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Molecular Mechanisms of Antitumor Activity of Niclosamide

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Abstract—The review summarizes literature data on the antitumor activity of niclosamide and its molecular mechanisms. Earlier niclosamide was used for treatment of intestinal parasitic infections. Recent screening of various drugs and chemical compounds, aimed at identifying putative agents with high antitumor activity, revealed that it possesses such activity. Niclosamide not only inhibits such signaling pathways as Wnt/β-catenin, mTORC1, Stat3, NF-κB and Notch, but also damages tumor cell mitochondria, inhibits proliferation and induces apoptosis. The effectiveness of niclosamide has been demonstrated in vitro experiments as well as in vivo models using xenotransplantation of human tumors to immunodeficient mice. Niclosamide was active not only against tumor cells but also cancer stem cells. Normal cells are resistant to niclosamide. The accumulated experimental data suggest that niclosamide is a promising pharmacological agent for the treatment of various types of cancer.

Keywords: niclosamide, antitumor agents, cancer stem cells, signaling pathways, mitochondrial damage

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INTRODUCTION

Modern trends in the search for new antitumor drugs are often based on screening of a large number of both new compounds and already known pharmacological agents with different therapeutic activities. According to the latest literature data, niclosamide, a drug used for 50 years for treatment of anthelminthic teniasis and disinfection of water in areas of high infestation with schistosomes, is one of such newly identified antitumor agents. Its antitumor activity has been detected in several recently performed screening studies, which have attracted much attention of researchers from various scientific centers. Creating modern anticancer drugs and new treatments of tumors are targeted not only to provide effective elimination of the bulk of the tumor cells, but to a much greater extent, to remove cancer stem cells (CSC) at the maximum preservation of normal stem cells (SC) and cells of critical organs. Recent publications show that niclosamide does not damage normal tissue, but exhibits activity against tumor cells and CSC. The aim of this review is to analyze new data on study of antitumor activity of this drug and its molecular mechanisms.

1. NICLOSAMIDE AND ITS PROPERTIES

Niclosamide (systematic name: 5-chloro-*N*-(2-chloro-4-nitrophenyl)-2-hydroxybenzamide, Fig. 1) is well known as an anthelmintic agent taken orally in the pill form. In Russia, it is known as phenasal [1]. Niclosamide acts as an uncoupler of mitochondrial respiration and oxidative phosphorylation in tapeworms [2], snails [3], and tumor cells [4].

Niclosamide pharmacokinetics has been investigated in rats after its intravenous (2 mg/kg) and oral (5 mg/kg) administration [5]. Since niclosamide is poorly soluble in water, for administration to animals it was dissolved in a solvent mixture of the following composition: dimethyl sulfoxide (DMSO)/Cremophor EL/water in the ratio of 3/15/82 (v/v/v). Under these conditions, the preparation gave a true niclosamide solution; the latter is quite important as niclosamide solubility in water is very low (especially in an acidic medium; in alkaline conditions it increases slightly). During oral administration niclosamide bioavailability was 10%; this is close to its bioavailability in humans. The study of acute toxicity of niclosamide has shown that in rats exposed to its oral administration LD_{50} values exceeded 5000 mg/kg, while studies

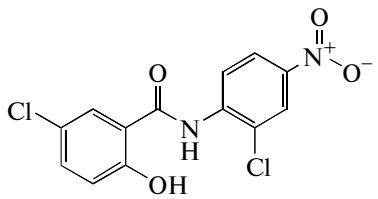

Fig. 1. Structural formula of niclosamide.

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of its subacute toxicity during administration of a daily dose of 2000 mg/kg for 4 weeks did not reveal any signs of toxicity [5]. Good tolerability of these high doses suggests a good therapeutic index and a wide safe range of niclosamide concentrations.

According to the literature, the targets of the antitumor effect of niclosamide are signaling pathway Wnt, NF-κB, Stat3, Notch, and mTORC1, which play an important role in the SC and CSC [6]. Below we analyze niclosamide action on different types of tumor cells.

2. THE ANTITUMOR ACTIVITY OF NICLOSAMIDE AND ITS MECHANISMS

2.1. Myeloid Leukemia

Acute myeloid leukemia (AML) is the most common type of malignant hematological diseases, and chemotherapy is the leading method used for its treatment. Over the last decades in this field no considerable progress in treatment of this disease has been achieved and the main chemotherapeutic agents are cytarabine (Ara-C), etoposide (VP-16) and daunorubicin (DNR) [7]. Therefore the search for new and more effective drugs for the treatment of AML is continued.

CSC with the $CD34^+CD38^-$ phenotype play a central role in the pathogenesis, development and recurrence of acute leukemia [8]. In AML, $CD34^+$ cells actively express nuclear factor NF-κB, while in normal unstimulated $CD34^+$ human cells its expression has not been detected. Importantly, the active form of NF-κB has antiapoptotic activity, and for some types of cancer it is considered as a key factor in survival of tumor cells. Thus, NF-κB is considered as one of the most promising targets for selective elimination of CSC in leukemia [9].

The study of the niclosamide effect on leukemia cells revealed two independent mechanisms: inhibition of the formation of active NF-κB and formation of reactive oxygen species (ROS) [10]. Niclosamide inhibited proliferation of various human leukemia cell lines in vitro and also in vivo experiments using a xenograft model of human tumors transplanted to immunodeficient nude mice. The water-soluble analogue (a phosphate pro-drug of niclosamide [6]) was injected intraperitoneally at a daily dose of 40 mg/kg for 15 days. This resulted in inhibition of HL-60 tumor growth and reduction of the NF-κB level. In the various myeloid leukemia cells niclosamide selectively induced apoptosis; the IC_{50} value for different cell lines varied in the range 0.4–0.9 μM in the case of determination of cell viability by the MTT assay and in the range 60–75 nM in the analysis of clonogenic activity. In the case of primary blast cells, stem and early progenitor cells of AML patients the IC_{50} value for different patients ranged from 8.5 nM to 718 nM in determining cell viability by the MTT assay. Normal cells were resistant to niclosamide. In the leukemic cell line HL-60 niclosamide decreased the level of the antiapoptotic factors MCL-1 and XIAP and increased the level of cytochrome *c* in the cytoplasm; it also caused depolarization of mitochondrial membranes.

In the case of combined application of niclosamide and classical chemotherapeutic agents it acted synergistically with Ara-C, VP-16, and DNR. After oral administration of niclosamide to rats (5 mg/kg), its maximal plasma concentration was about 1 μM [5, 10]. As follows from the above data, this concentration is sufficient for effective killing of leukemic cells. The results obtained in these studies suggest that niclosamide is an effective drug not only for selective elimination of tumor cells but also myeloid leukemia CSC with the phenotype $CD34^+CD38^-$.

2.2. Erythroleukemia

Different types of human tumors are frequently characterized by impaired transduction of the Notch signal [11].

Mammals express four genes encoding Notch ligands (NOTCH 1–4). Ligand (Delta or Serrate) binding to the Notch receptor on the cell surface results in release of the intracellular portion of the receptor (Notch-IC) followed by its translocation to the nucleus where Notch-IC influences function of the DNA binding transcription factor RBP-J, by binding to this factor and also to number of other proteins (Fig. 2). This results in conversion of RBP-J from the transcription repressor to the transcription activator of the Notch target genes. It is extremely difficult to detect Notch-IC in normal cells, although even in very low concentrations it causes biological effects [12, 13].

The stimulation of tumor growth in response to activation of the Notch signaling pathway is based on increased expression of Notch ligands and receptors and activation of other signaling pathways that stimulate tumor cell growth [11].

To screen drugs capable of controlling the activity of the Notch signaling pathway, a luciferase-based reporter system was generated. Using this system the time- and dose-dependent inhibition of the Notch-dependent reporter gene expression by niclosamide was found in the K562 erythroleukemia cell line [14]. Based on these results authors concluded that niclosamide is involved in regulation of the Notch signaling pathway [14].

2.3. Multiple Myeloma

Multiple myeloma (generalized plasmacytoma) is a malignant tumor derived from plasma cells (differentiated B-lymphocytes producing antibodies). This disease of the blood system is referred to paraproteinemic leukemia. The name of the disease and tumor cells

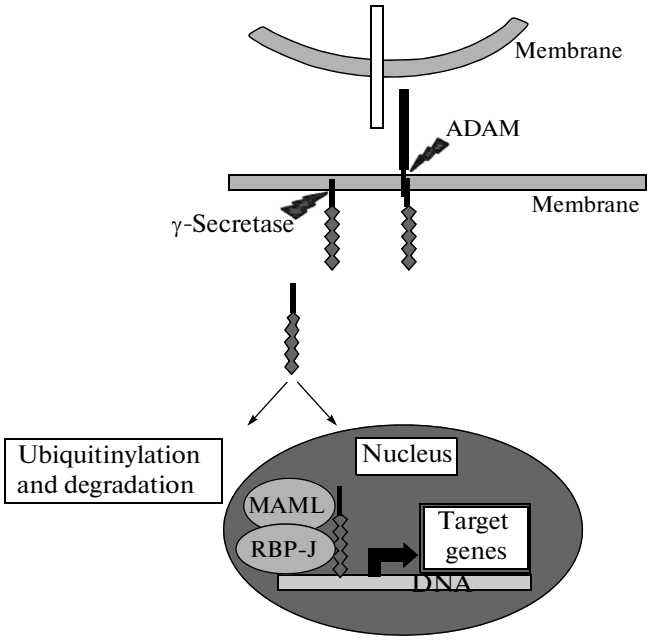

Fig. 2. Notch signaling pathways (adapted from [12, 13]). The Notch ligands and receptors are transmembrane proteins localized on the surface of adjacent cells. Notch receptors are heterodimers with one transmembrane domain. Receptor extracellular domains bind specific ligands presented on the surface of the adjacent cells, while cytoplasmic domains are involved in the formation and transduction of the signal to the cell nucleus. Ligand binding to the Notch receptor is accompanied by receptor cleavage by metalloproteinases ADAM10 and ADAM1 and γ-secretase in the transmembrane domain. The intracellular domain is translocated to the nucleus where it binds to the transcription factor RBP-J and other proteins and activates the appropriate target genes, or ubiquitinylates and subjected to proteasome degradation.

reflects the predominant localization of the process in the bone marrow. The most frequently affected areas of the bone marrow include the spine and pelvis, as well as in the ribs, and skull [15]. Multiple myeloma is the malignant disease characterized by the presence in the bone marrow of about 10% of the tumor plasma cells, the accumulation of monoclonal immunoglobulins in serum and urine, high incidence of fractures, anemia, and infections associated with immunodeficiencies. The majority of patients develop renal failure [16]. To search for new effective drugs for the treatment of multiple myeloma, Khanim et al. screened more than 100 different known pharmacological agents with different therapeutic specificity; they were looking for compounds possessing cytotoxic activity against three human myeloma cell lines at concentrations achievable in serum during their clinical use [17]. They found that niclosamide exhibited high activity against myeloma cells. In vitro, at concentrations of 1–4 μM it induced apoptosis and decreased formation of free immunoglobulin light chains in various myeloma cell lines and myeloma cells obtained from patients. Studying the mechanism of niclosamide action, the authors demonstrated that it induced formation of ROS, including superoxide anion (SOX) in the mitochondria of human multiple myeloma cells [17].

Niclosamide induced dose-dependent mitochondrial formation of SOX, which also depended on the time of its incubation with cells. In all cell lines tested 1 μM niclosamide significantly increased mitochondrial SOX production. However, dynamics of SOX increase in examined cell lines was different. For example, in JJN3 and H929 cell lines this increase continued for 18 h, while in the U266 cell line characterized by a low rate of proliferation, the increase mitochondrial SOX lasted much longer.

Analysis of other ROS assayed by means of carboxy-H_2DCFDA failed to detect a significant increase in ROS at any niclosamide concentration used. In these experiments, authors also examined cell viability and an inverse correlation between cell survival and mitochondrial SOX formation was found. Authors concluded that the cytotoxic effect of niclosamide on myeloma cells is mediated by excessive formation of SOX in mitochondria.

Uncouplers of oxidative phosphorylation and mitochondria respiration impair coupling between electron transport chain functioning and ATP synthesis [18]. Usually, this occurs due to increased permeability of the inner mitochondrial membrane for protons leading to the decrease in electrochemical potential difference. Niclosamide quickly, within 1 min, decreased mitochondrial membrane potential and uncoupled oxidative phosphorylation and respiration in the myeloma cells lines H929 and JJN [17]. These data suggest that mitochondria are the main target during niclosamide action on cells. The decrease in the mitochondrial membrane potential leads to the increase in ROS production, the decrease in the ATP level and release of proapoptotic factors such as cytochrome *c*, Smac and apoptosis-inducing factor (AIF) from the mitochondrial intermembrane space into the cytoplasm; these events finally result in caspase mediated apoptosis [17].

The same study also demonstrated existence of the second mechanism of niclosamide action on myeloma cells, associated with inhibition of NF-κB and STAT3 activation; however, this mechanism does not depend on the ROS level [17].

2.4. Glioblastoma

Glioblastoma is the most malignant tumor that develops in the brain. This tumor originates from astrocytes, which in contrast to neurons are capable of reproduction. Impairments in the regulation of this process may cause their uncontrolled division and active tissue growth. Screening of a library containing 160 natural and toxic compounds for antitumor activ-

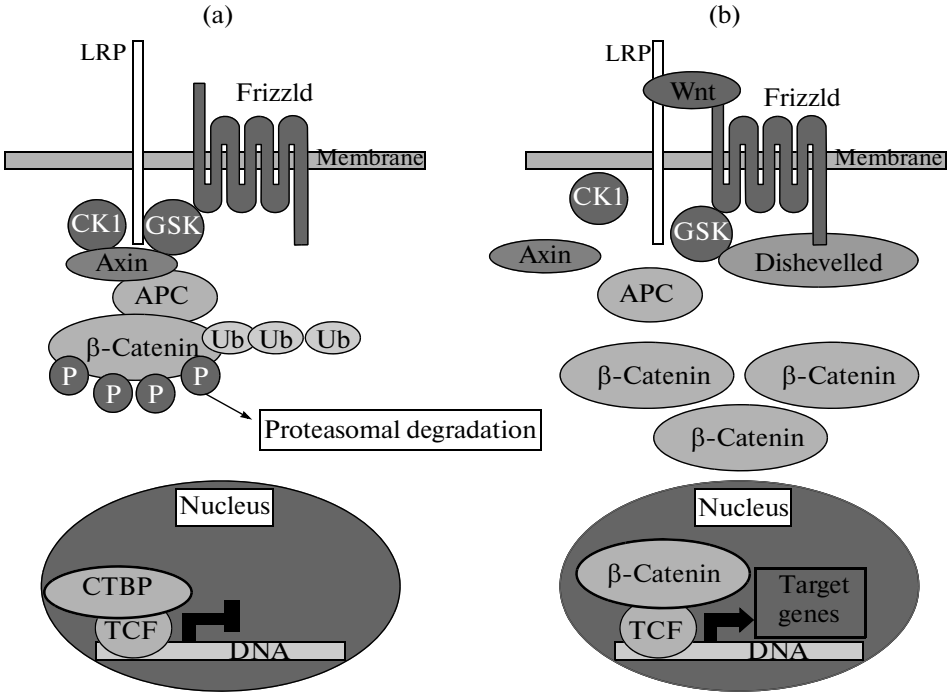

Fig. 3. Wnt signaling pathway (adapted from [20, 21]). Explanations are given in the text.

ity resulted in selection of niclosamide as a putative candidate for clinical trials [19]. Wieland et al. showed that niclosamide exhibited cytostatic and cytotoxic effects; it suppressed migration of tumor cells and decreased their malignant potential in vivo [19]. The mechanism of niclosamide action on glioblastoma cells is associated with inhibition of the signaling cascades WNT/β-catenin, NOTCH, mTOR and NF-κB. Niclosamide potentiated temozolomide action during their combined application. The authors believe that in the near future niclosamide will be introduced in the clinical practice for the treatment of glioblastomas [19].

2.5. Hepatoma

The signaling pathway Wnt (Fig. 3) plays an important role in embryogenesis and development of malignant tumors, including hepatocarcinogenesis [20, 21].

Wnt proteins (19 in mammals) interact with the Wnt receptor, known as Frizzled, and a co-receptor LRP 5/6 [22]. Frizzled is a transmembrane protein spanning the cell membrane seven times, co-receptor LRP 5/6 are transmembrane proteins with a single transmembrane domain. Wnt interaction with its receptor induces formation of a receptor complex, which activates the canonical signal transduction pathway β-catenin/TCF.

In the absence of Wnt proteins (Fig. 3a), the level of cytoplasmic β-catenin is maintained at a very low level due to the action of a protein complex, which phosphorylates it and thus prepares for subsequent ubiquitylation followed by degradation in proteasomes. This complex consists of two regulatory protein kinases (glycogen synthase kinase 3β (GSK-3β) and casein kinase) and β-catenin binding proteins: Axin1 or Axin protein 2 and so-called APC (adenomatous polyposis coli) protein. Receptor binding of Wnt protein (Fig. 3b) results in decomposition of the protein complex determining β-catenin degradation; this results in its accumulation in the cytoplasm and subsequent translocation into the nucleus where it binds to transcription factors of the TCF/LEF (T-cell factor/lymphoid enhancer factor) family and activates the expression of other, more distant targets of the Wnt signaling pathway, including cyclins, C-myc and bone morphogenetic proteins.

Transduction of the receptor mediated Wnt signal involves Dishevelled (Dvl) protein, which moves to the membrane, interacts with the Frizzled receptor and forms a polymer complex with other Dishevelled molecules. Phosphorylation of the cytoplasmic domain of LRP5 or LRP6 co-receptor and formation of the Dishevelled polymeric complex serves as a signal for translocation of Axin protein to the plasma membrane and inactivation of the complex, degrading β-catenin.

Activation of the signaling pathway induced by Wnt was found in various tumor cells. Mutations in destabilizing complex proteins also resulted in increased levels of β-catenin and the development of cancer [23, 24].

Tomizawa et al. evaluated the effect of Wnt3A protein and niclosamide on proliferation of hepatoma Huh-6 and Hep3B cell lines [25]. They found that Wnt3A protein stimulated proliferation of hepatoma Huh-6 and Hep3B cells, increased the content of cyclin D1, β-catenin, Dishevelled-2 protein, enhanced activity of promoter of T cell factor. These data indicate that the Wnt signaling pathway regulates proliferation of hepatomas studied. Niclosamide (1 μM) inhibited spontaneous proliferation of Huh-6 and Hep3B cells and also proliferation stimulated by Wnt3A [25]. These results demonstrate that niclosamide suppresses proliferation of hepatoma cells by inhibiting the Wnt signaling pathway.

2.6. Prostate Cancer

Prostate cancer is a malignant tumor arising from of the alveolar-cellular elements of the prostate gland epithelium; it occurs with high frequency: in Russia about 14000 new cases are diagnosed annually [26]. Prostate cancer is the cause of almost 10% of cancer deaths in men. It was shown that niclosamide inhibits proliferation of prostate cancer cells in vitro and tumor growth in animal models in vivo [27].

The scheme of prostate cancer treatment includes anti-androgenic drugs as important elements. Enzalutamide is a second generation anti-androgen agent. It is used for treatment of drug-resistant forms of prostate cancer in patients, which do not respond to docetaxel ((2R,3S)-*N*-carboxy-3-phenylisoserine, *N*-tert-butyl ester, 13-ester with 5β, 20-epoxy-1,2α,4,7β, 10β,13α-hexahydroxytax-11-en-9-one 4-acetate 2-benzoate, trihydrate, a semi-synthetic antitumor agent of plant origin obtained by chemical synthesis from natural raw materials (European yew needles)). It is used for treatment of various types of cancers, including metastatic prostate cancer. However, this therapy is often accompanied by development of resistance to enzalutamide, associated with the presence of splice variants of the androgen receptor AR-V7. Screening of about 1120 preparations revealed that niclosamide is a potent inhibitor of AR-V7, as it causes reduction in the amount of the protein due to its enhanced proteasomal degradation and inhibition of transcription [27]. During combined action of niclosamide and enzalutamid tumor cells lost their resistance to enzalutamide [27].

Thus, niclosamide is an inhibitor of splice variants of the androgen receptor and may be a promising pharmacological agent for the treatment of advanced prostate cancer both as monotherapy and in combination with antiandrogenic agents, including combination with enzalutamide to overcome the resistance of tumor cells to this drug [27].

2.7. Colon Cancer

Colon cancer is one of the most common causes of cancer death worldwide. Progression of the disease is associated with the formation of metastases. In the case of stage 1 non-metastatic tumor, survival of patients for 5 years is 90% and only 10% when distant metastases are detected at the diagnosis of this disease [30]. Significant efforts are undertaken for both active search of drugs for treatment of this disease and studies of molecular mechanisms regulating target genes, which promote formation of metastases.

Osada et al. [31] studied the effect of niclosamide on proliferation of colorectal tumor cells (HT29, HCT116, Caco-2 cell lines); they showed that niclosamide inhibited the Wnt signaling pathway, which is active in about 80% of cases of colon cancer due to the presence of mutations in the APC gene, or (less often) activating mutations in the β-catenin gene [31]. Certain evidence exists that ligands and inhibitors of the Wnt signaling pathway may affect growth and survival of tumor cells despite the presence of such mutations.

It was shown [32] that niclosamide accelerated endocytosis of Frizzled1 receptors, decreased the content of the Dishevelled-2 protein, blocked Wnt3A induced stabilization of β-catenin, and inhibited distant stages of transcription activation involving LEF/TCF transcription factors (see Fig. 3). Furthermore, niclosamide increased the antitumor effect of oxaliplatin. Importantly, the antiproliferative effect of niclosamide was recognized in experiments with cells derived from human colon tumor metastases to the liver [31].

These authors also investigated pharmacokinetics of niclosamide orally administrated to NOD/SCID mice at a dose of 200 mg/kg. Furthermore, they investigated niclosamide accumulation in mice with inoculated tumor HCT116: the drug was administered orally by a gavage at a dose of 200 mg/kg for three weeks, starting on the fourth day after tumor inoculation. Osada et al. have shown [31] that the maximal plasma concentration of niclosamide was observed 15 min after administration (893.7 ng/mL), while after 30–45 min and 1.5 h it decreased to 50–40 ng/mL and 78 ng/mL, respectively; the elimination half-life time was 3.5 h. At the end of the experiment the niclosamide concentration in the tumor and plasma was 37 ng/g tissue and 38 ng/mL, respectively.

Niclosamide-induced inhibition of tumor growth was observed after oral niclosamide administration in doses of 100–200 mg/kg (depending on the sensitivity of tumor cells) for 2–3 weeks, 6 days per week starting on the fourth day after tumor inoculation. Decreased levels of Dishevelled-2 and β-catenin found in the tumor tissue suggest that niclosamide treatment suppressed the Wnt signaling system (Fig. 3). Under these conditions no signs of toxicity were detected in control animals.

Impairments in the canonical WNT/β-catenin signaling pathway present in almost 90% of colon tumors, thus causing constitutively active expression of relevant target genes [29], including those responsible for the formation of metastases. Formation of metastases involves S100 protein, calcium-binding protein A4 (S100A4) with molecular mass of 11 kDa [30].

Elevated expression of S100A4 was found in many types of cancer: gall bladder cancer, urinary bladder cancer, breast cancer, esophagus cancer, stomach cancer, pancreas cancer, hepatocellular carcinoma, small cell lung cancer and especially colon cancer. High expression of S100A4 is closely associated with the aggressiveness of the tumor, its ability to metastasize and low patient survival [33].

S100A4 plays an important role in cellular processes determining migration, invasion, adhesion, and angiogenesis, which represent the basis of the formation of metastases. It was shown that in colon cancer S100A4 expression in tumors is a prognostic factor for the development of metastases [34]. Inhibitors of S100A4 expression, which would suppress S100A4-mediated metastatic activity, remain unknown. Screening 1280 compounds for detection of inhibitors of S100A4 expression, Sack et al. [35] found that niclosamide inhibited interaction of β-catenin with the T-cell factor TCF; this impaired S100A4 expression and S100A4-dependent metastasis. Treatment of various human colon tumor cell lines (HCT116, SW620, LS174T, SW480, DLD-1) with niclosamide in vitro decreased S100A4 expression at the mRNA and protein levels and inhibited migration, invasiveness, cell proliferation, and colony formation. In vivo experiments have shown that treatment with niclosamide (daily dose of 20 mg/kg) resulted in inhibition of metastasis and prolongation of life of mice with tumor grafts.

It is believed that the antimetastatic activity of niclosamide will help to develop a new method for the treatment and prevention of metastases in colon cancer.

2.8. Ovarian Cancer

Ovarian cancer remains the most common cause of death from cancers of female reproductive organs and is the fifth leading cause of death in women.

In model experiments on mice Yoshioka et al. have shown [36] that the ovarian cancer tumor growth is regulated by the level of WNT7A protein, and that malignant transformation of epithelial ovarian cells occurs under control of the signaling pathway WNT. In cell culture experiments, it was shown that proliferation of ovarian cancer cells, adhesion, and invasion are regulated by WNT7A protein. WNT7A overexpression stimulated activity of the TCF/LEF transcription factor. In the subsequent study these authors demonstrated that the WNT7A/β-catenin signaling system controls expression of the FGF1 gene, encoding fibroblast growth factor [37]. The level of this protein is a risk factor for ovarian cancer and correlates with a low survival rate of patients [37]. Niclosamide effectively blocked the WNT7A/β-catenin signaling pathway, inhibited β-catenin activity as a transcription factor, reduced survival and migration of cells. Niclosamide action was more effective in cells overexpressing WNT7A. During oral administration of niclosamide to mice it inhibited growth of the tumor, growing intraperitoneally (a representative model of ovarian cancer) [37].

Arend et al. investigated the antitumor activity of niclosamide against the SKOV3.ip1 ovarian cancer cell line. They found that in the range of concentrations 1–8 μM niclosamide inhibited the Wnt signaling pathway. Niclosamide also caused a significant decrease in the content of the dephosphorylated co-receptor protein LRP6 and its phosphorylated form, Axin2 protein, cyclin D1, survivin, β-catenin, as well as death of SKOV3.ip1 cells [38].

Yo et al. [39] investigated the effect of niclosamide on human ovarian CSC. According to their data, 3 μM niclosamide reduces CSC survival to 10–60%. In animal experiments, intraperitoneal administration of niclosamide at a daily dose of 5 mg/kg and 10 mg/kg daily, starting from the next day after inoculation of tumor cells, decreased CSC ability to induce tumors during their administration to animals. Administration of niclosamide altered the expression profile of 2928 genes in tumor cells (including genes encoding proteins forming mitochondrial electron transport chain). Experiments on cells showed that niclosamide caused a 20%-increase of ROS formation in CSC, mitochondrial damage, accompanied by release of cytochrome *c* and apoptosis-inducing factor, as well as DNA fragmentation due to CSC apoptosis.

2.9. Head and Neck Cancer

Squamous cell carcinoma (SCC) of the upper gastrointestinal tract and respiratory tract, including the oral cavity, pharynx, larynx and sinuses, accounts for more than 90% cases of head and neck cancers. Despite the success of surgery, radiotherapy, and chemotherapy, achieved in recent years, the survival rate of patients with SCC of the head and neck remains low [40, 41]. Since SCC cells are characterized by high expression of the epidermal growth factor receptor (EGFR) it was reasonable to investigate the possibility of erlotinib use for SCC treatment; erlotinib is the anticancer agent acting as human inhibitor of EGFR tyrosine kinase, which after activation causes EGFR autophosphorylation and initiates a signaling cascade resulting in stimulation of cell proliferation. Inhibition of this process inhibits growth of tumor cells and/or causes their death. However, erlotinib efficacy against SCC was low [42]; this can be associated with the acti-

vation of several signaling pathways, increasing viability of tumor cells and reducing erlotinib efficacy against SCC. Indeed, SCC cells have higher levels of STAT3 protein compared to normal cells [43]. STAT3 is a member of a family of STAT transcription factors; its activity is often increased in many tumor cell types [43]. STAT3 is localized in the cell cytoplasm and is activated by phosphorylation at Tyr705 by Janus associated kinase (JAK) or receptor tyrosine kinase Src associated with growth factor receptors [43]. Phosphorylation is accompanied by pSTAT3 dimerization and translocation of the dimer to the nucleus where it activates transcription of many genes, including Bcl2/Bcl-XL, cyclin D1, c-Myc, and other genes promoting cell survival [44]. STAT3 activity is also regulated by dephosphorylation, which involves recently discovered protein tyrosine phosphatase PTPMeg2, dephosphorylating Tyr705 residue in pSTAT3 [45]. In the animal model using xenografted tumors in immunodeficient mice it has been shown that STAT3 blockade leads to inhibition of SCC tumor growth in vivo. This explains intensive search for such small molecule STAT3 inhibitors, which would be applicable in the clinic practice.

Li et al. have shown [46] that niclosamide in vitro and in vivo inhibited formation of phosphorylated STAT3 (pSTAT3) in tumor cells, and its combined application with erlotinib blocked pSTAT3 formation (attributed to erlotinib action) caused complete absence of Bcl2 and Bcl-XL proteins analyzed in cell lysates. This determined the synergistic actions of these drugs and resulted in almost complete suppression of the SCC growth during combined use of erlotinib and niclosamide.

2.10. Non-Small Cell Lung Cancer

EGFR is the molecular target for treatment not only of SCC but also non-small cell lung cancer (NSCLC), as up to 20% of patients have somatic mutations in the tyrosine kinase domain region of the EGFR gene, which promote tumor cells growth, and therefore it was expected that inhibitors of EGFR tyrosine kinase activity such as erlotinib and gefitinib would be effective in NSCLC therapy.

However, it appeared that after 6–12 months of such treatment, patients develop resistance to these drugs, and this significantly limits this treatment in clinical practice.

Mechanisms of NSCLC cell resistance to EGFR tyrosine kinase inhibitors are also associated with activation of the STAT3 signaling pathway: STAT3 is a transcription factor for Bcl2 and Bcl-XL, and STAT3 activation by erlotinib leads to upregulation of Bcl2, and Bcl-XL genes (see above), thus contributing to the development of resistance to erlotinib in tumor cells. Li et al. [46] have shown that in NSCLC cells erlotinib increased STAT3 phosphorylation by inhibiting phosphatase PTPMeg2; this resulted in increased levels of Bcl2 and Bcl-XL and subsequent loss of sensitivity to erlotinib. In this context, niclosamide blocked erlotinib-induced activation of the STAT3/Bcl2/Bcl-XL signaling pathway in NSCLC cells and thus abolished resistance to erlotinib in vitro and in vivo [47].

Using niclosamide it was possible to overcome developed resistance of the HCC827/ER NSCLC cell line to erlotinib, including in vivo in mice with xenografted tumors (niclosamide 20 mg/kg/day + erlotinib 40 mg/kg/day during 32 days of observation). The authors suggest that combined therapy with erlotinib and niclosamide can be a new and effective strategy for the treatment of NSCLC, including those patients who developed resistance to standard therapy with inhibitors of EGFR tyrosine kinase activity.

2.11. Breast Cancer

Breast cancer is the most frequent form of cancer in women. However, despite the significant progress in diagnostics and treatment, the disease ranks the second in cancer mortality in women, mainly due to the high incidence of metastasis. Therefore, the search for new therapeutic approaches for treating this form of cancer continues.

Ye et al. studied niclosamide efficacy against mammary tumor cells in vitro and in vivo [48]; they demonstrated that niclosamide inhibited proliferation of the tumor cells lines MDA-MB-231, MCF-7, MDA-MB-468 and 4T1. According to the MTT assay, after 72 h of incubation with niclosamide the following IC_{50} values were obtained for the cell lines studied: 0.95 μM for MDA-MB-231, 1.05 μM for MCF-7, and 1.88 μM for MDA-MB-468. Analysis of the colony forming activity performed 12 days after niclosamide addition revealed the IC_{50} value of 0.2 μM for the niclosamide action to the 4T1 cell line. In this cell line niclosamide induced apoptosis, which was accompanied by appearance of activated caspase and decreased levels of the anti-apoptotic proteins Bcl-2, Mcl-1 and survivin. It was also found that in tumor cells niclosamide inhibited the STAT3 signaling pathway, reduced the level of phosphorylated (at Tyr705) STAT3, phosphorylated (at Tyr495) FAK kinase, and phosphorylated (at Tyr416) Src protein. Niclosamide inhibited migration and invasion of breast tumor cells.

In experiments employing the model of xenotransplantation of human tumor cell lines 4T1 to mice, it was demonstrated that intraperitoneal injections of niclosamide at daily doses of 10 and 20 mg/kg for three weeks, starting on day 7 after tumor cell inoculation inhibited tumor growth, formation of lung metastases and angiogenesis. Niclosamide reduced levels of myeloid suppressor cells in tumor tissue [48]. Toxicological studies performed using hematological, histological, and general parameters revealed no toxic effects in these conditions of niclosamide administration.

Niclosamide exhibited high antitumor activity against breast cancer CSC [49]. Using the SP fraction of MCF-7 cells, cultivated as spheroids as a representative model for evaluating breast cancer CSC, Wang and coworkers have shown that intraperitoneal administration of niclosamide (at daily dose of 10 mg/kg 5 days a week for 8 weeks starting on the next day after SP cell inoculation) to NOD/SCID mice transplanted with SP cells from MCF-7 spheroids resulted in growth of smaller tumors than in animals of control group. The tumors extracted from niclosamide treated animals had lower expression levels of cyclin D1, Hes1, and PTCH genes, controlled by the signaling systems Wnt, Notch and Hedgehog, respectively. This demonstrates the CSC suppression under these experimental conditions.

2.12. Cancer Stem Cells as a Target for Niclosamide Action

Studies performed during the last decade in the field of SC changed modern concepts of the mechanisms of carcinogenesis, which are currently associated with the emergence and existence of the CSC [50]. The results accumulated during studies of SC and characteristic features of tumor development suggest that the tumor develops from CSC, which obviously appear due to accumulation of mutations in somatic SC [50]. Biology and features of CSC have been considered in numerous reviews [50–56], and so below we will cite only some references relevant to this area.

The first evidence for the existence of CSC was obtained by Dick et al. in 1997, in the study of AML [8]. They found that human myeloid leukemia is carried only by a small proportion of cells with properties of the CSC phenotype of $CD34^+CD38^-$. In the general population of tumor cells, they represented just 0.1%, but after administration of these particular cells to immunodeficient mice NOD/SCID they divided and differentiated into various types of leukemia cells.

In 2003, using the same methodology and breast cancer as an example, Al-Hajj et al. for the first time demonstrated experimental evidence for existence of CSC in solid tumors [57]; they showed that only cells with a particular phenotype, $CD44^+CD24^{-/low}ESA^+$ (ESA—epithelial-specific antigen), were able to induce human breast cancer in immunodeficient mice. Using this approach, convincing evidence of the existence of CSC was demonstrated for a large number of human solid tumors [58, 59]. CSC of different origin as well as normal SC demonstrate the ability to exclude the lipophilic fluorescent dyes Hoechst 33342 and Rhodamine-123 and form so-called side population (SP) [59].

CSC are characterized by high resistance to the damaging effects due to expression of gene encoding such ABC transporters as ABCG2, ABCB1, and ABCC1; they encode proteins responsible for transport of both hydrophobic and hydrophilic compounds from the cells into the medium [60, 61], and also perform efflux of Hoechst and Rhodamine dyes; the latter is used for identification of cells constituting SP. Furthermore, CSC are characterized by high levels of DNA repair, and a high content of antioxidants that make them resistant to ionizing radiation [62, 63]. Therefore, high sensitivity of CSC to the niclosamide action gives certain hope that this drug may represent a good basis for the development of an effective anticancer agent.

Thus, based on results of experimental studies of the molecular mechanisms responsible for stimulation of proliferation and differentiation of SC there are reasons to suggest that regulation of these processes in CSC is controlled by the same signaling pathways as in normal SC and includes Wnt, Notch and Hedgehog signaling pathways [38, 39, 64].

Dysregulation of SC activity by mutation may underlie the development of benign tumors. Uterine leiomyoma is a frequent benign estrogen- and progesterone-dependent tumor of the myometrium, which can cause uterine bleeding, severe anemia and miscarriage. The tumor arises from myometrium smooth muscle mutant SC. It has been shown that myometrium and leiomyoma mature cells respond to estrogen and progesterone by secretion of WNT family proteins, which cause β-catenin-dependent stimulation of the leiomyoma SC thus determining tumor growth [65]. It was found that niclosamide inhibits the WNT signaling pathway and proliferation of leiomyoma cells [66]. Based on these data author recommend niclosamide for clinical trials, which would evaluate the possibility of niclosamide application for treatment of uterine leiomyoma.

Summarizing experimental results obtained in different studies, it should be noted that niclosamide exhibits high antitumor activity not only against a broad spectrum of tumors, but also, more importantly, with respect to CSC. The effectiveness of its action on CSC has been convincingly demonstrated for myeloid leukemia [10], ovarian cancer [39], and breast cancer [49]. In the case of other tumors the action of niclosamide on CSC can be evaluated by its ability to inhibit signaling pathways specific to CSC. The inhibition of the signaling pathways Wnt, Notch, and Hedgehog by niclosamide has been shown in many forms of cancer.

3. RADIO-SENSITIZING ACTIVITY OF NICLOSAMIDE

Screening of more than thousands compounds for radio-sensitizing activity revealed that niclosamide effectively sensitized tumor cells (NSCLC cell line H1299) to ionizing radiation. Pretreatment of the H1299 cells with niclosamide increased the level of their apoptosis, induced by gamma radiation, to

higher values than in the case of action of each of these factors alone. Combined use also caused a significantly higher level of phosphorylation of p38 and c-Jun in H1299cells. These results suggest that niclosamide is a promising radiosensitizer, which can be rapidly introduced into clinical practice for the treatment of patients with lung cancer, as this drug for about 50 years is used to treat teniasis and does not cause side effects [67].

4. REGULATION OF THE ACTIVITY OF CERTAIN METABOLIC PROCESSES BY NICLOSAMIDE AS THE APPROACH TO TREAT NON-TUMOR DISEASES

4.1. Uncoupling of Oxidative Phosphorylation and Mitochondrial Respiration

Anthelmintic activity of niclosamide is associated with its ability to cause uncoupling of oxidative phosphorylation and mitochondrial respiratory in parasites. Good evidence now exists that a niclosamide derivative, niclosamide ethanolamine (possessing higher solubility), at nanomolar concentrations cause uncoupling in mammalian cells [68]. Oral administration of niclosamide ethanolamine increased energy dissipation and intensified lipid metabolism in mice. This preparation was also effective in the prevention and treatment of hepatic steatosis and insulin resistance caused by a high fat diet [68]. The authors suggest that niclosamide ethanolamine can be used for the treatment of type 2 diabetes mellitus.

4.2. Inhibition of mTORC1 Activity and Stimulation of Autophagy

Treatment of certain diseases requires the search for active compounds capable of regulating autophagy; this process of "cell self-eating," is activated by a lack of nutrients, and is often regarded as one of the options of programmed cell death [69].

In mammalian cells, this process is controlled by the protein complex known as the mammalian target of rapamycin complex 1 (mTORC1), which includes mTOR serine/threonine kinase. This kinase transduces signals about availability of nutrients and controls a number of cellular processes. mTORC1 regulates the stage of protein synthesis initiation by phosphorylation of proteins binding to the eukaryotic initiation factor 4E (4E-BPS), and by phosphorylation of ribosomal S6 kinases. Under conditions of sufficient nutrient supply the complex mTORC1 is active; it increases ribosome biogenesis and protein synthesis and inhibits autophagy. Autophagy induction is accompanied by translocation of the cytosolic protein LC3 (also known as Atg8) to the membrane of growing autophagosomes, and it controls their increase [70].

Targeted regulation of mTORC1 signaling and autophagy requires the search for active compounds with desired pharmacological properties.

In this context, a screening system based on the breast adenocarcinoma cell line MCF-7 was created [71].

In the process of screening of 3500 compounds Balgi et al. [71] found four compounds with the desired properties, one of them was niclosamide. It rapidly increased the content of autophagosomes. Niclosamide-induced inhibition of mTORC1 and accumulation of autophagosomes was reversible and rapidly restored after removal of the drug. The study of the mechanism of niclosamide-induced inhibition of the mTORC1 signaling pathway has shown that niclosamide had no effect on the activity of the signaling system PI3K/Akt, it did not inhibit the mTORC1 kinase activity and did not affect the structure of the mTORC1 complex; however, it exhibits protonophore activity in tumor cells and in cell free lysates in vitro [71]. In breast cancer cells niclosamide decreased the proton gradient between the cytoplasm and lysosomes, leading to acidification of the cell cytoplasm. However, the relationship of the mechanism of niclosamide action and its effect on the mTORC1 activity remains unclear.

The authors suggest that because niclosamide has already been used in clinical practice and it is active at the concentrations, already used for treatment of other diseases, its preclinical and clinical trials can be accelerated. The use of this drug may be promising in the treatment of diseases such as tuberous sclerosis, diabetes mellitus, cardiovascular diseases, diseases caused by protein misfolding, and cancer.

CONCLUSIONS

Summarizing all results obtained by different groups it appears that niclosamide possesses high antitumor activity towards various tumor cells (including CSC) and inhibits metastasis. Table summarizes quantitative data of niclosamide activity against various tumor lines and normal human cells. They indicate that leukemia cells exhibit the highest sensitivity to niclosamide: the IC_{50} values ranged from 0.4 μM to 1 μM. In the case of solid tumors IC_{50} values ranged from 0.5 μM to 12 μM, for different types of normal cells IC_{50} values were in the range 4.5–9 μM and in the case of peripheral blood mononuclear leukocytes IC_{50} values exceeded 1 μM. Taking into consideration a low toxicity these data clearly indicate that niclosamide may be considered as a promising anticancer agent applicable for the treatment of a wide range of cancers, including advanced forms of cancer. Recognized synergism of niclosamide and many commonly used anticancer drugs and its radiosensitizing activity expand prospects of niclosamide application in the treatment of cancer. At the same time it should be noted that

Antitumor activity of niclosamide against various tumor lines and normal human cells, estimated by the value of IC_{50}*

Tumor		Cell line	IC_{50}, μM	Reference
Lung cancer	Adenocarcinoma	HCC827	>0.50**	47
	Adenocarcinoma	HCC827/ER[1]	>0.50**	47
	Carcinoma	A549	3.00	6
Breast cancer	Adenocarcinoma	MCF-7	1.05	48
			7.30**	49
			0.80**	72
			1.06	73
	Adenocarcinoma	MDA-MB-231	0.95	48
			4.77**	49
			0.79	73
	Adenocarcinoma	MDA-MB-468	1.88	48
Pancreatic cancer	Adenocarcinoma	AsPC1	1.47	73
	Epithelioid carcinoma	Panc-1	1.73	73
Malignant ovarian tumors	Carcinoma	CP70[2]	4.41**	74
			>3.00**	39
		CP70sps[3]	1.95**	39
	Adenocarcinoma	SKOV-3	>12.00**	74
		SKOV3.ip1[4]	1.20	37
			1.00	38
		TCP[5]	1.29	38
		CSC from TCP[6]	<3.00**	39
Malignant tumors of lymphoid, hematopoietic and related tissues	Promyelocytic leukemia	HL-60	0.48	10
	Histiocytic lymphoma	U937	0.41	10
	Myeloid leukemia	OCI-AML3	0.79	10
	Myeloid leukemia	Molm13	0.72	10
	Myelomonocytic leukemia	MV4-11	1.00	10
Colorectal cancer	Colon carcinoma	HTC116	2.20	34
			< 2.00**	31
	Colon carcinoma	HT-29	7.20	6
			4.84**	31
	Colon adenocarcinoma	Caco-2	0.85**	31
Prostate cancer	Carcinoma	Du145	0.70	6
	Adenocarcinoma	PC3	11.70	6
	Carcinoma	CWR22Rv1	0.50	27
Cervical cancer	Adenocarcinoma	HeLa	1.40	6
Skin squamous cell carcinoma	Carcinoma	A431	8.80	6
Multiple myeloma	Myeloma	JJN3	1.41**	17
	Plasmacytoma	H929	0.77**	17
	Plasmacytoma	U266	1.21**	17
	Plasmacytoma	RPMI 8226	1.05**	17
		TCP[7]	0.60–1.60**	17
Glioblastoma	Glioblastoma	LN229	2.53	19
	Glioblastoma multiforme	T98G	2.35	19
	Glioblastoma	U87	1.59	19
	Astrocytoma	U138	2.78	19
	Glioblastoma	U373	2.05	19
		TCP[8]	0.66	19

Table. (Contd.)

Tumor		Cell line	IC_{50}, μM	Reference
Hepatoma	Hepatoblastoma	Huh-6	0.22	25
	Carcinoma	Hep3B	0.37	25
Head and neck squamous cell carcinoma		Tu212[9]	>0.50*	46
		Tu686[9]	>0.50*	
Uterine leiomyoma		TCP[10]	0.21*	66
Normal cells		MEF	5.78	10
		NHF	4.54	10
		BMMC	9.00	10
		PBML	>1.00	17

* IC_{50}—the concentration of the drug causing death of 50% of the cells,

** The value calculated based on image data of the authors.

Abbreviations: TCP—tumor cells from patients; CSC—cancer stem cells; MEF— mouse embryonic fibroblasts; NHF—normal human fibroblasts; BMMC—bone marrow mononuclear cells of healthy volunteers; PBML—mononuclear leukocytes of peripheral blood of healthy volunteers.

1—cell line HCC827/ER, resistant to erlotinib, derived from the cell line HCC827;

2—cell line CP70, resistant to cisplatin, derived from the cell line A2780;

3—cells CP70sps (side population spheroid) in the form of spheroids obtained from the fraction SP (side population) cell line CP70;

4—SKOV3.ip1 cell line derived from ascites of nude mice and nude, which was administered intraperitoneally cell line SKOV3;

5—ovarian cancer cells obtained from the ascites of ovarian cancer patients during surgery, neoadjuvant chemotherapy or during relapse;

6—cancer stem cells grown as spheroids derived from patients with primary malignant ovarian tumors;

7—mononuclear cells of patients with primary multiple myeloma;

8—cells obtained during surgery from patients with primary glioblastoma;

9—Tu212 cell line derived from primary tumor subesophageal space; Tu686 line derived from a lymph node metastasis patients with primary squamous cell carcinoma of the tongue;

10—samples from patients with uterine leiomyoma not receiving hormonal drugs and did not have malignant tumors of the uterus. Obtained during hysterectomy or myomectomy.

most of the data have been obtained on the tumor cells in vitro and in model experiments with human tumor cells xenografted in immunodeficient mice in vivo. In addition, the mechanism of antitumor action of niclosamide still need better understanding, which is hampered by low solubility of this drug. However, more soluble derivatives have been already synthesized [6, 73], and methods for niclosamide incorporation into polymeric carriers have been developed [74] and their properties are actively studied. It appears that mitochondria are the important target for niclosamide action and therefore it requires further investigation, which mechanisms is primarily responsible for blockade of signaling pathways, specific for tumor cells and CSC contacting with this drug. This allows to expect appearance of new information about the mechanisms of the antitumor action of niclosamide and its effective analogues in the near future.

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