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Subcellular Targeting Strategies Using Killer Red in Leukemia: Investigating Chemotherapy-Induced Ros

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Abstract: Reactive oxygen species (ROS) in leukemia checks the potential target molecules of cancer therapy. A photosensitizer called KillerRed is used to control ROS to gain a deeper understanding of ROS-induced cellular mechanisms, ROS effects on disease advancement, and the effects of therapy. Patients with leukemia are facing one of the greatest challenges in oncology due to the fast oxidative course of the disease and a high tendency to develop drug resistance. The study focuses on the application of ROS to overcome these challenges by causing cytotoxic effects in leukemia cells. It describes the development and characterization of a plasmid encoding an NLS-fused KillerRed, which favors the production of ROS in the nucleus. The effectiveness proved to be functional through different experiments, including a series of cytotoxicity assays, live-cell imaging, and gel electrophoresis, showing that the construct was precisely integrated and performed as expected in generating higher ROS levels and inducing apoptosis. This suggests that specific modulation of ROS leads to reduced viability of the leukemia cells and contributes to understanding new approaches to improve existing therapeutic options by overcoming the mechanisms of resistance to drugs. In particular, the results emphasize the fundamental role of ROS both as signaling molecules and mediators of damage to the cell and corroborate the relevance of ROS in the design of targeted therapeutic strategies for cancer. It also contributes to a deeper knowledge of the dynamic of ROS in cancer and to the possibility of intervening in the balance of ROS to achieve a therapeutic action in leukemia.

Keywords: Reactive Oxygen Species; ROS; Leukemia; KillerRed; Photosensitizer

Introduction

Leukemia, particularly acute myeloid leukemia (AML), is a hematological malignancy characterized by the uncontrolled proliferation of abnormal white blood cells, often leading to poor prognosis and treatment resistance (Juliusson et al., 2009; Döhner et al., 2015). Conventional therapies like chemotherapy and hematopoietic stem cell transplantation have limitations, including relapse and treatment-associated toxicity (Barbosa & Deshpande, 2023). Consequently, novel therapeutic targets are under investigation. One such target is reactive oxygen species (ROS), which, while traditionally considered metabolic byproducts, are now recognized as key regulators of cellular signaling and cancer progression (Trachootham et al., 2009; Reczek & Chandel, 2015). Elevated ROS levels in leukemic cells contribute to genomic instability and tumor progression, but excessive ROS can also induce cell death, making them a double-edged sword in cancer biology (Sallmyr et al., 2008). This study explores the use of KillerRed, a genetically encoded photosensitizer that generates ROS upon light activation, enabling precise modulation of intracellular ROS (Bulina et al., 2006). By employing KillerRed in leukemic models, we aim to investigate ROS-mediated signaling mechanisms and assess its potential in overcoming drug resistance in AML. Modulating reactive oxygen species (ROS) has become a promising strategy in cancer therapy, particularly in leukemia, where elevated ROS levels can selectively induce apoptosis in malignant cells without harming normal tissue. Therapeutic agents such as arsenic trioxide and anthracyclines exploit this mechanism by disrupting mitochondrial function and altering redox balance, overwhelming the antioxidant capacity of leuke-

mic cells and promoting their death (Gorrini et al., 2013; Kim et al., 2019). This approach has proven especially effective in drug-resistant leukemia, such as acute promyelocytic leukemia (APL), where arsenic trioxide induces cell death even in cases unresponsive to all-trans retinoic acid (ATRA) (Mannan et al., 2021). Additionally, ROS modulation impacts the leukemic microenvironment, enhancing therapeutic response by reducing resistance and relapse through microenvironmental remodeling (Gao et al., 2020). A key tool for studying ROS dynamics is KillerRed, a genetically encoded photosensitizer that generates singlet oxygen upon green light exposure (540–580 nm), allowing for precise spatiotemporal control of oxidative stress in live cells. KillerRed serves both as a fluorescent marker and a ROS inducer, enabling researchers to investigate ROS-mediated cell signaling, damage, and apoptosis in targeted subcellular regions (Bulina et al., 2006). Its utility extends beyond cancer to neurodegenerative diseases, underscoring its versatility in biomedical research (Liu et al., 2021). By harnessing such tools, researchers can deepen their understanding of redox biology and develop targeted therapies that exploit the oxidative vulnerabilities of leukemia cells. Therefore, the current research is designed to investigate the potential of a genetically engineered KillerRed plasmid, specifically targeted to mitochondria, for the controlled modulation of reactive oxygen species (ROS) in leukemia cells. By combining light-activated ROS generation with molecular targeting, this study aims to explore a novel approach for inducing selective cytotoxicity in malignant cells. Through a combination of cytotoxicity assays, live-cell imaging, and transcriptomic profiling, the research seeks to uncover the therapeutic potential of ROS modulation in leukemia and identify molecular signatures that could serve as biomarkers or targets for future treatments.

Materials and Methods

2.1 Plasmid Construction with KillerRed and NLS Tag

A pTWIST plasmid containing the ROS-sensitive fluorescent protein KillerRed was designed, incorporating a nuclear localization signal (NLS) to enable mitochondrial targeting. NLS-encoding oligonucleotides were synthesized with overhangs compatible for ligation into the pTWIST vector. Oligos were annealed in a buffer containing 10 mM Tris, 50 mM NaCl, and 1 mM EDTA to a final concentration of 1000 ng/μL. Forward and reverse primers were added, vortexed, pulse-spun, incubated in a water bath at 95 °C for 5 minutes, and then slowly cooled to room temperature. The annealed oligos were phosphorylated using T4 polynucleotide kinase and ligated into the pTWIST vector using T4 DNA ligase.

2.2 Transformation of Competent E. coli

The ligated plasmid was introduced into competent E. coli K12 cells via the heat shock method. Briefly, 5–10 μL (200 ng/μL) of ligated plasmid was mixed with 50 μL of competent cells and incubated on ice for 30 minutes. Cells were then heat-shocked at 42 °C for 1 minute and immediately returned to ice for 2 minutes. SOC medium (250 μL) was added, followed by incubation at 37 °C for 2 hours to allow expression of the antibiotic resistance marker.

2.3 Selection, Culturing, and Miniprep

Transformed cells were plated on LB agar supplemented with ampicillin (100 μg/mL) and incubated at 37 °C for 24 hours. Single colonies were inoculated into ampicillin-containing LB broth and incubated overnight with shaking. For plasmid isolation, 1.5 mL of overnight culture was centrifuged at 13,000 rpm, and pellets were resuspended in resuspension buffer containing Tris-HCl, EDTA, and RNase A. Cells were lysed with alkaline lysis buffer, and plasmid DNA was purified using the ThermoFisher GeneJET miniprep kit. DNA was eluted in 40 μL of nuclease-free water and stored on ice.

2.4 Sanger Sequencing

Plasmid DNA was quantified using a Nanodrop spectrophotometer and diluted to 50–100 ng/μL. Samples were prepared with sequencing primers and submitted to Eurofins Genomics for Sanger sequencing. Sequence analysis confirmed successful integration of the NLS sequence into the pTWIST-KillerRed construct.

2.5 Diagnostic Digestion

To confirm successful plasmid construction, diagnostic restriction digestion was performed using NheI and HindIII. Plasmid concentration was determined using a Nanodrop spectrophotometer, and 1 μg of DNA was prepared for digestion. Digestion reactions contained 1 μL plasmid DNA, 1 μL of each enzyme, and 2 μL ddH_2O.

A parallel uncut sample served as a control. Samples were incubated at 37 °C for 30 minutes and heat-inactivated at 65 °C for 5 minutes prior to electrophoresis.

2.6 Gel Electrophoresis

A 1% agarose gel was prepared using TBE buffer and poured into a casting tray. Both digested and undigested plasmid samples, along with a DNA ladder, were loaded into wells. Electrophoresis was performed at 80–100 V for 40–60 minutes. DNA fragments were visualized under UV light to verify the presence and size of the insert.

2.7 Cytotoxicity Assay, Live-Cell Imaging, and Survival Analysis

Blood cancer cells were cultured in RPMI-1640 medium with 10% FBS and 1% penicillin-streptomycin at 37 °C, 5% CO_2. Cells were seeded in 96-well plates and treated with varying concentrations of paclitaxel (0.1, 1, 10, 100 μM) or DMSO control. ROS production was measured using a fluorescent ROS dye. Cell viability and IC_{50} values were assessed using the Incucyte live-cell imaging system. Survival analysis was conducted for both modified and unmodified cells, and Kaplan-Meier (KM) plots were generated using GraphPad Prism.

2.8 Fluorescent Imaging

Post-treatment, cells were fixed with 4% paraformaldehyde (PFA) for 10 minutes, washed with PBS, and stained with DAPI. Coverslips were mounted and sealed with liquid cement. Fluorescence was visualized using an EVOS microscope to assess ROS localization and intensity.

2.9 Bio-Portal Analysis

Genetic alterations in ROS-associated genes in blood cancer were explored using BioPortal tools. Copy number variations, mutations, and mRNA expression levels were analyzed and correlated with ROS signaling pathways to identify potential biomarkers.

Results

3.1 Genetic Alterations and Protein Analysis

3.1.1 BioPortal Analysis of ROS-Related Proteins

BioPortal analysis revealed significant genetic alterations in key ROS-associated proteins, including NADPH oxidase (NOX), superoxide dismutase (SOD), and thioredoxin (TRX) in leukemia. These proteins play critical roles in modulating oxidative stress and influence disease progression and treatment response. Notably, TP53, a central tumor suppressor gene known as the "guardian of the genome," regulates antioxidant and pro-oxidant genes such as SOD2 and GPX1. TP53 induces apoptosis, halts cell cycle progression, and facilitates DNA repair. Mutations in TP53, commonly observed in leukemia, disrupt this regulatory network, impair ROS homeostasis, and promote oxidative damage. This dysregulation enhances leukemic cell survival, contributes to disease progression, and undermines therapeutic outcomes. Altered expression of ROS-generating NOX and antioxidant enzymes SOD and TRX highlights the dynamic interplay between oxidative stress pathways and TP53 in maintaining redox homeostasis.

3.1.2 Functional Role of ROS-Associated Proteins in Leukemia

Proteins such as NOX, SOD, TRX, catalase (CAT), and glutathione peroxidase (GPx) serve as biomarkers in leukemia due to their direct involvement in ROS regulation. Mutations in TP53 impair the balance between oxidative and antioxidative signaling, leading to elevated ROS levels, enhanced leukemic cell proliferation, and resistance to apoptosis. This dysregulation correlates with poor prognosis and increased treatment resistance. TP53-mutant cells often escape oxidative stress-induced apoptosis, enabling disease progression.

Table 1 summarizes the biological functions, alterations, and therapeutic relevance of these ROS-related proteins in leukemia.

Protein	Function	Role in Leukemia	Alteration in Leukemia	Considered as a Therapeutic Target	Reference
NADPH Oxidase (NOX)	Enzyme that generates ROS by transferring electrons from NADPH to oxygen	Overexpression leads to increased ROS levels, promoting leukemic cell survival	Overexpression and increased activity in various leukemia types	Yes, targeting NOX can reduce ROS production and leukemia progression	(Bedard and Krause, 2007)
Superoxide Dismutase (SOD)	Converts superoxide radicals into hydrogen peroxide and oxygen, reducing oxidative stress	Protects leukemic cells from oxidative stress, aiding in their survival	Altered expression levels in some leukemia types	Yes, inhibitors are being explored to increase ROS and induce apoptosis in leukemic cells	(Zelko et al., 2002)
Thioredoxin (TRX)	Antioxidant protein that reduces oxidized proteins and maintains redox balance	Promotes survival and proliferation of leukemic cells by reducing ROS levels	Overexpression associated with poor prognosis in leukemia	Yes, targeting TRX is considered to increase ROS-induced apoptosis in leukemia cells	(Lincoln et al., 2003)
Catalase (CAT)	Enzyme that decomposes hydrogen peroxide into water and oxygen, reducing oxidative stress	Helps leukemic cells survive by neutralizing ROS and preventing cell damage	Altered expression in various leukemia types, potentially contributing to resistance	Yes, inhibition of CAT is being studied to increase ROS and promote apoptosis in leukemia cells	(Glorieux and Calderon, 2017)
Glutathione Peroxidase (GPx)	Enzyme that reduces hydrogen peroxide to water, detoxifying ROS	Helps in maintaining low ROS levels, aiding leukemic cell survival	Increased activity in certain leukemias, leading to resistance to oxidative stress	Yes, targeting GPx could enhance ROS-mediated cell death in leukemia	(Brigelius-Flohé and Maiorino, 2013)

Plasmid Construction and Validation

Cloning of the Tagged KillerRed

The cloning of the tagged KillerRed used the vector pTwist_CMV_OriP with 7159 bp. The KillerRed gene was inserted into a specific site within the vector, facilitated by restriction enzyme sites that allow for precise cutting and ligation of DNA fragments. Key restriction sites around the KillerRed insertion include HindIII, which provide multiple options for modular cloning and subcloning, ensuring that KillerRed integrates in the correct orientation and frame for proper expression under the CMV *(Cytomegalovirus)* promoter.

Validation of Plasmid

Validation of the plasmid construct was critical to confirm the successful incorporation and functionality of the KillerRed tag. Its structure is shown in Figure 2. This process involved:

Restriction Digest Analysis: Using the mapped sites, specific enzyme (such as HindIII) was used to cut the plasmid, followed by gel electrophoresis to confirm the presence of expected fragment sizes. The pattern of bands observed on the gel should correspond to predicted sizes based on the plasmid map, confirming the correct structure of the plasmid.

Sequencing

To further verify the sequence integrity and correct orientation of the KillerRed insert sequencing was performed from multiple locations using primers flanking the insertion site. This ensures that the KillerRed gene is intact and in the correct reading frame.

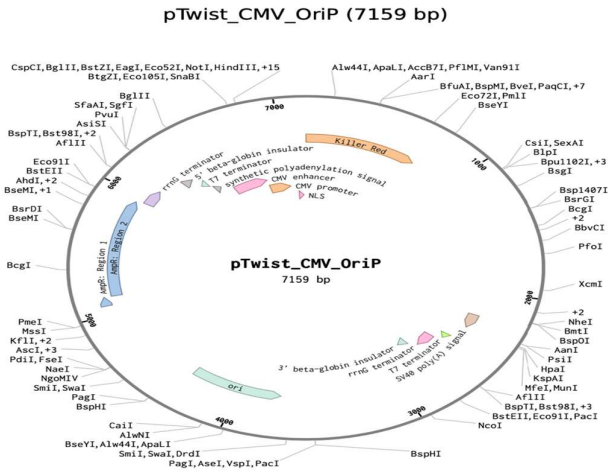

Figure 1: pTWIST plasmid construction using the snap gene for validation and identification of restriction sites for the presence of inserted Killer Red sequence.

Gel Electrophoresis

The gel image represents the diagnostic digestion of the modified plasmid to confirm the precise integration of the NLS tag into the plasmid. The gel displays distinct bands for both cut and uncut plasmid samples. The first lane contains the molecular weight marker, serving as a reference for plasmid DNA. Lanes 2 and 4 show the uncut plasmid DNA (KR uncut-NLS), while lanes 3 and 5 display the digested plasmid DNA (KR-NLS). The molecular weight markers at 8000 bp and 6000 bp in the ladder were used as reference points to estimate the size of the DNA fragments in the experimental lanes. The single smooth band is about 8000 bp in the uncut plasmid lanes (2 and 4). Figure 3 shows that when the putative add-back plasmid is digested with BamHI/Sall, a cleavage site is located between the plasmid backbone and the NLS insert. The bands in the digested plasmid sample (lanes 3 and 5) are distinct: one at about 8000 bp and another at 6000 bp. The upper band at 8000 bp is the linearized backbone of the plasmid, and the band at 6000 bp reflects the presence of the NLS insert. These two bands with the higher intensity 6000bp band in the digested lanes (3 and 5) and its absence in the uncut lanes (2 and 4) clearly show that the 6000bp band is the NLS insert. Hence, this confirms the successful transformation of the plasmid and the precise integration of the NLS tag into it. Moreover, the observed band size of 5000bp in lanes 3 and 4, which matches the calculated size of the NLS insert (6000 bp), further establishes the success of restriction cloning and ligation. Furthermore, the absence of smearing in the digested samples and clear-cut bands also confirms the efficiency of the restriction digestion and effective cleaving of the plasmid at their expected restriction sites.

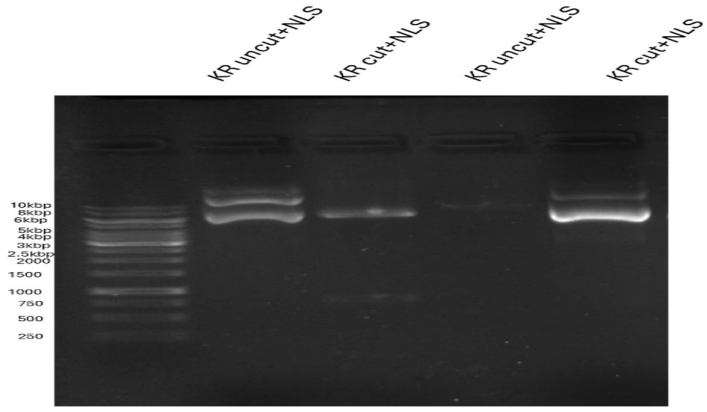

Figure 2: Gel electrophoresis was used to confirm NLS tag integration. Uncut plasmids showed a single band at ~8000 bp, while digested plasmids revealed two bands at ~8000 bp and ~6000 bp, confirming successful transformation and NLS insertion.

Validation of the KillerRed-NLS Construct via Sequencing and BLAST Analysis

The pTWIST plasmid, engineered to express the KillerRed fluorescent protein, was designed to target mitochondrial and nuclear locations via the nuclear localization sequence (NLS). The sequencing results validated the synthesis and modification of the construct. Specifically, a segment of 776 base pairs aligns perfectly with the expected sequence for the NLS and the flanking regions necessary for accurate integration and expression, confirming that the plasmid will effectively localize within the cellular mitochondria. Additionally, a detailed sequencing outcome of 1046 base pairs encompasses the region encoding KillerRed and associated regulatory elements under the CMV promoter as shown in Fig 4, ensuring that the fusion of KillerRed with the NLS enables the protein to be localized within the nucleus.

This was further validated by DNA sequencing followed by BLAST analysis. The complete insert sequence, as determined by sequencing, was subjected to a BLAST search to confirm its fidelity and integrity in the pTwist_CMV_OriP vector. The alignment information shows that the sequence of the KillerRed-NLS construct has several stretches that align closely with reference sequences with minor occasional gaps, indicating almost perfect alignment.

These observations confirmed correct sequence integrity and also implied that the construct was assembled correctly by the presence of KillerRed correctly integrated into the vector between the promoter and terminator. The validation of correct sequence insertion is important as this verifies the NLS tag function and, therefore, its ability to localize and generate ROS in the nuclear compartment, which is poised for further experimental use. The representative chromatogram from the sequencing results is attached in Figure 4.

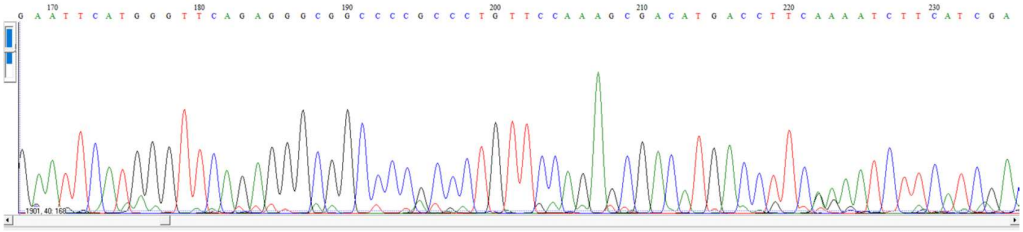

Figure 3: Representative sequencing chromatogram of the KillerRed-NLS construct. Sequencing and BLAST analysis confirm correct integration of KillerRed-NLS in the pTWIST plasmid, showing high sequence identity and accurate nuclear localization.

Validation of constructs in cells and cytotoxic Effects of KillerRed-Induced ROS

Cell Viability Assessments

The cell viability assessments over six days shows highlight the cytotoxic effects of paclitaxel on cancer cells, as reflected by the significant differences in IC50 values in Fig 5. On day 2, the IC50 was 0.8905 μM, indicating a relatively high sensitivity of the cells to the drug. By day 4, the IC50 slightly increased to 1.082 nM as shown in Table 2, suggesting a decrease in drug sensitivity, possibly due to adaptive responses within the cancer cells. This elevation continued onto day 6, where the IC50 further increased more than twice to 1.997 nM. These results suggest the development of resistance by leukemia against paclitaxel over time, showcasing the decreased drug efficacy due to prolonged exposure. The increasing IC50 values demonstrate the dynamic nature of drug-cell interactions.

Table 1: IC50 values of paclitaxel at different time points (Day 2, Day 4, and Day 6), showing the progressive increase in drug resistance in cancer cells over time. The table provides the data points used to generate the corresponding cytotoxicity plots, reflecting the reduced sensitivity of cancer cells to paclitaxel with prolonged exposure.

PAC day 2		**PAC day 4**		**PAC day 6**	
Concentration	Response	Concentartion	Response	Concentration	Response
0mg/ml	100	0mg/ml	100	0mg/ml	100
0.1nM	93.67773	0.1nM	95.41259	0.1nM	99.38464

1nM	80.56698	1nM	74.24319	1nM	75.88832
10nM	62.67001	10nM	46.73303	10nM	43.05827
100nM	63.71496	100nM	48.94725	100nM	44.81096

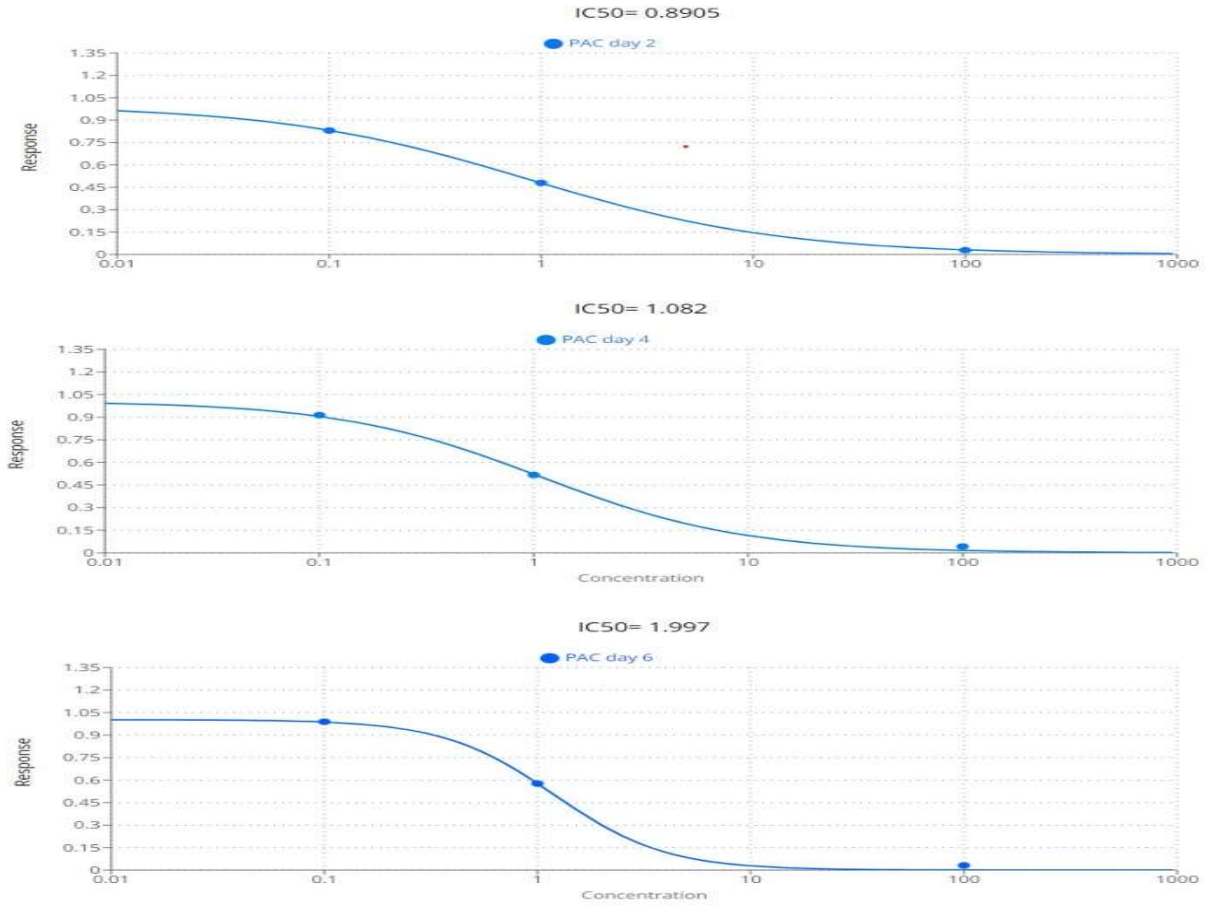

Figure 4: IC50 values of paclitaxel showing increasing resistance in cancer cells over six days, with a significant rise from 0.8905 μM to 1.997 μM, indicating reduced drug sensitivity over time.

Effects of Paclitaxel on Cancer Cell Viability

The assay represents the effects of paclitaxel on KR_NLS cancer cells at different time intervals. Fig 6 shows that KR_NLS cells exhibited their highest growth at around 24 hours with nearly 7 growth measurements. However, this growth declined to approximately 2 at 100-hour time, suggesting the increasing effectiveness of paclitaxel over time. In contrast, control (DMSO) treated cells showed even higher growth than KR_NLS cells, which remained the highest over time. It can be concluded from the results that paclitaxel is more effective than DMSO against cancer cells, particularly KR_ NLS cells.

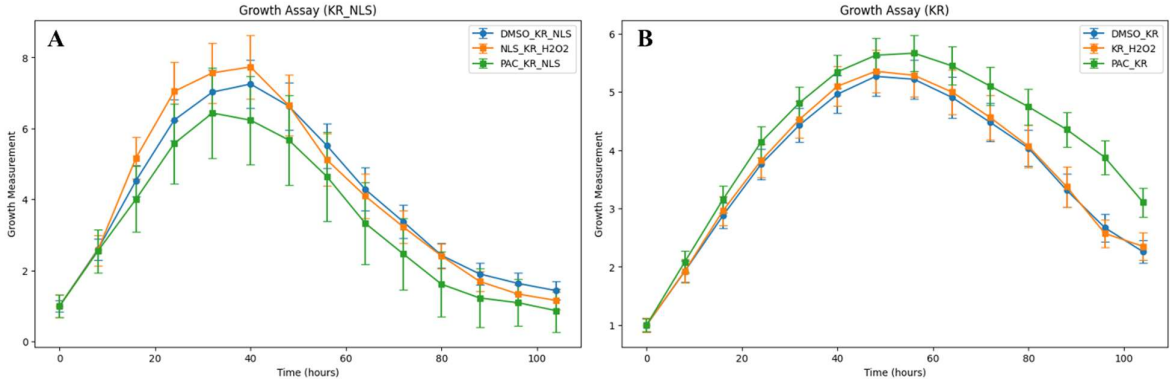

Figure 5: Growth inhibition assay showing the effects of paclitaxel on KR_NLS cells. Paclitaxel-treated cells (red line) show a significant decline in growth from a peak of 7 at 24 hours to 2 by 100 hours, indicating strong cytotoxic effects. The DMSO control (blue line) maintains a higher growth rate, peaking at 8, while H_2O_2-treated cells (orange line) display a moderate decline, with paclitaxel demonstrating the strongest growth inhibition.

Fluorescent Imaging

Paclitaxel-induced reactive oxygen species (ROS) in cancer cells were evaluated through fluorescence imaging to understand the localization of the construct. The blue spots in Fig 7 represent healthy cells, while the red fluorescence shows the presence of ROS in the cells, which are further responsible for causing oxidative damage to the macromolecules in cancer cells. The fluorescence intensity is quantified in relative calibration units (RCU) in specific pixels. It can be observed from the images that paclitaxel has induced relatively higher concentrations of ROS as the fluorescence appears to be distinct sports rather than uniform distribution, indicating the localization of ROS generation around cellular organelles such as mitochondria. The red dot intensity shows that ROS has successfully penetrated and affected the cytoplasm significantly, suggesting the widespread oxidative effects of ROS within the cellular environment.

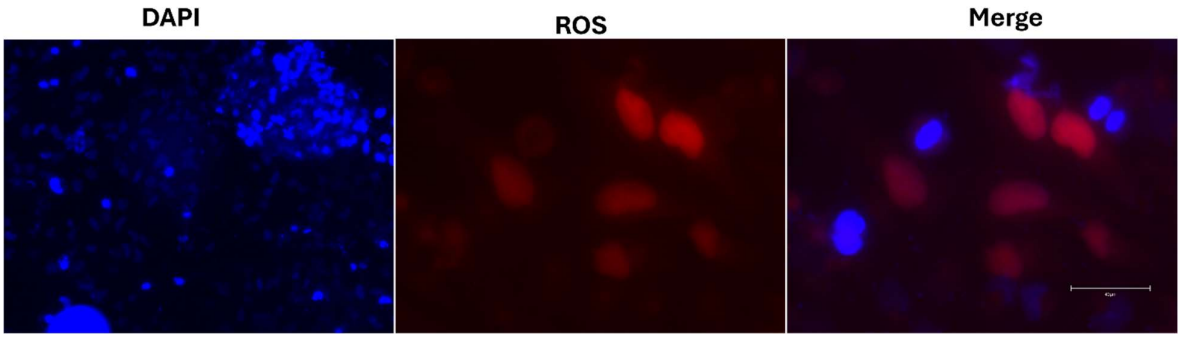

Figure 6: Fluorescent imaging of paclitaxel-induced ROS in cancer cells, with red spots indicating high ROS concentrations and blue spots representing healthy cells. The increased ROS levels highlight oxidative damage and localization around cellular organelles.

Kaplan-Meier Survival Analysis

The Kaplan-Meier (KM) plot represents the comparison of survival probabilities over time for two distinct groups of leukemia patients categorized by their gene expression levels. In Fig 8, the plot distinctly shows that patients with higher gene expression, outlined as the 'High Expression' group, exhibit a higher survival probability over time, as represented on the y-axis, against time on the x-axis. The data points (Number at Risk) show how many patients remain under observation at various periods. The Hazard Ratio (HR) of 0.72, with a confidence interval ranging from 0.64 to 0.81, statistically exhibits a 28% reduction in the risk of death for patients with

high gene expression compared to those with lower expression, affirming a protective benefit of higher expression levels. Furthermore, the extremely low log-rank P-value of 6.8e-08 verifies the significance of these findings, confirming that the survival difference between high and low-expression groups is highly unlikely to be due to random variation. This comprehensive analysis effectively shows that higher gene expression is associated with improved prognosis in leukemia.

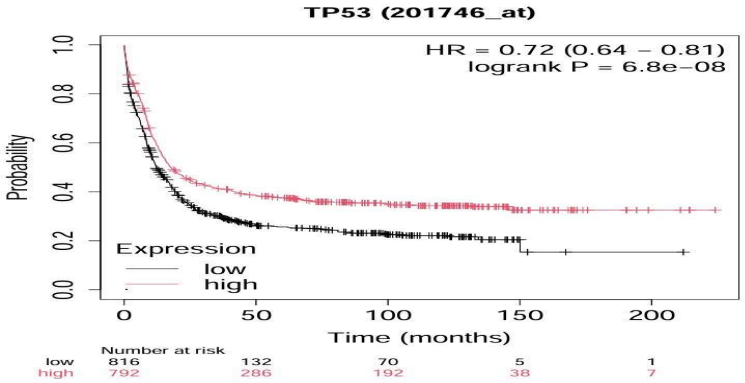

Figure 7: Kaplan-Meier plot comparing survival probabilities for leukemia patients with high versus low TP53 expression. Higher TP53 expression is associated with improved survival (HR = 0.72, p < 6.8e-08), indicating a significant protective benefit

Discussion

This study explored the role of reactive oxygen species (ROS) in cancer, emphasizing their signaling behaviors and potential as therapeutic targets. The involvement of ROS in nearly every aspect of cellular function, along with their role in disease pathologies and mechanistic interactions, presents both a challenge and an opportunity in cancer therapy. In this context, the effect of ROS modulation inside cancer cells was examined using the engineered photosensitizer KillerRed, which generates ROS in a controllable manner, enabling the monitoring of cellular responses to ROS-induced events. The study uncovered crucial ROS-triggered pathways linked to cancer progression and tumor responses to various treatment modalities. These insights advance our understanding of ROS dynamics in cancer and open new avenues for enhancing anticancer drug responses by targeting ROS homeostasis in cancer cells. A KillerRed plasmid was constructed to contain a nuclear localization signal (NLS), targeting the fluorescent protein to mitochondria while remaining sensitive to ROS. The plasmid, customized using a pTWIST backbone, included KillerRed flanked by restriction sites for precise cloning and expression localization. Integrating the NLS into this construct was critical, as it allowed selective nuclear targeting, an active site for ROS production and cellular signaling. Successful uptake and expression of the plasmid construct were confirmed via antibiotic selection on ampicillin media, plasmid purification, and sequencing. The structural integrity of the construct was validated through diagnostic digestions and gel electrophoresis, confirming the proper insertion of KillerRed and its fusion with the NLS sequence. This selective expression of KillerRed in the nucleus enabled targeted apoptosis at the cellular origin. Nuclear targeting through the NLS relied on the sequence's ability to interact with the nuclear import machinery and transport the fusion protein through the nuclear pore complex. Literature, such as the work by Marfori et al. (2011), supports how NLSs localizes proteins to the nucleus to regulate gene expression and other nuclear functions. Fluorescence microscopy validated the localization of the NLS-tagged KillerRed construct to the nucleus, as demonstrated by Kosugi et al. (2009), underscoring the need to confirm therapeutic proteins' correct compartmentalization. Functional assays assessed ROS generation in the nucleus and its effects on cell viability, apoptosis, and senescence. Compared to mitochondrial-targeted ROS generation, nuclear ROS production had unique therapeutic effects by modulating transcriptional responses and inducing DNA damage directly. Studies like Huang et al. (2021) reported nuclear ROS generators inducing apoptosis in cancer cells by triggering DNA damage responses. The current findings align with these conclusions, showing that nuclear-targeted ROS via the NLS-KillerRed construct impairs nuclear function and boosts therapeutic outcomes. To functionally characterize KillerRed-tagged proteins, cytotoxicity assays, live-cell imaging, and Kaplan-Meier (KM) survival plots were performed to evaluate the effects of ROS on

blood cancer cells. KillerRed's real-time ROS generation enabled visualization of oxidative changes over time, providing insights into cellular responses. Cytotoxicity assays assessed the impact of paclitaxel, a chemotherapeutic that induces oxidative stress, on both wild-type and control-expressing blood cancer cells. A clear dose-dependent reduction in cell viability demonstrated the drug's efficacy. Dose-response studies are fundamental for identifying optimal drug concentrations to maximize therapeutic effects while minimizing toxicity. In line with existing literature, paclitaxel's known mechanism involves binding to tubulin, stabilizing microtubules, and inducing cell death. These effects were further studied via cell cycle analysis, as supported by Weaver (2014). IC50 values were tracked over time, revealing evolving drug sensitivity. Initially, low IC50 values indicated high sensitivity to paclitaxel, but increased values over time suggested adaptation or resistance. This trend matches findings from Vadlapatla et al. (2013), who documented similar adaptive responses involving drug efflux pumps or altered drug targets. Live-cell imaging allowed direct observation of ROS dynamics, with KillerRed enabling real-time visualization post-paclitaxel treatment. ROS levels increased shortly after drug administration, confirming paclitaxel's role in enhancing oxidative stress. Zhao et al. (2017) showed that targeting ROS-generating compounds increased apoptosis in cancer cells, consistent with these observations. Fluorescence imaging pinpointed localized ROS production, emphasizing the benefit of specific ROS generators in reducing resistance and minimizing off-target effects. In contrast to general fluorescent probes used in studies like Lee et al. (2012), KillerRed provides continuous, specific monitoring without interfering with cellular physiology. Widespread dose-dependent cytotoxic effects, well documented in pharmacological studies of paclitaxel, support the broader applicability of these findings. Similar resistance dynamics in breast cancer cells, as reported by Smith et al. (2010), reinforce the value of studying ROS modulation in cancer. Temporal studies using KillerRed in dynamic culture systems revealed significant ROS fluctuations post-treatment, helping decode oxidative signaling in cancer physiology. Elevated ROS levels triggered by paclitaxel align with known oxidative stress-induced damage mechanisms, as discussed by Trachootham et al. (2009). Bulina et al. (2006b) demonstrated KillerRed's capacity to cause localized oxidative stress and apoptosis without systemic toxicity. This study further compared diffuse (paclitaxel) and specific (KillerRed) ROS strategies, revealing that while paclitaxel indiscriminately raised ROS in all cells, KillerRed induced apoptosis selectively in cancer cells. The study also aimed to identify key molecular players in ROS modulation, focusing on proteins like NADPH Oxidase (NOX) and Superoxide Dismutase (SOD). NOX is a primary source of intracellular ROS, whereas SOD neutralizes superoxide radicals into less toxic hydrogen peroxide. Results indicate paclitaxel-induced oxidative stress may modulate these proteins, altering ROS dynamics. This aligns with findings by Chasara et al. (2023), who noted the therapeutic potential of targeting oxidative stress responses. Identifying proteins upregulated or downregulated in response to ROS offers a strategy to develop novel therapies or enhance existing treatments. KillerRed was further employed to investigate the impact of nuclear oxidative stress in cancer cells. Elevated ROS production upon KillerRed activation overwhelmed cellular antioxidant defenses, resulting in oxidative cell death. Numerous studies have proposed targeted ROS elevation as a viable cancer therapy. The ability of KillerRed to kill disease cells while sparing normal ones supports its potential to reduce side effects associated with conventional chemotherapy. Trachootham et al. (2009) demonstrated that increasing ROS levels selectively eliminates cancer cells due to their inherent oxidative vulnerability. Altogether, these findings underscore the potential of ROS modulation in cancer therapy. This study showed that precisely controlled ROS production through KillerRed can induce cancer cell death and suppress tumor progression. The data not only validates the approach's novelty and effectiveness but also justifies future research targeting ROS. Despite promising results, further optimization of KillerRed's specificity and efficiency is needed for application across various cancer types. Potential toxicity from prolonged ROS exposure requires investigation, and targeting strategies must be refined to limit ROS production to tumor sites. Expanding the range of molecular probes and testing additional chemotherapeutics could reveal synergistic effects, leading to more effective treatment protocols. The evidence gathered provides a solid foundation for future development of ROS-based therapies in cancer.

Conclusion

This study provides a comprehensive understanding of the dynamic role of reactive oxygen species (ROS) in leukemia progression and therapy, underscoring their dual function as both cellular signaling molecules and mediators of oxidative damage. Through integrated bioinformatics analysis and experimental validation, we

demonstrated that dysregulation of ROS-related proteins such as NADPH Oxidase (NOX), Superoxide Dismutase (SOD), and Thioredoxin (TRX) especially in the context of TP53 mutations plays a pivotal role in altering the redox homeostasis of leukemic cells, thereby contributing to enhanced survival, proliferation, and treatment resistance. The novel application of a nucleus-targeted KillerRed photosensitizer represents a significant advancement in ROS modulation technology. Our plasmid constructs efficiently induced ROS in the nuclear region, triggering apoptosis in leukemia cells as confirmed by cytotoxicity assays and live-cell imaging. These findings suggest that carefully controlled elevation of ROS levels can selectively induce cancer cell death, offering a potential strategy to overcome chemoresistance in acute myeloid leukemia (AML). By pushing ROS levels beyond the antioxidant buffering capacity of leukemic cells—while sparing normal cells. this study opens new avenues for targeted cancer therapies. Moreover, our work aligns with and extends existing literature by proposing ROS not only as a marker of disease state but also as a promising therapeutic target. The methodologies and insights presented here lay the groundwork for future research combining ROS-inducing agents with other treatment modalities, paving the way toward more effective and less toxic interventions for leukemia patients.

References

1. Barbosa, K. and Deshpande, A. J. (2023) 'Therapeutic targeting of leukemia stem cells in acute myeloid leukemia', *Front Oncol,* 13, pp. 1204895. DOI: 10.3389/fonc.2023.1204895.
2. Bedard, K. and Krause, K. H. (2007) 'The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology', *Physiol Rev,* 87(1), pp. 245-313. DOI: 10.1152/physrev.00044.2005.
3. Belousov, V. V., Fradkov, A. F., Lukyanov, K. A., Staroverov, D. B., Shakhbazov, K. S., Terskikh, A. V. and Lukyanov, S. (2006) 'Genetically encoded fluorescent indicator for intracellular hydrogen peroxide', *Nature methods,* 3(4), pp. 281-286.
4. Brigelius-Flohé, R. and Maiorino, M. (2013) 'Glutathione peroxidases', *Biochim Biophys Acta,* 1830(5), pp. 3289-303. DOI: 10.1016/j.bbagen.2012.11.020.
5. Brown, D. I. and Griendling, K. K. (2015) 'Regulation of signal transduction by reactive oxygen species in the cardiovascular system', *Circ Res,* 116(3), pp. 531-49. DOI: 10.1161/circresaha.116.303584.
6. Bulina, M. E., Chudakov, D. M., Britanova, O. V., Yanushevich, Y. G., Staroverov, D. B., Chepurnykh, T. V., Merzlyak, E. M., Shkrob, M. A., Lukyanov, S. and Lukyanov, K. A. (2006a) 'A genetically encoded photosensitizer', *Nature biotechnology,* 24(1), pp. 95-99.
7. Bulina, M. E., Chudakov, D. M., Britanova, O. V., Yanushevich, Y. G., Staroverov, D. B., Chepurnykh, T. V., Merzlyak, E. M., Shkrob, M. A., Lukyanov, S. and Lukyanov, K. A. (2006b) 'A genetically encoded photosensitizer', *Nat Biotechnol,* 24(1), pp. 95-9. DOI: 10.1038/nbt1175.
8. Chasara, R. S., Ajayi, T. O., Leshilo, D. M., Poka, M. S. and Witika, B. A. (2023) 'Exploring novel strategies to improve anti-tumour efficiency: The potential for targeting reactive oxygen species', *Heliyon,* 9(9), pp. e19896. DOI: 10.1016/j.heliyon.2023.e19896.
9. Döhner, H., Weisdorf, D. J. and Bloomfield, C. D. (2015) 'Acute myeloid leukemia', *New England Journal of Medicine,* 373(12), pp. 1136-1152.
10. Gao, L., Loveless, J., Shay, C. and Teng, Y. (2020) 'Targeting ROS-mediated crosstalk between autophagy and apoptosis in cancer', *Reviews on new drug targets in age-related disorders*, pp. 1-12.
11. Glorieux, C. and Calderon, P. B. (2017) 'Catalase, a remarkable enzyme: targeting the oldest antioxidant enzyme to find a new cancer treatment approach', *Biol Chem,* 398(10), pp. 1095-1108. DOI: 10.1515/hsz-2017-0131.
12. Gorrini, C., Harris, I. S. and Mak, T. W. (2013) 'Modulation of oxidative stress as an anticancer strategy', *Nat Rev Drug Discov,* 12(12), pp. 931-47. DOI: 10.1038/nrd4002.
13. Huang, R., Chen, H., Liang, J., Li, Y., Yang, J., Luo, C., Tang, Y., Ding, Y., Liu, X., Yuan, Q., Yu, H., Ye, Y., Xu, W. and Xie, X. (2021) 'Dual Role of Reactive Oxygen Species and their Application in Cancer Therapy', *J Cancer,* 12(18), pp. 5543-5561. DOI: 10.7150/jca.54699.
14. Juliusson, G., Antunovic, P., Derolf, Å., Lehmann, S., Möllgård, L., Stockelberg, D., Tidefelt, U., Wahlin, A. and Höglund, M. (2009) 'Age and acute myeloid leukemia: real world data on decision to treat and outcomes from the Swedish Acute Leukemia Registry', *Blood, The Journal of the American Society of Hematology,* 113(18), pp. 4179-4187.
15. Kim, S. J., Kim, H. S. and Seo, Y. R. (2019) 'Understanding of ROS-Inducing Strategy in Anticancer Therapy', *Oxid Med Cell Longev,* 2019, pp. 5381692. DOI: 10.1155/2019/5381692.
16. Koren, S. A., Ahmed Selim, N., De la Rosa, L., Horn, J., Farooqi, M. A., Wei, A. Y., Müller-Eigner, A., Emerson, J., Johnson, G. V. W. and Wojtovich, A. P. (2023) 'All-optical spatiotemporal mapping of ROS dynamics across mitochondrial microdomains in situ', *Nat Commun,* 14(1), pp. 6036. DOI: 10.1038/s41467-023-41682-z.
17. Kosugi, S., Hasebe, M., Tomita, M. and Yanagawa, H. (2009) 'Systematic identification of cell cycle-dependent yeast nucleocytoplasmic shuttling proteins by prediction of composite motifs', *Proc Natl Acad Sci U S A,* 106(25), pp. 10171-6. DOI: 10.1073/pnas.0900604106.
18. Lee, J., Giordano, S. and Zhang, J. (2012) 'Autophagy, mitochondria and oxidative stress: cross-talk and redox signalling', *Biochem J,* 441(2), pp. 523-40. DOI: 10.1042/bj20111451.

19. Lincoln, D. T., Ali Emadi, E. M., Tonissen, K. F. and Clarke, F. M. (2003) 'The thioredoxin-thioredoxin reductase system: over-expression in human cancer', *Anticancer Res,* 23(3b), pp. 2425-33.
20. Liu, J., Wang, F., Qin, Y. and Feng, X. (2021) 'Advances in the Genetically Engineered KillerRed for Photodynamic Therapy Applications', *Int J Mol Sci,* 22(18). DOI: 10.3390/ijms221810130.
21. Mannan, A., Germon, Z. P., Chamberlain, J., Sillar, J. R., Nixon, B. and Dun, M. D. (2021) 'Reactive Oxygen Species in Acute Lymphoblastic Leukaemia: Reducing Radicals to Refine Responses', *Antioxidants (Basel),* 10(10). DOI: 10.3390/antiox10101616.
22. Marfori, M., Mynott, A., Ellis, J. J., Mehdi, A. M., Saunders, N. F., Curmi, P. M., Forwood, J. K., Bodén, M. and Kobe, B. (2011) 'Molecular basis for specificity of nuclear import and prediction of nuclear localization', *Biochim Biophys Acta,* 1813(9), pp. 1562-77. DOI: 10.1016/j.bbamcr.2010.10.013.
23. Pak, V. V., Ezeriņa, D., Lyublinskaya, O. G., Pedre, B., Tyurin-Kuzmin, P. A., Mishina, N. M., Thauvin, M., Young, D., Wahni, K. and Gache, S. A. M. (2020) 'Ultrasensitive genetically encoded indicator for hydrogen peroxide identifies roles for the oxidant in cell migration and mitochondrial function', *Cell metabolism,* 31(3), pp. 642-653. e6.
24. Pelicano, H., Carney, D. and Huang, P. (2004) 'ROS stress in cancer cells and therapeutic implications', *Drug Resist Updat,* 7(2), pp. 97-110. DOI: 10.1016/j.drup.2004.01.004.
25. Reczek, C. R. and Chandel, N. S. (2015) 'ROS-dependent signal transduction', *Curr Opin Cell Biol,* 33, pp. 8-13. DOI: 10.1016/j.ceb.2014.09.010.
26. Rhee, S. G., Kang, S. W., Jeong, W., Chang, T. S., Yang, K. S. and Woo, H. A. (2005) 'Intracellular messenger function of hydrogen peroxide and its regulation by peroxiredoxins', *Curr Opin Cell Biol,* 17(2), pp. 183-9. DOI: 10.1016/j.ceb.2005.02.004.
27. Sai, D. L., Lee, J., Nguyen, D. L. and Kim, Y.-P. (2021) 'Tailoring photosensitive ROS for advanced photodynamic therapy', *Experimental & Molecular Medicine,* 53(4), pp. 495-504.
28. Sallmyr, A., Fan, J., Datta, K., Kim, K. T., Grosu, D., Shapiro, P., Small, D. and Rassool, F. (2008) 'Internal tandem duplication of FLT3 (FLT3/ITD) induces increased ROS production, DNA damage, and misrepair: implications for poor prognosis in AML', *Blood,* 111(6), pp. 3173-82. DOI: 10.1182/blood-2007-05-092510.
29. Serebrovskaya, E. O., Ryumina, A. P., Boulina, M. E., Shirmanova, M. V., Zagaynova, E. V., Bogdanova, E. A., Lukyanov, S. A. and Lukyanov, K. A. (2014) 'Phototoxic effects of lysosome-associated genetically encoded photosensitizer KillerRed', *J Biomed Opt,* 19(7), pp. 071403. DOI: 10.1117/1.Jbo.19.7.071403.
30. Smith, J., Tho, L. M., Xu, N. and Gillespie, D. A. (2010) 'The ATM-Chk2 and ATR-Chk1 pathways in DNA damage signaling and cancer', *Adv Cancer Res,* 108, pp. 73-112. DOI: 10.1016/b978-0-12-380888-2.00003-0.
31. Trachootham, D., Alexandre, J. and Huang, P. (2009) 'Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach?', *Nat Rev Drug Discov,* 8(7), pp. 579-91. DOI: 10.1038/nrd2803.
32. Vadlapatla, R. K., Vadlapudi, A. D., Pal, D. and Mitra, A. K. (2013) 'Mechanisms of drug resistance in cancer chemotherapy: coordinated role and regulation of efflux transporters and metabolizing enzymes', *Curr Pharm Des,* 19(40), pp. 7126-40. DOI: 10.2174/13816128113199990493.
33. Weaver, B. A. (2014) 'How Taxol/paclitaxel kills cancer cells', *Mol Biol Cell,* 25(18), pp. 2677-81. DOI: 10.1091/mbc.E14-04-0916.
34. Yang, H., Villani, R. M., Wang, H., Simpson, M. J., Roberts, M. S., Tang, M. and Liang, X. (2018) 'The role of cellular reactive oxygen species in cancer chemotherapy', *J Exp Clin Cancer Res,* 37(1), pp. 266. DOI: 10.1186/s13046-018-0909-x.
35. Zelko, I. N., Mariani, T. J. and Folz, R. J. (2002) 'Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression', *Free Radic Biol Med,* 33(3), pp. 337-49. DOI: 10.1016/s0891-5849(02)00905-x.

36. Zhang, J., Wang, X., Vikash, V., Ye, Q., Wu, D., Liu, Y. and Dong, W. (2016) 'ROS and ROS-Mediated Cellular Signaling', *Oxid Med Cell Longev,* 2016, pp. 4350965. DOI: 10.1155/2016/4350965.
37. Zhao, Y., Hu, X., Liu, Y., Dong, S., Wen, Z., He, W., Zhang, S., Huang, Q. and Shi, M. (2017) 'ROS signaling under metabolic stress: cross-talk between AMPK and AKT pathway', *Molecular Cancer,* 16(1), pp. 79. DOI: 10.1186/s12943-017-0648-1.