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ORIGINAL PAPER

Pharmacological Insights: Mitochondrial ROS Generation by FNC (Azvudine) in Dalton's Lymphoma Cells Revealed by Super Resolution Imaging

Naveen Kumar[1] · Vikram Delu[2] · Ilya Ulasov[3] · Sanjay Kumar[4] · Rishi Kant Singh[4] · Sandeep Kumar[4] · Alok Shukla[4] · Anand Kumar Patel[4] · Lokesh Yadav[4] · Ruchi Tiwari[4] · Kumari Rachana[4] · Shivashish Priyadarshi Mohanta[4] · Varsha Singh[4] · Anuradha Yadav[4] · Kaushalendra Kaushalendra[5] · Arbind Acharya[4]

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Abstract

Nucleoside analogs are a common form of chemotherapy that disrupts DNA replication and repair, leading to cell cycle arrest and apoptosis. Reactive oxygen species (ROS) production is a significant mechanism through which these drugs exert their anticancer effects. This study investigated a new nucleoside analog called FNC or Azvudine, and its impact on ROS production and cell viability in Dalton's lymphoma (DL) cells. The study found that FNC treatment resulted in a time- and dose-dependent increase in ROS levels in DL cells. After 15 and 30 min of treatment with 2 and 1 mg/ml of FNC, mitochondrial ROS production was observed in DL cells. Furthermore, prolonged exposure to FNC caused structural alterations and DNA damage in DL cells. The results suggest that FNC's ability to impair DL cell viability may be due to its induction of ROS production and indicate a need for further investigation.

Keywords Cell survival · Azvudine · DNA damage · DNA replication · Nucleoside analogs

Introduction

Nucleoside analogs have revolutionized antiviral and cancer therapy by interfering with DNA synthesis, a critical process for cancer cells. ROS, highly reactive molecules generated in cells during normal metabolism, can damage DNA, proteins, and lipids, leading to cell death [1]. In cancer cells, nucleoside analogs can increase ROS production by inhibiting the enzymes involved in nucleotide synthesis, leading to a decrease in nucleotide levels available for DNA synthesis [2]. This decrease leads to the activation of the ROS-producing enzyme, NADPH oxidase, which generates superoxide radicals that converted into other ROS, such as hydrogen peroxide and hydroxyl radicals. The increased levels of ROS generated by nucleoside analogs can cause oxidative stress in cancer cells, resulting in cell death. Moreover, nucleoside analogs can disrupt the mitochondrial electron transport chain, leading to an increase in ROS production in the mitochondria [2, 3]. This increase can damage mitochondrial DNA and proteins, causing mitochondrial dysfunction and cell death. The timing of ROS generation can vary depending on the specific nucleoside analog being used and can be classified as immediate (within seconds to minutes) or delayed (hours to days) [3, 4]. The classification of ROS generation can also be based on the mechanism of action of the nucleoside analog. Nucleoside analogs gemcitabine induces ROS within 10 min of exposure to pancreatic cancer, which in long-term precedes the nuclear accumulation of NF-κB and HIF-1α [5]. Rapamycin another nucleoside analogs induced

✉ Arbind Acharya
acharya@bhu.ac.in

1 Department of Zoology, School of Basic and Applied Sciences, Raffles University, Neemrana, Rajasthan 301705, India

2 Senior Analyst, Pashmina Certification Centre, Wildlife Institute of India (WII), Dehradun, Uttarakhand 248001, India

3 Group of Experimental Biotherapy and Diagnostic, Department of Advanced Materials, Institute for Regenerative Medicine, Sechenov First Moscow State Medical University, Moscow 119991, Russia

4 Department of Zoology, Institute of Science, Banaras Hindu University (BHU), Varanasi, Uttar Pradesh 221005, India

5 Department of Zoology, Pachhunga University College Campus, Mizoram University, Aizawl 796001, India

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ROS in gastric cancer cells in time dependent manner, from 10 min of exposure to upto 6 h [6]. Moreover, nucleoside analog nelarabine induces ROS by targeting aberrant PI3K/AKT/mTOR signaling pathway in the T cell acute lymphoblastic leukemia [7]. Nucleoside analogs like gemcitabine and cytarabine, require intracellular phosphorylation to generate ROS and activate the mitochondrial respiratory chain [8, 9]. Nucleoside analog prodrugs such as didanosine and ribavirin directly target the mitochondrial respiratory chain, leading to immediate ROS generation [10, 11]. The timing of ROS generation and the mechanisms involved depend on the cell type, dose, and exposure time of the nucleoside analog.

The novel nucleoside analog, FNC, 4′-azido-2′-deoxy-2′-fluoro(arbino)cytidine, a modified version of the nucleoside cytidine, has been studied for its potential as a therapeutic agent against retroviral infections and various cancer cell lines [12, 13]. It has been approved for high-load or drug resistant HIV patients under the name Azvudine. However, little is known about the ROS generating capacity of FNC, which is critical to understanding its potential influence and might be key mechanism of FNC action. In this study, we investigate the ROS generation capacity of FNC and its activation site in DL cells, shedding light on the potential effects of FNC on cellular health and the implications for its use in antiviral and cancer therapies. Understanding the ROS mechanism is essential to elucidating the potential therapeutic implications of FNC and its impact on cellular health. The activation of FNC might be dependent on the release or interaction of the azido or fluoro group, and it is unknown whether the activated ROS is of immediate type or delayed type.

Materials and Methods

Materials

For this study, FNC obtained from Granlen Inc. located in China, and dissolved in DMSO (dimethyl sulfoxide). The DCFDA (2'-7'-Dichlorodihydrofluorescein diacetate), DAPI (4',6-diamidino-2-phenylindole), and Mito-Tracker sourced from Thermo-Fisher Scientific in the USA. Other chemicals procured from Hi-media or SRL, India. The quality and purity of reagents ensured through proper checking before ordering and usage. All experiments conducted using high-quality, standardized materials and procedures to guarantee the accuracy and reproducibility of the results.

Tumor Model

For developing cancer model in this study, pathogen-free Balb/c (H2d) mice aged 8–12 weeks and weighing 25 ± 2 gm used. To mimic the characteristics of T-cell lymphoma in humans, the DL tumor model selected as a dependable model for the assessment of treatment efficacy. To induce DL, mice were intraperitoneally (i.p.) transplanted with 1×10^6 DL cells in 1 ml chilled PBS, following the protocol described elsewhere [14]. The use of i.p. transplantation ensures high rates of DL proliferation and growth, making it an ideal model for assessing the effectiveness of potential treatments. Additionally, it allows for easy monitoring of tumor growth and metastasis.

ROS Assay through Live Cell Imaging

To measure ROS generation in DL cells, DCFH-DA used as a fluorescent probe. DCFH-DA is cleaved by nonspecific esterase to generate DCFH and is then quantitatively oxidized by ROS to form fluorescent DCF [15]. To determine the intracellular ROS, the following protocol used. DL cells extracted from mice, washed thrice with complete RPMI-1640 media, and dissolved in PBS (extracted cells) and used throughout the study. The cells incubated with DCFH-DA for 10 min, and then FNC added, followed by immediate photography of the cells (0 min) through live cell imaging (cell discoverer 7, Carl Zeiss). The cells then tracked for 60 min, and their fluorescence images captured at 15-min intervals (15, 30, and 60) through live cell imaging, however cells were only excited at given intervals to escalate artifact-induced emission. Excitation of the cells performed at 473 nm and their emission detected at 520 nm. Three hundred cells manually classified based on the type of ROS present in the cell, as observed from the photographs obtained. All assay performed in triplicate in three independent experiments.

Flow Cytometry for ROS Assessment

ROS generation further complemented using flow cytometry (CytoFLEX LX, Backman coulter) at the same time intervals. Briefly, DL cells treated with 1 and 2 mg/ml of FNC for 15, 30, and 60 min in separate wells, washed twice with PBS, followed by incubation with DCF-DA for 10 min prior to flow cytometry analysis. The flow cytometry data obtained in this study used to complement the live cell imaging data, providing a more comprehensive understanding of the cellular response to FNC exposure and the quantitative generation of ROS in DL cells.

ROS Assay through Laser Scanning Super-resolution Microscopy (LS-SRM)

Intracellular ROS quantified using DCFH-DA as described in the (Akira Onodera et al., 2015). The extracted DL

cells treated with 1 µM Mito Tracker for 30 min. After washing twice with PBS kept in the incubator chamber of LS-SRM (SP8 STED, Leica Microsystems), and visualized at 0, 15, 30, and 60 min after FNC exposure. The microscopic data analyzed using Zen mode, 3.0 software. This method provided a quantitative analysis of the ROS generated in the cells, offering a more comprehensive understanding of the impact of FNC on ROS production in DL cells. DCF or Mito Tracker were excited at 473 nm or 559 nm, respectively, and their emission detected at 520 nm or 598 nm, respectively. This LS-SRM allowed for accurate measurement of the levels and location of intracellular ROS and provided valuable insight into the mechanism of FNC. LS-SRM is a powerful technique that enables visualization of localized fluorescence with spatial resolution beyond the diffraction limit of optical microscopy [16]. This method offers distinct advantages over traditional fluorescence and live cell imaging, including the ability to generate high-resolution images of living cells without requiring complex optical setups, and facilitates more comprehensive investigations of nano-cell interactions.

Mitochondrial Membrane Potential (MMP) Assessment through Fluorescence Microscopy and Flow Cytometry

To evaluate the impact of FNC on the MMP of DL cells, we employed a combination of fluorescence microscopy and flow cytometry techniques. Specifically, we extracted DL cells and treated them with FNC at concentrations of 1 and 2 mg/ml, followed by three washes with PBS. Next, we stained the cells with Rhodamine-123 (Rh-123) (100 µg/ml) for 10 min and analyzed them using fluorescence microscopy (BX63, Olympus) and flow cytometry. These methods allowed us to gain valuable insights into the potential impact of FNC on MMP in DL cells.

Cell Viability Analysis through Dilution-Cum-Trypan (DCT) Assay

To evaluate the impact of FNC exposure on cellular viability, DL cells treated with FNC for varying time points ranging from 15 to 60 min, followed by incubation for up to 6 h. Viability of the cells assessed using our recently developed DCT assay. Specifically, cells stained with a standard trypan blue concentration (0.4%) for 3 min, washed thrice with PBS, and the number of viable cells calculated by determining the ratio of live cells to total cells (unpublished protocol). This method provides a quantitative assessment of cell viability for impact of FNC on cell viability.

$$\text{Cell viability} = \frac{\text{Number of live cells}}{\text{Total number of cells}} \times 100$$

Scanning Electron Microscopy for Cellular Structure

The impact of intracellular FNC-induced ROS on cell morphology analyzed using scanning electron microscopy (SEM). The cells treated with FNC washed at 0, 15, 30 and 60 min, up to 6 h with chilled PBS and then procced for sample preparation for SEM. To ensure accurate and reliable results, a stringent sample preparation protocol was implemented, were treated with glutaraldehyde in 0.1 M PBS for 30 min and post-fixed with OsO4 overnight [17]. Subsequently, the sample underwent dehydration with a series of ethanol and coated with gold-palladium (Quantum technology-SC7620) to facilitate SEM imaging at 25 kV at LV under a Zeiss-EVO LS-10 microscope located at the Department of Zoology, Banaras Hindu University, India. This meticulous and detailed sample preparation procedure enabled a thorough analysis and characterization of the cellular structure and morphology, thereby offering valuable insights into the structural changes induced by ROS.

Fluorescence Microscopy for Apoptosis and ROS Colocalization

Following treatment with FNC, DL cells incubated with DAPI and DCF-DA for 10 min and subjected to fluorescence microscopy to assess cellular morphology, chromatin status and ROS for timepoints 15, 30, 60 min and up to 6 h. Specifically, cells were excited at 473 nm and emission detected at 520 nm for DCF-DA, while DAPI was excited at 358 nm and emission detected at 461 nm. This approach enables visualization and quantification of cellular changes induced by FNC exposure.

Statistical Analysis

The data obtained from the experiments analyzed using GraphPad Prism 5.01 software (GraphPad Software, CA). The results presented as mean ± standard deviation (SD) for each group, unless otherwise mentioned. The statistical significance of the differences assessed by a one-way ANOVA followed by Tukey post-test. The significance level of $*p < 0.05$ was set to indicate statistically significant. The utilization of the statistical analyses ensured robustness and reliability of the results, thereby facilitating a clear interpretation of the experimental outcomes.

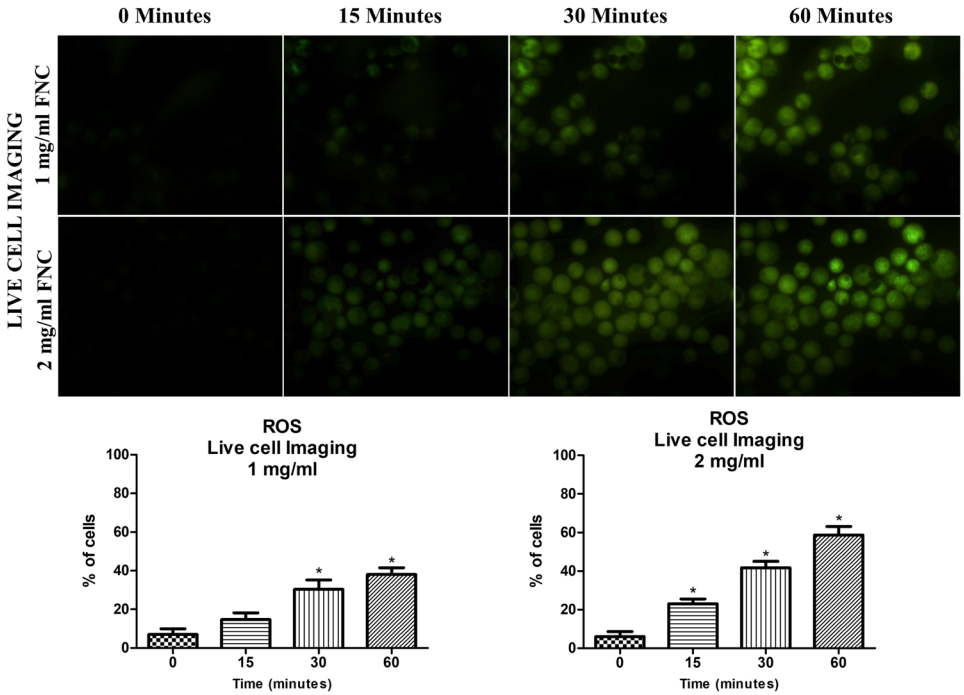

Fig. 1 Depicts ROS levels in DL cells treated with FNC at concentrations of 1 and 2 mg/ml, captured through live cell imaging over a time course (0, 15, 30, 60 min) at a consistent site. ROS quantification involved counting a total of three hundred cells. Error bars represent standard deviations. Significance was determined at $p < 0.05\%$ compared to the initial time point (0)

Results

FNC Induces Time and Dose Dependent ROS Generation in DL Cells: Evidence from Live Cell Imaging and Flow Cytometry

To explore the impact of FNC on ROS production in DL cells, we conducted DCF-DA assay and visualized ROS levels through live-cell imaging. We monitored ROS levels in 300 cells at four different time points (0, 15, 30, and 60 min) after treatment with FNC at two different doses (1 and 2 mg/ml) and observed a dose- and time-dependent increase in ROS levels in FNC-treated DL cells (Fig. 1). At both doses, FNC triggered a gradual increase in ROS fluorescence that became significant after 15 and 30 min after treatment, respectively at 2 and 1 mg/ml. These findings were corroborated by flow cytometry, which revealed a significant elevation in ROS levels (after 15 and 30 min respectively at 2 and 1 mg/ml) in FNC-treated DL cells compared to ROS at 0 min, with 1 and 2 mg/ml FNC inducing 45%, and 53% ROS levels at 60 min, respectively (Fig. 2).

FNC Induces Mitochondrial ROS in DL Cells

Confocal and flow cytometry analyses demonstrated that FNC induced significant ROS in DL cells. However, the subcellular localization of ROS induction remained unclear. To address this, LS-SRM used to visualize the colocalization of DCF-DA and Mito tracker dyes. DCF-DA and Mito Tracker are fluorescent dyes commonly used to study oxidative stress and mitochondrial function, respectively. Mito Tracker is a family of fluorescent dyes that selectively stain mitochondria in live cells. These dyes taken up by cells and accumulate in active mitochondria, binding to the membrane and emitting red fluorescence. While normal fluorescence microscopy at the highest resolution cannot differentiate localized fluorescence, LS-SRM can visualize fluorescence in specific cellular locations. Our results showed that FNC induced ROS in the mitochondria after 15 and 30 min of treatment at both concentrations, with higher ROS levels at later time points (Fig. 3). LS-SRM revealed the mitochondrial ROS induction was dose and time-dependent, these findings suggest that FNC-induced ROS generation occurs through mitochondrial pathways (delayed type), and the potential pathways involved in drug-induced ROS generation.

FNC Induced Mitochondrial ROS Leads to Decrease in MMP of DL Cells

LS-SRM showed that FNC induced mitochondrial ROS levels in DL cells. Given these findings, we aimed to examine whether the observed increase in mitochondrial ROS resulted in changes to the MMP in DL cells. To achieve this, we employed fluorescence microscopy and flow cytometry techniques (Fig. 4). Our results indicate that the induction of mitochondrial ROS by FNC led to a significant decrease in MMP in DL cells, as evidenced by fluorescence microscopy. Furthermore, flow cytometry analyses revealed that FNC exposure at concentrations of 2

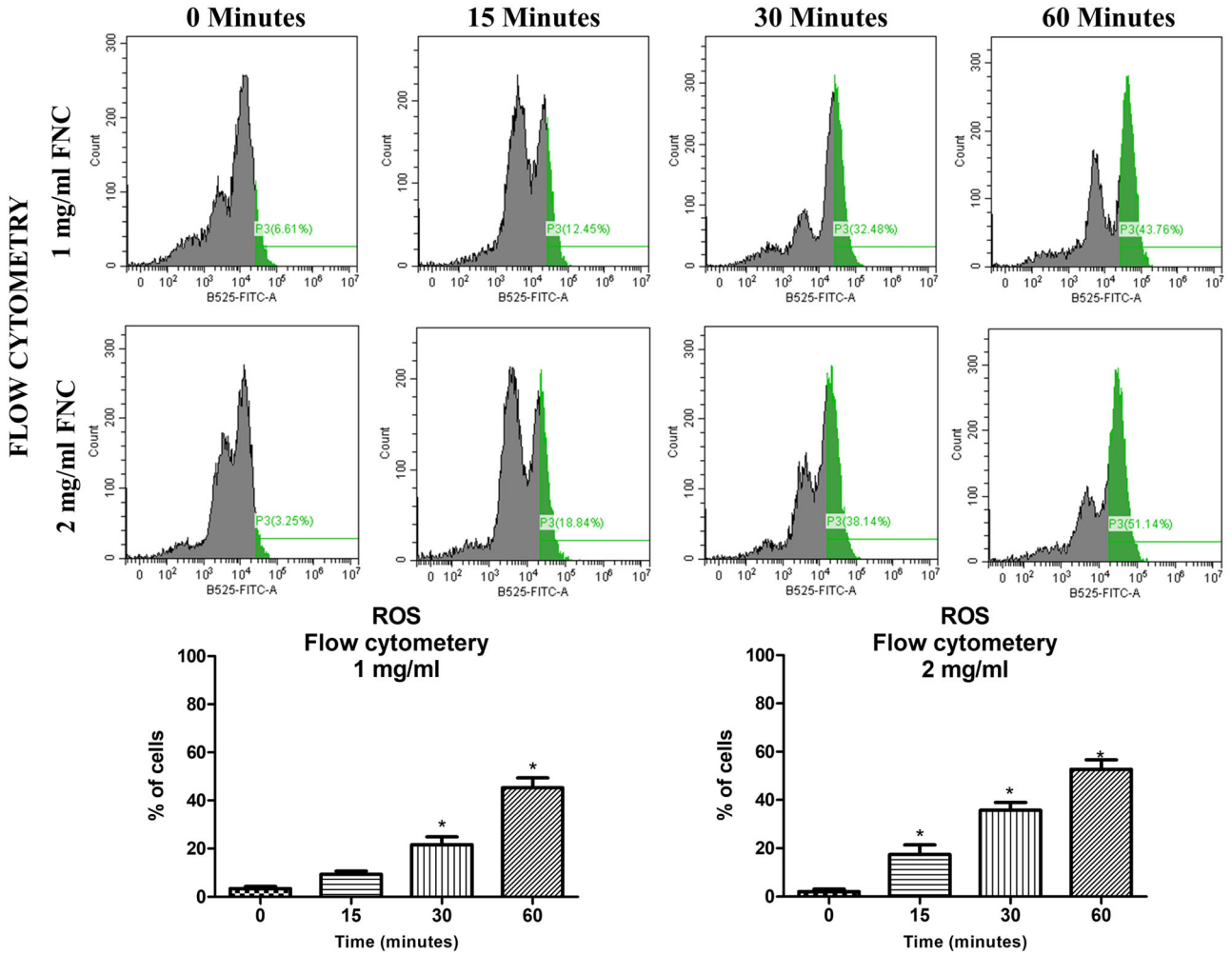

Fig. 2 Quantification of ROS in DL cells using flow cytometry. DL cells treated with FNC at concentrations of 1 and 2 mg/ml and analyzed at 0, 15, 30, and 60 min. Error bars represent standard deviations. Significance was determined at $p < 0.05\%$ compared to the initial time point (0)

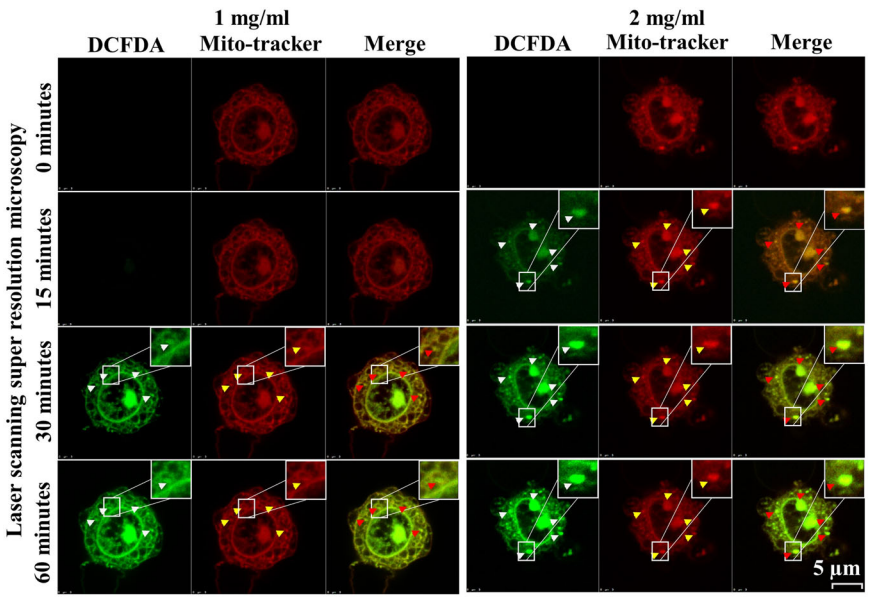

Fig. 3 FNC-induced ROS source in DL cells identified using laser scanning super resolution microscopy. A single cell (60× and then zoomed for single cell) stained with DCFDA and Mito Tracker and imaged at the same site at 0, 15, 30, 60 min after treatment with FNC at concentrations of 1 and 2 mg/ml. The inset depicts the localization of ROS generation in mitochondria

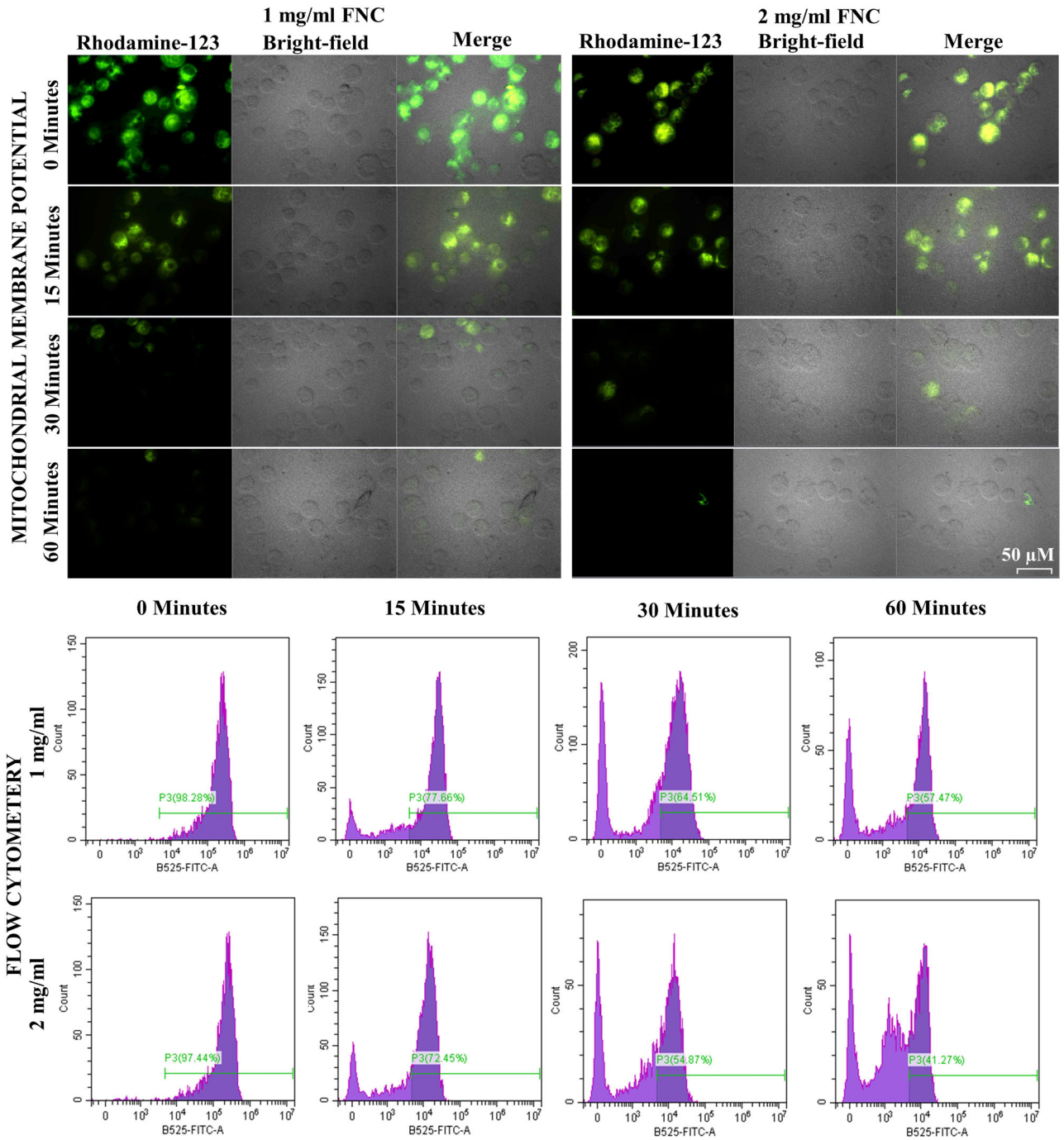

Fig. 4 The impact of FNC on the mitochondrial function in DL cells assayed using fluorescence microscopy and flow cytometry. Our results demonstrate that FNC induced ROS in the mitochondria, leading to a notable change in the mitochondrial membrane potential at both 1 and 2 mg/ml concentrations of FNC

and 1 mg/ml resulted in a 41 and 57% decrease in MMP, respectively, in DL cells.

FNC Alters the DL Cells Viability in Long Term

Next, to investigate the potential dose- and time-dependent influence of FNC on the viability of DL cells, we performed DCT assays. The principle of DCT assay is based on the fact that live cells with intact membranes will not be stained, while cells with compromised membranes will allow the entry of trypan blue dye, resulting in staining. Therefore, staining by DCT can used to identify and quantify dead or dying cells in a sample. FNC has found to induce mitochondrial ROS production in DL cells, we

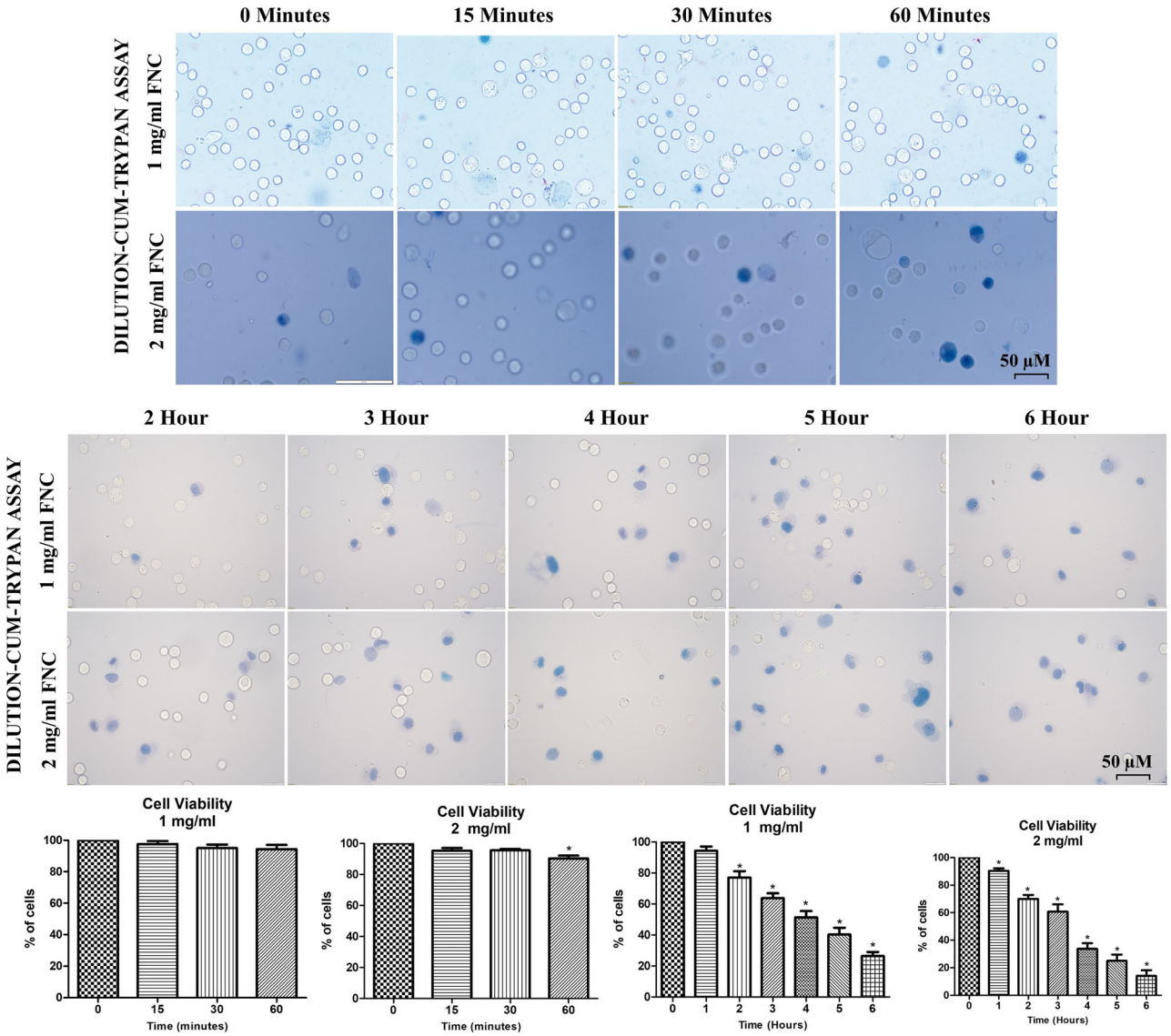

Fig. 5 FNC treated DL cells viability assessed using the dilution-cum-trypan assay at 0, 15, 30, 60 min up to 6 h. The white and blue cells, respectively indicates live and dead cells. A total of three hundred cells counted for calculating cell viability. Error bars represent standard deviations. Significance was determined at $p < 0.05\%$ compared to the initial time point (0)

hypothesized that its exposure would lead to a decline in cellular viability. We first exposed DL cells to 1 or 2 mg/ml of FNC for 15, 30, and 60 min, and up to 6 h, before measuring their viability using DCT. Interestingly, while we observed no significant changes in viability at 60 min in the 1 mg/ml group, the 2 mg/ml group showed a small but significant decline at this time point, despite the observed increase in ROS levels after 15 and 30 min of FNC exposure (Fig. 5). We then extended the exposure time up to 6 h and repeated the DCT assay. Our results revealed a progressive decline in cellular viability, starting at 1 h for 2 mg/ml and 2 h for 1 mg/ml of FNC exposure. The viability continued to decrease with longer exposure times, reaching 14 and 26% at 6 h for 2 and 1 mg/ml of FNC, respectively. These findings suggest that FNC impairs the viability of DL cells in a dose- and time-dependent manner, through ROS-mediated mechanisms.

FNC Treatment Induces Structural Alteration in DL Cells in Long-term

Further, to investigate whether FNC-induced ROS generation alters the structure and viability of DL cells, we performed a time course study of FNC exposure. We already reported that FNC did not affect cell viability for up to 30 and 60 min in the 2 and 1 mg/ml FNC group, with a continued decrease up to 6 h. Here we show that FNC treatment did not induce any structural alteration in DL cells for up to

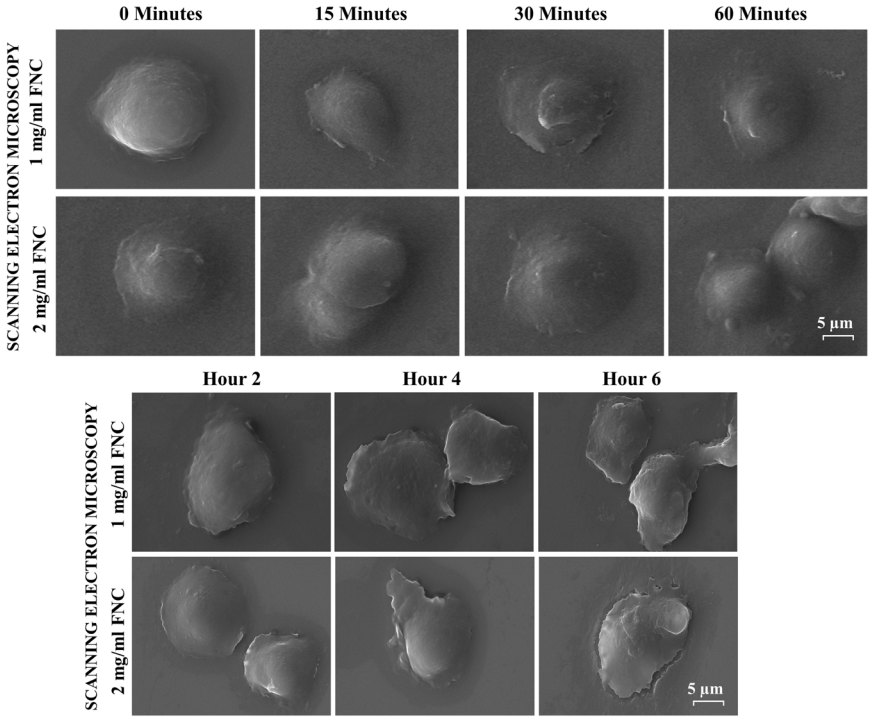

Fig. 6 The impact of FNC-induced ROS on cellular structure assessed by scanning electron microscopy. DL cells treated with FNC at a concentration of 1 and 2 mg/ml and visualized at various time points ranging from 0, 15, 30, 60 min up to 6 h

1 h at both concentrations, as assessed by SEM (Fig. 6). However, after 2 h for both concentrations, we observed structural changes in the cells exposed to FNC, suggesting that FNC triggers ROS production in DL cells but takes longer to affect their viability and morphology. These results indicate that ROS might be a key factor in FNC-mediated cytotoxicity in DL cells.

FNC Mediated ROS Generation Cause Chromatin Condensation or Nuclear Fragmentation

Next, we aimed to investigate the effects of FNC-induced oxidative stress on DNA integrity of DL cells. FNC is a nucleoside analog that can incorporated into DNA and result in DNA or nuclear fragmentation, which might be key reason behind the generated ROS. To assess the impact of FNC on DNA integrity, we conducted a DAPI/DCF-DA assay up to 1 h of exposure to FNC. Surprisingly, our results showed that FNC induced a significant increase in ROS levels without causing significant DNA or nuclear fragmentation in DL cells up to 1 h (Fig. 7). This led us to hypothesize that FNC-induced ROS generation may take longer to cause DNA fragmentation or nuclear condensation, as a significant higher viability reduction observed in later time points, as shown in the bottom of Fig. 7. To evaluate this hypothesis, we extended the duration of the DAPI/DCF-DA assay up to 6 h. Our results indicated that a significant DNA fragmentation observed in cells after 2 h at both 1 and 2 mg/ml of FNC concentration. This suggests that FNC follows a similar mechanism of action, where after phosphorylation, it incorporated into DNA and leads to chain termination, causing DNA fragmentation, however further study is required.

Discussion

Nucleoside analogs have widely used in antiviral and anticancer therapies. Nucleoside analogs are structurally similar to naturally occurring nucleotides and can interfere with DNA replication and repair by incorporating into DNA or RNA, leading to cell cycle arrest and apoptosis. The ability of nucleoside analogs to induce ROS remained an important mechanism for their anticancer or antiviral effects [3]. Nucleoside analogs have also shown to induce ROS production, especially in cancer cells [2, 3]. ROS are highly reactive oxygen-containing molecules that can cause oxidative damage to cellular macromolecules, such as proteins, lipids, and DNA, leading to cell death. The ability of nucleoside analogs to induce ROS has recently gained attention as an important mechanism underlying their anticancer effects [2, 3]. Other nucleoside analogs have studied for their ROS-inducing properties and potential use in cancer treatment. For example, gemcitabine (di-fluorinated at 2'-position), a widely used chemotherapeutic drug, has been shown to induce ROS generation and apoptosis in cancer cells [5]. Similarly, 5-fluorouracil (fluorinated at 5'-position), another commonly used chemotherapeutic drug, has been shown to induce ROS generation and DNA damage in cancer cells [2, 18]. Other nucleoside analogs

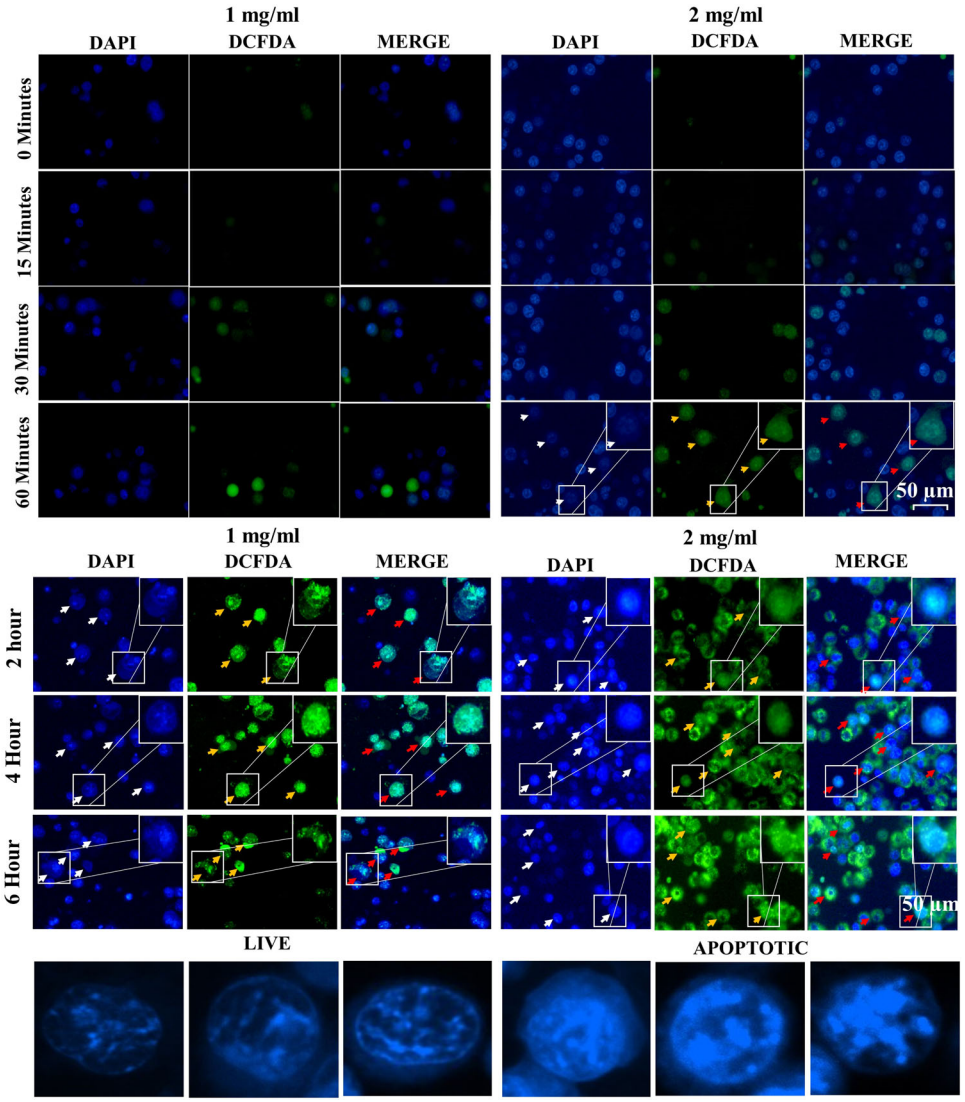

Fig. 7 Evaluation of the impact of FNC-induced ROS on the DNA of DL cells conducted using DAPI/DCFDA staining. DL cells treated with FNC at concentrations of 1 and 2 mg/ml and visualized at 0, 15, 30, 60 min up to 6 h. The inset illustrates a cell exhibiting increased ROS levels, along with DNA fragmentation and chromatin condensation

such as cladribine, cytarabine, and fludarabine have also been reported to induce ROS generation and cytotoxicity in cancer cells [19–21]. These findings suggest that nucleoside analogs with ROS-inducing properties may have broad potential for cancer treatment.

In our previous investigation, our findings elucidate that FNC induces dose-dependent mitochondrial-mediated apoptosis and triggers reactive oxygen species (ROS) activation in DL cells in vitro [22] as well as in vivo [23]. Nevertheless, the precise site of ROS activation remains to be comprehensively elucidated. In this study, the ROS inducing ability of novel nucleoside analog FNC in T-cell Lymphoma, specially, DL cells investigated, along with potential mechanism, and impact on cell viability etc. Live cell imaging and flow cytometry revealed that FNC induced a dose- and time-dependent increase in ROS levels in DL cells, with significant ROS production observed after 15 and 30 min at 2 and 1 mg/ml of FNC, respectively (Figs. 1 and 2). Moreover, LS-SRM demonstrated the source of this ROS and showed that FNC induced mitochondrial ROS production in DL cells after 15 and 30 min of treatment at 2 and 1 mg/ml of FNC (Fig. 3). Moreover, Rh-123 study showed that FNC treatments lead to decreases in MMP of cells (Fig. 4). Next, we want to assess the impact of FNC induced mitochondrial ROS on cell viability and DCT assays revealed a significant reduction in viability starting from 1 h at 2 mg/ml and 2 h at 1 mg/ml of FNC, with the viability decreasing progressively with longer exposure times (Fig. 5). SEM revealed that FNC did not induce any structural alteration in DL cells for up to 1 h at both concentrations (Fig. 6). However, after 2 h, structural changes observed in the cells exposed to FNC, indicating that FNC triggers ROS production causes structural changes in DL cells. Lastly, DAPI/DCF-DA staining revealed that FNC induced ROS causes DNA damage or nuclear fragmentation after 2 h at both concentrations (Fig. 7).

FNC contains two functional groups on sugar, Azido at 4'-poistion and fluorine at 2'-poistion, which could be the reason behind the mitochondrial ROS induction observed in FNC treatment. Azido and fluorine groups, when present on sugar molecules, can induce the generation of ROS in cancer cells, particularly in the mitochondria [24]. Azido group, which contains three nitrogen atoms, is a strong electron-withdrawing group that can interact with mitochondrial respiratory complexes and disrupt the electron transport chain. This can lead to the production of superoxide radicals and other ROS. For example, azidothymidine (Azido at 3'-poistion), a nucleoside analog used to treat HIV, has been found to induce mitochondrial ROS generation and trigger apoptosis in cancer cells [25]. Fluorine group, on the other hand, can interact with oxygen molecules and form highly reactive fluorine species that can cause oxidative damage to cellular components [25, 26]. Fluorinated nucleoside analogs, such as 5-fluorouracil, have shown to induce mitochondrial ROS generation in cancer cells. 5-fluorouracil has been found to increase mitochondrial ROS production by inhibiting the activity of mitochondrial respiratory complexes [27]. Furthermore, the presence of both azido and fluorine groups on sugar molecules can enhance the ROS-generating ability of the molecule. Therefore, mitochondrial ROS induction by FNC can attributed to its azido and fluorine group. These Nucleoside analogs, commonly used in cancer chemotherapy, induce ROS generation in cancer cells, leading to mitochondrial dysfunction and cell death. They disrupt the mitochondrial electron transport chain by inhibiting the activity of respiratory complexes, leading to electron accumulation and ROS production. Additionally, nucleoside analogs can directly interact with mitochondrial DNA and proteins, causing ROS generation. Mitochondrial dysfunction and ROS generation have linked to drug resistance in cancer cells, prompting the search for novel strategies to target resistant cancer cells.

ROS activation within mitochondria is a crucial trigger for the intrinsic pathway of apoptosis. Elevated ROS, primarily induced by cellular stress, prompts mitochondrial dysfunction, resulting in the release of cytochrome c [28, 29]. The subsequent formation of the apoptosome, involving cytochrome c and apoptotic protease-activating factor 1 (Apaf-1), activates caspase-9, initiating a cascade leading to DNA fragmentation and cellular dismantling [30]. Our recent study reinforces this connection, showing that FNC induces mitochondrial-mediated apoptosis [22, 23]. FNC treatment led to a notable release of cytochrome c, downregulation of anti-apoptotic genes Bcl-2 and Bcl-xl, and upregulation of pro-apoptotic gene Bax. Elevated caspase-3 and caspase-9 levels further underscore the role of ROS in orchestrating mitochondrial-mediated apoptosis, highlighting its ROS mediated activation of mitochondrial-mediated apoptosis maintaining in DL cells.

Conclusion

This study provides evidence that FNC induces time- and dose-dependent ROS generation in DL cells, with mitochondrial ROS being a key source of FNC-induced ROS. Furthermore, FNC impairs the viability of DL cells in a dose- and time-dependent manner, through ROS-mediated mechanisms, inducing significant structural alterations or DNA damage at longer exposure times. These findings suggest that FNC or other nucleoside analogs with ROS-inducing properties may hold potential as therapeutic agents for cancer treatment.

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Author Contributions Author contributions N.K. - Conceptualization, Methodology, Investigation, Writing- Original Draft, Writing-Review & Editing, Data analysis; V.D., I.U., S.K., R.K.S., S.K., A.S., A.K.P., L.Y., R.T., K.R., S.P.M., V.S., A.Y., K.K. - Methodology, Writing-Review & Editing; A.A., -Writing Original Draft, Writing-Review & Editing; Conceptualization, Supervision.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

Abbreviations

DAPI	4′,6-diamidino-2-phenylindole
DCFDA	2'-7'-Dichlorodihydrofluorescein diacetate
DCT	Dilution-cum-trypan assay
DL	Dalton's Lymphoma
FNC	4′-azido-2′-deoxy-2′-fluoro(arbino)cytidine
Rh-123	Rhodamine-123
ROS	Reactive oxygen species

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