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Review

Niclosamide, an old antihelminthic agent, demonstrates antitumor activity by blocking multiple signaling pathways of cancer stem cells

Jing-Xuan Pan[1], Ke Ding[2] and Cheng-Yan Wang[1]

Abstract

Niclosamide, an oral antihelminthic drug, has been used to treat tapeworm infection for about 50 years. Niclosamide is also used as a molluscicide for water treatment in schistosomiasis control programs. Recently, several groups have independently discovered that niclosamide is also active against cancer cells, but its precise mechanism of antitumor action is not fully understood. Evidence supports that niclosamide targets multiple signaling pathways (NF-κB, Wnt/β-catenin, Notch, ROS, mTORC1, and Stat3), most of which are closely involved with cancer stem cells. The exciting advances in elucidating the antitumor activity and the molecular targets of this drug will be discussed. A method for synthesizing a phosphate pro-drug of niclosamide is provided. Given its potential antitumor activity, clinical trials for niclosamide and its derivatives are warranted for cancer treatment.

Key words Niclosamide, cancer, cancer stem cells, signal transduction

Niclosamide, whose systematic name is (5-chloro-N-2-chloro-4-nitrophenyl)-2-hydroxybenzamide, is an oral antihelminthic drug used for approximately 50 years for treating most tapeworm infections. It is also used as a molluscicide for water treatment in schistosomiasis control programs. The activity of niclosamide against these parasites is believed to be mediated by inhibition of mitochondrial oxidative phosphorylation and anaerobic ATP production[1]. Recently, several groups have independently discovered that niclosamide is active against cancer cells[2-4], though its precise mechanism of antitumor action is not fully understood. Accumulating evidence suggests that niclosamide targets multiple signaling pathways such as nuclear factor-kappaB (NF-kB), Wnt/β-catenin, and Notch, most of which are closely involved with cancer stem cell proliferation. This mini-review focuses on the exciting progress in elucidating the antitumor activity and the molecular targets of this drug.

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Niclosamide Inhibits Tumor Cell Growth

Niclosamide inhibits the proliferation of tumor cells with minimal effect on normal cells

Niclosamide has shown antiproliferative activity in a broad spectrum of cancer cells including hematologic cancer cells (e.g., acute myeloid leukemia, AML) and solid tumor cells (e.g., colon cancer, breast cancer, and prostate cancer)[2-5]. The 50% inhibition concentration (IC_{50}) values ranged from 0.18 to 1 μmol/L for AML cells (Table 1). Furthermore, niclosamide showed impressive *in vivo* antitumor activity in xenograft nude mouse models[2,3].

Drug resistance and relapse are challenges for chemotherapy, but combining agents that target different pathways may help prevent these challenges. We have reported that niclosamide may be useful not only as a monotherapy but also in combination therapy. Indeed, we found that niclosamide showed synergistic effects with frontline chemotherapeutic agents for AML (e.g., cytarabine, etoposide, and daunorubicin)[2]. Similarly, the additive anti-proliferative activity of niclosamide when combined with the cytotoxic agent oxaliplatin was noted

Table 1. The half maximal inhibition concentration (IC_{50}) values of niclosamide in some tumor cell lines and normal cells

Tumor type	Cell line	IC_{50} (μmol/L)	Reference
Leukemia			
Acute myeloid leukemia	U937	0.41	[2]
	OCI-AML3	0.79	[2]
	HL-60	0.48	[2]
	Molm13	0.72	[2]
	MV4-11	1.0	[2]
Solid tumor			
Prostrate cancer	Du145	0.7	[5]
	PC3	11.7±2.3	[5]
Colorectal cancer	HT29	7.2±1.2	[5]
	HCT116	2.2	[4]
Cervical carcinoma	HeLa	0.25±0.07	[5]
Lung adenocarcinoma	A549	3.0±0.2	[5]
Epithelial carcinoma	A431	8.8±0.9	[5]
Normal cell			
	MEF	4.54	[2]
	NHFB	5.78	[2]
	Bone marrow	9.00±4.87	[2]

in colorectal cancer cell lines HCT116, HT29, and $CaCO_2$[3].

Although niclosamide has a potent activity against tumor cells, it has minimal effects on the viability of normal cells. We reported that the IC_{50} of niclosamide in mouse embryonic fibroblast (MEF) cells and normal human fibroblast (NHFB) cells was 4.54 and 5.78 μmol/L, respectively, based on MTS assay after drug treatment for 72 h [2]. In addition, the IC_{50} value for bone marrow cells from four healthy donors was (9.00 ± 4.87) μmol/L [2]. Osada *et al.* [3] also reported that niclosamide did not have significant toxicity against nonmalignant cancer cells such as mammary epithelial cells (MCF-10A) and peripheral blood mononuclear cells from healthy individuals. These findings from us and others suggest that niclosamide possesses a therapeutic window for its antitumor effects.

Niclosamide inhibits cancer cell metastasis and migration

Sack *et al.*[4] evaluated the effect of niclosamide on the migration and invasion of cells overexpressing S100 calcium-binding protein A4 (S100A4), an 11-kDa protein originally identified as metastasin 1 (MTS1) that is essential for metastasis in colon cancer[6]. Exposure of colon cancer HCT116 cells to 1 μmol/L niclosamide for 24 h reduced cell migration to less than 50% of solvent-treated control cells as measured by Boyden chamber and scratch wound healing assays; however, ectopic expression of S100A4 counteracted the effect of niclosamide on cell migration[4]. In an assay conducted in Matrigel-covered Boyden chambers, niclosamide also showed potent activity against invasion of HCT116 cells (30% of control). Similarly, S100A4 overcame the niclosamide-mediated inhibition of cell motility, suggesting the involvement of S100A4 in this process.

By using noninvasive *in vivo* luminescence imaging, Sack *et al.* [4] monitored the effect of niclosamide on metastasis in a mouse colon cancer xenograft model. The results showed that niclosamide treatment inhibited S100A4-induced metastasis *in vivo*.

Niclosamide eradicates cancer stem cells

Human cancers are believed to originate from a small fraction of cancer stem cells capable of uncontrolled self-renewal and aberrant differentiation. The persistence of cancer stem cells is an important mechanism of chemotherapeutic resistance and relapse after conventional chemotherapy. Cancer stem cells share some key properties including signaling pathways (e.g., Wnt, Notch, and hedgehog) with normal stem cells[7]. Notably, several signaling pathways critical for self-renewal and proliferation of cancer stem cells (e.g., NF-κB, Notch, and Wnt/β-catenin) are targets of niclosamide[2,4,8]. Thus, we hypothesized that niclosamide is effective against cancer stem cells, and we indeed

discovered that niclosamide at nanomolar cencentrations killed AML stem cells ($CD34^+CD38^-$) with minimal cytotoxicity against the progenitor cells in normal bone marrow cells from three healthy donors[2]. In this sense, niclosamide may be a promising chemotherapeutic agent to effectively eradicate leukemic stem cells while sparing normal hematopoietic tissue. This aspect of clinical efficacy remains to be investigated in clinical trials. In addition, future work should investigate whether niclosamide inhibits cancer stem cells in solid tumors and its effect on the "stemness" molecules.

Molecular Targets of Niclosamide

Niclosamide inhibits the NF-κB pathway

The transcription factor NF-κB has been demonstrated to promote cancer growth, angiogenesis, escape from apoptosis, and tumorigenesis. NF-κB is sequestered in the cytosol of resting cells through binding the inhibitory subunit IκBα. IκBα can be phosphorylated by the IκB kinase (IKK) complex (containing IKKα, IKKβ, and NEMO/IKKγ) subunits in response to diverse stimuli[9]. Phosphorylated IκBα is then degraded by the ubiquitin-proteosome pathway, allowing NF-κB translocation into the nucleus and transactivation of its target genes. IKK is regulated predominantly by TGF-β–activated kinase-1 (TAK1) in the canonical pathway of cytokine-induced NF-κB activation[10,11]. Niclosamide blocked TNFα-induced IκBα phosphorylation, translocation of p65, and the expression of NF-κB–regulated genes (Figure 1). The inhibitory effects of niclosamide involved two steps: TAK1 →IKK and IKK →IκBα. Niclosamide also inhibited the DNA binding of NF-κB to the promoter of its target genes[2]. Inhibition of NF-κB pathway was a key molecular mechanism for the antileukemic action of niclosamide. Of note, leukemia stem cells but not normal hematopoietic stem cells display constitutive activation of NF-κB, and blocking the NF-κB pathway is believed to selectively kill leukemia stem cells.

Niclosamide elevates ROS levels

Reactive oxygen species (ROS) generation is an important mechanism by which certain chemotherapeutic agents (e.g., arsenic tetroxide, farnesyltransferase inhibitors, and phenethyl isothiocyanate) kill tumor cells[12]. Because niclosamide was reported to inhibit NADH → $NADP^+$ transhydrogenation in the *Hymenolepis diminuta* submitochondrial particles assay, we tested whether niclosamide induces ROS generation and discovered that niclosamide treatment in AML cells led to a ~20-fold elevation of ROS[2].

Quenching ROS by N-acetyl-cysteine (NAC) attenuated but did not completely abrogate niclosamide-mediated apoptosis[2]. In a separate approach, the mitochondrial respiration-deficient ρ-cells (C6F/HL-60 cells), which presumably generate less ROS, underwent the same extent of IκBα phosphorylation and p65 translocation as parental HL-60 cells. Therefore, niclosamide inhibited the TNFα-induced NF-κB activation in a ROS-independent manner. Conversely, TNFα treatment did not influence the niclosamide-induced ROS generation[2]. Taken together, our data support that niclosamide has two independent effects: NF-kB activation and ROS elevation (Figure 1).

Niclosamide inhibits the Wnt/β-catenin pathway

The Wnt signaling pathway plays fundamental roles in directing tissue patterning in embryonic development, in maintaining tissue homeostasis in differentiated tissue, and in tumorigenesis. In canonical Wnt signaling, Wnt binds and activates Frizzled receptor and low-density lipoprotein-related protein receptors 5 and 6 (LRP5 and LRP6) to regulate the stability of the downstream protein β-catenin by modulating its destruction complex containing axin, adenomatous polyposis kinase (APC), glycogen synthase kinase 3β (GSK3β), and casein kinase. Stabilized β-catenin then translocates to the nucleus to bind and initiate the transcriptional activity of TCF/LEF transcription factors.

In a recent study using a high-throughput screening approach, Chen *et al.*[13] validated that niclosamide is a potent inhibitor of the Wnt/β-catenin pathway (Figure 2). Niclosamide promoted Frizzled1 internalization, down-regulated the expression of Dishevelled-2 protein, and inhibited β-catenin stabilization[3]. Independently, Sack *et al.*[4] demonstrated that niclosamide inhibited β-catenin/TCF transcription-activating complex and diminished the expression of downstream target S100A4, which is a driving force of metastasis in colon cancer cells. Interestingly, a single treatment of 1 μmol/L niclosamide reduced S100A4 at levels of mRNA and protein *in vitro*, and discontinuous niclosamide treatment still had a major effect of prolonging overall survival in a xenograft mouse model[4].

Niclosamide inhibits the Notch pathway

The Notch signaling pathway plays important roles in a variety of cellular processes such as proliferation, differentiation, apoptosis, cell fate decisions, and maintenance of stem cells. Upon activation, Notch receptor regulates downstream target genes through both C promoter-binding factor-1 (CBF-1)–dependent and CBF-1-independent pathways. Wang *et al*[8] reported that niclosamide potently suppresses the luciferase activity of

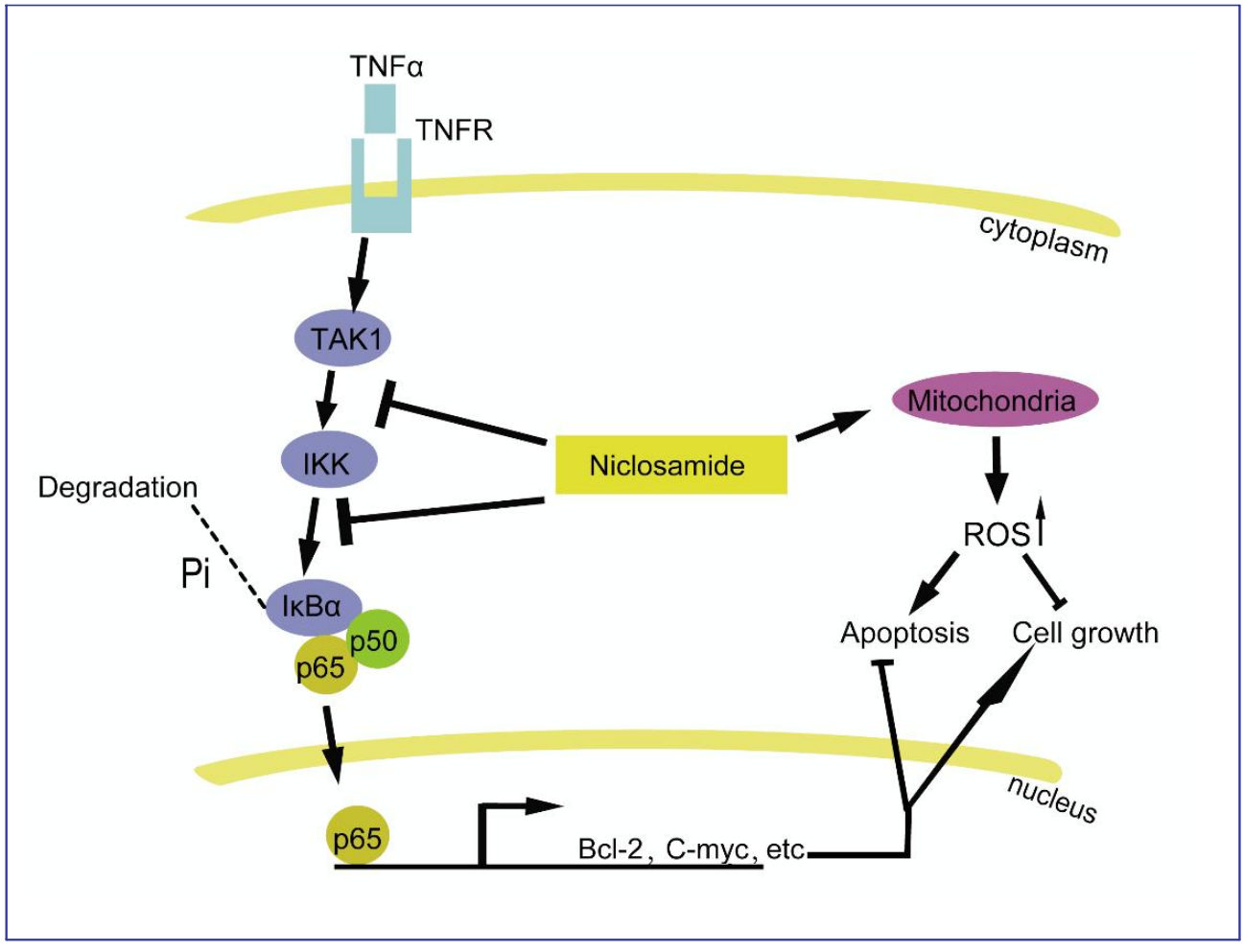

Figure 1. **Niclosamide targets nuclear factor-kappaB (NF-κB) and elevates reactive oxygen species (ROS) levels.** Niclosamide blocks the tumor necrosis factor-α (TNFα)-induced I B phosphorylation, translocation of p65, and the expression of NF-κB–regulated genes. On the other hand, niclosamide elevates the intracellular ROS content. TNFR, tumor necrosis factor receptor; TAK, TGF-β-activated kinase; IKK, IκB kinase.

a CBF-1–dependent reporter gene in both a dose-dependent and a time-dependent manners in K562 leukemia cells (Figure 2). Taken together, these observations suggest that niclosamide has the potential to treat Notch signaling pathway-associated diseases.

Niclosamide inhibits the Stat3 pathway

Ren *et al.*[5] reported that niclosamide is a selective inhibitor of Stat3 but not the closely related Stat1 or Stat5. Namely, niclosamide treatment abrogated the epidermal growth factor (EGF)–stimulated dimerization and nuclear translocation and transcriptional activity of Stat3, and induced cell growth inhibition and apoptosis in several types of cancer cells (e.g. Du145, Hela, A549) that exhibit relatively higher levels of Stat3 constitutive activation[5].

Niclosamide inhibits the mTORC1 pathway and the proteasome

Using an automated cell-based screening assay, Balgi *et al.*[14] identified that niclosamide can rapidly increase autophagosome formation. Biochemical assays showed that niclosamide induced autophagy and inhibited mammalian target of rapamycin complex 1 (mTORC1) but not mTORC2 signaling in cells maintained in nutrient-rich conditions (Figure 2). Furthermore, niclosamide prevented the formation of large ubiquitin-containing aggregates caused by proteasome inhibition, suggesting that niclosamide may inhibit protein ubiquitination or promote the selective clearance of ubiquitinated proteins in the absence of proteasome activity.

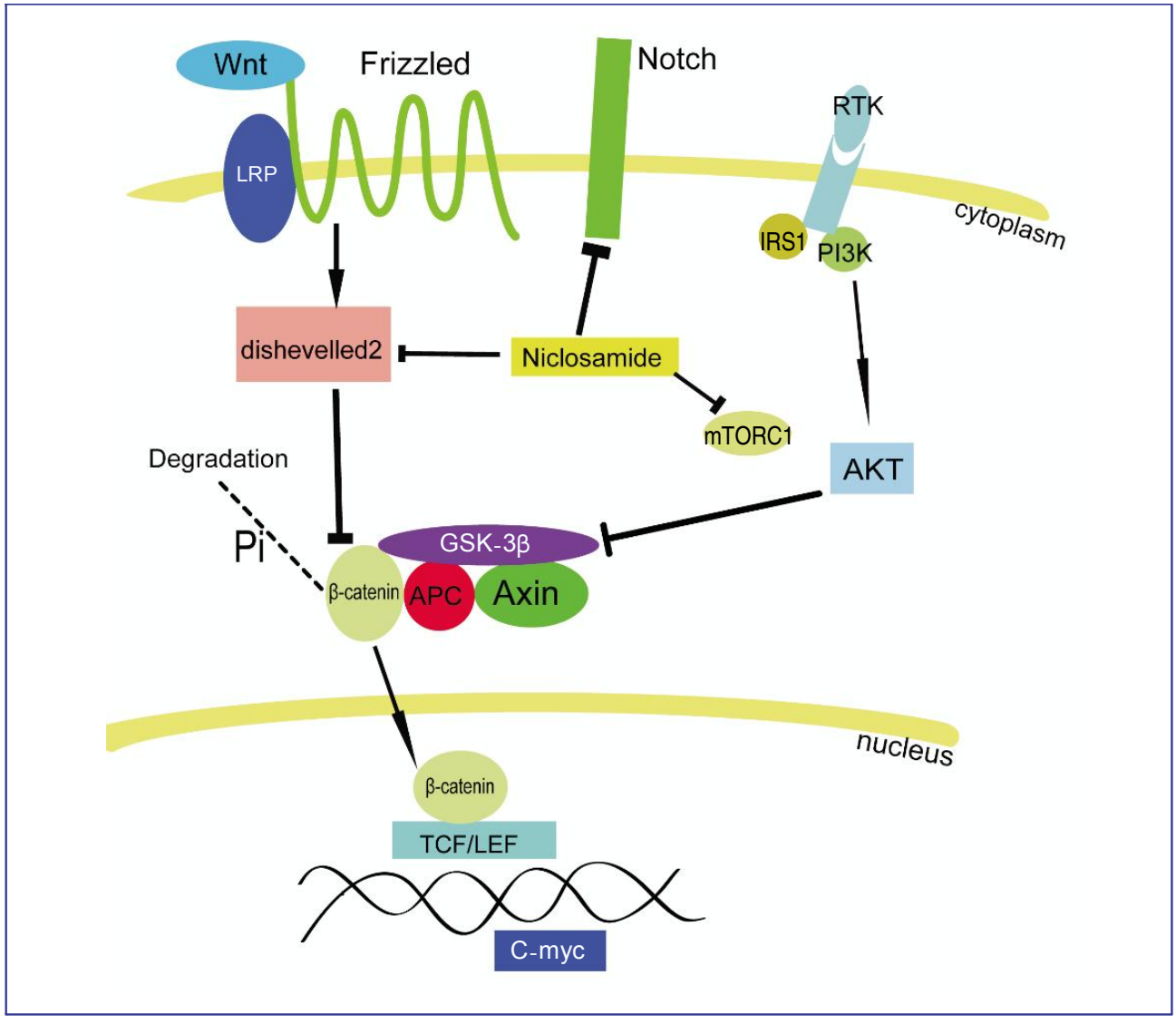

Figure 2. **Niclosamide targets the Wnt/β-catenin, Notch, and mTORC1 pathways.** Niclosamide down-regulates the expression of Dishevelled-2 and inhibits the stabilization of β-catenin. Niclosamide also inhibits Notch signaling pathway and mTORC1. LRP, low-density lipoprotein-related protein receptor; IRS1, insulin receptor substrate 1; RTK, receptor tyrosine kinase.

Pharmacokinetics and Safety

Niclosamide is a Food and Drug Administration (FDA)–approved drug that is given orally for helminthosis. A single 5 mg/kg oral administration of niclosamide in rats could generate a maximal plasma concentration of 1.08 μmol/L [15], which was sufficient to kill AML cells [2]. However, poor bioavailability of niclosamide because of its limited water solubility and absorption is a challenge. Indeed, niclosamide is practically insoluble in water, but the solubility does increase sparingly over the pH range of 8–10. Preparation of solid dispersions with PEG-6000 and addition of polyamidoamine (PAMAM) dendrimers were reported to improve the solubility and *in vitro* dissolution properties of niclosamide[16].

To improve the water solubility of niclosamide, we modified this drug to a phosphate pro-drug [2]. In the addendum of this article, the procedure for synthesis of water-soluble p-niclosamide was made available, upon the request of many readers.

Niclosamide has low toxicity in mammals (oral median lethal dose in rats >5000 mg/kg) [17], which is obviously associated with poor absorption. The safety of niclosamide needs to be re-evaluated if it is translated to chemotherapy for cancer patients by systemic intravenous administration.

Conclusions

Niclosamide is active against cancer cells such as AML and colorectal cancer cells, not only as a monotherapy but also as part of combination therapy, in which it has been found to be synergistic with frontline chemotherapeutic agents (e.g., oxaliplatin, cytarabine, etoposide, and daunorubicin). Because niclosamide targets multiple signaling pathways (e.g., NF-κB, Wnt/β-catenin, and Notch), most of which are closely involved with cancer stem cells, it holds promise in eradicating cancer stem cells. These exciting findings warrant clinical trials of niclosamide in cancer patients.

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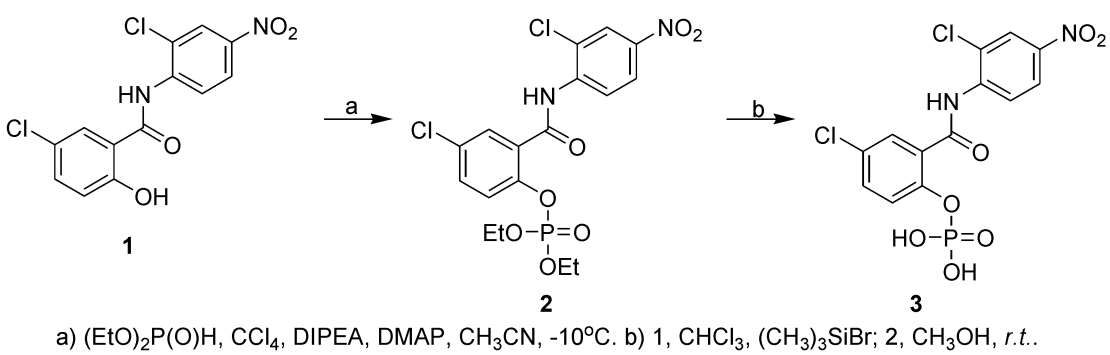
Addendum

In order to improve the water solubility of niclosamide and hence to improve its bioavailability *in vivo*, we have synthesized the phosphate of niclosamide (Scheme 1).



a) $(EtO)_2P(O)H$, CCl_4, DIPEA, DMAP, CH_3CN, -10°C. b) 1, $CHCl_3$, $(CH_3)_3SiBr$; 2, CH_3OH, *r.t.*.

Scheme 1. **Synthesis of water-soluble p-niclosamide.** First, niclosamide (compound 1) was phosphorylated with diethyl phosphite to yield compound 2. The designed compound 3 was obtained by a simple deprotection of compound 2 with an excellent yield (Tetrahedron Letters, 1996, 37:771). To the stirred suspension of niclosamide (654 mg, 2 mmol) in anhydrous CH_3CN (10 mL), CCl_4 (1.54 g, 10 mmol), DIPEA (N,N-Diisopropylethylamine) (516 mg, 4 mmol), and DMAP (4-Dimethylaminopyridine) (24 mg, 0.2 mmol) were added at –10℃. After stirring for a further 10 min, diethyl phosphate (414 mg, 3 mmol) was added dropwise at the same temperature. The reaction was completed within approximately 1 h (as determined by thin layer chromatography). Aqueous KH_2PO_4 (0.5 mol/L) was then added to quench the reaction, and the mixture was allowed to warm to room temperature. The mixture was extracted three times with ethyl acetate. The organic phase was then combined and washed successively with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The product was used in the next step without any further purification. The product obtained was dissolved in $CHCl_3$ (20 mL), followed by addition of $(CH_3)_3SiBr$ (3.06 g, 20 mmol) at room temperature. After stirring for 24 h, the mixture was concentrated under reduced pressure. CH_3OH (20 mL) was then added, and the mixture was stirred for 30 min at room temperature. After the reaction was completed, the solvent was removed *in vacuo* and yielded a white solid (660 mg, 81% in two steps).

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