Extracellular Metabolic Profiling of Gemcitabine-responsive Cholangiocarcinoma Cell Line
แบบแผนเมตาบอไลต์ในน้ำเสียเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดีที่ตอบสนองต่อยาเจมไซตาบีน

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ABSTRACT

Gemcitabine has been long considered as a standard anti-cancer for the palliative treatment of advanced cholangiocarcinoma (CCA). However, the low efficacy was found in some cases. To explore the insight of the metabolic changes in relation to gemcitabine treatment, we investigated gemcitabine-induced metabolic alteration in CCA cells using UHPLC-MS/MS analysis. We found that gemcitabine treatment leads to the decreased levels of extracellular C20 diterpenoids, C15 sesquiterpenoids, C9 fatty acyls and 10-hydroxy-3,7-dimethyl-2E,6E-decadienoic acid in CCA cell culture media. Therefore, the reduced levels of such metabolites may indicate CCA cells-responsiveness to gemcitabine treatment and may serve as potential panel biomarker of gemcitabine efficacy in CCA cells.

บทคัดย่อ

เจมไซตาบีนเป็นยาต้านมะเร็งมาตรฐานที่ใช้ในการรักษาแบบประคับประคองในผู้ป่วยมะเร็งท่อน้ำดีระยะลุกลามอย่างไรก็ตามการรักษาด้วยเจมไซตาบีนพบว่ามีประสิทธิภาพที่ต่ำในผู้ป่วยมะเร็งท่อน้ำดีบางราย ดังนั้นการศึกษาครั้งนี้จึงมีวัตถุประสงค์เพื่อที่จะหากริยาต้านมะเร็งที่จะทำงานร่วมกับเจมไซตาบีน ในการตรวจสอบผลกระทบของเจมไซตาบีนต่อการเปลี่ยนแปลงของสารเมตาบอไลต์ในเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดี โดยใช้เทคนิคผลิตสกอเทคทริโอฟิวเอลย์xef ระบบใหม่ ผลการศึกษาพบว่าสารตามรายวัตถุได้แก่ C20 diterpenoids C15 sesquiterpenoids C9 fatty acyls และ 10-hydroxy-3,7-dimethyl-2E,6E-decadienoic acid มีระดับที่ลดลงในน้ำเสียเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดีเมื่อได้รับยาเจมไซตาบีน แสดงผลว่าการใช้เจมไซตาบีนในมะเร็งท่อน้ำดีนั้นจะมีความเกี่ยวข้องกับการตอบสนองของเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดีต่อเจมไซต์และอาจสามารถเป็นตัวบ่งชี้เพื่อติดตามประสิทธิภาพของเจมไซต์ในมะเร็งท่อน้ำดีได้

Keywords: Cholangiocarcinoma, Gemcitabine, Pharmacometabonomics

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Introduction

Cholangiocarcinoma (CCA) is the most common biliary tract cancer and the second most common primary hepatic cancer worldwide. The incidence of CCA is found highest in Southeast Asia, especially in Thailand (Blechacz, 2017; Khuntikeo et al., 2018). The liver fluke infection, *Opisthorchis viverrini* is regarded as a major risk factor for CCA in Thailand, and other regions in Southeast Asia (Khuntikeo et al., 2018). A surgical resection is considered as an only potentially curative treatment for CCA (Vogel et al., 2014). The symptoms of CCA are usually present at the late stages, thus the resection is consequently unavailable for most of patients (Khuntikeo et al., 2018). Therefore, additional adjuvant treatments such as radiotherapy, chemotherapy, or combined treatment, are considered as the potential options for supporting the survival of patients (Toschi et al., 2005; Luvira et al., 2016; Vogel et al., 2014).

Gemcitabine is an active anti-cancer agent with pyrimidine nucleoside anti-metabolite property, which provides effective treatment for various types of solid tumour (Toschi et al., 2005) including CCA (Pasetto et al., 2007). Gemcitabine monotherapy has been long considered as a standard regimen for the palliative treatment of advanced CCA due to the satisfied outcomes (Charoentum et al., 2007; Hezel, Zhu, 2008; Okusaka et al., 20105; Thongprasert et al., 2005; Butthongkomvong, 2013). However, low efficacy of chemotherapy with gemcitabine was found in some cases (Wirasorn et al., 2013).

One of the major drawbacks to the successful clinical translation of the promising therapeutic agents is a lack of biomarkers to reveal an early read out of biological activity and understand whether therapeutic agents have reached the targets to trigger a biologic function (Brown and Rufini, 2015). There are several types of biomarkers, including cellular, molecular, imaging and histopathological parameters that are widely used in preclinical and early phase clinical trials (Sarker, Workman, 2006). Moreover, the pharmacological biomarkers, such as pharmacodynamic (PD) biomarkers, are important to investigate the effects of drugs or interventions on patient’s bodies in order to improve efficacy and reduce costs implicated in drug development (Sarker, Workman, 2006). Recently, pharmacometabonomics has been an emerging field of metabonomics that reveals the mechanisms of drug effects through alterations of metabolites (Clayton et al., 2006), which are the downstream products of transcription and translation and is thus closest to phenotypic traits (Mamas et al., 2011). With the distinguished characteristics of sensitive and appropriate measurement using high-throughput platform, the metabolites, one of the most favourable biomarkers of drug efficacy monitoring, can be holistically analysed (Mamas et al., 2011; Sarker, Workman, 2006). In addition, there are several evidences showing that gemcitabine treatment exhibits metabolic change implication in various cancers (Yang et al., 2019; Mehrmohamadi et al., 2017). Thus, metabonomics may serve as a potential tool for revealing biomarkers or metabolic patterns of gemcitabine-response in CCA.
Objective of the study

The aim of this study was to reveal the metabolic change patterns or biomarkers of drug efficacy which may associate with the response of gemcitabine treatment in CCA cell line using ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) analysis.

Materials and methods

Cell culture

CCA cell lines including KKU-055 (JCRB1551), KKU-213A (JCRB 1557) and KKU-100 (JCRB 1568) were obtained from Cholangiocarcinoma Research Institute (CARI), Khon Kaen University, Thailand. Cell Line Certificates of Analysis were obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank, Osaka, Japan. All cells were cultured in Ham’s F12 media (Gibco™, USA) mixed with 10% fetal bovine serum and 100 IU/ml of penicillin-streptomycin. Cell lines were cultured at 37°C in a humidified incubator with 5% CO₂ atmosphere (Promraksa et al., 2019).

Gemcitabine preparation

Gemcitabine (℞Gemita, India) was dissolved in 0.9% (w/v) normal saline to obtain a stock solution of approximately 11.0 mM. Then, desired concentrations of gemcitabine (0.000, 0.001, 0.01, 0.1, 1, 10, 100 and 1,000 μM) were subsequently diluted with the cell culture media. The drug solution stock was freshly prepared.

Sulforhodamine B (SRB) assay

The standard protocol of SRB assay was previously described as follows (Vichai and Kirtikara, 2006). Briefly, 10% (w/v) cold trichloroacetic acid was added to fix cells cultured for 1 hour, followed by washing with deionized water, and 0.4% (w/v) SRB dye staining. 1% (v/v) acetic acid was used to remove unbound dye. Then, the plates were allowed to hot air-dry at 60°C for 30 minutes. After that, 10 mM Tris base solution (pH 10.5) was added to solubilize the protein-bound dye. Then, the absorbance was measured at 540 nm in a microplate reader (Tecan, Switzerland).

Cytotoxicity test

CCA cell lines were seeded at 2,000 cells/well (2,500 cells/well for KKU-100) in 96 well-plate. After 20 hours of culture, cells were treated with 100 μl of gemcitabine solutions in designing concentrations for a further 48 hours culture. The non-treatment baseline (day 0) was prepared. The percentage of cell-growth inhibition against gemcitabine treatment cells was calculated based on the following formula:

\[
\% \text{ cell growth} = \left( \frac{\text{Absorbance sample}}{\text{Absorbance negative control or non-treated}} \right) \times 100
\]

When % growth inhibition = 100 - % cell growth

The experiment was conducted in five replicates of three independent experiments.

Cell and cultured media samples preparation for UHPLC-MS/MS analysis

The KKU-055 cell line was cultured until passage number 2. Cells were then counted to set at the density of 2x10⁶ cells in 15-cm culture dishes for a further 48 hours cultured. After that, the used
media were removed followed by 20 ml of gemcitabine-mixed culture media and the control vehicle were added into cultured cells. The dose of gemcitabine was selected on the basis of the maximal inhibitory concentrations (IC₀ and IC₅₀) from the cytotoxicity test. Cells were then cultured for additional time periods of 24 and 48 hours, respectively. After that, the whole cultured media and a total of 1x10⁶ cells were collected through centrifugation at 671 x g, 4°C for 5 minutes. Then, supernatant was stored at -80°C until used. Cell pellets were washed with PBS and stored at -80°C prior to analysis.

For the metabolite extraction, 0.5 ml of cold methanol were added to cell pellets to quench metabolites. Mixture was snap-frozen in liquid nitrogen and subsequently thawed on ice. Cells were then lysed using sonication of pulse on 30 seconds and off 5 seconds for 3 cycles at maximum power. For phase extraction step, 0.5 ml of cold water (HPLC grade) and 1.5 ml of cold chloroform were added into cell lysates (total volume = 2.5 ml). Samples were incubated on ice for 20 minutes with frequent vortex, and centrifuged at 4,000 x g for 20 minutes at 4°C. The mixture was separated into an aqueous phase (water-soluble metabolite) composed of macromolecules and proteins, and an organic phase (lipid-soluble metabolite). The entire aqueous phase supernatant was collected and transferred to clean microcentrifuge tube. To remove unwanted solvents, supernatant was spun using a vacuum concentrator at 333 x g, 45°C for 12 hours.

For reconstitution, 200 µl of reconstitution buffer (water and acetonitrile, 1:1 ratio) were added to dissolve metabolites, and sonicated for 10 minutes, 3 cycles using ultrasonic bath (Sonsics & Materials, USA). Reconstituted samples were then centrifuged at 15,300 x g for 10 minutes at 4°C. A total of 150 µl of supernatant was transferred into glass insert located in the glass vial. To prepare quality control (QC), 20 µl of each sample was taken from each sample and pooled.

Ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) acquisition

The aqueous phase extracts of samples were analysed on the reverse-phase platform. The initial separation was performed using UHPLC system (Bruker, Germany) with Bruker intensity solo HPLC C18 2.1 x 100 mm, 2 µm column (Bruker, Germany). The column was used at optimal 40°C, and the autosampler temperature was set at 4°C. Mobile phase A was 100% water with 0.1% formic acid (FA), and mobile phase B was 100% acetonitrile with 0.1% FA. The elution was performed at 0.35 ml/minute flow rate. The elution gradient was set as follow: 99% A (0.0-2.0 minutes, 0.25 ml/minute), 99-1% A (2.0-17.0 minutes, 0.25 ml/minute), 1% A (17.0-20.0 minutes, 0.25 ml/minute), 1-99% A (20.0-20.1 minutes, 0.25-0.35 ml/minute), 99% A (20.1-22.0 minutes, 0.35 ml/minute), 99% A (22.0-30.0 minutes, 0.35 ml/minute). Seven-microlitre of injection volume was applied for both positive and negative ionization polarity modes. The compact ESI-Q-TOF system (Bruker, Germany) was used for mass detection. The external calibrant was sodium formate (2 mM sodium hydroxide, 0.1% FA, 50% IPA) with 0.5 µl/minute of flow rate. The optimal conditions of positive ionization polarity mode were set at followed: mass range: 50-1500 m/z, cone voltage 31V, capillary voltage 4500V, source temperature 220°C, desolvation
temperature 220°C, desolvation gas flow 8 L/minute. The negative ionization polarity mode was performed in following conditions: m/z range: 50-1200 m/z, cone voltage 30V, capillary voltage 4500V, source temperature 220°C, desolvation temperature 220°C, desolvation gas flow 8 L/minute. The standard QC strategy was applied to estimate the instrument stability and analysis reproducibility. To detect complements and impurity of the extraction and reconstitution solvent, QC sample: the reconstitution buffers were applied in MS/MS mode. Extraction blank samples and reconstitution blanks were also performed in MS mode after sample analysis.

Statistical analysis

The cytotoxicity test of all cell lines was presented as mean ± standard deviation (S.D.) from 3 independent experiments. For metabolomic analysis, univariate analysis was performed using GraphPad Prism 8.0.1 software (GraphPad Software, USA). The Kolmoğorov-smirnov test was used to investigate normality of data set. The Kruskal-Wallis test was used to determine statistically significant differences between groups of a feature. Metabolic alterations among different ICs and time points of treatment were elucidated. The p-value <0.05 was considered as the cut-off statistical significance. Metabolite assignment was conducted using MS-DIAL software (RIKEN Centre for Sustainable Resource Science, Japan), and compared against the human metabolome database (HMDB) and METLIN database.

Results

To investigate the cytotoxic effect of gemcitabine on CCA cell lines, the growth inhibition of gemcitabine on CCA cell lines was determined using SRB assay. Figure 1 showed inhibitory effects of gemcitabine on KKU-055, KKU-100 and KKU-213A cell lines, respectively. The half-maximal inhibitory concentrations (IC_{50}) of gemcitabine were as follows, 0.164±0.038 µM for KKU-055, 0.153±0.015 µM for KKU-100 and 0.241±0.026 µM for KKU-213A. Then, we investigated the alteration of extracellular metabolites upon gemcitabine treatment using UHPLC-MS/MS analysis. Then, covariance of variation of QC (CV_{QC}) was applied to the dataset in order to assess the acceptable variation of each feature upon the performance of analytical platform. The features with CV_{QC} > 30% were removed from the dataset. After that, multivariate analyses, principal component analysis (PCA) and orthogonal signal correction-projection to latent structures-discriminant analysis (O-PLS-DA), were then conducted to visualize the pattern of data and to identify metabolites with significantly changing concentrations as a result of the treatment, respectively. However, no validity or significance of statistical model was found based on PCA and O-PLS-DA analysis (data not shown). Therefore, univariate analyses were performed in order to determine statistically significant differences between groups of metabolic alterations. The 1.2-fold-change (FC) cutoff of intensity values was applied to assess the magnitude of difference between the two groups. The Kruskal-Wallis test was then used to determine statistically significant differences between groups, 95% confidence level was applied to models (Table 1). The results showed a significant decrease in extracellular levels of four metabolites, including C20 diterpenoids, C15 sesquiterpenoids,
C9 fatty acyls and 10-hydroxy-3,7-dimethyl-2E,6E-decadienoic acid, following the treatment of gemcitabine at IC<sub>50</sub> compared to IC<sub>0</sub>. The C20 diterpenoids, C15 sesquiterpenoids and 10-hydroxy-3,7-dimethyl-2E,6E-decadienoic acid were significantly decreased at IC<sub>50</sub> after 48 hours of treatment, while no significant difference was observed at 24 hours of treatment. In addition, C9 fatty acyls was significantly decreased at 24 hours, whereas no significant alteration was observed at 48 hours (Figure 2).

![Figure 1](image_url)  
**Figure 1** Cytotoxic effects of gemcitabine treatment on CCA cell lines at 48 hours.

**Table 1** Statistical summary of extracellular metabolites using UHPLC-MS/MS analysis. Absolute log<sub>2</sub>(fold-change) of values equal or greater than 0.263 were retained. The Kruskal-Wallis test was used for non-parametric comparison. The p-value < 0.05 was considered as the cut-off statistical significance.

<table>
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<th>Features</th>
<th>IC&lt;sub&gt;0&lt;/sub&gt; vs IC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>24 hrs</th>
<th>48 hrs</th>
<th>IC&lt;sub&gt;0&lt;/sub&gt; vs IC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>24 hrs</th>
<th>48 hrs</th>
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<tbody>
<tr>
<td>C20 diterpenoids</td>
<td>0.034</td>
<td>0.397</td>
<td>0.6689</td>
<td>0.0325*</td>
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<tr>
<td>C15 sesquiterpenoids</td>
<td>0.061</td>
<td>0.341</td>
<td>0.6689</td>
<td>0.0103*</td>
<td></td>
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<tr>
<td>C9 fatty acyls</td>
<td>0.315</td>
<td>0.124</td>
<td>0.0064**</td>
<td>0.3098</td>
<td></td>
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<tr>
<td>10-hydroxy-3,7-dimethyl-2E,6E-decadienoic acid</td>
<td>0.094</td>
<td>0.348</td>
<td>0.4543</td>
<td>0.0139*</td>
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Discussion and Conclusions

There are many studies demonstrating that gemcitabine either in combinations or monochemotherapy is an effective anti-cancer agent in a variety of solid tumors. Our study showed that gemcitabine treatment has effects on the viability of CCA cell lines in a dose-dependent manner. According to IC$_{50}$ values, all three cell lines showed fewer dose-different effect to establish a resistance cell as normally greater than the 10-fold increase of resistant cells (Sherman-Baust et al., 2011; Barr et al., 2013). Therefore, all three cells are defined as equally responsive cell lines so that only KKU-055 was selected for investigating metabolic changes using UHPLC-MS/MS platform. The investigation of alteration of extracellular metabolites showed that gemcitabine treatment has influence in both dose- and time-dependent manners in KKU-055 cell line. At 48 hours and IC$_{50}$ of gemcitabine treatment, the levels of C20 diterpenoids, C15 sesquiterpenoids and 10-hydroxy-3,7-dimethyl-2E,6E-decadienoic acid were significantly decreased compared to IC$_{0}$. In addition, the level of C9 fatty acyl was significantly decreased at 24 hours after IC$_{50}$ dose treatment. The 10-hydroxy-3,7-dimethyl-2E,6E-decadienoic acid is a member of unsaturated fatty acids, which involves in various energy and lipid metabolisms (Schonfeld et al., 2016). The intermediates of unsaturated fatty acid oxidation such as 5-dodecenoic acid 12:1(n-7),
4-decenoic acid 10:1(n-6), 5,8-tetradecadienoic acid 14:2(n-6), 7,10-hexadecadienoic acid 16:2(n-6) and 5-tetradecenoic acid 14:1(n-9) were found to be increased in patients with medium-chain, long-chain and multiple acyl-CoA dehydrogenase deficiency (Onkenhout et al., 2001). The acyl-CoA dehydrogenase is the key enzyme at the initial step of mitochondrial beta-oxidation (Lea et al., 2000). The low expression of long-chain acyl-CoA dehydrogenase was significantly associated with poor prognosis of hepatocellular carcinoma patients, whereas restoring the expression of this enzyme resulted in arrest of cell cycle and growth inhibition (Zhao et al., 2020). Fatty acyls are the class of lipid molecules which include monounsaturated (MUFA), polyunsaturated (PUFA) and saturated (SFA) fatty acids, synthesised from chain elongation of an acetyl-CoA in fatty acid synthesis (Xicoy et al., 2019). Diterpenoids and sesquiterpenoids are the members of isoprenoids class produced in the mevalonate pathway which is initiated with condensation of three molecules of acetyl-CoA (George et al., 2015). In addition, mitochondrial beta-oxidation of fatty acids in cancer cells was found to be able to generate acetyl-CoA through citrate conversion from the tricarboxylic acid (TCA) cycle. Moreover, upregulation of beta-oxidation serves to deviate acetyl-CoA from the fatty acid synthesis pathway (Gruenbacher, Thurnher, 2017). Accordingly, upregulation of de novo lipid synthesis was correlated to gemcitabine resistance in pancreatic cancer (Tadros et al., 2017). It has been known that cancers can retain their progression through cholesterol accumulation. As correlated with mevalonate pathway, cholesterol is subsequently generated from isoprenoids which are the product of the mevalonate pathway. Interestingly, inhibition of both cholesterol and mevalonate pathway is considered as cancer prevention and treatment strategies (Murai, 2015; Karlic et al., 2015). Accordingly, inhibition of mevalonate pathway is a potential strategy to recover drug sensitivity in resistant breast cancer cells (Sethunath et al., 2019). Concurrent decreased of those four metabolites which are mainly found in lipid metabolism, may have some implications in CCA cells-responsiveness to gemcitabine treatment, according to the previous evidence. However, further study of intracellular metabolites in CCA cells is needed to shed brighter lights on the drug mechanism understanding.

In summary, the significantly low levels of extracellular C20 diterpenoids, C15 sesquiterpenoids, C9 fatty acyls and 10-hydroxy-3,7-dimethyl-2E,6E-decadienoic acid may relate to CCA cells-responsiveness to gemcitabine treatment and may serve as potential panel biomarker of gemcitabine efficacy in CCA cells. However, the alteration of intracellular metabolizes upon gemcitabine treatment needs to be further investigated.

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References


