Niclosamide induces mitochondria fragmentation and promotes both apoptotic and autophagic cell death

So Jung Park1, Ji Hyun Shin1, Hee Kang1, Jung Jin Hwang2 & Dong-Hyung Cho1*

1Graduate School of East-West Medical Science, Kyung Hee University, Yongin 446-701, 2Institute for Innovative Cancer Research, Asan Medical Center, Seoul 138-736, Korea

Mitochondrial dynamics not only involves mitochondrial morphology but also mitochondrial biogenesis, mitochondrial distribution, and cell death. To identify specific regulators to mitochondrial dynamics, we screened a chemical library and identified niclosamide as a potent inducer of mitochondria fission. Niclosamide promoted mitochondrial fragmentation but this was blocked by down-regulation of Drp1. Niclosamide treatment resulted in the disruption of mitochondria membrane potential and reduction of ATP levels. Moreover, niclosamide led to apoptotic cell death by caspase-3 activation. Interestingly, niclosamide also increased autophagic activity. Inhibition of autophagy suppressed niclosamide-induced cell death. Therefore, our findings suggest that niclosamide induces mitochondria fragmentation and may contribute to apoptotic and autophagic cell death. [BMB reports 2011; 44(8): 517-522]

INTRODUCTION

Mitochondria are highly dynamic organelles that continuously divide and fuse in healthy cells (1, 2). Mitochondria dynamics (fission and fusion) control all processes related to mitochondria biogenesis, subcellular localization, and distribution as well as their morphology. Disruption of the balance is highly associated with many diseases such as neurodegeneration, aging, and cancer (3-6).

Mitochondria fission is regulated by dynamin-related protein 1 (Drp1). Drp1 translocation from the cytosol into the outer mitochondria membrane (OMM) initiates membrane fragmentation (7). siRNA against Drp1 or a dominant negative Drp1 mutant (K38A, defective GTPase activity) inhibits fission and suppresses cytochrome c release and cell death (8, 9). On the other hand, the mitochondria fusion is mediated by mitofusin1 and 2 (Mfn1/2) and optic atrophy protein 1 (Opa1) (10). OMM fusion is mediated by Mfn1/2 while inner mitochondria membrane (IMM) fusion is regulated by Opa1. In contrast to fission, mitochondria fusion functions as a cell-protective mechanism. Silencing Mfn1/2 results in mitochondria fragmentation and increases susceptibility to apoptotic stimuli. Similarly, loss of Opa1 induces the disruption of cristae as well as spontaneous apoptotic cell death (11, 12). Interestingly overexpression of Fis1 selectively reduces mitochondrial mass and triggers autophagy (13). In addition, overexpression of both Opa1 and a K38A Drp1 efficiently suppresses autophagy (14). Although mitochondria dynamics are associated with autophagy, the precise underlying mechanisms are poorly understood. Recently Cassidy-Stone et al. screened mdivi-1 as a Drp1 chemical inhibitor (15). Mdivi-1 suppresses OMM permeabilization, the release of cytochrome C, and eventually prevents apoptotic cell death in HeLa cells (15). Therefore, identification of regulator of mitochondria dynamics should lead to the development of new therapeutic strategies for treating mitochondria associated diseases. However, chemicals modulating mitochondria dynamics are not well known yet.

In the present study, we used HeLa cells stably expressing a fluorescence mito-tracker protein to identify mitochondria dynamics regulators. We identified niclosamide as a factor that promotes the fragmentation of mitochondria. Niclosamide treatment decreased cell viability and proliferation rate. We also investigated both apoptotic and autophagic cell death mechanisms are involved in niclosamide-induced cell death.

RESULTS

Niclosamide induces Drp1 dependent mitochondria fragmentation in HeLa cells

Mitochondria play a pivotal role in cell death by mediating both intrinsic and extrinsic signaling pathways. Additionally, mitochondria dynamics not only control morphology of these organelles but also regulate mitochondria-related functions including cell death. To identify chemical modulators of mitochondria dynamics, we developed a cell-based screening system using HeLa cells (HeLa/Mito-YFP) stably expressing a YFP protein fused with a mitochondria tracker (mito-YFP) (16). Using this assay, we screened a Lopac 1280 chemical library (a collection of 1,280 pharmacologically active compounds). Based on the screening
Niclosamide induces cell death via mitochondria fragmentation
So Jung Park, et al.

Fig. 1. Niclosamide induces Drp1-dependent mitochondrial fission. (A) Representative fluorescence micrographs of mitochondrial morphology before and after niclosamide treatment. HeLa cells stably expressing mito-YFP (HeLa/mito-YFP) were exposed to in the presence or absence of niclosamide for 3 hr and then were imaged using fluorescence microscope. (B) HeLa/mito-YFP cells were exposed to increasing concentrations of niclosamide (0.5, 1, 5, and 10 uM) and then mitochondrial fragmentation was monitored after 6 hr. (C) Niclosamide (10 uM) induced mitochondria fragmentation in a time dependent manner. (D) HeLa/mito-YFP cells were transfected with control scrambled siRNA (Scram) or specific siRNA for Drp1 (siDrp1). After 2 days the cells were treated with niclosamide (10 uM) for 1.5 hr and mitochondrial fragmentation was observed. Reduced expression of Drp1 by siRNA was confirmed by Western blotting. (E) HeLa/mito-YFP cells transfected with a control empty vector (pcDNA) or a Drp1 dominant negative mutant (K38A) were treated with niclosamide (10 uM) for 1.5 hr and then mitochondrial fragmentation was monitored. Data are represented by the mean ± SEM (n > 3).

results, we selected niclosamide (5-chloro-N-(2-chloro-4-nitrophenyl)-2-hydroxybenzamide, C_{13}H_{8}Cl_{2}N_{2}O_{4}) for further analysis as a potent inducer of mitochondria fragmentation (Fig. 1A). To verify the screening results, HeLa/mito-YFP cells were exposed to different doses of niclosamide (0.5 uM to 10 uM) for different periods of time (0.5 hour to 6 hour). And we confirmed mitochondria were dramatically fragmented by niclosamide (Fig. 1B, C). Drp1, a GTPase protein regulates the mitochondria fission machinery. Therefore, we next examined the effects of Drp1 on niclosamide-induced mitochondria fragmentation. Both down-regulation of Drp1 with siRNA and overexpression of a dominant negative Drp1 mutant (K38A) inhibited mitochondria fragmentation induced by niclosamide (Fig. 1D, E). These results show mitochondria fragmentation by niclosamide is dependent on Drp1 activity.

Niclosamide induces mitochondrial dysfunctions in HeLa cells
Mitochondria undergo excessive fragmentation during apoptosis. Down-regulation of Drp1 suppresses cell death, implicating that mitochondria fission is directly involved in apoptosis. Recently, it was shown that niclosamide increases the levels of reactive oxygen species (ROS), resulting in introduction of apoptosis in acute myelogenous leukemia (AML) cells (17). Thus, we next examined the effect of niclosamide on mitochondria functions related to cell death. Niclosamide functions to inhibit NADH → NADP+ transhydrogenation driven by electron transport-dependent NADH oxidation and increases ROS level (18). Excessive ROS disrupts the mitochondrial permeability transition that is an important step for the induction of mitochondria-mediated cell death. Therefore, JC-1, a lipophilic cationic dye, was used to investigate mitochondrial membrane collapse. Mitochondria depolarization was highly induced (14% to 60%) by treatment with niclosamide (Fig. 2A, B). The electrochemical gradient of mitochondria is important to generate cellular ATP level. Next, we examined cellular ATP levels in cells treated with niclosamide. Cellular ATP levels were also gradually decreased by niclosamide, suggesting that niclosamide disrupts mitochondrial membrane potential (Fig. 2C). Recently it was reported that niclosamide inhibits the growth of AML cell xenografts in nude mice (17). We examined the effect of niclosamide on proliferation in HeLa cells. Similar to the previous studies, niclosamide efficiently suppressed cell proliferation in HeLa cells (Fig. 2D). These results indicate that niclosamide actively contributes to cytotoxicity in HeLa cells.
Niclosamide induces apoptotic cell death in HeLa cells

Since excessive mitochondria fragmentation impacts many cellular processes including cell death, our previous findings led us to investigate the mode of niclosamide-mediated cell death. Activation of caspase is a typical property of apoptotic cell death. To examine whether or not niclosamide induces apoptotic cell death, we examined caspase-3 activation in HeLa cells treated with this compound (Fig. 3). Cleaved caspase-3 was detected following niclosamide treatment, and this was significantly suppressed by zVAD, a pan-caspase inhibitor, suggesting that niclosamide induces apoptotic cell death in HeLa cells.

Niclosamide induces autophagic cell death in HeLa cells

Mitochondrial dynamics are associated not only with apoptosis but also autophagy. Niclosamide has been previously screened as a potent inducer of autophagy. Balgi et al. showed that niclosamide induces autophagosome formation and inhibits mTORC1 signaling in MCF-7 cells (19). To study the effect of niclosamide on autophagy, HeLa cells (HeLa/GFP-LC3) stably expressing GFP-LC3 were exposed to niclosamide after which autophagy was subsequently observed (Fig. 4A, B). Similar to MCF-7 cells, niclosamide efficiently induced autophagy in HeLa cells. Autophagy has roles in both cell protective and cell death responses. ATG5 is an essential component of autophagy regulation. To address the roles of autophagy in niclosamide-mediated cell death, ATG5 knock-out MEF cells were employed. Niclosamide failed to induce autophagy in the ATG5 knock-out MEF cells while it dramatically activated autophagy in wild type MEF cells (Fig. 4C). More importantly, down-regulation of ATG5 significantly suppressed niclosamide-induced cell death compared to the control cells (Fig. 4D), indicating that cell death induced by niclosamide is also associated with autophagic cell death in HeLa cells.

**DISCUSSION**

Mitochondria continuously move, divide, and fuse together. The balance between fusion and fission regulates mitochondria functions. Accumulating evidence has indicated that imbalances of mitochondria dynamics contribute to the development many diseases including neurodegenerative and metabolic disorders and...
Niclosamide induces cell death via mitochondria fragmentation
So Jung Park, et al.

Niclosamide induces cell death via mitochondria fragmentation

So Jung Park, et al.

Fig. 4. Deletion of ATG5 blocks ARP101-induced autophagy. (A) HeLa cells stably expressing GFP-LC3 (HeLa' GFP-LC3) cells were treated with niclosamide (10 μM) for 3 hr. The cells were fixed and examined by fluorescence microscopy. Conversion of LC3I to LC3II was increased in niclosamide-treated cells. (B) HeLa/GFP-LC3 cells were exposed to niclosamide (1 μM, 10 μM) and cells with punctate GFP-LC3 dots were counted under a fluorescence microscope. (C) Wild type MEF (W) or ATG5 knock out MEF (K) cells were treated with niclosamide (1 μM, 10 μM) for 3 hr and autophagy induction was examined by measuring the conversion of LC3I to LC3II. (D) Wild type MEF (WT) or ATG5 knock out MEF (KO) cells were treated with niclosamide (1 μM, 10 μM) for 16 h. Cell viability was determined by MTT assay. The data means ± SEM (n > 3); (*P < 0.02).

cancer (6, 20). Indeed mutations in fusion proteins cause two distinct types of neurodegenerative disease, Charcot Marie Tooth 2A and dominant optic atrophy. Thus, small compounds that regulate mitochondria dynamics may be of interest in developing new therapeutic approaches. In this study, we screened bioactive chemicals and identified several known modulators such as thapsigargin, a compound that leads to cellular calcium release, and gossypol, a bcl-2 inhibitor (21, 22). Niclosamide has been used as an anti-helminthic drug for treating infections of beef, dwarf, and dog tapeworms through uncoupling oxidative phosphorylation and blocking the transhydrogenation of NADH to NADP⁺ (23, 24). Interestingly, niclosamide was shown to exert potent anti-neoplastic effects in acute myelogenous leukemia stem cells by increasing ROS levels and decreasing NF-kB activation (17). Although it was suggested that niclosamide possesses anti-neoplastic functions, the precise mechanisms have not been fully elucidated. In the present study, we found evidence that niclosamide largely induces mitochondria fragmentation which eventually leads to the activation of cell death pathway. Mitochondria fission processes are governed by Drp1 activity. Thus, we first investigated the effect of Drp1 on niclosamide-induced mitochondria fragmentation. Inhibition of Drp1 by both siRNA and a dominant negative mutant strongly abolished niclosamide-induced mitochondria fragmentation. Drp1 is also an important player in niclosamide-induced mitochondria fission. Mitochondria fragmentation leads to the depletion of mitochondria membrane potential (25, 26). Dissipation of mitochondria membrane potential results in increased ROS generation, reduced ATP levels, and the release of several pro-apoptotic factors, including cytochrome C, Smac and apoptosis-inducing factor (AIF), ultimately leading to caspase-mediated apoptosis (27, 28). In this study, we observed that HeLa cells treated with niclosamide showed mitochondrial membrane depolarization, reduction of ATP levels, and increased rates of cell death. These results suggest that niclosamide evokes mitochondria dysfunction by extensive fragmentation in cancer cells.

Interestingly, we found that a pan caspase inhibitor did not completely block niclosamide-induced cell death in HeLa cells. Three types of cell death have been classified based on their distinct characteristics; apoptosis, necrosis and autophagy (29, 30). Excessive mitochondria fragmentation leads to autophagy. Indeed, overexpression of Fis1 selectively reduces mitochondrial mass and triggers autophagy (13). In addition, overexpression of either Opa1 or a Drp1 dominant negative mutant reduces the level of autophagy (14). These reports suggest that dysfunctional mitochondria promote autophagy. Recently niclosamide was identified as an autophagy inducer and it prevents ubiquitin-containing aggregates by autophagy induction (19, 31). Therefore, we examined effect of niclosamide on autophagy and autophagic cell death. Consistent with previous studies, we observed niclosamide-induced autophagy in HeLa cells. The effects of autophagy depend on the stress stimuli and status of cells and may lead to cell protection or cell death. Deletion of ATG5 blocked autophagy and suppressed niclosamide-mediated cell death, thereby demonstrating that autophagy induced by niclosamide is associated with cell death pathways.

In summary, our studies showed that niclosamide efficiently induces mitochondria fragmentation and dysfunctions. Further-
more, niclosamide induces both apoptotic and autophagic cell death. Although more studies are needed, our results suggest that niclosamide is a potential chemotherapeutic agent for treating cancer.

MATERIALS AND METHODS

Chemical screening for regulator of mitochondria dynamics
HeLa cells stably expressing mito-YFP were seeded in 96-well plates at 1500 cells per well. 24 hours after seeding, chemicals were added to each well at a final concentration of 10 μM. And change of mitochondrial morphology was observed under fluorescence microscopy (Olympus X71).

Reagents
The Lopac 1280 chemical library was from Sigma (St. Louis, MO). The expression plasmid GFP-LC3 and Drp1 dominant negative (K38A) were a kind gift from Noboru Mizushima (Tokyo Medical and Dental University, Japan) and van der Bliek AM (University of California at Los Angeles, CA). mito-YFP plasmid was kindly provided from Dr. Gyeooon Yoon (Ajou University, Korea). siRNA targeting Drp1 (5'- GAG GUU AUU GAA GGA CUA A) and negative scrambled siRNA (5'- CUC ACG CCA CCA CUA AUU UC) were synthesized from Bioneer (Daejeon, Korea). Niclosamide and 3-methyladenine (3MA) were purchased from Sigma (St. Louis, MO, USA). zVAD-FMK; pan caspase inhibitor was obtained from R&D Systems (Minneapolis, MN).

Cell culture and stable transfection
HeLa cells were obtained from the American Type Culture Collection (ATCC). Wild type MEF and ATG5 knock out MEF cells were kindly provided by Noboru Mizushima (Tokyo Medical and Dental University, Japan). All cells were cultured at 37°C in a 5% CO2 incubator and maintained in DMEM containing 10% FBS and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA). HeLa cells were transfected with pmito-YFP or pEFG-LC3 using Lipofectamine according to manufacturer’s protocol (Invitrogen, Carlsbad, CA). Stable transfectants were selected by growth in selection medium containing 1 mg/ml of G418 for 10 days. After single cell cloning, the stable clones were obtained under fluorescence microscope.

Western blotting
Cells were extracted with 2X Laemmli sample buffer (Bio-Rad, Hercules, CA), separated by SDS-polyacrylamide gel electrophoresis, and then transferred to PVDF membrane. After blocking with skim milk in TBST, the membranes were incubated with HRP-conjugated secondary antibodies (Pierce, Rockford, IL). Anti-Drp1 was from BD (San Jose, CA); anti-caspase-3 antibody was from Cell Signaling Technology (Beverly, MA); anti-ATG5 antibody was from Abcam (Cambridge, UK); anti-LC3 antibody was from NOVUS Biologicals (Littleton, CO); anti-Actin antibody was from Millipore (Temecula, CA).

Measurement of mitochondria membrane potential and ATP level
HeLa/mito-YFP cells (1 × 10^5) were treated with niclosamide (1, 10 μM) for 8 h. The Mitochondrial membrane potential detection kit uses a unique fluorescent cationic dye, JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide), to signal the loss of mitochondrial membrane potential (BD, San Jose, CA) and the depolarized mitochondria was measured with NucleoCounter NC-3000 (Chemometec, Denmark).

HeLa/mito-YFP cells (1 × 10^5) were treated with niclosamide (1, 10 μM) for 8 h. and total ATP level was measured with ATP Fluorometric Assay kit (Biovision, San Diego, CA) according to manufacturer’s protocol.

Cell viability assay and autophagy analysis
Cell death was measured by MTT assay (Sigma, St. Louis, MO). Cells seeded in 96-well plates were incubated at 37°C in 5% CO2 for 24 h, the medium was replaced with 100 μl of fresh medium with drugs. At the end of the incubation, 10 μl of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], 5 mg/ml was added to each well and the plate was incubated at 37°C for 1 h. The medium was then removed and DMSO was added. The absorbance of the solution was read spectrophotometrically at 540 nm with a reference at 650 nm using a micro plate reader (VictorX3, Perkinelmer). Autophagy was determined by counting the number of cells with GFP-LC3 punctate structure under fluorescence microscopy. The results were expressed as the means ± SD. The probability of statistical differences between experimental groups was determined by the Student’s t test.

Acknowledgments
We thank Dr. N. Mizushima (Tokyo Medical and Dental University, Japan) for providing of wild type and ATG5−/− MEF cells and LC3 plasmid. And we also thank Dr. van der Bliek AM (UCAL, CA) and G Yoon (Ajou University, Korea) for providing Drp1 and mito-YFP plasmid.

This study was supported by a grant of the Korean Health 21 R&D Project, Ministry of Health & Welfare Republic of Korea (A090013).

REFERENCES


