DNA-PK inhibitor reduces cell viability on colorectal cancer

Bui Khac Cuong[1,2], Tran Phuong Linh[1], *Nguyen Thi Mai Ly[3]*
(1) Laboratory Animal Research Center, Vietnam Military Medical University
(2) Department of Pathophysiology, Vietnam Military Medical University
(3) Department of Biochemistry, Military Hospital 103, Vietnam Military Medical University

Abstract

Background and Objectives: Colorectal cancer (CRC) is one of the most common malignant cancers of the colon worldwide. Several novel approaches to cancer treatment have emerged, including gene therapy, targeted therapy, and adjuvant therapies. One of the most dangerous effects of chemotherapy is the induction of double-strand DNA breaks, which can lead to cell cycle arrest and cell death. Inhibition of DNA-PK can increase cellular sensitivity to radiotherapy and DNA-damaging agents. The present study aimed to evaluate the anti-cancer effect of the DNA-PK inhibitor (DNA-PKi) NU7441 on the HCT116 cell line. **Method:** HCT116 colorectal cancer cells were cultured under standard conditions. The effects of NU7441, a DNA-PKi, on cell viability and cell cycle were evaluated. WST-1 and FACS cell cycle assays were used to assess cell proliferation and cell cycle. Data were analyzed using GraphPad Prism 8.4. **Results:** DNA-PKi showed dose-dependent effects on cell death and proliferation inhibition in HCT116 cells *in vitro*. The groups treated with DNA-PKi at concentrations of 0.125, 0.250, and 0.500 µM all had significantly lower cell survival rates than the control group ($p < 0.05$). However, DNA-PKi did not affect cell cycle distribution in HCT116 colorectal cancer cells. **Conclusion:** DNA-PKi NU7441 exerted a dose-dependent effect on cell death and proliferation inhibition in HCT116 cells *in vitro.*

Keywords*: colorectal cancer, DNA-PK, DNA repair, Double-strand DNA breaks, target therapy, non-homologous end joining.*

1. INTRODUCTION

Colorectal cancer (CRC) remains a formidable global health challenge and ranks among the leading causes of cancer-related morbidity and mortality. Colorectal cancer (CRC) is a significant health burden. Globally, it is the third most common cancer in males and the second most common in females, ranking as the third leading cause of cancer-related deaths [1]. Colorectal cancer is trending toward younger ages, with cases as young as 15 to 18 years [1]. Data from 2018 in Vietnam showed that colorectal cancer ranked fifth among the top 10 most common cancers in both genders, with 14,733 new cases and 7,856 deaths. By the year 2020, in Vietnam, the incidence of new cases of colorectal cancer had risen to the fourth position in males and the third position in females, with a new incidence rate of 9%, totaling 16,426 cases [2].

The conventional treatment methods often applied in the comprehensive management of colorectal cancer include surgery, chemotherapy, radiation therapy, or a combination of these approaches. However, the prognosis of colorectal cancer remains limited, with a 5-year survival rate of less than 20% [3]. Depending on the condition and progression of the disease, treatment methods can be used in combination. For localized malignancies, surgical removal of the entire colon tumor is often an option, and any tumor location requires therapy. However, not all cancer cells can be eradicated entirely. Approximately 66% of patients with colon cancer undergo additional adjuvant treatment with chemotherapy and/or radiotherapy [4]. These treatments have many side effects because they are nonspecific and toxic to healthy cells. Additionally, even after receiving adjuvant therapy, up to 54% of patients relapsed [4]. Therefore, the development of more effective alternative therapies to treat patients with CRC is urgently needed.

Targeted therapy is a type of cancer treatment that focuses on specific molecules involved in the growth and survival of cancer cells. The goal of targeted therapy is to block the growth and spread of cancer cells while minimizing damage to normal cells. DNA double-strand breaks (DSBs) are considered the most deleterious form of DNA damage. The DNA damage response (DDR) pathway encompasses a collection of intricate mechanisms, including DNA damage repair, DNA damage tolerance mechanisms, and cell-cycle checkpoint control. This intricate system governs the accurate execution of DNA replication and proliferation and, subsequently, cell viability. The DDR pathway plays a pivotal role in

Corresponding author: Nguyen Thi Mai Ly; Email: dr.nguyenmaily@gmail.com
DOI: 10.34071/jmp.2024.4.4
Received: 20/4/2024; Accepted:18/6/2024; Published: 25/6/2024

preserving genomic integrity and stability through DNA damage repair. Strand breakage resulting from base alterations, single-strand breaks (SSBs), or double-strand breaks (DSBs) can lead to chromosome breakage, resulting in gene loss. It is mostly caused by altered DNA replication forks, ROS, ionizing radiation, and physical or mechanical stress [5]. DNA-dependent protein kinase (DNA-PK) is an important enzyme in DDR pathways that plays a crucial role in the repair of DSBs via non-homologous end joining (NHEJ) [6]. NU7441 represents a new generation of selective DNA-PK inhibitors. The aim of the present study was to investigate the anti-colorectal cancer effect of the DNA-PK inhibitor NU7441, which inhibits DNA-DSB repair by blocking the NHEJ pathway, in the HCT116 cell line.

2. MATERIALS AND METHODS

2.1. Cell lines and culture conditions

The antitumor efficacy of DNA-PKi was assessed using HCT116 cells as an experimental model. The HCT116 cells, sourced from the American Type Culture, USA, were cultured in Dulbecco's Modified Eagle Medium (DMEM) obtained from Cytiva, USA. The culture medium was supplemented with 10% fetal bovine serum (FBS) provided by Cytiva and 1% penicillin-streptomycin (Sigma-Aldrich, USA). Cells were incubated in an atmosphere of 5% CO2 and 95% air at 37°C.

2.2. Reagent:

DNA-PKi NU7441 (KU-57788, Catalog No.S2638, Selleckchem) was stored at -20°C for subsequent experiments. The dosage was determined during the experiments.

2.3. WST-1 assay:

Cells were seeded at a density of 1000 cells/well in a 96-well plate and cultured at 37 °C overnight. Cells were treated with DNA-PKi for 72h at increasing concentrations of 0.000, 0.125, 0.250, and 0.500 µM. Next, 100 µL of 2% WST-1 solution (Roche, Switzerland) was added to each well, and the plate was incubated at 37°C for 2h. A microplate reader (Tecan, Switzerland) was used to measure the absorbance at 450 nm and 620 nm was used as the reference wavelength. Cell viability was assessed with Excel ver. 2016 (Microsoft, USA) using the following formula:

$$\% \text{ Cell viability } = \frac{absorbance\ of\ treatment\ group}{absorbance\ of\ control\ group} \text{ x } 100\%$$

2.4. FACS cell cycle assay:

HCT116 cells were cultured in 6-well plates of 500,000 cells each for 24 h. After the cells reached the appropriate amount, they were treated with DNA-PKi at different temperatures (0.125 µM/ml; 0.25 µM/ml) and the control group was the normal cultured without treatment. Cells were imaged at 0, 24, and 48 h after drug testing. After 48 h, cells were scraped off the plate, cycled with EtOH, and incubated overnight at -30°C. The cells were stained with Propidium Iodide (PI, 10 µg/ml), incubated for 30 min, and analyzed using a flow cytometer. PI is a common fluorescent red nuclear pigment antagonist. Because propidium iodide does not permeate living cells, it is commonly used to detect dead cells in populations. Stain PI provides information about cell cycle and amount of DNA of population. The flow cytometer will analyze the data and charts from the obtained cells.

2.5. Statistical analysis:

GraphPad Prism version 8.4 (GraphPad Software, Inc., California, USA) was used for statistical analysis. The study results are presented as mean ± standard error (SD). One-way ANOVA was used to test the difference in the mean when comparing more than two groups. Differences were considered statistically significant when the p-value was less than 0.05.

3. RESULTS

3.1. Cell culture and proliferation assay

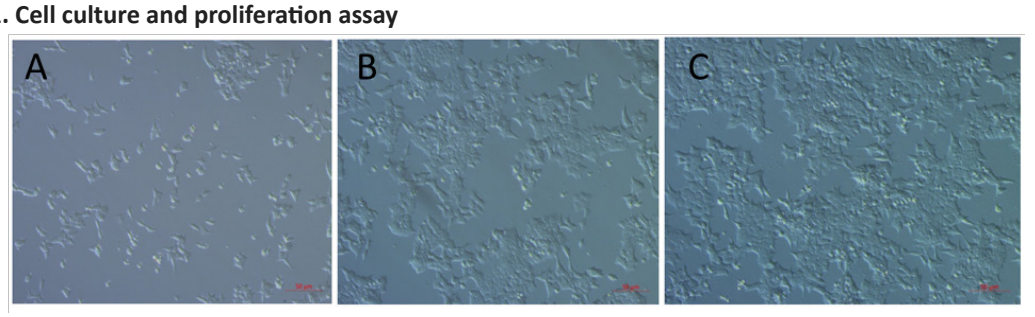

Figure 1. Morphological characteristics of HCT116 cell line in culture: (A) HCT116 cells on day 1; (B) HCT116 cells on day 2; (C) HCT116 cells on day 7. After 24h, cells adhered to the bottom 40 - 50% of the culture area. After seven days, the cultured cells covered 80% of the culture area.

The images in *Figure 1* show the status, form, and density of HCT116 cells cultured after 1, 2, and 7 days. HCT116 cells appeared as basal epithelial cells, and monolayer growth, having many different shapes from oval to polygonal, was approximately 20 - 25 µm in size, although this size could vary depending on the stage of the cell cycle. In addition, HCT116 cells had large nuclei that occupied most of their cytoplasm. After 24h of culturing, the HCT116 cells adhered to the bottom of the culture dish and showed robust proliferation. The proliferation rate of HCT116 was about 40 - 50% within 24 hours. After seven days, the cultured cells covered 80% of the flask area. The cell proliferation rate was monitored, and when it reached approximately 80% of the plate area, the cells were transferred to a new culture plate.

3.2. Evaluation of the impact of DNA-PKi on the viability of colorectal cancer cells using the WST-1 assay

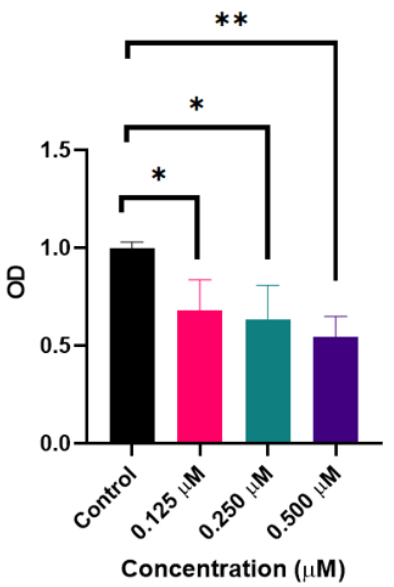

Figure 2. Percentage of viable cells 72 h after DNA-PKi treatment at concentrations of 0.125, 0.250, and 0.500 µM. Data were analyzed by one-way ANOVA and post-hoc Tukey's test; results are shown as mean ± SD. *P < 0.05, **P < 0.01

Based on the WST-1 results, the number of living cells was proportional to the OD index displayed on the spectrophotometer. The OD index after reading was calibrated by the index at 650 nm wavelength minus the index at 450 nm wavelength. The higher the OD index, the higher is the percentage of living cells.

To investigate the effect of DNA-PKi on the viability of colorectal cancer cells, HCT116 cells were treated with DNA-PKi at concentrations: 0.125 µM, 0.250 µM and 0.500 µM and the control group was not treated with DNA-PKi. Employing one-way ANOVA and post hoc Tukey's pairwise comparison tests, we observed that at the 72-hour time point after DNA-PKi treatment, the control group exhibited the highest cell survival rate. In contrast, the groups treated with DNA-PKi at concentrations of 0.125 µM, 0.250 µM, and 0.500 µM all had significantly lower cell survival rates compared to the control group ($p < 0.05$, *Figure 2*). This indicates that DNA-PKi at concentrations of 0.125, 0.250, and 0.500 µM can induce cell death and inhibit *in vitro* proliferation in HCT116 colorectal cancer cell lines. Additionally, DNA-PKi showed a dose-dependent effect on cell death and proliferation inhibition in HCT116 cells *in vitro*. Therefore, we concluded that the inhibitory effect of DNA-PKi on HCT116 cells was dose-dependent.

3.3. Evaluation of the effect of DNA-PKi on the cell cycle was performed on colorectal cancer cells

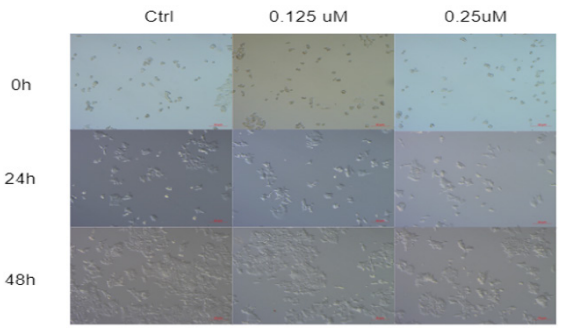

Figure 3. HCT116 colorectal cancer cells in the control group and DNA-PKI treatment groups at concentrations of 0.125 µM and 0.250 µM at 0h, 24h and 48h after treatment. Cells in the control group and the 0.125 µM treatment group grew in large patches and spread evenly on the surface while the 0.25µM treatment group grew in small discrete clusters.

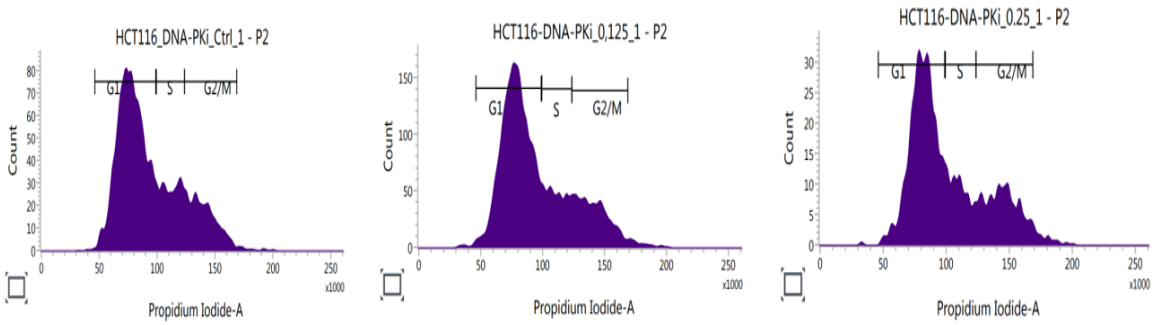

Figure 4. Flow cytometry results of cell cycle assay between the control group and DNA-PKi treatment groups at concentrations of 0.125 µM and 0.250 µM on HCT116 cell line after 48 h of treatment. Nuclear DNA was stained with Propidium Iodide and analyzed by Flow cytometry. If PI penetrates the cell membrane, it can cause damage.

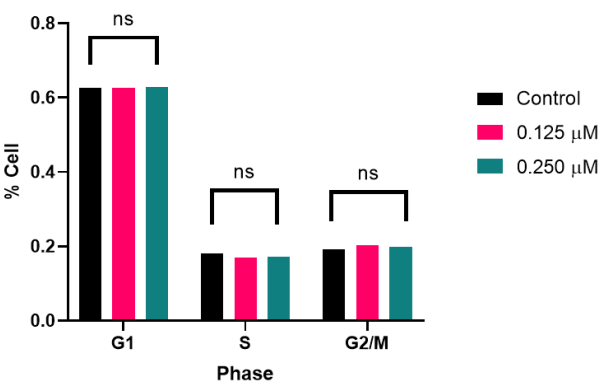

Figure 5. Comparison of cell cycle test results between the control group and the DNA-PKi treatment groups at concentrations of 0.125 µM and 0.250 µM on the human colorectal cancer cell line HCT116 after 48 h of treatment. Data were analyzed by one-way ANOVA test and post-hoc Tukey test, and the results are presented as mean ± SD.

To analyze the effect of DNA-PKi on the phases of the division cycle of HCT116 cells, cells were treated with different concentrations (0.125 µM and 0.25 µM), and then nuclear DNA was stained with Propidium Iodide and analyzed by Flow cytometry. Cells in the control group and the 0.125 µM treatment group grew in large patches and spread evenly on the surface while the 0.25 µM treatment

group grew in small discrete clusters (*Figure 3*).

Two-way ANOVA and Dunnett's post-test were used to compare the proportion of cells in each stage between the groups. We did not observe a difference in the cell cycle distribution after treatment with DNA-PKi compared to that in the control group in all groups. Specifically, the average percentage of cells in S phase in all groups ranged between 17 - 18%. Similarly, the ratio of cells in the G1 and G2/M phases was the same in all groups (*Figure 4-5*). Therefore, DNA-PKi did not affect the cell cycle.

4. DISCUSSION

Colorectal cancer is one of the leading causes of cancer in the world, so there is increasing appreciation for the development of novel methods for the treatment of colorectal cancer. DNA-PK is an essential component of the NHEJ pathway, and its activation is necessary for DNA repair. Inhibiting the phosphorylation process of DNA-PK prevents the repair of DNA damage, leading to programmed cell death. NU7441, a potent and specific DNA-PKcs inhibitor, is predicted to enhance the effectiveness of disease treatment. A previous study has shown a combination of pharmacological agents with specific chemotherapeutic agents against lung cancer. Inhibition of DNA-PKcs counteracts cell proliferation in A549 lung cancer cells when topoisomerase inhibitors are applied . NU7441 inhibits the repair of DNA damage caused by topoisomerase inhibitors in non-small cell lung carcinoma (NCSLC) cells, subsequently leading to programmed cell death. Therefore, the results indicate that the combination of traditional therapy inhibitors and NU7441 is a potential treatment option for NCSLC [7].

Targeting DNA is a promising approach for cancer treatment, but it has some limitations. Despite promising findings *in vitro*, DNA-PK inhibitors have failed to induce changes in animal studies. One reason for these findings may be related to the limited solubility and poor pharmacokinetic properties of DNA-PK inhibitors. NU7441 is poorly absorbed and rapidly metabolized in mice, which hinders its clinical application [8]. In addition, its poor water solubility hampers the possibility of higher dosing. Based on promising pharmacokinetics, studies on the effectiveness of combined therapies have been conducted, showing that NU7441 doubles the delay in tumor development caused by etoposide without increasing toxicity to unacceptable levels.

Targeting DNA-PK as a therapeutic intervention in human malignancy began entering clinical trials, especially in combination with chemotherapy or radiotherapy. CC-122, an inhibitor of DNA-PK, currently undergoing phase I clinical trial for solid tumors, non-Hodgkin lymphoma, and multiple myeloma. Similarly, CC-115, designed as a dual inhibitor of DNA-PK and mTOR, is in phase I trial for advanced solid tumors and hematologic malignancies. Notably, phase I studies have revealed that oral formulations of CC-122 and CC-115 demonstrate favorable tolerability profiles, with no unexpected toxicities or adverse effects reported [9], [10]. The utilization of CC-122 in brain cancer has yielded promising outcomes[10]. Additionally, ZSTK474, primarily a PI3K inhibitor with DNA-PK inhibitory activity, is undergoing phase I trials for advanced solid malignancies [11]; the DNA-PKi MSC2490484A, is being investigated in combination with radiotherapy for the treatment of advanced solid tumors or chronic lymphocytic leukemia [12]. These clinically significant DNA-PKi hold promise for potentially offering therapeutic benefits in the fight against cancer. Peposertib is a powerful and selective small molecule DNA-PK inhibitor that is taken orally [13]. It has been shown to have radio sensitizing and anticancer action in xenograft models and to be well-tolerated when used in monotherapy. However, enrollment was discontinued because of insufficient exposure at that dose, and the RP2D was not formally declared [14].

DNA-dependent protein kinase inhibitors (DNA-PKis) have significant effects on the cell cycle. In response to DNA damage, cells often undergo cell cycle arrest to allow time for DNA repair. DNA-PKi-induced inhibition of DNA-PK can lead to G1 phase arrest [15]. In this phase, the cell checks for DNA damage and either repairs it or initiates apoptosis (programmed cell death) if the damage is too severe to be repaired. DNA-PK also plays a role in regulating cell progression through the S phase of the cell cycle, during which DNA replication occurs. Inhibition of DNA-PK can slow down or delay progression through the S phase as the cell attempts to repair DNA lesions before replicating the damaged DNA [16].

Jarah showed that DNA-PKI can induce cell cycle arrest at G2/M phase in glioblastoma cells. DNA demethylating agents such as 5-Aza-CdR and Zebularine induce G2/M arrest in various cancer cell types [17], and it has been shown that the release from Zebularine-induced G2/M arrest and DNA repair efficiency correlates well with phosphorylation of ATM, p53, and Chk1 proteins. If cells with persistent DNA damage undergo mitosis due to a G2/M checkpoint deficiency, mitotic

catastrophe is expected to occur [18]. G2/M cell cycle arrest is a classical cellular response to DNA damage, allowing DSB repair before the cell enters mitosis and thus maintaining the stability cellular genome. Our data showed that DNA-PKi inhibition does not affect the cell cycle, and the cell cycle arrest ability of DNA-PKi should be further studied. But in previous research, Wang found that M3814- a novel DNA-PKi promotes G2/M cell cycle arrest induced in NSCLC cell [13]. The results in this study provide a scientific basis for our research on the mechanism of action of DNA-PKi - a type of drug that inhibits the enzyme DNA-dependent protein kinase (DNA-PK) on colorectal cancer cells HCT116. Based on the promising results *in vitro*, we suggest that the anticancer effects of NU7441 on colorectal cancer could be tested in preclinical studies and clinical trials in the future.

5. CONCLUSION

In summary, DNA-PKi NU7441 exerted a dose-dependent effect on cell death and proliferation inhibition in HCT116 cells *in vitro.*

Funding: This research is funded by Vietnam National Foundation for Science and Technology Development (NAFOSTED) under grant number 108.02-2019.324. The funder had no role in the study design, data collection and analysis, decision to publish, or manuscript preparation.

Declaration of Interest Statement: The authors declare no potential conflicts of interest.

REFERENCEs

[1] P. Dharwadkar, T. A. Zaki, and C. C. Murphy, 'Colorectal cancer in younger adults', *Hematol. Oncol. Clin. North Am.*, vol. 36, no. 3, pp. 449–470, Jun. 2022, doi: 10.1016/j.hoc.2022.02.005.

[2] N. Le and H. Dao, 'Colorectal Cancer in Vietnam', 2021. doi: 10.5772/intechopen.93730.

[3] P. Rawla, T. Sunkara, and A. Barsouk, 'Epidemiology of colorectal cancer: incidence, mortality, survival, and risk factors', *Przegląd Gastroenterol.*, vol. 14, no. 2, pp. 89–103, 2019, doi: 10.5114/pg.2018.81072.

[4] S. Kosmider and L. Lipton, 'Adjuvant therapies for colorectal cancer', *World J. Gastroenterol. WJG*, vol. 13, no. 28, pp. 3799–3805, Jul. 2007, doi: 10.3748/wjg.v13.i28.3799.

[5] H. Arai *et al.*, 'The Landscape of Alterations in DNA Damage Response Pathways in Colorectal Cancer', *Clin. Cancer Res.*, vol. 27, no. 11, pp. 3234–3242, Jun. 2021, doi: 10.1158/1078-0432.CCR-20-3635.

[6] H. H. Y. Chang, N. R. Pannunzio, N. Adachi, and M. R. Lieber, 'Non-homologous DNA end joining and alternative pathways to double-strand break repair', *Nat. Rev. Mol. Cell Biol.*, vol. 18, no. 8, Art. no. 8, Aug. 2017, doi: 10.1038/nrm.2017.48.

[7] M. Yanai *et al.*, 'DNA-PK Inhibition by NU7441 Enhances Chemosensitivity to Topoisomerase Inhibitor in Non-Small Cell Lung Carcinoma Cells by Blocking DNA Damage Repair', *Yonago Acta Med.*, vol. 60, no. 1, pp. 9–15, Mar. 2017.

[8] Y. Zhao *et al.*, 'Preclinical Evaluation of a Potent Novel DNA-Dependent Protein Kinase Inhibitor NU7441', *Cancer Res.*, vol. 66, pp. 5354–62, Jun. 2006, doi: 10.1158/0008-5472.CAN-05-4275.

[9] C. Carpio *et al.*, 'Avadomide monotherapy in relapsed/refractory DLBCL: safety, efficacy, and a predictive gene classifier', *Blood*, vol. 135, no. 13, pp. 996–1007, Mar. 2020, doi: 10.1182/blood.2019002395.

[10] D. W. Rasco *et al.*, 'A First-in-Human Study of Novel Cereblon Modulator Avadomide (CC-122) in Advanced Malignancies', *Clin. Cancer Res.*, vol. 25, no. 1, pp. 90–98, Jan. 2019, doi: 10.1158/1078-0432.CCR-18-1203.

[11] A. C. Lockhart *et al.*, 'Abstract B271: A first-in-human Phase I study of ZSTK474, an oral pan-PI3K inhibitor, in patients with advanced solid malignancies.', *Mol. Cancer Ther.*, vol. 12, no. 11_Supplement, p. B271, Nov. 2013, doi: 10.1158/1535-7163.TARG-13-B271.

[12] M. T. J. van Bussel *et al.*, 'A first-in-man phase 1 study of the DNA-dependent protein kinase inhibitor peposertib (formerly M3814) in patients with advanced solid tumours', *Br. J. Cancer*, vol. 124, no. 4, pp. 728–735, Feb. 2021, doi: 10.1038/s41416-020-01151-6.

[13] M. Wang, S. Chen, Y. Wei, and X. Wei, 'DNA-PK inhibition by M3814 enhances chemosensitivity in non-small cell lung cancer', *Acta Pharm. Sin. B*, vol. 11, no. 12, pp. 3935–3949, Dec. 2021, doi: 10.1016/j.apsb.2021.07.029.

[14] M. Samuels *et al.*, 'A Phase 1 Study of the DNA-PK Inhibitor Peposertib in Combination With Radiation Therapy With or Without Cisplatin in Patients With Advanced Head and Neck Tumors', *Int. J. Radiat. Oncol. Biol. Phys.*, vol. 118, no. 3, pp. 743–756, Mar. 2024, doi: 10.1016/j.ijrobp.2023.09.024.

[15] E. A. Hendrickson, 'Cell-Cycle Regulation of Mammalian DNA Double-Strand-Break Repair', *Am. J. Hum. Genet.*, vol. 61, no. 4, pp. 795–800, Oct. 1997, doi: 10.1086/514895.

[16] B. P. C. Chen *et al.*, 'Cell Cycle Dependence of DNA-dependent Protein Kinase Phosphorylation in Response to DNA Double Strand Breaks*', *J. Biol. Chem.*, vol. 280, no. 15, pp. 14709–14715, Apr. 2005, doi: 10.1074/jbc.M408827200.

[17] H. Dote *et al.*, 'Enhancement of In vitro and In

vivo Tumor Cell Radiosensitivity by the DNA Methylation Inhibitor Zebularine', *Clin. Cancer Res.*, vol. 11, no. 12, pp. 4571–4579, Jun. 2005, doi: 10.1158/1078-0432.CCR-05-0050.

[18] J. A. Meador, Y. Su, J.-L. Ravanat, and A. S. Balajee, 'DNA-dependent protein kinase (DNA-PK)-deficient human glioblastoma cells are preferentially sensitized by Zebularine', *Carcinogenesis*, vol. 31, no. 2, pp. 184–191, Feb. 2010, doi: 10.1093/carcin/bgp284.