Investigating the effects of Apaziquone (EO9) in solid tumour Cancer Treatment: A focus on NAD(P)H:quinone oxidoreductase (NQO1) expression in Skin, Nasopharyngeal and Cervical cancers.

A research project presented by

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for the degree of MSc by Research in Biomedical Sciences

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September 2024

Signature: _____ Submission date: 30/09/2023

The candidate confirms that the work submitted is their own and that appropriate credit has been given where reference has been made to the work of others.

I dedicate this paper to my ever-patient partner who supported me through this MRes journey. From supplying me with constant RedBulls, to pulling me away when I needed it most, I couldn't have done this without you. A Palliative care nurse by day and a caring partner by night thank you for being there when I needed you most this degree is as much yours as it is mine.

Acknowledgement

Firstly, I would like to express my deepest thanks to my supervisor, Dr. Owen Kavanagh. His unwavering support, insightful guidance, and encouragement throughout this degree has been invaluable. Owens expertise and dedication to my development as a researcher have profoundly shaped this work, and I am incredibly fortunate to have had him as my mentor and look forward to him supervising my PhD.

I am also immensely grateful to my secondary supervisor, Dr. Scott Dawson, whose practical expertise and problem-solving skills were crucial in troubleshooting the various challenges encountered in the lab. Scott's hands on approach and willingness to engage with the technical difficulties of this project provided much needed clarity and direction during some of the most critical moments. His support has been instrumental in ensuring that the research progressed smoothly.

My sincere thanks go to my external advisor, Dr. Roger Phillips, for his continuous support and wise counsel. Dr. Phillips has provided me with invaluable advice and direction, helping me to navigate the challenges of this project with confidence and clarity.

I would like to extend my appreciation to the talented undergraduate team: Hollie, Honor, Laura, and Scott: whom I had the pleasure of supervising. Your contributions to the project and insights have been crucial to the success of this project. It has been a joy to work alongside such dedicated and enthusiastic individuals.

I am also deeply thankful to the lab technicians, Andy, Thelma, Amy, and Ash, for their understanding, patience, and guidance in the laboratory. Your expertise and support have been vital in overcoming the practical challenges of this research, and I am grateful for the collaborative spirit you have brought to the lab environment.

Finally, I would like to acknowledge my family, friends and the MBs for their unwavering support and encouragement throughout this journey. Your belief in me has been a constant source of strength and motivation.

This thesis would not have been possible without the contributions and support of each one of you. Thank you.

Abstract

Solid tumour cancers pose a significant issue within the research and medical field due to the increase in resistance to conventional treatment options that are often attributed to the presence of hypoxia found within tumour microenvironments. This study investigates the therapeutic potential of EO9 (Apaziquone), a bio reductive alkylating agent previously explored for the treatment of NMIBC, focusing on its activation by the enzyme NAD(P)H:quinone 1 (NQO1). Prior research has established a link between NQO1 activity and EO9 cytotoxicity, this study builds on the idea by looking at the correlation between NQO1 activity levels and EO9's half-maximal lethal dose (LD50) across cervical, nasopharyngeal and melanoma solid tumour cancer cell lines. Additionally, the research explores the use of catalase inhibitors to enhance the therapeutic efficacy, potentially lowering the drug concentration required for an effective dose. These findings highlight a conjunctive approach that could improve the clinical outcome of EO9, specifically in tumours that express both elevated levels of NQO1 and catalase activity.

The results show that EO9 exhibits a lower LD50 in tumour cells with higher NQO1 expression, supporting the idea of the enzyme's role in EO9's bioactivation and selective cytotoxicity. The combined use of catalase inhibitors with EO9 was shown to further reduce the lethal dose of EO9 required for similar therapeutic effect, suggesting that inhibiting catalase can increase the killing efficacy of EO9, potentially minimizing its off-target effects and improving patient outcomes. The in vitro findings outlined in this study suggest EO9 has potential therapeutic effect in Cervical, Nasopharyngeal and Melanoma cancers and may also exhibit greater therapeutic effect when used in conjunction with catalase inhibitors such as 3-AT, Cannaflavin A, ECG+ and myricetin. This data supports the need for further investigation into whether similar therapeutic effects would be seen in other sarcoma, carcinoma, and lymphoma cancers.

In conclusion, this study provides compelling evidence for the potential of EO9, particularly in combination with catalase inhibitors, as a new therapeutic approach for solid tumour cancers. This innovative strategy could lead to more effective and less toxic treatments, offering hope for improved patient outcomes.

Contents

Abbreviations

Shorthand	Full hand
3-AT	3-Amino-1,2,4-triazole
5FU	fluorouracil
ABC	ATP-binding cassette
BRCA1/2	Breast cancer Gene 1/2
DCPIP	2,6-Dichlorophenolindophenol
dH2O	Distilled H2O
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ECG+	(+)-epicatechin gallate
EMT	epithelial mesenchymal transition
EO9	Apaziquone
FBS	Fetal Bovine Serum
GPx	Glutathione peroxidase
HIFs	Hypoxia-Inducible Factors
H_2O_2	Hydrogen Peroxide
I.V	Intravenous
LD50	Lethal Dose 50
MAPK	Mitogen-Activated Protein Kinase.
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAD	Nicotinamide adenine dinucleotide
NMIBC	Non-Muscular Invasive Bladder Cancer
NQO1	NAD(P)H:quinone oxidoreductase
Nrf2	Nuclear factor erythroid 2-related factor 2
O_2^-	superoxide anions
PBS	Phosphate Buffer Solution
ROS	reactive oxygen species
SOD	Superoxide dismutase
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organisation

1. Introduction

1.1: Solid tumour cancers

1.1.1 overview

Solid tumour cancers are a group of cancers defined by abnormal cells growth, proliferation, and formation of mass in different tissues and organs such as the brain, lung, prostate, colon, and breast. These tumours can be classified into two groups, malignant (cancerous) or benign (non-cancerous) (Pascual et al., 2018). Several key characteristics seen within solid tumour cancers include uncontrolled growth, metastasis, and invading into nearby tissues (Suhail et al., 2019), these factors are driven by genetic mutation and adaptations within cellular pathways that regulate cell division, apoptosis, and DNA repair (Fancello et al., 2019). Another key factor in tumour progression is the presence of a tumour microenvironment which plays a critical role in response to treatment (Marabelle et al., 2020). A tumour microenvironment often exhibiting a range of hallmarks (figure 1) to aid in the survivability of the tumour often leading to tumours outgrowing existing blood vessels and being unable to undergo adequate angiogenesis, contributing to the development of hypoxic regions, a condition of low oxygen levels within the tumour (C. Wang et al., 2024) in turn contributing to increased susceptibility of treatment resistance and further promotes the hallmarks, leading to a feedback loop continually contributing to the growth, survival and metastasis of tumours.

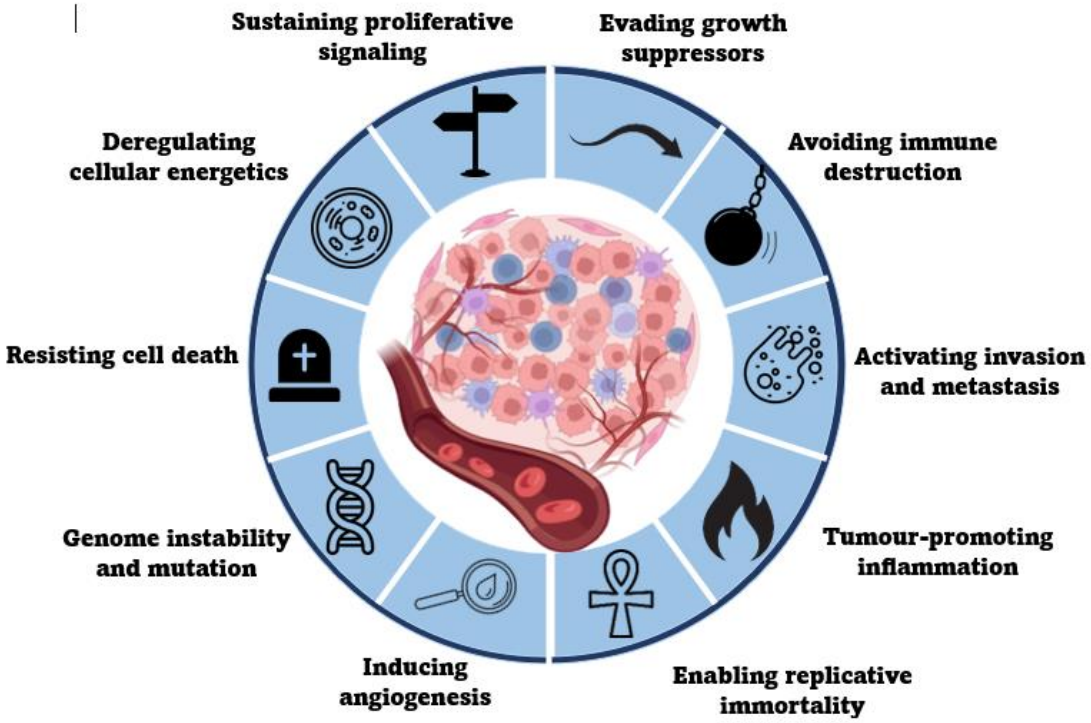

Figure 1: The Hallmarks of Cancer, adaptations of tumour microenvironment to sustain tumour survival (figure created in bio render)

Solid tumours present a significant burden on global health, accounting for most of the cancer related morbidity and mortality worldwide. According to the World Health Organization (WHO), cancer is one of the leading causes of death globally, with solid tumours such as lung, breast, colorectal, and prostate cancers among the most diagnosed types (Contiero et al., 2023). In 2020 alone, there were an estimated 19.3 million

new cancer cases and nearly 10 million cancer related deaths globally, with solid tumours forming a majority of these cases, with lung, prostate, skin, colon, and stomach cancer accounting for 42% of new cases alone (Sung et al., 2021). The global impact of solid tumour cancers extends beyond mortality rates, significantly affecting quality of life, healthcare costs, and economic productivity (Haier & Schaefers, 2022). The high incidence and mortality rates associated with these cancers pose a substantial challenge to healthcare systems, particularly in low- and middle-income countries where access to early detection and advanced treatments is often limited or difficult to obtain (Fitzmaurice et al., 2017).

1.1.2 issues with treatment of solid tumour cancers

Treatment of solid tumour cancers typically involves a combination of surgery, chemotherapeutic drugs, and radiotherapy, each aimed at removing or destroying cancerous cells (Kaur et al., 2023). Surgery is often the first line treatment, particularly for localized tumours, where the primary goal is to remove the tumour mass and surrounding tissue to minimize the risk of recurrence (Wyld et al., 2015).

Chemotherapy, while often effective at treating cancer, comes with its own set of issues, often resulting in severe side effect and challenges that affect both the treatment outcomes and patients' quality of life (Altun & Sonkaya, 2018). Cisplatin, commonly used in solid tumour cancer treatment, is known to have severe side effects such as nephrotoxicity (kidney damage), ototoxicity (hearing loss) and neurotoxicity due to its inability to specifically target cancerous cell on its own (Barabas et al., 2008). Radiotherapy, on the other hand, uses high energy radiation to damage the DNA of cancer cells, leading to cell death. It is particularly effective for locoregional therapy of solid tumours that surgery or chemotherapy alone will not successfully treat, therefore is often combined with surgery or chemotherapy to enhance therapeutic outcomes (Vinod & Hau, 2020)

Despite the widespread use of these treatments, several significant challenges limit their effectiveness in treating solid tumour cancers with one of the main limitations being drug resistance. Over time, cancer cells can develop mechanisms to resist the effects of chemotherapy, making these drugs less effective (Eisenberg et al., 2020). This resistance is caused by many factors, including genetic mutations, alterations in drug targets, and the activation of alternative survival pathways within the cancer cells (Assaraf et al., 2019). Cancer cells may change the expression or activity of drug transporters, such as ATP-binding cassette (ABC) transporters, which actively remove chemotherapeutic compounds out of the cell, lowering intracellular drug concentrations (Chien et al., 2025). As a result, many patients experience tumour recurrence and further neoplastic growth after a first response to treatment, which significantly complicates long term management and increases economic burden (Ward et al., 2021).

Another major challenge in the treatment of solid tumours is toxicity of the chemotherapeutics. Both chemotherapy and radiotherapy, while effective at targeting cancer cells, also affect normal, healthy cells, leading to a range of off target effects (Devlin et al., 2017). These can include nausea, fatigue, hair loss, immunosuppression, and enhanced susceptibility to infections; these effects are mostly due to non-specificity of the drugs on healthy tissues. In some cases, the toxicity of treatment is so severe due to a narrow therapeutic index that it limits the dose that can be safely administered, potentially compromising the overall efficacy of the treatment (Devlin et al., 2019). There are also documented cases of cumulative toxicity from repeated treatments leading to long term health issues, further complicating patient care (Sohl et al., 2009). Incomplete resection of the tumour is another critical challenge when it comes to cancer treatment (Miszczyk et al., 2017). Even with the most advanced surgical techniques, it can be difficult to remove all cancerous cells, especially in cases where the tumour has invaded surrounding tissues or spread to distant sites (Marsidi et al., 2022), some residual cancerous cells may be left behind in surgical sites,

leading to relapse and metastasis. Additionally, the presence of tumour hypoxia, areas of low oxygen within the tumour, can contribute to resistance to both chemotherapy and radiotherapy, as hypoxic cells are less responsive to these treatments due to them being able to go into a quiescent state, making drugs that target rapidly dividing cells less effective (Codony & Tavassoli, 2021).

In summary, while current treatment options for solid tumour cancers, surgery, chemotherapy, and radiotherapy, are essential components of cancer treatment regimes, their effectiveness is often limited by issues such as drug resistance, treatment related toxicity, and the challenge of achieving complete tumour removal. These limitations highlight the urgent need for new therapeutic strategies that can overcome these barriers and improve outcomes for patients with solid tumours.

1.1.3 need for new treatment options

The treatment of solid tumour cancers stays a formidable challenge, despite advancements in conventional therapies such as surgery, chemotherapy, and radiotherapy. These standard approaches often fall short due to inherent limitations mentioned above (Sorino et al., 2024). The presence of tumour hypoxic cores further complicates treatment, contributing to therapeutic resistance and the emergence of more aggressive cancer phenotypes. As a result, there is an urgent need for novel therapeutic approaches that can overcome these barriers and improve patient outcomes (Peterman et al., 2024).

One of the most promising directions for new cancer treatments lies in the development of therapies that target tumour specific vulnerabilities instead of healthy cells. Solid tumours show a range of unique characteristics, such as altered metabolic pathways, specific genetic mutations, and abnormal microenvironments, including hypoxia. These features can be exploited to create therapies that selectively target cancer cells while sparing normal tissues, thereby reducing toxicity and improving efficacy (Weber et al., 2020). For example, bio reductive drugs that are activated under hypoxic conditions or therapies that inhibit enzymes critical to tumour survival offer the potential to selectively eradicate tumour cells, particularly in cases where conventional therapies are ineffective (C. Zhang et al., 2022).

The importance of targeting tumour specific vulnerabilities cannot be overstated. By focusing on the distinct properties of cancer cells, novel therapies have the potential to not only enhance the precision of cancer treatment but also to overcome the significant challenges posed by tumour heterogeneity and resistance mechanisms (Gauthier & Yakoub-Agha, 2017). This approach is a critical shift towards more personalized and effective cancer care, addressing the unmet needs of patients with solid tumour cancers (Mohanty et al., 2019).

Given the complexity and heterogeneity of solid tumours, ongoing research is crucial to understanding the underlying mechanisms driving these cancers and to developing more effective, targeted therapies (Q. Wang & Tan, 2023). Advances in personalized medicine, immunotherapy, and molecular diagnostics hold promise for improving outcomes for patients with solid tumour cancers, but significant challenges stay, specifically in addressing therapy resistance, tumour recurrence, and the management of metastatic disease (Kaur et al., 2023).

1.2 EO9

1.2.1 Introduction of EO9 as a promising Solid tumour cancer treatment

Apaziquone (EO9) is a bio reductive alkylating agent that has shown promise as a targeted treatment for solid tumour cancers, due to its ability to exploit two key features of the tumour microenvironment: hypoxia and the overexpression of the enzyme NAD(P)H:oxidoreductase 1 (NQO1) (Caramés Masana & de Reijke, 2017; Phillips et al., 2017). Structurally derived from the indolequinone family, EO9 stays inactive under normal oxygen (normoxic) conditions, thereby minimizing its cytotoxic effects on healthy tissues. However, the hypoxic conditions prevalent within solid tumours Favor EO9's activation, allowing the drug to selectively target cancer cells (Li et al., 2021). This is facilitated by the elevated levels of NQO1 often found in many solid tumours, which is particularly important because NQO1 serves as the primary enzyme responsible for converting EO9 into its active hydroquinone form (Ma et al., 2014).

NQO1 overexpression is observed in a variety of tumour types, including those of the lung, colon, and breast, and serves as a critical determinant of EO9's tumour specificity (Joseph et al., 1994). The enzyme catalyses a two-electron reduction of EO9, transforming it into a highly reactive hydroquinone form, with a semiquinone intermediate, which then induces cytotoxic effects (see Figure 2). This reduction process not only activates EO9 but also ensures that the drug's cytotoxic activity is concentrated in tumour cells with high NQO1 expression, sparing normal cells and thereby reducing the likelihood of systemic toxicity (Yutkin & Chin, 2012). This targeted activation mechanism enhances EO9's therapeutic index, as it allows for selective killing of cancer cells while minimizing damage to healthy tissues.

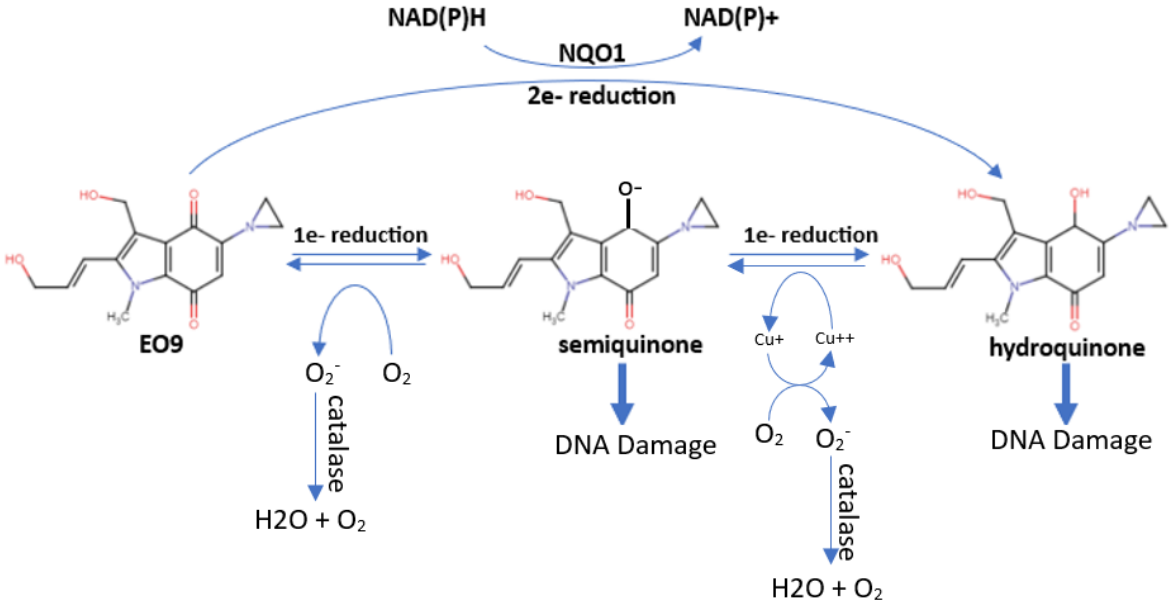

Figure 2 ***Mechanism of action of EO9,*** *the reduction to hydroquinone with the intermediate step of a semiquinone. The 2-electron reduction facilitated by NQO1 bypasses the intermediate step, both the semiquinone and hydroquinone contribute to direct DNA damage whereas their oxidation leads to an increase in ROS, that catalase breaks down into H_2O and O_2 (Figure created in bio render).*

Once activated, EO9 exerts its cytotoxic effects primarily by inducing DNA damage. The activated form of EO9 can form DNA cross links (Bailey et al., 1997). These cross links prevent the separation of DNA strands, thereby blocking DNA replication and transcription, both processes crucial for cell division and survival. As a result, cancer cells are unable to proliferate and are driven towards cell death, through apoptosis (Rycenga & Long, 2018). The reduction of EO9 is accompanied by the generation of reactive oxygen species (ROS), which are byproducts of the redox cycling process. These ROS further enhance the drug's cytotoxicity by causing oxidative damage to cellular components, including lipids, proteins, and nucleic acids, which amplifies the DNA damage and interferes with vital cellular pathways (Villalpando-Rodriguez & Gibson, 2021; Clarke, 1990). This accumulation of oxidative stress is particularly lethal to tumour cells, as it destabilizes various cellular processes, leading to further disruption and eventual cell death.

EO9's selective activation is intricately linked to the tumour's hypoxic microenvironment. Hypoxia, a characteristic feature of many solid tumours, triggers the stabilization and activation of hypoxia inducible factors (HIFs), which play a key role in the cellular response to low oxygen levels. HIFs upregulate a variety of genes involved in survival, angiogenesis, and metabolic adaptation, including NQO1 (Baek et al., 2005). The increased expression of NQO1 under hypoxic conditions enhances the reduction and activation of EO9 within tumour cells, providing a dual targeting mechanism that maximizes the drug's specificity. This makes EO9 particularly effective in targeting hypoxic tumour cells, which are often resistant to conventional therapies like radiation and chemotherapy (Sullivan et al., 2008).

In addition to its selective activation under hypoxic conditions, EO9's cytotoxicity is further amplified by the production of ROS within tumour cells. Tumour cells often possess elevated levels of antioxidant defences, such as glutathione and catalase, to protect against oxidative stress. However, the excessive ROS generated during EO9 activation can overwhelm these defences, particularly in cancer cells that are already under oxidative stress due to their rapid metabolism and hypoxic environment (Schieber & Chandel, 2014). This makes the tumour cells more susceptible to oxidative damage, pushing them toward a state of lethal oxidative stress. This feature of EO9's mechanism of action also opens potential for combination therapies;

for instance, combining EO9 with agents that inhibit antioxidant pathways could further increase ROS levels within the tumour cells, thereby enhancing the drug's cytotoxic effects.

In summary, EO9 is a promising bioreductive prodrug that selectively targets solid tumour cells through a dual mechanism of hypoxia driven activation and NQO1 mediated reduction. This dual targeting strategy allows EO9 to induce DNA damage and generate ROS specifically in cancer cells, thereby maximizing its therapeutic effects while minimizing toxicity to healthy tissues. By leveraging the hypoxic tumour environment and elevated NQO1 activity levels, EO9 presents a targeted approach to cancer therapy that addresses key challenges in conventional treatment, such as systemic toxicity and drug resistance. Further research is warranted to refine its clinical application and explore its potential in combination with other therapeutic agents, to fully exploit its capabilities in the fight against solid tumours (Arends & Alfred Witjes, 2020; Phillips et al., 2017).

1.2.2 NQO1 over expression in solid tumour cancer

NAD(P)H:Oxidoreductase 1 (NQO1) is a cytosolic enzyme that plays a crucial role in the cellular defence against oxidative stress by catalysing the two-electron reduction of quinone to hydroquinone (Dinkova-Kostova & Talalay, 2010). This reduction process helps to prevent the formation of reactive oxygen species (ROS) that can result from the one-electron reduction of quinones, which is typically catalysed by other enzymes like cytochrome P450 reductase. NQO1's activity is particularly significant in the context of cancer therapy because it can be exploited for the bioactivation of prodrugs, such as EO9 (Apaziquone) (C. Zhang et al., 2020).

NQO1 catalyses the two-electron reduction of EO9, converting it into a highly reactive, cytotoxic form. This bioactivation process is key to EO9's mechanism of action, as the reduced form of the drug can alkylate DNA, leading to cross linking, disruption of replication and transcription, and cell death (Smitskamp-Wilms et al., 1996).

The overexpression of NQO1 in many solid tumours is a critical factor that contributes to EO9's tumour selectivity (Choudry et al., 2001). In normal tissues, NQO1 activity levels are low, meaning that EO9 remains inactive, thereby reducing the risk of systemic toxicity (Verweij et al., 1994). In contrast, various tumour types, including those of the lung, pancreas, breast, and colon, exhibit significantly higher levels of NQO1, this overexpression is often a response to the oxidative stress characteristic of the tumour microenvironment and is regulated by the transcription factor Nrf2, which is frequently activated in cancer cells (Nioi et al., 2003). NQO1's ability to selectively activate EO9 in tumour cells provides a therapeutic advantage, as it allows the drug to target cancer cells with minimal impact on healthy tissues.

In summary, NQO1 is integral to the bioactivation of EO9, transforming it from an inactive prodrug into a potent DNA damaging agent within tumour cells. The enzyme's overexpression in many solid tumours underlies EO9's selective cytotoxicity, targeting cancer cells while sparing normal tissues. This tumour selectivity, driven by NQO1, enhances the therapeutic potential of EO9 and highlights the importance of NQO1 as both a therapeutic target and a biomarker in the treatment of solid tumours.

1.2.3 Hypoxia in solid tumour cancer and its effects on Quinone based drugs

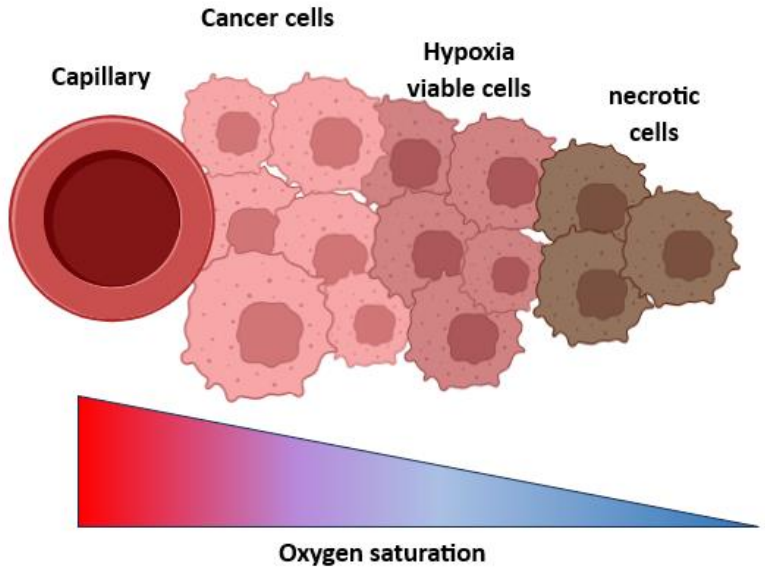

Figure 3: Tumour Oxygen saturation in relation to proximity to blood supply, showing the progression of hypoxia (figure created in Bio render)

Hypoxia is a condition that occurs in solid tumours where regions of the tumour mass experience significantly reduced oxygen levels, leading areas of necrotic cells due to low oxygen saturation and areas surrounding these necrotic cores being hypoxia able (figure 3). This occurs because the rapid and uncontrolled growth of cancer cells outpace the development of new blood vessels, leading to an insufficient supply of oxygen to all areas of the tumour (C. Wang et al., 2024). Additionally, the abnormal structure and function of the tumour vasculature contribute to irregular blood flow, further adding to oxygen deprivation in certain regions (Petrova et al., 2018). Hypoxia is a hallmark of solid tumours and is characterized by oxygen levels that are lower than those found in normal tissues. While normal tissues typically maintain oxygen levels around 5-10%, hypoxic regions within tumours can experience oxygen concentrations as low as 0.1-1% (Y. Zhang et al., 2021). This severe oxygen deficiency induces a range of adaptive responses in tumour cells, primarily mediated by hypoxia inducible factors (HIFs), which are transcription factors that regulate the expression of genes involved in survival, metabolism, angiogenesis, and metastasis under low oxygen conditions (Chiu et al., 2017).

Tumour hypoxia plays a critical role in promoting cancer progression and complicating treatment efforts (Lundgren et al., 2007). One of the most significant impacts of hypoxia is its ability to drive tumour aggressiveness. Hypoxic conditions within the tumour microenvironment stimulate the expression of genes that promote angiogenesis (the formation of new blood vessels), such as vascular endothelial growth factor (VEGF) (Finger & Giaccia, 2010). While this neovascularisation attempts to supply the tumour with more oxygen, the newly formed vessels are often structurally and functionally abnormal, leading to further hypoxia and creating a vicious cycle that supports continued tumour growth and survival (Liu et al., 2023). Hypoxia contributes to the selection of more aggressive cancer cell phenotypes. Hypoxic tumour cells often undergo metabolic reprogramming, shifting from oxidative phosphorylation to glycolysis, even in the presence of oxygen which is known as the Warburg effect (Semenza, 2017). This metabolic shift not only supports survival in low oxygen conditions but also enhances the cells' ability to metastasize to distant organs (T. Zhang et al., 2019). Hypoxia induced alterations in gene expression also promote epithelial mesenchymal transition (EMT), a process by which cancer cells gain migratory and invasive properties, further facilitating metastasis (Pastushenko et al., 2018).

In terms of treatment resistance, hypoxia poses significant challenges. Hypoxic tumour cells are inherently more resistant to radiotherapy, as the efficacy of radiation depends on the presence of oxygen to generate reactive oxygen species (ROS) that cause DNA damage and cell death. In the absence of sufficient oxygen, the therapeutic effects of radiation are markedly reduced (Sørensen & Horsman, 2020). Similarly, hypoxia induced alterations in cell survival pathways and drug metabolism can confer resistance to chemotherapy, making it difficult to achieve effective drug concentrations in hypoxic regions of the tumour (Kopecka et al., 2021).

However, there is an emergence of hypoxia favourable chemotherapeutic drugs that have shown higher efficacy in hypoxic conditions (Rauth et al., 1993). An example of this is Quinone based drugs such as Mitomycin c and Apaziquone, that favour hypoxia due to their break down into cytotoxic hydroquinone under hypoxic conditions, due to the lack of oxygen to reverse redox reactions intracellularly (Song et al., 2015). The reduced metabolites of quinone drugs, specifically hydroquinone's, are highly reactive and can cause DNA cross linking, generation of reactive oxygen species (ROS), and alkylation of cellular components as mentioned in section 1.21. reduced quinones rely on oxygen to return it to a stable form, thus the presence of hypoxia keeps the drug in its cytotoxic form, allowing it to exert a more potent therapeutic effect on hypoxic tumour cells (Iyanagi & Yamazaki, 1970).

Overall, the presence of hypoxia in solid tumours is a major barrier to effective cancer treatment. By promoting tumour progression, metastasis, and resistance to conventional therapies, hypoxia contributes to the poor prognosis often associated with solid tumour cancers. These challenges highlight the need for therapeutic strategies that specifically target hypoxic tumour regions or modulate the hypoxic response to improve treatment outcomes.

1.2.4 clinical history and limitations of EO9

One of the crucial issues faced when translating pre-clinical use of EO9 into patients was the lack of bioavailability to the site of the tumour, this was due to a multitude of factors including rapid metabolism, systemic availability, and hypoxic selectivity (Connors, 1996). Systemic delivery did not achieve the necessary concentration within bladder cancer only achieving a Cmax of 4.92uM (+/-2.16) when administered via I.V (Schellens et al., 1994) compared to intravesical administration which reached a Cmax of 347uM (Puri et al., 2006) due to it being rapidly metabolised within the blood stream. This led to EO9 going through further pre-clinical trials to refine the pharmacological profile before retrying clinical trials.

The preclinical studies of EO9 demonstrated its potent cytotoxic effects within Bladder cancer and have provided compelling evidence of its ability to induce DNA damage selectively in hypoxic tumour cells (Puri et al., 2006; Hendricksen et al., 2012). This preclinical success has led to early phase clinical trials aimed at evaluating EO9's safety, pharmacokinetics, and preliminary efficacy in human patients with solid tumours (Puri et al., 2006).

However more recent studies, including phase III trials, have yielded mixed outcomes. Studies such as those by Hendericksen et al. (2012) and Karsh *et al.* (2018) assessed EO9 in non-muscular-invasive bladder cancer (NMIBC) but reported limited benefit over conventional therapies such mitomycin C. these trials highlighted issues relating to dose frequency and variability in patient response with most side effects being grade 1 to 2. These findings highlight the need for more targeted therapies and refined patient selection, particularly in relation to NQO1 expression to establish EO9s full therapeutic potential.

While the clinical results have been mixed, primarily due to challenges related to drug stability and delivery, the concept of targeting hypoxic tumour cells through NQO1 activation remains a compelling strategy

(Phillips et al., 2013). Improvements in EO9's formulation, such as enhancing its stability and optimizing its delivery to tumours, are active areas of research aimed at maximizing its clinical utility.

1.2.5 comparison between EO9 and current use chemotherapeutics

EO9 is designed to be selectively activated in hypoxic tumour environments, offering a more targeted approach that could reduce toxicity to healthy cells (Robertson et al., 1994). Compared to other widely used chemotherapeutic drugs like Cisplatin and Doxorubicin, which have broad mechanisms of action and significant systemic toxicity, EO9 may present a lower risk of severe side effects, such as the nephrotoxicity associated with Cisplatin (Pabla & Dong, 2008) or the cardiotoxicity linked to Doxorubicin (Octavia et al., 2012).

EO9 is particularly effective when administered directly into the tumour site (intravesical therapy), allowing for high local concentrations while minimizing systemic exposure (Puri et al., 2006). This localized delivery can offer an advantage over drugs like Mitomycin C, which, even when given intravesically, can still cause systemic off target effects (Nissenkorn et al., 1981). Additionally, EO9's rapid cellular uptake can potentially lead to a quicker therapeutic response, making it effective against rapidly dividing cancer cells (Verweij et al., 1994b).

However, EO9's quick clearance from the body poses challenges. Unlike agents such as Mitomycin C or Gemcitabine, EO9 has a short half-life, which limits its duration in the bloodstream and necessitates frequent dosing to maintain therapeutic levels (McLeod et al., 1996). This characteristic is particularly problematic for systemic use, as it fails to provide adequate exposure to circulating tumour cells or metastases, restricting EO9's effectiveness primarily to direct delivery. The inconsistent hypoxic regions and variability in NQO1 enzyme expression within tumours result in unpredictable activation and effectiveness, limiting EO9's utility as a standalone therapy (Siegel et al., 2011).

In summary, while EO9 has advantages over some circulating drugs, its effectiveness depends on specific tumour parameters. Establishing a clear correlation between EO9 efficacy and intracellular factors, such as NQO1 activity, could improve pre-treatment strategies, allowing for correct dosing to minimize off-target effects and enhance cancer cell eradication.

1.3 combatting off target effects

1.3.1 catalase

Cells respond to an increase in reactive oxygen species through a range of protective mechanisms. Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen, such as superoxide ions, hydrogen peroxide and hydroxyl radicals, when intracellular levels of ROS rise beyond a normal level (oxidative stress) they can cause DNA, protein, and lipid damage (Srinivas et al., 2018). Redox sensitive transcription factors such as Nrf2 detect this change in oxidative state and activate antioxidant defence mechanisms (Xue et al., 2020), such as Superoxide dismutase (SOD) which Converts superoxide anions (O_2^-) to hydrogen peroxide (H_2O_2) (Hileman et al., 2001). Catalase which Converts hydrogen peroxide (H_2O_2) to water and oxygen (Glorieux & Calderon, 2017) and Glutathione peroxidase (GPx) which Reduces hydrogen peroxide and lipid peroxides using glutathione as a co factor (Chen et al., 2016).

Catalase plays a crucial role in cancer progression due to its role in controlling the levels of ROS within tumours, catalase is an enzyme that breaks down hydrogen peroxide (H_2O_2), a major ROS, into water (H_2O) and oxygen (O_2) (Glorieux & Calderon, 2017). Tumour microenvironments often up regulate catalase to protect cells from oxidative damage and apoptosis, ensuring survival (Hemachandra et al., 2016). Thus, lowering of ROS levels in the tumour helps protect cancerous cells from immune detection and destruction.

ROS plays a role in activating signalling pathways that promote cell proliferation, such as the MAPK and PI3K/AKT pathways (Averill-Bates, 2024). Low levels of ROS can stimulate these pathways, leading to enhanced growth and survival of cancer cells, so catalase's regulation of H_2O_2 ensures that ROS levels remain in a range that supports these pro-oncogenic signalling pathways without causing lethal damage to the cells (Martindale & Holbrook, 2002).

As seen in figure 2 the redox reaction of quinones causes ionisation of oxygen molecules that lead to the increased formation of hydrogen peroxide, catalase is an essential enzyme in breaking this molecule down to counteract potential DNA damage. This in turn would lower the effectiveness of EO9 as it would only rely on direct cross link DNA damage as opposed to indirect damage through ROS production

1.3.2 catalase inhibition and current use in chemotherapy

Catalase inhibition is a promising strategy in cancer therapy due to its potential to increase reactive oxygen species (ROS) in cancer cells, leading to heightened oxidative stress and cell death (Majumder et al., 2017). Many cancers overexpress catalase to counteract ROS and avoid apoptosis, allowing them to resist treatments like chemotherapy and radiotherapy, which rely on ROS mediated damage as discussed above (Glorieux et al., 2011). By inhibiting catalase, ROS levels can rise beyond cellular tolerance making cancer cells more susceptible to treatment (de Oliveira et al., 2016).

Research, such as the study by Xiao et al. (2015), demonstrates that catalase reduces oxidative stress caused by ionizing radiation in cancer cells (Xiao et al., n.d.). This protective effect indicates that catalase helps tumour cells counteract ROS inducing therapies, suggesting that catalase inhibitors could enhance treatment efficacy by preventing ROS breakdown.

Catalase inhibitors such as 3-amino-1,2,4-triazole (3-AT) are being used to enhance the cytotoxicity of amyloid beta peptide damage in myeloma cell lines. A study done by Milton et al (2001) showed a significant ($p<0.05$) increase in cellular death when 3-AT was used in conjunction with Aβ 1-42 and 25-35 compared to the Aβ on its own, the research then looks at 3-ATs, indicating that inhibiting catalase can improve the effectiveness of chemotherapeutic drugs when used in conjunction (Milton, 2001).

While catalase inhibition shows promise, challenges remain in developing selective inhibitors that target tumour cells without harming normal tissues. Future research focuses on developing potent inhibitors, novel delivery systems, and combination therapies to exploit this strategy effectively in cancer treatment.

2. Aims and objectives

2.1 Primary Aim

The primary aim of this study is to investigate the potential of EO9 (Apaziquone) as a targeted treatment option for solid tumour cancers. Given the current limitations of existing therapies such as drug resistance, toxicity, and incomplete eradication of tumours, there is a need for novel approaches that can selectively target cancer cells while minimizing harm to normal tissues. EO9, a bio reductive alkylating agent, presents a promising therapeutic candidate due to its selective activation in hypoxic tumour environments and its reliance on the enzyme NAD(P)Oxidoreductase 1 (NQO1) for bioactivation. This study aims to explore EO9's efficacy in the context of solid tumour survival, particularly focusing on enhancing its therapeutic effect by inhibiting oxidative stress pathways, specifically the catalase pathway, within tumour cells.

2.2 Specific Objectives

Objective 1: To Evaluate the Levels of NQO1 in Solid Tumour Cancers and Correlate with EO9's LD50

The first objective of this study is to assess the expression levels of NQO1 in various solid tumour types and attempt to correlate these levels with the lethal dose 50 (LD50) of EO9. NQO1 plays a critical role in the bioactivation of EO9, converting it into its cytotoxic form within the tumour microenvironment. By correlating NQO1 activity levels with the LD50 of EO9, this study seeks to determine whether higher NQO1 expression is associated with increased sensitivity to EO9, which would support the enzyme's role as a biomarker for patient stratification and treatment planning. The findings from this objective will help to identify tumour subtypes that are most likely to benefit from EO9 therapy and could inform the development of more personalized treatment strategies.

Objective 2: To Investigate Catalase Inhibitors as a Strategy to Reduce the Therapeutic Dose of EO9

The second objective is to explore the potential of catalase inhibitors to enhance EO9's efficacy, thereby reducing the therapeutic dose required for effective tumour cell killing. Catalase is an antioxidant enzyme that mitigates oxidative stress by breaking down hydrogen peroxide, a reactive oxygen species (ROS) generated during EO9 activation. By inhibiting catalase, this study hypothesizes that the accumulation of ROS within tumour cells can be increased, leading to heightened oxidative stress and enhanced cytotoxicity at lower doses of EO9. This objective involves testing various catalase inhibitors in combination with EO9 *in vitro*, assessing their impact on ROS levels, tumour cell viability, and EO9's LD50. The goal is to determine whether catalase inhibition can effectively lower the required dosage of EO9, thereby minimizing its off-target effects and improving its therapeutic index.

Objective 3: To identify optimal Catalase inhibitor

The third objective focuses on evaluating the viability and effectiveness of known catalase inhibitors *in vitro* as potential adjuvants to EO9 therapy. This objective involves screening a range of catalase inhibitors, including established pharmacological agents and novel compounds, to assess their ability to inhibit catalase activity and potentiate EO9's cytotoxic effects in cancer cell lines. Key parameters to be measured include catalase activity, independent cell viability, and effective use with EO9. The study aims to identify the most promising catalase inhibitors that can be combined with EO9 to enhance its efficacy, reduce required doses, and achieve selective tumour targeting with minimal impact on healthy cells.

The successful completion of these objectives will provide critical insights into the mechanisms underlying EO9's selective cytotoxicity and the potential for enhancing its therapeutic effectiveness through combination strategies. By identifying key biomarkers such as NQO1 and exploring the role of oxidative stress modulation, this study aims to contribute to the development of more effective and safer treatment options for patients with solid tumour cancers.

3. Methods and materials

3.1 Cell Culture

Cell Lines

The human cancer cell lines A431 Skin Melanoma (ATCC no. CRL-1555), FaDu Nasopharyngeal Carcinoma (ATCC no. HTB-43), C33a Cervical Carcinoma (ATCC no. HTB-31), and SiHa Cervical Carcinoma (ATCC no. HTB-35) were used to represent different solid tumours. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and 1% penicillin streptomycin. Cells were maintained in adherent T75 cell culture flasks at 37°C in a humidified atmosphere with 5% CO_2. For experiments, cells were seeded at a density of 1x10^4 cells in 200uL of complete media per well in 96-well plates.

3.2 Measurement of NQO1 Activity

Preparation of Cell Cultures

Adherent Cells were trypsinised to remove them from a stock flask, and the cell number was determined using a haemocytometer. Approximately 1x10^4 cells were inoculated into T25 flasks containing 10 mL of complete media and incubated at 37°C until the adherent cells reached late exponential growth phase (equal to or greater than 90%), typically over 5 days.

Harvesting Cells for Enzyme Assays

To determine enzyme activity levels; cell supernatants need to be harvested by sonication. When the cells were ready for the experiment, the monolayer was washed extensively with phosphate buffered saline (PBS). Two millilitres of homogenization buffer (0.25M Sucrose) were added to prevents osmotic shock and helps preserve the integrity of organelles and subcellular vesicles, the cells were then scraped from the flask. The cell suspension was transferred into a 25 mL universal tube and placed on ice for 5 minutes to bring to 4°C. Cells were then sonicated on ice with three bursts of 20 seconds, with 30 second intervals between bursts. The resulting cell homogenates were used immediately in the NQO1 enzyme assay.

NQO1 Enzyme Assay

The activity of the NQO1 enzyme was measured using a spectrophotometric method that involved monitoring the dicumarol sensitive reduction of 2,6-dichlorophenolindophenol (DCPIP) by NQO1 in the presence or absence of an NQO1 inhibitor, dicumarol. To perform this assay, several cell supernatant solutions were prepared in advance. A 10 mM nicotinamide adenine dinucleotide (NAD) + hydrogen (H) solution was freshly prepared daily by dissolving 6.63 mg of NADH in distilled water (dH2O). For the DCPIP solution, a 2 mM concentration was made by dissolving 0.536 mg of DCPIP in dH2O, and this solution was stored at 4°C for up to one week to maintain its stability. Additionally, a 1 mM dicumarol solution was prepared by dissolving 0.336 mg of dicumarol in 0.1N NaOH, and this solution was also stored at 4°C for up to one week to preserve its activity. These solutions were essential for accurately measuring NQO1 activity through spectrophotometric analysis.

Assay Conditions

The reaction was performed in 1 mL cuvettes, with a carefully measured combination of components to ensure precise measurement of NQO1 activity. The reaction mixture included 0.89 mL of reaction buffer, consisting of 50 mM Tris at pH 7.4 and containing 0.7% w/v bovine serum albumin (BSA). To this, 50 µL of the sample, 20 µL of 10mM NADH, 20 µL of 2mM DCPIP, and 20 µL of either 1mM dicumarol or buffer were added. The reaction was initiated by adding 20 µl NADH (final concentration 200 µM) and mixing thoroughly using repeat pipetting to ensure even distribution of the reagents. Once initiated, the change in absorbance at 600 nm (ΔA/min) was monitored spectrophotometrically over a 30 second period. To determine the

dicumarol sensitive reduction of DCPIP, the ΔA/min in the presence of dicumarol was subtracted from the ΔA/min in its absence, allowing the calculation of enzyme activity.

Measurement of Protein Concentration

Protein concentration in the samples was determined using the Bradford assay according to a standard protocol.

Determination of Specific Enzyme Activity

The specific activity of NQO1 was calculated using the following formula:

Specific activity = ΔA/min / ε x P

where ΔA/min is the dicumarol sensitive rate of reduction of DCPIP, ε is the molar extinction coefficient for DCPIP (21 mM/cm), and P is the amount of protein in the cuvette in mg. Each assay was performed in triplicate, and the mean ± standard deviation was reported.

3.3 EO9 Sensitivity Assay

Seeding Cells

Cells were trypsinized from T25 flasks at 80-90% confluency and resuspended in complete media. Cells were counted using a haemocytometer, and an appropriate volume of the cell suspension was diluted to achieve a final density of 1x10^4 cells per well in 96-well plates. Cells were incubated for 24 hours at 37°C with 5% CO_2 to adhere to the surface.

EO9 Treatment

A two-fold serial dilution of EO9 was prepared, starting from a 1000uM stock solution (100-0.001uM). After checking that cells were evenly attached, the media in each well was carefully removed, and 200 µL of the EO9 dilution series was added to the appropriate wells for 1 hour at 37°C. After incubation, the EO9 containing media was gently removed, and fresh complete media was added to each well then placed into the incubator for 1 hour to replicate treatment conditions in patients, as seen in the use of topical Imiquimod (Aldara, Zyclara) and Tirbanibulin (Klisyri) in treating actinic keratosis (Sendín-Martín & Conejo-Mir Sánchez, 2023).

MTT Assay

The purpose of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is to measure cell viability by assessing the metabolic activity of living cells, MTT is reduced by mitochondrial dehydrogenase to form formazan crystals, these crystals when dissolved in DMSO form a blue/purple dye, the optical density of this dye directly correlates to the number of viable cells. MTT reagent was prepared fresh in complete media to a final concentration suitable for the assay (5 mg/mL) centrifuged to remove excess particles and protected from light. After removing the media from the wells, 200 µL of MTT complete media was added and cells were incubated for 4 hours at 37°C to allow for intracellular incorporation of the dye. Following incubation, plates were centrifuged at 1000g for 5 minutes, and the media was carefully dispensed. MTT Formazan crystals were dissolved by adding 150 µL of DMSO to each well. Absorbance was read at 540 nm using a Varioskan™ LUX multimode microplate reader, with a shake step added before reading to ensure crystal solubility.

3.4 Catalase Inhibition Assay (Aebi Method)

Catalase Inhibitors

The Purpose of the Aebi catalase inhibition assay was to assess the in vitro effects of known catalase

inhibitors had on Bovine catalase to validate their use in catalase inhibition. The catalase assay was conducted by monitoring the decrease in absorbance of H_2O_2 by spectrophotometric analysis using 7305 UV-VIS Spectrophotometer, 198-1000nm (JENWAY) at 240 nm in quartz cuvettes to stop interference. The decrease in absorbance of H_2O_2 directly correlates with the decomposition via catalase (Aebi 1984). The catalase inhibitors (Table 1) were tested in a range of concentrations: Cannflavin A was tested at concentrations between 100nM and 2000nM, (+)-epicatechin gallate (ECG+) was tested from 10nM to 1000nM, 3-AminoTriazole (3-AT) was tested between 10nM and 500nM, and Myricetin was tested at concentrations ranging from 1.0 to 100nM. All inhibitors were prepared from dry powder stocks, under sterile conditions, with each stock dissolved to a concentration of 10µM in dimethyl sulfoxide (DMSO) through a microfilter before further dilutions were made to achieve the desired working concentrations for testing. Storage of prepared Stock solution were all kept at –20°C, ECG+ was kept at -80°C

Catalase	Type	Reported IC50 (µM)	Catalase source	reference
3-Amino Triazole	Heterocyclic	0.013 ± 0.002	*trypanosomatid*	(Chmelová et al., 2022)
Epicatechin Gallate	polyphenol	0.029 ± 0.002	Bovine	(Krych & Gebicka, 2013)
Myricetin	Flavonoid	0.014 ± 0.001	Bovine	(Krych & Gebicka, 2013)
Cannflavin A	Flavonoid	NA	NA	NA

Table 1 ***Catalase inhibitor used in this study****: 3-AminoTriazole, Epicatechin Gallate, Myricetin and Cannflavin A. Their respective known IC50 in catalase inhibition, Cannflavin A is a derivative of cannabinoids, how its own specific inhibition of catalase has not previously been recorded*

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Reagent preparation

The phosphate buffer (50 mM, pH 7.0) was prepared by mixing solutions of KH_2PO_4 and $Na2HPO_4.2H_2O$ in a 1:1.5 ratio. The assay was run at 4°C to stabilise the enzyme. A 30 mM solution of H_2O_2 was freshly prepared by diluting a 30% hydrogen peroxide stock in the phosphate buffer. Bovine catalase (20,000units/mg) was prepared as a 10 mg/mL solution in cold phosphate buffer, mixed with glycerol to permit storage at -20°C without enzyme denaturation, and stored at -20°C.

Procedure

The reaction setup included a blank containing 4 mL of phosphate buffer and a sample containing 40 µL of catalase, 2000 µL of assay buffer, and 2000 µL of 30 mM H_2O_2 (14.9mM final H_2O_2 concentration). Absorbance was recorded at 240 nm from 0 to 60 seconds. The catalase activity was calculated by determining the rate of H_2O_2 decomposition, based on the changes in absorbance over time.

3.5 Combination Cytotoxicity Assay

Assay Setup

A combination of EO9 and catalase inhibitors was tested in a cytotoxicity assay to examine the effects of catalase inhibition in conjunction with a cytotoxic agent. A 10-fold serial dilution of EO9 (10mM) was prepared at 100 µM, and three concentrations of catalase inhibitors (10-fold dilution) up to 25 µM was prepared. Both EO9 and inhibitors were added to the 96-well plates containing pre seeded cells, followed by incubation for 4 days.

Data Analysis

Cell viability was assessed using the MTT assay as described above. The results were analysed to determine the combined effect of EO9 and catalase inhibitors on cell viability, focusing on any synergistic or additive effects.

3.6 Statistical Analysis

All experiments were performed in triplicate, and the resulting data were expressed as mean values ± standard deviation. To assess statistical significance between groups, an independent two-tailed Student's t-test was employed. This test was applied to compare specific conditions, including NQO1 activity levels between different cell lines, the sensitivity of EO9 in the presence and absence of catalase inhibitors, and cell viability in treated groups versus control groups. A p-value of less than 0.05 was considered statistically significant, indicating that differences between the compared groups were unlikely due to chance.

4. Results

4.1 Cytotoxic Effects of EO9 Against Solid Tumour Cell Lines

To determine the cytotoxic effects of EO9 on different solid tumour cell lines, the following tissue types and cell lines were used: SiHa (cervical cancer), FaDu (nasopharyngeal carcinoma), A431 (skin melanoma), and C33a (cervical carcinoma) cells. Cells were treated with EO9 for 1 hour, followed by 4 days of incubation in fresh media. The anti-cancer efficacy of EO9 was assessed by determining the LD50 for each cell line, which represents the concentration required to kill 50% of the cells.

Among the four cell lines tested, the cervical (SiHa) cells were the most sensitive to EO9, with an LD50 of 0.15µM (+/-0.008µM) (figure 4 - SiHa). This indicates that even at low concentrations, EO9 is potent in killing SiHa cells. The second cervical (C33a) cell line, was the least sensitive to EO9, with an LD50 of 33.98µM (figure 4- C33a). The Nasopharyngeal (FaDu) cell line exhibited moderate sensitivity to EO9, with an LD50 of 0.36µM (+/-0.02µM) (figure 4- Fadu). While less sensitive than SiHa cells, the low LD50 still suggests that EO9 has a cytotoxic effect on nasopharyngeal carcinoma cells. The skin melanoma (A431) cells showed lower sensitivity to EO9, with an LD50 of 1.83µM (figure 4- A431).

These results demonstrate that EO9 is effective against solid tumour cancers, though its efficacy varies significantly depending on the cell line. The next question is to determine is this sensitivity proportional to NQO1 activity levels.

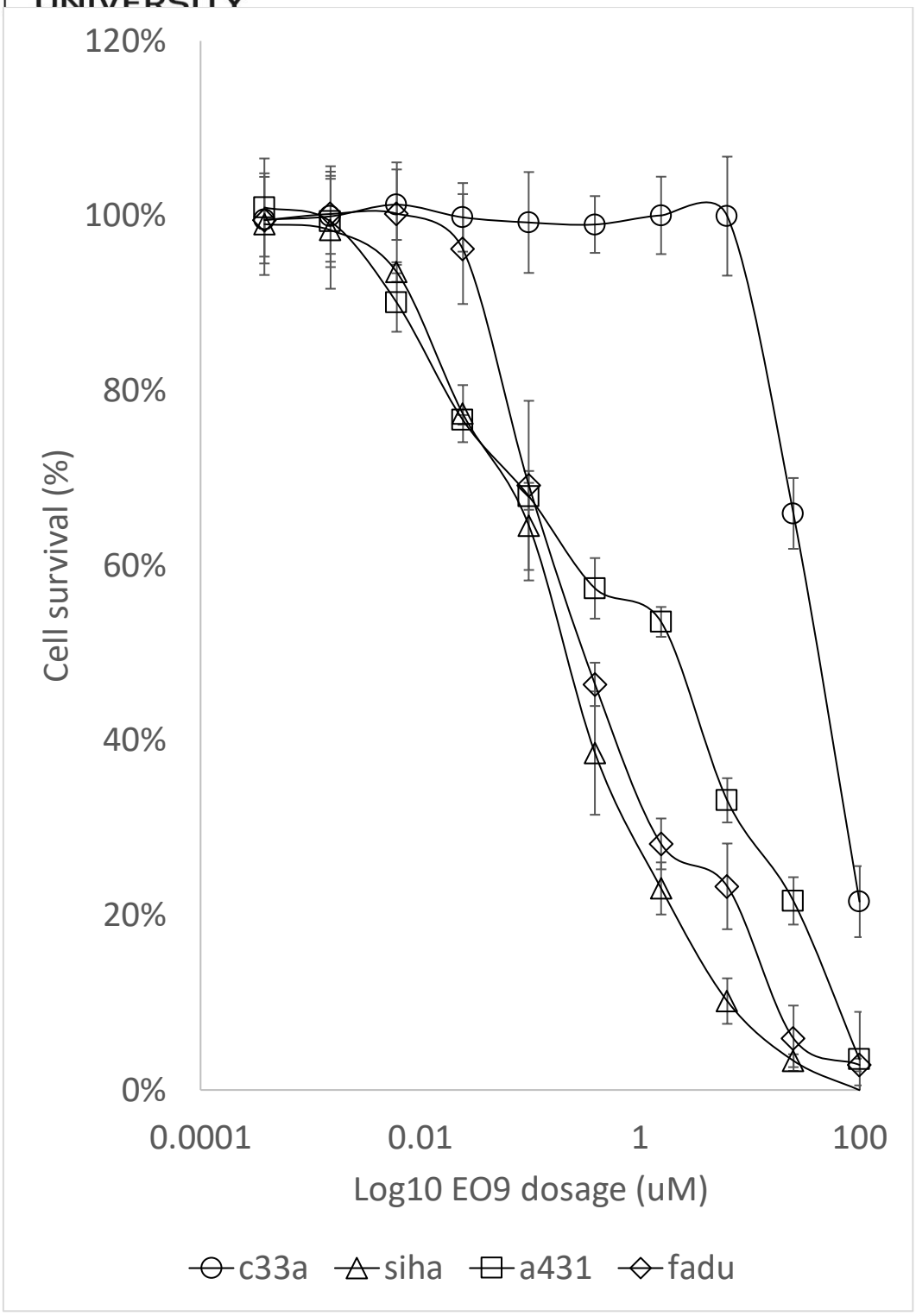


*Figure **4 dose-response curves for the cytotoxic effect of EO9 on cell survival** across four different human solid cancer cell lines: C33a (cervical carcinoma), SiHa (cervical carcinoma), A431 (skin melanoma), and FaDu (nasopharyngeal carcinoma). Cells were treated with 10-fold serial dilutions of EO9 (0.001-100μM), and cell survival was assessed after 72 hours using the MTT assay. The x-axis represents the logarithmic scale of EO9 concentration (μM), while the y-axis shows the percentage of cell survival relative to untreated controls. Data points represent the mean ± standard deviation from three independent experiments.*

4.2 NQO1 Specific Activity in Solid Tumour Cell Lines

Given the known role of NAD(P)Hoxidoreductase 1 (NQO1) in the activation of EO9, the specific activity of NQO1 was measured in the same solid tumour cell lines used in the cytotoxicity assays to determine if there is a correlation between NQO1 activity and EO9 sensitivity. This was done via spectrophotometric DCPIP reduction assay, in which the reduction directly corresponds with the activity of NQO1, this change in absorbance was then used to calculate the specific enzyme activity of NQO1 by measuring the protein expression in all cell lines and using the formula seen in Section 3.2.

The specific enzyme activity of NQO1 was assessed across four different cell lines, revealing variations in activity levels (Figure 5). In Cervical cancer (SiHa) cells, the NQO1 activity was the highest among all cell lines, at 0.0431 µmol/mg/min, suggesting a high presence of this enzyme in these cells. Nasopharyngeal cancer (FaDu) cells exhibited a moderate level of NQO1 activity at 0.0258 µmol/mg/min, indicating a moderate expression of the enzyme. In contrast, Skin melanoma (A431) cells had a much lower NQO1 specific activity, measured at 0.0065 µmol/mg/min, reflecting a reduced enzyme presence. Cervical cancer (C33a) cells had no detectable NQO1 activity, with a recorded value of 0 µmol/mg/min. A statistically significant difference in NQO1 specific activity was observed between the two Cervical cancers SiHa and C33a, with a p-value of 0.0004, indicating a substantial difference in enzyme activity between these two cell types.

The observed differences in NQO1 activity across these cell lines correspond closely with the observed differences in EO9 sensitivity, reinforcing the importance of NQO1 activity in mediating the cytotoxic effects of EO9.

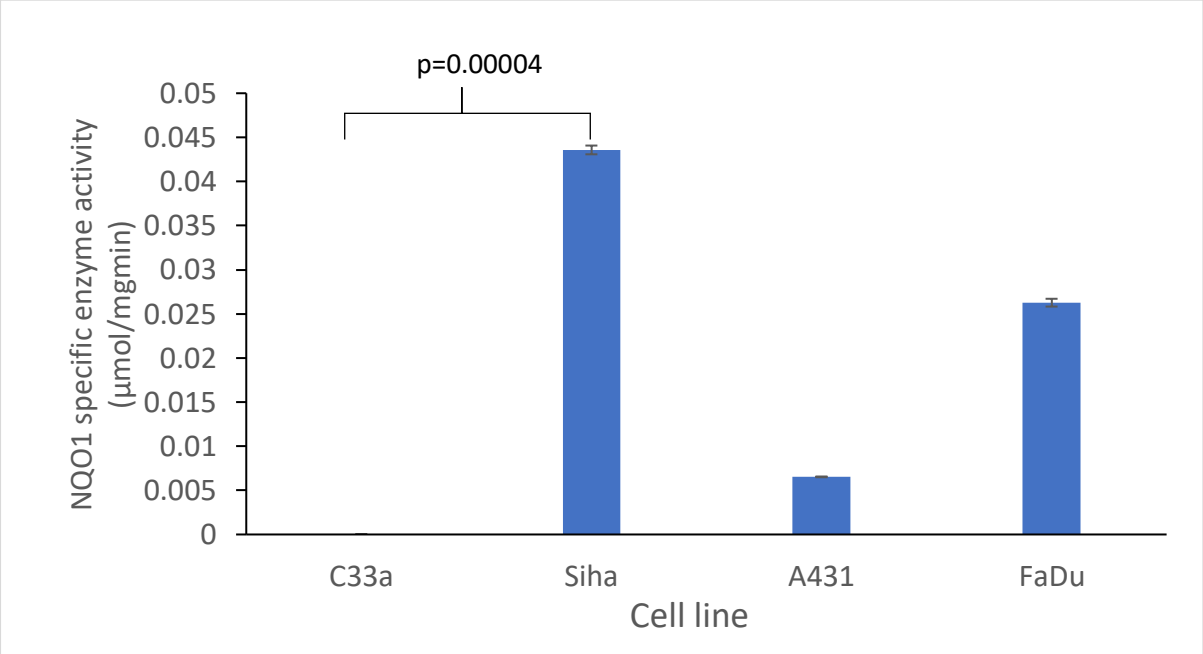


Figure 5 ***the specific enzyme activity of NAD(P)Hoxidoreductase 1 (NQO1) measured in four human cancer cell lines: C33a (cervical carcinoma), SiHa (cervical carcinoma), A431 (skin melanoma), and FaDu (nasopharyngeal carcinoma).*** *The y-axis represents the NQO1 specific enzyme activity (µmol/min/mg protein), and the x-axis denotes the different cell lines tested. Data are presented as mean ± standard deviation from three independent experiments. SiHa cells exhibited the highest NQO1 activity, which was significantly greater than that observed in C33a cells (p= 0.00004), as indicated by the statistical comparison. A431 cells showed the lowest NQO1 activity, while FaDu cells had moderate NQO1 activity levels. These results suggest a differential expression of NQO1 across the tested cell lines, which may correlate with their sensitivity to EO9.*

4.3 Putative inhibitors Chemosensitivity Assay results

The purpose of the chemosensitivity assay was to determine the effect that 3-AT, Myricetin, ECG+, and Cannflavin A independently had effect on cell survival. Cells were seeded at 1x10^4 cells per well on a 96 well plate then incubated in a treated media for 4 days with each of the drugs up to a max concentration of 50uM in a 2-fold dilution, these cells were then assessed via MTT assay to determine the cell survivability in comparison to untreated cells. This was to see if the drugs them self, independent of EO9, were cytotoxic to cells.

3-AT did not affect cell survival on its own up to 50 µM with cell exhibiting 100% survivability at this dose (figure 6a). Like 3-AT, Myricetin (figure 6b) did not substantially reduce cell survival at concentrations up to 50 µM. in Figure 3c it is shown that ECG+ also showed no impact on cell survival up to 50uM. However, in figure 3d, it is shown that at higher doses of Cannflavin A cell survival had a significant decrease in cell survivability in cervical (SiHa) and nasopharyngeal (FaDu) cell lines (figure 6d).

Cannflavin A use on SiHa showed a significant decrease to 88% cell survival at 25uM ($p=0.00000014$) and a further reduction to 79% at 50uM ($p=0.000000004$), its effect on FaDu also showed a significant decrease in cell survival at 25uM (89% cell survival) and 50uM (79% cell survival), with a significance of $p=0.00000013$ and $p=0.000000004$ respectively.

These results indicate that any change in cell survivability seen in combination with EO9 in the next section is due EO9 alone, based on the change in cell survival seen in Cannflavin A, the max dosage of all putative inhibitors will be capped at a maximum dose of 25uM in combination with EO9 to ensure all change is due to EO9s cytotoxicity.

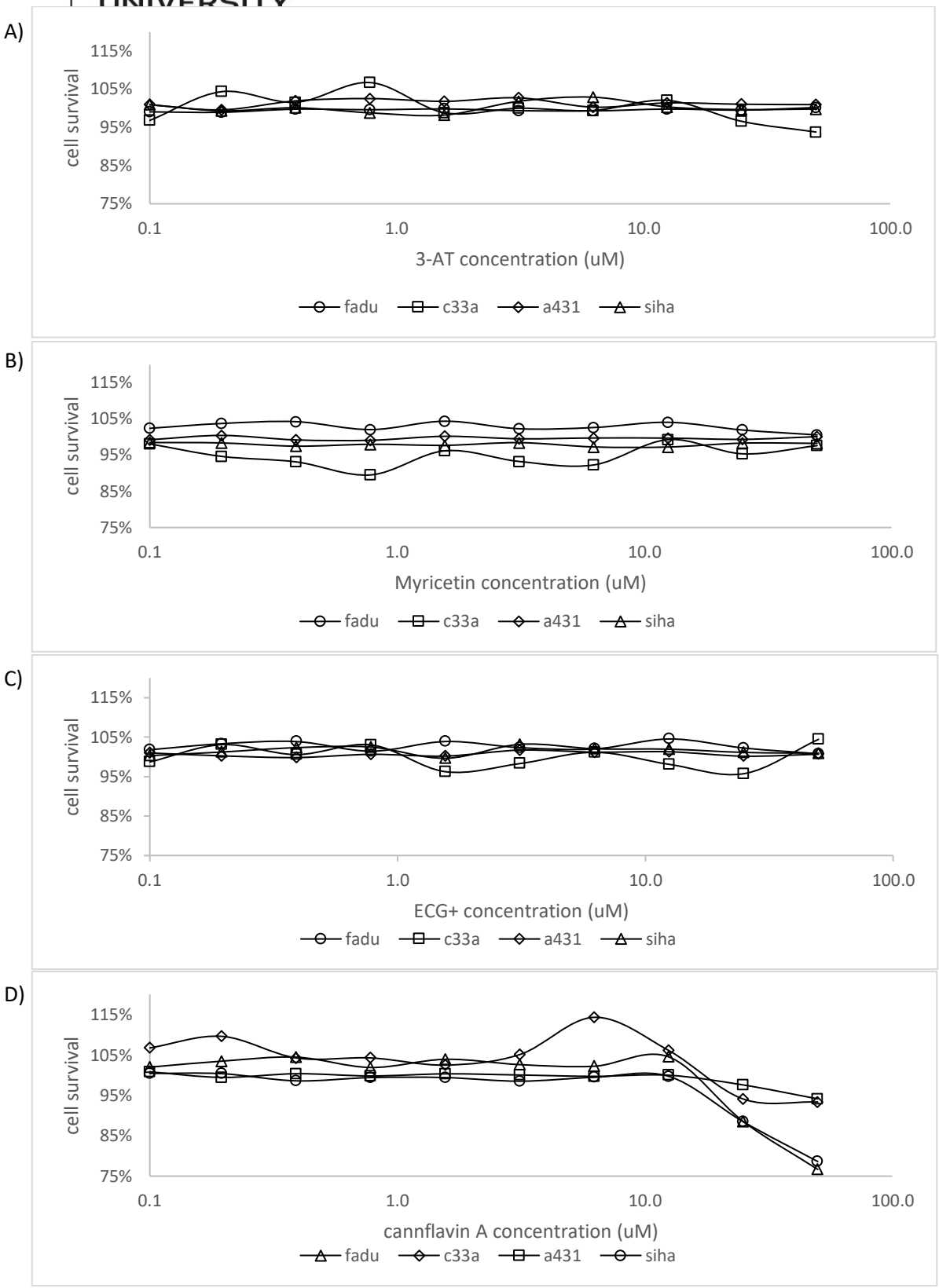


Figure 6 ***the effects of 3-AT (A), Myricetin (B), ECG+ (C), and Cannflavin A (D) on the survival of four cell lines: cervical cancer (SiHa and C33a), Skin melanoma (A431) and Nasopharyngeal cancer (FaDu)*** *across a concentration range of 0.1 to 50 µM. For all compounds, cell survival remained largely consistent around 100%, indicating minimal cytotoxic effects. Only Cannflavin A exhibited a slight reduction in cell viability at higher concentrations, while Myricetin, ECG+, and 3-AT had no significant impact on cell survival within the tested range.*

4.4 Combination Assay: EO9 and Catalase Inhibitors

To explore the potential for putative inhibitors to enhance the cytotoxic effects of EO9, combination assays were conducted in which EO9 was used in conjunction with the various putative inhibitors. The objective was to determine if these inhibitors could reduce the LD50 of EO9, thereby enhancing its therapeutic efficacy.

In Cervical cancer (SiHa) cells (figure 7a), the control LD50 of EO9 alone was 0.15 µM. When combined with 3-AT at 25 µM, the LD50 significantly decreased to 0.0289 µM ($p < 0.001$), suggesting a substantial increase in EO9's effectiveness. This significant reduction was maintained across lower EO9 concentrations (2.5 µM and 0.25 µM), with p-values remaining highly significant (0.0377 and 0.0592, respectively, both $p < 0.001$). The other inhibitors (Myricetin, ECG+, and Cannflavin A) also caused notable reductions in the EO9 LD50 at 25 µM (0.0332 µM, 0.0340 µM, and 0.0323 µM, respectively, all with $p < 0.001$), and this pattern persisted at 2.5 µM (all $p < 0.001$), but their effect was slightly less pronounced compared to 3-AT.

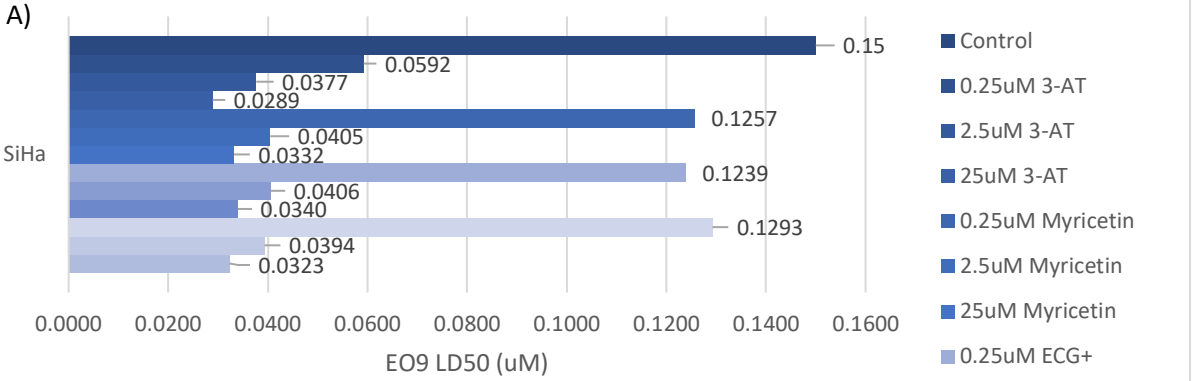
For Cervical cancer (C33a) cells (figure 7b), the control EO9 LD50 was 33.98 µM. However, the addition of inhibitors at all concentrations tested (25 µM to 0.25 µM) did not significantly affect the LD50 values, as indicated by p-values > 0.05 across all inhibitors and concentrations. This suggests that the inhibitors have minimal impact on EO9's activity in C33a cells.

In Skin melanoma (A431) cells (figure 7c), the control EO9 LD50 was 1.83 µM. The combination with 3-AT (25 µM) significantly lowered the LD50 to 0.0361 µM ($p < 0.001$), indicating a strong enhancement of EO9's cytotoxicity. At 2.5 µM and 0.25 µM, the LD50 reductions were also statistically significant (0.0642 µM, $p < 0.01$ and 0.2279 µM, $p < 0.05$, respectively). The other inhibitors (Myricetin, ECG+, and Cannflavin A) produced less pronounced reductions in LD50 at 25 µM (0.1210 µM, 0.1016 µM, and 0.1134 µM, respectively), with significant p-values ranging from $p < 0.05$ to $p < 0.001$. At lower concentrations (2.5 µM and 0.25 µM), reductions in LD50 were still significant but less substantial.

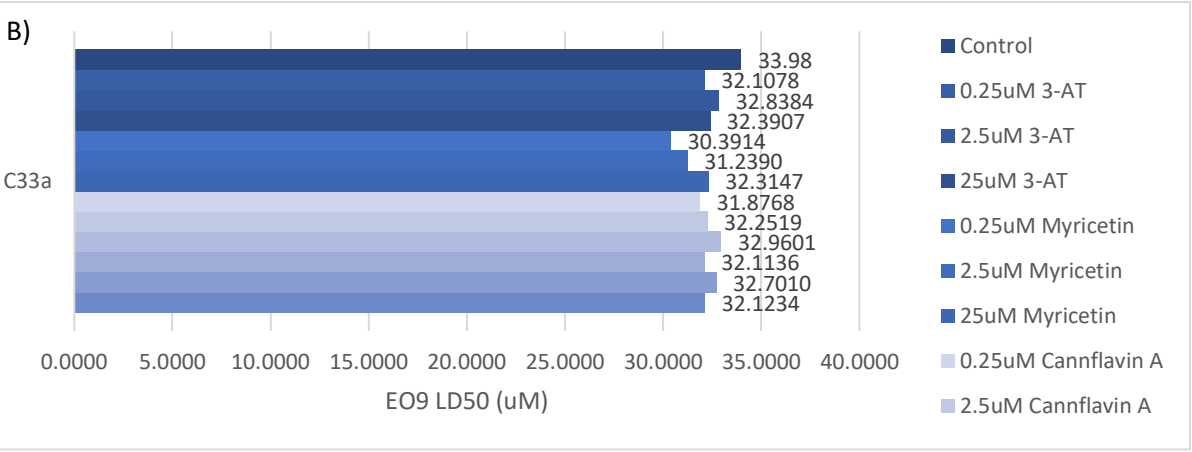
Nasopharyngeal cancer (FaDu) cells (figure 7d), with a control EO9 LD50 of 0.36 µM, showed reductions in LD50 with 3-AT treatment (25 µM) to 0.1288 µM ($p < 0.01$) and modest reductions at 2.5 µM and 0.25 µM (0.1432 µM, $p < 0.05$ and 0.1711 µM, $p < 0.05$, respectively). The other inhibitors also caused reductions in EO9 LD50 at 25 µM (Myricetin at 0.1465 µM, ECG+ at 0.1373 µM, Cannflavin A at 0.1525 µM), all with $p < 0.05$, suggesting that these inhibitors enhance EO9 cytotoxicity in FaDu cells, though less potently than 3-AT.

Overall, the data highlight that 3-AT was the most potent inhibitor across all cell lines, significantly reducing EO9 LD50 at all tested concentrations. Other inhibitors, including Myricetin, ECG+, and Cannflavin A, also showed reductions in LD50, although their effects were generally less pronounced compared to 3-AT. C33a cells demonstrated resistance to all inhibitors, as the LD50 remained largely unaffected regardless of the inhibitor used. These results suggest that catalase inhibitors, particularly 3-AT, can enhance EO9 cytotoxicity by lowering its LD50 in several cancer cell lines, except for C33a, where inhibitors showed minimal efficacy.

A)


SiHa
0.15
0.0592
0.0377
0.0289
0.1257
0.0405
0.0332
0.1239
0.0406
0.0340
0.1293
0.0394
0.0323
0.0000 0.0200 0.0400 0.0600 0.0800 0.1000 0.1200 0.1400 0.1600
EO9 LD50 (uM)
Control
0.25uM 3-AT
2.5uM 3-AT
25uM 3-AT
0.25uM Myricetin
2.5uM Myricetin
25uM Myricetin
0.25uM ECG+

B)


C33a
33.98
32.1078
32.8384
32.3907
30.3914
31.2390
32.3147
31.8768
32.2519
32.9601
32.1136
32.7010
32.1234
0.0000 5.0000 10.0000 15.0000 20.0000 25.0000 30.0000 35.0000 40.0000
EO9 LD50 (uM)
Control
0.25uM 3-AT
2.5uM 3-AT
25uM 3-AT
0.25uM Myricetin
2.5uM Myricetin
25uM Myricetin
0.25uM Cannflavin A
2.5uM Cannflavin A

C)

D)

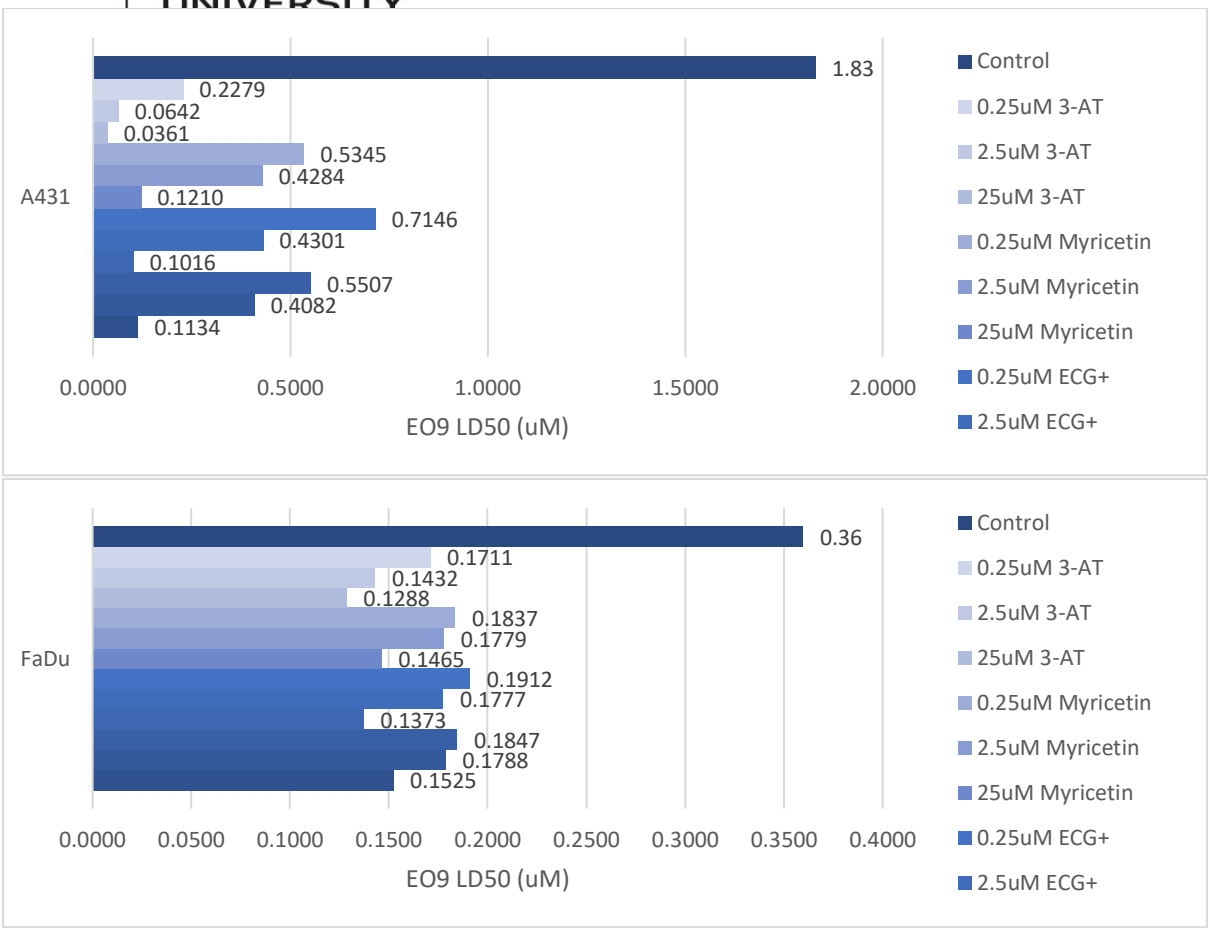


Figure 7 ***LD50 values of EO9 in combination with various catalase inhibitors*** *across SiHa (a), C33a (b), A431 (c), and FaDu (d) cell lines. The graph illustrates the impact of combining EO9 with putative catalase inhibitors on cell survival, showing a significant reduction in the LD50 of EO9 for three of the four cell lines. In SiHa (a), A431 (c), and FaDu (d) cells, the combination of EO9 with catalase inhibitors markedly lowers the LD50, indicating enhanced sensitivity and increased cytotoxicity compared to EO9 treatment alone. This suggests that catalase inhibition potentiates EO9's effectiveness. In contrast, C33a (b) cells exhibit little to no change in EO9 LD50 with the addition of catalase inhibitors, consistent with their lower baseline sensitivity to EO9 and potentially lower NQO1 activity. The figure highlights the differential response of each cell line to the combination treatment, emphasizing the potential role of catalase inhibitors in improving EO9's therapeutic efficacy in certain cancer types. Error bars represent the standard deviation from triplicate experiments, and asterisks indicate statistically significant differences compared to control (EO9 only) treatments ($p < 0.05$).*

4.5 Catalase Inhibition and IC50 determination

The purpose of the Aebi assay was to validate the change in cell survival LD50 seen in 4.4 was due to the putative inhibitor inhibiting catalase activity. The rationale behind this approach is that catalase metabolises the E09 generated reactive oxygen species (ROS) that cause cytotoxicity, thus suggesting inhibition of catalase would increases the amount of ROS resulting in improved cytotoxicity of EO9.

3-AT was the most effective, with an IC50 of 0.025 µM (+/-0.001), indicating it significantly reduced catalase activity at low concentrations. Myricetin showed an IC50 of 0.040 µM (+/- 0.003), making it the second most effective catalase inhibitor. Like 3-AT. ECG+ was less effective, with an IC50 of 0.209 µM (+/-0.02). Suggesting that while it does inhibit catalase, its overall impact on cellular ROS levels might be less significant compared to 3-AT and Myricetin. Cannflavin A was the least effective, with an IC50 of 0.295 µM (+/-0.03). Interestingly, Cannflavin A did impact cell survival at higher concentrations (25 µM and 50 µM) in SiHa, FaDu, this suggests that Cannflavin A might have additional off-target effects or that it increases ROS levels sufficiently to induce cell death in certain contexts.

These results (table 2) indicate that while all tested inhibitors are effective at inhibiting catalase, their impact on cell survival varies when combined with EO9, with 3-AT indicating the most promise as a potential enhancer of EO9 cytotoxicity by selectively increasing ROS levels without directly harming the cells. This data also suggests that the reduction in cell survival in combination with EO9 could be due to the inhibition of the antioxidant catalase pathway.

Catalase inhibitor	IC50 (uM)
3-AT	0.025
Myricetin	0.040
ECG+	0.209
Cannflavin A	0.295

Table 2 ***Calculated IC50 of Putative inhibitors*** *as measured by the decomposition of hydrogen peroxide (H_2O_2) over time. The optical density (OD) of H_2O_2, indicative of its concentration, was monitored at its lambda max OD (240 nm) over a 30-second period. 3-Aminotriazole (3-AT): Myricetin: The effect of this putative inhibitor on catalase activity was assessed at 50 nM, 10 nM, 5 nM, and 1 nM concentrations. Cannflavin A: Cannflavin A was tested at concentrations of 2000 nM, 1000 nM, 500 nM, and 100 nm. I Epicatechin Gallate (ECG+): ECG+ was evaluated at concentrations of 500 nM, 200 nM, 50 nM, and 10 nM Inhibitory effects on catalase were observed across all concentrations, with a more pronounced effect at higher concentrations. This Change in absorbance was then used to calculate the IC50 of each inhibitor.*

5. Discussion

This study explores the therapeutic potential of EO9 (Apaziquone) as a targeted treatment for solid tumours, specifically assessing its efficacy against cervical cancer (SiHa and C33a), nasopharyngeal cancer (FaDu), and skin carcinoma (A431) cell lines. A key focus is to determine whether EO9's cytotoxicity is correlated with the activity of the NQO1 enzyme, which is known to bioactivate the drug. By evaluating the levels of NQO1 activity in these tumour cells, the study aims to identify whether higher enzyme activity corresponds with increased EO9 induced cell death. Additionally, the research investigates the role of putative inhibitors in enhancing EO9's efficacy by inhibiting catalase activity, which is expected to elevate oxidative stress and improve cancer cell killing efficacy.

5.1 Key findings and Implications for solid tumour cancer therapy

EO9 sensitivity and suggested correlation with NQO1

EO9 has demonstrated significant efficacy across four previously untested cell lines, with varying degrees of sensitivity observed. Among these, the cervical cancer cell line SiHa exhibited the highest sensitivity to EO9, with a LD50 of 0.15 µM (±0.008 µM) (figure 4), indicating potent cytotoxicity even at low concentrations The high sensitivity could be linked to specific molecular characteristics of SiHa cells, such as the higher NQO1 activity of 0.0431 µmol/mg/min. In contrast, the other cervical cancer cell line, C33a, showed little to no sensitivity to EO9, with an LD50 of 33.98 µM, suggesting and had an enzyme specific activity of 0µmol/mg/min further suggesting this changing in EO9 sensitivity is due to NQO1.

The correlation between NQO1 expression and EO9 efficacy highlights the potential for EO9 to be integrated into personalized medicine strategies for solid tumour cancers where patients with high NQO1 expressing tumours could be identified as ideal candidates for EO9 therapy, thus improving the likelihood of treatment success and reducing unnecessary exposure to ineffective treatments. The use of biomarkers to guide treatment decisions is increasingly being recognized to enhance the precision and efficacy of cancer therapies (Mandal et al., 2013), and these findings support the incorporation of NQO1 testing into clinical protocols for cancers considered for EO9 treatment. This idea of biomarkers for predictive treatment outcome can be seen in breast cancer patients, specifically looking at breast cancer genes 1 and 2 (BRCA1/2) which are used to predict platinum sensitivity in ovarian cancer (Ledermann et al., 2012)

The integration of biomarkers like NQO1 to guide treatment decisions is increasingly recognized for enhancing the precision and efficacy of cancer therapies (Mandal et al., 2013). These findings support the potential inclusion of NQO1 testing in clinical protocols to identify patients with solid tumours that are most likely to respond to EO9, thereby optimizing treatment success.

Effectiveness of Catalase Inhibitors in Reducing EO9 Dosage

Catalase is an enzyme that protects tumour cells by breaking down hydrogen peroxide, a reactive oxygen species (ROS), into water and oxygen, thereby reducing oxidative stress. Since EO9 exerts its cytotoxic effect through ROS generation, the presence of catalase can limit the accumulation of ROS within tumour cells, diminishing EO9's effectiveness. This approach could not only improve the efficacy of EO9 but also reduce its off target effects by minimizing the dosage necessary for tumour eradication.

In our study, various putative catalase inhibitors were tested, with 3-amino-1,2,4-triazole (3-AT) showing the most promise in enhancing EO9's activity (figure 7). At a concentration of 0.25 µM, 3-AT significantly reduced the LD50 of EO9 across three different cell lines: SiHa, A431, and FaDu. Specifically, the LD50 values for EO9 in the presence of 3-AT were 0.592 µM for SiHa, 0.2279 µM for A431, and 0.1711 µM for FaDu (table 2). These results demonstrate a substantial decrease in the EO9 dose required to achieve 50% cell death when

catalase activity is inhibited, supporting the hypothesis that catalase inhibition can potentiate the cytotoxic effects of EO9.

This idea of 3-AT increasing cellular death is mirrored in a study done by Milton et al (2001), where cellular death caused by Amyloid-β (Aβ) showed a significant increase in efficacy when used in combination with 3-AT. By increasing intracellular ROS through catalase inhibition, EO9's DNA-damaging and cytotoxic effects are amplified, which may present a promising strategy for developing combination therapies.

5.2 Limitations of the Study

Methodological Constraints

While this study has provided valuable insights, several limitations must be acknowledged. Firstly, the in vitro nature of the assays used to assess EO9 sensitivity and catalase inhibition limits the direct applicability of the results to in vivo settings. Cell culture conditions do not fully replicate the complexity of the tumour microenvironment (van Staveren et al., 2009), Consequently, the efficacy and safety of EO9 in combination with catalase inhibitors observed in vitro may differ in clinical scenarios so using in vivo models would allow us to understand the effects of EO9 *in situ*, using genetic modification of mice would provide a controlled view the levels of expression of NQO1 giving a definitive idea of correlation between enzymatic activity and dose response.

Sample Diversity and Generalizability

Another limitation is the number and diversity of the tumour cell lines used. Although cell lines with varying levels of NQO1 expression were selected to represent different tumour types, the results may not be generalizable to all solid tumours. The heterogeneity of tumours, both within and between patients, means that further studies using a broader range of cell lines and primary tumour samples are necessary to confirm the findings. Broadening the scope and variety of cell lines and tumour types used with different biochemical profiles would give a more holistic view into what specific parameters the use of EO9 would work for.

Potential Off target effects of Catalase Inhibition

The inhibition of catalase, while beneficial in enhancing EO9 efficacy, could also lead to unintended off target effects due to increased oxidative stress in non-cancerous cells (Scheit & Bauer, 2014). The systemic effects of catalase inhibition were not explored in this study, and further research is needed to evaluate the safety of this approach in vivo. Additionally, the potential for cancer cells to develop resistance to oxidative stress by upregulating other antioxidant pathways poses a challenge that warrants further investigation (Crawford & Davies, 1994). To investigate the up regulation of other antioxidant pathways and the effects the used putative inhibitors would have on them, using in vitro models like that done in this study with catalase would give a clearer insight into how effective long-term use of these inhibitors would be.

5.3 Future Research Directions

In Vivo Studies and Clinical Trials

To build on the findings of this study, future research should focus on spheroid models to validate the efficacy and safety of EO9 in combination with catalase inhibitors. Spheroid models that more accurately mimic the tumour microenvironment will be crucial in determining greater therapeutic potential and see if EO9 in combination is able to enter a tumour core (Pinto et al., 2020). Additionally, due to the poor bioavailability seen in I.V administration looking at alternative delivery systems that better suite patient need, as seen with fluorouracil (5FU) a topical cream for skin melanoma (Prince et al., 2018).

Finally, understanding the mechanisms by which tumour cells may develop resistance to EO9 and catalase inhibition will be essential for ensuring the long-term efficacy of this therapy. Studies focused on the adaptive responses of cancer cells to oxidative stress and the identification of biomarkers for resistance could inform the development of second line therapies or combination approaches to prevent or overcome resistance.

6. Conclusion

The treatment of solid tumour cancers remains a significant challenge in oncology, due to the complex nature of these tumours and the limitations of existing therapies. Despite advances in surgery, chemotherapy, and radiotherapy, the persistence of hypoxia within tumour microenvironments continues to drive resistance to conventional treatments and contributes to poor patient outcomes. This highlights the urgent need for novel therapeutic strategies that can effectively target the unique vulnerabilities of solid tumours while minimizing systemic toxicity.

In this study, EO9 (Apaziquone) was investigated as a potential therapeutic possibility for solid tumours, focusing on its mechanism of action through the bioactivation by NQO1, an enzyme overexpressed in many tumour types. these assays proved that EO9 exhibits significant cytotoxicity in tumour cells with elevated levels of NQO1, as shown by the lower LD50 values seen in these cells. This correlation suggests that EO9's efficacy is closely tied to NQO1 expression, making it a promising candidate for targeted cancer therapy, particularly in tumours where NQO1 is abundantly present.

This study explored the potential of combining EO9 with catalase inhibitors to further enhance its therapeutic index. The rationale behind this approach is that catalase, an enzyme responsible for neutralizing reactive oxygen species (ROS), may protect tumour cells from the oxidative damage induced by EO9. By inhibiting catalase, the intracellular levels of ROS increase, leading to enhanced cytotoxicity at lower doses of EO9. These findings confirm that the use of catalase inhibitors does in fact reduce the LD50 of EO9, implying that lower doses of the drug can achieve the same therapeutic effect, potentially reduce the risk of off target effects and improve patient response.

The implications of these findings are significant. By combining EO9 with catalase inhibitors, it may be possible to design a more effective treatment regimen that not only targets tumour cells with high specificity but also reduces the required dosage, thereby limiting adverse effects. This strategy holds promise for improving outcomes in patients with solid tumours, particularly those who are less responsive to traditional therapies.

However, while these results are encouraging, further research is necessary to confirm the efficacy and safety of this approach. More in vivo studies are needed to assess the long-term effects of EO9 in combination with catalase inhibitors, as well as to explore the potential for resistance development. Clinical trials will be essential to figure out the best dosing regimens and to evaluate the overall benefit in a real-world patient population.

In conclusion, this study highlights the potential of EO9 as a targeted therapy for solid tumour cancers, particularly when used in conjunction with catalase inhibitors. The observed reduction in LD50 with increased NQO1 expression and catalase inhibition offers a promising avenue for enhancing the effectiveness of cancer treatment while minimizing off target effects. Continued research in this area is crucial to fully realize the therapeutic potential of EO9 and to bring this innovative approach from the laboratory to clinical practice.

Word Count:10,473

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