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Structure–Activity Relationship of Niclosamide Derivatives

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Abstract. *Background/Aim: Cancer is a leading cause of death. Hence, this study aimed at the optimization of niclosamide derivatives for the development of new potential anticancer agents. Materials and Methods: Niclosamide derivatives were synthesized and tested against a panel of human cancer cells: MDA and MCF7 breast cancer cells, PC3 and DU-145 prostate cancer cells, Hela cervical cancer cells, and HL-60 acute promyelocytic leukemia cells. They were also tested in nuclear factor-κappa B (NFκB), V-Ki-ras2 Kirsten rat sarcoma viral oncogene (KRAS), and mitochondria transmembrane potential (MTP) assays. Results: N-(3,5-Bis(trifluoromethyl)phenyl)-5-chloro-2-hydroxybenzamide exhibited the most significant cytotoxicity against HL-60 cells, while 5-chloro-N-(2-chlorophenyl)-2-hydroxybenzamide was the most active in the NFκB assay and 5-chloro-N-(3,5-difluorophenyl)-2-hydroxybenzamide in the MTP assay. 5-chloro-N-(2-chloro-4-(trifluoromethyl) phenyl)-2-hydroxybenzamide and 5-chloro-2-hydroxy-N-(4-hydroxyphenyl)benzamide inhibited both HL-60 cell proliferation and NFκB. Conclusion: In-depth study of the most promising compounds is highly encouraged to further develop into potential anticancer agents those derivatives found to be significantly active.*

Niclosamide is a salicylanilide with anticestodal activity that was discovered in 1958. It was the drug-of-choice before the discovery of praziquantel. As part of our drug discovery program, we decided to examine at the potential of this drug highly valued by the World Health Organization (WHO) as it is part of the WHO list of essential medicines (1). Because the mechanism of action of niclosamide is associated with the uncoupling of oxidative phosphorylation from electron transport by allowing protons to translocate through the inner mitochondria membrane, there is potential for an impact on cancer cells (1). Multiple targets might be part of the oxidative phosphorylation process in cancer cells, including nuclear factor-κappa B (NFκB) and V-Ki-ras2 Kirsten rat sarcoma viral oncogene (KRAS). NFκB is an important transcription factor as there is increasing evidence that its activation is essential for survival and proliferation of malignant cancer cells. If activated, it is translocated to the nucleus where it regulates more than 200 genes that control cell growth and inflammation (2). NFκB activation has been linked to initiation and progression of several human cancer types (3). *KRAS*-mutated adenocarcinomas have been shown to have significantly higher levels of nuclear NFκB expression compared to wild-type tumors (4). Moreover, oncogenic *KRAS* mutations are commonly found in certain cancer types (5, 6). Hence, the epidermal growth factor (EGF) was used in our assay to stimulate the KRAS pathway. EGF is the ligand of the cell membrane-bound receptor EGFR, and induces intracellular KRAS protein downstream. According to previous studies, KRAS may provide a possible target for prevention of the progression of cancer (7, 8). Furthermore, a recent study showed that suppression of NFκB activation induces mitochondrial dysfunction and promotes cell death (9).

Niclosamide also works by blocking the uptake of sugar in helminths (10), another pathway that could be exploited in therapy of cancer by developing new agents able to block cancer glucose-regulated metabolism and signaling. This type of agent would have a significant impact on cancer cell proliferation and viability as cancer cells have been found to exhibit increased levels of glucose uptake.

The suggested mechanism of action of niclosamide on cancer cells stimulated the idea of chemically optimizing this molecule for the development of new anticancer drugs. As a result, 13 derivatives of niclosamide were synthesized (Figure 1). This project focused on evaluating the potential

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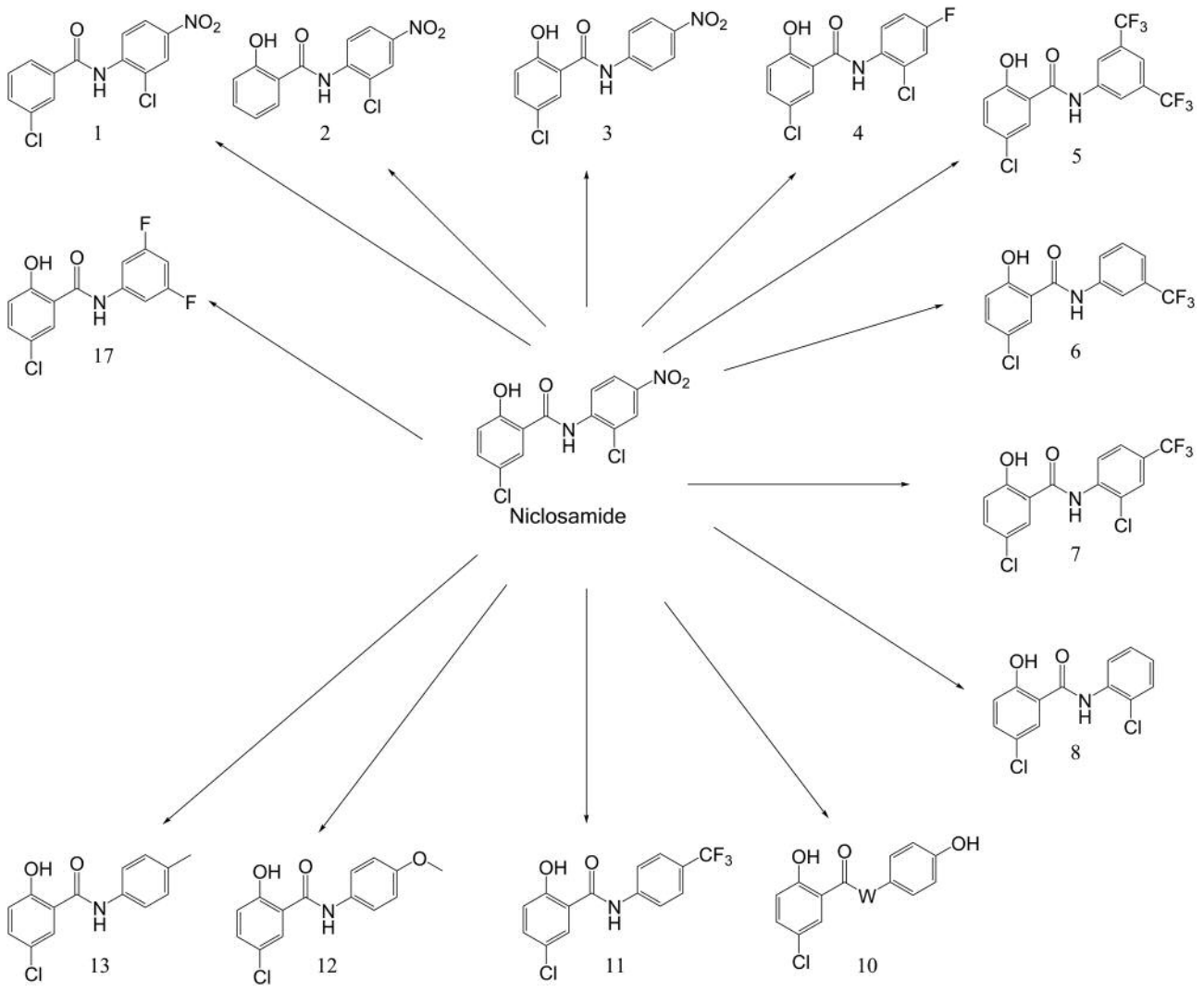

Figure 1. *Niclosamide and its derivatives*.

effects of niclosamide derivatives on cytotoxicity towards cancer cells, and on NFκB, mitochondria membrane potential, and KRAS.

Materials and Methods

Chemicals, reagents, antibodies and working solutions. Staurosporine and epidermal growth factor (EGF) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Rocaglamide was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). Bradford protein assay kit and human recombinant tumor necrosis factor α (TNFα) were obtained from Thermo Scientific (Rockford, IL, USA).

Preparation of compounds. Niclosamide and its derivatives (compounds 1-8, 10-13, 17) were synthesized according to a previously reported procedure (11).

Cell culture. MDA and MCF7 human breast cancer cells, PC3 and DU-145 prostate cancer cells, HeLa cervical cancer cells, and HL-60 acute promyelocytic leukemia cells were obtained from the American Type Culture Collection, Manassas, VA, USA. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) or Roswell Park Memorial Institute Media (RPMI-1640) containing 10% fetal bovine serum (FBS) and 10% antibiotic-antimycotic from Gibco (Rockville, MD, USA). The cells were grown as a monolayer in T75 tissue culture flasks and kept at 37˚C and in an atmosphere with 5% CO_2.

Sulforhodamine (SRB) cytotoxicity assay. The antiproliferative effects of niclosamide and its derivatives were tested on human cell lines, using a previously published protocol (12). The cells were seeded in a 96-well plate (1×10^5 cells per well). The compounds were diluted in 10% dimethylsulfoxide and cells treated with a concentration range of 0.25 ng/ml-20 μg/ml. The cell suspension

was incubated for 72 h. Cells were then fixed with 100 μl of ice-cold trichloroacetic acid (20%) for 30 min at 4˚C. After drying, cells were stained by adding 100 μ of SRB (0.4%) and kept at room temperature for 30 min, before washing the wells three times with acetic acid (1%) then letting them air-dry. Unbound dye was solubilized by adding 200 μl Tris base solution (10 mM) to each well. Paclitaxel was used as a positive control. Absorbance was read at a wavelength of 515 nm using a FLUOstar Optima plate reader (BMG Labtech Inc, Durham, NC, USA). The absorbance values were compared to those of the untreated control samples and the effective dose (ED_{50}, μM) was calculated using Table Curve 2Dv4 (System Software Inc., San Jose, CA, USA). The assay was run in triplicate.

NFκB assay. The EZ-Detect™ Transcription Factor Assay kit (Pierce Biotechnology, Rockford, IL, USA) protocol was used to evaluate the ability of niclosamide derivatives to interfere with the specific binding between the biotinylated-consensus sequence for the corresponding factor and active form of NFκB, NFκB-p65. The experiment was performed following the manufacturer's instructions with few modifications as previously reported (13). Nuclear extracts of HeLa cells treated with different concentrations of each compound and TNFα were used to evaluate NFκB-p65 binding. TNFα-stimulated nuclear extract was used as a control and rocaglamide served as a positive control. The calculation of NFκB activity was based on the measurement of a chemiluminescent signal in a plate reader (FLUOstar Optima; BMG Labtechnologies GmbH, Inc.) and results are presented as the half maximal inhibitory concentration (IC_{50}, μM).

Mitochondrial transmembrane potential (MTP) assay. The MTP assay kit from Cayman Chemical Company (Ann Arbor, MI, USA) was used to assess the MTP in colon cancer cells following a previously published protocol (14, 15). In brief, cells were seeded in a 96-well plate and treated with the test compounds at different concentrations. After incubation, cells were washed in phosphate-buffered saline (PBS). The potentiometric dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazoylcarbocyanine iodide (JC-1) was used to stain the cells. A volume of 10 μl of JC-1 stain (1/20 dilution) was added to the cells, followed by 15 min incubation at 37˚C in 5% CO_2. At a high MTP, red fluorescent J-aggregates are formed in healthy cells; however, in cells with a low MTP, such as those in an apoptotic state, JC-1 remains in the monomeric form, which exhibits green fluorescence. Mitochondrial function was evaluated by detecting J-aggregates using an excitation wavelength of 520-570 nm, and emission of 570-610 nm, respectively. Analysis was performed using a plate reader (FLUOstar Optima; BMG Labtechnologies GmbH, Inc) and results are presented as the half maximal inhibitory concentration (IC_{50}) in μM.

KRAS inhibitory assay. The assay was performed following a previously published protocol (11, 15). Human colorectal HT-29 cells, purchased from the ATCC, were cultured in RPMI-1640 supplemented with 10% FBS and 100 IU/ml antibiotic-antimycotic. The cells were treated and incubated at 37˚C and 5% CO_2 for 3 h. This was followed by removal of the medium and washing three times with phosphate-buffered saline (PBS). The cells were then treated with EGF solution (5 ng/ml) for 2 min. Thereafter, protease inhibitor was added and cells were lysed. Aliquots were stored at a temperature of −80˚C. Protein concentration was determined using BCA kit and then KRAS activity was assessed by using the Ras GTPase Chemi ELISA kit from Activity Motif (Carlsbad, CA, USA). The luminescence was read using Fluostar Optima plate reader (BMG Labtech Inc) and results are presented as the half maximal inhibitory concentration (IC_{50}) in μM.

Statistical analysis. Experimental results are presented as means±standard error of the mean (SEM) and all measurements and analyses were carried out in triplicate. TableCurve 2D 4v (System Software Inc.) was used for statistical evaluations.

Table I. *Cytotoxic activity of niclosamide derivatives. All compounds exhibited <50% inhibition when tested against MDA-MB-231, MCF-7, HeLa, PC-3, and DU-145 cells (data not shown).*

SRB assay[3]

Compound	HL-60 cells	
	Inhibition at 20 μg/ml	ED_{50} (μM)
1	<50%	--
2	<50%	--
3	>50%	8.79
4	>50%	7.29
5	>50%	1.94
6	<50%	--
7	>50%	7.63
8	<50%	--
10	>50%	12.26
11	<50%	--
12	<50%	--
13	<50%	--
17	<50%	--
Niclosamide	<50%	--

-- Not tested. Positive control for assay was taxol. ED_{50}:effective dose, determined for compounds exhibiting >50% inhibition.

Results

Our efforts toward chemical and biological optimization of niclosamide derivatives identified compound **5** as the chemical derivative that exhibited the most significant cytotoxicity against HL-60 cells (Table I). None of the compounds were active against the other cell lines at 20 μg/ml. In the target-based assays, compound **8** was the most significantly active in the NFκB assay and compound **17** in the MTP assay (Table II). However, several derivatives exhibited activity in more than one assay. Compounds **7** and **10** inhibited HL-60 cell proliferation as well as NFκB activation, suggesting that its antiproliferative effect may be related to its inhibition of NFκB activation (Tables I and II). Some compounds inhibited NFκB activity, but did not inhibit the proliferation of cells, suggesting that the mechanism may involve other cancer pathways. All compounds were tested in the KRAS assay and

none of them exhibited >50% inhibition. Tables I and II summarize the preliminary analysis of cytotoxicity, MTP, KRAS, and NFκB screening for niclosamide and its derivatives.

Discussion

The mechanism of action of niclosamide has been associated with the uncoupling of oxidative phosphorylation from electron transport by allowing protons to translocate through the inner mitochondria membrane. This mechanism could have a potential impact on cancer cell proliferation (1). Hence, compounds from this research project were screened in a panel of *in vitro* bioassays, including a MTP assay, to determine their potential as leads for cancer treatment.

Compounds **3, 4, 5, 7** and **10** were found to inhibit the proliferation and growth of HL-60 acute promyelocytic leukemia cells. The results showed that compound 5 had the best inhibitory effect on HL-60 cells. In 2014, leukemia was among the five leading cancer types by sex and age and in 2017, cancer continues to be the second leading cause of death in the U.S. (16). Thus, the results obtained from this assay encourage further chemical optimization of niclosamide derivatives to improve their cytotoxic activity.

NFκB, an important transcription factor, has been shown to have increased activity in most tumor cells. A variety of carcinogenic factors can be activated by NFκB pathway to promote cell growth, malignant transformation of cells and promote tumor cell metastasis. Inhibition of NFκB activity can inhibit tumor cell proliferation, promote apoptosis, increase the sensitivity of cells to chemotherapy, inhibit inflammation, and reduce tumor angiogenesis. Therefore, we further studied the effect of compounds on NFκB activity, and found that nine compounds (namely **6, 7, 8, 10, 11, 12, 13, 17** and niclosamide) significantly inhibited the activity of NFκB (Table II). Compounds **7** and **10** also inhibited the proliferation of HL-60 cells. Hence, further analysis is recommended to confirm if the inhibitory effect on proliferation of HL-60 cells may be related to their inhibition of NFκB activity.The other three compounds (namely **3, 4,** and **5**) that inhibited the proliferation of leukemic HL-60 cells did not inhibit the activity of NFκB. Thus, further in-depth study would also be worth considering in order to uncover if the effects of these compounds may involve other unrelated mechanistic pathways.

MTP plays an important role in the oxidative processes of cells as well as in producing energy required for cell homeostasis. The results presented in Table II shows that all the compounds reduced the MTP, among which six compounds had strong activity, namely compounds **3, 4, 8, 11, 12, 13** and **17**. Compounds **8, 11, 12, 13** and **17** also inhibited the activity of NFκB, suggesting that these compounds may have a better potential in the management of oxidative phosphorylation (a previously reported mechanism for this class of compounds) when compared to the parent compound niclosamide.

Table II. *Target-based activity of niclosamide derivatives against HL-60 cells.*

	NFκB assay		MTP assay		KRAS assay
Compound	%*	IC_{50} (μM)	%*	IC_{50} (μM)	%*
1	<50	--	>50	17.96	<50
2	<50	--	>50	1.13	<50
3	<50	--	>50	0.05	<50
4	<50	--	>50	0.12	<50
5	<50	--	>50	2.89	<50
6	>50	1.84	>50	6.92	<50
7	>50	1.98	>50	8.13	<50
8	>50	0.97	>50	0.11	<50
10	>50	3.32	>50	0.50	<50
11	>50	1.52	>50	0.09	<50
12	>50	>10	>50	0.03	<50
13	>50	>10	>50	0.05	<50
17	>50	>10	>50	0.01	<50
Niclosamide	>50	>10	>50	0.03	<50

*Inhibition at 50 μg/ml (%). -- Not tested; NFκB: nuclear factor κappa B; MTP: mitochondria transmembrane potential; KRAS: V-Ki-ras2 Kirsten rat sarcoma viral oncogene; IC_{50}: half maximal inhibitory concentration, determined only for compounds exhibiting >50% inhibition. Positive controls: NFκB, rocaglamide; MTP: staurosporine; KRAS: methylrocaglate.

Scrutiny of the structure–activity relationship (SAR) of niclosamide derivatives indicates that the activity of the different derivatives is dependent on both the nature and the location of the different substituents. In summary, the replacement of the nitro ($-NO_2$) substituent in the anilide group of the niclosamide molecule by fluorine or trifluoromethyl substituents, as well as the loss of chlorine atoms greatly influences the MTP and NFκB activity of the different niclosamide derivatives. Briefly, the loss of a chlorine atom and the substitution of the $-NO_2$ group by two -CF3 (Figure 1) in compound **5** greatly influence the cytotoxic activity of this derivative when compared to the parent compound niclosamide. A similar trend was observed for compound 7, through the replacement of the $-NO_2$ group by a $-CF_3$ (Figure 1). On the other hand, a single replacement of a nitro group by the trifluoromethyl in C7 derivative led to decreased activity on the MTP when compared to niclosamide (Table II). The opposite is observed for C17 with the replacement of one $-NO_2$ group and one chlorine by two fluorine. C17 has increased activity on MTP when compared with niclosamide. In relation to the NFκB activity, the loss of a nitro group (compound C8) increased NFκB-inhibitory activity. Substitutions in the –NH group by

a tungsten group and the nitro group by the hydroxyl group (compound **10)** resulted in reduced activity when compared to the other four derivatives that exhibited significant NFκB-inhibitory activity. Even though these substitutions in the niclosamide molecule resulted in increased activity in terms of MTP and NFκB, they did not improve the overall KRAS activity profile (Table II). Thus, in order to target KRAS signaling pathway in cancer cells, another type of substitution may need to be considered.

Conclusion

Using a panel of *in vitro* bioassays, this study analyzed differential activity of semi-synthetic derivatives of niclosamide. Compounds **7** and **10** inhibited both HL-60 cell proliferation and NFκB activation, suggesting further studies to confirm whether its inhibitory effect on cell proliferation is due to the inhibition of NFκB activation. Compounds **6, 8,** and **11** inhibited NFκB, but did not inhibit the proliferation of leukemia HL-60 cells, suggesting the potential for a different mechanism of action, which requires further in-depth study. The overall SAR analysis suggests that replacement of the nitro group (-NO_2) in the anilide group by the trifluomethyl group and fluorine, as well as the loss of chlorine, greatly influence the MTP and NFκB activity of the different niclosamide derivatives. The results and analysis of this study suggest high potential for further development of these niclosamide derivatives into safe and effective anticancer agents.

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