Proteomic analysis of anti-cancer effects by 5'-deoxy-5'-methylthioadenosine treatment in cholangiocarcinoma cell line

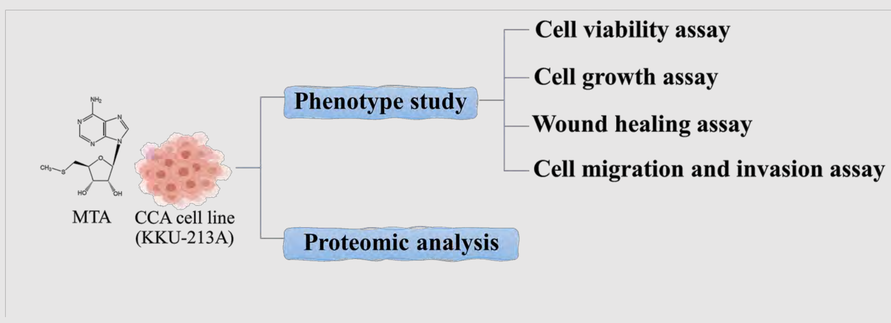
Sutthipong Nanarong[1], Sittiruk Roytrakul[2], Thanawat Pitakpornpreecha[1], Sumalee Obchoei[1, *]

[1]Division of Health and Applied Science, Faculty of Science, Prince of Songkla University, Songkhla 90110, Thailand
[2]National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Pathumtani 12120, Thailand
*Email: sumalee.o@psu.ac.th

ABSTRACT

The treatment of advanced cholangiocarcinoma (CCA) is mostly ineffective due to the intrinsic and acquired chemotherapeutic drug resistance phenotypes. Clearly, a novel and effective therapies are urgently needed. 5'-deoxy-5'-methylthioadenosine (MTA) is a natural-derived bioactive compound that has been shown to possess anti-cancer activity in various human cancers. In the present study we demonstrated the MTA exhibited a potent cytotoxic effect against a CCA cell line, KKU-213A in a dose dependent manner. In addition, it strongly inhibited cell migration and invasion of CCA cells. Proteomic analysis using LC/MS/MS identified the total of 4,117 proteins. In which, 10 were identified only in untreated cells, 30 were found only in MTA treated cells, whereas 4,077 were expressed in both. The 25 most up-regulated and down-regulated proteins in MTA-treated KKU-213A cell line were displayed using heatmap analysis. The results showed that MTA significantly suppressed numbers of oncoproteins such as RYR1, RCOR2, KLC1, GRAMD1C, and HLA-DQB1. Protein-protein interaction analysis of 25 most down-regulated proteins predicted 3 major clusters namely calcium ion transmembrane transport, antigen processing and presentation of exogenous peptide, and microtubule motor activity. These clusters were reported to involved in tumor progression and they might be the main target of MTA. The detailed study of molecular mechanisms underlying MTA effect on CCA cells both *in vitro* and *in vivo* should be further explored with the anticipation that this promising natural bioactive compound might be an alternative treatment for CCA in the future.

METHODS



RESULTS

MTA inhibits CCA cell viability and growth of CCA line in vitro

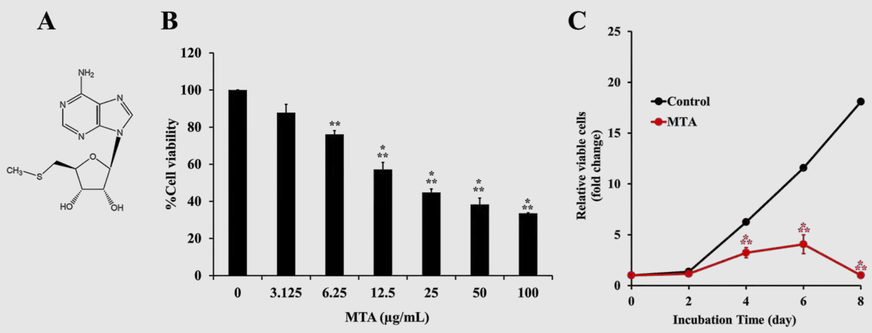


Figure 1 Effect of MTA on cell viability and growth of CCA cell line. (A) chemical structure of MTA. (B) Cell cytotoxicity assay. KKU-213A cells were treated with various concentrations of MTA for 72 h and were determined by MTT. (C) Cell growth assay. Cells were treated with 25 µg/mL MTA and the MTT assay was carried out at day 0, 2, 4, 6, and 8. Data are represented as the mean ± SEM of three independent experiments. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

To determine the cytotoxic effect of MTA on CCA cell line and to obtain the optimal concentration of MTA for use in the subsequent experiments, KKU-213A were treated with various concentrations of MTA and then the cell cytotoxicity assay was performed using MTT test. The results showed that MTA reduced cell viability in a dose-dependent manner **Fig. 1B**. The results showed that MTA at 25 µg/mL inhibited growth of KKU-213A significantly at day 2, 4, 6 and 8 as compared with untreated control **Fig. 1C**.

Effect of MTA on cell migration and invasion.

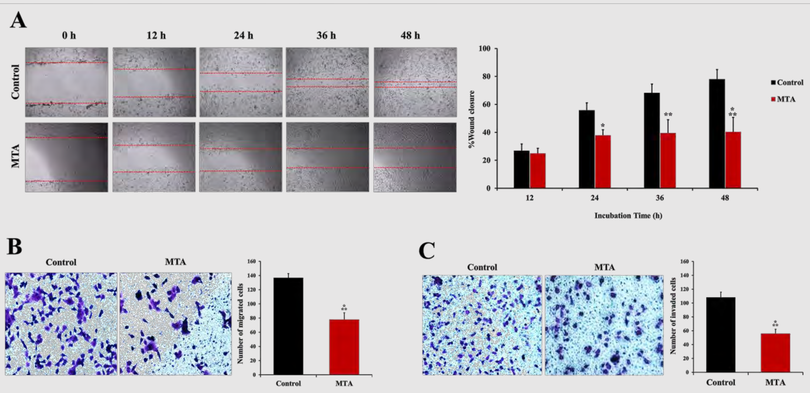


Figure 2 Effects of MTA on cell migration and invasion. (A) Wound healing assay. Confluent monolayer of KKU-213A cells was scratched and treated with MTA, representative images were taken from the same field every 12 h. Percent wound closure was calculated by comparing with 0 h (B) Migration assay. Treated cells were allowed to migrate through Transwell® for 12 h (C) Matrigel invasion assay. Treated cells were allowed to invade Matrigel-coated Transwell® for 12 h. Cells were fixed, stained and the representative images are shown. The data are represented as the mean ± SEM of three independent experiments. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

Wound healing assay was performed to determine the effect of MTA on cell migration. The results showed that the wound closure rate was significantly slower in MTA-treated cells. Percent wound closure in MTA-treated vs control cells were 24.9 vs 26.7, 39.5 68.1, and 40.3 vs 77.9 at 24, 36, and 48 h time point respectively **Fig. 2A**. Similarly, using modified Boyden chamber assay, MTA significantly suppressed cell migration to 57% **Fig. 2B** and suppressed cell invasion to 52% **Fig. 2C**. These results suggest that MTA could inhibit migration of CCA cells in *in vitro*.

ACKNOWLEDGEMMENTS

This study was supported by the budget revenue of Prince of Songkla University to S. Obchoei (SCI6202093S). Sutthipong Nanarong was supported by the Research Grant for Thesis in the Fiscal Year 2020 Scholarships, Graduate School of Prince of Songkla University, Thailand

Identification of differentially expressed proteins by proteomic analysis

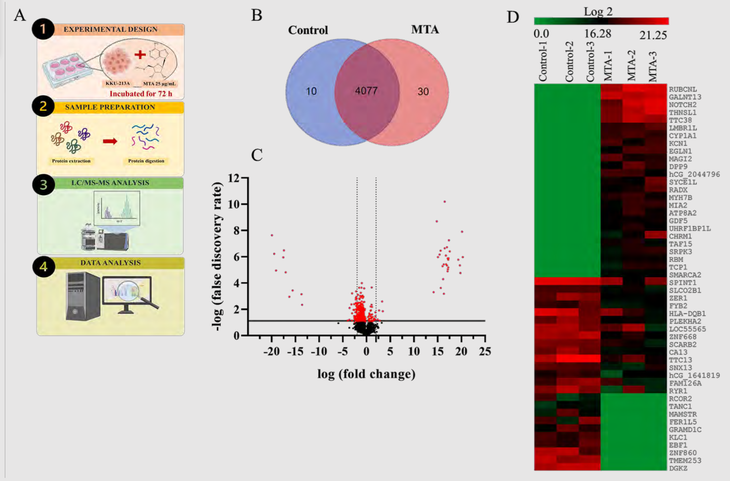


Figure 3 Identification of differentially expressed proteins by proteomics analysis. (A) Illustration of experimental workflow (B) Venn diagram. Total number of identified proteins in all samples were displayed by Venn diagram (C) Volcano plot of all proteins identified in the proteome database. (D) Heatmap analysis of 25 most up- and down-regulated proteins.

To further investigate the anti-cancer effects and protein targets of MTA, LC/MS/MS was carried out to identify differentially expressed proteins. The summary workflow of the proteomic and data analysis is shown in **Fig. 3A**. The Venn diagram showed that the total of 4,188 unique non-redundant proteins were identified in all 3 biological triplicates samples. In which, 10 proteins were identified only in untreated control cells, 30 proteins were found only in MTA-treated cells, whereas 4,148 were expressed in both treated and untreated samples **Fig. 3B**. Volcano plot showed that using p-value < 0.05 the total of 4,117 proteins. In which un-changes 4,026 protein and 91 protein changes were found in protein expression were identified between the control and MTA treated **Fig. 3C.** Heatmap was generated from 25 most down- and up-regulated proteins using log 2 values of protein expression data **Fig. 3**D.

Gene ontology and protein-protein interaction analyses

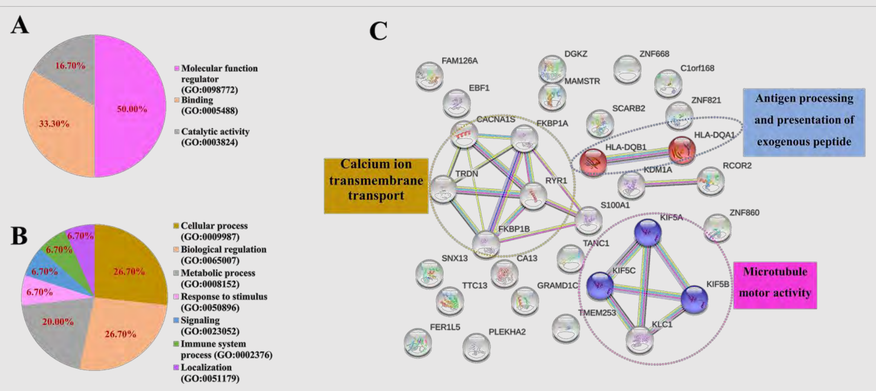


Figure 4 Bioinformatic analyses of the 25 most down-regulated proteins (≥ 4 folds). (A) Gene ontology analysis based on molecular function (B) Gene ontology analysis based on molecular function (C) Prediction of protein-protein interaction using STRING (v.11.0) software.

The 25 most down-regulated proteins were further classified by molecular function and biological process. The results showed that the down-regulated proteins are involved in molecular function regulator (50.0%), binding (33.30%), and catalytic activity (16.70%) respectively **Fig. 4A**. Annotation via biological process, revealed that the down-regulated proteins are involved in biological regulation (26.70%), cellular process (26.70%), metabolic process (20.00%), response to stimulus (6.70%), signaling (6.70%), immune system process (6.70%), and localization (6.70%) respectively **Fig. 4B.**

Furthermore, protein-protein interaction was analyzed by STRING software. The results showed interaction between top 25 down-regulated proteins and their predicted protein partners (HLA-DQA1, FKBP1A, KIF5B, CACNA1S, TRDN, KIF5C, FKBP1B, KDM1A, KIF5A, and S100A1). As shown in **Fig. 4C**, the three main protein clusters were, 1) calcium ion transmembrane transport (RYR1, CACNA1S, FKBP1A, FKBP1B, TRDN, S100A1), 2) protein antigen processing and presentation of exogenous peptide (HLA-DQB1, HLA-DQA1), and 3) protein microtubule motor activity (KIF5A, KIF5C, KLC1 KIF5B).

DISCUSSION AND CONCLUSION

Currently, the only effective treatment for CCA is curative surgery, but more than 80% of the patients are inoperable due to late presentation and delayed diagnosis [1]. Chemotherapy is therefore the most common treatment option for patients with advanced CCA [2]. This study showed that MTA exhibited anti-proliferative activity of this compound is well known in many cancers [3]. Here in, we demonstrated that MTA strongly suppressed CCA cell migration and invasion capability. Concomitant with the previous reports in other cancers such as melanoma [4] and liver cancer [5]. Analysis of 25 most down-regulated proteins predicted 3 major clusters namely calcium ion transmembrane transport, antigen processing and presentation of exogenous peptide, and microtubule motor activity. These clusters were reported to involved in tumor progression and they might be the main target of MTA. In summary, this study shows for the first time that MTA exhibits strong anti-cancer effect against CCA cells in *in vitro*. Proteomic analysis demonstrates differentially expressed protein profile in MTA treated cells. More detailed study focusing on these differentially expressed proteins will bring a better knowledge on mechanism of action of MTA. In the future, MTA might serve as a potential anti-cancer agent.

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