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Inhibition of DNA-dependent Protein Kinase by Blocking Interaction between Ku Complex and Catalytic Subunit of DNA-dependent Protein Kinase

Chung-Hui Kim[1], Dang Van Cuong[1], Jong-Su Kim[2], Nari Kim[1], Euiyong Kim[1], and Jin Han[1]

[1]Department of Physiology and Biophysics, College of Medicine, Inje University, Busan 614-735; [2]Department of Pharmacology & Toxicology, College of Veterinary Medicine, Gyeongsang National University, Chinju 660−701, Korea

Recent studies indicated that cancer cells become resistant to ionizing radiation (IR) and chemotherapy drugs by enhanced DNA repair of the lesions. Therefore, it is expected to increase the killing of cancer cells and reduce drug resistance by inhibiting DNA repair pathways that tumor cells rely on to escape chemotherapy. There are a number of key human DNA repair pathways which depend on multimeric polypeptide activities. For example, Ku heterodimer regulatory DNA binding subunits (Ku70/Ku80) on binding to double strand DNA breaks (DSBs) are able to interact with 470-kDa DNA-dependent protein kinase catalytic subunit (DNA-PKcs), and are essential for DNA-dependent protein kinase (DNA-PK) activity. It has been known that DNA-PK is an important factor for DNA repair and also is a sensor-transmitting damage signal to downstream targets, leading to cell cycles arrest. Our ultimate goal is to develop a treatment of breast tumors by targeting proteins involved in damage-signaling pathway and/or DNA repair. This would greatly facilitate tumor cell cytotoxic activity and programmed cell death through DNA damaging drug treatment. Therefore, we designed a domain of Ku80 mutants that binds to Ku70 but not DNA end binding activity and used the peptide in co-therapy strategy to see whether the targeted inhibition of DNA-PK activity sensitized breast cancer cells to irradiation or chemotherapy drug. We observed that the synthesized peptide (HNI-38) prevented DNA-PKcs from binding to Ku70/Ku80, thus resulting in inactivation of DNA-PK activity. Consequently, the peptide treated cells exhibited poor to no DNA repair, and became highly sensitive to IR or chemotherapy drugs, and the growth of breast cancer cells was inhibited. Additionally, the results obtained in the present study also support the physiological role of resistance of cancer cells to IR or chemotherapy.

Key Words: DNA-dependent protein kinase (DNA-PK), Ku70/Ku80 complex, Ionizing radiation, Breast cancer cells, DNA repair

INTRODUCTION

DNA is constantly damaged by radiation and chemotherapy drugs, which must be repaired to prevent genomic alterations. Unless repaired, damaged DNA contributes to cancer progression and/or generation of cancer. For this reason, cells maintain various mechanisms to repair DNA, such as double-strand break (DSB) repair or nucleotide excision repair (NER) pathways. Ionizing radiation (IR) and chemicals activate DNA-dependent protein kinase (DNA-PK), which has homologous kinase domain. DNA-PK is a nuclear serine/threonine protein kinase composed of DNA binding and catalytic subunits. The DNA binding subunit is heterodimer of Ku70 and Ku80, that binds DNA ends and nicks, and has structures containing a transition fork between dsDNA and two single strand. The catalytic subunit of DNA-PK (DNA-PKcs) is a 470-kDa polypeptide, and is sufficient for the kinase activity of the enzyme (Jin et al, 1997; Lieber et al, 1997). DNA-PK is also a key component of the non-homologous end joining (NHEJ) pathway and V(D)J recombination (Blunt et al, 1995) with the unique property of being activated by DNA ends (Featherstone & Jackson, 1999). It has been suggested to be involved in sensing and transmitting DNA damage signals to the downstream targets (Lee & Kim, 2002). A number of evidence have indicated that it is essential for activation of p53 through ATM kinase phosphorylation (Woo et al, 1998), nucleotide excision repair (Muller et al, 1998) and damage-induced S-phase arrest (Park et al, 1999) in response to DNA damage. These reactions contribute to protection of cells form genetic alterations as well as chemotherapy drug resistance. DNA-PK mutant cells are sensitive to IR (ionizing irradiation) and chemotherapy drugs, and deficient in correct and efficiency repair of DNA

Corresponding to: Jin Han, Department of Physiology and Biophysics, College of Medicine, Inje University, 633-165 Gaegeum-Dong, Busanjin-Gu, Busan 614-735, Korea. (Tel) +82-51-890-6714, (Fax) +82-51-894-4500, (E-mail) phyhanj@ijnc.inje.ac.kr

ABBREVIATIONS: BER, base excision repair; DNA-PK, DNA-dependent protein kinase; DNA-PKcs, catalytic subunit of DNA-dependent protein kinase; DSB, double-stranded DNA break; NER, nucleotide excision repair; NHEJ, non-homologous end-joining.

damage, suggesting a positive role of DNA-PKcs in DNA repair (Frit et al, 1999). Previous studies also used cells with drug-sensitive phenotype (Shen et al, 1998), showing their link to cell death via accumulation of damaged DNA.

Studies also showed that, without DNA, DNA-PKcs is inactive and incapable of binding Ku (Suwa et al, 1994; Hartley et al, 1995). It is, however, unclear how the Ku/DNA complex activates the kinase activity of DNA-PKcs. One hypothesis is that DNA-PKcs undergoes a conformational change upon association with the Ku/DNA complex, and this conformational change accounts for the activation of kinase activity. The kinase activity associated with DNA-PK is needed for DNA repair *in vivo*, since expression of a kinase-inactive form of DNA-PKcs failed to complement the radiosensitive phenotype of a mammalian cell line, lacking the DNA-PK *in vivo*. The DNA-PK complex can physically tether two ends of a DSB in close proximity *in vitro*, suggesting that the DNA-PK complex acts as a scaffold to assemble the NHEJ pathway proteins at a DSB (Cary et al, 1997).

From the above considerations, DNA-PK is highly likely to play an important role of resistance of cancer cells to ionizing radiation or anticancer DNA damaging drugs. Furthermore, viewing from a different standpoint, selective inhibition of DNA-PK may reduce drug-resistance of cancer cells, thereby facilitating cell killing. Using peptides that can directly interfere with DNA-PK activity, one might be able to develop a novel co-therapy that can selectively target and disrupt IR-induced DSBs repair pathway: It will enhance the efficacy of currently available treatments and also broaden the usefulness of chemotherapeutic agents in cancer treatment. Therefore, in the present study, we tested whether a synthesized peptide (HNI38), which mimicked the domain of Ku80 essential for interaction with DNA-PKcs, could selectively target and disrupt DNA-PK activity necessary for DSBs repair, and also whether DNA-repair inhibition could potentiate the effect of chemotherapy drug in cancer treatment.

METHODS

Cell lines and chemicals

Two human breast cancer cells, MDA435 and SKBR3, were obtained from Dr. Lee (Indiana University Cancer Center, Indianapolis, IN) and maintained in MEM supplemented with 10 % fetal bovine serum at 37°C in a CO_2 incubator. [γ-^{32}P]ATP (4500 Ci/mmol) was from ICN, and dsDNA cellulose and cisplatin were obtained from Sigma Chemical Co (St. Louis, MO).

DNA-PK kinase assay

Reaction mixtures (20 μl) contained 20 mM HEPES-KOH (pH7.5), 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 10 mM $MgCl_2$, 7 mM $MnCl_2$, 5 mM NaF, 1 mM $NaVO_4$, 50 μl of ^{32}P-ATP, 150 μl of substrate peptide, 0.4 μg of DNase I-activated calf-thymus DNA (Sigma Chemical Co., St. Louis, MO), and 100 ng of partially purified DNA-PK complex. DNA-PK complex was obtained from Dr. Lee (Indiana University Cancer Center, Indianapolis, IN). Substrate peptide (EPPLSQEAFDLWKK), representing amino acid residues of # 11-24 of p53, was used for DNA-PK assay (Lees-Miller et al, 1990). To find out whether the peptide interfered with DNA-PK kinase activity, various amounts of the inhibitor peptide were added to the reaction mixture. After incubation at 30°C for 30 min, the reaction was stopped with 30% acetic acid, and a portion of the reaction mixtures (5 μl) was spotted onto a P81-strip. After extensive washing, radioactivity was measured. DNA-PK activity was expressed as pmol of ^{32}P transferred to the substrate peptide.

Cell survival assay

Cells (1.0×10^4 cells/well) were seeded in a 96 well plate in the presence of control or target peptide and incubated for 24 hrs prior to the treatment of cells with either ionizing radiation or cisplatin. After further incubation for 72 hrs at 37°C in 5% CO_2, cell survival was measured using a colorimetric cell survival assay kit purchased from Boehringer Mannheim (MTT Cell Proliferation Kit). Alternatively, clonogenic assay was used to measure the ability of cells to form colonies on 100 mm^2 tissue culture dishes, following treatment with ionizing radiation or cisplatin. Controls consisted of cells untreated with peptides, DNA damaging agent, or neither with. Cells were continuously exposed to the indicated concentrations of the peptide for 5 days, and colonies were then stained with crystal violet and colonies with more than 50 cells were counted. Each point represents mean value$\pm$SE, each conducted with triplicate plates.

Double-stranded DNA breaks (DSBs) repair assay

Kinetics of rejoining of radiation-induced damaged DNA in breast cancer cells following exposure of cells to 40 Gy gamma irradiation (^{137}Cs) were measured by pulsed field gel electrophoresis. Thus, breast cancer cells were grown in the presenceof 2.5 μM [^{14}C]-thymidine (0.1 μCi/ml) (DiBiase et al, 2000) and then treated with control peptide or target peptide. Following irradiation (40 Gy), the cells were further incubated in pre-warmed (42°C) fresh medium at 37°C to allow DSB repair to take place and then harvested at various times and re-suspended in serum-free medium at a concentration of $2\sim5\times10^6$ cells/ml. Cells were mixed with an equal volume of 2% agarose, and the solidified cell-agarose suspension was lysed with 10 mM Tris (pH 8.0) buffer containing 50 mM NaCl, 0.5 M EDTA, 2% N-lauryl sarcosyl, and proteinase E & O (0.1 mg/ml) for 16~18 hrat 50°C (DiBiase et al, 2000). DNA double-strand breaks were analyzed by asymmetric field inversion gel electrophoresis (AFIGE) using 0.5% agarose gel in 0.5$\times$ TBE at 10°C for 36 hr. After electrophoresis, gels were analyzed by fluorography. For quantification of damaged DNA repair, intact chromosome and damaged DNA were separately removed from gel and measured for [^{14}C] using liquid scintillation counter.

Statistical analysis

Data were statistically analyzed using either the Student's unpaired t test when two treatment groups were compared, or one-way analysis of variance (ANOVA) followed by a post hoc Student-Newman-Keuls test when all pairwise comparisons among the different treatment groups were made. Tests were considered significant when $P<0.05$.

RESULTS

Targeted inhibition of DNA-PK

Heterodimeric complex (Ku70 and Ku80) is important for DNA-termini binding, in which the extreme C-terminus of Ku80 mediates a highly specific interaction with the domain of DNA-PKcs; Neither Ku70 nor Ku80 alone is active in DNA binding activity (Wu & Lieber, 1996; Gell & Jackson, 1999). The DNA-PKcs interacting domain is likely involved in the DNA-PKcs-Ku complex interaction, heterodimer assembly, and DNA termini binding. In the present study, this region containing 12 amino acids (720~732) of Ku80 was selected for a target peptide that would prevent DNA-PKcs from binding to Ku70/Ku80 regulatory subunits. To deliver a peptide into the cancer cells, a cell-permeable peptide import domain and the nuclear localization domain were added to the target peptide to obviate the need for permeabilization or microinjection of individual cells (Lin et al, 1995; Fig. 1).

HN-26 *AAVALLPAVLLALLAP***VQRKRQKLM**Y

HNI-38 *AAVALLPAVLLALLAP***VQRKRQKLM**YEGGDVDDLLDMI

Fig. 1. Synthetic peptide used for co-therapy study. Sequences of synthetic peptide-based inhibitor and control peptides (single-letter amino acid code). The membrane-translocation hydrophobic signal sequence is indicated in *italic* letter and the nuclear localization sequence is shown in bold face. Twelve residues of peptide inhibitor region is indicated at the C-terminus of HNI-38.

Effect of target peptide (HNI-38) on DNA-PK kinase activity

The interaction between DNA-PKcs and Ku complex activates its kinase activity (Hartley et al, 1995). Therefore, the efficacy of the target peptide was analyzed by measuring DNA-PK kinase activity *in vitro* in the presence of either control peptide (HN-26) or target peptide (HNI-38) as shown in Fig. 2. DNA-PK kinase activity was inhibited up to 50% in the presence of HNI-38 under the conditions where the control peptide (HN-26) showed minimal effect on MDA435 (Fig. 2A) and SKBR3 cells (Fig. 2B), strongly suggesting that the target peptide specifically bound to DNA-PKcs and consequently interfered with interaction between DNA-PKcs and Ku complex. Even at a low concentration of the peptide, the inhibitory effect of the target

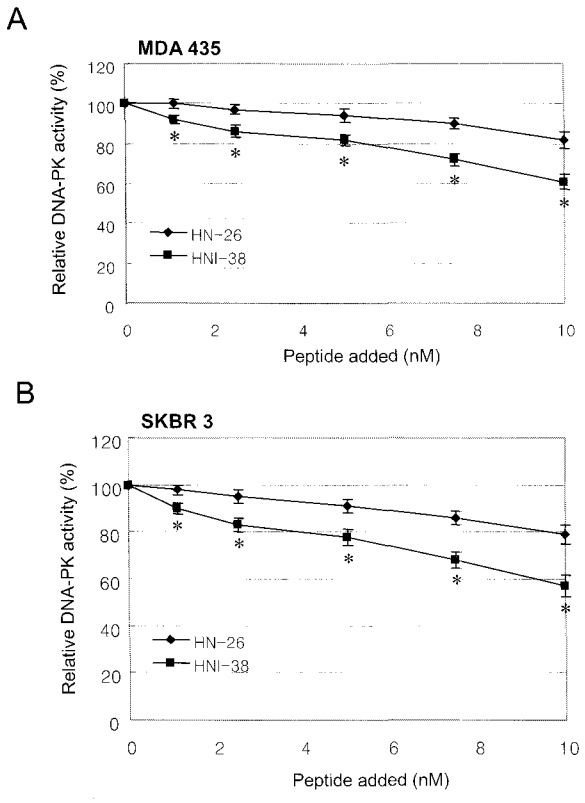

Fig. 2. *In vitro* effect of target peptide on DNA-PK kinase activity isolated from MDA435 (A) and SKBR3 cells (B). Partially purified DNA-PK fraction was incubated with various concentrations of either control peptide or target peptide prior to the addition of substrate peptides and other components for DNA-PK kinase assay (see the Methods section for the details). DNA-PK activity was measured as the relative amounts of ^{32}P transferred to the substrate peptide. *, significantly different from that in the presence of HI-26 ($P<0.05$).

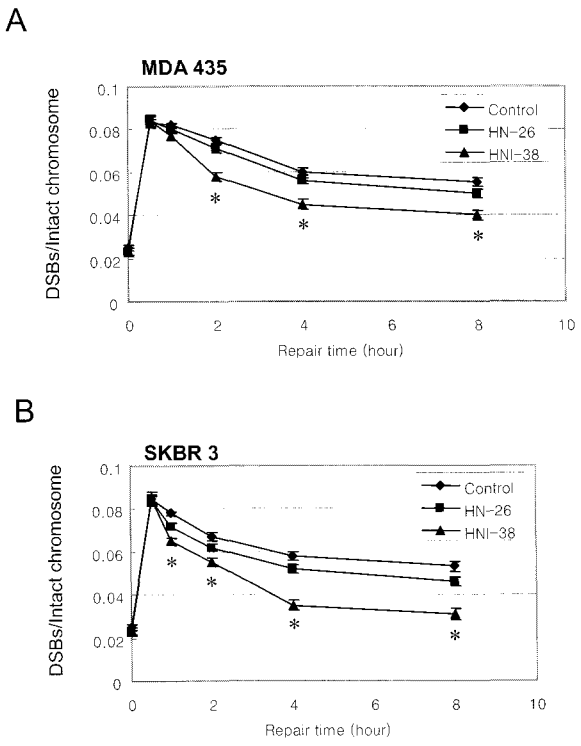

Fig. 3. Effect of target peptide on double-stranded DNA breaks (DSB) repair. MDA435 (A) and SKBR3 cells (B) grown in ^{14}C-containing media were treated with ionizing radiation (40 Gy) in the presence of no peptide, 50 nM control peptide, or 50 nM target peptide. After harvesting the cells at various time points, intact chromosomes and double-stranded DNA breaks (DSBs) were quantified by liquid scintillation counter. *, significantly different from control and the presence of HI-26 ($P<0.05$).

peptide on DNA-PK still occurred.

Target peptide interferes with repair of dsDNA breaks induced by IR

Ionizing Radiation (IR) induced dsDNA breaks are efficiently repaired by non-homologous end-joining (NHEJ) process. Genetic and biochemical studies strongly indicated that DNA-PK plays an essential role in NHEJ (Jin et al, 1997; Blunt et al, 1995). Hence, an alternative way to determine the efficacy of the peptide inhibitor is to measure the repair of double-stranded DNA breaks following IR. Therefore, breast cancer cells (MDA 435 and SKBR3) grown in the presence of [^{14}C]-thymidine (DiBiase et al, 2000) were treated with either a control or target peptide for 24 hrs. Following irradiation (40 Gy), the cells were harvested at various time points, and intact chromosomal DNA and DSBs were separated by pulsed field gel electrophoresis (0.5% agarose).Treatment of MDA435 (Fig. 3A) and SKBR3 (Fig. 3B) cells with IR (40 Gy) induced substantial amounts of dsDNA breaks, most of which were repaired within 4 hrs. The cells treated with the target or control peptides did not reveal any difference in generation of DSBs following IR (Fig. 3). On the other hand, the cells treated with the target peptide showed a noticeable decrease in DSBs repair activity, compared with those treated with the control peptide. This result suggests that the target peptide interfered with DSBs repair *in vivo,* through the targeted inhibition of DNA-PK activity.

Breast cancer cell growth is inhibited by target peptide only in the presence of DNA damage

The cells which lack of DNA-PK catalyic subunit show increased sensitivity to DNA damaging drugs or ionizing radiation (IR) (Lees-Miller et al, 1995), suggesting that DNA-PK activity is essential for DNA repair and cell survival after DNA damage. We, therefore, examined whether the targeted inhibition of DNA-PK by the peptide HNI-38 would sensitize SKBR3 (Fig. 4A) and MDA435 (Fig. 4B) cells, using standard colony count cell survival assay. In the absence of IR (0 Gy), both the control and target peptides did not show any effect on cell growth. However, the cells treated with IR (2 and 5 Gy) showed significant cell growth inhibition in the presence of the target peptide, but not treated with the control peptide (Fig. 4), suggesting that cell growth inhibition by the target peptide occurred through targeting the DNA-PK activity. The cells treated

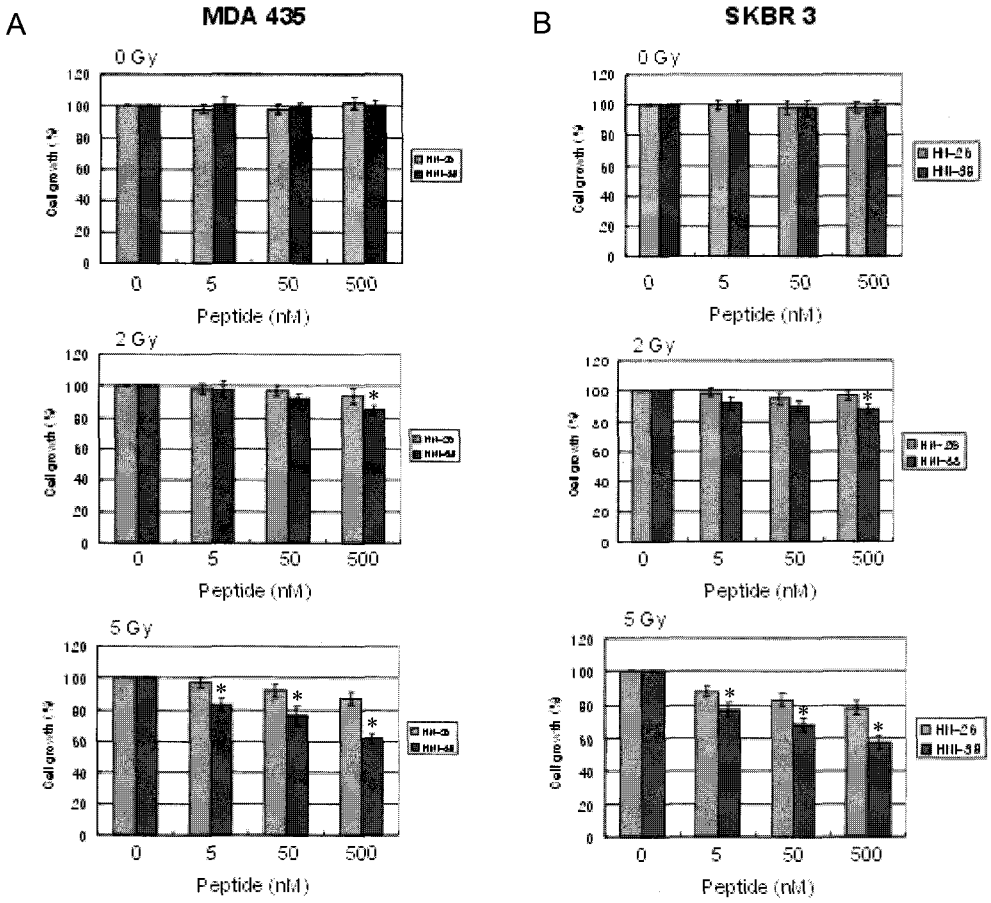

Fig. 4. Effect of control (HN-26) and target (HNI-38) peptides on the growth of MDA435 (A) and SKBR3 cells (B) treated with ionizing radiation (0, 2, and 5 Gy). Values expressed means±SE of four replications (*, $P<0.05$). The clonogenic assay was used for the cells treated with ionizing radiation (see Methods section for detailed procedure).

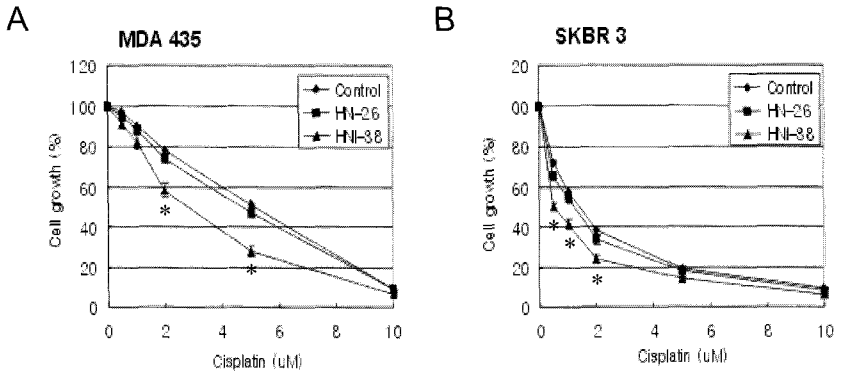

Fig. 5. Effect of control (HN-26) and target (HNI-38) peptides on the growth of MDA435 (A) and SKBR3 cells (B), treated with cisplatin. The cell survival assay (MTT) was employed for those treated with cisplatin (see the Methods section for detailed procedure). *, significantly different from control and the presence of HN-26 ($P<0.05$).

with cisplatin were not as effective as those treated with ionizing radiation (Fig. 5). HNI-38 also showed an inhibitory effect on cell growth.

DISCUSSIONS

When DNA is damaged by IR or chemotherapy drugs, it must be repaired to prevent genomic alterations. Unless repaired, the damaged DNA could give rise to cancer cells. There are a number of key human DNA repair pathways which depend on multimeric polypeptide activities (Friedberg, 1996; Lee, 2001). Damage recognition proteins interact with those proteins to report the damage to downstream repair activities, and they are crucial for DNA repair. DNA-PK is a key component of the con-homologous end joining (NHEJ) pathway with an unique property of being activated by double-stranded DNA breaks (Blunt et al, 1995). Previous studies with drug-resistant and-sensitive cancer cells suggested that high level expression of DNA-PK leads to drug-resistant cells, whereas low DNA-PK activity was associated with drug-sensitive phenotype (Muller et al, 1998; Tew et al, 1998; Shen et al, 1998; Kim et al, 2000), implicating a role of DNA-PK in conferring cells with drug resistance in response to anticancer DNA damaging drug. The interaction between DNA-PKcs with its regulatory subunits, (Ku70/Ku80) is crucial for its function in DNA repair, and a targeted inhibition of DNA-PK would sensitize drug-resistant cancer cells and facilitate cell killing. Based on the consideration of this important pathway, we attempted to develop a peptide co-therapy strategy, by which a low molecular weight peptide-based inhibitor specifically interferes with interaction between DNA-PKcs and Ku complex (Ku70/ Ku80).

In the present study, a target peptide (HNI-38) which contained the C-terminus of Ku80 was found to interfere with the interaction between DNA-PKcs and Ku complex. This was quite anticipated, since the C-terminus of Ku80 was previously identified as DNA-PKcs interaction domain (Gell & Jackson, 1999). Under the condition where the control peptide (HN-26) showed very little effect, the targeted peptide (HNI-38) directly inhibited the interaction between DNA-PKcs and Ku70/Ku80 and affected its kinase activity, inhibiting the DNA-PK activity up to 50% (Figure 2). Addition of the excess amount of the target peptide, however, did not further inhibit DNA-PK kinase activity (data not shown), suggesting that DNA-PKcs without Ku complex can still function, even though at low activity level.

When DNA is damaged by IR or chemotherapy drugs, the damaged DNA activates DNA-PK, which has homologous kinase domain, essential for DNA repair as well as cell cycle arrest in response to DNA damage. This consequently contributes to cell survival by protecting cells from apoptosis. The cells treated with the target peptide, but not control peptide, showed a noticeable decrease in double strand breaks (DSB) repair, following high dose (40 Gy) of IR, suggesting that HNI-38 specifically targeted DNA-PK *in vivo* and interfered with DSB repair activity through inhibition of DNA-PK activity. Targeted inhibition of DNA-PK by HNI-38 also caused cell growth inhibition only, when the cells were treated with IR, suggesting that HNI-38 targeted DNA-PK *in vivo* and lowered resistance of cells to ionizing radiation, which eventually resulted in growth inhibition of both SKBR3 and MDA435. The cells treated with HNI-38 also showed additive effect on cell growth inhibition in response to cisplatin treatment. This observation confirms previous findings that DNA-PK is directly involved in NER action in mammals (Muller et al, 1998). It also indicates that a targeted inhibition of DNA-PK would sensitize cancer cells to chemotherapeutic drugs such as cisplatin. Furthermore, our study not only validates DNA-PK as a useful molecular target for the treatment of drug resistant cancer cells, but also supports the physiological role of DNA-PK in resistance of cancer cells to IR or chemotherapy

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