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Synergistic antitumor activity of STI571 and camptothecin in human cancer cells

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The *in vitro* activity of STI571, an inhibitor of the Abl group of protein-tyrosine kinases, alone or in combination with camptothecin (CPT), a specific topoisomerase I inhibitor, was evaluated against human cancer cells with different metastatic capacity and drug resistance potency. These cell lines showed different sensitivity to STI571 on growth inhibition, and the expression of DNA-dependent protein kinase (DNA-PK), which interacts constitutively with c-Abl, was significantly decreased in drug sensitive CEM and MCF-7 cells and poorly metastatic PC3 and KM12 cells as compared with that of multidrug resistant CEM/MDR and MCF-7/MDR cells and highly metastatic PC3-MM2 and KM/L4a cells, respectively. These results suggest differential modulation of DNA-PK by STI571 treatment in drug resistance and metastatic degree dependent manner. We showed that CPT as well as STI571 significantly inhibits the expression of DNA-PK. The combined treatment with STI571 and CPT revealed synergistic effect, and the effect was accompanied by inhibition of cell proliferation due to significant reduced expression of DNA-PK components, which resulted in CPT sensitizes human cancer cells resistant to STI571. Therefore, the results of our study suggested that the suppression of DNA-PK using combination of STI571 and CPT could be a novel molecular target for against drug resistant and metastatic cancer cells.

Key words – STI571, DNA-PK, metastatic cells, camptothecin, synergistic effect

Introduction

Despite the improved strategies of cancer therapy, the metastasis and development of multidrug resistance of cancer cells are the major obstacle for successful cancer treatment, since recurrent or metastatic cancers occurred after initial treatment with radiotherapy and chemotherapy are generally refractory to second treatments with these anticancer therapies [1]. Therefore, it is necessary to develop effective therapeutic modalities against metastatic and/or multidrug-resistant cancers.

In the previous study it has been shown that an enhanced expression of DNA-dependent protein kinase (DNA-PK), which composed of 460-kDa catalytic subunit (DNA-PKcs) and a heterodimer of Ku70 and Ku80 regulatory subunits and plays role in repairing double strand breaks, participates in the development of multidrug resistance (MDR) that protects cancer cells from a variety of drugs with different structure and function [2,3], and radioresistance [4]. In addition, various metastatic cancer cells showed consistently higher levels of antiapoptotic proteins including DNA-PK, Bcl-2, NF-κB, MDM2 and epidermal growth factor receptor (EGFR) than low metastatic parental cells [3]. The inhibition of DNA-PK with wortmannin potentiated the effectiveness of anticancer drugs and ionizing radiation and thus could partially overcome therapy resistance in tumor cells [2].

Recently, it has been demonstrated that expression of DNA-PK catalytic subunit was suppressed by STI571 (Imatinib mesylate) not by anti-bcr/abl siRNA in K562 chronic myelogenous leukemic cells [5]. STI571 is a small molecule drug that in vitro inhibits the Abelson (Abl), Arg (abl-related gene), stem cell factor receptor (Kit), and platelet-derived growth factor receptor A and B tyrosine kinases and consequently is being studied for treatment of solid tumors such as gastrointestinal tumors and prostate cancer as well as various hematologic malignancies [6-8].

Therefore, in the present study, it was examined if STI571 can be used as an enhancer of cytotoxicity of camptothecin through down-regulation of DNA-PK.

Materials and Methods

Reagents

The following reagents were obtained from the listed sources and used at the concentrations indicated in the text. STI571 were kindly donated by Dr. I.J. Fidler (UT M.

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D. Anderson Cancer Center, Huston, USA). Camptothecin (CPT) was provided by Dr. Lee. N. J (Kosin University, Pusan, South Korea). The following antibodies were used in this study. The purified antibodies specific to the 70- and 86-kDa Ku protein, Rad51, p53, Bcl-2, NF-κB and β-tubulin were from Santa Cruz Biotechnology (USA) and the anti-DNA-PKcs and c-Abl antibodies were from NeoMarkers (USA). Secondary antibodies were obtained from Amersham. Pharmacia Biotech. The following antibodies were used in these studies.

Cell Lines and culture conditions

The poorly metastatic KM12 cell line was established from a primary colorectal carcinoma classified as Dukes B2. The highly metastatic cell line, KM12L4a (KM/L4a), was derived from a liver metastasis after injection of KM12 into the spleen of athymic mice and repletion of this procedure three times. Human androgen-independent PC3P prostate adenocarcinoma cells and PC3-MM2 cells, variants of PC3 selected for their highly metastatic potential were provided by Dr. I.J. Fidler. Human breast carcinoma MCF-7 (provided by Dr. I. J. Fidler) and the multidrug-resistant (MDR) variants, MCF-7/MDR cells were isolated from MCF-7. These cell lines were maintained in culture (5% CO_2 and 95% air at 37°C) as adherent monolayers in DMEM supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, and vitamin solution. CCRF-CEM (CEM) cells, the multidrug-resistant CEM/MDR which isolated from CEM and chronic myelogenous leukemia K562 cells were maintained in RPMI supplemented with 10% fetal bovine serum.

Drug sensitivity assay

In case of drug sensitivity, exponentially growing cells ($5x10^3$ cells/well) were plated in 96 well and incubated in growth medium at 37°C for 96 hr the culture medium containing STI571 and CPT at various concentrations. It followed by the addition of 100 μl of MTT [3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] solution (5 mg/ml), and incubation for 4 hr in the dark room. The water-insoluble MTT-formazan crystals were dissolved in dimethyl sulfoxide, and reduction of MTT was determined at 570 nm using ELISA reader (Bio-Tec Instruments). The concentration of each anticancer drug which reduced cell growth by 50% after 96 hr treatment (IC_{50}) was determined from the growth inhibition plots.

Cell extract preparation and electrophoretic mobility shift assay

Cells (2×10^6 cells/ml) were treated with exposed to graded single dose and in the presence or absence of a STI571 for indicated times, and nuclear extracts of the cells were prepared from the cells. In brief, the cells were washed with cold phosphate buffered saline and harvested quickly and resuspended in 300 μl of lysis buffer [10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulphonyl fluoride (PMSF)]. The cells allowed swelling in ice for 10 min. After 0.05% Nonidet P40 was added, the tube was vigorously mixed 3 times for 3 sec on a vortex, and centrifuged at 250 × g for 10 min to pellet the nuclei. The nuclear pellet was resuspened in 30 μl of ice-cold nuclear extraction buffer (5 mM HEPES, pH 7.9, and 26% glycerol (v/v), 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF). Then this pellet incubated on ice for 30 min with intermittent mixing, and centrifuged at 24,000 × g for 20 min at 4°C. The nuclear extract was either used immediately or stored at -70°C for later use. Ten μg of nuclear extract was incubated with ^{32}P-labeled double-stranded oligonucleotide, 5'-AGTTGAGGGGACTTTCCCAGGC-3' for Ku binding (Santa Cruz Biotechnology, Inc) in binding buffer (10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 4% glycerol) containing 50 μg/ml of poly (dI-dC). The DNA-protein complex was separated from free oligonucleotide on 4% nondenaturing polyacrylamide gel using 0.5×TBE buffer (44.4 mM Tris-HCl, pH 8.0, 44.5 mM boric acid, 1 mM EDTA) for 3 hr at 120 V. The gels were dried and autoradiographed.

Western blot analysis

Protein samples (80 μg of whole cell extracts or nuclear extracts) were separated by SDS-PAGE and blotted to nitrocellulose membrane. Immunoblots were blocked in Tris-buffered saline-Tween (TBS-T, 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20) containing 5% nonfat dry milk for 2 hr at room temperature. The membrane was incubated with antibody as specified, followed by secondary antibody conjugated with horseradish peroxidase. Membranes were washed 3 times with TBS-T. Specific antigen-antibody complexes were detected by enhanced chemiluminescence (Pierce).

DNA-PK kinase assay

The kinase activity of DNA-PK was determined using

the Signa TECT™ DNA-dependent Protein Kinase Assay System from Promega. DNA-PK was assayed by measuring phosphorylation of a p53 peptide substrate (EPPLSQE AFADLWKKR). In brief, 15 μg of nuclear extract was incubated with activator DNA, a biotinylated p53-derived peptide substrate, and [γ-^{32}P] ATP for 5 min at 30°C. The sample was terminated by adding termination buffer. Each termination reaction sample was spotted onto SAM^{2TM} Biotin Capture Membrane and then washed with 2 M NaCl and 2 M NaCl in 1% H_3PO_4. The SAM2™ membrane squares were analyzed using Molecular Imager System (Bio-Rad, Model GS 525).

Results

Modulation of DNA-PK by STI571 treatment in various cancer cells

Recently, it has been reported that DNA repair protein, Rad51 could be regulated by STI571 [9,10]. To additionally assess the impact of STI571 on DNA repair protein, K562 cells were treated with STI571 and the expression and activity of DNA-PK were compared with those of untreated control cells. As shown in Fig. 1, the expression levels of DNA-PKcs and Ku70/80, as well as both Ku-DNA binding and DNA-PK kinase activities were down-regulated by STI571 in K562 cells, along with decreased expression level of Bcr/Abl. These data indicated the possibility that DNA-PK could be regulated by STI571.

Since the enhanced DNA-PK expression could contribute to metastatic potential [3], the sensitivity of STI571 in various cancer cells with different metastatic capacity and tissue origin and their parental cells were compared. The highly metastatic KML4a and PCMM2 cells and their poorly metastatic parental KM12 and PC3 cells, respectively were treated with various concentration of STI571 for 96 hr, and the growth inhibition was determined by MTT assay. KML4a and PCMM2 cells were 2.0- and 3.3-fold resistant to STI571 than each parental KM12 and PC3 cells respectively, suggesting metastatic capacity could be related with sensitivity of STI571 in cancer cells. Since DNA-PK expression was decreased by STI571 in K562 cells, to examine whether a similar effect occurs in cells originated from solid tumors, and to determine whether expression of DNA-PK could be modulated in highly metastatic cancer cells, which resistant to STI571 compared to parental cancer cells, the effect of STI571 on DNA-PK expression in

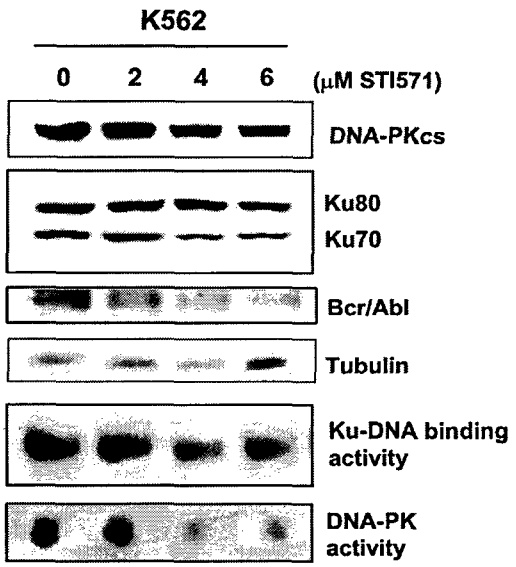

Fig. 1. Modulation of DNA-PK activity by STI571 in K562 cells. The expression of DNA-PKcs, Ku and Bcr/Abl in whole cell extracts of the K562 cells treated with indicated doses of STI571 for 9 hr was determined by Western blot analysis. Data were normalized to β -tubulin (tubulin) in order to show relative expression and loading differences. Ku-DNA binding and DNA-PK kinase activities of the cells were analyzed by EMSA and DNA-PK Kinase Assay System, respectively.

KM12 and KM/L4a cells were analyzed by Western blotting and EMSA (Fig. 2). After treatment of indicated dose of STI571, DNA-PKcs level and Ku-DNA binding activity were down-regulated in KM12 cells in a dose dependent manner, whereas the expression of DNA-PKcs and Ku-DNA binding activity were not decreased in KM/L4a cells by STI571. To examine if the down-regulated Ku activity and DNA-PKcs expression in KM12 cells could lead to decrease the kinase activity of whole DNA-PK complex, DNA-PK activity was analyzed in KM12 and KM/L4a cells treated with STI571. As expected, the DNA-PK activity was decreased in KM12 cells while it was not decreased in KM/L4a cells. The reduction of DNA-PK activity at a high concentration (9 μM) of STI571 might be due to the cytotoxic effect of the drug. To confirm these results, other metastatic PCMM2 and parental PC3 cells were treated with indicated dose of STI571. As shown in Fig. 3, DNA-PKcs and Ku expression and Ku-DNA binding activity decreased in PC3 cells but increased in PCMM2 cells. These results suggest that STI571-resistance would be associated with DNA-PK activation in metastatic cancer cells, and thus DNA-PK could be possible molecular targets for therapy against STI571-resistant cancer cells.

Inhibition of DNA-PK expression by the treatment of camptothecin

Recent study reported that nonhomologous end-joining (NHEJ), which depends on the DNA-PK, could be a cytotoxic pathway in the presence of camptothecin (CPT) [11]. Thus, it was examined whether CPT could inhibit DNA-PK expression in KM12 and KM/L4a cells. As shown in Fig. 3, the expression of Ku protein and DNA-PKcs and activity of Ku-DNA binding were significantly reduced in KM/L4a cells, but slightly reduced in KM12 cells. As a result, the KM/L4a cells appeared to be susceptible to CPT-induced suppression of DNA-PK activity in KM/L4a,while DNA-PK activity of KM12 cells was not modulated by CPT.

These results providethe possibility that CPT is helpful to be sensitivity to STI571 in highly metastatic cancer cells by modulation of DNA-PK activity, and thus could overcome resistant to STI571.

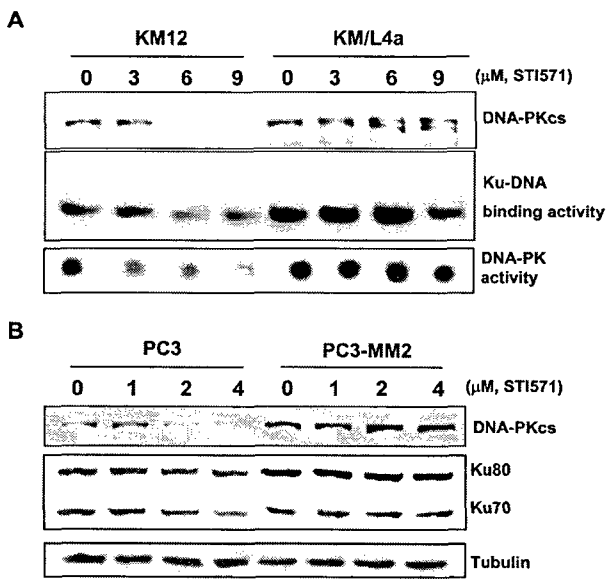

Fig. 2. Differential effect of STI571 on DNA-PK activity of between highly metastatic and poorly metastatic parental cells. (A) The alteration of DNA-PKcs expression, Ku-DNA binding and DNA-PK kinase activities of highly metastatic KM/L4a and their parental KM12 cells treated with indicated doses of STI571 for 6 hr were assayed by Western blot analysis, EMSA and DNA-PK Kinase Assay System, respectively. (B) The level of DNA-PKcs and Ku70/80 in whole cell extracts of the PC3 and its highly metastatic PCMM2 cells treated with indicated doses of STI571 for 6 hr was determined by Western blot analysis. Data were normalized to tubulin in order to show relative expression and loading differences.

Chemosensitization of STI571 by treatment of CPT in metastatic cancer cells

Since increased expression of DNA-PK appears to be involved in development of STI571-resistance, it was investigated if CPT also could affect the STI571-modulated DNA-PK activity in metastatic cancers. KM12 and KM/L4a cells were treated with STI571 (3 μM) in the presence or absence of CPT (0.1 μg/ml) for 9 hr (Fig. 4). The combination effect of CPT and STI571 on the expressions of Ku and DNA-PKcs in KM/L4a cells was significantly higher than that of KM12 cells, suggesting the efficiency of combined treatment of CPT with STI571 to metastatic cancer cells.

Therefore, to investigate whether synergistic suppression

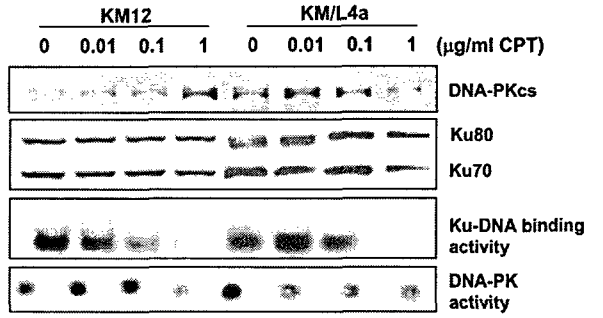

Fig. 3. Effect of camptothecin (CPT) on DNA-PK activity in metastatic cancer cells. The expression of DNA-PKcs and Ku70/80 of KM12 and its highly metastatic KML4a cells treated with indicated doses of CPT for 6 hr was determined by Western blot analysis. Also, the nuclear extracts were analyzed for DNA binding activity by EMSA using the Ku consensus oligonucleotide and determined DNA-PK kinase activity.

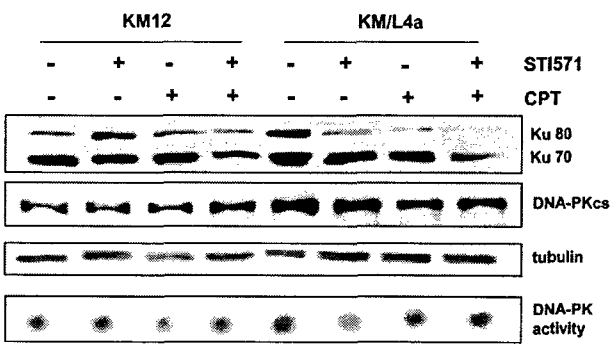

Fig. 4. Synergistic effect of STI571 and CPT on DNA-PK activity. The KM12 and its highly metastatic KM/L4a cells treated with 3 μM STI571 and 0.01 μg/ml CPT or treated with STI571 or CPT alone for 9 hr, respectively. The level of DNA-PKcs and Ku70/80 was determined by Western blot analysis. Data were normalized to β-tubulin in order to show relative expression and loading differences.

of DNA-PK by treatment of CPT leadsto enhance cytotoxicity of STI571, KM12 and KM/L4a cells were treated with various concentration of STI571 and 0.01 ng/ml CPT. As shown in Table 1, treatment of CPT sensitized KM/L4a cells 13.6-fold to STI571, while co-treatment of STI571 and CPT for KM12 cells resulted in only 2.8-fold increase in cytotoxicity of the STI571. This data indicated that the combination effect was more effective in KM/L4a cells that have a higher DNA-PK activity than parental cells. Consistently, PCMM2 cells showed 17.8-fold enhanced cytotoxicity of STI571 by CPT, whereas PC3P cells exhibited 4.0-fold combination effect under the same condition, suggesting that CPT enhanced the cytotoxicity of STI571, thus could reverse resistance to STI571 in metastatic cancer by suppression of DNA-PK activity.

As expected, the cytotoxicity of CPT was significantly enhanced by STI571treatment in metastatic cancer cells. The highly metastatic PCMM2 and KM/L4a cells as well as its parental PC3 and KM12 cells were significantly sensitized to CPT by co-treatment of STI571 (Fig. 5). These results suggest STI571 could be as an important modulator of CPT cytotoxicity against metastatic cancer cells.

Chemosensitization of STI571 by CPT in MDR cells

It has been reported that K562/MDR cells displayed resistance of STI571 [12,13]. To determine whether other MDR variants CEM/MDR and MCF/MDR cells exhibit resistance of STI571, theses MDR cells and their parental CEM and MCF-7 cells were treated with STI571, CEM/MDR and MCF/MDR cells were 1.6- and 1.8-fold resistant to STI571 than their parental CEM and MCF-7 cells.

After treatment of STI571, the levels of DNA-PKcs and

Table 1. Potentiation of STI571-sensitivity by CPT in metastatic cancer cells.

	IC_{50} (μM) of STI571	
Cells	- CPT	+ 0.01 ng/ml CPT
KM12	2.8±0.1	0.5±0.02 (5.6)
KML4a	6.8±0.4	0.5±0.01 (13.6)
PC3	1.6±0.1	0.4±0.03 (4.0)
PCMM2	5.35±0.3	0.3±0.02 (17.8)

Each cell line (1×10^4 cell/ml) was treated with STI571 for 96 hr in the presence or absence of CPT. Growth inhibition assay was performed by MTT method. Values in parenthese indicate the ratio of IC_{50} for STI571 alone to the IC_{50} for STI571 in the presence of CPT. Values are the average of (at least) two independent experiments and triplicate determinants in each experiment.

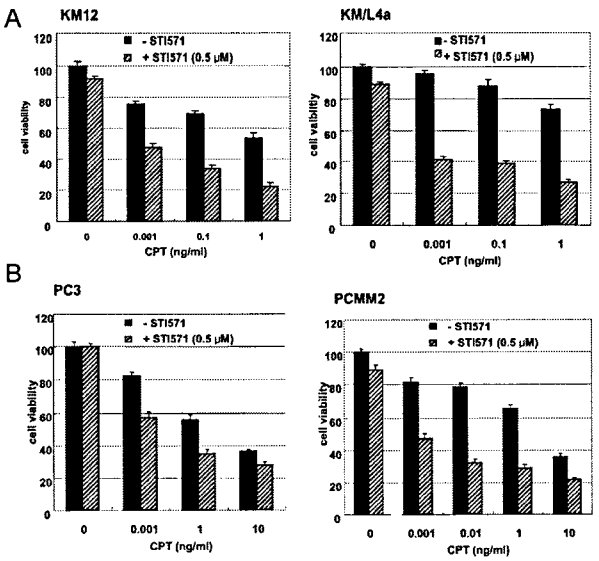

Fig. 5. Synergistic effect of STI571 on CPT cytotoxicity in metastatic cells KM12 and KM/ L4a cells (A) or PC3 and PCMM2 cells (B) were treated with indicated doses of CPT and 0.5 μM STI571. The cell viability relative to untreated control cells was determined after 4 days incubation using the MTT assay.

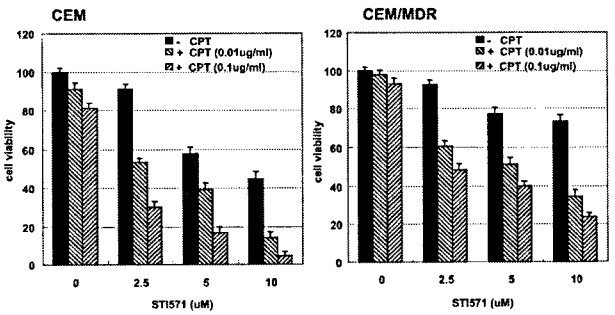

Fig. 6. Synergistic effect of CPT on STI571 cytotoxicity in MDR cells. CEM and its MDR CEM/MDR cells were treated with indicated doses of STI571 and CPT (0.01- or 0.1, ng/ml). The cell viability relative to untreated control cells was determined after 4 days incubation using the MTT assay.

Ku70/80 were increased in CEM/MDR cells, whereas the levels of DNA-PKcs and Ku70/80 were decreased in CEM cells (data not shown). This phenomenon was very similar to STI571 sensitivity of highly metastatic KM/L4a or PCMM2 cells. Therefore, to examine whether resistance to STI571 could be reversed by CPT in MDR cells, CEM and CEM/MDR cells were treated with various concentrations of STI571 and CPT (Fig. 6). The CEM cells were 1.4-fold sensitized to STI571 by co-treatment of CPT (0.01 ng/ml) and 3.2-fold sensitized by co-treatment of CPT (0.1 ng/ml), also CEM/MDR cells were sensitized 2.3- and 5.0-fold to

STI571 under the same concentrations of CPT. These results indicated that resistant to STI571 could be overcome by combined treatment with CPT in MDR variants as well as metastatic cancer cells.

Discussion

The emergence of resistance to STI571 is an obstacle in treatment of cancer and affects the curability of the patients. The reason for STI571 resistance was explained by several factors including up-regulation of Bcr/Abl, mutations in the kinase domain of Bcr/Abl and increased expression of multidrug resistance gene (MDR) [6,14].

In this study, it was demonstrated that resistance to STI571 was associated with DNA-PK in human metastatic cancer cells and multidrug resistant cancer cells. The present study showed that the expression levels of DNA-PKcs and Bcr/Abl were decreased by STI571 in K562 cells. This observation is in agreement with the report that transcripts of DNA-PKcs significantly suppressed in STI571-treated K562 cells [5]. Although the expression of DNA-PK components and activity of Ku-DNA binding were decreased by STI571 in poorly metastatic cancer cells, the highly metastatic cancer cells and MDR variants, which are resistant to STI571, showed constitutively increased DNA-PK activity, suggesting that resistance to STI571 could be modulated by DNA-PK activity.

Because increased efficiency of DNA repair significantly reduce the sensitivity of cell to therapeutic treatment, it is considered as one of the major obstacles of anti-cancer therapy [1,15,16]. Several studies have shown that the kinase activity of DNA-PK is up-regulated following exposure to ionizing radiation (IR) or other genotoxic agents. Recent work has established that inhibition of DNA-PK activity by wortmannin enhanced IR and drug sensitivity [2,17,18].

The Abl tyrosine kinase is also activated by different genotoxic treatments including IR and anticancer agents. It has been reported that DNA-PK phosphorylates and activates c-Abl in response to DNA damage induced by IR [19]. However, it remains to be studied whether resistance to STI571 induced by DNA-PK involves with the Abl tyrosine kinase activation directly or indirectly remains to be studied.

On the other hand, combination of STI571 with several anticancer agents has been reported in K562 cells [20,21]. The synergistic potential of STI571 combined with CPT demonstrated in this study suggests that the combination of STI571 and CPT is a promising regimen for patients to STI571 resistance. CPT, the topoisomerase I inhibitor, is a potent DNA-targeting drug in patients with lung and colorectal cancer [22,23]. It has been reported that CPT strongly inhibits DNA synthesis in $Ku80^{-/-}$ cells than its parental cells. [24]. These results revealed that expression level of DNA-PK components and activity of Ku-DNA binding and DNA-PK kinase activity were decreased by CPT in metastatic cancer.

Since the expression and activation of DNA-PK were the important determining factors for sensitivity of the cells to STI571, modulation of DNA-PK by CPT might be responsible for the synergistic effect between STI571 and CPT. Present results showed that expression of DNA-PK components and activity of Ku-DNA binding were decreased by combination of STI571 and CPT in metastatic cancer and MDR variants, along with an enhancement of STI571 cytotoxicity.

In conclusion, this study suggested that the DNA-PK could contribute to acquirement of resistance of STI571 and combination with CPT and STI571 may be helpful to improve the cytotoxicity effect of STI571 in metastatic cancer and MDR variants via inhibition of DNA-PK activity.

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초록 : Camptothecin 에 의한 STI571 의 항암 활성 증강

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본 연구에서는 전이성 암세포와 항암제 다제내성 세포에 있어서 항암제 내성에 영향을 미치는 것으로 알려진 DNA-dependent protein kinase (DNA-PK) 가 Abl protein-tyrosine kinases저해제인 STI571 내성에도 연관되어 있는지에 대하여 조사하였다. 또한 STI571 과 topoisomerase I 저해제인 camptothecin (CPT) 의 단독 및 병용처리에 의한 항암 활성을 전이성 암세포와 항암제 다제내성 세포를 대상으로 조사하였다. 세포의 전이도와 내성정도에 따라 STI571 의 감수성이 다르게 나타났다. 이와 함께 STI571의 처리후 농도에 따라 전이도가 낮은 KM12, PC3 세포와 항암제 감수성인 CEM, MCF-7 세포에서는 DNA-PK 의 발현이 감소하는 반면, 전이도가 높은 KML4a, PC-MM2 세포와 다제내성 CEM/MDR 및 MCR/MDR 세포에서는 그 발현이 증가되어 있음을 알 수 있었다. 이는 DNA-PK 의 발현이 STI571 의 내성에 관여한다는 것을 시사한다. 이와 같은 결과에 근거하여 DNA-PK 의 발현을 감소시키는 CPT 를 STI571 내성을 나타내는 암세포에 대하여 STI571 과 병용처리 하였다. 그 결과 DNA-PK 의 발현이 감소되고 세포증식이 억제됨으로써 STI571 의 감수성이 CPT 에 의해 증가하는 것을 알 수 있었다. 따라서 본 연구에서는 DNA-PK 가 STI571 의 내성을 극복하는데 있어서 새로운 표적이 될 수 있으며, STI571 의 치료내성 극복에 CPT 와의 병용처리가 유효함을 알 수 있었다.