Use of nicosamide as a treatment of hepatoma, therefore, may have limitations when translating the results of this study to the clinic.

In conclusion, nicosamide suppressed the proliferation of hepatoma cells and may be a potential candidate agent for the treatment of hepatoma.

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Disclosure

The authors report no conflicts of interest in this work.

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