นิพนธ์ต้นฉบับ • Original Article

การเปรียบเทียบฤทธิ์ระหว่างตำรับยาสามัญ และตำรับยาอ้างอิง gemcitabine ต่อเซลล์เพาะเลี้ยงมะเร็งหลายชนิดในหลอดทดลอง

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**ศูนย์วิจัยพยาธิใบไม้ตับและมะเร็งท่อน้ำดี มหาวิทยาลัยขอนแก่น จ.ขอนแก่น 40002*

Comparative *in vitro* Cytotoxicity of the Generic and Reference Products of Gemcitabine on Various Cancer Cell Lines

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หลักการและวัตถุประสงค์: Gemcitabine เป็นยาเคมีบำบัดที่สามารถใช้ในการรักษาโรคมะเร็งหลายชนิด จากรายงานการวิจัยทางคลินิกเมื่อเร็วๆ นี้พบว่า gemcitabine เมื่อใช้เป็นยาเดี่ยว หรือให้ร่วมกับ cisplatin สามารถรักษามะเร็งท่อน้ำดีได้ผลดี ปัจจุบันมียา gemcitabine ในรูปตำรับยาสามัญหลายขนานวางจำหน่ายในประเทศไทย ซึ่งตำรับยาสามัญเหล่านั้นมีราคาประหยัดกว่าตำรับยาต้นแบบค่อนข้างมาก การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาเปรียบเทียบฤทธิ์ของยา gemcitabine ในรูปตำรับยาสามัญ (Gemita®, Fresenius Kabi Oncology Ltd.; เดิมเป็นของบริษัท Dabur Pharma Ltd.) และตำรับยาอ้างอิง (Gemzar®, Eli Lilly) ในการยับยั้งการเจริญของเซลล์เพาะเลี้ยงมะเร็งหลายชนิดในหลอดทดลอง

วิธีการศึกษา: เซลล์เพาะเลี้ยงมะเร็งที่ใช้ในการศึกษาครั้งนี้ได้แก่ เซลล์เพาะเลี้ยงมะเร็งท่อน้ำดี (KKU-M055, KKU-M213, KKU-M214) มะเร็งปากมดลูก (HeLa) มะเร็งลำไส้ใหญ่ (SW620) และ มะเร็งปอด (A549) ตรวจวัดฤทธิ์ของ gemcitabine ในการยับยั้งการเจริญเติบโตของเซลล์เพาะเลี้ยงมะเร็งด้วยวิธี sulforhodamine B ค่าความเข้มข้นของยาที่สามารถยับยั้งการเจริญเติบโตของเซลล์เพาะเลี้ยง

Background and objectives: Gemcitabine is a chemotherapeutic agent which can be used for the treatment of several cancers. Recent clinical studies demonstrated that gemcitabine alone or in combination with cisplatin are effective for treatment of cholangiocarcinoma (CCA). To date, several generic products of gemcitabine are now available in Thailand and the price of these products are lower than the reference product. This study aimed to determine the cytotoxic activity of gemcitabine on several human cancer cell lines as well as to compare the *in vitro* cytotoxicity of the generic gemcitabine product (Gemita®, Fresenius Kabi Oncology Ltd.; formerly Dabur Pharma Ltd.) with its reference product (Gemzar®, Eli Lilly) in the similar experimental conditions.

Methods: Cancer cell lines used in the present study were CCA cell lines (KKU-M055, KKU-M213, KKU-M214), cervical cancer cell line (HeLa), colon cancer cell line (SW620) and the lung cancer cell line (A549). The growth inhibitory effect of gemcitabine on these cancer cell lines was measured by the sulforhodamine B assay. The concentration of drug required to inhibit cell proliferation by

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มะเร็งได้ร้อยละ 50 (IC_{50}) คำนวณจากกราฟร้อยละของเซลล์ที่มีชีวิต กับความเข้มข้นของยา เปรียบเทียบค่า IC_{50} ของตำรับยาสามัญและตำรับยาอ้างอิงโดยใช้วิธี Student's t-test

ผลการศึกษา: Gemcitabine มีฤทธิ์ยับยั้งการเจริญเติบโตของเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดีทั้ง 3 ชนิด KKU-M055, KKU-M213 และ KKU-M214 แตกต่างกัน โดยมีค่า IC_{50} ตั้งแต่ 52 ถึง 1,832 nM โดยออกฤทธิ์ยับยั้งได้ดีที่สุดกับเซลล์ KKU-M055 แต่มีฤทธิ์ยับยั้งการเจริญเติบโตของเซลล์เพาะเลี้ยงมะเร็งปากมดลูก (HeLa) มะเร็งลำไส้ใหญ่ (SW620) และมะเร็งปอด (A549) ใกล้เคียงกัน (IC_{50} 13.44 ถึง 22.97 nM) เมื่อเปรียบเทียบค่า IC_{50} ของตำรับยาสามัญ (Gemita ®) และตำรับยาอ้างอิง (Gemzar®) ในเซลล์เพาะเลี้ยงมะเร็งชนิดต่างๆ ไม่พบว่ามีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ ($p>0.05$)

สรุป: Gemcitabine มีฤทธิ์ดีในการยับยั้งการเจริญเติบโตของเซลล์เพาะเลี้ยงมะเร็งท่อนำดี มะเร็งปากมดลูก มะเร็งลำไส้ใหญ่ และมะเร็งปอด ไม่พบความแตกต่างของค่า IC_{50} ระหว่างตำรับยาสามัญและตำรับยาอ้างอิงเมื่อทำการทดสอบกับเซลล์เพาะเลี้ยงมะเร็งเหล่านั้นซึ่งบ่งชี้ว่าฤทธิ์ในการยับยั้งการเจริญเติบโตของเซลล์มะเร็งในหลอดทดลองของยาทั้งสองตำรับไม่แตกต่างกันอย่างมีนัยสำคัญทางสถิติ

50% (IC_{50}) was determined by plotting the percentage of cell viability versus the drug concentrations. Comparisons of IC_{50} values of the generic product with reference product were analyzed using independent *t*-test.

Results: The sensitivities to gemcitabine were varied in the 3 different human CCA cell lines, KKU-M055, KKU-M213 and KKU-M214, with the IC_{50} values ranging from 52-1,832 nM and KKU-M055 was the most sensitive. The cytotoxic effects of these products on cervical cancer (HeLa), colon cancer (SW620) and lung cancer (A549) cell lines were very similar (IC_{50} 13.44 to 22.97 nM). No statistically significant differences were found between the IC_{50} values for the test product (Gemita®) and the reference product (Gemzar®) on these cancer cell lines ($p>0.05$).

Conclusions: Several human cancer cell lines including CCA, cervical cancer, colon cancer and lung cancer cell lines were sensitive to gemcitabine. The IC_{50} values of the generic product, Gemita®, of all the cancer cell lines tested in the present study, were not significantly different from its reference product, Gemzar®. This suggests that the *in vitro* cytotoxicity of these two products on cancer cell lines are not statistical significant different.

Keywords: Gemcitabine, Gemita®, Gemzar®, Human cancer cell lines

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Introduction

Gemcitabine (2',2'-difluorudeoxycytidine, dFdC) is a nucleoside cytidine analogue that exhibits antitumor activity. This drug exhibits cell phase specificity by primarily inhibiting cell proliferation in DNA synthesis (S-phase) and on the G1/S-phase boundary. Gemcitabine is intracellularly metabolized to the active diphosphate and triphosphate nucleosides by nucleoside kinases. The mechanisms of action of gemcitabine involve competition for incorporation into DNA thereby inhibiting the synthesis of DNA; preventing DNA repair by masked termination; and undergoes self-potentiation[1] (Figure 1). Gemcitabine undergoes phosphorylation by deoxycytidine kinase to gemicitabine di- and then tri-phosphate. Gemcitabine diphosphate inhibits ribonucleotide reductase, which is the primary enzyme involved in the formation of deoxycytidine triphosphate (dCTP) that is a natural substrate in DNA replication thus allowing the incorporation of gemcitabine triphosphate nucleotides into the DNA chain during replication. In addition, gemcitabine permits more nucleotides to pair before termination of the replication process. This means that gemcitabine nucleotide is less susceptible to excision repair by exonuclease enzymes, making DNA repair more difficult (masked termination)[1]. This drug is inactivated mainly by deoxycytidine monophospate deaminase to uridine metabolite and then excreted from the body[1].

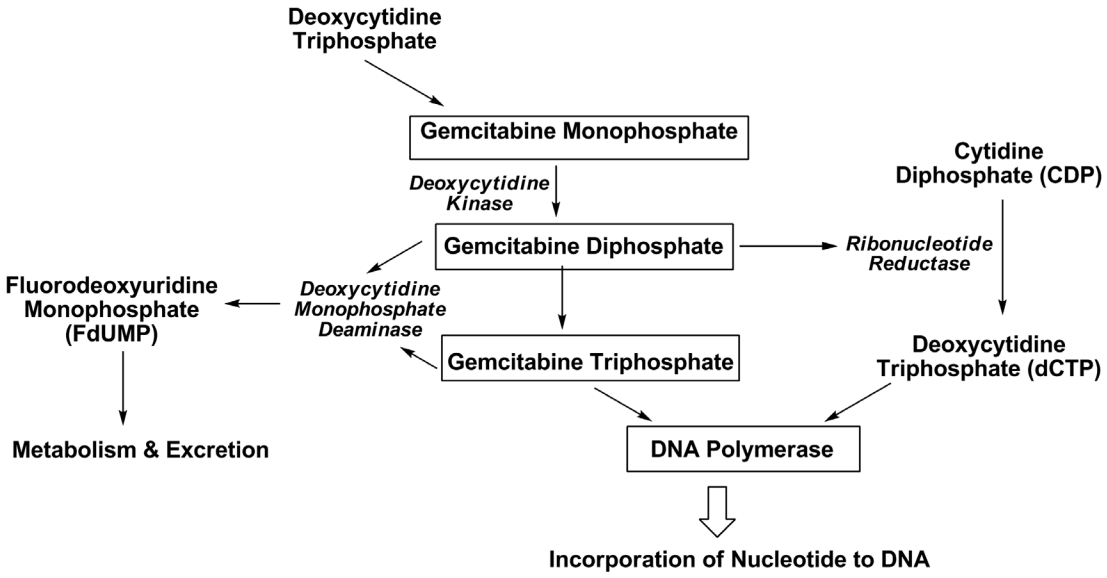

Figure 1 Mechanism of action of gemcitabine[1].

Gemcitabine has been approved by the US-FDA as the first-line treatment for patients with locally advanced or metastatic adenocarcinoma of the pancreas. In addition, this drug in combination with cisplatin or with paclitaxel is indicated as the first-line treatment of patients with inoperable, locally advanced or metastatic non-small cell lung cancer and or with metastatic breast cancer after failure of prior anthracycline-containing adjuvant chemotherapy.

It has recently been reported that gemcitabine may be beneficial for the treatment of cholangiocarcinoma (CCA). The use of gemcitabine as a single agent for treatment of CCA showed a response rate of about 30% with generally mild hematologic toxicity. [2-4] Recent data from a multicenter study revealed that cisplatin plus gemcitabine is an effective treatment option for locally advanced or metastatic biliary tract cancer[5]. The median progression-free survival was 8.0 months in the cisplatin-gemcitabine group and 5.0 months in the gemcitabine-only group and the rate of tumor control among patients in the cisplatin-gemcitabine group was significantly increased (81.4% vs. 71.8%).[5]

To date, several generic gemcitabine products are now commercially available in Thailand. In general, the efficacy of the generic products should be evaluated by an *in vivo* bioequivalence in healthy volunteers. High cytotoxicity of chemotherapeutic drugs, however, preclude the feasibility of conducting the *in vivo* bioequivalence study in healthy volunteers. In addition, conducting *in vivo* bioequivalence or therapeutic studies of chemotherapeutic drugs in cancer patients seems not feasible due to many blood samplings and difficulty to control confounding factors that may arise from the heterogeneity of patients. The *in vitro* efficacy of generic chemotherapeutic products may be demonstrated by comparisons *in vitro* cytotoxicity studies between the generic and the reference product using cancer cell lines.[6-9] Compared to the pharmaceutical equivalence method which measures only the amount of the drug in the pharmaceutical preparations, this *in vitro* cytotoxicity study may provide more information on the *in vitro* efficacy of generic products. Due to the difference in the cost between reference and generic products, cost effectiveness is one of the key factors that need to be considered when prescribing the drug for patients. The aims of this study were to determine the cytotoxic activity of gemcitabine on several human cancer cell lines as well as to compare the *in vitro* cytotoxicity of the generic gemcitabine product (Gemita®, Dabur Ltd.) with its reference product (Gemzar®, Eli Lilly).

Methods

Chemicals

RPMI-1640 medium, 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.

Generic and reference products of gemcitabine used in this study were Gemita® (Fresenius Kabi Oncology Ltd.; formerly Dabur Pharma Ltd., India Lot no. BKH105; Mfg date 04/2008; Exp date 03/2010) and Gemzar® (Lilly France S.A.S, France; Lot no. A447931A; Mfg date 21/01/2008; Exp Date 19/01/2010). The lyophilized powder of Gemita® or Gemzar® contained 200 mg gemictabine HCl, 200 mg manitol and 12.5 mg sodium acetate. Both products were dissolved with normal saline to 20 mg/ml and then diluted about 10^6 fold with culture medium before adding to microtiter plates.

Human cancer cell lines

Various types of human cancer cell lines, including CCA cell lines (i.e. KKU-M055; moderately differentiated adenocarcinoma, KKU-M213; adenosquamous and KKU-M214; moderately to poorly differentiated adenocarcinoma), HeLa (cervical cancer cell line), SW620 (colon cancer cell line) and A549 (lung adenocarcinoma cell line) were used in this study. CCA cell lines were established by the Liver Fluke and Cholangiocarcinoma Research Center from CCA patients residing in northeastern Thailand while HeLa, SW620 and A549 cell lines were obtained from Faculty of Medicine, Thammasat University, Thailand. Cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml of penicillin and 100 µg/ml of streptomycin at 37°C with 5% CO_2.

Cytotoxicity assay

Sulforhodamine B assay was used to determine cell viability as described previously[6] with some minor modifications. In brief, cancer cell lines (5×10^4 cells/ml) at exponential growth phase were 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 of medium with or without drug was added and incubated at 37°C for 72 h. The culture medium was subsequently removed and 200 µl of 10% (w/v) ice-cold TCA was added to each well. The plates were then incubated at 4°C for 60 min. Trichloro acetic acid (TCA)-treated cells were stained 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 unbound dye. The protein-bound dye was solubilized with 200 µl of 10 mM Tris base buffer (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 viability versus the drug concentrations.

Statistical analysis

Tests were performed in 3 independent experiments. Data are expressed as mean of $IC_{50} \pm SD$. Comparison of IC_{50} values of the generic product with reference product was analyzed using the independent *t*-test.

Results

The cytotoxic effects of the generic product (Gemita®) and the reference product (Gemzar®) of gemcitabine against 3 human CCA cell lines are shown in Figure 2. There were no statistically significant differences in the IC_{50} values observed for generic and reference products on these human CCA cell lines (Table 1). The sensitivities of gemcitabine were varied in the 3 different human CCA cell lines with the IC_{50} values ranging from 52-1,832 nM. KKU-M055, a moderately differentiated adenocarcinoma, was the most sensitive CCA cell line for gemcitabine with an IC_{50} value of 52-54 nM while KKU-M214, a moderately to poorly differentiated adenocarcinoma, was the most resistant CCA cell line with the IC_{50} value of 1,567-1,832 nM (Table 1).

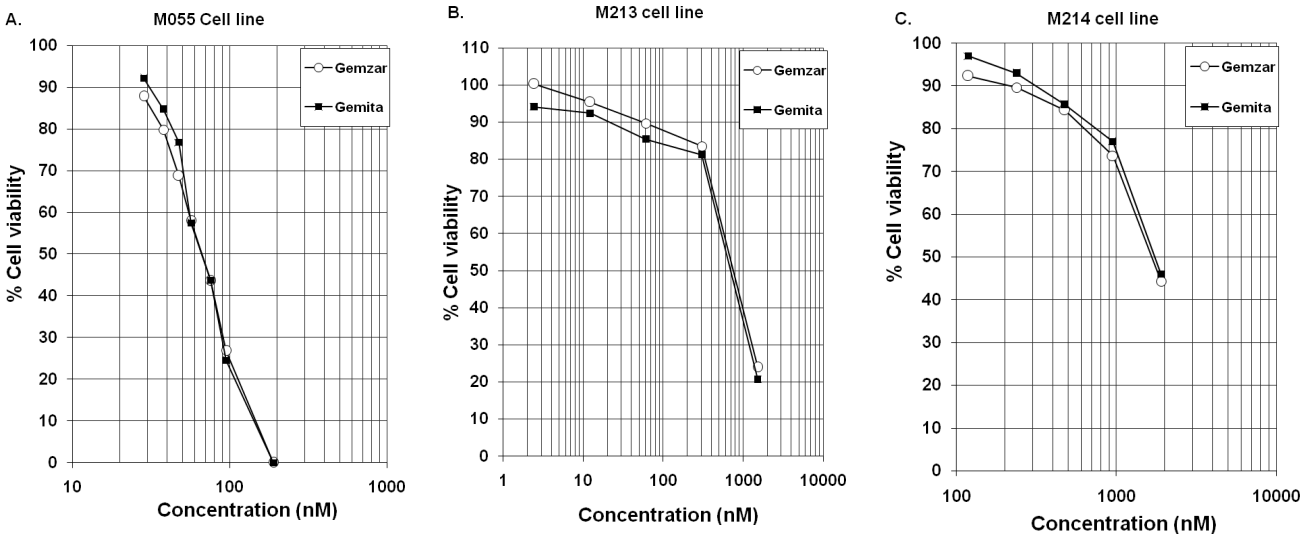

Figure 2 Effects of Gemzar® and Gemita® on various human CCA cell lines

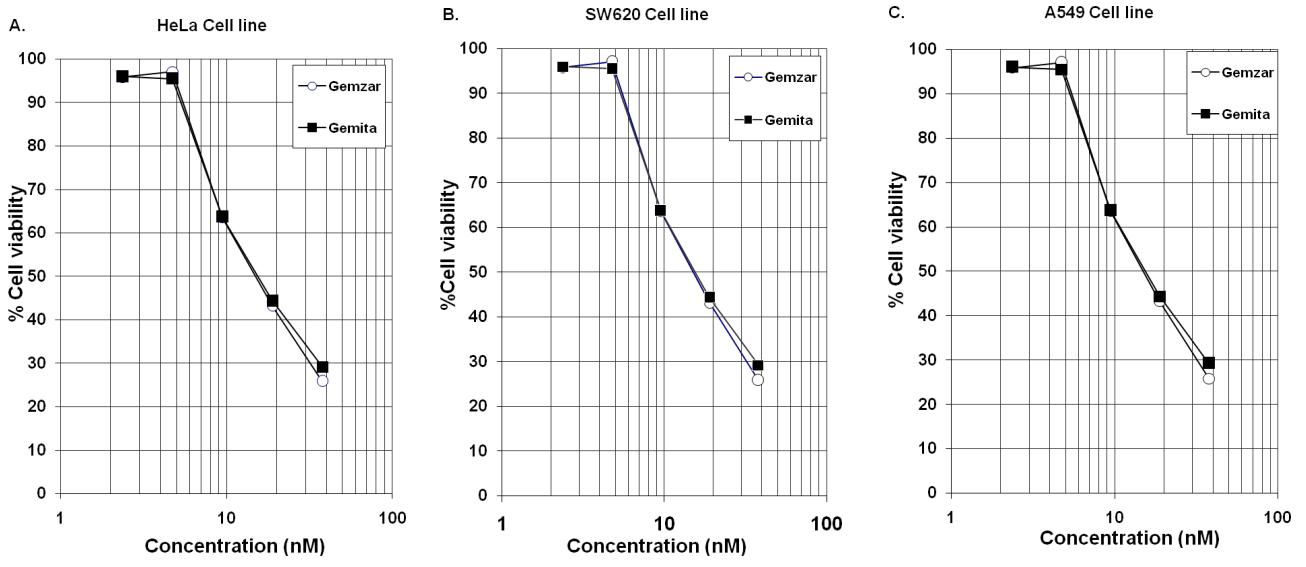

Figure 3 Effects of Gemzar® and Gemita® on several human cancer cell lines

The cytotoxic effect of the generic product (Gemita®) and the reference product (Gemzar®) of gemcitabine against HeLa cervical cancer cell line, SW620 colon cancer cell line and A549 lung adenocarcinoma cell line are shown in Figures 3. There were no statistically significant differences in the IC_{50} values observed for the generic and reference products among these human cancer cell lines (Table 2). When compared to human CCA cell lines, HeLa, SW620 and A549 cell lines were more sensitive to gemcitabine. The IC_{50} values of gemcitabine in these 3 cell lines were approximately 13-23 nM (Table 2).

Table 1 Mean IC_{50} values of Gemzar® and Gemita® on various human CCA cell lines

Cell line	IC_{50} values (nM)		
	Reference Product (Gemzar®)	Test Product (Gemita®)	P Value
KKU-M055	52.86 ± 2.49	54.41 ± 2.31	> 0.05
KKU-M214	1,832.50 ± 206.78	1,567.82 ± 306.36	> 0.05
KKU-M213	548.36 ± 74.29	695.26 ± 247.71	> 0.05

Data represents mean ± SD of three independent experiments.
P > 0.05 means that it was not statistically significant different.

Table 2 Mean IC_{50} values of Gemzar® and Gemita® on several cancer cell lines

Cell line	IC_{50} values (nM)		
	Reference Product (Gemzar®)	Test Product (Gemita®)	p-value
HeLa (cervical cancer cell line)	18.73 ± 4.84	17.10 ± 3.22	> 0.05
SW620 (colon cancer cell line)	22.73 ± 3.01	22.97 ± 1.10	> 0.05
A549 (lung cancer cell line)	13.44 ± 2.90	14.23 ± 0.21	> 0.05

Data represents mean ± SD of at least three independent experiments.
P > 0.05 means that it was not statistically significant difference.

Discussion

The results from this *in vitro* study clearly showed that several human cancer cell lines including cervical cancer (HeLa), colon cancer (SW620) and lung cancer (A549) cell lines were sensitive to gemcitabine with IC_{50} values of 13.44 to 22.97 nM. The sensitivity of gemcitabine toward 3 human CCA cell lines varied about 30 folds (with the IC_{50} values varying from 52 to 1,832 nM). Among, the 3 human CCA cell lines, KKU-M214 was appeared to be the most resistant cell line to gemciabine. Consistent with the previously reported data, KKU-M055 was the most sensitive cell line for gemcitabine.[6] These results suggest that sensitivity of gemcitabine are vary among human CCA cell lines. These 3 CCA cell lines are different histological type, however, it has been previously reported that the sensitivity of CCA cell lines to chemotherapeutic agents were not related to the histological type of cell lines.[7] The IC_{50} values of gemcitabine toward CCA cell lines, particularly KKU-M213 and KKU-M214 were about 30-140 higher than other cancer cell lines. This result suggest that HeLa, SW620, A549 cell lines appeared to be more sensitive to gemcitabine than CCA cell lines.

It should be noted, however, that IC_{50} values of gemcitabine on KKU-M055, KKU-M213 and KKU-M214 reported by Dokduang[6] were much higher than those reported in the present study. The discrepancy in the different IC_{50} values may be partly due to the source of the human CCA cell lines used in the studies. The cell lines used in Dokduang's study had been treated with a mixture of antibiotics in order to eliminate mycoplasma contamination but the cell lines used in the present study were not exposed to those antibiotics.

In addition, the IC_{50} values of the generic product, Gemita®, for all of the cancer cell lines tested in the present study were not significantly different than those

obtained from its reference product, Gemzar®. This suggests that the *in vitro* cytotoxic effects of these two products are not statistically significant different.

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