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Multi-targeted therapy of cancer by niclosamide: a new application for an old drug

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Abstract

The rapid development of new anticancer drugs that are safe and effective is a common goal shared by basic scientists, clinicians and patients. The current review discusses one such agent, namely niclosamide, which has been used in the clinic for the treatment of intestinal parasite infections. Recent studies repeatedly identified niclosamide as a potential anticancer agent by various high-throughput screening campaigns. Niclosamide not only inhibits the Wnt/ β -catenin, mTORC1, STAT3, NF- κ B and Notch signaling pathways, but also targets mitochondria in cancer cells to induce cell cycle arrest, growth inhibition and apoptosis. A number of studies have established the anticancer activities of niclosamide in both *in vitro* and *in vivo* models. Moreover, the inhibitory effects of niclosamide on cancer stem cells provide further evidence for its consideration as a promising drug for cancer therapy. This article reviews various aspects of niclosamide as they relate to its efficacy against cancer and associated molecular mechanisms.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Keywords

niclosamide; FDA-approved drug; multi-targeted therapy; drug discovery; cancer stem cells

1. Introduction

Niclosamide (trade name Niclocide), a teniacide in the anthelmintic family which is especially effective against cestodes, has been approved for use in humans for nearly 50 years (Fig. 1) [1, 2]. Niclosamide inhibits oxidative phosphorylation and stimulates adenosine triphosphatase activity in the mitochondria of cestodes (eg. tapeworm), killing the scolex and proximal segments of the tapeworm both *in vitro* and *in vivo* [2]. Niclosamide is well tolerated in humans. The treatment of *T. saginata* (beef tapeworm), *D. latum* (fish tapeworm) and *Dipylidium caninum* (dog tapeworm) in adult is 2 g as a single oral dose. For the treatment of *H. nana* (dwarf tapeworm), the same oral dose is used for 7 days [2].

Drug development, from the initial lead discovery to the final medication, is an expensive, lengthy and incremental process [3]. Finding new uses for old or failed drugs is much faster and more economical than inventing a new drug from scratch, as existing drugs have known pharmacokinetics and safety profiles and have often been approved for human use, therefore any newly identified use(s) can be rapidly evaluated in clinical trials [4]. In the last 5 years niclosamide has been identified as a potential anticancer agent by various high-throughput screening campaigns. This article reviews the current studies regarding various aspects of niclosamide as they relate to its potential new use in cancer therapy.

2. Niclosamide – a multiple pathway inhibitor for anti-cancer efficacy

Recently, several studies reported the inhibitory effects of niclosamide on multiple intracellular signaling pathways. The signaling molecules in these pathways are either over-expressed, constitutively active or mutated in many cancer cells, and thus render niclosamide as a potential anticancer agent. The effects of niclosamide on these pathways are described below.

2.1. The Wnt/ β -catenin pathway

The Wnt/ β -catenin signaling pathway regulates cancer progression, including tumor initiation, tumor growth, cell senescence, cell death, differentiation and metastasis [5-7]. In the absence of Wnt, β -catenin is sequestered in a complex that consists of the adenomatous polyposis coli (APC) tumor suppressor, axin, glycogen synthase kinase-3 β (GSK3 β), and casein kinase 1 (CK1). This complex formation induces the phosphorylation of β -catenin by CK1 and GSK3 β , which results in the ubiquitination and subsequent degradation of β -catenin by the 26S proteasome. Conversely, when Wnt proteins form a ternary complex with the cell surface receptors, low-density lipoprotein receptor-related protein5/6 (LRP5/6) and Frizzled (Fzd), signaling from Wnt receptors proceeds through the proteins dishevelled (Dvl) and axin, leading to the inhibition of GSK3 β and the stabilization of cytosolic β -catenin. The β -catenin then translocates into the nucleus where it interacts with T-cell factor/lymphoid enhancing factor (TCF/LEF) to induce the expression of specific target genes [5-7] (Fig. 2A).

Chen *et al.* performed a high-throughput screening of a library containing approximately 1200 FDA-approved drugs and drug-like molecules with a primary imaged-based green fluorescent protein (GFP) fluorescence assay that used Fzd1 endocytosis as the readout in human osteosarcoma U2OS cells, and identified niclosamide as a small molecule inhibitor of Wnt/ β -catenin signaling [8]. Niclosamide promoted Wnt receptor Fzd1 endocytosis, downregulated Dvl2 protein, and inhibited Wnt3A-stimulated β -catenin stabilization and TCF/LEF reporter activity in U2OS cells [8]. It also decreased the cytosolic expression of endogenous Dvl2 and β -catenin in human colorectal cancer cell lines and in human colorectal cancer cells isolated by surgical resection of metastatic disease, regardless of mutations in APC [9]. Moreover, we recently demonstrated that niclosamide was able to inhibit Wnt/ β -catenin signaling by promoting Wnt co-receptor LRP6 degradation, but had no effect on Dvl2 expression in prostate and breast cancer cells [10, 11], suggesting that the mechanism underlying niclosamide-mediated inhibition of Wnt/ β -catenin signaling could be cell type-dependent (Fig. 2A). In addition, the inhibitory effects of niclosamide on Wnt/ β -catenin signaling were also demonstrated in primary human glioblastoma cells [12].

More than 90% of colorectal cancers bear mutations that result in the activation of the Wnt/ β -catenin pathway [13]. S100 calcium binding protein A4 (S100A4) is a target gene of the Wnt/ β -catenin pathway in colorectal cancers [14]. Sack *et al.* performed a high-throughput screening of the LOPAC chemical library containing 1280 compounds with a cell-based luminescence assay that used S100A4 promoter-driven luciferase activity as the readout in human colorectal cancer HCT116 cells, and identified niclosamide as an inhibitor of S100A4 through reduction of S100A4 mRNA and protein expression in colorectal cancer cells [15]. It was proposed that niclosamide inhibits β -catenin/TCF complex formation and thereby interrupts target gene transcription, an alternative model of Wnt/ β -catenin inhibition by niclosamide in colorectal cancer cells [15] (Fig. 2A).

2.2. The mTORC1 pathway

The mammalian target of rapamycin complex 1 (mTORC1) is a heterotrimeric protein kinase that consists of the mTOR catalytic subunit and two associated proteins, raptor (regulatory associated protein of mTOR) and mLST8 (mammalian lethal with sec-13). The mTORC1 activity is regulated by upstream signals from growth factors, amino acids, stresses and energy state, and its activation induces the phosphorylation of p70S6 kinase (p70S6K) and eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4E-BP1), leading to the enhanced translation of a subset of mRNAs that are critical for cell growth and metabolism [16, 17] (Fig. 2B). Activation of either the serine/threonine protein kinase Akt (also known as protein kinase B or PKB) or the extracellular signal-regulated kinase (ERK) pathway, or inhibition of the adenosine monophosphate-activated protein kinase (AMPK) pathway, leads to activated mTORC1 signaling. As a downstream effector of Akt, mTORC1 has been described as the most essential effector in driving cell proliferation and susceptibility to oncogenic transformation. This leads to the targeting of mTORC1 as a therapeutic strategy in many types of cancer [16, 17].

Balgi *et al.* performed a high-throughput screening of a collection of >3,500 chemicals with a primary imaged-based enhanced GFP (EGFP) fluorescence assay that used EGFP-LC3

punctate staining as the readout in human breast cancer MCF-7 cells, and identified niclosamide as an inhibitor of mTORC1 signaling in cells maintained in nutrient-rich conditions [18]. Niclosamide did not inhibit mTORC2, which also contains mTOR as a catalytic subunit, suggesting that niclosamide does not inhibit mTOR catalytic activity but rather inhibits signaling to mTORC1 [18]. Tuberous sclerosis complex (TSC2), a negative regulator of mTORC1, was not required for the inhibition of mTORC1 signaling by niclosamide, as niclosamide was able to suppress mTORC1 signaling in TSC2-deficient cells where mTORC1 activity was elevated [18]. Further studies demonstrated that niclosamide does not impair PI3K/Akt signaling, nor does it interfere with mTORC1 assembly and mTORC1 kinase activity [19]. It had been proposed that increased cytosolic acidification is responsible for the mTORC1 inhibition, and that the structural features of niclosamide required for protonophoric activity are essential for the mTORC1 inhibition [19]. Lysosome is the degrading machine for autophagy, but has also been linked to mTORC1 activation through the Rag/RRAG GTPase pathway [20]. More recently, Li *et al.* proposed that mTORC1 inhibition by niclosamide is caused by lysosomal dysfunction [21]. Niclosamide inhibits the lysosomal degradative function likely by altering lysosomal permeability and the pH gradient [21], which is consistent with the finding that niclosamide can cause dispersion of protons from the lysosomes to the cytosol, leading to cytosolic acidification [19] (Fig. 2B).

2.3. The STAT3 pathway

Signal transducers and activators of transcription 3 (STAT3), a member of a family of six different transcription factors, is one of the down-stream signaling proteins for cytokine and growth factor receptors [22]. Engagement of cell-surface cytokine or growth factor receptors activates the janus kinase (JAK) family of protein kinases, which in turn phosphorylates STAT3 at tyrosine residue 705, leading to the dimerization of two STAT3 monomers through the SH2 domains of the proteins [23, 24]. The activated STAT3 dimers then translocate into the nucleus and activate the transcription of a panel of genes that control cell proliferation, apoptosis, angiogenesis and other cell functions [25, 26]. This JAK2/STAT3 signaling pathway is one of the three major modules that play an essential role in transmitting external signals from the surface membrane to target genes in the nucleus, controlling processes such as growth, differentiation, senescence and apoptosis [27, 28]. Importantly, STAT3 is the major intrinsic pathway for cancer inflammation owing to its frequent activation in malignant cells and key role in regulating many genes crucial for cancer inflammation in the tumor microenvironment [22].

Ren *et al.* demonstrated that niclosamide is a potent STAT3 inhibitor that suppresses STAT3 transcriptional activity by blocking its phosphorylation and nuclear translocation in prostate cancer DU145 cells [29] (Fig. 2C). The inhibitory effect of niclosamide on STAT3 signaling was subsequently reported in multiple myeloma cells [30]. STAT3 can mediate cancer cell survival by activating antiapoptotic genes such as *Bcl-2* and *Bcl-XL*. Recently, it was reported that inhibition of STAT3 by niclosamide blocked the erlotinib activated STAT3/*Bcl-2*/*Bcl-XL* pathway and reversed erlotinib resistance of human head and neck cancer cells and non-small cell lung cancer cells [31, 32]. Furthermore, niclosamide not only blocked the ionizing radiation-induced activation of the STAT3/*Bcl-2*/*Bcl-XL* pathway in

both radiosensitive and radioresistant human lung cancer cells, but also reversed the radioresistance and restored the sensitivity of radioresistant cells to ionizing radiation [33].

2.4. The NF- κ B pathway

Nuclear factor-kappaB (NF- κ B) is an important transcription factor associated with cancer, and has been implicated in many hallmarks of cancer development, including growth factor independent proliferation, inhibition of apoptosis, tumor angiogenesis, limitless replicative potential and tissue invasion and metastasis [34, 35]. NF- κ B is activated by several agents, including cytokines, oxidant free radicals, inhaled particles, ultraviolet irradiation, and bacterial or viral products. In response to these stimuli, I κ B kinase (IKK) is activated and can phosphorylate the inhibitory I κ B α subunit of the NF- κ B-I κ B α complex in the cytoplasm. This phosphorylation marks I κ B α for degradation by the proteasome and releases NF- κ B from the inhibitory complex, and the freed NF- κ B proteins are then transported into the nucleus where they bind to their target sequences and activate gene transcription [36, 37].

Jin *et al.* demonstrated that niclosamide blocked tumor necrosis factor α (TNF- α)-induced I κ B α phosphorylation, translocation of NF- κ B p65 subunit, and expression of NF- κ B-regulated genes in acute myelogenous leukemia cells [38]. The inhibitory effects of niclosamide on NF- κ B signaling were also reported in multiple myeloma cells [30] and primary human glioblastoma cells [12]. Transforming growth factor- β (TGF- β) activated kinase1 (TAK1) mediates IKK activation in the TNF- α signaling pathway [39, 40]. It was demonstrated that niclosamide inhibited the steps of TAK1 $\rightarrow$ IKK and IKK $\rightarrow$ I κ B α of the NF- κ B activation [38] (Fig. 2D). In addition, niclosamide inhibited the DNA binding of NF- κ B to the promoter of its target genes [38].

2.5. The Notch pathway

The Notch signaling pathway represents a critical component in the molecular circuits that control cell fate during development. Under physiological conditions, the Notch ligand binds to its receptor to initiate Notch signaling by releasing the intracellular domain of the Notch receptor (Notch-IC) through a cascade of proteolytic cleavages by both α -secretase and γ -secretase. The released intracellular Notch-IC then translocates into the nucleus where it modulates gene expression primarily by binding to a ubiquitous transcription factor, CBF1, and suppressor of hairless, Lag-1 (CSL). In cancers, molecular genetic alterations cause increased levels of intracellular Notch-IC and subsequent constitutive activation of the Notch pathway. Aberrant activation of this pathway contributes to tumorigenesis, and Notch inhibitory agents, such as γ -secretase inhibitors, are being investigated as candidate cancer therapeutic agents [41-43].

Wang *et al.* developed a cell based luciferase reporter gene assay to measure the endogenous CBF1-dependent Notch signaling in human erythroleukemia K562 cells, and identified niclosamide as a potent inhibitor of Notch signaling [44]. Niclosamide inhibited the luciferase activity of CBF1-dependent reporter gene, and suppressed the expression of cleaved activated Notch1 receptor and Notch target Hes-1 in K562 cells [44] (Fig. 2E). In

addition, the inhibitory effects of niclosamide on Notch signaling were also reported in primary human glioblastoma cells [12].

3. Niclosamide targeting of mitochondria

Mitochondria are vital for cellular bioenergetics and play a central role in determining the point-of-no-return of the apoptotic process. It has been proposed that targeting mitochondria is an efficient strategy for cancer chemotherapy [45]. Khanim *et al.* screened a panel of 100 off-patent licensed oral drugs for anti-myeloma activity, and identified niclosamide as a killer of multiple myeloma cell lines and primary multiple myeloma cells [30]. Interestingly, niclosamide anti-multiple myeloma activity could be mainly mediated through the mitochondria with rapid loss of mitochondrial membrane potential, uncoupling of oxidative phosphorylation and production of mitochondrial superoxide [30]. Moreover, Park *et al.* screened the LOPAC chemical library and identified niclosamide as a potent inducer of mitochondrial fission, which induced mitochondria fragmentation and promoted both apoptotic and autophagic cell death [46]. In addition, it was reported that inactivation of NF- κ B by niclosamide caused mitochondrial damage and the generation of reactive oxygen species (ROS), leading to apoptosis of acute myelogenous leukemia cells [38]. Finally, Yo *et al.* performed a genome-wide gene expression array and demonstrated that niclosamide was able to disrupt multiple metabolic pathways affecting biogenetics, biogenesis, and redox regulation in ovarian-cancer-initiating cells. These disruptions presumably lead to the activation of the intrinsic mitochondrial apoptosis pathway, loss of tumor stemness, and growth inhibition [47].

4. Anti-cancer activity of niclosamide: *in vitro* studies

Anti-cancer activity of niclosamide has been demonstrated in human breast cancer [10, 11, 18, 48, 49], prostate cancer [10, 29], colon cancer [9, 15], ovarian cancer [47], multiple myeloma [30], acute myelogenous leukemia [38], glioblastoma [12], head and neck cancer [21] and lung cancer cells [31]. As summarized in the earlier sections, niclosamide is able to block the multiple signaling pathways that govern cancer initiation and progression, thus it is not surprising that niclosamide has a potent activity to induce cancer cell cycle arrest, growth inhibition and apoptotic death across multiple cancer types [50].

The NCI-60 human tumor cell line anticancer drug screen was developed in the late 1980s as an *in vitro* drug-discovery tool, and was rapidly recognized as a rich source of information about the mechanisms of growth inhibition and tumor-cell kill [51]. To further characterize the anticancer activities of niclosamide, we performed a 60 human tumor cell line anticancer drug screen with niclosamide. We found that niclosamide inhibited cell proliferation of all tested cancer cell lines, and that the IC₅₀ values for most cell lines were less than 1 μ M (Table 1). These results indicate that niclosamide has a significant broad-spectrum and potent *in vitro* anticancer activity.

Niclosamide is able to potentiate the cytotoxicity towards tumor cells of different anticancer agents in several *in vitro* cancer models. It enhanced the anti-proliferative activity of oxaliplatin, a commonly used drug for colorectal cancer, in human colorectal cancer Caco2 cells and colorectal cancer explant cells [9]. Furthermore, inhibition of STAT3 by

niclosamide blocked erlotinib-induced STAT3 phosphorylation and sensitized human head and neck cancer cells and non-small cell lung cancer cells to erlotinib [31, 32]. In addition, combinatorial drug testing established that a heterozygous deletion of the *NFKB1A* locus in glioblastoma samples could serve as a genomic biomarker for predicting synergistic activity of niclosamide with temozolomide, the current standard in glioblastoma therapy [12]. Finally, synergistic effects of niclosamide in combination with Ara-C, VP-16, and DNR, the frontline chemotherapeutic agents for acute myelogenous leukemia, have been reported in acute myelogenous leukemia stem cells [38].

5. Anti-cancer activity of niclosamide: *in vivo* studies

The efficacy of niclosamide has been shown against tumor growth and metastases in several xenograft models. Jin *et al.* demonstrated for the first time that niclosamide has *in vivo* anti-cancer activities [38]. As niclosamide has limited solubility in water, Jin *et al.* synthesized a niclosamide analog – phosphate of niclosamide (p-niclosamide), and found p-niclosamide showed significant inhibition of xenograft tumor growth of acute myeloid leukemia HL-60 cells by suppressing the NF- κ B pathway [38]. Subsequently, Osada *et al.* reported that niclosamide inhibited the growth of colorectal cancer cells in NOD/SCID mice by down-regulating Dvl2 and β -catenin expression [9]. Moreover, niclosamide in combination with erlotinib potently repressed erlotinib-resistant head and neck cancer xenografts and lung cancer xenografts by inhibiting STAT3 signaling [31, 32]. In addition, niclosamide alone or in combination with radiation overcame radioresistance in lung cancer xenografts [33]. Niclosamide was also able to significantly diminish the malignant potential of primary human glioblastoma cells *in vivo* by suppressing intracellular Wnt/ β -catenin-, NOTCH-, mTORC1-, and NF- κ B signaling cascades [12]. TRA-8 is an agonistic monoclonal antibody (mAb) to TRAIL death receptor 5 (DR5) [52, 53]. We recently found that niclosamide in combination with TRA-8 suppressed the growth of basal-like breast cancer 2LMP orthotopic tumor xenografts by inhibiting Wnt/ β -catenin and STAT3 signaling [11]. Importantly, niclosamide was able to inhibit the tumor formation of ovarian-cancer-initiating cells derived from cancer cell lines *in vivo* [47] and tumor growth of breast cancer stem-like cell subpopulations *in vivo* [49]. Finally, it was found that niclosamide significantly reduced liver metastasis formation in mice bearing xenografted intrasplenic colon tumors by suppressing the expression of the Wnt/ β -catenin signaling target S100A4 [15], as S100A4 plays a critical role in metastasis formation in colon cancer [14, 54-56]. Overall, these studies reveal the *in vivo* anticancer activities of niclosamide, and provide a rationale for its use in clinical trials.

6. Niclosamide as a drug for targeting CSCs

Current cancer treatments such as chemotherapy, targeted therapy and radiotherapy are successful at destroying bulk cancer cells, but fail to eliminate cancer stem cells (CSCs). CSCs are characterized by tumorigenic properties and the ability to self-renew, form differentiated progeny, and develop resistance to therapy. The inability to eradicate CSCs is thought to be the reason for cancer relapse and chemo-resistance, the major obstacles in current cancer therapy [57-59]. The Wnt/ β -catenin, Notch, and Hedgehog pathways are three major pathways which have been implicated in CSC tumorigenic properties and the ability

to self-renew, form differentiated progeny, and develop resistance to therapy [43, 60-62]. However, many other pathways such as the mTORC1, STAT3 and NF- κ B pathways are also involved in CSC self-renewal and tumor initiation [63].

Many studies have been performed on surface markers for potential identification and isolation of CSCs [59]. In some cases, stem-like cancer cells were identified using the flow cytometry-based side population (SP) technique [64, 65]. Wang *et al.* isolated the SP of breast cancer MCF-7 cells, generated SP spheres, and performed a high-throughput drug screening using these SP spheres and the LOPAC chemical library, and identified niclosamide as an inhibitor of breast cancer stem-like cells [49]. Niclosamide downregulated several stem cell signaling pathways including the Wnt/ β -catenin, Notch, and Hedgehog pathways, inhibited the formation of spheroids, and induced apoptosis in breast cancer SP spheres. Animal studies also confirmed this therapeutic effect [49]. Using a similar approach, the same group further demonstrated that niclosamide was an inhibitor of stem-like ovarian-cancer-initiating cells, and suppressed the tumor formation of ovarian-cancer-initiating cells *in vivo* [47]. Moreover, it was reported that niclosamide was effective in killing acute myeloid leukemia (AML) stem cells (CD34⁺CD38⁻), but had minimal cytotoxicity against progenitor cells in normal bone marrow [38]. In addition, STAT3-mediated stem cell marker *OCT-4* gene expression was effectively suppressed by treatment with niclosamide during mammosphere culture of breast cancer MDA-MB-231 cells [48]. Aldehyde dehydrogenase (ALDH) is a known marker of CSCs [66, 67]. Recently, we isolated a non-adherent population of cells that have high ALDH expression, and found that niclosamide suppressed Wnt/ β -catenin and STAT3 signaling, and showed cytotoxicity against these non-adherent ALDH expressing cells in addition to the adherent cells from four basal-like breast cancer cell lines [11]. The inhibitory effects of niclosamide on CSCs provide compelling evidence for its consideration as a promising drug for cancer therapy.

Salinomycin, a polyether ionophore antibiotic isolated from *Streptomyces albus*, is used as an antibiotic in animal husbandry [68]. Recently, Gupta *et al.* performed a high throughput screening to discover agents with specific toxicity for epithelial CSCs, and identified salinomycin as a selective inhibitor of breast CSCs [69]. Promising results from preclinical trials in human xenograft mice and a few clinical pilot studies reveal that salinomycin could be a promising novel anti-cancer agent [70-72]. Ketola *et al.* performed connectivity map analysis to identify compounds with similar or opposite effects to salinomycin [73]. The differentially expressed genes in response to salinomycin exposure for 6 h in prostate carcinoma VCaP cells were compared with the 47,000 expression profiles representing drug responses to 41,309 compounds. Interestingly, niclosamide was one of the two top enriched compounds altering gene expression in the same direction as salinomycin [73], providing further evidence that niclosamide, like salinomycin, can work as a CSC killer.

7. Challenges to using niclosamide as an anti-cancer agent in humans

The oral dose of niclosamide for adult in cestocidal treatment is 2 g as a single dose, leading to maximal serum concentrations of 0.25 to 6.0 μ g/ml (corresponding to 0.76 – 18.35 μ M) [1], which is well within the anti-cancer active concentration range. The reason for this wide range of serum concentrations is considered to be due to intra-individual difference in

absorption rate [1]. Niclosamide has poor water solubility, and the oral bioavailability of niclosamide was only 10% in male Sprague-Dawley rats [74]. The plasma half-life ($t_{1/2}$) of niclosamide was 6.7 ± 2.0 hr in rats [74]. However, no specific data for pharmacokinetic parameters of niclosamide in humans is available in the literature [2]. Overall, the low oral bioavailability and wide range of serum concentrations of niclosamide could result in variation of its anti-cancer efficacy when it is used in clinical studies.

Studies in animals have suggested that niclosamide has no mutagenic, oncogenic, or embryotoxic activity [1]. Long term experiments in rats (1,000 or 2,500 mg/kg b.w. oral daily for 55 or 64 days, thereafter 10,000 and 25,000 mg/kg feed; total duration 365-381 days) and dogs (100 mg/kg b.w. orally in capsules for 366-393 days) found no indication of a higher incidence of tumor occurrence [1]. Niclosamide has been proven to be without effect on hematological and urinalysis parameters and not to influence liver and kidney function tests [1]. Recent studies have also indicated that no significant toxicity was shown against non-tumor cells *in vitro* and no obvious side effects were observed in niclosamide-treated mice [9, 30, 38]. The fate of niclosamide in humans, which closely resembles that in animals, and the low acute toxicity in humans and animals suggest that even a prolonged contact with niclosamide will not result in cumulative toxic effects in humans [1]. However, niclosamide is poorly absorbed from the intestinal tract, which explains its good tolerability in humans [1, 2]. Therefore, additional safety studies on niclosamide with a well absorbed formulation are required before definitive conclusions can be drawn for cancer therapy.

8. Future directions

While considerable high-throughput screening campaigns have identified niclosamide is a potent inhibitor of a number of biological signaling pathways that mediate niclosamide's anticancer effects *in vitro* and *in vivo*, direct targets of niclosamide still remain unclear. Niclosamide was tested *in vitro* against a panel of 95 protein kinases, and it did not significantly inhibit any of these kinases at concentrations (1 or 10 μ M) efficiently inhibiting the Wnt/ β -catenin, mTORC1, STAT3, NF- κ B and Notch signaling pathways in cancer cells, indicating that the mechanism of action of niclosamide is probably not direct kinase inhibition [19]. Among the techniques currently available, protein affinity isolation using suitable small-molecule probes (pulldown) and subsequent mass spectrometric analysis of the isolated proteins appears to be the most powerful and most frequently applied [75]. Therefore, the targets of niclosamide could be identified using affinity reagents – biotinylated derivatives of niclosamide in the future.

Although many studies have demonstrated that niclosamide has potent *in vitro* and *in vivo* anti-tumor growth activities, the cancer chemopreventive efficacy of niclosamide has not been studied yet. Therefore, there is a great need for further research to examine cancer chemopreventive activity of niclosamide in various animal carcinogenesis models and transgenic models.

Niclosamide is an FDA-approved drug that is given orally to helminthosis patients, however its applications in cancer therapy could be limited, as niclosamide is only partially absorbed from the gastrointestinal tract. Thus, there is great interest in conducting studies to elucidate

the structure-activity relationship of niclosamide and to identify novel derivatives of niclosamide with improved bioavailability as additional clinical candidates for cancer therapy [76, 77].

Finally, intravenous administration of niclosamide to rats gave rise to a peak plasma concentration of 25 μ M [74]. Thus, an intravenous injectable niclosamide formulation would be desirable. However, the safety and possibility of systemic intravenous application of niclosamide needs to be comprehensively investigated before niclosamide could be administered intravenously in the clinic.

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Fig. 1.
The chemical structure of niclosamide.

Fig. 2.

The intracellular signaling pathways inhibited by niclosamide in cancer cells. (A) Niclosamide blocks Wnt/ β -catenin signaling by enhancing Wnt receptor Fzd1 internalization, promoting Wnt co-receptor LRP6 degradation, suppressing Wnt signaling regulator Dvl2 expression, and inhibiting β -catenin/TCF complex formation. (B) mTORC1 inhibition by niclosamide is associated with cytosolic acidification and lysosomal dysfunction. (C) Niclosamide inhibits STAT3 transcriptional activity by blocking its phosphorylation and nuclear translocation. (D) Niclosamide ablates TNF-induced NF- κ B activation at TAK1 and IKK steps. (E) Niclosamide inhibits Notch signaling by suppressing the expression of cleaved activated Notch1 receptor.

Table 1

Effects of niclosamide on growth of 60 human tumor cell lines

IC ₅₀ (μM)		IC ₅₀ (μM)	
Leukemia		Ovarian	
Leukemia	CCRF-CEM	Renal	IGR-OV1
	HL-60 (TB)	786-0	OVCAR-3
	K562	A498	OVCAR-4
	MOLT-4	ACHN	OVCAR-5
	RPMI-8226	CAKI-1	OVCAR-8
Leukemia	SR	RXF 393	NCI/ADR-RES
		SN12C	SK-OV-3
		TK-10	
		UO-31	
Non-Small Cell Lung		Melanoma	
Non-Small Cell Lung	A549/ATCC	Colon	LOX IMVI
	EKVX	COLO 205	MALME-3M
	HOP-62	HCC-2998	MI4
	HOP-92	HCT-116	MDA-MB-435
	NCI-H226	HCT-15	SK-MEL-2
	NCI-23	HT29	SK-MEL-28
	NCI-H322M	KM12	SK-MEL-5
	NCI-H460	SW-620	UACC-257
	NCI-H522		UACC-62
Breast	MCF-7	CNS	Prostate
	MDA-MB-231	SF-268	PC-3
	MDA-MB-468	SF-295	DU-145
	HS 578T	SF-539	
	BT-549	SNB-19	
	T-47D	SNB-75	
		U251	

Cells were seeded into 96-well tissue culture treated microtiter plates at a density of 5,000-20,000 cells/well (depending on cell line) in a total volume of 50 μ l. After overnight incubation, the cells were treated with niclosamide for 72 h in triplicate by adding 50 μ l of 2X stock solutions to appropriate wells already containing 50 μ l of cells and medium to expose cells to the final concentrations of niclosamide required. Cell viability was measured by the Cell Titer Glo Assay (Promega).