นิพนธ์ต้นฉบับ • **Original Article**

ฤทธิ์ยับยั้งการเจริญเติบโตของยา Gemcitabine ต่อเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดี และการเปรียบเทียบผลการยับยั้งระหว่างยาตำรับสามัญกับตำรับอ้างอิง

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Determination of Growth Inhibitory Effect of Gemcitabine on Human Intrahepatic Cholangiocarcinoma Cell lines and Comparison of its Inhibition Between the Generic and Reference Formulation

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หลักการและเหตุผล: Gemcitabine เป็นยาเคมีบำบัดที่นิยมใช้ในการรักษาโรคมะเร็งท่อน้ำดี อย่างไรก็ตามยังไม่มีรายงานการศึกษาเกี่ยวกับการออกฤทธิ์ของยา gemcitabine ในการยับยั้งการเจริญเติบโตของเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดีจำนวนหลายชนิดที่แยกได้จากผู้ป่วยมะเร็งท่อน้ำดีของไทย การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อทดสอบและเปรียบเทียบฤทธิ์ในการยับยั้งการเจริญเติบโตของ gemcitabine ในรูปตำรับยาสามัญกับตำรับยาอ้างอิงต่อเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดี

วิธีการศึกษา: ใช้เซลล์เพาะเลี้ยงมะเร็งท่อน้ำดีจำนวน 7 ชนิดที่เพาะเลี้ยงได้ในโรงพยาบาลศรีนครินทร์ มหาวิทยาลัยขอนแก่น ตรวจวัดค่าการยับยั้งการเจริญเติบโตของ gemcitabine ต่อเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดี ด้วยวิธี sulforhodamine B (SRB) โดยรายงานเป็นค่าความเข้มข้นของยา (μM) ที่สามารถยับยั้งการเจริญเติบโตของเซลล์ได้ร้อยละ 50 (IC_{50}) เทียบกับกลุ่มควบคุมที่ไม่ใส่ยา และเปรียบเทียบค่า IC_{50}

Background and Objective: Gemcitabine is one of the most popular drug-of-choices that is currently used for the treatment of cholangiocarcinoma (CCA). However, the study revealing the inhibitory effect of this agent in the series of CCA cell lines established from Thai patients has not been reported. We aim to determine and compare the growth inhibitory effect of generic gemcitabine formulation with the reference formulation on CCA cell lines.

Methods: Seven CCA cell lines established in Srinagarind Hospital, Khon Kaen University were used. A cell growth inhibition by gemcitabine was determined by sulforhodamine B. The IC_{50} value was expressed as the concentration of drug that caused a 50% growth inhibition comparing with untreated control. The IC_{50} values of those two formulations were compared using independent *t*-test.

Results: Growths of KKU-M055, KKU-OCA17 and KKU-M139 CCA cell lines were highly inhibited by gemcitabine (IC_{50} = 13.35-16.0 μM) whereas KKU-M214 was

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ระหว่างยาที่เป็นตำรับสามัญกับตำรับอ้างอิงด้วยวิธีการทางสถิติ independent *t*-test

ผลการศึกษา: ยา gemcitabine สามารถออกฤทธิ์ยับยั้งการเจริญเติบโตของเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดีจำนวน 3 ชนิด คือ KKU-M055, KKU-OCA17 และ KKU-M139 ได้สูง โดยมีค่า IC_{50} อยู่ในช่วง 13.35-16.0 μM ในขณะที่ออกฤทธิ์ยับยั้งได้ระดับปานกลางกับเซลล์ชนิด KKU-M214 (IC_{50} = 36.7 μM) และยาชนิดนี้ออกฤทธิ์ในการยับยั้งการเจริญเติบโตของเซลล์ชนิด KKU-100, KKU-M156 และ KKU-M213 (IC_{50} = 406-4629 μM) ได้ต่ำ ยาตำรับสามัญ (Gramagen®) และตำรับอ้างอิง (Gemza®) มีฤทธิ์ยับยั้งการเจริญเติบโตของเซลล์มะเร็งท่อน้ำดีจำนวน 7 ชนิดไม่แตกต่างกัน ($P > 0.05$)

สรุป: ถึงแม้ว่าฤทธิ์ของ gemcitabine ในการยับยั้งการเจริญเติบโตของเซลล์มะเร็งท่อน้ำดีมีค่าแตกต่างกัน แต่ไม่พบความแตกต่างระหว่างค่า IC_{50} ของยาที่เป็นตำรับสามัญกับตำรับอ้างอิง ผลการศึกษานี้บ่งชี้ว่าประสิทธิผลของยาทั้งสองตำรับมีค่าเท่ากันเมื่อทดสอบในหลอดทดลอง

moderately inhibited by this drug (IC_{50} = 36.7 μM). These least inhibited growths were found on KKU-100, KKU-M156 and KKU-M213 (IC_{50} = 406-4629 μM). The generic (Gramagen®) and the reference product (Gemza®) formulations were not significantly different in their inhibitory effects on the all seven CCA cell lines.

Conclusions: Although the inhibitory effect of gemcitabine was varied towards seven CCA cell lines, there was no difference in the IC_{50} values of the generic and reference formulations. Our findings indicate that the *in vitro* efficacy of these two formulations is similar.

Keywords: growth inhibitory effect, gemcitabine, cholangiocarcinoma, generic formulation

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Introduction

Cholangiocarcinoma (CCA) is an uncommon cancer worldwide, but its incidence rate is highest in Northeastern Thailand[1]. There are evidences from clinical studies demonstrating that response of this cancer to conventional chemotherapy is relatively poor[2]. Preclinical study using the *in vitro* drug testing on CCA cells should be the urgent task to screen new anticancer agents in order to provide the data for selecting the potential chemotherapeutic drugs on treatment CCA[3,4]. Our recent *in vitro* study has shown that cytotoxicities of various anticancer agents including anthracyclines, platinum derivatives, 5-fluorouracil, taxanes, vinca-alkaloids, etoposide, irinotecan and mitomycin C on five human intrahepatic CCA cell lines isolated from Thai patients show different degrees of potency varied from high to low sensitivities among those cell lines[4]. To date, various types of anticancer drugs are commercially attainable as a low-cost generic formulation. Recently, Namwat and coworkers[5] have demonstrated that generic formulation of two anticancer drugs, paclitaxel and irinotecan, has a similar efficacy towards CCA cell lines to the corresponding reference formulations.

Gemcitabine (20,20-difluorodeoxycytidine, dFdC, Gemza®) is a nucleoside analogue that shows activity against solid tumors and hematologic malignancies both as a single agent[6] and in combination with other chemotherapeutic agents[7]. In Thai CCA patients, gemcitabine seems to be the most promising new agent with consistent data supporting efficacy and tolerability[8]. Therapy with gemcitabine and cisplatin is a well-tolerated therapeutic option for patients with unresectable and metastatic cholangiocarcinoma by achieving a response rate of 22% plus an additional disease stabilization rate of 26% giving an overall disease control rate of 48%[8]. To enhance the effectiveness of gemcitabine in CCA treatment may need a preclinical study including *in vitro* sensitivity testing and the mechanism of drug response. In addition, the introduction of gemcitabine in treatment of CCA patients who admitted at Srinagarind Hospital is fiscally desirable due to the high-cost problem. Therefore, this study aims to determine and compare the growth inhibitory effect of gemcitabine between its generic and reference formulations in seven human intrahepatic cholangiocarcinoma cell lines. These data may provide an

alternative for physicians and pharmacists in choosing the chemotherapeutic drug for their patients, particularly when cost-effectiveness is a matter of concern.

Materials and Methods

Chemicals

Generic formulation of gemcitabine (Gramagen®) was from Sandoz S.A: Buenos Aires, Argentina (lot no. 00104, mfg date 02/2008, exp. date 02/2010). Reference formulation of gemcitabine (Gemza®) was from Lilly France S.A.S, Fegersheim, France (lot no. A447931A, mfg date 21/01/2008, exp date: 19/01/2011).

Ham's F12, penicillin, streptomycin and trypsin-EDTA were purchased from Invitrogen Co., California, USA, fetal bovine serum from Seromed, Germany, and sulforhodamine B (SRB) from Sigma Chemical Co., USA. Tissue culture plates were obtained from Nunc, Denmark. All other chemicals were analytical grade.

Human CCA cell lines

Seven human intrahepatic CCA cell lines, namely, KKU-100 (poorly differentiated adenocarcinoma), KKU-M055 and KKU-M156 (moderately differentiated adenocarcinoma), KKU-M139 (squamous carcinoma), KKU-M213 (adenosquamous), KKU-M214 (moderately to poorly differentiated adenocarcinoma) and KKU-OCA17 (well differentiated adenocarcinoma) were used in this study. All cell lines were established in the Faculty of Medicine, Khon Kaen University, from CCA patients residing in Northeastern Thailand. Cells were cultured in Ham's F12 supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml of penicillin and 100 mg/ml of streptomycin at 37 °C with 5% CO_2. The presence of mycoplasma contamination was periodically examined.

Growth inhibition assay

Sulforhodamine B (SRB) assay was used to determine growth inhibition as described previously[5]. In brief, CCA cell lines (4 x 10^4 cells/ml) at exponential growth phase were trypsinized with 0.25% (v/v) trypsin and seeded in triplicate in 96-well flat-bottom microtiter plates and incubated for 24 h at 37 °C in a humidified 5% CO_2 atmosphere. Then 100 μl aliquot of medium containing drug (from 0.0003 μg/ml to 300 μg/ml) or no drug as control was added to the 96-well plates and incubated at 37 °C for 72 h. The culture medium was subsequently removed and 200 μl aliquot of 10% (w/v) ice-cold TCA was added to each culture well. The plates were then incubated at 4 °C for 60 min. TCA-treated cells were stained for 30 min with 0.4% (w/v) SRB in 1% (v/v) acetic acid for 30 min, and subsequently washed five times with 1% (v/v) acetic acid to remove the unbound stain. The plates were left to dry and the protein-bound stain was solubilized with 200 μl of 10 mM Tris base (pH 10.5) for 60 min. Absorbance was measured at 540 nm using a microplate reader (Tecan Austria GmbH, Austria). The concentration of drug required to inhibit cell proliferation by 50% (IC_{50}) was determined by plotting the percentage of cell growth inhibition versus the drug concentration.

Table 1 Growth inhibitory activities of test formulation and reference formulation on CCA cell lines.

CCA Cell line	Histological type	Mean IC_{50} (μM) of gemcitabine	
		Gramagen®	Gemza®
KKU-M055	Moderately differentiated	13.3±0.00	13.3±0.00
KKU-OCA17	Well differentiated	13.3±0.00	13.3±0.00
KKU-M139	Squamous	16.6±0.00	16.6±0.00
KKU-M214	Moderately differentiated	36.7±14.5	26.7±3.34
KKU-100	Poorly differentiated	406±92	482±70
KKU-M156	Moderately differentiated	599±119	478±363
KKU-M213	Adenosquamous	4629±655	4808±852

Data represents mean±SD of at least three experiments. Statistical analysis between two formulations is not different ($P > 0.05$) in all tested cell lines.

Statistical analysis

Tests were performed in 3 independent experiments. Data were expressed as mean±S.D. IC_{50} value represented the concentration of drugs that inhibited 50% cell growth. Comparison of IC_{50} values of generic formulation with reference formulation was analyzed using independent *t*-test.

Results

The growth inhibitory effect of the generic formulations of gemcitabine and its reference formulation, Gemza® against the seven CCA cell lines presented as IC_{50} values were shown in Table 1. Results obtained from seven CCA cell lines clearly revealed that the degree of sensitivity to this drug was different. Among these cell lines, KKU-OCA17, KKU-M055 and KKU-M139 showed high sensitivity to gemcitabine with IC_{50} value less than 20 μM whereas KKU-M214 was moderately sensitive (IC_{50} values of 36.72 μM). The cell lines with the least sensitivity were as KKU-100, KKU-M156 and KKU-M213 showing the IC_{50} values of 406.0, 598.6 and 4629 μM, respectively. Histological types of CCA were not related to gemcitabine sensitivity. Comparison between Gramagen® and Gemza® demonstrated that there was no significant difference in the IC_{50} values observed between the generic and the reference formulations.

Discussion

The growth inhibitory effect of gemcitabine in the seven human intrahepatic CCA cell lines established from Thai CCA patients is firstly demonstrated in this study. Our results revealed that CCA cell lines possed various degrees of drug sensitivity - from high (less than 20 μM) to extremely low (up to 4000 μM). These data may imply that tumor cells obtained from different patients are distinctly responsible to this drug depending upon the intratumoral drug metabolism[9]. In addition, cell lines such as KKU-M213 and KKU-M156 that have a low degree of sensitivity to gemcitabine in this study are also less sensitive to paclitaxel and irinotecan[5]. On the other hands, KKU-M055 cell lines that are highly sensitive to most chemotherapeutic drugs[4] also showed the great inhibitory effect on gemcitabine. In addition, when compared the growth inhibitory effect of gemcitabine between its generic and reference formulations we clearly found that both formulations contain a similar *in vitro* efficacy in all seven CCA cell lines.

In conclusion, the growth inhibitory effects of gemcitabine in seven CCA cell lines are different from high to low degrees. No difference in the IC_{50} values of the generic and reference formulations suggests that the *in vitro* efficacies of these two formulations are very similar. Further investigation such as the histoculture drug response assay may be essential for evaluating the effectiveness of this drug on CCA chemotherapy.

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