DNA-DEPENDENT PROTEIN KINASE INHIBITOR INDUCES APOPTOSIS IN COLON CANCER CELLS

Tran Phuong Linh[1], *Bui Khac Cuong*[1,2*]

Abstract

Objectives: Among all types of DNA damage, DNA double-strand breaks (DSBs) are considered the most deleterious form induced by either endogenous factors (oxi-dative damages, mismatches, altered chromatin structures, and missing, or modified nucleotides) or exogenous factors, i.e., ultraviolet (UV) radiation, ionizing radiation (IR), and chemicals or drugs. DNA-dependent protein kinase (DNA-PK) plays a crucial role in repairing DSBs through non-homologous end joining (NHEJ). Cells lacking DNA-PK exhibit heightened sensitivity to IR and various DNA-damaging agents. The inhibition of DNA-PK further intensifies cellular susceptibility to IR and DNA-damaging agents. Several small molecules that inhibit DNA-PK have been developed. This study aimed to evaluate the effect of DNA-PK inhibitor (DNA-PKi) NU7441 on the HCT116 cell line. ***Methods:*** DNA-PKi NU7441 was used to assess the effect on anti-proliferation and induction of apoptosis on the HCT116 colorectal cancer cell line. Cells were cultured under standard conditions; crystal violet and apoptosis assay were applied to evaluate cell proliferation and apoptosis. Data were analysed using GraphPad Prism 8.4. ***Results:*** DNA-PKi effectively inhibited HCT116 colon cancer cell growth via crystal violet assay ($p < 0.01$). In addition, DNA-PKi also induced programmed cell death in the HCT116 cell line ($p < 0.05$). ***Conclusion:*** DNA-PKi NU7441 suppressed cell proliferation and induced apoptosis in the HCT116 colon cancer cell line.

Keywords: DNA-PK; DNA-PKi; DNA damage repair; Non-homologous end joining; Colon cancer.

---

[1]**Laboratory Animal Research Center, Vietnam Military Medical University**
[2]**Department of Pathophysiology, Vietnam Military Medical University**
[*]**Corresponding author: Bui Khac Cuong (buikhaccuong@gmail.com)**
Date received: 23/01/2024
Date accepted: 15/3/2024
http://doi.org/10.56535/jmpm.v49i4.726

INTRODUCTION

DNA damages are caused by either endogenous factors (oxi-dative damages, mismatches, altered chromatin structures, and missing, or modified nucleotides) or exogenous factors, i.e., UV radiation, IR, and gen-otoxic chemicals, or drugs [1]. IR is thought to exert a variety of biological effects by causing damage to DNA. DNA damage repair (DDR) is a complex of different mechanisms including DDR, DNA damage tolerance mechanisms, and cell-cycle control checkpoint control. This complex system regulates the proper efficiency of DNA replication, proliferation, and cell survival. The role of the DDR pathway is crucial in maintaining genome integrity and stability by repairing DNA damage. If the damage is not repaired, it will cause instability and gene mutation, which is one of the hallmarks of cancer. In eukaryotes, DSBs are repaired mainly through homologous recombination (HR) and NHEJ [2]. DSBs are considered the most dangerous among the different types of DNA damage. Unlike HR, NHEJ does not require a DNA template (sister chromatid) for repair. Instead, NHEJ operates by modifying the free ends of DNA located on either side of the break by using various nucleases so that the ends become compatible (i.e. 3'-hydroxyl and a 5'-phosphate), followed by ligation with the enzyme DNA ligase 4. However, NHEJ is a relatively quick but intrinsically error-prone process, and its excessive use can lead to gene rearrangements, deletions, and mutations, all of which can cause post-replicative cells to be more vulnerable to DSBs [2].

DNA-PK is a serine/threonine protein kinase located in the nucleus. Its activation occurs upon interaction with its target DNA. DNA-PK forms a complex consisting of a sizable catalytic subunit, DNA-PKcs, and a regulatory factor, Ku proteins (Ku70/80), which form a heterodimer. Numerous studies highlight the pivotal role of mammalian DNA-PK in responding to DNA damage, particularly in the repair and recombination of DNA DSBs. It collaborates with ATM and ATR to phosphorylate related proteins, contributing to the DNA damage response and subsequent processes [3]. Furthermore, DNA-PK participates in modulating chromatin structure and maintaining telomeres [4].

The human genome encodes Ku70 and Ku80 (also known as Ku86), which are products of the XRCC6 and XRCC5 genes, respectively. These

proteins exhibit a strong affinity for the exposed ends of double-stranded DNA, and their binding is independent of the DNA sequence, focusing on the sugar backbone rather than the bases. Ku, particularly in its heterodimeric form (Ku70/80), plays a crucial role in NHEJ. Ku70/80 senses and binds double-strand breaks, which initiates NHEJ. Acting as a scaffold, it recruits the NHEJ machinery to the damaged DNA site and directly interacts with canonical NHEJ factors such as DNA-PKcs, XRCC4, DNA ligase IV, and XLF [5], along with most DNA end processing factors. Beyond its primary recruitment function, the Ku heterodimer serves a secondary role in maintaining the stability of the broken DNA molecule's ends when a double-strand break occurs. Forming a ring-shaped protein structure, the Ku heterodimer slides onto the ends of the fractured DNA, keeping them together and preventing nonspecific processing.

After induction of the DNA-strand break, the Ku70/80 heterodimer promptly identifies and binds to the damaged DNA, forming a ring-like protein complex at the site of the lesion. This complex plays a crucial role in stabilizing the broken double strands of DNA. Simultaneously, the Ku heterodimer recruits various processing proteins, including DNA-PKcs (the other component of DNA-PK), XRCC4, DNA ligase IV, XLF, and APL [6, 7].

The Ku heterodimer also contributes to maintaining the stability of the NHEJ complex at the DNA damage site. The NHEJ complex, once assembled, bridges the DNA ends and further enhances end stability. The interaction between DNA-PKcs and the Ku heterodimer triggers the inward translocation of the Ku heterodimer along the double-stranded DNA, ultimately activating the kinase activity of DNA-PKcs. The activated DNA-PKcs then phosphorylates numerous factors beyond the Ku heterodimers, including Artemis, polynucleotide kinase/ phosphatase (PNKP), Werner syndrome ATP-dependent helicase (WRN), DNA polymerase, and Aprataxin [8].

DNA-PK plays a crucial role in repairing DSBs through NHEJ. Cells lacking DNA-PK exhibit heightened sensitivity to IR and various DNA-damaging agents. The inhibition of DNA-PK further intensifies cellular susceptibility to IR and DNA-damaging agents. Therefore, inhibition of DNA-PK could impact the cell fate. Several small molecules that inhibit DNA-PK have been developed. This study aimed: *To evaluate the effect of DNA-PKi NU7441 on the HCT116 cell line.*

MATERIALS AND METHODS

1. Materials

** Cell line and cell culture condition:*

In this experiment, HCT116 cells were used as an experimental model to evaluate the antitumor activity of DNA-PKi NU7441 (Catalog No.S2638; purity: 99.94%; Selleckchem), subsequently referred to as DNA-PKi. HCT116 cells originated from an American Type Culture Collection, Manassas, Virginia, USA. These cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) obtained from Cytiva, Massachusetts, USA, supplemented with 10% fetal bovine serum (FBS) also from Cytiva, Massachusetts, USA, and 1% Penicillin-Streptomycin sourced from Sigma-Aldrich, Missouri, USA. The cells were grown in an environment with 5% CO_2 and 95% air at 37ºC.

2. Methods

** Crystal Violet:*

After harvesting, cells were transferred to 12 wells plate, each well with a density of 300 cells/well and 5mL medium, and cultured in an incubator with 37ºC and 5% CO_2. After 24 hours, cells were treated with control, 0.125µM or 0.250µM of DNA-PKi, and monitored until the cell colonies were visible by eye (approximately 7 - 8 days), then proceed to stain the cells with a specialized purple dye (Crystal Violet). Then, the plates were scanned to obtain cell colony images. Cell images were analyzed using ImageJ software and histograms were constructed based on the measured number of cell colonies and color intensity (n = 4).

** FACS apoptosis assay:*

Cells were treated and stained according to the procedure included with Annexin V Apoptosis Detection kit: HCT116 cells were cultured in 6-well plates of 300,000 cells per well for 2h. After the cells had reached the appropriate number, the DNA-PKi was treated with different concentrations (0.125µM; 0.250µM), and the control group was cultured without treatment. After treatment, the cells were washed with PBS (phosphate-buffered saline), and counted the cell number. Cells were scraped off the plate, stained with Propidium Iodide (PI, 10 ug/mL) and Annexin V, and incubated for 15 min to label the cells. After incubation, cells were analyzed on a flow cytometer.

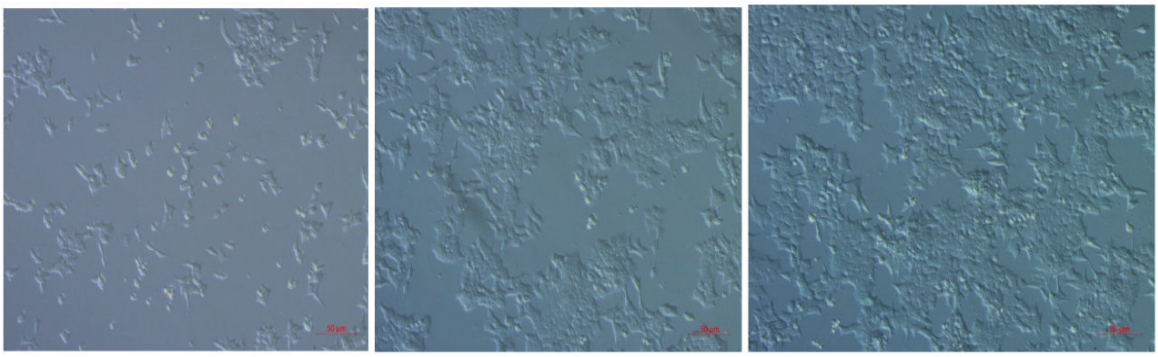
** Statistical analysis method:*

GraphPad Prism version 8.4 (GraphPad Software, Inc., California,

USA) was used for statistical analysis. The study results were presented as Mean ± Standard Deviation (SD). Using One-Way ANOVA to test the difference of mean when comparing more than 2 groups. The difference is considered statistically significant when $p < 0.05$.

RESULTS

1. Cell culture and proliferation



A B C

Figure 1. Morphological characteristics of HCT116 cell line in culture.

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

The images in figure 1 recorded the status, form and density of HCT116 cultured after 1 day, 2 days, and 7 days. HCT116 cells appeared as basal epithelial cells, monolayer growing, having many different shapes from oval to polygonal, were about 20 - 25µM in size, although this size could vary depending on the stage of the cell cycle. In addition, HCT116 cells had large nuclei occupied most of the 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 7 days, the cultured cells had 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.

2. Evaluation of cell proliferation by Crystal Violet assay

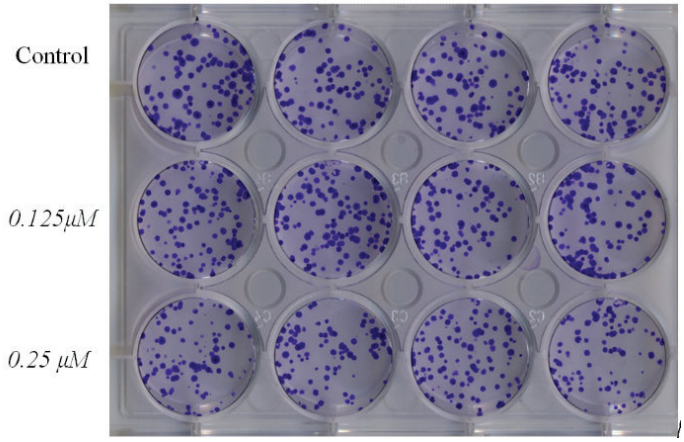


Figure 2. Changes in the results of staining HCT116 cells with Crystal violet between the control and DNA-PKi treatment groups after 2 weeks.

Cells were treated with control, 0.125μM or 0.250μM of DNA-PKi. After that, treatment with DNA-PKi was maintained until the cell colonies were visible by eye (approximately 7 - 8 days), then proceed to stain the cells with a specialized purple dye (Crystal Violet).

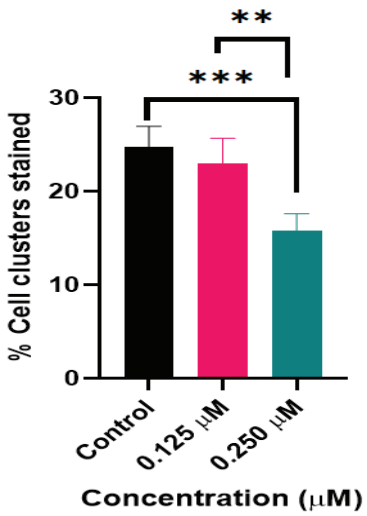


Figure 3. Quantitative analysis of cell of Crystal Violet assay after 2 weeks between control and treatment groups at concentrations: 0.125μM and 0.250μM.

*(p** < 0.0008, p*** < 0.003. Relative staining intensities were measured by using ImageJ software)*

The results of Crystal Violet assay were presented in figure 2 and 3. In figure 2, after stained with Crystal Violet, the control group had large, intensely colored, and densely packed cell clusters, whereas the group treated with DNA-PKi at a concentration of 0.125µM showed smaller, paler, and sparser cell clusters. Meanwhile, the group treated with DNA-PKi at 0.250µM appeared lighter and smaller clusters compared to the control group. We observed that HCT116 at concentrations of 0.250µM exhibited proliferation inhibitory activity on HCT116 cells *in vitro* especially at concentrations 0.250µM (the average stained area of cell clusters for the control group was 24.78%, significantly higher than 0.250µM treatment group (15.75%), with p*** < 0.001). At a concentration of 0.125µM, there was inhibition of proliferation but it is not really clear. Furthermore, the inhibitory effect on proliferation was dose-dependent when comparing the 0.125µM treatment group and the 0.250µM treatment group (the average stained area of cell clusters for the 0.125µM treatment group was significantly higher than the corresponding value for the 0.250µM treatment group, with p** < 0.01, *Figure 3*).

3. Evaluation of the ability of DNA-PKi to induce programmed cell death (apoptosis)

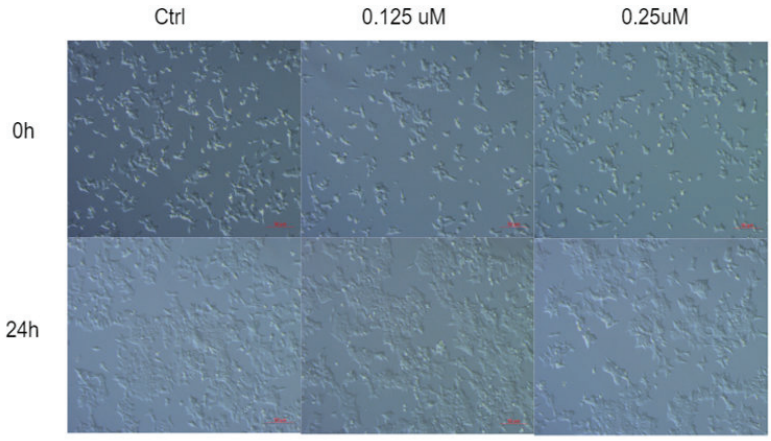


Figure 4. DNA-PKi stimulates apoptosis on HCT116 cells. The cells were treated with DNA-PKi at different concentrations: 0; 0.125µM; 0.250µM.

(At 0h, the cells grew in small dicrete cluster while cells at 24h on all group grew in large patches, spreading evenly on the surface).

HCT116 cells were treated with DNA-PKi at concentrations of 0.125µM, 0.250µM for 48h to evaluate the stimulation of DNA-PKi to apoptosis. After 24h, the number of dead cells increased significantly in both treatment groups compared with the control group (*Figure 4*). The cells were then analyzed on a flow cytometer with the AnnexinV/PI indicators.

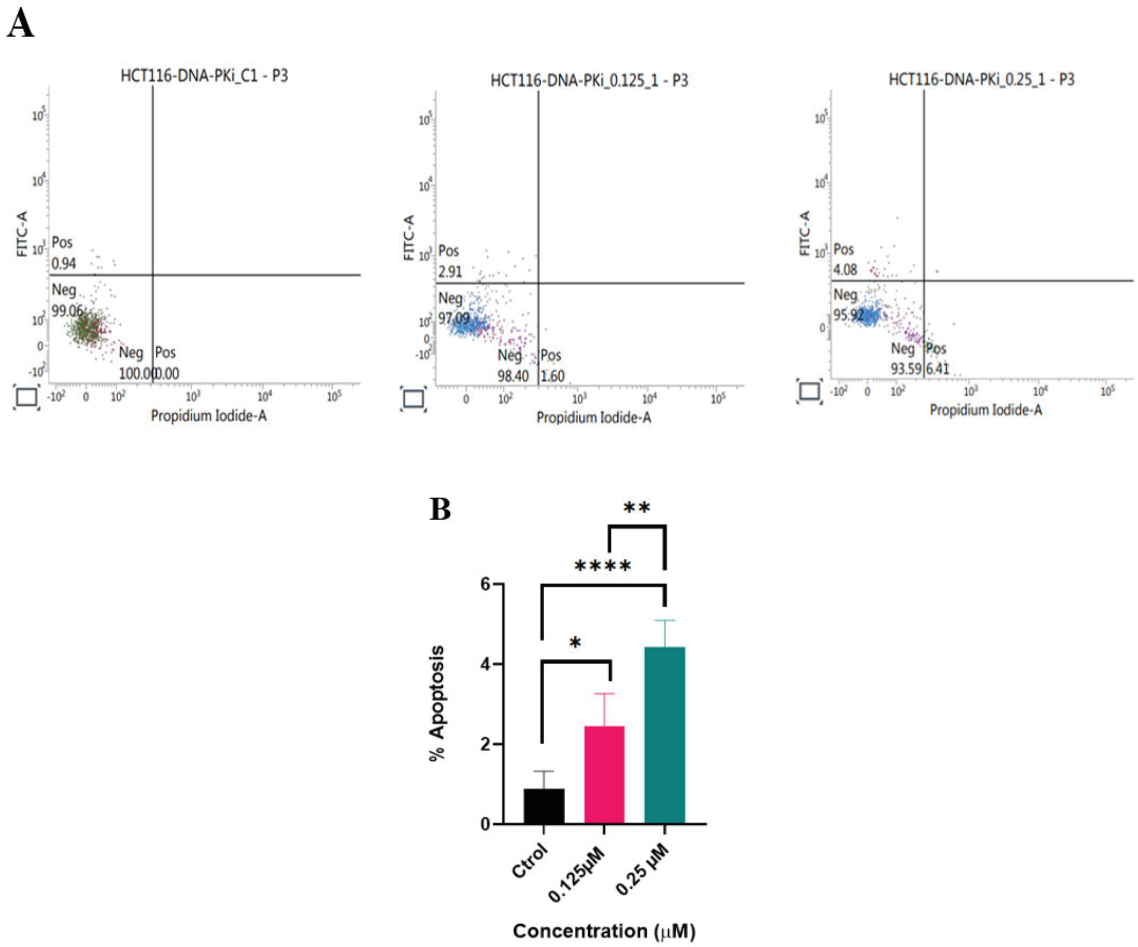


Figure 5. DNA-PKi induces apoptosis on HCT116 cells.

(A: HCT116 cells were treated with DNA-PKi at different concentrations for 24h and stained with Annexin V - FITC; cells were then counted by flow cytometry for apoptosis analysis. Upper right quadrant: Late apoptosis; upper left quadrant: Early apoptosis. Lower right quadrant: Necrosis; lower left quadrant: Viable cells.

B: Quantification of apoptotic cells. Data were analyzed by one-way ANOVA test and post-hoc Tukey test, $^{*}p < 0.01$, $^{****}p < 0.001$).

Cells were treated with DNA-PKi, evaluated with FITC Annexin V-PI staining, and analyzed by flow cytometry. Cells in the upper right quadrant represent late apoptosis, and cells in the upper left quadrant represent early apoptosis. Besides that, cells in the lower right quadrant represent necrosis cells, and cells in the lower left quadrant represent viable cells.

The impact of DNA-PKi on apoptosis was demonstrated in figure 5. The statistical analysis results revealed that the group treated with DNA-PKi at a concentration of 0.125µM after 24 hours had an average apoptosis rate of 2.46%, significantly higher than the corresponding value of the control group at 0.88% ($p = 0.0196$). However, a 4-fold increase in the total number of apoptosis cells was observed when treatment with DNA-PKi 0.25µM ($p < 0.0001$, *Figure 5*). The results of this study serve as preliminary data for future investigations into the anticancer potential of DNA-PKi.

DISCUSSION

The apoptosis process in cancer cells leads to the destruction of the cell's nuclear DNA, rendering the cells incapable of division, thereby reducing the proliferative potential of cancer cells. For cancer, promoting the apoptosis cell death pathway has been considered a strategy for anticancer drug development. In this regard, studies on the anticancer have shown KU-57788 to be possible to prevent cancer cell growth through activation of cell death pathways apoptosis. The results showed that DNA-PKi initially suppresses cell proliferation by induction the apoptosis pathway. A study indicated that inhibition of DNA-PK kinase activity increases apoptosis without affecting DNA repair in proliferating cancer cells [9]. These results were surprising because the percentage of apoptotic cells significantly varied with cell conditions. The presence of NU7026 DNA-PKi, which significantly decreased the ability to repair DNA damage, will likely undergo cell death. These studies corroborate DNA-PK's ability to inhibit apoptosis without affecting DNA repair activity in proliferating cells, where many DNA repair mechanisms are in use. However, in neural cells, where NHEJ is primarily responsible for repairing DNA damage (more precisely, DSBs), DNA-PK complex repair activity may be crucial to the survival of the cell.

Targeting DNA holds promise as an approach for future cancer therapies; however, DNA-PKi also presents certain

limitations. DNA-PKi have failed to induce significant changes in animal studies despite promising laboratory findings. One reason for these observations may be related to the limited solubility and poor pharmacokinetic properties of DNA-PKi. For instance, NU7441 exhibits poor absorption and rapid metabolism in mice, hindering its clinical application as a DNA-PKi [10]. Animal studies have shown that the concentration of NU7441 required for sensitizing cells to chemotherapy and radiation *in vitro* can be achieved and maintained within tumor tissue for at least 4 hours after administration. However, the compound's limited aqueous solubility has impeded further dose escalation. Based on promising pharmacokinetics, combination efficacy studies have been conducted, revealing that NU7441 effectively doubles the delay in tumor growth caused by etoposide without increasing toxicity to unacceptable levels. Similar results have been reported when using the colorectal cancer xenograft system, where IC86621 resulted in a fourfold enhancement of IR, leading to slowed tumor development and increased survival rates. However, due to its pharmacokinetic properties, this compound requires dosing every 4 hours, limiting its dose escalation potential [10].

CONCLUSION

DNA-PKi NU7441 suppressed cell proliferation and induced apoptosis in the HCT116 colon cancer cell line.

Acknowledgment: This research is funded by the 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, analysis, or decision to publish or prepare the manuscript. The authors declare to have no potential conflicts of interest.

REFERENCES

1. S Hu, F Lirussi, C Garrido, XY Ye, and T Xie. Small molecule DNA-PK inhibitors as potential cancer therapy: A patent review (2010-present). Feb 2021. DOI: 10.1080/13543776.2021.1866540.

2. Y Matsumoto. Development and evolution of DNA-Dependent Protein kinase inhibitors toward cancer therapy. *Int. J. Mol. Sci.* Jan 2022; 23(8). DOI: 10.3390/ijms23084264.

3. Double-stranded DNA induces the phosphorylation of several proteins including the 90 000 mol. wt. heat-shock protein in animal cell extracts. Accessed: Jan. 17, 2024. [Online]. Available: https://www.embopress.org/doi/epdf/10.1002/j.1460-2075.1985.tb02328.x.

4. BL Ruis, KR Fattah, and EA Hendrickson. The catalytic subunit of DNA-dependent protein kinase regulates proliferation. Telomere length, and genomic stability in human somatic cells. *Mol. Cell. Biol.* Oct 2008; 28(20):6182-6195. DOI: 10.1128/MCB. 00355-08.

5. JM Sekiguchi and DO Ferguson. DNA Double-strand break repair: A relentless hunt uncovers new prey. *Cell*. Jan 2006; 124(2):260-262. DOI: 10.1016/j.cell.2006.01.010.

6. M O'Driscoll et al. DNA ligase IV mutations identified in patients exhibiting developmental delay and immunodeficiency. *Mol. Cell.* Dec 2001; 8(6):1175-1185. DOI: 10.1016/S1097-2765(01)00408-7.

7. Identification of a defect in DNA ligase IV in a radiosensitive leukaemia patient - ScienceDirect. Accessed: Jan. 17, 2024. [Online]. Available: https://www.sciencedirect.com/science /article/pii/S096098229980311X.

8. D Moshous et al. Artemis, a Novel DNA Double-Strand Break Repair/V(D)J recombination protein, is mutated in human with severe combined immune deficiency. *Cell*. Apr 2001; 105(2):177-186. DOI: 10.1016/S0092-8674(01)00309-9.

9. A Cardinale et al. DNA repair protein DNA-PK protects PC12 cells from oxidative stress-induced apoptosis involving AKT phosphorylation. *Mol Biol Rep*. Feb 2022; 49(2):1089-1101. DOI: 10.1007/s11033-021-06934-5.

10. Y Zhao et al. Preclinical evaluation of a potent novel DNA-dependent protein kinase inhibitor NU7441. *Cancer Res*. May 2006; 66(10):5354-5362. DOI: 10.1158/ 0008-5472.CAN-05-4275.