Novel targets for increasing radiosensitivity in hypoxic tumours

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Table of Contents

Abstract

Radiotherapy resistance remains a major barrier to durable responses within solid tumours, particularly within lung and colorectal cancers, where hypoxia limits treatment efficacy. Dyskerin pseudouridine synthase 1 (DKC1) is frequently overexpressed in these cancers; its implication in ribosome biogenesis, telomerase activity, and stress signalling suggesting a potential role in increasing tumour radioresistance. This study examined the consequences of DKC1 inhibition using the pharmacological inhibitor Pyrazofurin and siRNA-mediated DKC1 depletion in A549 lung and HCT116 colorectal cancer cell lines. Assays of cell viability, stress signalling, DNA damage, and cell cycle distribution were examined in addition to clonogenic survival following irradiation under normoxia and radiobiological hypoxia (<0.1% 0_2). Pyrazofurin treatment had variable and modest reductions in viability, activated p53 stress responses, and redistributed cells away from G2/M without inducing DNA damage. Importantly, Pyrazofurin sensitised A549 cells to irradiation under normoxia, but this effect was completely abrogated under hypoxia, highlighting the dominant role of oxygen availability in radiosensitivity. These findings establish DKC1 inhibition as an oxygen-dependent modulator of radiosensitivity. As Pyrazofurin itself has limited clinical application due to historical toxicity, and its radiosensitising activity is oxygen dependent, its use in terms of further study does not have a strong case; however, there are avenues of research, such as looking at effects within mild hypoxia, which are yet to be explored.

Acknowledgements

I would like to say a massive thanks to my supervisor, Ester, and everyone in the lab this year who have helped me in big and small ways. I am very grateful for all the time spent!

Thanks to my parents for talking to me over the phone whenever I needed some company, and to all the other people I love who now know what hypoxic tumours are.

List of tables

Methods

List of Figures

Introduction

Methods

Results

List of abbreviations

% 0_2	-	percentage of oxygen
°C	-	degree Celsius
53BP1	-	p53 binding protein 1
ATM	-	ataxia-telangiectasia mutated
ATR	-	ataxia-telangiectasia mutated and Rad3-related
Chk1	-	checkpoint kinase 1
Chk2	-	checkpoint kinase 2
CPT	-	camptothecin
DAPI	-	4',6-diamino-2-phenylindole
DDR	-	DNA damage response
DKC1	-	dyskerin pseudouridine synthase 1
DMEM	-	Dulbecco's Modified Eagle's Medium
DMSO	-	dimethylsulphoxide
DNA	-	deoxyribonucleic acid
dNTP	-	deoxyribonucleoside triphosphate
DSB	-	double-strand break
dsDNA	-	double-stranded DNA
ETOP	-	etoposide
FBS	-	foetal bovine serum
H	-	hour (s)
H2AX	-	H2A histone family member X
HIF	-	hypoxia-inducible factor
HIF-1α	-	hypoxia-Inducible Factor-1 alpha
HR	-	homologous recombination
IF	-	immunofluorescence
IP	-	immunoprecipitation
IR	-	ionising radiation
mRNA	-	messenger RNA
NFκB	-	nuclear factor kappa B
NHEJ	-	non-homologous end joining
ns	-	not significant

OER	-	oxygen enhancement ratio
PBS	-	phosphate-buffered saline
PCR	-	polymerase chain reaction
PFA	-	paraformaldehyde
Pyr	-	Pyrazofurin
RNA	-	ribonucleic acid
SSB	-	single-strand break
ssDNA	-	single-stranded DNA
UPR	-	unfolded protein response
γH2AX	-	phosphorylation of the serine-139 residue of H2AX histone family member X
TBS	-	tris-buffered saline
TBS-T	-	TBS-tween
WB	-	western blot

Introduction

Radiotherapy and resistance

Despite the dynamic field of modern cancer research, the mainstay of treatment continues to be surgery, chemotherapy, immunotherapy and radiotherapy. Radiotherapy is given to around 50% of all cancer patients throughout their illness, so improvements on side effects or resistance have the potential for widespread impact (Baskar *et al.*, 2012; Akhunzianov *et al.*, 2025). Increasing the radiation dose is often limited by the tolerance level of surrounding organs and tissues, with higher doses resulting in more normal tissue damage (Baker *et al.*, 2016; Giaj-Levra, Ricchetti and Alongi, 2016). Therefore, novel solutions are needed for addressing radioresistance to ultimately avoid radiotherapy failure and cancer recurrence.

Lung, colorectal cancer and radiotherapy resistance

Worldwide, lung cancer is the leading cause of cancer mortality, with around 1.8 million deaths associated in 2022 (Bray *et al.*, 2024). Within lung cancer, the most common form is non-small cell lung cancer (NSCLC), which makes up around 80% of all cases (American Cancer Society, 2025). Following this, colorectal cancer has the second-highest cause of mortality, associated with 903,859 deaths each year (Bray *et al.*, 2024).

Radiotherapy is often a cornerstone of treatment for patients with colorectal and lung cancers. However, even following combination with surgery and chemotherapy, resistance to radiotherapy persists (Clifford *et al.*, 2018; George *et al.*, 2019; Buckley *et al.*, 2020; Césaire *et al.*, 2022). Within NSCLC patients, around 35% of inoperable

tumours have a 5-year survival of 10-15% from radiotherapy and chemotherapy combination (Aupérin *et al.*, 2010). Colorectal cancer still has a 5-10% local recurrence rate even when using neoadjuvant radiotherapy, which has been seen to reduce recurrence (Folkesson et al., 2005; Gijn et al., 2011).

Research working to decrease radiotherapy resistance has resulted in the development of agents to increase the radiosensitivity of cancer cells or protect non-cancer cells from radiation. Radiosensitisers are pharmaceutical or chemical agents that are able to increase the killing of cancer cells by increasing DNA damage and producing free radicals, with the ideal radiosensitisers having less effect on normal tissues (Gong et al., 2021). The development of radiosensitisers dates back decades, with the main mechanisms being: inhibition of radiation-induced repair of DNA damage, increasing DNA damage, disturbing the cell cycle to improve cytotoxicity, inhibiting the expression of resistance genes or promoting the expression of radiation-sensitive genes (Gong et al., 2021). Radiosensitisers already in use include common chemotherapy agents, which, when used concurrently, can work as radiosensitisers; these include cisplatin, 5-fluorouracil (5-FU), and gemcitabine (Wardman, 2007). There are more specific chemotherapy drugs used as radiosensitisers such as nicotinamide and nimorazole (for acute hypoxia), which are in limited but routine use clinically (Low, Rodriguez-Berriguete and Higgins, 2024). Despite these radiosensitisers already in use, they have limits to their effectiveness, and there is a need for novel radiosensitisers and new mechanisms to increase sensitisation (Zakeri *et al.*, 2020; Low, Rodriguez-Berriguete and Higgins, 2024).

Intra-tumour heterogeneity, when there is non-uniformity in cancer cells or within the tumour, is associated with therapeutic resistance (Michor and Polyak, 2010; Visvader, 2011). Heterogeneity of oxygen concentration within tumours is an example of this, where regions of low oxygen concentration (hypoxia) have high radiotherapy resistance (Hammond *et al.*, 2014).

Hypoxia

The term hypoxia, while generally describing insufficient oxygen, in this context refers to a region of low oxygen in the body and tissues. There can be hypoxic regions in the wound healing of normal tissues or as part of pathologies such as metabolic, heart, or reproductive diseases, as well as cancer (Hammond *et al.*, 2014; Lindholm and Rundqvist, 2016; Lee, Chandel and Simon, 2020). Normoxia describes the atmospheric level of oxygen (21% 0_2); the concentration at which the bulk of scientific research is undertaken (Place, Domann and Case, 2017). However, the level of oxygen needed within the body can be different between organs and tissues, with some sustained by oxygen conditions which would be considered hypoxic by others (Hammond *et al.*, 2014; Keeley and Mann, 2019). It is generally acknowledged that there is a difference between actual tissue concentrations of oxygen and those used for normoxic scientific experiments (Keeley and Mann, 2019). To study hypoxic conditions, the levels used in research vary between <0.1% and 2% 0_2. With 0.5 - 2% 0_2 considered ‘mild hypoxia’, within which HIF hypoxia-inducible factors (HIFs) are stabilised, and radiobiological hypoxia being when 0_2 is <0.1 (Hammond *et al.*, 2014).

Hypoxic tumours

During the development of tumours, there is a marked increase in the oxygen needed from the blood to support this rapid cell proliferation (Nagy *et al.*, 2009). This is quickly more than the existing vasculature of the body, leaving the cancer cells and tumour with deficiencies (Vaupel, Kallinowski and Okunieff, 1989; Bristow and Hill, 2008). As a result of this abnormal vasculature and high metabolic demand, there are areas of hypoxia within most solid tumours, forming a common feature of the tumour microenvironment (TME). Figure 1.1 shows this decrease in oxygen with distance from blood vessels, creating variable regions of oxygen concentration.

Areas of hypoxia within solid tumours are associated with poor patient outcomes and resistance to chemotherapy, radiotherapy and immunotherapy treatments (Brown and Giaccia, 1998; Vaupel and Harrison, 2004; Nordsmark *et al.*, 2005; Hammond *et al.*, 2014; O'Donnell, Teng and Smyth, 2019; Saleh and Elkord, 2020; Vesely, Zhang and Chen, 2022). Additionally, tumour metastasis (the most aggressive presentation of cancer) is more likely if a patient's primary tumour was hypoxic (Bristow and Hill, 2008). Oxygen levels in TME hypoxia are also very dynamic, changing spatially and temporally. The reduction of oxygen followed by reoxygenation is known as cyclic or intermittent hypoxia, and is also associated with therapy resistance and poor patient outcomes (Dewhirst, Cao and Moeller, 2008; Celora *et al.*, 2022). This state of cyclic hypoxia leads to higher levels of free radicals in the cell, and subsequent activation of stress response and tissue damage (Prabhakar, 2001; Bader, Dewhirst and Hammond, 2020). This pathogenic acceleration and resistance to treatment have created an appeal to targeting hypoxia for potential therapeutics; however, despite

considerable research within this area, there is still no effective and standardly used treatment to exploit hypoxia within tumours (Wilson and Hay, 2011).

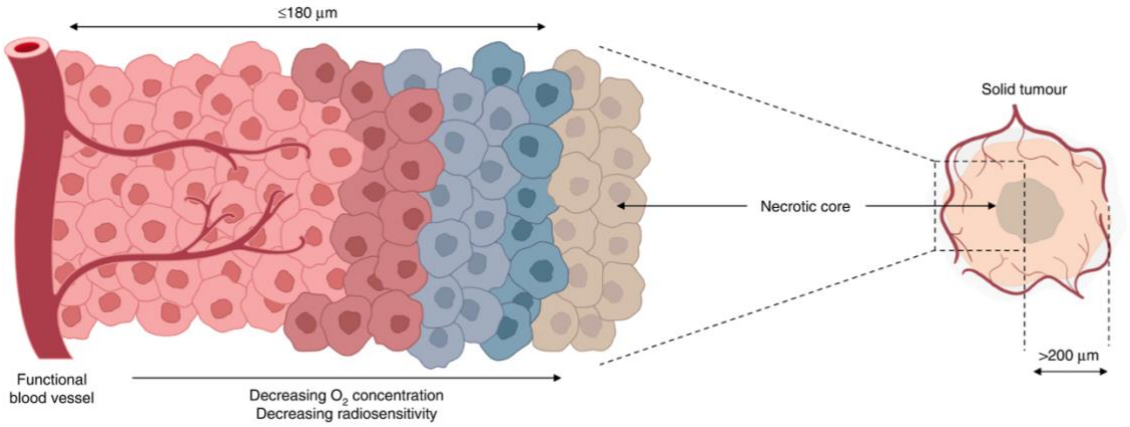


Figure 1.1. Schematic outlining decreasing oxygen within a tumour with increasing distance to the blood vessel. From (Worth, Papandreou and Hammond, 2023).

Novel treatments to try and decrease therapy resistance by targeting hypoxia have been researched extensively; however, many have limitations which have stopped their progression clinically. These have included hyperbaric oxygen, which got to clinical trials but showed toxicity, seizures and was impractical for standard practice (Fernández *et al.*, 2021). Another example is hypoxia-activated pro-drugs (HAPs), biologically inactive compounds which can be reduced selectively in hypoxia, creating a cytotoxic response. Despite pre-clinical and early-stage success, they did not improve overall survival, and no further trials were opened, something which is attributed to the HAPs lack of ability to reach hypoxic tumour cells and an absence of patient stratification (Hay, Hicks and Wang, 2014; Hunter, Wouters and Wilson, 2016; Spiegelberg *et al.*, 2019).

Hypoxia is a common feature of both lung and colorectal tumours, presenting the expected issues with radiotherapy resistance (Ancel *et al.*, 2021; Fletcher *et al.*, 2022). Within the most common form of lung cancer NSCLC, efforts to exploit hypoxia in therapeutic trials have not yet made it to standard patient treatment (Brustugun, 2015; Dewhirst and Birer, 2016). However, there are promising therapeutics currently being explored, an example of which is the repurposing of the anti-malarial drug atovaquone, which has been shown to reduce hypoxia and decrease hypoxic tumour volumes clinically for NSCLC patients (Skwarski *et al.*, 2021). Numerous clinical trials have been carried out or are currently in the process of examining atovaquone as an anti-cancer drug (https://clinicaltrials.gov/search?intr=Atovaquone) (NCT04648033).

Poor prognosis and tumour invasion within colorectal cancer have also been linked to hypoxia and HIF-1α overexpression, but colorectal cancer like NSCLC, does not yet have a standardly used treatment against hypoxia to improve radiotherapy resistance (Rasheed *et al.*, 2009; Zong *et al.*, 2017; Fletcher *et al.*, 2022). Nicotinamide and nimorazole, as mentioned above, are two examples of drug agents which have been used to target hypoxia for improving radiotherapy resistance. For nicotinamide, it has been used in conjunction with carbogen, a hyperoxic gas and has led to improvements in laryngeal and urinary bladder cancers; however, in glioblastoma and NSCLC patients, there was no therapeutic benefit seen and high gastrointestinal toxicity (Kaanders, Bussink and van der Kogel, 2002; Miralbell *et al.*, 1999; Simon *et al.*, 2003; Bernier *et al.*, 1999). Nimorazole has been seen to improve outcomes within hypoxia tumours, but current use is only with head and neck cancer and in Denmark (Overgaard *et al.*, 1998). Therefore, despite work within this area, there is a lack of

current therapeutics to improve hypoxia-induced radiotherapy resistance within colorectal and lung cancers, as well as within solid tumour cancers more generally.

Mechanistic response to hypoxia

The adaptation of tumour cells to hypoxia involves the modification of already existing mechanisms through gene expression, including the activation of hypoxia-inducible factors (HIFs) (Semenza, 1999; Koh and Powis, 2012). The transcription factor, hypoxia inducible factor-1 (HIF-1α), sees increased expression and stabilisation in hypoxic conditions (Hammond *et al.*, 2014). HIF-1α is the most well-characterised hypoxia-associated protein, and when it comes together with HIF-1β, it promotes the transcription of genes that increase hypoxic tumour cell survival through metastasis, angiogenesis and metabolism (Wang *et al.*, 1995; Koh and Powis, 2012). This HIF-1 protein complex affects gene transcription by binding to hypoxia response elements (HREs) of target genes (Figure 1.2) (Weidemann and Johnson, 2008).

However, in normoxic conditions, the von Hippel-Lindau tumour suppressor protein (VHL) binds to HIF-1α at the protein stabilisation domain, which leads to it being polyubiquitinated and marked for degradation by the proteasome (Ohh *et al.*, 2000; Ravi *et al.*, 2000; Liu and Semenza, 2007). For VHL to bind, there needs to be hydroxylation of two proline residues on HIF (Ivan *et al.*, 2001; Jaakkola *et al.*, 2001). The enzymes which catalyse this hydroxylation are prolyl hydroxylase domain proteins (PHDs), and as they use molecular oxygen within the catalysis, it is an oxygen dependent process (Epstein *et al.*, 2001).

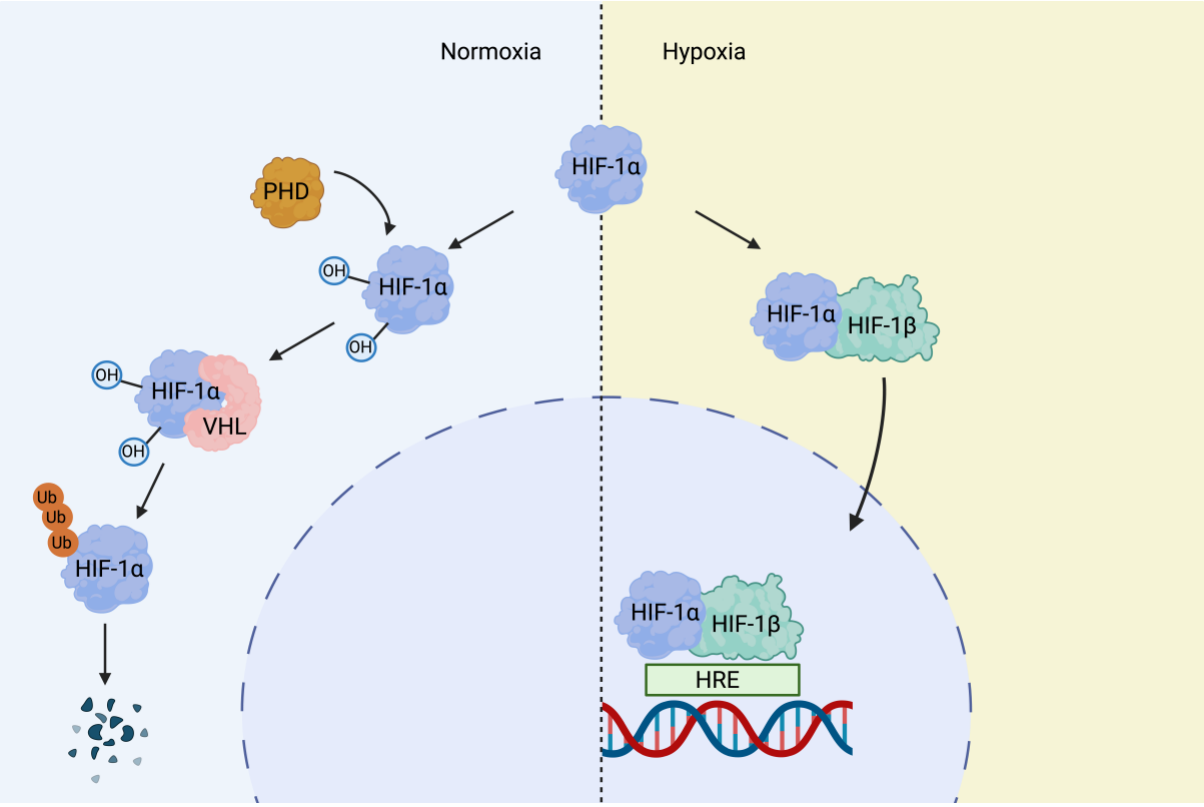


Figure 1.2. HIF regulation in normoxic and hypoxic conditions. In normoxic conditions, PHD hydroxylates HIF-α in the presence of oxygen. Then VHL marks the protein for proteasomal degradation. Within hypoxic conditions, PHD is inactive and unable to hydroxylate; therefore, HIF-α binds to HIF-1β, which is then able to translocate as the HIF-1 complex to HREs and activate target genes. Created with Biorender.

Hypoxia and the DNA damage response

To ensure accurate inheritance of genetic material, the cell has many functions to guard the integrity of DNA; one of these is the DNA damage response (DDR). The DDR is a system which enables the detection, prevention and ultimate repair of DNA damage (Surova and Zhivotovsky, 2013; Huang and Zhou, 2021). A trio of kinases control the DDR: ataxia telangiectasia and Rad3-related (ATR), ataxia telangiectasia mutated (ATM) and DNA-dependent protein kinase (DNA-PK) (Shiloh and Ziv, 2013; Smith *et al.*, 2020). These kinases are at the top of the signal transduction pathway, which can induce DNA repair, cell cycle arrest and apoptosis (Shiloh and Ziv, 2013; Smith *et al.*, 2020). Hypoxia is a DSB-independent way that ATM and its subsequent

pathway are activated (Olcina, Grand and Hammond, 2014). Hypoxia triggers ATM-dependent and ATR-dependent phosphorylation of the downstream targets (Chk1, Chk2, KAP1, RPA, p53 and H2AX) (Smith *et al.*, 2010, 2020; Shiloh and Ziv, 2013). Hypoxia has been associated with driving downregulation of DNA repair pathways, which include homologous recombination, non-homologous end joining and mismatch repair (Kaplan and Glazer, 2020).

The p53 protein is a pivotal factor in the DDR and cell death pathways of normal cells, with many important roles including cell cycle arrest, autophagy, apoptosis, and senescence (Senturk and Manfredi, 2013; Chen, 2016; Fischer and Sammons, 2024). The gene which encodes p53 (TP53) is the most frequently mutated in cancer. More specifically, in response to chronic hypoxia, there is a selection bias for cancer cells with inactive TP53, as those with active p53 tend to undergo apoptosis in those conditions (Hammond and Giaccia, 2005; Hammond *et al.*, 2006). An example of a protein implicated in this p53 cell cycle control is p53 binding protein 1 (53BP1), which forms foci at DSBs, functioning as a useful marker for DNA damage (Redon *et al.*, 2011; Rothkamm *et al.*, 2015). Another common DNA damage marker is gamma H2AX (γH2AX), which ATM phosphorylates in response to double-strand DNA breaks (DSBs) (Redon *et al.*, 2011). The foci γH2AX form on chromatin are useful for detection as they can be quantified (Redon *et al.*, 2011). Under milder 1% 0_2 and radiobiological hypoxia <0.1 0_2, γH2AX has been seen to be induced in an ATR-dependent manner (Hammond, Dorie and Giaccia, 2003; Hammond, Green and Giaccia, 2003; Economopoulou *et al.*, 2009; Wrann *et al.*, 2013).

Radiobiological hypoxia

Severe hypoxia (<0.1% 0_2), also known as radiobiological hypoxia, is the hypoxic level at which significant resistance to radiotherapy is observed (Hammond *et al.*, 2014). When oxygen is present in high enough levels, the absorption of radiation leads to a chain of events resulting in the production of free radicals. These free radicals are what are largely responsible for the chemical bonds and changes that result in cell damage from irradiation, with the accumulated unrepaired DNA damage then leading to tumour cell death (Little, 2003). In the absence of oxygen, much of this DNA can repair itself, with this resistance to radiotherapy reducing the effectiveness of treatment (Brown, 2020; Singleton, Macann and Wilson, 2021). This resistance to radiotherapy was something initially recognised about hypoxia in solid tumours in the 1950s (Gray *et al.*, 1953; Thomlinson and Gray, 1955).

Radiobiological hypoxia leads to a unique and targetable biological response, involving several stress pathways, including the unfolded protein response (UPR), transcriptional stress and a replication stress-induced DNA damage response (DDR) (Wouters and Koritzinsky, 2008; Hammond *et al.*, 2014; Bolland *et al.*, 2021). This is different to the HIF-mediated response seen in hypoxia with higher oxygen levels, as the replication stress-induced DDR and UPR are uniquely activated in radiobiological hypoxia (Ramachandran *et al.*, 2021). Normal DDR activation is a result of damaged DNA, for example, single or DSBs; however, DDR in radiobiological hypoxic conditions occurs in the absence of detectable levels of this DNA damage (Ng *et al.*, 2018). The accumulation of R-loops has been implicated in the transcriptional stress response from radiobiological hypoxia (Figure 1.3) (Ma *et al.*, 2023).

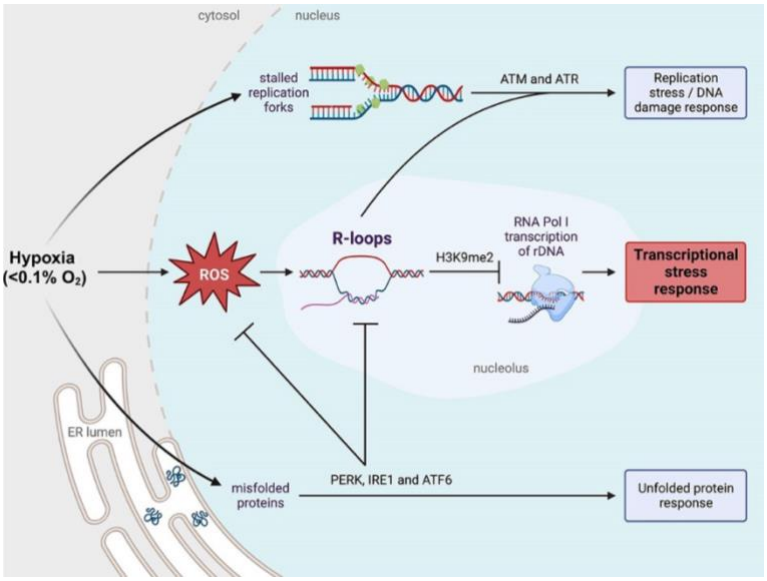


Figure 1.3. Schematic outlining radiobiological hypoxia downstream stress. Radiobiological hypoxia (<0.1 0_2) stimulates replication stress, DDR, and the UPR. It has also been observed to result in the transcriptional stress response, within which the accumulation of R-loops is implicated. From (Ma *et al.*, 2023).

R-loops

R-loops are three-stranded nucleic acid structures composed of an RNA/DNA hybrid and an ssDNA (Figure 1.4). While the terms R-loops and RNA/DNA hybrids have been used synonymously, there are some who believe that RNA/DNA hybrids are different. Instead, suggesting that RNA/DNA hybrids represent short structures that can form during ribonucleotide triphosphate incorporation, transcription at the active site of RNA polymerase or lagging-strand DNA synthesis (Santos-Pereira and Aguilera, 2015).

First observed in 1976, R-loops have recently become a more popular area of research (Thomas, White and Davis, 1976). While they are a common feature of normal gene regulation in cells, aberrant unresolved R-loops have been seen to contribute to genomic instability (Aguilera and García-Muse, 2012; Crossley, Bocek and Cimprich, 2019). R-loops are conserved and widely prevalent across a range of

organisms, and the heightened interest can likely be attributed to their pathological implications in cancer and neurodegeneration (Richard and Manley, 2009; Al-Hadid and Yang, 2016). The rapid and uncontrolled proliferation of cancer cells is thought to make it more likely for R-loop accumulation to occur (Helmrich, Ballarino and Tora, 2011; Kotsantis *et al.*, 2016)

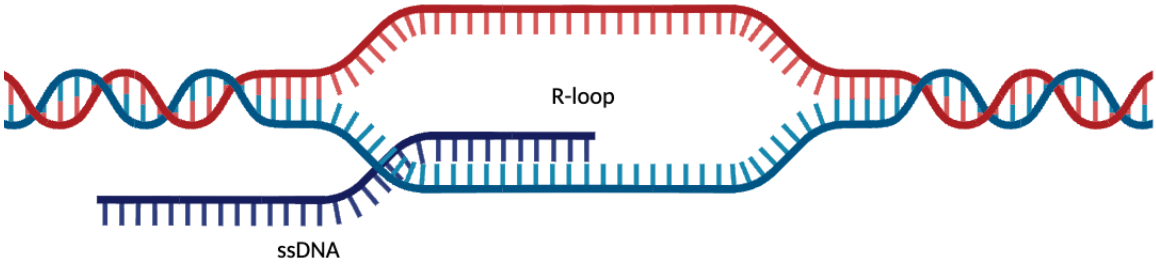


Figure 1.4. Schematic of R-loop with dsDNA and ssDNA. Made using BioRender.

Non-pathogenic roles for R-loops include regulating gene expression at promoters, transcription termination, and aspects of DNA repair (Stavnezer, Guikema and Schrader, 2008; Skourti-Stathaki, Proudfoot and Gromak, 2011; Nadel *et al.*, 2015; Chen *et al.*, 2017; Dumelie and Jaffrey, 2017; Zhang *et al.*, 2017; Cristini *et al.*, 2018; Petermann, Lan and Zou, 2022). However, the pathological description of R-loops has instead been associated with DNA damage, dysregulation of DNA replication as well as DSBs, senescence and cell death (Santos-Pereira and Aguilera, 2015; Sollier and Cimprich, 2015; Al-Hadid and Yang, 2016; Crossley, Bocek and Cimprich, 2019). A large amount of this genomic instability caused by R-loops is attributed to replication fork stalling and transcription-replication collision (TCRs) (Kotsantis *et al.*, 2016; Stork *et al.*, 2016). The formation of R-loops is more likely to occur at repetitive sequences, including telomeres, centromeres, rDNA and transposable elements or when certain

features are present for DNA, such as negative DNA supercoiling, DNA nicks, and G-content (Roy *et al.*, 2010; Kuzminov, 2018; Allison and Wang, 2019).

R-loops, with their accumulation in radiobiological hypoxia, have been observed to have a critical role in the induced transcriptional stress response (Figure 1.5) (Ma *et al.*, 2023). Furthermore, it has been shown that the mechanism of hypoxic R-loop accumulation is dependent on non-DNA-damaging levels of reactive oxygen species (ROS) (Ma *et al.*, 2023). The R-loop accumulation in hypoxia has also been seen to downregulate ribosome biogenesis by repressing Pol I-mediated production of rRNA (Ma *et al.*, 2023).

R-loops as therapeutic potential

R-loops are an area of great potential for new therapeutic targets in multiple cancers, especially in the context of tumour radiosensitivity, because of their observed role in genome instability (Boros-Oláh *et al.*, 2019). This is particularly as tumour cells are very tolerant of increased R-loops; meaning there is potential for chemotherapeutics to increase the sensitivity of tumours to treatment through decreasing this tolerance to R-loops (Boros-Oláh *et al.*, 2019). Thereby making the tumours more sensitive to DNA-damaging agents and apoptosis within combinational therapy. The targeting of R-loop binding proteins have been seen to increase R-loops within the cytoplasm, stimulating a subsequent immune response, which increased apoptosis (Crossley *et al.*, 2023).

With regards to clinical application, R-loop–associated proteins have emerged as promising therapeutic targets, as exemplified by the helicase senataxin, which has

been shown to impair tumour cell survival through increasing both nuclear and cytoplasmic DNA damage (Skourti-Stathaki, Proudfoot and Gromak, 2011; Ramachandran *et al.*, 2021; Gatti *et al.*, 2023). Other R-loop binding factors currently being explored as potential therapeutic targets include DDX9; which is being explored following observations that it triggers a tumour-intrinsic interferon response (Lee and Pelletier, 2016; Murayama *et al.*, 2024). In summary, these observations suggest that further analysis of R-loop interactomes could uncover novel candidate proteins with the potential to enhance radiosensitivity in hypoxic tumours.

DKC1

The DKC1 gene (Dyskerin Pseudouridine Synthase 1), located on the X chromosome, encodes the protein dyskerin (Hassock, Vetrie and Giannelli, 1999; Knight *et al.*, 1999). Dyskerin is a highly conserved pseudouridine synthase composed of 514 amino acids and plays a central role in the telomerase complex (Heiss *et al.*, 1998; Mitchell, Wood and Collins, 1999). DKC1 catalyses the pseudouridylation of RNA, a common post-transcriptional modification that enhances RNA stability and is also known by the terms NAP57, Cbf5 (yeast and archaea) and TruB (bacteria) (Grozdanov *et al.*, 2009; Dokal *et al.*, 2011; Mason and Bessler, 2011; Miracco and Mueller, 2011; McMahon, Bellodi and Ruggero, 2013).

DKC1 functions at telomerase and ribosomes

The control of telomere stability is vital for the preservation of DNA integrity, to maintain cell proliferation and to regulate cellular senescence (Blasco, 2005; Blackburn, Epel and Lin, 2015; Chakravarti, LaBella and DePinho, 2021). Telomeres, being the

protective end regions of eukaryotic chromosomes, are made up of 5-15 kb of double-stranded tandem repeats (Makarov, Hirose and Langmore, 1997; Wright *et al.*, 1997). This stability is aided by the protective mechanisms of the telomerase and shelterin complex (Greider, 1991; de Lange, 2005; Xin, Liu and Songyang, 2008; Roake and Artandi, 2020). The telomerase complex has many components, including the telomerase RNA component (TERC), the catalytic subunit telomerase reverse transcriptase (TERT) and proteins from the H/ACA ribonucleoprotein (RNP) family (Figure 1.5) (Xin, Liu and Songyang, 2008; Martínez and Blasco, 2011). Dyskerin is a member of the H/ACA ribonucleoprotein family, in association with the factors NHP2, NOP10, and GAR1 (Figure 1.5. A) (Lafontaine *et al.*, 1998; Xin, Liu and Songyang, 2008). Telomerase and telomere stability are strongly associated with cancer, as dysregulation of either function is linked to malignant tumours (Nakayama *et al.*, 1998; Nassour *et al.*, 2023). Additionally, as telomerase activity is lower in most normal tissues, the inhibition of telomerase could prove an attractive strategy for cancer therapeutics (Herbert, Wright and Shay, 2001; Ouellette, Wright and Shay, 2011; Kan, Wang, Sheng, Yao, *et al.*, 2021).

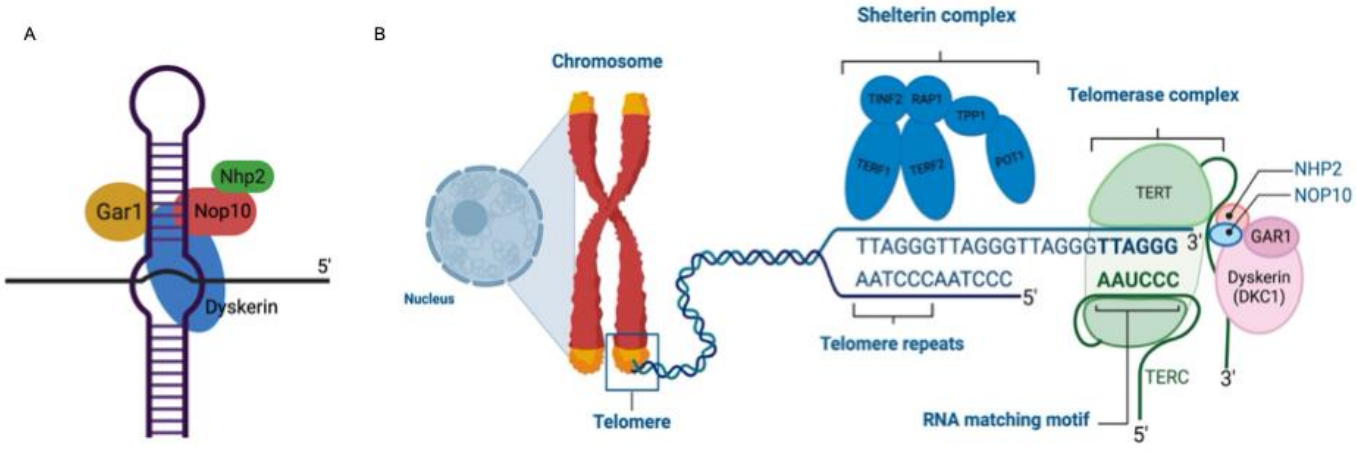


Figure 1.5. Diagrams outlining DKC1 functions at telomeres. A) Composition of the H/ ACA family of ribonucleoproteins. Created using BioRender. B) Telomerase is comprised of TERT and TERC. Telomerase, as a ribonucleoprotein, maintains telomere length using an RNA template to reverse transcribe and synthesise telomeric DNA repeats. The H/ACA RNP complex proteins are shown RHS including Dyskerin, GAR1, NOP10 and NHP2. From (Loukopoulou *et al.*, 2024).

Dyskerin has key roles in the maintenance of this system, ranging from the maturation of the telomerase complex, the accumulation of TERC, and the activation of telomerase (Lafontaine *et al.*, 1998; Mitchell, Wood and Collins, 1999). The precise details of the various roles of dyskerin in telomerase assembly, maintenance and TERC processing, however, are not yet fully elucidated (MacNeil, Lambert-Lanteigne and Autexier, 2019).

Together with its binding partners NHP2 and NOP10, DKC1 also performs non-telomeric functions, playing a central role in the pseudouridylation required for the maturation of rRNA, tRNA, and snoRNA (Trahan, Martel and Dragon, 2010; Ibáñez-Cabellos *et al.*, 2018). Specifically, dyskerin acts as an RNA-guided pseudouridine synthase, binding to snoRNAs and then catalysing the isomerisation of uridine residues to pseudouridine (Ψ) in 18S and 28S rRNAs (Figure 1.6) (Ge and Yu, 2013; Schwartz *et al.*, 2014; Penzo and Montanaro, 2018; Garus and Autexier, 2021).

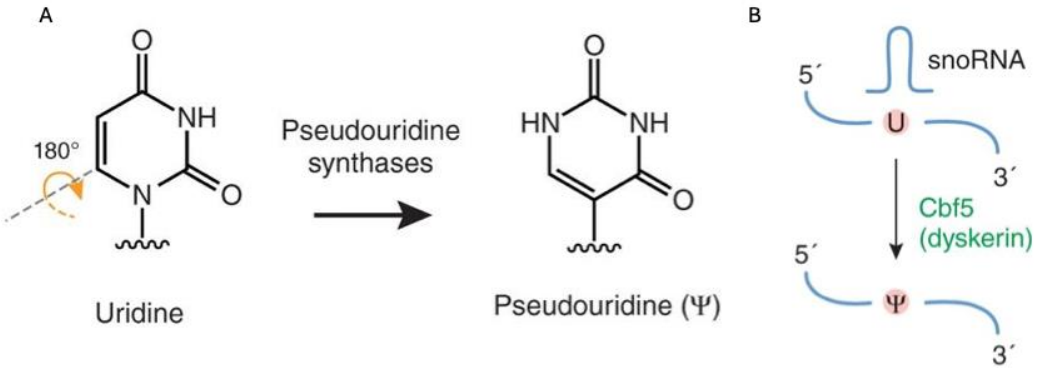


Figure 1.6. Dyskerin acts as an RNA-guided pseudouridine synthase. A) Schematic outlining the 180° flip of the uracil base that pseudouridine synthases facilitate. B) Dyskerin specifically needs a snoRNA to undertake the enzymatic mediation; it works as a guide bringing the dyskerin complex to a target RNA. Adapted from (Jaffrey, 2014).

Dyskerin has also been observed to have a role in ribosome biogenesis, and its dysregulation has been associated with defects in this process, including a decrease

in rRNA pseudouridylation (Ruggero *et al.*, 2003; Elsharawy *et al.*, 2020; Richards *et al.*, 2020). Resultingly, mutations of DKC1 have been linked to impairments in both ribosome maturation and pseudouridylation (Penzo and Montanaro, 2018). As well as these known functions, it has also been suggested that there are likely roles of DKC1 yet to be elucidated (Kirwan and Dokal, 2009; Liu, Tan and Li, 2023).

Dyskeratosis congenita

Dyskeratosis congenita (DC) is a rare inherited disorder of telomere maintenance, it is marked by malformation in the outer ectoderm, involving abnormalities of the mucus membranes, skin and nails (Kirwan and Dokal, 2008; Armanios and Blackburn, 2012). It is linked to an increased risk of disorders, including cancer, gastrointestinal issues, immunological issues and bone marrow failure (Mitchell, Wood and Collins, 1999; Kirwan and Dokal, 2008; Armanios and Blackburn, 2012).

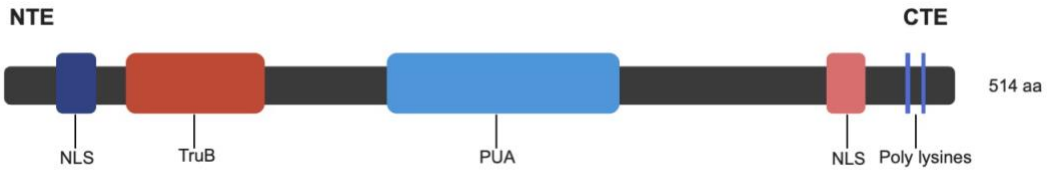


Figure 1.7. Schematic detailing the functional domains of DKC1.

DKC1 was first discovered as mutations in the DKC1 gene are linked to X-linked recessive dyskeratosis congenita (X-DC), the most prevalent form of the disorder (Knight *et al.*, 1999). There are two key regions of DKC1 within which pathogenic mutations in X-DC mainly occur; these are the N-terminal extension (NTE) and another domain near the C-terminus (Figure 1.7) (Li and Ye, 2006; Rashid *et al.*, 2006). In humans, a null mutation for Dyskerin has never been observed, and it is lethal in mice

(He *et al.*, 2002). Therefore, the mutations described for DKC1 only impair function. Observations around how DKC1 functions are impaired by dyskeratosis congenita mutations remain a point of uncertainty, as studies emphasise the difficulty of disentangling ribosomal, telomerase, and RNA processing contributions to disease pathology (Mochizuki *et al.*, 2004; Wong and Collins, 2006; Walne *et al.*, 2007; Kirwan *et al.*, 2011; Tummala, Walne and Dokal, 2022). However, it has been shown that impairment of telomere maintenance is a key part of the pathology of the disease, with DC patients generally having short telomeres (Vulliamy *et al.*, 2001; Yamaguchi *et al.*, 2005; Wong and Collins, 2006; Alter *et al.*, 2007; Savage *et al.*, 2008).

Dyskerin and cancer

In addition to X-DC-related cancer, studies have seen dyskerin overexpression in breast, lung, prostate cancers, colorectal cancers, hepatocellular carcinoma and neuroblastoma (Montanaro *et al.*, 2006; Sieron *et al.*, 2009; Bellodi *et al.*, 2010; Alawi and Lin, 2011; Liu *et al.*, 2012; Penzo *et al.*, 2015; O'Brien *et al.*, 2016; Alnafakh *et al.*, 2019; Elsharawy *et al.*, 2020). Many of these results have also seen inhibition of DKC1 within various models result in slower cell and tumour growth as well as reduced motility (O'Brien *et al.*, 2016; Miao *et al.*, 2019; Hou *et al.*, 2020). With others having the overexpression of DKC1 associated with shorter survival (Elsharawy *et al.*, 2020; Kan, Wang, Sheng, Chen, *et al.*, 2021; Kan, Wang, Sheng, Yao, *et al.*, 2021).

Further connections to cancer have been suggested through observations of DKC1 linked to c-MYC as a conserved transcriptional target, where c-MYC is a transcription factor implicated in many cancers (Adhikary and Eilers, 2005; Alawi and Lee, 2007). Inhibition of dyskerin shows slowed cell proliferation in telomerase-independent

neuroblastoma cells in MYC-driven neuroblastomas (O'Brien *et al.*, 2016). The cumulative findings highlight that alterations of DKC1 expression appear to have roles in tumour activation or suppression.

DKC1 and hypoxia

There has been one study to link DKC1 with HIF-1α, where they observed a positive correlation between DKC1 and HIF-1α expression in normoxic and hypoxic conditions (Hou et al., 2020). The paper suggested that DKC1 was able to bind to the promoter region of HIF-1α and increase HIF-1α transcription, therefore enhancing HIF-1 expression (Hou et al., 2020). This study was the first and currently only research linking hypoxia and DKC1, but the suggestion of a strong link opens further interest in DKC1 as a possible target to reduce hypoxia-related therapeutic resistance.

DKC1 and the DDR

There is no solid consensus on the effects of DKC1 mutations or depletion on the DDR. The p53 pathway has been seen to be activated and altered following DKC1 depletion through siRNA silencing of DKC1 or NOP10 (its telomerase binding partner) (Ibáñez-Cabellos *et al.*, 2020). It has also been observed in DC cells with mutations for telomere-binding proteins or telomerase components that a DDR is triggered through p53 and p21 (a downstream mediator) (Westin *et al.*, 2011). Another study examining pathogenic DKC1 mutations in mice saw that they caused an enhanced DNA damage response in normal telomeres (Gu, Bessler and Mason, 2008; Kirwan et al., 2011). Contrary to this, siDKC1 colorectal cells saw a decrease in DDR compared to control (Tammy, 2015). This was similarly seen in U2Os osteosarcoma cells, where

siDKC1 cells were seen to limit DNA damage accumulation (Lin, Mobasher and Alawi, 2014).

Examinations of siDKC1 cells showed no clear changes in cellular viability, cell cycle or γH2AX foci even following H_2O_2 (Lin, Mobasher and Alawi, 2014; Ibáñez-Cabellos *et al.*, 2018). This has been theorised to be a result of the low number of DSBs at 48 h of cell culture, or that the cellular model being used may have an impaired DDR. (Ibáñez-Cabellos *et al.*, 2018). In addition, it was suggested that H_2O_2 is not an effective inducer of DSBs as it mainly causes SSBs (Ibáñez-Cabellos *et al.*, 2018). Additionally, studies examining dyskeratosis congenita patient cells with DKC1 mutations have seen either higher levels of γH2AX or no significant difference within mutated cells (Kirwan *et al.*, 2011; Manguan-Garcia *et al.*, 2014; Pereboeva *et al.*, 2016; Ibáñez-Cabellos *et al.*, 2018).

DKC1 and oxidative stress

The loss of DKC1 and/or its associated protein NOP10 has been observed to trigger oxidative stress and impair ribosome biogenesis through activation of p53 and NPM1 pathways (Ibáñez-Cabellos *et al.*, 2018, 2020). This was seen independently and before telomere shortening (Ibáñez-Cabellos *et al.*, 2018, 2020). One hypothesis being that as DKC1 is depleted, there is a triggering of DDR dysregulation and the accumulation of DNA damage may cause this increase in oxidative stress (Ibáñez-Cabellos *et al.*, 2018, 2020).

Reactive oxygen species (ROS) have various roles physiologically and can be produced as byproducts of oxygen metabolism (Sies, Berndt and Jones, 2017).

However, ROS levels can increase in response to various factors, for example, inflammation, environmental stressors, UV ionising radiation or pollutants (Sies, Berndt and Jones, 2017). When this leads to an accumulation of ROS in cells and tissues, we get oxidative stress, which can then induce oxidative DNA damage, DSBs, and telomere dysfunction (Park and Larsson, 2011; Massudi *et al.*, 2012; Moskalev *et al.*, 2013; Barnes, Fouquerel and Opresko, 2019). The mitochondria are where the bulk of ROS is produced within the cell, and it was seen that in oxidative stress conditions DKC1 translocates from the nucleus to the mitochondria (Scialò et al., 2018). Leading to a theory for DKC1 playing a role in regulating energy metabolism and ROS response (Scialò et al., 2018). Additionally, oxidative stress has been implicated in the pathogenesis of DC, with some DC gene mutations seen to increase ROS and decrease antioxidant expression in cells (Pereboeva *et al.*, 2013; Manguan-Garcia *et al.*, 2014; Ibáñez-Cabellos *et al.*, 2018, 2020). It has been suggested that these observations are the result of telomere dysfunction, causing initiation of the DDR and a pro-oxidant environment (Pereboeva *et al.*, 2013, 2016; Ibáñez-Cabellos *et al.*, 2018, 2020). Another suggested model involves a positive feedback loop of disruptions to oxidative stress pathways promoting telomere attrition to sustain elevated levels of ROS (Pereboeva *et al.*, 2016).

The findings here regarding DC show how possible DNA damage could be induced through changes to DKC1. Particularly with oxidative stress and the DDR heavily implicated in cancer, these results highlight the possibilities of the inhibition of DKC1 as a radiosensitising target, through increasing DNA damage and oxidative stress.

Pyrazofurin

Pyrazofurin was identified as an inhibitor for dyskerin through computational docking and then real-time PCR assays to detect dyskerin pseudouridylation activity *in vitro* (Rocchi et al., 2014). First isolated during a fermentation of *Streptomyces candidus* in 1969, Pyrazofurin is a C-nucleoside initially utilised as an antiviral drug against both RNA and DNA viruses *in vitro* and *in vivo* (Gutowski *et al.*, 1975; De Clercq, 2010). Within *Streptomyces candidus*, Pyrazofurin functions as an antimetabolite, stopping the biosynthesis of pyrimidines and nucleotides through inhibition of orotidine-5′-monophosphate decarboxylase (ODCase); this results in the halting of cellular replication and DNA synthesis (Cadman, Dix and Handschumacher, 1978; Olah *et al.*, 1980; Christopherson, Lyons and Wilson, 2002). As a small molecule inhibitor, the action of Pyrazofurin has been previously seen to follow this original role, inhibiting ODCase (Cadman, Dix and Handschumacher, 1978; Christopherson, Lyons and Wilson, 2002)

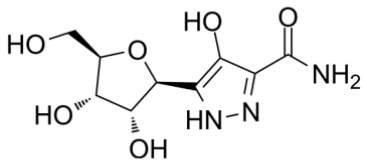


Figure 1.8. Chemical structure of Pyrazofurin.

The first published study investigating Pyrazofurin for its antitumour activities, saw a decrease in tumour size across 13 tumours using *in vitro* and rat studies (Sweeney *et al.*, 1973). This led to a variety of further studies of Pyrazofurin and antitumour effects, and eventually to it being tested in clinical trials for several different cancers, these have included sarcomas, colorectal carcinoma, breast cancer and acute myelogenous

leukaemia (Salem *et al.*, 1977; Cadman, Dix and Handschumacher, 1978; Gralla, Sordillo and Magill, 1978; Nichols *et al.*, 1978; Carroll, Kemeny and Gralla, 1979). While Pyrazofurin did make it to stage 3 clinical trials in some of the cancers, there was limited therapeutic potential. However, these studies did not consider DKC1 level within the cancers, something that Rocchi et al., (2014) highlights might mean lower levels of the drug could be used to be effective, therefore decreasing its toxicity.

Pyrazofurin as a DKC1 inhibitor

This identification of Pyrazofurin as a DKC1 inhibitor was carried out via computational docking of small molecules, and then the effects of selected compounds were tested to detect the dyskerin pseudouridylation activity *in vitro* by using a real-time PCR assay (Rocchi et al., 2014). This study renewed interest in Pyrazofurin, and since there have been two key studies which have looked further into the use of Pyrazofurin against DKC1, within both colorectal cancer and lung adenocarcinoma (Kan, Wang, Sheng, Chen, *et al.*, 2021; Kan, Wang, Sheng, Yao, *et al.*, 2021). Pyrazofurin was seen to decrease cell growth and colony numbers within these cancer types (Kan, Wang, Sheng, Chen, *et al.*, 2021; Kan, Wang, Sheng, Yao, *et al.*, 2021). Additionally, they observed that high expression levels of DKC1 can increase the expression of ribosomal proteins, depending on pseudouridine synthase activity and subsequently promote colorectal cancer progression (Kan, Wang, Sheng, Chen, *et al.*, 2021). Additionally, when Pyrazofurin was combined with trametinib in a combination therapy, there was synergistic anticancer activity seen within CRC cells (Kan, Wang, Sheng, Chen, *et al.*, 2021). These studies further emphasised the potential of utilising Pyrazofurin in cancers with very high DKC1 expression (Kan, Wang, Sheng, Chen, *et al.*, 2021).

As the most recent papers examining DKC1 as a target focused on lung and colorectal cancers, this project looked within these cancer types (Hou *et al.*, 2020; Kan, Wang, Sheng, Chen, *et al.*, 2021; Kan, Wang, Sheng, Yao, *et al.*, 2021). This is both useful for comparability to the results within these studies, but also as both lung and colorectal cancers have persistent radiotherapy resistance and hypoxic solid tumours (Clifford *et al.*, 2018; George *et al.*, 2019; Buckley *et al.*, 2020; Ancel *et al.*, 2021; Césaire *et al.*, 2022; Fletcher *et al.*, 2022). This project focused on identifying a novel target to address hypoxic radioresistance, and to do this, looked to identify a novel R-loop-associated protein. Which, as far as we are aware, has not yet been explored within published work on hypoxic radioresistance. Within this study, the protein DKC1 was identified when examining R-loop interactomes. DKC1 is a promising candidate given its roles in telomerase, ribosome biogenesis, and RNA processing, but its role in radiosensitivity under hypoxia has not yet been tested.

Aim

Since radiotherapy resistance remains a critical clinical challenge in lung and colorectal cancers, identifying R-loop–associated proteins as radiosensitising targets could provide a new therapeutic strategy to improve treatment outcomes in these patient populations. Therefore, this project aimed to identify a novel target for increasing sensitivity to radiotherapy, potentially through targeting R-loops, in hypoxic tumours.

Methods and Materials

Cell culture

Cell lines listed were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS). All cell lines were incubated in a humidified incubator at 37°C in 5% CO_2 and exponentially grown. Routine tissue culture involved media removal, washing with 1x phosphate-buffered saline (PBS) and trypsinisation for approximately 5 min at 37°C using 0.25% Trypsin-EDTA (Sigma Aldrich). The trypsinised cells were observed under a microscope to confirm detachment and subsequently resuspended in 2x trypsin volume of fresh pre-warmed media to inactivate the trypsin. Cells were counted manually using a Hawksley haemocytometer counting chamber. All cell lines were verified to be negative for mycoplasma using a Lonza MycoAlert® mycoplasma detection assay (LT07-318, Lonza).

Hypoxia treatment

Hypoxia treatments of <0.1% 0_2 were carried out in an A35 anaerobic workstation (Don Whitley Ltd) and treatments of 2% 0_2 in an M35 workstation (Don Whitley Ltd). For all <0.1% 0_2 experiments, cells were plated on glass dishes. Oxygen concentrations were confirmed using an Oxylite probe (Oxford Optronix) and anaerobic indicator strips (Fisher Scientific).

Cell lysis and protein sample preparation

Cells were washed twice with PBS and harvested in UTB lysis buffer (9 M urea, 75 mM Tris- HCl pH 7.5, 0.15 M β-mercaptoethanol). Samples were sonicated for 20 seconds (s) twice with 10 s on ice in between before followed by centrifugation at 16000 x *g* for 10 min at 4°C. The protein concentration of the supernatant was quantified using a NanoDrop One/OneC Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific).

Western blotting

10-100 µg of protein was mixed with sample buffer (3.3% SDS, 6 M urea, 17 mM Tris-HCl (pH 7.5), 0.07 M β-mercaptoethanol, 0.01% Bromophenol blue) and denatured at 100°C for 10 min. 5 µL of protein ladder (Dual Colour Precision Plus Protein Standards kaleidoscope protein ladder, BioRad) was loaded for indication of molecular weight of the band followed by the prepared samples. Samples were electrophoresed in Tris-glycine running buffer (25 mM Tris base, 192 mM glycine, 0.1% (w/v) SDS) on 4-20% or 7.5% Mini- PROTEAN TGXTM pre-cast gels at 100-120 V for 60-90 min. Gels were electro-transferred onto a 0.2 µM nitrocellulose membrane transblot (BioRad) on a Transblot-Turbo machine (BioRad) for 7 min in transfer buffer (25 mM Tris base, 150 mM glycine, methanol). The membranes were placed in Intercept® Blocking Buffer (LI-COR) diluted 1:1 in Tris-Buffered Saline (TBS) (50 mM Tris-HCl, pH 7.6, 150 mM NaCl) for 1 h of blocking on a belly dancer shaker (Stovall Life Science). Membranes were then incubated with primary antibody (diluted in 1:1 of Intercept® Blocking Buffer (LI-COR): TBS-Tween) overnight at 4°C while shaking. Primary antibodies used are listed in Table 1.5. Membranes were washed 3 times in TBS-Tween (TBST) for 5 min each time. Membranes were probed with secondary antibody, (diluted in 1:1 of Intercept® Blocking Buffer (LI-COR): TBS-Tween) for 1.5 h at RT on belly dancer

shielded from light. Secondary antibodies used are listed in Table 1.6. The membranes were then washed 3 times for 5 min in TBST before being stored in TBS for imaging. The blots were developed using the LI-COR Biosciences Odyssey Infrared imaging system and Image Studio™ Version 5.2 software.

MTT assay

Cells were seeded at the appropriate density, depending on the cell line (Table. 1.1) in clear, flat bottomed 96 well plates to give a total volume of 100 µL per well. Each treatment condition was performed in triplicate. After treatment, 3-(4,5-Dimethyl-2-yl)-2,5-diphenyltetrazolium bromide (10 µL, 0.5 mg/mL) was added to each well and then the plate was incubated (37°C, 2-3 h) away from light. The plate was checked under a light microscope to ensure that purple crystals had formed inside the cells within the untreated wells. Media was carefully removed from the wells and DMSO (100 µL/well) was added before incubating the plate (37°C, 15 min) away from light. The contents of each well were mixed, and the absorbance (570 nm) was read immediately (POLARstar plate reader, BMG LabTech). Cell viability was calculated as a percentage relative to the untreated control.

Table 1.1. Seeding numbers for MTT

Cell line	24 h treatment (48 h from seeding)
HCT116	3,500
A549	4,000

MTT cell optimisation number assay

Between 0 and 6,000 cells were seeded. The resulting absorbances were plotted as shown in Figure 2.1. with the number closest to an absorbance of 1 chosen in each case.

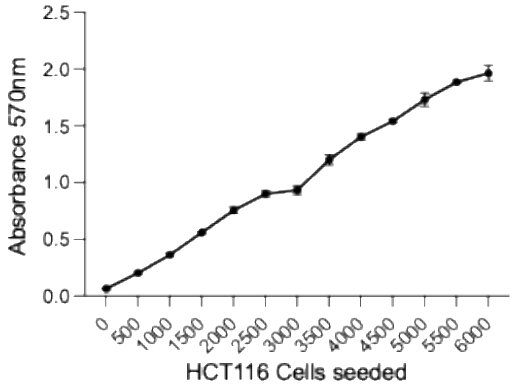


Figure 2.1. Optimisation of cell number for the MTT assay in HCT116 cells. 3,500 cells gave an absorbance reading of approximately 1; therefore, 3,500 was used going forward.

Radiation treatment

Irradiation was undertaken using g-rays from a Cs-137 irradiator (GSM: GSR D1) at a dose rate of 1.7 Gy/min, at rt. For irradiating experiments in hypoxia, cells were transferred to airtight Perspex plastic boxes to allow for transportation to and from the irradiator. The OER was assessed for every experiment to ensure that no reoxygenation had occurred.

Clonogenic survival assay

Cells were trypsinised into a single-cell suspension and counted. 200 cells (A549 and HCT116) were plated into each well in 6-well plates, with 3 technical replicates per treatment. Cells were left to settle for 3-5 h before drug treatment for 16 h. After treatment, media containing the drug was replaced with fresh media and the cells were

left to form colonies for 7-9 days in a humidified incubator at 37°C. The media was removed, and crystal violet (0.5% w/v) in 50% methanol and 20% ethanol was added to the wells to stain and visualise the colonies. Colonies containing at least 50 cells were counted using a colony counter.

Immunofluorescence microscopy

Cells on coverslips were washed once with PBS and fixed in 4% (w/v) paraformaldehyde in PBS for 10 min. The cover slips were washed 3 times with PBS to remove the paraformaldehyde. Cells were then permeabilised in 1% Triton-X-100 in PBS for 10 min, then blocked with 2% (w/v) BSA in 0.1% Tween-20 in PBS for 10 min, then blocked with 2% (w/v) BSA in 0.1% tween-20 in PBS for 10 min, then blocked with 2% (w/v) BSA) in 0.1% Tween-20 in PBS for 1 h. Primary antibodies were incubated on coverslips for 2 h at 37°C or overnight at 4°C. Coverslips were then washed 3 times with PBS before being incubated in secondary antibody for 1 h at 37°C. Cells were mounted onto slides using ProLong Gold Antifade Mountant with DAPI (Thermo Fisher). Cells were imaged using LSM710 confocal microscope ((Carl Zeiss Microscopy Ltd). Antibodies used are listed in Tables 1.5 and 1.6.

Short interfering RNA (siRNA) transfection

The Dharmafect-1 Transfection Reagent (Horizon Discovery) was used to transfect cells following the manufacturer's protocol. Cells were seeded in a 6-well plate using the appropriate cell density for the cell line and left overnight. The siRNA was

incubated in FBS-free and antibiotic-free media. Separately, the Dharmafect-1 Transfection reagent was also incubated in FBS-free and antibiotic-free media. Both mixtures were left to incubate at RT for 5 min. The siRNA mixture was then mixed with the transfection reagent mixture and incubated at RT for 20 min. The siRNA transfection mixture was then diluted in complete antibiotic-free media to a final concentration of 50 nM. The media on the seeded cells were discarded, and the siRNA transfection mixture was then added to the cells. Cells were incubated for 24 h in a standard humidified incubator at 37°C, after which the media was replaced with fresh complete antibiotic-free media. Catalogue ID of siRNA [L-013639-00-0005].

Flow cytometry using PI

5 x 10^5 cells were seeded overnight and allowed to adhere. Pyrazofurin (1 or 2 μM) or CPT (0.5 μM) was added and left for 24 h. Cells were trypsinised, resuspended in 1 mL of fresh media and centrifuged at 250 x g. The supernatant was removed, and the cell pellet was fixed in 1 mL of 70% ethanol while being vortexed and then chilled on ice for 30 min. Samples were centrifuged at 250 x g for 2 min, the ethanol supernatant removed, and were washed in 1ml PBS and re-centrifuged at 250 x g for 2 min. The supernatant was removed, and the cell pellet was resuspended in propidium iodide (PI) staining solution containing PI (10 μg/ml) and RNase A (100 μg/ml) mixture and incubated at 37°C for 15 min. Data acquisition was carried out for 10,000 cells per experimental condition using CytoFLEX flow cytometer (Beckman Coulter) using CytExpert software. Analysis was carried out for a cell count of 10,000 events for each treatment condition. FloJo software (BD Biosciences) was used to analyse the data.

Materials

Table 1.2. Drugs used in the study

Name	Stock concentration and solvent; storage temperature	Final working concentration	Supplier
Camptothecin (CPT)	12 mM; DMSO; -20°C	10 μM	Sigma
Pyrazofurin	10 mM; Water; -20°C	0.5 -10 μM	Merck
Etoposide	25 mM; DMSO; - 20 °C	10 mM	Sigma
Remodelin	10 mM; DMSO, 4 °C	0 - 200 mM	Medchemexpress

Table 1.3. Cancer cell lines and specifications

Cell Line	Cell Type	Media	Source
A549	Lung carcinoma	DMEM	American Type Culture Collection (ATCC)
BT474	Oestrogen and progesterone receptor positive breast carcinoma	RPMI	Dr Ejung Moon, Oxford University
BT549	Triple-negative breast carcinoma	RPMI	Prof Katherine Vallis, Oxford University
FLO1	Oesophageal adenocarcinoma	DMEM	Prof Ricky Sharma, UCL
H460	Non-small cell lung cancer	DMEM	Prof Geoff Higgins, University of Oxford
HCC1395	Triple-negative breast cancer	DMEM	Dr Ejung Moon, University of Oxford
HCT116	Colon carcinoma	DMEM	Prof Bert Vogelstein, Johns Hopkins University
MDA-MB-231	Triple-negative breast adenocarcinoma	DMEM	Prof Anne Kiltie, Oxford University
MDA-MB-453	Triple-negative breast carcinoma	DMEM	IP

RKO	Colorectal carcinoma	DMEM	American Type Culture Collection (ATCC)

Table 1.4. Chemicals and cell culture reagents

Name	Producer/UK distributor
β-mercaptoethanol	Fisher Scientific
Blocking Buffer LI-COR	LI-COR
Dimethylsulphoxide (DMSO)	Fisher Scientific
Dulbecco's modified Eagle's medium (DMEM)	Gibco
Ethanol	Fisher Scientific
Foetal bovine serum (FBS)	Gibco
Gel Loading Buffer II	Fisher Scientific
Glycerol	Fisher Scientific
Glycine	Sigma-Aldrich UK
Hydrochloride acid (HCl)	Fisher Scientific
Methanol	Fisher Scientific
Penicillin-streptomycin (P/S)	Sigma-Aldrich
PFA Fisher Scientific	PFA Fisher Scientific
Phosphate buffer saline (PBS)	Fisher Scientific
ProLong™ Diamond antifade mountant (with Dapi)	Invitrogen/Life technologies
Propidium iodide (PI)	Sigma-Aldrich UK
Protease inhibitor (Pi)	Roche
Protein ladder Precision plus protein standards	Bio-Rad

RNAse A (ribonuclease A)	Sigma-Aldrich UK
Sodium chloride (NaCl)	Sigma-Aldrich UK
Sodium dodecyl sulphate (SDS)	Fisher Scientific
SYBR® Green	Applied Biosystems
Triton-X-100	Fisher Scientific
TRIzol®	Invitrogen/ Life Technologies
Trypan blue	Invitrogen
Trypsin 0.05% EDTA	Sigma-Aldrich UK
Tween 20	Fisher Scientific

Table 1.5 Primary antibodies

Name	Species	Dilution	Application	Producer	Product code
ZNF598	Rabbit	1:1,000	WB/IP	Genetex	GTX119245
β-Actin	Mouse	1:10,000	WB	Santa Cruz Biotechnology	AC-69879
HIF-1α	Mouse	1:1,000	WB	BD Biosciences	NB100-105
IgG	Mouse	1:1,000	IP	Abcam	Ab-37355
Lamin B2	Mouse	1:1,000	WB	Invitrogen	MA1-06103
S9.6	Rabbit	1:100/ 10 µg: 20 µg	IP	Kerafast	Kf-Ab01137- 23.0
Dyskerin	Mouse	1:1,000/ 1:250	WB/IF	Santa Cruz Biotechnology	SC-373956
P53-S15	Rabbit	1:1,000	WB	Cell signalling technology	9284S
53BP1	Rabbit	1:250	IF	Novus Biologics	NB100-305
P53	Mouse	1:1,000	WB	Santa Cruz Biotechnology	SC-126

Rpa32	RAT	1:250	IF	Cell signalling technology	2208S
Tubulin	Rabbit	1:1,000	WB	Abcam	AB6046
γH2AX	Rabbit	1:1,000	WB	Millipore Sigma	07-627
GLUT1	Rabbit	1:1,000	WB	Abcam	AB14683

Table 1.6 Secondary antibodies

Name	Species	Dilution	Application	Producer	Product code
IRDye 680RD Goat anti-Mouse IgG	Mouse	1:10000	WB	LI-COR	926-68070
IRDye 800CW Goat anti-Rabbit IgG	Rabbit	1:10000	WB	LI-COR	926-32211
Alexa Fluor 594-conjugated goat anti-rat IgG	Rat	1:250	IF	Invitrogen	A11006
Alexa Fluor 488-conjugated goat anti-rabbit IgG	Rabbit	1:250	IF	Invitrogen	A32731
Alexa Fluor 488-conjugated goat anti-mouse F(ab')2 fragment	Mouse	1:250	IF	Invitrogen	A11017

Results

ZNF598

ZNF598 has not been observed at R-loops, with the only tentative link to hypoxia being that ZNF598 was downregulated in pre-eclampsia (hypoxia is a pathological factor of pre-eclampsia) (Shabani *et al.*, 2024). However, ZNF598 has been linked to several cancer-related factors. For example, it has been observed to function as a radiation-regulated gene, with ZNF598 loss increasing resistance to UV-induced apoptosis (Yang and Gupta, 2017). Other links include observations of ZNF598 knock-out cells increasing sensitivity to the chemotherapy agent 5-Fluorouracil, and a positive correlation between ZNF598 presence and invasive bladder cancer linked to the ribosome quality control pathway (Khaket *et al.*, 2024; Ojha *et al.*, 2024; McGirr, Onar and Jafarnejad, 2025).

To begin examining ZNF598, a cell line was chosen. Whole cell lysates were run on a western blot, and ZNF598 was found to be uniformly expressed across the range of cell lines (Figure 3.1). As subsequent experiments had been optimised in human lung adenocarcinoma A549 cells, and ZNF598 showed similar expression in this cell line compared to the others, it was used going forward.

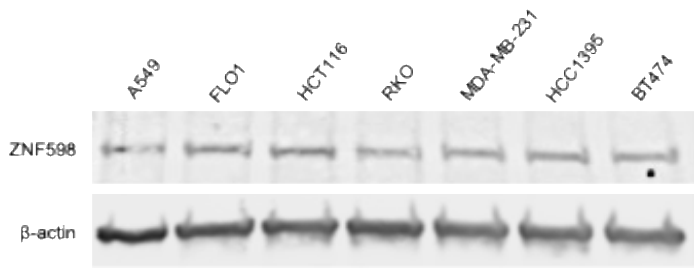


Figure 3.1. ZNF598 is expressed in a range of cell lines. Using a lung cancer cell line (A549), an oesophageal cancer cell line (FLO1), colorectal cell lines (HCT116 and RKO) and breast cancer cell lines (MDA-MB-231, HCC1395, BT474), western blotting was carried out for ZNF598. β-actin was used as a loading control. n=1

ZNF598 expression did not change in an oxygen-dependent manner

To study if there was a change in ZNF598 expression level between normoxia and hypoxia, a time course was carried out in 21%, 2% and <0.1% 0_2 (Figure 3.2.A). The quantified plot shows that there is no observable difference in ZNF598 expression level between the different oxygen conditions (Figure 3.2.B). As expected, HIF-1α was induced following hypoxia as a positive control for the conditions. Next, we examined the cellular localisation of ZNF598.

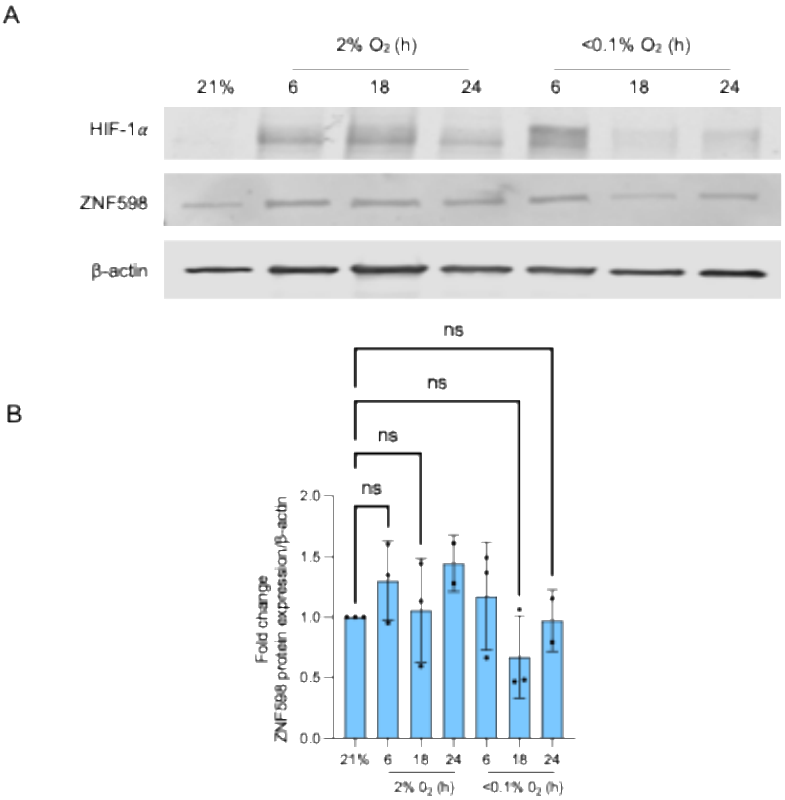


Figure 3.2. ZNF598 does not change in an oxygen-dependent manner. A) A549 cells were exposed to normoxic and hypoxic conditions (21%, 2% and <0.1% 0_2) followed by western blotting. HIF-1α was used as a positive control for hypoxic conditions. β-actin was a loading control. B) Quantification of the western blot normalised to β-actin. Statistics used were one-way ANOVA, where ns = p-value >0.05. For 6 h and 18 h, n=3, For 24 h, n=2

Most R-loop formation and R-loop factors are found in the nucleus (Hegazy, Fernando and Tran, 2020; Brickner, Garzon and Cimprich, 2022). To examine if ZNF598 was present in the nucleus, cellular fractionation was carried out in 21% and 2% 0_2. Preliminary data showed ZNF598 was only present in the whole cell and cytosolic fractions, with no clear protein expression in the nuclear fraction (Figure 3.3). Due to R-loops being mainly nuclear proteins and ZNF598 appearing almost exclusively cytoplasmic, it was decided to carry out analysis to identify a novel target.

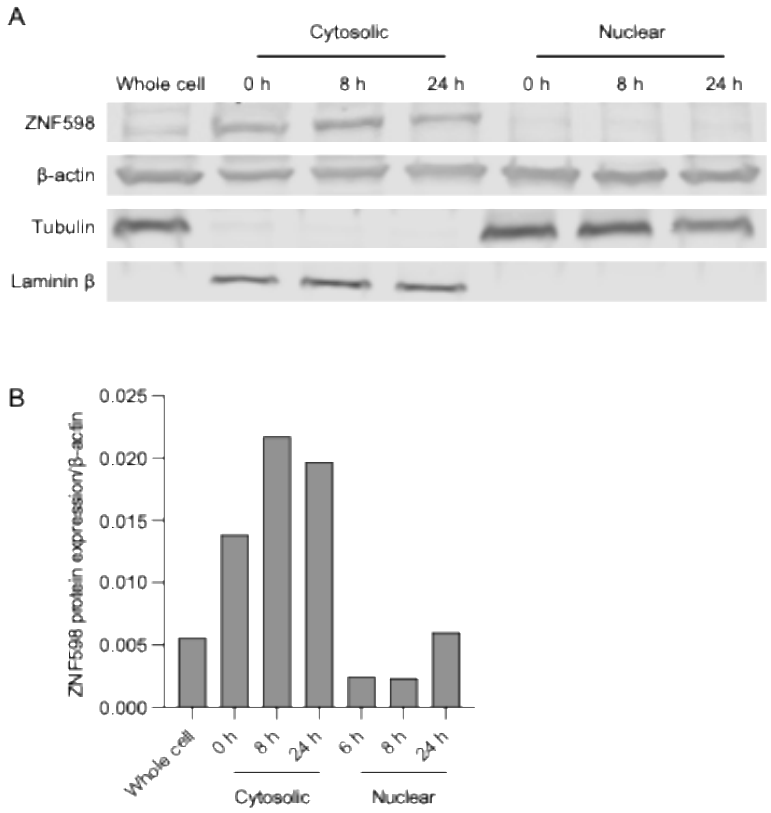


Figure 3.3. Fractionation blot shows ZNF598 present in the control and cytosolic fractions. A) H460 cells were exposed to hypoxia (2% 0_2) for the times indicated, followed by cellular fractionation and western blotting. β-actin was used as a loading control. Tubulin was the control for nuclear fractionation, and laminin β was the control for cytosolic fractionation. Samples were generated by Eduard Petrosyan. B) Quantification of the Western blot normalised to β-actin. n=1

Analysis of interactome data for a possible R-loop binding protein

At the time of writing, there were four published R-loop interactomes (Cristini et al., 2018; Wang et al., 2018; Wu et al., 2021 and Yan et al., 2022). These were analysed alongside an unpublished R-loop interactome (Petrosyan/Hammond). Utilising the Multiple List Comparator from molbiotools (http://www.molbiotools.com/listcompare.html), overlaps were identified between the data sets available (Figure 3.4).

From this ranked list, 19 possible targets were identified as present in all 5 interactomes, and 84 in 4 interactomes. Taking the 103 proteins from these two rankings, the cellular localisation was considered. As outlined above, R-loops have mostly been observed in the nucleus; therefore, the search focused only on targets that have been observed in the nucleus. Initial investigation was done through The Human Protein Atlas, then if thought to be nuclear, through confirmation of the associated literature (*The Human Protein Atlas*). The second consideration was the existence of a commercially available inhibitor for the target, leaving 10 possible targets. It was decided to exclude helicases and splicing factors, as their role in R-loop biology is the focus of significant research within the field, leaving three potential targets shown (Figure 3.4). One of these 3, NPM1, has been widely studied and well characterised in cancer therapeutics, and as the focus is on the identification of a novel target, it was excluded going forward (Loubeau *et al.*, 2014; Qin *et al.*, 2020; Karimi Dermani *et al.*, 2021; Khan and Gartel, 2022; Patel, 2023; Wang *et al.*, 2024). Following this, the two remaining proteins with commercially available inhibitors, DKC1 and NAT10, were chosen as targets for further examination.

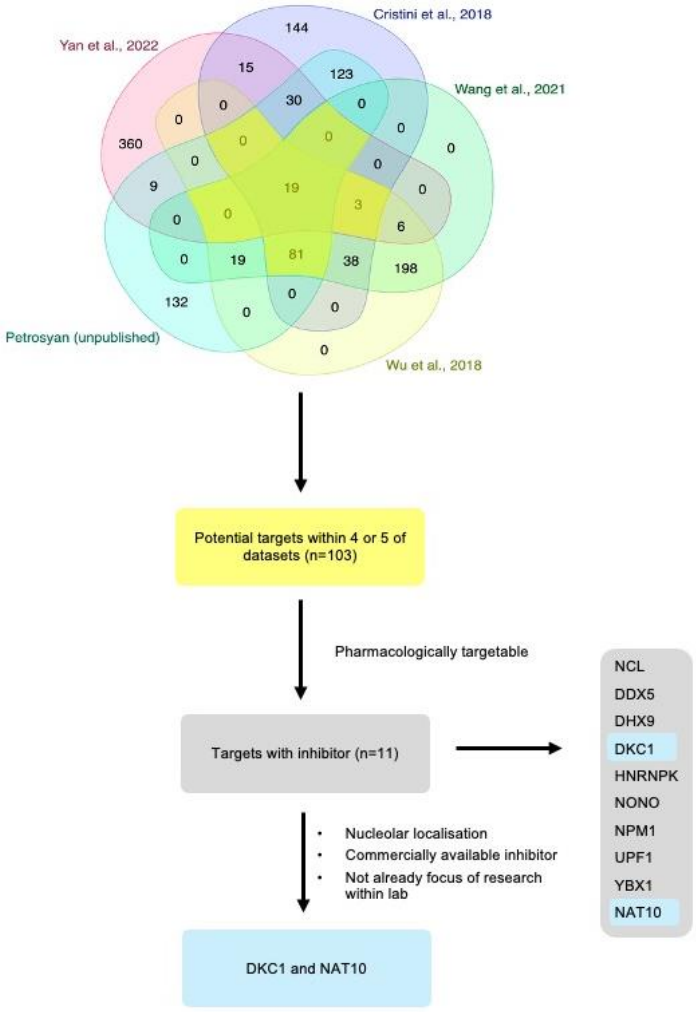


Figure 3.4. The target selection process. R-loop interactome lists from Yan et al.,22, Petrosyan/Hammond (unpublished), Wu et al., 2021, Wang et al., and Cristini et al., 2018 were overlapped to give potential targets in 4 or 5 of the interactomes analysed, diagram at top. These 103 were then taken forward and considered against the metrics listed in the diagram to select a potential target. The top diagram was created using Molbiotool.

Remodelin is a small-molecule inhibitor of NAT10

The nucleolar protein NAT10 is an N-acetyltransferase and an ac4C-associated protein responsible for governing RNA modification mechanisms (Arango *et al.*, 2018). In cancer, NAT10 is expressed at high levels to maintain mRNA acetylation, enabling mRNA stability and translation (Sinclair *et al.*, 2017; Arango *et al.*, 2018; Guo *et al.*, 2020; Sas-Chen *et al.*, 2020; Dalhat *et al.*, 2021). To examine NAT10 as a target, the response to its inhibitor, Remodelin, was analysed using MTT viability assays where

HCT116 cells were exposed to a range of doses of Remodelin for 24 h in normoxia (Figure 3.5.B). Compared to no treatment, the cell viability was seen to increase significantly at 100 and 200 μM Remodelin (Figure 3.5.B). This increased cell viability was not expected, and following a study showing NAT10 at R-loops, NAT10 was put aside for the other chosen target, DKC1 (Su *et al.*, 2024).

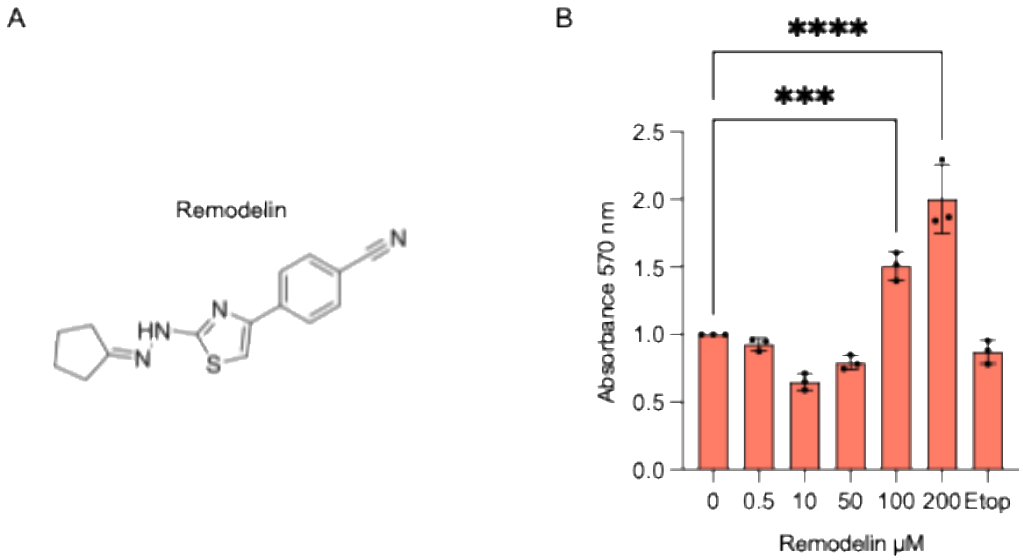


Figure 3.5. Remodelin led to increased cell viability. A) Chemical structure of Remodelin, a NAT10 small molecule inhibitor. B) HCT116 cells exposed to Remodelin for 24 h at doses indicated. Etoposide (Etop) was used at 25 μM for 6 h. An MTT assay was carried out. Statistics used were one-way ANOVA, where *** = p-value <0.001 and **** = p-value <0.0001. n=3

Validation of DKC1 siRNA and antibody

The siRNA DKC1 was used in tandem with the DKC1 small-molecule inhibitor Pyrazofurin. We transfected A549 cells with siSc (scramble) or siDKC1 (siRNA to DKC1). An efficient knockdown of DKC1 was observed compared to the siSc (Figure 3.6.A). A549 cells were treated with siDKC1 or siSc, followed by immunofluorescence staining with DAPI (blue) and DKC1 (green). DKC1 appeared predominantly nucleolar and reduced when treated with siDKC1 (Figure 3.6.B). This validated the use of the DKC1 antibody and DKC1 siRNA.

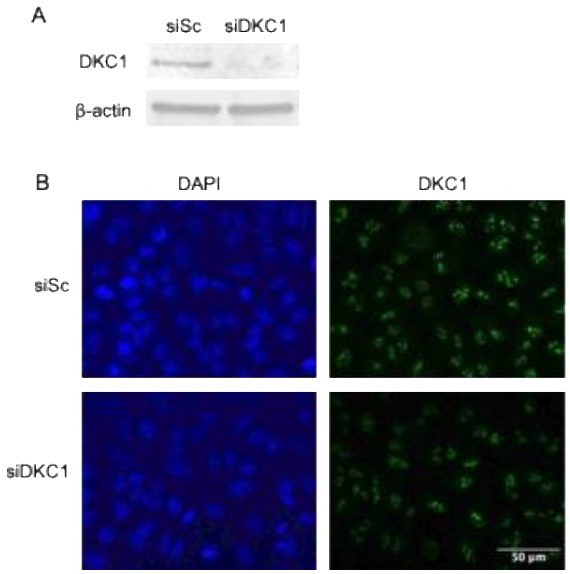


Figure 3.6. Depletion of DKC1 levels using siRNA. A) DKC1 antibody was applied to a western blot of A549 cells treated with siDKC1 or siSc (scramble) for 24 h. β-actin was used as a loading control. B) A549 cells were treated with siDKC1 or siSc for 24 h, followed by immunofluorescence staining with DAPI (blue) and DKC1 (green). n=1

To study the DKC1 expression level between normoxia and hypoxia, mRNA expression was examined. Evidence of links between hypoxia and DKC1 in cancer patients were analysed using GEPIA2 (Gene Expression Profiling Interactive Analysis 2) for The Cancer Genome Atlas (TCGA) in colorectal and lung cancer data sets against the hypoxia buffa signature (Buffa *et al.*, 2010). For LUSC and COAD, no correlation was seen (Figure 3.7. A & B). However, for both LUAD and READ data sets, there were R values of >0.6, suggesting a positive correlation (3.7. C & D). Together, these data suggest that in some tumour types, DKC1 could be induced in response to hypoxia at the mRNA level.

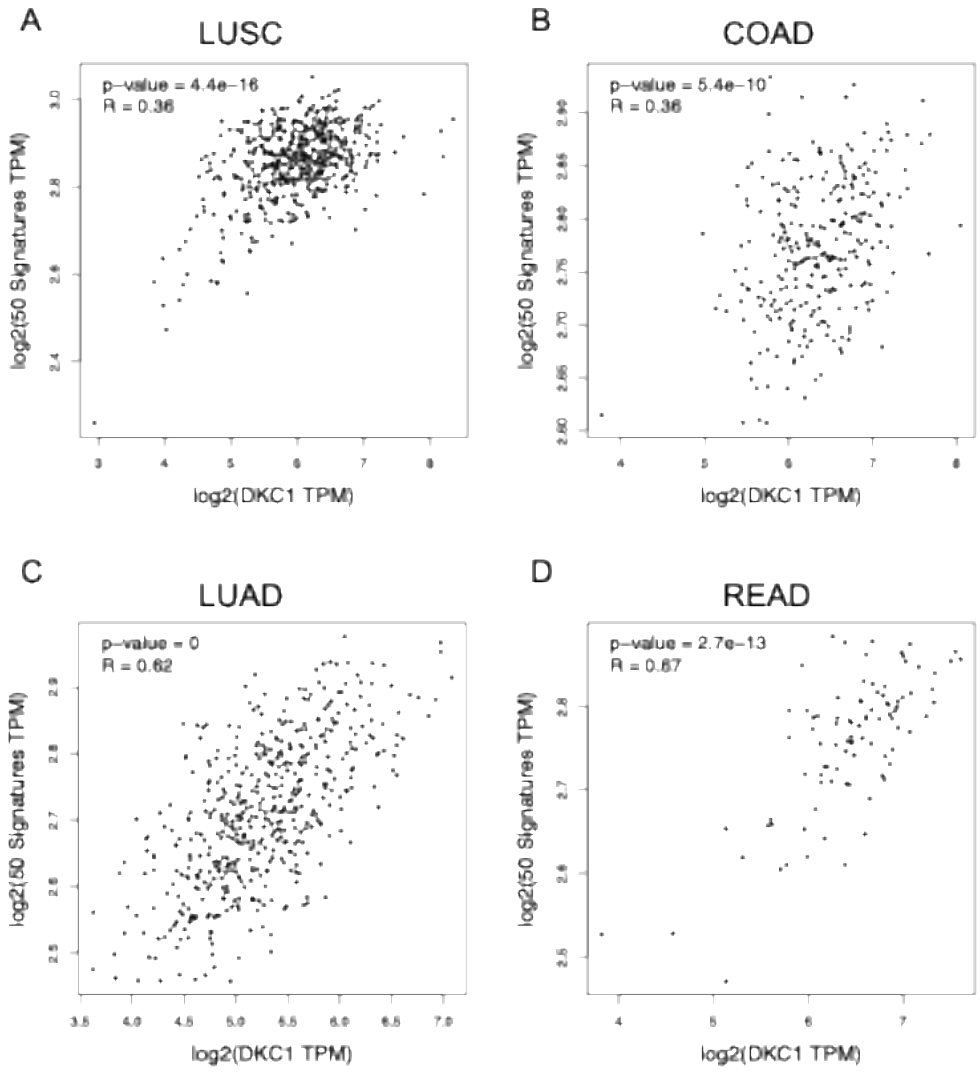


Figure 3.7. Correlation of DKC1 with hypoxia signatures and cancer cell types. Using Geipia 2 buffa hypoxia signature genes were compared with DKC1 to different cancer cell subtypes from the TCGA database to give correlation expression analysis. Pearson's correlation coefficient was used, where the non-log scale was used for calculation and the log-scale axis for visualisation. A) LUSC - lung squamous cell carcinoma. B) COAD - colon adenocarcinoma. C) LUAD - lung adenocarcinoma. D) READ - rectum adenocarcinoma.

DKC1 protein expression level does not change in hypoxic conditions

To examine DKC1 protein expression in hypoxic conditions, a time-course in HCT116 cells at 21%, 2% and <0.1% 0_2 was carried out to examine DKC1 protein expression in hypoxic conditions (Figure 3.8.A). HIF-1α is the positive control for hypoxia, and as expected, it accumulated in response to hypoxia (Figure 3.8.A). DKC1 expression did not change in 2% and <0.1% 0_2 even after 24 h (Figure 3.8.B). Additionally, a variety

of breast cancer cell lines were exposed to 16 h of cyclic hypoxia. Through creating <0.1% 0_2 conditions and then reoxygenating to 2% 0_2 in dynamic cycles within a chamber, cyclic hypoxia has been seen in cancer cells to accumulate DNA damage as a result of increased ROS (Bader *et al.*, 2021). No change in DKC1 protein expression was seen in cyclic hypoxic conditions (Figure 3.8.C).

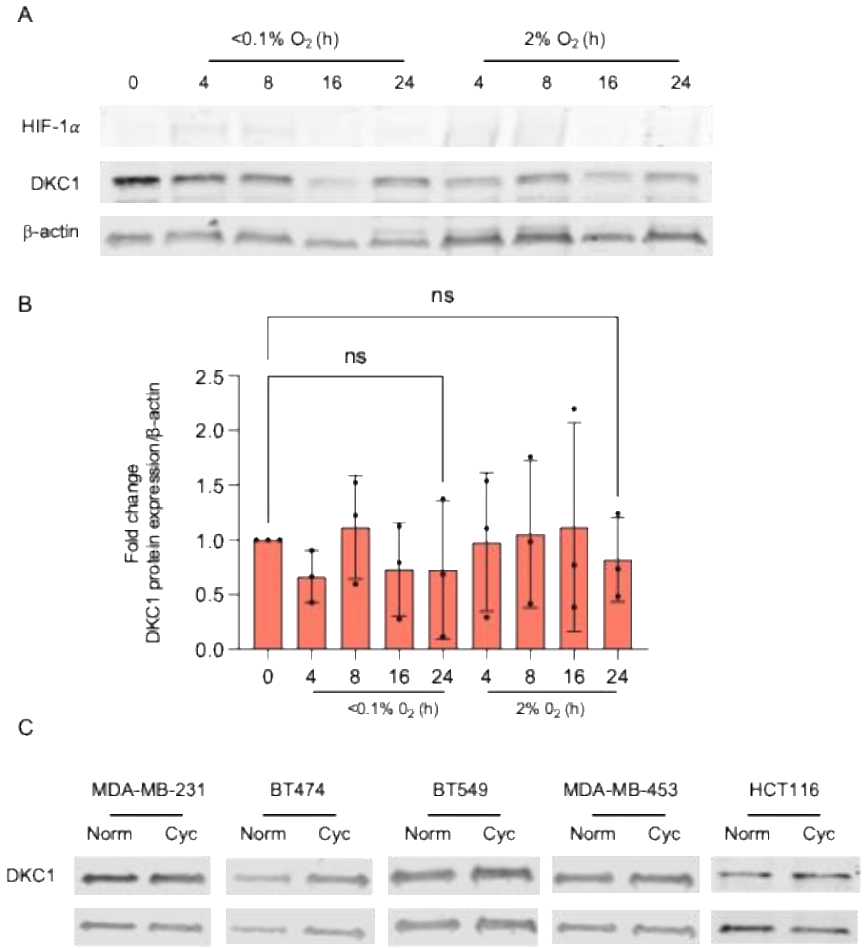


Figure 3.8. DKC1 expression does not change in hypoxia. A) HCT116 cells were exposed to hypoxia (<0.1 or 2% 0_2) followed by western blotting. β-actin was used as a loading control. B) Quantification of the Western blot normalised to β-actin. Statistics used were one-way ANOVA, where ns = p-value >0.05. n=3. C) Cell lines exposed to 16 h cyclic hypoxia between <0.1 and 2% O_2. DKC1. β-actin was used as a loading control. n=1

Pyrazofurin does not change HIF-1α protein expression

A previous study showed DKC1 knockdown decreased HIF-1α expression in hypoxia (Hou *et al.*, 2020). To examine if the DKC1 inhibitor Pyrazofurin would affect the accumulation of HIF-1α protein expression, A549 cells were exposed to Pyrazofurin (1 μM) for 24 h and then incubated at either 21% or <0.1% 0_2. HIF1-α is the positive control for hypoxia, and as expected, was seen to increase compared to normoxic conditions (Figure 3.9.B). However, for both 2 and 6 h of hypoxia exposure, no significant difference in HIF-1α protein expression was seen following Pyrazofurin treatment compared to untreated hypoxic control (Figure 3.9.C). These data suggest that DKC1 inhibition by Pyrazofurin does not vary within hypoxic conditions and does not modify HIF-1α stabilisation. We next looked to examine cell viability following Pyrazofurin treatment at various concentrations.

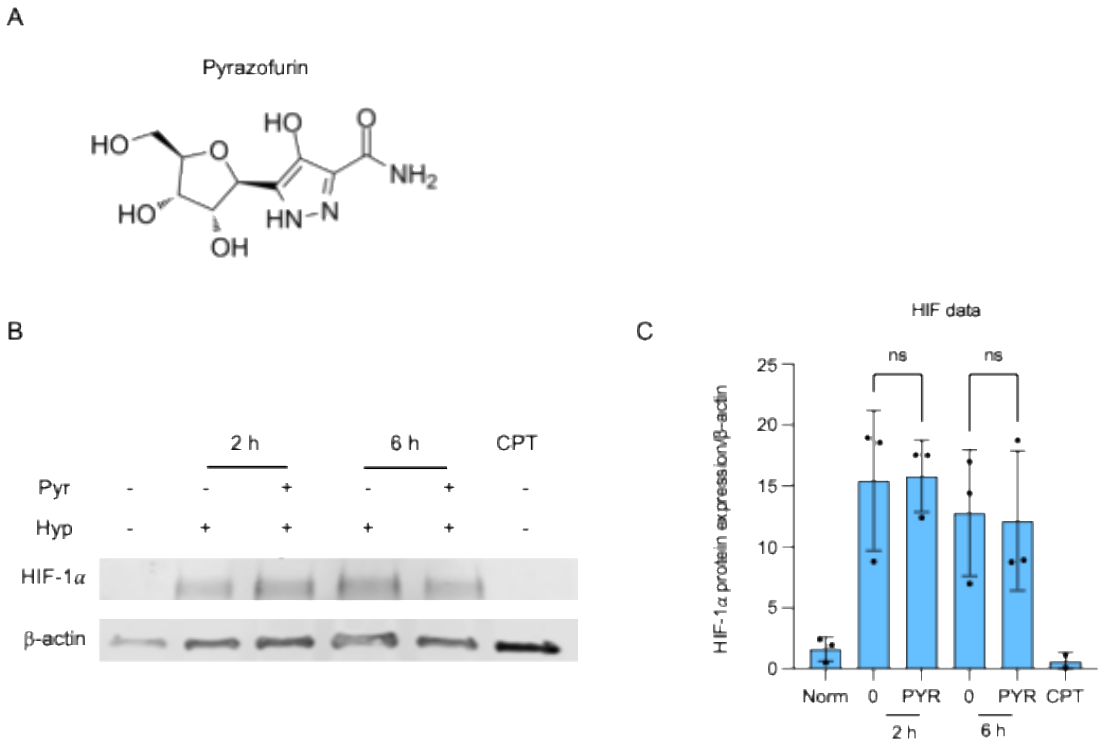


Figure 3.9. Pyrazofurin does not change HIF-1α expression. A) Chemical structure of Pyrazofurin. B) A549 cells were exposed to Pyrazofurin (1 μM) for 24 h and then spent 2 h or 6 h at either 21% or <0.1% 0_2, followed by western blotting. CPT (Camptothecin) was used at 25 μM for 6 h. β-actin was used as a loading control. C) Quantification of the western blots normalised to β-actin. Statistics used were one-way ANOVA, where ns = p-value >0.05. n=3

Pyrazofurin does not affect cell viability

To test the effect of Pyrazofurin on cell viability, a range of concentrations were applied to A549 and HCT116 cells for 24 h, followed by an MTT assay. Etoposide (Etop) was used as the control, and as expected, showed a significant decrease in cell viability. Between the concentrations of 0.5-5 μM Pyrazofurin, no change in cell viability was seen for HCT116 and A549 cells (Figure 3.10.A & B). For HCT116, there was again no change in cell viability at 10 μM Pyrazofurin (Figure 3.10. A). However, in A549 cells, a decrease in cell viability was seen at 10 μM Pyrazofurin (Figure 3.10.B).

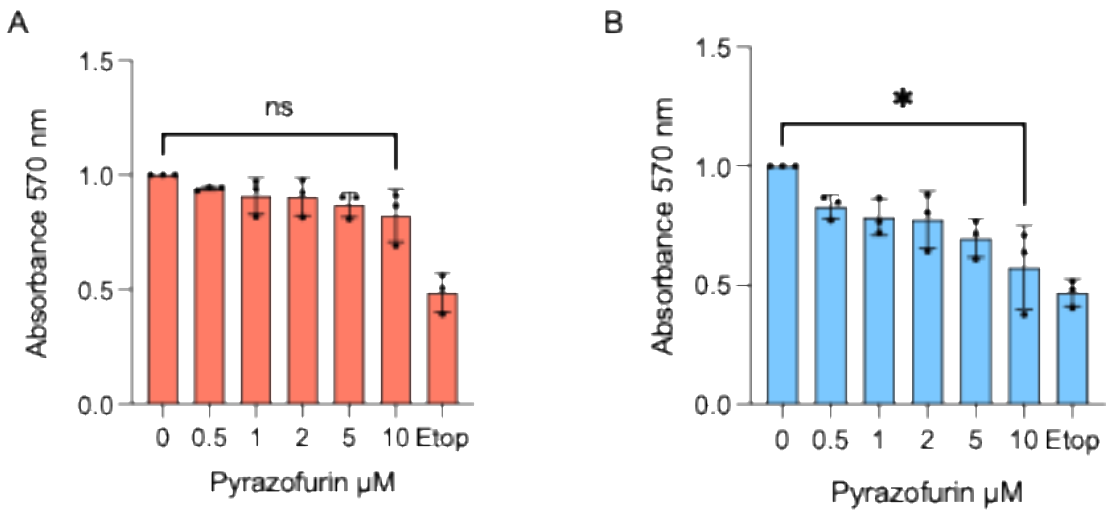


Figure 3.10. Pyrazofurin decreases MTT cell viability for A549 but not HCT116 cells. A) HCT116 cells exposed to Pyrazofurin for 24 h for MTT in 21% 0_2. B) A549 cells exposed to Pyrazofurin for 24 h for MTT in 21% 0_2. Etoposide (ETOP) was used at 25 μM for 6 h as a positive control for damage. Statistics used were unpaired t-test, where * = p-value <0.05 and ns = p-value >0.05. n=3

Colony survival assays are the gold standard for the determination of cell viability. Therefore, to study the effect of Pyrazofurin on cell viability further, clonogenic survival assays were carried out in 21% 0_2 in A549 and HCT116 cells. Pyrazofurin was applied for 16 h and media was then changed before the plates were left to grow for 7-9 days (Figure 3.11.A). At 10 μM Pyrazofurin, as seen with the MTT, there was no significant

difference between the untreated control and Pyrazofurin for HCT116 cells (Figure 3.11.B). For A549 cells, again as seen with the MTT, 10 μM Pyrazofurin gave a decrease in cell viability compared to the untreated control (Figure 3.11.C).

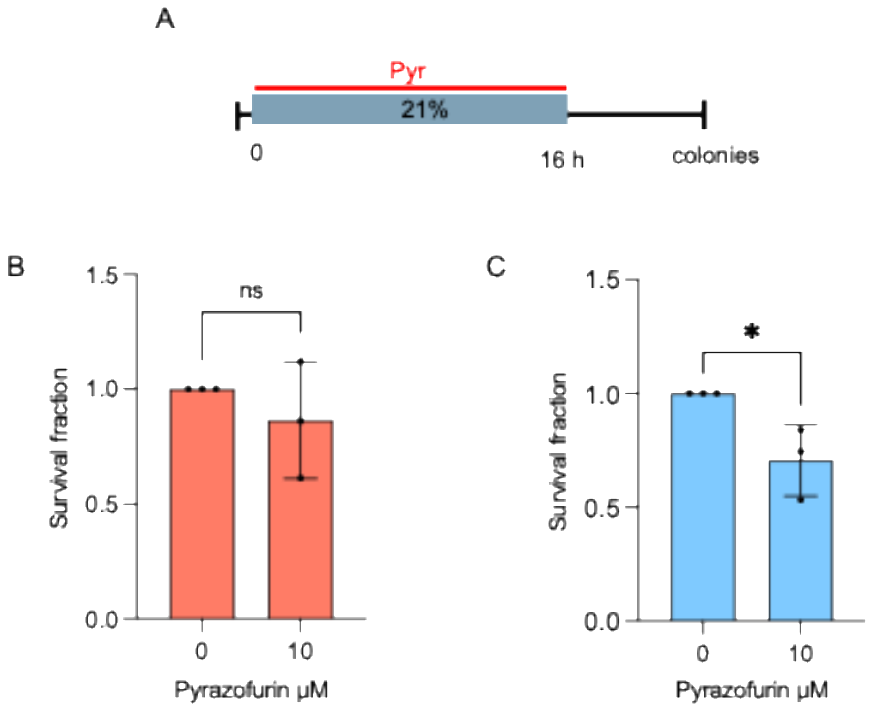


Figure 3.11. Pyrazofurin decreases clonogenic survival assay in normoxia for A549 but not HCT116 cells. A) HCT116 cells were exposed to different concentrations of Pyrazofurin for 16 h, the media was then changed, and the cells were left to form colonies. B) A549 cells were exposed to different concentrations of Pyrazofurin for 16 h, the media was then changed, and the cells were left to form colonies. Statistics used were an unpaired t-test, where * = p-value <0.05 and ns = p-value >0.05. n = 3

As we were interested in an agent that would be effective in hypoxia, the clonogenic survival assays were carried out again; this time with exposure to hypoxic conditions (<0.1% 0_2) in A549 cells. As before, Pyrazofurin was applied for 16 h; however, during the 16 h of Pyrazofurin application, plates were placed into a <0.1% 0_2 chamber. Following the 16 h, plates were removed from the hypoxia chamber, and the media was changed, with the plates then left to grow for 7-9 days (Figure 3.12.A). The same cell death at 10 μM Pyrazofurin was seen for normoxia as above (Figure 3.12.B);

however, this decrease in cell viability was not seen in hypoxic conditions (Figure 3.12.B).

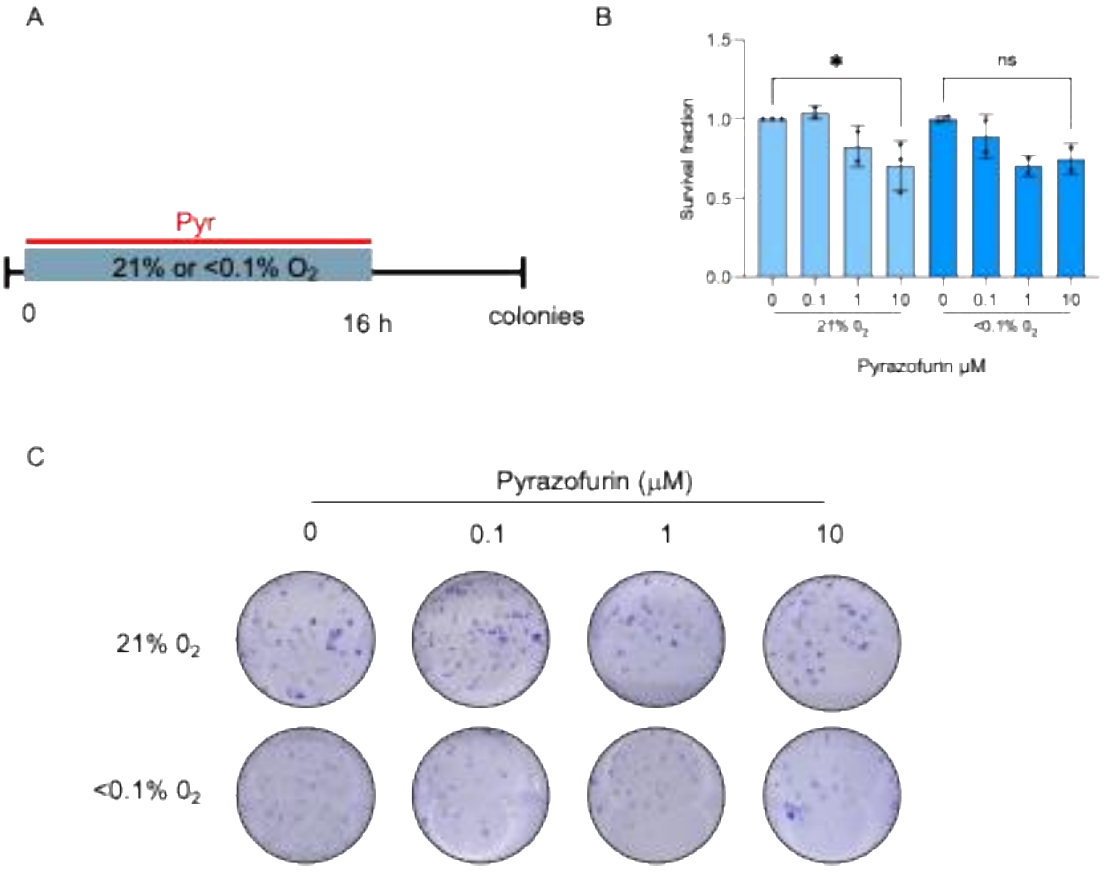


Figure 3.12. Pyrazofurin has an insignificant effect on clonogenic survival assays in hypoxia. A) Schematic representation of the clonogenic survival assay. B) A549 cells exposed to different concentrations of Pyrazofurin for 16 h in 21% 0_2 or <0.1% 0_2 and then left to form colonies. C) Representative A549 colonies exposed to Pyrazofurin (µM) at different oxygen concentrations. Statistics used were a one-way ANOVA, where * = p-value <0.05 and ns = p-value >0.05. n=3

Pyrazofurin increases p53 and p53-S15 in normoxic but not hypoxic conditions

Cell stress, which can include replicative stress, ER stress or oxidative stress, can significantly change a cell's susceptibility to radiotherapy. To examine the effects of DKC1 inhibition on cell stress, we examined p53 and p53-S15 levels following Pyrazofurin exposure, as p53 and its phosphorylation at serine-15 (p53-S15) are indicators of cellular stress (Ljungman, 2000; Loughery *et al.*, 2014).

A549 cells were exposed to Pyrazofurin (1 μM) for 8,16 and 24 h, then prepared and analysed by western blot (Figure 3.13. A). Camptothecin (CPT) was used as a positive control, and as expected, resulted in increased p53 and p53-S15. Following Pyrazofurin application, p53 was seen to be significantly increased after 16 and 24 h when compared to untreated control (Figure 3.13.B). An increase in p53-S15 was also observed after 24 h when compared to the untreated control (Figure 3.13.C). The increase in p53-S15 and p53 was also seen preliminarily in DKC1-depleted siDKC1 compared to undepleted control (Figure 3.13.D).

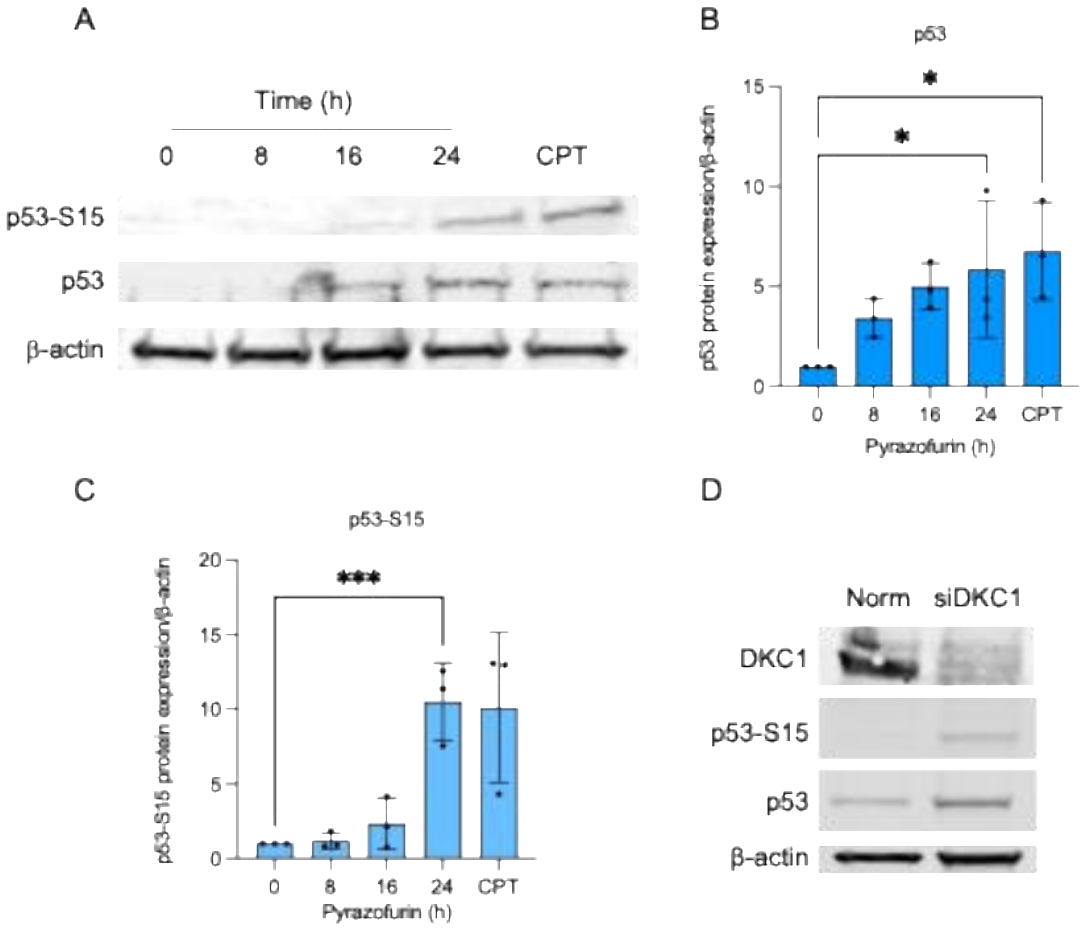


Figure 3.13. Pyrazofurin increased p53 and p53-S15 levels. A) A549 cells were exposed to Pyrazofurin (1 μM) for different lengths of time. CPT (Camptothecin) was used at 25 μM for 6 h as a positive control for damage. β-actin was used as a loading control. B) Quantification of p53 normalised to β-actin. Statistics used were one-way ANOVA, where * = p-value <0.05 and *** = p-value <0.001. n=3. C) Quantification of p53-S15 normalised to β-actin. D) DKC1, p53, and p53-S15 antibodies were applied to a western blot of A549 cells treated with siDKC1 for 24 h. n=1

To explore the effect of Pyrazofurin on p53-S15 and p53 in hypoxia, A549 cells were exposed to Pyrazofurin (1 μM) for 24 h in 21% and <0.1% 0_2 (Figure 3.14.A). HIF-1α was the control for hypoxic conditions. We saw the same increase in p53-S15 and p53 at 21% 0_2 as was the case in Figure 3.13; however, in the absence or presence of Pyrazofurin, there was no increase in p53-S15 and p53 protein expression at <0.1% 0_2 concentration (Figure 3.14.C & D). This was surprising as hypoxia (<0.1% 0_2) has been described to increase p53 and p53-S15 levels (Koumenis *et al.*, 2001).

As these increases in p53-S15 and p53 following Pyrazofurin suggest that the cells are experiencing stress, we therefore looked to examine any DNA damage following Pyrazofurin treatment.

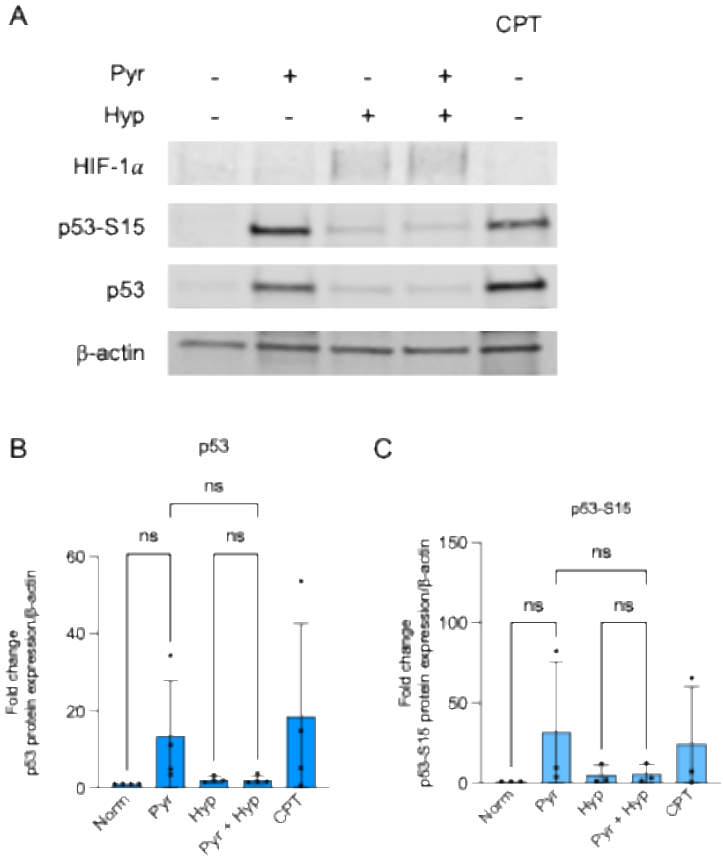


Figure 3.14. Pyrazofurin did not increase p53 and p53-S15 levels in hypoxic conditions. A) p53-S15 and p53 antibodies were applied to a Western blot of A549 cells exposed to Pyrazofurin (Pyr) (1 μM) for 24 h in either 21% (Norm) or <0.1% 0_2 (Hyp). CPT (Camptothecin) was used at 25 μM for 6 h as a positive control for damage. β-actin was a loading control. B) Quantification of the p53 on the Western blots, normalised to β-actin. n=3.C) Quantification of the p53-S15 on the western blots, normalised to β-actin. n=3

Pyrazofurin did not increase DNA damage in normoxic or hypoxic conditions

The main target for cell death from radiotherapy is DNA damage (Ward, 1981). Accumulating DNA damage through chemotherapy can increase the effectiveness of radiotherapy by increasing the susceptibility of the cell to more damage by irradiation (Morgan and Lawrence, 2015; Reuvers, Kanaar and Nonnekens, 2020; Nickoloff *et al.*, 2021).

Therefore, we examined the impact of Pyrazofurin on DNA damage in normoxia. A549 cells were exposed to Pyrazofurin (1 μM) for 8,16, and 24 h, then prepared and run on a western blot (Figure 3.15.A). Camptothecin (CPT) was used as a positive control and resulted in the expected increase in γH2AX. There was no significant difference seen in γH2AX expression following any of the Pyrazofurin timepoints when compared to the untreated control (Figure 3.15.B). However, following DKC1 knockdown with siDKC1, preliminary data shows increased γH2AX when compared to the undepleted sample (Figure 3.15.C).

To study the effect of Pyrazofurin on γH2AX in hypoxia, A549 cells were exposed to Pyrazofurin (1 μM) for 24 h in 21% and <0.1% 0_2 (Figure 3.15.D). Following exposure to <0.1% 0_2, γH2AX expression levels increased in Pyrazofurin treated and untreated cells compared to 21% 0_2. However, preliminary data show there is no difference between untreated and Pyrazofurin treatment in 21% or <0.1% 0_2 (Figure 3.15.E). This absence of γH2AX accumulation suggested a lack of DSBs, so we examined 53BP1 foci as an additional measure of DNA damage.

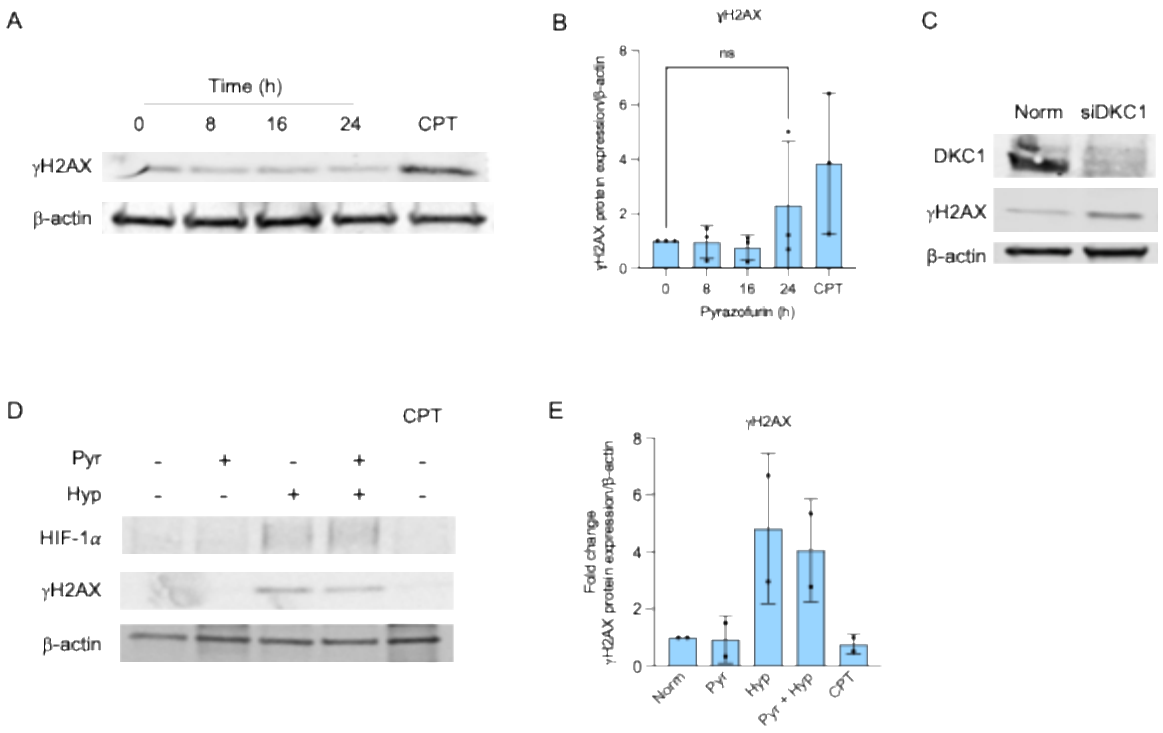


Figure 3.15. Pyrazofurin did not change γH2AX expression levels in normoxic or hypoxic conditions. A) A549 cells were exposed to Pyrazofurin (Pyr) (1 μM) for different lengths of time. CPT (Camptothecin) was used at 25 μM for 6 h as a positive control for damage. β-actin was used as a loading control. B) Quantification of γH2AX normalised to β-actin. Statistics used were one-way ANOVA, where * = p-value <0.05 and *** = p-value <0.001. n=3. C) γH2AX antibodies were applied to a western blot of A549 cells treated with siDKC1 for 24 h. n=1. D) DKC1, HIF-1α and γH2AX antibodies were applied to a Western blot of A549 cells exposed to Pyrazofurin (1 μM) for 24 h in either 21% (Norm) or <0.1% 0_2 (Hyp). CPT (Camptothecin) was used at 25 μM for 6 h as a positive control for damage. β-actin was a loading control. E) Quantification of the γH2AX on the Western blots, normalised to β-actin. n=2

Immunofluorescence was used to quantify 53BP1 foci following Pyrazofurin treatment, with 53BP1 being another marker of DNA damage from DSBs. This was carried out in A549 following 24 h of Pyrazofurin (1 μM) in either 21% or <0.1% 0_2 (Figure 3.16). Etoposide (Etop) was the positive control and had a significant difference compared to the untreated control. When looking at untreated and Pyrazofurin treated, there was no significant difference in 53BP1 foci numbers when compared in either 21% or <0.1% 0_2 (Figure 3.16.A). This lack of change to 53BP1 or γH2AX suggests a lack of

DNA damage following inhibition of DKC1 by Pyrazofurin. We next decided to examine whether Pyrazofurin treatment might change the phases of the cell cycle.

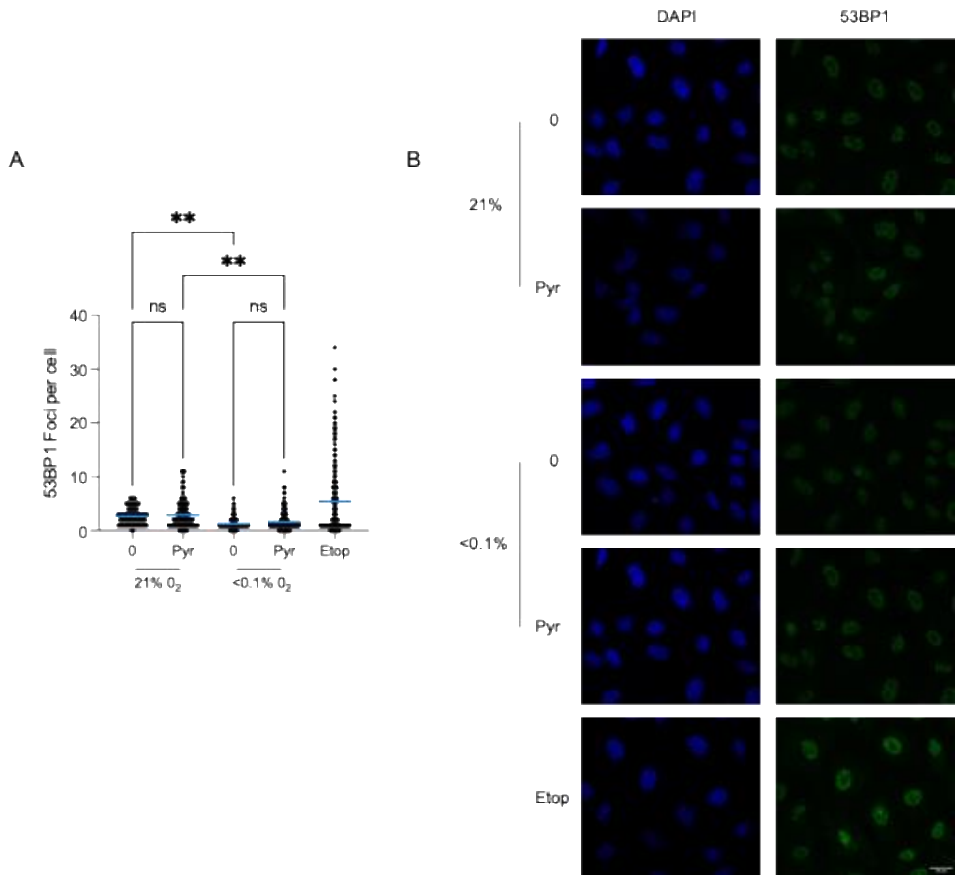
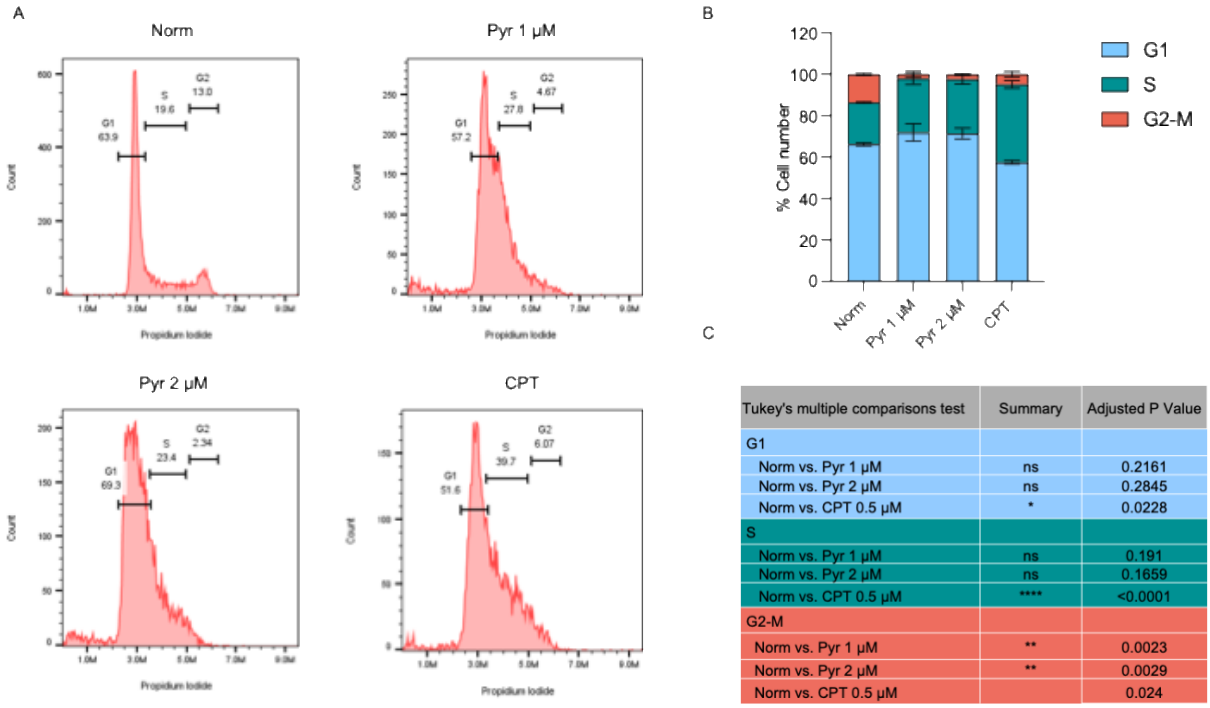


Figure 3.16. Pyrazofurin does not increase 53BP1 foci in normoxia or hypoxia. A) 53BP1 antibody was applied to A549 cells following exposure to Pyrazofurin (Pyr) at 1 μM for 24 h in either 21% or <0.1% 0_2, then examined using Immunofluorescence for foci number, where the blue line represents the mean. Etoposide (ETOP) was used at 25 μM for 6 h as a positive control for damage. Analysed by Image J (Fiji). Statistics used were one-way ANOVA, where ns = p-value >0.05. n =3. B) Representative Immunofluorescence images of the foci. Analysed by Image J (Fiji).

Pyrazofurin significantly decreases G2/M in A549 cells

Cells in different phases of the cell cycle have different radiosensitivity; therefore, it was examined whether treatment with Pyrazofurin changed the cell cycle distribution into a more or less radiosensitive phase. A549 cells were used to examine the cell cycle using Propidium iodide; the cells were pre-treated for 24 h with Pyrazofurin before analysis (Figure 3.17. A & B). CPT was the positive control, showing a change in cell cycle with significant differences in G1, S and G2/M when compared to

untreated (Figure 3.17.C). When looking at Pyrazofurin, we saw a significant decrease in the proportion of G2/M when compared to the untreated control for both 1 and 2 μM of Pyrazofurin (Figure 3.17.C). G2/M are the phases with the highest radiosensitivity (Pawlik and Keyomarsi, 2004); this decrease suggests that treatment with irradiation might be less effective following Pyrazofurin.



Tukey's multiple comparisons test	Summary	Adjusted P Value
G1		
Norm vs. Pyr 1 μM	ns	0.2161
Norm vs. Pyr 2 μM	ns	0.2845
Norm vs. CPT 0.5 μM	*	0.0228
S		
Norm vs. Pyr 1 μM	ns	0.191
Norm vs. Pyr 2 μM	ns	0.1659
Norm vs. CPT 0.5 μM	****	<0.0001
G2-M		
Norm vs. Pyr 1 μM	**	0.0023
Norm vs. Pyr 2 μM	**	0.0029
Norm vs. CPT 0.5 μM		0.024

Figure 3.17. Decreased G2/M arrest upon 24 h treatment of Pyrazofurin. A) A549 cells were exposed to 1 or 2 μM of Pyrazofurin for 24 h. Cells were labelled with Propidium iodide and analysed by FACS. CPT was treated at 0.5 μM for 24 h. Graphs were created in Flow Jo. B) Quantification of the data in A showing the percentage of cells in each phase of the cell cycle. n=3 for Pyrazofurin concentrations. n=4 for Norm and CPT concentrations. C) Table of statistics for quantified [B], statistic carried out was two-way ANOVA.

Pyrazofurin increases irradiation sensitivity in normoxic conditions

Cells in radiobiological hypoxia have been observed to be significantly more resistant to radiation than cells in normoxia (Brown, Evans and Kovacs, 1992; Wang, Biedermann and Brown, 1992; Terris *et al.*, 2002; Olive and Banáth, 2004). An inhibitor

which increases radiosensitivity within radiobiological hypoxic conditions could therefore have a large impact on radiotherapy efficacy.

To test the potential radiosensitivity of Pyrazofurin, clonogenic survival assays were carried out to examine cell viability in normoxic and hypoxic conditions. These involved 24 h pre-treatment of A549 cells with Pyrazofurin, before 16 h exposure to 21% 0_2 or <0.1% 0_2 followed by irradiation and 7-9 days to form colonies (Figure 3.18.A). The control comparing no treatment at 21% and <0.1% 0_2 showed the decrease in survival expected in 21% 0_2, validating that the hypoxic conditions were maintained during irradiation (Figure 3.18.B). Within 21% 0_2 conditions, Pyrazofurin was seen to decrease the survival fraction when compared to untreated (Figure 3.18.C). However, when comparing Pyrazofurin and untreated in <0.1% 0_2 there was no difference in colony survival (Figure 3.18.D). The results of these clonogenic assays suggest that Pyrazofurin can increase radiosensitivity in A549 cells in normoxic conditions but not in hypoxia (<0.1% 0_2).

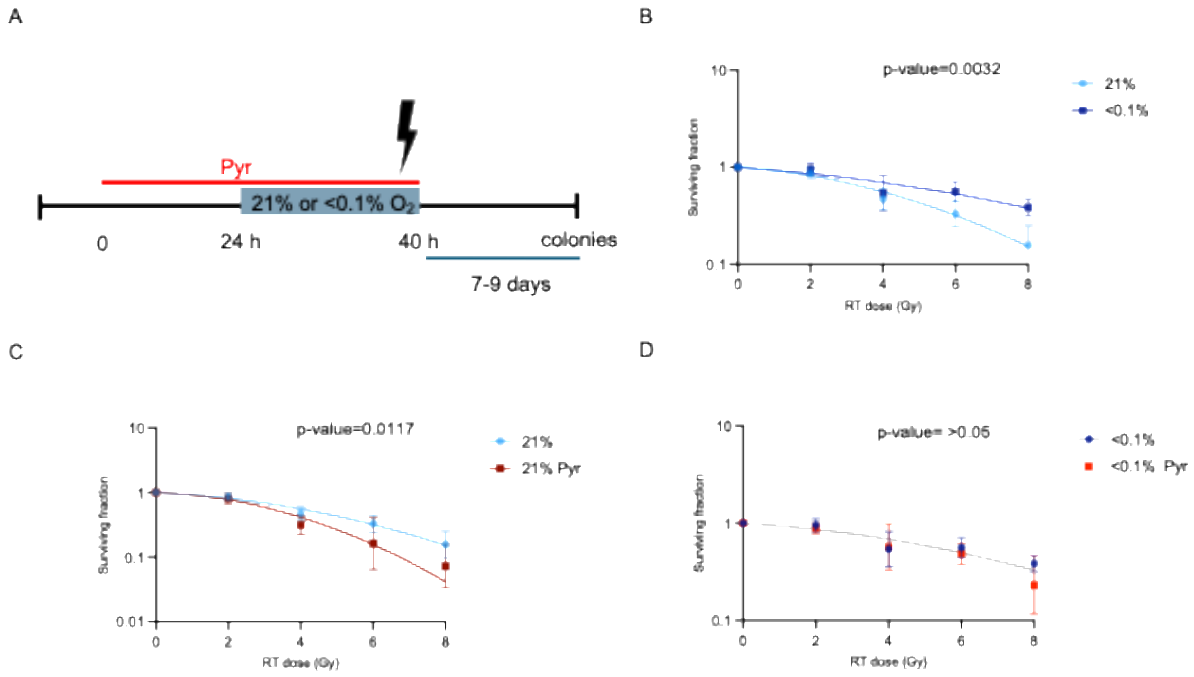


Figure 3.18. Pyrazofurin increases sensitivity with increasing irradiation in normoxic conditions but not in <0.1% 0_2. A) Schematic representation of the irradiated colony survival assay. A549 cells exposed to 1 μM of Pyrazofurin (Pyr) for 40 h in 21% 0_2 or <0.1% 0_2 as according to the schematic,

were irradiated and then left to form colonies. B) Quantification of clonogenic assay, control for hypoxic resistance at <0.1% and 21% 0_2, p-value = 0.0032, n=4. C) Quantification of clonogenic assay, for treatment with or without 1 μM Pyrazofurin at 21% 0_2 and p-value = 0.0117, n=4. D) Quantification of clonogenic assay, for treatment with or without 1 μM Pyrazofurin at <0.1% 0_2 and p-value = >0.05, n=4

Discussion

With resistance to radiotherapy a common feature of hypoxia, this project aimed to identify a novel target for increasing radiosensitivity in hypoxic tumours. We looked to identify this novel target within R-loop-associated proteins, as these have been seen to be promising for therapeutics. The initial potential target was the protein ZNF598, but because of its cytoplasmic localisation, it was not further explored.

To choose a new target, the analysis of R-loop interactomes was narrowed down to 10 possible targets, of which 4 proteins have already been implicated in R-loops (DHX9, DDX5, NONO and UPF1) (Chakraborty and Grosse, 2011; Chakraborty, Huang and Hiom, 2018; Petti *et al.*, 2019; Yu *et al.*, 2020; Saha *et al.*, 2022; Moriggi *et al.*, 2025). This high proportion of known R-loop binding proteins increased confidence in the list, with the two initially selected being NAT10 and DKC1.

NAT10 was quickly dismissed as its small-molecule inhibitor Remodelin, was seen to increase cell viability. Additionally, as there was conflicting literature around whether remodelin does, in fact, inhibit NAT10 activity, and NAT10 had already been implicated at R-loops, it was decided to change focus to the second chosen possibility, DKC1 (Dalhat *et al.*, 2021; Shrimp *et al.*, 2021; Gao *et al.*, 2024; Su *et al.*, 2024).

DKC1 and hypoxia: mRNA vs protein evidence

Transcriptomic analyses revealed that the association between DKC1 mRNA expression and hypoxia was highly context-dependent, with positive correlations in LUAD and READ but no association in LUSC or COAD. This heterogeneity suggests

that hypoxic regulation of DKC1 is not a universal feature across tumour types but rather may reflect tumour type-specific transcriptional mechanisms.

The lack of change in DKC1 protein expression in hypoxia was somewhat expected, considering prior work saw no change in DKC1 protein levels at 1% 0_2 (Hou *et al.*, 2020). The HCT116 cells tested most closely correspond to the COAD dataset, which also showed no correlation at the mRNA level, meaning the absence of protein expression change in this context is not surprising, considering there was no change transcriptionally. While the positive mRNA correlations in LUAD and READ imply that DKC1 upregulation under hypoxia may be clinically relevant in certain cancers, this cannot be generalised across all tumour types.

No change in HIF-1α protein expression was seen following Pyrazofurin treatment, which contrasts with Hou et al. (2020), who reported that DKC1 knockdown reduced HIF-1α expression, potentially through direct promoter binding. Taken together, the findings suggest that DKC1 is not uniformly regulated by hypoxia and that there might be variable effects depending on inhibition/depletion.

Functional effects of DKC1 inhibition

Pyrazofurin produced only slight reductions in cell viability, with effects varying by cell line. Previous studies show that longer Pyrazofurin exposures (7–9 days) reduced colony survival, suggesting that the limited cell killing observed here may reflect the shorter treatment duration (Kan, Wang, Sheng, Chen, *et al.*, 2021; Kan, Wang, Sheng, Yao, *et al.*, 2021). This also shows a discrepancy between the *in vitro* tolerability compared to the historical clinical toxicity of Pyrazofurin, highlighting the complexity of extrapolating from culture systems to patients, where off-target effects play a greater role (Gralla, Sordillo and Magill, 1978; Carroll, Kemeny and Gralla, 1979; Nichols *et*

al., 1978; Cadman, Dix and Handschumacher, 1978; Peterson and Houghton, 2004; Lin *et al.*, 2019). Our observation that this decrease in cell viability was not seen within hypoxic cells is also consistent with the well-documented resistance of hypoxic tumours to therapeutics (Brown and Giaccia, 1998; Hammond *et al.*, 2014). The limited viability effects observed are consistent with previous *in vitro* studies, with longer exposures appearing necessary for more significant reductions.

Cell stress and p53 activation

Pyrazofurin treatment increased expression of p53 and p53-S15 under normoxic conditions, with a similar trend seen following siDKC1 depletion. This aligns with previous observations that DKC1 silencing activates p53 signalling, potentially through ribosomal stress pathways (Ibáñez-Cabellos *et al.*, 2020). The absence of p53 induction under severe hypoxia (<0.1% 0_2) was unexpected, given prior evidence that hypoxia itself stabilises p53 (Hammond *et al.*, 2002). This surprising lack of p53 raises questions around why the effects of DKC1 inhibition might be different within hypoxic conditions.

DNA damage response

Despite the induction of stress markers, Pyrazofurin exposure did not increase γH2AX or 53BP1 foci, suggesting that acute inhibition does not immediately generate DSBs; however, in contrast, siDKC1 depletion produced preliminary evidence of γH2AX accumulation. This puts our results in line with the literature, showing less DNA damage than expected following DKC1 depletion/inhibition, with two studies seeing no clear changes in γH2AX foci with siDKC1 (Lin, Mobasher and Alawi, 2014; Ibáñez-

Cabellos *et al.*, 2018). However, within these studies, there was speculation over whether DKC1 depletion was long enough to have accumulated observable DNA damage, suggesting that sufficient damage might only occur after a period of oxidative stress (Ibáñez-Cabellos *et al.*, 2018, 2020). The evidence from dyskeratosis congenita patient cells (specifically those with DKC1 mutations) shows elevated γH2AX, further supporting a model in which chronic DKC1 loss contributes to cumulative genomic instability, even if acute inhibition does not (Manguan-Garcia *et al.*, 2014; Pereboeva *et al.*, 2016).

This idea of a longer period of depletion/inhibition of DKC1 being needed to induce damage is a useful theory to explain the lack of DNA damage seen here and in other studies. It would also explain the clear cell stress seen, with the increase in p53 occurring pre-accumulation of DNA damage. The studies around DC are useful for possible routes through which the DNA damage might be induced, including ribosomal or telomeric dysfunction. Particularly regarding radiosensitivity, knowing this might help us figure out how DKC1 inhibition might be best exploited.

Cell cycle progression

Cell cycle examination of Pyrazofurin treatment resulted in reduced proportions of cells in G2/M, with a corresponding increase in earlier phases of the cycle, matching previous and historic studies (Hill and Whelan, 1980; Kan, Wang, Sheng, Yao, *et al.*, 2021). This pattern was also observed following siDKC1 silencing, although not universally (Hou *et al.*, 2020; Kan, Wang, Sheng, Yao, *et al.*, 2021; Wang *et al.*, 2025).

The shift away from G2/M is mechanistically significant in the context of radiosensitivity, as cells in G2/M are most sensitive to radiation (Pawlik and Keyomarsi, 2004). Thus, the reduction in the G2/M fraction may counteract any radiosensitising actions of Pyrazofurin, a hypothesis that was subsequently addressed in clonogenic assays.

Radiosensitivity and hypoxic modulation

The central question of this project was whether DKC1 inhibition could enhance tumour radiosensitivity. In clonogenic survival assays, Pyrazofurin treatment sensitised A549 cells to irradiation under normoxic conditions. This represents, to our knowledge, the first demonstration that pharmacological inhibition of DKC1 can modulate radiosensitivity in lung cancer cells. However, the radiosensitising effect of Pyrazofurin was completely lost under radiobiological hypoxia (<0.1% 0_2), which is consistent with the well-established phenomenon that hypoxia confers radiotherapy resistance, and suggests that Pyrazofurin is unable to overcome the hypoxic barrier to radiotherapy efficacy (Brown and Giaccia, 1998; Hammond *et al.*, 2014). Literature linking Pyrazofurin and radiotherapy has only one historic clinical report noting increased toxicity in patients previously exposed to radiation (Ohnuma and Holland, 1977). Our results therefore provide the first preclinical evidence that Pyrazofurin can function as a radiosensitiser, albeit in an oxygen-dependent manner. A key implication is that the translational utility of DKC1 inhibition may be limited to more oxygenated tumour regions, whereas hypoxic niches, which are most relevant for radiotherapy resistance, remain unaffected.

Conclusion

This study identifies DKC1 inhibition as an oxygen-dependent strategy for enhancing tumour radiosensitivity. Pyrazofurin increased radiosensitivity in lung cancer cells under normoxia but not under radiobiological hypoxia, the key driver of clinical radiotherapy resistance. While DKC1 is often overexpressed in cancer and its inhibition activates stress responses, Pyrazofurin's lack of efficacy in hypoxia and concerns over specificity and toxicity limit its therapeutic value. Nonetheless, there might be use in further investigation of DKC1 as a radiosensitising target, particularly in combination with approaches that mitigate hypoxia, to improve radiotherapy outcomes across cancer types.

Limitations

One major limitation of the study was that the work was conducted predominantly in two cell lines, A549 and HCT116, selected for their relevance to lung and colorectal cancers and comparability with prior DKC1 studies (Hou *et al.*, 2020; Kan, Wang, Sheng, Chen, *et al.*, 2021; Kan, Wang, Sheng, Yao, *et al.*, 2021). While this provided consistency, the cell line-specific responses observed here limit the generalisation of the findings, highlighting the need to extend analyses across a broader panel of tumour models. This is particularly the case for the experiments where only one cell line was used. An example includes when we explored DKC1 protein expression in hypoxia only within HCT116 cells, the results may have shown differential expression within a lung adenocarcinoma cell line such as A549, particularly considering the mRNA expression correlation being different between the tumour types.

When working with hypoxia, there is always a necessary limitation of choosing only certain concentrations to work with, as there are extensive possibilities. The reasoning for the use of radiobiological hypoxia for most experiments was due to it being the hypoxia level with the highest resistance to radiotherapy. However, it means results in relation to the radiosensitising activity of Pyrazofurin may be variable in 2% or 1% 0_2, as the mechanistic, stress and damage responses are very different. Additionally, as hypoxia concentrations are heterogeneous in tumours, testing at only <0.1% 0_2 will not have fully captured *in vivo* conditions of the variable hypoxic levels.

Another limitation of the study is Pyrazofurin and its exposure time, as highlighted above, studies have seen that longer exposure times may induce more cell stress and DNA damage. Wider studies also suggest that cell stress and subsequent damage take a while to accumulate within DKC1-depleted cells, and by extension, there is the speculation that this might be the same for DKC1 inhibition; therefore, it would have been useful to have extended the timescales for Pyrazofurin exposure for cell viability assays, cell stress and DNA damage protein expression. However, the timings used here were justified based on when we saw decreased cell viability in this project as well as in others (Kan, Wang, Sheng, Chen, *et al.*, 2021; Kan, Wang, Sheng, Yao, *et al.*, 2021).

When comparing to the pre-existing studies, one important consideration is siDKC1. Without the direct comparison to DKC1 depletion, it means that observations of Pyrazofurin DKC1 inhibition in this study cannot be said to be a direct effect of DKC1 but could result from off-target drug effects. While used sparingly here, for further work,

more extensive use of siDKC1 would make for easier comparison to the literature and verification that the effects of Pyrazofurin are a result of DKC1 inhibition.

Future research directions.

Based on the results of this study, there are several paths for future research. Firstly, the examination of how the radiosensitivity of Pyrazofurin within normoxia is mediated. There are multiple avenues to examine, such as whether the sensitisation is due to ribosome biogenesis, telomerase dysfunction or unrelated stress signalling. To create a clearer picture, it would be useful to directly compare depletion and pharmacological inhibition across matched experimental conditions. For depletion, this could include siRNA over extended time courses or even a CRISPR-based depletion model. Additionally, the use of the neutral comet assays, considered the gold standard for recognising DSBs, could also help provide more information on how DKC1 contributes to DNA damage responses.

Another path for future research would be expanding the range of cell lines and conditions tested. Given the cell line–specific responses observed, future studies should incorporate a panel of lung and colorectal cancer models, alongside other tumour types where DKC1 is overexpressed, such as breast and liver cancers. This would allow assessment of whether radiotherapy sensitisation is a generalisable feature of DKC1 inhibition or restricted to specific genetic or microenvironmental contexts.

Regarding hypoxia, the absence of the radiosensitising effect of Pyrazofurin at (<0.1% 0_2) does not exclude a role for DKC1 at (1–2% 0_2) or under conditions of cyclic

hypoxia, which more closely mimic the fluctuating oxygenation of solid tumours. Systematic evaluation across these levels would help determine whether DKC1 inhibition can overcome intermediate levels of hypoxic radiotherapy resistance.

Finally, to evaluate the more translational aspect, we could examine DKC1 inhibition in more relevant therapeutic environments. This could include *in vivo* studies examining Pyrazofurin in xenograft or patient-derived tumour models to evaluate therapeutic efficacy and toxicity. Additionally, combination strategies that target hypoxia, such as agents modulating oxygenation, could be explored alongside Pyrazofurin to see if it counteracts the oxygen-dependent activity. Such combinatorial approaches could offer the most realistic path to overcoming hypoxic radiotherapy resistance in lung and colorectal cancers. If results suggested that DKC1 inhibition was a good therapeutic target, then there might also be a call for the construction of more selective small-molecule inhibitors of DKC1, considering that Pyrazofurin has high levels of toxicity associated with it clinically.

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