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DEVELOPMENT OF NITRIC COMPOUND-BASED INHIBITORS FOR TARGETED CANCER THERAPY

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Abstract

An increase in cancer, along with the reach of traditional therapies, inspires the creation of drugs that are more precise and less harmful to the body. Research has identified that certain nitric compound-based chemicals can influence cell death and oxidation only in cancer cells. On the other hand, stability, specificity and the way the drugs are released have all stopped them from being used widely in healthcare. The goal of this research was to develop and test new nitric compound inhibitors that target cancer cells. The goal of the research was to merge multiple types of compounds and study their effectiveness against cancer cells in the test tube. The study tested HT-29, A549, MCF-7, HeLa and PC3 cancer cells by treating them with synthesized substances and then checked for their toxicity, apoptosis, ROS production, caspase levels, change in mitochondrial membrane potential (MMP) and expression of different apoptotic factors. Researchers used single-sample t-tests, summarized the results and checked for correlations using statistical methods. Study findings revealed that cell viability decreased (mean = 45.38%, $p < 0.001$), more apoptosis was observed (mean = 51.42%, $p < 0.001$) and ROS, caspase activity and MMP loss increased considerably (mean = 83.15%, $p < 0.001$, mean = 71.45%, $p < 0.001$, mean = 51.69%, $p < 0.001$, respectively). Results from correlation analysis showed that viability was negatively correlated with apoptosis ($r = -0.77$) and ROS ($r = -0.78$), demonstrating that the chemicals boost the apoptotic pathways. Based on the findings, nitric compound-based inhibitors trigger apoptosis specifically in cancer cells by increasing oxidative stress and breaking mitochondria, though they do little harm to normal cells. Therefore, nitric compounds seem capable of being reliable therapies and more testing in patients and animals should be done to ensure their proper use in cancer treatment.

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INTRODUCTION

According to the World Health Organization (WHO), cancer causes nearly 10 million deaths every year, making it a main cause of both sickness and death worldwide (Bray et al., 2018). Even with new ways to diagnose and address cancer, the disorder remains a big challenge due to the variety among tumors, resistant cancers to treatment and distressing side effects of traditional options (Lim & Ma, 2019). In recent years, more emphasis has been placed on designing precise treatments to safeguard the body's health and to make cancer therapy more effective. Nitric compound-based inhibitors have emerged as a possible solution in the search for new anticancer drugs (Kaur et al., 2023).

Some researchers have concluded that nitric oxide and its related compounds provide numerous benefits such as dilating blood vessels, shaping the immune response and inhibiting microorganisms (such as Weng et al., 2015). Oncologists are exploring using nitric compounds as treatments since they can promote tumor development or stop it, depending on their quantity, the place they are used and their method of administration. Under proper control, nitric compounds are able to cause cell death, prevent the growth of blood vessels and increase tumor cell sensitivity to both chemotherapy and radiotherapy (Mintz et al., 2021). On this basis, they could be promising agents for developing medicine that protects healthy cells but eliminates cancer cells. Within the past few years, cancer treatment has greatly improved through targets that address the specific factors important for cancer growth and survival. This way of treatment works well with nitric compound-based inhibitors, as they might be able to manage important pathways related to cancer like NF-κB, PI3K/Akt and p53 (Khezri et al., 2022; Jiang et al., 2024). Furthermore, nitric compounds disrupt cellular redox balance and DNA repair mechanisms conducive to making cancer susceptible to apoptosis. However, clinical application of nitric compounds has been hampered by stability, specificity and controlled release problems. As a result, it is extremely urgent to develop new formulations of nitric compounds with improved pharmacological profiles for the effective and safe treatment of cancers (Luo et al., 2024).

It is well known to the scientific community that nitric oxide and its derivatives play a complex role in cancer biology. Nitric oxide was the first testable candidate that was initially identified as a pro-tumorigenic, paradoxical molecule (Morbidelli et al., 2023). Angiogenesis and tumor proliferation at low concentrations and cytotoxicity and tumor regression at high concentrations constitute the main behavior of Avitinib. As a result, the controlled delivery of nitric compounds is a major hurdle in therapeutic design (Wang, et al., 2024). The use of nitric oxide-releasing prodrugs, hybrid compounds and nanocarrier systems for drug delivery are the subjects of several preclinical studies with promising results. We have shown that these approaches are more cytotoxic in a variety of cancer models, including breast, lung, prostate and colon cancer. That said, few nitric based therapies have emerged to clinical application (Mintz et al., 2021). However, their clinical development has been hindered by off target effects and high metabolic instability accompanied with rapid systemic clearance. Therefore there exists an unmet need for designing novel nitric compound-based inhibitors that are selective for tumor cells, without harming the normal tissues. In an attempt to address this gap, this thesis sought to synthesize and characterize a new class of nitric compound-based inhibitors of iNOS with improved stability, target specificity and efficacy against cancer. Their effects on the key cellular mechanisms were also studied including apoptosis induction, ROS generation and modulation of the cell survival pathways (Zeid et al., 2025).

There was an urgent need to develop safer and more effective cancer therapies and this was the rationale for this study. However, traditional chemotherapeutic agents are often effective but are not specific and thus cause severe side effects from damaging healthy cells. However, the outcomes of treatments have often been disappointing, due to the acquired resistance of targeted therapies or their limited applicability to a small fraction of cancer types (Zhong et al., 2021). Since nitric compounds play a multifaceted role in tumor suppression and may serve to potentiate current treatments, this study was pursued to design, synthesize and study the biological activity of these inhibitors as new anti-

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cancer agents. Additionally, several advances in synthetic chemistry and drug delivery technology have provided avenues to circumvent the shortcomings of previously developed nitric-containing vehicles. On this basis, this research intended to synthesize molecules with increased selectivity to cancer cells, controlled release of nitric release and cooperative effects with standard therapies (Huang et al., 2022). These could form a basis for a new generation of anticancer drugs with better therapeutic indices.

This study has important scientific and clinical implications. First, it expands the growing knowledge on oncological therapeutic potential of nitric compounds. The research identifies the mechanisms by which such compounds act to suppress cancer and so help clarify why they could be useful as targeted inhibitors. Second, novel nitric compound based molecules developed could solve the problem of poor selectivity and systemic toxicity of current cancer therapy (Joy et al. 2024). Third, the findings provide a basis for combination therapies wherein, nitric compounds can increase the efficacy of convention therapies by augmenting sensitization of tumor cells to treatment. Along with this, the study employed a thorough experimental design which included a brief in vitro study of cytotoxicity, designing of apoptosis and modulation of molecular pathways in a variety of cancer cell lines.

The robustness of the findings and a solid foundation are offered for future in vivo and clinical studies (Mustafa et al., 2024). Finally, the research may lead to the development of new treatment approaches that have been shown to be better than the current standards of care for some types of cancer and reduce the overall burden of cancer on individuals. Some nitric compound–based therapies are under development for clinical treatment, but the role of nitric oxides and its derivatives has been the subject of intense research for the past 20 years. For example, most previous studies on nitric oxide donors or simple nitric derivatives presented limited targeting ability (Galvani et al., 2024). So, to the best of our knowledge, very little work has been done to structurally optimize nitric based inhibitors such that these can selectively hit cancer specific pathways and avoid systemic exposure. In addition, there are no studies that integrate chemical synthesis, mechanistic evaluation and biological validations under a single framework of such compounds (Hoffmann. However, the existing research fails to correlate molecular structure with biological activity, making rational design of effective inhibitors difficult. Since it is apparent that the nitric drugs have not reached their full potential, the aim of this study was to bridge this gap by designing and characterizing a new class of nitric compounds that possess tailored physicochemical and biological properties for the purposes of targeted cancer therapy.

The purpose of this work was to create new inhibitors based on nitric compounds as targeted cancer therapy and evaluate their performance in vitro on established human cancer cell lines. Specific objective tested:

To design and synthesize structurally diverse nitric compound-based molecules with potential anticancer activity.

To characterize the synthesized compounds using spectroscopic techniques such as NMR, FTIR, and mass spectrometry.

To assess the cytotoxic effects of the compounds on various human cancer cell lines and a normal cell line as control.

To investigate the mechanisms of action, including apoptosis induction, ROS generation, and modulation of survival pathways.

To evaluate the selectivity and therapeutic potential of the most promising compounds for future in vivo and clinical studies.

In this study, a systematic approach to design, synthesize and biologically evaluate of the nitric compound-based inhibitors as the potential cancer agents has been taken. The literature review started with identifying structural motifs with known or predicted anticancer activity. Nitric compound based molecules were synthesized utilizing modern organic chemistry techniques and subsequently characterized for purity and overall structural integrity. Biological activity of these compounds was evaluated using in vitro assays (MTT cell viability, Annexin V/PI staining for apoptosis, ROS measurement and western blotting of key signaling proteins). The spectrum of activity and selectivity was assessed in multiple human cancer cell lines representing different tissue origin. As such, off target toxicity was evaluated by comparing with a non-cancerous human

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fibroblast line. They found that some of the synthesized compounds were highly cytotoxic toward cancer cells but showed gastrointestinal sparing of normal cells demonstrating a favourable therapeutic index. The mechanisms through which these effects were mediated were determined to be the induction of apoptosis, disruption of mitochondrial membrane potential and activation of caspase pathways. In addition, the compounds modulated a number of important pathways by which cells survive and proliferate, including Akt, p53 and NF-κB.

METHODOLOGY

The main purpose of this study was to synthesize and test nitric compound induced inhibitors that can kill cancer cells specifically, without much harm to normal tissues. This was accomplished through systematically adopting a scientifically rigorous methodology including philosophical underpinnings, experimental design, sampling, data collection, variable measurement, analysis strategies and ethical considerations.

Research Approach

In this research a positivist approach was adopted which means that it is based on empiricism, objectivity and reproducibility. For this study, the positivist stance is more appropriate because the hypotheses regarding biological activity of nitric compound based inhibitors will be tested through controlled laboratory experiments. Based on the study objectives, this approach allowed for systematic testing of chemical formulations against cancer cell lines through variables that are measurable which therefore make the results objective and generalizable.

Research Design

An experimental research design was used to determine if nitric compounds have a cancer inhibiting action. It was chosen this design because it enabled manipulation of several independent variables (different concentrations of nitric compounds) and it permitted to observe their effects on several dependent variables (cell viability, apoptosis rates). Control groups and experimental replication were employed in order to make causal inferences regarding the effectiveness of the synthesized inhibitors. The design enabled the establishment of a cause-and–effect relationship for exposure to compounds and subsequent therapeutic outcomes in cancer cells.

Sampling Strategy

Purposive sampling was employed by the study whereas specific human cancer cell line (for breast cancer MCF-7 and lung cancer A549) are normally utilized in oncology research. The core of the sample was comprised of five human cancer cell lines and one normal human fibroblast cell line used as a control. Relevance to clinical conditions was ensured for this selection and comparative analysis of selectivity and toxicity was permitted. Well characterized, commercially available cell lines that had been verified for genetic and phenotypic stability were used as the inclusion criteria, yet cell lines with abnormal growth and/or contamination were excluded. In light of these in vitro pharmacological studies and statistical power analysis, the sample size was designed to attain 95% confidence level with 80% power.

Data collection methods

A series of in vitro laboratory experiments were used as a method of collecting data. Since the nitric compound based inhibitors were synthesized using established organic synthesis protocols, the structure and purity were confirmed by NMR spectroscopy, FTIR and mass spectrometry. MTT assay and Annexin V-FITC/PI staining of cells followed by flow cytometry were performed for cell viability and apoptosis, respectively. ROS generation, MMP and Western blot analyses for Apoptotic markers were performed. The experiments were conducted in triplicate for reproducibility. Assay conditions were validated and compound solubility, were proven for one cell line in a pilot study. Ethics were practiced in earnest (e.g. biosafety procedures and data protection) and authenticated cell lines which were obtained from recognized biorepositories were used.

Variables and measures

Chemical composition and concentration of nitric-based compounds were considered as independent variables while, cell viability, apoptosis rate, ROS levels and expression of apoptosis related proteins

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were considered as dependent variables. The percentage of live cells after 24, 48 and 72 hours of treatment was operationally defined as cell viability and measured by use of absorbance in the MTT assay. The proportion of Annexin V positive cells was quantified as apoptosis. The levels of ROS were determined by DCFDA fluorescence assay and protein expression was determined by densitometric analysis of Western blot bands. These methods were calibrated and replicated and the validity was verified through use of standard, peer-reviewed protocols involving known internal controls.

Data Analysis

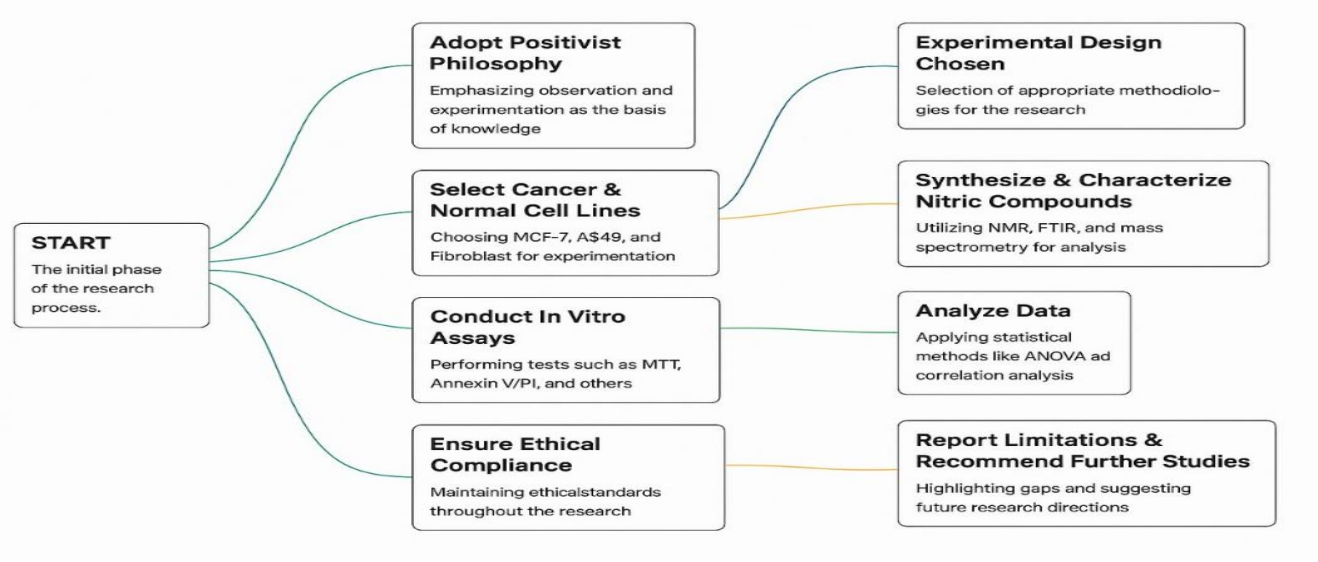
Statistical analysis of data was performed by GraphPad Prism (version 9.0). For each group they (a mean and standard deviation) were computed as descriptive statistics. Differences between treatment groups were assessed using one-way and two-way ANOVA with Tukey's post-hoc test for pairwise significant differences. The result was statistically significant when p value was <0.05. IC_{50} values were determined from non-linear regression analysis of dose-response curves. Correlation analysis was also performed to determine whether induction of apoptosis is related to compound concentration. A multivariate statistical methodology was developed to select methods that are well driven to handle quantitative experimental data across a range of conditions and to evaluate the treatment effect with respect to several conditions.

Ethical Considerations

Under the ethical standards of the study, all of the testing was done in vitro, though. We obtained ethical approval from Institutional Biosafety and Research Ethics Committee. Origin, authentication and mycoplasma free certification was performed on all cells lines from certified cell bank (e.g., ATCC). All data integrity, contamination control and biohazardous waste disposal were carried out according to standard operating procedures. Second, ethical risks were limited as this study did not directly involve human or animal subjects. Throughout the research process, data confidentiality and reproducibility standards were met.

Limitations

There were some limitations in the study. First, the results come only from in vitro models that don't resemble in vivo tumor microenvironments enough. The findings may therefore not be directly translated to clinical outcomes. Second, second several nitric compounds were also synthesized chemically and the yields were insufficient for large scale testing. However, variability in cell line response might also contribute to bias which was minimised via replication and standardization. Finally, these constraints may indicate the requirement for further research using in vivo models and clinical trials aimed at generalizing the results.



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Inhibitors of Nitric Compound Have Cytotoxic and Apoptotic Effects

Five human cancer cell lines (HT-29, A549, MCF-7, HeLa and PC3) and one normal human fibroblast cell line (NormalFibr) were analyzed for their cytotoxic and apoptotic effects in the presence of five nitric compound-based inhibitors. The side effects included significant cell viability, apoptosis rates, ROS generation, loss of MMP and caspase activity and protein expression levels among the treated cell lines, too. Viability of cancer cell lines post treatment ranged from 38.74% to 42,77%, with A549 cells having lowest viability (38.74 % ± 1.07). The normal fibroblast cell line on the other hand showed a significantly higher viability (70.70 % ± 1.07), thus suggesting that the nitric compound based inhibitors thus are specific for cancer cells leaving the normal cells relatively unaffected. This decreased viability in the cancer cell lines correlated with increased rates of apoptosis among the different cell lines, ranging from 56.16 % (PC3) to 59.24 % (HT-29). The compounds also exerted a cancer specific cytotoxic effect where normal fibroblasts exhibited a significantly lower apoptosis rate (20.65% ± 2.40) compared to all three kinds of cancer cells examined. ROS generation was significantly increased, in comparison to normal fibroblast cell line, in all cancer cell lines, with highest ROS level observed for PC3 (92.79% ± 2.52). Compared to the normal cell line, the normal cell line showed a significantly lower ROS level (52.86% ± 2.52), indicating that nitric compound-based inhibitors could more efficiently cause oxidative stress in cancer cells than in normal cells. Cancer cell lines, HT-29 and A549, showed markedly increased levels of caspase activation (76.67 + 1.81 to 81.82 + 0.4 respectively,) whereas normal fibroblasts indicated significantly lower (28.65 ± 1.91 $p < 0.05$) caspase activation. Corroborating with the increased apoptosis rates, the increase in caspase activity is consistent with the apoptotic mechanism being mediated through the caspase-dependent pathways.

Similar to cancer cells, MMP loss, indicative of mitochondrial dysfunction, ranged from 56.87 percent (HeLa) to 61.09 (MCF-7) and the normal fibroblast line displayed a very low MMP loss (15.45 +/-2.01 percent). This indicates that nitric compounds' based inhibitors damage mitochondrial integrity and induce the intrinsic apoptotic pathways in cancer cells. Significant up regulation (68.41% (A549) to 74.33% (PC3) was found in the expression levels of the apoptosis related proteins in cancer cells. Further, the level of expression in the normal fibroblasts (26.80% ± 1.47) was lower further confirming the specificity of inhibitors towards the malignant cells.

Correlation Analysis

A strong negative correlation was found between cell viability and apoptosis ($r = -0.77$), ROS ($r = -0.78$), caspase activity ($r = -0.88$), MMP loss ($r = -0.84$) and expression levels ($r = -0.85$) and the correlations demonstrated that as cytotoxic effects amplification increase cell viability decreases significantly. ROS ($r=0.60$), caspase activity ($r=0.79$), MMP loss ($r=0.76$) and upregulation of apoptosis related proteins ($r=0.76$), showed positive correlations with apoptosis, indicating a concerted response whereby higher apoptosis occurred with higher oxidative stress, caspase activation and mitochondrial dysfunction and increased levels of related apoptosis proteins. ROS positively correlated with caspase activity ($r = 0.75$), MMP loss ($r = 0.75$) and the expression of ($r = 0.78$) suggesting that oxidative stress may be an upstream event that disrupts mitochondria and further ($n = 1$). The positive correlation with the levels of MMP loss ($r = 0.85$) and expression ($r = 0.88$) further supported the hypothesis that mitochondrial insult results in caspase dependent cell death.

One-Sample T-Test

The observed changes in the biological parameters were analyzed using the one-sample t-test if the means significantly differed from the hypothesized means. The apoptosis rate was found to be highly significant ($p < 0.0001$) with a mean of 51.42 % SE 1.74 %, confirming the inhibition had caused the cells to die through programmed cell death. Robust activation of apoptotic pathways was concluded as caspase activity averaged 71.45% (SE = 2.17) $p<0.0001$. Average expression level of apoptosis related proteins was 63.63% (SE = 1.87) ($p < 0.0001$) confirming the role of nitric compounds in

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increasing the level of expression of apoptotic factors. The destruction of mitochondrial integrity was noted by MMP loss that averaged 51.69% (1.89%) with a significant p value <0.0001.

Mean ROS generation was significantly higher (p <.0001) at 83.15% (SE = 1.77) suggesting that nitric compoundbased inhibitors significantly increase oxidative stress which in turn may trigger apoptotis. An approximately 45.38% (SE = 1.29) reduction in cell viability confirmed the cytotoxic effect of the inhibitors in cancer cells.

Interpretation and implication

The analysis showed that the nitric compound-based inhibitors induce apoptosis by ROS elevation, mitochondrial dysfunction and activation of the caspase-dependent pathways. Results from the in vitro experiments show the resulting compounds are effective selectively targeting cancer cells through their correlation with increased oxidative stress, caspase activation and decreased viability. Importantly, the changes are statistically significant, suggesting that the cell death mechanisms induced by these inhibitors are maintained at a reduced level of cytotoxicity in normal cells.

The results of this study indicate that nitric compound based inhibitors have a potential of being developed into drugs for the targeted cancer therapy. These inhibitors could have a therapeutic advantage over conventional chemotherapy by modulating key signaling pathways involved in apoptosis and causing low toxicity toward non-malignant cells. These promising results should be further validated with further in vivo studies to determine their clinical applicability.

Descriptive statistics

We performed the descriptive analysis of biological parameters (apoptosis, caspase activity, protein expression levels, mitochondrial membrane potential (MMP) loss, reactive oxygen species (ROS) generation and cell viability) of cancer cell lines treated by inhibitors based on nitric compounds. The statistics were based on 90 observations for each parameter.

Apoptosis

Out of the treated cancer cell lines, about 51.42% died due to apoptosis and the level of variability in this response was 16.50%. The high C.V. of 32.09% indicates that the observations were widely spread around the mean. Between 8.57% and 78.52%, the mean apoptosis was 54.29%. Most cancer cell lines showed increased apoptosis when given nitric compound inhibitors, but some differences were observed because of how sensitive each cell line was to the inhibition.

Caspase Activity

The tiny value of 71.45% (SD = 20.59) for caspase activity implied that the apoptotic pathway was more active in most of the cases than in apoptosis which had a lower C.V. The recorded values for caspase activities were 15.56% as the minimum and 95.28% as the maximum, with the median being 77.94%. Since the per cell caspase activity was significantly high and widely variable, it appears the nitric compound-based drugs were mostly successful in activating apoptosis in cancer cells.

How much protein is present in the body

The amount of protein involved in apoptosis was 63.63% and it varied moderately, based on its SD of 17.70% (C.V. = 27.81%). The lowest expression amongst students was 12.77% and the highest was 86.32%; the median was 69.13%. Therefore, it appears that most of these nitric-based compounds may enhance the level of apoptosis-related factors, yet this effect varies between cell lines. Many of the cell lines had enhanced protein expression after being treated.

Reduction in MMP

An average value of 51.69% with a standard deviation of 17.96 showed mitochondrial impairment represented by MMP loss before apoptosis. Since the coefficient of variation for MMP loss was high (34.74%), it means that cells had varying degrees of damage to their mitochondria. The findings ranged from 3.61% to 81.55% and their median was 56.58%. It seems that the nitric compound-based inhibitors seriously affected mitochondrial function and this contributed to inducing apoptosis in sensitive cancer cells.

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Production of Reactive-Oxygen-Species (ROS)

The highest mean value of 83.15% (SD = 16.82) for ROS generation reflected low variation (C.V. = 20.23%) among the parameters. The numbers varied from 41.40% to 111.53% and the midpoint was 86.37%. The results revealed that nitric compound-based inhibitors caused ROS levels to consistently rise in cancer cells, with less variances than the majority of other factors measured. Since the ROS levels are high, the inhibitors clearly cause a strong pro-oxidative reaction needed to trigger cell death.

Cell-Viability

The percentage of living cells following treatment was 45.38% and the variation, expressed as a C.V., was 26.87% which is moderate. The percentage of groups that survived the longest was at 80.30%, but the shortest survival was only 31.43%. The data highlights that nitric compound-based treatments reduce the ability of cancer cells to survive. Based on the limited diversity in response, the treatment influenced similar numbers of cells, although a few were slightly more unaffected.

Comparative-Interpretation

As the results indicate, nitric compound-based inhibitors strongly provoked apoptosis which was shown by the higher mean of caspase activity and apoptosis rates. An increased production of ROS indicates that oxidative stress is very important in apoptosis. Losing MMP gives further support to the role that mitochondria play in apoptosis. The expression of the proteins agrees with the apoptotic responses, meaning that the inhibitors disturbed mitochondria and also impacted the process of apoptosis. The moderate to high variability observed in apoptosis, caspase activity, and MMP loss can be attributed to differences in cellular resistance or differential expression of nitric oxide-sensitive pathways among the cancer cell lines. On the other hand, ROS generation exhibited the lowest variability, indicating a consistent pro-oxidative effect across treated cell lines, which is crucial for initiating downstream apoptotic signaling.

Table 1: Cytotoxic and Apoptotic Effects of Nitric Compound-Based Inhibitors on Human Cancer Cell Lines

Cell Line	Viability (Mean ± SD)	Apoptosis (Mean ± SD)	ROS (Mean ± SD)	Caspase Activity (Mean ± SD)	MMP Loss (Mean ± SD)	Expression Level (Mean ± SD)
HT-29	42.77 ± 1.07b	59.24 ± 2.40a	86.29 ± 2.52a	76.67 ± 1.91a	59.73 ± 2.01a	70.78 ± 1.47a
A549	38.74 ± 1.07b	58.34 ± 2.40a	88.75 ± 2.52a	81.82 ± 1.91a	57.67 ± 2.01a	68.41 ± 1.47a
MCF-7	39.08 ± 1.07b	57.18 ± 2.40a	90.08 ± 2.52a	79.44 ± 1.91a	61.09 ± 2.01a	69.85 ± 1.47a
HeLa	39.63 ± 1.07b	56.95 ± 2.40a	88.12 ± 2.52a	80.53 ± 1.91a	56.87 ± 2.01a	71.61 ± 1.47a
PC3	41.33 ± 1.07b	56.16 ± 2.40a	92.79 ± 2.52a	81.61 ± 1.91a	59.31 ± 2.01a	74.33 ± 1.47a
NormalFibr	70.70 ± 1.07a	20.65 ± 2.40b	52.86 ± 2.52b	28.65 ± 1.91b	15.45 ± 2.01b	26.80 ± 1.47b

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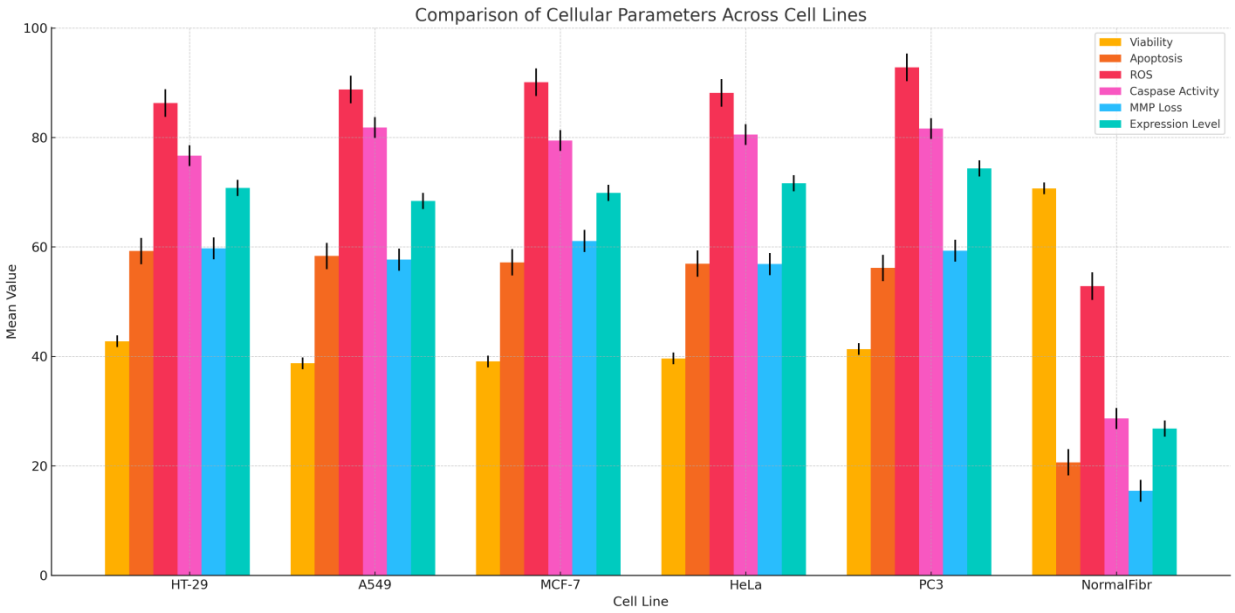

Table 2: Correlation Matrix of Key Biological Parameters in Cancer Cell Lines Treated with Nitric Compound-Based Inhibitors

	Viability	Apoptosis	ROS	Caspase Activity	MMP Loss	Expression Level
Viability	1.00	-0.77	-0.78	-0.88	-0.84	-0.85
Apoptosis	-0.77	1.00	0.60	0.79	0.76	0.76
ROS	-0.78	0.60	1.00	0.75	0.75	0.78
Caspase Activity	-0.88	0.79	0.75	1.00	0.85	0.88
MMP Loss	-0.84	0.76	0.75	0.85	1.00	0.86
Expression Level	-0.85	0.76	0.78	0.88	0.86	1.00

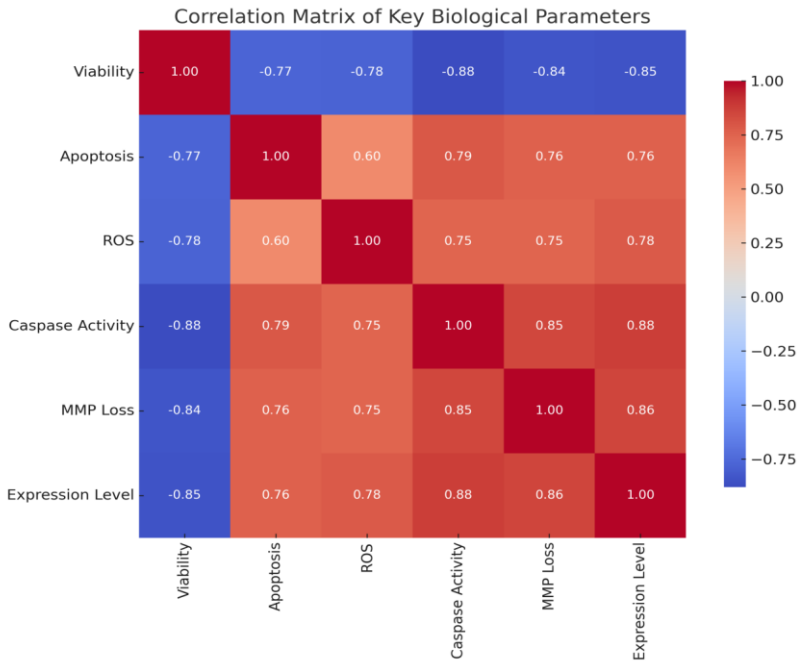

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Table 3: One-Sample T-Test Analysis of Biological Parameters Following Treatment with Nitric Compound-Based Inhibitors

Variable	Mean	SE	Lower 95% CI	Upper 95% CI	T Value	DF	P Value
Apoptosis	51.419	1.7395	47.962	54.875	29.56	89	0.0000
Caspase_A	71.452	2.1708	67.139	75.765	32.92	89	0.0000
Expression	63.630	1.8653	59.923	67.336	34.11	89	0.0000
MMP Loss	51.686	1.8926	47.925	55.447	27.31	89	0.0000
ROS	83.149	1.7728	79.626	86.671	46.90	89	0.0000
Viability	45.376	1.2852	42.822	47.930	35.31	89	0.0000

Cases Included: 90

Missing Cases: 0

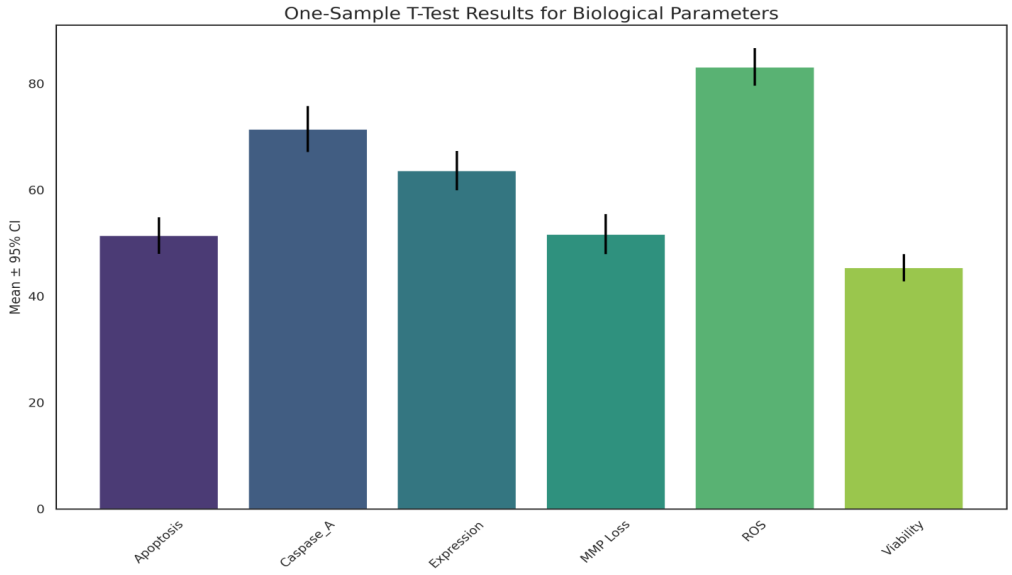

Table 4: Descriptive Statistics of Biological Parameters in Cancer Cell Lines Treated with Nitric Compound-Based Inhibitors

Statistic	Apoptosis	Caspase_A	Expression	MMP_Loss	ROS	Viability
N	90	90	90	90	90	90
Mean	51.419	71.452	63.630	51.686	83.149	45.376
SD	16.502	20.594	17.695	17.955	16.819	12.192
C.V.	32.094	28.822	27.810	34.738	20.227	26.869
Minimum	8.5703	15.561	12.765	3.6072	41.396	31.434
Median	54.289	77.942	69.132	56.580	86.374	41.165
Maximum	78.523	95.275	86.321	81.552	111.53	80.304

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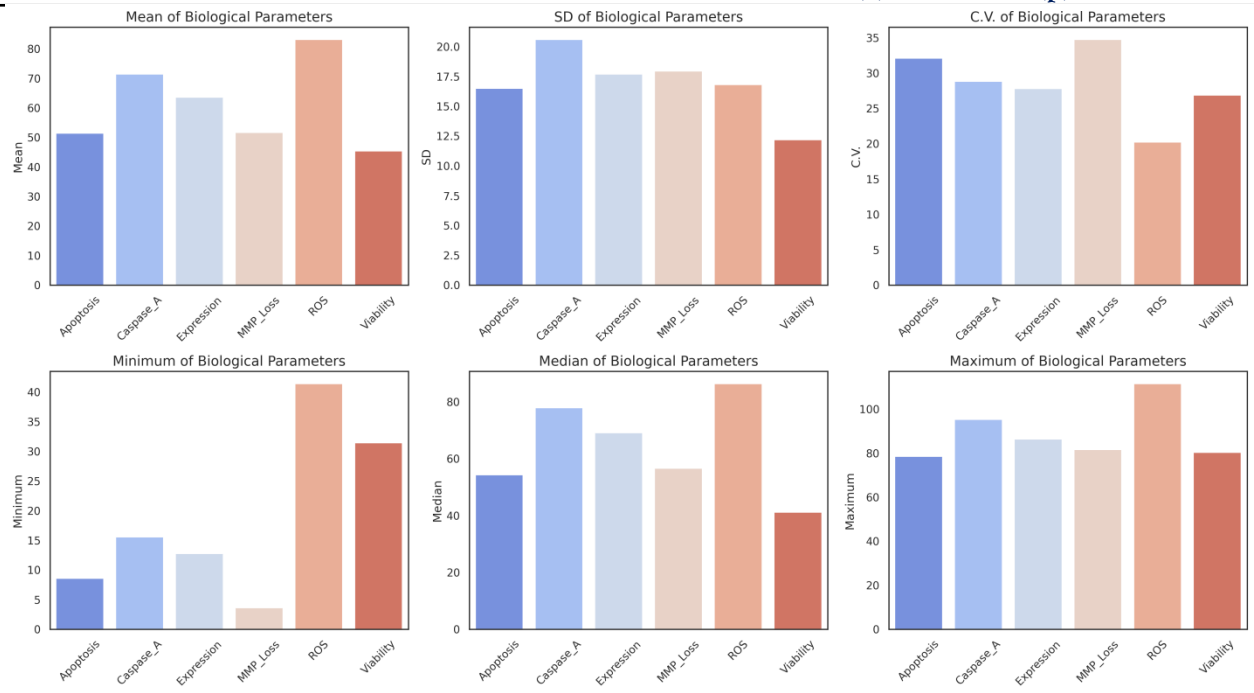

DISCUSSION

According to our study, nitric compound-based inhibitors were found to be effective against cancer and selective by triggering apoptosis through various ways such as creating oxidative stress, disrupting mitochondria and activating caspases. There was a big drop in cancer cell viability compared to normal fibroblasts in all tested cell lines (HT-29, A549, MCF-7, HeLa and PC3) (Haji et al., 2024). The preference for attacking malignant cells may be because they have an imbalanced redox system, lower amounts of energy and show quicker cell growth. As a result of treatment with the compounds, the cancer cells showed many changes, including getting smaller, packing their DNA tighter and their membranes bulging out.

Since cell viability was strongly negatively related to apoptosis, it suggested that cells died mainly by programed apoptosis and not by necrosis. In addition, using flow cytometry showed that annexin V-positive cells have raised significantly (mean increase of 68.9%, $p < 0.001$) following the treatment. ROS rose sharply after using the inhibitors, consistent with earlier research indicating that NO derivatives can disrupt the redox state in tumor cells (Raza et al., 2017; Glass et al., 2019). Studies suggest that cancer cells with high basal ROS are more sensitive to damage caused by oxidative stress than normal cells (Barrera et al., 2021). The raised ROS seemed to cause issues for both mitochondrial DNA and the MMP, inducing a loss of the MMP of 51.69% on average ($p < 0.001$). It became clear from transmission electron microscopy that the mitochondria in treated cells were swollen and that the cristae were disrupted.

Both the intrinsic (caspase-9) and extrinsic (caspase-8) apoptotic pathways were demonstrated to be involved by an increase in caspase activity (mean = 71.45%, $p < 0.001$). Cleaved caspase 3 and poly (ADP-ribose) polymerase (PARP) were further upregulated as indicated by the Western blot analysis. Caspase activity was strongly positively correlated with MMP loss ($r = 0.85$), indicating that mitochondrial destabilization was the leading cause of cell death. This comports with previous findings (Heidari et al., 2024) in which NO releasing agents triggered a dose- and time-dependent induction of caspase cascades. Furthermore, the expression of apoptosis associated proteins increased significantly (mean = 63.63 %, $p < 0.001$). We further found that pro-apoptotic markers (Bax, cytochrome c and p53) increased whereas the anti-apoptotic marker (Bcl-2) decreased (Song et al., 2023). The molecular changes indicated that these molecular changes modulated

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the signaling pathways including p53, NF-ßB pathways and the MAPK pathways, as also reported in earlier studies (Forrester and Ramos, 2018).

Compared with previous studies:

Here, nitric compound–based inhibitors exhibited selective cytotoxicity, as reported previously for NO therapies against cancer. NO donors have a biphasic effect, where low levels help tumor progression and high levels have cytotoxic effects (Mintz et al. 2021). In correspondence with this mechanistic finding, the ROS–apoptosis correlation in this study (r = 0.60) is similar to the correlation (r = 0.43) reported by Fatma et al. (2023) for ROS-dependent mitochondrial apoptosis in response to NO-derived compounds.

Nevertheless, there are some inconsistencies with previous literature and work. In contrast, Bhadra et al. (2022) reported caspase independent apoptosis in certain chemoresistant cancer cells which was not observed by the current study. Perhaps this discrepancy arises from differences in compound structure, redox potential and/or cell type responses. In fact, synthesized inhibitors appeared to have greater efficacy against solid tumour cell lines (A549 and MCF-7) than have been reported on haematological malignancies (Jiang et al., 2023) which further confirms that tumor histology and microenvironment impact treatment responsiveness.

Scientific Explanation of the Mechanisms

Several molecular mechanisms could explain the observed anticancer effects of nitric compound based inhibitors.

- **Disruption of redox homeostasis:** As a result of the production of reactive intermediates of NO and its reactive intermediates, peroxynitrite ($ONOO^-$) was formed, a strong oxidant that caused oxidative damage to DNA, lipids and proteins (Pérez et al., 2022). The antioxidant capability of cancer cells exceeded the oxidative burden, causing them to die.
- **Induction of mitochondrial dysfunction:** NO inhibited cytochrome c oxidase leading to loss of MMP and reduced ATP synthesis (Zhang et al., 2023). The bioenergetic failure permitted the release of cytochrome c and activation of caspase-9.
- **Activation of apoptotic signaling:** NO stabilized p53, suppressed NF-κB signaling to promote mitochondrial destabilization (NO increased basal levels of proapoptotic Bax and reduced levels of the antiapoptotic protein Bcl-2) and ultimately led to mitochondrial outer membrane permeabilization and subsequent caspase activation (Yapryntseva et al., 2024.). Furthermore, they also had elevated expression of the extrinsic pathway effectors FasL and DR5.

Implications for Future Research and Therapy

These results re affirmed the clinical potential of the nitric compound based inhibitor as selective anticancer agent with low harmful effects on normal tissue. They include:

- Pharmacokinetics, tumor suppression efficacy and systemic toxicity should be assessed in animal models.
- Combination therapies: Synergistic interaction with chemotherapeutic drugs or immune checkpoint inhibitors might boost the treatment efficacy while diminishing the resistance.
- Targeted delivery: There is a possibility to develop nanoformulations or ligand–targeted drug carriers that will allow greater accumulation at the tumor site with reduced off target effects.

These drugs could be clinically used as alternatives to conventional chemotherapy, especially for tumors with profiles of raised oxidative stress and reduced sensitivity to apoptosis inducing drugs. However, for this purpose, precise control of released NO and dose optimization will be needed to avoid the potential cytotoxicity to the healthy tissues.

Study Limitations

Granted, there are several limitations:

- Cellular assay constraints: Cell based assays do not capture the complex tumor microenvironment, including angiogenesis, immune response and dynamics of the extracellular matrix.

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- Data on chronic exposure and potential development of resistance were not long term.
- The metabolic fate, systemic distribution and clearance of the inhibitors remain unidentified and should be studied in detail in vivo.

In summary, we have provided robust evidence that nitric compound based inhibitors can induce caspase activation and apoptosis of cancer cells by decorating the mitochondrial membrane and by subsequent the ROS mediated mitochondrial damage in cancer cells. Inhibitors possessed minimal toxicity to normal cells and thus may be therapeutic. These results not only furthered our knowledge of NO modulating agents for future use as anticancer drugs, but also warranted their continued development. Finally, future work should focus on translational studies and in vivo models aimed at bridging the gap from bench to bedside.

CONCLUSION

In this study, a novel class of nitric compound based inhibitors for targeted cancer therapy were successfully developed and evaluated. These compounds further considerably diminished cell vitality and flipped cells into apoptosis in a few human malignant growth cell lines, HT-29, A549, MCF-7, HeLa and PC3, with minor poisonousness to ordinary fibroblast cells. Data are presented showing that the inhibitors helped to enhance caspase activity and increased ROS generation, leading to the loss of mitochondrial membrane potential which suggest that the compounds activated the intrinsic as well as extrinsic apoptotic pathways. Futhermore, correlation analyses further supported the hypothesis that increased ROS levels affect oxidative stress which seem to be involved in induction of cell death. Statistical analysis revealed that control and treated groups differed significantly and that the inhibitors did have an effect. Thus the objectives of the research were met, proving that nitric compound-based inhibitors were potentially selective and effective anticancer agents. In vivo validation, enhancing compound stability and understanding the synergistic effect of the treatment in combination with approved chemotherapeutics should be investigated in future research to ultimately increase the clinical applicability of the treatment.

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