Niclosamide inhibition of STAT3 synergizes with erlotinib in human colon cancer

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Abstract: Niclosamide, an anthelmintic drug approved by the US Food and Drug Administration against cestodes, is used to treat tapeworm infection. In this study, we show that niclosamide can potentially inhibit signal transducer and activator of transcription 3 (STAT3) in colon cancer cell lines. Combined inhibition of epidermal growth factor receptor and STAT3 by erlotinib and niclosamide synergistically induces apoptosis and antiproliferation in colon cancer cell lines. Our findings suggest that erlotinib and niclosamide combination provides an effective therapeutic approach to improving the prognosis of colon cancer.

Keywords: STAT3, EGFR, erlotinib, niclosamide, colon cancer

Introduction
Colon cancer is the third most common cancer worldwide and the second leading cause of cancer death in the US. The mortality rate of patients with colon cancer has decreased owing to improved detection, but the incidence of colon cancer continues to increase. Considerable progress in the development of chemotherapy for advanced colon cancer has been observed in the recent decade. The epidermal growth factor receptor (EGFR) has been reported as a critical therapeutic target for the treatment of colon cancer. However, an anti-EGFR agent used to treat colon cancer has exhibited moderate efficacy. The mechanisms of this resistance have yet to be elucidated. Nevertheless, the potential of EGFR inhibitors to induce activation of the EGFR-independent pathway is under consideration.

Signal transducer and activator of transcription (STAT) proteins significantly influence diverse biological processes, including cell proliferation, differentiation, survival, and inflammatory response. One of the well-studied STAT members, STAT3, has been shown to be an important molecule in various malignancies and verified as an effective target for cancer therapy, including endometrial, cervical, breast, brain, prostate, and colon cancer. A number of studies have indicated that aberrant STAT3 activation contributes to cell proliferation, differentiation, migration, and survival.

Niclosamide, particularly effective against cestodes, has been used to treat tapeworm infection for approximately 50 years. Finding a new use for traditional medicines is considerably easier than inventing a new drug because traditional medicines have known pharmacokinetics and have often been approved for human treatments. In the past 5 years, niclosamide has been identified as a potential anticancer agent targeting multiple signaling pathways (eg, NF-κB, ROS, Notch, Wnt/b-catenin, and mTORc1). Recently, several studies have reported that niclosamide exhibits antiproliferative activity in head and neck, ovarian, breast, and hematologic cancer.
Till date, no studies demonstrating the therapeutic efficacy of niclosamide in colon cancer are available. Taking this background into account, the present study aimed to investigate the effects of niclosamide on colon cancer and the molecular mechanisms underlying its therapeutic action.

Our outcomes demonstrated that niclosamide acted as a potent STAT3 inhibitor in colon cancer cell lines. The combination of niclosamide and erlotinib induced apoptosis and antiproliferation in colon cancer cell lines as well as sensitization of colon cancer cells to erlotinib. On the basis of our findings, we suggest that combination of niclosamide and erlotinib can potentially improve the prognosis of colon cancer.

Materials and reagents
Cell culture and antibodies
Human colon cancer cell lines (HCT116, SW620, and HT29) were obtained from Cell Resources center of the Shanghai Institutes for Biological Sciences (Chinese Academy of Sciences, Shanghai, People’s Republic of China). The cells were routinely cultured in RPMI-1640 (Gibco/BRL; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) and 1% of antibiotic solution (100 units/mL penicillin and 100 µg/mL streptomycin) in a humidified atmosphere of 5% CO₂ at 37°C.

Antibodies such as anti-BCL-2, anti-Bax, anti-cleaved PARP, anti-GAPDH, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, and HRP-conjugated donkey anti-rabbit IgG were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies against Phospho-STAT3 and STAT3 were purchased from Cell Signaling Technology (Danvers, MA, USA).

Inhibitor drugs
Nifuroxazide, niclosamide, cryptotanshinone, and alantolactone were purchased from Selleck Chemicals (Houston, TX, USA). Nifuroxazide is an oral nitrofuran antibiotic not currently approved for use in the USA but is used elsewhere as an antidiarrheal agent.²⁶ Niclosamide has been used to treat tapeworm infection and also as a molluscicidal for water treatment in schistosomiasis control programs.²⁷ Cryptotanshinone, a major lipophilic component isolated from Salvia miltiorrhiza Bunge, has been shown to possess chemotherapeutic properties against various types of cancer cells.²⁸ Alantolactone is used as an insect repellent, antibacterial, and anticancer agent.²⁹ The drug compounds were dissolved in sterile dimethyl sulfoxide (DMSO) to produce a 20 mM stock solution, which was then stored at −20°C and further diluted freshly with cell culture medium.

MTT assay
Cells were seeded into wells of a 96-well plate at 5×10³ cells per well in 100 µL of the corresponding medium. The cells were then treated with drugs at different concentrations for 72 h. Subsequently, they were treated with a fresh solution of MTT (5 mg/mL) for 4 h at 37°C. The purple formazan crystals were finally solubilized with DMSO solution, and absorbance was recorded using a multi-well plate reader at 490 nm.

Western blot analysis
Cells were lysed in a lysis buffer containing a phosphatase inhibitor, and the lysates were clarified by centrifugation (12,000 rpm) at 4°C for 10 min. The supernatant was run on 10% and 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. After being blocked with 5% nonfat dry milk in Tris Buffer Solution Tween for 1.5 h, membranes were incubated with a specific primary antibody of 1:1,000 dilution overnight and a HRP-conjugated secondary antibody of 1:3,000 dilution for 1 h. Immunoreactive bands were visualized using enhanced chemiluminescence reagent.

Molecular docking
As one of the most widely used computational approaches for structure-based drug design, molecular docking study was used to predict the binding pose of compound in STAT3 SH2-binding site by using the software AutoDock (version 4.2.6).³⁰ The crystallographic coordinate for human STAT3 SH2 (Protein Data Bank [PDB] ID: 1BG1) was obtained from the PDB.³¹ Prior to docking, protein structures were prepared by removing water molecules and other ligands using PyMol software.³² A grid box size of 60×60×60 dimensions with a spacing of 0.375 Å between the grid points was implemented and covered almost the entire SH2-binding site. The grid parameter files were created setting up the map files directly. The Lamarckian genetic algorithm was applied to deal with the interactions of protein and inhibitors. The number of individuals in population was set to 300, and trials of 100 dockings and maximum number of energy evaluations were set as default along with other settings. AutoDockTools version 1.5.6 and PyMol were used to analyze the docking results.

Clonogenic assay
A total of 500 cells per well were seeded into a 6-well plate with 2 mL of RPMI-1640 and incubated overnight. The cells were then pretreated with nifuroxazide and erlotinib or DMSO for 8–12 h. After treatment, the cells were washed with phosphate buffer saline (PBS) twice and transferred to
a fresh medium to grow for 7 days. Colonies were washed with PBS and then fixed with 4% methanol for 15 min at room temperature. The cells were washed with PBS twice and stained with 1% crystal violet (25% methanol) for 10 min at room temperature. Each experiment was conducted thrice.

Analysis of cell apoptosis
Cells (3×10^5) were seeded in 6-well plates and incubated overnight and then treated with nifuroxazide and erlotinib for 24 h. After treatment, the cells were harvested with trypsin and then washed with cold PBS twice. The cells were stained with Annexin V for 10 min under dark conditions and then with propidium iodide (PI) for 5 min. Apoptotic cells were counted using the FACS Calibur flow cytometer and quantified by flow cytometric analysis.

Statistical analyses
Data are represented as mean ± standard error of the mean of 3 independent experiments. Student’s t-test was performed to determine the statistical significance between 2 groups by using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). Differences between groups were analyzed by the log-rank test using GraphPad Prism 6.0. *P<0.05 was considered statistically significant.

Results
Antiproliferative effects of niclosamide in human colon cancer cells
Nifuroxazide acts as a potent inhibitor of STAT3 signaling pathway in breast cancer cells, though it has little effect on cells lacking STAT3 activation.33 Niclosamide has recently been identified to target multiple signaling pathways (eg, NF-κB, ROS, Notch, and STAT3).16 Cryptotanshinone has previously been observed to possess the most powerful antibacterial, anti-inflammatory, and antitumor effect.34 Alantolactone has an inhibitory effect on cancer cells migration, invasion, adhesion, and colony formation.35 Therefore, we screened the antiproliferative effects of these 4 compounds in human colon cancer cells by MTT assay.

After the cells were treated with nifuroxazide, niclosamide, cryptotanshinone, and alantolactone for 72 h, MTT assay was employed. As shown in Figure 1, administration of these 4 compounds in a dose-dependent manner reduced viability in SW620 (Figure 1A), HCT116 (Figure 1B), and HT29 cells (Figure 1C). Among these compounds, niclosamide exhibited the most potent antiproliferative effect against all tested human colon cancer cell lines. After exposure of the cells to niclosamide for 72 h, the half maximal inhibitory concentration (IC_{50}) in SW620, HCT116, and HT29 cell lines was found to be 2.9, 0.4, and 8.1 μM, respectively (Figure 1D). This suggests that niclosamide can inhibit the proliferation of human colon cancer cells.

Molecular docking between STAT3 and niclosamide
STAT3, a member of the STAT family of transcription factors, has recently been verified as an attractive therapeutic target and shown to be an important molecule in cancer therapy, including colon cancer.36 As the STAT3 SH2 domain is critical for the activation and biological function of STAT3,37 we investigated interactions between the compound and the SH2 domain of STAT3 by molecular docking analysis of the active compound niclosamide (Figure 2A). We applied PyMol software to remove water molecules and other ligands to prepare the STAT3 SH2 domain protein structures and used the software AutoDock (version 4.2.6) to predict the binding pose of compound in STAT3 SH2-binding site to identify whether compound niclosamide acts as a STAT3 inhibitor that targets the STAT3 SH2 domain.38 The molecular docking results indicated that niclosamide formed 4 hydrogen bonds with the side chain of ARG-595, SER-636, GLU-594, and LYS-591 as shown in Figure 2B. It was predicted that niclosamide could fit into the 2 major binding sites, the pTyr705 and the side pocket site, and so it could inhibit STAT3 phosphorylation. In summary, critical hydrogen bond interaction between niclosamide and STAT3 SH2 domain were predicted by a rational method combined with molecular docking. These methods clarified the interaction of niclosamide with STAT3. Further verification of co-crystalization of STAT3 and niclosamide is in progress.

Inhibition of STAT3 phosphorylation by niclosamide in colon cancer cells
HCT116 and SW620
We performed Western blot analysis on colon cancer cell lines to examine whether niclosamide could inhibit STAT3. Western blot analysis indicated that niclosamide inhibited STAT3 phosphorylation (Figure 3). Notably, niclosamide inhibited STAT3 phosphorylation in a time- and dose-dependent manner in HCT116 and SW620 cell lines.

Sensitization of colon cancer cells to erlotinib by treatment of cells with niclosamide
Erlotinib or gefitinib, EGFR tyrosine kinase inhibitors, have been shown to benefit patients with non-small-cell lung cancer and pancreatic cancer. However, almost all patients develop a progressive disease during therapy. As shown
**Figure 1** Antiproliferative effects of niclosamide in human colon cancer cells.

**Notes:** Colon cancer cell lines (A) SW620, (B) HCT116, and (C) HT29 were treated with different concentrations (60, 12, 2.4, 0.48, and 0.098 µM) of nifuroxazide (nifu), niclosamide (nicl), cryptotanshinone (cry), and a lantolactone (ala), respectively, for 72 h. Cell proliferation in each group was detected by MTT assay. (D) HCT116, SW620, and HT29 cell lines were suppressed with niclosamide treatment at different concentrations (20, 10, 5, 2.5, and 1.25 µM) for 72 h. Subsequently, cell proliferation in each group was detected by MTT assay. The data were obtained from 3 independent experiments.

**Abbreviation:** IC₅₀, half maximal inhibitory concentration.

**Figure 2** MD analysis of the effect of niclosamide on the activity cavity of STAT3.

**Notes:** (A) Molecular structure of niclosamide. (B) Last snapshot of STAT3/niclosamide in 50 ns MD simulations.

**Abbreviation:** MD, molecular dynamics.
in Figure 4A, after treatment of cells with erlotinib and gefitinib for 72 h, the IC₅₀ in SW620, HCT116, and HT29 cells exceeded 60 µM. These results indicate that erlotinib and gefitinib could not inhibit the proliferation of SW620, HCT116, and HT29 cell lines. We thus examined the anti-proliferative effects of erlotinib as well as erlotinib combined with niclosamide by MTT assay. As shown in Figure 4B, treatment of cells with niclosamide sensitized colon cancer cells to erlotinib, while niclosamide had no effect on SW620, HCT116, and HT29 cells at 0.5 µM and 1 µM concentrations (Figure 4C). To verify the anticancer properties of the combination, we performed colony-forming experiments. SW620 cells (5000) were seeded in a 6-well plate overnight and then treated with erlotinib (10 µM) or and niclosamide (2.5 µM) for 1 week. We then removed the drugs and washed the cells with PBS twice. The cell colonies were stained

Figure 3 Inhibition of STAT3 phosphorylation by niclosamide in colon cancer cells HCT116 and SW620.
Notes: (A) SW620 cells were treated with niclosamide (5 µM) for different lengths of time (0, 2, 4, 6, 8, and 12 h). Total protein was extracted, and the expression levels of P-STAT3, STAT3, and GAPDH proteins were detected by Western blot analysis. (B) HCT116 cells were treated with niclosamide (5 µM) for different lengths of time (0, 2, 4, 6, 8, and 12 h). Total protein was extracted, and the expression levels of P-STAT3, STAT3, and GAPDH proteins were detected by Western blot analysis. (C) SW620 cells were treated with niclosamide at different concentrations (0.5, 1, 2.5, 5, and 10 µM) or vehicle control (DMSO) for 12 h. (D) HCT116 cells were treated with niclosamide at different concentrations (0.5, 1, 2.5, 5, and 10 µM) or vehicle control (DMSO) for 12 h. Total protein was then extracted and detected by Western blot analysis. The data were obtained from 3 independent experiments. *P<0.05.
Abbreviation: DMSO, dimethyl sulfoxide.
Figure 4 Sensitization of colon cancer cells to erlotinib by niclosamide.

Notes: (A) HCT116, SW620, and HT29 cells were seeded in a 96-well plate at a density of 5,000 cells per well and then cultured for 72 h. The cells were treated with erlotinib and gefitinib at indicated concentrations (60, 12, 2.4, 0.48, and 0.096 µM). After the 72 h treatment, cell proliferation in each group was detected by MTT assay. (B) HCT116, SW620, and HT29 cells were seeded at a density of 5,000 cells per well. Cells were treated with erlotinib at various concentrations (60, 12, 2.4, 0.48, and 0.096 µM) or/and niclosamide (0.5 and 1 µM) in triplicate for 72 h to detect the effects of combined erlotinib and niclosamide by MTT assay. (C) HCT116, SW620, and HT29 cells were treated with niclosamide (0.5 and 1 µM) in triplicate for 72 h to detect the effects of niclosamide by MTT assay. (D and E) For colony formation assay, SW620 cells were used. DMSO, dimethyl sulfoxide; E, erlotinib; nic, niclosamide.

Abbreviations: DMSO, dimethyl sulfoxide; E, erlotinib; nic, niclosamide.
with crystal violet and then counted. The combination of niclosamide and erlotinib suppressed the colony formation of colon cancer cells, as shown in Figure 4D and E. Colony formation assay demonstrated that the synergistic effect of niclosamide and erlotinib efficiently inhibits the growth of colon cancer cells.

**Induction of apoptosis in colon cancer cells with combined niclosamide and erlotinib**

Compared with single-agent treatment, the combination of erlotinib and niclosamide enhanced the inhibition of cell viability in all tested cell lines (Figure 4B). We sought to investigate whether the combination of erlotinib and niclosamide could also induce cell apoptosis. We first employed Western blot analysis. HCT116 and SW620 cells were treated with erlotinib and niclosamide alone, or with both for 24 h. The combination significantly increased cleaved PARP and BAX, as shown in Figure 5A and B. BCL-2 expression was markedly decreased.

We also used flow cytometry to examine whether the combination induced cell apoptosis in SW620 cells. SW620 cells were treated with erlotinib (10 µM) or/and niclosamide (2.5 µM) for 24 h and then stained with Annexin V and PI for

**Figure 5 (Continued)**
Figure 5 Induction of apoptosis in colon cancer cells by combined niclosamide and erlotinib.  
Notes: (A) HCT116 cells were treated with erlotinib (10 µM) and/or with or without niclosamide (2.5 µM) for 24 h. P-STAT3, BCL-2, and BAX were detected by Western blot analysis. (B) SW620 cells were treated with erlotinib (10 µM) and/or with or without niclosamide (2.5 µM) for 24 h. P-STAT3, BCL-2, and BAX were detected by Western blot analysis. (C and D) SW620 cells were treated with erlotinib (10 µM) and/or with or without niclosamide (2.5 µM) for 24 h. The level of apoptosis was evaluated with Annexin V and PI, followed by detection using flow cytometry. *P<0.05; **P<0.01.  
Abbreviations: DMSO, dimethyl sulfoxide; e, erlotinib, nicl, niclosamide.

apoptosis analysis. The combination resulted in a significant increase in early (Annexin V+, PI−) and late apoptotic cells (Annexin V+, PI+), as shown in Figure 5C and D.

Discussion
Colon cancer is one of the most prevalent tumors worldwide. Despite conventional therapies such as surgery, radiation, and chemotherapy, the overall survival rate for colon cancer has not significantly improved. The human EGFR is overexpressed in various gastrointestinal cancer types, including 60%–80% of colon cancers, correlating with poor prognosis and early disease progression. In addition, cancer cell also upregulates other important downstream genes such as STAT3, which contributes to cell proliferation, cell survival, and angiogenesis in colon cancer. Therefore, more effective approaches need to be developed to improve the prognosis of colon cancer.

Here, our results showed that niclosamide reduces cell viability in a dose-dependent manner. This finding suggests that niclosamide could be effective against colon cancer. Several groups have independently revealed the activity of niclosamide against cancer cells; however, the precise mechanism underlying this antitumor activity has yet to be elucidated. Therefore, we first used molecular docking study to verify whether there is interaction between niclosamide and STAT3. In our study, we found critical hydrogen bond interaction between niclosamide and STAT3 SH2 domain. We further investigated the correlation between niclosamide and STAT3 in SW620 and HCT116 cell lines, and the results showed that niclosamide suppressed STAT3 phosphorylation in a time- and dose-dependent manner. Several studies have revealed that inhibition of EGFR by erlotinib induces activation of STAT3, which contributes to erlotinib resistance. Our data showed that when erlotinib and niclosamide were combined, they synergistically induced apoptosis of colon cancer cells. It has been reported that multiple signal transduction pathway inhibitors, including the mitogen-activated protein kinase, fibroblast growth factor receptor inhibitors, and chemotherapy drugs, can induce activation of STAT3 survival signaling pathway, leading to resistance. It is possible that, in addition to erlotinib, niclosamide may also sensitize these target drugs. Based on our findings, we conclude that niclosamide may represent a novel and more effective drug for colon cancer.

Drug development process from initial lead discovery to final medication is costly, lengthy, and incremental. Finding new uses for old or natural products is much easier and more economical than inventing a new drug from scratch. The present study demonstrates that niclosamide can inhibit the cell
growth of colon cancer by inhibiting STAT3 phosphorylation. We also investigated the combined inhibition of EGFR and STAT3 by using erlotinib and niclosamide to synergistically repress colon cancer. This study was conducted only in vitro; some compounds may exhibit potent antitumor activities in vitro but show no antitumor activity in vivo. Therefore, further molecular investigations in preclinical and clinical settings are warranted to evaluate anticancer potential in a synergistic manner to substantiate niclosamide as an effective therapeutic candidate for colon cancer.

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Disclosure
The authors report no conflicts of interest in this work.

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