A CDK1 inhibitor, BEY-A, controls cell cycle and induces apoptosis in biliary tract cancer

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This certifies that the Master's Thesis
of Chanyang Kim is approved.

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ABSTRACT

A CDK1 inhibitor, BEY-A, controls cell cycle and induces apoptosis in Biliary Tract Cancer

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(Directed by Professor Si Young Song)

Cyclin dependent kinase 1 (CDK1) is cell cycle related protein that regulates the G2 phase as part of a complex with cyclin A and is involved in G2/M transition by forming a complex with cyclin B. CDK1 is highly expressed in various cancers and is known to be closely related with cancer progression. BEY-A is a recently developed CDK1 inhibitor to arrest G2/M but its role in biliary tract cancer is not clear. In this study, we explored the expression of CDK1 in biliary tract cancer patients tissues and analyzed its possible role in cell cycle and cell apoptosis. More, the combined use with other chemo-drugs or radiation was assessed. Using immunohistochemistry(IHC), the expression of CDK1 was observed in both normal and cancer tissues. However, the expression was only observed in cytoplasm in normal but both in cytoplasm and nucleus in cancer tissues. The active CDK1 was analyzed by IHC and the expression was only observed in cancer nucleus but not in normal cell nucleus. We performed MTT assay to access the cytotoxicity in biliary tract cancer cells and IC_{50} values were determined as 0.38μM for HuCCT-1 and 0.528μM for SNU-1196. We next examined whether BEY-A induce apoptosis in biliary tract cancer cells by western blot analysis and observed that BEY-A increased

cleaved caspase-3 and cleaved PARP expression in time-dependent manner. Cell cycle analysis was demonstrated using FACS analysis and BEY-A induced G2/M arrest was observed. The effect of BEY-A on the level of G2/M phase-related proteins using western blot analysis and increased expression of cyclin A, cyclin B1, and CDK1 phosphorylation on Thr161, Thr14, Tyr15 whereas decreased expression of cdc25c was observed in time and dose-dependent manner. Combination with other chemo-drugs including gemcitabine and cisplatin was performed to observe cell viability and combination with radiation was also performed. Neither additive effect nor synergistic effect in combined use with other chemo-drugs was observed but combination with radiation resulted in enhanced to inhibit in cell survival. In this study, we first observed BEY-A controls cell cycle, induces apoptosis and effective with radiation and suggest BEY-A as a putative biliary tract cancer chemo-drug.

Key words : CDK1 inhibitor, biliary tract cancer, G2/M cell cycle arrest, apoptosis

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I. INTRODUCTION

Biliary tract cancers (BTCs) encompass a group of invasive carcinomas, including cholangiocarcinoma, which refers to cancers arising in the intrahepatic, perihilar, or extrahepatic biliary tree, and gallbladder carcinoma[1]. They originate from the epithelium of the gallbladder and the bile ducts[2]. Biliary tract cancers are rare, approximately 90% of patients present with advanced, unresectable disease and have a poor prognosis[3].

The cell-division cycle of eukaryotic cells is divided into four phases of G1, S, G2 and mitosis (M)[4]. Cyclin-dependent kinases (CDKs) are important cell cycle-regulating proteins, which belong to a serine/threonine kinase family that comprise of a catalytic kinase subunit, together with cyclin protein partners. There are at least 13 different CDKs and more than 25 cyclin proteins dentified to date[5]. However, only CDK1, 2, 4, and 6 are directly involved in the cell cycle, and of these, CDK2, 4, and 6 are not essential to the cell cycle. Furthermore, unlike other CDKs, CDK1 can promote the cell cycle alone and is essential for cell cycle progression and cell division[5, 6].

CDK1 regulates the G2 phase as part of a complex with cyclin A and is involved in G2/M transition by forming a complex with cyclin B. Knockdown of CDK1 increases G2/M arrest during the cell cycle and results in polyploid cells[5, 6].

Cyclin dependent kinase 1 (CDK1), controls the cell cycle entry from the G2 to the M phase and promotes the commencement of mitotic phase events[7].

CDK1 activation also depends on the phosphorylation of Thr161, and CDC25-mediated dephosphorylation at Thr 14 and Tyr 15, which exhibits enzymatic activity when only phospho-Thr161 remains[8]. CDK1 is highly expressed in various cancers. It has been reported that CDK1 expression or activity is elevated in Hodgkin's lymphomas[9], human colorectal cancer[10], prostate cancer[11], gastric lymphoma[12], childhood acute lymphoblastic leukemia[13]. CDK1 is closely related with cancer progression. Therefore, CDK1 has been introduced as a promising gene for targeted therapy that could inhibit progress of the cell cycle in various cancers and induce apoptosis. A number of CDKs inhibitors including CDK1 inhibitors have been developed to date and their effects have also been studied[14-16]. BEY-A is a recently developed CDK1 inhibitor to arrest cells at G2/M but its role with biliary tract cancer is not clear.

In this study, we explored the expression of CDK1 in biliary tract cancer tissue and examined whether BEY-A induce apoptosis and regulates cell cycle in biliary tract cancer cells. We also performed combination treatment with other chemo-drugs and radiation.

II. MATERIALS AND METHODS

1. Chemicals and cell culture

Human biliary tract cancer cell HuCCT-1 was obtained from Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan), SNU-1196 was purchased from the Korean Cell Line Bank (Seoul, Korea) and maintained in RPMI1640 medium respectively, containing 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) and 1% antibiotics. The cells were trypsinized with 0.25% trypsin-EDTA (Gibco by Life Technologies, Grand Island, NY, USA). Both cell line were incubated in a 5% CO_2 atmosphere at 37℃.

BEY-A is recently developed CDK1 inhibitor to G2/M arrest by BeyondBio. It was dissolved in Dimethyl Sulphoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) to 10 mM stock solution, stored at -20℃ and diluted with media for treatment.

2. Assessment of cell viability

The viability of the cells was measured by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Amresco, Solon, OH, USA) assay. The two cell lines were plated at $1x10^3$ cells per well in 96-well plates. After 72 h they were treated with various concentration of BEY-A, the cytotoxicity of BEY-A was then measured by the MTT Assay. MTT

was dissolved at a concentration of 5 mg/ml in phosphate buffer saline (PBS) and filter sterilized. MTT stock solution was diluted at a final concentration of 0.5 mg/ml and 100 μl of diluted MTT solution was added to each well to be anlyzed. The cells were incubated for 3 h at 37℃ in CO_2 incubation. After 3 h incubation, MTT solution was washed and 100 μl of DMSO was added to each well to dissolve formazan produced. Optical density was measure at 570 nm with a ELISA reader (Versamax tunable microplate reader, Sunnyvale, CA, USA). The sensitivity of HuCCT-1 and SNU-1196 cells to BEY-A, entinostat, and vorinostat was determined based on half-maximal inhibitory concentration (IC_{50}) values.

3. Combination treatment

The effect of BEY-A combined with gemcitabine, cisplatin or gemcitabine plus cisplatin on cancer cell viability was evaluated in HuCCT-1, SNU-1196 cells using the fixed-ratio method. Cells were treated with BEY-A, gemcitabine, cisplatin individually or in combination. The combination treatment consisted of 10-fold serial dilutions of gemcitabine and 5-fold dilutions of cisplatin with IC_{10} or IC_{30} BEY-A. For triple combination treatment, gemcitabine and cisplatin were combined at a concentration ratio of 1:1 (gemcitabine IC_{50} :cisplatin IC_{50} and 2-fold serial dilutions were prepared with IC_{10} or IC_{30} BEY-A. After treatment, cell viability was determined with the MTT assay.

Cells were treated with MTT solution, incubated for 3 h at 37℃ in CO_2

incubation. After 3 h incubation, MTT solution was washed and 100 μl of DMSO was added to each well to dissolve formazan produced. Optical density was measure at 570 nm with a ELISA reader (Versamax tunable microplate reader, USA).

4. Fluorescence activated cell sorter (FACS) analysis for cell cycle

The two cell lines were seeded $2x10^5$ per well in 6-well plates, treated with IC_{50} of BEY-A and DMSO as control group for 0, 12, 24 and 48 h. After treatment, cell culture medium were collected in a 15ml conical tube and the cells were washed once with PBS (pH 7.4), and centrifuged. The supernatant was removed and the cell pellets were washed twice using PBS and centrifuged. The cell pellets were suspended with 1 ml PBS, transferred to FACS tube, added 1 ml ethanol with vortexing and added also 1ml ethanol for a fixation. The cells were washed twice with 1 ml PBS, after pellet down. They were incubated with 300μ l FxCycle™PI/RNase staining solution (Invitrogen, Waltham, Massachusetts, USA) for 20 min at room temperature (RT) and analyzed by flow cytometry. Flow cytometry was performed on a FACS Caliber system equipped with argon-ion laser (Becton Dickinson, Franklin Lakes, NJ , USA). Percentages of cells in each phase were calculated using software programs.

5. Cell extractions

Cell culture media were collected in a 15 ml conical tube and the cells were washed once using 1 ml PBS. The cells were trypsinized with 0.25% trypsin-EDTA (Gibco by Life Technologies, USA) and collected in a 15 ml conical tube with their cell culture medium. The tubes were centrifuged at 100 xg, RT for 3 min. The cells were washed once with PBS and collected in a 1.5 ml eppendorf tube and then quickly centrifuged at 15,700 xg, 4℃ for 3min. Cell pellets were resuspened with protein lysis buffer containing 1M β-glycerophosphate (pH 7.2), 50mM sodium vanadate, 0.5M $MgCl_2$, 0.2M ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 1M dithiothreitol (DTT), 100% triton, 100 mM Phenylmethylsulfonyl Fluoride (PMSF), and protease inhibitors and incubated on ice for 1 hour then centrifuged at 15,700 xg, 4℃ for 1 hour. The supernatant was collected in a 1.5 ml microcentrifuge tube and stored at -70℃ for further analysis or directly used for determination.

6. Western blot analysis

Protein concentration was determined by using Bicinchoninic acid assay (BCA) (Thermo Scientific) and bovine serum albumin (BSA) was used as the standard. 50μg of protein were separated by 10% or 15% SDS polyacrylamide gel electrophoresis (PAGE), transferred to PVDF membrane (Amersham Life Science, Buckinghamshire, UK), stained for 5 min with

Ponceau S, blocked for 1 hour in 5% nonfat dry milk (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in Tris-buffered saline with 0.05% tween-20 (TBST) and incubated over nights at 4℃ with a primary antibody in TBS-T. The primary antibodies were diluted in 1 to 500 or 1000 ratio with TBST. Antibodies used were as follows: cyclin B1 (Santa Cruz Biotechnology, Dallas, TX, USA), cyclin A (Santa Cruz Biotechnology, USA), cdc25c (Cell Signalling Technology, Danvers, MA, USA,), cdc2 (Cell Signalling Technology, USA), p-cdc2 (Tyr14) (Cell Signalling Technology, USA), p-cdc2 (Thr15) (Santa Cruz Biotechnology, USA), p-cdc2 (Tyr161) (Cell Signalling Technology, USA), PARP (Cell Signalling Technology, USA), Bax (Santa Cruz Biotechnology, USA). The secondary antibodies were blotted for 1 h at RT, diluted in 1 to 5000 ratio with TSBT and visualized with enhanced chemoilluminescent substrate (Thermo Scientific, Rockford, IL, USA).

7. Cell clonogenic Assay

HuCCT-1 cells ($1x10^6$) were treated with 0.38μM of DMSO as a control and 0.38μM of BEY-A for 12 h after seeding into 100mm culture dish. SNU-1196 cells ($1.5x10^6$) were treated with 0.528μM of DMSO as a control and 0.528 μM of BEY-A for 24 h after seeding into 100mm culture dish. Each time-point was determined by resulting of FACS data in most G2/M arrested cell populations. After BEY-A treatment, the two cell lines were trypsinized, counted to equivalent cells number in e-tube for 5 radiation grade (0, 2, 4, 6

or 8 Gy) and performed radiation. Then, the cells were plated $1x10^3$ per well in 6-well plates for clonogenic assay. Ten days after radiation, the cells were fixed and a clonogenic assay was performed. Cells were washed once with 1 ml PBS . The cells were then fixed using 1ml fixation solution that was made of 75% methanol and 25% acetic acied (v/v) for 15 min at RT. After fixation, colonies were stained for 20 min with 1 ml BBL™ Gram Crystal Violet (Becton Dickinson, East Rutherford, NJ, USA). The stained cell were washed twice using 1 ml water and dried for over-night. After washing and drying, the colonies were counted.

8. Immunohistochemistry (IHC)

Tumor xenografts were fixed in formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin(H&E). Immunohistochemistry (IHC) with the anti-CDK1 antibody was performed according to standard protocols. They were incubated in 60℃ oven for 1 hour, deparaffinized twice in xylene for 20 min and three times in absolute ethanol. They were rehydrated in a graded series of 95% to 30% ethanol for 1 min each step, washed three times with PBS, submerged the slides in peroxidase quenching solution for 20 min. The peroxidase quenching solution was made of 0.3 % H_2O_2 in methanol. After quenching, they were washed three time using PBS, boiled for antigen retrieval for 3 min in 10 mM citrate buffer (pH 6.0), cooled down for 20 min in running water, washed three times with PBS, blocked with 10 % normal donkey serum in PBS for 1 hour at RT, washed three times with PBS and

they were incubated for over nights at 4℃ with a rabbit anti-CDK1 phospho-Thr161 Ab (LSBio, 1:200). The slides were washed three times using PBS, incubated with Dako REAL™ EnVision™ HRP RABBIT/MOUSE (Dako, Santa Clara, CA, USA) for 20 min at RT. Horse radish peroxidase (HRP)-conjugated secondary antibody was used and colored using DAB solution. Slides were counterstained with hematoxylin, and mounted with mounting solution.

9. Irradiations

Irradiations were carried out using a Gammacell® 3000 Elan (Theratronics, Ontario, Canada) at a dose grade of 2, 4, 6 and 8 Gy.

III. RESULTS

1. Cyclin-dependent kinase 1 (CDK1) expression in biliary tract cancer patients

To investigate CDK1 could be a target gene for biliary tract cancer cells, we performed immunohistochemical (IHC) staining using biliary tract cancer patients tissues to verify the expression of active form of CDK1, phospho-Thr161 (p-Thr161). Three patient's non-cancer and cancer specimen slides were immune-statined with CDK1 (p-Thr161) antibody. The CDK1 (p-Thr161) expression was consistently observed in cytoplasm in non-cancer tissues but both in cytoplasm and nucleus in cancer tissue slides. The representative figures are presented in Figure 1. Based on the previous studies, CDK1 becomes active when 161th amino acid threonine (Thr161) becomes phosphorylated (p-Thr161) and translocated into the nucleus[17]. The IHC staining showed that majority of cancer cells express p-Thr161 in the nucleus whereas the expression of p-Thr161 was mostly expressed in cytoplasm in non-cancer cells. Collectively, the results suggested that CDK1 was active in cancer tissues and CDK1 could be a putative target gene for in biliary tract cancer treatment.

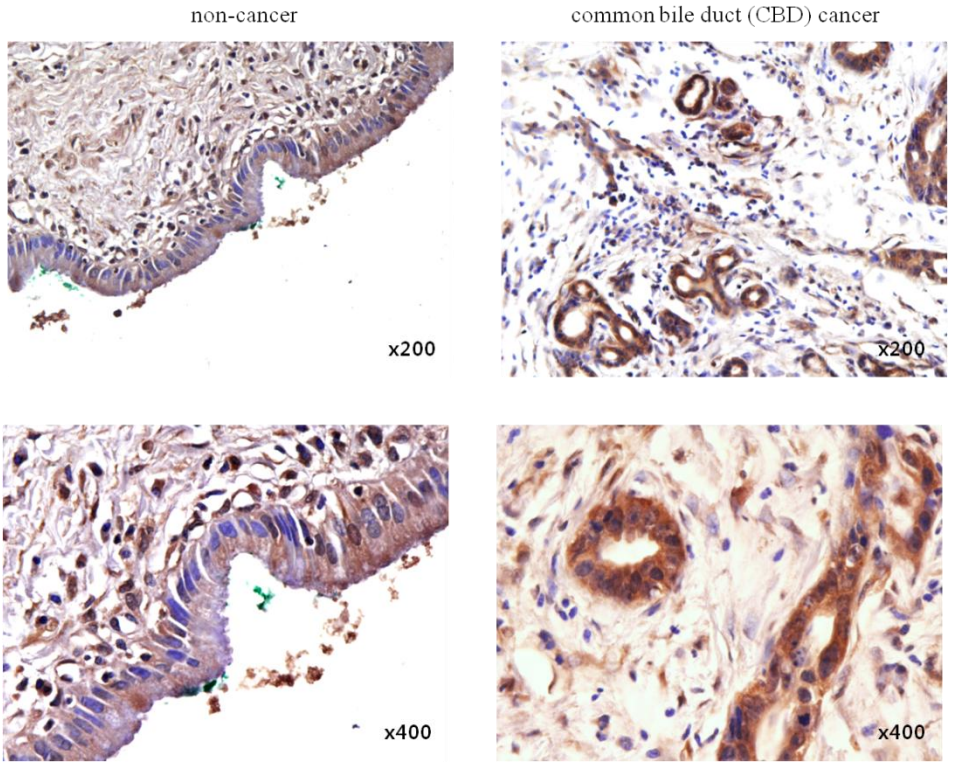


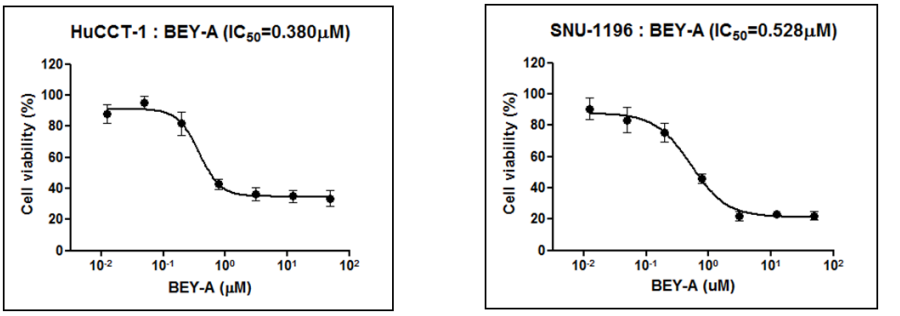
Figure 1. CDK1 is expressed in Biliary Tract cancer tissues. Immunohistochemical staining of a biliary tract cancer patient tissue slides with an anti-CDK1 phospho-Thr161 antibody. Left panels are non-cancer tissue and right panels are common bile duct (CBD) cancer tissue. Both tissue are same patient. Images magnified by a factor of 200 or 400.

2. BEY-A induces apoptosis in human biliary tract cancer cell lines; HuCCT-1 and SNU-1196

To assess the cytotoxicity of BEY-A, we performed MTT assay to measured the IC_{50} values of BEY-A in two cell lines. The treatment consisted of 4-fold serial dilutions starting from 50 μM BEY-A. After 72 h, the cytotoxicity of BEY-A was then measured. IC_{50} values were determined as 0.38 μM for HuCCT-1 and 0.528 μM for SNU-1196. (Figure 2A)

Then we examined whether BEY-A induce apoptosis in biliary tract cancer cells on levels of apoptosis-related proteins. As the activation of caspase plays a central role in the process of apoptosis, cleaved (active) forms of caspase-3 in BEY-A treated two cell lines were measured by Western blotting. Each cell was treated with DMSO and BEY-A IC_{50} values for 12, 24 and 48 h and harvested on the indicated time-point. After HuCCT-1 and SNU-1196 cells are exposed to BEY-A for 0, 12, 24 and 48 h, the expressions of cleaved caspase-3 were sharply up-regulated in the time-dependent manner. Simultaneously, the cleavage of poly-ADP ribose polymerase (PARP) protein, one intracellular substrate of activated caspase-3, gradually increased in BEY-A treated two cell lines, confirming that caspase-3 was activated by BEY-A treatment. (Figure 2B) These result indicated that the mitochondrial-mediated apoptotic pathway is involved in BEY-A induced apoptosis in biliary tract cancer cells lines.

A



B

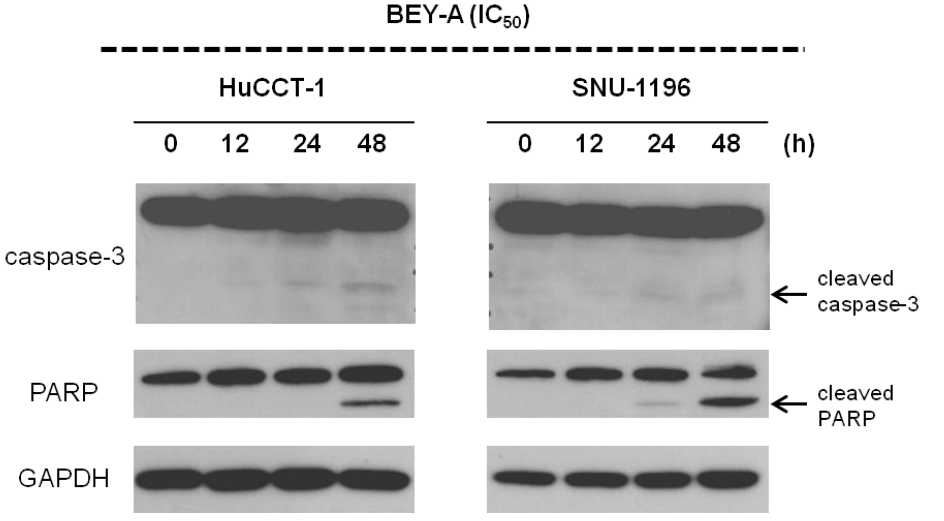


Figure 2. Anti-proliferative and pro-apoptotic activities of BEY-A against biliary tract cancer cells. (A) Cell viability curve based on the BEY-A concentration in two biliary tract cancer cells lines. BEY-A inhibits the proliferation of two cell lines. Their IC_{50} values were 0.38μM in HuCCT-1 and 0.528μM in SNU-1196. (B) Effects of BEY-A on cleaved caspase-3 and cleaved PARP expression. Cells were treated BEY-A at the their IC_{50} values for 0, 12, 24 and 48 h. GAPDH was used as an internal control.

3. BEY-A induced G2/M arrest in human biliary tract cancer cell lines; HuCCT-1 and SNU-1196

We examined the effects of BEY-A on cell cycle. Cell cycle analysis was performed using flow cytometry. HuCCT-1 and SNU-1196 cells were treated with DMSO and BEY-A (IC_{50}) for 12 h, 24 h and 48 h. Cells were then harvested and fixed in every time-point. Based on the flow cytometry analysis, HuCCT-1 cells arrested and accumulated in G2/M phase for 45% in 12 h, 37.6% in 24 h, and 24.8% after 48 h of BEY-A treatment whereas 26.8% of cells were accumulated in G2/M phase with DMSO treatment for 48 h (Figure 3). SNU-1196 cells were also arrested and accumulated G2/M cell populations to 30.8% of cells at control DMSO, 38.7% of cells at 12 h, 46.4% of cells at 24 h and 42.7% of cells at 48 h after treatment BEY-A (Figure 3). The maximal effect of BEY-A induced G2/M arrest and accumulation was observed at 12 h in HuCCT-1 and at 24 h in SNU-1196 treated with respective BEY-A IC_{50} values. And these time-point were used for the further anlaysis of treatment BEY-A.

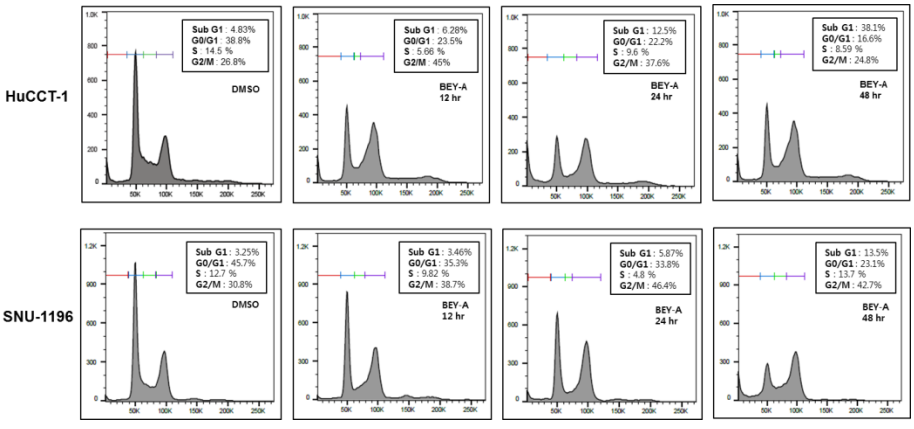


Figure 3. Cell cycle analysis of HuCCT-1 and SNU-1196 cells treated with BEY-A IC_{50}. Cells were treated with DMSO and BEY-A (IC_{50}) for 12 h, 24 h and 48 h. The cell cycle distributions of cells were analyzed by flow cytometry.

4. BEY-A induced G2/M arrest through inhibition of CDK1 activity-related protein expressions

To drive cell cycle progression, CDK1 needs to form complex with cyclin B1[18, 19]. CDK1, also known as cdc2, is activated by binding cyclin B1 followed by phosphorylation of CDK1 on the Thr161 site and negatively regulated by the rapid dephosphorylation on both Thr14 and Tyr15 sites[18]. To examine whether BEY-A affected CDK1 activity of treated cells, the level of CDK1-related proteins was analyzed using Western blotting. Both HuCCT-1 and SNU-1196 cells were treated with each IC_{50} value of BEY-A for 0, 12, 24 and 48 h.

BEY-A induced increase in the protein levels of cyclin A, cyclin B1, and CDK1 phosphorylation on Thr161, Thr14, Tyr15 and a significant decrease in the protein levels of cdc25c. We also examined the effect of BEY-A in dose-dependent manner (Figure 4). Both cell lines were treated with BEY-A of 0, 0.125, 0.25, 0.5, 1.0 and 2.0μM. HuCCT-1 was harvested after 12 h of BEY-A treatment and SNU-1196 was harvested after 24 h of BEY-A treatment. The level of CDK1-related proteins increased or decreased in dose-dependent manner (Figure 5). These results may indicate that BEY-A induced cell cycle arrest in G2/M phase through blocking binding site of phophorylate-Thr14, -Thr15 of CDK1 and cdc25c that leads to CDK1 remaining its inactive form in G2 phase (Figure 6).

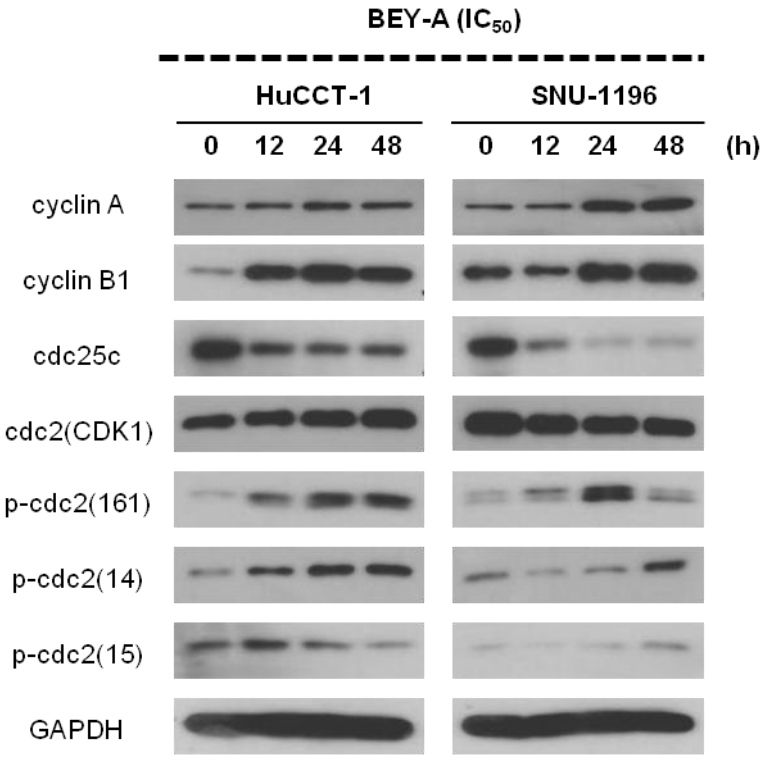


Figure 4. Time dependent effect of BEY-A in HuCCT-1 and SNU-1196 cells on the expression of CDK1 activity-related molecules. HuCCT-1 and SNU-1196 cells were treated with DMSO and BEY-A for 12 h, 24 h and 48 h. The protein levels of cyclin A, cyclin B1, cdc25c, cdc2 (CDK1), Thr16, Thr14 and Tyr15 phosphorylated cdc2 were examined using western blot aanlysis.

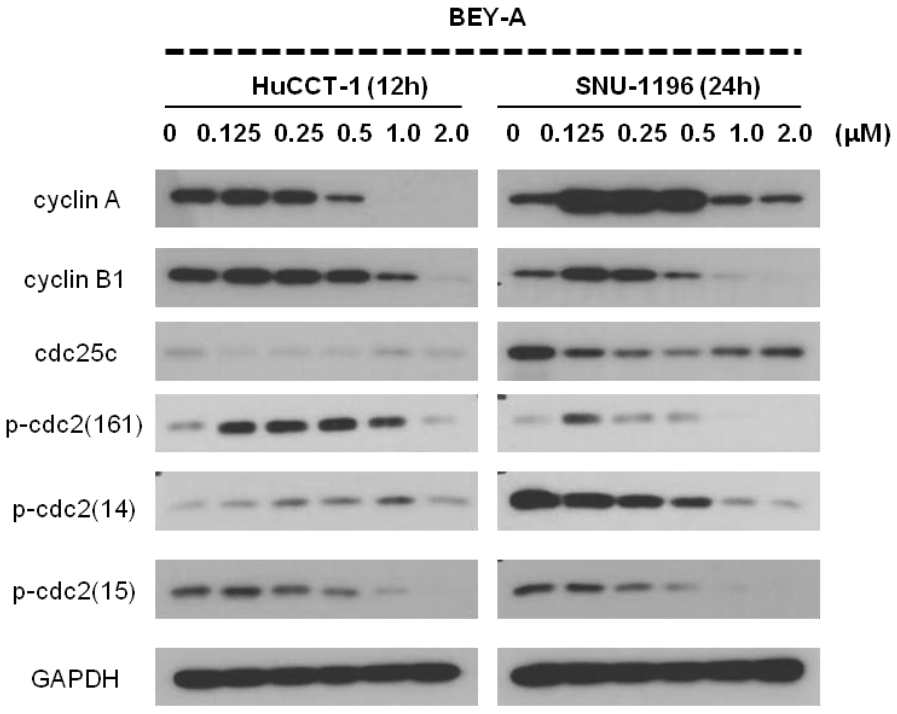


Figure 5. Dose dependent effect of BEY-A in HuCCT-1 and SNU-1196 cells on the expression of cell cycle realted molecules. Cells were treated with DMSO and BEY-A for 12 h for HuCCT-1 or, 24 h for SNU-1196 using indicated concentration of BEY-A. The protein levels of cyclin A, cyclin B1, cdc25c, cdc2 (CDK1), Thr16, Thr14 and Tyr15 phosphorylated cdc2 were determined using western blot assay.

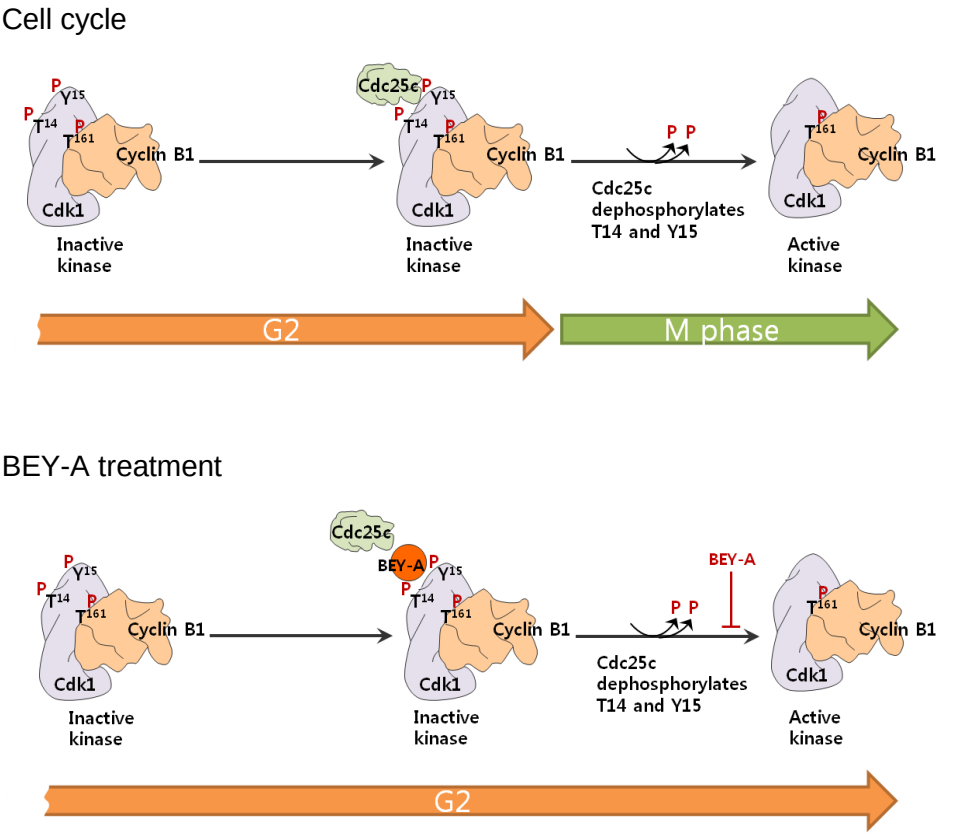


Figure 6. Regulation of CDK1 by clin binding and protein phosphorylation from late G2 phase to M phase.

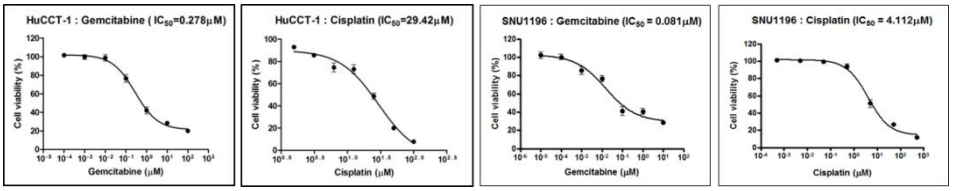
5. Combination with Gemcitabine and Cisplatin is no synergy effect

To examine the combination cytotoxic effect with other chemo-drugs, we first measured the IC_{50} values of gemcitabine and cisplatin in two biliary tract cancer cell lines by the MTT assay.

Based on the MTT assay, IC_{50} values of gemcitabine were measured at 0.278 μM in HuCCT-1 and 0.081 μM in SNU-1196. IC_{50} values of cisplatin were also measured at 29.42 μM in HuCCT-1 and 4.112 μM in SNU-1196 (Figure 7A).

We then investigated the anti-tumor effect of BEY-A combined with gemcitabine and cisplatin in biliary cancer cell lines. HuCCT-1 and SNU-1196 were treated with gemcitabine, cisplatin and BEY-A at the same time. The results from the cell viability, no synergistic inhibitory effect was observed when BEY-A with gemcitabine and cisplatin was treated (Figure 7B). To validate whether the sequential treatment may give synergistic effect, we treated BEY-A for 10 days at sublethal doses[20]. The sublethal doses were IC_{10} values for HuCCT-1 and SNU-1196. After 10 days of treatment with BEY-A, cells were treated with gemcitabine and cisplatin and cytotoxicity was analyzed with MTT assay. This method, however, showed no synergistic inhibitory as well (Figure 7C). Based on the result of two combination treatment methods, we concluded that BEY-A treated with gemcitabine and cisplatin shows neither synergistic nor additive effects.

A



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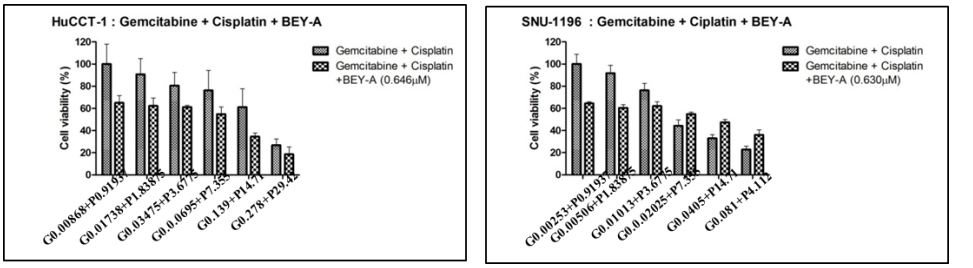

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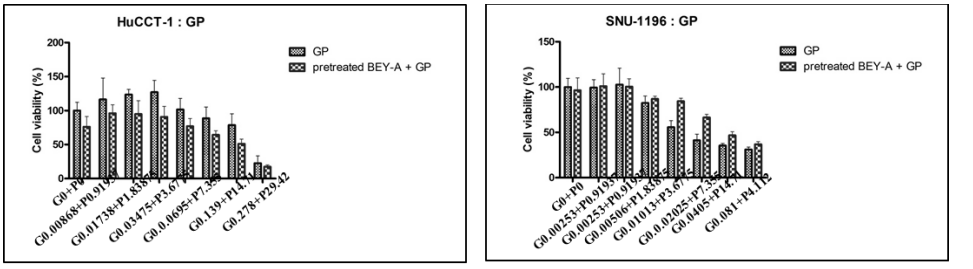

Figure 7. Gemcitabine and cisplatin inhibits the proliferation of Biliary Tract cancer cells. (A) Cell viability curve based on the gemcitabine and cisplatin concentration in two Biliary Tract cancer cells lines. (B,C) The results from the cell viability indicated that the anti-proliferative effect of BEY-A with gemcitabine and cisplatin was no synergistic inhibitory.

6. Combination with radiation is synergy effect

To examine the combination effect with radiation, two biliary tract cancer cells were treated with BEY-A for 12 or 24 h, respectively, and irradiated at 5 radiation doses (0, 2, 4, 6, 8 Gy).

HuCCT-1 was treated with 0.380μM of BEY-A for 12 h after seeded $1x10^6$ into 100 mm culture dish. Cells were treated with DMSO as a control. After BEY-A treatment, cells were trypsinized and counted to equivalent cell number and irradiated for indicated doses 0, 2, 4, 6 and 8 Gy (Table 1). Then, $1x10^3$cells were plated per well in 6-well plates for clonogenic assay. Ten days after the radiation, the cells were fixed in 75% methanol and 25% acetic acid (vol/vol), stained with crystal violet and a colony counting was carried out. In the HuCCT-1 cells, BEY-A significantly sensitized cells to radiation that survival and proliferation were significantly decreased by BEY-A in combination with radiation compare to that of cell irradiated without BEY-A (Figure 8A, B). The effects of BEY-A on radiosensitivity were expressed as the RER (radiation enhancement ratio), defined as (MID of control group)/(MID of treatment group). These results indicate that BEY-A in combination with radiation is effective on biliary tract cancer cells. When HuCCT-1 were exposed to indicated doses 0, 2, 4, 6 and 8 Gy with treated DMSO or BEY-A, each RER were measured to 1.0, 1.14, 1.28, 1.9 and 1.12 respectively. Based on the RER, treatment of HuCCT-1 cells with a dose of 6 Gy resulted in a significant enhancement in radiation-induced cell killing (RER=1.9) (Figure 8C).

Table 1. Design for combination treatment of BEY-A and radiation

Cell lines	Treatment	Time(G2/M arrest)	IR dose (Gy), 10 days
HuCCT-1	DMSO BEY-A (IC_{50})	12 hr	0, 2, 4, 6, 8

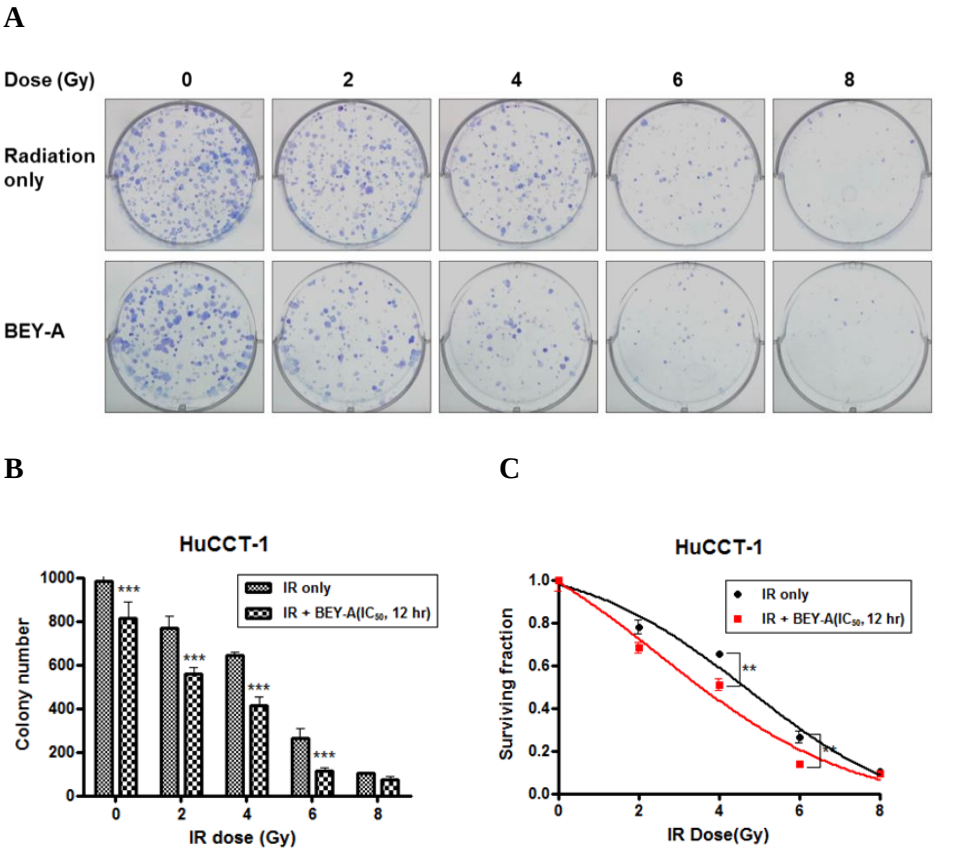

Figure 8. Analysis of clonogenic survival in human biliary tract cancer cell lines exposed to radiation and/or BEY-A *in vitro*. (A) Cells were seeded into 6-well plates at a density of 1,000 cells per well. After attachment, BEY-A (IC_{50}) or the DMSO control was added and the plates were irradiated (0, 2, 4, 6, 8 Gy) after 12 h or 24 h. Ten days after radiation, the cells were fixed and a clonogenic assay was carried out. (B) The bar graph demonstrates that BEY-A with the indicated irradiation doses effects on HuCCT-1 (C) Radiation enhancement was calculated as the ratio of the mean inactivation dose for BEY-A treated cells to DMSO treated cells.

IV. DISCUSSION

The cell-division cycle of eukaryotic cells is conventionally divided into four phases of G1, S, G2 and mitosis (M)[4]. Cyclin-dependent kinases (CDKs) are important cell cycle-regulating proteins, which belong to a serine/threonine kinase family that comprise of a catalytic kinase subunit, together with cyclin protein partners. There are at least 13 different CDKs and more than 25 cyclin proteins dentified to date[5].

Unlike other CDKs, CDK1 can promote the cell cycle alone and is essential for cell cycle progression and cell division[5, 6]. CDK1 regulates the G2 phase as part of a complex with cyclin A and is involved in G2/M transition by forming a complex with cyclin B. Knockdown of CDK1 increases G2/M arrest during the cell cycle and results in polyploid cells[5, 6]. The kinase is completely inactive without its cyclin partner, but in addition to the binding of cyclin, activation of the holoenzyme requires the phosphorylation of a key residue in the activation loop of the kinase subunit [21].

CDK1 kinase plays a crucial role in regulation of cells from G2 phase into M phase[19]. Activation of CDK1 kinase is a highly ordered process. First, binding of cdc2 to cyclin B1 is essential for activation of this kinase. Then, phosphorylations of Thr14, Tyr15 and Thr161 of cdc2, which are mediated by upstream kinases such as WEE1, Myt1 and CDK7, are required for activation of the cdc2-cyclin B1 complexs. Finally, dephosphorylation of Thr14 and Try14 of cdc2 by cell division cycle protein (cdc25c) phosphatase activated the cdc2-cyclin B1 complex and triggers the initiation of mitosis[19, 22, 23]. Hence,

cdc2 is very important in the process of activation of CDK1.

In late G2, CDK1 is activated by cdc25c phosphatase through dephosphorylation upon both Thr14 and Tyr15, as an obligate step for the G2/M transition. To activate CDK1, the phosphorylation of Thr161 by CDK1 activating kinase (CAK) is required. Therefore, phosphorylation upon Thr161 of CDK1 is activated forms. To examine possibility of BEY-1 as a CDK1 inhibitor in biliary tract cancer, we investigated expression of CDK1 in biliary cancer patients tissues using IHC and our data shown expression of CDK1 is in both normal and cancer tissues in cytoplasm and nucleus but the active form of CDK1 (p-cdc2 Thr161) was only detected in nucleus of biliary cancer patients tissues.

A number of CDK inhibitors have been developed to date and their effects have also been studied. Inhibition of CDK1 and Cdk2 by 6,7,4′-trihydroxyisoflavone inhibited growth of HCT-116, a human colon cancer cell line[14], and JNJ-7706621, an inhibitor of CDK1/cyclinB1 and aurora kinase effectively reduced growth of transplantable liver tumor in combination treatment with paclitaxel[15]. Knockdown of cdc2 in glioblastoma U87 and U251 raised cell sensitivity to anticancer drugs such as temozolomide, resulting in reduction of cell proliferation and promotion of apoptosis [16].

BEY-A is recently developed CDK1 inhibitor to G2/M arrest. Mitochondrial apoptotic pathway has been described as an important signaling of cell death for mammalian cells and activated caspase 3 is the key roles of apoptosis[24,25]. In the present study, we first examined that BEY-A induced apoptosis through the mitochondrial-mediated apoptotic pathway in two biliary tract cancer cell

lines. Western blot analysis demonstrates that cleaved (active) forms of caspase-3 in two cell lines with treated BEY-A were activated in a time-dependent manner. Simultaneously, the cleavage of poly-ADP ribose polymerase (PARP) protein, one intracellular substrate of activated caspase-3, gradually increased in two cell lines with treated BEY-A, confirming that caspase-3 was activated by BEY-A treatment. Moreover, we first investigated the effects of BEY-A which inhibits the dephosphorylations of cdc2 at both Thr14 and Tyr15. The inhibitory effect of dephosphorylations of cdc2 at Thr14 and Tyr15 were associated with the decrease in cdc25c protein. Nevertheless there were some reports that various CDK1 inhibitors induce cell cycle arrest in some phases, this study is the first report that reveal inhibitory effect on two biliary tract cancer cells.

Based on clinical data suggesting that cisplatin combined with gemcitabine produced a survival benefit in patients with biliary tract cancer[26], we designed a study combining BEY-A with gemcitabine and cisplatin. We expected BEY-A have synergistic effects with other chemo-drugs in two biliary tract cancer cell lines but the our MTT assay data indicates that BEY-A is no effective with other chemo-drugs using the methods both simultaneous treatment and additional treatment.

A number of studies have been conducted in an effort to improve chemo-radiotherapy by adding other chemotherapeutic agents[27]. In the present study, we also expected BEY-A can improve in combination with radiation treatment and result of clonogenic assay shown the survival and proliferation were significantly decreased by BEY-A in combination with radiation more than

caused by the radiation alone. BEY-A was treated for 12 or 24 h before irradiation because arrested cell population at G2/M phase is the most at 12 h in HuCCT-1 and at 24 h in SNU-1196 which were determined by resulting of FACS data. The difference of synergistic effects with combination between other chemo-drugs and radiation is considered that BEY-A can increase radiosensitivity to arrested cells at G2/M phase.

Based on our *in vitro* studies, we believe that the new schedule needs for *in vivo* study available to most combination effect and also synergistic effective in mouse injected biliary tract cancer cell lines.

V. CONCLUSION

In conclusion, we obseved active CDK1 form (p-Thr161) expression in biliary tract cancer patients. CDK1 p-Thr161 expression was observed in cytoplasm and nucleus in both non-cancer and cancer cells but majority of cancer cells express p-Thr161 in the nucleus whereas the expression of p-Thr161 was mostly expressed in cytoplasm in non-cancer cells. In this result, we indicated possibility that CDK1 can be targeted gene of BEY-A in biliary tract cancer. We also observed that BEY-A inhibited cell growth and induced apoptosis. It appears that BEY-A dose-dependently decreased cell viability in biliary tract cancer cells. Increased level of cleaved caspase-3 and cleaved PARP indicated BEY-A induced apoptosis The treatment of BEY-A also arrested and accumulated more than 40% of cells in G2/M phase, maintained up to 12 h in HuCCT-1, 24 h in SNU-1196. Therefore, BEY-A caused G2/M arrest of the cell cycle in two different biliary tract cancer cell lines. Based on the previous experiment cell cycle analysis, we examined the effect of BEY-A on the level of CDK1-related proteins using Western blot analyses. In the two cell lines, expression levels in various proteins were changed by BEY-A with time-dependent and also dose-dependent. Effects of combination with gemcitabine and cisplatin were no synergistic but treatment of BEY-A with radiation had effective more than only with treated radiation alone on cells.

Many CDK1 inhibitors have been developed that could inhibit progress of the cell cycle in various cancers and induce apoptosis. Here, we first report BEY-A induces G2/M arrest in cell cycle and apoptosis in biliary tract cancer cells. This drugs is more useful through combination treatment with radiation

therapy. Moreover, the CDK1 inhibitor BEY-A plays a role of cancer growth in biliary tract cancer and effective with radiation treatment.

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ABSTRACT(IN KOREAN)

BEY-A의 담도암에서 CDK1 저해제로서의 효능 연구

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김찬양

담도암(biliary tract cancer)은 전체 종양의 1-2%를 차지하는 비교적 드문 종양이지만, 국내의 경우 서구에 비하여 발생이 비교적 높아 전체 종양 중 약 4%를 차지한다. 담도암은 담도 상피세포에서 기원하는 종양으로 해부학적인 위치에 따라 간내담관암, 간외담관암으로 나뉘며, 근치적 절제술만이 완치를 기대할 수 유일한 치료법이다. 그러나 근치적 수술이 가능한 환자도 50-80%에서 국소 재발과 원격전이가 흔하기 때문에 예후는 매우 불량하다.

세포주기는 G1-S-G2-M phase의 4단계로 나눌 수 있는데, 세포주기의 진행을 책임지는 것은 cyclin과 CDK의 복합체이다. 각 phase에 따라 특정 복합체들이 순차적으로 활성화되면서 세포주기가 진행된다. Cyclin-dependent kinases (CDKs) 은 serine/threonine kinase family 에 속하는 주요 세포 주기 조절 단백질로 알려져있다. CDK1은 cyclin A와 복합체를 이루어 G2 phase 를 조절하고, cyclin B 와 복합체를 형성하여 G2/M 전이를 진행하는 역할을 하기때문에 결과적으로 CDK1의 발현을 감소시키면 G2/M arrest가 증가된다. 현재까지 CDK1은 다양한 암종에서 증가된 표현과 활성을 보이는 것으로 보고되었고, 이러한 암 유발과의 밀접한 관련성으로 CDK1은 암 유발과 세포 사멸을 유도하여 세포 주기 진행을 억제하는

target therapy의 표적 유전자로서 소개되고 있다.

BEY-A는 Beyond-bio사에서 CDK1 저해제로써 G2/M 단계를 정체하는 약물로 개발되었다. 그러나 아직까지 BEY-A의 담도암에서의 효능이 아직 밝혀지지 않았기 때문에 본 연구에서는 BEY-A가 담도암 세포주에서 어떤 역할을 하는지에 대해 조사하였다. 먼저 담도암 환자 조직에서 CDK1의 활성화 형태인 CDK1 p-Thr161의 핵 안에서의 발현을 확인하고, 담도암에 BEY-A를 처리하였을 때 세포사멸도와 세포주기 분석을 수행하였다. 이후 기존 담도암 항암약제와 병용사용 효과를 확인하고 마지막으로 BEY-A와 방사선을 함께 처리했을때의 효과를 조사하였다. 그 결과, 담도암 환자에서 정상조직과 암조직 모두에서 CDK1 p-Thr161가 발현하지만 상대적으로 암조직의 핵에서 CDK1 p-Thr161의 발현이 증가되어 있는 것을 확인하였다. BEY-A가 처리된 담도암 세포주에서 세포 생장이 억제되며 세포사멸을 유도하는 것을 확인하였고, 세포주기 분석 결과로 G2/M 기의 억제를 유도하는 것을 관찰하였다. 기존 담도암 항암약제와의 병용사용에서는 효과가 없었으나 방사선과의 병용 사용에서는 병용효과가 있음을 확인하였다. 본 연구를 통해 향후 담도암의 새로운 항암제로서 BEY-A가 사용될 수 있을 것이라 사료된다.

핵심되는 말 : CDK1 저해제, 담도암, 세포주기, 세포사멸