Effect of Niclosamide on Metabolic Changes in Cholangiocarcinoma Cells

ผลกระทบของยานิโคลซาไมด์ต่อการเปลี่ยนแปลงระดับเมทาบอไลต์ในเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดี

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ABSTRACT

Niclosamide is an antihelmintic drug of which its mode of action includes mitochondrial uncoupling. Recent studies revealed its strong anticancer effects against many cancer cells. However, the molecular mechanisms of niclosamide affecting cholangiocarcinoma (CCA) cell functions remain questionable. We investigated niclosamide-induced metabolic changes in CCA cells using NMR-based metabolomic analysis. We found that higher concentrations of extracellular glycine, alpha-ketoglutarate, branched-chain amino acids and aromatic amino acids were detected, together with decreased levels of alanine and methylamine. Collectively, our findings indicate the suppressed uptake of glycine that may affect the biosynthesis of several molecules including glutathione, an essential molecule in cancer cell proliferation and drug metabolism.

บทคัดย่อ

ยานิโคลซาไมด์ เป็นยาถ่ายพยาธิที่มีกลไกการทำงานคือการยับยั้งการทำงานควบคู่ในไมโตคอนเดรีย การศึกษาที่ผ่านมาพบว่ายานิโคลซาไมด์ มีฤทธิ์ต้านมะเร็งอย่างมีประสิทธิภาพในเซลล์มะเร็งหลายชนิด อย่างไรก็ตามยังไม่ทราบถึงฤทธิ์ต้านมะเร็งและกลไกระดับโมเลกุลของยานิโคลซาไมด์ ที่ส่งผลกระทบต่อเมแทบอลิซึมของเซลล์มะเร็งท่อน้ำดี การศึกษาครั้งนี้ มีวัตถุประสงค์เพื่อตรวจสอบผลกระทบของยานิโคลซาไมด์ต่อการเปลี่ยนแปลงของสารเมแทบอไลต์ในเซลล์มะเร็งท่อน้ำดี โดยใช้การศึกษาด้านเมแทโบโลมิกส์ด้วยเทคนิคนิวเคลียร์แมกเนติกเรโซแนนซ์สเปกโทรสโกปี ผลการศึกษาพบว่า ไกลซีน, แอลฟา-คีโตกลูตาเรท, กรดอะมิโนโซ่กิ่ง และกรดอะมิโนแอโรแมติก นอกเซลล์มีความเข้มข้นสูงขึ้น ส่วนอาลานีนและเมธิลามีน นอกเซลล์มีความเข้มข้นต่ำลง ทั้งนี้ผลการวิเคราะห์ชี้ให้เห็นว่า การยับยั้งการนำเข้าของไกลซีนอาจส่งผลกระทบต่อการสังเคราะห์สารชีวโมเลกุลหลายชนิด รวมถึงกลูตาไธโอนที่เป็นโมเลกุลสำคัญในกระบวนการเพิ่มจำนวนและการเมทาบอไลซ์ยาของเซลล์มะเร็ง

Keywords: Cholangiocarcinoma, Niclosamide, Metabolomics
คำสำคัญ: มะเร็งท่อน้ำดี นิโคลซาไมด์ เมแทโบโลมิกส์

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Introduction

Cholangiocarcinoma (CCA) is a bile duct cancer described as a malignant tumor developing from the bile duct epithelium. CCA has been reported with the highest mortality rate, especially in the Northeastern Thailand. The study about carcinogenesis of CCA in Thailand has pointed that CCA is correlated with liver fluke (*Opisthorchis viverrini*) infection that causes chronic inflammation (Songserm et al., 2009; Sripa, Pairojkul, 2008). The treatment of CCA is challenging because of the difficulty to diagnose at early stages. Surgery is an only potential strategy for curative treatment of CCA (Ghouri et al., 2015). At present, the development of new anticancer drugs is a goal of cancer research. Drug development from the beginning to the final step is expensive and prolonged. Instead of discovering the novel drugs, it is much faster and more economical to employ the existing drugs of which their pharmacokinetics and safety information have already been known, and have usually been approved for human use. In 1953, Bayer chemotherapy research laboratory discovered and developed niclosamide as a drug to kill snails (Andrews et al., 1982). In 1960, niclosamide had been used as anti-helminthic drug to treat the tapeworm infection in human and was marketed as Yomesan (Pearson, Hewlett, 1985). In 1982, niclosamide was approved by the USFDA for using in human (World Health Organization [WHO], 2007). Niclosamide has been reported to act as the mitochondrial uncoupler of the electron transport chain leading to the inhibition of oxidative phosphorylation and stimulation of adenosine triphosphatase activity. Recent studies have found that niclosamide has a strong *in vitro* anticancer activity against a wide range of cancer cells such as colon, breast, prostate, and many other cancer types (Al-Hadiya, 2005; Li et al., 2014). Thus, this study aimed to investigate the metabolic changes upon niclosamide treatment in CCA cell using NMR-based metabolomics.

Objectives of the study

The aim of this study was to investigate the metabolic changes upon niclosamide treatment in KKU-213 CCA cells using NMR-based metabolomics.

Methodology

Cell culture

The human CCA cell line, KKU-213 was obtained from Cholangiocarcinoma Research Institute, Khon Kaen University, Thailand. CCA cells were cultured in HAM's F12 complete media (Gibco™, USA) with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 g/mL streptomycin at 37 °C in a humidified incubator with 5% CO_2. The media was changed every other day.

Niclosamide preparation

Niclosamide (Sigma-Aldrich, USA) was dissolved in methanol and acetone at ratio 1:1 to obtain a stock solution of niclosamide 9.17 x 10^3 µM, which was freshly prepared. The different concentrations of niclosamide was diluted in the complete media.

Cell proliferation assay

The cell viability of KKU-213 cell line was assessed using Sulforhodamine B (SRB) assay. Cell line (2 x 10^3 cells) in media 100 µl are seeded in 96-well flat-bottom in triplicate. Cell were treated with various concentrations of niclosamide from 0.1 µM to 1 µM. Then, treated cells were incubated for 24, 48 and 72 hours at 37°C with 5% CO_2 atmosphere. The 10% of TCA were added to each well to fix cells. Then, the plates are incubated for 1 hour at 4 °C. TCA-treated cells were washed three times with deionized water and air dried and stained with 0.4% (w/v) SRB in 1% (v/v) acetic acid for 30 minutes at room temperature. Then, the unbound stain was washed with 1% (v/v) acetic acid for three times. After cultured plates are dry, 200 µl of 10 mM Tris base was added to solubilize the protein-bound stain for 60 minutes on shaker. The absorbance was detected at 540 nm using a microplate reader (Tecan Austria GmbH, Austria).

Cell sample preparation for NMR spectroscopy

CCA cells were seeded at 2 x 10^6 cells in 15-cm dish and cultured at 37 °C in a humidified incubator with 5% CO_2. Then, cells were treated with various concentrations of niclosamide for 48 hours. The cultured media was collected and centrifuged at 400 xg at 4 °C for 5 minutes and stored at -80 °C. The cells were trypsinized and harvested for 15 x 10^6 cells, followed by washing with 1X PBS 2 times and centrifuged at 400 xg at 4 °C for 5 minutes. Then, cell pellet was resuspended with 1 ml of 1X PBS and transferred to 1.5 ml tube, centrifuged at 2,300 xg at 4 °C for 5 minutes and stored at -80 °C

Metabolite extraction

Cell metabolites were extracted, 5.4 ml of 1X PBS were added and sample tubes were dipped into the liquid nitrogen until completely frozen then let slowly thawed on ice. Then, cell lysate was sonicated for 3 cycles of pulse on 30 seconds and off 5 seconds at maximum power. At the phase extraction step, 6 ml of cold methanol (-20 °C) and 6 ml of chloroform were added into an original sample tube (total volume = 17.4 ml) and the sample was incubated for 20 minutes on ice with frequent vortexing. Then, the sample tube was centrifuged at 4,000 xg for 20 minutes at 4 °C. The solution was separated to 3 layers including an aqueous phase (water soluble metabolite) containing protein and macromolecules and an organic phase (lipid soluble metabolite). All of aqueous phase were transferred into 1.5 ml tube and the solvents were removed by a vacuum concentrator until completely dried. Cell metabolites were dissolved in 600 µl of 100% D_2O with 0.1 % (w/v) of TSP (3-(Trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt) then centrifuged at 15,300 xg for 10 minutes at 4 °C. The 550 µl of sample was transferred to an NMR tube.

^{1}H NMR-based metabolomic analysis

^{1}H NMR spectra was acquired on 400 MHz NMR spectrometer (Bruker Avance III, Bruker Biospin, Germany) and was then exported to MATLAB software (MathWorks, USA) for data pre-processing and

multivariate statistical analysis. Orthogonal signal correction-projection to latent structures-discriminant analysis (O-PLS-DA) was conducted with Pareto scaling method. Assignment of metabolites were confirmed using statistical total correlation spectroscopy (STOCSY) and compared with the public database, human metabolome database (HMDB) and in-house database.

Correlation network analysis

Metabolic network was constructed using MetaboNetworks, a tool to create custom sub-networks using main reaction pairs as defined by KEGG (Kyoto Encyclopedia of Genes and Genomes). It calculates the shortest pathway between a set of metabolites and plots the connectivity between metabolites as link in a network graph (Posma et al., 2013).

Results

To investigate the inhibitory effect on cell proliferation upon niclosamide treatment in CCA cells, the cell viability of KKU-213 was assessed using SRB assay following 24, 48 and 72 hours with concentration between 0.1-1 μM. The result showed the inhibitory effect of niclosamide on cell proliferation in dose-dependent manner (Figure 1). The half-maximal inhibitory concentration (IC_{50}) of niclosamide on KKU-213 were 0.52 ± 0.04 μM, 0.42 ± 0.02 μM and 0.28 ± 0.02 μM after 24-, 48- and 72-hour treatments. To investigate the metabolic changes upon niclosamide treatment, the ^{1}H NMR data sets of all intracellular and extracellular metabolites were analyzed using O-PLS-DA analysis. The validity of the models was determined using the permutation p-value, assessing the class-predictability of the model and further confirmed the validity of the O-PLSDA model ($p < 0.05$). Interestingly, the O-PLS-DA score plot (Figure 2) of extracellular metabolic changes of control compared with 1 μM niclosamide group showed that these two groups were clearly separated. The coefficient loading plots of the O-PLS-DA models displayed significant metabolites that distinguished two groups (Figure 3). The result showed that many metabolites were altered when cells were treated with niclosamide, including higher concentration of isoleucine, leucine, valine, alpha-ketoglutarate, methylamine, glycine, tyrosine, and tryptophan together with lower concentration of alanine when compared with the control. To investigate and construct the metabolic sub-networks, we used main reaction pairs as defined by KEGG (MetaboNetworks). It calculated the shortest pathway between a set of metabolites and plotted the connectivity between metabolites as linked into a network graph (Figure 4). According to the network analysis, we found that extracellular alpha-ketogutarate and glycine were significantly increased. We hypothesized that the accumulation of extracellular alpha-ketogutarate could result from the incomplete ATP synthesis that may cause cancer cell death, as being evident by lower concentration of citrate and succinate (Figure 5) that are the other two major TCA cycle intermediates. Moreover, the metabolic network shows that glycine was inversely correlated with glutathione. So, we assume that the accumulation of extracellular glycine could be due to its less uptake that may affect the biosynthesis

of several molecules including glutathione which was previously reported as an essential molecule in cancer cell proliferation through inhibition of ROS (Reactive oxygen species).

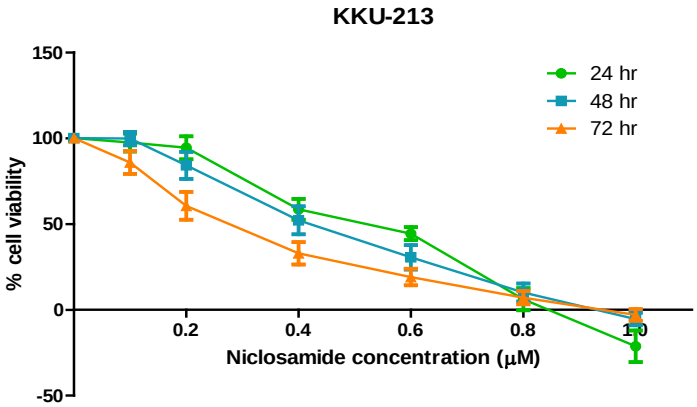


Figure 1 Percentage of KKU-213 cell viability after niclosamide treatment for 24, 48 and 72 hours.

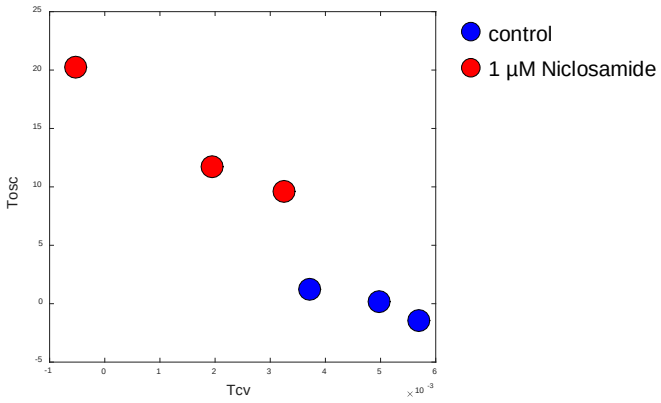
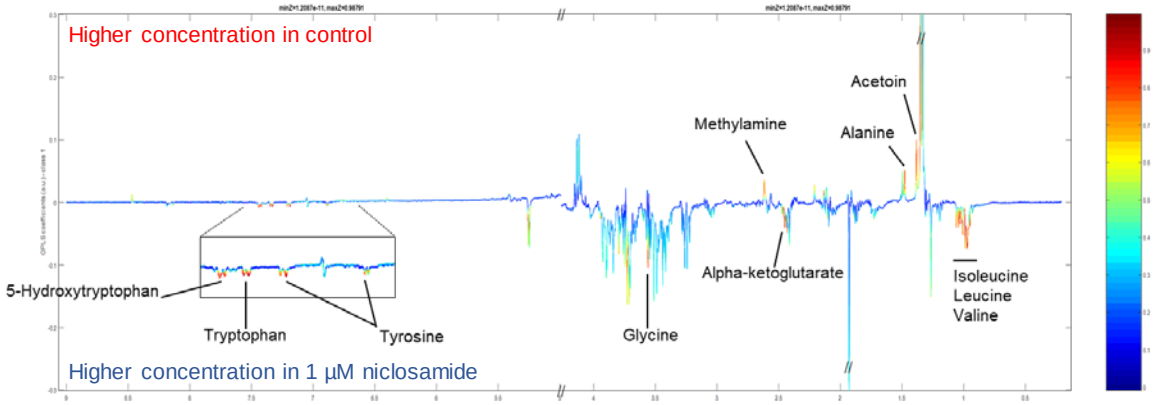


Figure 2 O-PLS-DA scores plot of ^{1}H NMR spectral data between control and 1 µM niclosamide treatment groups.



$Q^2Y = 0.7266$, $R^2X = 0.8933$, P-value = 0.05

Figure 3 O-PLS-DA loading plot of ^{1}H NMR spectral data between control and 1 µM niclosamide treatment groups. Resonances pointing upwards indicate metabolites that have higher concentrations in control group, whereas resonances pointing downwards indicate metabolites that have higher concentrations in 1 µM niclosamide group.

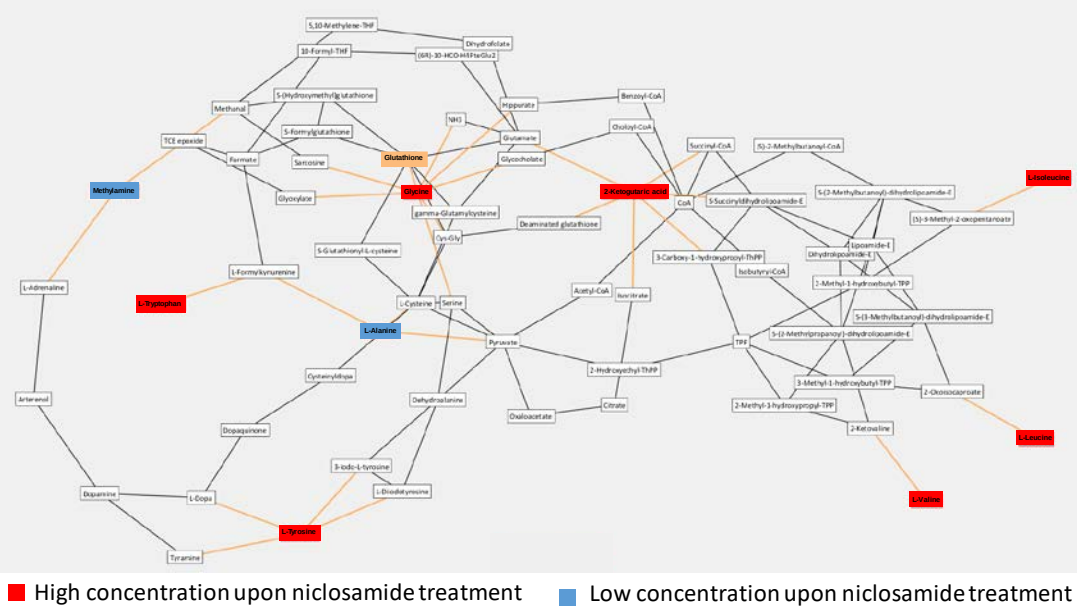


Figure 4 A metabolic network shows metabolites involved in extracellular metabolic changes using MetaboNetwork based on KEGG pathway. Red box indicates metabolites with higher concentrations, whereas blue box indicates metabolites with lower concentrations after niclosamide treatment. White box indicates the backbone of metabolic pathways involved in the metabolic alterations.

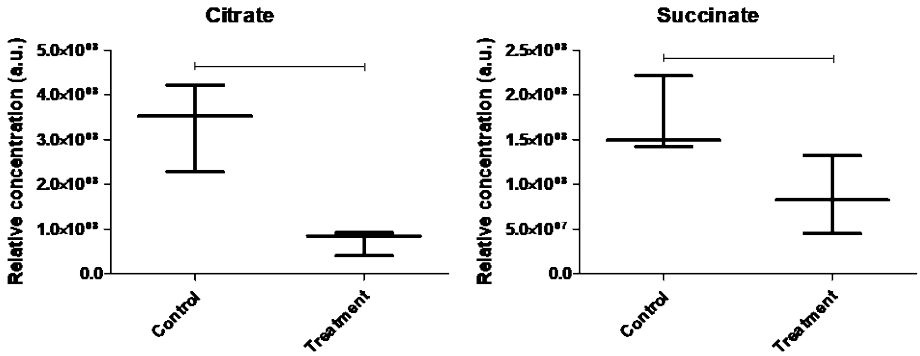


Figure 5 Box and whisker plot demonstrates relative concentrations of citrate and succinate of control and the treatment groups.

Discussion and Conclusions

Recent studies have found that niclosamide has a strong in *vitro* anticancer activity against a wide range of cancer cells. Our result showed that niclosamide affects CCA cells proliferation in dose-dependent manner. The IC_{50} value of niclosamide on KKU-213 was in the same range as those of other cancer cells (Li et al., 2014). This study investigated that the metabolic changes upon niclosamide treatment in CCA cells using NMR-based metabolomics. It has been proved that many metabolites were altered after niclosamide treatment including higher concentrations of isoleucine, leucine, valine, alpha-ketoglutarate, methylamine, glycine, tyrosine, and tryptophan, together with lower concentration of

alanine when compared with control. As a consequence of Warburg effect in cancer cells, a majority of pyruvate is converted into lactate, yielding less Kreb's cycle intermediates (Vander Heiden et al., 2009) that is consistent with our findings of lower relative concentration of citrate in the current study. In addition, niclosamide mode of action involves the inhibition of ATP synthesis as previously reported that niclosamide inhibits oxidative phosphorylation and stimulates adenosine triphosphatese activity (Al-Hadiya, 2005) but they did not elucidate, at that time, which specific pathway is the major target of this drug. In regards with our findings, we assume that the increase of alpha-ketoglutarate could be due to the incomplete ATP synthesis, resulting in the decrease of ATP. Moreover, the metabolic network shows that glycine is associated with glutathione. Glutathione plays an important role in cell proliferation (Traverso, 2013) and drug metabolism (Orrenius, 1984). In addition, the process of glutathione synthesis requires ATP. Therefore, when ATP decreased, it may lead to the accumulation of extracellular glycine, possibly due to its less uptake that may affect the glutathione synthesis that plays an important role in the prevention of drug toxicity and may inhibit cancer cell proliferation leading to cancer cell death. Our results suggest that niclosamide can be potentially used for CCA chemotherapy.

In the current study, the authors are aware that our findings may be cell type-specific metabolic characterization as only KKU-213 has been solely employed. Therefore, we have been investigating such effects of niclosamide on the other cholangiocarcinoma cell lines in order to maximize more meaningful data of the metabolic alterations in regards with niclosamide treatment for future publication.

Acknowledgements

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