Inhibition of LEF1-Mediated DCLK1 by Niclosamide Attenuates Colorectal Cancer Stemness

So-Yeon Park1,2, Ji-Young Kim3, Jang-Hyun Choi1, Jee-Heun Kim1, Choong-Jae Lee1, Pomila Singh4, Shubhashish Sarkar4, Jeong-Heum Baek5, and Jeong-Seok Nam1,2,6

Abstract

**Purpose:** Niclosamide, an FDA-approved anthelmintic drug, has been characterized as a potent Wnt inhibitor that can suppress tumor growth and cancer stem-like cell (CSC) populations. However, the underlying molecular mechanisms remain poorly understood. This study aimed to examine how Wnt inhibition by niclosamide preferentially targets CSCs.

**Experimental Design:** The mechanistic role of niclosamide in CSC inhibition was examined in public databases, human colorectal cancer cells, colorectal cancer xenografts, and azoxymethane/dextran sulfate sodium (AOM/DSS)-induced colorectal cancer model.

**Results:** Niclosamide suppresses CSC populations and their self-renewal activities in colorectal cancer cells, and this CSC-targeting effect leads to irreversible disruption of tumor-initiating potential in vivo. Mechanistically, niclosamide downregulates multiple signaling components of the Wnt pathway, specifically lymphoid enhancer-binding factor 1 (LEF1) expression, which is critical for regulating stemness. Subsequently, we identified that the doublecortin-like kinase 1 (DCLK1)-B is a target of LEF1 and upregulates cancer stemness in colorectal cancer cells. We first documented that niclosamide blocks the transcription of DCLK1-B by interrupting the binding of LEF1 to DCLK1-B promoter. DCLK1-B depletion impairs cancer stemness resulting in reduced survival potential and increased apoptosis, thus sensitizing colorectal cancer to chemoradiation.

**Conclusions:** Disruption of the LEF1/DCLK1-B axis by niclosamide eradicates cancer stemness and elicits therapeutic effects on colorectal cancer initiation, progression, and resistance. These findings provide a preclinical rationale to broaden the clinical evaluation of niclosamide for the treatment of colorectal cancer.

Introduction

Colorectal cancer is a major health problem worldwide owing to its high prevalence and mortality rates (1, 2). Although earlier diagnosis by advanced technology and new treatment regimens have considerably improved the survival of patients with colorectal cancer in the past decades, nearly 50% of patients with colorectal cancer still face recurrence at local or distant sites after conventional therapy (3). Initially, conventional therapy kills most cancer cells and immediately shrinks tumor while sparing a small subpopulation, collectively referred to as cancer stem-like cells (CSC), which re-initiate tumor growth and colonize distant organs resulting in relapse and metastasis (4, 5). CSCs engage in self-renewal, induce tumors at low-cell density, and produce tumors with differentiated and heterogeneous cell profiles. An increasing number of studies suggest that CSCs promote cancer progression at various stages including tumor initiation, growth, invasion, and metastasis, and notably, they are more resistant to anticancer therapy than are differentiated non-CSCs (5). In this regard, compounds or designed drugs that hinder CSC-specific pathways or interfere with CSC-specific targets have been recently recognized as promising combinatorial therapies for permanent cure. To actualize this possibility, we need to understand molecular mechanism by which CSCs maintain stemness and promote resistance, leading to recurrence after chemoradiotherapy.

CSCs display many features of embryonic or tissue stem cells and typically demonstrate persistent activation of one or more highly conserved signal transduction pathways involved in development and tissue homeostasis, including Notch, Hedgehog, and Wnt pathways. In particular, the aberrant activation of Wnt/β-catenin signaling pathway is involved in both development and progression of colorectal cancer. During colon carcinogenesis, hyperactivation of Wnt/β-catenin signaling drives adenomatous polyp formation (6, 7), and differentiated colon epithelial cells with constitutive activation of Wnt/β-catenin signaling can re-acquire stem cell-like properties and give rise to poorly differentiated colorectal cancer (8, 9). Colorectal cancer tissues display more intense nuclear accumulation of β-catenin than do normal tissues. Moreover, CSC populations harbor more enhanced
activated Wnt/β-catenin has been implicated in cancer stem cells (CSC), leading to cancer initiation and progression. A number of Wnt inhibitors have been proposed as CSC-targeting drugs; yet, a lack of understanding of molecular regulation on CSC survival and self-renewal remains an obstacle. Niclosamide inhibits multiple components of Wnt signaling and CSC; thus we explored the downstream targets of niclosamide by using public genomic databases, human colorectal cancer cells, and colorectal cancer mouse models. We found that doublecortin-like kinase 1 (DCLK1)-B is predominantly expressed in colorectal cancer cells, and it is more enriched in CSCs than in non-CSCs. DCLK1-B is transcriptionally activated by lymphoid enhancer-binding factor 1 (LEF1) and this LEF1/DCLK1-B axis is critical for CSC survival and self-renewal activity. Disruption of the LEF1/DCLK1-B axis by niclosamide can impair the tumor-initiating and survival potential of CSCs. These findings between LEF1 and DCLK1-B may improve understanding of the signaling network of CSCs.

Translational Relevance

Constitutive activation of Wnt signaling has been implicated in cancer stem cells (CSC), leading to cancer initiation and progression. A number of Wnt inhibitors have been proposed as CSC-targeting drugs; yet, a lack of understanding of molecular regulation on CSC survival and self-renewal remains an obstacle. Niclosamide inhibits multiple components of Wnt signaling and CSC; thus we explored the downstream targets of niclosamide by using public genomic databases, human colorectal cancer cells, and colorectal cancer mouse models. We found that doublecortin-like kinase 1 (DCLK1)-B is predominantly expressed in colorectal cancer cells, and it is more enriched in CSCs than in non-CSCs. DCLK1-B is transcriptionally activated by lymphoid enhancer-binding factor 1 (LEF1) and this LEF1/DCLK1-B axis is critical for CSC survival and self-renewal activity. Disruption of the LEF1/DCLK1-B axis by niclosamide can impair the tumor-initiating and survival potential of CSCs. These findings between LEF1 and DCLK1-B may improve understanding of the signaling network of CSCs.

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Materials and Methods.

Knockdown/knockout cell lines, is provided in Supplementary information assay, the limiting dilution assay, the establishment of inhibiting Wnt/C3/C3/C3

More information about methods, including the cell proliferation assay, the limiting dilution assay, the establishment of knockdown/knockout cell lines, is provided in Supplementary Materials and Methods.

Results

Niclosamide inhibits cancer stemness and therapy resistance by inhibiting Wnt/β-catenin signaling in colorectal cancer cells

To examine whether niclosamide inhibits the colorectal cancer proliferation, we evaluated the IC50 values in multiple cells including HCT116, HT29, SW480, and patient-derived colorectal cancer cells (P#21257113 and P#14005083). Patient-derived colorectal cancer cells were isolated from primary tumors of patients with colorectal cancer and pathologically validated based on IHC markers. They were determined to express the colon-specific cytokeratin (CK) pattern, CK20+/CK7− (31), but not to express the fibroblast marker vimentin (Supplementary Fig. S1A; ref. 32). The IC50 values of niclosamide were below 6 μmol/L in all tested colorectal cancer cells, whereas niclosamide showed minimal effect in a normal colon epithelial cell line (CDD-18Co) even at a maximum concentration 20 μmol/L (Fig. 1A). These data are consistent with previous report that niclosamide does not exert a significant effect against proliferation in normal cells (20), suggesting that niclosamide possesses a therapeutic window for its antitumor effects. Next, to determine whether niclosamide inhibits Wnt/β-catenin signaling, we performed TOP/FOP assay at lower concentrations than IC50 (Fig. 1B). Indeed, niclosamide dose-dependently decreased the transcriptional activity of Wnt/β-catenin signaling by more than 50% at lower concentrations than IC50. At approximately one-third of IC50 concentration (0.4 μmol/L in HCT116 and 2 μmol/L in HT29), niclosamide decreased Wnt/β-catenin transcriptional activity by 74.5% and 63.3% in HCT116 and HT29 cells, respectively. We used this concentration in functional and molecular analyses to minimize the effect on cellular proliferation. Previously, we have discovered that Wnt/β-catenin signaling is activated to a greater extent in tumor cells than in normal cells and that it is further enhanced in CSC populations for regulating self-renewal activity and chemoresistance (29, 33, 34). Therefore, we hypothesized that niclosamide might target CSCs by inhibiting Wnt/β-catenin signaling. Consistently, niclosamide treatment effectively decreased various CSC populations such as LGR5+, CD44v6+, and Aldefluor+ CSCs in multiple colorectal cancer cells (Fig. 1C; Supplementary Fig. S1B and S1C). Additionally, niclosamide inhibited the self-renewal ability of CSC populations (Fig. 1D; Supplementary Fig. S1D). Moreover, niclosamide-exposed CSCs could not fully restore their self-renewal activity even under niclosamide-free conditions. These effects were accompanied by a shift of mRNA patterns in which a set of stemness-related transcription factors was decreased, whereas differentiation marker ANPEP was enhanced by niclosamide (Supplementary Fig. S1E). Next, we investigated whether CSC-targeting niclosamide could suppress the therapy resistance of CSCs. Indeed, the surviving colorectal cancer cells following 5-fluorouracil (5-FU) or radiation exposure, the first-line therapies for colorectal cancer, displayed the increased CD44v6+ CSC populations (Fig. 1E; Supplementary Fig. S1F) with the enhanced self-renewal activities (Fig. 1F; Supplementary Fig. S1G). Surprisingly, combinatorial treatment of niclosamide prevented the increase of stemness in surviving cells following conventional anticancer. Of note, these CSC-targeting effects of niclosamide finally resulted in sensitization of colorectal cancer cells to chemotherapy (Fig. 1G and H) and radiotherapy (Fig. 1I). Therefore, niclosamide inhibits CSC-like properties by inhibiting Wnt/β-catenin signaling and thus can be a potential agent to overcome therapy resistance in colorectal cancer cells.

LEF1, a regulator of cancer stemness, is a potent target of niclosamide

Next, to investigate the molecular mechanism of niclosamide, we performed qPCR screening of Wnt/β-catenin pathway genes in niclosamide-treated HCT116 cells (Fig. 2A). Various Wnt components were suppressed by niclosamide, and LEF1 showed the most potent reduction following niclosamide treatment. In further experiments, niclosamide dose-dependently reduced both mRNA and protein levels of LEF1 in multiple colorectal cancer cells (Fig. 2B and C). Consistently, immunofluorescence analysis revealed that LEF1 were increased in nuclear regions by Wnt activation, and niclosamide could reduce both the basal and Wnt-induced nuclear LEF1 (Fig. 2D). In fact, histopathologic analysis determined that colorectal cells expressed LEF1 more intensely in cancerous tissues than in normal tissues (Fig. 2E). And fluorescence-activated cell sorting (FACS) analysis indicated that LEF1 were significantly enriched in LGR5+, CD44v6+, and ALDH1A1+ CSC populations (Fig. 2F). These data suggest that LEF1 expression is higher in cancer than in normal tissue and further enriched in CSC populations. Therefore, we investigated whether LEF1 was critical for regulating CSC functions. First, we established LEF1 knockout HCT116 cells using the CRISPR-Cas9 genome editing system. We generated multiple clones of LEF1-knockout cells by using two different gRNA sequences. LEF1 knockout clone #1 (sequence I) and clone #3 (sequence II) were validated to lose LEF1 expression (Supplementary Fig. S2A), thus used for further analysis. We discovered that various CSC populations were significantly decreased in both LEF1-knockout clones (Fig. 2G). Moreover, the reduction of tumor sphere forming efficiency was observed in LEF1-knockout cells, convincing the impaired self-renewal activity in the absence of LEF1 (Fig. 2H). Also, we found that high LEF1 expression was associated with poor prognosis such as low relapse-free survival and overall survival in patients with colorectal cancer (Fig. 2I), in consistent with previous report that high expression of LEF1 serves as a poor prognostic marker in patients with colorectal cancer (35). Additionally, we found that LEF1 expression was more elevated in metastatic colorectal cancer than in nonmetastatic colorectal cancer (Fig. 2J), and that chemoresistant colorectal cancer tumors displayed higher expression of LEF1 than chemosensitive colorectal cancer tumors (Fig. 2K). Given that metastasis and chemoresistance are typical features of CSCs resulting in poor prognosis in patients with colorectal cancer (4, 5), these findings suggest that LEF1 is positively correlated with cancer stemness in patients with colorectal cancer. Taken together, we first documented that LEF1, a potent target of niclosamide, is highly expressed in...
Niclosamide attenuates stemness and therapy resistance in colorectal cancer cells. A, Proliferation inhibition by niclosamide treatment was determined for 48 hours by the MTT assay in multiple colorectal cancer cells, patient-derived primary cells (P#21257113 and P#14005083), and normal epithelial cells (CCD-18Co).

Figure 1.
Niclosamide treatment
Without treatment

(Continued on the following page.)
DCLK1 is a target of LEF1 and positively correlated with cancer progression and CSCs in patients with colorectal cancer

To elucidate the LEF1-mediated downstream target of niclosamide, we compared gene expression profiles between metastatic and nonmetastatic primary colorectal cancer samples (GSE41258) and applied gene expression data to gene set enrichment analysis. In these computations, a group of genes that are upregulated by LEF1 were significantly enriched in metastatic colorectal cancers (FDR q-value < 0.005; Fig. 3A). Twenty LEF1 target genes were determined as the leading-edge subset, which contributed the most to the LEF1 enrichment signal in metastatic colorectal cancers. Next, we compared these genes with a set of stemness-related genes (n = 3,401), which were generated by combining multiple gene sets from three categories, namely, adult stem cell, embryonic stem cell, and CSC-related genes. Seven LEF1 target genes were related to stemness and upregulated in metastasis colorectal cancer versus nonmetastatic colorectal cancer tissues (Fig. 3B). Among them, DCLK1 was decreased the most in a dose-dependent manner after niclosamide treatment (Fig. 3B). In silico analysis showed that DCLK1 expression was upregulated in chemoresistant colorectal cancer cells (Fig. 3C) and clinical evidence strongly suggested that DCLK1 expression is positively correlated with LEF1 expression in patients with colorectal cancer (GSE37892 and GSE14333), and with poor prognosis in patients with colorectal cancer (GSE17538 and GSE14333; Fig. 3D and E). Additionally, the stem cell transcription factor POU5F1 was positively correlated with DCLK1 in colorectal cancer patient tumors (GSE17538 and GSE14333; Supplementary Fig. S3A). Consistent with genomic analyses, IHC results revealed that the number of DCLK1+ cells was markedly increased in the epithelial region of cancerous tissues than in that of normal tissues (Fig. 3F). Previously, DCLK1+ colorectal cancer cells were determined to be CSCs originating from LGR5+ stem cells, suggesting that DCLK1 can be a potential CSC-specific target (8). Our comprehensive genomic analysis indicating DCLK1 as a potential target of niclosamide that regulates Wnt-induced stemlike properties in colorectal cancer was thus promising. Therefore, we decided to perform further investigations on DCLK1 and its regulation by niclosamide. According to Uniprot database (https://www.uniprot.org), there are two types of DCLK1 protein isoforms generated by two distinct promoter regions (Supplementary Fig. S3B; refs. 36–38). The A promoter (α-promoter) regulates the transcription of ~82 kDa DCLK1 (DCLK1-A), which contains two N-terminal doublecortin (DC) domains, a C-terminal serine/threonine kinase domain, and a middle serine/proline-rich domain. The B promoter (β-promoter) exists in downstream of the type A promoter and it regulates transcription of ~47 kDa DCLK1 (DCLK1-B), which lacks two N-terminal DC domains. Thus, we quantified DCLK1 isoforms in various cells (Fig. 3C).

Western blot analysis determined that all colorectal cancer cells predominantly expressed DCLK1-B rather than DCLK1-A, in accordance with previous report that DCLK1-B is highly expressed in human colorectal cancer cells and tissues (30). Indeed, DCLK1-B promoter was about 30-fold more activated than that of DCLK1-A promoter in HCT116 cells, suggesting that the major expression of DCLK1-B may be derived from its highly active transcription (Supplementary Fig. S3C). Consistently, we confirmed that DCLK1-B was principally expressed in colorectal cancer tissues and its expression was elevated in colorectal cancer tissues than normal tissues (Fig. 3H; Supplementary Fig. S3D), as described in previous report (30). Then, we enriched CSCs by cultivating multiple colorectal cancer cells in suspension condition where CSCs were allowed to survive and proliferate to form spheres while non-CSCs were not. Then, immunofluorescence revealed that CSC-enriched spheres expressed DCLK1 at higher levels than did monolayer bulk cancer cells (Fig. 3I). In multiple colorectal cancer cells, we newly discovered that DCLK1-B was far more predominant than DCLK1-A in CSCs. Additionally, DCLK1-A was also increased in colorectal cancer tissues than in normal tissue (Fig. 3H; Supplementary Fig. S3D) and it was further enhanced in CSCs (Fig. 3J) in accordance with previous reports that DCLK1-A marks CSCs contributing to colorectal cancer progression in multiple colorectal cancer animal models (8, 39). However, in these previous reports, DCLK1-B has been overlooked, and more recently, overexpression of DCLK1-B has emerged as a prognostic factor in patients with colorectal cancer (30). Consistently, we observed the principal expression of DCLK1-B in colorectal CSCs. Therefore, we tried to figure out whether DCLK1-B is critical for cancer stemness and whether it could be a target for niclosamide in colorectal cancer cells.

Niclosamide effectively inhibits DCLK1-B expression via the Wnt/β-catenin-LEF1 axis

First, we investigated whether DCLK1-B was regulated by Wnt/β-catenin signaling using multiple colorectal cancer cells. DCLK1-B was increased following Wnt activation at both mRNA and protein levels (Fig. 4A). Next, because comprehensive genomic analysis demonstrated that DCLK1 might be a target of LEF1, we validated the molecular relationship between LEF1 and DCLK1-B using LEF1-knockout HCT116 cells (Fig. 4B). LEF1 was upregulated by Wnt3a in wild-type and control (empty vector-transfected) cells, whereas it was not in LEF1-knockout cells. Interestingly, DCLK1-B expression was significantly diminished in LEF1-knockout cells and the upregulation of DCLK1-B by Wnt3a was disrupted in the absence of LEF1, suggesting that...
Niclosamide inhibits cancer stemness by attenuating LEF1 in colorectal cancer cells. A, Inhibitory effect of niclosamide on the expression of Wnt/β-catenin pathway-related genes was determined by RT-PCR in HCT116 cells after 48-hour treatment of niclosamide. Quantitation was performed by RT-PCR, and data are presented as fold change relative to control (log2). (Continued on the following page.)
DCLK1-B was regulated by Wnt/b-catenin signaling and LEF1 might be involved in DCLK1-B regulation. Next, we discovered that DCLK1-B was significantly decreased by niacinamide at both mRNA and protein levels in a dose-dependent manner (Fig. 4C and D). Given that niacinamide treatment led to a significant suppression in DCLK1-B promoter activity (Fig. 4E), niacinamide might regulate DCLK1-B at transcription level. Therefore, we subsequently investigated whether Wnt/b-catenin signaling directly regulates the transcription of DCLK1-B on its promoter region. To this end, we searched the LEF1-binding sites on DCLK1-B promoter using a prediction tool, ALLGEN PROMO database version 3.0.2 (http://allgen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3). We found three potential LEF1-binding sites at −1743, −1595, and −1412 bp (Fig. 4F). Then chromatin immunoprecipitation assay revealed that LEF1 bound to DCLK1-B promoter between −1895 and −1329 regions. Notably, niacinamide inhibited the binding of LEF1 to DCLK1-B promoter (Fig. 4G). Collectively, we first demonstrated that niacinamide could target DCLK1-B through abrogating its transcription on its promoter region. In further investigation, we found that two siRNAs targeting LEF1 (siLEF1) showed different efficacy on LEF1-knockdown, and that the more the siLEF1 decreased LEF1 expression, the more DCLK1-B expression was reduced (Supplementary Fig. S4A). Similarly, DCLK1-B reduction was far more significant in LEF1-knockout cells than in LEF1-knockdown cells. Thus, these data convinced our theory that DCLK1-B expression is dependent on LEF1 expression. In fact, LEF1 mediates nuclear responses to Wnt signals by forming the transcriptional activity of Tcf4 complex with other coactivators such as b-catenin and T-cell transcription factor (Tcf7) families. Indeed, we found that silencing of b-catenin or Tcf4 reduced DCLK1-B expression (Supplementary Fig. S4B and S4C), proposing that a LEF1/b-catenin/Tcf4 complex may be involved in DCLK1-B regulation. Also, DCLK1-A was suppressed by silencing of LEF1, b-catenin, or Tcf4 (Supplementary Fig. S4A-S4C). These data were in consistent with previous report that DCLK1-A is transcriptionally regulated by a direct binding of LEF1/b-catenin/Tcf4 complex to its DCLK1-A promoter (30). Furthermore, we firstly determined that Wnt3a ligand could upregulate DCLK1-A in multiple colorectal cancer cells (Supplementary Fig. S4D) and provided new evidences that niacinamide could suppress DCLK1-A expression at transcription level (Supplementary Fig. S4E-S4G). Taken together, our data newly discovered that DCLK1-A is a downstream target of Wnt/b-catenin signaling, therefore, a potent target of niacinamide.

DCLK1-B expression is a critical driver of initiation and maintenance of colorectal cancer stemness

To examine whether DCLK1-B is critical for regulating cancer stemness in colorectal CSCs, we designed siRNAs specifically targeting DCLK1-B and validated that two siRNA sequences (siDCLK1-B #1 and siDCLK1-B #2) efficiently decreased DCLK1-B in colorectal cancer cells without affecting DCLK1-A (Supplementary Fig. S5A-S5C). Limiting dilution assay demonstrated that DCLK1-B-knockdown impaired self-renewal abilities of CSCs (Fig. 5A), resulting in a significant decrease in survival potential of CSCs (Fig. 5B; Supplementary Fig. S5D). Moreover, DCLK1-B-knockdown increased AnnexinV+ apoptotic cells (Fig. 5C) by activating apoptotic cascades, such as cleaved PARP and cleaved caspase 3 (Supplementary Fig. S5E and S5F). In addition, CSC inhibitory effects of DCLK1-B-knockdown led to sensitization of colorectal cancer cells to anticancer therapy such as 5-FU and radiation (Fig. 5D and E). Next, to estimate in vivo function of DCLK1-B, we established DCLK1-B-knockout HCT116 cells by CRISPR-Cas9 system. Multiple DCLK1-B-knockout clones were generated using two different gRNA sequences (Supplementary Fig. S5G). Clone #1 (sequence I) and clone #3 (sequence II) were validated to lose DCLK1-B expression without affecting DCLK1-A. In similar with DCLK1-B-knockdown effects (Fig. 5A), the frequency of self-renewing CSC and the proportion of CSC populations were diminished by DCLK1-B-knockout (Supplementary Fig. S5H and S5I). Next, we evaluated in vivo CSC properties of DCLK1-B-knockout cells (clone #1) using two different colorectal cancer mouse models. Because growing evidence suggests that CSCs display greater tumorigenic and metastatic potential in vitro and in vivo than non-CSC cancer cells (4), we estimated both tumor-initiating and metastatic potential of DCLK1-B-knockout cells. First, we subcutaneously transplanted limiting dilutions of DCLK1-B-knockout mice and found that the frequency of tumor-initiating cells was decreased with a statistically significance (Fig. 5F; Supplementary Fig. S5J). Moreover, DCLK1-B-knockout significantly reduced primary tumor volume (Fig. 5G; Supplementary Fig. S5K). Next, we used a tail vein injection model which is a common model for studying lung metastasis. Tail vein injection of HCT116 cells resulted primarily in pulmonary metastases. Interestingly, DCLK1-B-knockout diminished both the number and size of metastatic colonies on lungs (Fig. 5H and I), suggesting that DCLK1-B expression appears to be critical for founding of metastatic colonies and their subsequent robust outgrowth. Finally, we examined whether the reduction of DCLK1-B mediates CSC-inhibitory effects of niacinamide by using DCLK1-B-overexpressing cells (Supplementary Fig. S5L). DCLK1-B-overexpressing cells displayed the increased CD44v6+ CSC population and self-renewal activities. More importantly, DCLK1-B overexpression rescued cancer stemness under niacinamide treatment (Fig. 5J and K). suggesting that suppressive effects of niacinamide on CSCs are achieved, at least in part, through disruption of DCLK1-B expression. Taken together, our results indicated that DCLK1-B, a
Tumors showed significant enrichment in LEF1 target genes (FDR q-value < 0.005; GSE41258). A, A set of leading-edge subset of LEF1 target genes and a set of stemness-related genes (described in the Supplementary Materials and Methods) were compared, and seven common genes were identified. mRNA levels of the seven genes were quantified by real-time PCR following 48-hour treatment of niclosamide. C, mRNA expression levels of DCLK1 were compared between chemo-sensitive and chemoresistant colorectal cancer cells from Oncomine (Gyorffy cell line). D, Significant positive correlations between DCLK1 and LEF1 were observed in colorectal cancer patient tumors (GSE37892 and GSE14333). Statistical significance was determined by correlation analysis using GraphPad software. E, Kaplan-Meier survival analyses were conducted based on DCLK1 expression in two independent colorectal cancer patients cohorts (GSE17538 and GSE14333). DCLK1 expression was determined by immunofluorescence assay in patient-derived colorectal cancer tissues and the paired normal adjacent to tumor (NAT) tissues from the patients with identical colorectal cancer. G, Protein levels of DCLK1-A (82 kDa) and DCLK1-B (47 kDa) isoforms were determined in normal mouse brain tissues, normal human colon epithelial cells, and human colorectal cancer cells by Western blot assays. Total amount of loaded protein is indicated above the wells. β-Actin was used as a loading control. H, DCLK1-A and DCLK1-B protein levels were determined in colorectal cancer tissues and the normal tissues obtained from the identical patients with colorectal cancer. I, HCT116 cells were cultured under attached monolayer condition or sphere-forming conditions to enrich for CSCs. Immunofluorescence assays were performed to visualize DCLK1 expression. Blue indicates nuclei, and red indicates DCLK1. Scale bar represents 100 μm. J, DCLK1-A and DCLK1-B protein levels were compared between attached monolayer colorectal cancer cells and CSC-enriched sphere colorectal cancer cells by Western blot assays. Bar graphs represent the mean ± SD, and statistical analyses were performed by one-way ANOVA with Dunnett’s multiple comparison. *, **, and *** indicate P < 0.05, P < 0.01, and P < 0.001, respectively.

Figure 3.
DCLK1 is a target of LEF1 and correlates with CSCs and poor prognosis in patients with colorectal cancer. A, GSEA of metastatic colorectal cancer patient tumors showed significant enrichment in LEF1 target genes (FDR q-value < 0.005; GSE41258). B, A set of leading-edge subset of LEF1 target genes and a set of stemness-related genes (described in the Supplementary Materials and Methods) were compared, and seven common genes were identified. mRNA levels of the seven genes were quantified by real-time PCR following 48-hour treatment of niclosamide. C, mRNA expression levels of DCLK1 were compared between chemo-sensitive and chemoresistant colorectal cancer cells from Oncomine (Gyorffy cell line). D, Significant positive correlations between DCLK1 and LEF1 were observed in colorectal cancer patient tumors (GSE37892 and GSE14333). Statistical significance was determined by correlation analysis using GraphPad software. E, Kaplan-Meier survival analyses were conducted based on DCLK1 expression in two independent colorectal cancer patients cohorts (GSE17538 and GSE14333). Statistical significance was determined by correlation analysis using GraphPad software. F, Kaplan-Meier survival analyses were conducted based on DCLK1 expression in two independent colorectal cancer patients cohorts (GSE17538 and GSE14333). Statistical significance was determined by correlation analysis using GraphPad software.
Niclosamide Targets DCLK1-Mediated Cancer Stem Functions

Figure 4.
Niclosamide effectively inhibits DCLK1-B expression via the Wnt/β-catenin-LEF1 axis. A, Time-dependent induction of DCLK1-B protein and mRNA levels following Wnt activation in multiple colorectal cancer cells. Cells were treated with Wnt3a (100 ng/mL) in the absence of serum for indicated times, then they were subjected to real-time PCR or Western blot analysis. B, Protein levels of DCLK1-B and LEF1 were determined in wild-type, control (empty vector-transfected), and LEF1-knockout cells with or without Wnt activation (Wnt3a, 100 ng/mL, 2 hours) by Western blot analysis. C and D, Inhibitory effects of niclosamide on DCLK1-B expression were determined in various human colorectal cancer cells by (C) RT-PCR and (D) Western blot assays. Cells were treated with niclosamide for 48 hours. E, Promoter reporter assay was performed against DCLK1-B promoter in HCT116 cells following 24-hour treatment of niclosamide in dose-dependent manner. F, Predicted binding sites of LEF1 on DCLK1-B promoter region according to ALLGEN PROMO database version 3.0.2. G, ChIP assay was conducted in HCT116 cells with or without niclosamide treatment (0.2 μmol/L). Immunoprecipitation was conducted against LEF1 and LEF1-bound DNA fragments amplified by PCR. PCR products were confirmed by gel electrophoresis. Bar graphs represent the mean ± SD (n = 3), and statistical analyses were performed by one-way ANOVA with Dunnett’s multiple comparison. *, **, and *** indicate P < 0.05, P < 0.01, and P < 0.001, respectively.
Figure 1A: Log normon without sphere (10^7 cells/well) of HCT116 cells transfected with siCTRL or siDCLK1-B #1.

Figure 1B: Tumor incidence and necropsy of S.C. injection of HCT116 cells (empty vector and DCLK1-B KO) over 21 days (Days).

Figure 1C: Tumor formation monitoring of Tail vein injection of HCT116 cells (empty vector and DCLK1-B KO) over 21 days (Days).

Figure 1D: Cell viability (%). WT siCTRL #1 #2 100 120 80 40 60 Number of cells inoculated (×10^5).

Figure 1E: Survival fraction (%). Radiation (Gy) NCRT (1 Gy) – + + + + + NS 0 1 2 4 6 8 10 Tumor volume (mm³).

Figure 1F: Cell no. of inoculation: 1 × 10^6.
downstream target of niclosamide, is critical for maintaining CSC populations and CSC properties both in vitro and in vivo.

Niclosamide exerts a potent in vivo antitumor effect in both HCT116 xenografts and AOM/DSS-induced spontaneous colorectal cancer models

Following mechanistic studies of niclosamide on CSC regulation, we investigated whether niclosamide can be a potential therapeutic agent for colorectal cancer using multiple in vivo systems. First, to investigate the tumor growth-inhibitory effect of niclosamide, we subcutaneously xenografted HCT116 cells and treated mice with niclosamide (10 or 40 mg/kg) when the mean primary tumor volume reached 100 mm³. Compared with vehicle, niclosamide significantly inhibited primary tumor growth (Fig. 6A–C). In addition, 10 mg/kg seemed to be sufficiently potent in exerting a maximum effect on primary tumor growth, because there was no significant difference between 10 and 40 mg/kg niclosamide-treated groups. Also, we evaluated therapeutic effect of niclosamide in patient-derived tumor xenograft (PDX) model (Supplementary Fig. S6A–S6C), which was authenticated by short tandem repeat profiling analysis (Supplementary Fig. S6D). The growth of PDX tumors were also inhibited by niclosamide treatment (40 mg/kg). Second, we used the classical model of spontaneous colorectal cancer, AOM/DSS model, to evaluate whether niclosamide can be used as a chemopreventive agent against colorectal cancer development (Fig. 6D). The niclosamide treatment (40 mg/kg) from week 1 to 13 significantly blocked colorectal cancer development, indicating the decreased number of colorectal polyps than control mice (Fig. 6E). Additionally, AOM/DSS treatment significantly reduced colon length, which is a classical symptom of inflammation; however, co-treatment with niclosamide blocked the AOM/DSS-induced shortening of colon (Fig. 6F). Microscopic observations revealed that niclosamide efficiently blocked the AOM/DSS-induced development of intermediate- or high-grade dysplasia (Fig. 6G). Immunofluorescence analysis demonstrated that AOM/DSS-induced dysplastic tissues expressed higher levels of DCLK1 than did normal tissues, and niclosamide reduced the number of DCLK1+ dysplastic regions. Next, to investigate whether this anticancer effect of niclosamide is derived from its CSC-targeting effect, we isolated HCT116 cells from vehicle- or niclosamide-treated primary tumors and evaluated their self-renewal ability through sphere-forming assays (Fig. 6H). HCT116 cells from niclosamide-treated mice did not form tumor spheres as efficiently as HCT116 cells from vehicle-treated mice did. These in vivo results are consistent with our in vitro data, showing that niclosamide-exposed colorectal cancer cells could not restore their self-renewal ability even under niclosamide-free conditions (Fig. 1D). Immunohistochemical analyses demonstrated that CD44v۶/CSC populations were decreased in niclosamide-treateed primary tumors (Fig. 6I), indicating the reduced expression of stemness-related transcription factor OCT4 (Fig. 6J). In the same manner, in vivo limiting dilution assay revealed that niclosamide treatment irreversibly reduced the frequency of tumor-initiating cells in PDX tumors (Supplementary Fig. S6E) resulting in reduction of tumor growth (Supplementary Fig. S6F). Accordingly, the molecular targets of niclosamide, LEF1 and DCLK1, were equally decreased in niclosamide-treated primary tumors (Fig. 6K and L, Supplementary Fig. S6G and S6H). Thus, CSC-targeting niclosamide efficiently attenuated both tumor growth and AOM/DSS-induced colonic dysplasia, suggesting that the inhibition of Wnt/LEF1/DCLK1 axis might be a new therapeutic strategy for colorectal cancer treatment as well as chemoprevention of colorectal cancer development.

Discussion

The use of niclosamide, an anthelmintic family drug, has now been extended to multiple disease models including Helicobacter pylori infection, Parkinson’s disease, and severe acute respiratory syndrome (40–42). Recently, this antiparasitic drug has been proposed as a promising anticancer agent in several types of cancer due to its remarkable ability to inhibit tumor growth (20–24). However, the potential mechanism of niclosamide during colorectal cancer initiation and progression is still elusive. CSCs are believed to initiate cancer and be resistant to chemoradiation by demonstrating higher survival potential and lower apoptosis than non-CSCs, thereby resulting in metastasis and poor prognosis (43). In this study, we reported that niclosamide efficiently decreases therapy resistance in colorectal cancers by reducing CSC populations and their self-renewal activity, thereby attenuating the survival potential of CSCs following...
A. Tumor volume (mm³) over time for different treatments.

B. Colon macroscopic view showing the effects of different treatments.

C. Box plot showing tumor weight (g) across different treatments.

D. Scheme illustrating the administration of AOM and Niclosamide treatments, followed by necropsy.

E. Number of polyps comparison between AOM/DSS and Niclosamide treatments.

F. Colon length comparison between AOM/DSS and Niclosamide treatments.

G. AOM/DSS treatment status and Niclosamide supplementation.

H. Schematic of HCT116 cells treated with Niclosamide, followed by CSC sphere formation evaluation.

I. CD44v6 expression level across different conditions.

J. OCT4 expression level across different conditions.

K. LEF1 expression level across different conditions.

L. DCLK1 expression level across different conditions.
Niclosamide Targets DCLK1-Mediated Cancer Stem Functions

Niclosamide exerts a potent antimetastatic effect in both HCT116 xenografts and AOM/DSS-induced spontaneous colorectal cancer models. A–C, Therapeutic effect of niclosamide was evaluated in colorectal cancer xenograft models. HCT116 cells were subcutaneously inoculated into NPG mice. When the volume of primary tumors reached approximately 100 mm³, tumor-bearing mice were treated with niclosamide (10 mg/kg or 40 mg/kg, daily, i.p.) or vehicle (PBS) (n = 6/group). A, Primary tumor volume was measured twice per week until the day of sacrifice. B, On the day of sacrifice, all primary tumors were isolated, and (C) the primary tumor weights were evaluated. D–G, Therapeutic effect of niclosamide was examined in an AOM/DSS-induced colorectal cancer mouse model. D, C57BL/6 mice were intraperitoneally injected with AOM (10 mg/kg). One week later, the mice were given 2.0% DSS in the drinking water for 2 weeks. Then, the mice were given drinking water without DSS for 2 weeks. This cycle was repeated three times. After injection with AOM, the mice were intraperitoneally treated with niclosamide (20 mg/kg) or vehicle twice a day for 12 weeks (n = 6/group) and sacrificed on the 13th week. On the day of sacrifice, (E) the number of intestinal polyps and (F) colon length were measured. G, DCLK1 expression levels in intestinal polyps were visualized by immunofluorescence and presented with matched H&E images. Blue indicates nuclei, and red indicates DCLK1. H, Colorectal cancer cells were isolated from vehicle- or niclosamide-treated HCT116 xenografts, and the tumor repopulating potential was evaluated by sphere-forming assay. I, CD44v6+ CSC populations within primary tumors of HCT116 xenografted mice were visualized by immunofluorescence assay, and the intensity of CD44v6 expression was quantified using Image-Pro Plus software. Nuclear staining was counterstained with DAPI and a matched region of an H&E-stained section is presented. Scale bar represents 100 μm. J–L, Visualization and quantification were performed as above against various target proteins including (J) OCT4, (K) LEF1, and (L) DCLK1. Bar graphs represent the mean ± SD. Statistical analyses were performed by one-way ANOVA with Dunnett’s multiple comparison among more than three groups or Student t test between two groups. * and ** indicate P < 0.05, P < 0.01, and P < 0.001, respectively.

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Authors’ Contributions
Conception and design: J.-S. Nam
Development of methodology: J.-H. Baek
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.-Y. Park, J.-Y. Kim, J.-H. Choi, J.-H. Kim, C.-J. Lee, J.-H. Baek
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.-Y. Park, J.-Y. Kim, J.-H. Choi, J.-H. Kim
Writing, review, and/or revision of the manuscript: S.-Y. Park, J.-Y. Kim, J.-S. Nam
Study supervision: J.-S. Nam
Other (material support): P. Singh, S. Sarkar

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Inhibition of LEF1-Mediated DCLK1 by Niclosamide Attenuates Colorectal Cancer Stemness

So-Yeon Park, Ji-Young Kim, Jang-Hyun Choi, et al.


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