

SYNTHESIS AND EVALUATION OF SOME NITRIC OXIDE DONORS FOR POTENTIAL BIOLOGICAL APPLICATIONS

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2026

DECLARATION

(To be filled by Scholar)

I, Kashish, bearing Registration No. 42000270, hereby declare that the research work embodied in the Ph.D. thesis titled "Synthesis and Evaluation of Some Nitric Oxide Donors for Potential Biological Applications" submitted to Lovely Professional University is the original and bonafide work carried out by me under the supervision of Dr. Harpreet Kaur, Professor, Department of Chemistry, Lovely Professional University and co-supervision of Dr. Amilan Jose, Assistant Professor, Department of Chemistry, National Institute of Technology, Kurukshetra.

I further declare that:

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- b) The research work has been conducted in accordance with the academic and ethical standards prescribed by the university.
- c) Due acknowledgements and references have been duly given wherever the work of others has been cited or used.
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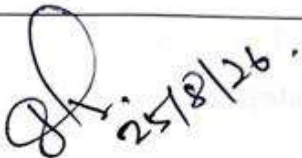
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CERTIFICATE

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This is to certify that the work reported in the Ph. D. thesis entitled "Synthesis and Evaluation of Some Nitric Oxide Donors for Potential Biological Applications" submitted in fulfillment of the requirement for the award of degree of **Doctor of Philosophy (Ph.D.)** in the Department of Chemistry is a research work carried out by Kashish, 42000270, is bonafide record of her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.

 Signature of Supervisor Name – Dr. Harpreet Kaur UID - 11724 Date 29.8.26	 Signature of Co-supervisor (if applicable) Name – Dr. Amilan Jose UID (if applicable) Date
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Abstract

A diatomic free radical with great reactivity, nitric oxide (NO) is an essential signaling molecule in many physiological and pathological processes. It plays essential roles in vasodilation, neurotransmission, immune response modulation, and the regulation of apoptosis. While NO has therapeutic potential, particularly in cardiovascular disease, wound healing, cancer, and antimicrobial applications, its clinical translation has been significantly limited by the lack of systems capable of delivering it in a controlled, targeted, and safe manner. Traditional NO donors often exhibit uncontrolled release profiles, leading to systemic toxicity, poor site-specific delivery, and undesired off-target effects. Consequently, there has been a pressing need for the development of NO delivery systems that offer spatiotemporal control, tunability, and biocompatibility.

In response to this challenge, novel Nitro-aniline-based nitric oxide (NO) donors namely N¹-(3-nitro-4-(trifluoromethyl)phenyl)ethane-1,2-diamine, N¹,N³,N⁵-tris(2-((3-nitro-4-(trifluoromethyl)phenyl)amino)ethyl)benzene-1,3,5-tricarboxamide, 5 - (dimethylamino) -N-(2-((4 - nitro -3- (trifluoromethyl) phenyl) amino) ethyl)naphthalene -1- sulphonamide, N-(2-((4 - nitro -3- (trifluoromethyl) phenyl) amino) ethyl)naphthalene -1- sulphonamide and N²,N⁶-bis(2-((4-nitro-3-(trifluoromethyl)phenyl)amino)ethyl)pyridine-2,6-dicarboxamide have been developed and reported for efficient, light-controlled NO release in both organic and aqueous media. These compounds are specifically designed to remain inert under ambient or dark conditions and become activated only upon exposure to a specific light stimulus - namely, blue light with a wavelength of 410 nm and 3W power. Under normal, dark conditions, the compounds remain stable and do not undergo any noticeable chemical change or decomposition, which significantly enhances their safety profile. However, upon exposure to blue light, a photochemical reaction is initiated within the Nitro-aniline scaffold. This reaction leads to the transformation of nitro (NO₂) to nitrito (ONO) bonds within the molecular structure and Consequently, this culminates in the release of nitric oxide accompanied by the formation of non-toxic, weakly absorbing photoproducts such as phenoxy and NO radicals. This light-triggered release mechanism ensures that NO delivery can be turned "on" or "off"

simply by controlling the light exposure. This light-triggered mechanism offers a unique approach to modulating NO release, allowing for external, non-invasive control over the time and location of therapeutic action. The photoreactivity of the Nitro-aniline core provides a precise method for achieving on-demand NO delivery, making these compounds highly promising for applications in photopharmacology, targeted therapy, and biological research, where the biological activity of a compound can be regulated precisely using light. The ability to activate NO release using specific wavelengths of light allows for an unprecedented level of spatial and temporal control in both in vitro and potentially in vivo systems. This innovation makes them suitable candidates for applications in therapeutic and diagnostic settings. Experimental evidence has shown that upon exposure to 410 nm blue light, these NO donors undergo a controlled and gradual release of NO. This release is not a burst or rapid reaction but follows a predictable, tunable trajectory. One of the key features of this system is its **dose-dependent control**: By adjusting the intensity and duration of the light exposure, as well as the concentration of the donor molecules, the amount and rate of NO released can be finely controlled. This level of tunability makes these compounds particularly suitable for applications where precise dosage and timing of NO delivery are critical, such as in **targeted cancer therapy** or localized infection control.

This offers a powerful level of control not typically achievable with conventional NO-donating compounds, which often release NO uncontrollably or spontaneously in physiological conditions, leading to undesired side effects or systemic toxicity. In contrast, the photochemical approach described here minimizes off-target effects and ensures that NO is released only at the desired site and time. Along with this the results of pH-dependent NO release studies confirmed that NO release is relatively fast in acidic medium as compared to other conditions studied.

The mechanism of NO release was investigated using UV-Vis spectroscopy and validated quantitatively via the Griess assay. **UV-Visible (UV-Vis) spectroscopy** has been used to monitor spectral changes corresponding to the photolysis of the Nitro-aniline-based donor molecules in real-time. The spectral changes observed upon light exposure indicated structural modifications within the NO donor molecule and thus validating the light-triggered release process. The Griess assay, a classical and widely

used method for detecting nitrite (a stable breakdown product of NO), provided quantitative evidence for NO production. In this assay, nitrite reacts with sulfanilic acid under acidic conditions to form a diazonium salt, which then couples with N-(1-naphthyl)ethylenediamine, producing a pink-colored azo dye. Spectrophotometric measurement of the color's intensity, correlates with the amount of nitrite and thus NO released.

The data obtained from these spectroscopic investigations further reinforced the conclusion that the release of NO is gradual and consistent and the NO release mechanism is both efficient and controlled, with minimal or no spontaneous release in the absence of light. The compatibility of this system with aqueous media also opens avenues for potential biomedical applications, including those involving physiological fluids and live-cell environments.

A critical requirement for the practical use of any therapeutic compound is its biocompatibility. To assess this, **in vitro cytotoxicity studies** were carried out on human cancer cells, namely human prostate cancer cells, HeLa cells, and breast cancer cells under both dark and light-irradiated conditions. The cytotoxicity assays involved treating cells with varying concentrations of the NO donor compounds and evaluating cell viability through a well established MTT assay method. Under dark conditions, where no NO release occurred, the compounds showed no significant cytotoxic effects even at concentrations as high as **20 µg/mL**. This finding confirms that the compound itself is not harmful unless it is activated via light thus reinforcing the **inertness and safety** of the compounds in the absence of light, making them suitable for systemic administration with minimal risk of off-target effects. This property is highly beneficial for applications where selective toxicity is required such as targeted cancer therapies.

In contrast to this a significant **reduction in cell viability** was detected when cancer cells were exposed to NO donors and then subjected to blue light for different time intervals. This confirms that the photoreleased NO exerts a toxic effect on the cancer cells, leading to cell death. The toxicity is believed to be a direct consequence of elevated intracellular levels of NO, which can interfere with critical cellular functions such as DNA replication, mitochondrial respiration, and oxidative stress regulation. NO at high concentrations can initiate both necrotic and apoptotic cell death

pathways, and in cancer cells, this property can be exploited for selective killing of malignant tissue while sparing normal, healthy cells.

The **selectivity** of the phototriggered toxicity is one of the most promising aspects of this delivery system. Since the NO donors are harmless in the absence of light, they can circulate in the body without damaging healthy tissues. Upon reaching the target site such as a tumor localized blue light irradiation can be applied externally or via minimally invasive fiber optic systems to activate the compound precisely where it is needed. This spatiotemporal control ensures that therapeutic effects are limited to diseased tissues, lowering adverse effects like immunological suppression, hair loss, and exhaustion commonly associated with conventional chemotherapy or radiation therapy. This makes the system adaptable to various clinical scenarios, including superficial tumors or deeper tissues with endoscopic light delivery. Future studies could focus on enhancing the penetration depth of the activation wavelength or using two-photon excitation for even deeper tissue access.

Furthermore, this platform opens up possibilities for **multi-modal therapeutic strategies**. For example, these NO donors could be co-administered with other chemotherapeutic agents, potentially enhancing their efficacy through synergistic mechanisms. Additionally, NO has been shown to sensitize tumor cells to radiation and certain drugs, suggesting potential applications in combination therapies

In conclusion, the development of light-sensitive Nitro-aniline-based NO donors that respond selectively to blue light represents a notable progress in the area of controlled nitric oxide release. Their unique ability to release NO in a gradual, consistent, and highly controllable manner upon blue light activation makes them excellent candidates for **precision medicine applications**. These compounds provide an elegant solution to the longstanding challenge of achieving precise, safe, and effective NO release. Their stability in the dark, high biocompatibility, efficient light-triggered activation, and dose-dependent control make them highly versatile and promising for therapeutic, diagnostic, and research applications. The work presented in this study not only confirms the practical feasibility of light-controlled NO delivery but also lays the groundwork for further **in vivo** studies, structural optimization for deeper tissue penetration, and eventual clinical translation. As the need for precision medicine

continues to grow, such stimuli-responsive systems offer a pathway toward safer and more effective treatments tailored to individual patient needs.

This thesis presents the design, synthesis, characterization, photochemical investigation, and biological assessment of the newly synthesised light-sensitive Nitro-aniline-based NO donors and establishes a foundation for their further development in translational biomedical research. Future work is required on **in vivo validation, optimization of light delivery methods**, and **structural modifications** to improve tissue penetration and specificity. Ultimately, this technology holds great promise for advancing both therapeutic and diagnostic capabilities in modern medicine.

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This thesis is dedicated to all those who inspired my curiosity, nurtured my learning, and stood by me in the pursuit of knowledge.

Abbreviations

1. SNP - Sodium nitroprusside
2. ISDN - Isosorbide dinitrate
3. iNOS - Inducible Nitric Oxide Synthase
4. Bcl – B-cell lymphoma
5. DETA-NONOate - Diethylenetriamine-NONOate
6. EMT - Epithelial-mesenchymal transition
7. OSCC - Oral squamous cell carcinoma
8. DLD - Duke's Large Bowel Disease
9. Apaf - Apoptotic Protease Activating Factor
10. Hsp - Heat-shock protein
11. T-cells - Thymus-derived cells
12. VEGF - Vascular Endothelial Growth Factor
13. p.o - Per os
14. i.p - Intraperitoneal
15. cGMP - Cyclic guanosine monophosphate
16. GTN - Glyceryl trinitrate
17. NMA - N-methyl-arginine
18. HIF - Hypoxia-inducing factor
19. SNAP - S-nitroso-N-acetylpenicillamine
20. GSNO - S-nitrosoglutathione
21. PARP - Poly-(ADP-ribose) polymerase
22. BCNU - 1,3-bis(2-chloroethyl)-1-nitro-sourea
23. SNVP - S-nitroso-N-valerylpenicillamine
24. UW - University of Wisconsin
25. NATs - N-acetyltransferases
26. DEA – Diethylamine
27. EGFR - Epidermal growth factor receptor
28. TGF - Transforming growth factor
29. HSG - Human salivary gland
30. LPS – Lipopolysaccharide

31. ODC - Ornithine decarboxylase
32. YY - Yin Yang
33. TNF - Tumor necrosis factor
34. NSAIDs - Non steroidal anti-inflammatory drugs
35. PSA - Prostate-specific antigen
36. NCI - National Cancer Institute
37. RAID - Rapid Access to Interventional Development
38. MSI - Microsatellite instability
39. HNPCC - Hereditary non-polyposis colon cancer
40. PAI - Plasminogen activator inhibitor
41. ACE - Angiotensin-converting enzyme
42. SNO-Cap - S-nitrosocaptopril
43. t-PA - type plasminogen activator
44. vWF - von Willebrand factor
45. GRMs – Gas releasing molecules
46. BNN - Bis-N-nitroso compound
47. PDA – Polydopamine
48. MSN - Mesoporous silica nanoparticle
49. hMSN - Hollow mesoporous silica nanoparticles
50. N-GQDs - N-doped graphene quantum dots
51. CDs - Carbon dots
52. ADT - Anetholedithiolethione
53. MDR - Multidrug resistance
54. DOX - Doxorubicin
55. TPZ - Tirapazamine
56. HMTN - Hollow mesoporous titanium dioxide nanoparticles
57. MMP - Matrix metalloproteinases
58. MDA - Malondialdehyde
59. Cox – Cyclooxygenase
60. AOM – Azoxymethane
61. ACF - Abberant crypt formation
62. LOX - Lipoxygenase

- 63. EGCG - Epigallocatechin gallate
- 64. AG - Aminoguanidine
- 65. CPA - Cyclophosphamide
- 66. B-NHL - B non-hodgkin's lymphoma cell line
- 67. TRAIL - TNF-related apoptosis-inducing ligand
- 68. ISMN - Isosorbide mononitrate
- 69. MtADH - Mitochondrial aldehyde dehydrogenase

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Chapter 1: Introduction

1.1 Nitric Oxide Donors

NO or NO donor-releasing agents are among the new agents that have recently surfaced. Compounds that release nitric oxide (NO) are known as nitric oxide donors. NO is a vital signaling molecule in the human body that plays an important role in numerous physiological functions, including controlling blood pressure and the immune system, wound healing, cancer treatment, antibacterial infections, neurotransmission, and vasodilation. [1] There are several types of NO donors namely - Organic Nitrates, S-Nitrosothiols (RSNOs), Nitrate Esters, Amino Acid Derivatives, SNP, NONOates and Diazeniumdiolates, further discussed in details in the following section.

1.2 Types of Nitric Oxide Donors

1.2.1. Organic Nitrates: These are widely used in cardiovascular therapy.

a) Nitroglycerin: Nitroglycerin, a well-known vasodilator that releases NO, is being studied for its ability to improve tumor oxygenation and chemotherapy effectiveness.

b) Isosorbide dinitrate (ISDN): Used in treating angina and heart failure, particularly in combination therapies.

1.2.2. S-Nitrosothiols (RSNOs): A class of chemicals in which a sulfur atom has a thiol group (NO) bonded to it. Release NO under physiological conditions.

1.2.3. Nitrate Esters: These are substances in which, in physiological conditions, the ester link cleaves, releasing NO. Compared to organic nitrates, their release mechanism is frequently more regulated and slower.

1.2.4. Amino Acid Derivatives: Some amino acids can also function as organic nitric oxide donors, especially those that have thiol groups. Under specific enzymatic circumstances, these compounds have the ability to release NO.

1.2.5. Metal-NO complexes [Sodium Nitroprusside (SNP)]: This substance releases NO and is being investigated for its potential to improve blood flow to tumors and make them more sensitive to radiation treatment.

1.2.6. NONOates: Unlike conventional NO donors, these chemical compounds release NO in a more regulated manner. Certain preclinical models have demonstrated their anti-cancer properties.

1.2.7. Diazeniumdiolates: A class of NO donors known as diazeniumdiolates releases NO in response to physiological stimuli, such as blood. By altering the tumor blood supply and causing tumor cells to undergo apoptosis, they have been investigated for their potential to cure a variety of malignancies.

1.3 Importance of fluorogenic/chromogenic group in NO donors

In incorporating fluorogenic or chromogenic groups into organic NO donors, the goal is to transform a single-purpose NO releaser into a dual-function molecular tool simultaneously delivering nitric oxide and providing a built-in visual or fluorescent signal to monitor release kinetics, location, and dose. Embedding fluorogenic or chromogenic groups into organic NO donors profoundly enhances their functionality, enabling self-reporting molecular systems that both release nitric oxide and visibly indicate when and where the release occurs. This smart design approach adds precision, control, and usefulness making these molecules valuable for synthesis monitoring, biological investigations, and therapeutic applications.

When fluorophores are integrated into organic NO donor molecules, they serve dual functions:

1.3.1 Acting as Optical Reporters

Upon exposure to light, the fluorophores undergo a photochemical transformation often structural change, cleavage, or oxidation that turns on fluorescence or induces a visible color shift. This transformation is linked to direct indication of NO release [2].

1.3.2. Functions as Light-Harvesting Antennae

Beyond reporting, the fluorophores also play a photophysical role: they absorb incident photons efficiently (typically in the visible spectrum, which many NO donor chromophores cannot) and transfer that energy into the NO-releasing core. This enhances the donor's photochemical efficiency, boosting the rate and yield of NO release under biologically friendly light. [2]

Thus the Fluorophores, Dansyl chloride and naphthalene have been incorporated in compound **3** & **4** respectively are known to not only serve as optical reporters, but

also play a secondary role in improving the overall molecular systems' capacity to harvest light, which promotes the photorelease of NO.

1.4 Role of light in NO delivery

Light plays a pivotal role in the controlled delivery of nitric oxide (NO) by enabling precise spatial and temporal regulation, enhanced photochemical efficiency, and real-time monitoring. Light-activated NO donors or photodonors remain inert until illuminated, allowing researchers and clinicians to turn NO release on and off with high precision, targeting specific tissues or even cellular subregions [3].

By incorporating visible or near-infrared (NIR)-absorbing antennae such as aza-BODIPY or rhodamine these molecules harvest longer-wavelength light efficiently, converting it into energy that triggers NO release without harming biological tissue [4]

Furthermore, many photodonors are self-reporting: they produce a fluorescent or photoacoustic signal upon activation, enabling real-time, in situ tracking of NO release [5]. For example, NIR-activated “photoNOD” compounds utilize aza-BODIPY-based chromophores that, upon irradiation, not only release NO but also generate a photoacoustic “turn-on” signal, facilitating deep-tissue imaging and guided therapy, including tumor treatment in animal models [5]. This synergy of controlled activation, efficient energy conversion, and simultaneous monitoring makes light-activated NO donors powerful tools for precision medicine applications such as vasodilation control, targeted cancer therapy, and antimicrobial treatment.

1.5 Therapeutic Applications

NO donors are used in cardiovascular disorders such as angina, hypertension, and heart failure due to their vasodilatory effects. They are also being explored in cancer therapy, wound healing, and anti-inflammatory applications.

1.5.1 Role of NO in cancer

NO exhibits both tumor-promoting and tumor-suppressing effects depending on concentration, exposure duration, and cellular environment [6].

1.5.1.1 Tumor-Promoting Roles of NO

- **Low to moderate NO levels** (nanomolar to micromolar), often arising from constitutive NOS (e.g., eNOS, nNOS), can foster tumor growth. Key mechanisms include:
 - **Angiogenesis:** NO enhances VEGF-driven blood vessel formation via cGMP/MAPK pathways [7].
 - **Cell proliferation & migration:** it increases DNA synthesis, cell division, and metastasis by activating pro-oncogenic signaling cascades like PI3K/Akt/mTOR and ERK [8].
 - **Suppression of apoptosis:** This process involves the reduction of pro-apoptotic proteins (such as Bax) and the enhancement of anti-apoptotic regulators, including Bcl-2. [9]
 - **Immune evasion & microenvironment modulation:** creates immunosuppressive conditions, stimulates stromal interactions, stabilizes HIF-1 α , and favors M2 macrophage polarization [10]

1.5.1.2 Tumor-Suppressive Roles of NO

- **High NO levels** (micromolar to tens of micromolar), typically resulting from inducible NOS (iNOS) in immune or inflamed cells, can counter cancer by:
 - **DNA damage and genotoxic stress:** generating peroxynitrite and other reactive nitrogen species that disrupt DNA integrity [11].
 - **Inducing apoptosis and necrosis:** via caspase activation, mitochondrial impairment, and p53 signaling. [12]
 - **Immune-mediated cytotoxicity:** increasing susceptibility to immune cell attacks and acting synergistically with chemo/radiotherapy [11]
 - **Impairing metabolic and proliferative machinery:** inhibiting ribonucleotide reductase, modulating mitochondrial function, and depleting iron reserves [13].
 - **Tumor vessel normalization:** low-dose NO from controlled release systems can transiently normalize vasculature, improving drug delivery and immune infiltration [14]

Through the creation of tools for NO donors, this dissection could lead to a unified mechanistic role of NO in oncology. Because NO can cause tumor cells to undergo

apoptosis, prevent angiogenesis, and increase the effectiveness of traditional chemotherapeutics in recent years, it has garnered a lot of attention in the field of cancer treatment.

A detailed mechanism of action of NO in oncology has been discussed below.

1.6 Mechanism of Action

NO is released by organic nitric oxide donors via chemical breakdown or enzymatic reduction. After being released, NO causes cGMP to rise by activating the enzyme guanylate cyclase. Smooth muscle cells, especially those in blood arteries, relax as a result of this cascade effect, improving blood flow and causing vasodilation. This procedure is essential for the treatment of cardiovascular diseases such as angina, hypertension, and heart failure. In order to overcome tumour cell resistance to traditional treatment drugs, nitric oxide (NO) is showing promise as an anti-carcinogenic agent.

NO is a common free radical gas that dissolves in water and has a variety of biological effects. Nitric oxide's effects in cancer are very diverse; findings in the literature have shown it to be both a pro- and anti-cancer agent. The kind of cell that is exposed to the drug, the redox state of the reaction, ultimate intracellular concentration, and the span of time that nitric oxide is exposed intracellularly comprise the working environment of NO in oncology, all affect the compound's ultimate activity. However, Nitric oxide's biphasic involvement in cancer lacks a unifying molecular explanation. In a broad variety Over a range of time intervals (seconds to days), nitric oxide donors simulate the constant production of NO. Therefore, NO donors exhibit a range of biological activities with both tumor-promoting and tumor-suppressing effects depending on the kind of cell exposed to the molecule and its half-life [15]. NO donors have drawn interest in cancer treatment because of their capacity to control tumour growth, improve the efficiency of radiation and chemotherapy, and stop the the development of cancer cells.

Here's a breakdown of how NO donors are being investigated in cancer therapy:

1.6.1. Regulation of Tumour Blood Flow: Nitric oxide is essential for controlling blood vessel dilatation and blood flow. Unusual blood arteries with uneven blood flow and poor perfusion are frequently developed by tumours. Increased blood flow to

tumours through the use of NO donors may improve the way chemotherapy or radiation treatments are administered.

1.6.2. Inducing Tumour Cell Death: NO can result in cancerous cells to undergo necrosis and apoptosis, two types of programmed cell death. Additionally, NO can make cancer cells more vulnerable to other therapies like radiation and chemotherapy.

1.6.3. Immunological Response Modulation: NO has a multifaceted role in the immunological system. Immune cells like T cells may become more active, which aid in locating and eliminating cancer cells, even at low quantities. At larger doses, NO can have immune-suppressive effects, but its precise role in cancer immunity is still being explored.

1.6.4. Chemoresistance and Radiosensitization: By altering the tumour microenvironment, NO has been demonstrated to sensitize tumours to radiation and chemotherapy, potentially making the cancer cells more vulnerable to these treatments.

1.6.5. Angiogenesis Inhibition: Although NO contributes to angiogenesis, or the development of new blood vessels, too much NO production can also prevent tumours from growing by disrupting the newly created blood vessels, which lowers the tumor's blood supply.

1.7 Preclinical and Clinical Research

1.7.1 Preclinical Research: NO donors have shown promise in preventing the formation of tumors in animal models, reduce metastasis, and increase the effectiveness of chemotherapy and radiation. For example, research has shown that the distribution of chemotherapy medications can be facilitated by NO donors to tumor sites by increasing vascular permeability.

1.7.2 Clinical Trials: Even though several early-phase clinical trials have shown promising outcomes, the systemic effects of NO continue to raise concerns, which can influence various biological systems and contribute to side effects like hypotension (low blood pressure) or undesired immune modulation. NO donors are used therapeutically to treat cancer, either by themselves or in conjunction with additional cytotoxic drug concentrations that are subtoxic, is strongly supported by a number of encouraging discoveries [16]. However, the difficulties associated with its controlled

and targeted delivery, as indiscriminate release can lead to cytotoxicity in healthy tissues frequently restrict the therapeutic use of NO.

These challenges have been elaborated in the following section.

1.8 Challenges and Considerations

1.8.1 Dose Control: NO release must be carefully regulated. While too much could >40 ppm have negative effects including hypotension or severe immune suppression, too little <5 ppm might have little therapeutic benefit.

1.8.2 Side Effects: Because NO has a wide variety of physiological effects, it must be carefully managed to prevent unintended systemic consequences. Furthermore, in some situations, excessive NO levels may promote tumor growth, thus time and dosage must be carefully considered.

1.8.3 Tumor Microenvironment: The tumor microenvironment, which includes elements like blood flow and oxygen levels, can affect how well NO donors work. Hypoxic areas are common in tumors, which can make NO donors' job more difficult.

1.8.4 Resistance Mechanisms: Due to changes in their immunological or vascular systems, certain cancers may become resistant to NO-based treatments. To comprehend resistance mechanisms and how to overcome them, more research is necessary.

These challenges have prompted the development of NO donor compounds capable of site-specific and stimuli-responsive NO release.

To overcome these challenges, a novel class of nitro-aniline based nitric oxide donors that exhibit light-triggered NO release in both aqueous and organic environments, representing a notable progress in the area of targeted cancer treatment and controlled drug delivery namely Compound **1**, **2**, **3**, **4** and **5** [Fig.1.1] have been reported here.

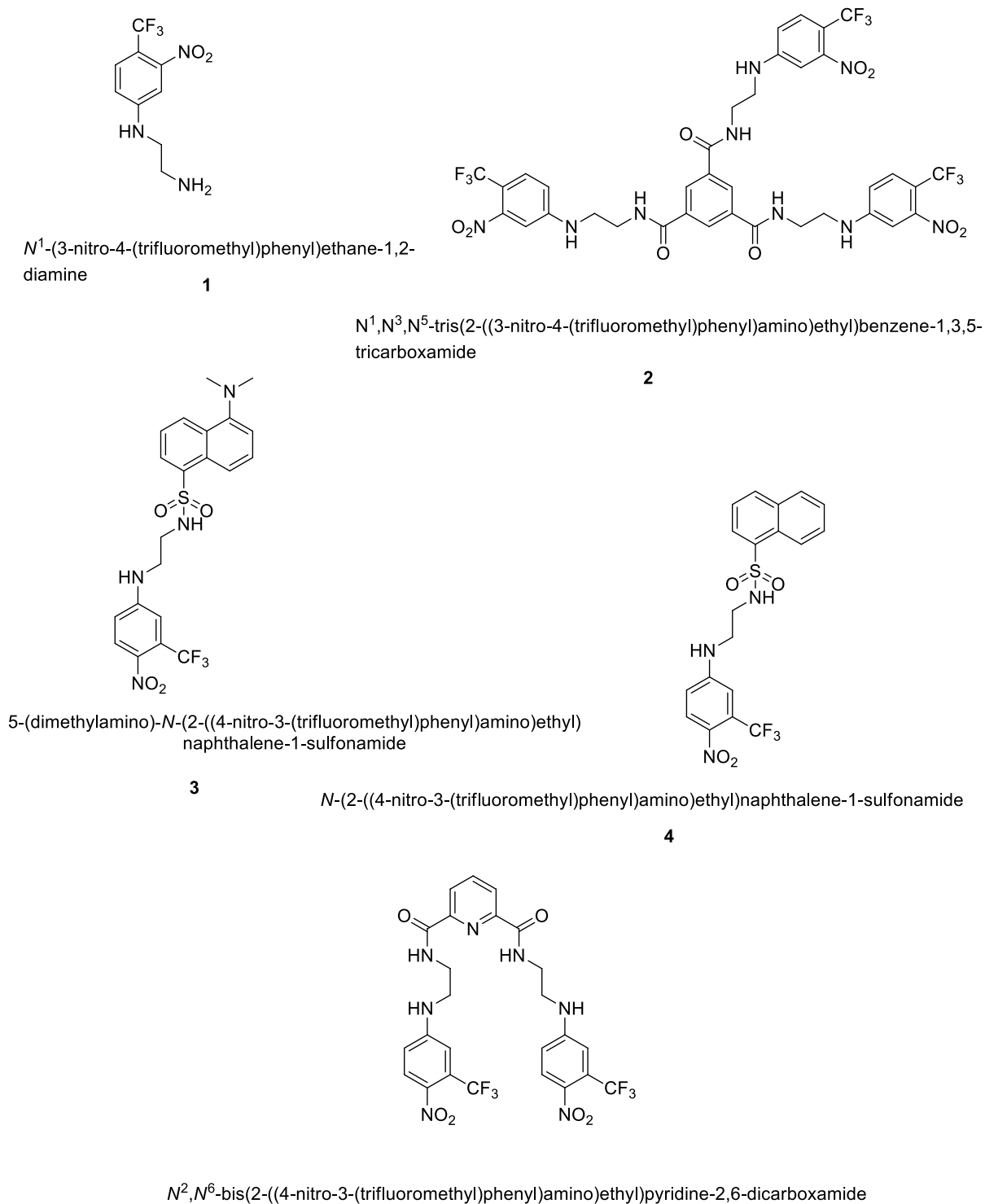


Figure 1.1 Showing the structures of synthesized nitro-aniline based nitric oxide donors

The key innovation lies in the design of novel nitro-aniline based compounds that enable exact temporal and spatial control of NO release with the use of an external, non-invasive trigger - blue light (410 nm). This level of control addresses a major limitation of conventional NO donors, which often lack selectivity and release NO indiscriminately, leading to potential toxicity in healthy tissues.

The ability to modulate NO release rates simply by adjusting donor concentration adds another layer of control, making these compounds highly versatile for therapeutic applications. This level of tunability makes these compounds particularly suitable for applications where precise dosage and timing of NO delivery are critical, such as in **targeted cancer therapy** or localized infection control. The light-triggered behavior not only ensures minimal background activity but also enables activation specifically at the disease site, reducing off-target effects.

This offers a powerful level of control not typically achievable with conventional NO-donating compounds, which often release NO uncontrollably or spontaneously in physiological conditions, leading to undesired side effects or systemic toxicity. In contrast, the photochemical approach described here minimizes off-target effects and ensures that NO is released only at the desired site and time. Along with this the results of studies on pH-dependent NO release verified that, in comparison to other circumstances examined, NO release occurs very quickly in acidic media.

The efficiency and consistency of NO release have been validated using the Griess assay and UV-Vis spectroscopic studies. Evaluations of in vitro cytotoxicity show that the compounds are safe for cells at up to 20 $\mu\text{g/mL}$ in dark conditions, confirming that NO release does not occur without light activation. This non-toxic nature of the donors in the absence of light ensures their safety under physiological conditions until deliberate activation is desired.

However, upon blue light irradiation, significant cytotoxic effects are observed in various cancer cell lines, namely HeLa, human prostate cancer and breast cancer cells. This light-induced cytotoxicity is directly correlated with NO release, highlighting the potential of these compounds as selective, light-activated anticancer agents.

This is particularly valuable for developing site-specific treatments where localized light exposure can be used to activate the therapeutic agent precisely within tumors, minimizing systemic toxicity.

Overall, this work adds to the expanding field of phototherapeutics and controlled NO delivery by offering a robust, tunable, and effective platform for NO release. It opens promising avenues for future research in non-invasive cancer treatments, photoactivated therapies, and broader applications in biomedicine where controlled gasotransmitter release is required.

With an emphasis on their use in targeted cancer therapy, this thesis describes development, preparation, structural analysis, and biological evaluation of these newly designed photoresponsive NO-releasing molecules based on nitro-aniline. The findings offer overwhelming backing for the creation of controlled, non-invasive medical procedures based on light-triggered NO administration.

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Chapter 2: Literature review

Some of the potential benefits of nitric oxide (NO) donors in cancer therapy include the following:

- i) a compound-dependent variable half-life;
- ii) compound-dependent spontaneous NO release;
- iii) regulated rate of NO release

When choosing the right NO donor for a given system, the following factors are crucial to take into account:

- i) the byproducts that are generated as the NO donor breaks down, such as sodium nitroprusside, which results in the byproduct cyanide;
- ii) The reaction's microenvironment, including temperature and pH, which may have an impact on the NO donor's half-life and release;
- iii) Depending on the NO donor and the reaction's microenvironment, It is possible to achieve a broad range of NO production times, from 1.8 seconds to 56 hours; and iv) the process of NO release, since enzymatic activation may be required for certain NO donors to produce NO. [1]

Overall, these factors collectively indicate that NO donor performance is highly context-dependent, which adds complexity to their selection for biomedical applications.

2.1 NO donor drugs

The inability to fully remove oxygen to stop oxidation to nitrogen dioxide makes NO gas notoriously challenging to handle. Nonetheless, the gas itself has therapeutic uses, particularly for pulmonary hypertension [2] and neonates [3], where it is inhaled into the lungs. Despite this particular application, we must rely on NO donor medications, or NO molecular carriers, these systems provide a means of stabilizing NO until activation under physiological or external stimuli.

Since NO was shown to be a crucial cardiovascular mediator in the 1980s, it is somewhat unexpected that only two types of NO donor medications are now being used in clinical settings and that no new NO donors have entered the market, considering the huge potential of NO in cardiovascular therapy.[4] Because NO donors may have systemic effects in vivo, their therapeutic usage has been restricted.

Among these negative effects is the buildup of hazardous metabolites like cyanide and vasodilation that causes severe hypotension [5]. These effects suggest that NO donor activity is not only therapeutic but also strongly dose-dependent, requiring careful regulation to achieve desired outcomes. The current goal is to find the best NO donor with the most potent anti-proliferative effects and the fewest side effects. By adding a NO moiety to an already-approved anti-cancer medication (NO-NSAID), NO-drug hybrids are created that reduce side effects while enhancing anti-tumor capabilities (NSAID-induced peptic ulcer disease, for example). This strategy reflects an effort to optimize therapeutic balance between efficacy and safety. Clinical trials for NO-NSAIDs, which have the potential to be anti-cancer medications (e.g., NO-releasing aspirin for individuals at elevated risk of developing colorectal cancer [6]), are presently being conducted as part of a phase I randomized study financed by the NCI. Out of all the significant types of NO donors, already discussed in section 1.2, this literature review focuses only on the light stimulated organic NO donors.

2.1.1 Organic nitrates

The earliest class of NO donors used in therapeutic applications are organic nitrates [7]. These substances have the general formula (RONO_2) and are nitric acid esters of mono and polyhydric alcohols. GTN (Fig. 2.1 a) and ISDN (Fig. 2.1 b) are two examples of frequently used organic nitrates. For more than a century, GTN has been utilized as a medicinal drug to relax the coronary arteries, which relieves chest pain [8]. As a prodrug, GTN needs to be denitrated in order to produce NO.

While glyceryl trinitrate (GTN) has a short plasma half-life of approximately 1–4 minutes, while its metabolites persist for longer durations (30–60 minutes) [8]. This rapid metabolism supports acute therapeutic use but also contributes to variability in sustained pharmacological response. [8] GTN has been used clinically in the cardiovascular system for more than a decade, and its relatively modest list of adverse effects, which includes headaches and hypotension, has demonstrated that it is a safe medication. The development of tolerance with prolonged use of nitrates is one of its main drawbacks [9]. Nonetheless, GTN is a desirable candidate to ascertain its characteristics in the field of oncology due to its present clinical administration. GTN stopped the malignant development of murine melanoma B16F10 cells brought on by hypoxia. Established tumor xenografts containing these cells decreased the ability of

murine melanoma cells to proliferate and form lung nodules in vivo if GTN had been administered to the naked mice [10]. By triggering caspases-1 and -10, GTN made colon cancer cells more vulnerable to Fas-mediated apoptosis [11] because of colon cancer cells' β -catenin-proteasome-dependent degradation. Additionally, GTN demonstrated β -catenin/TGF signaling pathway down-regulation [12]. Under hypoxic circumstances (0.5% O_2), Prostate cancer TRAMP-C2 and PC-3 cells were chemosensitized to DOX (12.5 μ M) by GTN at a low concentration (0.1 nM) [13]. GTN transdermal patches have been used to treat angina in the United States, since the 1980s [8].

Interestingly, the development of tolerance in cardiovascular settings may have dual implications, as it can limit systemic NO exposure while still allowing transient sensitization effects in experimental cancer models [14].

Recent studies have shown that GTN patches may enhance chemotherapy outcomes in prostate and lung cancer models with limited adverse effects. [15,16,17] However, standardized dosing strategies for oncological applications have not yet been established.



Figure 2.1 Structures of Organic nitrates – GTN and ISDN

Caruso et al. [18] synthesized water soluble MPC-3 (Fig. 2.2 a) by subsequently modifying monolayer-protected platinum nanoparticles these ultra-small (~ 1 nm) water-soluble and thermally stable nanoparticles were functionalized with nitroaromatic NO-donor groups on their surface. MPC-3 have been reported to release NO only after irradiation with a light of 420 nm, in buffer solution at neutral pH with a rate constant = 1.5 pmol s^{-1} and quantum yield = 1.9×10^{-3} . The photogenerated NO intermediates were found to be non-toxic, making these nanomaterials particularly suitable for biological applications.

Fraix et al. [19] synthesized a polymeric nanoparticle “nanoplatfrom” co-encapsulating two distinct photosensitive agents - one for singlet oxygen ($^1\text{O}_2$) generation and another nitric oxide (NO) photodonor (Fig. 2.2 b), specifically for nitric oxide (NO) release. Both the reactive species were generated simultaneously, only after irradiation with a light of 405 nm, aqueous dispersion and the quantum yield has been reported as 0.03. The biological application of the synthesized compound has also been studied on melanoma cells and its application in the cytotoxicity has been found in the presence of irradiated compound. The NO donor component exhibit dual-color fluorescence, enabling real-time optical monitoring of its activation alongside the photodynamic action of the singlet oxygen photosensitizer. This elegant integration of bimodal phototherapy with built-in fluorescent feedback showcases a powerful strategy for precision cancer treatment.

Caruso et al. [20] reported a multifunctional nanoassembly comprising mesogen-bearing amphiphiles co-assembled with porphyrin photosensitizers into ~ 40 nm spherical aggregates, containing nitric oxide (NO) photodonor (Fig. 2.2 c) to release NO only after irradiation with a light of 450 nm in aqueous solution with a quantum yield of 0.6. The intrinsic fluorescence and compact size of the system enhanced its optical tracking and can have potential impact in cancer treatment.

Kandoth et al. [21] synthesized a host-guest supramolecular complex comprising a tailored nitric oxide (NO) photodonor (Fig. 2.2 d) non-covalently encapsulated within a rhodamine-labeled β -cyclodextrin. It has been reported to release NO only after irradiation with a light of 420 nm in Phosphate buffer with a quantum yield of 0.21, while simultaneously exhibiting distinct red fluorescence from the rhodamine tag. It was found to be non toxic to HeLa cancer cells without irradiation with light and caused 50% cell mortality after irradiation, thus acts as a photoactivatable, theranostic platform, enabling controlled NO delivery and optical imaging in cancer cells laying the foundation for advanced light-triggered nanotherapeutics.

A molecular hybrid that combines two chromogenic centers a porphyrin unit and a nitroaniline derivative (Fig. 2.2 e) connected via an alkyl spacer, has been developed by Fraix et al. [22].

This design enables the hybrid to generate both singlet oxygen and nitric oxide (NO) upon visible light activation. The NO donor component, derived from the nitroaniline

moiety, releases nitric oxide in methanol with a quantum yield of 0.06 when exposed to light of 405 nm, contributing to the cytotoxic effects on A375 melanoma cancer cells. It has been reported to show Dual modal (therapeutic photoaction and imaging) photoinactivation of cancer cells.

Nocito et al. [23] reported a NO photodonor, β CD-NOPD (Fig. 2.2 f) tethered to a cyclodextrin-based polymer, which upon irradiation with light of 420 nm releases nitric oxide in aqueous medium with a quantum yield of 0.005. Studies performed on sarcoma cancer cells demonstrated that the compound shows significant cells mortality after irradiation but remains non toxic without irradiation.

Parisi et al. [24] synthesized a compound DXNO-GR (Fig. 2.2 g) that combines doxorubicin (DOX) with an N-nitroso group, which remains inert in the dark but upon exposure to green light 532 nm releases nitric oxide (NO), at pH 7.4 with a rate constant $3.3 \times 10^{12} \text{ M}^{-1} \text{ s}^{-1}$. This light-triggered activation leads to potent cell killing in both DOX-sensitive (MCF-7) and DOX-resistant (MDA-MB-231) breast cancer cell lines. Compared with free DOX, the hybrid accumulates more efficiently inside cells likely due to strong binding to serum albumin bypasses efflux mechanisms in resistant cells, and preferentially localizes in the Golgi and endoplasmic reticulum rather than the nucleus, thus avoiding DNA-based toxicity when unactivated. After green-light exposure, NO release occurs (confirmed via DAF-FM fluorescence), which alongside DOX's phototoxicity markedly reduces cell viability ($\text{IC}_{50} \sim 0.46 \mu\text{M}$ in MCF-7; $\sim 3.9 \mu\text{M}$ in MDA-MB-231) and inhibits MDR1 efflux pump activity. In essence, this hybrid remains safe in the dark, but becomes a powerful, spatially controlled dual-action theranostic agent under green light offering a promising tool to overcome chemotherapy resistance while minimizing side effects.

Navacchia et al. [25] synthesized photocage-SdAdo (Fig. 2.2 h) a set of bioconjugates by linking a visible-light-sensitive nitric oxide (NO) donor (a 4-nitro-2-(trifluoromethyl)aniline "photocage") to biologically relevant carriers: 2'-deoxyadenosine and bile acids (ursodeoxycholic and chenodeoxycholic acid) via flexible thioalkyl or triazole linkers. Under 420 nm light, the deoxyadenosine and ursodeoxycholic hybrids efficiently released NO in aqueous medium, demonstrating strict photo-control over delivery. When tested on cancer cell models, all conjugates showed antiproliferative activity against leukemia (K562) and colon carcinoma

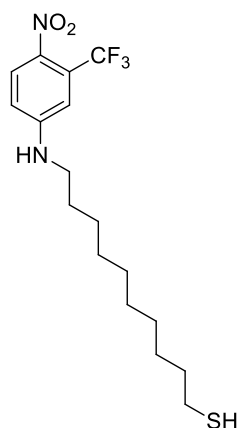
(HCT116), with the ursodeoxycholic derivative triggering pronounced cytotoxicity under light highlighting its potential as a novel photochemotherapeutic agent.

Benkovics et al. [26] synthesized AN- β CD-NOPD (Fig. 2.2 i), water-soluble β -cyclodextrin derivative equipped with two distinct functional groups on its primary rim: an anthracene fluorophore and a nitroaniline-based nitric oxide (NO) photodonor. When exposed to visible light, the nitroaniline unit releases NO in aqueous medium, which in turn initiates an energy-transfer process that restores anthracene fluorescence - an optical "turn-on" signal that reports the release event in real time. Remarkably, after NO release, the anthracene moiety also acts as a singlet oxygen ($^1\text{O}_2$) photosensitizer, offering a secondary reactive oxygen species capable of therapeutic action. Additionally, the cyclodextrin cavity remains free to host guest molecules, illustrating the system's potential as a multifunctional theranostic nanoplatform capable of controlled NO delivery, fluorescence signaling, subsequent ROS generation and can be used for photodynamic therapy.

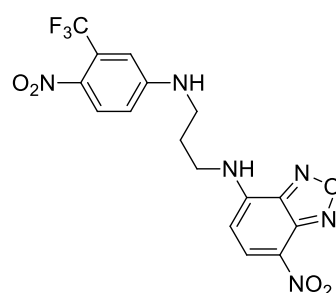
Fraix et al. [27] synthesized a dual-function molecule combining a green-light-sensitive BODIPY unit (for $^1\text{O}_2$ generation) with a blue-light-responsive nitroaniline-based NO donor (Fig. 2.2 j) connected by a short alkyl chain. The two chromophores maintain distinct absorption and photoreactivity profiles when linked. Under green-light (~ 532 nm) irradiation, the BODIPY unit efficiently produces singlet oxygen, while blue-light ($\sim 405\text{--}420$ nm) exposure selectively triggers nitric oxide release in acetonitrile with a quantum yield of 0.028, offering precise photomodulation of reactive oxygen and nitrogen species. In melanoma cells, this wavelength-controlled activation led to significantly greater cell killing compared to a similar compound without the NO-donating arm. This work showcases a refined, tunable phototherapeutic approach, where the desired cytotoxic agent ($^1\text{O}_2$, NO, or both) can be chosen by simply adjusting the light color.

Vittorino et al. [28] synthesized thin multilayer films by combining a visible-light-sensitive NO donor (Fig. 2.2 k) with a zinc-porphyrin dimer. Using Langmuir-Schäfer deposition, they transferred these organized layers onto quartz surfaces. The high loading of NO donor kept the porphyrins from clustering, enabling each chromophore to act independently under light exposure. When illuminated with visible light, the films produced both nitric oxide and singlet oxygen efficiently in

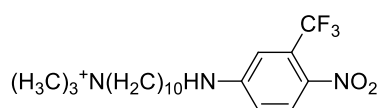
aqueous medium with a rate constant = $2 \times 10^3 \text{ s}^{-1}$ directly observed in real time using sensitive electrodes and infrared measurements. This dual-generation of reactive species, tightly controlled by light, opens avenues for materials that perform precise, light-triggered therapeutic or sensing functions.



MPC-3
a

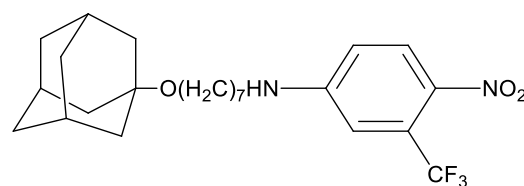


4-(N-(aminopropyl)-3-(trifluoromethyl)-4-nitrobenzenamine)-7-nitrobenzofurazan
b



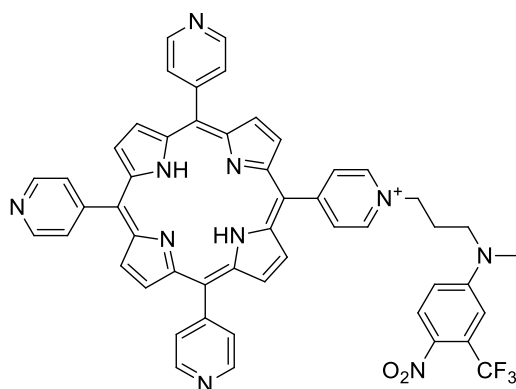
N,N,N-trimethyl-10-[[4-nitro-3-(trifluoromethyl)phenyl]amino]decan-1-aminium bromide

c



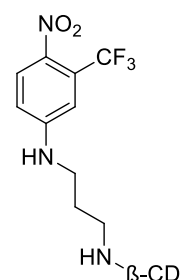
[7-(adamantan-1-yloxy)heptyl]-[4-nitro-3-(trifluoromethyl)phenyl]amine

d



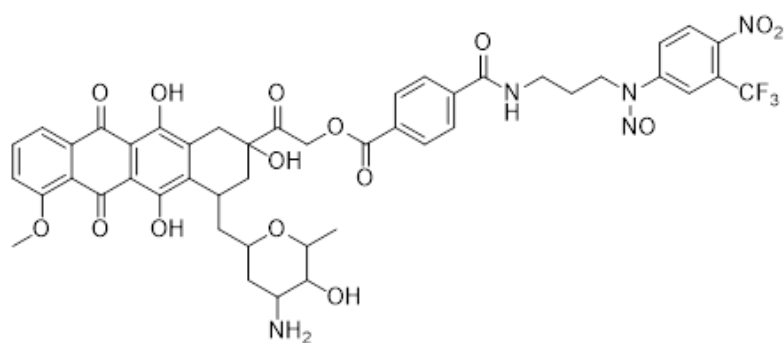
1-(3-{methyl[4-nitro-3-(trifluoromethyl)phenyl]amino}propyl)-504-(10,15,20-tripyridin-4-yl)porphyrin-5-yl)pyridinium bromide

e



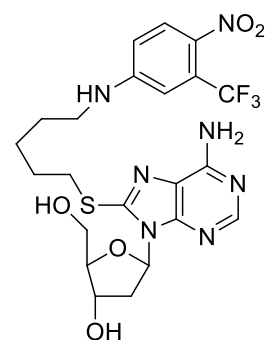
βCD-NOPD

f



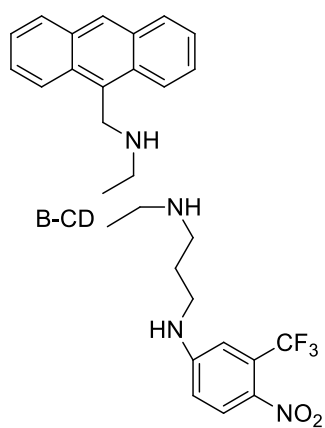
DXNO-GR

g



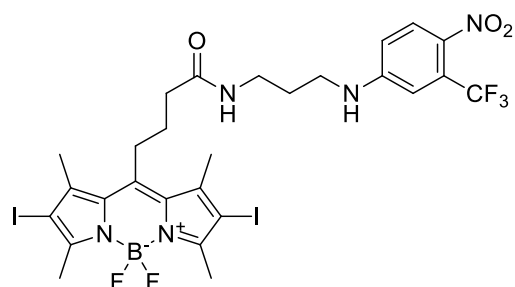
Photocage-SdAdo

h



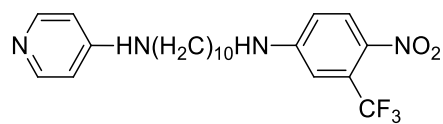
AN-βCD-NOPD

i



4-(4,4-Difluoro-2,6-diiodo-1,3,5,7-tetramethyl-4-bora-3,4-diaza-s-indacen-8-yl)-N-(3-((4-nitro-3-(trifluoromethyl)phenyl)-amino)propyl)butanamide

j



+

Ethane bridged Zn-porphyrin dimer

k

Figure 2.2 Structures of Nitro Anilline NO donors

2.2 A list of engineering techniques for creating sophisticated gas-releasing nanomedicine has been compiled.

2.2.1 Techniques for creating gas-releasing, stimuli-responsive nanomedicines (grm)

Therapeutic gas release at the tumor site is a crucial strategy to boost gas therapy effectiveness and prevent potential blood poisoning from NO, H₂S and CO. Catalytic gas generation and nanomedicines that release gas in response to a stimuli are the two most effective techniques for regulated gas release [29]. For potential therapeutic applications, we are focusing on creating stimuli-responsive gas-releasing nanomedicines.

2.2.2 Engineering stimuli-responsive GRMs to create gas-releasing nanomedicines:

Stimuli can either endogenous or exogenous. When exposed to visible light and/or UV, the coordination attraction between transition metals and carbonyls stabilizes the family of generic CO donors (GRMs) known as metal carbonyls, which includes Mn-CO and Ru-CO are vulnerable to photochemical degradation into CO. [30,31] Graphene oxide nanomedicines that are MnCO-coordinated and BNN-stacked (MnCO-GON and BNN-GON) [32,33] NIR-photothermal-responsive CO and NO release characteristics were demonstrated by PDA@MSN-SNO and FeCO@MCN-PEG nanomedicines, which clearly offers a better alternative to UV and visible light-sensitive GRMs [34,35]. Endogenous triggers for the production of stimuli-responsive gases can be H₂O₂, lactic acid, glucose, and enzymes, all of which are overexpressed in the TME [29]. MnCO@hMSN nanomedicines and Arg@hMSN were created to produce H₂O₂-responsive NO and CO release, respectively [36, 37].

2.2.3 Methods for delivering gas-releasing nanomedicines to specific tumors:

Gas delivery that targets tumors is the process of delivering a gas (or its prodrug) to a tumor. Only a small portion of gasses and their small-molecular prodrugs can truly take action at the site of the tumor, once they have entered the body. This lessens the effectiveness of cancer treatment and may even have a negative impact on blood and healthy tissues, particularly for extremely poisonous SO₂, H₂S, CO, and NO. By using nanotechnology to create nanomedicines that target tumors specifically, this problem

can be remedied. There are three sorts of tumor-targeted delivery systems based on various techniques: passive, active, and physical targeting [29].

2.2.4 Administration of gas to tumor tissues

Artificially improved retention, the EPR effect, as well as active identification and attachment of tumor arteries (integrin α_3) through local modification of the nanomedicine size are all ways to deliver nanomedicine to target tumor tissue [29]. Cyclic decapeptide (CGLIIQKNEC) with Arg -encapsulated MSN nanomedicine (Arg@hMSN-CLT1-G) enables high-efficacy tumor-targeted NO delivery by specifically binding fibrinogen in the extracellular matrix [36].

2.2.5 Gas delivery that targets tumor cell membranes

The membranes of cancer cells exhibit overexpression of certain markers, including integrin α_3 , CD44, and receptors for folic acid, galactose and transferrin. By attaching specific indicator ligands to the surface of gas-releasing nanomedicines, these therapies can effectively recognize and target cancer cells. In order to deliver NO to the 4T1 tumor membrane (N-GQDs, CDs and TiO_2 nanoparticles), Liu et al. co-conjugated RuNO-type NO prodrugs with a variety of ligands (receptors for folic acid and galactose) on a variety of multifunctional nano platforms [38].

2.2.6 Gas delivery with lysosome specificity

The lysosome is a vital organelle where nanoparticles are frequently endocytosed. Hazardous materials may be given and released with lysosomes in mind in order to damage them and cause cell mortality. Elevated levels of sulfur dioxide (SO_2), nitric oxide (NO), and reactive oxygen species (ROS) can disrupt the integrity of tumor cell and lysosomal membranes through oxidative stress, particularly the highly oxidative $\bullet\text{O}_2$ and $\bullet\text{OH}$ [29].

2.2.7 Delivery of gas to the mitochondria

In order to get rid of cancerous cells and shield normal cells from damage, several gaseous signaling molecules, including as CO, H_2S , H_2 , and NO modify cellular energy [29]. The most important target site for these substances is the mitochondrion.

2.2.8 Using magnets for gas delivery

In order to use external fields including electric, ultrasonic, and magnetic fields to control nanomedicines so they build up in tumors, responsive nanoplateforms are essential for field management [29]. A magnetic liposome with a diameter of roughly

200 nm was created by Liu et al. by encasing Fe_2O_3 nanoparticles in the hydrophilic core of liposomes (AMLs) and ADT, a hydrophobic H_2S prodrug, respectively [39].

2.2.9 Techniques for combining gas treatment

Among many recently developed, novel therapeutic strategies to treat cancer are chemotherapy, radiation, dynamic therapy, thermal therapy, gene therapy, immunotherapy, and gas therapy. Problems like side effects, the emergence of drug resistance and metastasis, and poor therapeutic efficacy are always present when one therapy technique is used as the sole choice. It has been empirically recognized that combining different therapeutic modalities might improve therapeutic outcomes [40,41]. One example of this is the typical integration of chemotherapy and radiation therapy.

2.2.10 Gasochemotherapy: The combination of chemotherapy and gas therapy

Combining gas treatment with chemotherapy may help address the issues and challenges related to chemotherapy, including metastasis, low specificity, and MDR. Min et al. created a DOX- CaCO_3 nanomedicine by in situ encapsulating DOX into CaCO_3 nanoparticles. This resulted in the realization of the straightforward CO_2 gas and DOX co-release in response to intratumoral acid-gas treatment and chemotherapy combined [42].

2.2.11 Gasoradiotherapy: combining radiation and gas therapy

Apart from chemotherapy, radiation therapy is an essential cancer treatment technique since it affects the DNA of cancerous cells. The most evident disadvantage of the radiation is the radio-resistance brought on by the TME's hypoxia. Combining radiotherapy and gas treatment as an anti-cancer technique can overcome the aforementioned problem by making radiotherapy more sensitive by adding O_2 and NO [29].

2.2.12 Gasothermaltherapy: Combining gas therapy with thermal therapy

Combining thermal therapy with gas therapy may help prevent any potential side effects. Hydrogenated Pd ($\text{PdH}_{0.2}$) nano cubes are developed on the basis of this idea in order to produce heat locally and provide hydrogen to tumors in a manner that is NIR-controlled and passively targeted for hydrogenothermal therapy. It was found to decrease heat damage to healthy cells and increase the H_2 efficacy of thermal therapy [43].

2.2.13 Gasophoto-/sono-/microwave-/chemodynamic treatment (combining these techniques with gas therapy):

Recently developed cancer treatments such as photodynamic, sonodynamic, and microwave therapy harm mitochondria, nuclei, endoplasmic reticulum and tumor cells' membranes before causing them to undergo apoptosis. Under the influence of light, sound, and microwave radiation, it converts oxygen into singlet oxygen ($^1\text{O}_2$) via photo-, sono-, and microwave-sensitizers. Feng et al. loaded the reduction-responsive anticancer medication TPZ is converted into HMTN by altering S-nitrosothiol (SNO) to create TPZ@HMTN-SNO nanomedicine [44].

By producing ROS for sonodynamic therapy, HMTN worked as a sonosensitizer when exposed to ultrasonic waves. These ROS were then able to convert SNO into NO for NO gas therapy due to the hypoxia produced by the sonodynamic therapy and TPZ was further activated to destroy tumor cells. Furthermore, stimulation sources like microwave, light and sound can be utilized to start the production of ROS and gas in order to combine dynamic therapy and gas therapy [45]. The following discusses the advantages of GRMs, which consist of three gas molecules (NO, CO, and H_2S), in the use of NIR lasers to treat cancers.

2.2.14 NO prodrug activated by NIR light

Upon its initial use NO, a gas signaling molecule, was discovered to be crucial for several physiological mechanisms, including immune response, formation of arteries and apoptosis [46–51]. Furthermore, by inducing MMP, which can deplete collagen, NO has been utilized to enhance the penetration of nanoparticles into solid tumors [52, 53]. The physiological role and therapeutic uses of NO have been the subject of more research than those of other gas molecules [54]. In tumor treatment, NO serves two purposes: It can encourage the growth of cancer at low concentrations (1 M) and at high concentrations (>1 M), it possesses anti-tumor properties [55–57]. Therefore, increasing the NO concentration in tumor tissues over the basic level can effectively stop tumor growth.

2.2.15 Photothermal therapy-triggered NO prodrug

In order to locally heat the tumor and encourage NO release during gas treatment, photothermal therapy uses Organic or inorganic nanoparticle lesions caused by NIR laser radiation [58, 59]. Numerous NO prodrugs are currently on the market,

including S-nitrosomercaptan, metal-NO complexes, nitrosamines, and organic nitrates and nitrites [1]. Table 2.1 has a list of nanogasprodrugs used in photothermal therapy.

Table 2.1: Nano-gas prodrugs used in Photothermal therapy

Nano - gas prodrugs	Biomedical applications	References
GO-BNN ₆	143B cells (Human osteosarcoma cells)	[33]
PTNGs	MCF-7/ADR cells (Human breast cancer cells)	[34]
RBS-UCNPs	MCF-7/DOXR cells (Human breast cancer cells)	[60]
Me-RBSs	4T1-LUC cells (Human breast cancer cells)	[61]
Nb ₂ C-MSNs-SNO	4T1 cells (Mouse breast cancer cells)	[62]
BNN-Bi ₂ S ₃	BEL-7402 cells (Human liver cancer cells)	[63]
Fe ₃ O ₄ @PDA@Ru-NO@FA	HeLa cells (Human cervical carcinoma cells)	[64]
PNOC-PDA/DOX	MCF-7/ADR cells (Human breast cancer cells)	[65]
DTX@m-PB-NO	4T1 cells (Mouse breast cancer cells)	[66]
SPNs PFTDPP	MCF-7 cells (Human breast cancer cells)	[67]

2.2.16 Prodrug of NO induced by photodynamic therapy

Additionally, PDT can be used in antineoplastic therapy to reduce P-gp protein expression and increase the chemotherapeutic drug susceptibility of cancer cells by causing the controlled release of NO [68, 69]. Moreover, highly active peroxynitrite (ONOO) molecules are produced by direct or indirect interactions between ROS and NO, which work better with PDT and gas therapy [68, 70]. Upon irradiation with an 808 nm laser, nanoparticles localized within lysosomes can undergo light-induced electron and energy transfer processes that prompt the release of reactive species or therapeutic agents. This will damage the lysosomes and accelerate the cancer cells'

inevitable demise because of the NO donor's and the surrounding O₂'s production of NO and ROS [71].

Table 2.2 shows the biological nano gas prodrugs used in Photodynamic therapy.

Table 2.2: Photodynamic therapy triggering nano gas prodrugs

Nano - gas prodrugs	Biomedical applications	References
Lyso-Ru-NO@FA@C-TiO ₂	MCF-7 cells (Human breast cancer cells)	[71]
L-Arg@PCn@Mem	4T1 cells (Mouse breast cancer cells)	[72]
DPP-NF	HeLa cells (Human cervical cancer cells)	[73]
ADAu@CuS YSNPs	MCF-7/ADR cells (Human breast cancer cells)	[74]

2.3 Effect of NO on cancer

2.3.1 Contribution of NO in the development of cancer

When leukocytes (also known as active macrophages) release free radicals like NO in response to increased oxygen absorption, both NO and its derivatives harm normally healthy nearby epithelial and stromal cells, which aids in the development of cancer. NO can harm DNA whether it is present alone or in conjunction with oxygen and superoxide, which can lead to carcinogenesis [75]. By interacting with superoxide to create ONOO⁻ and N₂O₃, NO can cause damage to DNA [76].

When nitric oxide (NO) is introduced either to TK6 lymphoblastoid cells or to isolated DNA in an oxygen-rich environment it consistently triggers the deamination of guanine and adenine, leading to the a significant rise around 40- to 50-fold in generation of xanthine and hypoxanthine, respectively, thus contributing to DNA alterations that can accumulate and promote carcinogenic mutations. Additionally, a study that exposed NO to Salmonella typhimurium revealed that the G:C to A:T alterations are the most frequent modifications brought on by NO-mediated deamination [78,79]. Mutant oncogenes and tumor suppressor genes frequently exhibit G:T transversions, which are caused by oxidation of guanine at the C8 site [80].

Single-strand breaks in DNA are produced either directly by peroxynitrite or indirectly through the creation of NO-mediated abasic sites, which are caused by DNA deamination [79]. The nuclear enzyme PARP, which acts as a "off" switch to stop DNA replication until base excision repair is completed by DNA repair enzymes, is activated by single strand breaks in DNA [81]. However, it has been demonstrated that in genotoxic conditions, NO permanently damages DNA repair enzymes, such as the zinc finger-containing enzyme formamidopyrimidine DNA glycosylase (Fpg protein) [82].

The most mutagenic substitution has been shown to be O₆, which also causes G:A conversions during DNA replication [83]. The synthesis of S-nitrosothiols is another way that NO indirectly damages DNA [84]. Hepatocytes cultured with NO donors such SNAP or V-PYRRO/NO suppressed caspase-3-like activity in the cytoplasm, according to a study by Kim et al. [85]. It has also been discovered that caspase-9 and caspase-8 are impacted by NO-mediated caspase inhibition [86].

Calmels et al. (1997) found that high concentrations of nitric oxide (NO) lead to an accumulation of p53 in mammalian cells while simultaneously diminishing its ability to bind to its specific DNA consensus sequence suggesting that NO compromises p53's tumor-suppressing function. Subsequent studies demonstrated that NO can chemically modify p53 either through S-nitrosylation or nitration of tyrosine residues resulting in impaired DNA binding and reduced transcriptional activity. These post-translational alterations hinder p53's regulatory control over cell proliferation and may indirectly affect the repression of proto-oncogenes such as c-FOS, c-JUN, and c-MYC, thereby potentially promoting carcinogenic processes [87].

Lipid peroxidation is also indirectly brought on by NO by damaging cellular membranes through radicals. Numerous compounds, including reactive electrophiles such aldehydes and epoxides, are produced by this process [88]. MDA, a highly electrophilic and nucleophilic tautomer that results from lipid degradation, has been shown to cause cancer [80].

2.3.2 NO in tumor progression

The ability to initiate angiogenesis, metastasis, growth and invasion metastasis are all results of a progressive sequence of genetic changes to tumor cells that occur during solid tumor evolution and can be impacted by NO signaling. Tumor cells and host-

derived cells, such as tumor vasculature cells and tumor-infiltrating leukocytes, make up a solid tumor [89]. Any of the active NOS iso-forms may be expressed by any of these different tumor components. Thomsen and Miles (1995) conducted one of the earliest investigations into nitric oxide synthase (NOS) activity in human breast tumors, revealing that both NOS activity and nitric oxide production are markedly higher in malignant breast tissue compared to benign or normal tissue. They also discovered a correlation between NOS activity and the degree of tumor growth.

Numerous experimental tumor models provide compelling evidence that NO directly promotes tumor growth and metastasis [89]. Upon introducing functioning NOS II into a human colon cancer cell line, Jenkins et al. [91] demonstrated that the tumors grew and became more vascular when transplanted into naked mice. Additionally, it was shown that both constitutively iNOS-expressing EMT6 tumors and a genetically edited cell line (iNOS-19) had their tumor growth rates decreased by a selective NOS II inhibitor (1400 W), indicating that NO plays a direct role in the growth and vascularity of tumors [92]. In a C3H/HeJ-derived murine mammary adenocarcinoma model, Orucevic et al. found that C3-L5 tumor cells, which naturally express endothelial nitric oxide synthase (eNOS), continuously generate nitric oxide (NO). This persistent NO production enhances the invasive behavior of these cancer cells in vitro, primarily by suppressing the expression of tissue inhibitors of metalloproteinases, TIMP-2 and TIMP-3. This underscores NO's role in promoting metastasis through eNOS activity in aggressive clonal variants. Furthermore, it was discovered that MMP-2 was elevated following the generation of endogenous NO by IFN-gamma and LPS, suggesting that the MMP-2/TIMP2 and TIMP3 systems are altered as part of a NO-mediated tumor invasiveness pathway [93].

2.3.3 NO and tumor angiogenesis

By advancing the proliferative and NO's migratory effects on endothelial cells have been shown to stimulate angiogenesis as tumors advance [91]. Because VEGF stimulates active NOS III, it is essential for endothelial cell motility. By upregulating NOS III mRNA/protein, increasing interaction with HSP90, activating PI3 kinase, or activating MAPK/PLC-, VEGF can activate NOS III [89]. In conclusion, tumor angiogenesis is caused by NOS-generated NO.

2.3.4 Function of NO in cancer chemoprevention

It has been discovered that NOS II inhibitors lessen metastases, inflammation, and tumor promotion [91]. A later study reported that male F344 rats with AOM-induced colon cancer were treated with NOS II-specific inhibitors (SC-51 and AG), either individually or in combination with the COX-2 inhibitor celecoxib [94]. These inhibitors reduce colon cancer's AOM-induced colonic ACF, a marker for the disease and concurrent treatment of SC-51 and celecoxib was reported to further lower the incidence of ACF [94].

In cancer chemoprevention, NOS II can be directly inhibited, but it can also be indirectly inhibited by targeting the NFB pathway, which is required for NOS II activation [95]. Curry and turmeric combine to form curcumin (diferuloylmethane), a yellow spice that has anti-tumor properties. PKC, EGF-receptor tyrosine kinase, and I κ B kinase all involved in the control of NO are significantly inhibited by curcumin [96]. Curcumin should therefore be taken into consideration that it prevents the activation of numerous NO-mediated signaling pathways, making it a promising therapeutic medication for cancer chemoprevention.

Grape seeds and skins contain the phytopolyphenol resveratrol (trans-3,4',5-trihydroxystibene). Tsai et al. investigating how resveratrol affects RAW 264's NOS induction. LPS was revealed to be the cause of the decrease in NOS II protein and to strongly limit NO generation in activated macrophages in 7 cells. [97]. Mutoh et al. looked into how resveratrol affected a human colon cancer cell line [98]. Additionally, it was shown to inhibit the activity of the COX-2 promoter. Resveratrol might be an effective chemopreventive medication for cancer by inhibiting NO, NF κ B, and COX-2 [99].

In a variety of in vitro and animal settings, the green tea polyphenol EGCG has chemopreventive and antioxidant qualities [100]. By suppressing the activity of NF κ B triggered by LPS in vitro, Lin and Lin [101] demonstrated that EGCG inhibited NOS II activation. In this study, EGCG treatment decreased NOS II's enzymatic activity, which in turn decreased NO generation. Additionally, by stopping I κ B from degrading, EGCG suppressed the binding activity of NF- κ B [101]. Additionally, EGCG prevented DNA damage and consequent carcinogenesis by inhibiting peroxynitrite-mediated tyrosine nitration and causing human cancer cells to undergo apoptosis and cell cycle arrest [102].

NO-NSAIDs have been shown by Kashfi et al. [103] to suppress the growth of tongue-derived cancer cell lines, prostate, lung, colon, and pancreas. Through S-nitrosylation and consequent suppression of caspase-1, Additionally, NO-NSAIDs can inhibit the production of cytokines such IFN- γ , IL-1 β , and IL-18, which in turn inhibits the formation of NOS II [104]. Furthermore, it was demonstrated by Williams et al. [105] that by binding to NFB and expressing NOS II, NO-releasing aspirin (ASA), and more specifically NO-NSAID, inhibited the growth of human colon cancer cells.

2.3.5 NO-mediated effects on tumorigenesis

NO has been shown to have anti-apoptotic and cytotoxic effects on tumor cells [106]. The tumor's destiny will depend on the ratio of apoptosis to NO-mediated cellular growth. The kind of tumor cell and its sensitivity to NO will determine, high but physiologically significant NO concentrations might cause apoptosis whereas lesser amounts can have the opposite effect [87].

TRAIL, Fas ligand, and death receptor ligands were overexpressed in the spleen and thymus of p53^{-/-} NOSII^{+/+} mice prior to tumor growth, together with a lower proliferative index and a higher apoptotic index than the double knockout mice [107]. Tumors grew more quickly in NOSII^{-/-} mice than in wild-type NOSII^{+/+} mice, according to a study [108]. According to this study, NO plays a significant part in inhibiting the growth of tumors. NO mediates the anti-tumor effect induced by IL-13, according to another study. Numerous solid tumors, including head and neck cancers, have elevated IL-13 receptors, AIDS Kaposi's sarcoma, renal cell carcinoma, and glioblastoma. The IL-13-mediated anti-tumor activity was markedly reduced in mice that had previously received a NO inhibitor [109]. Tumor cells' local NO generation and cellular origin seem to be crucial factors in the metastatic process. The propensity for lung metastasis varied across two clones of breast cancer cells that produced varying amounts of NOSII and NO. Tumor formation by both clones was greatly reduced when mice were given AG and a NOSII inhibitor [110].

It has been shown that dendritic cells' anti-cancer efficacy is increased by the in vivo administration of NO or NO-generating medications [111]. According to recent research, following radiation therapy, NOSII-induced NO contributes to the development of estrogen-dependent mammary adeno-carcinoma [112]. Rats given the

estrogen diethylstilbestrol after being weaned were immediately exposed to whole-body radiation.

When compared to the non-irradiated mice (11.1%), the tumor incidence rose 7.6 times (85%) and decreased to 25.6% in the rats given an i.p injection of a NOSII inhibitor.

2.4 Function of NO in immunosensitizing and chemosensitizing tumors to trigger apoptosis

2.4.1 Role of NO against tumors

Through elevated NOS II expression, NO affects subsequent carcinogenesis processes that support anti-tumor actions, specifically apoptosis. According to a study by Xie et al. [113],

in vitro studies demonstrated that murine melanoma cells overexpressing NOS II underwent nitric oxide-mediated apoptosis, which limited their tumor growth and viability, preventing their spread in vivo. Conversely, deletion of NOS II in mice has been reported to accelerate colon tumor development in the Apc (Min/+) model of colorectal cancer [114]. Furthermore, p53 accumulation, tumor cell death and cell cycle halt can be brought on by NO-mediated DNA damage.

In tumor cells, heightened p53 transcriptional activity—and its post-transcriptional regulation—promotes increased NOS II (iNOS) expression, elevating nitric oxide (NO) levels and triggering p53-mediated cell-cycle arrest and cell death [76]. Concurrently, wild-type p53 directly represses NOS II (transrepression), forming a feedback loop that helps limit NO-driven DNA damage, as demonstrated in both in vitro and in vivo studies [115]. It's crucial to remember that that resistance to NO-mediated tumor growth is conferred by COX-2 activity. NO-sensitive cells were either pretreated with low NO levels to activate COX-2 by NFκB or AP-1 activation, Alternatively a COX-2 expression vector was used to transfect them. As a result cells become resistant to elevated NO levels [116]. The mechanisms of NO-mediated apoptosis include DNA damage, caspase signaling activation, and modifications in the expression of Bcl-2 family members [117].

2.4.2 Function of NO in chemosensitization

Research indicates that nitric oxide significantly enhances the sensitivity of tumor cells to chemotherapeutic agents. The development of treatment resistance in cancer cells has been linked to hypoxia [118]. In an experimental study using human MDA-MB-231 breast cancer and mouse B16F10 melanoma cells exposed to low-oxygen conditions, blocking endogenous nitric oxide production intensified their resistance to chemotherapy, indicating that NO serves as a chemosensor in tumor cells and that hypoxia-mediated drug resistance is most likely brought on by the inhibition of endogenous NO generation [118].

In a study utilizing human PC-3 and murine TRAMP-C2 prostate cancer cells exposed to low oxygen levels (0.5% O₂), treatment with a low concentration of glyceryl trinitrate (GTN, 0.1 nM) effectively reversed the hypoxia-caused resistance to doxorubicin [13].

Furthermore, a study by Evig et al. [119] showed that when human breast cancer cells MCF-7 are exposed to an aqueous solution of NO that is administered (as a bolus) before DOX, NO-mediated chemosensitization takes place. They discovered that cell survival was 40% when DOX was used alone and only 5% when NO and DOX were combined. A different study used a DOX-resistant epithelial colon cell line (HT29-dx) to demonstrate an equivalent role for NO in chemosensitization.

The partial reversion of doxorubicin resistance in HT29-dx cells was achieved by administering several nitric oxide (NO) donors namely SNAP, GSNO, and SNP as well as NO-inducing agents such as atorvastatin [120]. This treatment enhanced both NOS activity and intracellular doxorubicin accumulation, while kinetic investigations revealed that NO (specifically via SNAP) reduced doxorubicin efflux and led to tyrosine nitration of multidrug-resistant transport proteins (particularly MRP3), thereby diminishing P-glycoprotein activity through an unspecified mechanism [120]. In a separate study by Konovalova et al. [121], co-injecting cyclophosphamide (CPA) together with an NO donor in P388 leukemic mice significantly boosted CPA efficacy. The NO donor also increased tumor sensitivity to doxorubicin and cisplatin and prevented metastasis formation in melanoma B16-bearing mice [121]. Another report showed that athymic nude mice with subcutaneous human prostate (PC-3) xenografts experienced notably suppressed tumor growth when treated concurrently with the NO donor DETA/NO₂Oate and cisplatin an effect greater than when either

agent was used alone [122]. Finally, NO and other chemosensitizing compounds may improve the anti-tumor action of chemotherapy medications. The full molecular interpretation of these anti-tumor actions, however, requires more research.

The literature is split in half due to the opposing roles that NO and RNS play in pathophysiology. Despite the fact that NO's anti-apoptotic function has been well documented [123], a cohesive idea is still lacking. NO and reactive nitrogen species protect cells by directly inhibiting caspases, promoting anti-apoptotic gene expression, activating the cGMP survival pathway, and interfering with harmful radicals although the specific outcomes depend on the cellular context. According to a recent study by Sebens-Muerkoster et al. [124], etoposide-mediated IL-1 β production and subsequent NO production appear to be responsible for the improved chemoresistance in pancreatic cancer cells, which further inactivates caspases. The hypothesis that S-nitrosation of essential reactive cysteine residues present in all members of the caspase family inactivates caspase function has been supported by a large body of evidence since 1997 [125]. It is challenging to explain these outcomes, nonetheless, given the activation of caspases linked to NO's pro-apoptotic function [86].

2.4.3 Contribution of NO in immuno-sensitization

Immunosensitization refers to making cells more vulnerable to immune-mediated killing. It has been suggested that apoptosis can be induced by molecular immunosensitization processes like pro-apoptotic proteins are transcriptionally up-regulated, while anti-apoptotic proteins are down-regulated. It's interesting to note that NO has been linked to cancerous cells becoming more perceptive of a variety of apoptotic triggers, including TNF- α , TRAIL, and FasL (APO-1/CD95). Fas-mediated apoptosis is one way by which cytotoxic immune lymphocytes eliminate tumor cells. Fukuo et al. [126] discovered that in aortic vascular smooth muscle cells, Nitric oxide elevated Fas receptor expression and heightened the cells' susceptibility to apoptosis via Fas ligand. In addition to TNF- α , IL-1 β , LPS, and numerous other pro-inflammatory cytokines, IFN- γ can promote the stimulation of NOS II and the ensuing generation of NO. Through the induction of NOS II and the resulting reactive nitrogen species-driven upregulation of Fas expression, IFN- γ and the NO donor SNAP (used individually or together) sensitized A2780 and AD10 ovarian carcinoma

cells to FasL-triggered apoptosis [127, 128]. These findings illustrate that NO and related species can modulate tumor cell susceptibility to cytotoxic immune responses. Similar to the findings on the function of NO as an immuno-sensitizer, in a work by Park et al. [129], Ionizing radiation, when combined with the NO donor SNAP, enhanced FasL-mediated apoptosis in HeLa cervical cancer cells by increasing Fas receptor expression. Additionally, the transcription factor YY1 naturally suppresses Fas by binding to its promoter's silencer region, thereby repressing Fas expression and reducing apoptotic susceptibility [128]. By increasing the expression of Fas, NO-mediated suppression of YY1 rendered ovarian cancer cells more vulnerable to FasL-induced apoptosis [128].

Recent research has shown that the B-NHL cell line, Ramos, can be rendered vulnerable to FasL-induced apoptosis by rituximab (chimeric anti-CD20 Ab), some NF- κ B inhibitors (such as Bay 11-7085 and DHMEQ), and/or YY1 inhibition (using the NO donor, DETA/NONOate) [130]. It is noteworthy that the NO-mediated suppression of YY1 activity (without rituximab) once more led to a notable increase in surface Fas expression and made Ramos cells more vulnerable to CH-11-induced apoptosis (Fas agonist mAb). However, Hongo et al. [131] showed that using DETA/NONOate to treat prostate cancer (PC-3) cells led to the S-nitrosation of YY1, this, via a direct NO-mediated mechanism, raised Fas expression and improved tumor cells' susceptibility to FasL-induced apoptosis.

The importance of NF- κ B's participation in cancer has been extensively documented in recent years [132]. Inflammatory cytokines like TNF- α are up-regulated when NF- κ B is improperly activated, which is of particular significance when it comes to NO. Tumor cells may become resistant to TNF- α -mediated apoptosis even though it can have a strong cytotoxic effect.

According to Han et al. [133], lowering NF- κ B activity may render colon cancer cells more susceptible to TNF- α -induced apoptosis. IFN- γ has been observed to stimulate NO production and subsequently increase the vulnerability of human ovarian cancer cell lines to TNF- α -mediated cell death [134]. In studies involving the ovarian cancer cell line AD10, both NOS inhibition with L-NMMA and NO donor treatment (e.g., SNAP) were examined. Miller et al. reported that NO donors, as well as blocking NOS with L-NAME, enhanced TNF- α cytotoxicity. Furthermore, they found that NO

donors interrupted NF- κ B's translocation into the nucleus, with this effect strongly correlating to intracellular H₂O₂ levels. The injection of exogenous H₂O₂ reversed this. Their findings demonstrated that anti-apoptotic/resistance signals are eliminated by NO-mediated suppression of NF- κ B activity, increasing tumor cells' susceptibility to lethal cytokines like TNF- α [134]. It has been demonstrated that the cytotoxic protein known as TRAIL preferentially suppresses the growth of tumors both in vivo and in vitro. While posing little risk to healthy tissues [135]. TRAIL-resistance can be reversed by a variety of sensitizing agents, including inhibitors, cytokines, and chemotherapeutic medications [136]. The NO donor DETA/NOOate increased the sensitivity of prostate cancer cell lines DU145, PC-3, CL-1, and LNCaP to apoptosis induced by TRAIL.

Current research investigating how DETA/NOOate made this sensitivity possible, DETA/NOOate S-nitrosylated NF- κ B (p50), thereby suppressing NF- κ B activity. Additionally, it was discovered that DETA/NOOate inhibited YY1 and caused TRAIL death receptor DR5 to be upregulated [137]. All things considered, NO's function in making cancer cells more susceptible to immune-driven cytotoxicity may offer significant medicinal promise. One important strategy to regulate demise of cancerous cells through chemo- and immunological mechanisms controlled by apoptosis may be the application of NO-based treatments.

2.5 NO donors in current clinical use

It is crucial to quickly discuss current medications before looking into novel NO donor therapies because a number of recent research have deepened our comprehension of the chemicals and could change clinical practice. The most often utilized NO donor medications are organic nitrates. However, ISMN (Fig. 2.3 a) and other delayed release preparations are utilized to treat chronic angina, the most researched nitrate, GTN (commonly known as nitroglycerin) is primarily utilized to relieve acute angina discomfort. Transdermal patches for heart failure and persistent angina and GTN ointments for anal fissures [138], may be beneficial for some subgroups with pulmonary hypertension, whereas nebulized GTN may be useful for others [139].

Although GTN possesses three nitro-oxy ester (nitrate) groups, only one mole-equivalent of nitric oxide is released from its terminal position following bioactivation

[140]. Whereas the necessity of NO release at all was recently contested in an intriguing work [141], it is now nearly unanimously accepted that certain enzymes mediate the conversion of GTN to NO, which has stringent criteria beyond simple chemical reduction [142]. For more than 30 years, the identification of the nitrate-activating enzyme or enzymes has been the focus of substantial investigation, yielding several potential candidates as well as a mountain of unclear and frequently contradicting data.

Chen et al.'s recent work [143] has generated a lot of interest since it provides compelling evidence that mtADH is a viable candidate [144]. Mitochondrial aldehyde dehydrogenase (mtALDH or ALDH2) fulfills the critical requirements for GTN bioactivation: it operates intracellularly, is highly sensitive to low (nanomolar) concentrations, uses redox-regulated thiol residues, and transforms GTN into nitrite and 1,2-glyceryl dinitrate which are subsequently converted into nitric oxide (NO) while its enzymatic activity declines with prolonged GTN exposure [145].

Furthermore, the strong affinity route of GTN vasodilatation observed in wild type mice is absent in transgenic mice deficient in the mtADH genes [146]. Considering that the main disadvantage of organic nitrates is the known emergence of tolerance with prolonged, continuous use, the latter two criteria are extremely important [147]. Utilizing nitrates to treat long-term conditions is obviously hindered by the need to incorporate a nitrate-free period into the treatment plan, as this is the only efficient way to prevent tolerance. Furthermore, in vivo research in animals [148] and humans [149] has demonstrated that endothelial dysfunction and elevated oxidative stress may be related to tolerance.

Reports showing long-term nitrate use paradoxically raises the risk of cardiac events [150] may be influenced by this. Once the nitrates' mechanism of action is completely understood, the root cause of tolerance and more effective ways to get around this issue may become apparent. Despite the strong evidence supporting mtADH's function, conflicting results have previously been reported [151], and several significant mechanistic concerns still need to be answered. Chief among them is why certain thiols only affect mtADH activity and not others, such as glutathione [65]. Which chemical processes enable the GTN nitrate moiety to interact with thiols and/or produce NO [61, 65]?

Why is nitrate PETN (Fig. 2.3 b) less prone to tolerance and less likely to desensitize mtADH's esterase activity [61]? Why do not all nitrates, such as ISDN [61], get inhibited by mtADH inhibitors? If nitrates are venoselective [65], is this explained by the cellular distribution of mtADH? [152] The availability of several NO donors that are excellent antiplatelet agents [153] and do not develop tolerance [152] may lead to a decline in the usage of these medications in clinical settings in favor of alternative therapies. The introduction of BiDil [154] in 2005 for the management of heart failure among African Americans [155] is the only other noteworthy development with this family of medications.

The controversy over the arena's targeting of a particular racial group [156] has sparked renewed interest in it more than any new therapeutic technique. The other therapeutically important NO donor now in use is SNP (Fig. 2.3c). In hospitals, SNP is used on-site to quickly reduce blood pressure during hypertensive crises. Although the intricate mechanism underlying NO release may suggest that some of the newly developed novel NO donors would be more appropriate for this use, as the gold standard NO-dependent but endothelium-independent vasodilator, SNP continues to be the medication of choice in clinical trials.

The process by which SNPs in biological tissue produce NO is more intricate than is frequently thought and is still unclear. As SNP is relatively stable and does not spontaneously release nitric oxide in physiological conditions, NO liberation depends on either light exposure or interaction with specific tissues [157]. A key safety concern with SNP is the potential release of cyanide from its five cyanide ligands [158]. Indeed, cyanidosis may infrequently arise from prolonged usage of this medication, according to a few isolated studies [159]. Other limitations on the use of SNP include its high potency, which can make dose titration difficult, sensitivity to photolysis once in solution, and requirement for intravenous delivery [160]. It is challenging to envision a broader use of this NO donor medication in light of these restrictions.

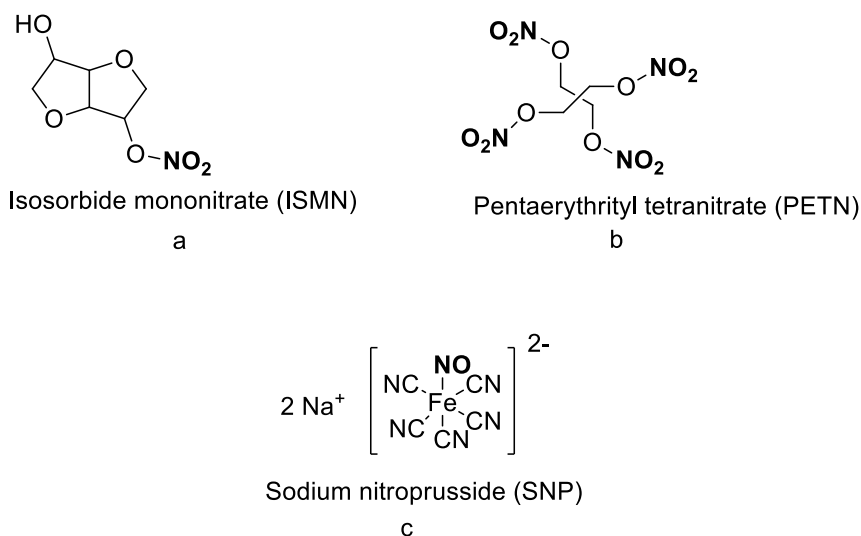


Figure 2.3 The chemical makeup of organic nitrates (a&b) and SNP (c), two NO donor medications used in therapeutic settings. The bolded portion is the NO-containing moiety.

2.6 Research gaps in nitric oxide donor-based therapeutic systems

Despite extensive research on nitric oxide donor compounds across cardiovascular and anticancer applications, several overarching challenges remain in the field that limit their broader clinical translation.

- One of the major gaps lies in the lack of precise spatial and temporal control over nitric oxide release in conventional NO donor systems. Many clinically used or early-generation NO donors release NO in a systemic and non-specific manner, making it difficult to achieve localized therapeutic effects while minimizing off-target toxicity.
- Another important limitation lies in the limited clinical advancement of NO donor systems, as most reported compounds demonstrate promising anticancer or therapeutic activity in preclinical models but have not progressed substantially into standardized clinical oncology applications.
- These combined limitations have driven the development of advanced NO delivery strategies, particularly light-responsive and nanostructured NO donor systems, which aim to improve targeting efficiency, control release behavior, and enhance therapeutic selectivity in cancer treatment.

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Chapter 3: Objectives and Methodology

3.1 Aim and Objectives of the thesis :

Aim : Synthesis and evaluation of some nitric oxide donors for potential biological applications.

Objectives to achieve the aim :

1. Synthesis and characterization of some nitric oxide (NO) donors.
2. To evaluate NO release behavior of synthesized molecules by light activation.
3. To evaluate biological potential of newly synthesized donors for anticancer studies on multiple cell lines.
4. To establish the mechanism of action, using computational and binding studies on synthesized NO donors.

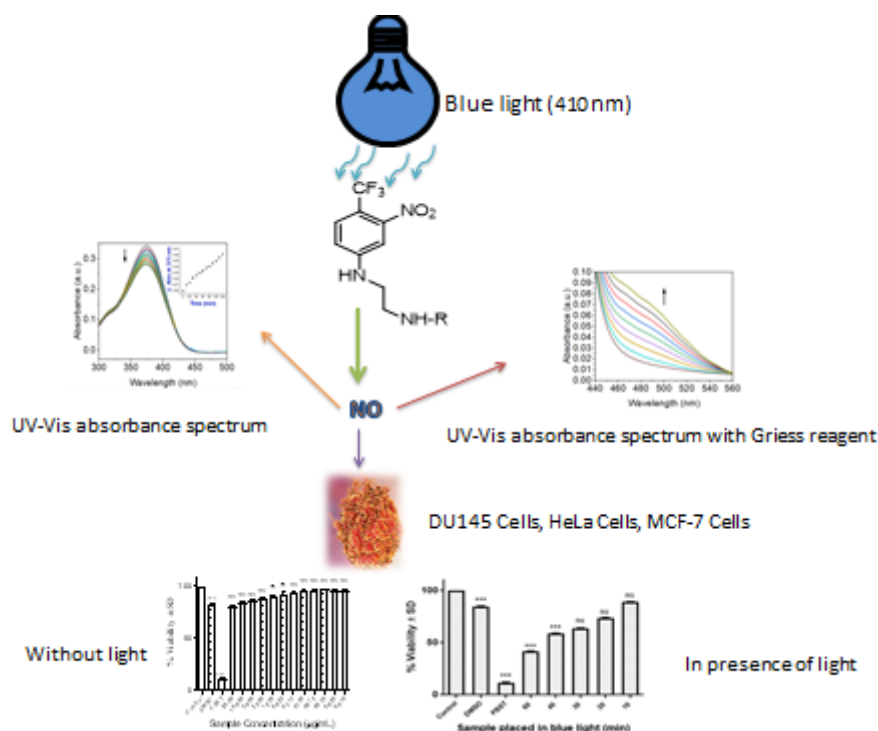


Figure 3.1 Schematic Workflow Diagram

3.2 Methodology for achievement of the objectives :

To achieve the above objectives following methodology has been used.

3.2.1 Synthesis and characterization of some nitric oxide (NO) donors

NO donors namely, Compound 1, 2, 3, 4 and 5 have been synthesised by following scheme 3.1, 3.2, 3.3, 3.4 and 3.5 respectively.

Scheme 3.1

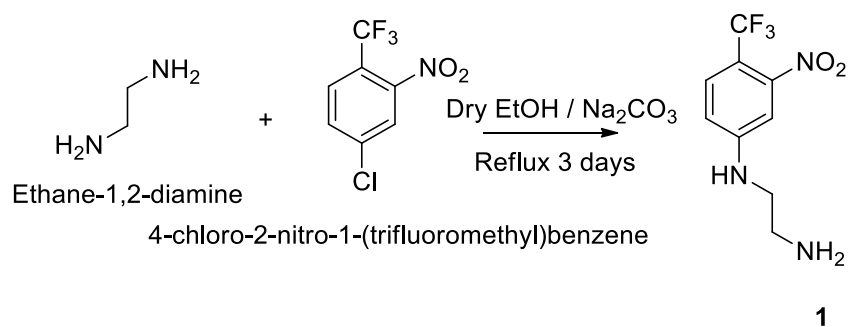


Figure 3.2 Scheme for the Synthesis of NO donor **1**

Scheme 3.2

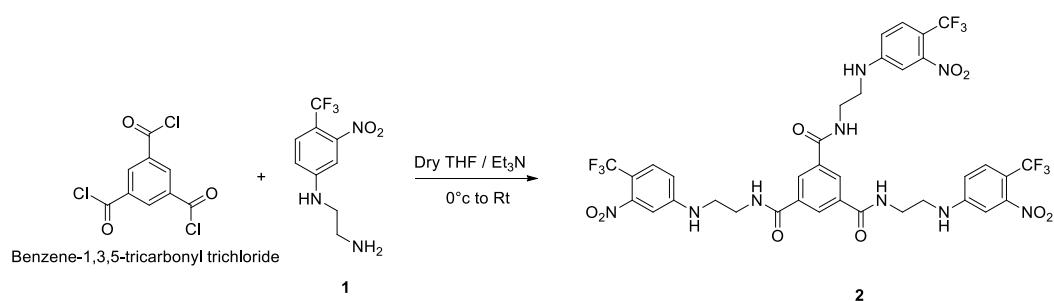


Figure 3.3 Scheme for the Synthesis of NO donor **2**

Scheme 3.3

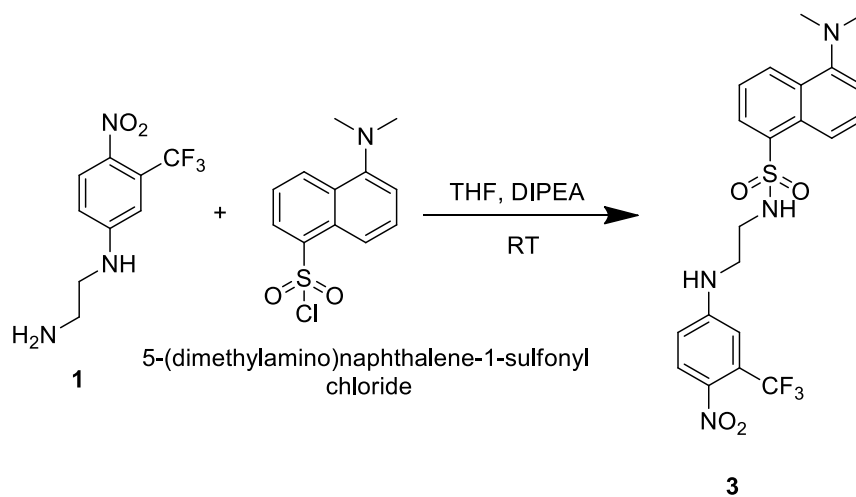


Figure 3.4 Scheme for the Synthesis of NO donor **3**

Scheme 3.4

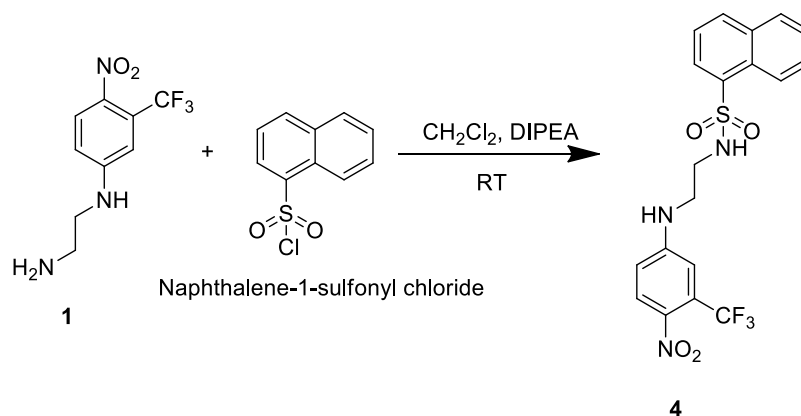


Figure 3.5 Scheme for the Synthesis of NO donor **4**

Scheme 3.5

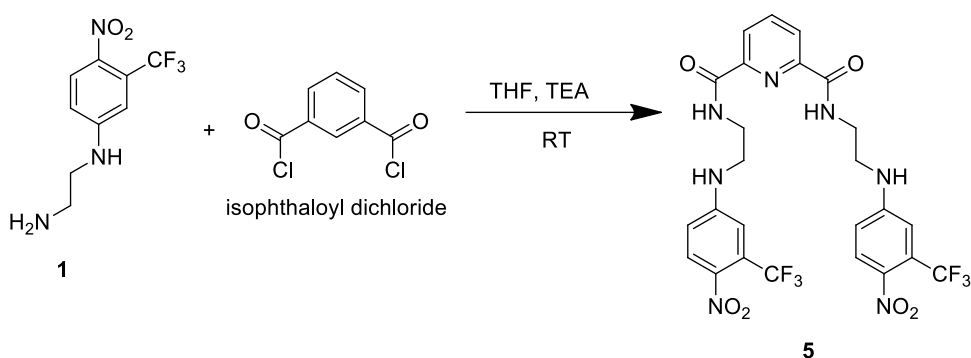


Figure 3.6 Scheme for the Synthesis of NO donor **5**

3.2.2 Instrumentation

All the synthesized compounds have been characterized by using standard analytical techniques such as High-resolution mass spectrometry (HR-MS), Electrospray ionization mass spectrometry (ESI-MS) Proton nuclear magnetic resonance (^1H NMR) and Carbon-13 nuclear magnetic resonance (^{13}C NMR). There are the instrumentation methods that have been used in carrying out the work described in the thesis. Proton NMR spectra have been performed on Bruker Advance 500 MHz instrument at room temperature using DMSO-d_6 as solvent. The values of chemical shift have been mentioned in ppm. Tetramethyl silane has been used as an internal reference; s = singlet, d = doublet, t = triplet and m = multiplet have been used as the abbreviations in describing the splitting patterns.

Waters Q-ToF mass spectrometer has been used to record ESI-mass spectrum using positive ion mode.

An Evolution 201 UV-Vis spectrophotometer has been used to record UV absorption spectra with quartz cells of 1.0 cm path length.

To record the fluorescence spectra, Cary Eclipse fluorescence spectrophotometer has been used. All origin graphs have been plotted using the absorption and fluorescence data saved as ASCII files.

3.2.3 To evaluate NO release behavior of synthesized molecules by light activation.

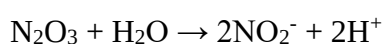
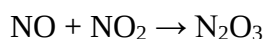
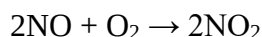
The NO gas release studies have been performed by recording UV–Vis Absorption Spectra on Agilent Technologies Cary 60 UV–Vis Spectrophotometer using quartz cells of 1.0 cm path length.

i) NO release studies by UV–Vis absorption spectroscopy

To ensure that the synthesized compounds are releasing NO, UV–Vis spectra of methanolic solution of all the compounds was monitored at different time interval upon irradiation with blue light (410 nm, 3 W). The light source was placed 1 cm away from the quartz cuvette for the irradiation experiment. The NO release was determined by monitoring the decrease in absorbance (λ_{max}).

ii) Quantisation of NO released by using Griess assay

After confirming the release of NO from the above synthesized compounds, next step is to calculate the amount of NO released and for that Griess assay method has been used [1]. It is one of the most commonly used analytical method for the detection and quantification of NO from nitric oxide donors based on the diazotization reaction. It is widely used over various disciplines due to its simplicity and inexpensive analytical instrumentation. It is an absorbance based spectroscopic method [2]. Griess assay is an indirect way of quantifying nitric oxide as it actually measures its by-product, nitrite (NO_2^-). NO undergoes oxidation under aerobic conditions to form nitrite as follows:



Griess reaction was first reported in 1879 by a German chemist, Johann Peter Griess. In the original griess reaction, diazonium cation is formed when amino group of the sulfanilic acid reacts with the nitrite under acidic condition which couples at the para-

position of α -naphthylamine to form the water soluble azo-dye derivative (Fig. 3.7) [3] Since then Griess reagents have been modified and are commercially accessible for quick and simple nitric oxide detection.

The standardization of griess assay is performed by preparing calibrationcurve with known concentrations of NaNO_2 .

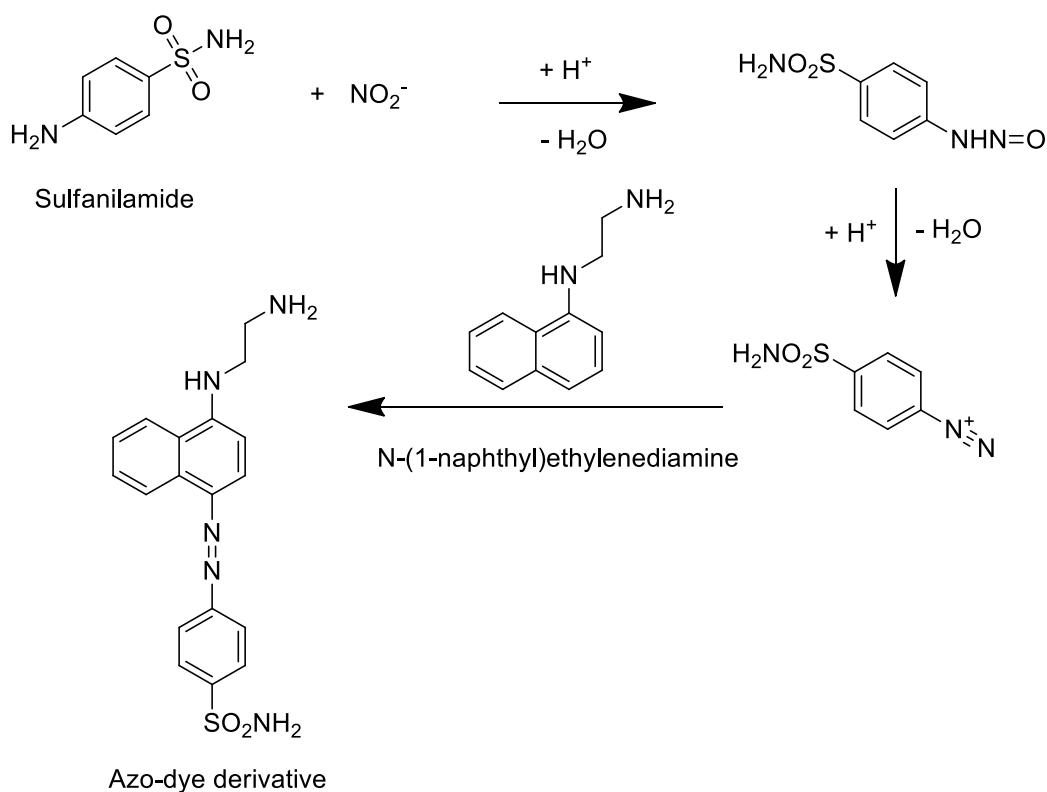


Figure 3.7 Schematic representation of Griess reaction

The release of nitric oxide from the synthesized NO photo-donor or the vesicular solution was quantified using colorimetric Griess assay. A pink-colored azo dye is formed in the Griess reaction with the free nitric oxide in aqueous solution [4]. A stock solution of Griess reagent was prepared by dissolving 400mg of modified Griess reagent (purchased from Sigma-Aldrich) in 10mL of phosphate buffer (10mM, pH = 7.2). The required volume of the Griess stock solution was added to the aqueous solution of NO photo-donor. UV-vis spectra were recorded after regular light irradiation of the solution for a definite period of time. The increase in UV-vis absorption intensity in the range 460–493 nm indicates the formation of a chromophoric azo-derivative due to nitric oxide release. The amount of nitric oxide released was estimated by tracking the rise in absorbance. Using an aqueous solution

of NaNO_2 at varying concentrations (0 - 130 μM), the standard curve (Fig. 3.8) was created to estimate the amount of nitric oxide released. Then the absorbance of the azo dye prepared from the NO photo-donor is compared to the prepared calibration curve.

For assay, 100 μL of this stock solution was added to a methanolic solution of synthesized compounds (2.1 mL) and the UV-Vis spectrum was recorded while exposed to blue light (410 nm, 3 W) at ambient temperature. The color shift and the rise in UV-Vis absorbance at 460 nm is evidence that NO was released from the donor NO molecules upon photolysis [5].

Amount of NO released has been calculated by using a simple formula, which is

$\text{NO released} = (\lambda_{\text{max}}) / \text{Slope of the Calibration curve}$

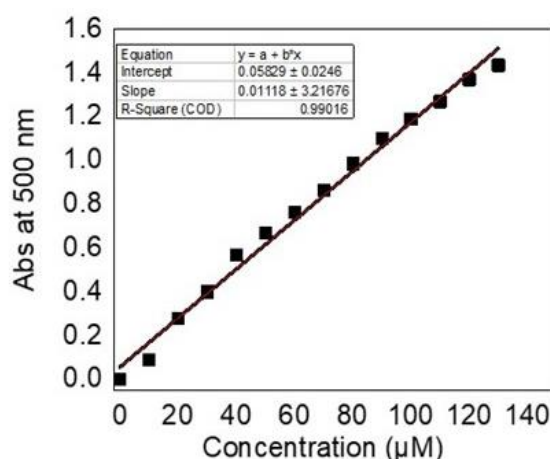


Figure 3.8 Calibration plot derived from aqueous sodium nitrite dilutions covering the 0–130 μM concentration range.

iii) Photochemical Quantum Yield and Rate Calculation

Photo-flow of the light source used has been determined using the ferrioxalate actinometer. A chemical actinometer is a photochemical system which undergoes a light-induced reaction for which quantum yield is well-known. This quantum yield is then used to estimate the absorbed photon flux for specific photochemical reactions [6]. Ferrioxalate actinometer is the most widely used chemical actinometer.

Photon flux (q_{in}) of the Blue LED light source (3W) has been calculated observing the procedure described in the literature [7,8]. For that, following solutions have been prepared: 6 mM 1,10-phenanthroline solution (X), 6 mM Potassium ferrioxalate

trihydrate solution (W) and sodium acetate buffer (Y). The solution of W (3mL) has been taken in a quartz cuvette and photo decomposed using the blue LED light source (3Watt) for different time intervals. A distance of 4 cm has been maintained between the cuvette and the light source during the photo-decomposition experiment. In a 10 mL standard flask, for one hour, 1 mL of photo-decomposed solution of W, was incubated in the dark with 7.5 mL of deionized water, 1 mL of solution X, and 0.5 mL of solution Y. In another 10 mL standard flask, a control solution has been prepared using 1 mL solution of non-photo decomposed W with other solutions and similarly incubated under dark as mentioned above. Absorbance at 510 nm has been recorded for these incubated solutions after 1 hr. The amount of $[\text{Fe}(\text{phen})_3]^{2+}$ present was determined using the Lambert Beer's law and molar extinction coefficient (ϵ) was used as $11100 \text{ M}^{-1}\text{cm}^{-1}$. Equation 3.1 has been used to calculate the formation of Fe^{2+} ions upon light irradiation.

$$n(\text{Fe}^{2+}) = \frac{V_1 V_3 \Delta A_{510}}{10^3 \times V_2 l \epsilon} \quad (3.1)$$

Where V_1 is volume of solution W, V_2 is volume of irradiated solution W taken out for incubation, V_3 is total volume incubated in dark, ΔA_{510} is the change in absorbance between irradiated sample and control sample after incubation, l is the pathlength of cuvette in cm and ϵ is molar extinction coefficient. The photon flux (q_{in}) was calculated by using equation 3.2.

$$q_{\text{in}} = \frac{n(\text{Fe}^{2+})}{t \times \Phi_\lambda} \quad (3.2)$$

where the Φ_λ value for the excitation wavelength of 407 nm was set at 1.19 and the slope of the plot of $n(\text{Fe}^{2+})$ vs. t is denoted by $n(\text{Fe}^{2+})/t$. Equation 3.2 has been used to calculate the photon flux (q_{in}), which came out to be 1.5×10^{-6} Einstein/min. Equation 3.3 has been used to calculate the quantum yield of photodecomposition experiment.

$$\Phi \approx \frac{([R]_0 - [R]_t)}{t} \times \frac{V \times N_A}{q_{\text{in}}} \times \frac{1}{f_m} \quad (3.3)$$

where V is the volume of the solution, t is the irradiation time, $[R]_0$ and $[R]_t$ are the molar concentrations of R at the start and finish of the irradiation period, and N_A is the Avogadro constant and f_m is the mean value of the fraction of light absorbed at time "t."

The solutions have been prepared under dim light to avoid their decomposition.

The rate constant (k_{NO}) for the photo-release of nitric oxide has been calculated by linear fitting of the plot of the natural logarithm of concentration of the compound versus time. The concentration of the compound has been calculated based on the change in absorbance at a particular wavelength where spectral changes have been observed. The half-life for nitric oxide release was calculated by applying equation: $t_{1/2} = 0.693 / k_{\text{NO}}$, where $t_{1/2}$ and k are half life and rate constant, respectively.

3.2.4 To evaluate biological potential of newly synthesized donors for anticancer studies on multiple cell lines

To achieve this objective, the effect of all the compounds on three different cell lines has been studied, namely **DU145, HeLa and MCF-7** cell lines both with and without irradiation with blue light (410 nm, 3 W). DU145, HeLa and MCF-7 (10×10^3 cells/well) were seeded in a 96-well plate in (Dulbecco's Modified Eagle Medium (DMEM) + 10 % fetal bovine serum (FBS)) media and incubated overnight. The following day, cells were treated with new media at varying concentrations (20.00–0.010 $\mu\text{g/mL}$) of the sample in triplicates after the old media was disposed of. The controls used were, a) only media (control), b) $1\times$ phosphate-buffered saline (PBS) + 0.05 % tween 20 (PBST) (positive control for cytotoxicity) and methanol used as Vehicle control. After 24 h of incubation, the media was removed and complete media along with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) solution was added along with the controls to the wells for the next 3 h. For the following two hours, 50 μL of 10% SDS with 0.01 M HCl was added to dissolve the formazan crystals and endpoint readings were taken at OD₅₇₀ (optical density) in an enzyme-linked immunosorbent assay (ELISA) reader. After this, following the same methods, five 96-well plates are prepared and placed in blue light for 10, 20, 30, 40, and 60 min. After a specific time of incubation, the media was removed and complete media along with MTT solution was added along with the controls to the wells for the next 3 h. Formazan crystals were solubilized by adding 50 μL of 10 % Sodium dodecyl sulfate (SDS) containing 0.01 M HCl for the next 2 h and endpoint readings were taken at OD₅₇₀ in an ELISA reader.

3.2.5 To establish the mechanism of action, using computational and binding studies on synthesized NO donors.

To determine physicochemical descriptors and predict ADME (Absorption, Distribution, Metabolism, Excretion) parameters, pharmacokinetic qualities, drug-like nature, and medicinal chemistry friendliness of the synthesized compounds, a variety of methods, such as Swiss ADME, have been employed for *insilico* studies. PyMol Molecular Graphic System version 2.4.1 has been used to prepare protein-ligand complexes after docking, and Discovery Studio 2016 has been used to visualize protein-ligand complexes and interactions in two dimensions. The UCSF-Chimera[®] software (version 1.13) has been used to prepare protein targets for docking and to perform molecular docking calculations.

As the level of Human sigma-1 receptor overexpression has been reported from in prostate cancer [9].

The three-dimensional protein structure of the human sigma-1 receptor was obtained using the resolution 2.10 Å criteria from the Protein Data Bank (PDB ID: 5HK1). The Dock preparation tool of UCSF-Chimera[®] (version 1.13) software was used to select and clean protein receptors from heteroatoms after importing the PDBs into Chimera. The UCSF-Chimera software's AMBER force field was used to minimize the receptors, with the conjugated gradient set to 10 and the steepest gradient set to 100. To confirm the accuracy and reliability of the docking results, the docking methodology was validated. The goal of the current *in silico* investigations is to accurately replicate the binding model and the molecular interaction of the co-crystallized ligand of the experimentally crystallized protein structures.

Consequently, all of the X-ray proteins' natural ligands were extracted from the protein and made ready for docking using UCSF-Chimera. The Auto Dock Vina module was then used to dock the ligands back into the active regions of the receptors [10]. Using the PyMOL molecular visualizer, the docked complexes were aligned with the X-rayed resolved crystals of proteins that contained the co-crystallized ligand in order to generate the root mean square deviation (RMSD) value. With the exception of the grid box, which was modified based on each protein molecule's active sites, all other parameters were left at their default settings. The receptor became stiff because all of the ligand's links were free to rotate. Ten configurations were produced for every protein-ligand combination after the molecular docking experiments were finished.

The ideal docking stance was thought to be the one with the lowest binding energy (BE, kcal/mol) and root mean square deviation (RMSD). In order to obtain more accurate and dependable results, an exhaustiveness of 10 was chosen for docking and the number of modes was set at 10 throughout this *in silico* experiment. PyMOL and Discovery Studio 2016 were then used to prepare, visualize, and analyze the ligand-protein interaction.

Furthermore, binding studies of all the compounds have also been done with BSA (10 μ M) to understand the interaction of the synthesized compounds with the Bovine Albumin Serum (BSA), to study the internalization process of the compounds [11]. The interaction of the compounds with BSA was done with the help of Fluorescence studies, a Cary Eclipse Fluorescence spectrophotometer was used to record the fluorescence spectra. The analysis of the fluorescence of BSA in the presence of increasing amounts of drug was carried out to obtain the binding properties of the protein with the compounds. When excited at 280 nm BSA shows an emission band with maximum at ca. 340 nm due to the tryptophan residue [12]. Addition of increasing concentrations of Synthesized samples significantly quenches this emission, taking into consideration an interaction between the two components.

3.2.5.1 Calculation of Binding Constant

Hill plot method was employed to analyze ligand-receptor interactions and to determine both the dissociation constant (K_d) and the cooperativity of binding, using hill equation –

$$\theta = [L]^n / K_d + [L]^n$$

Where:

- θ is the fraction of occupied binding sites,
- $[L]$ is the free ligand concentration,
- K_d is the apparent dissociation constant,
- n is the Hill coefficient, indicating the degree of cooperativity.

To linearize the data for easier analysis, the equation is transformed into its logarithmic form:

$$\log (\theta / 1 - \theta) = n \log [L] - \log K_d$$

Plotting $\log (\theta/1-\theta)$ against $\log [L]$ yields a straight line, known as the Hill plot (Fig. 4.70 – Fig. 4.74). The slope of this line corresponds to the Hill coefficient n , and the x-intercept provides the value of $\log K_d$ and as we know binding constant/ association constant K_a is inversely proportional to the dissociation constant K_d so binding constant $K_a = 1/ K_d$

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green light to overcome resistance in breast cancer cells”, *ACS Omega*, 7, 7452-7459 (2022).

Chapter 4: Results and discussion

This chapter includes the results and discussion of synthesis, characterization, NO release studies and cytotoxicity of all the synthesized compounds.

4.1 Synthesis, Qualitative analysis and Biological studies of NO donor N¹-(3-nitro-4-(trifluoromethyl)phenyl)ethane-1,2-diamine (Compound 1)

Synthesis of compound **1** was done by following scheme 3.1 and the compound was analysed using mass and NMR spectroscopy and the results have been compiled.

4.1.1 Synthesis of **1**

Ethylene diamine (5.91 mL, 88.6 mmol) and Na₂CO₃ (5.6 g, 53.16 mmol) was dissolved in dry ethanol (50 mL) at 75 °C and stirred for 15 min. 5-Chloro-2-nitro-benzotrifluoride (1.29 mL, 8.86 mmol) was added to this solution and stirred for 3 days at 75 °C. The resultant solution was cooled, filtered, and evaporated to solid under vacuum. The crude compound was purified by 100–200 mesh silica gel column chromatography using CHCl₃/methanol (90/10, v/v) as eluent. The desired product **1** was isolated as a yellow color solid. Yield = 40.0 % (0.895 g). m.p. 85–88 °C; ESI-MS m/z: calculated 249.19; found [M + H]⁺ 250.08. HR-MS: [M + H]⁺ calculated; 250.0803 observed; 250.0802. (Fig. A.1). ¹H NMR (500 MHz, DMSO-D₆) δ 8.09 (d, J = 9.1 Hz, 1H) 8.00 (s, 1H), 7.83 (t, J = 5.5 Hz, 2H, –NH), 7.10 (d, J = 2.5 Hz, 1H), 6.93 (t, J = 2.5 Hz, 1H, –NH), 3.48 (t, J = 5.6 Hz, 2H), 2.99 (m, 2H). (Fig. B.1 (a)) ¹³C NMR (125 MHz, DMSO-D₆) δ 46.4, 40.9, 40.7, 40.5, 40.2, 40.0, 39.8, 39.6, 39.4. (Fig. B.1 (b))

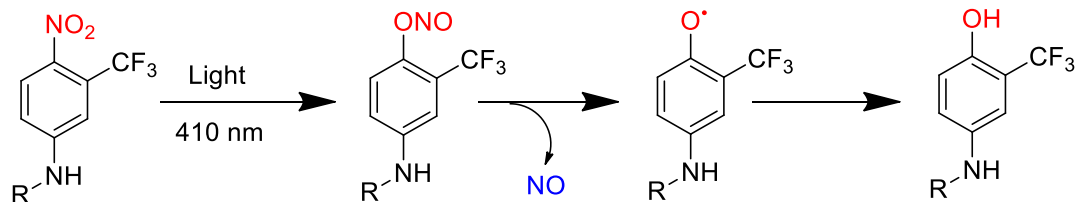
4.1.2 Qualitative Analysis

The compound was used for further NO releasing studies with the help of UV-Vis spectrophotometer.

4.1.2.1 UV-Vis Studies

The NO release behavior of **1** was observed using time-dependent UV-Vis studies. The absorbance at 375 nm decreases continuously in the presence of blue light (410 nm, 3 W) (Fig. 4.1), but there is no change in absorbance in its absence. Light was found to photobleach **1**, which is entirely compatible with the photochemical process that releases NO. According to the literature [1], the transformation of **1** from nitro (NO₂) to nitrito (ONO) allowed for the release of NO. The reduced absorbance in the

visible area is caused by the potential production of phenoxy and NO radicals, which are non-toxic and weakly absorbing photoproducts (Scheme 4.1).



R – varies with compound

Scheme 4.1 Showing mechanism of NO release.

NO was released only under blue light irradiation, the UV-Vis spectrum did not alter in the absence of light and even after keeping under daylight for a prolonged time.

UV-Vis spectra of methanolic solution of **1** (39.3 μM) was monitored at different time interval upon irradiation with blue light (410 nm, 3 W). The quartz cuvette was placed 1 cm away from the light source for the irradiation experiment. The NO release was determined by monitoring the decrease in absorbance (λ_{max}) at 375 nm.

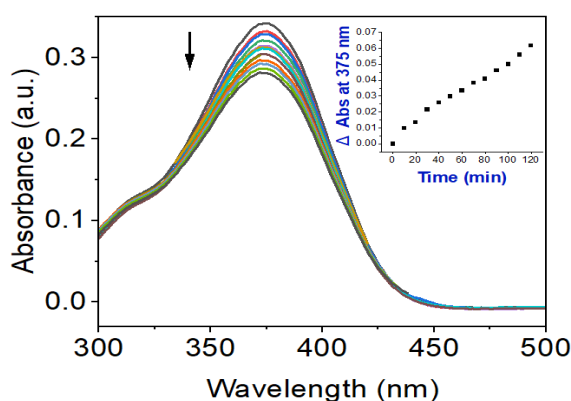


Figure 4.1 UV–Visible absorbance of Compound **1** monitored under blue (410 nm, 3 W) illumination over a 0–120 min interval, with an inset depicting the temporal absorbance changes at 375 nm

4.1.2.2 Griess Assay:

Using the Griess assay with the standard protocol, the amount of NO released from **1** has been estimated [2]. The NO donor and Griess reagents were mixed and exposed to blue light (410 nm, 3 W) for 4 hours, and the change in the UV-Vis absorbance was measured at different times. The Griess assay study states that the chromophoric azo-derivative production with a strong absorbance band in the 460–493 nm range and the

color change are proof that NO was released from the donor NO molecules or materials [3].

There was a color shift from pale yellow to pink and an increase in absorbance at 485 nm (Fig. 4.2). The release of NO from **1** is responsible for the formation of an azo dye in solution, as indicated by the new UV-Vis peak.

800 mg of Griess reagent were dissolved in 20 mL of 10 mM phosphate buffer to prepare the stock Griess reagent solution. A methanolic solution of **1** (39.3 μM , 2.1 mL) was mixed with 100 μL of this stock solution for the experiment, and the UV-Vis spectra was recorded at room temperature while exposed to light. The release of NO from **1** following photolysis is indicated by the increase in UV-Vis absorbance at 485 nm.

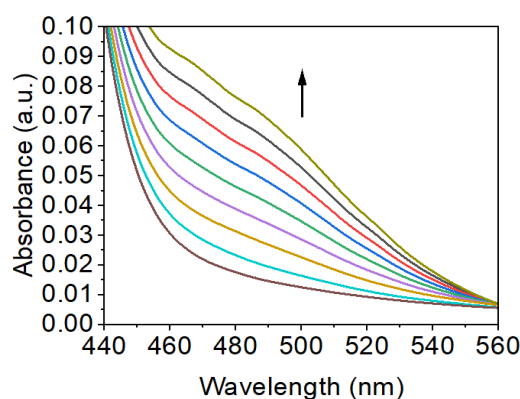


Figure 4.2 UV-Vis spectral changes of Compound **1** in the presence of Griess reagent over a 0 - 300 min period of irradiation with blue light (410 nm, 3 W).

Based on the calibration plot (Fig. 3.8), the amount of NO released was found to be 3.88 μM .

4.1.2.3 Calculation of rate constant

A graph was plotted between the natural logarithm of reactant concentration and time, which gave a straight line, thus making it clear that the reaction follows first-order kinetic hence slope of the curve = Rate constant which was found to be 0.0015 min^{-1} (Fig. 4.3) with $t_{1/2} = 462$ min and a quantum yield of 0.0003 [2].

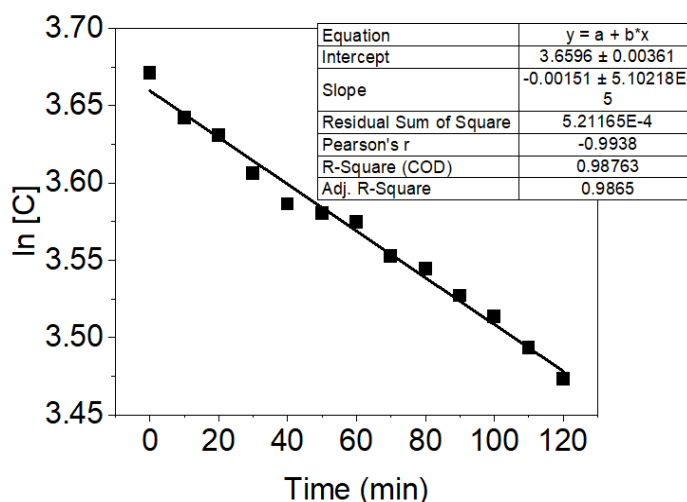


Figure 4.3 Graph used to calculate rate constant of **1** in Methanol

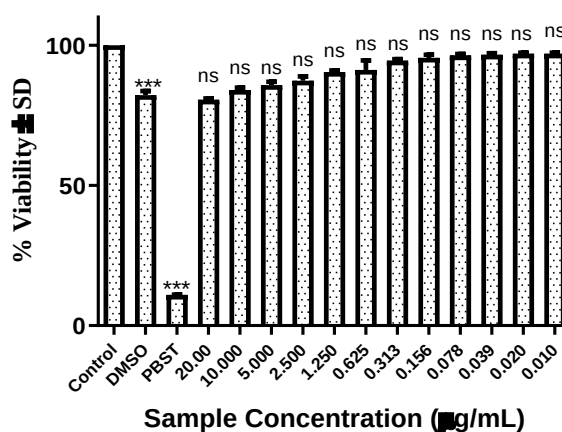
4.1.3 Evaluation of cytotoxicity of compound using Human Prostate Cancer Cells (DU145), Cervical Cancer Cells (HeLa cells) and Breast Cancer Cells (MCF-7) in vitro

NO is a powerful pro-apoptotic agent in prostate, cervical cancer, and breast cancer cells primarily by disrupting mitochondria, unleashing ROS, triggering caspases, and inhibiting survival/proliferation signals. However, dose and exposure duration also play a crucial role. Therefore, an in-vitro cytotoxicity experiment was performed to know the suitability of NO donor for a potential prostate cancer, cervical cancer and breast cancer therapeutic application.

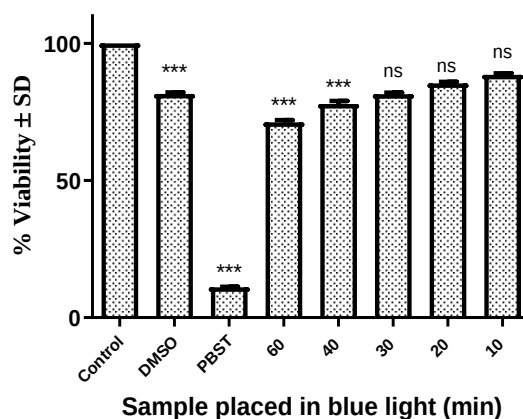
Human prostate cancer cells (DU145), cervical cancer (HeLa cells), and breast cancer cells (MCF-7) were used to study the safety profiles of **1** in methanol before NO release and during NO release at different concentrations 0.010–20.00 $\mu\text{g/mL}$ (Fig. 4.4 (a), Fig. 4.5 (a), and Fig. 4.6 (a)) respectively. The compound can be used safely for medicinal purposes because the MTT assay demonstrates that it is not harmful to cells even at concentrations of up to 20.00 $\mu\text{g/mL}$. This prompted us to perform preliminary studies on the killing of cancer cells caused by light. Therefore, in order to estimate cell mortality following NO release, DU145, HeLa, and MCF-7 cells were treated with **1** and exposed to blue light for 10, 20, 30, 40, and 60 minutes.

This experiment suggests that the observed cancer cell mortality is caused by the light-induced release of NO. The results show that the compound becomes toxic to the DU145, HeLa, and MCF-7 cancer cells after 60 minutes of irradiation with blue light,

resulting in 29% (Fig. 4.4 (b)), 30% (Fig. 4.5 (b)), and 32% (Fig. 4.6 (b)) of cell death, respectively. The control experiments clearly show that the compound is toxic to the cells only in the presence of blue light after irradiation of more than 40 minutes.

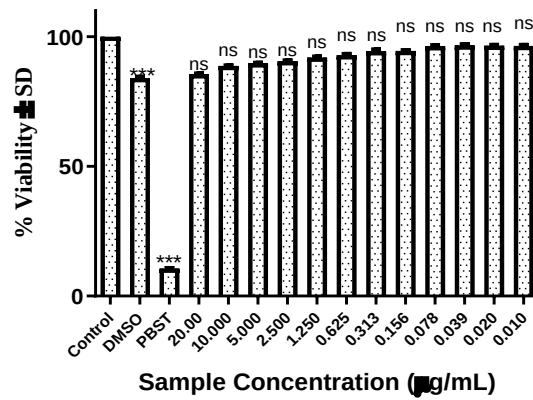


(a)

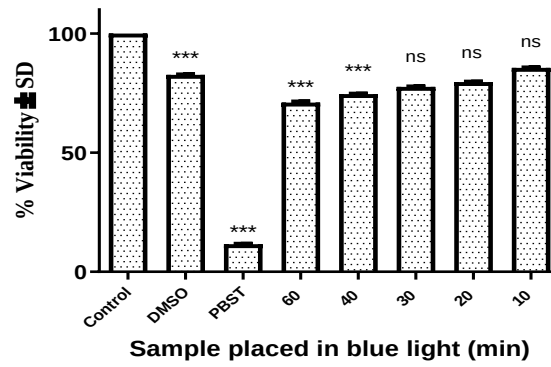


(b)

Figure 4.4 (a) & (b) Effect of Sample 1 on DU145 cells without blue light or with blue light. (***) $p < 0.001$, ns-nonsignificant).

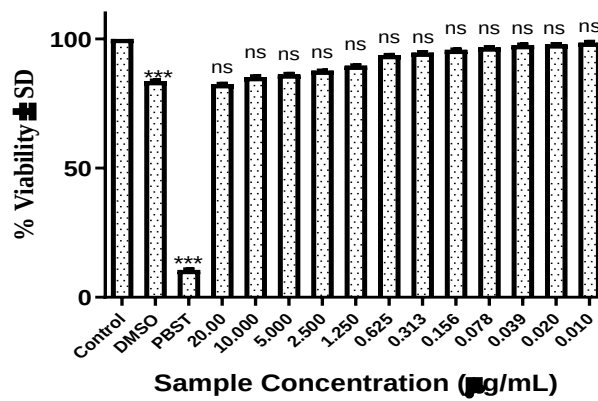


(a)

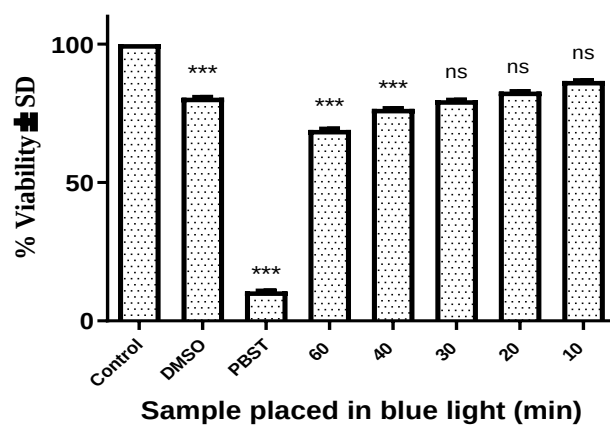


(b)

Figure 4.5 (a) & (b) Effect of Sample 1 on HeLa cells without blue light or with blue light. (***) $p < 0.001$, ns-nonsignificant).



(a)



(b)

Figure 4.6 (a) & (b) Effect of Sample 1 on MCF-7 cells without blue light or with blue light. (*** $p < 0.001$, ns-nonsignificant).

4.2 Synthesis, Qualitative analysis and Biological studies of NO donor N¹,N³,N⁵-tris(2-((3-nitro-4-(trifluoromethyl)phenyl)amino)ethyl)benzene-1,3,5-tricarboxamide (Compound 2)

Synthesis of compound **2** was done by following scheme 3.2 and the compound was analysed using mass and NMR spectroscopy and the results have been compiled.

4.2.1 Synthesis of 2

Compound **1** (300 mg, 1.11 mmol) was dissolved in dry THF (70 mL) to this solution dry Et₃N (515 μ L, 3.7 mmol) was added at 0 °C with continuous stirring. After 10 min., benzene-1,3,5- tricarbonyl trichloride (100 mg, 0.37 mmol) was added dropwise to the reaction mixture and stirred at rt for 8 hrs. The solution was filtered to remove the Et₃N.HCl salt and evaporated under vacuum. The obtained solid was washed with distilled H₂O and the compound **2** was obtained as yellow color solid. m.p. 210-212 °C. Yield: 44 % (150 mg); ESI-MS m/z: calculated 903.66; found [M + H]⁺ 904.2672 (Fig. A.2 (a)) HR-MS: [M + H]⁺ calculated; 904.2101 observed; 904.2095. (Fig. A.2 (b)) ¹H NMR (500 MHz, DMSO-*D*₆) δ 8.89 (t, *J* = 5 Hz, 3H-NH), 8.44 (s, 3H), 8.07 (d, *J* = 10 Hz, 3H), 7.69 (d, *J* = 5 Hz, 3H), 7.10 (s, 3H), 6.91 (t, *J* = 10 Hz, 3H-NH), 3.47 (t, *J* = 5 Hz, 6H), 3.42 (t, *J* = 5 Hz, 6H) (Fig. B.2 (a)). ¹³C NMR (125 MHz, DMSO-*D*₆) δ 165.5, 154.3, 153.0, 149.6, 134.5, 133.7, 129.7, 128.6, 115.5, 114.3, 45.4, 39.8. (Fig. B.2 (b))

4.2.2 Qualitative Analysis

The compound was used for further NO releasing studies with the help of UV-Vis spectrophotometer.

4.2.2.1 UV-Vis Studies

The NO release behavior of **2** was observed using time-dependent UV-Vis studies. The absorbance at 379 nm decreases continuously in the presence of blue light (410 nm, 3 W) (Fig. 4.7), but there is no change in absorbance in its absence. Light was found to photobleach **2**, which is entirely compatible with the photochemical process that releases NO. The reduced absorbance in the visible area is caused by the potential production of phenoxy and NO radicals, which are non-toxic and weakly absorbing photoproducts (Scheme 4.1).

NO was released only under blue light irradiation, the UV-Vis spectrum did not alter in the absence of light and even after keeping under daylight for a prolonged time.

UV-Vis spectra of methanolic solution of **2** (105 μM) was monitored at different time interval upon irradiation with blue light (410 nm, 3 W). The quartz cuvette was placed 1 cm away from the light source for the irradiation experiment. The NO release was determined by monitoring the decrease in absorbance (λ_{max}) at 379 nm.

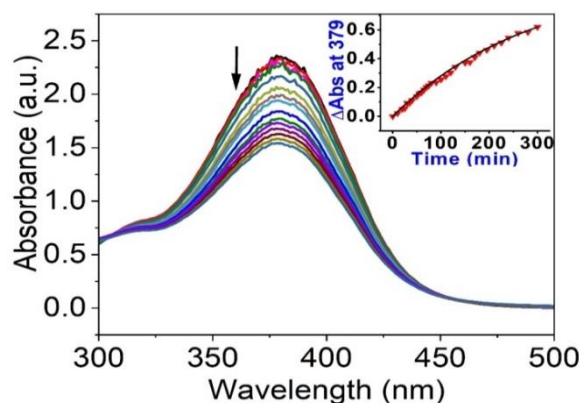


Figure 4.7 UV–Visible absorbance of Compound **2** monitored under blue (410 nm, 3 W) illumination over a 0–300 min interval, with an inset depicting the temporal absorbance changes at 379 nm.

4.2.2.2 Griess Assay:

Using the Griess assay with the standard protocol, the amount of NO released from **2** has been estimated [2]. The NO donor and Griess reagents were mixed and exposed to blue light (410 nm) for 7 hours, and the change in the UV-Vis absorbance was measured at different times. The Griess assay study states that the chromophoric azo-derivative production with a strong absorbance band in the range 460–493 nm and the color change are proof that NO was released from the donor NO molecules or materials.[3]

There was a color shift from pale yellow to pink and an increase in absorbance at 490 nm (Fig. 4.8). The release of NO from **2** is responsible for the formation of an azo dye in solution, as indicated by the new UV-Vis peak.

800 mg of Griess reagent were dissolved in 20 mL of 10 mM phosphate buffer to prepare the stock Griess reagent solution. A methanolic solution of **2** (105 μM , 2.1 mL) was mixed with 100 μL of this stock solution for the experiment, and the UV-Vis spectra was recorded at room temperature while exposed to light. The release of NO

from **2** following photolysis is indicated by the increase in UV-Vis absorbance at 490 nm.

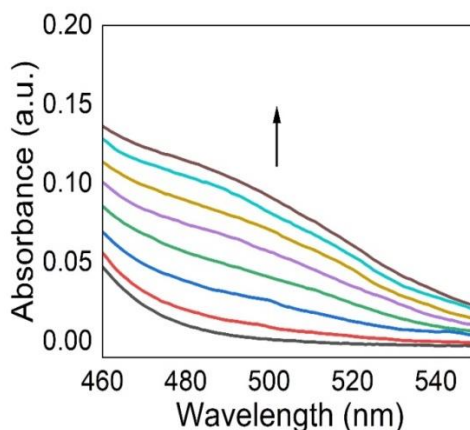


Figure 4.8 UV-Vis spectral changes of Compound **2** in the presence of Griess reagent over a 0 - 300 min period of irradiation with blue light (410 nm, 3 W).

Based on the calibration plot (Fig. 3.8), the amount of NO released was found to be 11.81 μ M, which is approximately thrice the amount of NO released by **1**. Thus we can say that the light-harvesting capabilities of the entire construct are significantly enhanced by the attachment of the multiple NO photo-releasing unit in a single scaffold; this finding is consistent with the previously documented calix [4] arene-based NO donor containing multiple NO donors. [5]

4.2.2.3 Effect of pH on NO release

The tissue's pH affects how cells function and plays a significant role in the onset and treatment of cancer. [6] It is commonly recognized that because of chronic hypoxia, high amounts of lactic acid, and insufficient blood flow, cancer cells have an extracellular pH that is more acidic (5.6 to 7.8) than normal cells. [7] Studying **2**'s NO release behavior under various pH levels is therefore crucial. It is important to assess how nitric oxide release varies with pH. To maintain the solution's pH, common buffers such as acetate (pH 5), phosphate (pH 7.4), and borate (pH 9) were employed. After being exposed to blue light for 120 minutes, the UV-Vis absorbance spectrum was used to track all three samples. To determine the rate of NO release, the absorbance change over time was tracked and plotted (Fig. 4.9).

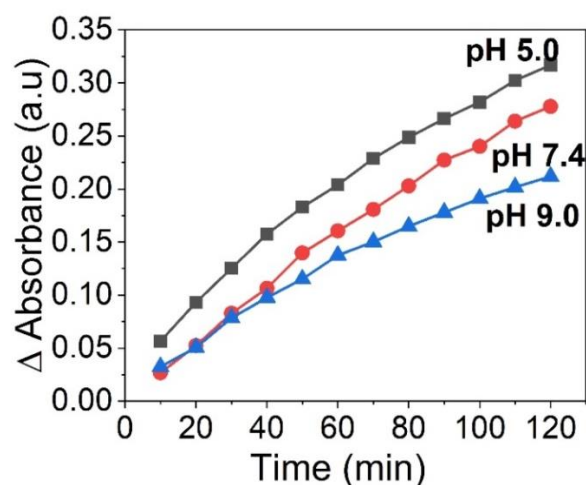
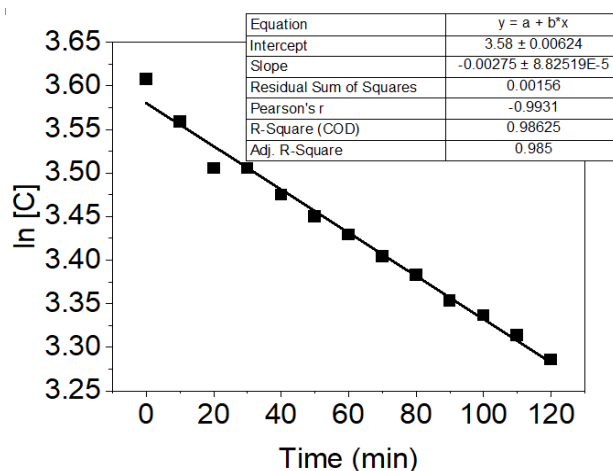


Figure 4.9 Change in absorbance of **2** (36.8 μM) with respect to time at different pH.

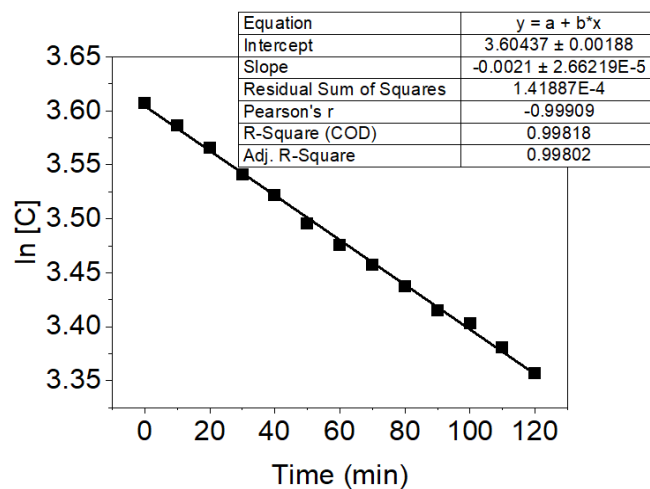
4.2.2.4 Calculation of rate constant

A graph was plotted between the natural logarithm of reactant concentration and time (Fig. 4.10) which gave a straight line, thus making it clear that the reaction follows first-order kinetic hence slope of the curve = Rate constant which was found to be 0.00198 min^{-1} which is greater than that of **1** (0.0015 min^{-1}) with $t_{1/2} = 350 \text{ min}$ which means **2** releases NO at a faster rate as compared to **1** and quantum yield (Φ) of 0.001 [2]. The quantum yield of **1** was found to be lower than Tris-NTA under the same conditions, this is due to the presence of three NO donors and the interaction of -OH and -NH intermolecular hydrogen bonding might be responsible for better NO releasing behavior.

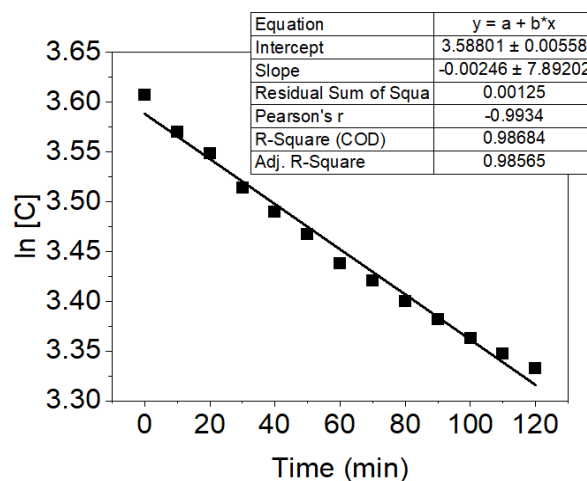
4.2.2.4.1 Graphs to calculate Rate constant



(a)



(b)



(c)

Figure 4.10 Graphs used to calculate rate constant of **2** at different pH (a) at pH 5.0 (b) at pH 7.4 (c) at pH 9.2

With the help of these graphs rate constant and half life has been calculated for the compound at different pH and has been presented in the table below.

Table 4.1: Rate constant (k_{NO}) and $t_{1/2}$ of **2** (36.8 μ M) at different pH conditions.

pH	Rate constant (min^{-1})	Half lifetime $t_{1/2}$ (min)
5.0	0.00275	252
7.4	0.0021	330
9	0.00246	281.7

Table 4.1 illustrates how minor changes in the solution's pH caused differences in the NO-releasing activity. **2** may release NO more quickly at the malignant spot than at

other cells since the rate of NO release, k_{NO} , is highest at an acidic pH of 5.0 and its $t_{1/2}$ is the lowest of all.

The protonation/deprotonation of the secondary amine moiety connected to the 1-nitro-2-(trifluoromethyl)benzene may be the cause of the data's delayed NO release at pH 7.4 in comparison to pH 5.0 and pH 9.0. The slight change in **2**'s UV-Vis absorption peak intensity (400 nm) at strong UV-Vis absorbance at pH 5.0 and pH 9.0 compared to pH 7.4 indicates that **2** may be able to absorb more photon energy upon light irradiation under acidic or basic conditions, which results in a slightly faster release of NO than at neutral pH. This is related to protonation or deprotonation at the -NH moiety.

4.2.2.5 Effect of Concentration

To study the relationship between concentration and NO release rate, different concentrations of NO donor **2** (5.26 μM – 36.86 μM) were irradiated with blue light and the change in UV-Vis absorbance was monitored for 120 min with a time interval of 10 min (Fig. 4.11).

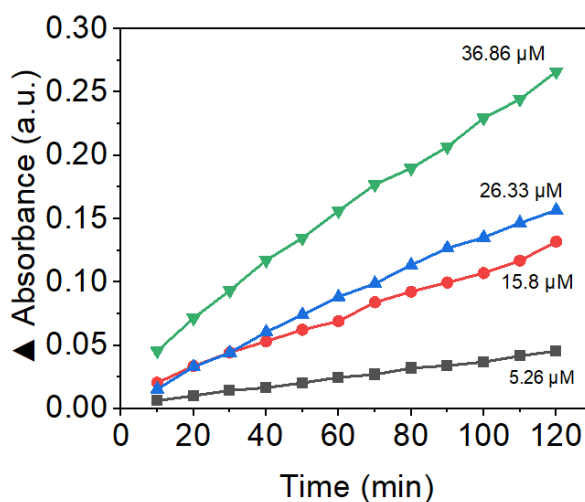
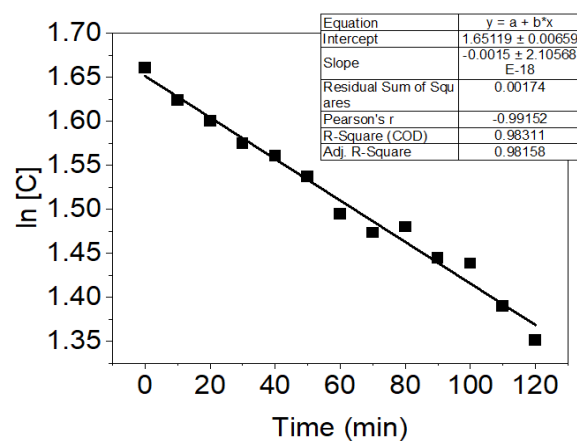


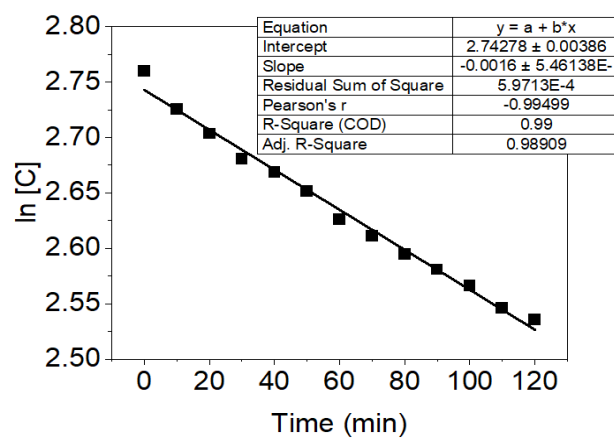
Figure 4.11 Temporal absorbance variation of Compound **2** at multiple concentrations under 410 nm (3 W) blue-light irradiation.

4.2.2.6 Calculation of rate constant

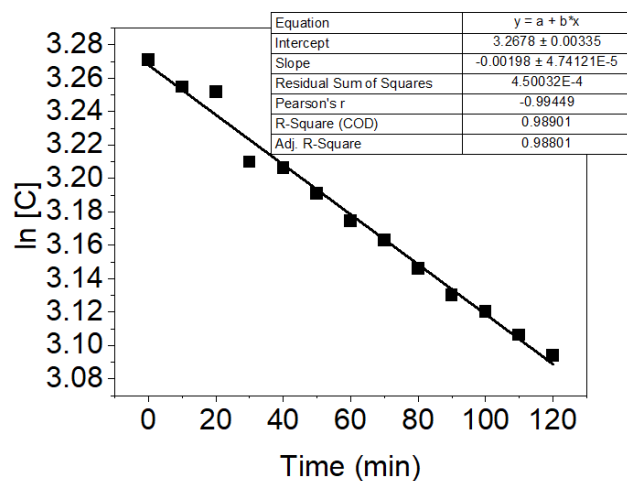
4.2.2.6.1 Graphs to calculate Rate constant



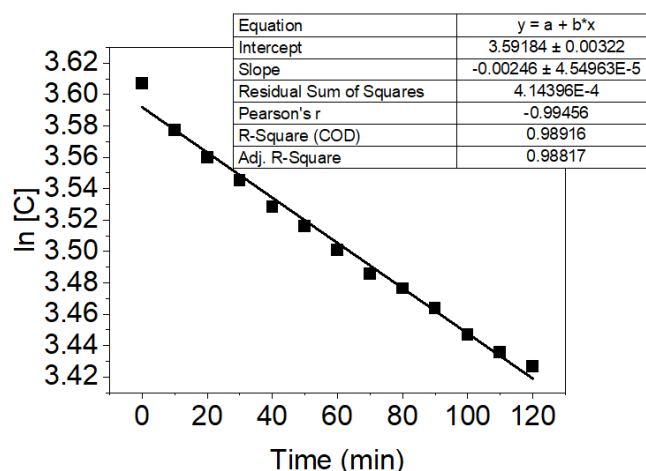
(a)



(b)



(c)



(d)

Figure 4.12 Graphs used to calculate rate constant of **2** at different concentrations (a) 5.26 μM (b) 15.8 μM (c) 26.33 μM (d) 36.86 μM

With the help of these graphs rate constant and half life has been calculated for the compound at different concentration and has been presented in the table below.

Table 4.2: Showing the values of k_{NO} , $t_{1/2}$ and Φ for different concentrations of the sample

Concentration (μM)	Rate Constant (min^{-1})	Half lifetime (min)	Quantum Yield
5.26 μM	0.0015	462	3.96×10^{-5}
15.8 μM	0.0016	433	1.26×10^{-4}
26.33 μM	0.00198	350	2.61×10^{-4}
36.86 μM	0.00246	281	4.55×10^{-4}

As indicated in table 4.2, it is also noted that the rate constant k_{NO} rises as concentration does. Increase in concentration means increase in number of molecules and thus increase in decomposition events per unit time. Moreover NO release follows first order kinetics, thus, increasing donor concentration leads to a directly proportional increase in the initial rate of NO release, as per the equation –

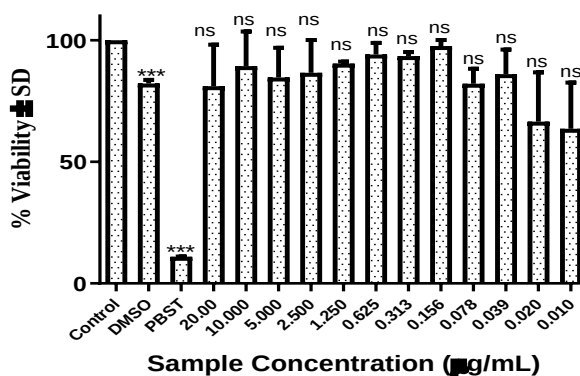
$$d[\text{NO}]/dt = k[\text{donor}]$$

where k is the rate constant and $d[\text{NO}]/dt$ signifies the concentration of NO donor per unit time.

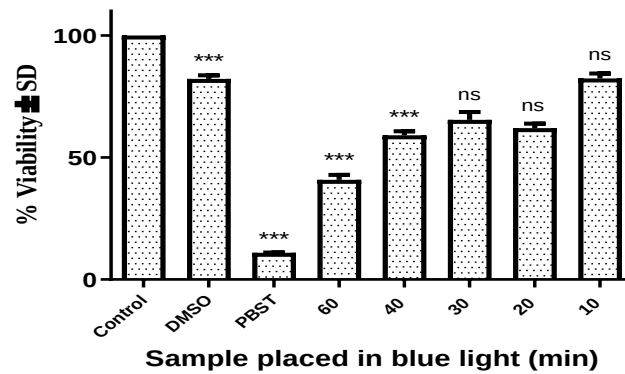
As a result, the concentration-dependent study demonstrates that altering the NO donor's concentration can regulate or control the light-induced release of NO from **2**.

4.2.3 Evaluation of cytotoxicity of compound using Human Prostate Cancer Cells (DU145), Cervical Cancer Cells (HeLa cells) and Breast Cancer Cells (MCF-7) in vitro

Human prostate cancer cells (DU145), cervical cancer (HeLa cells), and breast cancer cells (MCF-7) were used to study the safety profiles of **2** in methanol before NO release and during NO release at different concentrations 0.010–20.00 µg/mL (Fig. 4.13 (a), Fig. 4.14 (a), and Fig. 4.15 (a)) respectively. The compound can be used safely for medicinal purposes because the MTT assay demonstrates that it is not harmful to cells even at concentrations of up to 20.00 µg/mL. This prompted us to perform preliminary studies on the killing of cancer cells caused by light. Therefore, in order to estimate cell mortality following NO release, DU145, HeLa, and MCF-7 cells were treated with **2** and exposed to blue light for 10, 20, 30, 40, and 60 minutes. This experiment suggests that the observed cancer cell mortality is caused by the light-induced release of NO. The results show that the compound becomes toxic to the DU145, HeLa, and MCF-7 cancer cells after 60 minutes of irradiation with blue light, resulting in 60% (Fig. 4.13 (b)), 57% (Fig. 4.14 (b)), and 55% (Fig. 4.15 (b)) of cell death, respectively. Hence it is evident from the mortality percentage that **2** is a better anti-cancerous agent as compared to **1** as it has three NO donor units. The control experiments clearly show that the compound is toxic to the cells only in the presence of blue light after irradiation of more than 40 minutes.

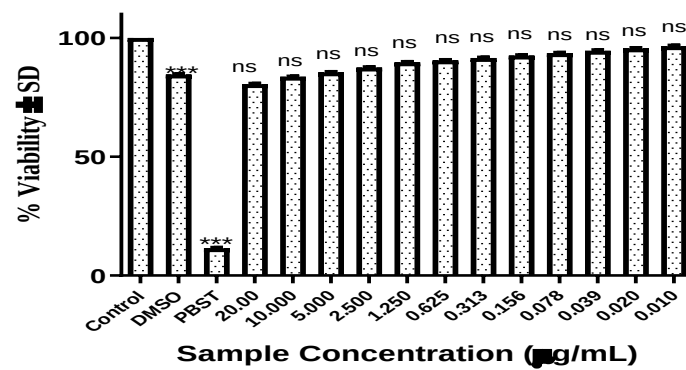


(a)

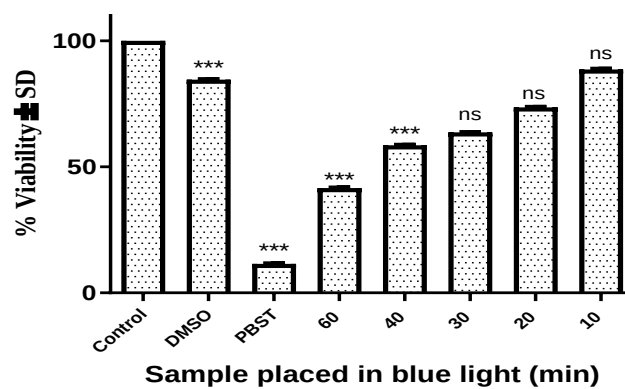


(b)

Figure 4.13 (a) & (b) Effect of Sample 2 on DU145 cells without blue light or with blue light. (***) $p < 0.001$, ns-nonsignificant).

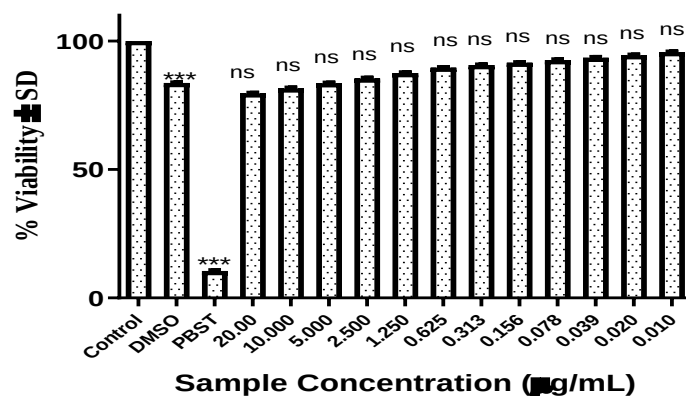


(a)

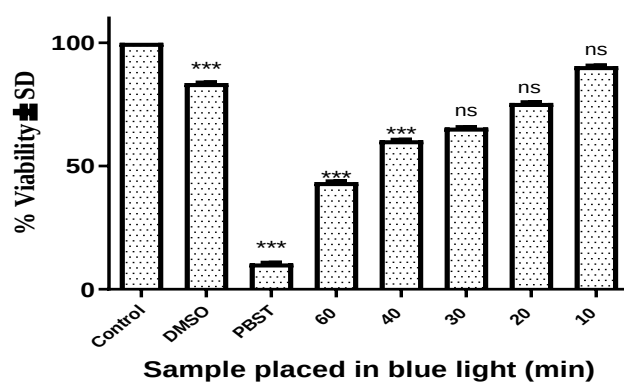


(b)

Figure 4.14 (a) & (b) Effect of Sample 2 on HeLa cells without blue light or with blue light. (***) $p < 0.001$, ns-nonsignificant).



(a)



(b)

Figure 4.15 (a) & (b) Effect of Sample 2 on MCF-7 cells without blue light or with blue light. (***) $p < 0.001$, ns-nonsignificant).

4.3 Synthesis, Qualitative analysis and Biological studies of NO donor 5 - (dimethylamino) -N-(2-((4 - nitro -3- (trifluoromethyl) phenyl) amino) ethyl)naphthalene -1- sulphonamide (Compound 3)

Synthesis of compound **3** was done by following scheme 3.3 and the compound was analysed using mass and NMR spectroscopy and the results have been compiled.

4.3.1 Synthesis of compound 3

First of all **1** (250 mg, 1.003 mmol) was dissolved in 65 mL dry THF and after that Diisopropylethylamine (524 μ L, 3.009 mmol) with continuous stirring under nitrogen atmosphere was added after some time Dansyl chloride (270 mg, 1.003 mmol) was added in the reaction mixture and the reaction mixture was left undisturbed for 20 hrs. The resulting mixture was filtered and concentrated by rotary evaporation. The crude product was recrystallised with methanol and **3** was obtained as yellow colored solid. Yield = 144 mg (30 %). ESI-MS m/z : calculated 482.48; found $[M + H]^+$ 483.13 (Fig. A.3 (a)) HR-MS: $[M + H]^+$ calculated; 483.1314 observed; 483.1323. (Fig. A.3 (b)) ^1H NMR (500 MHz, DMSO- D_6) δ 8.38 (d, J = 8Hz, 1H), 8.19 (d, J = 8Hz, 1H), 8.05 (1H, 1H-NH), 7.90 (1H), 7.53 (2H), 7.38 (1H), 7.16 (d J = 8Hz, 1H), 6.87 (s, 1H), 6.57 (1H-NH), 3.51 (2H), 3.17 (2H), 2.91 (6H) (Fig. B.3 (a)). ^{13}C NMR (125 MHz, DMSO- D_6) δ 152.57, 151.21, 135.54, 133.68, 129.44, 129.37, 128.90, 128.83, 128.22, 127.73, 123.42, 123.36, 118.80, 114.90, 44.86, 41.97, 40.98 (Fig. B.3 (b))

4.3.2 Qualitative Analysis

The compound was used for further NO releasing studies with the help of UV-Vis spectrophotometer.

4.3.2.1 UV-Vis Studies

The NO release behavior of **3** was observed using time-dependent UV-Vis studies. The absorbance at 376 nm decreases continuously in the presence of blue light (410 nm, 3 W) (Fig. 4.16), but there is no change in absorbance in its absence. Light was found to photobleach **3**, which is entirely compatible with the photochemical process that releases NO. The reduced absorbance in the visible area is caused by the potential production of phenoxy and NO radicals, which are non-toxic and weakly absorbing photoproducts (Scheme 4.1).

NO was released only under blue light irradiation, the UV-Vis spectrum did not alter in the absence of light and even after keeping under daylight for a prolonged time.

UV-Vis spectra of methanolic solution of **3** (9.86 μM) was monitored at different time interval upon irradiation with blue light (410 nm, 3 W). The quartz cuvette was placed 1 cm away from the light source for the irradiation experiment. The NO release was determined by monitoring the decrease in absorbance (λ_{max}) at 376 nm.

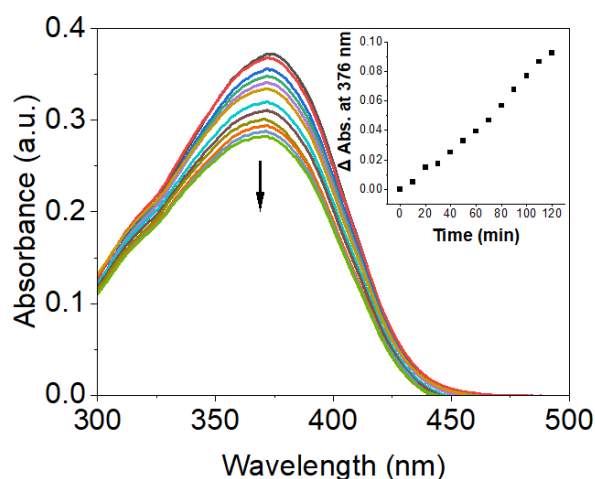


Figure 4.16 UV–Visible absorbance of Compound **3** monitored under blue (410 nm, 3 W) illumination over a 0–250 min interval, with an inset depicting the temporal absorbance changes at 376 nm.

4.3.2.2 Griess Assay:

Using the Griess assay with the standard protocol, the amount of NO released from **3** has been estimated [2]. The NO donor and Griess reagents were mixed and exposed to blue light (410 nm) for 6 hours, and the change in the UV-Vis absorbance was measured at different times. The Griess assay study states that the chromophoric azo-derivative production with a strong absorbance band in the range 460-493 nm and the color change are proof that NO was released from the donor NO molecules or materials.[3]

There was a color shift from pale yellow to pink and an increase in absorbance at 480 nm (Fig. 4.17). The release of NO from **3** is responsible for the formation of an azo dye in solution, as indicated by the new UV-Vis peak.

800 mg of Griess reagent were dissolved in 20 mL of 10 mM phosphate buffer to prepare the stock Griess reagent solution. A methanolic solution of **3** (98.6 μM , 2.1 mL) was mixed with 100 μL of this stock solution for the experiment, and the UV-Vis spectra was recorded at room temperature while exposed to light. The release of NO

from **3** following photolysis is indicated by the increase in UV-Vis absorbance at 480 nm.

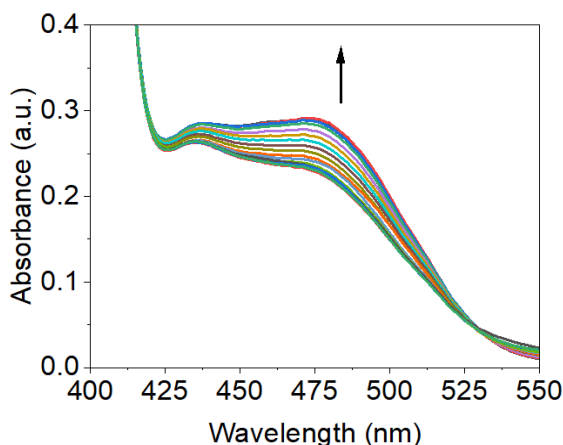


Figure 4.17 UV-Vis spectral changes of Compound **3** in the presence of Griess reagent over a 0-320 min period of irradiation with blue light (410 nm, 3 W).

Based on the calibration plot (Fig. 3.8), the amount of NO released was found to be 28 μ M.

4.3.2.3 Effect of pH on NO release

To maintain the solution's pH, common buffers such as acetate (pH 5), phosphate (pH 7.4), and borate (pH 9) were employed. After being exposed to blue light for 120 minutes, the UV-Vis absorbance spectrum was used to track all three samples. To determine the rate of NO release, the absorbance change over time was tracked and plotted (Fig. 4.18).

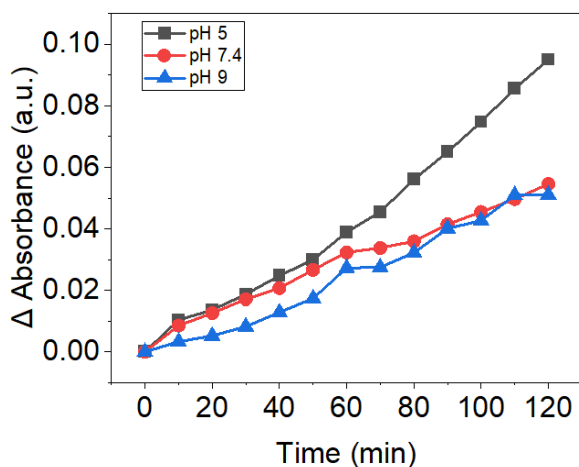
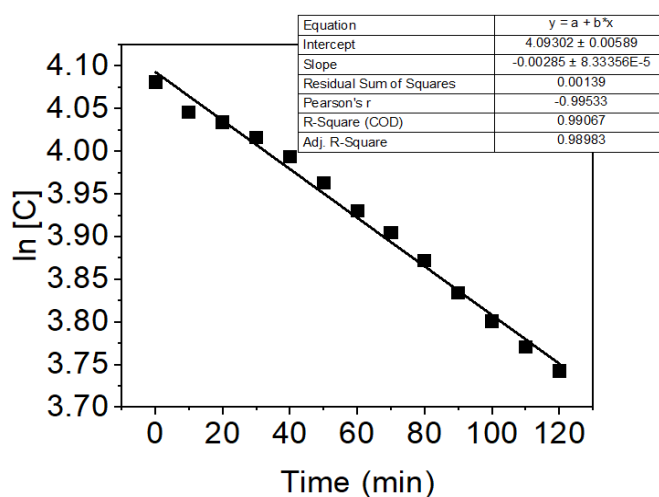


Figure 4.18 Change in absorbance of **3** (56.2 μ M) with respect to time at different pH

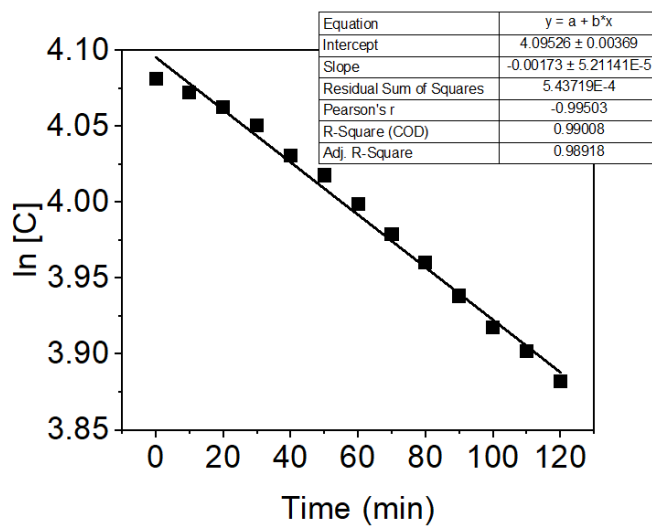
4.3.2.4 Calculation of rate constant

A graph was plotted between the natural logarithm of reactant concentration and time (Fig. 4.19) which gave a straight line, thus making it clear that the reaction follows first-order kinetic hence slope of the curve = Rate constant which was found to be 0.0034 min^{-1} with $t_{1/2} = 203.82 \text{ min}$ and quantum yield (Φ) of 0.0005 [2].

4.3.2.4.1 Graphs to calculate Rate constant



(a)



(b)

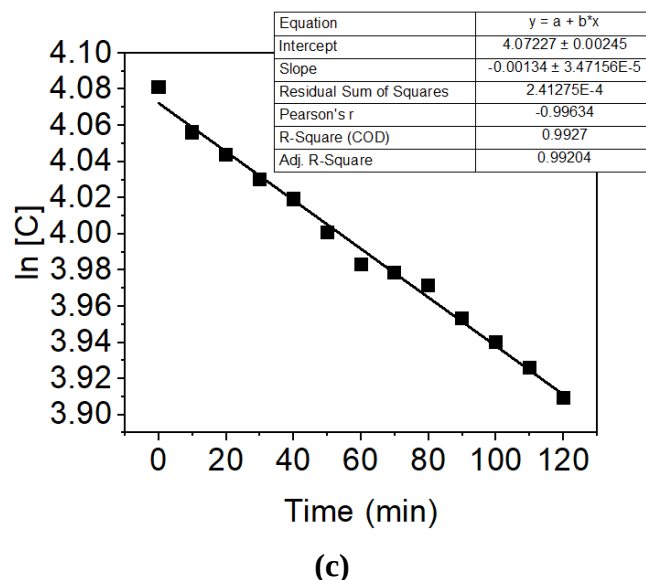


Figure 4.19 Graphs used to calculate rate constant of **3** at different pH (a) at pH 5.0 (b) at pH 7.4 (c) at pH 9.2

With the help of these graphs rate constant and half life has been calculated for the compound at different pH and has been presented in the table below.

Table 4.3: Rate constant (k_{NO}) and $t_{1/2}$ of **3** (56.2 μ M) at different pH conditions.

pH	Rate constant (min^{-1})	Half lifetime $t_{1/2}$ (min)
5.0	0.0028	247.5
7.4	0.0017	407.64
9	0.00134	517.16

Table 4.3 illustrates how minor changes in the solution's pH caused differences in the NO-releasing activity. **3** may release NO more quickly at the malignant spot than at other cells since the rate of NO release, k_{NO} , is highest at an acidic pH of 5.0 and its $t_{1/2}$ is the lowest of all. While the data indicates that the release of NO is moderate at pH 7.4 and slowest at pH 9, respectively.

4.3.2.5 Effect of Concentration

To study the relationship between concentration and NO release rate, different concentrations of NO donor **3** (9.86 μ M – 59.2 μ M) were irradiated with blue light and the change in UV-Vis absorbance was monitored for 120 min with a time interval of 10 min (Fig. 4.20).

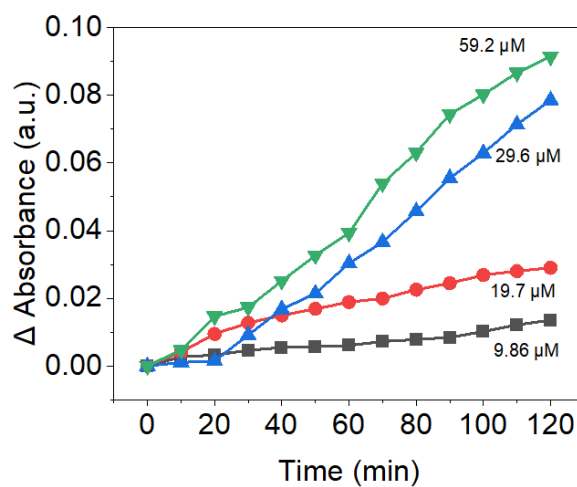
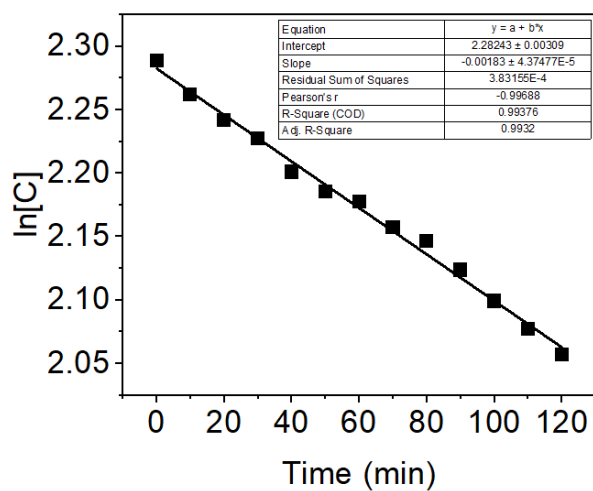


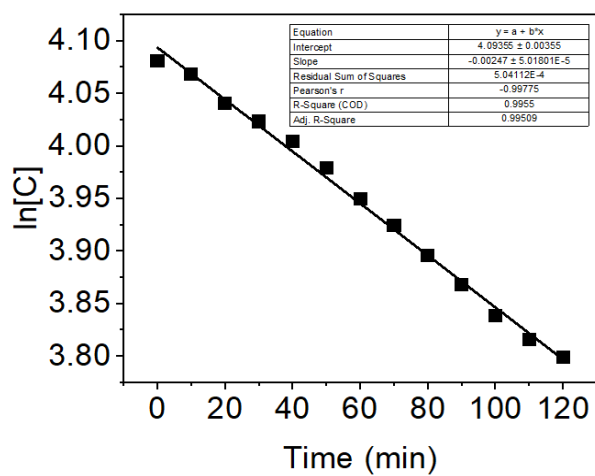
Figure 4.20 Temporal absorbance variation of Compound **3** at multiple concentrations under 410 nm (3 W) blue-light irradiation.

4.3.2.6 Calculation of rate constant

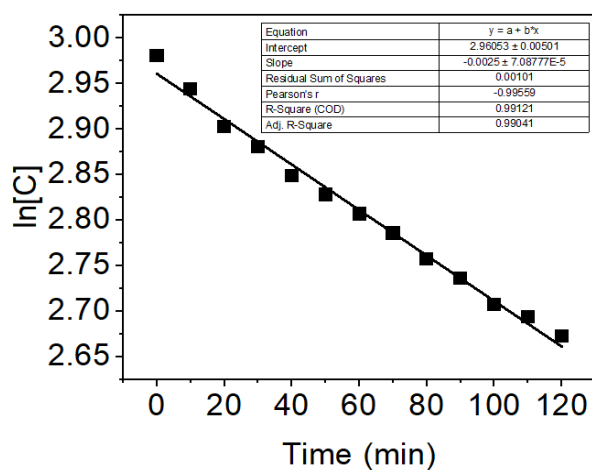
4.3.2.6.1 Graphs to calculate Rate constant



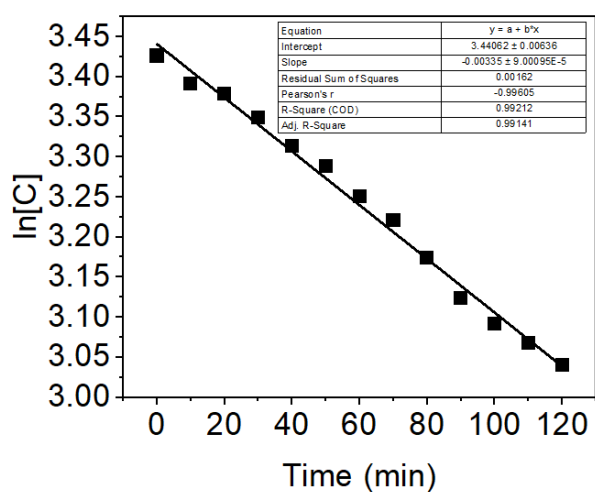
(a)



(b)



(c)



(d)

Figure 4.21 Graphs used to calculate rate constant of **3** at different concentrations (a) 9.86 μM (b) 19.7 μM (c) 29.6 μM (d) 59.2 μM

With the help of these graphs rate constant and half life has been calculated for the compound at different concentration and has been presented in the table below.

Table 4.4: Showing the values of k_{NO} , $t_{1/2}$ and Φ for different concentrations of the sample

Concentration (μM)	Rate Constant (min^{-1})	Half lifetime (min)	Quantum Yield
9.86 μM	0.00183	378.68	9.0×10^{-5}
19.7 μM	0.0024	288.75	2.3×10^{-4}
29.6 μM	0.0025	277.2	3.7×10^{-4}
59.2 μM	0.00335	206.86	9.9×10^{-4}

As indicated in table 4.4, it is also noted that the rate constant k_{NO} rises as concentration does. As a result, the concentration-dependent study demonstrates that altering the NO donor's concentration can regulate or control the light-induced release of NO from **3**.

4.3.3 Fluorescence Studies of **3**

The fluorescence studies of **3** (Fig. 4.22) were carried out in methanol and water mixture taken in the ratio of 20:80 respectively. For this 59.2 μM of **3** was taken in 20:80 methanol and HPLC water and was irradiated with blue light and was monitored at regular time intervals. The NO donor showed an increase in the intensity at 493 nm. As the fluorescence of the naphthalene unit is efficiently suppressed by photoinduced energy transfer between the naphthalene and the nitroaniline. When the nitroaniline chromophore is excited with blue light, nitric oxide (NO) is released, and the naphthalene fluorescence is also revived. These two phenomena serve as optical reporters to measure the amount of NO emitted [8].

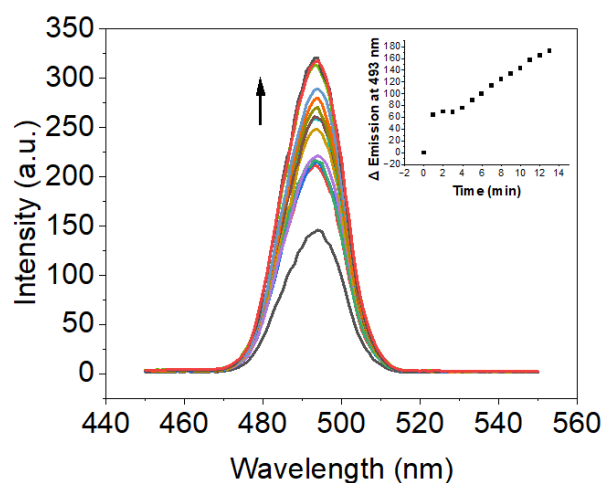
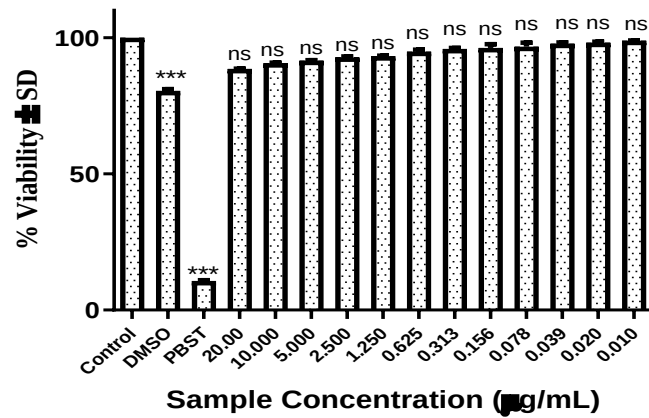


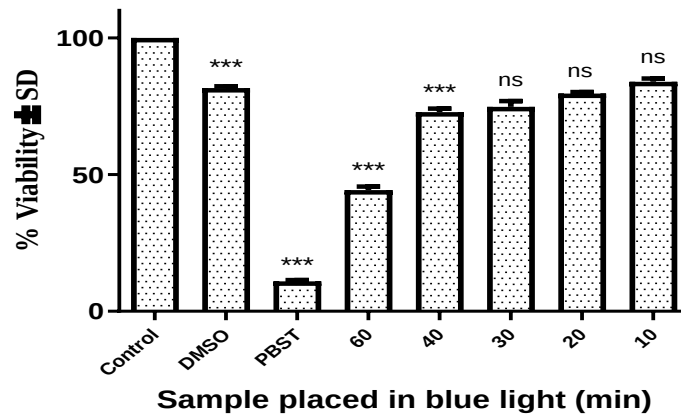
Figure 4.22 Fluorescence studies of **3** in Methanol Water mixture (Inset) Change in intensity at 493 nm with respect to time.

4.3.4 Evaluation of cytotoxicity of compound using Human Prostate Cancer Cells (DU145), Cervical Cancer Cells (HeLa cells) and Breast Cancer Cells (MCF-7) in vitro

Human prostate cancer cells (DU145), cervical cancer (HeLa cells), and breast cancer cells (MCF-7) were used to study the safety profiles of **3** in methanol before NO release and during NO release at different concentrations 0.010–20.00 $\mu\text{g/mL}$ (Fig. 4.23 (a), Fig. 4.24 (a), and Fig. 4.25 (a)) respectively. The compound can be used safely for medicinal purposes because the MTT assay demonstrates that it is not harmful to cells even at concentrations of up to 20.00 $\mu\text{g/mL}$. This prompted us to perform preliminary studies on the killing of cancer cells caused by light. Therefore, in order to estimate cell mortality following NO release, DU145, HeLa, and MCF-7 cells were treated with **3** and exposed to blue light for 10, 20, 30, 40, and 60 minutes. This experiment suggests that the observed cancer cell mortality is caused by the light-induced release of NO. The results show that the compound becomes toxic to the DU145, HeLa, and MCF-7 cancer cells after 60 minutes of irradiation with blue light, resulting in 56% (Fig. 4.23 (b)), 51% (Fig. 4.24 (b)), and 53% (Fig. 4.25 (b)) of cell death, respectively. The control experiments clearly show that the compound is toxic to the cells only in the presence of blue light after irradiation of more than 40 minutes.

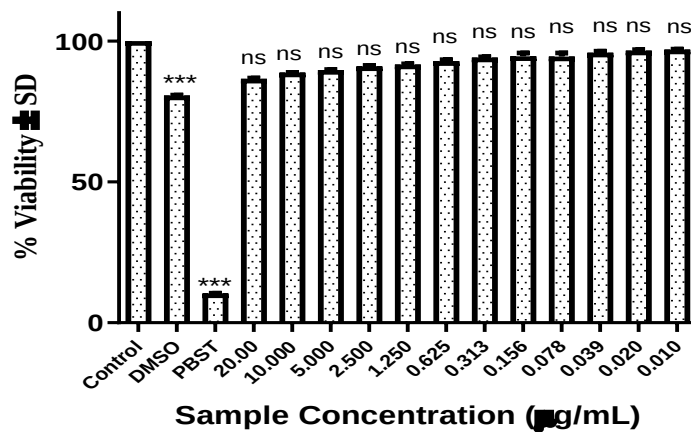


(a)

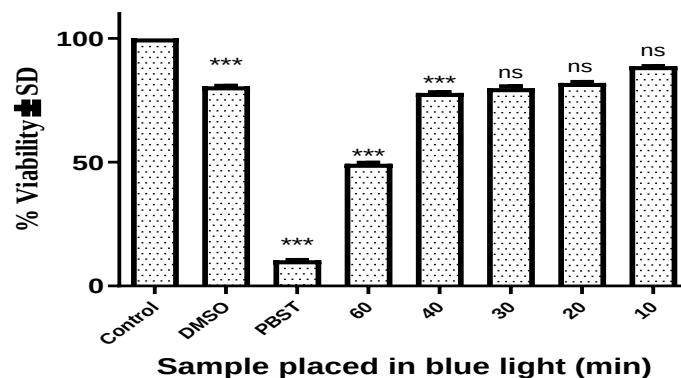


(b)

Figure 4.23 (a) & (b) Effect of Sample 3 on DU145 cells without blue light or with blue light. (***) $p < 0.001$, ns-nonsignificant).

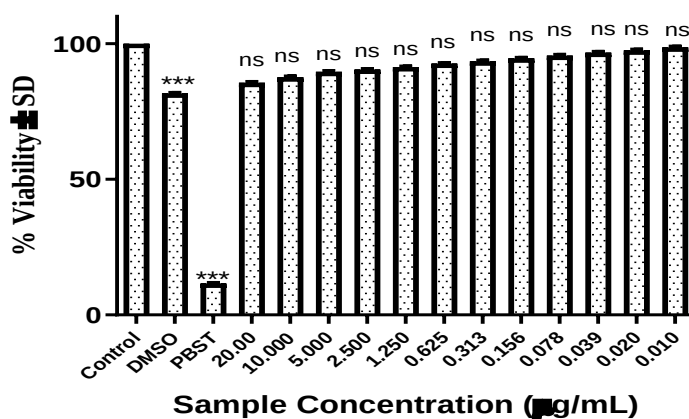


(a)

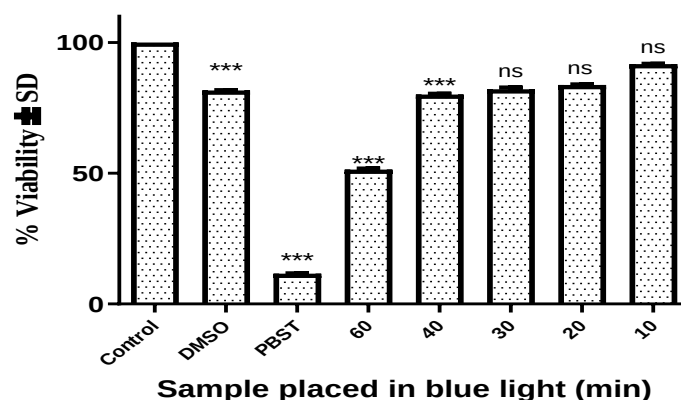


(b)

Figure 4.24 (a) & (b) Effect of Sample 3 on HeLa cells without blue light or with blue light. (***) $p < 0.001$, ns-nonsignificant).



(a)



(b)

Figure 4.25 (a) & (b) Effect of Sample 3 on MCF-7 cells without blue light or with blue light. (***) $p < 0.001$, ns-nonsignificant).

4.4 Synthesis, Qualitative analysis and Biological studies of NO donor N-(2-((4 - nitro -3- (trifluoromethyl) phenyl) amino) ethyl)naphthalene -1- sulphonamide (Compound 4)

Synthesis of compound **4** was done by following scheme 3.4 and the compound was analysed using mass and NMR spectroscopy and the results have been compiled.

4.4.1 Synthesis of compound 4

100 mg of **1** (0.40 mmol) was dissolved in 40 mL CH₂Cl₂ after that Diisopropylethylamine (742 μ L, 1.2 mmol) with continuous stirring under nitrogen atmosphere was added after some time 2- Naphthalene sulfonyl chloride (90.6 mg, 0.40 mmol) was added in the reaction mixture and the reaction mixture was left undisturbed for 20 hrs. The resulting mixture was filtered and concentrated by rotary evaporation. The crude product was recrystallised with methanol and was obtained as yellow colored solid. Yield = 70 mg (40 %). ESI-MS m/z: calculated 439.41; found [M + H]⁺ 440.06 (Fig. A.4 (a)) HR-MS: [M +H]⁺ calculated; 440.0892 observed; 440.0882. (Fig. A.4 (b)) ¹H NMR (500 MHz, DMSO-*D*₆) δ 8.42 (1H-NH) 8.09 (dd, 2H), 8.00 (1H), 7.90 (2H), 7.81 (d, *J* = 10 Hz, 1H), 7.66 (2H), 7.50 (1H) 6.97 (1H), 6.70 (1H-NH), 3.30-3.27 (q, 2H), 2.99-2.96 (q, 2H) (Fig. B.4 (a)). ¹³C NMR (125 MHz, DMSO-*D*₆) δ 152.60, 137.04, 133.97, 133.71, 131.52, 129.35, 129.26, 128.96, 128.55, 127.63, 127.41, 127.27, 122.02, 41.86, 41.14.(Figure B.4 (b))

4.4.2 Qualitative Analysis

The compound was used for further NO releasing studies with the help of UV-Vis spectrophotometer.

4.4.2.1 UV-Vis Studies

The NO release behavior of **4** was observed using time-dependent UV-Vis studies. The absorbance at 378 nm decreases continuously in the presence of blue light (410 nm, 3 W) (Fig. 4.26), but there is no change in absorbance in its absence. Light was found to photobleach **4**, which is entirely compatible with the photochemical process that releases NO. The reduced absorbance in the visible area is caused by the potential production of phenoxy and NO radicals, which are non-toxic and weakly absorbing photoproducts (Scheme 4.1).

NO was released only under blue light irradiation, the UV-Vis spectrum did not alter in the absence of light and even after keeping under daylight for a prolonged time.

UV-Vis spectra of methanolic solution of **4** (43.2 μM) was monitored at different time interval upon irradiation with blue light (410 nm, 3 W). The quartz cuvette was placed 1 cm away from the light source for the irradiation experiment. The NO release was determined by monitoring the decrease in absorbance (λ_{max}) at 378 nm.

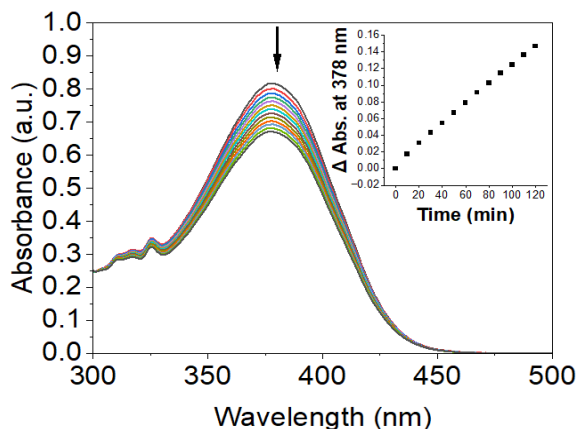


Figure 4.26 UV–Visible absorbance of Compound **4** monitored under blue (410 nm, 3 W) illumination over a 0–120 min interval, with an inset depicting the temporal absorbance changes at 378 nm.

4.4.2.2 Griess Assay:

Using the Griess assay with the standard protocol, the amount of NO released from **4** has been estimated [2]. The NO donor and Griess reagents were mixed and exposed to blue light (410 nm) for 5 hours and 20 minutes, and the change in the UV-Vis absorbance was measured at different times. The Griess assay study states that the chromophoric azo-derivative production with a strong absorbance band in the range 460–500 nm and the color change are proof that NO was released from the donor NO molecules or materials.[3]

There was a color shift from pale yellow to pink and an increase in absorbance at 497 nm (Fig. 4.27). The release of NO from **4** is responsible for the formation of an azo dye in solution, as indicated by the new UV-Vis peak.

800 mg of Griess reagent were dissolved in 20 mL of 10 mM phosphate buffer to prepare the stock Griess reagent solution. A methanolic solution of **4** (43.2 μM , 2.1 mL) was mixed with 100 μL of this stock solution for the experiment, and the UV-Vis spectra was recorded at room temperature while exposed to light. The release of NO from **4** following photolysis is indicated by the increase in UV-Vis absorbance at 497 nm.

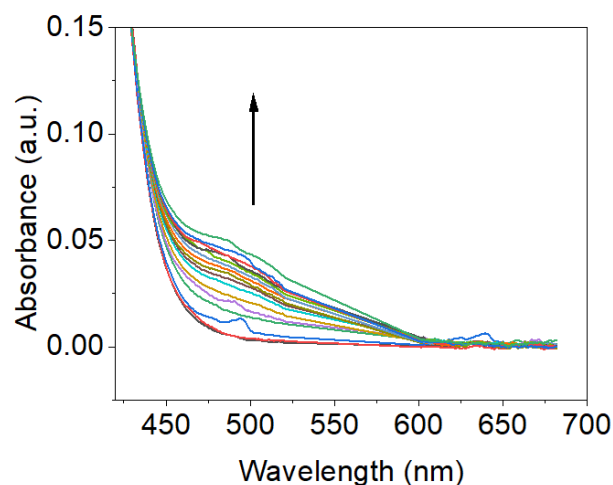


Figure 4.27 UV-Vis spectral changes of Compound **4** in the presence of Griess reagent over a 0-320 min period of irradiation with blue light (410 nm, 3 W).

Based on the calibration plot (Fig. 3.8), the amount of NO released was found to be 26 μ M, which is just 2 units less than that of **3** (28 μ M) thus we can say both the NO donors **3** & **4** release approximately same amount of NO which is indeed quite greater than the other synthesized NO donors i.e **1** (3.88 μ M), **2** (11.81 μ M) & **5** (7.72 μ M).

4.4.2.3 Effect of pH on NO release

To maintain the solution's pH, common buffers such as acetate (pH 5), phosphate (pH 7.4), and borate (pH 9) were employed. After being exposed to blue light for 120 minutes, the UV-Vis absorbance spectrum was used to track all three samples. To determine the rate of NO release, the absorbance change over time was tracked and plotted (Fig. 4.28).

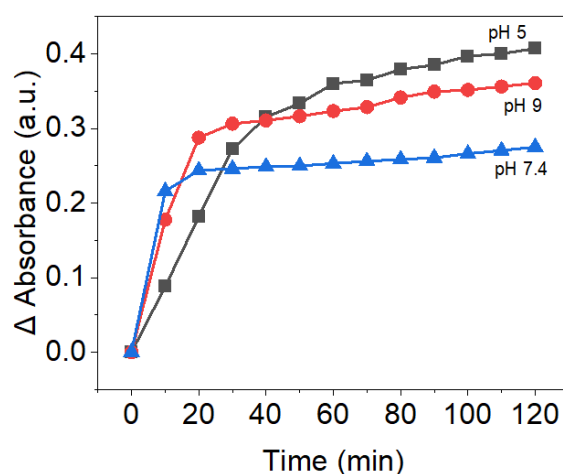
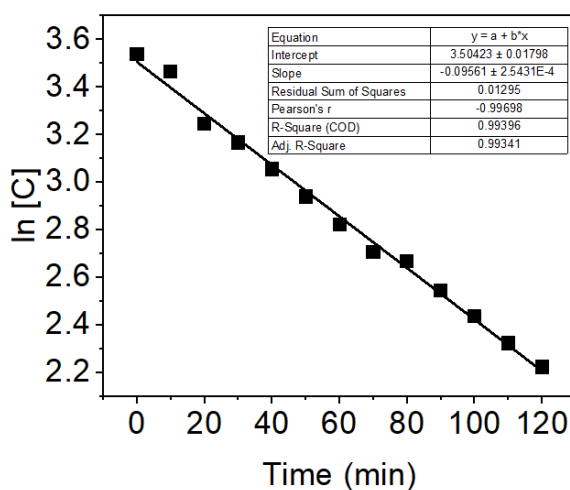


Figure 4.28 Change in absorbance of **4** (43.2 μ M) with respect to time at different pH

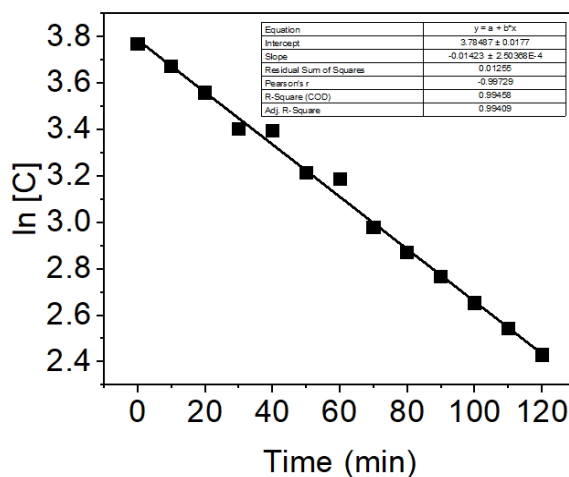
4.4.2.4 Calculation of rate constant

A graph was plotted between the natural logarithm of reactant concentration and time (Fig. 4.29) which gave a straight line, thus making it clear that the reaction follows first-order kinetic hence slope of the curve = Rate constant which was found to be 0.0016 min^{-1} which is approximately three times less than that of **3** (0.0034 min^{-1}) with $t_{1/2} = 427.77 \text{ min}$ which means **3** will release NO three times faster as compared to **4** and quantum yield (Φ) of 0.00035 [2] which is again less than that of **3** (0.0005) that means more molecules undergo the intended chemical transformation per photon absorbed in case of **3** as compared to **4**. This suggests a more efficient reaction pathway with fewer competing side reactions or non-productive energy losses.

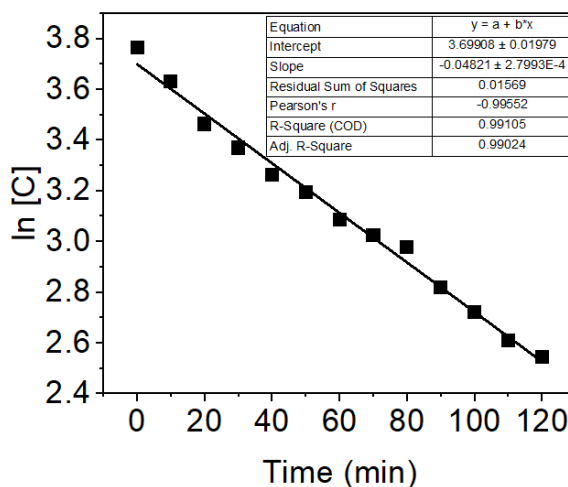
4.4.2.4.1 Graphs to calculate Rate constant



(a)



(b)



(c)

Figure 4.29 Graphs used to calculate rate constant of **4** at different pH (a) at pH 5.0 (b) at pH 7.4 (c) at pH 9.2

With the help of these graphs rate constant and half life has been calculated for the compound at different pH and has been presented in the table below.

Table 4.5: Rate constant (k_{NO}) and $t_{1/2}$ of **4** (43.2 μM) at different pH conditions.

Ph	Rate constant (min^{-1})	Half lifetime $t_{1/2}$ (min)
5.0	0.09561	7.24
7.4	0.01423	48.69
9	0.04821	14.37

Table 4.5 illustrates how minor changes in the solution's pH caused differences in the NO-releasing activity. **4** may release NO more quickly at the malignant spot than at other cells since the rate of NO release, k_{NO} , is highest at an acidic pH of 5.0 and its $t_{1/2}$ is the lowest of all. While the data indicates that the release of NO is moderate at pH 9 and slowest at pH 7.4, respectively.

4.4.2.5 Effect of Concentration

To study the relationship between concentration and NO release rate, different concentrations of NO donor **4** (10.8 μM – 43.2 μM) were irradiated with blue light and the change in UV-Vis absorbance was monitored for 120 min with a time interval of 10 min (Fig. 4.30).

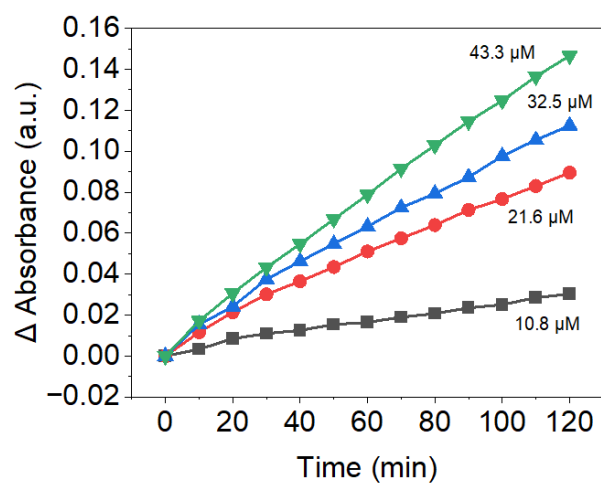
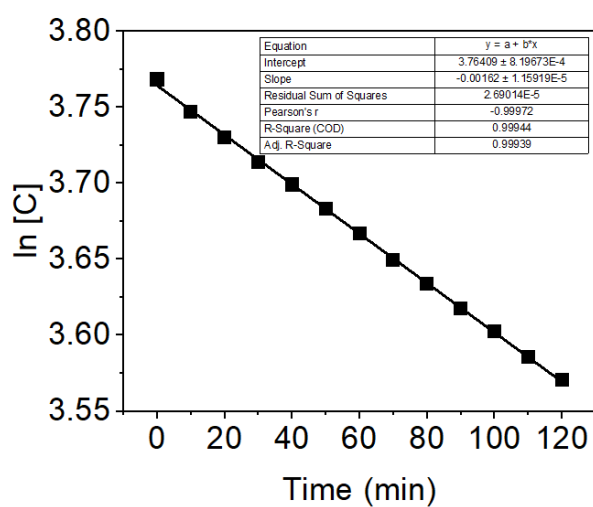


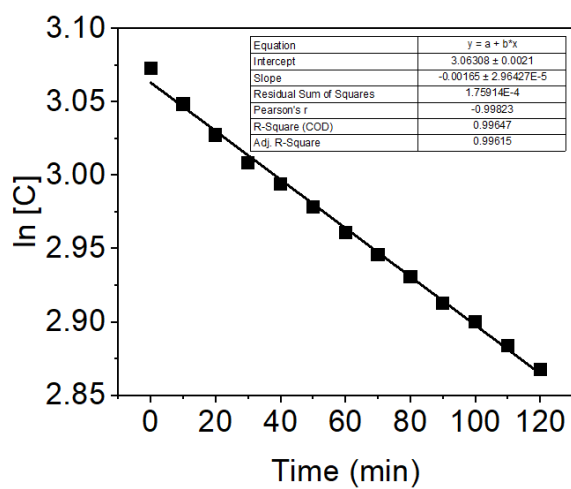
Figure 4.30 Temporal absorbance variation of Compound **4** at multiple concentrations under 410 nm (3 W) blue-light irradiation.

4.4.2.6 Calculation of rate constant

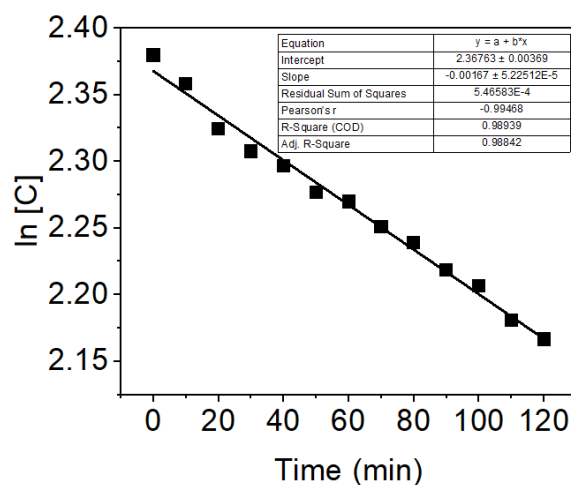
4.4.2.6.1 Graphs to calculate Rate constant



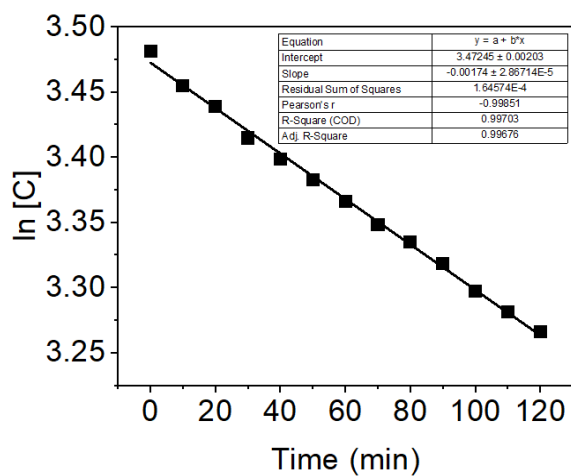
(a)



(b)



(c)



(d)

Figure 4.31 Graphs used to calculate rate constant of **4** at different concentrations (a) 10.8 μM (b) 21.6 μM (c) 32.5 μM (d) 43.3 μM

With the help of these graphs rate constant and half life has been calculated for the compound at different concentration and has been presented in the table below.

Table 4.6: Showing the values of k_{NO} , $t_{1/2}$ and Φ for different concentrations of the sample

Concentration (μM)	Rate Constant (min^{-1})	Half lifetime (min)	Quantum Yield
10.8 μM	0.00162	427.77	8.7×10^{-5}
21.6 μM	0.00165	420	1.7×10^{-4}
32.5 μM	0.00167	414.97	2.7×10^{-4}
43.3 μM	0.00174	398.27	3.7×10^{-4}

As indicated in table 4.6, it is also noted that the rate constant k_{NO} rises as concentration does. As a result, the concentration-dependent study demonstrates that altering the NO donor's concentration can regulate or control the light-induced release of NO from **4**.

4.4.3 Fluorescence Studies of **4**

The fluorescence studies of **4** (Fig. 4.32) were carried out in methanol and water mixture taken in the ratio of 20:80 respectively. A 54.4 μM solution of compound **4** was prepared in a 20:80 methanol-HPLC water mixture and subjected to blue-light irradiation. The NO donor's fluorescence intensity at 340 nm increased over time, due to photoinduced energy transfer between the dansyl and nitroaniline chromophores. Upon blue-light excitation, nitric oxide is released from the nitroaniline group, which restores the dansyl fluorescence - serving as an optical indicator of NO release. [8].

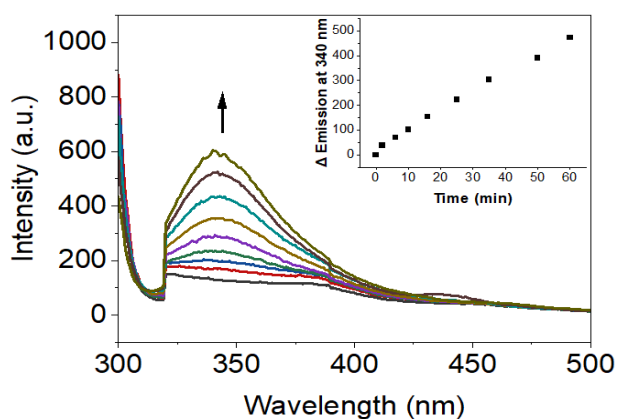
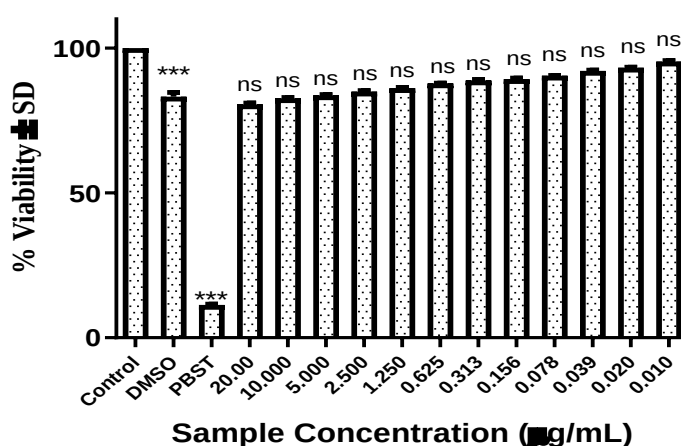


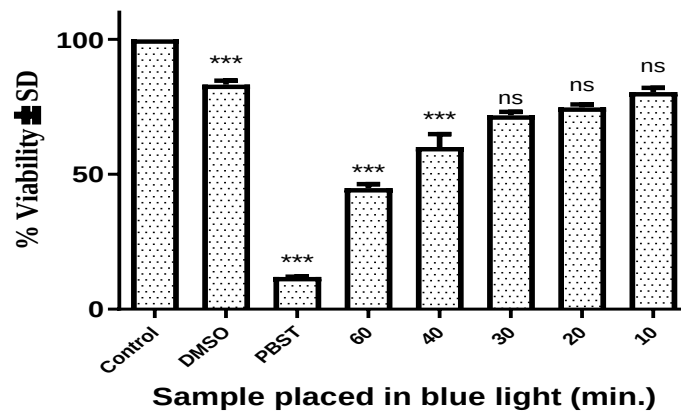
Figure 4.32 Fluorescence studies of **4** in Methanol Water mixture (Inset) Change in intensity at 340 nm with respect to time.

4.4.4 Evaluation of cytotoxicity of compound using Human Prostate Cancer Cells (DU145), Cervical Cancer Cells (HeLa cells) and Breast Cancer Cells (MCF-7) in vitro

Human prostate cancer cells (DU145), cervical cancer (HeLa cells), and breast cancer cells (MCF-7) were used to study the safety profiles of **4** in methanol before NO release and during NO release at different concentrations 0.010–20.00 $\mu\text{g/mL}$ (Fig. 4.33 (a), Fig. 4.34 (a), and Fig. 4.35 (a)) respectively. The compound can be used safely for medicinal purposes because the MTT assay demonstrates that it is not harmful to cells even at concentrations of up to 20.00 $\mu\text{g/mL}$. This prompted us to perform preliminary studies on the killing of cancer cells caused by light. Therefore, in order to estimate cell mortality following NO release, DU145, HeLa, and MCF-7 cells were treated with **4** and exposed to blue light for 10, 20, 30, 40, and 60 minutes. This experiment suggests that the observed cancer cell mortality is caused by the light-induced release of NO. The results show that the compound becomes toxic to the DU145, HeLa, and MCF-7 cancer cells after 60 minutes of irradiation with blue light, resulting in 55% (Fig. 4.33 (b)), 50% (Fig. 4.34 (b)), and 48% (Fig. 4.35 (b)) of cell death, respectively. The control experiments clearly show that the compound is toxic to the cells only in the presence of blue light after irradiation of more than 40 minutes.

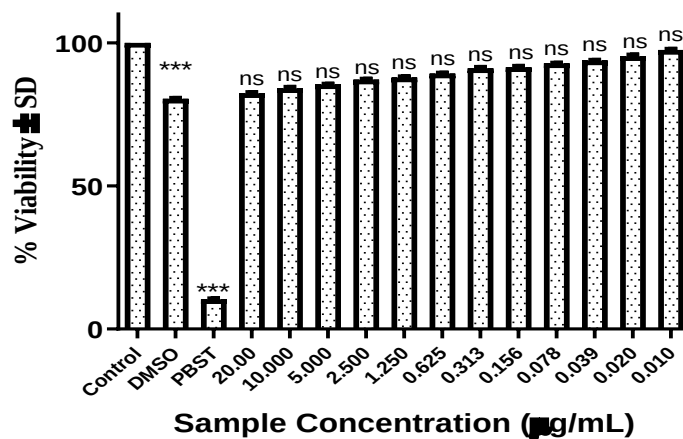


(a)

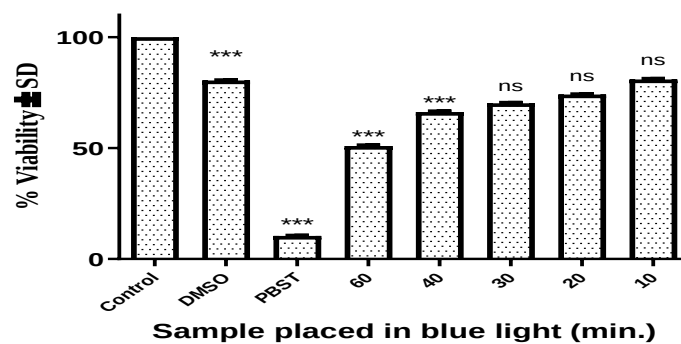


(b)

Figure 4.33 (a) & (b) Effect of Sample 4 on DU145 cells without blue light or with blue light. (***) $p < 0.001$, ns-nonsignificant).

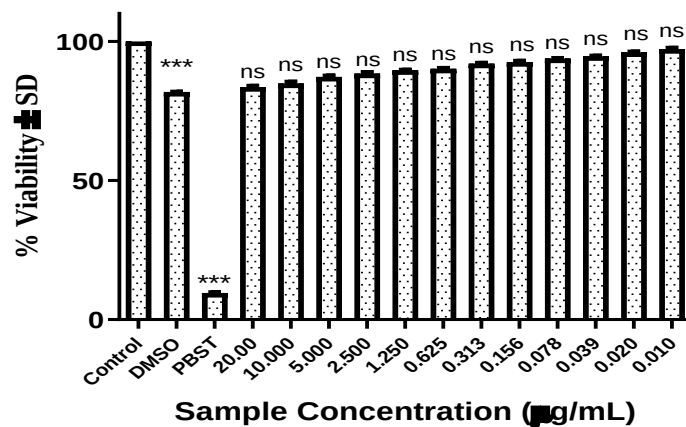


(a)

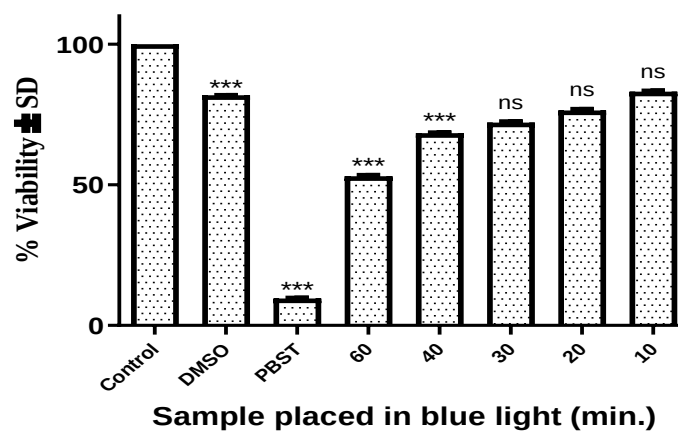


(b)

Figure 4.34 (a) & (b) Effect of Sample 4 on HeLa cells without blue light or with blue light. (***) $p < 0.001$, ns-nonsignificant).



(a)



(b)

Figure 4.35 (a) & (b) Effect of Sample 4 on MCF-7 cells without blue light or with blue light. (***) $p < 0.001$, ns-nonsignificant).

4.5 Synthesis, Qualitative analysis and Biological studies of NO donor N²,N⁶-bis(2-((4-nitro-3-(trifluoromethyl)phenyl)amino)ethyl)pyridine-2,6-dicarboxamide (Compound 5)

Synthesis of compound **5** was done by following scheme 3.5 and the compound was analysed using mass and NMR spectroscopy and the results have been compiled.

4.5.1 Synthesis of compound 5

Compound **1** (250 mg, 1.003 mmol) was dissolved in dry THF (70 mL) to this solution dry Et₃N (515 μ L, 3.7 mmol) was added at 0 °C with continuous stirring. After 10 min., isophthaloyl dichloride (102.3 mg, 0.501 mmol) was added dropwise to the reaction mixture and stirred at rt for 8 hrs. The solution was filtered to remove the Et₃N.HCl salt and evaporated under vacuum. The obtained solid was washed with distilled H₂O and the final product was obtained as yellow color solid. Yield: 44 % (150 mg). ESI-MS *m/z*: calculated 629; found [M + H]⁺ 630.14 (Fig. A.5 (a)) HR-MS: [M + H]⁺ calculated; 630.1536 observed; 630.1541. (Fig. A.5 (b)) ¹H NMR (500 MHz, DMSO-*D*₆) δ 9.41-8.21 (q, 2H), 8.18-8.15 (m, 2H-NH), 8.19 (t, *J* = 15 Hz, 1H), 8.06-7.70 (q, 2H), 7.69-6.95 (q, 2H), 6.93 (d, *J* = 10 Hz, 2H), 3.57-3.54 (m, 2H-NH), 3.53-3.48 (m, 4H), 3.47-3.31 (m, 4H). (Fig. B.5 (a)). ¹³C NMR (125 MHz, DMSO-*D*₆) δ 163.5, 153.0, 146.3, 139.4, 133.7, 129.5, 124.1, 41.5, 39.9, 39.7, 39.6, 39.5, 39.4, 39.2, 39.0, 38.9, 38.0 (Fig. B.5 (b))

4.5.2 Qualitative Analysis

The compound was used for further NO releasing studies with the help of UV-Vis spectrophotometer.

4.5.2.1 UV-Vis Studies

The NO release behavior of **5** was observed using time-dependent UV-Vis studies. The absorbance at 379 nm decreases continuously in the presence of blue light (410 nm, 3W) (Fig. 4.36), but there is no change in absorbance in its absence. Light was found to photobleach **5** which is entirely compatible with the photochemical process that releases NO. The reduced absorbance in the visible area is caused by the potential production of phenoxy and NO radicals, which are non-toxic and weakly absorbing photoproducts (Scheme 4.1).

NO was released only under blue light irradiation, the UV-Vis spectrum did not alter in the absence of light and even after keeping under daylight for a prolonged time.

UV-Vis spectra of methanolic solution of **5** (30 μ M) was monitored at different time interval upon irradiation with blue light (410 nm, 3 W). The quartz cuvette was placed 1 cm away from the light source for the irradiation experiment. The NO release was determined by monitoring the decrease in absorbance (λ_{max}) at 379 nm.

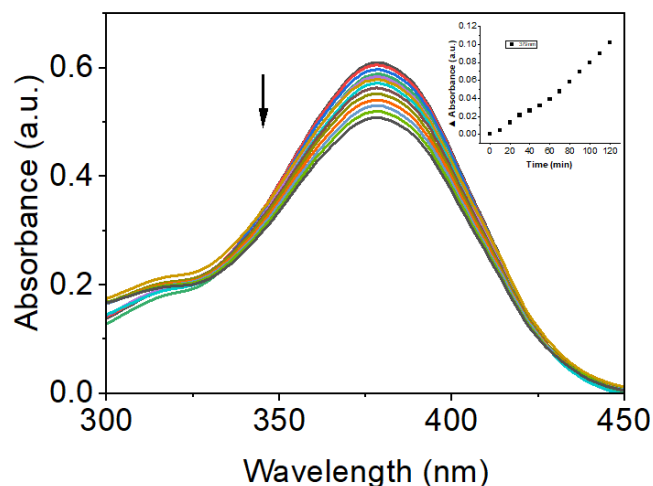


Figure 4.36 UV–Visible absorbance of Compound **5** monitored under blue (410 nm, 3 W) illumination over a 0–120 min interval, with an inset depicting the temporal absorbance changes at 379 nm.

4.5.2.2 Griess Assay:

Using the Griess assay with the standard protocol, the amount of NO released from **5** has been estimated [2]. The NO donor and Griess reagents were mixed and exposed to blue light (410 nm) for 4 hours, and the change in the UV-Vis absorbance was measured at different times. The Griess assay study states that the chromophoric azo-derivative production with a strong absorbance band in the range 460–493 nm and the color change are proof that NO was released from the donor NO molecules or materials.[3]

There was a color shift from pale yellow to pink and an increase in absorbance at 474 nm (Fig. 4.37). The release of NO from **5** is responsible for the formation of an azo dye in solution, as indicated by the new UV-Vis peak.

800 mg of Griess reagent were dissolved in 20 mL of 10 mM phosphate buffer to prepare the stock Griess reagent solution. A methanolic solution of **5** (52 μ M, 2.1 mL) was mixed with 100 μ L of this stock solution for the experiment, and the UV-Vis spectra was recorded at room temperature while exposed to light. The release of NO

from **5** following photolysis is indicated by the increase in UV-Vis absorbance at 474 nm.

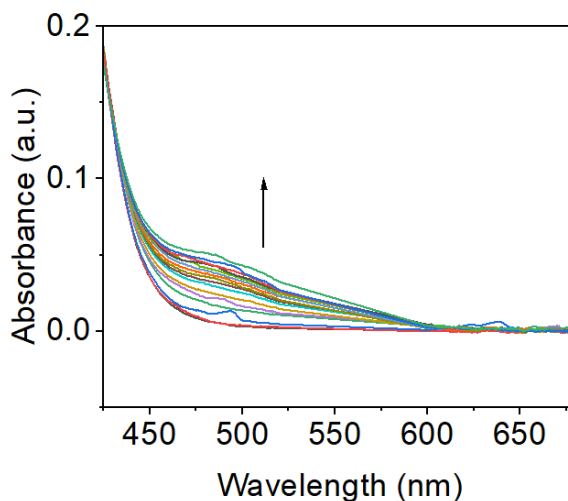


Figure 4.37 UV-Vis spectral changes of Compound **5** in the presence of Griess reagent over a 0-240 min period of irradiation with blue light (410 nm, 3 W).

Based on the calibration plot (Fig. 3.8), the amount of NO released was found to be $7.72\ \mu\text{M}$ which is more than that of **1** ($3.88\ \mu\text{M}$) and less than that of **2** ($11.81\ \mu\text{M}$) this implies that the amount of NO released is directly dependent on the number of NO photo-releasing units present in the molecule i.e. 1 in **1**, 2 in **5** and 3 in **2** and same is the order of NO released which is $\mathbf{1} < \mathbf{5} < \mathbf{2}$.

4.5.2.3 Effect of pH on NO release

To maintain the solution's pH, common buffers such as acetate (pH 5), phosphate (pH 7.4), and borate (pH 9) were employed. After being exposed to blue light for 120 minutes, the UV-Vis absorbance spectrum was used to track all three samples. To determine the rate of NO release, the absorbance change over time was tracked and plotted (Fig. 4.38).

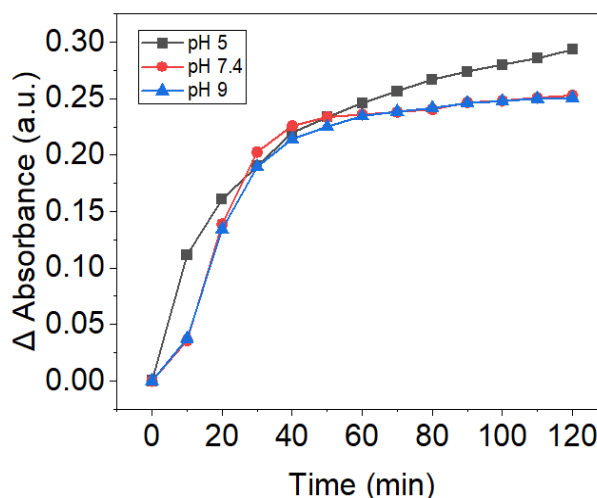
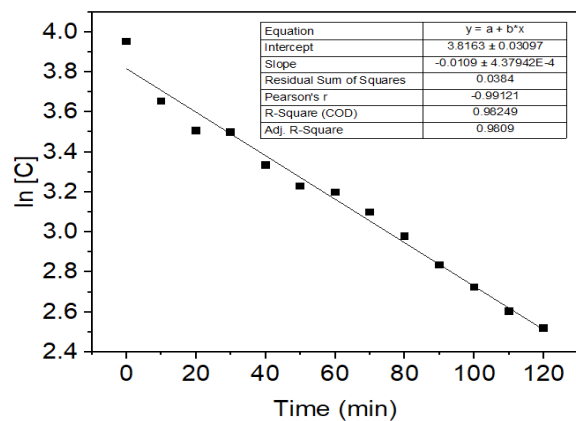


Figure 4.38 Change in absorbance of **5** (52 μM) with respect to time at different pH.

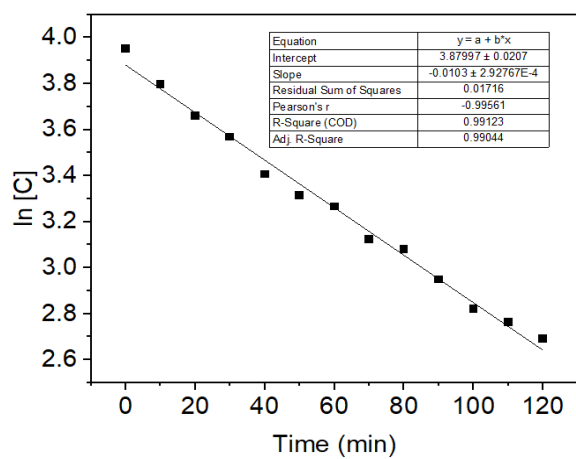
4.5.2.4 Calculation of rate constant

A graph was plotted between the natural logarithm of reactant concentration and time (Fig. 4.39) which gave a straight line, thus making it clear that the reaction follows first-order kinetic hence slope of the curve = Rate constant which was found to be 0.0016 min^{-1} which is greater than that of **1** (0.0015 min^{-1}) and less than that of **2** (0.00198 min^{-1}) that means **5** will release NO with a moderate speed due to the presence of two NO donors and the interaction of -OH and -NH intermolecular hydrogen bonding might be responsible for better NO releasing behaviour as compared to **1** and that of **2** is even better than **5** with $t_{1/2} = 433.12 \text{ min}$ and quantum yield (Φ) of 0.00056 [2] which is again an intermediate value between that of **1** (0.0003) and **2** (0.001) which in accordance with the above condition. The quantum yield and NO release behavior of all the synthesized NO donors were also compared with the previously reported NO donors (section 2.1.1) and it they were found to be slow and sustained, stable in dark NO-releasing molecules.

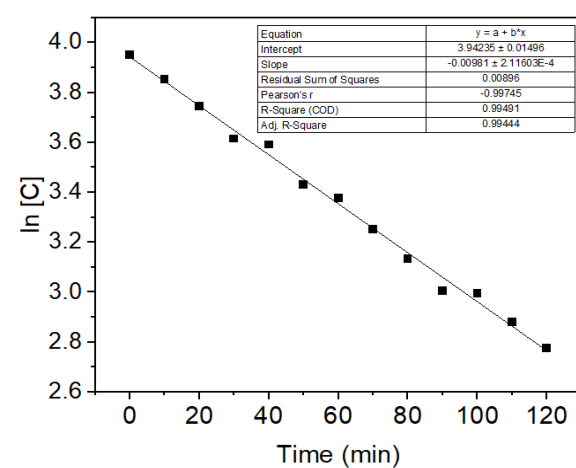
4.5.2.4.1 Graphs to calculate Rate constant



(a)



(b)



(c)

Figure 4.39 Graphs used to calculate rate constant of 5 at different pH (a) at pH 5.0 (b) at pH 7.4 (c) at pH 9.2

With the help of these graphs rate constant and half life has been calculated for the compound at different pH and has been presented in the table below.

Table 4.7: Rate constant (k_{NO}) and $t_{1/2}$ of **5** (52 μM) at different pH conditions.

pH	Rate constant (min^{-1})	Half lifetime $t_{1/2}$ (min)
5.0	0.0109	63.6
7.4	0.0103	67.3
9	0.0098	70.7

Table 4.7 illustrates how minor changes in the solution's pH caused differences in the NO-releasing activity. **5** may release NO more quickly at the malignant spot than at other cells since the rate of NO release, k_{NO} , is highest at an acidic pH of 5.0 and its $t_{1/2}$ is the lowest of all. While the data indicates that the release of NO is moderate at pH 7.4 and slowest at pH 9, respectively.

4.5.2.5 Effect of Concentration

To study the relationship between concentration and NO release rate, different concentrations of NO donor **5** (7.5 μM – 75 μM) were irradiated with blue light and the change in UV-Vis absorbance was monitored for 120 min with a time interval of 10 min (Fig. 4.40).

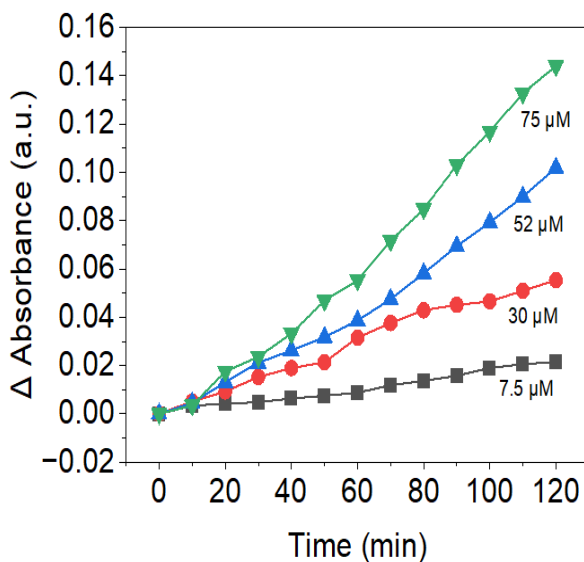
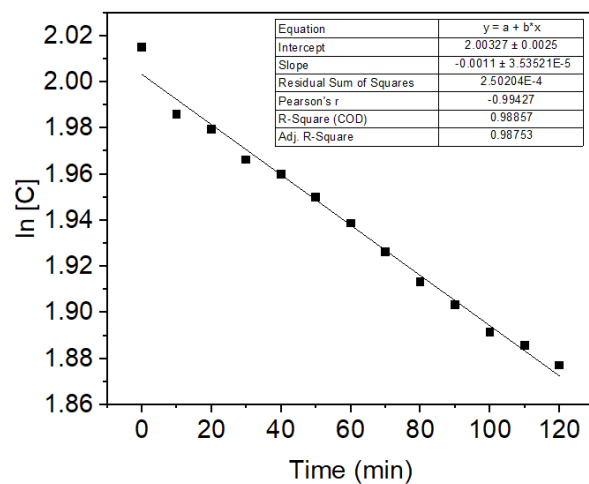


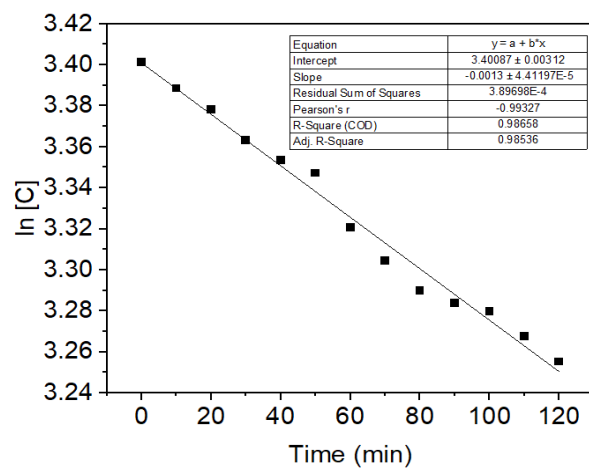
Figure 4.40 Temporal absorbance variation of Compound **5** at multiple concentrations under 410 nm (3 W) blue-light irradiation.

4.5.2.6 Calculation of rate constant

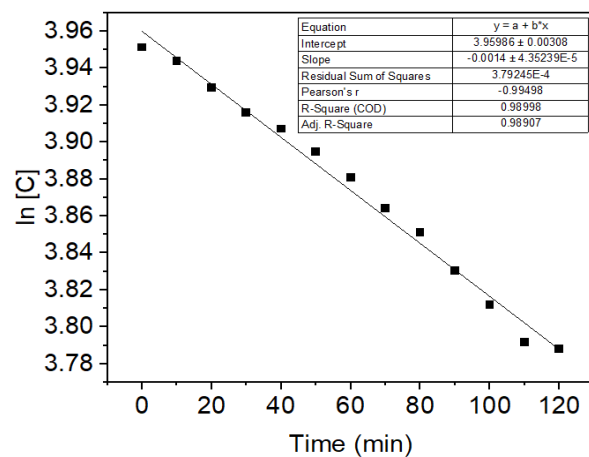
4.5.2.6.1 Graphs to calculate Rate constant



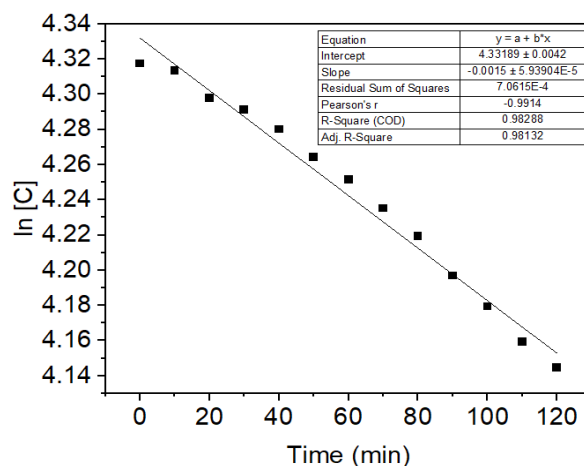
(a)



(b)



(c)



(d)

Figure 4.41 Graphs used to calculate rate constant of **5** at different concentrations (a) 7.5 μM (b) 30 μM (c) 52 μM (d) 75 μM

With the help of these graphs rate constant and half life has been calculated for the compound at different concentration and has been presented in the table below.

Table 4.8: Showing the values of k_{NO} , $t_{1/2}$ and Φ for different concentrations of the sample

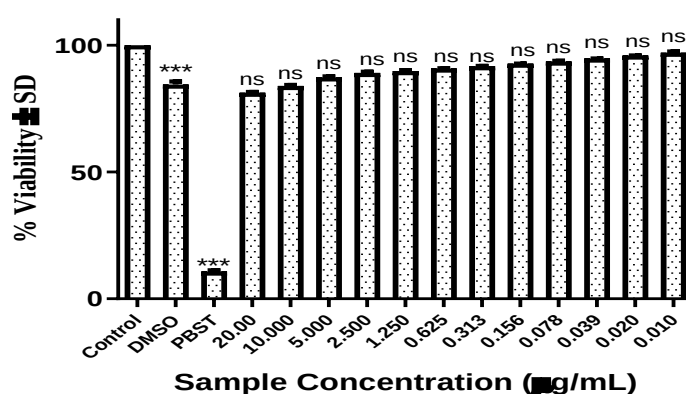
Concentration (μM)	Rate Constant (min^{-1})	Half lifetime (min)	Quantum Yield
7.5 μM	0.0011	630	4.14×10^{-5}
30 μM	0.0013	533	1.95×10^{-4}
52 μM	0.0014	495	3.65×10^{-4}
75 μM	0.0015	462	5.64×10^{-4}

As indicated in table 4.8, it is also noted that the rate constant k_{NO} rises as concentration does. As a result, the concentration-dependent study demonstrates that altering the NO donor's concentration can regulate or control the light-induced release of NO from **5**.

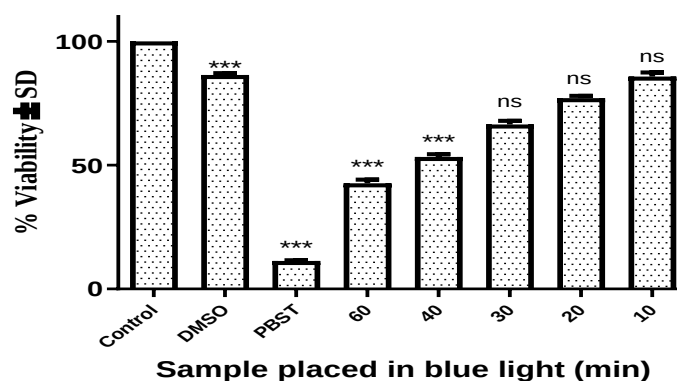
4.5.3 Evaluation of cytotoxicity of compound using Human Prostate Cancer Cells (DU145), Cervical Cancer Cells (HeLa cells) and Breast Cancer Cells (MCF-7) in vitro

Human prostate cancer cells (DU145), cervical cancer (HeLa cells), and breast cancer cells (MCF-7) were used to study the safety profiles of **5** in methanol before NO release and during NO release at different concentrations 0.010–20.00 $\mu\text{g/mL}$ (Fig. 4.42 (a), Fig. 4.43 (a), and Fig. 4.44 (a)) respectively. The compound can be used

safely for medicinal purposes because the MTT assay demonstrates that it is not harmful to cells even at concentrations of up to 20.00 $\mu\text{g/mL}$. This prompted us to perform preliminary studies on the killing of cancer cells caused by light. Therefore, in order to estimate cell mortality following NO release, DU145, HeLa, and MCF-7 cells were treated with 5 and exposed to blue light for 10, 20, 30, 40, and 60 minutes. This experiment suggests that the observed cancer cell mortality is caused by the light-induced release of NO. The results show that the compound becomes toxic to the DU145, HeLa, and MCF-7 cancer cells after 60 minutes of irradiation with blue light, resulting in 57% (Fig. 4.42 (b)), 53% (Fig. 4.43 (b)), and 52% (Fig. 4.44 (b)) of cell death, respectively. The control experiments clearly show that the compound is toxic to the cells only in the presence of blue light after irradiation of more than 40 minutes.

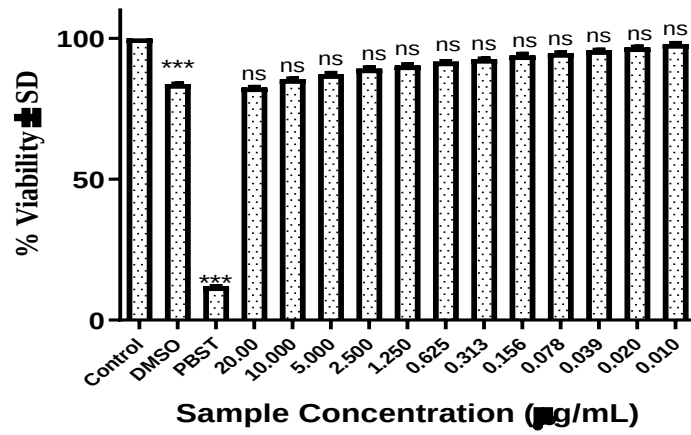


(a)

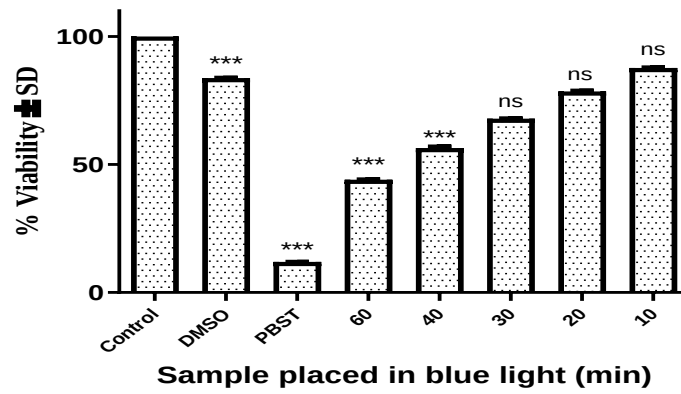


(b)

Figure 4.42 (a) & (b) Effect of Sample 5 on DU145 cells without blue light or with blue light. (***) $p < 0.001$, ns-nonsignificant).

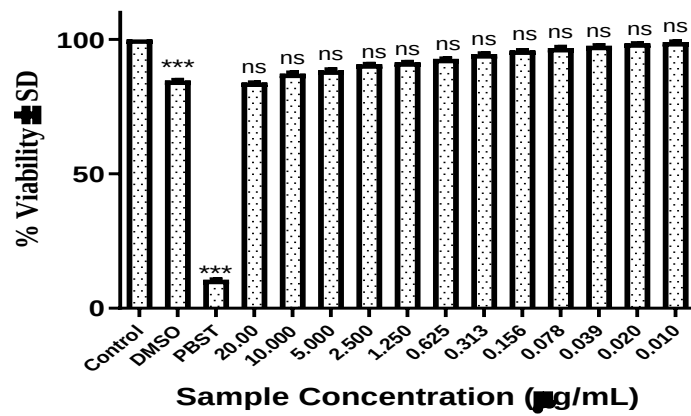


(a)

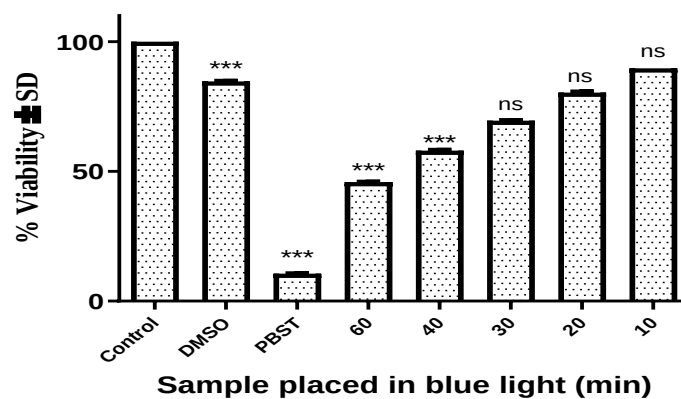


(b)

Figure 4.43 (a) & (b) Effect of Sample 5 on HeLa cells without blue light or with blue light. (***) $p < 0.001$, ns-nonsignificant).



(a)



(b)

Figure 4.44 (a) & (b) Effect of Sample 5 on MCF-7 cells without blue light or with blue light. (*** $p < 0.001$, ns-nonsignificant).

4.6 In silico studies

Potential treatments can be screened for efficacy against molecular targets using *in silico* approaches, which aids in choosing the most promising ones for additional *in vitro* and *in vivo* research. To determine physicochemical properties, lipophilicity, water solubility, pharmacokinetics, drug likeness and medicinal chemistry, *in silico* studies have been performed on the designed compounds and the results have been compiled.

Table 4.9: Physicochemical and structural properties of synthesized compounds

Compound	Formula	Molecular weight	Num. heavy atoms	Num. arom. heavy atoms	Fraction Csp3	Num. rotatable bonds	Molar Refractivity	TPSA
1	C ₉ H ₁₀ F ₃ N ₃ O ₂	249.19 g/mol	17	6	0.33	5	57.09	83.87 Å ²
2	C ₃₆ H ₃₀ F ₉ N ₉ O ₉	903.66 g/mol	63	24	0.25	24	207.80	260.85 Å ²
3	C ₂₁ H ₂₁ F ₃ N ₄ O ₄ S	482.48 g/mol	33	16	0.24	9	121.51	115.64 Å ²
4	C ₁₉ H ₁₆ F ₃ N ₃ O ₄ S	439.41 g/mol	30	16	0.16	8	107.30	112.40 Å ²
5	C ₂₅ H ₂₁ F ₆ N ₇ O ₆	629.47 g/mol	44	18	0.24	16	145.14	186.79 Å ²

Table 4.9 depicts the values for various physicochemical properties. According to Ghose, for a molecule to possess a good absorption and oral bioavailability, its molar refractivity value should be between 40 and 130. Hence the compounds, **1**, **3** and **4** are predicted to possess strong oral bioavailability and intestinal absorption. Whereas the compounds **2** and **5** can be absorbed by alternative route.

According to Lipinski's rule of five, a molecule should have the TPSA (topological polar surface area) value to be ≤ 140 Å² to have better pharmacological flexibility and permeability. Hence the compounds **1**, **3** and **4** are predicted to show more permeability as compared to the compounds **2** and **5** (table 4.9).

Table 4.10: Comparison of computational lipophilicity (log P) predictions across multiple predictive models

Compound	Log $P_{o/w}$ (iLOGP)	Log $P_{o/w}$ (XL OGP3)	Log $P_{o/w}$ (WL OGP)	Log $P_{o/w}$ (ML OGP)	Log $P_{o/w}$ (SIL ICOS-IT)	Consensus Log $P_{o/w}$
1	1.33	1.68	2.95	1.15	-0.03	1.41
2	2.92	6.93	9.88	2.55	1.32	4.72
3	2.65	4.65	6.27	2.47	1.18	3.44
4	2.13	4.53	6.20	3.37	1.45	3.54
5	2.69	4.86	6.54	1.66	0.82	3.31

Table 4.10 depicts the log P values of the synthesized compounds.

According to Lipinski's rule of five, a compound should have IlogP values less than five to possess great oral absorption qualities and better interaction with their biological targets and as shown in table 4.10 the IlogP values are in the range of 1.33-2.92, hence all the molecules can be predicted to have good oral absorption and interaction with their biological targets (Table 4.10).

Table 4.11: Comparison of predicted aqueous solubility (Log S) using Log S (ESOL) and Log S (Ali)

Compound	Log S (ESOL)	Solubility	Class	Log S (Ali)	Solubility	Class
1	-2.37	1.05e ⁺⁰⁰ mg/ml ; 4.22e ⁻⁰³ mol/l	Soluble	-3.06	2.19e ⁻⁰¹ mg/ml ; 8.79e ⁻⁰⁴ mol/l	Soluble
2	-8.51	2.82e ⁻⁰⁶ mg/ml ; 3.12e ⁻⁰⁹ mol/l	Poorly soluble	-12.22	5.44e ⁻¹⁰ mg/ml ; 6.02e ⁻¹³ mol/l	Insoluble
3	-5.53	1.44e ⁻⁰³ mg/ml ; 2.98e ⁻⁰⁶ mol/l	Moderately soluble	-6.80	7.56e ⁻⁰⁵ mg/ml ; 1.57e ⁻⁰⁷ mol/l	Poorly soluble
4	-5.28	2.28e ⁻⁰³ mg/ml ; 5.19e ⁻⁰⁶ mol/l	Moderately Soluble	-6.61	1.07e ⁻⁰⁴ mg/ml ; 2.44e ⁻⁰⁷ mol/l	Poorly soluble
5	-6.05	5.59e ⁻⁰⁴ mg/ml ; 8.89e ⁻⁰⁷ mol/l	Poorly soluble	-8.52	1.91e ⁻⁰⁶ mg/ml ; 3.04e ⁻⁰⁹ mol/l	Poorly soluble

Table 4.11 depicts the values of water solubility of all the compounds.

Amongst all the compounds, **1** has been predicted to be as soluble, **2** and **5** have been predicted to be poorly soluble and **3** and **4** have been predicted to be moderately soluble in water (Table 4.11).

Table 4.12: *In Silico* pharmacokinetic profiling: ADME and dermal permeation of synthesized compounds

Compound	GI absorption	BBB permeant	P-gp substrate	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2D6 inhibitor	CYP3A4 inhibitor	Log K_p (skin permeation)
1	High	No	No	Yes	No	No	No	-6.63 cm/s
2	Low	No	No	No	No	No	No	-6.89 cm/s
3	Low	No	No	No	Yes	Yes	Yes	-5.94 cm/s
4	Low	No	No	No	Yes	No	Yes	-5.76 cm/s
5	Low	No	No	No	Yes	Yes	Yes	-6.69 cm/s

Table 4.12 depicts the Pharmacokinetics properties of all compounds.

All compounds have been predicted to be non-substrate of P-glycoprotein, which reduced the chances of resistance as per the data provided in table 4.12. As the compound **1** showed high GI absorption so it can be formulated for oral intake. However, compounds **2**, **3**, **5** and **4** showed low GI absorption and, thus can be ingested via alternate route in future studies. Along with this all the 5 compounds were predicted as impermeant to the blood brain barrier.

Table 4.13: Drug-Likeness compliance and predicted oral bioavailability of synthesized compounds

Compound	Lipinski	Ghose	Veber	Egan	Bioavailability Score
1	Yes; 0 violation	Yes	Yes	Yes	0.55
2	No; 3 violations: MW>500, NorO>10,	No; 4 violations: MW>480, WLOGP>5.6,	No; 2 violations: Rotors>10, TPSA>140	No; 2 violations: WLOGP>5.88, TPSA>131.6	0.17

	NH or OH > 5	MR > 130, #atoms > 70			
3	Yes; 0 violation	No; 2 violations: WLOGP > 5.6, MW > 480,	Yes	No; 1 violation: WLOGP > 5.88	0.55
4	Yes; 0 violation:	No; 1 violation: WLOGP > 5.6	Yes	No; 1 violation: WLOGP > 5.88	0.55
5	No; 2 violations: MW > 500, NorO > 10,	No; 3 violations: MW > 480, WLOGP > 5.6, MR > 130,	No; 2 violations: Rotors > 10, TPSA > 140	No; 2 violations: WLOGP > 5.88, TPSA > 131.6	0.17

Table 4.13 depicts the Druglikeness of all the compounds on the basis of various rules proposed by different scientists and bioavailability score.

The drug likeness predictions showed that all the 5 compounds can be used as good drug candidates for further evaluation, and derivatization (table 4.13).

Table 4.14: PAINS Alerts, Lead-Likeness Violations, and Synthetic Accessibility Scores of synthesized compounds

Compound	PAINS	Leadlikeness	Synthetic accessibility
1	0 alert	No; 1 violation: MW < 250	2.32
2	0 alert	No; 3 violations: MW > 350, Rotors > 7, XLOGP3 > 3.5	4.76
3	0 alert	No; 3 violations: MW > 350, Rotors > 7, XLOGP3 > 3.5	3.40
4	0 alert	No; 3 violations: MW > 350, Rotors > 7, XLOGP3 > 3.5	3.09
5	0 alert	No; 3 violations: MW > 350, Rotors > 7, XLOGP3 > 3.5	3.57

Table 4.14 depicts the Medicinal Chemistry of all the compounds on the basis of PAINS alert, Leadlikeness and Synthetic accessibility.

All the compounds showed 0 PAINS (pan assay interference compounds) alert and thus have been predicted to not interfere with the biological screening methods.

In Silico prediction studies have been performed on the synthesized compounds to determine physicochemical properties, lipophilicity, water solubility, pharmacokinetics, drug likeness and medicinal chemistry. These predictions were performed to ascertain the drug likeness and lead likeness so that these compounds could be analyzed further for therapeutic evaluation.

The molar refractivity value (Table 4.9) predicted that the compounds, **1**, **3** and **4** have good intestinal absorption and oral bioavailability. Whereas the compounds **2** and **5** can be absorbed by alternative route along with that the compounds **1**, **3** and **4** are predicted to show more permeability as compared to the compounds **2** and **5**, as predicted by TPSA (topological polar surface area) (Table 4.9).

The synthesized compounds have been predicted to possess excellent oral absorption properties, according to the estimated values of *I*logP (Table 4.10).

Amongst the above synthesized compound, **1** was predicted to be as soluble, **2** and **5** were predicted to be poorly soluble and **3** and **4** were predicted to be moderately soluble in water (Table 4.11). All compounds were predicted to be non-substrate of P-glycoprotein, which reduced the chances of resistance as per the data provided in Table 4.12. As the compound **1** showed high GI absorption so can be formulated for oral intake. However, compounds **2**, **3**, **5** and **4** showed low GI absorption and, thus can be ingested via alternate route in future studies. Along with this all the 5 compounds were predicted as impermeant to the blood brain barrier (Table 4.12).

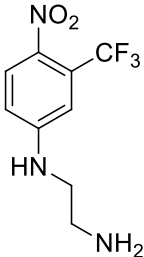
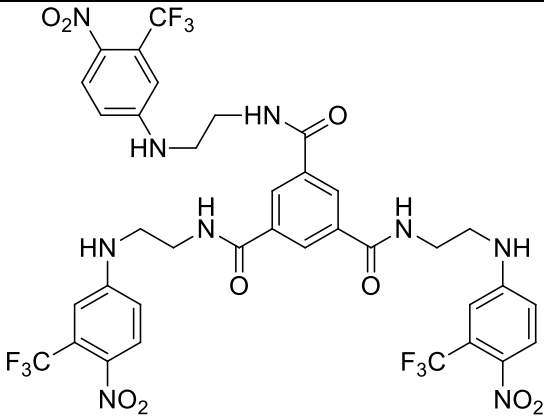
In addition, the drug likeness predictions showed that all the 5 compounds can be used as good drug candidates for further evaluation, and derivatization (Table 4.13). As predicted, all the compounds showed 0 PAINS alert and would not interfere with the biological screening methods (Table 4.14). Thus, most of the compounds showed drug likeness, lead likeness, good GI absorption and synthetic accessibility. These compounds will be analyzed by docking studies and on the basis of their binding affinity and will be sent for further in vitro evaluations.

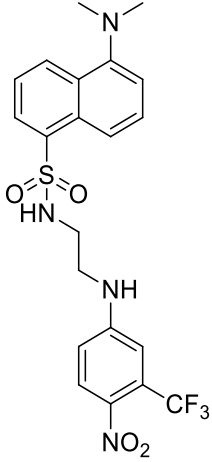
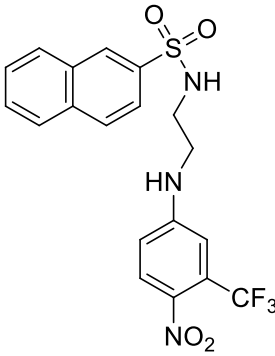
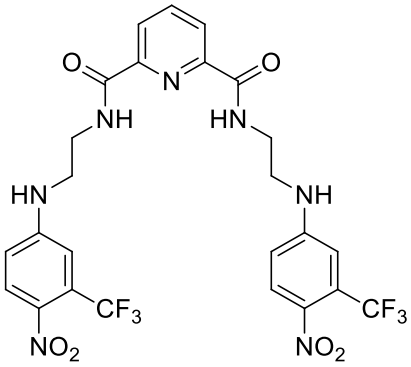
4.7 Elucidation of Anti-cancer Activity

4.7.1 Docking Studies with σ_1 receptor

By chemically linking a nitrate-based NO donor to σ receptor ligands, dual-function compounds that hold promise for targeted cancer therapies have been created [9]. Thus to prove the binding affinity of the designed compounds with σ_1 receptor (PDB id - 5HK1) docking studies (Fig. 4.45 – Fig. 4.49) have been performed and are tabulated as follows.

Table 4.15: Demonstrates the interaction strength between the compounds and the σ_1 receptor (PDB ID: 5HK1)

Compound	Structure	Docking Score
1		-91.29
2		-164.21

3		-89.908
4		-141.006
5		-175.89

As per the docking score values shown in table 4.15, **5** shows the highest affinity to bind with σ_1 receptor (PDB id - 5HK1) with a docking score value of **-175.89** followed by **2** with a value of **-164.21**, whereas, **3** shows the lowest binding affinity with the σ_1 receptor with a docking score of **-89.908**.

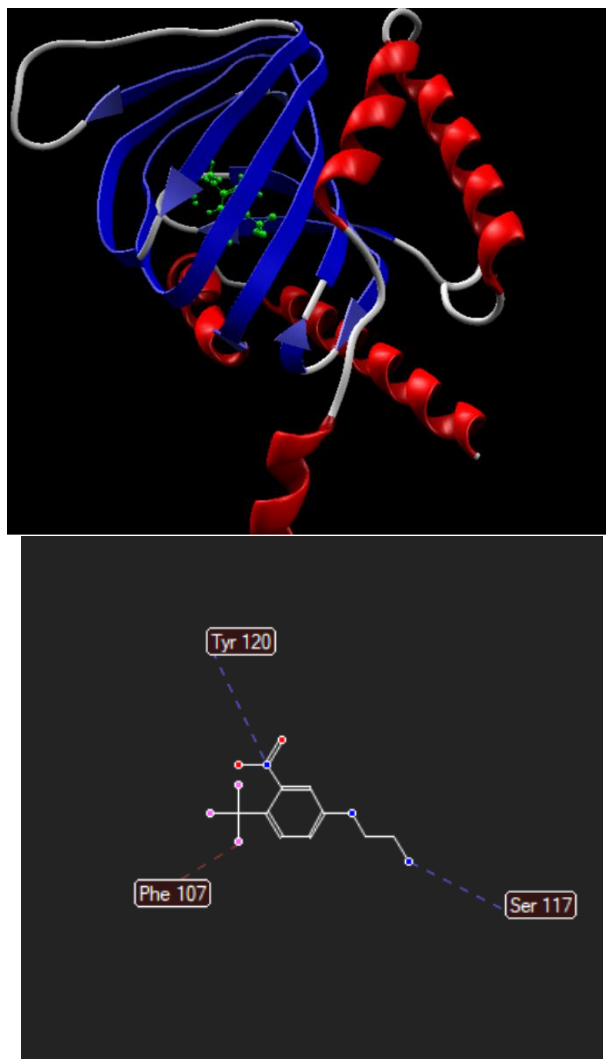


Figure 4.45 Docking of compound **1** with sigma-1 receptor protein (PDB:5HK1).

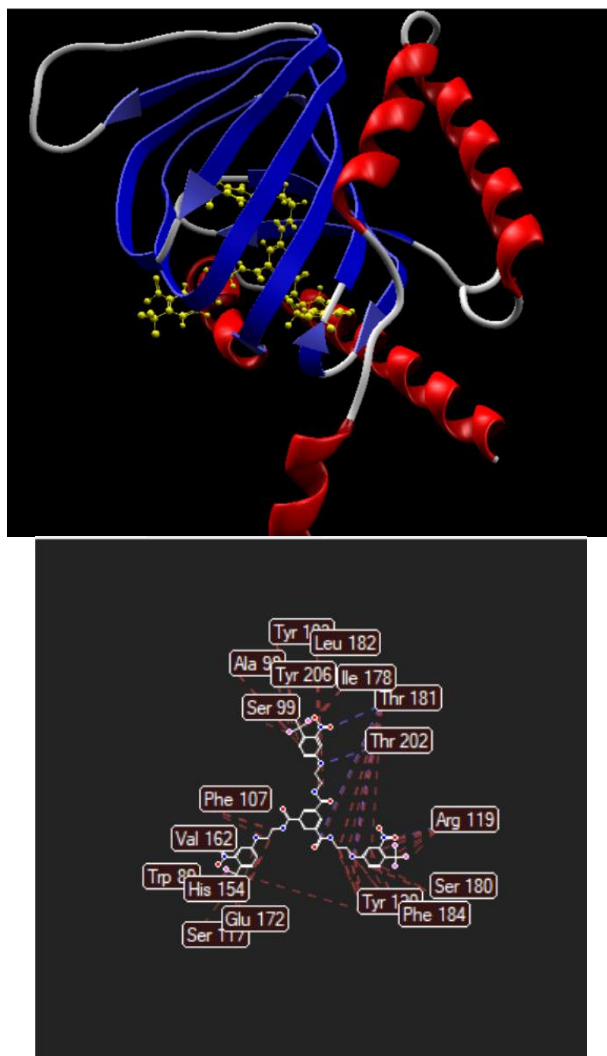


Figure 4.46 Docking of compound 2 with sigma-1 receptor protein (PDB:5HK1).

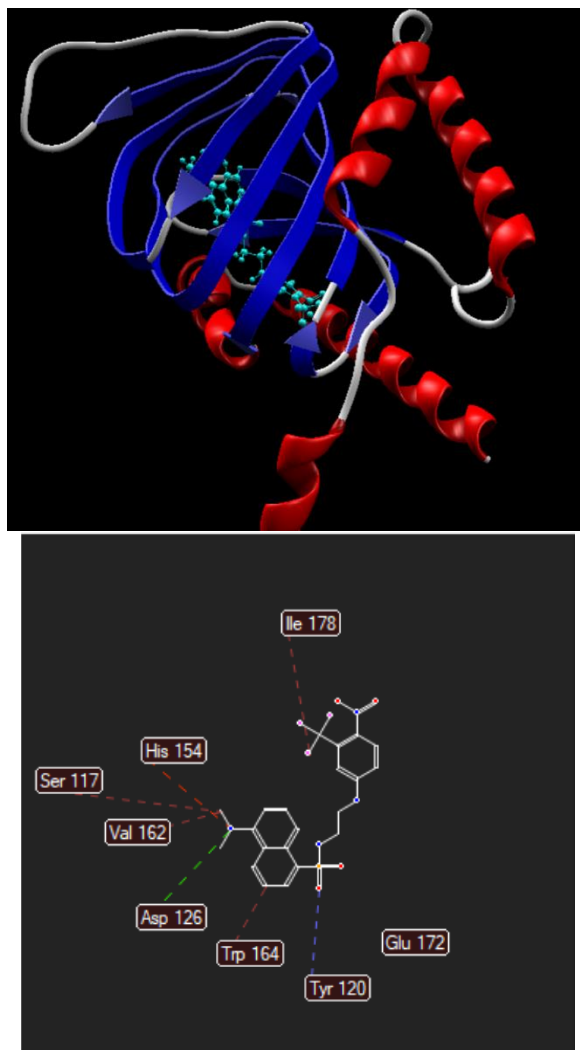


Figure 4.47 Docking of compound **3** with sigma-1 receptor protein (PDB:5HK1).

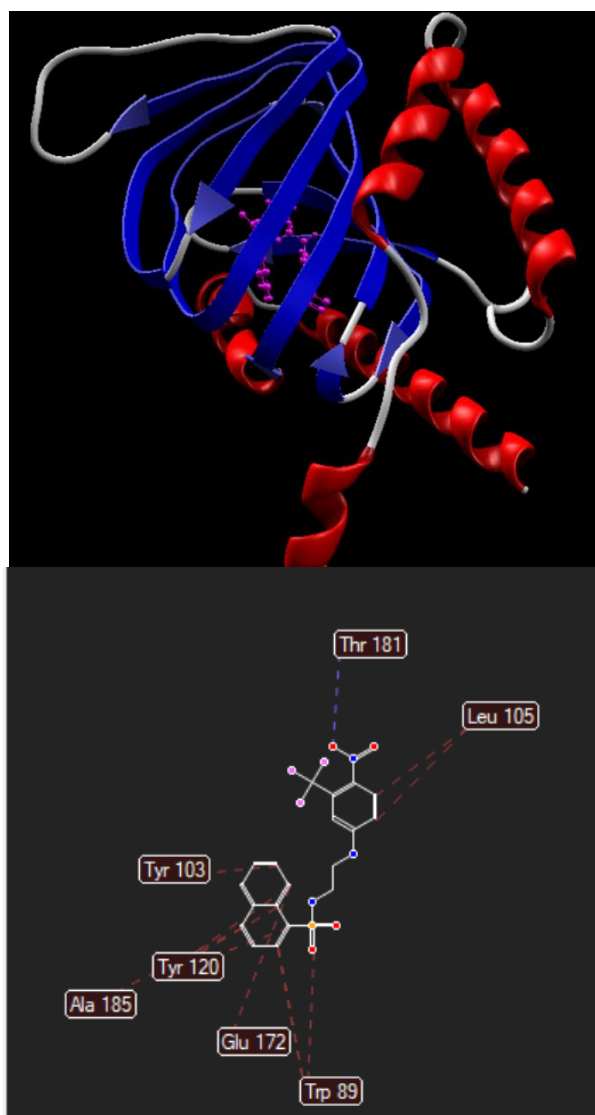


Figure 4.48 Docking of compound **4** with sigma-1 receptor protein (PDB:5HK1).

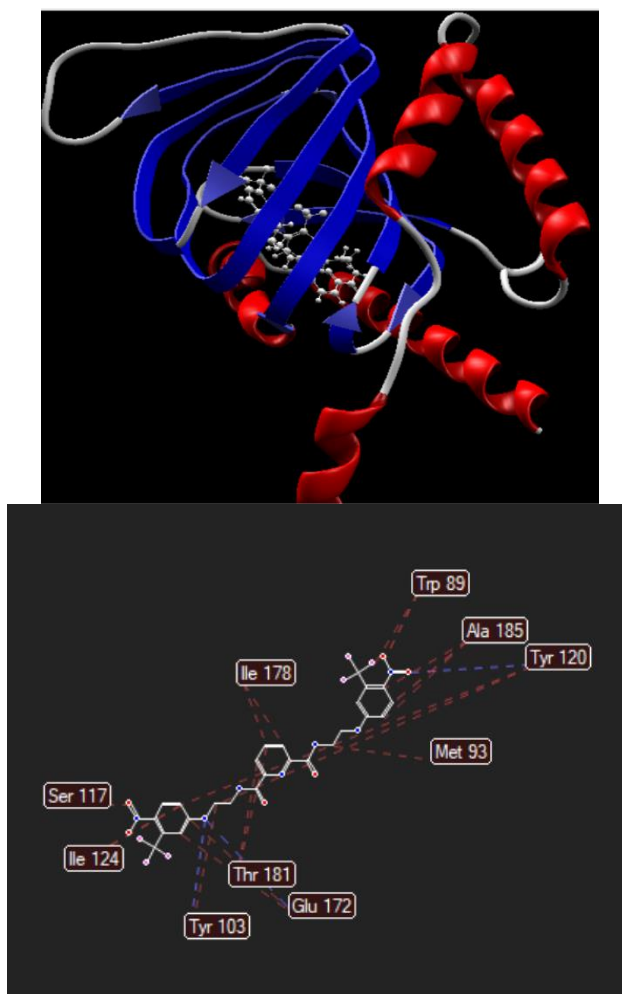


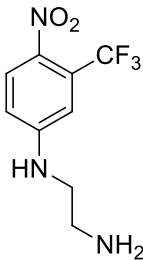
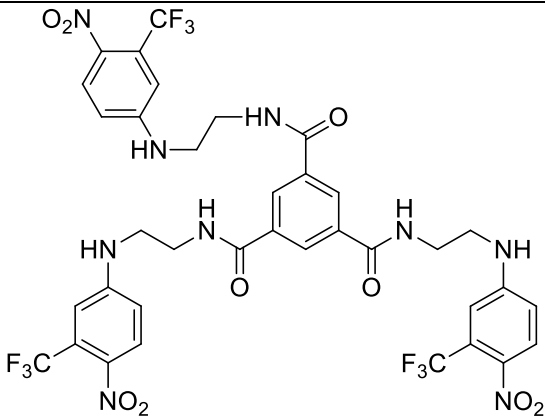
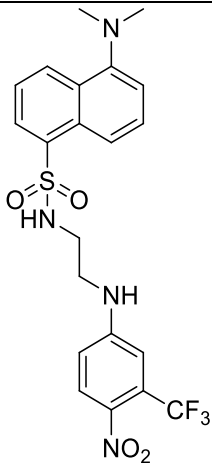
Figure 4.49 Docking of compound 5 with sigma-1 receptor protein (PDB:5HK1).

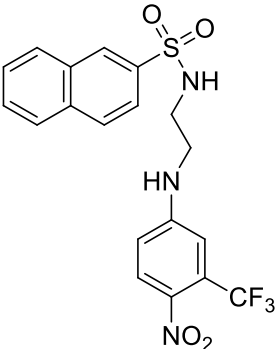
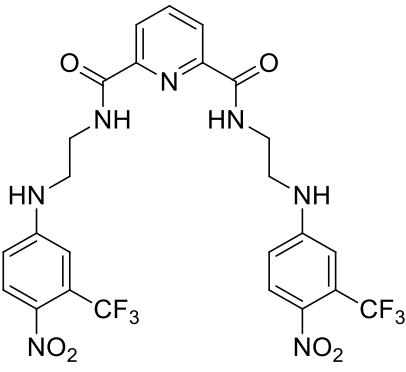
*Blue-Hbond interaction, Green- electrostatic interaction, Red- steric interaction.

4.7.2 Docking Studies of the synthesized compounds with BSA

Evidences show that bovine serum albumin (BSA) plays a vital role in the internalization process and thus it can help medications move through endocytic pathways into cancer cells [10]. Additionally, because albumin is the predominant serum component in the culture medium (in cell culture experiments - such as when serum accounts for 10% of the medium) drug binding to serum components can substantially diminish internalization and weaken cytotoxic efficacy. Thus to prove the binding affinity of the designed compounds with BSA (PDB id - 3VO3) docking studies (Fig. 4.50 – Fig. 4.54) have been performed and are tabulated as follows.

Table 4.16: Demonstrates the interaction strength between the compounds and BSA (PDB ID: 3VO3)

Compound	Structure	Docking Score
1		-99.74
2		-218.57
3		-144.49

4		-140.09
5		-182.78

As per the docking score values shown in table 4.16, **2** shows the highest affinity to bind with BSA (PDB id - 3VO3) with a docking score value of **-218.57** followed by **5** with a value of **-182.78**, whereas, **1** shows the lowest binding affinity with BSA with a docking score of **-99.74**.

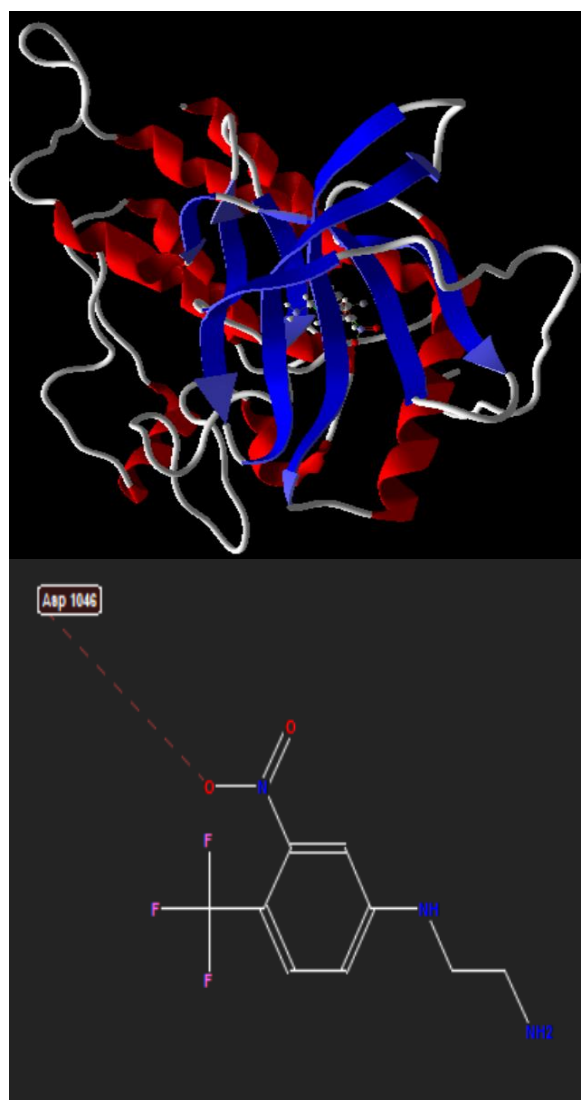


Figure 4.50 Docking of compound **1** with Bovine Serum albumin (3VO3).

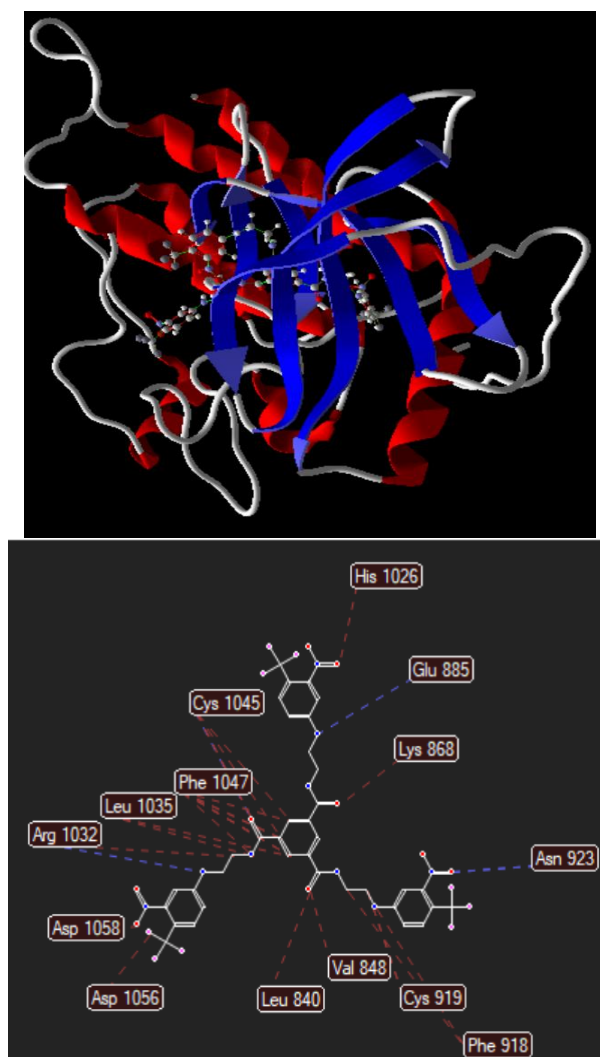


Figure 4.51 Docking of compound 2 with Bovine Serum albumin (3VO3).

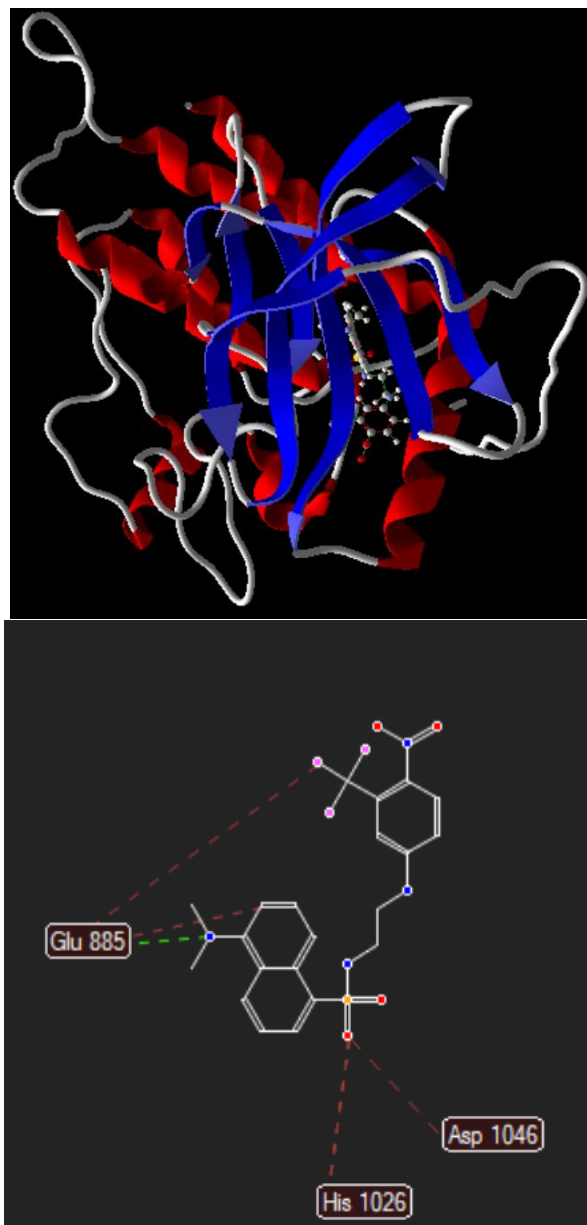


Figure 4.52 Docking of compound 3 with Bovine Serum albumin (3VO3).

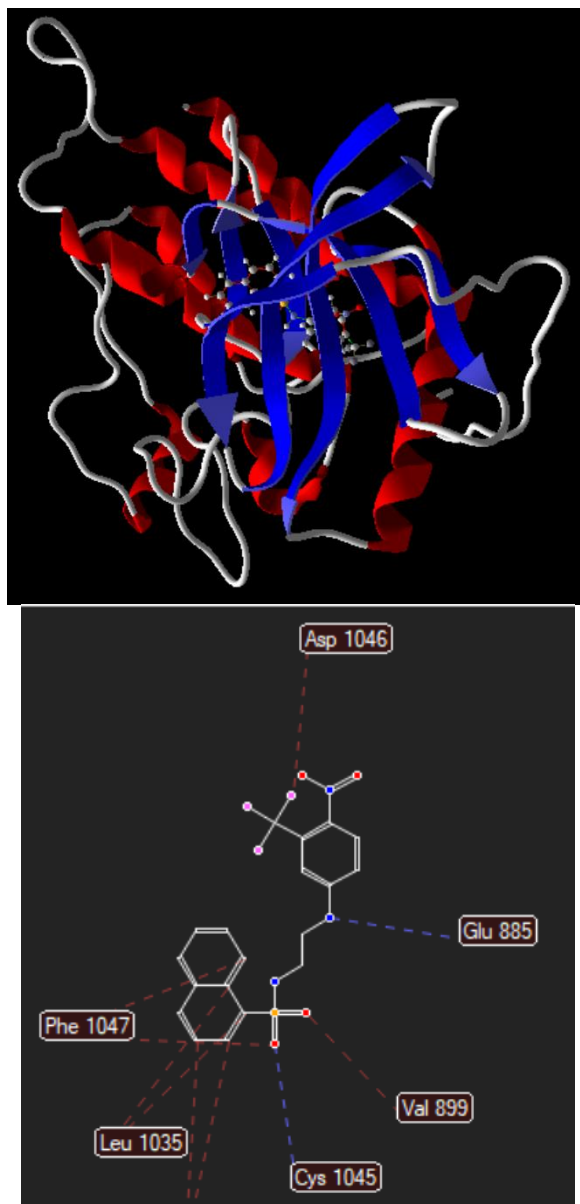


Figure 4.53 Docking of compound 4 with Bovine Serum albumin (3VO3).

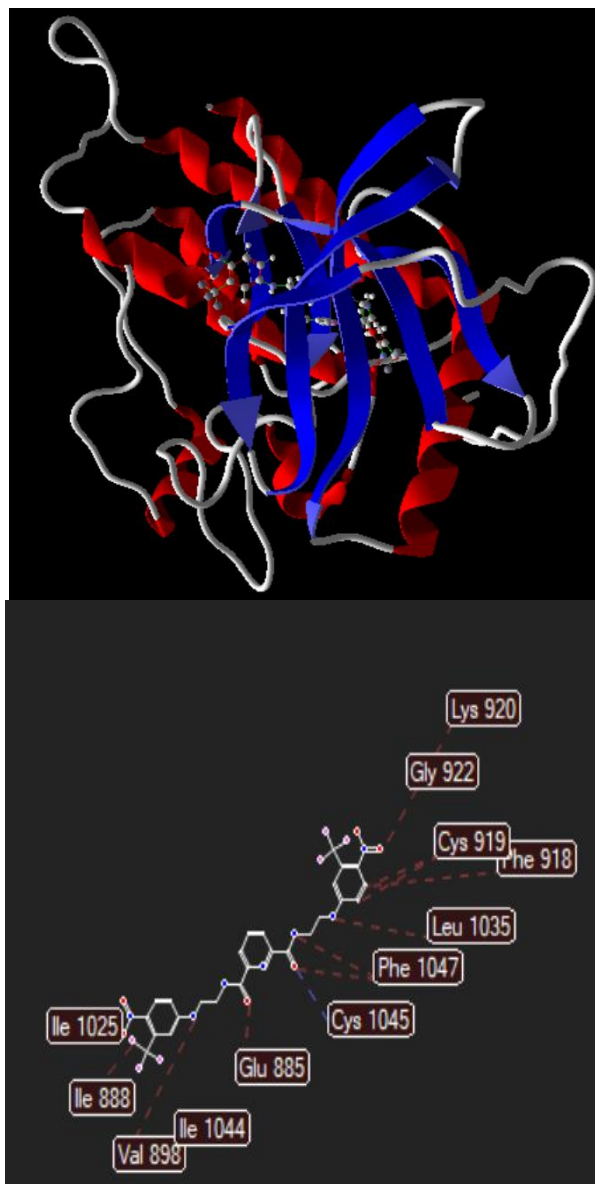


Figure 4.54 Docking of compound 5 with Bovine Serum albumin (3VO3).

*Blue-H bond interaction, Green- electrostatic interaction, Red- steric interaction.

The synthesized compounds **1-5** were subjected to comparative *in silico* docking studies on sigma-1 receptor protein (Fig. 4.45 – Fig. 4.49), which were evaluated in prostate cancer as sigma-1 receptor inhibitors and as bovine serum albumin binders. The docking scores have been mentioned in tables 4.15 and 4.16 for both the proteins respectively. It was observed that **2** and **5** showed maximum binding affinity on sigma receptors with docking scores -164.21 and -175.89 respectively, and **2** and **5** showed maximum binding affinity on bovine serum albumin with docking scores -218.57 and -182.78 respectively at zero deviation pose. A molecule's binding affinity is

determined by its negative docking score; a higher negative score indicates a higher binding affinity. In figures 4.45 - 4.54, the docking of the synthesised molecules have been shown along with their interactions with various amino acids at the active site of the protein. The blue lines in the images show the H bonding interactions, Green lines- electrostatic and Red lines- steric interactions. The stability of the ligand-receptor complex has been significantly enhanced by these interactions. Based on the *in silico* results, **2** and **5** were found to possess the significant binding affinity/docking score on selected sigma-1 receptor protein and **2** showed the maximum in vitro cytotoxic effect (60%) as per the results obtained cytotoxicity studies done on various cancer cell lines including Human Prostate Cancer Cells (DU145), and may act as its inhibitor in prostate cancer. In case of prediction of binding to Bovine Serum Albumin, **2** and **5** showed best binding affinity, and **5** showed highest binding constant in fluorescence studies. This proved our hypothesis, as *in silico* predictions were in alignment with the in vitro results obtained (Fig. 4.55 – Fig. 4.59). Thus, **2** and **5** could get better internalised through endocytic pathways also, to facilitate cytotoxic effect.

4.8 Binding studies with BSA

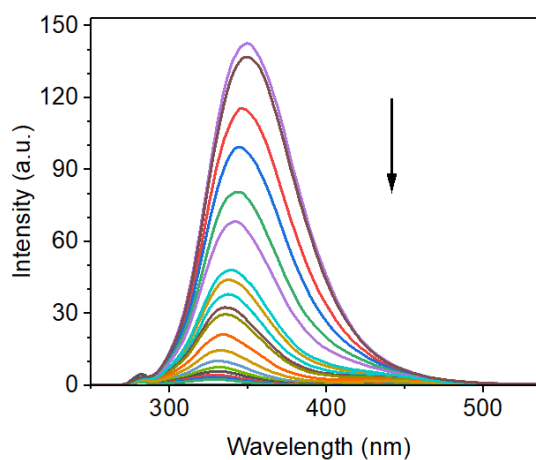


Figure 4.55 Fluorescence emission profiles of 10 μM bovine serum albumin (excited at 280 nm) were recorded while gradually increasing the concentration of compound **1** from 0 to 38.09 μM .

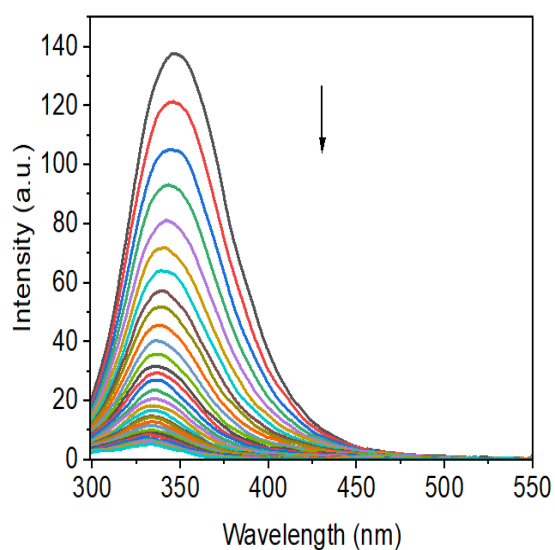


Figure 4.56 Fluorescence emission profiles of 10 μM bovine serum albumin (excited at 280 nm) were recorded while gradually increasing the concentration of compound **2** from 0 μM – 16.59 μM .

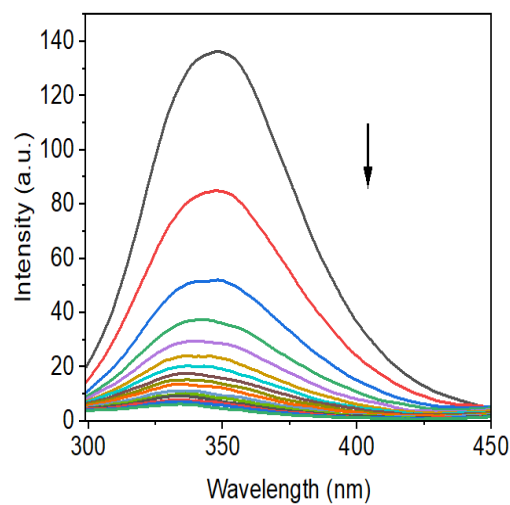


Figure 4.57 Fluorescence emission profiles of 10 μM bovine serum albumin (excited at 280 nm) were recorded while gradually increasing the concentration of compound **3** from 0 μM – 15.54 μM .

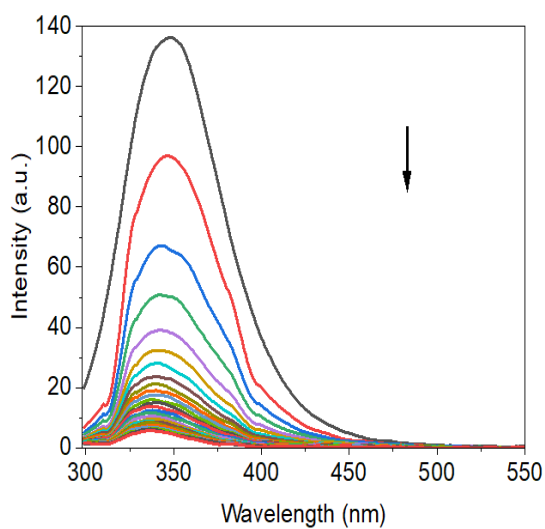


Figure 4.58 Fluorescence emission profiles of 10 μM bovine serum albumin (excited at 280 nm) were recorded while gradually increasing the concentration of compound **4** from 0 μM – 28.37 μM .

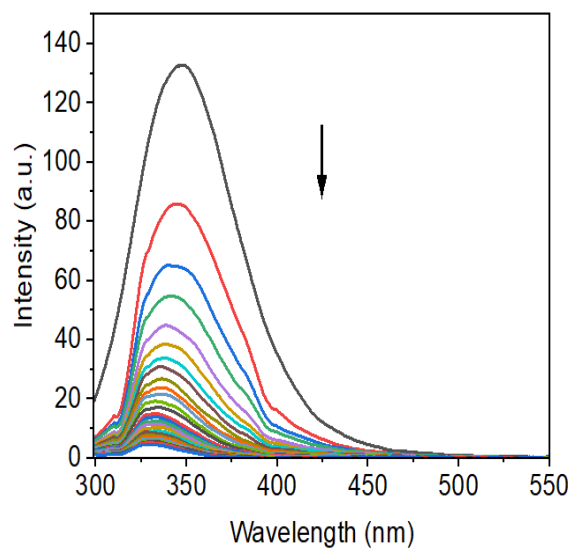


Figure 4.59 Fluorescence emission profiles of 10 μM bovine serum albumin (excited at 280 nm) were recorded while gradually increasing the concentration of compound 5 from 0 μM – 20.54 μM .

4.8.1 Determination of Binding Constant

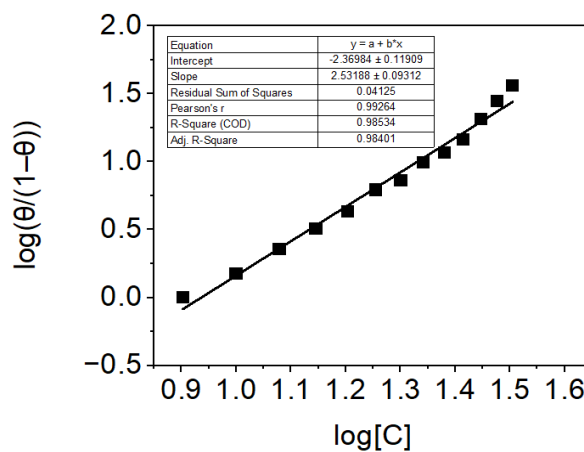


Figure 4.60 Linear Hill plot illustrating the binding interaction of Compound 1 with BSA

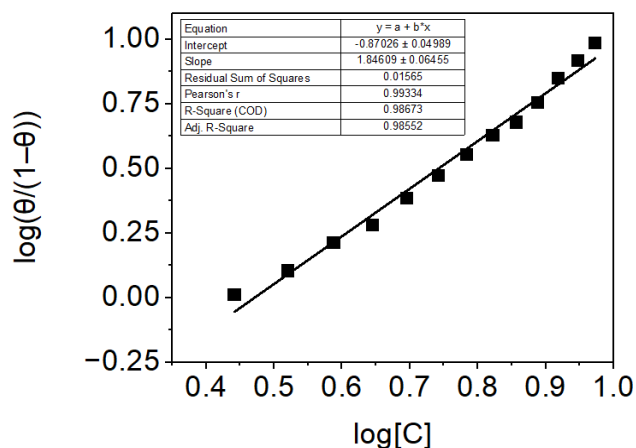


Figure 4.61 Linear Hill plot illustrating the binding interaction of Compound **2** with BSA

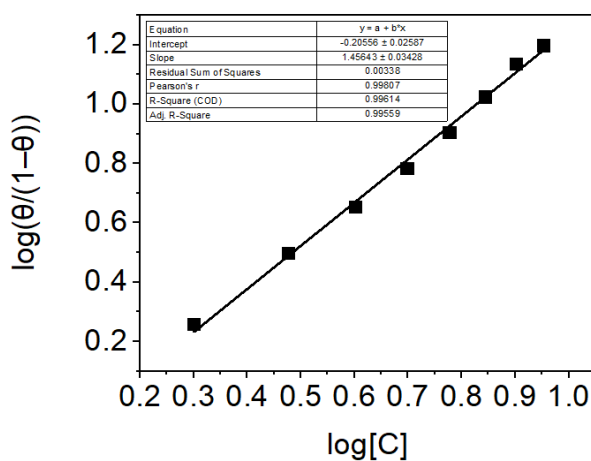


Figure 4.62 Linear Hill plot illustrating the binding interaction of Compound **3** with BSA

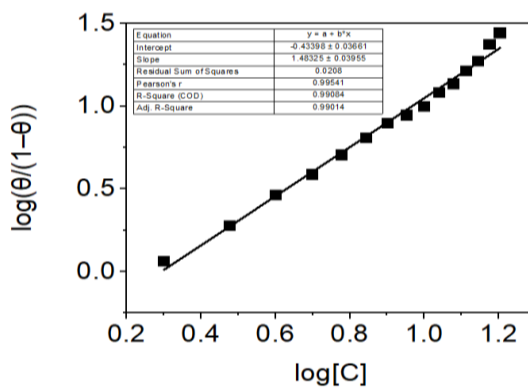


Figure 4.63 Linear Hill plot illustrating the binding interaction of Compound **4** with BSA

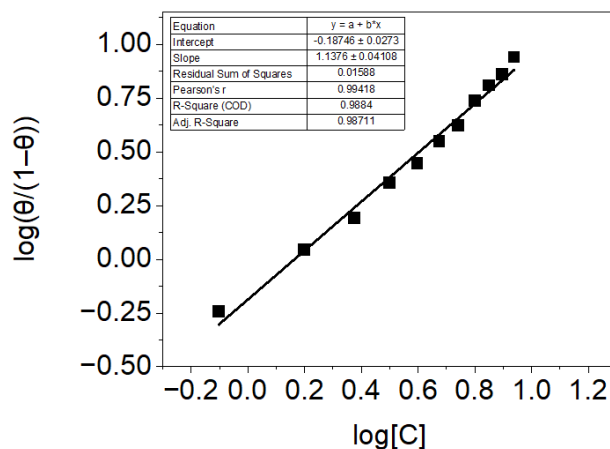


Figure 4.64 Linear Hill plot illustrating the binding interaction of Compound 5 with BSA

The values of binding constant (K_a) and Hill coefficient (n) have been calculated for all the compounds by using above linear hill plots and have been summarised in the Table 4.17.

Table 4.17: Showing the values of Hill coefficient (n), dissociation constant (K_d) and binding constant (K_a) for different compounds.

Compound	Hill coefficient (n)	Dissociation constant (K_d)	Binding constant (K_a)
1	2.53	234.34	0.0043
2	1.85	7.42	0.1348
3	1.46	1.60	0.625
4	1.48	2.72	0.3676
5	1.14	1.54	0.6493

The binding affinities and cooperativity of the five compounds show significant variation. **Compound 1** exhibits the highest Hill coefficient ($n = 2.53$), indicating **strong positive cooperativity** among binding sites, but it also has the **highest dissociation constant ($K_d = 234.34 \mu\text{M}$)**, suggesting **weak overall binding affinity** (low $K_a = 0.0043 \mu\text{M}^{-1}$).

On the other hand, **Compound 5** shows **the highest binding affinity** (lowest $K_d = 1.54 \mu\text{M}$ and highest $K_a = 0.6493 \mu\text{M}^{-1}$), but the **Hill coefficient is close to 1 ($n = 1.14$)**, indicating **mild** positive cooperativity.

Compound 3 and **Compound 4** demonstrate a **moderate degree of cooperativity** ($n \approx 1.46\text{--}1.48$) and relatively **strong binding affinities** ($K_a = 0.625$ and $0.3676 \mu\text{M}^{-1}$, respectively), making them balanced in both aspects.

Compound 2 shows a higher Hill coefficient ($n = 1.85$) than Compounds **3–5**, indicating **stronger cooperativity**, but its binding affinity ($K_a = 0.1348 \mu\text{M}^{-1}$) is moderate compared to others.

These binding constant values are in good agreement with the docking score values (Table 4.16) of the compounds with BSA (3VO3).

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Chapter 5: Conclusion

NO is a quite stable, free radical gas that easily permeates cells and cell membranes where it interacts with molecular targets. The exact NO reactions are determined by the concentration of NO attained as well as minute changes in the intracellular and extracellular milieu's makeup. Regarding the function of nitric oxide in oncology, there is a significant dichotomy that divides the literature in two parts. NO has been reported to act as both pro-neoplastic and anti-neoplastic agent. The fact that whether NO will act as a pro-neoplastic or anti-neoplastic agent depends on the factors such as the microenvironment, the cellular background, the type of cell exposed, the redox status, the final intracellular concentration, and the amount and duration of exposure of NO to the targets. Numerous NO donors with varying modes and rates of NO release have been synthesized as a result of chemical versatility of NO. subsequently NO donor medications can be selected or customized to fit the disease target. The important classes of NO donors that are being examined in cancer research include: i) Organic nitrates; ii) Metal-NO complexes [sodium nitroprusside (SNP)]; iii) S-nitrosothiols; iv) Sydnonimines; v) Diazeniumdiolates (NONOates); and vi) NO-drug hybrids, whereas the long-term use of current NO donors is limited by development of tolerance and toxicity issues, ensuring that there is a clinical need for novel alternatives. Generation of the correct amount of NO in the right place for the right length of time is the major obstacle in using NO gas therapy in treatment of cancer and various other diseases. To overcome this obstacle light triggered NO donors can work wonders in the treatment of various diseases. NO donors provide an excellent approach for their study in oncology in a controlled manner.

Compounds 1,2,3,4 and 5 NO donors have been synthesised for the light-controlled release of NO in aqueous medium. They release NO only in the presence of blue light of 410 nm and 3 W and remain inactive in the absence of light. The effective release of NO from these donors have been confirmed by UV–Vis and Griess assay studies. It has been discovered that the rate of NO emission is remarkably consistent and sluggish. All the five synthesised NO donors, exhibit distinct nitric oxide (NO) release efficiencies and photochemical behaviors. Among all compounds, Compound **3** showed the highest NO release (28 μM) along with the highest rate constant (0.0034 min^{-1}), suggesting the fastest and most efficient NO-release kinetics. Compound **4**

also demonstrated substantial NO release (26 μM), although with a lower rate constant, indicating comparatively slower release despite high overall NO generation. Compound **2** displayed moderate NO release (11.81 μM) but possessed the highest quantum yield (0.001), implying superior photochemical efficiency relative to the other compounds. In contrast, Compounds **1** and **5** released comparatively lower amounts of NO, corresponding to lower rate constants and reduced efficiencies. Overall, the results suggest that NO release is influenced by both kinetic parameters and photochemical efficiency, with Compound **3** emerging as the most promising NO donor in terms of rapid and high NO generation, while Compound **2** exhibits the most favorable quantum efficiency (Table 5.1). Studies on pH-dependent NO release verify that, in comparison to other circumstances examined, NO release occurs very quickly in acidic media. The concentration of the NO donors also affects how quickly NO is released.

Table 5.1: Showing amount of NO released, Rate constant and Quantum yield of different compounds.

Compound	Amount of NO released	Rate constant (min^{-1})	Quantum yield
1	3.88 μM	0.0015	0.0003
2	11.81 μM	0.00198	0.001
3	28 μM	0.0034	0.0005
4	26 μM	0.0016	0.00035
5	7.72 μM	0.0016	0.00056

The safety of NO donors for living cells up to a concentration of 20 $\mu\text{g/mL}$ is confirmed by in-vitro MTT tests.

However, Cell viability studies on human prostate cancer cells, HeLa cells and breast cancer cells in the presence of light at different times suggest that the compounds are toxic to these cells especially from 40 to 60 min of irradiation with light. Hence it's evident that the NO released from NO donors is responsible for the cell death. Out of the 5 synthesised NO donors, compound **2** was found to show the maximum %age of cell mortality, followed by compound **5**, followed by compound **3** and compound **4**, whereas compound **1** was found to show the minimum %age of cell mortality. (Table 5.2) Which is in accordance with the number of NO units present in the compounds.

This finding points to the possible benefit of employing organic NO donors in therapeutic applications. NO can sensitize tumor cells to chemotherapy, radiation, or immunotherapy, making it a unique potential therapeutic agent for patients with resistant cancer. To create NO-based cancer preventive and therapy strategies, however, more validation and experimental/clinical trials are needed. Future clinical translation of NO-based therapies could be accelerated by regular reporting of NO release parameters, which would improve human disease therapy.

Table 5.2: Showing cell mortality in %age of different compounds.

Compound	DU145 Cells	HeLa cells	MCF-7 Cells
1	29%	30%	32%
2	60%	57%	55%
3	56%	51%	53%
4	55%	50%	48%
5	57%	53%	52%

Appendix A: Mass Spectra

Elemental Composition Report

Page 1

Single Mass Analysis

Tolerance = 100.0 PPM / DBE: min = -1.5, max = 50.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

44 formula(e) evaluated with 1 results within limits (up to 100 best isotopic matches for each mass)

Elements Used:

C: 9-9 H: 1-100 N: 0-4 O: 0-2 F: 1-3

Sample Name : 12:23:47KM01B

IITRPR

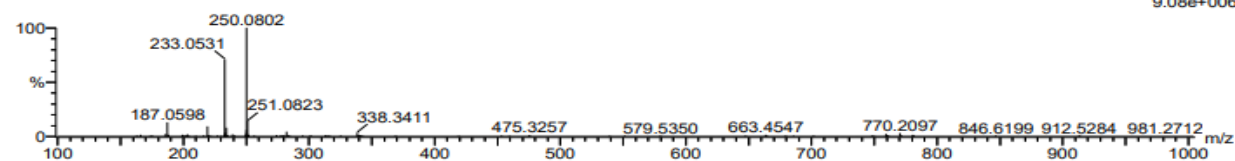
XEVO G2-XS QTOF

Test Name :

041022_KM01B 19 (0.214)

1: TOF MS ES+

9.08e+006



Minimum: 10.0 100.0 -1.5
Maximum: 10.0 100.0 50.0

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
250.0802	250.0803	-0.1	-0.4	4.5	1285.6	n/a	n/a	C9 H11 N3 O2 F3

Figure A.1 High-resolution mass spectrometry (HR-MS) of Compound **1**, acquired using a Waters Q-ToF mass spectrometer. The spectrum displays a prominent molecular ion peak at m/z 250.08 $[M+H]^+$ corresponding to Compound **1**.

Sample Name : 14:37:06KM04 II
Test Name :

IITRPR

XEVO G2-XS QTOF

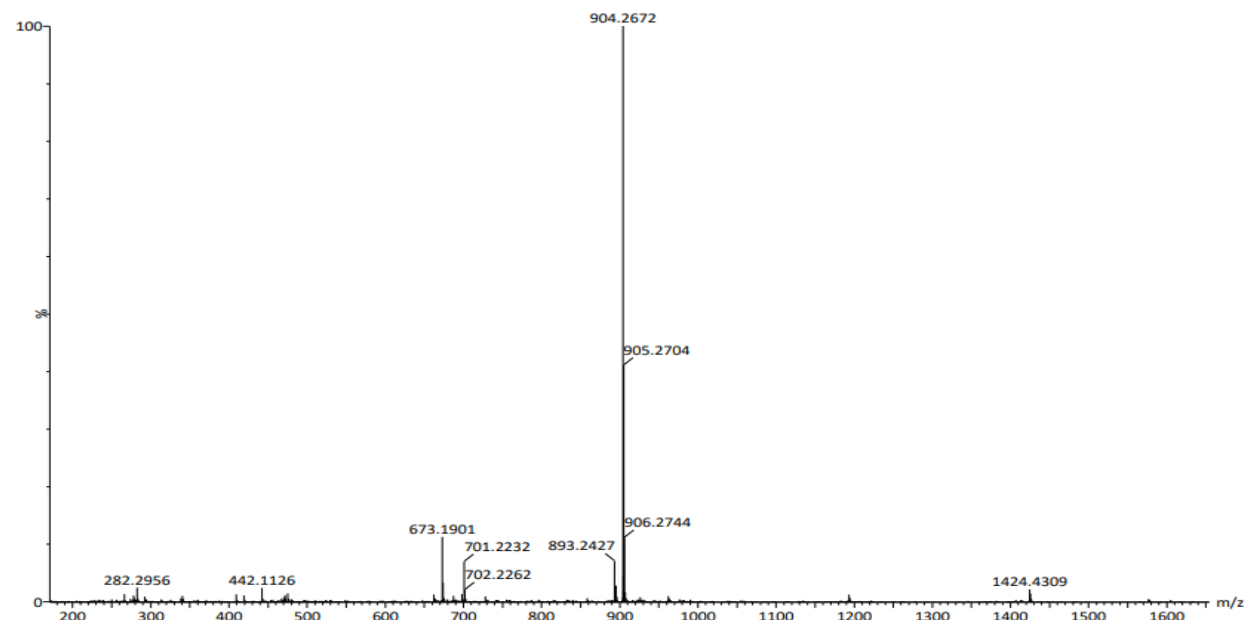


Figure A.2 (a) Electrospray Ionization Mass Spectrometry (ESI-MS) of Compound **2**, acquired using a Waters Q-ToF mass spectrometer. The spectrum displays a prominent molecular ion peak at m/z 904.26 $[M+H]^+$ corresponding to Compound **2**.

Elemental Composition Report

Page 1

Single Mass Analysis

Tolerance = 5.0 mDa / DBE: min = -1.5, max = 50.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

982 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)

Elements Used:

C: 0-36 H: 0-40 N: 0-9 O: 0-9 F: 0-9

290323_KM04 41 (0.437)

Test Name :

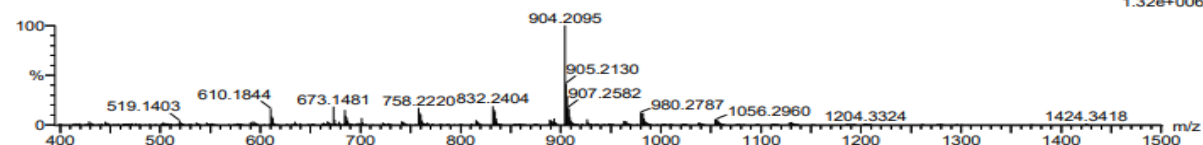
1: TOF MS ES+

IITRPR

XEVO G2-XS QTOF

290323_KM04

1.32e+006



Minimum: -1.5
Maximum: 5.0 10.0 50.0

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf (%)	Formula
904.2095	904.2101	-0.6	-0.7	21.5	410.4	n/a	n/a	C36 H31 N9 O9 F9

Figure A.2 (b) High-resolution mass spectrometry (HR-MS) of Compound 2, acquired using a Waters Q-ToF mass spectrometer. The spectrum displays a prominent molecular ion peak at m/z 904.20 $[M+H]^+$ corresponding to Compound 2.

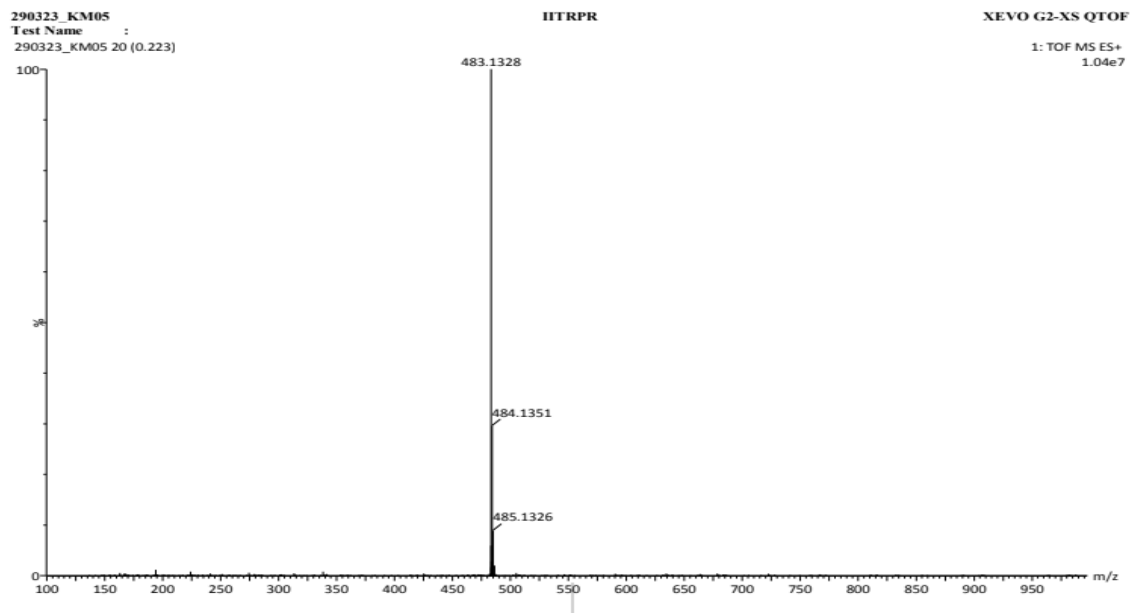


Figure A.3 (a) Electrospray Ionization Mass Spectrometry (ESI-MS) of Compound 3, acquired using a Waters Q-ToF mass spectrometer. The spectrum displays a prominent molecular ion peak at m/z 483.13 $[M+H]^+$ corresponding to Compound 3.

Elemental Composition Report

Page 1

Single Mass Analysis

Tolerance = 5.0 mDa / DBE: min = -1.5, max = 50.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

195 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)

Elements Used:

C: 0-21 H: 0-40 N: 0-4 O: 0-4 S: 0-1 F: 0-3

290323_KM05 21 (0.231)

Test Name :

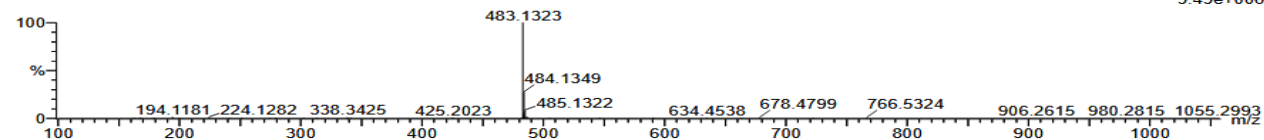
1: TOF MS ES+

IITRPR

XEVO G2-XS QTOF

290323_KM05

9.49e+006



Minimum:

Maximum:

5.0 10.0 -1.5

50.0

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf (%)	Formula
483.1323	483.1314	0.9	1.9	11.5	807.2	n/a	n/a	C21 H22 N4 O4 S F3

Figure A.3 (b) High-resolution mass spectrometry (HR-MS) of Compound **3**, acquired using a Waters Q-ToF mass spectrometer. The spectrum displays a prominent molecular ion peak at m/z 483.13 $[M+H]^+$ corresponding to Compound **3**.

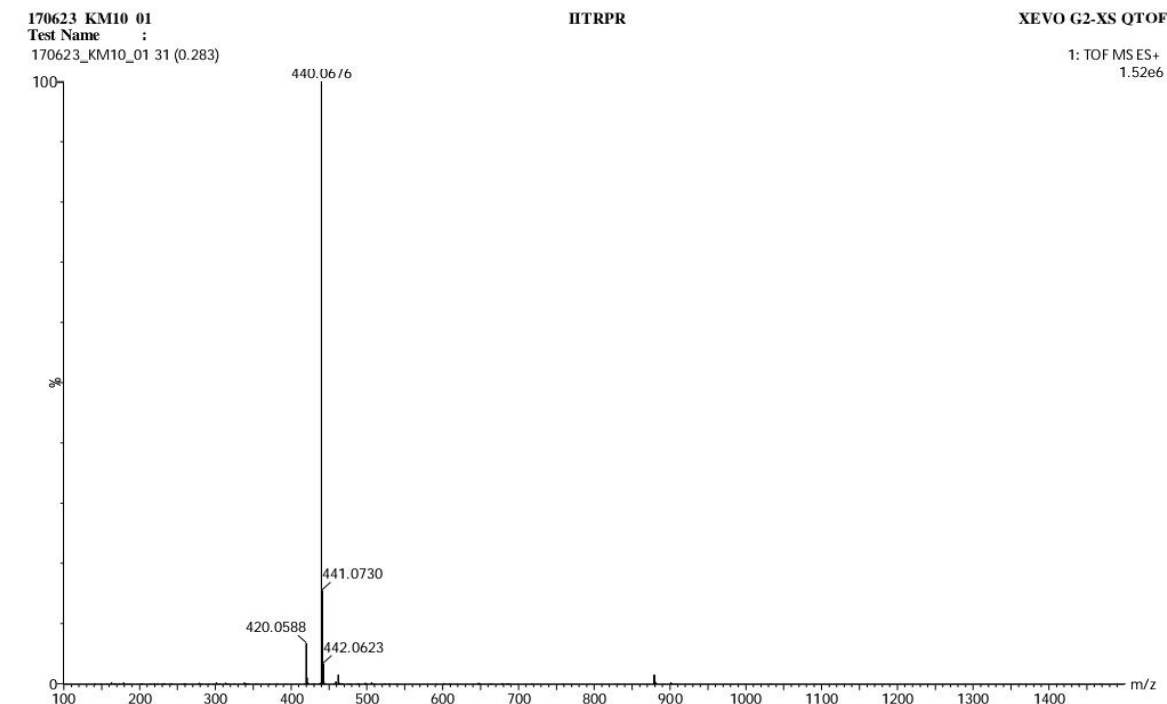


Figure A.4 (a) Electrospray Ionization Mass Spectrometry (ESI-MS) of Compound **4**, acquired using a Waters Q-ToF mass spectrometer. The spectrum displays a prominent molecular ion peak at m/z 440.06 $[M+H]^+$ corresponding to Compound **4**.

Single Mass Analysis

Tolerance = 20.0 PPM / DBE: min = -1.5, max = 50.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

77 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)

Elements Used:

C: 1-19 H: 1-100 N: 1-3 O: 1-4 S: 0-1 F: 1-3

170623_KM10_01 29 (0.311)

IITRPR

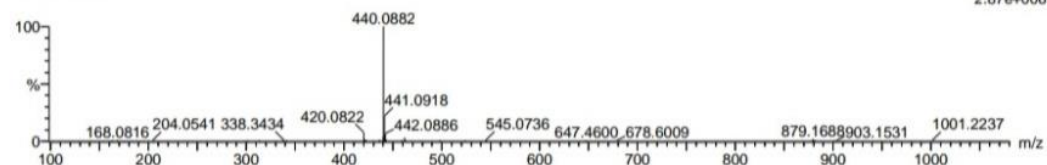
XEVO G2-XS QTOF

Test Name :

170623_KM10_01

1: TOF MS ES+

2.87e+006



Minimum:

Maximum:

5.0 20.0 -1.5
50.0

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
440.0882	440.0892	-1.0	-2.3	11.5	692.4	n/a	n/a	C19 H17 N3 O4 S F3

Figure A.4 (b) High-resolution mass spectrometry (HR-MS) of Compound **4**, acquired using a Waters Q-ToF mass spectrometer. The spectrum displays a prominent molecular ion peak at m/z 440.08 $[M+H]^+$ corresponding to Compound **4**.

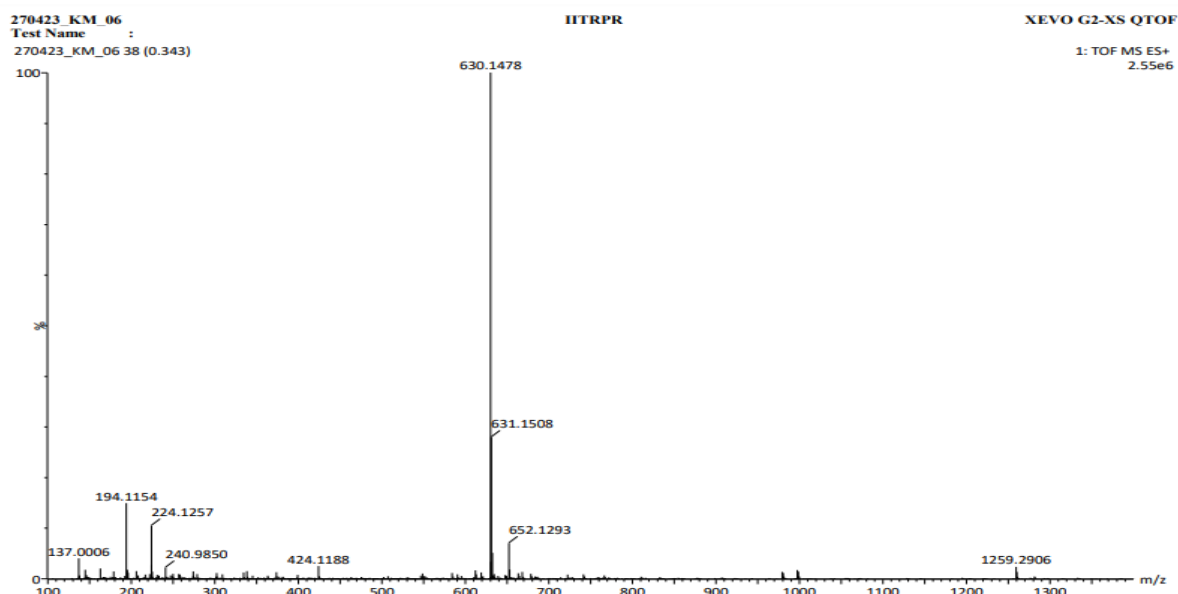


Figure A.5 (a) Electrospray Ionization Mass Spectrometry (ESI-MS) of Compound **5**, acquired using a Waters Q-ToF mass spectrometer. The spectrum displays a prominent molecular ion peak at m/z 630.14 $[M+H]^+$ corresponding to Compound **5**.

Elemental Composition Report

Page 1

Single Mass Analysis

Tolerance = 20.0 PPM / DBE: min = -1.5, max = 50.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

397 formula(e) evaluated with 1 results within limits (up to 50 closest results for each mass)

Elements Used:

C: 0-25 H: 0-70 N: 0-7 O: 0-6 F: 0-6

270423_KM_06 34 (0.366)

IITRPR

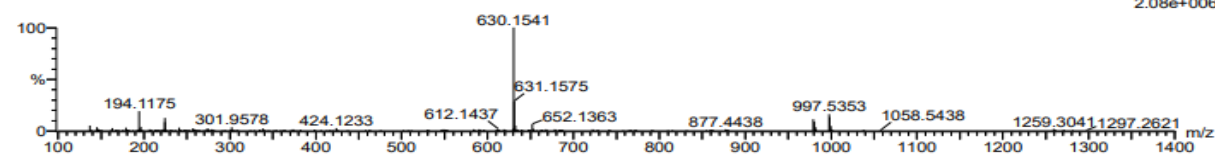
XEVO G2-XS QTOF

Test Name :

270423_KM_06

1: TOF MS ES+

2.08e+006



Minimum: -1.5
Maximum: 50.0

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf (%)	Formula
630.1541	630.1536	0.5	0.8	15.5	542.8	n/a	n/a	C25 H22 N7 O6 F6

Figure A.5 (b) High-resolution mass spectrometry (HR-MS) of Compound 5, acquired using a Waters Q-ToF mass spectrometer. The spectrum displays a prominent molecular ion peak at m/z 630.15 $[M+H]^+$ corresponding to Compound 5.

Appendix B: NMR Spectra

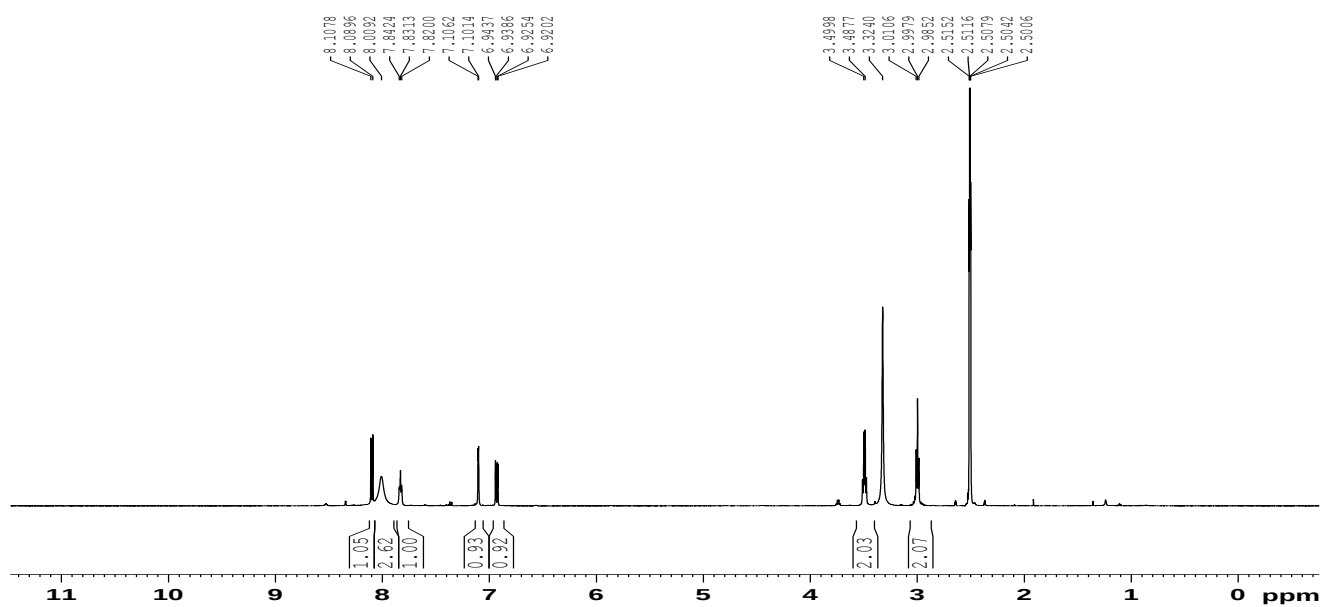


Figure B.1 (a) Proton (^1H) NMR of Compound **1** in DMSO- d_6 at 500 MHz

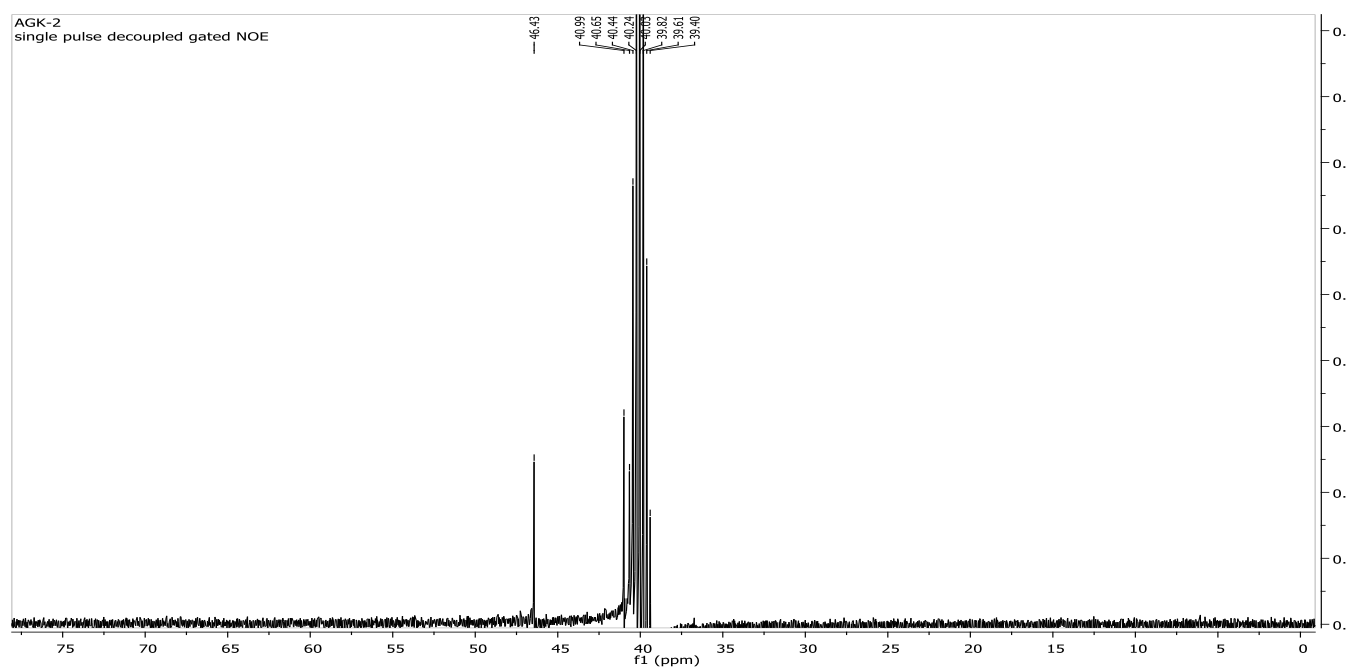


Figure B.1 (b) Carbon-13 (^{13}C) NMR of Compound **1** in DMSO- d_6 at 125 MHz

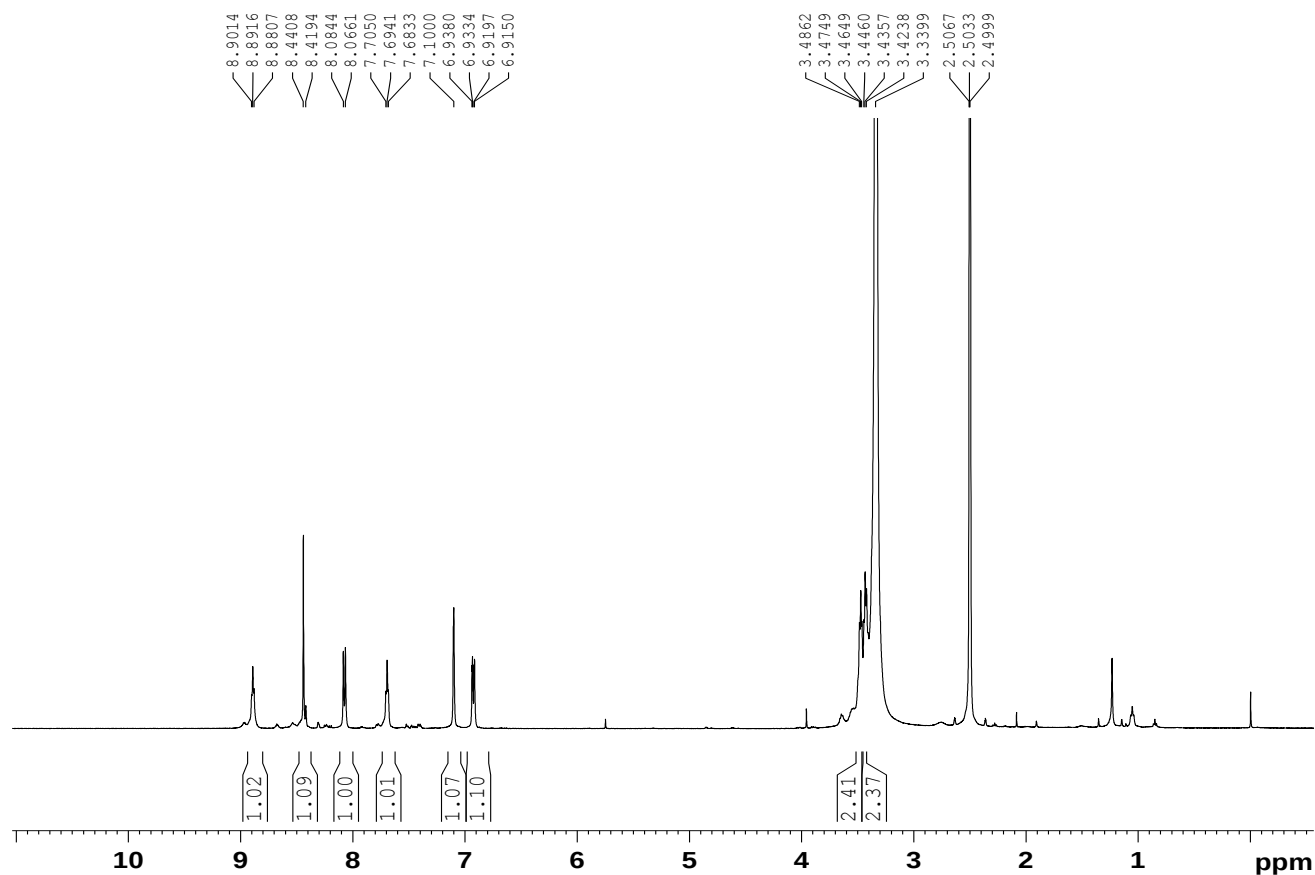


Figure B.2 (a) Proton (^1H) NMR of Compound **2** in DMSO- d_6 at 500 MHz

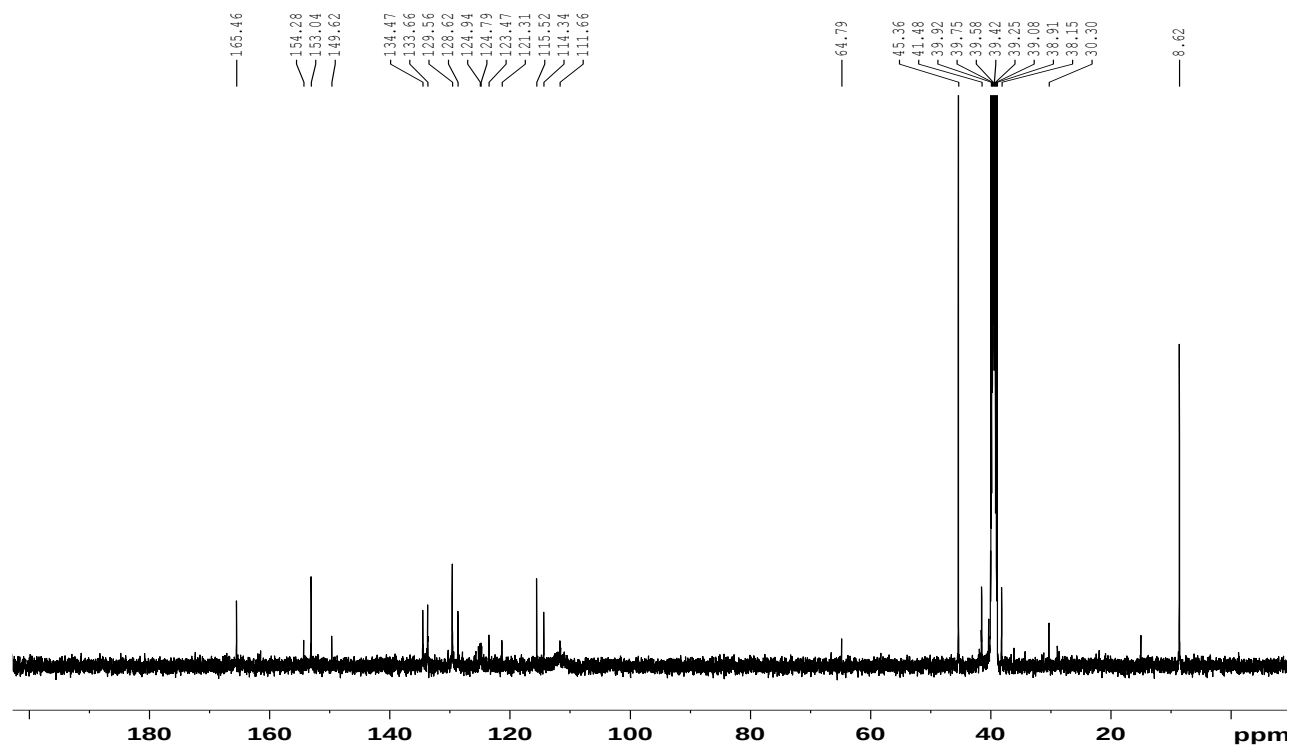


Figure B.2 (b) Carbon-13 (^{13}C) NMR of Compound **2** in DMSO- d_6 at 125 MHz

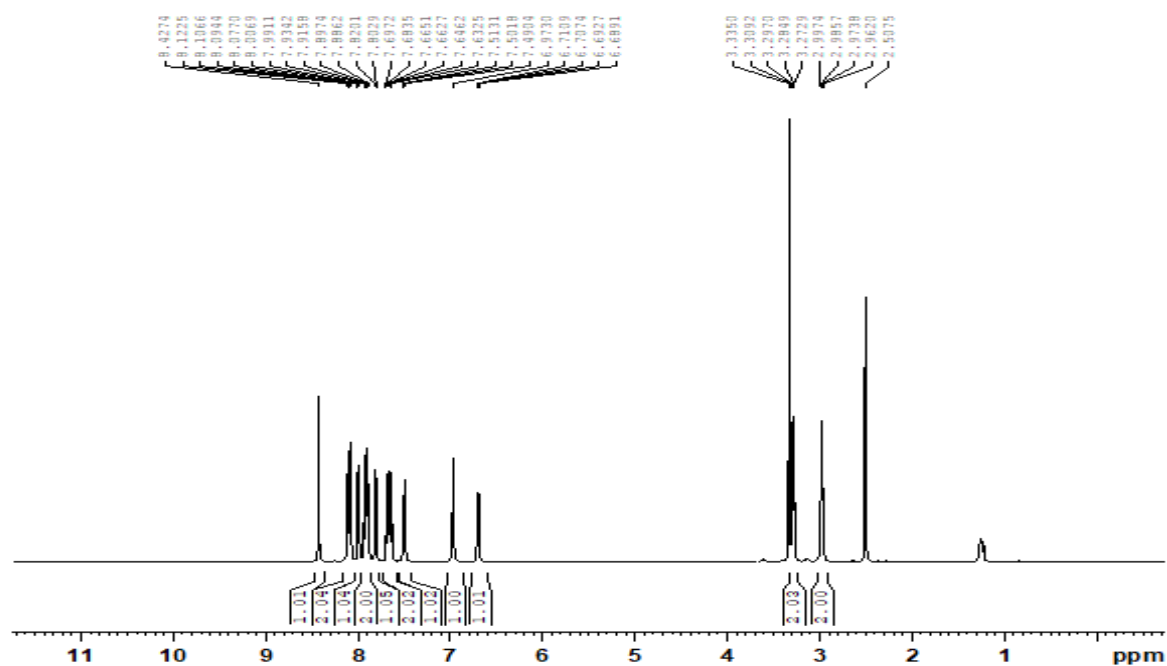


Figure B.4 (a) Proton (^1H) NMR of Compound **4** in DMSO- d_6 at 500 MHz

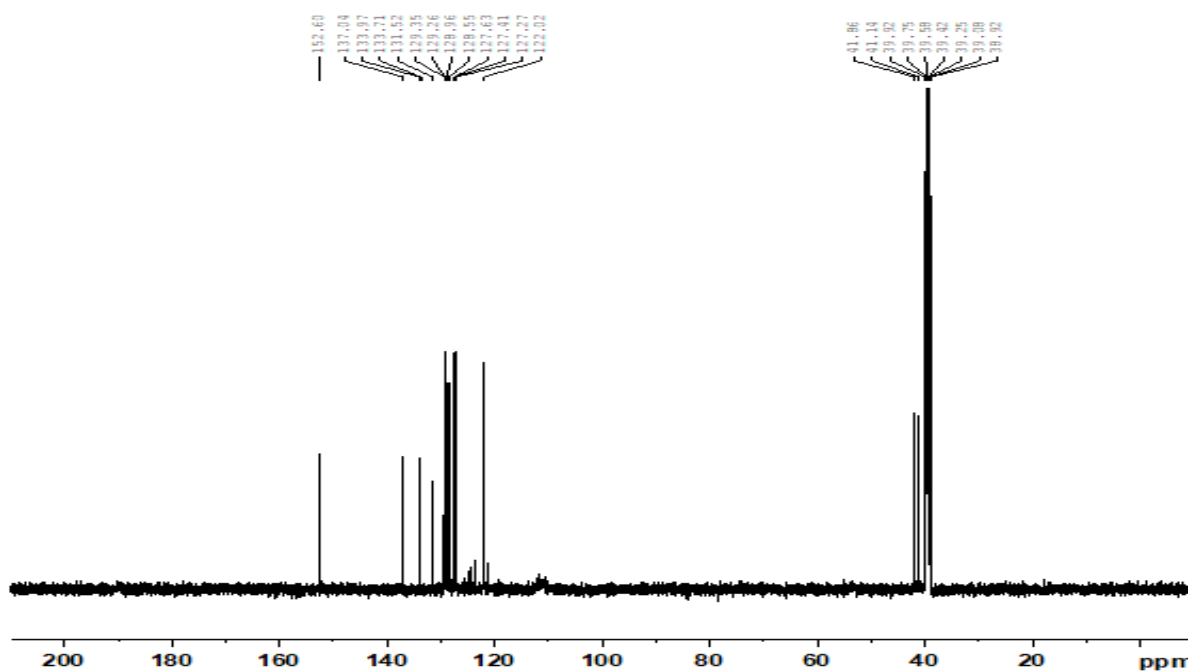


Figure B.4 (b) Carbon-13 (^{13}C) NMR of Compound **4** in DMSO- d_6 at 125 MHz

KM06
1H_8scan DMSO {D:\Spectra} nmr 9

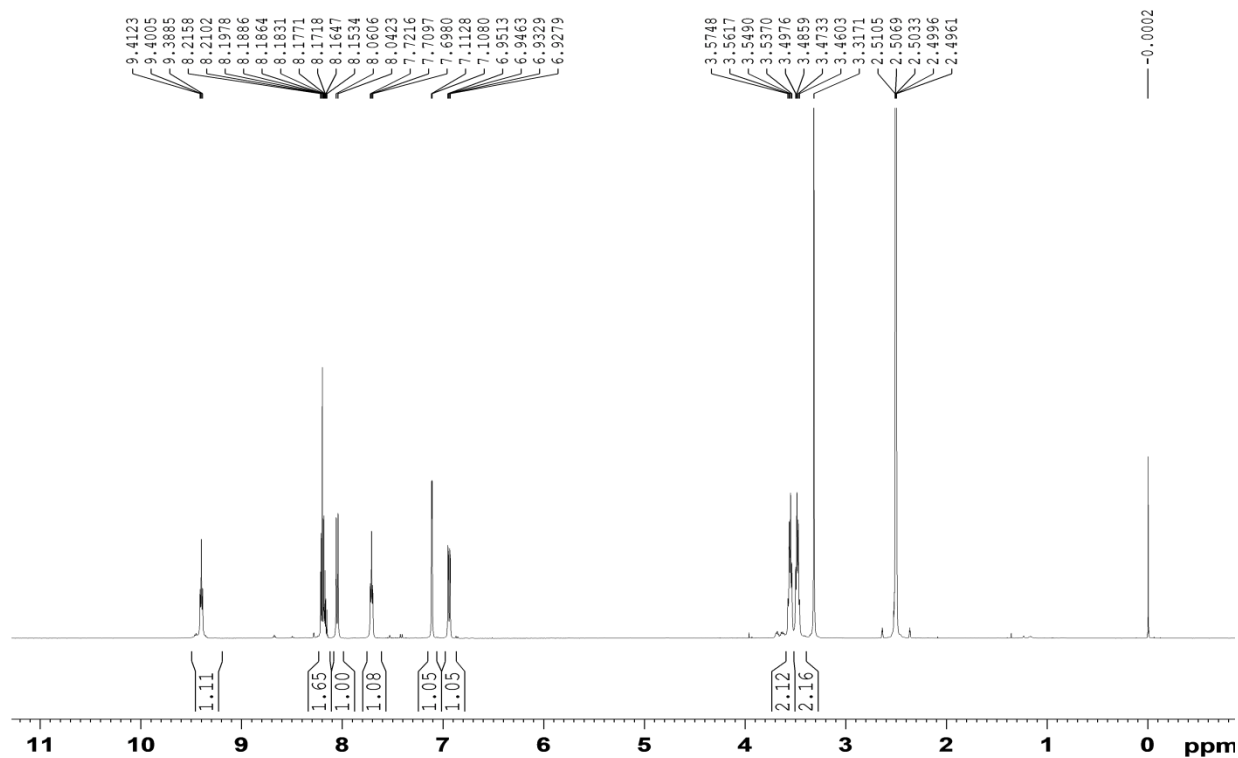


Figure B.5 (a) Proton (^1H) NMR of Compound 5 in DMSO- d_6 at 500 MHz

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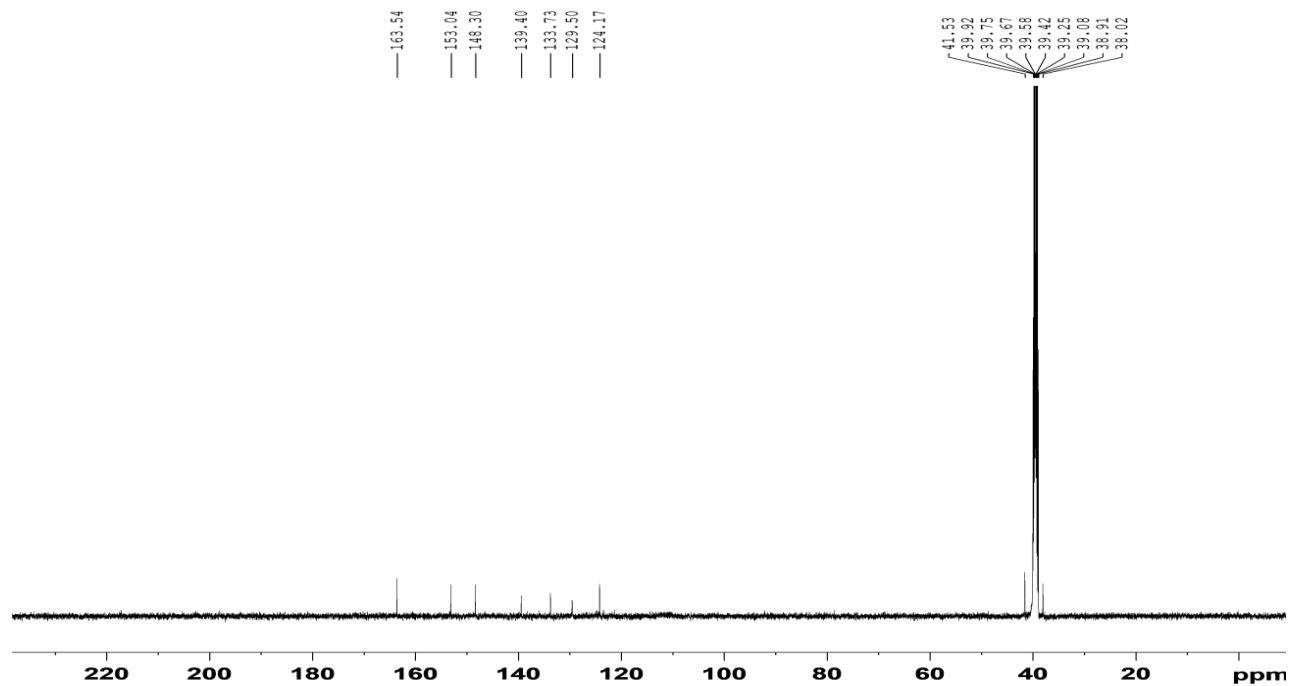


Figure B.5 (b) Carbon-13 (^{13}C) NMR of Compound 5 in DMSO- d_6 at 125 MHz

List of Publications

1. K.Mehla, H.Kaur “A comprehensive study of therapeutic entities for treatment of cataract”, *AIP Conf. Proc.* **2800**, 1–12 (2023)
2. K.Mehla, H.Kaur, D.A.Jose “Nitro-aniline based tripodal nitric oxide (NO) releasing molecule and light-triggered cytotoxicity in cancer cells”, *Tetrahedron* **162**, 1-6 (2024)
3. K.Mehla, H.Kaur “A Review on Aldose Reductase Inhibitors: Chemistry and Pharmacological Activity”, *Lett. Appl. NanoBioScience* **14**, 1-16 (2024)

List of conferences

1. **Recent Advances in Fundamental and Applied Sciences (RAFAS-2021)**, Lovely Professional University, Punjab, India, *June 25–26, 2021*. Poster presented: “*A Comprehensive Study of Therapeutic Entities for Treatment of Cataract*”.
2. **Recent Advances in Fundamental and Applied Sciences (RAFAS-2023)**, Lovely Professional University, Punjab, India, *March 24–25, 2023*. Poster presented: “*Photo Responsive Nitric Oxide Donors for Cancer Gas Therapy*”.
3. **International Conference on Molecules and Materials Technology (MMT-2023)**, National Institute of Technology, Kurukshetra, India, *April 21-22, 2023*. Poster presented: “*Trimesic Acid Based Nitro-Aniline Compound as Light Controllable NO Releasing Molecule for Cancer Gas Therapy*”.