Niclosamide (5-chloro-N-2-chloro-4-nitrophenyl)-2-hydrobenzamide (Fig. 1) is an antihelminthic drug used for over 50 years in the treatment of intestinal tapeworm infections (1). In the last decade, niclosamide has been identified as a potential anti-cancer drug. This compound suppresses the growth of cancer cells in vitro (2-5) and inhibits tumor growth in vivo models (6). Moreover, when combined with conventional chemotherapeutic drugs it enhances their therapeutic effect (7).

Among the possible mechanisms of the anti-cancer activity of niclosamide, the inhibition of Wnt/β-catenin signaling was pointed out (6). This pathway regulates all the stages of cancer development including tumor initiation, tumor growth and metastasis (8, 9). In the absence of Wnt ligands, β-catenin is sequestered by a complex formed by Adenomatous Polyposis Coli (APC) tumor suppressor, Axin, Glycogen Synthase Kinase-3β (GSK-3β) and Casein Kinase 1α (CK-1α). The formation of this complex leads to the phosphorylation of β-catenin and its subsequent degradation by the 26S proteasome. During pathway activation, the Wnt ligands bind to and form a ternary complex with the cell surface Frizzled (Fzd) receptor and its co-receptor, Low-density Lipoprotein Receptor-related Protein 5/6 (LRP5/6), what results in signal transduction through Dishevelled (Dvl) and Axin proteins, leading to the inhibition of GSK-3β, the stabilization of β-catenin and its translocation into the nucleus where it interacts with T-cell Factor/Lymphoid Enhancing Factor (TCF/LEF) to induce the expression of specific target genes, such as MMP7 encoding Matrix Metalloproteinase 7, CCND1 encoding the cell cycle-related protein Cyclin D1 and MET encoding the receptor kinase Met (6, 10).

Niclosamide may interact with several crucial elements of Wnt canonical signaling. Initially, niclosamide was thought to promote Fzd-1 receptor endocytosis, downregulate Dvl-2 protein and inhibit Wnt 3A-stimulated β-catenin stabilization and TCF/LEF reporter activity (11). Later on, it was also demonstrated that niclosamide is able to inhibit
Wnt/β-catenin signaling by promoting the degradation of the LRP6 co-receptor without affecting Dvl-2 expression (4, 12). The recent study of Monin et al. (2016) confirmed that the transcription promoting activity of TCF/LEF was significantly decreased after the treatment of colorectal cancer cells with niclosamide. The target genes (CCND1, Met, MMP7), as well as the co-activating factor Bcl9, were downregulated (10). Based on these observations, it was proposed that niclosamide reduces the expression of the co-activator Bcl9 and therefore impairs the formation of the complex with active β-catenin and TCF/LEF. Moreover, available data on the mechanisms underlying the modulation of Wnt signaling by niclosamide indicate that this effect may be cell-type dependent (6).

As oxidative phosphorylation inhibition in Cestodes is responsible for the antihelminthic effect of niclosamide, it might be expected that the drug’s anti-cancer activity could be mediated through mitochondrial disruption. Indeed, rapid loss of mitochondrial potential, uncoupling of oxidative phosphorylation and the production of superoxide was observed in multiple-myeloma cell lines (3). Moreover, in ovarian cancer, the shift from oxidative phosphorylation to aerobic glycolysis was found as the result of niclosamide treatment (13).

Cancer cells possess unique metabolic properties, known as the Warburg effect. In contrast to normal cells, cancer cells are more dependent on aerobic glycolysis. Thus, the inhibition of metabolic, particularly glycolytic, enzymes, is considered as a promising strategy to improve the effectiveness of cancer therapy and to overcome therapeutic resistance (14). Many enzymes and proteins are involved in glycolytic pathway and current researches are focused on finding the inhibitors of its selected components in cancer cells.

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide and is usually associated with unfavorable prognosis. Since Wnt pathway is frequently hyper-activated in HNSCC (15-18), its inhibition may be a good strategy for blocking cell proliferation. Moreover, the enhancement of the activity of this pathway is higher in HN carcinoma cells showing increased invasiveness (19). Thus, targeting Wnt may not only affect the survival of the bulk of cancer cells but also of cancer stem cells which are believed to be responsible for tumor recurrence. Besides modulation of Wnt pathway, downregulation of glycolysis might be considered as equally important in improving HN cancer therapy (14).

Although the anti-cancer activity of niclosamide was shown in various tumor models, its efficacy in HNSCC has not been described so far. In this study, we investigated the effect of niclosamide on the viability, cell cycle distribution and apoptosis in HNSCC cell lines of different origin and the expression of key components of Wnt and glycolysis pathways.

![Figure 1. The effect of niclosamide on the viability of BICR6, H314 and FaDu cells. The viability of vehicle-treated cells was considered 100%. Mean values ± SEM from three independent experiments are shown.](image-url)
EXPERIMENTAL

Cell culture

Three HNSCC cell lines: BICR6, FaDu (hypopharyngeal carcinoma cells) and H314 (derived from the floor of mouth) were purchased from American Type Culture Collection (ATCC). The cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% FBS (Biowest SAS, France) with the addition of antibiotics – penicillin and streptomycin 1% (Sigma-Aldrich, USA) at 37°C in 95% humidified atmosphere containing 5% CO₂.
MTT assay for cell viability

The effect of niclosamide (Cayman Chemical Company, MI, USA) on HNSCC cells viability was assessed by the MTT assay, providing data on the sum of cell viability and proliferation, using a standard protocol. Briefly, $1 \times 10^4$ cells (FaDu cell line) or $2 \times 10^4$ cells (BICR6 and H314 cell lines) were seeded per well in a 96-well plate. After 24 h of pre-incubation in DMEM supplemented with 5% FBS and antibiotics (200 µL/well), niclosamide was added to the culture medium in a concentration range of 0.05-2 µM and the cells were incubated for additional 48 h. Subsequently, the cells were washed with pre-warmed PBS buffer (200 µL/well), and fresh medium containing MTT salt (0.5 mg/mL; 200 µL/well) was added. After 4 h of incubation, formazan crystals were dissolved in acidic isopropanol (200 µL/well) in order to measure the absorbance at 570 and 690 nm. All the experiments were repeated three times with three measurements per assay. Sub-toxic doses of niclosamide were applied in further studies.

Cell treatment, isolation of total RNA and cDNA synthesis

$1 \times 10^6$ cells (FaDu) or $2 \times 10^6$ cells (BICR6 and H314) were seeded in 100 mm culture dishes and pre-incubated for 24 h in 10 mL of DMEM containing 5% FBS and antibiotics. Afterward, cells were treated with niclosamide at the following concentrations: 0.1 µM and 0.2 µM – FaDu cells or 0.2 µM and 0.5 µM – BICR6 and H314 cells. Cells treated with 0.05% DMSO were used as a control. After 48 h of incubation, total RNA was isolated using Universal RNA Purification Kit (EURx, Poland) and subjected to reverse transcription using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., MA, USA), according to manufacturer’s protocol.

Quantitative real-time PCR

Quantitative real-time PCR was performed using 5 × HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne, Estonia) on the LightCycler® 96 System (Roche, Germany). Primer sequences, which are listed in Table 1, were generated using Beacon Designer software. All the reactions were run in triplicate. The protocol started with a 15 min enzyme activation at 95°C, followed by 40 cycles of 95°C for 15 s; 56°C for 20 s; 72°C for 20 s and the final elongation at 72°C for 5 min. The melting curve analysis (60-90°C) was used to confirm the generation of a single amplification product. Experiments were normalized for the mean expression of the TATA-box binding protein (TBP) and porphobilinogen deaminase (PBGD). The Pfaffl relative method was used for fold-change quantification.

![Figure 2. The effect of niclosamide on the expression of CTNNB1, GSK-3β and β-catenin target genes (CCND1, c-MYC, MMP7, BIRC5, Axin2) in BICR6, H314 and FaDu cell lines after 48 h incubation with niclosamide at the concentrations: a = 0.1 µM (FaDu) or 0.2 µM (BICR6, H314), b = 0.2 µM (FaDu) or 0.5 µM (BICR6, H314). The level of each transcript was calculated in relation to cells treated with the vehicle (DMSO), where expression was equal to 1. Mean values ± SD from two independent experiments are shown. Asterisk above bars denotes statistically significant changes, p ≤ 0.05](image-url)
Flow cytometric cell cycle analysis in FaDu cells

The analysis of the cell cycle was performed using Muse Cell Cycle Kit (Merck, Germany) according to the manufacturer’s protocol. Briefly, $1 \times 10^5$/well FaDu cells were seeded in 12-well plate and pre-incubated for 24 h in DMEM (1 mL/well) containing 5% FBS and antibiotics. Afterward, fresh medium containing niclosamide, topotecan or 0.1% DMSO was added and cells were incubated for additional 48 h. Then cells were collected by trypsination, washed with 1 mL/well pre-warmed PBS buffer, fixed in 1 mL 70% ethanol and stored at -20°C for at least 24 h. Subsequently, cells were once again washed with 1 mL PBS buffer and stained with 200 µL propidium iodide solution in the presence of RNase A. After 30 min incubation at room temperature in the dark, fluorescence of cells was analyzed with flow cytometry on Muse Cell Analyzer. Data analysis was performed using Muse 1.5 Analysis software. All the experiments were done in triplicate. Cells treated with topotecan were used as a positive control.

The evaluation of apoptosis induction in FaDu cells

As a marker of apoptosis, the externalization of phosphatidylserine was assessed by Annexin V staining (Muse Annexin V & Dead Cell Kit, Merck, Germany) according to the manufacturer’s protocol. 7-Aminoactinomycin D (7-AAD) stain was used as a counterstain to discriminate between early and late apoptotic cells.

Briefly, $1 \times 10^5$/well FaDu cells were seeded in 12-well plate and pre-incubated for 24 h in DMEM (1 mL/well) containing 5% FBS and antibiotics. Afterward, fresh medium containing niclosamide, topotecan or 0.1% DMSO was added and cells were incubated for additional 48 h. Subsequently, cells were collected by trypsination, resuspended in 100 µL of fresh medium and stained with a solution of Annexin V and 7-AAD (100 µL/well). After 20 min incubation at room temperature in the dark, fluorescence of cells was detected by flow cytometry on Muse Cell Analyzer and data were analyzed using Muse 1.5 Analysis software. All the experiments were done in triplicate. Cells treated with topotecan were used as a positive control.

Mitochondrial transmembrane potential (MTP, $\Delta \Psi_m$) in FaDu cells

The measurement of the changes in mitochondrial membrane potential ($\Delta \Psi_m$) was performed with the Muse MitoPotential Assay Kit (Merck, Germany) according to the manufacturer’s protocol. Briefly, $1 \times 10^5$/well FaDu cells were seeded in 12-well plate and pre-incubated for 24 h in DMEM (1 mL/well) containing 5% FBS and antibiotics. Afterward, fresh medium containing niclosamide, topotecan or 0.1% DMSO was added and cells were incubated for additional 48 h. Then, cells were collected by trypsination, resuspended in 100 µL of 1X Assay Buffer and 95 µL of MitoPotential working solution and incubated for 20 min at 37°C in CO2 incubator. 5 µL of Muse MitoPotential 7-AAD reagent was added to each well and after 5 min incubation at room temperature in the dark, fluorescence was analyzed by flow cytometry on Muse Cell Analyzer. Data were analyzed using Muse 1.5 Analysis software. All the experiments were done in triplicate. Cells treated with topotecan were used as a positive control.

Statistical analysis

The statistically significant changes between the experimental groups and their respective controls were assessed using Student’s t-test using STATISTICA software (version 11), with $p \leq 0.05$ considered as significant.

RESULTS

The effect of niclosamide on the HNSCC cells viability

Cells viability was assessed by MTT assay (Fig. 1). In general, the dose-dependent effect was observed in all three cell lines. However, significant differences in the susceptibility to niclosamide cytotoxic and/or antiproliferative effect were found. The hypopharyngeal FaDu cells were the most sensitive with IC50 value of 0.40 µM, while H314 cells derived from the floor of mouth were the least sensitive (IC50 = 0.94 µM).

The effect of niclosamide on the expression of genes associated with Wnt signaling

Figure 2 presents the results of quantitative PCR performed to assess the expression of genes associated with the canonical Wnt pathway elements, CTNNB1 and GSK-3β, as well as Wnt signaling target genes, CCND1, transcription factor c-MYC, MMP7, survivin – BIRC5 and Axin2. Treatment with niclosamide did not change the expression of CTNNB1 or GSK-3β genes in any of the cell lines tested. However, the reduction in the expression of MMP7 was observed in H314 and FaDu cell lines, while the expression of CCND1 was also slightly reduced in H314 cells. The expression of Axin2, a feedback repressor of β-catenin-depend-
ent signaling, is strongly related to TCF/LEF transcription factor activity. Although there were no statistically important changes in Axin2 transcript level, a tendency was observed to reduce its expression in FaDu and H314 cells.

**The effect of niclosamide on the expression of genes encoding glycolysis-related enzymes and transporters**

The expression of genes encoding glucose and lactate transporters GLUT1 and MCT1, and enzymes involved in glycolysis, hexokinase 2 – HK2, phosphofructokinase M – PFKM, pyruvate kinase M2 – PKM2, pyruvate dehydrogenase A1 – PDHA1, pyruvate dehydrogenase kinase 1 – PDK1, lactate dehydrogenase A – LDHA, was assessed (Fig. 3).

In FaDu cells, which were most sensitive to niclosamide treatment, the expression of the genes encoding glycolytic enzymes was generally reduced, except for overexpressed LDHA gene. In contrast to FaDu cells, the expression of the glycolysis-related genes was rather increased in BICR6 cells. In H314 cells, which were most resistant to niclosamide treatment, the expression of all the assessed genes remained unchanged or was increased in the case of GLUT1, PDK1, LDHA, and MCT1.

**Flow cytometric analysis of cell cycle distribution, apoptosis induction and mitochondrial transmembrane potential changes in FaDu cells**

Since FaDu cells were the most sensitive to niclosamide cytotoxic activity, additional analyses were performed in order to evaluate this effect in detail. Figure 4 presents data illustrating the effect of niclosamide on cell cycle distribution, apoptosis induction and mitochondrial potential changes.

The treatment with niclosamide at 1 µM concentration showed the strongest cell cycle inhibition, with a significant increase in the number of cells in S phase. Additionally, changes in the number of cells in G0/G1 and G2/M phases after 0.5 µM and 1 µM niclosamide treatment were also observed. Niclosamide at the highest concentration also doubled the percentage of total apoptotic cells accompanied by enhanced depolarization of the mitochondrial membrane. The latter, leading to mitochondrial dysfunction, is considered as an indicator of early stages of apoptosis and intrinsic pathway origin of apoptosis.

**DISCUSSION**

Among the various aspects of the possible anticancer activity of niclosamide, several studies point-
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ed to the modulation of Wnt/β-catenin signaling pathway (10). Moreover, as the result of niclosamide treatment, the shift from oxidative phosphorylation to enhanced aerobic glycolysis was also observed (13). Although the anti-cancer activity of niclosamide was described in several in vitro and in vivo models, there is no data available on its effect on HNSCC cells.

Thus, the aim of this study was threefold, i/ to evaluate the effect of niclosamide on HNSCC cells survival, ii/ to find out whether niclosamide may affect the expression of selected Wnt pathway-related genes in HNSCC, iii/ to check if the expression of genes encoding key enzymes of glycolysis may be also affected.

The cytotoxic and antiproliferative effect of niclosamide in HNSCC cells was confirmed by MTT assay (Fig. 1). Among the three cell lines tested, FaDu cells derived from hypopharyngeal SCC were the most sensitive to the treatment with...
niclosamide, while H314 cells derived from the floor of mouth were the most resistant (IC$_{50}$ = 0.4 µM and 0.94 µM, respectively). These values were lower than those reported in colorectal cancer cells, but in the latter case, the viability was assessed after 12 or 24 h (10), while in our study the viability was evaluated after 48 h of exposure to niclosamide. On the other hand, in ovarian cancer cells after 72 h of exposure to niclosamide, the IC$_{50}$ value for the original compound and nano-drug formulation was assessed as approximately 6.0 µM and 3.5 µM, respectively (13).

The more detailed analysis of possible mechanisms of niclosamide cytotoxic effect in FaDu cells showed its ability to induce cell cycle arrest in the S phase (Fig. 4A) and the induction of apoptosis (Fig. 4B), at least at the concentrations above IC$_{50}$. The induction of apoptosis in part may be related to mitochondrial dysfunction resulting from depolarization of its membranes as indicated by the results of mitopotential of its membranes as indicated by the results of the more detailed analysis of possible mechanisms of niclosamide cytotoxic effect in FaDu cells showed its ability to induce cell cycle arrest in the S phase (Fig. 4A) and the induction of apoptosis (Fig. 4B), at least at the concentrations above IC$_{50}$. The induction of apoptosis in part may be related to mitochondrial dysfunction resulting from depolarization of its membranes as indicated by the results of mitopotential assay (Fig. 4C).

Similarly, as in CC531 colorectal cancer cells, niclosamide did not affect the expression of β-catenin or its destruction complex element GSK-3β in any of the tested HNSCC cells, however, it reduced the canonical Wnt target genes MMP7 (FaDu and H314 cells) and CCND1 (H314 cells). Also, in these two cell lines, Axin2 expression, which is a good marker of TCF/LEF transcription activity (20), tended to be reduced (Fig. 2). Thus, it is possible that, at least in FaDu and H314 cells, niclosamide can affect canonical Wnt signaling, although in a limited extent. Metalloproteinases have been long associated with cancer cell invasion and metastasis, but their involvement in tumor initiation and promotion was also reported. Of them, MMP7 overexpression was detected in 50% of cancer tissues and was associated with aggressiveness (21). Moreover, it has recently been shown that altered expression of MMP7 controlled by SIPA1 (Signal-induced Proliferation-associated Protein 1) plays a crucial role in metastatic oral SCC (22). Thus, reduced expression of MMP7 in FaDu and H314 cells resulting from niclosamide treatment may suggest its potential anti-metastatic activity in HNSCC.

In FaDu cells also the transcript levels of glycolytic genes were generally reduced as the result of niclosamide treatment, except for the transcript of LDHA, which was increased, and HK2 and PKM2, whose expression was unchanged (Fig. 3). The increased expression of LDHA may potentially lead to higher production of lactate and lower pH in cell environment.

It has recently been shown that in pH 6.5 or lower the viability of prostate cancer cells treated with niclosamide significantly decreased, while relative resistance to niclosamide toxicity at alkaline pH was observed. Thus, it is possible that the higher toxicity of niclosamide in FaDu cells may be related to acidic environment similarly as in prostate cancer cells (23).

Such a hypothesis may be further supported by the observation that in H314 cells, which were the most resistant to niclosamide treatment, the expression of genes encoding glycolytic enzymes and transporters was mostly unchanged. Moreover, in BICR6 cells, which showed intermediate susceptibility to the toxic effect of niclosamide, the up-regulation of glucose transporter GLUT1 and many other genes encoding glycolytic enzymes was found. The only exception was PDHA1, which was downregulated most probably as the result of the up-regulation of PDK1 gene, whose protein product inactivates PDH via phosphorylation.

These observations suggest that the cytotoxic and antiproliferative activity of niclosamide in HNSCC may be related to its effect on the expression of genes encoding glycolytic enzymes and proteins. The loss of mitochondrial membrane potential observed in our study may support this assumption. Further functional studies are necessary in order to confirm this suggestion.

CONCLUSION

Our study showed that niclosamide inhibits the growth of HNSCC cells. This effect strongly depends on the cell type. In FaDu cells, which were the most sensitive to the antiproliferative and cytotoxic effect of niclosamide, this might be, at least in part, related to the modulation of canonical Wnt signaling pathway and increased expression of LDHA gene.

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Conflict of interest

The authors declare that they have no conflict of interest.
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