SYNTHESIS AND CHARACTERISATION OF NEW FLUORESCENT PYRAZOLINE- PIPERAZINYL LINKED PACLITAXEL AS PRODRUGS: PHOTODYNAMIC THERAPY AND ANTICANCER ACTIVITY AGAINST MCF-7 AND MCF-10A CELL LINES

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by

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LIST OF SYMBOLS

MHz	Mega Hertz
t	Triplet
q	Quartet
m	Multiplet
ppm	Parts per million
cm^{-1}	Per centimeter
δ	Chemical shift
µg	Micrograms
mg	Milligram
g	Gram
mL	Milliliter
s	Singlet
d	Doublet
nm	Nanometer
°C	Degree Celsius
h	Hour
min	Minutes
conc.	Concentration
cm	Centimeter
μgmL^{-1}	Microgram per milliliter
µL	Microliter
µM	Micromole
IC_{50}	The half maximal inhibitory concentration
EC_{50}	The half maximal effective concentration
λ_{max}	Lambda (Maximum Wavelength)

LIST OF ABBREVIATIONS

^{13}C-NMR	Carbon Nuclear Magnetic Resonance
1D-NMR	One Dimensional Nuclear Magnetic Resonance
^{1}H -NMR	Proton Nuclear Magnetic Resonance
2D-NMR	Two-Dimensional Nuclear Magnetic Resonance
br.*d*	Broad Doublet
br.*s*	Broad Singlet
C_5D_5N	Deuterated Pyridine
$CDCl_3$	Deuterated Chloroform
COSY	Correlation Spectroscopy
DEPT	Distortionless enhancement by Polarization Transfer
DMSO- d_6	Deuterated Dimethyl sulfoxide
FT-IR	Fourier Transformer Infrared
HCl	Hydrochloric Acid
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Correlation
HSQC	Heteronuclear Single Quantum Correlation
IR	Infra-Red
MeOD-d_4	Deuterated Methanol
MIC	Minimum Inhibitory Concentration
Mol. Wt.	Molecular weight
PDT	Photodynamic Therapy
Ps	photosensitizer
PTX	Paclitaxel
R_f	Retention factor
TLC	Thin layer chromatography

WHO	World Health Organisation

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SINTESIS DAN PENCIRIAN SEBATIAN BERPENDARFLOUR BAHARU PIRAZOLINA-PIPERAZINIL YANG BERANGKAI DENGAN *PACLITAXEL* SEBAGAI PRODUG: AKTIVITI TERAPI FOTODINAMIK DAN ANTIKANSER TERHADAP TITISAN SEL MCF-7 DAN MCF-10A

ABSTRAK

Kajian ini melibatkan sintesis sebatian pendarflour baharu dengan gabungan terapi fotodinamik (PDT) dan aktiviti kemoterapi terhadap MCF-7 (sel kanser) dan MCF-10A (sel normal) yang merupakan strategi berkesan untuk merawat kanser. Prodrug photosensitizer **(1-17)a** (PS)-terkonjugat paclitaxel (PTX) **(1-15)c** yang unik telah dibangunkan, yang mana PS teruja oleh cahaya panjang gelombang dekat-inframerah bagi membebaskan PTX secara khusus sambil menjana oksigen singlet (SO) untuk membunuh sel-sel kanser. Mekanisme ini melibatkan penggunaan oksigen singlet untuk membelah penghubung jenis aminoakrilat dalam prodrug. Ini menghasilkan spesies oksigen reaktif (ROS) yang mendorong kerosakan oksidatif dan kematian sel. Kematian sel MCF-7 dicetuskan oleh ROS yang melebihi kapasiti redoks sel, yang membawa kepada apoptosis, disahkan oleh ujian caspase Glo-3/7. Konjugat fotosensitif berasaskan paclitaxel (PTX) telah dibangunkan, di mana pendedahan cahaya membebaskan PTX utuh, menggabungkan kemoterapi dengan tindakan segera PDT. Pirazolina (PS) **(1-17)a**, komponen utama konjugat, didapati tidak toksik dan berkesan dalam PDT. Penyerapan UV/Vis dan ciri pelepasan pendarfluor dinilai, mendedahkan kestabilan dan kereaktifan fotosensitizer. Kajian *in vitro* menunjukkan bahawa Py-L-PTX menyebabkan kematian sel dengan kehadiran cahaya tanpa menjejaskan sel dalam gelap. Konjugat menunjukkan hasil yang menjanjikan, dengan pelepasan PTX yang berjaya selepas penyinaran, disahkan oleh analisis TLC dan

HPLC. Selain itu, sifat ADME bagi terbitan pirazolina telah dinilai, menunjukkan keserupaan dadah yang positif, keterlarutan, dan potensi farmakologi yang menggalakkan. Kajian pengedokan molekul menunjukkan interaksi yang kuat dengan sasaran colchicine, menyokong reka bentuk calon ubat masa depan. Sistem penyampaian ubat yang inovatif ini menawarkan platform yang fleksibel untuk menggabungkan PDT dan kemoterapi, dengan aplikasi yang berpotensi dalam terapi kanser dan penyakit lain. Sifat terapeutik yang boleh dilaraskan meluaskan kemungkinan strategi rawatan yang disasarkan

SYNTHESIS AND CHARACTERISATION OF NEW FLUORESCENT PYRAZOLINE- PIPERAZINYL LINKED PACLITAXEL AS PRODRUGS: PHOTODYNAMIC THERAPY AND ANTICANCER ACTIVITY AGAINST MCF-7 AND MCF-10A CELL LINES

ABSTRACT

This study involves the synthesis of new pendarflour compounds with combination of photodynamic therapy (PDT) and chemotherapy activity against MCF-7 (cancer cells) and MCF-10A (normal cells) which is an effective strategy to treat cancers. A unique photosensitizer **(1-17)a** (PS)-conjugated paclitaxel (PTX) **(1-15)c** prodrug has been developed, in which a PS is excited by near-infrared wavelength light to specifically release PTX while generating singlet oxygen (SO) to kill cancer cells. The mechanism involves the use of singlet oxygen to cleave aminoacrylate-type linkers in the prodrug. This generates reactive oxygen species (ROS) that induce oxidative damage and cell death. MCF-7 cell death was triggered by ROS surpassing the cell's redox capacity, leading to apoptosis, confirmed by caspase Glo-3/7 assays. A paclitaxel (PTX)-based photosensitive conjugate was developed, where light exposure releases intact PTX, combining chemotherapy with immediate PDT action. Pyrazoline (PS) **(1-17)a**, a key component of the conjugate, was found to be non-toxic and effective in PDT. UV/Vis absorption and fluorescence emission characteristics were evaluated, revealing the stability and reactivity of the photosensitizer. *In vitro* studies demonstrated that Py-L-PTX induced cell death in the presence of light without affecting cells in the dark. The conjugate showed promising results, with successful PTX release upon irradiation, confirmed by TLC and HPLC analysis. Additionally,

the ADME properties of pyrazoline derivatives were assessed, showing positive drug-likeness, solubility, and favourable pharmacological potential. Molecular docking studies indicated strong interactions with the colchicine target, supporting the design of future drug candidates. This innovative drug delivery system offers a flexible platform for combining PDT and chemotherapy, with potential applications in cancer therapy and other diseases. The tunable nature of the therapeutic effects expands the possibilities of targeted treatment strategies.

CHAPTER 1

INTRODUCTION

1.1 Background of research

Cancer is a condition characterised by the body's cells' uncontrollable growth and spread to other body parts. (Figure 1.1). Death can occur if these abnormal cell growth is not prevented. Cancer is the second most common cause of mortality, following heart disease. According to data from the American Cancer Society, in 2024 an estimated over 2,001,140 new cases cancer-related deaths and almost 611,720 people will die from the disease common forms of cancer include breast, bladder, colon, rectal, endometrial, kidney, leukaemia, lung, melanoma, lymphoma, pancreatic, prostate, and thyroid cancer. For women the three most common cancer breast, lung and colorectal they will estimate 51% of all new cancer diagnoses in women 2024, breast cancer is the most prevalent malignancy among women and a significant contributor to global mortality. Breast cancer is made up 34.1% of female from 2012 to 2016, which increased from 32.1% in the previous period (2007–2011) (Azizah et al., 2019). About 1 out of 19 women in Malaysia are susceptible to breast cancer each year, and around 50% of them are less than 50 years old.

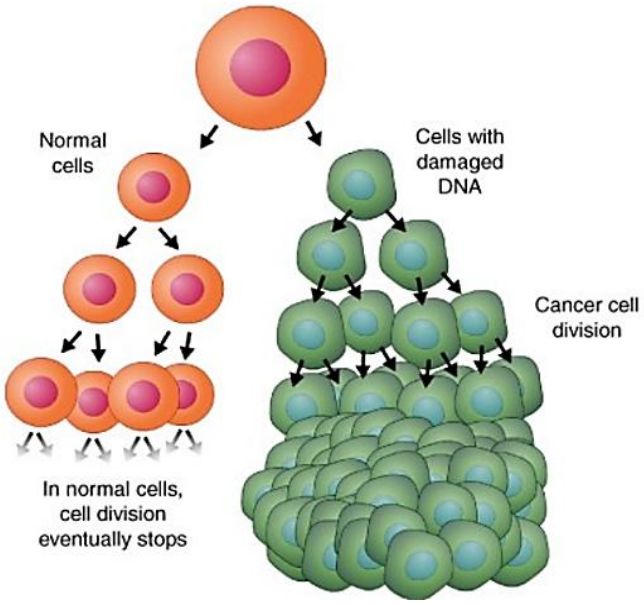


Figure 1.1 Uncontrolled growth of cancer cells (Mitchison et al., 1992).

Cancer cells respond to treatment in different ways. However, the common practice uses a combination of treatments such as surgery, chemotherapy, and radiation. These drugs can also harm healthy cells and cause bad side effects (Mokhtari et al., 2017). Over time, these cancer cells develop resistance to treatments, causing the cancer to reoccur and other complications, including relapse, metastasis, and death. There are no effective cytotoxic medicines that selectively target cancer cells, despite advancements in chemotherapy. Due to the limitations and side effects of traditional treatments, ongoing research focuses on developing new methods to treat cancer that are more accurate and effective with fewer side effects.

Photodynamic therapy (PDT) and various chemotherapy agents are among the key focus areas of this research. Light has been used for therapeutic purposes for more than 3,000 years. Light is utilised in the therapy of several illnesses, such as psoriasis, rickets, vitiligo, and skin cancer by ancient Chinese, Indian and Egyptian civilizations (Chilakamarthi et al., 2017). In the late 1800s, Dr. Niels Ryberg Finsen, a Danish physician and scientist developed phototherapy, which uses light to treat disease. He

was awarded Nobel Prize in 1903 for his work. PDT has since become a well-studied therapy for cancer as well as for other non-malignant disorders. PDT is a minimally invasive treatment that kills target cells in several types of cancer, malignancies, and other "non-neoplastic" diseases (Abrahamse & Hamblin, 2016). In PDT, a photosensitizer (PS) (light-sensitive molecule) is administered to a patient, and during exposure to specific wavelengths of light, it becomes excited and produce reactive oxygen species (ROS), which damage and kills cells. PSs can be designed to absorb light, allowing for deeper tissue penetration and reducing damage to healthy tissues.

This research focuses on Photodynamic Therapy (PDT) for cancer treatment, utilizing small-molecule photosensitizers, particular visible light, and endogenous molecular oxygen. Individually, these components are not harmful. However, when combined, the activated PS's phototoxic properties enable the generation of cytotoxic ROS. PDT has a less side effect compared to conventional cancer treatments like radiation and chemotherapy because it uses spatially controlled low-energy light to generate ROS at the tumour site for targeted tumour killing. (Bio et al., 2012; Bio et al., 2013; Bio et al., 2014; Dos Santos et al., 2019). Unlike conventional cancer therapies that show toxicity based on dosage, many rounds of PS dosage and irradiation can be given to a patient without inducing resistance or long-term adverse effects, hence improving its efficacy against tumours. Furthermore, PSs are naturally fluorescent molecules, which makes them beneficial for real-time fluorescence-guided surgery and diagnostic imaging (Hossion et al., 2013; Sarbadhikary et al., 2021). Additionally, unlike the mechanisms associated with conventional drug resistance, PDT demonstrates effectiveness against chemo-resistant tumours. Both PDT and chemotherapy agents represent areas of active research and hold great promise for revolutionising cancer treatment (Chen et al., 2021). The goal is to develop more

effective and personalised therapies that can enhance the quality of life and outcomes for patients while reducing the burden of side effects associated with cancer treatment.

Heterocyclic compounds, in particular nitrogen atom rings, have been of great interest due to their remarkable pharmaceutical activities, especially their anticancer properties. Most medicinal compounds contain nitrogen heterocycles, particularly pyrazoline, which are crucial in the creation of novel and efficient cancer therapy drugs (Kerru et al., 2020). Pyrazoline is a class of organic compounds that can exhibit interesting photophysical and photochemical properties (Adamus-Grabicka et al., 2020; Jumaah et al., 2022a). In the context of PDT, pyrazoline-based PSs are designed to be inactive or minimally active in their native state, but upon exposure to specific conditions, such as light of a particular wavelength, they become highly active and generate ROS that can cause cell damage and ultimately induce cell death (Mori et al., 2016; Wang et al., 2023). These pyrazoline-based PSs can be attached to specific molecules, such as antibodies or peptides, which enable them to selectively accumulate in the target cells, such as cancer cells. This enhances the specificity of the treatment. Depending on the kind and location of the target tissue, the pyrazoline-based PS is applied topically or by injection to the cancer. It accumulates in the target tissue over time due to its targeting strategy and its preferential retention in the abnormal tissue (e.g., cancer cells). This targeted approach reduces side effects and enhances the treatment's overall (Thapa et al., 2016).

Traditional chemotherapy, while effective at killing cancer cells, often affects healthy cells as well, leading to a range of severe side effects. Researchers have been actively working on developing new chemotherapy agents and treatment strategies that are more selective in targeting cancer cells, minimising damage to healthy tissues and organs, while maximising their effectiveness against cancer (Zhong et al., 2021).

Paclitaxel (PTX), also known as Taxol, isolated for the first time from the Pacific yew (Taxus brevifolia) in 1993. Has been a crucial chemotherapy drug to treat various cancers, particularly lung, ovarian, and breast cancer (Gallego-Jara et al., 2020). Modification of the structure of PTX has motivated medicinal chemists to pursue their studies to discover new bioactive lead molecules for drug discovery and development (Socinski et al., 2012).

PDT and site-specific chemotherapy when combined using novel PSs such as pyrazoline compounds and established anticancer drugs such as PTX proposed an innovative approach in the field of cancer treatment. This combination therapy has the potential to target the localised tumours effectively while minimising systemic adverse effects. This can methodology therapy also achieve a synergistic effect whereby the PDT can prime the tumour by disrupting the structure and making it more susceptible to chemotherapy (Grin et al., 20220). Both PDT and chemotherapy agents represent areas of active research and hold great promise in revolutionising cancer treatment to develop more effective and personalised therapies that can lead to improved patient outcomes and quality of life while reducing the burden of side effects (Mokhtari et al., 2017).

1.2 Problem statement

Since cancer is a significant global issue, finding and developing novel, effective, and selective anticancer medications is crucial (Link et al., 2019). Despite the numerous chemotherapeutic and PDT drugs for medical usage available, increasing resistance has made the continued search for new compounds with biological potential mandatory (Lee et al., 2018). Compared to other PSs and classic PSs, 1,3,5-triarylpyrazolines show a lot of potential in PDT. However, under

ultraviolet (UV) light irradiation is the only way that 1,3,5-triarylpyrazolines can become active. Short-wavelength UV radiation can cause DNA structural disruption by generating radicals and has a poor tissue-penetrating ability. Acute photokeratitis, additional cellular damage, and rapid skin ageing that encourages skin cancer are among the harmful health effects that result from this. 1,3,5-triarylpyrazolines are capable of absorbing visible light, due to their surface defects. Additionally, the classic PSs that are now in use are extensively deposited on the skin, producing photosensitivity, and have poor selectivity towards cancer cells. Their absorption peaks are at visible wavelengths of 630 nm which restricts their ability to penetrate tissues deeply. This is due to the fact that the majority of tissue chromophores, including melanin, fat, and haemoglobin, substantially absorb visible light (Shi & Sadler, 2020). Incomplete therapy and cancer recurrence may result from this, particularly if the tumour is situated in a deeper location. Therefore, when applied to solid or deeply ingrained tumours, such as breast tissue, light stimulus with wavelengths in the near-infrared (NIR) range (700–1300 nm) would be advantageous in enhancing the penetrability of the PDT agent. Pyrazolines potential toxicity also makes them difficult to be used as PSs in PDT; instead, they need to be non-toxic or incapable of degrading to produce hazardous degradation products in the absence of light activation (Correia et al., 2021). Due to pyrazolines special physicochemical characteristics and dual-function nature in PDT, PTX can be used to alter the surface characteristics and reduce their toxicity. Pyrazoline dispersibility is enhanced by PTX with strong biocompatibility characteristics. Pyrazoline PDT agent based on PTX may guarantees accurate localisation of the possible medication to the cancer site without substantially impairing healthy cells (Zhou et al., 2021).

1.3 Research objectives

The objectives of the research are as follows:.

1) To design and synthesise chalcone and pyrazoline compounds as potential photosensitizer.
2) To investigate the photophysical properties and solvatochromism of pyrazoline dye and pH-sensitive by absorption and fluorescence.
3) To evaluate the cytotoxcity of pyrazoline against MCF-7 and MCF-10A cells.
4) To design and synthesise new pyrazoline linked with Paclitaxel (PS-L-PTX) as prodrug photodynamic therapy (PDT).
5) To investigate the cytotoxicity of Py-L-PTX by combining the effects of photodynamic therapy (PDT) and anticancer activity against MCF-7 and MCF-10A cell lines by using cytotoxic reactive oxygen species (ROS) and chemotherapy (PTX). Caspase Glo-3/7 was utilised to investigate potential cell death pathways.

1.4 Scope of the study

This study explores the potential of novel prodrugs (**1c-15c**) combining photodynamic therapy (PDT) and targeted chemotherapy using paclitaxel (PTX) derivatives. It integrates comprehensive chemical characterisation techniques FT-IR, 1D/2D NMR, CHNS elemental analysis, HPLC, UV/Vis absorption and fluorescence emission characteristics of the pyrazoline compounds which are investigated to assess the suitability of the compounds as potential photosensitizer pyrazoline to link with paclitaxel (PTX) prodrug. These compounds can respond to changes in the acidity or basicity of its environment which are especially useful in biological systems where pH can vary between different compartments, such as between the inside of a cell and the

extracellular space. Biological evaluations include *in vitro* anti-cancer activity assays on MCF-7 (cancerous) and MCF-10A (non-cancerous) cell lines are monitored via MTT assays under both illuminated and non-illuminated conditions using semiconductor diode lasers (690 nm). The study also utilises molecular docking simulations (AutoDock Vina) to predict binding affinities and inhibition constants, comparing the results to the colchicine crystal structure (PDB code: 1SA0) for structural insights. Furthermore, cell death pathways are investigated through Caspase Glo-3/7 assays, differentiating apoptotic and necrotic responses. The study aims to synthesise and characterise novel PS-L-PTX prodrugs and the evaluation of their cytotoxicity and phototoxicity against breast cancer cells (MCF-7) and normal breast cells (MCF-10A). This assesses the role of reactive oxygen species (ROS) in PDT-induced cell death and explores the intracellular behaviour of the photosensitizer via fluorescence microscopy. Molecular interactions and binding efficiencies are predicted using molecular docking study. Despite the extensive analytical approach, this study has several limitations. The *in vitro* research is confined to MCF-7 and MCF-10A cell lines, which may not fully represent the *in vivo* tumour environments or account for factors such as immune response and tumour micro-environment heterogeneity. Single-wavelength PDT the PDT processes rely on a 690 nm semiconductor diode laser which might limit the applicability to other photosensitizers or wavelengths better suited for deeper tissue penetration. ROS dependency the effectiveness of PDT heavily depends on oxygen availability and photosensitizer localisation factors that are difficult to control uniformly in biological systems. Docking simulation of molecular docking study assumes rigid receptor structures and ideal binding scenarios. Real-world molecular dynamics (flexibility, solvation effects, etc.) are not fully captured. Limited structural comparison of the docking analysis is

benchmarked against colchicine (PDB 1SA0) as a reference, which may not comprehensively represent other relevant tubulin or protein targets. Hardware constraints of AutoDock Vina simulations were conducted on a Windows Intel® Pentium® Core i5-2450M CPU system. More advanced computational resources could provide faster, more accurate molecular modeling. Cell death pathway analysis while Caspase Glo-3/7 assays suggest the apoptotic pathways while other forms of cell death (e.g., autophagy, ferroptosis) are not extensively investigated.

CHAPTER 2

LITERATURE REVIEW

2.1 Principles of PDT

Photodynamic Therapy (PDT) is a non-invasive medical procedure that uses light, oxygen, and photosensitizing chemicals to specifically kill cancer cells. The process begins by administering a Photosensitizer (PS), which is a light-sensitive drug, into the patient's bloodstream. The drug tends to accumulate more in cancer cells than in healthy cells. After a specific period, a light of specific wavelength (generally within the infrared region, $\lambda \geq 600$ nm) is applied to the tumour area (Figure 2.1). This activates the PS, generating ROS, which then starts a cascade of biochemical events and causes the cell death of cancer cells, while sparing healthy surrounding tissues (Banerjee et al., 2017). PDT is a versatile and targeted therapy that continues to be a subject of research and development for various medical applications, with the potential for fewer side effects compared to traditional treatments. Additionally, it can be used repeatedly without causing significant resistance.

PDT has its limitations, such as a limited penetration depth of light, making it more suitable for superficial or localised tumours. Combination therapies, where multiple drugs are used simultaneously or sequentially, have also been investigated to improve treatment outcomes while minimising side effects. By combining drugs with different mechanisms of action, their effectiveness can be enhanced to overcome drug resistance (Figure 2.1).

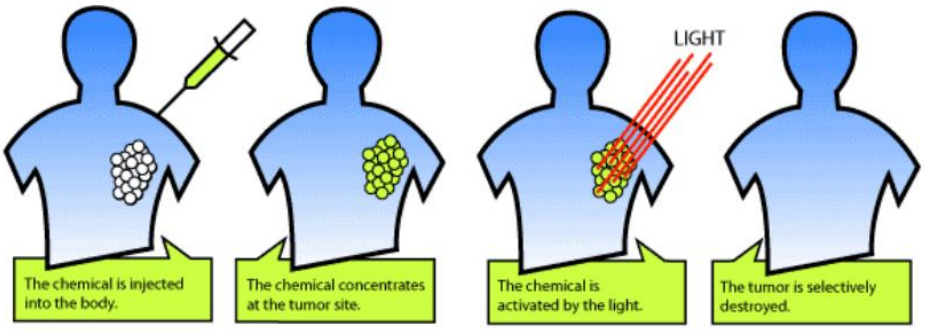


Figure 2.1 The process of PDT (Wyld et al., 2002).

2.1.1 Mechanism of PDT

The fundamental process of PDT is illustrated in Figure 2.2. (Chilakamarthi et al., 2017; Gunaydin et al., 2021). PDT is a therapy which utilises PSs agents in a dormant state till they are exposed to specific light (Moore et al., 2009). Their activity depends on the presence of oxygen. Activation of PS under light generates ROS, which is responsible for killing cancer cells (Castano et al., 2006). A low-energy light with a specific wavelength illuminates the singlet ground state (S0) PS, leading to the excitation of one electron in the PS to the higher-energy first singlet excited state (S1) due to opposing spins. The unstable and excited photosensitizer can relax to its ground state (S0) through non-radiative decay via internal conversion, photon emission (fluorescence), or by transferring energy to surrounding molecules to create cytotoxic species. An intersystem crossing that follows produces a triplet state. Reactive singlet oxygen ($^1O_2$) is produced when the triplet PS transfers energy to the triplet oxygen. Due to its high reactivity, singlet oxygen ($^1O_2$) can react with various biological substances, leading to oxidative damage and, ultimately, cell mortality. If the PS in its excited state directly interacts with a substrate like a cell membrane or a molecule, it can lead to processes involving hydrogen atom abstraction (hydrogen free radical) or electron transfer, resulting in the production of free radicals and radical ions in the

form of superoxide anions (O_2−•), hydroxide radicals (HO•), or hydrogen peroxide (H2O2). Reactive oxygen species (ROS) induce oxidative damage in the form of of biological lesions, which can result in cell death via apoptotic or necrotic pathways (Beharry et al., 2018; Martemucci et al., 2022). ROS play a crucial role in biological systems due to their chemical reactivity. Just like cancer cells, a consistent rise in ROS levels can promote the growth and survival of healthy cells. High levels of ROS can lead to oxidative harm to biological elements such as lipids, proteins, and DNA. Thus, it is crucial to maintain ROS homeostasis for the survival and proliferation of cells. Cancer cells are more susceptible to cell death when exposed to external stimuli that promote oxidative stress, unlike healthy cells. This event indicates an imbalance in redox homeostasis, after either increased ROS production or reduced ROS scavenging capacity due to impaired antioxidant system in the cancer cells (De Oliveira et al., 2012). Cells that have low amounts of ROS can uphold redox homeostasis by utilising an antioxidant defence system composed primarily of antioxidants (glutathione) and enzymes (superoxide dismutase, catalase, glutathione peroxidase, and glutathione S-transferase). This defense system can remove excess ROS and maintain stable ROS levels in normal settings (Trachootham et al., 2009; Hasanuzzaman et al., 2020).

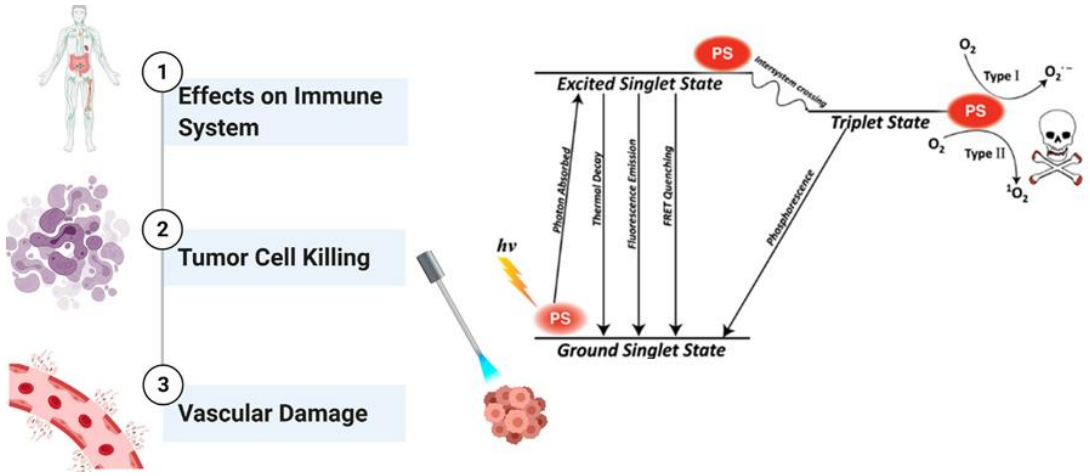


Figure 2.2 Basic mechanisms of action of PDT on tumours (Agostini et al., 2011; Gunaydin et al., 2021)

2.1.2 Classes of photosensitizers (PSs)

PSs are dyes, either natural or manufactured, that are light-sensitive and can absorb light within a certain wavelength range of 630 to 700 nm. They then convert this light into fluorescence emission to generate deadly ROS (Abrahamse & Hamblin, 2016). An essential factor for the effectiveness of photodynamic therapy is the PS's capability to activate fluorescence selectively in biological environments. Fluorescence brightness is a function that enables efficient fluorescence imaging. The optimal light absorption for a photosensitizer happens in the red spectrum (600-800 nm) because longer wavelengths allow for deeper tissue penetration (Lovell et al., 2010). Several endogenous and bodily tissues, such as haemoglobin and myoglobin, absorb light wavelengths below 600 nm, whereas water absorbs light wavelengths above 1000 nm. A photosystem absorbs light within the wavelength range of 600 to 1000 nanometers. Fast buildup and elimination of PS are required to reduce skin sensitivity to light and extend the time between doses. Furthermore, a key attribute of PSs that makes them beneficial for PDT is their increased accumulation in cancerous tissues as a result of their higher permeability and retention effect. This allows for gentle specific fluorescence, reactive oxygen species (ROS) generation, and light-induced damage to specific tissues.

Most light-sensitive PSs absorb light at wavelengths shorter than 700 nm. Even though visible light does not penetrate deep into tissues, the 380-780 nm light range is often used for PDT. PSs such as chlorine, bacteriochlorins, and porphyrins have different ranges of absorption, in which chlorine absorbs at 650 nm, bacteriochlorin at

710 nm and porphyrins at 430 nm. PDT is now considered a new treatment with cancer vaccines, virotherapy, and immunotherapy (Raschpichler et al., 2020; Cuenca et al., 2004). Since PDT shows a better ability to target cancer cells than surgery, chemotherapy, or radiation, with fewer side effects, it provides a good choice for many cancers. Furthermore, it provides a good option when surgery or radiation cannot be done. It involves a quick procedure (a shot of the drug, then a short burst of light), and it destroys tumours without hurting the nearby healthy tissue and provides fast healing (Agostinis et al., 2011).

Several important properties to take into account when designing PS are photophysical characteristics that focus on deep tissue penetration, strong absorption in the near-infrared or red region (620-850 nm) and minimal dye photobleaching decomposition due to light. Another set of properties are chemical attributes that emphasise efficient and high-yield synthesis, high quantum yield of $^1O_2$, triplet state formation with a high quantum yield, high triplet state energy, high singlet-to-triplet intersystem crossing efficiencies, and stable-characterised compounds with constant composition. The final properties are clinical characteristics, which encompass the least possible side effects such as photosensitivity of the skin and pain, being soluble in biological media, high retention and aggregation in sick tissue, minimal dark toxicity, and fast elimination from the body post-treatment. (Garland et al., 2009).

Even though PDT is a non-intrusive procedure for treatment of cancer, some of the drawbacks include normal cells being destroyed during the killing of tumour cells. This can be resolved by enhancing the selectivity of PSs within the tumour (Baskaran et al., 2018; Deng et al., 2021). A substantial amount of effort is still required to enhance this promising new cancer therapy. Various inorganic and organic compounds have been used as photosensitizing agents in photodynamic therapies for

the treatment of cancer. Some inorganic materials are chlorin e6, cadmium selenide, hydroxyapatite and hypocrellin A (Montaseri et al., 2021). Most of the organic PSs are built on porphyrin molecules because they possess several important photophysical, photochemical, and biological properties (Huang et al., 2015; Kou et al., 2017). Porphyrins are compounds with four interconnected pyrrole rings, which are usually hydrophobic. Due to their high level of conjugation, they are known to absorb visible light. Porphyrins are fluorescent in the dark and often non-toxic. They have a strong affinity for serum proteins, which increases their tumour-specificity (Allison et al., 2010). They are chemically stable, both as free compounds and as compounds bound to metals, while still being able to find tumours in living animals (Escudero et al., 2021).

2.1.3 Photodynamic Therapy (PDT) in breast cancer treatment

Over the past years, PDT has been the most promising therapy, in particular for breast cancer treatment. The use of organic compounds in PDT for breast cancer is a new avenue in breast cancer treatment. Several in vitro PDTs on breast cancer cells are summarised in Table 2.1. In the early drug discovery stage, the development of small-molecule drugs that exhibit pharmacological activity within an acceptable pharmacokinetic and toxicological criteria is a great challenge (Hughes et al., 2011). The relationship between the molecular structure and its biological activity can be understood by analysing the lipophilic, electronic, and steric properties of the functional groups. In PDT, 1,3,5-triarylpyrazolines have shown promising properties over traditional PSs (Ahmed et al., 2017; Jumaah et al., 2022b). However, the limitation of these compounds is their activation only under UV light, which raised concern about the potential harmful effects of UV light on human tissues, such as DNA

damage and skin-related issues such as premature ageing and cancer promotion. In contrast, conventional PSs often lack selectivity for targeting tumour cells, leading to unwanted accumulation in the skin and subsequent photosensitivity issues. Moreover, their absorption peaks fall within the region of visible light, which is freely absorbed by tissue chromophores which includes melanin, fat, and haemoglobin, limiting their effectiveness in deep-seated tumours (Ostańska et al., 2021).

Table 2.1 PDT on breast cancer in vitro.

Cell line	Photosensitizer (PS)	Wavelength & density	Reference
MCF-7, MDA-MB-231, MDA-MB-453, SkBr3, Hs578T	5-aminolevulinic acid 1 m	633 nm, 5 mW/cm^2, 3.0 J/cm^2	Palasuberniam et al. (2019)
MCF-7	5-aminolevulinic acid 0.5 and 1 nM	633 nm, 0.25 mW/cm^2	Abo-Zeid et al. (2018)
MCF-7	5-aminolevulinic acid 0.1 and 2.0 nM	633 nm, 16 mW/cm^2	Alkarakooly et al. (2018)
MCF-7	5-aminolevulinic acid 0.5 and 1 M	350- 450 nm, 86.5 mW/cm^2, 3.0 J/cm^2	Wyld et al. (2002)
MCF-7 MDA-MB-231 MCF-10A	Phenotiazonium salt of methylene 0.2 or 20 µM	640 nm, 4.5 J/cm^2	Dos Santos et al. (2017)
4T1	Methylene blue 0.8 or 16 µM	660 nm, 5 mW	Wu et al. (2018)
MCF-7	Methylene blue 85 µM	664 nm, 0.25 mW/cm^2	Khanal et al. (2014)
MDA-MB-231	Methylene blue curcumin 0.75 µM	Red 630 nm	Hosseinzadeh et al. , 2019
4T1, NIH, 3T3	Nano Methylene blue 85 µM	Led 660 nm	Dos Santos et al., 2018
MDA-MB-231	Methylene blue curcumin Methylene blue salicylic acid	Red led 630 nm, 30 mW/cm^2	Khorsandi et al. (2019a)
MCF-7	5-aminolevulinic acid 0.5 and 2 mW	633 nm, 0.25 mW/cm^2	Zakaria et al. (2014)
MCF-7	Chlorin 0.5 mg/ml	650 nm, 3 Mw/cm^2	Hayashi et al. (2015)

MCF-7	curcumin 0.15 µM.	650 nm, 86.5 mW/cm^2, 3.0 J/cm^2	Yang et al. (2017)
MCF-7	Curcumin	670 nm, 50 mW	Kamel et al. (2019)
4T1	Carrier free Curcumin	450 nm, blue 640 mW	Sun et al. (2019)
MDA-MB-231	Curcumin layered double hydroxide	465 nm, 30 mW/cm^2	Khorsandi et al. (2019)
MCF-7	Sulfonaic zinc phthalocyanine 0.25 µM.	681 nm, 4.53 mW/cm^2	Aniogo et al. (2017a)
MCF-7	5-aminolevulinic acid 0.5 mW	633 nm, 0.25 mW	Zakaria et al. (2014)

Table 2.1 (Continued)

Cell line	**Photosensitizer (PS)**	**Wavelength**	**Reference**
MCF-7	Methylene blue 1 mg	670 nm, 30 mW/cm^2	Kamel et al. (2019)
MDA-MB-231	Diiodo-squaraine 3.125 µM.	630 nm, 700 mW/cm^2	Fu et al. (2020)
MDA-MB-468	Chlorin 0.2 mg/mL	660 nm, 25 mW/cm^2	Seong et al. (2015)
4T1	Rose Bengal 230 µM.	Visible light, 3 mW	Borodziuk et al. (2020)
MCF-7	mitoxantrone 250 mW	660 nm, 75 mW	Khorsandi et al. (2019b)
MDA-MB-231	Methyl pyropheophenylchorin	Red light, 30 mW	Zhu et al. (2018)
MDA-MB-468 BT-549 HCC-1806	Indocyanine 15-38 µM.	450 nm, 25 mW/cm^2 1.14 J/cm^2	Zhao et al. (2020)

2.1.4 Pyrazoline Photosensitizer (PS) for activatable PDT

The increasing interest in utilising the bioactive heterocycle for cancer treatment has reported numerous pyrazoline structures as anticancer agents with great potential (Matiadis & Sagnou, 2020). The majority of the published research addressed mechanistic insights, PDT PSs, design methodologies, synthesis, in vitro and in vivo screening, and structure-activity correlations (SARs) (Nepali et al., 2014; Varghese et

al., 2017; Bansal et al., 2020). Pyrazoline compounds (Figure 2.3) stand out among existing organic compounds due to the significant research attention it has garnered (Jumaah et al., 2022c; Matiadis et al., 2020; Nehra et al., 2020; Tok et al., 2020). Pyrazoline derivatives have gained considerable attention as PSs for PDT. Among potential alternatives, pyrazoline dyes have been extensively employed as fluorophores owing to their favourable photophysical characteristics and biocompatibility.

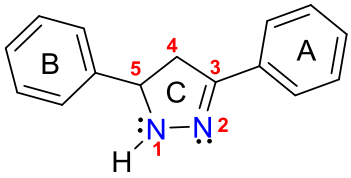


Figure 2.3 Basic structure of pyrazoline.

The design and synthesis of pyrazoline-based PSs with specific properties include minimal activity in their ground state and enhanced activity in their excited state upon light activation. The PSs can be conjugated or attached to specific targeting molecules, such as antibodies or peptides, which enable them to selectively accumulate in cancer cells. This enhances the specificity of the treatment (Matiadis & Sagnou, 2020). The PSs is administered through injection or topical application, depending on the location and type of cancer tissue. The PS accumulates in the cancer tissue over time due to its targeting strategy and its preferential retention in the cancer cells after which, it is activated using light at a specific wavelength. Upon light activation, the pyrazoline-based PS enters an excited state and interacts with oxygen molecules present in the surrounding tissue. This interaction leads to the generation of highly toxic ROS, such as $^1O_2$, which are toxic to cells. The ROS generated by the PS cause damage to nearby cells, leading to the destruction of cancer cells. After the PDT

treatment, the damaged tissue is cleared by the body's natural processes and goes through the recovery phase (Konan et al., 2002).

The advantage of using activatable PDT with pyrazoline PSs lies in its ability to minimise damage to healthy tissues until light activation occurs. This targeted approach reduces side effects and enhances the overall effectiveness of the treatment. However, concerns have been raised about their potential toxicity, despite their excellent performance as PSs. To address these concerns, a cell viability assay known as the MTT assay on MCF-7 and MCF-10A cells was conducted to study the drug conjugate efficacy in suppressing the proliferation of both cell lines.

The multi-stage operation of PDT is illustrated in Figure 2.4 (Hopper, 2000; Josefsen & Boyle, 2008). It comprises of administration of PS systematically or topically to the targeted area in the absence of light and it is left for a certain period which is known as the drug-light interval. During that period, the PS is selectively accumulated into the cancerous tissue. It is followed by illumination on the targeted cancerous tissues with the light of a specific wavelength for a particular duration, generating cytotoxic ROS. The cancerous tissues are eradicated as the targeted cancer cells are damaged via cellular apoptosis, necrosis or autophagy. The sequence of photochemical and photophysical processes resulted in PDT causes irreversible photodamage to the cancer cells (Sharman et al., 1999). Upon irradiation, the light-sensitive molecules of PS absorb photons and get excited from the ground state (S_o) to the short-lived excited singlet state (S_n). The excited PS relaxes to the triplet state (T_1) via non-radiative intersystem crossing. Portions of the excited PSs go back to the S_o by emitting fluorescence. Typically, the triplet state has a longer lifetime that enables interaction between PS and the surrounding environment. The triplet state can react through either Type I or Type II mechanisms. Type I involves hydrogen-atom

abstraction or electron-transfer between PS in the triplet state and a substrate, generating free radicals and radical ions. Meanwhile, the Type II mechanism is based on the energy transfer between PS in its excited triplet state and ground-state molecular oxygen ($^3O_2$), to yield singlet oxygen ($^1O_2$). The generated singlet oxygen species is extremely reactive, and it is generally accepted as the major cytotoxic agent responsible in interacting with the large number of biological substrates which results in biological effects (Josefsen & Boyle, 2008). Meanwhile, the Type I reaction is favoured in more polar environments or surroundings with low oxygen concentration (Ochsner, 1997). Both Type I and Type II reactions can occur simultaneously depending on the type of PS delivered and its surrounding environment. The overall effect of both reactions leads to oxidative damage and chain-reactions involving free radicals in the presence of oxygen. Thus, ultimately resulting in targeted cancer cell destruction (Banerjee et al., 2017).

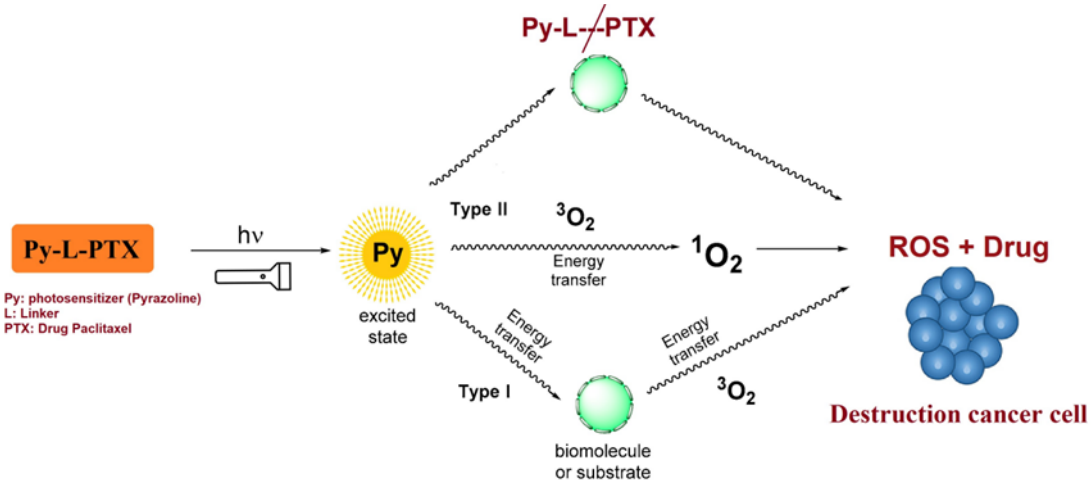


Figure 2.4 The multi-stage operation of PDT

In this therapeutic application, the primary role of PDT is to achieve cell death of unwanted cancer cells. The classical forms of cell death triggered by PDT are

classified into apoptosis, necrosis or autophagy, whereby they exhibit distinct morphological characteristics by activating particular signalling pathways. The cellular response to the photodamage relies on the concentration of PS, light dose applied, subcellular localisation of the PS and the type of the cells. The localisation of the PS strongly depends on the characteristics of the PS. Typically, it localises into subcellular organelles such as mitochondria, lysosomes, Golgi apparatus, endoplasmic reticulum (ER), and plasma membrane (Mroz et al., 2011). Traditionally, apoptosis has been considered as active cell death which is associated with activation of cysteine proteases, also known as caspases (Blank & Shiloh, 2007). As a programmed cell death, apoptotic cells are characterised by condensation of nuclear chromatin, cell shrinkage, nucleus fragmentation, membrane blebbing and segregation of cells into apoptotic bodies. Meanwhile, necrosis is known as a passive and unprogrammed form of cell death Necrotic cell death occurs as a consequence of extreme external conditions (high temperature, pressure, pH, mechanical stress) or extensive cellular damage caused by chemical processes (Fink & Cookson, 2005). Unlike apoptosis, necrosis typically does not require activation of any specific signalling pathways. This process is generally accompanied by swelling of organelles and cells, rupture of the plasma membrane and loss of cellular contents. Meanwhile, autophagy is a fundamental catabolic cellular mechanism that allows natural degradation process of dysfunctional and undesired cellular components to maintain cellular homeostasis during various stresses. This includes nutrient starvation, hypoxia, ROS, organelle damage and ER stress. Autophagy involves the lysosomal degradation of the cellular organelles and proteins (D'Arcy, 2019). In comparison with other cell death types, autophagy possesses the highest survival rate, followed by apoptosis and then necrosis.

The morphology of cells that undergo apoptosis, necrosis and autophagy are illustrated in Figure 2.5.

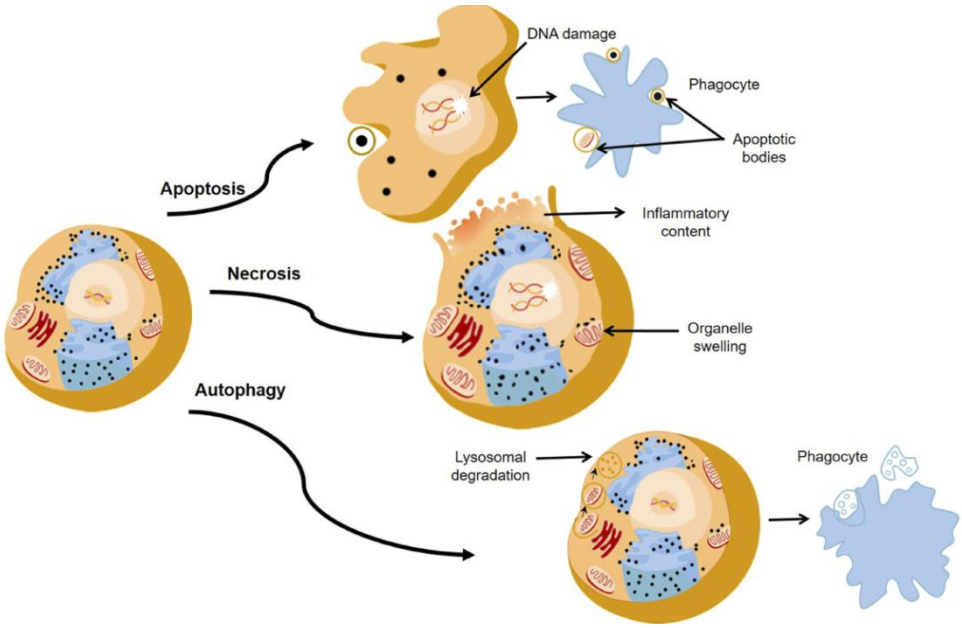


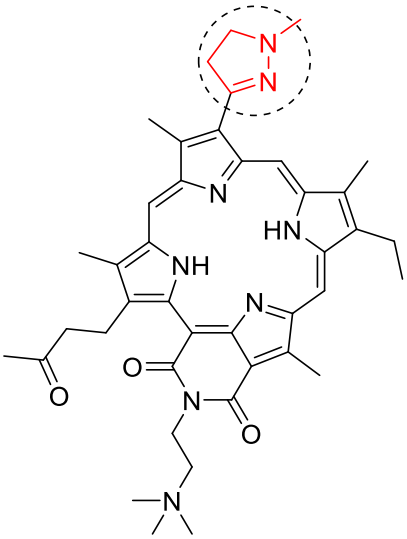
Figure 2.5 The morphology of cells undergoing apoptosis, necrosis and autophagy (Montaseri et al., 2020)

The literature indicates that PDT is successful at inducing apoptosis in many cancer cells, despite its ability to produce apoptosis or necrosis. Moreover, apoptosis is a multi-step cell death pathway. Apoptosis can be triggered by death receptors activation or mitochondrial cytochrome c release, known as extrinsic and intrinsic apoptosis, respectively (Jan & Chaudhry. 2019). Both pathways of apoptosis lead to activation of executioner caspase known as caspase cascade (caspase-3, 6 and 7) which subsequently execute apoptosis (Gougeon & Kroemer, 2003; Brentnall et al., 2013). The activated executioner caspase results in demolishing or cleaving of key structural proteins (nuclear lamins, cytoskeletal proteins) which leads to morphological and characteristic biochemical associated with dying cells. Besides, apoptotic bodies formed from the apoptotic cells are subsequently signalled for the clearance via

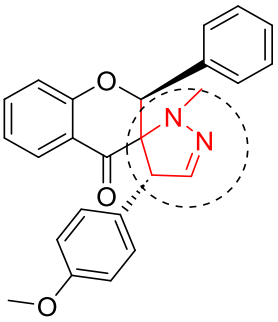
phagocytosis, limiting the release of their cellular constituents into surrounding interstitial tissues and therefore, reduce the occurrence of the inflammatory reaction. Meanwhile, during necrosis as the cellular membrane is damaged, it leads to the release of intracellular contents, thus inducing inflammation. Inflammation can cause further damage to the surrounding tissues and it is also detrimental to health (Yang et al., 2015).

2.1.5 Pyrazoline dyes as PSs

Pyrazoline dyes' appropriate photophysical characteristics and biocompatibility have led to their widespread usage as fluorophores (Adamus-Grabicka et al., 2020; Jumaah et al., 2022d). Cui et al. (2010) reported the use of purinimide-pyrazoline compounds and their polyoxometalate complexes as PSs in PDT for cancer treatment.



purinimide-pyrazoline



polyoxometalate

The 1,3,5-triarylpyrazolines displayed in Figure 2.6 have a π-system of fluorophore that can quench the fluorescence through photoinduced electron transfer

(PET) upon adding a cation, resulting in enhanced emission intensity. It can selectively destroy cancer cells and treat certain medical conditions (Fahrni et al., 2003; Cody et al., 2008; De Silva et al., 2009; Jumaah et al., 2022d). As illustrated with the orbital density plots, the most occupied molecular orbital (HOMO) is located in the 1-aryl ring and the least unoccupied molecular orbital (LUMO) is situated on the 3-aryl ring, which implies that the fluorophore moiety is made up of two aryl rings that electronically communicate through the pyrazoline π-system, while the third ring in the 5-position is decoupled electronically by an sp^3 hybridised carbon, which can be utilised as an electron-donating cation receptor for PET switching. Fluorescence can be quenched via PET by the fluorophore π-system, producing increasing emission intensity when the cation is coordinated by suppressing PET (Cody et al., 2008; Chaudhry et al., 2010; Mori et al., 2016; Wang et al., 2016).

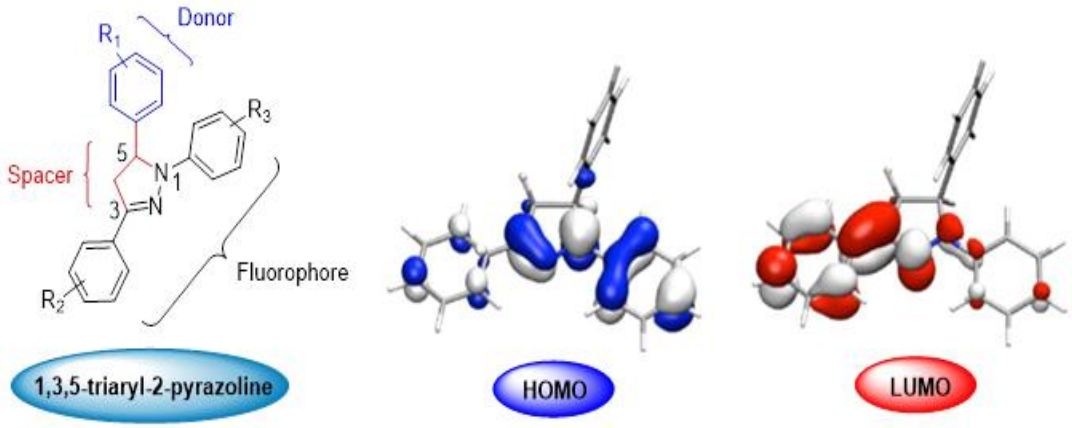


Figure 2.6 Electron transfer probes based on 1,3,5-triarylpyrazoline fluorophores. The HOMO and LUMO densities of 1,3,5-triphenylpyrazoline

Protonation-mediated PET is made possible by the addition of substituents at the 5-aryl unit that are pH-sensitive. The efficiency is influenced by the redox potential of the substituents and the excited singlet state of the pyrazoline core. The design of