EVALUATION OF A PHENOTYPIC APPROACH TO THE DISCOVERY OF DRUGS WITH ACTIVITY AGAINST CELLS THAT RESIDE IN THE MICROENVIRONMENT OF TUMOURS

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A thesis submitted to the University of Huddersfield in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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Contents

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Abbreviations

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide – (MTT)

5 – Fluorouracil – (5-FU)

Adenosine triphosphate – (ATP)

American type culture collection – (ATCC)

Ammonium persulfate - *APS)

Cancer associated fibroblasts – (CAFs)

Chinese hamster ovary – (CHO)

Chronic myeloid leukaemia – (CML)

CXC – chemokine ligand – (CXCL12)

Deoxyribonucleic acid – (DNA)

Dianhydrogalactitol – (DAC)

Dibromogalactitol – (DBG)

Dibromomannitol – (DBM)

Dimethyl sulfoxide – (DMSO)

Ethylenediaminetetraacetic acid – (EDTA)

Extracellular matrix – (ECM)

Extracellular pH – (pHe)

Fluorodeoxyuridine monophosphate – (FdUMP)

Fluorodeoxyuridine triphosphate – (FdUTP)

Fluorouridine triphosphate – (FUTP)

Foetal bovine serum – (FBS)

Food and drug administration – (FDA)

Hypoxia activated prodrugs – (HAPs)

Hypoxia inducible factor – (HIF)

Hypoxic cytotoxicity ratio – (HCR)

Interleukin 6 – (IL-6)

Intracellular pH – (pHi)

Investigational new drug – (IND)

Minor groove binder – (MGB)

N – heterocyclic carbene – (NHC)

National cancer institute – (NCI)

New drug application – (NDA)

Nicotinamide adenine dinucleotide phosphate – (NADP)

Non-small cell lung cancer – (NSCLC)

Nuclear factor $_{k}$B – (Nf-$_{k}$B)

pH cytotoxicity ratio – (pHR)

Phosphate buffered solution – (PBS)

Quinone oxidoreducatse 1 – (NQO1)

Ribonucleic acid – (RNA)

Selectivity index – (Si)

The concentration of compound required to kill 50% of cells – (LC_{50})

The concentration of test compound required to inhibit the growth of the cells by 50% - (GI_{50})

The concentration required to inhibit 50% growth – (IC_{50})

Thymidine triphosphate – (TTP)

Transforming growth factor β – (TGF-β)

Tumour growth inhibition – (TGI)

Tumour microenvironment – (TME)

Tyrosine kinase inhibitors – (TKI's)

Vascular endothelial growth factor – (VEGF)

Abstract

The hallmarks of cancer have paved the way for the development of target approached agents which are more selective and active towards cancer cells. Irrespective of this paradigm shift, there are still many hindrances to effective drug treatment which cause drug resistance such as hypoxia and mild acidity which are found in the tumour microenvironment (TME). One way to circumvent this issue is by the use of hypoxia activated prodrugs (HAPs), but with over 40 years of research there is still no HAP that is approved for use in humans. A new strategy to overcome this barrier is to apply a phenotypic screening approach to test compounds under those conditions which are favourable to the TME such as the combination of hypoxia and mild acidity.

The aim of this study is to evaluate a series of compounds using a phenotypic evaluation strategy based upon testing compounds under (i) normoxia / pHe 7.4, (ii) normoxia / pHe 6.5, (iii) hypoxia / pHe 7.4 and (iv) hypoxia / pHe 6.5 and (v) compare activity in cancer cells to non-cancer cells. The objective of this study is to identify compounds that preferentially and selectively kill cancer cells that reside within the TME that would not have been identified using standard cell culture conditions.

In this study, a series of novel compounds (i) ruthenium, iridium and rhodium based napthyridines, thiourea derivatives, benzylthiourea ligands and aroylthiourea compounds, (ii) Bromo-hexitol prodrugs, (iii) NHC-Ag based complexes, (iv) Minor groove binders and (v) Tyrosine kinase inhibitor (dasatinib) with the attachment of 2-nitroimidazole have been provided by collaborators which will be screened under TME conditions to determine the potency (IC_{50}), under hypoxic conditions (0.1% O_2) and in combination of mild acidity (pHe 6.5). As well as potency the selectivity index (SI) which is defined as the ratio of IC_{50} values in non-cancer cells divided by the IC_{50} of cancer cells will also be determined. Those compounds that pass these tests are then to be subjected for further mechanistic studies.

The initial studies focused on screening three classic chemotherapeutic drugs, cisplatin, 5-FU, etoposide and the classic HAP tirapazamine under the TME conditions. All three classic chemotherapeutic drugs displayed high activity under normoxic conditions but showed resistance under TME conditions (i.e. hypoxia, mild acidity and the combination of both) with moderate to poor selectivity towards cancer cells. As expected, tirapazamine displayed HAP like abilities. These results were set as the ‘gold standard’ throughout this PhD study and were used to compare with the new novel compounds that were screened. A total of 72 compounds were tested using this screen where 40 (55%) compounds were selective towards the cancer cell lines, 8 (11%) compounds were further activated under normoxic extracellular pH (pHe) 6.5 conditions, 24 (33%) compounds exhibited HAP like properties and finally 3 (4.1%) compounds displayed enhanced toxicity with the combination of hypoxia and pHe 6.5 conditions. The compounds screened in some instances did display superior selectivity and preferential activity towards cells under TME conditions in comparison to the classic chemotherapeutic drugs which is promising.

In conclusion, the phenotypic based screen employed throughout this study demonstrates potential in identifying compounds that show preferential activity and resistance to cells under TME conditions. Both results are valuable in terms of the decision making process for the selection of lead compounds and further studies are required to develop this concept further.

List of publications

These publications contributed towards partial aspects of this PhD.

1. Johansson, H., **Hussain, O**., Allison, S.J., Robinson, T.V., Phillips, R.M., and Pedersen, D.S. Revisiting Bromohexitols as a Novel Class of Microenvironment-Activated Prodrugs for Cancer Therapy. ChemMedChem. **2019**

2. Nichol, R.J., Khalaf, A.I., Sooda, K., **Hussain, O**., Griffiths, H.B., Phillips, R., Javid, F.A., Suckling, C.J., Allison, S.J., and Scott, F.J. Selective in vitro anti-cancer activity of non-alkylating minor groove binders. MedChemComm 10, 1620-1634. **2019**

3. Lapasam, A., **Hussain, O**., Phillips, R.M., Kaminsky, W., and Kollipara, M.R Synthesis, characterization and chemosensitivity studies of half-sandwich ruthenium, rhodium and iridium complexes containing κ1 (S) and κ2 (N, S) aroylthiourea ligands. Journal of Organometallic Chemistry 880, 272-280. **2019**

4. Sanjay Adhikari, **Omar Hussain**, Roger M. Phillips, Werner Kaminsky, Mohan Rao Kollipara. Neutral and cationic half-sandwich arene d6 metal complexes containing pyridyl and pyrimidyl thiourea ligands with interesting bonding modes: Synthesis, structural and anti-cancer studies. Journal of Organometallic Chemistry. **2018**.

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6. Dr. Simon J. Allison, Dr. David Cooke, Francesca S. Davidson, Prof. Paul I. P. Elliott, Dr. Robert A. Faulkner, Hollie B. S. Griffiths, Owen J. Harper, **Omar Hussain**, Prof. P. Jane Owen-Lynch, Prof. Roger M. Phillips, Prof. Craig R. Rice, Samantha L. Shepherd, Dr. Richard T. Wheelhouse. Ruthenium-Containing Linear Helicates and Mesocates with Tuneable p53-Selective Cytotoxicity in Colorectal Cancer Cells. Angewandte Chemie. **2018**.

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with unexpected bonding modes: Synthesis, structural and anti-cancer studies Journal of Organometallic Chemistry. **2018**.

Publications prior to PhD

1. Andrew Collett, Wafaa Al-Tameemi, Chris Dunnill, **Omar Hussain**, Nikolaos Georgopoulos Review: The role of scalp cooling in the prevention of chemotherapy induced alopecia European Journal of Clinical & Medical Oncology. **2014**.

2. Al-Tameemi W, Dunnill C, **Hussain O**, Komen M M, van den Hurk C J, Collett A, Georgopoulos N T. Use of in vitro human keratinocyte models to study the effect of cooling on chemotherapy drug-induced cytotoxicity. Toxicol In Vitro. **2014**.

Scientific meetings

Poster Presentation - **Hussain, O**, Johansson, H., Allison, S.J., Robinson, T.V., Phillips, R.M., and Pedersen, D.S. Revisiting Bromohexitols as a Novel Class of Microenvironment-Activated Prodrugs for Cancer Therapy Conference, AACR, Chicago, 2018, USA

Session Chair - Conference, NECB, University of Hull 2017, Hull, UK

Poster Presentation - Conference, University of Huddersfield 2017, Huddersfield, UK

Chapter 1: General introduction

1.1 Introduction to cancer

Cancer is characterised by the abnormal proliferation of cells caused by multiple changes in gene expression which leads to an imbalance between cell growth and cell death. These cells ultimately become a population that have the ability to metastasize and invade other tissues causing significant morbidity and if left untreated, may lead to mortality (Ruddon, 2007). This can be caused by several different factors including smoking, overweight and obesity, alcohol, lack of physical activity, poor diet, sun and UV (ultra-violet) light, air pollution, hormones and inherited genes (Ruddon, 2007). During the development of cancer, cells acquire a number of characteristics or 'hallmarks', details of which are summarised below.

1.2 The hallmarks of cancer

With the emergence of the field of molecular biology in the 1980's, a rich and complex body of knowledge has been generated revealing cancer to be a disease involving dynamic changes in the genome leading to the acquisition of multiple genetic and/or epigenetic lesions. In a landmark review article, Hanahan and Weinberg (2000), have described the development of cancer as the acquisition of a series of characteristics or 'Hallmarks'. This concept essentially describes the key features that tumours acquire during their journey from normal to malignant cells (Hanahan and Weinberg, 2000, 2011). The six original hallmarks are illustrated in figure 1.1 and they include: (i) sustained proliferative signalling (ii) evading growth suppressors (iii) enabling replicative immortality (iv) activating invasion and metastasis (v) angiogenesis and (vi) resisting cell death.

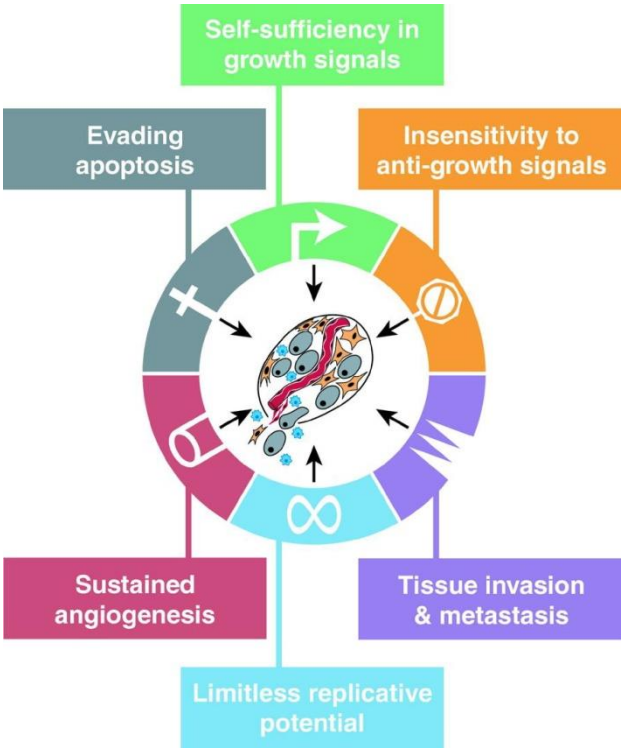


Figure 1.1 The hallmarks of cancer as defined by Hanahan and Weinberg (Hanahan and Weinberg, 2000).

Following on from their original review article, Hanahan and Weinberg reviewed the progress that had been made in the following decade and in 2011, they published the 'next generation' of hallmarks (Hanahan and Weinberg, 2011). As illustrated in figure 1.2, they identified two emerging hallmarks of cancer and two enabling characteristics that aid the development of cancers. In this article, they also illustrate the importance of how understanding of tumour biology has led to the development of novel therapeutics and describe the challenges posed by the emergence of new mechanisms of drug resistance caused by an unstable genome and tumour cell heterogeneity (Hanahan and Weinberg, 2011).

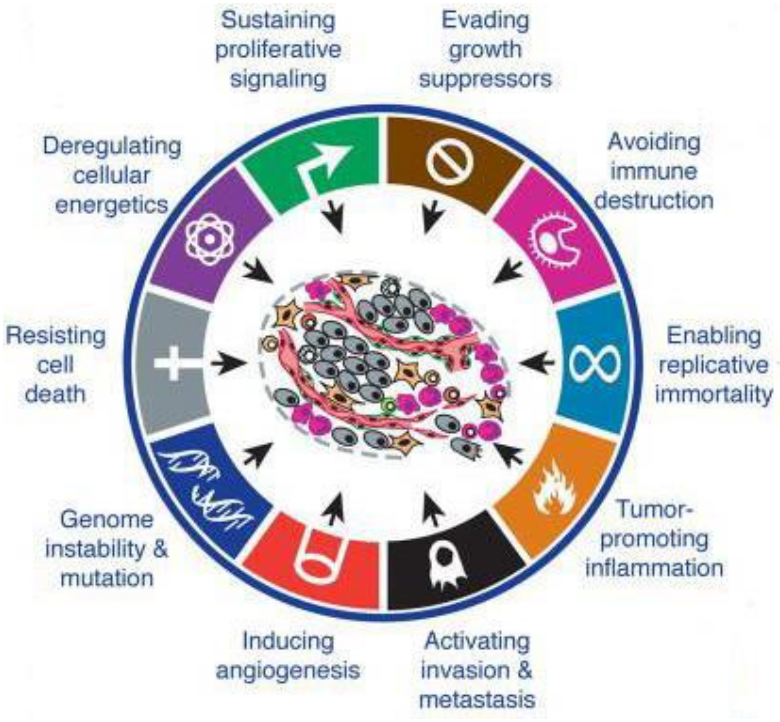


Figure 1.2 The next generation of the hallmarks of cancer as defined by Hanahan and Weinberg (Hanahan and Weinberg, 2011). This concept is built upon their original paper in 2000 with the addition of two emerging hallmarks (avoiding immune destruction and deregulating cellular energetics) and two enabling characteristics (genome instability and mutation and tumour promoting inflammation).

1.3 Treatment of cancer

The treatment of cancer involves three main strategies; surgery, radiotherapy and chemotherapy. Chemotherapy is typically used in combination with surgery or radiotherapy and in some instances both. Chemotherapy is given alone to leukaemia patients where the disease is blood borne from the onset and therefore not amendable to surgery. Chemotherapy is defined as the use of drugs to eradicate cancer cells that may have spread around the body or to treat disease that is disseminated from the onset.

Chemotherapy drugs can be categorised into two main groups: (i) traditional cytotoxic drugs and (ii) the more modern targeted therapeutics. Cytotoxic drugs consist of chemicals which target various aspects of DNA synthesis and cell replication and therefore have preferential activity against dividing cells. Targeted therapeutics target specific biochemical pathways that are implicated in the growth and survival of cancer cells. Cytotoxic drugs are classified based upon (i) their mechanism of action and (ii) their effect on cells at different stages of the cell cycle. Both classifications are illustrated in figure 1.3.

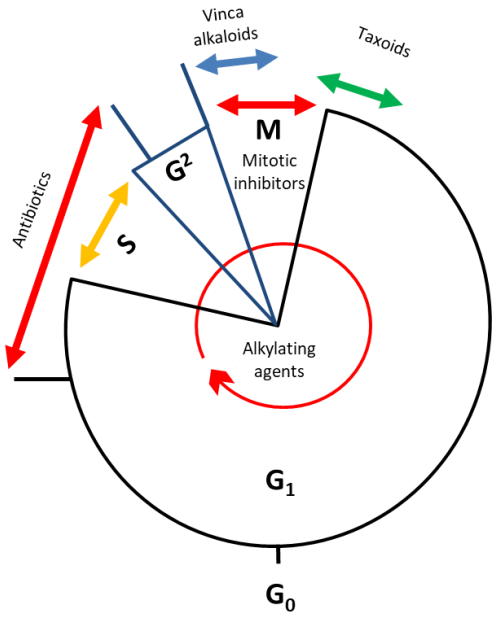


Figure 1.3 The cell cycle and the different types of chemotherapeutic agents targeting specific check points of the cell cycle. There are four main stages of the cell cycle which are (i) G_0 – cell growth and preparation for new cells, (ii) S – DNA and RNA synthesis, (iii) G^2 – checkpoint for DNA error and (iv) M – Microtubule assembly (Mills et al., 2018).

The cell cycle is divided into different stages, namely G1, S, G2 and M phases. The four stage cell cycle is coordinated accurately by cyclin-dependent kinases (CDKs),

checkpoint proteins as well as regulatory cyclins which all detect if there are any errors in order to preserve genome integrity (Hsieh and Yamane, 2008). The cytotoxic chemotherapy drugs target different phases of the cell cycle as illustrated in table 1.1 but their ability to target replicating cells is a fundamental characteristic of the majority of cytotoxic drugs. It is known however that certain alkylating agents are equally effective against dividing and non-dividing cells but in general, these drugs typically target dividing cells.

	Cell cycle stage			
Non-cell cycle specific	G_1	S	G_2	M
Alkylating agents	Hormonal	Anti-metabolites	Anti-tumour antibiotics	Microtubule Inhibitors
Cyclophosphamide	Tamoxifen		Bleomycin	Taxanes
Busulfan		5-FU		
Dacarbazine		Methotrexate		Docetaxel
Temozolomide		Mercaptopurine	Podophyllo-toxin	Paclitaxel
Ifosfamide		Gemcitabine		
Carmustine		6-Thioguanine	Etoposide	Vinca Alkaloids
		Cytarabine		
Platinum-based				
			Topoisomerase	Vincristine
Cisplatin				Vinblastine
Oxaliplatin			Irinotecan	
Carboplatin			Topotecan	

Table 1.1 Therapies designed to target the cell cycle (Mills et al., 2018). There are some chemotherapy drugs which are not cell cycle specific such as alkylating and platinum-based agents. The remainder of the therapies preferentially kill cells in certain phases of the cell cycle but there is some overlap as illustrated in figure 1.3.

Targeted therapeutics represent the product of modern-day approaches to drug discovery based upon the identification of biochemical targets and pathways that drive cancer progression and development (Jaibu et al., 2018). They can be broadly

characterised as being either small molecule inhibitors of key enzymes involved in aberrant cell signalling pathways (such as tyrosine kinase inhibitors) or large monoclonal antibodies that target either ligand or receptor biology. A detailed description of these therapeutics is beyond the scope of this thesis but for a more comprehensive review of targeted anti-cancer drugs, the reader is referred to excellent reviews on the topic (Seebacher et al., 2019), (Falzone et al., 2018).

1.4 Chemotherapeutic side effects and resistance

As stated above, one of the major characteristic features of tumours is unregulated cell proliferation and cytotoxic chemotherapy that work by targeting rapidly proliferating cells. Unfortunately, this class of anti-cancer agents are not able to distinguish between rapidly dividing normal and cancerous cells leading to unwanted side effects, many of which are dose limiting and potentially life threatening. The hair matrix keratinocytes, bone stem cells, immunocytes and gastrointestinal cells are some prime examples of normal cells that are rapidly dividing cells leading to unwanted side effects in these tissues and organs (Paus et al., 2013). Cytotoxic drugs therefore have poor selectivity for cancer cells and typically have a narrow therapeutic index.

To circumvent this issue, the targeted therapeutics introduced above were developed with the aim of specifically targeting the cancer cells and widening their therapeutic index (A Baudino, 2015). Examples of these are small molecule inhibitors such as the tyrosine kinase inhibitors (TKI's), monoclonal antibodies, naked monoclonal antibodies, conjugated monoclonal antibodies and bispecific monoclonal antibodies (Jaibu et al., 2018). Despite the promising concept of targeting cancerous cells specifically, these antibodies/ inhibitors also have their

limitations and are often associated with adverse effects. Examples of these adverse effects are, vomiting, rashes, diarrhoea and fever for the antibodies and bleeding, fever, hypertension, constipation, hypothyroidism for the small molecule inhibitors (Jaibu et al., 2018). However, their side effects are generally not as severe in comparison to conventional cytotoxic chemotherapeutic agents.

As well as unwanted side effects, another limitation of both cytotoxic and targeted anti-cancer drugs is the problem of resistance. There are three major forms of resistance; (i) inherent resistance where cells are insensitive to drugs (ii) apparent resistance where cells are inherently sensitive to the drugs but don't respond because of poor drug delivery and (iii) acquired resistance where cells initially respond to treatment but when relapse occurs, the tumours are not only resistant to the drugs they were originally treated with but have developed a multidrug resistance phenotype. Multiple mechanisms of resistance are known for cytotoxic drugs (increased drug efflux, decreased drug uptake, increased detoxification, increased DNA repair etc) and new mechanisms of resistance to targeted therapeutics are being reported including mutational changes to the target leading to steric hindrance of inhibitors etc (Barbuti and Chen, 2015) (Zhang et al., 2015).

Whilst significant improvements in the treatment of cancer patients using drugs has been achieved, it is debatable as to whether there has been a paradigm shift in the successful treatment of cancer has occurred. In addition, the high cost of the new generation of targeted drugs has led to 'financial toxicity' with patients and clinicians having to make very difficult decisions based upon the availability of funds (Drummond and Mason, 2007). Furthermore, the dual problems of tumour cell heterogeneity and genomic instability are significant barriers to the successful treatment of cancer and there is therefore still a need to develop novel therapeutics

that addresses these key issues. There is a need to develop new therapies mainly to overcome the evolving nature of cancer and one way to overcome this is by the phenotypic screening of novel drugs for the identification of new therapies that target multiple pathways. Before discussing this in more detail, a general overview of the drug discovery process is provided below.

1.5 The drug discovery process

The drug discovery and development process are lengthy and inefficient and it is estimated that only one compound from tens of thousands tested will become licensed treatments. There is a vigorous screening process as illustrated in figure 1.4 where new drugs can be rejected at any given time on the following basis, safety, effectiveness and quality (Lipsky and Sharp, 2001).

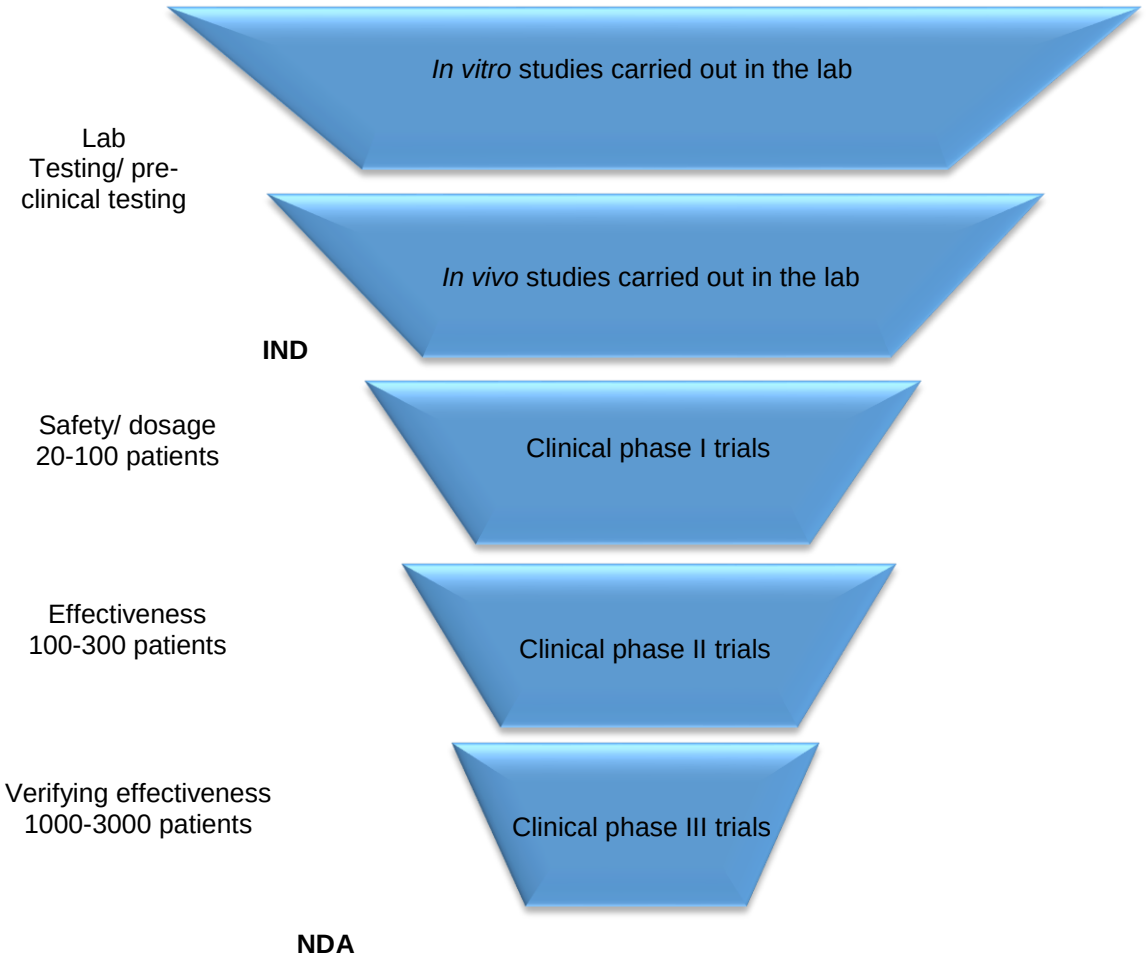


Figure 1.4 Diagram of the major processes involved from getting a new drug from lab to approval (Lipsky and Sharp, 2001). The blue boxes represent the filtering process of the thousands of drugs which are screened. IND (investigational new drug) allows pharmaceutical companies to start human clinical trials. NDA (new drug application).

There are two main approaches to the drug discovery process; (i) target orientated drug development and (ii) random screening or 'phenotypic' drug development. Target orientated drug discovery relies on an understanding of tumour biology to identify and validate key proteins or pathways that drive the cancer process as targets for therapeutic intervention. The synthesis of molecules or the screening of

compound libraries against the target will lead to the identification of ‘hits’ or lead compounds which then undergo optimisation steps to enhance their ‘drug like’ properties. These lead compounds are then evaluated *in vivo* and if successful, they enter phase I clinical evaluation. At all stages of the process, establishing ‘proof of principle’ is essential where any anti-cancer activity is assigned to drug target interactions. An example of targeted therapy are tyrosine kinase inhibitors such as imatinib (Jaibu et al., 2018).

The second approach of random drug screening has been traditionally run by the National Cancer Institute (NCI) in the USA and it assumes that the ‘magic bullet’ has been either synthesised by chemists or is present somewhere in the natural world. By using high capacity cell screening approaches, the magic bullet will eventually be found. In simple terms, the active compound is found first and then the mechanism of action is determined later in a process called target deconvolution. The NCI’s approach to random drug screening has evolved over the years and currently relies on testing compounds against 60 cell lines, details of which are described below.

1.6 The NCI60 approach to random drug screening

The NCI60 screening method was first utilised in the 1990’s and consists of 9 different distinct panels of cell lines including lung, brain, colon, ovary, prostate, breast, leukaemia, melanoma and kidney cells consisting of 60 cell lines in total (Shoemaker, 2006). Initial testing is done against 3 cell lines, the purpose of which is to eliminate those compounds that are inactive. If activity is obtained in the three-cell line panel, the compound progresses to the full 60 cell line panel and here, the test compound is added to cells for a 48hour time period using a 5-log concentration range. Upon completion, 60 dose response curves are obtained which can be analysed and three end point calculations are determined for each cell line. The

three end point calculations are (i) GI_{50} which is the concentration of test compound required to inhibit the growth of the cell line by 50% (ii) complete tumour growth inhibition (TGI) which is defined as the concentration of drug that completely prevents the growth of the cell line (i.e. the cell number at the end of the assay is the same as the starting cell number) and (iii) LC_{50} which is the concentration of compound required to kill 50% of the cells (Damia and D'Incalci, 2009). The completion of this study creates a unique 'fingerprint' of cellular response which allows the researcher to profile and characterise the data in terms of the sensitivity and resistance of each cell line to the test compound relative to the mean endpoint parameter used (GI_{50}, TGI, LC_{50}). The compounds which exhibit similar mechanism of actions will depict a similar pattern of growth inhibition across the 60 different cell lines screened against (Damia and D'Incalci, 2009). The COMPARE algorithm which was created by Paull et al., (1989) is a computer software programme that can be used to interpret chemosensitivity fingerprints by comparing the unique fingerprint of test compounds with that of established compounds for which the mechanism of action is known. If the unique fingerprint does not match the COMPARE algorithm database, then it is deemed as a COMPARE negative result which is more appealing to researchers as this is used for the identification of compounds with new mechanism of actions (Paull et al., 1989) (Damia and D'Incalci, 2009).

The NCI60 screening strategy has been very successful in the identification of new therapies such as bortezomib which is used in the treatment of multiple myeloma (Shoemaker, 2006). As with all *in vitro* based assays however, there are limitations but the widely accepted view is that the NCI60 has provided useful information, particularly with regards to the identification of compounds with novel mechanisms of

action. In addition, the characterisation of molecular targets in the cell line panel has enabled comparisons to be made between target expression and chemosensitivity patterns which aids the process of target deconvolution (Paull et al., 1989) (Shoemaker, 2006). Once lead compounds have been identified, they progress into *in vivo* testing, details of which are outlined below.

1.7 *In vivo* testing of novel compounds: The 'hollow fibre' assay

The NCI developed the *in vivo* hollow fibre assay as a bridge between the *in vitro* assays and the human xenograft models in immune-deficient mice (Decker et al., 2004; Hall et al., 2000). This intermediary assay was utilised for the better prediction of which lead compound observed in the *in vitro* study would be most appropriate to use in the subsequent xenograft models. The general cost of animals required, time constraints and the financial commitment essential for the *in vivo* studies justified the use of the hollow fibre assays as a faster means of screening these compounds (Damia and D'Incalci, 2009). The hollow fibre assay is devised in a manner where biocompatible hollow fibres are used as a 'vessel' for tumour cells to grow in an *in vitro* setting and later (24-48hours), these are implanted subcutaneously or intraperitoneally into nude mice. The pore size of the hollow fibres are not only small enough to prevent cancer cells escaping but they allow the entry of drug of interest with a molecular weight less than 500kDa (this also includes large proteins or peptides). The hollow fibre assay has also been used to investigate other important parameters such as the pharmacodynamics of the drug (Suggitt et al., 2006) (Temmink et al., 2007). The end point assays used in these pharmacodynamics studies are, apoptosis, comet assay and cell cycle analysis. If compounds are active in the hollow fibre assay, progression into xenograft studies is warranted.

1.8 *In vivo* testing of novel compounds: human tumour xenografts

The *in vivo* studies are carried out in an animal model setting in order to demonstrate (i) that the test compound has activity (at the maximum tolerated dose) and (ii) the pharmacokinetic properties (i.e. drug distribution, excretion, metabolism, drug-drug interactions, and absorption) of the compound are satisfactory (Venditti et al., 1984). There are different types of *in vivo* models that can be used such as (i) tumour xenograft models which are important for the testing of efficacy where the target is known or present (Venditti et al., 1984) (ii) the hollow fibre assay which has been previously mentioned, (iii) the use of orthotopic and metastatic tumour models to overcome the limitation of tumour xenograft models which are grown in a tissue environment that is different to the origin organ of the tumour (Bibby, 2004) and (iv) the use of transgenic mice and genetically engineered (GEM) cancer models (Damia and D'Incalci, 2009). If compounds are active *in vivo*, they must obtain 'investigational new drug' (IND) approval before any studies can take place in human subjects. As FDA highlights "safety first," it is important that the first 4 stages or phases of a clinical trial is planned to assess the maximum tolerated dose and safety of a drug, the pharmacokinetics and pharmacodynamics involved in the process.

1.9 Clinical testing: Phase I studies

The purpose of a phase I trial is to (i) determine the maximum tolerated dose (ii) define the toxic side effects that are encountered (iii) analyse the pharmacokinetic properties of a drug and (iv) conduct biomarker studies to determine if the drug is targeting the desired biochemical pathway (Lipsky and Sharp, 2001). This study is small in number and usually consists of around 20-100 patients with cancerous disease (Lipsky and Sharp, 2001).

1.10 Clinical testing: Phase II studies

The clinical phase II trial usually focuses on the efficacy of the drug against a cancer type (i.e. lung or breast) or tumours that carry a specific mutation/oncogenic driver that the drug targets. The primary objective is to establish whether or not the drug has anti-tumour activity (and if it works via the mechanism proposed). The number of patients in the phase II trials are normally larger than the phase I studies and involve patients with the disease of interest. The experimental design is also essential for planning the next phase (III studies) to determine, (i) optimum dose, (ii) how frequent the dose should be administered, (iii) the route of administration and (iv) endpoints (Umscheid et al., 2011). The number of participants in this trial limits the potential of a strong efficacy report and therefore warrants a study with a much larger participant number which is the clinical phase III studies (Umscheid et al., 2011).

1.11 Clinical testing: Phase III studies

The clinical phase III study focuses on confirming the data obtained from the previous studies which were focused on both safety and efficacy. The clinical phase III study also compares the results obtained with the test compound against the existing methods for treating patients, with the purpose of determining whether or not the new drug works better than existing therapies. This study is much larger and more diverse than the previous two and usually involves between 300-3000 participants (Umscheid et al., 2011). The statistical power established from these numbers of participants based on Hanley's 'Rule of 3' for an adverse event rate is no less than 1 in 100 participants (Eypasch et al., 1995). This signifies the importance of a clinical phase IV study as more than one clinical phase III study is sometimes required for the establishment of efficacy, drug safety and to identify less common adverse reactions (Umscheid et al., 2011).

1.12 Clinical testing: Phase IV studies

By this stage the drug is usually approved and is required to go through some additional testing, (i) to determine if the common adverse reactions can be reduced, (ii) if there are any limitations in pre-marketing as previous statistics have shown that approximately 20% of new drugs require new warnings post-marketing and roughly 4% of the drugs are consequently withdrawn due to safety reasons (iii) evaluate the costs of the drug and (iv) determine the effectiveness of the drug doses in diseases different to the original population (Bakke et al., 1995) (Lasser et al., 2002).

1.13 Targeted or phenotypic drug discovery?

Returning to the issue of preclinical drug development, the initial stages of the drug discovery process is challenging and each approach has its strengths and limitations. If the molecular target is known, then a much more focused targeted approach can be taken by using both cell-free and cell-based assays to identify new novel lead molecules which have a potent effect on either the pathway or biochemical target (Shoemaker et al., 2002). The cell-free based assay is (i) very amendable to high through put screening, (ii) precise, (iii) simple and (iv) target focused therefore able to generate many hit leads (Zhang et al., 1999) (Zhang et al., 2000). Whereas the cell-based assay can (i) present other targets as well as the target in focus, (ii) include membrane bound receptors, (iii) characterization of the mechanistic of the drug and (iv) include cellular metabolism (Zhang et al., 1999). The molecular target of interest does not have to be limited to one specific site as some compounds may have the ability to target multiple proteins, a process that is known as poly-pharmacological (Ramsay et al., 2018). In our laboratory, Ag8 is a silver based compound that has this ability to target multiple sites in the cell which is very

attractive as it represents one approach to tackling the problem of tumour heterogeneity and biochemical plasticity (Allison et al., 2017).

A review by Swinney and Anthony et al., (2011) have collated data from 1998-2011 where 259 new compounds were approved by the food and drug administration (FDA). From these compounds 28 were discovered by the phenotypic approach whereas 17 of these were by the target drug discovery approach (Swinney and Anthony, 2011). It is also the case that more 'first in class' compounds have been identified using a phenotypic screening approach than target orientated approaches (Swinney and Anthony, 2011) and this is particularly attractive. Furthermore, the phenotypic screen is inherently flexible in its design and there is a strong case to re-evaluate compounds using cell-based assays that more accurately reflect key features of tumour biology. This thesis addresses one such approach which is designed to identify compounds that selectively exploit physiological conditions that exist within the solid tumour, details of which are outlined below.

1.14 The tumour microenvironment (TME)

Solid tumours have been described as a complex 'rogue' organ which contain a heterogeneous population of tumour and non-malignant cells (Saggar et al., 2013). Within the tumour, there are microenvironments that can broadly be described as either cellular and/or physiological. Whilst the cellular tumour microenvironment (TME) is beyond the scope of this thesis, it is important to acknowledge that the cellular and physiological TME co-exist and ideally, they should not be viewed as distinct or isolated microenvironments. A brief overview of the cellular TME and its links to the physiological TME is provided below and summarised in figure 1.5.

1.15 The cellular TME

Within tumours, the cellular TME consists of endothelial cells, smooth-muscle cells, pericytes, eosinophils and basophils, cancer associated fibroblasts (CAFs), myofibroblasts, T, B lymphocytes, myeloid cells, dendritic cells, macrophages, myeloid cells and mast cells (Albini and Sporn, 2007). The role that these cells play in the TME is complex and it is influenced by physiological conditions including hypoxia. A brief account of CAF cells and macrophages in the TME is provided below and summarised in figure 1.5.

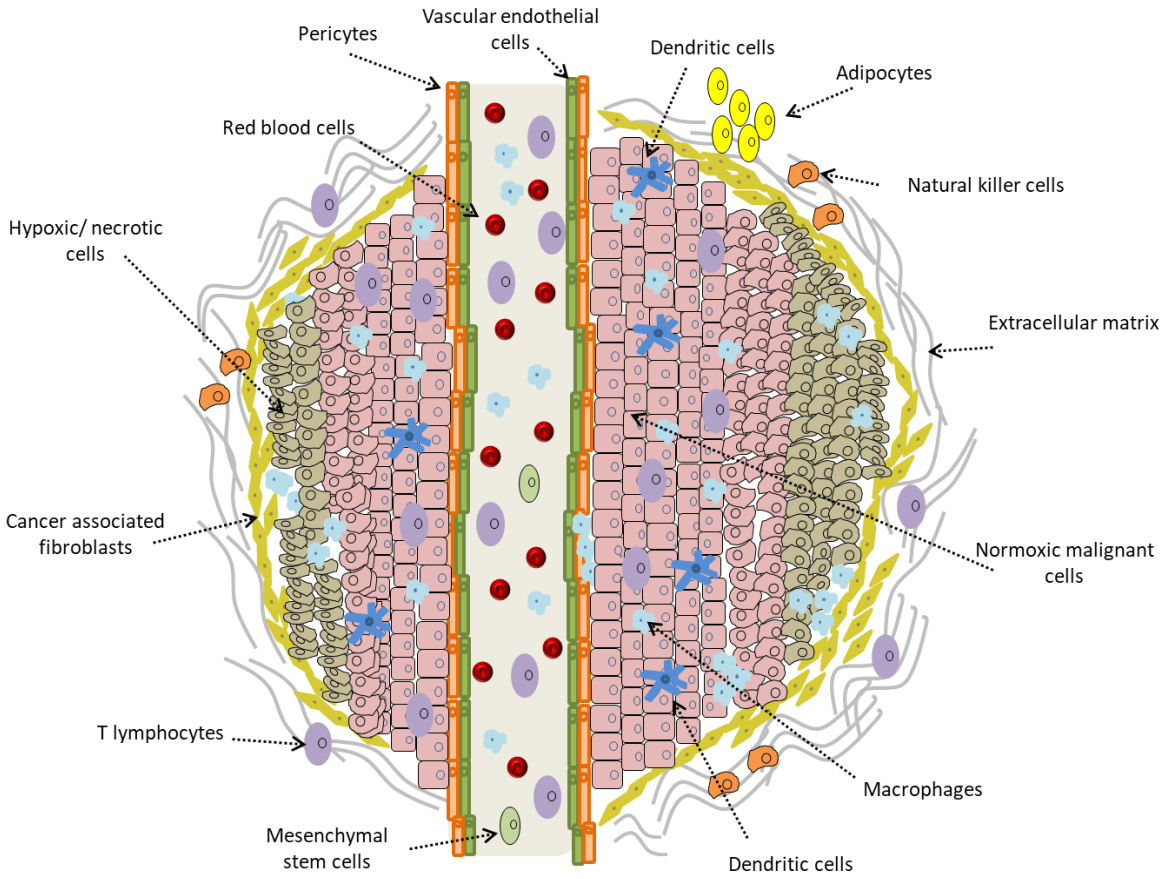


Figure 1.5 Cartoon illustration of the cells associated with the tumour microenvironment (Balkwill et al., 2012).

1.16 The biological importance of CAFs

As shown in figure 1.5, the tumour contains numerous CAF cells which play an important role in supporting the growth of the tumour. In normal tissues, fibroblasts are referred to as mesenchymal cells which are embedded in the physiological extracellular matrix (ECM). If there is an injury or wound to the tissue, these fibroblasts can be activated so that they can repair tissues and regeneration can occur during the healing process of tissue inflammation or fibrosis (Liu et al., 2019). CAFs when compared with quiescent fibroblasts are much larger in size and have distinct indented nuclei (De Wever et al., 2008). What also separates CAFs from their normal counterparts is that activated CAFs can inhibit migratory and proliferative properties (Chen and Song, 2019; Kobayashi et al., 2019). A small portion of CAFs also have the ability to trans-differentiate from a fibroblastic cell to blood vessels, pericytes, epithelial cells, adipocytes and smooth muscle cells (Dulauroy et al., 2012; Jotzu et al., 2011; Potenta et al., 2008; Rhim et al., 2012; Wikström et al., 2009).

CAFs are much more metabolically active in comparison to fibroblasts in normal tissue and are unique for their ability to produce an ECM (Kalluri, 2016). CAFs can also recruit immunosuppressive cells into the TME which can assist with immune evasion or promote angiogenesis by the secretion of the following pro-inflammatory cytokines: (i) vascular endothelial growth factor (VEGF), (ii) CXC-chemokine ligand (CXCL12), (iii) transforming growth factor-β (TGF-β) and (iv) interleukin-6 (IL-6) (Ahmadzadeh and Rosenberg, 2005; Feig et al., 2013). There is clear evidence that CAF cells are heterogeneous in population and these cells can either restrain or promote tumour growth (Chen and Song, 2019; Kalluri, 2016; Kobayashi et al., 2019). CAFs also represent a novel approach for cancer therapeutic intervention due

to their exertion of anti-tumour immunity which itself makes them a promising therapeutic target.

1.17 The role of macrophages in the TME

The TME also contains macrophages (figure 1.5) which are phagocytic cells that are important effectors of innate immunity to pathogens. The other key duties of macrophages are their role in both chronic and acute inflammatory responses (Egners et al., 2016). Hypoxia (low O_2 concentration - which will be covered in more depth later) is a key driver of multiple pathological processes involving the macrophages such as, (i) wound healing, (ii) tumour inflammation and (iii) atherosclerosis (Semenza, 2014). Hypoxia inducible factor (HIF) is a transcription factor, the alpha subunit of which is only stable under low levels of oxygen concentration. Previous studies have demonstrated that HIF-1 is stable in various stages in macrophages which are located in the hypoxic regions of the TME throughout. (Fang et al., 2009; Talks et al., 2000). Inhibiting HIF has an effect on various macrophage functions such as (i) aggregation and (ii) migration and invasion (Cramer et al., 2003; Peyssonnaux et al., 2005).

1.18 Generation of the physiological TME

The origin of the physiological TME stems from the tumour vasculature that develops within tumours. Angiogenesis is a hallmark of cancer which is a process that generates new blood vessels from existing blood vessels and it is required for the supply of oxygen, nutrients to tumours and for the removal of waste products (Folkman, 1971). Angiogenesis is stimulated by tumour tissues when there is a requirement of oxygen and nutrients (Nishida et al., 2006). Hypoxia-inducible factor (HIF-1α) which is a major transcription factor (discovered by the Nobel Prize laureate

Greg Semenza) that is stabilised at low levels (0.1-5%) of O_2 and plays a vital role in tumour proliferation (Ziello et al., 2007). The development of new blood vessels from existing blood vessels can be induced by HIF-1α stabilisation under hypoxic conditions resulting in the formation of functional HIF1 complexes leading to the increased transcriptional activity of a variety of genes including those encoding for vascular endothelial growth factors (VEGF) and its receptors (Bottaro and Liotta, 2003). The formation of the new blood supply to tumours not only enables the tumour to grow but provides the route for the metastatic spread of the disease to other organs (Folkman, 1971; Nishida et al., 2006).

In contrast to capillary beds in normal tissues which are well organised and structured, the vascular supply that develops in tumours is chaotic and structurally abnormal (Balkwill et al., 2012) leading to regions that are poorly perfused with blood. Cells close to blood vessels are therefore able to receive nutrients and oxygen but as distance from a supporting blood vessel increase, gradients of oxygen tension, nutrients, extracellular pH (pHe), proliferation rates decrease which are all characteristic features of the TME (Phillips, 2016). These characteristics can affect tumour cell biology and therapeutic response to anti-cancer drugs (Vaupel and Harrison, 2004) as illustrated in figure 1.6.

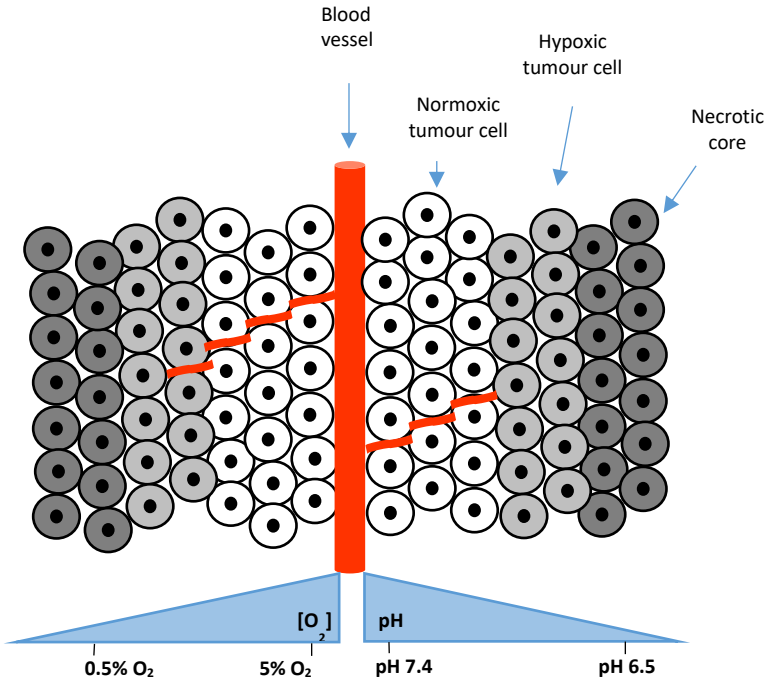


Figure 1.6 Depiction of the different fractions present in a TME. This image includes details of the normoxic, hypoxic and necrotic regions of the TME as well the changes in extracellular pH (pHe)

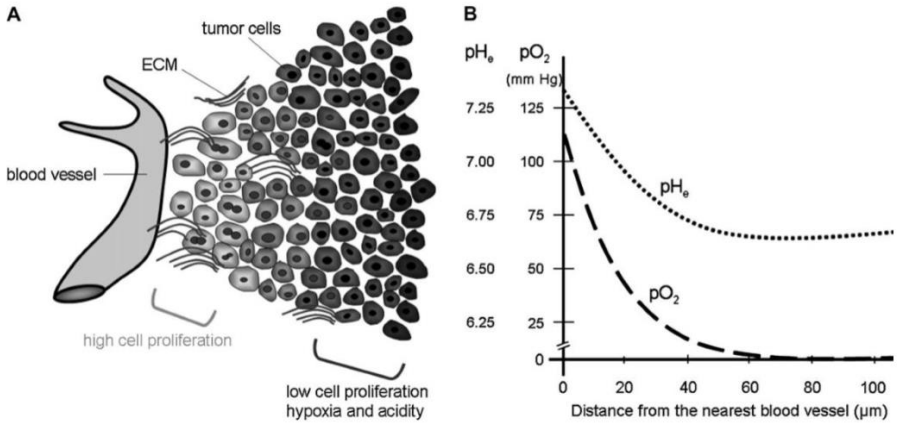


Figure 1.7 Tumour microenvironment and cross link of the hypoxic region in correspondence with the distance of the capillary. A) Diagram representation of tumour cells which are situated next to the capillary, showing the expression of hypoxia throughout the TME B) Graph representation of the level of hypoxia (dotted line) in relation with pH (dashed line) in a hypoxic and acidic tumour microenvironment. (Trédan et al., 2007).

As the distance from a supporting blood vessel increases, both acidic and pHe increases (Figure 1.7) (Vaupel and Harrison, 2004). In order to survive in these environments, cells have to biochemically adapt and numerous changes mediated primarily by HIF transcription factors occur (Semenza, 2012). This includes changes to cellular metabolism in order to generate sufficient ATP for cell survival (Ziello et al., 2007). Cells within the hypoxic regions proliferate slowly and this leads to resistance to cytotoxic anti-cancer drugs, many of which target the process of cell replication (Höckel and Vaupel, 2001). Hypoxic cells can remain viable due to their quiescent state which has been linked to the low levels of ATP production. These cells can re-enter cell proliferation following the eradication of aerobic cells by chemotherapy or radiotherapy caused by the improved supply of oxygen and nutrients (Saggar et al., 2013).

Another feature of the hypoxic tumour microenvironment is the creation of an acidic extracellular pH (Kato et al., 2013). The Warburg effect which was first described by Otto Heinrich Warburg in 1924 states that tumours have the tendency of producing lactate through the anaerobic glycolytic pathway despite the presence of adequate amounts of oxygen for the production of energy (in form of ATP) (Warburg et al., 1924). In aerobic glycolysis, the last step of which is the formation of lactate from pyruvate results in a build-up of lactate in tumour cells which is exported into the extracellular space by monocarboxylate transporters. High levels of lactate have been associated with poor clinical outcomes such as (i) poor prognosis in cancer patients, (ii) tumour reoccurrence and (iii) metastasis (Brizel et al., 2001; McFate et al., 2008; Walenta et al., 1997; Walenta et al., 2003). The molecular mechanisms for these poor clinical outcomes have been linked to effects on T cell function including effecting on the immune response, T-cells function themselves and also its

metabolism (Kato et al., 2013). The lactate produced by the tumour cells also has an effect on angiogenesis by the nuclear factor-$_K$B (NF-$_K$B) as well as the induction of VEGF (Végran et al., 2011).

1.19 Therapeutic implications of the TME

There is considerable evidence demonstrating that the presence of the TME adversely influences the response of tumours to chemotherapy. The causes or resistance can be either direct or indirect. As an example of a direct cause of resistance, drugs such as bleomycin require oxygen as a key component of their mechanism of action and therefore in hypoxic conditions, the efficacy of such drugs is reduced (Burger et al., 1981). The TME can also indirectly affect the efficacy of drugs, a key mechanism being the fact that the majority of cells that reside in the TME are not proliferating. As described earlier, most cytotoxic anti-cancer drugs work against dividing cells and therefore, non-proliferating tumour cells within the TME will inherently be less sensitive to chemotherapy than proliferating and well oxygenated cells. Another mechanism of resistance is poor drug delivery and reduced drug uptake. For an anti-cancer drug to be effective, it must have the ability to penetrate through the solid tumour by moving through multi-cell layers and be effectively taken up into cells within the TME (Minchinton and Tannock, 2006). This occurs best when the anti-cancer drug is in its uncharged form (Trédan et al., 2007). The mild acidic environment can mediate drug resistance as drug uptake into the cells is influenced by low pHe, leaving less chemotherapeutic drug to target tumour cells and produce the desired anti-tumour effect (Mayer et al., 1986). Whilst the intracellular pH (pHi) of tumour cells remains close to neutral or is slightly alkaline, the acidic pHe can have an effect on certain well-established anti-cancer drugs such mitoxantrone, vincristine, vinblastine and doxorubicin. All of these have pKa values

ranging between 7.5-9.5 meaning they become protonated under acidic conditions which in essence decreases their uptake ability into cells (Gerweck et al., 2006; Tannock and Rotin, 1989). If the pHe shifts towards (slightly) alkaline conditions, then the uptake of doxorubicin and mitoxantrone is enhanced (Raghunand et al., 1999; Raghunand et al., 2003). The mild acidic pHe does not only affect drug uptake but also plays a critical role on drug efflux by upregulating the expression of p-glycoprotein (Lotz et al., 2007).

The pHe also has a direct impact on the expression of other genes which directly correlate with pro-metastatic factors (Kato et al., 2013). Rofstad et al., 2006, carried out *in vivo* studies to determine the influence of pHe on metastasis using athymic nude mice. These mice were injected (via the tail vein) with melanoma cells which were pre-treated with mildly acidic medium and the results demonstrated that a lot of these cells managed to metastasize to the lungs (Rofstad et al., 2006). This demonstrates that the acidic pHe is associated with tumour metastasis (Kato et al., 2013). Tumour cells have to adapt to their environment (Swietach, 2019) and the presence of an acidic pHe is associated with not only resistance to certain drugs but it also appear to drive the malignant spread of the disease (Kato et al., 2013). There is therefore a need to develop therapeutic strategies that target hypoxic cells that reside within regions of tumours with acidic pHe.

1.20 Therapeutic targeting of the tumour microenvironment

As described above, there is evidence of poor clinical outcomes for tumours which are associated with hypoxia when compared to patients who lack hypoxic regions in their tumours (Höckel et al., 1996). Numerous well established anti-cancer drugs

have failed to adequately diminish tumour cells which reside within the hypoxic regions and this chemo/radiation resistance contributes to the poor outcome in the clinic (Ikeda et al., 2016). The poor therapeutic outcome may also be explained by the following major biological factors; resistance to apoptosis (Welsh et al., 1996), increased invasion and metastasis and induction of angiogenesis which are all associated with the hypoxia-inducible factors (HIF-1) (Phillips, 2016). With the increasing evidence that hypoxia can unfavourably affect clinical outcome, new chemotherapeutic strategies have been evaluated to eradicate the tumour cells which reside in the hypoxic regions. One such approach is the development of bioreductive drugs or hypoxia activated prodrugs (HAPs). The seminal work conducted in this field was done by Sartorelli in the 1970's following the demonstration that the activation of mitomycin C preferentially occurred under the reducing conditions present within the hypoxic regions of tumours (Lin et al., 1972) (Phillips, 2016). A brief account of HAPs and their efficacy against hypoxic cells is provided below.

1.21 The discovery of Hypoxia activated prodrugs (HAPs) or Bioreductive drugs.

As the prototypical bioreductive drug, mitomycin C is an anti-tumour antibiotic which is extracted from actinobacteria species Stretomyces caespitosus (Verweij and Pinedo, 1990). The mechanism of action of mitomycin C is by an initial enzymatic bioreduction which converts mitomycin C to a bi-electrophilic intermediate that is highly reactive leading to alkylation with cellular nucleophiles including DNA (Paz et al., 2012). The DNA-DNA interstrand cross link is the main cause for the cytotoxic

effect on tumour cells (Palom et al., 2002) and this has previously been reported to be most effective in the S phase and late G1 part of the cycle (Verweij and Pinedo, 1990).

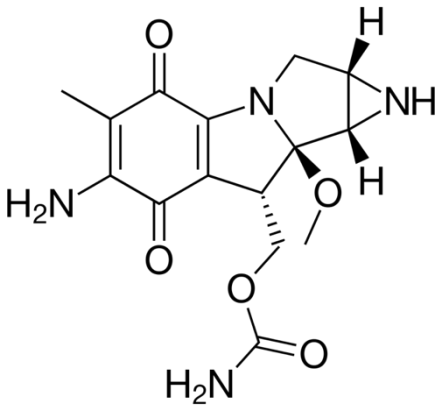


Figure 1.8. Chemical structure of mitomycin C (Paz et al., 2012)

Mitomycin C is considered to be the first original bioreductive prodrug because of its preferential cytotoxic abilities under hypoxic conditions. This can be achieved by either the one or two electron reduction of mitomycin C (under hypoxia) which spontaneously loses methanol and leads to a very unstable but highly reactive intermediate (Verweij and Pinedo, 1990). An *in vitro* study carried out by Sartorelli showcased this cytotoxic ability of mitomycin C against S-180 and EMT-6 murine cell lines under hypoxic conditions (Kennedy et al., 1980). Interestingly under normoxic conditions, mitomycin C displayed increased levels of cytotoxicity at pHe 6.6 against the Chinese hamster ovary cells when compared to pHe 7.2. This effect was only observed under normoxic conditions and when repeated under hypoxic conditions using the same pHe levels there was no difference in cytotoxicity observed (Begleiter and Leith, 1993). The limitations of mitomycin C are its severe side effects such as bone marrow toxicity, myelosuppression, anaemia as well as other less severe adverse effects; alopecia, nausea and vomiting (Verweij and Pinedo, 1990). The

bioreductive properties of mitomycin C have nevertheless encouraged researchers to develop compounds that are activated under hypoxic conditions to circumvent the issue of targeting all the cells in the TME.

1.22 Hypoxia activated prodrugs: General properties

As well as mitomycin C, misonidazole has also been shown to exert selective toxicity towards cells under hypoxic conditions when compared to normoxic (Adams and Stratford, 1994). This has triggered extensive research into investigating a large series of compounds with different chemical attributes for their ability to work as bio-reductive drugs or HAPs. Compounds within this class are typically inactive prodrugs in the presence of oxygen, but are enzymatically activated by reduction by either the one or two electron oxidoreductases which under hypoxic conditions, generate a cytotoxic agent (Denny et al., 1996). Denny has previously described a generalised modular design of HAPs which consists of a trigger, linker and an effector.

Trigger – The trigger unit is vital for the selectivity of the compound and must be capable of undergoing efficient metabolism by precise tumour mechanisms (Denny et al., 1996). These reactions are typically reversible in the presence of oxygen.

Linker – In the oxidised form the effector is inactive, however, when the trigger group is reduced under hypoxic conditions, the linker conveys this message to the effector leading to either its activation or release from the HAP which leads to the cytotoxic effects (Denny et al., 1996).

Effector – The effectors have been reported as being the cytotoxic module which has been previously reported as the potent DNA-interactive agents that produce the cytotoxicity (Phillips, 2016).

There are five different groups of HAPs which are able to selectively target the hypoxic cells and these include (i) aromatic and (ii) aliphatic N-oxides, (iii) transition metal compounds, (iv) nitro-cyclic compounds and (v) quinones (Guise et al., 2014). For a HAP to be successful it has to have the following properties; (i) penetrate to all those cells that are distant to the blood vessels within its pharmacokinetic lifetime, (ii) be selective enough to target only the tumour cells and not the normal tissue, (iii) to be able to target the aerobic fraction as well as the hypoxic fraction via a bystander effect and (iv) to be able to kill those hypoxic cells which are slow dividing which typically behave like quiescent cells (Phillips, 2016). The following sections describe several HAPs focusing on their mechanism of action, their successes and their limitations.

1.23 Tirapazamine

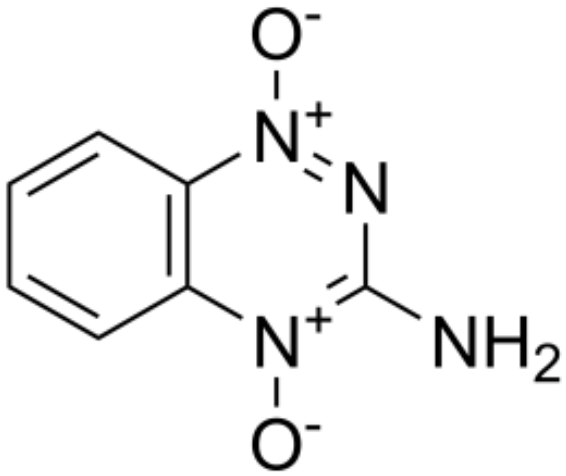


Figure 1.9 Chemical structure of the HAP tirapazamine (Brown, 1993)

Tirapazamine (figure 1.9) was developed back in the mid 1980's and was the first HAP to be extensively evaluated both pre-clinically and clinically (Brown, 1993; Spiegelberg et al., 2019). Tirapazamine consists of an aromatic N-oxide and becomes activated in the absence of oxygen following one electron reduction by

enzymes such as cytochrome P450 reductase (Shinde et al., 2009). This results in the formation of active/ cytotoxic hydroxyl and benzotriazinyl radicals (Shinde et al., 2009; Wilson and Hay, 2011). It can further undergo a two-electron reduction to form a tirapazamine mono N-oxide which is not toxic. Despite displaying very promising HAP like properties in the early pre-clinical tests (Brown, 1993; Brown and Wang, 1998), it became apparent in the clinical phase III studies that this did not offer any additional benefits to existing chemotherapeutic drugs alone or chemoradiotherapy (Reddy and Williamson, 2009). Tirapazamine also displayed a lot of side effects as this HAP required mild hypoxia to become active and activation occurred in the bone marrow, gastrointestinal tract and liver which may have contributed to these adverse side effects (Spiegelberg et al., 2019). Several different analogues of tirapazamine have been developed and tested pre-clinically but have not yet progressed to clinical trials (Wardman et al., 1995). Examples of these analogues are SN30000 and SN29751 which are both activated under hypoxia by one electron reduction. SN30000 in particular displayed high levels of potency and cancer selectivity towards the colorectal cancer cell line HT29 in comparison to tirapazamine (Hicks et al., 2010). The vascular penetration and diffusion of SN30000 was much greater in multi-cell layer models in the HT29 and SiHa cell lines than that of tirapazamine which was another limiting factor for its poor clinical outcome. Both these tirapazamine analogues were also much more active than tirapazamine in an *in vivo* setting too (Hicks et al., 2010), however both compounds are yet to enter clinical trials.

1.24 TH-302 (Evofosfamide)

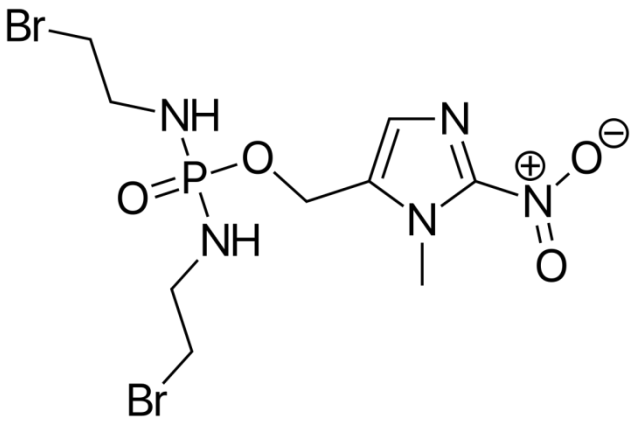


Figure 1.10 Chemical structure of the HAP TH-302

TH-302 is a nitrogen mustard (2-nitroimidazole-based nitrogen) which undergoes one electron reduction to release the active DNA alkylating isophosphoramide mustard (Millis et al., 1995). TH-302 was first described in 2007-2008 through clonogenic cytotoxicity studies from a panel of drug candidates (Hart et al., 2007). The results demonstrated that TH-302 was active under hypoxic conditions in the H460 lung cancer cell lines with clonogenic IC_{50} values of 0.1, 5 and 30μM at oxygen tensions of 0, 6 and 21% respectively (Hart et al., 2007). The advantage of TH-302 was that this HAP was tested as a monotherapy and in combinatorial studies with the likes of doxorubicin, docetaxel and gemcitabine (Liu et al., 2012). It has also been tested in conjunction with radiotherapy (Guise et al., 2014). Phase I and Phase II clinical safety and efficacy studies with TH-302 has shown promising results (Borad et al., 2015; Chawla et al., 2014; Ganjoo et al., 2011; Weiss et al., 2011). Moving forward, the next step was phase III clinical studies where two large trials consisting of 600 patients with advanced pancreatic cancer and soft tissue sarcoma were treated with doxorubicin, gemcitabine with the addition of TH-302 (Tap et al.,

2017). Unfortunately both trials did not perform well and the overall endpoint of improved overall survival was not met (Spiegelberg et al., 2019). The researchers tried justifying the overall performance of TH-302 by stating that the placebo group had performed better than they initially believed but TH-302 ultimately failed. One of the major reasons why TH-302 may have not performed as well as it should have is due to a poor understanding of the different oxygen tensions that may be present in the tumours. It has been previously reported that pancreatic cancer oxygen tensions can fluctuate between 0-26% which may have affected the performance of TH-302 activation (Dhani et al., 2015). Basic *in vitro* studies assessing the cytotoxicity of TH-302 in combination with the classic anti-cancer drugs against a panel of pancreatic cancer cell lines with fluctuating or even oxygen cycling from 0-26% may give insightful information. Unfortunately however, TH-302 also follows the same footsteps as tirapazamine in that clinical activity did not reflect the promising activity seen in preclinical models.

1.25 EO9 (Apaziquone)

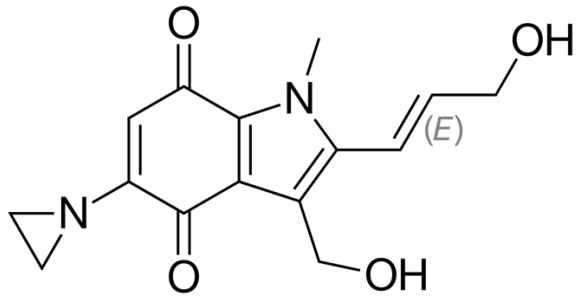


Figure 1.11 Chemical structure of the HAP EO9

EO9 was synthesised as a derivative of mitomycin C and undergoes activation by either one or two electron reduction. This creates DNA damaging species under both normoxic and hypoxic conditions (Oostveen and Speckamp, 1987; Phillips, 2016). The one electron reduction route generates a semi-quinone intermediate which can recycle back to the parent quinone in the presence of oxygen. This generates toxic reactive oxygen species in the presence of oxygen but in hypoxia, the semi-quinone is stabilised which can lead to further reduction to form the hydroquinone. The semi-quinone or the hydroquinone moiety could then generate toxic lesions in cells leading to hypoxia selectivity. These reactions are carried out by one electron oxidoreductases such as cytochrome P450 reductase (Plumb and Workman, 1994; Workman, 1994). The quinone can be directly converted to the hydroquinone by the 2 electron oxidoreductase NAD(P)H:quinone oxidoreductase 1 (NQO1) which is also known as DT-diaphorase. This bypasses the semi-quinone step and this reaction is effectively oxygen insensitive. The generation of the hydroquinone leads to the formation of DNA damage which is typically in the form of single strand breaks in the DNA although alkylation of DNA has also been reported (Workman, 1994).

There is a complex relationship between the enzymology of cells and the ability of EO9 to eradicate hypoxic cells. In the case of NQO1 rich cells, NQO1 tends to dominate over one electron oxidoreductases and this leads to both aerobic and hypoxic cell kill with low hypoxic cytotoxicity ratios (HCR). In cells that have low levels of NQO1, one electron reductases predominate and as this is an oxygen sensitive reaction, preferential activity against hypoxic cells has been reported (Plumb and Workman, 1994). EO9 was selected for clinical trials in the 1990's but failed to show any efficacy when administered intravenously (Boyer, 1997). Reasoning behind this was due to poor/ impaired pharmacokinetics and poor

penetration through avascular tissue (Phillips et al., 2013). Connors et al., 1996 went on to further discuss other limitations and design flaws of the phase II clinical trial by noting that levels of NQO1 and hypoxia were not measured from the patient samples. The pHe also has an effect on the cytotoxic ability of EO9 under normoxic conditions. A study carried out by Phillips et al., (1992) demonstrated that when EO9 was tested against the colorectal cancer DLD-1 cell line under pHe 7.4 resulted in 40% cell death, however when the pHe was reduced to 5.8 this enhanced the cytotoxic ability of EO9 killing 95% of the cells (Phillips et al., 1992).

Based on an understanding of the reasons why EO9 failed following intravenous administration, a further clinical trial of EO9 against non-muscle invasive bladder cancer was proposed (Phillips et al., 2013). The rational being that intravesical administration directly into the bladder would circumvent the drug delivery problem and any drug reaching the systemic circulation would be rapidly eliminated (EO9's poor pharmacokinetic properties would paradoxically be favourable in terms of reducing the risk of systemic side effects). Both phase I and II clinical trials demonstrated substantial anti-cancer activity (Puri et al., 2006) and EO9 entered phase III clinical trials. The results of these trials have been reported recently (Hendricksen et al., 2008) and both trials failed to reach their primary endpoint of delayed two-year recurrence rates. There were significant changes to the protocols used in the phase III and phase II trials and these ultimately contributed to its failure. In particular, the administration of EO9 immediately after surgery when haematuria was common led to the inactivation of EO9 by blood (Hendricksen et al., 2008).

1.26 AQ4N (Banoxantrone)

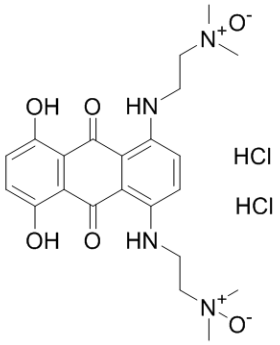


Figure 1.12 Chemical structure of the HAP AQ4N

AQ4N is an aliphatic N-oxide that is activated under low oxygen concentrations by sequential 1-electron reduction by cytochrome p450 resulting in the generation of the potent topoisomerase II inhibitor alkylaminoanthraquinone (AQ4) (Steward et al., 2007). AQ4N has shown promise in *in vivo* studies where it has good activity against mouse models (Loadman et al., 2001). AQ4N was also very effective in a phase I clinical trials where it demonstrated hypoxic selectivity in head and neck cancer patients (Albertella et al., 2008). In total there have been three clinical phase I studies where 2 have been as a single drug study and the other in combination with radiation (Albertella et al., 2008; Papadopoulos et al., 2008; Steward et al., 2007). Despite promising results from the clinical phase I studies, AQ4N did not progress any further than the clinical phase II trials which was initially carried out in 2006 (NCT00394628) where the results have not been published (Mistry et al., 2017). The reason for its lack of progression was commercial rather than performance based results and novel analogues of AQ4N are being developed to circumvent this issue (Nesbitt et al., 2017).

1.27 Current status of HAPs and the need for new therapeutic approaches to targeting the physiological TME of solid tumours

As described above, the development of HAPs has been an active area of research for many years but the attractive concepts underpinning this approach have so far proved difficult to translate into clinical activity. The reality of the situation is that despite 4 decades of research, no HAP has so far been approved for use in humans. Nevertheless, HAPs remain an active area of research and it is hoped that the new generation of drugs under development will perform better in clinical trials. In view of the lack of success of the HAP programme, it is also prudent to investigate alternative approaches to the development of drugs that target the physiological TME. The majority of HAPs that have been developed focus almost exclusively on tumour hypoxia but as described earlier, tumour hypoxia is only one component part of the physiological TME. Cells that reside in these regions are exposed to other environmental challenges including low pHe. The majority of preclinical studies conducted in this field only focus on hypoxia and the importance of both hypoxia and acidic pHe combined has largely been overlooked. It is therefore possible that better effects may be obtained if preclinical models reflected the complexity of the TME more closely that just looking at hypoxia in isolation. The hypothesis that will be tested in this thesis therefore is that novel molecules will be discovered that exploit both hypoxia and acidic pHe will be discovered compared to hypoxia alone.

1.3 Aims of this thesis

The aim of this study is to determine whether or not a phenotypic screen based on the evaluation of compounds under hypoxic and acidic pHe conditions will identify compounds that have superior activity compared to hypoxia alone. The phenotypic screen described here is based on testing compounds under (i) aerobic (ii) hypoxic (iii) aerobic plus acidic pHe and (iv) hypoxic plus acidic pHe conditions. These experiments will be conducted in a range of cell lines including cancer and non-cancer cells with the additional aim of determining selectivity indices (by comparing the response of cancer and non-cancer cells), hypoxic cytotoxicity ratios (comparing the response of tumour cells under aerobic and hypoxic conditions) and a microenvironment enhancement ratio (comparing the response of cells under aerobic and neutral pHe with hypoxic and acidic pHe conditions). A range of test compounds was obtained from collaborators from various institutions across the world and these are listed below;

i. Ruthenium, Iridium and Rhodium based napthyridines, thiourea derivatives, benzylthiourea ligands and aroylthiourea compounds
ii. Bromo-hexitol prodrugs
iii. NHC-Ag based complexes
iv. Minor groove binders
v. Tyrosine kinase inhibitor (dasatinib) with the attachment of N-oxide

Chapter 2: General Materials and Methods

2.1 Cell lines and cell culture conditions

The following cell lines were all obtained from American Type Culture Collection (ATCC): HCT116 $p53^{+/+}$, HCT116 $p53^{-/-}$, HCT116 KRAS mutant, HCT116 KRAS wildtype, HT29, Mia-PaCa-2, MDA-MB-231 and H460. In addition to these cancer cell lines, a non-cancer cell line (ARPE-19) was also included in order to assess selectivity *in vitro*. All the cells were routinely cultured in base media (table 2.1) containing 10% v/v foetal bovine serum (FBS) (Sigma), 2mM L-glutamine (Sigma) and 1mM sodium pyruvate (Sigma). Non-essential amino acids were used for some cell lines (H460 and ARPE-19). The final composition of media plus supplements used was termed 'complete media' and this terminology is widely used throughout the thesis. Cells were maintained at 37°C in a CO_2 enriched (5%) and humidified atmosphere. All the cell lines were routinely passaged when reaching 70-80% confluency.

Cell line	Disease	Base Media	Reference
HCT116 p53$^{+/+}$	Colorectal Carcinoma	DMEM	(Brattain et al., 1981)
HCT116 p53$^{-/-}$	Colorectal Carcinoma	DMEM	(Brattain et al., 1981)
HCT116 KRAS mutant	Colorectal Carcinoma	DMEM	(Yun et al., 2009)
HCT116 KRAS wildtype	Colorectal Carcinoma	DMEM	(Yun et al., 2009)
HT29	Colorectal Carcinoma	DMEM	(Godefroy et al., 1988)
Mia-PaCa-2	Pancreatic Carcinoma	DMEM	(Yunis et al., 1977)
MDA-MB-231	Breast Adenocarcinoma	DMEM	(Cailleau et al., 1974)
H460	Non-small cell lung cancer	RPMI	(Banks-Schlegel et al., 1985)
ARPE-19	Normal Eye; retinal pigmented epithelium; retina	DMEM	(Dunn et al., 1996)

Table 2.1 List of all the different types of cancerous cell lines used throughout this PhD study, type of disease and media used.

2.2 Sub culturing of cells

Cells were grown as 2D monolayers and were sub-cultured upon reaching confluency. This was carried out by washing cells with 10mL phosphate buffered saline (PBS) solution (Sigma) followed by the addition of 3-4mL of trypsin-EDTA (Sigma). Cells were placed in a humidified incubator for approximately 2 minutes. Cells were further checked under the light microscope to ensure cells were detached

from the surface of the flask. Occasionally the flask would be gently tapped to ensure all the cells have detached. To stop the trypsinisation process, complete media containing 10% FBS was added to the flask (10mL per flask). Cells were collected in a 25mL universal tube (Sarstedt) and centrifuged for 3 minutes at 1200 rpm. Upon completion, media containing trypsin was removed and the cell pellet was re-suspended in complete media. The cell suspension was diluted 1:3 or 1:10 depending on cell type and new cultures were established.

2.3 Cell counting and determination of cell number

Cells were counted using an improved Neubauer haemocytometer following the procedure recommended by ThermoFisher,

(www.thermofisher.com/uk/en/home/references/gibco-cell-culture-basics/cell-culture-protocols/counting-cells-in-a-hemacytometer.com).

Briefly, the cell suspension (10µL) was loaded onto the haemocytometer and cells were counted in each of the chambers (n=5 for each chamber). The average cell number was determined and multiplied by 10^4 to obtain the number of cells/mL.

2.4 Cryo-preservation

To protect the cultures from genetic drift, cells were routinely frozen down and stored in liquid nitrogen. Cell suspensions obtained from cultures in mid to late exponential growth were placed into media containing 20% FBS and 10% DMSO and aliquoted into cryo-tubes (1mL per tube). A Mr Frosty (Nalgene) was filled with isopropanol and each cryo-vial was labelled, inserted into the Mr Frosty and placed in the -80°C freezer overnight. The following day the cryo-vials were removed and placed into the liquid nitrogen Dewer storage system.

To defrost cells and establish fresh cultures, cells were rapidly thawed at 37°C and transferred to universal tubes containing 9mL of media. Cells were centrifuged for 3 minutes at 1200rpm and the cell pellet was re-suspended in complete media before being placed into a T25 flask and cultured as described above. Cells were routinely passaged up to ten times after which they were discarded and fresh cultures established from the liquid nitrogen bank.

2.5 Compounds

All of the drugs were made up in DMSO with the exception of cisplatin which was dissolved in PBS solution. The stock concentration of each compound varied between 10 and 100mM, details of which are described in the results chapters for each of the compounds evaluated.

2.6 Chemosensitivity studies under aerobic conditions

Upon completion of cell counting, the cells were inoculated into a 96 well plate at a cell density of 1.5×10^3 cells per well in a volume of 100µL complete media. Lane one had no cells but just contained media (100µL) as a blank. 96 well plate containing cells were placed at 37°C with 5% CO_2 in a humidified incubator for 24hours to allow cells to adhere to the base of the wells. The media was aspirated, a serial dilution of the drug was made and 200µL was added to each well. Where compounds were dissolved in DMSO, one lane was used as a vehicle control at a concentration of 0.1%v/v which is known not to be toxic to the cells. The layout of the plate is depicted in figure 2.1.

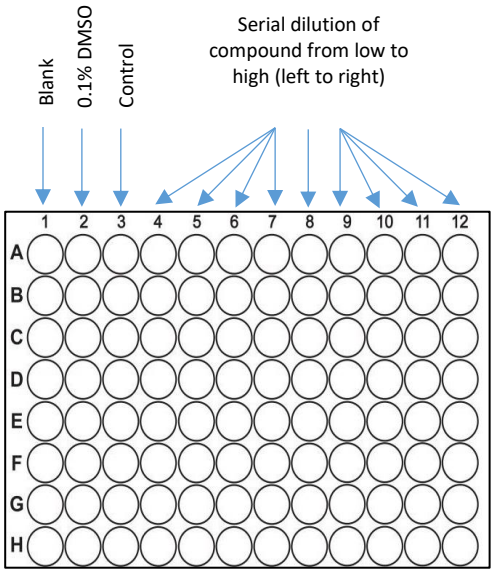


Figure 2.1 Representation of the layout of a standard 96 well plate for chemosensitivity studies.

Cells were exposed to the test compound for 96 hours before the addition of 20µL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5mg/mL) and incubated further for four hours at 37°C in a CO_2 enriched atmosphere (5%) in a humidified incubator**.** All media and MTT was removed and the formazan crystals formed were dissolved in 150µL of DMSO per well. 96 well plates were then inserted into a Tecan microplate photometer to measure absorbance which was set at 540nm. The true absorbance of the solution was obtained by subtracting the blank absorbance and % cell survival was determined as the true absorbance of treated lanes divided by the true absorbance of the control and expressed as a percentage. Dose response curves were plotted and each assay was performed in triplicate. IC_{50} values were determined directly from the dose response curve and the results were expressed as the mean ± standard deviation for three independent experiments.

2.7 The MTT assay – chemosensitivity studies under hypoxic conditions

This was carried out as described above with the exception that the 96 well plates were placed in a Whitley H35 Hypoxystation which is humidified at 37°C with 5% CO_2, and 0.1% oxygen tension. An oxygen tension of 0.1% oxygen was chosen to mimic severe hypoxia. Appropriate stocks of complete media were placed in the Hypoxystation 24h prior to carrying out any work to ensure the media was fully equilibrated to the hypoxic conditions. All subsequent steps of the procedure were carried out in hypoxic conditions up until the step where MTT was added which was carried out in a normal class II biological safety cabinet and then further subjected to a normal incubator at 37°C with 5% CO_2 in a humidified incubator for four hours before analysing using a Tecan microplate photometer to measure absorbance at 540nm**.** The data was analysed as described previously.

2.8 pH dependent chemosensitivity studies under aerobic and hypoxic conditions

The chemosensitivity study was carried out as mentioned in 2.6. When treating the cells with compound, the pH of the media was adjusted to pH 6.5 using 0.1M HCL in conjunction with a Jenway pH calibrator. For all hypoxia work, this was placed in the Hypoxystation 24h prior to carrying out any work to ensure the media is equilibrated to the hypoxic conditions. Serial dilution of drugs was performed in media at pH 7.4 or 6.5 and the remaining steps were conducted under hypoxia or normoxic conditions as described above (section 2.7).

2.9 Cell harvesting for Western Blot

Cells were seeded into T25 flasks at a cell density of 1 x10^6 cells and placed in either a humidified incubator at 37°C with 5% CO_2 or the Hypoxystation at 37°C with 5% CO_2, and 0.1% oxygen tension. Cells were exposed to various drug concentrations (as described in figure legends) and allowed to recover for 24 hours. Media was removed and cells were washed with 5mL of PBS solution and trypsinised as described above. All media and PBS including cells with trypsin were all placed in a universal tube and centrifuged at 1200rpm for 3 minutes. Media containing trypsin and PBS was discarded and the cell pellet was further washed with PBS twice to ensure all media and trypsin has been removed. To lyse the cells, 10µL of phosphatase inhibitor (Sigma) and 25µL of protease cocktail inhibitor (Sigma) was added to 965µL RIPA buffer (Sigma) and placed on ice until further use. 100µL of the RIPA buffer cocktail was added to the cell pellet and gently re-suspended to ensure all cells have been lysed before putting them into eppendorf tubes. Cell lysates were either frozen or used directly for protein quantification by the BCA assay. Samples were sonicated using a Ultrasonic cleaner (model: GT sonic G-2) for 30 seconds and then placed on ice for 2 minutes. This was repeated three times.

2.10 BCA assay

Protein concentrations in cell lysates were measured using the BCA assay (Thermofisher). A set of Bovine Serum Albumin standards was made ranging from 0.2 to 2mg/mL. 10µL of each of the standards was added to a 96 well plate in 4 wells with 10µL of the cell lysates to 4 separate wells. The BCA working solution was made up by adding Reagent A to B to a ratio of 50:1 respectively and 190 µL added to each well. The 96 well plate was left for 30minutes before the absorbance was

measured using a spectrophotometer at 562nm. The unknown concentrations of the cell lysates were calculated using the regression equation y=mx+c generated from the calibration curve. Any values falling outside of the linear range of the calibration curve were either diluted so that absorbance values fell within the linear range or were discarded if absorbance values were at or below the limit of detection of the assay.

2.11 SDS-PAGE Gel Electrophoresis

Laemmli buffer was prepared by adding 900µL 4 x laemmli buffer (Bio-Rad) to 100µL of β-mercaptoethanol (355mM). 40µg of protein lysate for each of the samples was diluted in laemmli buffer and the cell lysate was vortexed. Samples were heated to 95°C for 5 minutes and further centrifuged. SDS-PAGE resolving gels at 15% were prepared (5.4mL distilled autoclaved water, 5mL 1M Tris pH 6.8, 6ml 40% acrylamide (Bio-Rad), 3.2mL 2% bis-acrylamide (Bio-Rad), 200µL 10% SDS (InVitrogen), 20µL TEMED (Sigma) and 200µL of ammonium persulfate (APS) solution 0.2M) and poured in between two glass plates and allowed to set for 1 hour. Isopropanol was added to ensure a clear boundary formed at the surface of the resolving gel. Once the resolving gel had fully polymerised, the isopropanol was removed and a 4% stacking gel (13.5mL distilled autoclaved water, 2.9mL 1M Tris pH 6.8, 2ml 40% acrylamide, 1.2mL 2% bis-acrylamide, 240µL 10% SDS, 20µL TEMED and 240µL of APS solution) was poured onto the resolving gel and a 1.5mm comb was inserted into each gel. This was further left for an additional 30 minutes to allow the gel to set. The combs were removed and the glass plates were transferred to a Bio-Rad gel electrophoresis tank. Running buffer (Tris Base, Glycine and water) was poured into the tank and protein ladder (Thermofisher Scientific) along with

samples were added into each well in a logical order. Samples were run at 70V for one hour (until samples had crossed the stacking gel and entered the resolving gel) and then at 120V until the tracking dye (bromophenol blue) reached the bottom of the gel.

2.12 Western Blot

The stacking gel was removed from the resolving gel and the latter was placed carefully onto a nitrocellulose transfer membrane (Whatman) in running buffer (10x transfer buffer, methanol, 10% SDS and water). 2 filter papers were placed on both outer parts of the membrane and 2 further fibre pads were placed on top of this on both sides. Finally this was all placed into a ‘sandwich cassette’ which was inserted into a Bio-Rad transfer tank. Electrophoretic transfer was performed at 35mA overnight. Upon completion of transfer, the nitrocellulose membrane was separated from the gel and left on tissue to allow the membrane to dry. The membrane was washed with PBST (0.1% Tween and PBS) for 5 minutes and then blocked using 5% Marvel in PBST or 5% BSA for 1 hour on a shaker set to 50rpm. The membrane was probed using primary antibodies (see table 2.2) in blocking buffer and incubated overnight at 4°C on a shaker. Membrane was washed using PBST 3 times for 5 minutes before adding the secondary (HRP conjugated) antibody (table 2.2) was added in blocking buffer for 1 hour. The membrane was washed further using PBST 3 times for 5 minutes. Chemiluminescent (Roche) reagents were prepared by adding reagent a to reagent b at a 1:100 ratio and added to the membrane and kept in the dark for 1 minute. The membrane was dried and put onto a Hyper-cassette and taken to the dark room immediately for exposure using a hyperfilm ECL (GE Healthcare). The duration of film exposure was dependent on the protein of interest.

Films were developed and fixed using the instant developer and fixer (Ilford multigrade developer and Ilford Rapid fixer, Harman technology ltd).

Primary antibody (species)	Dilution primary antibody	Primary Antibody Supplier
ϒH2AX (rabbit)	1:1000	Abcam (ab11174)
p53 (rabbit)	1:10000	Abcam (ab131442)
Src (rabbit)	1:1000	Cell signalling (# 2108S)
pSrc 527 (rabbit)	1:1000	Cell signalling (# 2105S)
β-actin (mouse)	1:25000	Millipore (MAB1501)

Table 2.2 Primary antibodies used in this PhD with species, dilution and supplier. Antibodies ϒH2AX, Src and pSrc 527 were prepared in 5% bovine serum albumin (BSA) PBST whereas p53 and β-actin were prepared in 5% marvel PBST.

Secondary antibody (species/ isotype)	Dilution secondary antibody	Secondary Antibody Supplier
Rabbit/ IgG	1:10000	Rockland (611-145-122)
Mouse/ IgG	1:10000	Life Technologies (A21057)

Table 2.3 Secondary antibodies used in this PhD with species, dilution and supplier.

2.13 Immunodetection using the LiCor Odyssey

The alternative way to read the membranes was by using the LiCor Odyssey imaging system. Prior to reading the membrane the complimentary secondary anti-

body was added and incubated for 1 hour. Upon completion the membrane was washed with PBST for 5 minutes before being transferred to the membrane reader. A full digital scan of the membrane was taken using the LiCor Odyssey infra-red imaging system which was visualised at either 680 or 800nm.

2.14 Statistical analysis

Statistical analysis was carried out using Graph Prism or Microsoft Excel (data analysis). All experiments were done in triplicate (n=3), and results were presented as the mean ± SD. Statistical significance was determined by means of a two-tailed Student's t-test for comparing two mean samples or a one-way ANOVA to compare mean samples of groups, where significance was only accepted when $p \leq 0.05$.

Chapter 3: Performance of selected established anti-cancer drugs and tirapazamine in the phenotypic screen

3.1 Introduction

As discussed in the introduction, this thesis will determine whether or not the use of a phenotypic screen will identify novel compounds that are preferentially activated against cells that reside within the tumour microenvironment. In order to provide a 'yardstick' against which novel compounds can be measured, the performance of selected established anti-cancer drugs and a classical HAP in this screen is required. The work described in this chapter focuses on cisplatin, 5-fluorouracil (5-FU) and etoposide which are well known and established classic anti-cancer agents that are approved for use against various cancers in humans (Hande, 1998; Longley et al., 2003; Wiltshaw, 1979). These three agents all have different mechanism of actions and have therefore been selected for this study to provide a 'baseline standard' for their cytotoxicity under normoxic, hypoxic and mild acidic pHe. In addition, tirapazamine has also been selected as a representative example of a classical HAP that is designed to work better under hypoxic conditions. Brief details of their mechanisms of action are as follows;

Cisplatin – This is an alkylating agent capable of inducing intra-strand and interstrand (albeit less frequent) cross links in DNA. Once inside the cell, the Cl groups become hydrated and this leads to nucleophilic attack at the N7 position of guanine in DNA. The subsequent damage to DNA results in apoptosis (Dasari and Tchounwou, 2014).

Etoposide – The mechanism of action involves the inhibition of topoisomerase II enzymes that play an important role in DNA replication. Etoposide is known to affect mainly the S and G2 phases of the cell cycle (Baldwin and Osheroff, 2005).

5-FU – This is a thymidylate synthase inhibitor which can enter the cells by either active or facilitated diffusion. Metabolic activation of 5-FU involves conversion into three different active metabolites which are, fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP). It is FdUMP which inhibits thymidylate synthase which depletes one of the nucleotides thymidine triphosphate (TTP) required for DNA synthesis. This effectively prevents DNA synthesis and ultimately results in cell death (Longley et al., 2003).

Tirapazamine – This compound belongs to the class of drugs known as bioreductive drugs or HAPs and has been included in the panel of compounds as a positive control. Its mechanism of action involved enzymatic reduction by oxidoreductases leading to the generation of free radical species. Preferential activity against hypoxic cells has been documented in the literature and this has been described earlier in the introduction to this thesis (see section 1.23). Tirapazamine has the ability to target DNA producing a single or double stranded break leading to apoptosis (Brown, 1993). As this is a phenotypic screening program, the rationale for selecting cell lines to use was not based on biochemical characteristics but on pragmatic reasons including ease and reliability of growth under aerobic and hypoxic conditions. In addition, previous studies from out group have used the HCT116 $P53^{+/+}$ colorectal cancer cells for phenotypic screening studies and therefore to maintain consistency, this cell line was used. The ARPE-19 non-cancer cells was also selected for use in this chapter for the same reasons as outlined above.

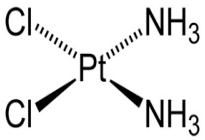
3.2 Aims and objectives

The aim of this study is to determine the activity of standard agents and a classic HAP against cancer cells under experimental conditions that mimic the hypoxic and acidic pHe conditions typically found within the physiological tumour microenvironment. This will provide a baseline against which the relative merits of novel test compounds can be compared with existing agents, the ultimate aim of which is to assess the validity of this approach for identifying novel compounds with activity against the TME. In addition, the ability of compounds to selectively target cancer cells will be determined by the inclusion of a non-cancer cell line (ARPE-19) in the study.

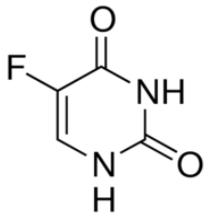
3.3 Materials and Methods

3.3.1 Drugs used in this chapter

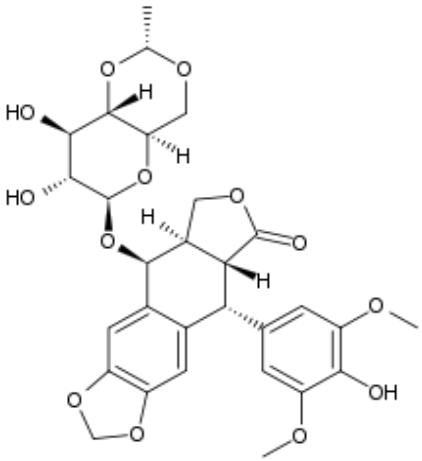
Anti-cancer agents were purchased from Sigma Aldrich and their chemical structures and molecular weights are presented in figure 3.1. These were prepared in dimethylsulphoxide (DMSO) with the exception of cisplatin which was dissolved in phosphate buffered saline (PBS) at 100mM, aliquoted (20µL) and stored at -20°C.



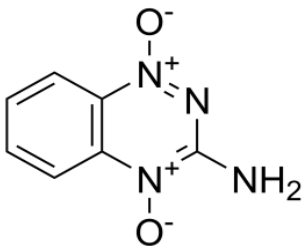
Cisplatin (RMM = 300.01)



5-Fluorouracil (RMM = 130.07)



Etoposide (RMM = 588.8)



Tirapazamine (RMM = 178.4)

Figure 3.1 Chemical structures and molecular weights of the drugs used in this chapter.

3.3.2 Validation of the MTT assay

In order for the MTT assay to provide a valid assessment of cell number, there needs to be a linear relationship between cell number and the absorbance of formazan. HCT116 $p53^{+/+}$ cells were inoculated into a 96 well plate ranging from 600-

12000 cells per well and treated with 20μL of MTT (5 mg/mL) immediately after plating. Lane 1 of the plate contained media only to serve as a blank for the spectrophotometer readings. The plate was incubated at 37°C and 5% CO_2 for 4 hours. Upon completion, media was carefully removed and the formazan crystals formed were dissolved in 150μL of dimethyl sulfoxide (DMSO) and the resulting solution mixed. The absorbance of the solution was measured at 540nm using a Tecan plate reader. The absorbance of the blank was subtracted from the absorbance of other lanes in order to obtain the true absorbance values and the results were graphically presented as mean absorbance ± standard deviation vs cell number (in each experiment, there were 8 replicates per cell number).

3.3.3 Cell culture and chemosensitivity

Cell culture and chemosensitivity studies under normoxic and hypoxic conditions were carried out as previously described in the general methods section (section 2.2, 2.6 and 2.7). Cell lines HCT116 $P53^{+/+}$ and HCT116 $P53^{-/-}$ were used in this chapter. The media was adjusted to the relevant pH and equilibrated to the correct oxygen concentration as described in the methods (section 2.8) 24 hours prior to any experiment. A series of parameters used to measure the effects of various conditions on efficacy were derived as follows:

HCR: This is the hypoxic cytotoxicity ratio defined as the ratio of IC_{50} values in normoxia divided by the IC_{50} value under hypoxia. Values greater than 1 indicate that the test compound has greater activity under hypoxic conditions.

pHR: This is the pH cytotoxicity ratio defined as the ratio of IC_{50} values under normal pHe conditions (pHe 7.4) divided by the IC_{50} value under acidic pHe (pHe 6.5)

conditions. Values greater than 1 indicate that the test compound has greater activity under acidic pHe conditions.

TME ratio: This is the tumour microenvironment ratio defined as the ratio of IC_{50} values obtained in normoxia (pHe 7.4) divided by the IC_{50} value under hypoxia (pHe 6.5). Values greater than 1 indicate that the test compound has greater activity under TME conditions.

Selectivity index (SI): This is defined as the IC_{50} value for the non-cancer ARPE-19 cell line (under normoxia, pHe 7.4) divided by the IC_{50} for cancer cells (under various experimental conditions of oxygen tension and pHe). Values greater than 1 indicate that the test compound has greater activity against cancer cells than non-cancer cells *in vitro*.

3.3.4 Statistical analysis

Statistical analysis was carried out using Microsoft Excel and Graph Prism. All experiments were done in triplicate (n=3), and results were presented as the mean ± SD. Statistical significance was determined by means of a two-tailed Student's t-test, or by One Way ANOVA where significance was only accepted when $p \leq 0.05$. The exact statistical analysis performed is mentioned in the figure legend.

3.4 Results

3.4.1 Validation of HCT116 for p53

HCT116 p53$^{+/+}$ is the main cell line used in this study. To confirm that this cell line expressed p53 and the HCT116 p53$^{-/-}$ did not, a western blot was carried out as described above. Instead of chemiluminescent detection using ECL reagents, the detection of p53 was performed using LiCor methodology (method section 2.13).

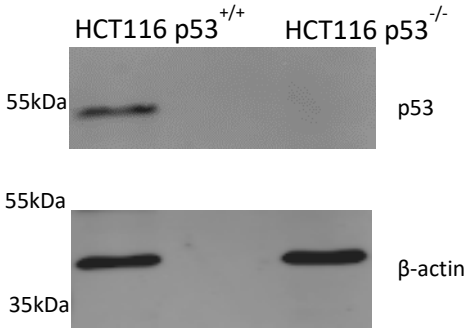


Figure 3.2 Western blot analysis of p53 on HCT116 p53$^{+/+}$ and HCT116 p53$^{-/-}$ cancer cells.

3.4.2 Validation of the MTT assay

The relationship between the number of cells and the absorbance of the solution obtained from the MTT assay is presented in figure 3.3. This relationship is linear demonstrating that the absorbance is directly related to the number of viable cells in the culture.

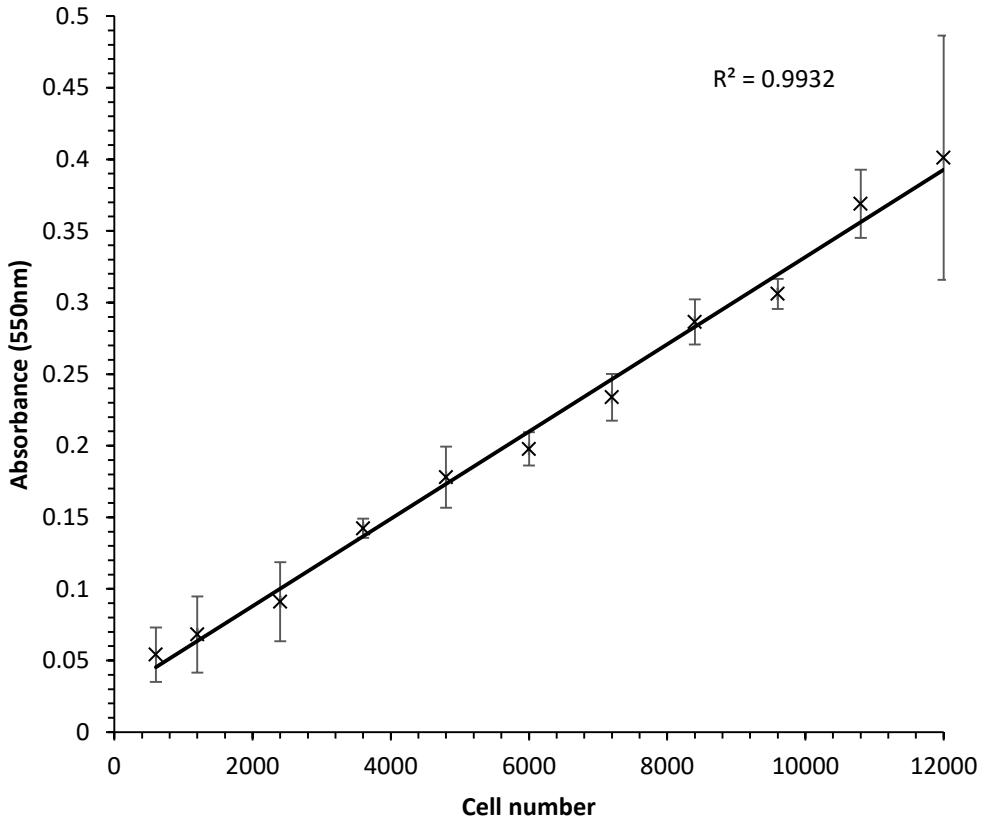


Figure 3.3 Validation of the MTT assay carried out on HCT116 p53$^{+/+}$ cells (n=3). HCT116 p53$^{+/+}$ cells were inoculated into a 96 well plate at different cell densities and treated with MTT straight away under normoxic and pHe 7.4. Each result represents the mean ± standard deviation of three independent experiments.

3.4.3 The effect of tirapazamine against the HCT116 p53$^{+/+}$ cell line under normoxic and hypoxic conditions

Tirapazamine, a well-established HAP was tested under both normoxic and hypoxic conditions (figure 3.4A). The IC_{50} values were 11.85 ± 0.63 µM and 1.24 ± 0.39 µM respectively (figure 3.4B) giving a HCR of 9.55. These results demonstrate that the hypoxic conditions used in this study are appropriate to identify HAPs.

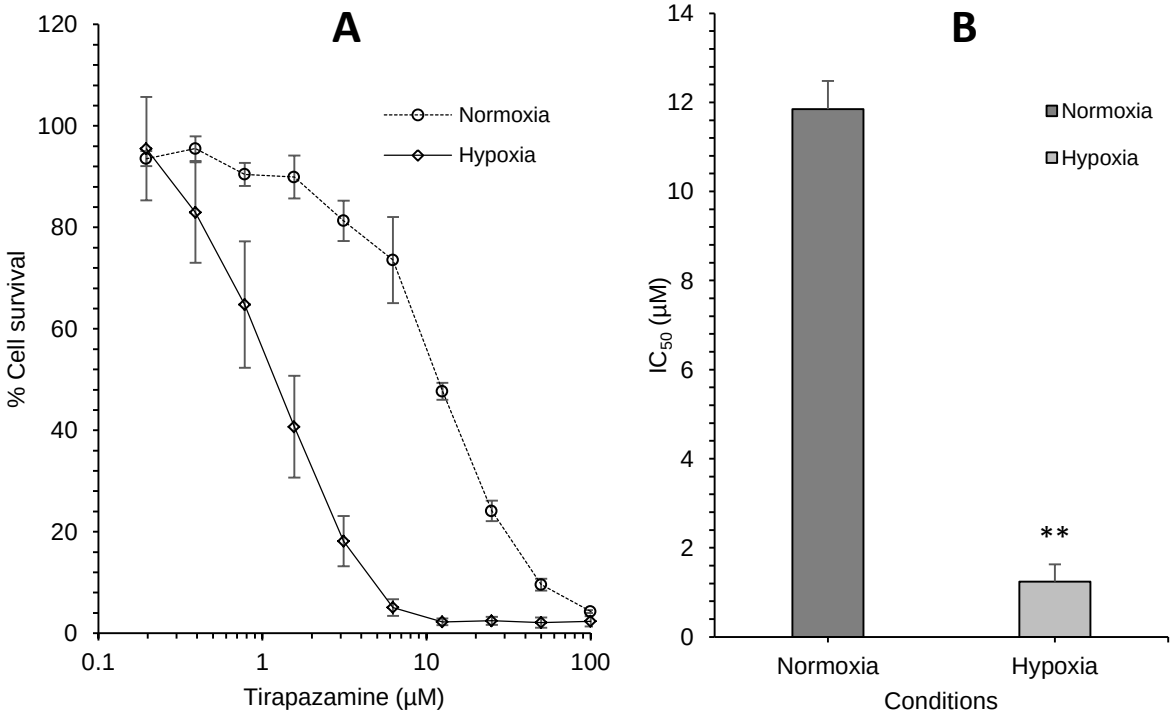


Figure 3.4 HCT116 p53$^{+/+}$ cells were drug treated for 96 hours with tirapazamine under hypoxic and normoxic conditions (pHe 7.4). Panel A: Dose response curves to tirapazamine under aerobic and hypoxic conditions. Panel B: IC_{50} value of tirapazamine under both normoxic and hypoxic conditions. Each result represents the mean ± standard deviation of three independent experiments. A students t-test was carried out to determine any significance when comparing hypoxia to normoxic conditions. ** represents a p value of < 0.01.

3.4.4 The effect of cisplatin, 5-FU and etoposide against the HCT116 $p53^{+/+}$ cell line under TME conditions

Dose response curves representing the response of HCT116 $p53^{+/+}$ under normoxic conditions (pHe 7.4) and under hypoxic (pHe 6.5) (TME conditions) are presented in figure 3.5. The response (IC_{50} values) obtained for all the conditions tested are presented graphically in figure 3.6 and numerically in table 3.1.

The results presented in figure 3.6 and table 3.1 demonstrate that the response of HCT116 $p53^{+/+}$ cells to standard cytotoxic drugs is adversely affected by TME conditions. In the case of cisplatin, the IC_{50} under normoxic conditions (pHe 7.4) was 2.03 (± 0.08) µM and this increased to 6.75 (± 1.33) µM under hypoxic (pHe 6.5) conditions. Similarly, the IC_{50} for 5-FU under normoxic conditions (pHe 7.4) was 1.01 (±0.06) µM and this increased to 3.73 (± 0.61) µM under hypoxic (pHe 6.5) conditions (table 3.1). Finally for etoposide, IC_{50} values increased from 0.52 (± 0.05) µM under normoxic conditions (pHe 7.4) to 0.91 (± 0.26) under hypoxic (pHe 6.5) conditions. The results for tirapazamine are also presented in table 3.1 and as mentioned previously, the activity of tirapazamine is significantly enhanced under hypoxic conditions (please note that the response of cells to tirapazamine under full TME conditions was not conducted).

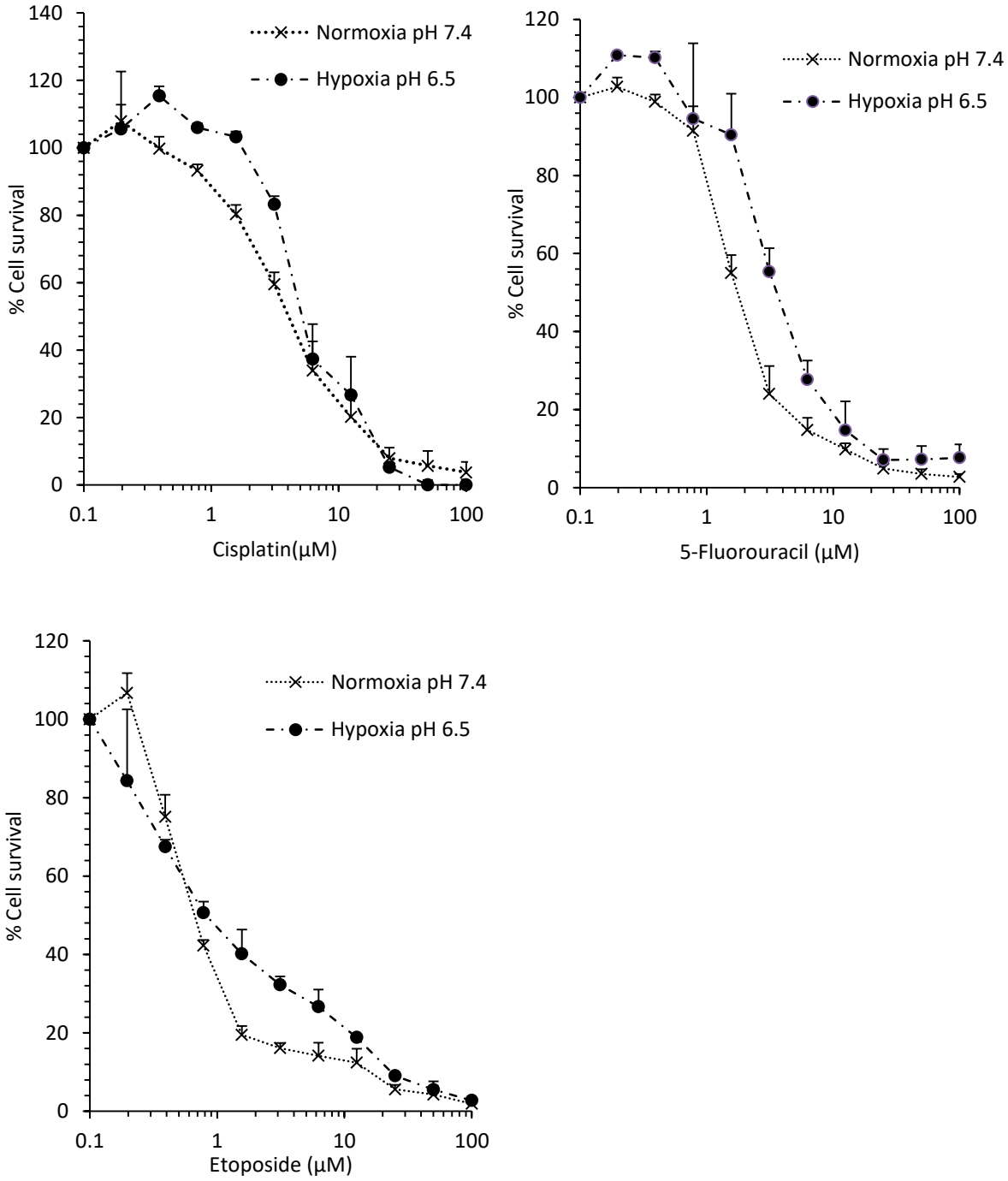


Figure 3.5 HCT116 p53$^{+/+}$ cells were drug treated for 96 hours with classic cytotoxic compounds under hypoxic (pHe 6.5) and normoxic conditions (pHe 7.4). Each result represents the mean ± standard deviation of three independent experiments.

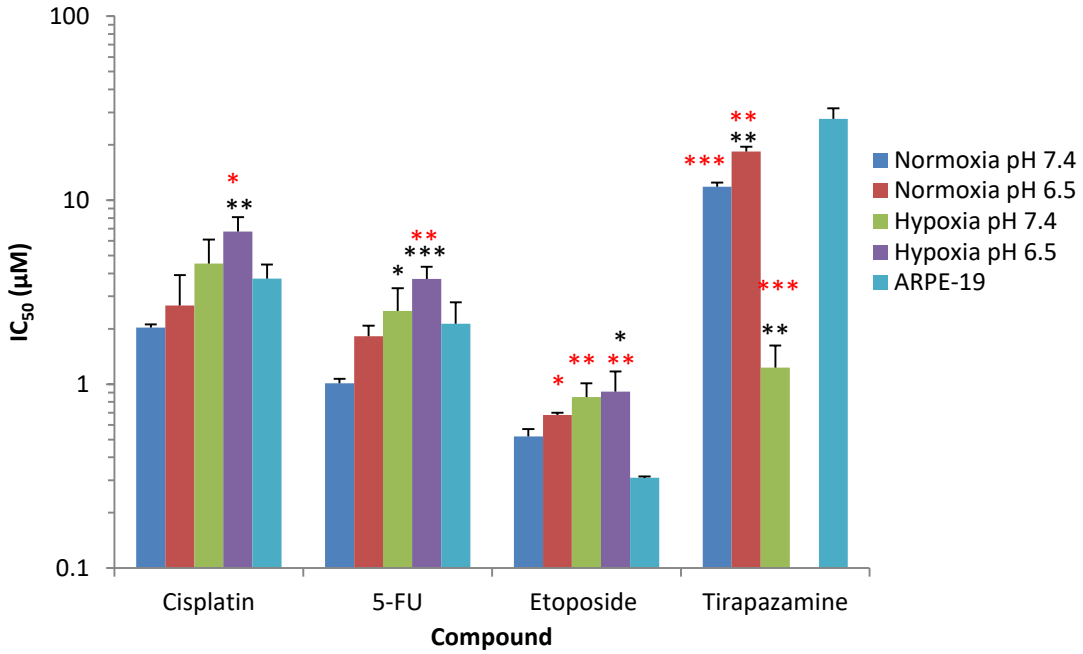


Figure 3.6 The response (IC_{50}) of HCT116 $p53^{+/+}$ and ARPE-19 cells to standard cytotoxic drugs and tirapazamine. Cells were exposed to test compounds for 96 hours using drug concentrations ranging from 0.3 to 100µM. Values represent the mean and standard deviation of three independent experiments (n=3). Statistical significance was worked out using the one-way ANOVA test to make multiple comparisons. The black asterisks show comparisons of different conditions to the normoxia pH 7.4 control, whilst the red asterisks show comparisons of different condition to ARPE-19. P-values are * ≤0.05, ** ≤0.001 and *** ≤0.0001.

Compound	IC_{50} (μM)				
	Normoxia pHe 7.4	Normoxia pHe 6.5	Hypoxia pHe 7.4	Hypoxia pHe 6.5	Normoxia pHe 7.4
	HCT116 $p53^{+/+}$ cells				ARPE-19
Cisplatin	2.03 (± 0.08)	2.68 (± 1.24)	4.52 (± 1.59)	6.75 (± 1.33)	3.75 (± 0.72)
5-FU	1.01 (±0.06)	1.82 (± 0.26)	2.5 (± 0.82)	3.73 (± 0.61)	2.13 (± 0.66)
Etoposide	0.52 (± 0.05)	0.68 (± 0.02)	0.85 (± 0.16)	0.91 (± 0.26)	0.31 (± 0.005)
Tirapazamine	11.85 (± 0.63)	18.41 (± 1.09)	1.23 (± 0.39)	n/a	27.63 (± 4.00)

Table 3.1 The response of HCT116 $p53^{+/+}$ and ARPE-19 cells to standard cytotoxic drugs and tirapazamine under various experimental conditions. All cells were exposed to test compounds for 96 hours over a concentration range from 0.3-100μM under normal and TME conditions. Each result represents the mean ± standard deviation of three independent experiments. N/a refers to no data available.

3.4.5 Analysis of the effect of the TME on cellular response to standard cytotoxic drugs and tirapazamine

Using the data presented in table 3.1, a series of parameters were derived to illustrate the effect that individual changes to experimental conditions had on the activity of the drugs tested. These results are presented in table 3.2. In the case of all compounds tested including tirapazamine, reducing the pHe to 6.5 reduced the efficacy of compounds tested under normoxic conditions with pHR values ranging from 0.55 to 0.76. In the case of cisplatin and 5-FU (and to a lesser extent etoposide), hypoxia (0.1% oxygen) has a more profound effect than pHe on cellular response with HCR values of 0.44 and 0.40 respectively. When both hypoxia and

acidic pHe conditions are combined, the response of cells is reduced further (TME values ranged from 0.27 to 0.57, table 3.2). When compared to the response of non-cancer ARPE-19 cells, the TME causes a clear and significant decrease in the selectivity ratios for cisplatin, 5-FU and to a lesser extent etoposide (table 3.2)

Compound	pHR	HCR	TME ratio	SI	SI (TME)
Cisplatin	0.75	0.44	0.3	1.84	0.55
5-FU	0.55	0.40	0.27	2.10	0.57
Etoposide	0.76	0.61	0.57	0.59	0.34
Tirapazamine	0.61	9.55	n/a	2.33	22.28*

Table 3.2 The effect of the TME on the response of cells to standard cytotoxic drugs and tirapazamine. pHR, HCR and TME represent the pH ratio, the hypoxic cytotoxicity ratio and tumour microenvironment ratio as defined in the methods section 3.3.3. * refers to the SI value for the hypoxic data only and not the combo of hypoxia and acidic pHe.

3.5 Discussion

The study was designed to test a novel phenotypic screening strategy utilising the physiological parameters of the TME which are normally not present *in vitro*. The screen was deployed on the classic chemotherapeutic compounds cisplatin, 5-FU and etoposide with the aim of generating some parameters that could be used as a 'yardstick' to measure the relative merits of the novel agents evaluated in this thesis.

Initially, two validation experiments were conducted. The first was to demonstrate that HCT116 cells either possessed p53 or not. The results of western blot analysis (figure 3.2) demonstrate that p53 is present in HCT116 $p53^{+/+}$ cells and absent from HCT116 $p53^{-/-}$ cells. The second was to validate the MTT assay. This validation assay was carried out to establish the relationship between cell number and absorbance. Figure 3.3 displays a linear relationship with an R^2 value of 0.9932 and demonstrates that the MTT assay is a reliable method for conducting chemosensitivity studies. This finding is similar to (Ahmadi et al., 2014) study where a linear relationship was also observed under both normoxic and hypoxic conditions which verifies the suitability of this method under both working conditions.

Before conducting experiments on the established drugs, initial studies were conducted on the HAP tirapazamine to ensure that the hypoxic conditions used in the H35 hypoxystation were suitable for subsequent studies. The results presented in figure 3.4 and table 3.1 and 3.2 clearly demonstrate that tirapazamine is preferentially cytotoxic under hypoxic conditions compared to aerobic conditions with IC_{50} values of 1.23µM (±0.39) and 11.85µM (±0.63) respectively. The HCR value of 9.55 is comparable to previously published values (Ahmadi et al., 2014) and therefore the conditions used in the hypoxystation are satisfactory. This was also

statistically proven by ways of a student's t-test where $p<0.01$. The results demonstrate that the hypoxic chamber was functioning correctly and the chemosensitivity testing continued under hypoxic conditions for the remainder of this PhD.

The results also demonstrates good cytotoxicity of the classic chemotherapy compounds against the HCT116 $p53^{+/+}$ cell lines under normoxic pHe 7.4 conditions. When the same compounds are subjected to hypoxia (pHe 7.4) there was notable resistance with HCR values all below 1 (table 3.2) reflecting an underlying problem of current anti-cancer chemotherapeutic compounds, namely ineffective efficacy against hypoxic cells. Furthermore, the increased IC_{-50} values under hypoxia reduce the selectivity index when the response of ARPE-19 cells are taken into account (Ahmadi et al., 2014; Nichol et al., 2019). The resistance of cisplatin to hypoxia displayed here is consistent with that of Song et al., 2006. They also demonstrate that manipulation of HIF in non-small cell lung cancer (NSCLC) cells by a lentiviral vector-mediated HIF-1α RNA interference (RNAi) strategy reverses the resistance exhibited by hypoxia (Song et al., 2006).

HCT116 $p53^{+/+}$ cells treated with classic chemotherapeutic compounds under pHe 6.5 under normoxic conditions also displayed resistance with a pHR ratio all below 1. The data expressed here is similar to the findings of Wachsberger et al. (1997) where Chinese hamster ovary (CHO) cells that were adapted in growth medium at pH 6.7 displayed resistance to cisplatin when compared to pH 7.3 (Wachsberger et al., 1997). The highest difference observed in treating HCT116 $p53^{+/+}$ cells with the chemotherapeutic compounds was in the combination of hypoxia and the pHe of 6.5. The TME ratio for all three classic chemotherapeutic compounds was below 1 and this is indicative that none of these compounds showcased any preferential activity

under the physiological TME conditions. This was statistically proven by the one way ANOVA parametric test between the classic compounds tested under normoxia pHe 7.4 vs hypoxia and pHe 6.5 combined (figure 3.4).

The classic chemotherapeutic compounds were also screened against the ARPE-19 cells where cisplatin and 5-FU (SI 1.84 and 2.10 respectively) displayed some levels of selectivity towards the HCT116 $P53^{+/+}$ cells. This was not the case for etoposide which was below 1. Under hypoxic or acidic pHe conditions, the SI values decrease even further and under TME conditions (combined hypoxia and acidic pHe), tumour cells are substantially more resistant than non-cancer ARPE-19 cells (SI values ranging from 0.34 to 0.57, table 3.2). In contrast, tirapazamine under hypoxic conditions has a substantially enhanced SI value of 22.46 which is consistent with the compound's mechanism of action.

Whilst the mechanistic reasons for the reduction in cellular response to cytotoxic drugs under hypoxia, acidic pHe or combinations of hypoxia and acidic pHe have not been explored in this thesis, the results provide a valuable baseline or yardstick for future studies. The results demonstrate that both hypoxia and acidic pHe can reduce the efficacy of the selected cytotoxic drugs and in the context of a phenotypic screening approach to drug discovery, these results are interesting. When combined with the results for tirapazamine, this approach has the potential to identify compounds that will (i) suffer a similar fate to the established anti-cancer drugs tested here in terms of resistance under TME conditions and (ii) be preferentially active under hypoxic, acidic pHe or combined TME conditions. In conclusion therefore, this chapter demonstrates that the phenotypic approach to drug discovery described here could identify compounds with preferential activity against cells that reside within the TME. Subsequent chapters of this thesis will explore this in detail

using a diverse range of novel compounds. The results presented in table 3.2 will be used in each of the subsequent chapters as the yardstick to judge the relative merits of each compound tested, the ultimate aim of which is to select promising lead compounds that have preferential activity against cancer cells in the TME.

Chapter 4: Bromo-Hexitols as a novel class of hypoxia-activated prodrugs for cancer therapy

4.1 Introduction

The basic design for a HAP is the 'trigger-linker-warhead' structure as described by Denny *et al* (Denny et al., 1996). There are many 'warheads' available which are capable of causing cell death but the general issue with these is lack of selectivity. One example of this is a class of compounds known as bromo-hexitols which are potent cytotoxins. The well-known bromo carbohydrate compounds which have been around for 40 years are dibromomannitol (DBM), dibromogalacticol (DBG) and threosulfane (figure 4.1). These compounds are designed to be cytotoxic under normal physiological conditions and are activated by the reduction of bromine *in vivo*. This releases the active DNA-alkylating agent bis-epoxide which are marked by the red circles (as shown in figure 4.2) and cross-link with DNA resulting in apoptosis.

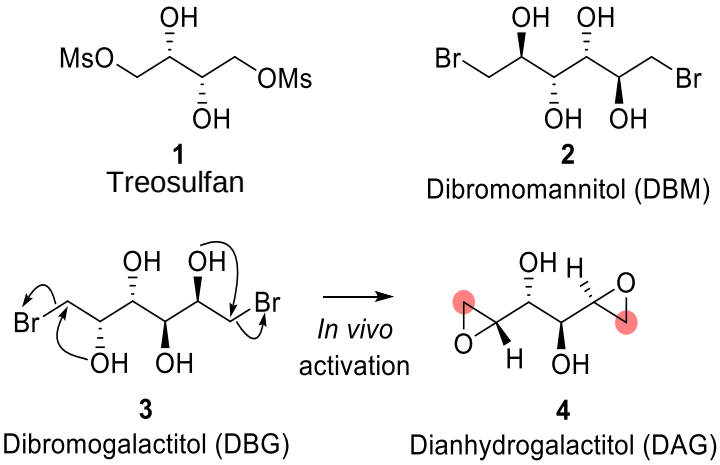


Figure 4.1 DNA-alkylating chemotherapeutics, treosulfan, DBM and DBG. Dianhydrogalactitol (DAG) is the DNA alkylating agent formed by the *in vivo* activation from DBG. The active sites are highlighted in red.

Irrespective of the small structural differences between DBM and DBG the cytotoxic activity of these are considerably different (Chiuten et al., 1981). These were not developed further because of toxicity to normal tissues (Chiuten et al., 1981). The most promising set of compounds were the bromo-hexitols and despite their cytotoxic properties, these compounds have been largely ignored in the past decade. There have been no attempts at exploring the correlation between structural activity of bromohexitols or in developing prodrugs which may be selective towards the hypoxic fraction of the tumour microenvironment.

The Pederson group (University of Copenhagen) have synthesised a total of 13 compounds (as shown in methods section 3.2.1) and have used a starting material known as endoperoxides which makes the synthesising process easier (Johansson et al., 2019). The endoperoxide is a closed ring carbohydrate structure consisting of bromine and due to the susceptibility of the endoperoxide linkage (O-O). It is hypothesised that this maybe cleaved under hypoxic conditions by way of one or two electron reduction which releases an activated form of the prodrug di-bromohexitol which is later converted into the DNA-alkylating chemotherapeutic agent as shown in figure 4.2.

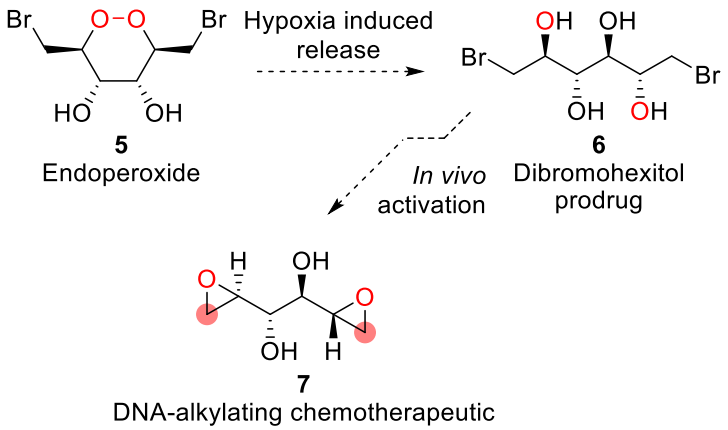


Figure 4.2 Schematic diagram indicating the hypoxic release of dibromohexitol from the endoperoxide. This is further activated to the active DNA-alkylating chemotherapeutic agent. The active sites are highlighted in red.

The synthesised compounds by Pedersons team are divided into two groups. The first group are all endoperoxides – DS1, DS2, DS3, DS4, DS5, DS6, DS9 and DS10 and these set of compounds are hypothesised to become activated by hypoxia and converted into the di-bromohexitol prodrug. The second group – DS11, DS12, DS13, DS14 and DS15 (see methods section 4.3.1) have been synthesised as the di-bromohexitols and therefore should not be activated by hypoxic conditions. As this is a phenotypic screening program, the rationale for selecting cell lines to use was not based on biochemical characteristics but on pragmatic reasons including ease and reliability of growth under aerobic and hypoxic conditions. In addition, previous studies from our group have used the HCT116p53+/+ colorectal cancer cells for phenotypic screening studies and therefore to maintain consistency, this cell line was used. The ARPE-19 non-cancer cells was also selected for use in this chapter for the same reasons as outlined above.

4.2. Aims and objectives

The aim of this study is to evaluate whether or not a series of bromo-hexitol derivatives are preferentially active under (i) hypoxia (0.1% oxygen), (ii) acidic extracellular pH (pHe 6.5) or (iii) a combination of hypoxia and acidic pHe. These studies were conducted on HCT116 $p53^{+/+}$ (human colorectal cancer cells) cells with the aim of identifying 'hit compounds'. To determine selectivity, these compounds were also evaluated against the non-cancer ARPE-19 cell line under conditions that reflect the 'normal conditions' found in healthy tissue (i.e. oxygenated and pHe 7.4). Following the identification of 'hit compounds', western blot analysis was conducted to determine if biomarkers of DNA damage induction (phosphorylated γH2AX and p53) are induced under different experimental conditions.

4.3 Material and methods

4.3.1 Compounds

All bromosugar HAPs were kindly provided by Professor Daniel Sejer Pederson (University of Copenhagen). These were prepared in dimethylsulphoxide (DMSO) at 100mM (figure 4.3) and aliquots of 20µL were stored at -20°C.

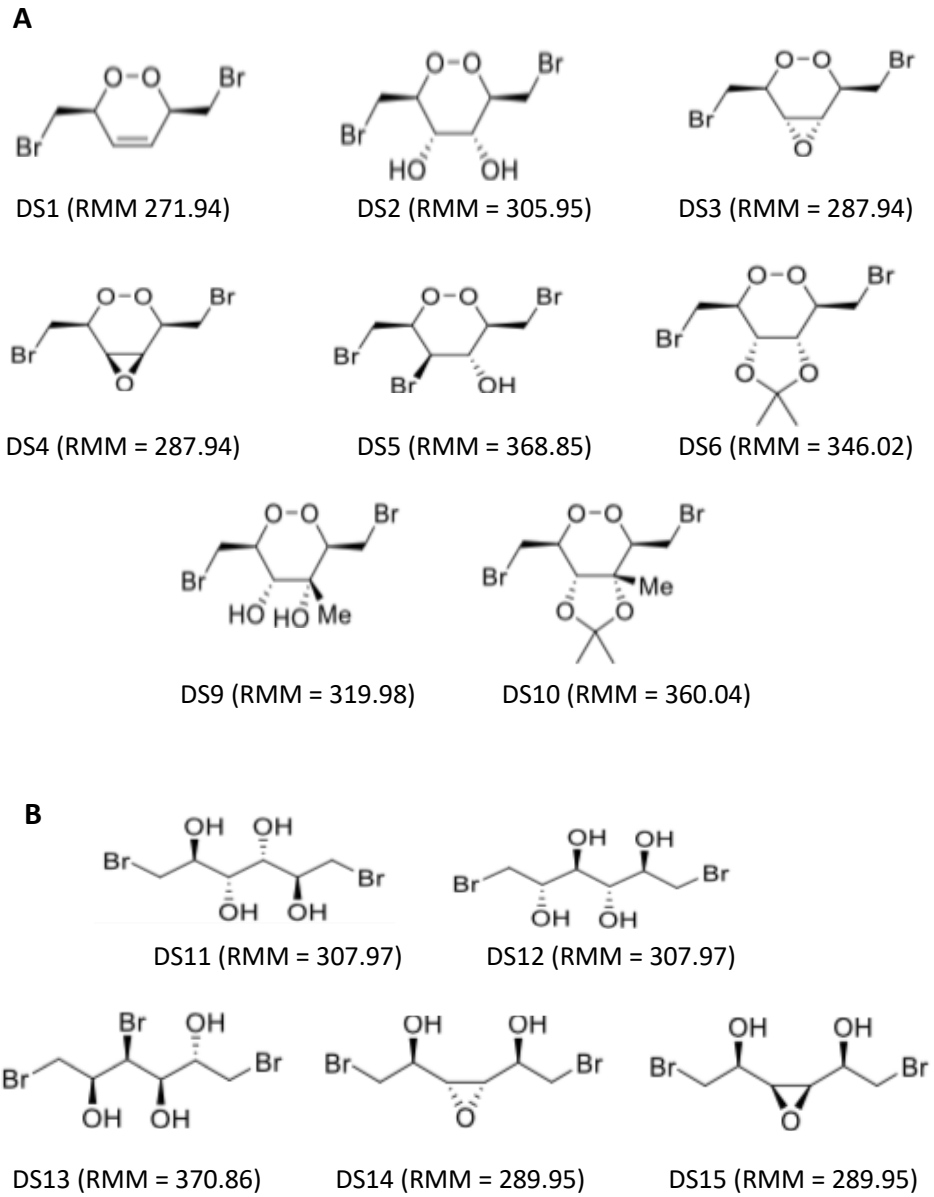


Figure 4.3 Chemical structure of bromosugar based compounds and molecular weight. Panel A: Endoperoxides and panel B: Di-bromohexitols

4.3.2 Cell culture and chemosensitivity

Cell culture, adjusting the pH of the media, drug dilutions and chemosensitivity studies were carried out as previously described in the general methods section (section 2.6-2.8). The HCT116 $P53^{+/+}$ cell line was used in this chapter. Any variation from these methods are indicated in the figure legends and described in the results section.

4.3.3 Western blot analysis

Cells were treated with hit compound for a duration of 24 hours at a drug concentration of 20μM (this concentration was chosen as it is a therapeutically relevant concentration (IC_{50} under hypoxic conditions was 25.93 ± 3.17 μM) and a 24 hour duration of drug exposure was selected as minimal cell death had occurred at this time point (as illiustrated in figure 4.6). Controls were treated with media alone or with vehicle control (DMSO 0.1% which has been previously reported not to be toxic to the cells) under the following conditions: (i) normoxia pHe 7.4, (ii) normoxia pHe 6.5, (iii) hypoxia pHe 7.4 and (iv) hypoxia pHe 6.4. Upon completion of treatment, cells were harvested for western blot analysis for DNA damage induction (phosphorylated γH2AX or p53 dependency). Details of western blot methodology and antibody dilutions are described in methods (section 2.9-2.13 and table 2.2).

4.3.4 Statistical analysis

Statistical analysis was carried out using Microsoft Excel and Graph Prism. All experiments were done in triplicate (n=3), and results were presented as the mean ± SD. Statistical significance was determined by means of a two-tailed Student's t-test, or by One Way ANOVA where significance was only accepted when $p \leq 0.05$. The exact statistical analysis performed is mentioned in the figure legend.

4.4 Results

4.4.1 Chemosensitivity studies

The response of HCT116 $p53^{+/+}$ and ARPE-19 cells following continuous exposure to test compounds under normoxic and hypoxic (pHe 7.4) are presented in table 4.1. Responses ranged from inactive at the highest dose tested (IC_{50} > 100 µM; DS6 and DS12) to active with the most potent compound (DS3) having an IC_{50} against HCT116 $p53^{+/+}$ cells of 4.50 ± 0.58 µM. The hypoxic cytotoxicity ratio (HCR) and selectivity index (SI) values are presented in table 4.2.

Of these compounds, only DS10 and surprisingly DS15 demonstrated statistically significant greater activity under hypoxic compared to normoxic conditions (HCR = 2.56 and 6.83 respectively (table 4.2)). Whilst the HCR was less than that of tirapazamine (HCR = 9.55), DS10 was the only endoperoxide compound to exhibit HAP like properties *in vitro* and was therefore the only compound subjected for further testing. It is not known why DS15 showed preferential activity under hypoxic conditions as it is in its inactive linear form under both conditions. Further studies are required to explore the mechanism(s) underpinning the hypoxia selectivity of DS15 but these were not possible due to a shortage of compound supply. In the context of selectivity for cancer as opposed to non-cancer cells, DS1, DS3, DS4 and DS14 showed a >2 fold selectivity towards HCT116 $p53^{+/+}$ cells compared to ARPE-19 cells under normoxic conditions (table 4.2). Under hypoxic conditions, DS10 also showed a degree of selectivity for HCT116 cells (SI = 1.65) compared to normoxic conditions (SI = 0.64). Based on the HCR and SI data (under hypoxia), a decision was taken to further explore the preclinical activity of DS10.

Compound	HCT116 p53$^{+/+}$ IC_{50} (µM)		ARPE-19 cells IC_{50} (µM)
	Normoxia pHe 7.4	Hypoxia pHe 7.4	Normoxia pHe 7.4
DS1	7.05 ± 1.74	13.26 ± 0.98	17.31 ± 5.96
DS2	27.70 ± 10.05	47.54 ± 3.82	34.18 ± 5.24
DS3	4.50 ± 0.58	21.56 ± 1.38	15.02 ± 4.32
DS4	4.90 ± 1.88	12.36 ± 1.02	9.85 ± 1.54
DS5	12.19 ± 2.17	19.16 ± 0.53	13.59 ± 1.43
DS6	IC_{50} >100	IC50 >100	77.22 ± 3.82
DS9	87.59 ± 5.37	72.15 ± 9.33	N/A
DS10	66.39 ± 8.10	25.93 ± 3.17*	42.95 ± 5.03
DS11	15.63 ± 4.30	25.90 ± 3.50	3.20 ± 0.89
DS12	IC_{50} >100	IC50 >100	IC_{50} >100
DS13	18.59 ± 3.00	16.18 ± 1.65	25.80 ± 9.75
DS14	22.02 ± 0.46	71.73 ± 5.20	35.93 ± 0.55
DS15	14.20 ± 2.32	2.08 ± 0.70**	15.13 ± 6.87
Tirapazamine	11.85 ± 0.63	1.24 ± 0.39**	27.63 ± 4.00

Table 4.1 The response of HCT116 p53$^{+/+}$ and ARPE-19 cells to endoperoxides, di-bromohexitols and tirapazamine under normoxic (pHe 7.4) and hypoxic (pHe 7.4) conditions. All cells were exposed to test compounds for 96 hours over a concentration range from 0.3-100µM under normal and hypoxic conditions. Each result represents the mean ± standard deviation of three independent experiments. A student's t-test was carried out to determine any significance when comparing hypoxia to normoxic conditions. p values of ≤0.05 and ≤0.01 are represented as * respectively **.

Compound	Normoxic SI	Hypoxic SI	HCR
DS1	2.45	1.30	0.53
DS2	1.23	0.71	0.58
DS3	3.34	0.69	0.21
DS4	2.01	0.79	0.39
DS5	1.11	0.70	0.63
DS6	-	0.77	-
DS9	-	-	1.21
DS10	0.64	1.65	2.56
DS11	0.20	0.12	0.60
DS12	-	-	-
DS13	1.38	1.59	1.14
DS14	1.43	0.50	0.30
DS15	1.06	7.27	6.83
Tirapazamine	2.33	22.28	9.55

Table 4.2 The SI and HCR of endoperoxides, di-bromohexitols and tirapazamine against the HCT116 p53$^{+/+}$ cells. The selectivity index (SI) is defined as the ratio of IC_{50} values for ARPE-19 divided by HCT116 p53$^{+/+}$ with values >1 indicating selectivity towards HCT116 p53$^{+/+}$ cancer cells. The HCR is defined as the ratio of IC_{50} values under normoxic conditions divided by the IC_{50} under hypoxic (0.1% oxygen) conditions. Values >1 indicate that compounds have selective toxicity towards cells under hypoxic conditions.

4.4.2 The effect of DS10 against the HCT116 $p53^{+/+}$ cell line under TME conditions

Under mildly acidic pHe conditions (pH 6.5), the activity of DS10 was significantly enhanced under normoxic conditions with IC_{50} values of 26.52 ± 7.01 and 66.39 ± 8.10 μM respectively (figures 4.4 and 4.5). Under both hypoxic and acidic pHe conditions, the potency of DS10 increased further (IC_{50} = 16.3 ± 4.11 μM) demonstrating that both hypoxia (0.1% oxygen) and acidic pHe (pH 6.5) generate additive effects when combined (figure 4.4 and 4.5).

Microenvironment enhancement ratios are presented in table 4.3 and this clearly shows the enhanced activity of DS10 under the combination of hypoxic and acidic pHe. This has a significant effect on the selectivity index (SI) which increases from 0.64 in normoxic pHe 7.4 to 2.63 in hypoxia pHe 6.5. The pHR is similar to the HCR (2.5 and 2.63 respectively) which may be indicative of DS10 potentially having a dual trigger mechanism, one under the activation of hypoxic conditions and secondly DS10 sensitivity under low pHe conditions.

DS10 displayed superiority over the classic compounds tested with preferential activity towards all of the physiological TME conditions tested for in this phenotypic screen (table 4.3). DS10 displayed a TME ratio of 4.07 which is more than 7 fold greater than etoposide and approximately 15 fold greater than 5-FU and cisplatin (table 4.3).

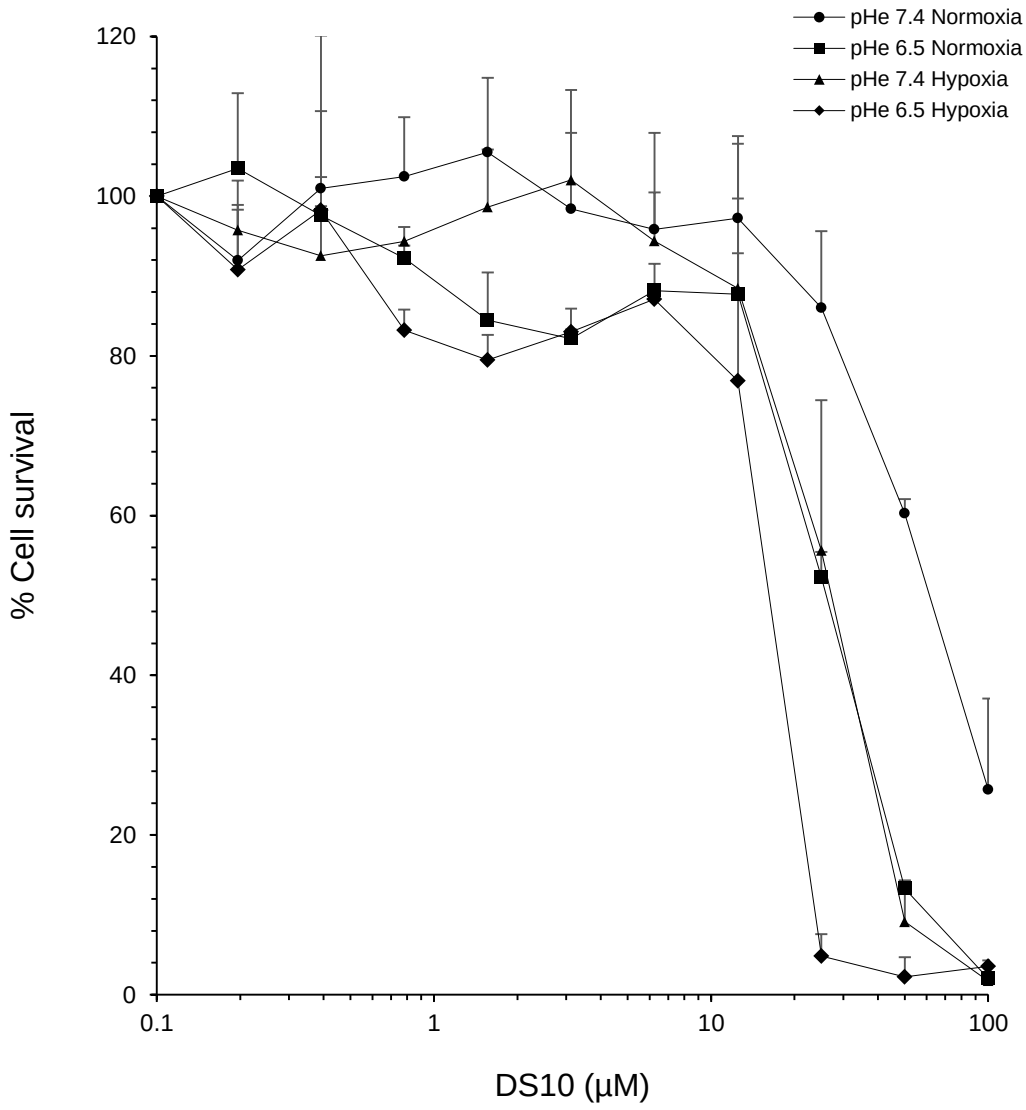


Figure 4.4 Dose response curve of HCT116 p53$^{+/+}$ cells exposed to DS10 under normoxic and tumour microenvironmental conditions. Cells were continuously exposed (96 hour exposure) to DS10 over a wide range of concentrations (0.3-100µM). HCT116 p53$^{+/+}$ cells were exposed to DS10 under the following conditions: (i) normoxia pHe 7.4, (ii) normoxia pHe 6.5, (iii) hypoxia pHe 7.4 and (iv) hypoxia pHe 6.5. Each result represents the mean ± standard deviation of three independent experiments.

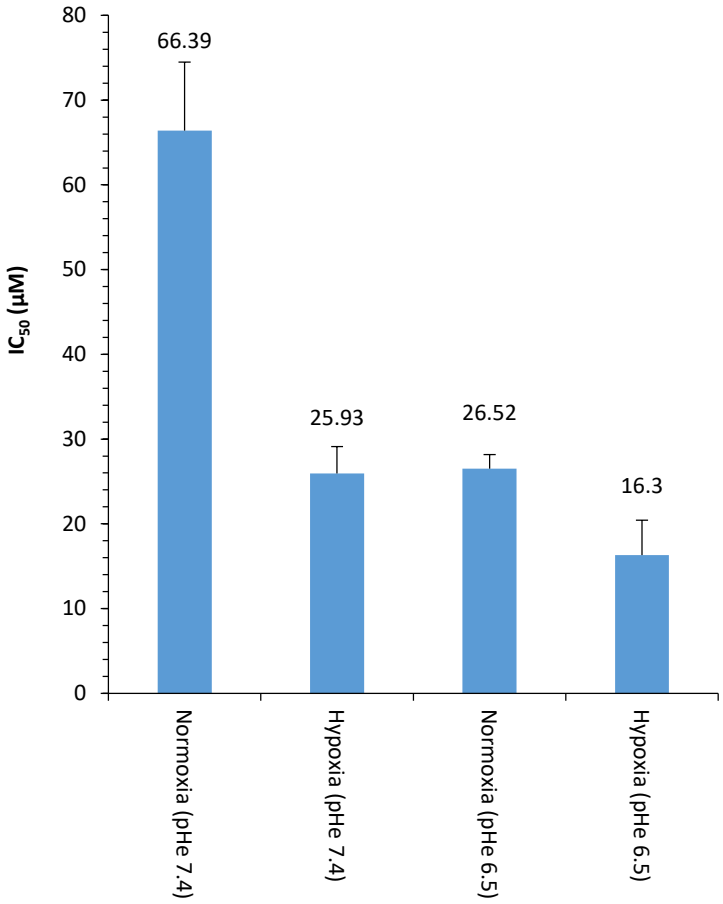


Figure 4.5 Response of HCT116 p53$^{+/+}$ cells to DS10 under normoxic and tumour microenvironmental conditions. IC_{50} values are presented in different combinations of oxygen concentration and pHe. Each value represents the mean IC_{50} ± SD for three independent experiments.

Compound	pHR	HCR	TME ratio	SI	SI (TME)
Cisplatin	0.75	0.44	0.3	1.84	0.55
5-FU	0.55	0.40	0.27	2.10	0.57
Etoposide	0.76	0.61	0.57	0.59	0.34
Tirapazamine	0.61	9.55	n/a	2.33	22.28*
DS10	2.50	2.56	4.07	0.64	2.63

Table 4.3 The effect of the TME on the response of cells to standard cytotoxic drugs, tirapazamine and DS10. pHR, HCR and TME represent the pH ratio, the hypoxic cytotoxicity ratio and tumour microenvironment ratio as defined in the methods section (see section 3.3.3). * refers to the SI value for the hypoxic data only and not the combo of hypoxia and acidic pHe. The IC_{50} values for the classic compounds are presented in chapter 3 (table 3.1).

4.4.3 Early signs of DNA damage by DS10 under hypoxic conditions in the HCT116 $p53^{+/+}$ cell line

As briefly discussed earlier, these set of compounds are designed to be activated to yield DNA damaging metabolites capable of alkylating DNA. To assess whether DS10 is activated under hypoxic conditions to generate DNA damage, western blot analysis for the detection of phosphorylated ϒH2AX, a protein marker which is used to determine the early signs of DNA damage, was used. The role of p53 dependency was also investigated.

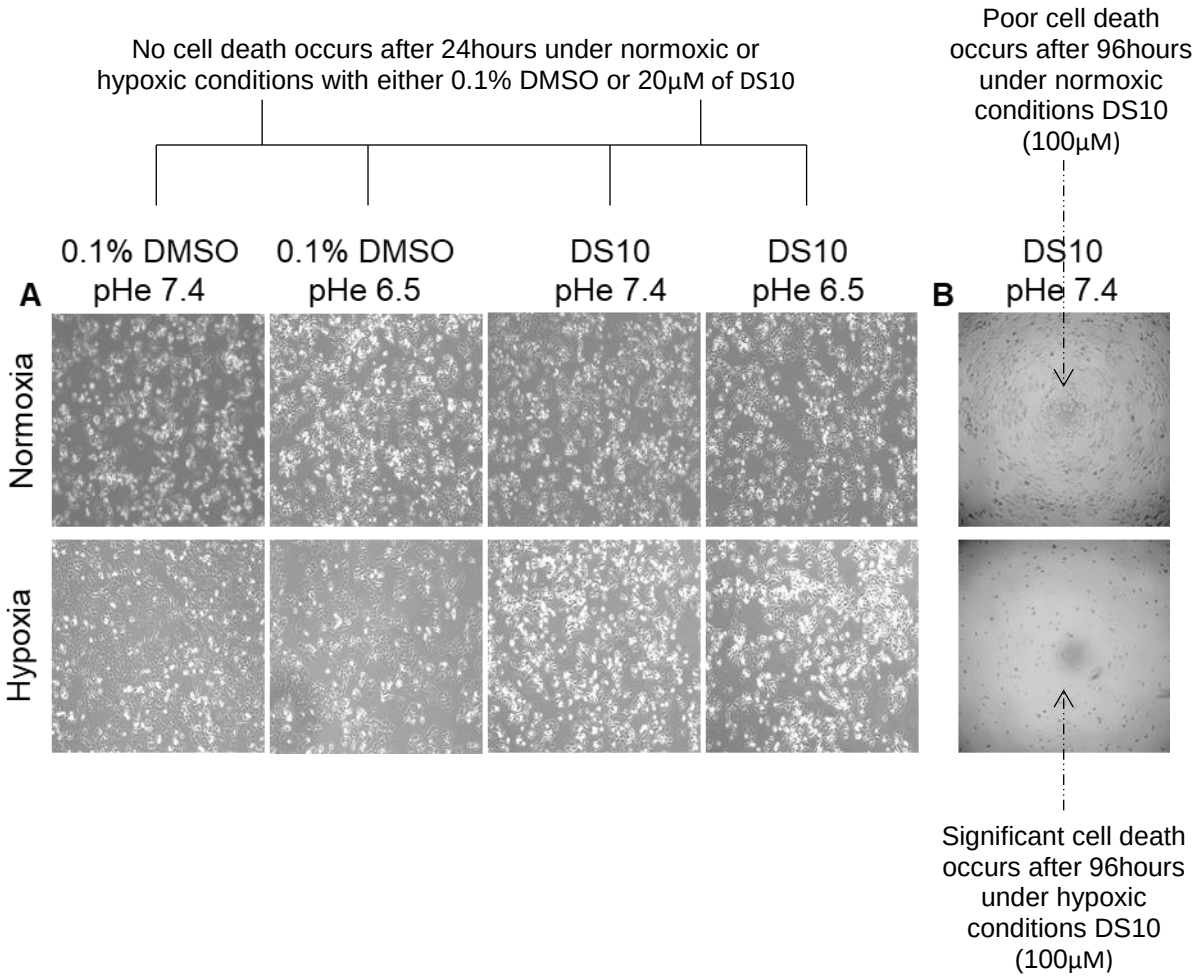


Figure 4.6 Post 24 hour treatment of HCT116 P53$^{+/+}$ cells treated with DS10 under the physiological and TME conditions. A. HCT116 p53$^{+/+}$ cells were treated with DS10 (20µM) for a duration of 24hours under the following conditions, normoxia pHe 7.4/ pHe 6.5 and hypoxia pHe 7.4/ pH 6.5. The vehicle control DMSO 0.1% was used as a control under each condition. B. HCT116 p53$^{+/+}$ cells were treated with DS10 (100µM) for a duration of 96hours under the following conditions, normoxia and hypoxia (both at pHe 7.4).

Choosing the correct concentration was important to determine whether DS10 was a DNA damaging agent or not. HCT116 p53$^{+/+}$ cells were subjected to 20µM of DS10 under both normoxic (pHe 7.4/ 6.5) and hypoxic (pHe 7.4/ 6.5) conditions. There was

no difference in cell morphology between drug treated and untreated under these conditions after 24hours (figure 4.6A). When a higher drug concentration of DS10 (100µM) was used there is a distinct difference in cell morphology between normoxic and hypoxic conditions indicating that cell death is imperative within 24hours (figure 4.6B). For a fair assessment of early signs of DNA damage by DS10, 20µM was used to verify if ϒH2AX was phosphorylated under hypoxic conditions.

Cells were immediately lysed and western blot analysis was carried out as described in the methodology section (see section 2.9-2.13). As depicted in figure 4.7, western blot analysis demonstrated that p-ϒH2AX is induced in cells treated with DS10 under hypoxic conditions only at pHe 7.4 and 6.5. No p-ϒH2AX was detected in DS10 treated cells under aerobic conditions. This would suggest that DS10 is being activated to a DNA damaging species under hypoxic conditions although the induction of DNA damage did not induce a significant p53 response (figure 4.7).

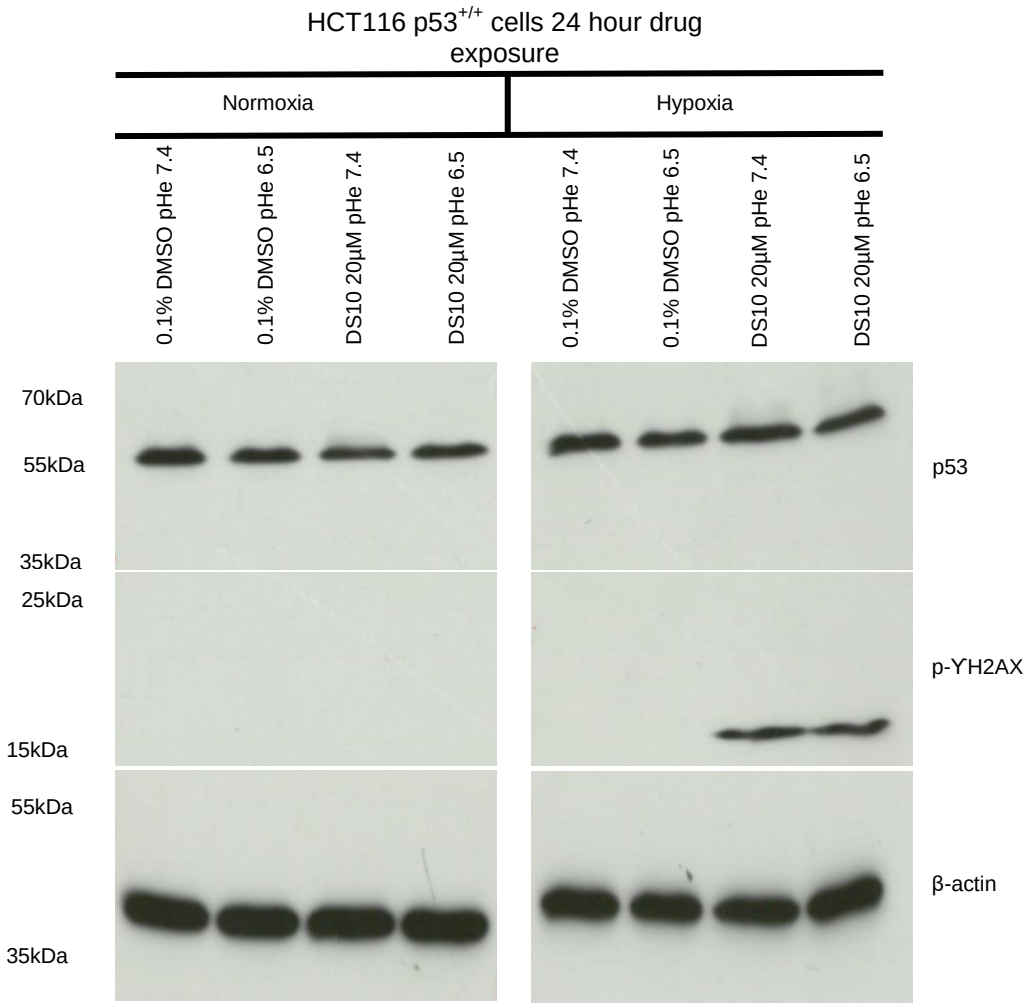


Figure 4.7 Influence of hypoxia and DS10 on induction of p-YH2AX and p53. Colorectal cancer HCT116 $p53^{+/+}$ cells were exposed to DS10 for 24 hours under normoxic and hypoxic (0.1% O_2) conditions, cell lysates prepared and subjected to Western blot analysis. β-actin was used as a loading control (n=1).

4.5 Discussion

In this chapter, a series of bromosugars that were designed as potential HAPs were evaluated in the phenotypic screen. Chemosensitivity studies with the bromosugar HAPs were carried out using HCT116 $p53^{+/+}$ and the normal human retinal epithelial cell line ARPE-19 was used to provide an indication of selectivity. The most potent bromo-sugars under normoxic conditions with IC_{50} less than 10μM were the endoperoxides, DS1 (IC_{50} = 7.05μM ± 1.74), DS3 (IC_{50} = 4.50μM ± 0.58) and DS4 (IC_{50} = 4.90μM ± 1.88) (table 4.1). From the compounds tested, the only endoperoxide that displayed preferential toxicity under hypoxic conditions was DS10 with a HCR value of 2.56 (table 4.2 and figures 4.4 - 4.5). A further chemosensitivity study carried out with DS10 under normoxic conditions at pHe 6.5 showed a similar increase in toxicity as that of DS10 under hypoxic conditions IC_{50} 26.53μM (±1.66) (figure 4.4 – 4.5). This warranted a final chemosensitivity study with DS10 under hypoxic conditions at pHe 6.5 to ascertain a TME ratio and the results were striking. Not only was this more toxic to the cancer cell line (2 fold decrease in IC_{50} values), the selectivity index had also increased by 4 fold (from 0.64 to 2.63). This is a desirable property for any new emerging chemotherapeutic compound as to be successful, it must have minimum or little toxicity to normal cells when compared to cancerous cells (Connors, 1982).

From the endoperoxides DS6 is the only compound which is structurally identical to DS10 with the exception of a methyl functional group attached to it. However both these compounds exhibit different levels of cytotoxicity. DS6 did not display any level of potency towards the HCT116 $p53^{+/+}$ cell line with IC_{50} values both greater than 100μM under normoxic and hypoxic conditions. With the removal of the methyl group to form DS10, not only is cytotoxic activity enhanced but hypoxia selectivity also

occurs. This data demonstrates that subtle changes to the compound can have an significant impact on the potency of these and how they behave under both normoxic and hypoxic conditions. Similar observations were made with compounds DBM, DBG and treosulfan under normoxic conditions (Chiuten et al., 1981; Mischler et al., 1979).

From the di-bromohexitols only DS12 displayed no toxicity against both cell lines and conditions tested with IC_{50} values all greater than 100µM. The other di-bromohexitols DS11 and DS14 were also less active under hypoxic conditions (HCR 0.6 and 0.3 respectively) and DS13 was slightly more toxic under hypoxic conditions (HCR 1.14). One interesting finding from this study was with the compound DS15 and its ability of behaving like a HAP. This may not be as potent (IC_{50} 14.20µM ± 2.32) as the classic chemotherapeutic drugs tested in chapter 3 under normoxic conditions but it was very toxic under hypoxic conditions (HCR 6.83). The HCR observed with this compound is less than the classic HAP agent tirapazamine (HCR 9.55) but 2 fold greater than DS10. With DS10 being a endoperoxide and DS15 a dibromohexitol (in conjunction with its limited supply), it was decided that DS10 will be subjected for further studies as this project investigates the roles of endoperoxides becoming active under hypoxic conditions. Further studies are nevertheless warranted to explore the unusual and unexpected observation of hypoxia selectivity for DS15.

When compared to the standard classic chemotherapeutic agents in results chapter 3 and table 4.3, the HCR of DS10 (2.56) is much greater than that of cisplatin, 5-FU and etoposide (0.44, 0.40 and 0.61 respectively). The SI under normoxic conditions at pHe 7.4 for DS10 is 0.64 which is similar to etoposide (0.59), but less than cisplatin and 5-FU (1.84 and 2.10). When DS10 is tested under hypoxic conditions at pHe 6.5 the SI is 2.63 which is much improved compared to the classic agents

cisplatin (SI = 0.55), 5-FU (SI = 0.57) and etoposide (SI = 0.34). This demonstrates that DS10 is more active and selective under tumour microenvironmental conditions in comparison to the leading classic chemotherapeutic drugs. Similar results were obtained with a 2,4-dinitrobenzamide mustard which showed that lowering the extracellular pH (6.5) enhanced the cytotoxicity of this compound under normoxic conditions in the KHT sarcoma cell line, an effect that was further enhanced when acidic pHe conditions were combined with hypoxia (Denny et al., 1996).

To further investigate the mechanism of action of DS10 under hypoxic conditions, western blot analysis was carried out for the screening of ϒH2AX and p53. Damaging the DNA, particularly the induction of double stranded DNA breaks, trigger the onset of several cellular response functions. One of these is the phosphorylation of the histone ϒH2AX to p-ϒH2AX (Podhorecka et al., 2010). P-ϒH2AX analysis is widely used to determine genotoxic effects of experimental compounds (Podhorecka et al., 2010) and has been used in a clinical setting where cancer patients receiving certain anti-cancer treatments are monitored for both toxicity and selectivity towards tumour cells (Downs, 2007; Taneja et al., 2004). As depicted in figure 4.7, ϒH2AX phosphorylation was only observed in HCT116 $p53^{+/+}$ cells treated with DS10 under hypoxic conditions with no ϒH2AX phosphorylation under normoxic conditions. This suggests that DS10 may be a DNA alkylating agent which is only active under hypoxic conditions. There was also no p53 dependency observed (figure 4.7). Further studies are warranted to explore the mechanism(s) of action of DS10 but the results presented here illustrates how endoperoxide linkage may be an important source of novel hypoxia triggers, the activity of which is further enhanced by acidic pHe conditions (figure 4.4 – 4.5 and table 4.3).

It is important to state that the results presented here have been conducted in monolayer cultures and further studies are required using experimental models that better mimic the tumour microenvironment. This includes the use of multi-cell layers to assess drug penetration (Phillips et al., 1998) and the use of multi-cell spheroids (Phillips, 2016). Due to the fact that limited supplies of the compounds were available (Prof Pederson's group moved to the Pharmaceutical Industry during the course of this study), further studies of this nature were not possible. However, if this was not the case then further studies assessing the role of DS10 to penetrate through a multi-cell layer or in 3D spheroids where both gradients of hypoxic and acidic pHe conditions exist would be the next step in their preclinical evaluation.

In conclusion, the phenotypic screen utilised in this results chapter has successfully identified a new novel di-bromohexitol prodrug DS10 which has shown preferential activity under all of the TME parameters tested. Within the broader context of current approaches to drug discovery, the US National Cancer Institute (NCI) 60 human tumour cell line screen plays a major role in random or phenotypic screening approaches. As stated previously, the NCI60 was developed in the late 1980's and has been used as a platform for screening compounds by the research community (Shoemaker, 2006). This screen essentially uses panels of cell lines exposed to test compounds under normoxic and neutral pHe conditions only. If the same screening strategy was implemented with these endoperoxides then the ability of DS10's to preferentially target cells under hypoxic and acidic pHe conditions would not be discovered. These results therefore demonstrate for the first time that the phenotypic approach to drug screening described in this study can identify novel compounds that are selectively toxic to cancer cells under conditions that partially mimic the TME.

Chapter 5: Performance of dasatinib and a HAP dasatinib derivative in the phenotypic screen

5.1 Introduction

Tyrosine kinase inhibitors (TKI's) are one of the most promising and most extensively studied class of targeted drugs for cancer treatment. Cancer therapy has changed significantly over the past two decades with the emergence of TKI's as a major 'weapon' in the war on cancer. These TKIs have continued to evolve over the past decades and we are currently on the third generation of TKI's. (Arora and Scholar, 2005; Minari et al., 2016). Most established cytotoxic anti-cancer agents present a narrow therapeutic index, resulting in undesirable and unwanted side effects (Arora and Scholar, 2005). The development of the TKI's was initially designed to circumvent these issues by targeting specific molecules and signalling pathways. TKI's are generally given in conjunction with other cytotoxic agents as well as radiotherapy (Arora and Scholar, 2005).

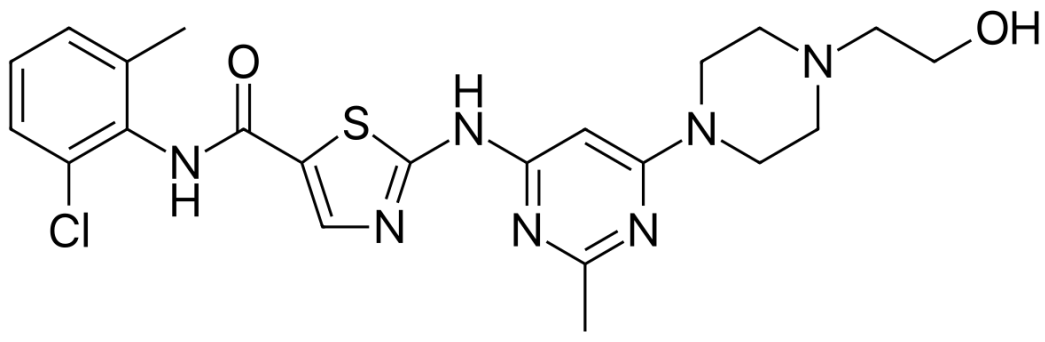


Figure 5.1 Chemical structure of Dasatinib (De Francia et al., 2009).

Dasatinib (figure 5.1) is a 2nd generation TKI which was developed by Bristol Myers Squibb (Kantarjian et al., 2006) and works by inhibiting the Src kinase family which are important proteins involved in the development of metastasis, angiogenesis, invasion, adhesion, growth and progression in human cancers (Frame, 2002; Schlessinger, 2000). In addition, dasatinib is widely used for the treatment of imatinib refractory chronic myeloid leukaemia (CML) by targeting the BCR-ABL oncogene. This 2nd generation TKI was developed as a successor to imatinib, a 1st generation TKI which is used in the treatment of CML. *In vitro* studies have clearly demonstrated that dasatinib is a highly potent ATP-competitive inhibitor and is 300 times more potent than imatinib. This could be because some patients can typically get a secondary resistance which is known to be linked to a mutation of the BCR-ABL 1 kinase domain referred to as the T315I mutation (Apperley, 2007). This mutation of the T315I site changes the conformation of the specific site where Imatinib would bind resulting in steric hindrance and reduced activity. TKIs such as dasatinib were designed to target imatinib resistant CML (Perrotti et al., 2010).

As stated previously, hypoxia has been shown to trigger a set of biochemical processes which are facilitated by the induction of the HIF factors (Rohwer and Cramer, 2011). Pham et al., (2009) has demonstrated this in cancer model xenografts by the up regulation of Src and phosphorylated Src proteins in the hypoxic regions of tumours (Pham et al., 2009). This has stimulated interest in this area to determine whether TKI's do possess HAP like abilities in cancer cells. A study carried out by Ahmadi et al., (2014) investigated this by testing a series of TKI's such as, sunitinib, sorafenib, gefitinib, masatinib, dasatinib, nilotinib, imatinib and erolitinib across a panel of cancer cell lines. The results demonstrated that dasatinib had increased potency under hypoxic conditions when compared to

normoxic in the MCF-7 and the triple negative MDA-MB-231, MDA-MB-468 and MDA-MB-453 breast cancer cell lines. In some cell lines this was not the case such as the non-small cell lung cancer cell line H460 which did not display any difference in potency (Ahmadi et al., 2014). The cell lines MDA-MB-231 and H460 were therefore chosen for this study. The selection of cell lines was based on previous studies within our group (Ahmadi et al 2014) which demonstrated that MDA-MB-231 was more sensitive to dasatinib under hypoxic conditions whereas H460 was not. The ARPE-19 non-cancer cells was also selected for use in this chapter for the same reasons as outlined above.

N-oxides have been previously used to increase potency under hypoxic conditions. AQ4N (a well-established HAP under clinical trials) has an N-oxide terminal group attached to the warhead AQ4 which undergoes a two electron reduction by metabolic processes under hypoxia thereby releasing AQ4 and increasing the potency of AQ4N up to 1000 times in the absence of air (Patterson, 1993). Consistent with this theory and rationale, nitroimidazole groups could also be used as bio-reductive triggers when combined with TKI agents such as dasatinib. Associate professor Adam Patterson from Auckland Cancer Society Research Centre, New Zealand has synthesised many novel compounds with the most well-known example being TH-4000 (tarloxotinib bromide). This compound releases a highly toxic EGFR-TKI under hypoxic conditions and is currently under phase 2 clinical studies (Patterson et al., 2015). As well as TH-4000, Patterson has also synthesised dasatinib which consists of an nitroimidazole trigger group as shown in figure 5.2 which is referred to as DAS-HAP.

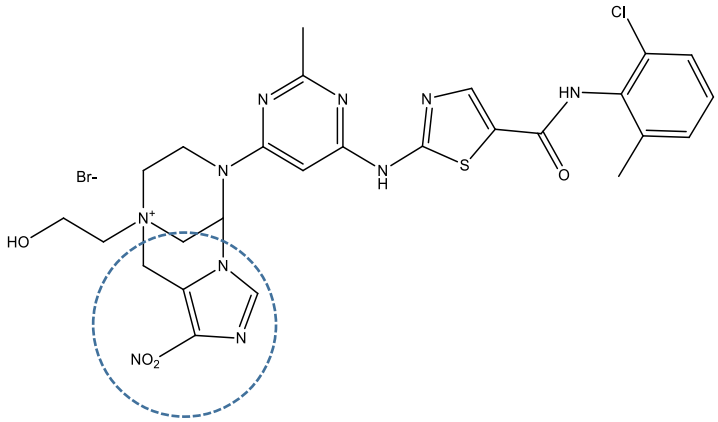


Figure 5.2 Chemical structure of DAS-HAP. The region outlined in blue is the hypoxia sensitive nitroimidazole ‘trigger’ group with the black region being dasatinib.

Dasatinib has previously demonstrated HAP like abilities (Ahmadi et al 2014) and to further test these inhibitors with the addition of nitroimidazole triggers could be beneficial in terms of providing an additional level of selectivity for targeting those cells in the hypoxic regions of the tumour microenvironment.

5.2 Aims

The aim of this study is to evaluate whether the addition of an nitroimidazole terminal to dasatinib is cytotoxic towards the triple negative breast cancer MDA-MB-231 and the non-small cell lung cancer H460 cell lines under TME conditions by determining preferential activity under (i) hypoxia (0.1% oxygen), (ii) acidic extracellular pH (pHe 6.5) and (iii) a combination of hypoxia and acidic pHe. To determine the selectivity, these compounds will also be evaluated against the non-cancerous ARPE-19 cell line under conditions that reflect the ‘normal conditions’ found in healthy tissue (i.e. oxygenated and pHe 7.4). Dasatinib will be used as the standard control.

5.3 Methods

5.3.1 Compounds

DAS-HAP was kindly provided by Associate Professor Adam Patterson (Auckland Cancer Society Research Centre, New Zealand). This compound along with dasatinib was dissolved in dimethylsulphoxide (DMSO) at 100mM and aliquots (20µL) were stored at -20°C.

5.3.2 Cell culture and chemosensitivity

Cell culture, adjusting the pH of the media, drug dilutions and chemosensitivity studies were carried out as previously described in methods (see section 2.6-2.8). The cell lines used in this study are the non-small cell lung cancer cell line H460 and the triple negative breast cancer cell line MDA-MB-231. Any variation from these methods are indicated in the figure legends and described in the results section.

5.3.3 Western blot analysis

Cells were treated with hit compounds for a duration of 24 hours at a drug concentration of 0.005, 0.05 and 0.5µM. These concentrations were chosen as it is a therapeutically relevant concentration (IC_{50} under hypoxic conditions was 0.036 ± 0.008 µM and 0.084 +/- 0.03 µM for dasatinib and DAS-HAP respectively as shown in table 5.12). A 24 hour duration of drug exposure was selected as minimal cell death had occurred at this time point. Controls were treated with media alone or with DMSO 0.1% vehicle control (which has been previously reported not to be toxic to the cells). Dasatinib was used as the positive control and these were carried out under the following tumour microenvironment conditions, normoxia pHe 7.4, normoxia pHe 6.5, hypoxia pHe 7.4 and hypoxia pHe 6.4. Upon completion of treatment, cells were harvested for western blot analysis for Src, and pSrc at tyrosine

527. Details of western blot methodology and antibody dilutions are described in the general methods (see section 2.9-2.13).

5.3.4 Statistical analysis

Statistical analysis was carried out using Microsoft Excel and Graph Prism. All experiments were done in triplicate (n=3), and results were presented as the mean ± SD. Statistical significance was determined by means of a two-tailed Student's t-test, or by One Way ANOVA where significance was only accepted when $p \leq 0.05$. The exact statistical analysis performed is mentioned in the figure legend.

5.4 Results

5.4.1 The effect of dasatinib against the H460 cell line under normoxic pHe 7.4

The non-small cell lung cancer H460 cell line was treated with a dose concentration range of dasatinib over 96 hours and achieved an IC_{50} value of 9.71µM ± 1.06 which is graphically displayed below (figure 5.3).

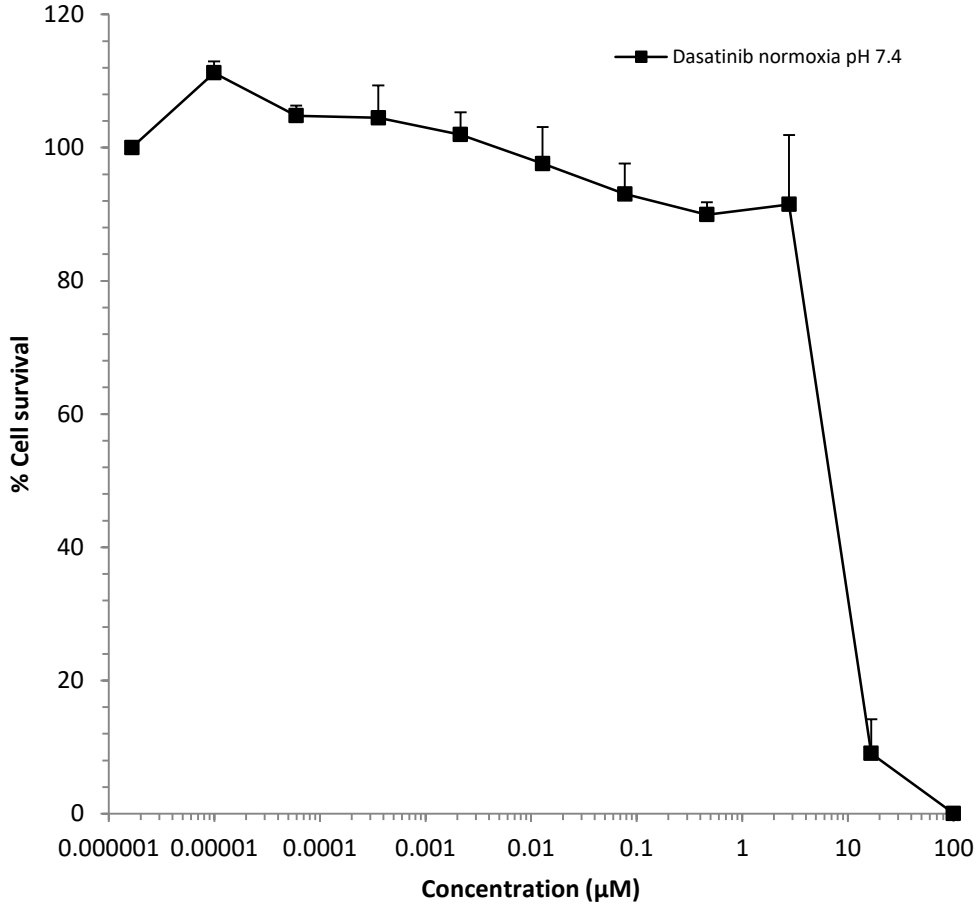


Figure 5.3 Response of H460 cells following a 96hour exposure to dasatinib under normoxic pHe 7.4. Each data point represents the mean ± standard deviation of three independent experiments.

5.4.2 The effect of dasatinib and DAS-HAP against the H460 cell line under normoxic and hypoxic pHe 7.4 conditions

The H460 cell line was treated with a dose concentration range of dasatinib and DAS-HAP under normoxic and hypoxic pHe 7.4 conditions over 96 hours. An IC_{50} value of 9.71µM ± 1.06 was ascertained for dasatinib and no IC_{50} value was reached for DAS-HAP (IC_{50} > 100µM) under normoxic pHe 7.4 conditions and this is graphically presented below (figure 5.4).

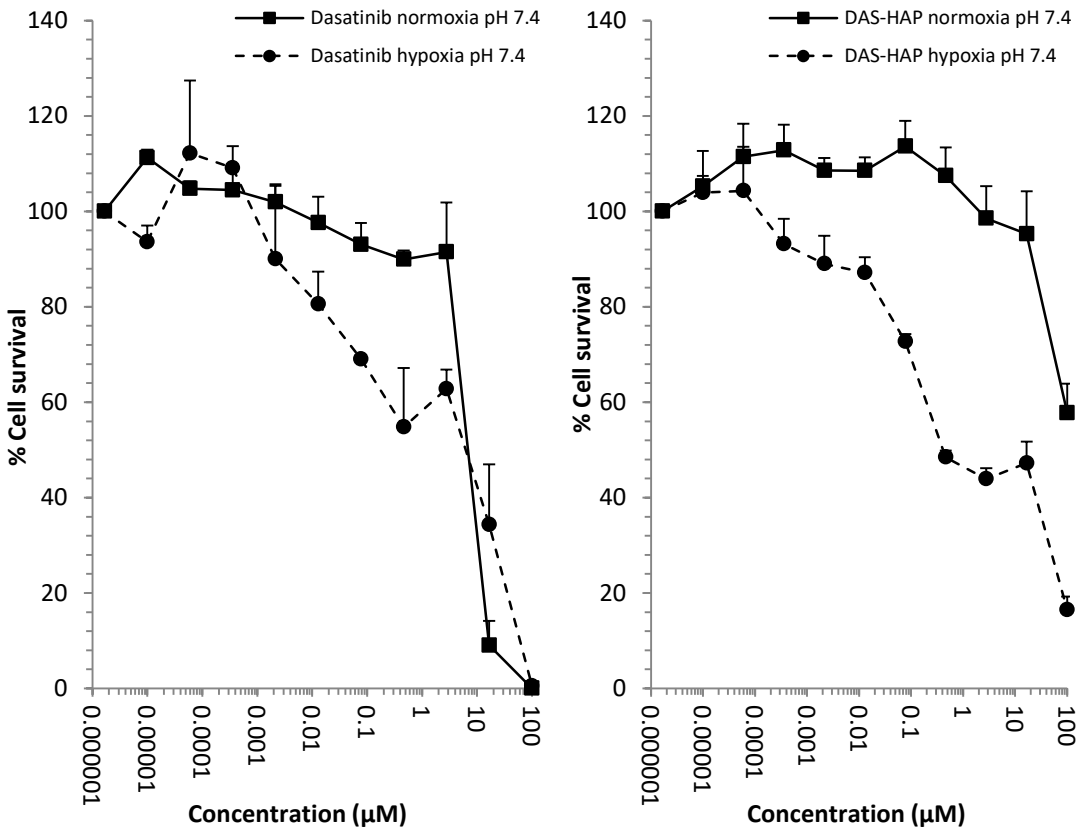


Figure 5.4 Response of H460 cells following a 96hour exposure to dasatinib and DAS-HAP under normoxic pHe 7.4. Each result represents the mean ± standard deviation of three independent experiments.

Under hypoxic pHe 7.4 conditions, the IC_{50} generated for the two compounds was 9.96μM ± 4.78 and 0.44 μM ± 0.02 (table 5.1) and an HCR ratio of 0.97 and 227.27 respectively. A comparison between dasatinib and DAS-HAP under these conditions is graphically shown in figure 5.4 and table 5.1.

	Normoxia pHe 7.4 H460 IC_{50} (μM)	**Hypoxia pHe 7.4 H460 IC_{50} (μM)**	**HCR**
Dasatinib	9.71 (± 1.06)	9.96 (± 4.78)	0.97
DAS-HAP	IC_{50} > 100	0.44 (± 0.02)	>222.27

Table 5.1. IC_{50} values and HCR for DAS-HAP and dasatinib against the H460 cell line. These are presented in different combinations of oxygen tension and extracellular pH. Each value represents the mean IC_{50} ± SD for three independent experiments.

5.4.3 The effect of dasatinib and DAS-HAP against the H460 cell line under normoxic pHe 7.4 and pHe 6.5 conditions

H460 cells were treated with a dose concentration range of dasatinib and DAS-HAP over 96 hours under normoxic conditions at pHe 7.4 and 6.5. The IC_{50} values for both compounds under physiological pH conditions have been reported below with IC_{50} values of 9.96μM ± 4.78 for dasatinib and >100 μM for Das-HAP. Under acidic pHe conditions, the IC_{50} generated for dasatinib was 10.34μM ± 0.94 for dasatinib but an IC_{50} value was not reached for DAS-HAP IC_{50} > 100μM (figure 5.5 and table 5.2). The pHR ratio for dasatinib and DAS-HAP are 0.93 and 1 respectively (table 5.2). A comparison between dasatinib and DAS-HAP under these conditions is graphically displayed below (figure 5.5).

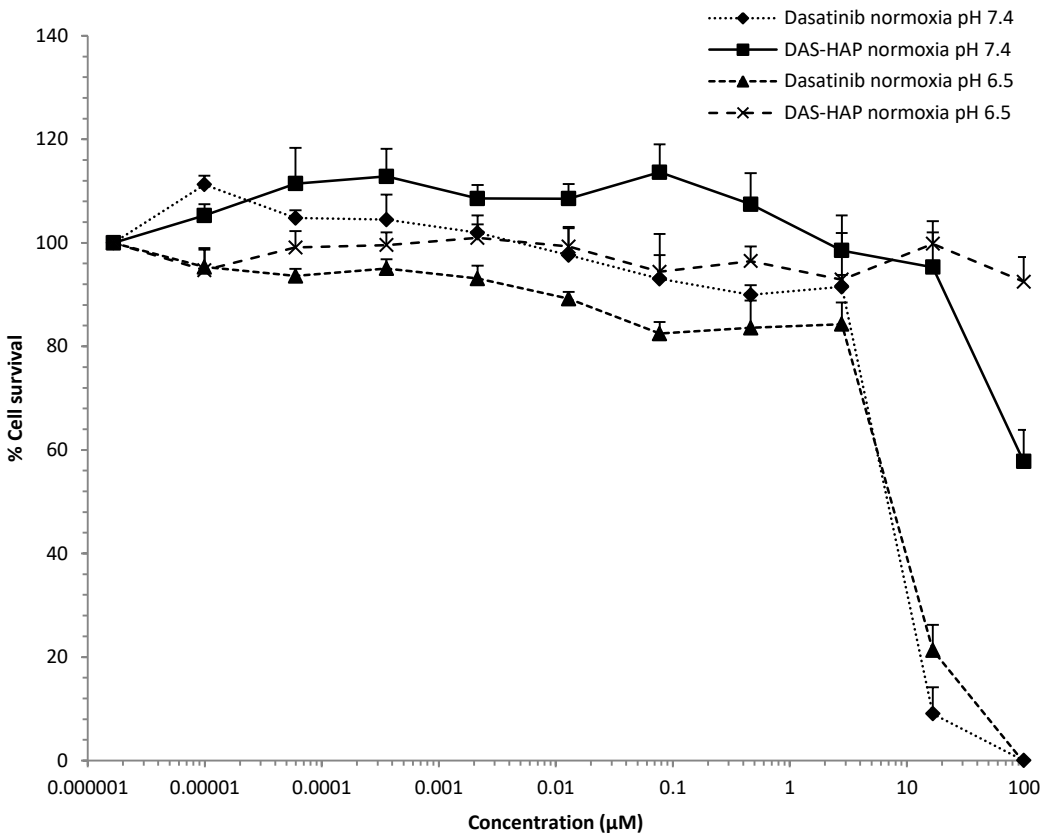


Figure 5.5 Response of H460 cells following a 96hour exposure to dasatinib and DAS-HAP under normoxic pHe 7.4 and pHe 6.5. Each result represents the mean ± standard deviation of three independent experiments.

	Normoxia pHe 7.4 **H460 IC_{50} (µM)**	**Normoxia pHe 6.5** **H460 IC_{50} (µM)**	**pHR**
Dasatinib	9.71 (± 1.06)	9.96 (± 4.78)	0.97
DAS-HAP	IC_{50} >100	IC_{50} >100	n/a

Table 5.2 IC_{50} values and pHR for DAS-HAP and dasatinib against the H460 cell line. These are presented in different combinations of extracellular pH under normoxic conditions. Each value represents the mean IC_{50} ± SD for three independent experiments. N/a = not applicable.

5.4.4 The effect of dasatinib and DAS-HAP against the H460 cell line under normoxic pHe 7.4 and hypoxia pHe 6.5 conditions

H460 cells were treated with a range of dasatinib and DAS-HAP concentrations over 96 hours under hypoxic and pHe 6.5 conditions. The IC_{50} generated for the two compounds was 6.76μM ± 1.31 for dasatinib and 67.73μM ± 0.003 for DAS-HAP (table 5.3). The TME enhancement ratio for dasatinib and DAS-HAP are 1.43 and 1.47 respectively (table 5.3) and demonstrates similar a potential for the TME conditions. A comparison between dasatinib and DAS-HAP under these conditions is graphically displayed below (figure 5.6).

	Normoxia pHe 7.4 H460 IC_{50} (μM)	**Hypoxia pHe 6.5 H460 IC_{50} (μM)**	**TME**
Dasatinib	9.71 (± 1.06)	6.76 ± 1.31	1.43
DAS-HAP	IC_{50} >100	67.73 ± 0.003	~ 1.47

Table 5.3 IC_{50} values and the TME ratio for DAS-HAP and dasatinib against the H460 cell line. These are presented in different combinations of oxygen tension and extracellular pH including the combination of hypoxia and acidic pHe. Each value represents the mean IC_{50} ± SD for three independent experiments. ~ = approximately.

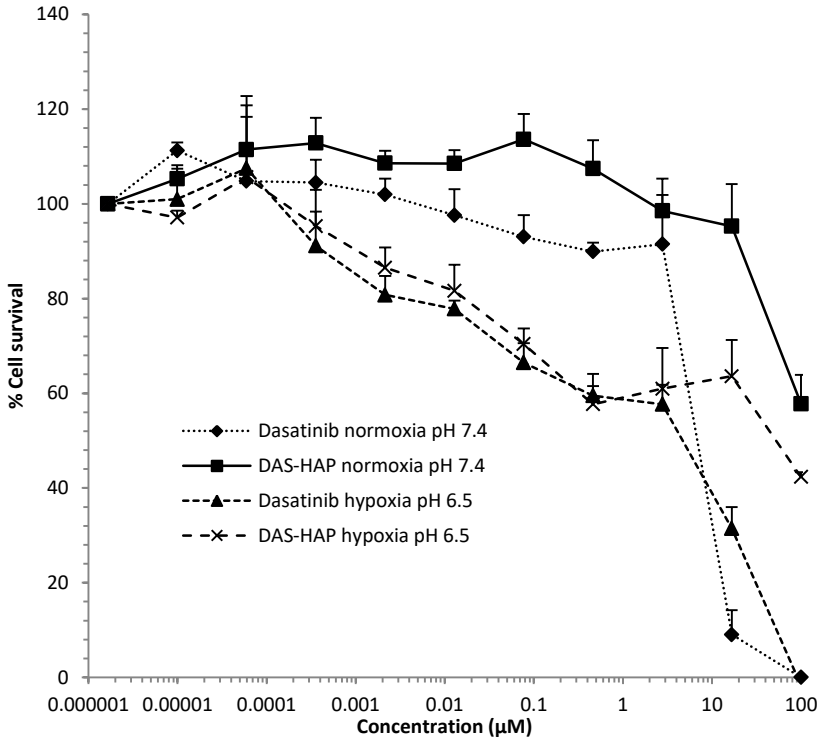


Figure 5.6 Response of H460 cells following a 96hour exposure to dasatinib and DAS-HAP under normoxic pHe 7.4 and hypoxia pHe 6.5. Each result represents the mean ± standard deviation of three independent experiments.

5.4.5 The effect of dasatinib and DAS-HAP against the H460 and ARPE-19 cell line under normoxic pHe 7.4

Dasatinib and DAS-HAP were also exposed to the normal human retinal epithelial cell line ARPE-19 cells under normoxic pHe 7.4 conditions for 96 hours. The IC_{50} values of these compounds towards this cell line were 0.12µM ±0.02 for dasatinib and 3.636µM ±0.56 for DAS-HAP (table 5.4). The SI index for dasatinib and DAS-HAP are 0.01 and 0.03 respectively (table 5.4). This indicates that DAS-HAP is less toxic towards the normal cells and more selective towards the H460 cancerous cell lines in comparison to dasatinib. This is graphically displayed below (figure 5.7).

	Normoxia pHe 7.4 H460 IC_{50} (µM)	ARPE-19 IC_{50} (µM)	SI
Dasatinib	9.71 (± 1.06)	0.12 (± 0.02)	0.01
DAS-HAP	IC_{50} >100	3.636 (± 0.56)	0.03

Table 5.4 IC_{50} values, SI for DAS-HAP and dasatinib against the H460 and ARPE-19 cell line. These are presented in different combinations of extracellular pH under normoxic conditions. Each value represents the mean IC_{50} ± SD for three independent experiments.

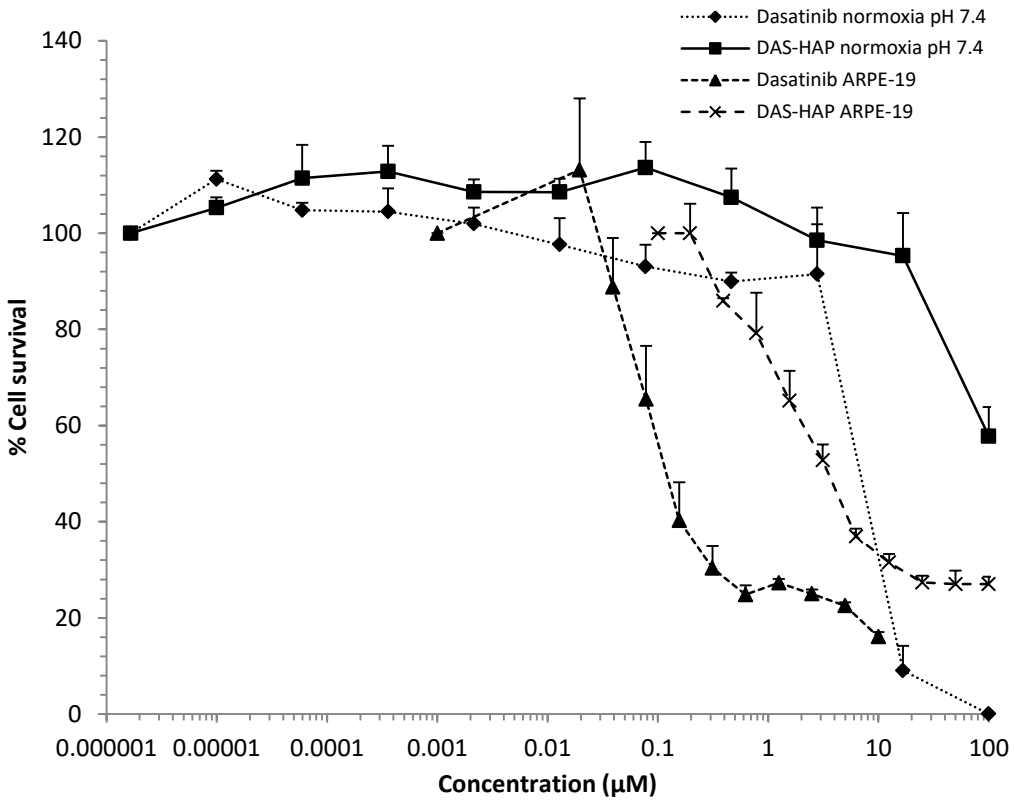


Figure 5.7 Response of H460 and ARPE-19 cells following a 96hour exposure to dasatinib and DAS-HAP under normoxic pHe 7.4. Each result represents the mean ± standard deviation of three independent experiments.

	Normoxia pHe 7.4 H460 IC_{50} (µM)	Normoxia pHe 6.5 H460 IC_{50} (µM)	Hypoxia pH e7.4 H460 IC_{50} (µM)	Hypoxia pHe 6.5 H460 IC_{50} (µM)	ARPE-19 IC_{50} (µM)
Dasatinib	9.71 (± 1.06)	10.34 (± 0.94)	9.96 (± 4.78)	6.76 (± 1.31)	0.12 (± 0.02)
DAS-HAP	IC_{50} >100	IC_{50} >100	0.44 (± 0.02)	67.73 (± 0.003)	3.636 (± 0.56)

Table 5.5 IC_{50} values for DAS-HAP and dasatinib against the H460 and ARPE-19 cell line. These are presented in different combinations of oxygen concentration and pHe. Each value represents the mean IC_{50} ± SD for three independent experiments

Compound	Cell	pHR	HCR	TME ratio	SI	SI (TME)
Dasatinib	H460	0.93	0.97	1.43	0.01	0.01
DAS-HAP	H460	1	227.27	1.47	0.03	0.05

Table 5.6 The effect of the TME on the response of H460 cells to dasatinib and DAS-HAP. pHR, HCR and TME represent the pH ratio, the hypoxic cytotoxicity ratio and tumour microenvironment ratio as defined in the methods section 3.3.3.

5.4.6 The effect of dasatinib against the MDA-MB-231 cell line under normoxic pHe 7.4

The triple negative MDA-MB-231 breast cancer cell line was treated with a dose concentration range of dasatinib over 96 hours and reached an IC_{50} value of

0.037μM ± 0.009. This is graphically displayed below (figure 5.8). This is more toxic towards the MDA-MB-231 in comparison to the H460 cell line.

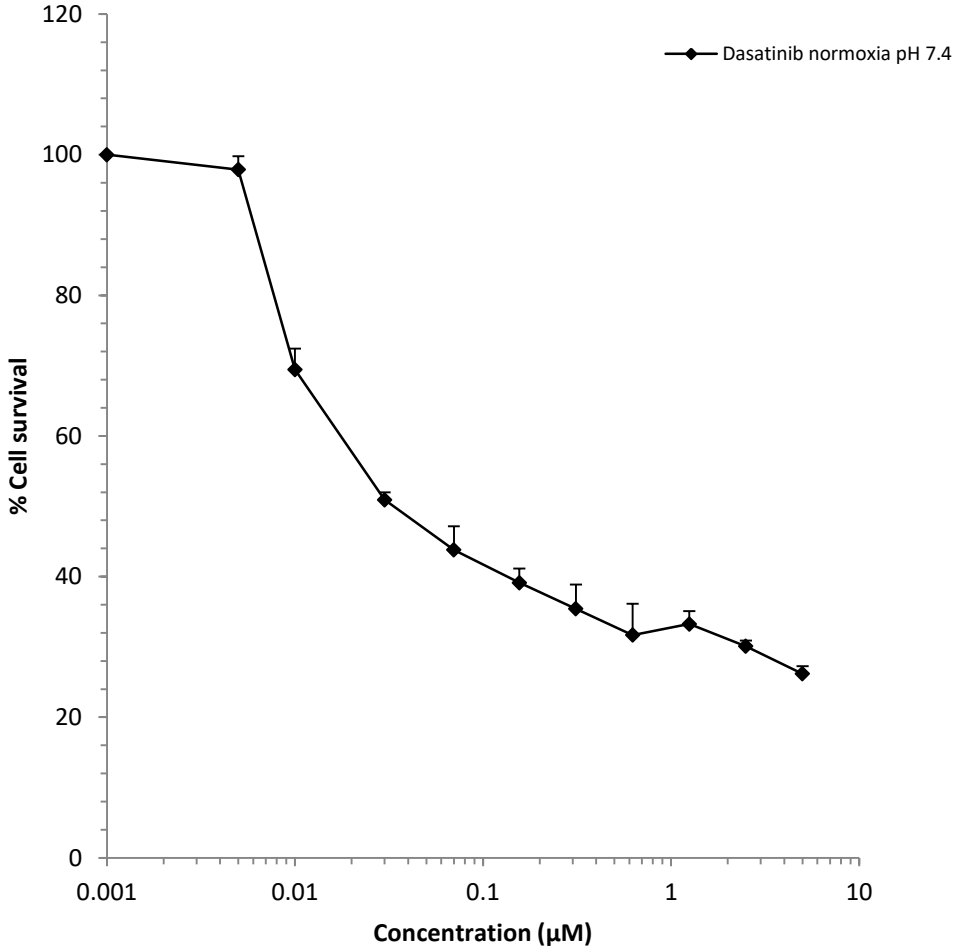


Figure 5.8 Response of MDA-MB-231 cells following a 96hour exposure to dasatinib under normoxic pHe 7.4. Each result represents the mean ± standard deviation of three independent experiments.

5.4.7 The effect of dasatinib and DAS-HAP against the MDA-MB-231 cell line under normoxic and hypoxic pHe 7.4

MDA-MB-231 cells were treated with a dose concentration range of DAS-HAP over 96 hours where an IC_{50} value of 1.644μM ±0.22 was reached for DAS-HAP and

0.037µM ± 0.009 for dasatinib under normoxia pHe 7.4 and is graphically displayed below (figure 5.9).

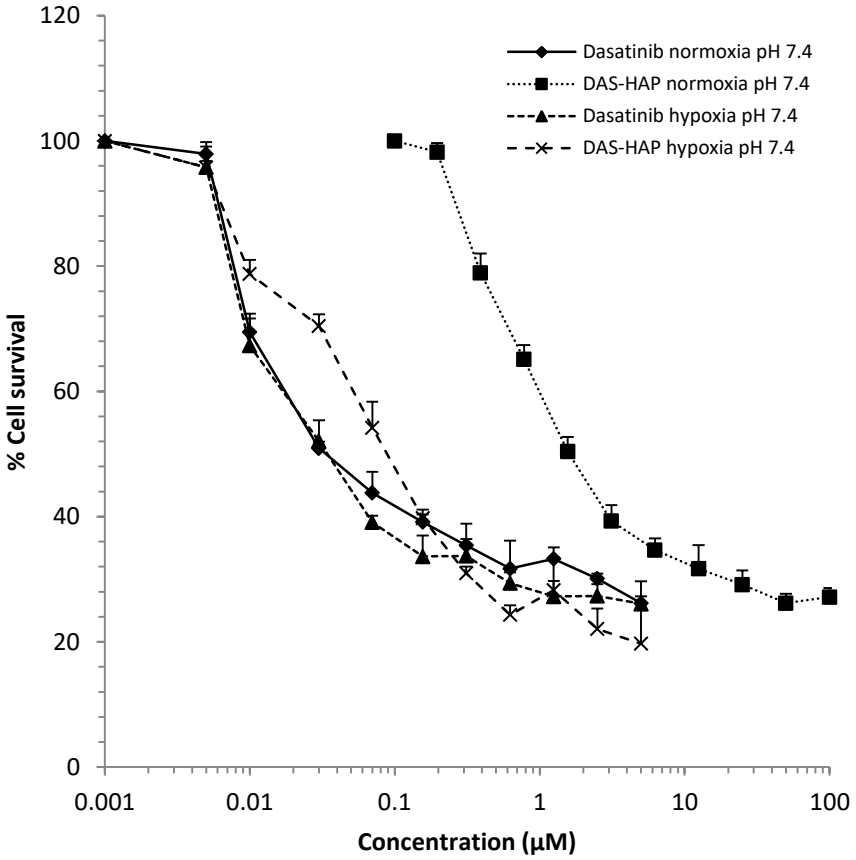


Figure 5.9 Response of MDA-MB-231 cells following a 96hour exposure to dasatinib and DAS-HAP under normoxic and hypoxic pHe 7.4. Each result represents the mean ± standard deviation of three independent experiments.

The IC_{50} generated for the two compounds under hypoxic pHe 7.4 conditions was 0.036µM ± 0.008 and 0.084µM ± 0.03 for dasatinib and DAS-HAP respectively. The HCR ratio for dasatinib and DAS-HAP was 1.02 and 19.57 respectively. A comparison between dasatinib and DAS-HAP under these conditions is graphically displayed in figure 5.9 and table 5.7

	Normoxia pHe 7.4 MDA-MB-231 IC_{50} (µM)	Hypoxia pHe 7.4 MDA-MB-231 IC_{50} (µM)	HCR
Dasatinib	0.037 (± 0.009)	0.036 ± 0.008	1.02
DAS-HAP	1.644 (± 0.22)	0.084 ± 0.03	19.57

Table 5.7 IC_{50} values and HCR for DAS-HAP and dasatinib against the H460 cell line. These are presented in different combinations of oxygen tension and extracellular pH. Each value represents the mean IC_{50} ± SD for three independent experiments

5.4.8 The effect of dasatinib and DAS-HAP against the MDA-MB-231 cell line under normoxic pHe 7.4 and pHe 6.5 conditions

MDA-MB-231 cells were treated with a dose concentration range of dasatinib and DAS-HAP over 96 hours under normoxic and pHe 6.5 conditions. The IC_{50} generated for the two compounds was 0.02μM ± 0.001 for dasatinib and 0.821μM ±0.18 for DAS-HAP (table 5.8). The pHR for dasatinib and DAS-HAP are 1.85 and 2 respectively (table 5.8). A comparison between dasatinib and DAS-HAP under these conditions is graphically displayed below (figure 5.10).

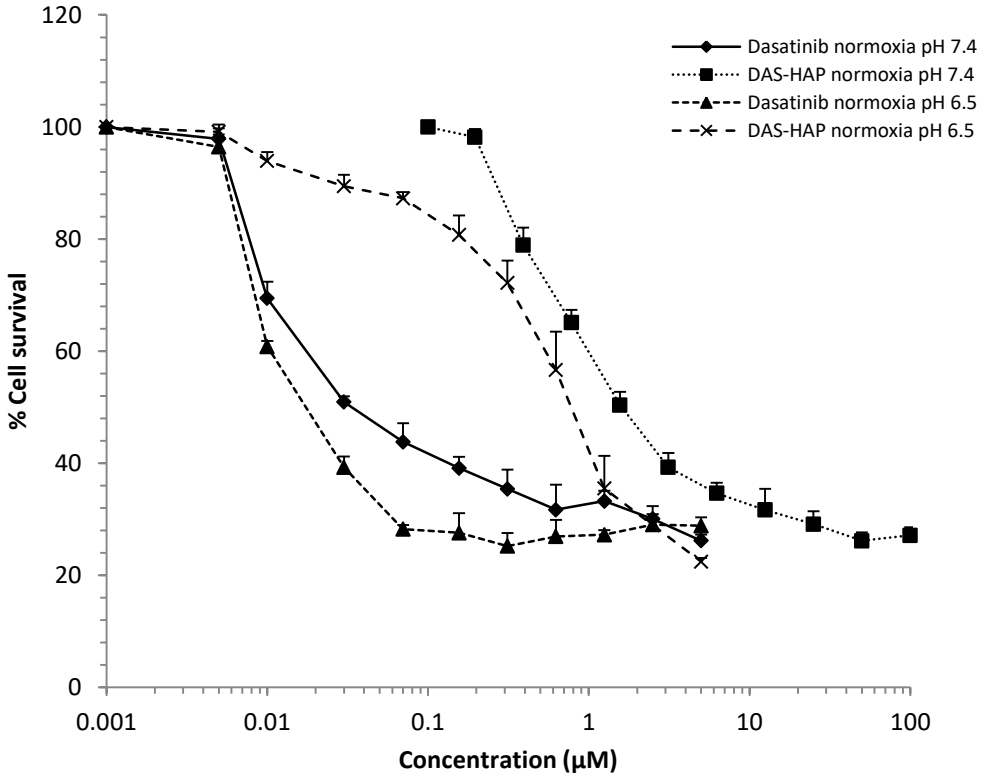


Figure 5.10 Response of MDA-MB-231 cells following a 96hour exposure to dasatinib and DAS-HAP under normoxic pHe 7.4 and pHe 6.5. Dose response curves to dasatinib and DAS-HAP (0.001-100μM) under normoxic pHe 7.4 and pHe 6.5. Each result represents the mean ± standard deviation of three independent experiments.

	Normoxia pHe 7.4 H460 IC_{50} (µM)	Normoxia pHe 6.5 H460 IC_{50} (µM)	pHR
Dasatinib	0.037 (± 0.009)	0.02 (± 0.001)	1.85
DAS-HAP	1.644 (± 0.22)	0.821 (±0.18)	2

Table 5.8 IC_{50} values and pHR for DAS-HAP and dasatinib against the H460 cell line. These are presented in different combinations of extracellular pH under normoxic conditions. Each value represents the mean IC_{50} ± SD for three independent experiments.

5.4.9 The effect of dasatinib and DAS-HAP against the MDA-MB-231 cell line under normoxic pHe 7.4 and hypoxia pHe 6.5 conditions

MDA-MB-231 cells were treated with a dose concentration range of dasatinib and DAS-HAP over 96 hours under hypoxic pHe 6.5 conditions. The IC_{50} generated for the two compounds was 0.016µM ± 0.004 for dasatinib and 0.02µM ± 0.003 for DAS-HAP (table 5.9). The TME enhancement ratio for dasatinib and DAS-HAP are 2.31 and 82.2 (table 5.9) respectively and demonstrates that DAS-HAP although not as toxic as dasatinib, but has a much higher selectivity for the TME conditions. A comparison between dasatinib and DAS-HAP under these conditions is graphically displayed below (figure 5.11).

	Normoxia pHe 7.4 H460 IC_{50} (µM)	Hypoxia pHe 6.5 H460 IC_{50} (µM)	TME
Dasatinib	0.037 (± 0.009)	0.016 ± 0.004	2.31
DAS-HAP	1.644 (± 0.22)	0.02 ± 0.003	82.2

Table 5.9 IC_{50} values and the TME ratio for DAS-HAP and dasatinib against the H460 cell line. These are presented in different combinations of oxygen concentration and pHe. Each value represents the mean IC_{50} ± SD for three independent experiments.

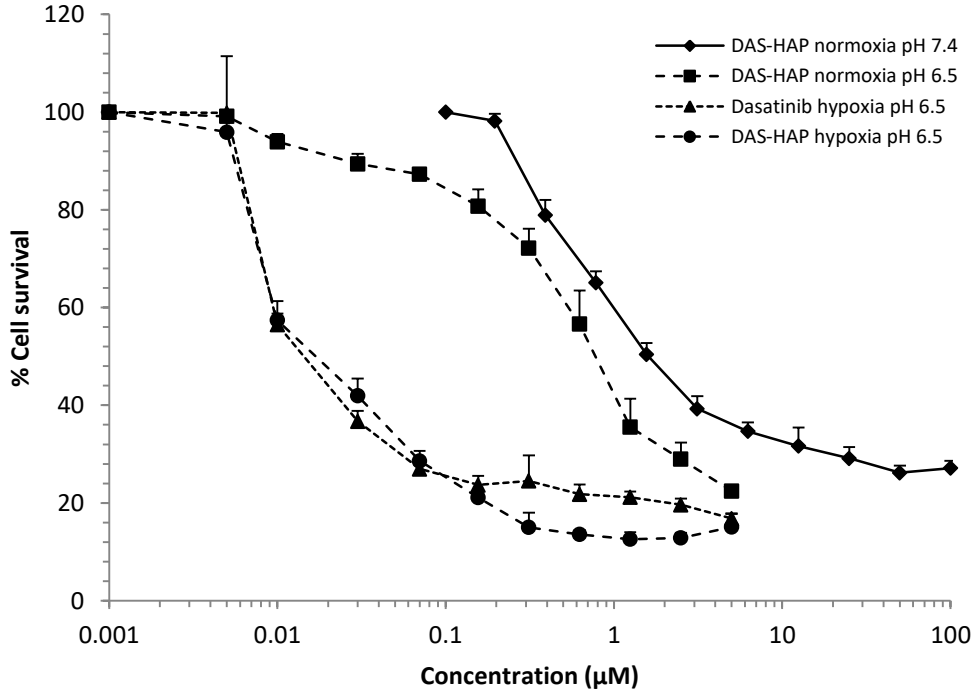


Figure 5.11 Response of MDA-MB-231 cells following a 96hour exposure to dasatinib and DAS-HAP under normoxic pHe 7.4 and hypoxia pHe 6.5. Each result represents the mean ± standard deviation of three independent experiments.

5.4.10 The effect of dasatinib and DAS-HAP against the MDA-MB-231 and ARPE-19 cell line under normoxic pHe 7.4

The SI index for dasatinib and DAS-HAP are 3.33 and 43.28 respectively (table 5.10). This indicates that DAS-HAP is less toxic towards the normal cells and more selective towards the MDA-MB-231 cancerous cell lines in comparison to dasatinib.

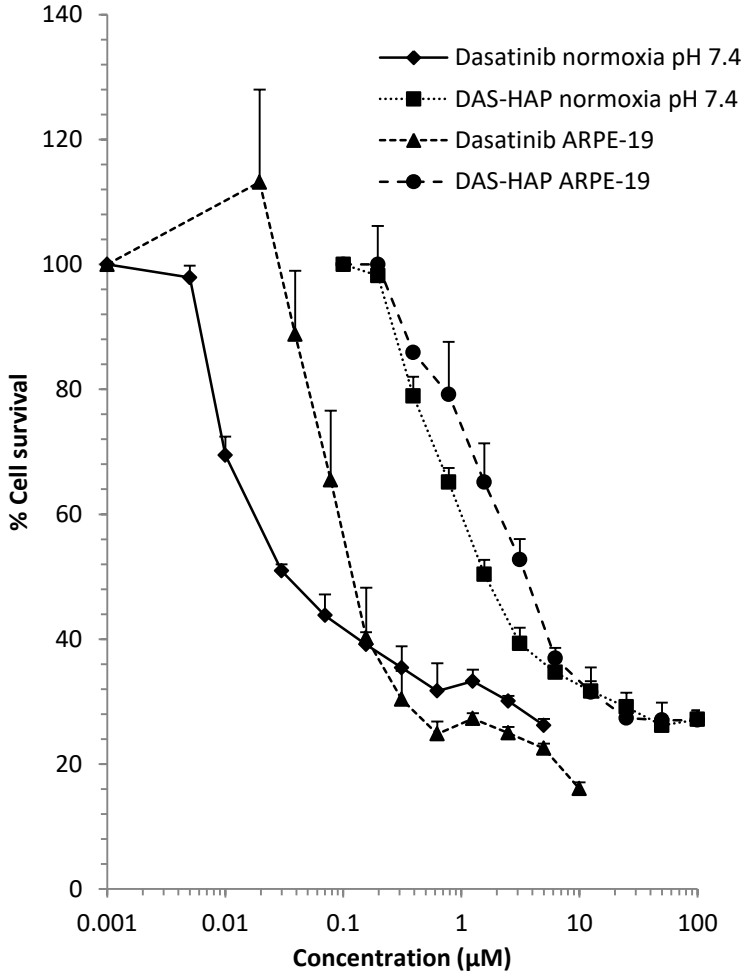


Figure 5.12 Response of MDA-MB-231 and ARPE-19 cells following a 96hour exposure to dasatinib and DAS-HAP under normoxic pHe 7.4. Each result represents the mean ± standard deviation of three independent experiments.

	Normoxia pHe 7.4 H460 IC_{50} (µM)	ARPE-19 IC_{50} (µM)	SI
Dasatinib	9.71 (± 1.06)	0.12 (± 0.02)	0.01
DAS-HAP	IC_{50} >100	3.636 (± 0.56)	0.03

Table 5.10 IC_{50} values, SI for DAS-HAP and dasatinib against the H460 and ARPE-19 cell line. These are presented in different combinations of extracellular pH under normoxic conditions. Each value represents the mean IC_{50} ± SD for three independent experiments.

5.4.11 Summary on the effect of dasatinib and DAS-HAP against the H460 and ARPE-19 cell line under TME conditions

	Normoxia pHe 7.4 H460 IC_{50} (µM)	Normoxia pHe 6.5 H460 IC_{50} (µM)	Hypoxia pH e7.4 H460 IC_{50} (µM)	Hypoxia pHe 6.5 H460 IC_{50} (µM)	ARPE-19 IC_{50} (µM)
Dasatinib	9.71 (± 1.06)	10.34 (± 0.94)	9.96 (± 4.78)	6.76 (± 1.31)	0.12 (± 0.02)
DAS-HAP	IC_{50} >100µM	IC_{50} >100µM	0.44 (± 0.02)	67.73 (± 0.003)	3.636 (± 0.56)

Table 5.11 Summary of IC_{50} values for DAS-HAP and dasatinib against the H460 cell line. These are presented in different combinations of oxygen tension and extracellular pH including the combination of hypoxia and acidic pHe. Each value represents the mean IC_{50} ± SD for three independent experiments.

	Normoxia pHe 7.4 MDA-MB-213 IC_{50} (µM)	**Normoxia pHe 6.5 MDA-MB-213 IC_{50} (µM)**	**Hypoxia pHe 7.4 MDA-MB-213 IC_{50} (µM)**	**Hypoxia pHe 6.5 MDA-MB-213 IC_{50} (µM)**	**ARPE-19 IC_{50} (µM)**
Dasatinib	0.037 (± 0.009)	0.02 (± 0.001)	0.036 (± 0.008)	0.016 (± 0.004)	0.12 (± 0.02)
DAS HAP	1.644 (± 0.22)	0.821 (± 0.18)	0.084 (± 0.03)	0.02 (± 0.003)	3.636 (± 0.56)

Table 5.12 Summary of IC_{50} values for DAS-HAP and dasatinib against the MDA-MB-231 cell line. These are presented in different combinations of oxygen concentration pHe. Each value represents the mean IC_{50} ± SD for three independent experiments.

Compound	Cell	pHR	HCR	TME ratio	SI	SI (TME)
Cisplatin	HCT116 $P53^{+/+}$	0.75	0.44	0.3	1.84	0.55
5-FU		0.55	0.40	0.27	2.10	0.57
Etoposide		0.76	0.61	0.57	0.59	0.34
Tirapazamine		0.61	9.55	n/a	2.33	22.46*
Dasatinib	MDA-MB-231	1.85	1.02	2.31	3.33	7.5
	H460	0.93	0.97	1.43	0.01	0.01
DAS-HAP	MDA-MB-231	2.00	19.57	82.2	43.28	181.8
	H460	1	227.27	1.47	0.03	0.05

Table 5.13 The effect of the TME on the response of cells to standard cytotoxic drugs, tirapazamine, dasatinib and DAS-HAP. pHR, HCR and TME represent the pH ratio, the hypoxic cytotoxicity ratio and tumour microenvironment ratio as defined in the methods section 3.3.3. * refers to the SI value for the hypoxic data only and not the combo of hypoxia and acidic pHe.

Although DAS-HAP was not as active as dasatinib, it did however display preferential activity towards all the TME parameters tested in this phenotypic screen in the MDA-MB-231 cells and this is defined by the high TME ratio of 82.2. In comparison, this is 35 fold greater than dasatinib and when compared to the classic chemotherapeutic compounds, it is 144 fold greater than etoposide, 274 fold greater than cisplatin and 304 fold greater than 5-FU (table 5.13). With regards to the selectivity indices, the DAS-HAP is superior to dasatinib alone with a SI value of 181.8 compared to 7.5 in the MDA-MB-231 cell line. In H460 cells however, the unexpected observation of reduced cytotoxicity under full TME conditions (both

hypoxia and acidic pHe) complicates matters and further experiments are required to explore this observation.

5.4.12 Western blot analysis for the detection of Src induction by dasatinib and DAS-HAP under TME condition

For further investigation of dasatinib and DAS-HAP on Src and pSrc only the MDA-MB-231 cells were used as dasatinib and the DAS-HAP showcased much more selectivity towards this cancer cell line under all the tumour microenvironment conditions tested. MDA-MB-231 cells were treated with either dasatinib or DAS-HAP with a concentration range of 0.005-0.5μM under the following conditions: (i) normoxia pHe 7.4, (ii) normoxia pHe 6.5, (iii) hypoxia pHe 7.4 and (iv) hypoxia pHe 6.5 (these conditions were the same as those used in the chemosensitivity studies). Following a 24 hour drug exposure, the cells were lysed in either the biological class II safety cabinet for normoxic conditions or the H35 hypoxystation for hypoxic conditions and Western blot analysis conducted for both Src and p527-Src, the results of which are presented in figures 5.13 and 5.14 respectively.

The western blot data for Src expression did not display much of a difference in activity under all concentrations tested for both dasatinib and the DAS-HAP. In general, the expression levels of Src were similar in treated samples and the DMSO 0.1% vehicle control (figure 5.13). In contrast however, dasatinib did inhibit pSrc 527 under normoxic conditions at pHe 7.4 at a concentration of 0.05μM. In comparison the concentration of DAS-HAP required to inhibit pSrc 527 was much higher (0.5μM) which is consistent with the prodrug nature of the DAS-HAP compound. Under hypoxic conditions, inhibition of pSrc 527 by DAS-HAP occurred at much lower

concentrations (figure 5.16). The results for DAS-HAP under hypoxic combined with acidic pHe conditions are less clear cut with evidence of inhibition of pSrc 527 at 0.005 μM conflicting with a much stronger band at 0.05 μM (figure 5.14). Further studies are required to address this but they were not possible due to time constraints in this project.

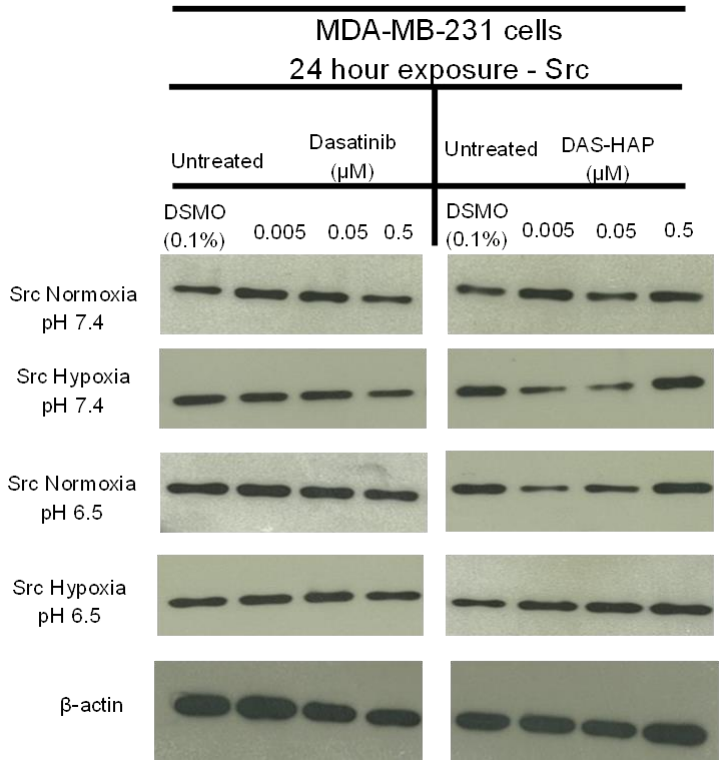


Figure 5.13 Western blot analysis of the effect of dasatinib or DAS-HAP (24 hour drug exposure) on SRC phosphorylation induction in MDA-MB-231 cancer cells under the tumour microenvironment conditions (n=1).

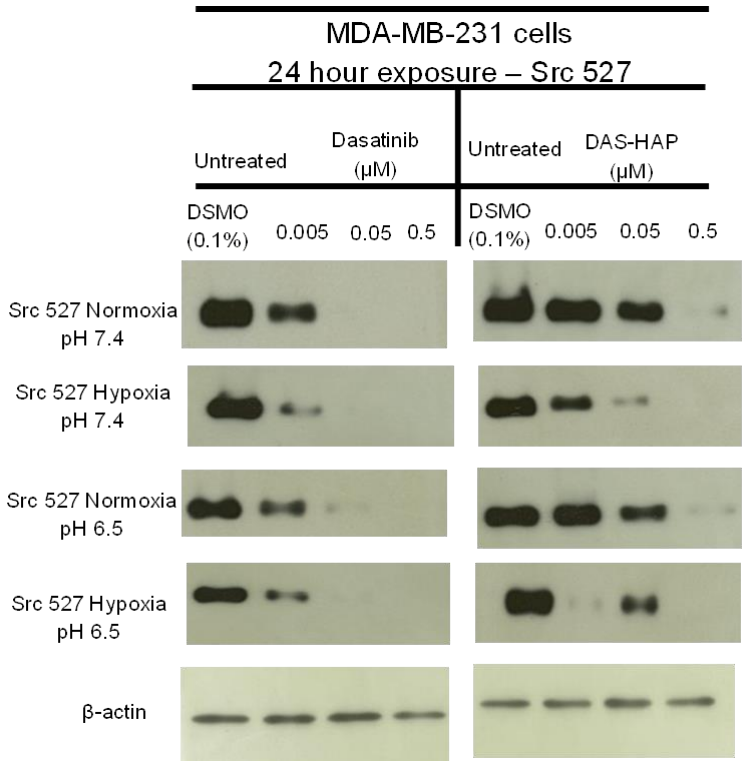


Figure 5.14 Western blot analysis of the effect of dasatinib or DAS-HAP (24 hour drug exposure) on SRC 527 phosphorylation induction in MDA-MB-231 cancer cells under the tumour microenvironment conditions (n=1).

5.5 Discussion

The purpose of this study was to evaluate the preferential TME activity of DAS-HAP that was provided by Associate Professor Adam Patterson (Auckland Cancer Society Research Centre, New Zealand) on the MDA-MB-231 and H460 cell lines by utilising the phenotypic screening strategy.

Structurally dasatinib and DAS-HAP are very similar; the only difference is that DAS-HAP has the addition of an 2-nitroimidazole as a hypoxia trigger. Dasatinib inhibits both the Src family tyrosine kinases and the BCR-ABL oncogene and is approved for the use of patients with imatinib refractory CML (Chen and Chen, 2015). As the tumour cell lines used in this study are MDA-MB-231 and H460 cell lines, then the main focus shall be on targeting of the Src tyrosine kinases. Dasatinib is a very potent TKI with IC_{50} values within the range of 0.037-0.016μM under the different parameters tested against the MDA-MB-231 cell lines as demonstrated in this study (table 5.12). This compound was more sensitive towards the MDA-MB-231 cell line in comparison to the H460 cell line under normoxic pHe 7.4 conditions. This data is in line with a study carried out by Ahmadi *et al* (2014) who tested the response of a panel of tumour cell lines to a series of well-established 1^{st} and 2^{nd} generation TKI's. The results displayed that the TKI's had a much more favourable response towards the triple negative breast cancer cell lines in comparison to the lung cancer cells (Ahmadi et al., 2014).

When both dasatinib and DAS-HAP were tested under hypoxic conditions, it was clear that these displayed HAP like properties. One of the most striking results was the response of DAS-HAP towards the H460 cells under the pHe 7.4 conditions which was 0.44μM with a HCR of 227.2 which is much higher than all of the

compounds tested in this study and those classic chemotherapeutic agents tested (table 5.13) including tirapazamine. This demonstrates that DAS-HAP is undergoing bioreductive activation under hypoxic pHe 7.4 conditions. Unfortunately, the significant increase in potency under hypoxia and pHe 7.4 was not observed under hypoxia combined with mild acidic conditions and in this case, it appears that DAS-HAP is becoming inactive under mild acidity. The TME enhancement ratio of DAS-HAP against the H460 and MDA-MB-231 cell line was 1.47 and 82.2 respectively which was much greater than dasatinib especially against the MDA-MB-231 cell line (1.43 and 2.31 – table 5.13). The reasons for the inhibitory effect of acidic pHe and hypoxia on DAS-HAP efficacy against H460 cells is not known and further studies are required to (i) confirm that this result is correct and not an experimental artefact and (ii) to understand the mechanism responsible for this observation. The results of this study nevertheless demonstrated that DAS-HAP has preferential activity towards the H460 cell line under hypoxic conditions although the inhibitory effects of acidic pHe results are a cause for concern.

A different picture emerged however when the same set of experiments were conducted against MDA-MB-231 cells. Although DAS-HAP is very potent, it is not as toxic as dasatinib towards the MDA-MB-231 cells (table 5.12). The enhancement ratios collectively put things into perspective by demonstrating its superior enhancement ratios compared to dasatinib. These were pHR of 2 against 1.85, a HCR of 19.57 against 2.31, a TME ratio of 82.2 against 2.31, a SI at pHe 7.4 of 43.28 against 3.33 and a SI (TME) of 181.8 against 7.5. This was not only superior against dasatinib which is a well-established TKI but also all of the classic chemotherapeutic compounds tested earlier (table 5.13). It is worth noting that the

inhibitory effect of acidic pHe observed with the H460 cell line was not observed in the MDA-MB-231 cell line.

Src plays many pivotal roles as intermediates in tumourigenesis such as, adhesion, invasion, metastasis, angiogenesis and proliferation (Frame, 2002). It was important to further understand the role of dasatinib and DAS-HAP on the Src kinases and its ability to inhibit these under TME conditions. The western blot data for MDA-MB-231 cells demonstrated that there was no major difference in Src expression between dasatinib and the DAS-HAP (figure 5.13). In contrast, there was a notable difference in the inhibition of pSrc 527 between normoxic and hypoxic conditions. Dasatinib showcased this at concentrations of 0.05μM for normoxic and 0.005μM for hypoxia conditions. In comparison, DAS-HAP showed inhibition of pSrc (527) at concentrations of 0.5μM for normoxic and 0.05μM for hypoxic (figure 5.14). This data is in line with the chemosensitivity studies carried out earlier (table 5.11 and 5.12) which demonstrate that dasatinib was more active than DAS-HAP. However this cannot be concluded with the investigation of pSrc 527 alone as pSrc 527 only inactivates c-Src. For full activation there is another Src which need to be phosphorylated which is Src 419 (Ahmadi et al., 2014) and further studies are required to assess the effects of these compounds on pSrc 419. The chemosensitivity data also demonstrated the selectivity of DAS-HAP towards the MDA-MB-231 cells and this could be due to the high expression of Src in cancer cells in comparison to the normal cells (Frame, 2002).

Through this phenotypic screening strategy, MDA-MB-231 cell line was considered for further studies as this compound displayed preferential activity towards all of the TME conditions tested for. The selectivity of DAS-HAP towards the MDA-MB-231

cells is much higher than those in comparison to classic chemotherapeutic drugs tested which demonstrates that this type of phenotypic screen is able to find new novel compounds which have more favourable towards cancer cells (the selectivity index) and preferential activity against cells under conditions that mimic the physiological TME.

Chapter 6: Phenotypic evaluation of novel organometallic compounds under TME conditions

6.1 Introduction

The previous chapters have described the establishment of a phenotypic screen to identify compounds with preferential activity against the tumour microenvironment. The work reported in the previous two chapters have focused on compounds that were designed to be preferentially activated under hypoxia but in this chapter and subsequent chapters will evaluate compounds that had not been designed to exploit the tumour microenvironment. This therefore this represents the first attempt to identify novel TME active agents in the true spirit of phenotypic screening. These compounds were synthesised by Professor Mohan Rao (North Eastern Hill University, Shillong, India) and they are all organometallic compounds. Brief details of the various types of compounds that have been evaluated are summarised below.

6.1.1 Napthyridines

Napthyridine consist of two pyridine rings which are fused together through two adjacent carbon atoms where each ring contains one nitrogen atom (Madaan et al., 2015).

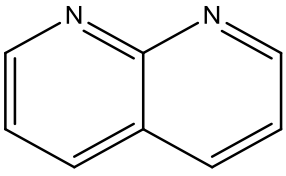


Figure 6.1 1,8-napthyridine structure (Adhikari et al., 2018c; Madaan et al., 2015).

There are six different isomers of napthyridines with the 1,8-napthyridine derivatives gaining the most interest for their numerous biological activities such as, anti-microbial, anti-viral, as well as anti-cancer properties (Brown, 1965; Daniels et al., 2007; Srivastava et al., 2007). There are only a few napthyridine metal compounds which consist of the half-sandwich metal platinum groups reported to date irrespective of its interesting chemistry and bonding modes (Sinha et al., 2013; Suzuki, 2006). Upon reflection on the different bonding modes corresponding with the napthyridine ligands it was hypothesised that if pyridine, phenol and aniline was substituted at the 2-position of the 1,8-napthyridine consisting of either ruthenium, iridium or rhodium it could behave as anti-cancer agents (Adhikari et al., 2018c).

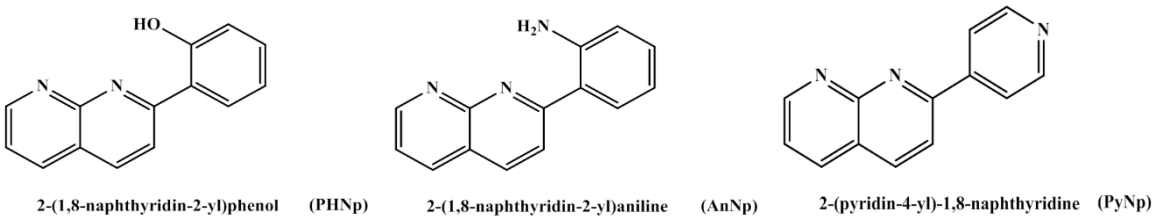


Figure 6.2 Ligands used in the synthesis process for napthyridine ligands with either phenol (PHNp), anailine (AnNp) or pyridine (PyNp), consisting of either ruthenium, iridium or rhodium which are known as KMR-SA-21-29 and KMR-LA-LC (Adhikari et al., 2018c).

6.1.2 Thiourea derivatives

Thiourea are a class of organic compounds having sulphur with the general formula (R1R2N)(R3R4N)C=S (Shakeel et al., 2016).

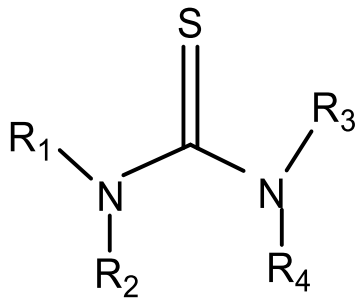
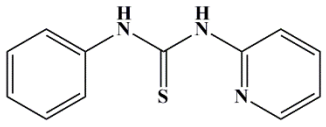


Figure 6.3 The general structure of Thiourea (Lapasam et al., 2019; Shakeel et al., 2016).

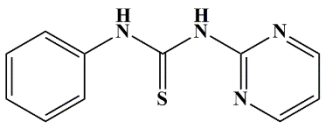
Half-sandwich arene d^6 compounds have been extensively researched for their organometallic metal based anti-cancer properties (Habtemariam et al., 2006). This has 'opened the lid' for new research into the synthesis of such compounds which consist of a half sandwich compound with an organometallic metal base for its anti-cancer properties.

Much interest has been paid towards the synthesis and development of transition metal compounds which consist of thiourea ligands due to their interesting binding modes (Lapasam et al., 2019). Thiourea derivatives are also known to exhibit a wide range of biological activities such as anti-bacterial, anti-fungal, anti-malarial and anti-tumour activities which is dependent on their ligands (Lin et al., 2013; Mahajan et al., 2007).

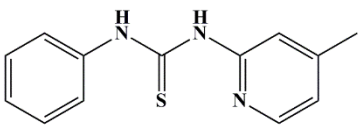
Professor Rao used 3 different pyridyl ligands with each one consisting of ruthenium, iridium or rhodium. The different pyridyl structures are shown below (figure 6.4)



1-phenyl-3-(pyridin-2-yl)thiourea (L1)



1-phenyl-3-(pyrimidin-2-yl)thiourea (L2)

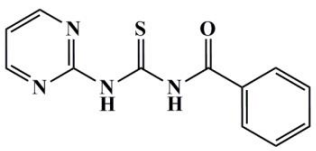


1-(4-methylpyridin-2-yl)-3-phenylthiourea (L3)

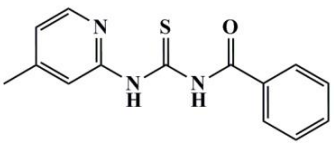
Figure 6.4 Ligands used in the synthesis process for transition metal half-sandwich compounds of pyridyl thiourea derivatives, consisting of either ruthenium, iridium or rhodium which are known as KMR-SA-1-9 and KMR-L1-L3 (Adhikari et al., 2018b).

6.1.3 Benzylthiourea

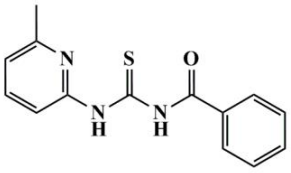
Benzolylthiourea ligands are of interest in regards to coordination chemistry due to their interesting behaviour towards various transition metals. In this particular study rhodium, iridium and ruthenium half-sandwich compounds consisting of pyrimidyl and pyridyl thiourea derivatives are tested for their chemotherapy like properties under TME conditions.



benzoyl(2-pyrimidyl)thiourea (L1)



benzoyl(4-picolyl)thiourea (L2)

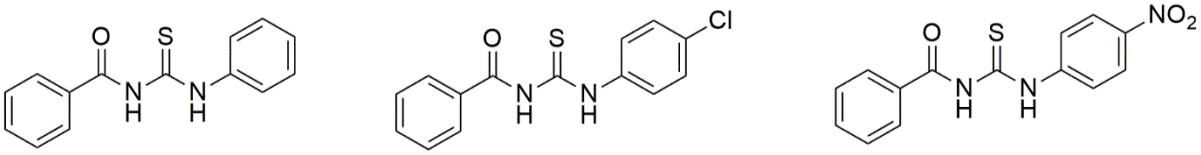


benzoyl(6-picolyl)thiourea (L3)

Figure 6.5 Ligands used in the synthesis process for transition metal half-sandwich compounds of pyrimidyl and pyridyl thiourea derivatives. These consist of either ruthenium, iridium or rhodium. These are known as KMR-SA-30-38 and KMR-L10-L12 (Adhikari et al., 2018a)

6.1.4 Aroylthiourea

Aroylthiourea are versatile in coordinating with a wide range of metal ions as neutral, dibasic or monobasic ligands (Lapasam et al., 2019). Therefore, the biological relevance of ruthenium-arene and aroylthiourea has warranted the biological evaluation of ruthenium-arene complexes which consist of the aroylthiourea ligand for their cytotoxic ability and selectivity towards cancer cells.



L1 L2 L3

Figure 6.6 Ligands used in the synthesis process for aroylthiourea compounds. These consist of either ruthenium, iridium or rhodium. These are known as KMR-AL-9-17 (Lapasam et al., 2019).

The compounds described above in this chapter were all used to treat the following different cell lines: colorectal cancer HCT116, HT29 and the pancreatic cancer Mia-PaCa-2. As in previous chapters, the selection of cell lines was not based on any biochemical rationale but for pragmatic reasons of easy and reliable growth in cell culture. The ARPE-19 non-cancer cells was also selected for use in this chapter.

6.2 Aims

The purpose of this chapter is the phenotypic evaluation of a series of organometallic compounds synthesised by Professor Mohan Rao (Eastern Hill University, India) with the aim of identifying novel compounds with preferential activity against cells under tumour microenvironmental conditions. The compounds evaluated included novel metal ruthenium, iridium and rhodium based napthyridines, thiourea derivatives, aroylthiourea ligands and benzoylthiourea compounds. All of the different compounds will be tested for their cytotoxic ability and selectivity towards the colorectal cancer HCT116, HT29 and the pancreatic cancer Mia-PaCa-2 cell lines compared to non-cancer ARPE-19 cells. The ability to become activated under hypoxic, mild pHe acidity or a combination of the two conditions using the HCT116 cell line will be determined and preliminary studies looking at the potential mechanism of action of ‘hit’ compounds will be reported.

6.3 Methods

6.3.1 Compounds

All synthesised compounds were synthesised by Professor Mohan Rao and his students (Eastern Hill University, India) and all compounds were dissolved in (DMSO) at 100mM (which are shown below in figures 6.7 to 6.10), aliquoted and stored at -20°C.

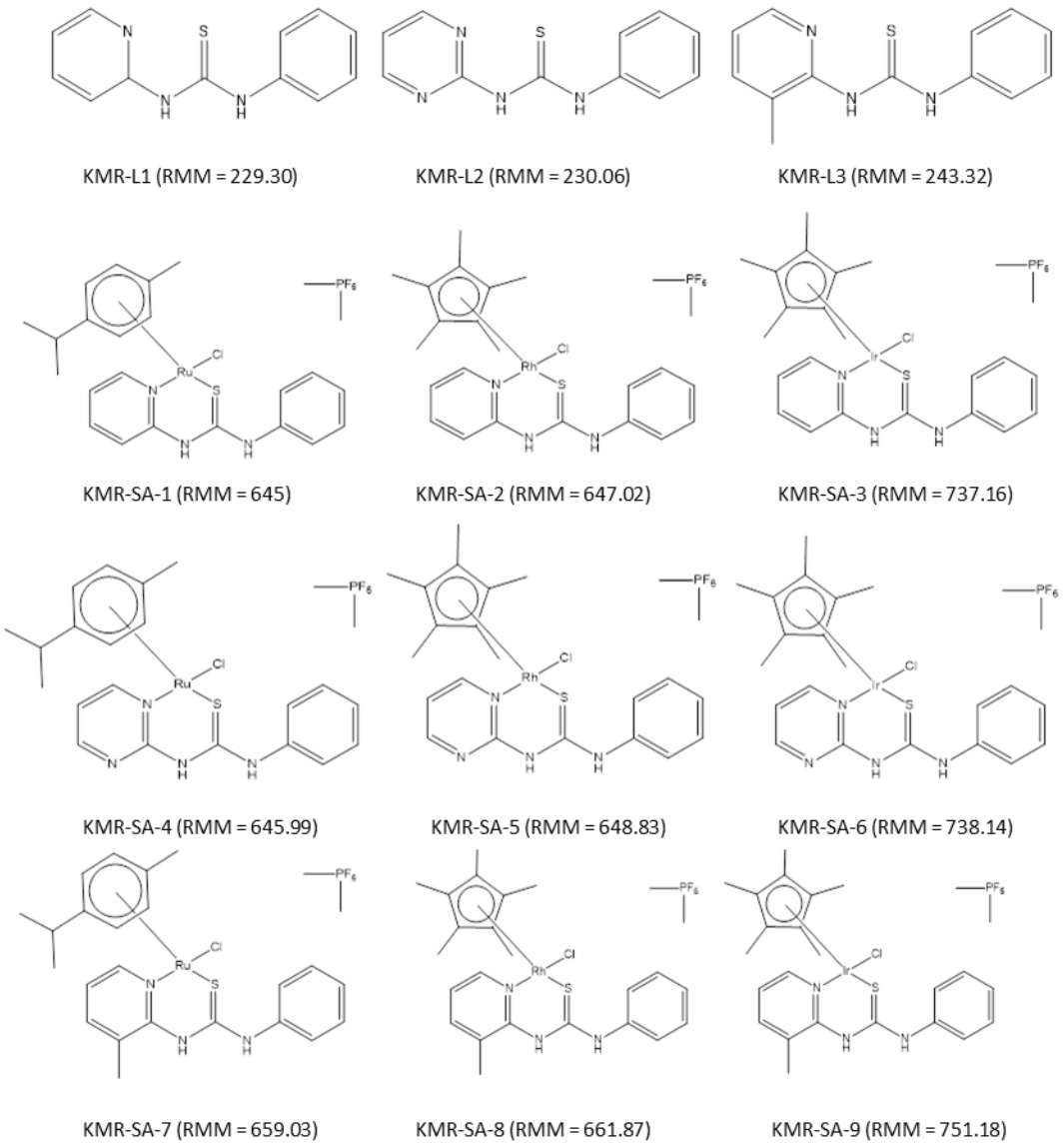


Figure 6.7 Chemical structures of thiourea ligands with molecular weights.

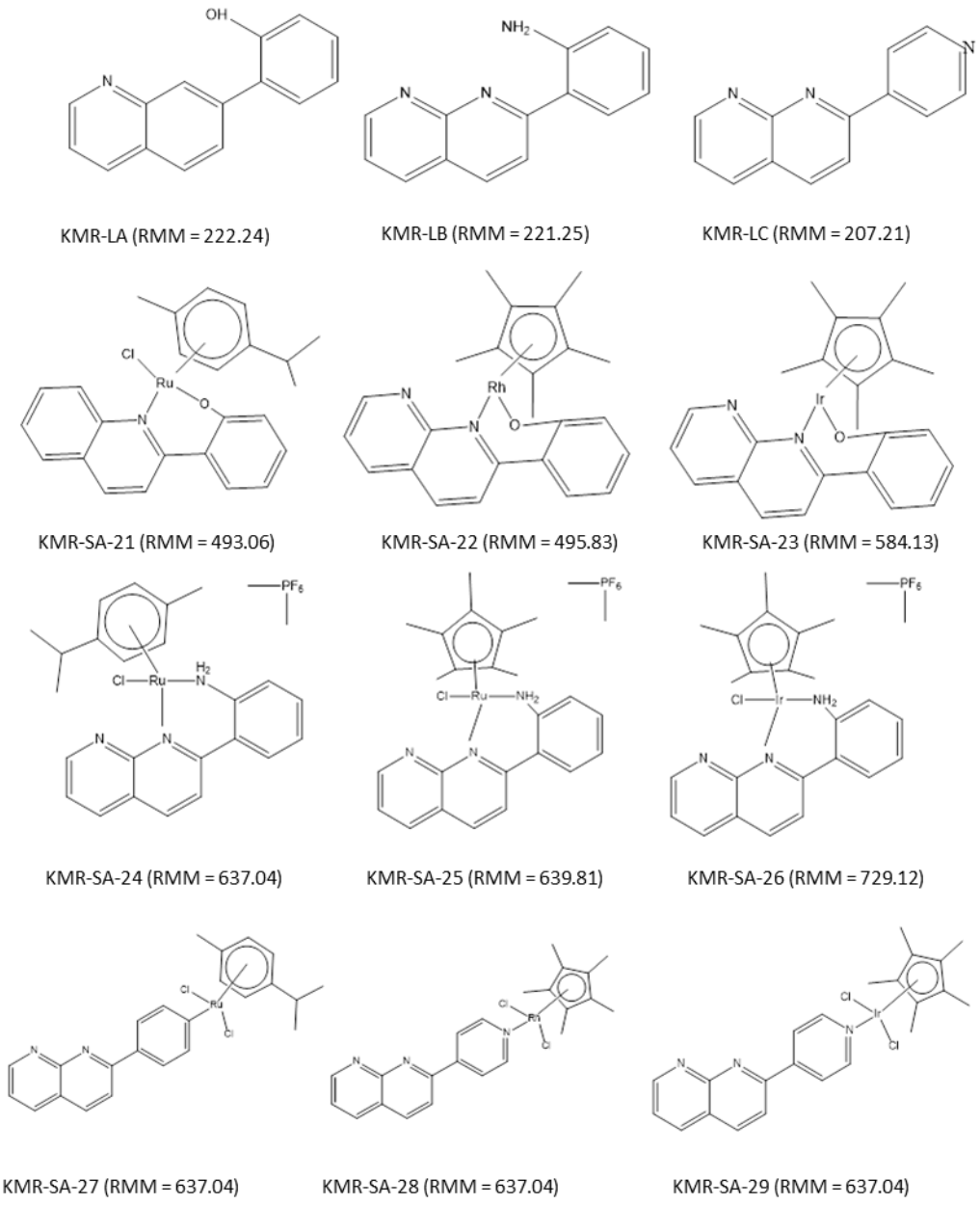


Figure 6.8 Chemical structures of napthyridines with molecular weights.

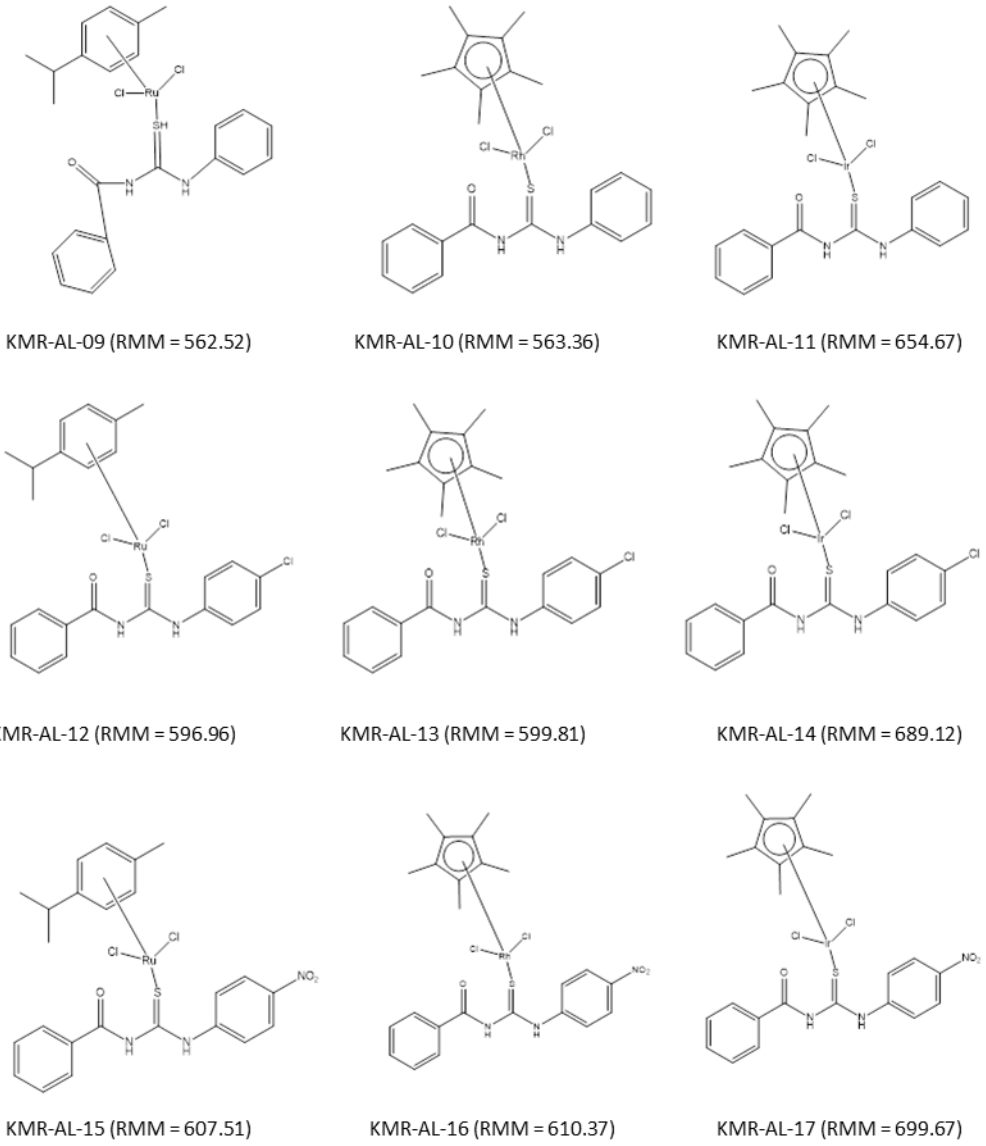
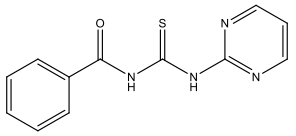
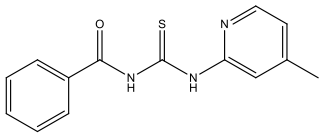


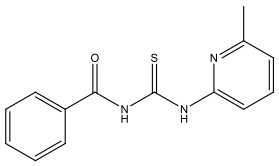
Figure 6.9 Chemical structures of aroylthiourea compounds with molecular weights.



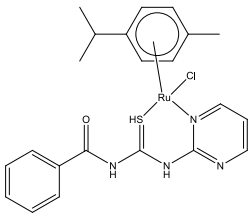
KMR-L10 (RMM = 258.31)



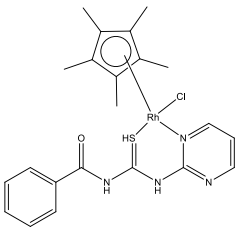
KMR-L11 (RMM = 271.33)



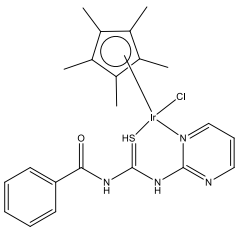
KMR-L12 (RMM = 271.33)



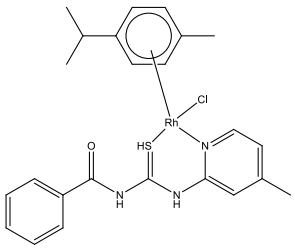
KMR-SA-30 (RMM = 564.50)



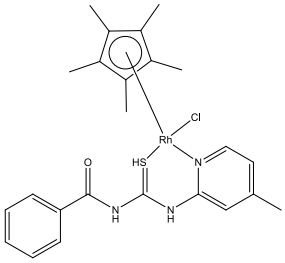
KMR-SA-31 (RMM = 567.33)



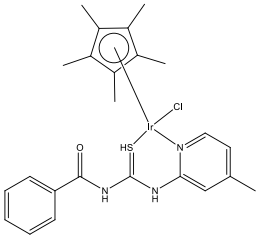
KMR-SA-32 (RMM = 656.64)



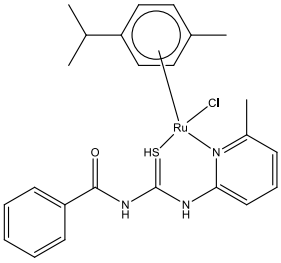
KMR-SA-33 (RMM = 577.53)



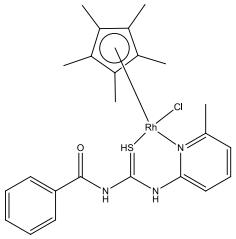
KMR-SA-34 (RMM = 579.03)



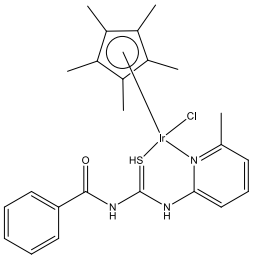
KMR-SA-35 (RMM = 669.68)



KMR-SA-36 (RMM = 577.53)



KMR-SA-37 (RMM = 579.03)



KMR-SA-38 (RMM = 669.68)

Figure 6.10 Chemical structures of benzoylthiourea compounds with molecular weights.

6.3.2 Cell culture and chemosensitivity

Cell culture, adjusting the pH of the media, drug dilutions and chemosensitivity studies were carried out as previously described in the general methods chapter (see sections 2.2, 2.6-2.8). The following cell lines were used for this study which were the colorectal cancer HCT116, HT29 and the pancreatic cancer Mia-PaCa-2 cell line. Any variation from these methods are indicated in the figure legends and described in the results section.

6.3.3 Western blot analysis

Cells were treated with hit compounds for a duration of 24 hours at a drug concentration of 12.5, 25 and 50μM. These concentrations were chosen as it is a therapeutically relevant concentration (IC_{50} under hypoxic conditions was 34.53 ± 13.86 μM) and a 24 hour duration of drug exposure was selected as minimal cell death had occurred at this time point (as illustrated in figure 6.12). Controls were treated with media alone or with DMSO (0.1% v/v) vehicle control (which has been previously reported not to be active to the cells). These were carried out under the following tumour microenvironment conditions, normoxia pHe 7.4, and hypoxia pHe 7. Upon completion of treatment, cells were harvested for western blot analysis of phosphorylated **γ**H2AX (as a marker of DNA damage). Details of western blot methodology and antibody dilutions are described in methods (see sections 2.12 and table 2.2).

6.3.4 Statistical analysis

Statistical analysis was carried out using Microsoft Excel data analysis. All experiments were done in triplicates (n=3) unless specified in the figure legend, and results were presented as the mean ± SD. Statistical significance was determined by

means of a two-tailed Student’s t-test, where significance was only accepted when $p \leq 0.05$.

6.4 Results

6.4.1 Chemosensitivity testing of thiourea ligand derivatives under normoxic pHe 7.4 conditions

Compounds KMR-L1 to KMR-L3 represent the ligand only (do not contain metal) and under standard cell culture conditions, these did not display any activity towards the cancer cell lines HCT116 and Mia-PaCa-2 tested with IC_{50} values all greater than 100μM (table 6.1).

	IC_{50} (μM)			Selectivity index	
Compound	HCT116	Mia-PaCa-2	ARPE-19	HCT116	Mia-PaCa-2
KMR-SA-1	17.53 ± 2.96	10.05 ± 0.17	21.31 ± 3.53	1.21	2.12
KMR-SA-2	9.69 ± 0.97	10.18 ± 0.38	19.47 ± 2.57	2.00	1.91
KMR-SA-3	15.38 ± 3.21	9.97 ± 0.11	24.14 ± 8.33	1.56	2.49
KMR-SA-4	68.44 ± 5.83	77.45 ± 2.71	67.52 ± 16.98	0.98	0.87
KMR-SA-5	44.83 ± 11.70	33.67 ± 3.97	84.41 ± 16.51	1.88	2.50
KMR-SA-6	35.60 ± 7.35	33.18 ± 0.40	66.28 ± 3.97	1.86	1.99
KMR-SA-7	18.23 ± 3.26	16.76 ± 0.42	20.82 ± 0.57	1.14	1.24
KMR-SA-8	9.16 ± 0.85	9.49 ± 0.33	15.75 ± 2.87	1.69	1.66
KMR-SA-9	16.03 ± 2.13	9.11 ± 0.09	19.78 ± 1.80	1.23	2.17
KMR-L1	IC_{50} > 100	IC_{50} > 100	IC_{50} > 100	-	-
KMR-L2	IC_{50} > 100	IC_{50} > 100	IC_{50} > 100	-	-
KMR-L3	IC_{50} > 100	IC_{50} > 100	IC_{50} > 100	-	-

Table 6.1 The response of HCT116, Mia-PaCa-2 and ARPE-19 cells following a 96hour exposure to thiourea ligand compounds under normoxic pHe 7.4 conditions. Each result represents the mean ± standard deviation of three independent experiments.

All the remainder thiourea compounds (KMR-SA-1-9) displayed varying degrees of activity towards the HCT116 and Mia-PaCa-2 cell lines. Thiourea ligands consisting of the L2 configuration (KMR-SA-4-6) were moderately active against both cell lines exhibiting an IC_{50} range of 33.18 (± 0.40) to 77.45µM (± 2.71). Compounds KMR-SA-1-3 (ligand 1) and KMR-SA-7-9 (ligand 2) also had IC_{50} values that were moderately active towards the HCT116 and Mia-PaCa-2 cell lines with IC_{50} values of 9.16 (± 0.85) – 18.23µM (± 3.26) respectively. The classic chemotherapeutic drugs tested earlier in chapter 3 were much more active than the thiourea ligand compounds tested in this study (KMR-SA-1-9). Although the selectivity of the thiourea ligand compounds was not high, these were much more selective towards the HCT116 and Mia-PaCa-2 cell lines in comparison to etoposide (table 6.1 and table 6.10). Only compounds KMR-SA-2 and KMR-SA-5 displayed a higher selectivity index than cisplatin towards the HCT116 cell line. All the thiourea ligand compounds displayed higher selectivity towards the Mia-PaCa-2 cell line (apart from KMR-SA-4). Compounds KMR-SA1, KMR-SA-3, KMR-SA-5 and KMR-SA-9 displayed a higher selectivity towards the Mia-PaCa-2 cell line in comparison to all of the classic compounds tested earlier (table 6.1 and table 6.10). Unfortunately these compounds were not tested any further due to limited time constraints.

6.4.2 Chemosensitivity testing of 1,8 napthyridine under normoxic pHe 7.4 conditions

KMR-LA displayed some moderate activity towards the HCT116 and Mia-PaCa-2 cell lines 39.90 (± 1.60) and 62.45µM (± 26.28) respectively. KMR-LB-LC did not display any activity (IC_{50} > 100µM).

1, 8 napthyridine compounds

Compound	IC_{50} (µM)			Selectivity index	
	HCT116 cells	Mia-PaCa-2 cells	ARPE-19 cells	HCT116	Mia-PaCa-2
KMR-SA-21	82.09 ± 3.55	IC_{50} > 100	IC_{50} > 100	1.21	-
KMR-SA-22	61.28 ± 13.80	25.13 ± 6.07	IC_{50} > 100	>1.61	>3.97
KMR-SA-23	37.80 ± 4.90	52.13 ± 12.28	IC_{50} > 100	>2.64	>1.91
KMR-SA-24	210.32 ± 3.44	IC_{50}> 100	678.55 ± 3.77†	3.22	-
KMR-SA-25	84.20 ± 7.30	IC_{50} > 100	436.53 ± 22.12†	5.18	-
KMR-SA-26	31.33 ± 12.93	82.09 ± 2.55	IC_{50} > 100	>3.19	>1.21
KMR-SA-27	IC_{50} > 100	IC_{50} > 100	IC_{50} > 100	-	-
KMR-SA-28	IC_{50} > 100	61.36 ± 3.25	IC_{50} > 100	-	1.62
KMR-SA-29	IC_{50} > 100	IC_{50} > 100	IC_{50} > 100	-	-
KMR-LA	39.90 ± 1.6	62.45 ± 26.28	IC_{50} > 100	>2.50	>1.60
KMR-LB	IC_{50} > 100	IC_{50} > 100	IC_{50} > 100	-	-
KMR-LC	IC_{50} > 100	IC_{50} > 100	IC_{50} > 100	-	-

Table 6.2 The response of HCT116, Mia-PaCa-2 and ARPE-19 cells following a 96hour exposure to 1,8 napthyridine compounds under normoxic pHe 7.4 conditions. Each result represents the mean ± standard deviation of three independent experiments. † refers to compounds that were re-screened using a wider concentration range (0.3-1000µM).

Napthyridines consisting of the KMR-LA configuration (KMR-SA-21-23) exhibited mild activity in contrast to napthyridines consisting the KMR-LC configuration which did not display any activity IC_{50} > 100μM. KMR-SA-26 was the most active napthyridine tested against the HCT116 cell line where as KMR-SA-22 was the most active towards the Mia-PaCa-2 cell line 31.33 (± 12.93) and 25.13 (± 6.07) respectively. All of the napthyridine compounds tested displayed different levels of activities against both the HCT116 and Mia-PaCa-2 cell lines which could be indicative of different biochemical processes occurring. Although the napthyridine compounds are not as active as the classic compounds tested earlier i.e cisplatin, 5-FU and etoposide as shown in table 6.10, what was an interesting finding was their selective ability for the cancer cell lines which was not the case for the classic chemotherapeutic drugs. So where the napthyridines lack in potency, they excel in selectivity under those same *in vitro* conditions used to test these compounds.

6.4.3 Chemosensitivity testing of 1,8 napthyridine against the HCT116 cells under hypoxic pHe 7.4 conditions

HCT116 cells were treated with a dose concentration range of 1,8 napthyridines over 96 hours under hypoxic pHe 7.4 conditions and the results are presented below (table 6.3).

Compound	IC_{50} (μM) Normoxia	IC_{50} (μM) Hypoxia	HCR	SI Hypoxia pHe 7.4
KMR-SA-21	82.098 ± 3.55	IC_{50} > 100	0.82	-
KMR-SA-22	61.282 ± 13.80	51.58 ±6.51	1.19	1.93
KMR-SA-23	37.80 ± 4.90	37.60 ± 11.32	1.01	2.65
KMR-SA-24	210.32 ± 3.44	34.53 ± 13.86**	6.09	19.65
KMR-SA-25	84.20 ± 7.30	39.67 ± 13.34*	2.12	11.00
KMR-SA-26	31.333 ± 12.93	26.44 ± 3.14	1.18	3.78
KMR-SA-27	IC_{50} > 100	IC_{50} > 100	-	-
KMR-SA-28	IC_{50} > 100	86.30 ±10.43	1.16	1.15
KMR-SA-29	IC_{50} > 100	IC_{50} > 100	-	-
KMR-LA	39.908 ± 1.6	26.75 ± 5.28*	1.49	3.73
KMR-LB	IC_{50} > 100	IC_{50} > 100	-	-
KMR-LC	IC_{50} > 100	IC_{50} > 100	-	-

Table 6.3 The response of HCT116 cells following a 96hour exposure to 1,8 napthyridine compounds under normoxic pHe 7.4 and hypoxic pHe conditions. A two-tail students t-test was applied where p values of ≤0.05 and ≤0.01 are represented as * and ** respectively. The SI hypoxia pHe 7.4 is defined as the ratio between the ARPE-19 IC_{50} value divided by the hypoxia pHe 7.4 IC_{50}. Each result represents the mean ± standard deviation of three independent experiments.

This aspect of the study was to ascertain if these compounds behaved as HAPs or not. Strikingly compounds KMR-SA-22-26, KMR-SA-28 and KMR-LA all exhibited HAP like properties with HCR all above 1 as shown in table 6.3. Compounds KMR-LA, KMR-SA-24 and 25 exhibited the best HAP like properties (HCR = 1.49, 6.09 and 2.12 respectively) which was statistically significant when comparing normoxia IC_{50} values against hypoxia IC_{50} as shown in figure 6.11 and table 6.3. KMR-SA-24 and 25 were also both selective towards the HCT116 cells under normoxic conditions (SI = 3.22 and 5.18 respectively). This shifts the selectivity indices drastically when comparing the IC_{50} with SI values increasing to 19.65 for KMR-SA-24 and 11.00 for KMR-SA-25. Cisplatin, etoposide and 5-FU displayed no HAP like properties as shown in table 3.2 and reproduced in table 6.10 and therefore novel compounds with HAP like activity have been identified.

6.4.4 Chemosensitivity testing of aroylthiourea ligand compounds under normoxic pHe 7.4 conditions

Chemosensitivity studies carried out under normoxic pHe 7.4 conditions with the aroylthiourea ligand compounds are displayed in table 6.4. The compounds tested were not as active as any of the classic compounds tested earlier and shown in table 6.10. From all the aroylthiourea ligand compounds only KMR-AL-11 was the most active with an IC_{50} value of 35.17µM ± 1.17.

Aroylthiourea ligand compounds IC_{50} (µM)			
Compound	Normoxia pHe 7.4		Selectivity index
	HCT116	ARPE-19	
KMR-AL-9	52.94 ± 4.81	33.52 ± 1.24	0.63
KMR-AL-10	63.18 ± 1.91	36.60 ± 1.07	0.57
KMR-AL-11	35.17 ± 1.17	37.94 ± 0.96	1.07
KMR-AL-12	43.75 ± 2.48	36.77 ± 0.16	0.84
KMR-AL-13	IC_{50} > 100	80.27 ± 1.21	0.80
KMR-AL-14	IC_{50} > 100	78.25 ± 1.50	0.78
KMR-AL-15	75.22 ± 4.73	46.91 ± 2.04	0.64
KMR-AL-16	IC_{50} > 100	IC_{50} > 100	-
KMR-AL-17	78.21 ± 8.66	69.30 ± 1.61	0.80

Table 6.4 The response of HCT116 cells following a 96hour exposure to aroylthiourea compoundsunder normoxic pHe 7.4 conditions. Each result represents the mean ± standard deviation of three independent experiments.

Compounds KMR-AL-13-14 and KMR-AL-17 displayed no activity. All of the compounds were more potent towards the normal human epidermal ARPE-19 cell line therefore displaying no selectivity for the cancer cell lines beside KMR-AL-11.

KMR-AL-11 had a selectivity index of 1.07 which is greater than etoposide and close to of cisplatin (selectivity = 1.83). This makes KMR-AL-11 the most promising aroylthiourea ligand compound from this series of

.

6.4.5 Chemosensitivity testing of aroylthiourea ligand compounds under hypoxic pHe 7.4 conditions

HCT116 cells were treated with a dose concentration range of aroylthiourea ligands over 96 hours under hypoxic pHe 7.4 conditions and the results are presented below (table 6.5)**.**

Compound	Normoxia	Hypoxia	HCR	SI Hypoxia pHe 7.4
	HCT116 cells IC_{50} (μM)			
KMR-AL-9	52.94 ± 4.81	28.22 ± 6.35*	1.88	1.18
KMR-AL-10	63.18 ± 1.91	40.83 ± 0.69**	1.54	0.89
KMR-AL-11	35.17 ± 1.17	39.03 ± 2.40	0.90	0.97
KMR-AL-12	43.75 ± 2.48	48.60 ± 4.59	0.90	0.75
KMR-AL-13	IC_{50} > 100	IC_{50} > 100	-	0.80
KMR-AL-14	IC_{50} > 100	71.80 ± 11.6	1.39	1.08
KMR-AL-15	75.22 ± 4.73	74.00 ± 11.4	1.01	0.63
KMR-AL-16	IC_{50} > 100	IC_{50} > 100	-	-
KMR-AL-17	78.21 ± 8.66	76.77 ± 2.83	1.01	0.90

Table 6.5 The response of HCT116 cells following a 96hour exposure to aroylthiourea compounds under normoxic pHe 7.4 conditions. A two-tail students t-test was applied where p values of ≤0.05 and ≤0.01 are represented as * and ** respectively. Each result represents the mean ± standard deviation of three independent experiments.

When carrying out chemosensitivity studies under hypoxic conditions, compounds KMR-AL-9, KMR-AL10, KMR-AL14, KMR-AL 15 and KMR-AL17 all displayed HAP like properties with HCR values all above 1. The aroylthiourea compound which exhibited the most HAP-like property from this group were KMR-AL9 and 10 (HCR =

1.88 and 1.54 respectively) which were both statistically significant p= ≤ 0.05 and 0.01 respectively). The trend towards the selectivity of ARPE-19 cells continued when comparing data with hypoxia pHe 7.4 values. Only KMR-AL-9 and KM4-AL-14 displayed some selectivity towards the HCT116 cells when evaluated under hypoxic conditions.

6.4.6 Chemosensitivity testing of benzoylthiourea compounds under normoxic pHe 7.4 conditions

Chemosensitivity studies were carried out using the benzoylthiourea derivatives which were tested on the following cell lines, HCT116, HT29, Mia-PaC-2 and ARPE-19. The IC_{50} data is displayed in table 6.6. Due to their hygroscopic nature compounds KM R-SA-30 and KMR-SA-33 were not tested. Compounds KMR-L10 to L12 displayed no activity towards all of the cell lines tested (IC_{50} > 100µM).

Compounds	Benzoylthiourea compounds IC_{50} (µM) Normoxia pHe 7.4			
	HCT-116 cells	Mia-PaCa-2 cells	HT29 cells	ARPE-19 cells
KMR-L10	IC_{50} > 100	IC_{50} > 100	IC_{50} > 100	IC_{50} > 100
KMR-L11	IC_{50} > 100	IC_{50} > 100	IC_{50} > 100	IC_{50} > 100
KMR-L12	IC_{50} > 100	IC_{50} > 100	IC_{50} > 100	IC_{50} > 100
KMR-SA-30	N/A	N/A	N/A	N/A
KMR-SA-31	24.9 ± 1.91	11.23 ± 0.49	23.27 ±3.57	47.23 ± 0.63
KMR-SA-32	1.37 ± 0.09	14.33 ± 0.79	4.89 ±0.56	18.26 ± 0.58
KMR-SA-33	N/A	N/A	N/A	N/A
KMR-SA-34	6.98 ± 0.50	4.01 ± 0.12	4.91 ±0.09	11.72 ± 0.30
KMR-SA-35	5.18 ± 0.10	4.48 ± 0.18	6.99 ±0.51	9.79 ± 0.05
KMR-SA-36	11.42 ± 1.86	9.02 ± 0.13	13.47 ±1.66	15.72 ± 0.83
KMR-SA-37	11.96 ± 2.19	6.21 ± 0.57	10.37 ±0.17	16.24 ± 1.16
KMR-SA-38	5.50 ± 1.86	6.22 ± 0.07	8.09 ±1.06	18.25 ± 0.48

Table 6.6 The response of HCT116, Mia-PaCa-2, HT29 and ARPE-19 cells following a 96hour exposure to benzoylthiourea compounds under normoxic pHe 7.4 conditions. Each result represents the mean ± standard deviation of three independent experiments.

Compounds KMR-SA-31, KMR-SA-36 and KMR-SA-37 were all moderately active against all the cell lines tested. KMR-SA-32, KMR-SA-34, KMR-SA-35 and KMR-SA-38 all displayed a good levels of activity. KMR-SA-32 was the most potent compound

tested with an IC_{50} value of 1.37 ± 0.09 µM against the HCT116 cell line. This was also the most selective compound exhibiting a selectivity index of 13.33 (table 6.7). Interestingly KMR-SA-32 is not only more potent than cisplatin (IC_{50} 2.03µM ± 0.08 and SI = 1.84) but also much more selective than all three classic compounds tested in chapter 3 and displayed in table 6.10. Another interesting finding was that all of these compounds displayed some level of selectivity towards all three cancer cell lines tested, which shows much promise for these set of compounds (table 6.7).

Benzoylthiourea compounds			
	Selectivity index		
Compounds	Normoxia		
	HCT-116 cells	Mia-PaCa-2 cells	HT29 cells
KMR-L10	-	-	-
KMR-L11	-	-	-
KMR-L12	-	-	-
KMR-SA-30	-	-	-
KMR-SA-31	1.89	4.21	2.02
KMR-SA-32	13.33	1.27	3.73
KMR-SA-33	-	-	-
KMR-SA-34	1.68	2.92	2.39
KMR-SA-35	1.89	2.18	1.40
KMR-SA-36	1.37	1.74	1.17
KMR-SA-37	1.36	2.62	1.56
KMR-SA-38	3.32	2.93	2.25

Table 6.7 The selective indices of benzoylthiourea compounds for HCT116, Mia-PaCa-2, HT29 cells under normoxic pHe 7.4 conditions. Each result represents the mean ± standard deviation of three independent experiments.

6.4.7 Chemosensitivity testing of benzoylthiourea compounds under hypoxic pHe 7.4 conditions

Further chemosensitivity studies were carried out under hypoxic conditions against the HCT116 cancer cells to determine if any of these compounds showcased HAP like properties. Unfortunately, despite their promising ability in terms of potency and selectivity, none of these set of compounds displayed any HAP like properties (SI =<1 for all compounds tested) and in the majority of cases, resistance under hypoxia was observed (table 6.8).

	Benzoylthiourea compounds IC_{50} (μM)			
Compounds	Normoxia	Hypoxia	HCR	Hypoxia SI pHe 7.4
	HCT-116 cells			
KMR-L10	IC50 > 100	IC50 > 100	-	-
KMR-L11	IC50 > 100	IC50 > 100	-	-
KMR-L12	IC50 > 100	IC50 > 100	-	-
KMR-SA-30	-	-	-	-
KMR-SA-31	24.92 ± 1.91	34.39 ± 1.16	0.72	1.37
KMR-SA-32	1.37 ± 0.09	33.47 ± 2.35	0.04	0.54
KMR-SA-33	-	-	-	-
KMR-SA-34	6.98 ± 0.50	9.81 ± 1.26	0.71	1.19
KMR-SA-35	5.18 ± 0.12	12.04 ± 0.24	0.43	0.81
KMR-SA-36	11.42 ± 1.86	18.01 ± 0.76	0.63	0.87
KMR-SA-37	11.96 ± 2.19	24.99 ± 1.34	0.48	0.64
KMR-SA-38	5.50 ± 1.86	14.85 ± 2.17	0.37	1.22

Table 6.8 The response of HCT116 cells following a 96hour exposure to benzoylthiourea compounds under hypoxic pHe 7.4 conditions, with HCR and hypoxia SI pHe 7.4. Each result represents the mean ± standard deviation of three independent experiments.

KMR-SA-32 was the most selective compound to come out of this set, but displayed resistance under hypoxic conditions with a HCR of 0.04 and hypoxia pHe 7.4 of 0.54. These set of compounds were more selective than the classic chemotherapeutic drugs tested earlier but had a similar HCR. None of these compounds demonstrated any preferential activity towards cells under hypoxic TME conditions.

6.4.8 Chemosensitivity testing of transition metal complexes under TME conditions

After thorough phenotypic screening of all the compounds tested in this results chapter, only those compounds which exhibited the best HAP like properties were further screened to assess the role of mild acidic pHe of 6.5. The short listed compounds were: the aroylthiourea ligand complexes KMR-AL-9 and 10 and the 1, 8 napthyridine complexes KMR-SA-24 and 25. These compounds displayed HCR values of 1.88, 1.54, 6.09 and 2.12 respectively.

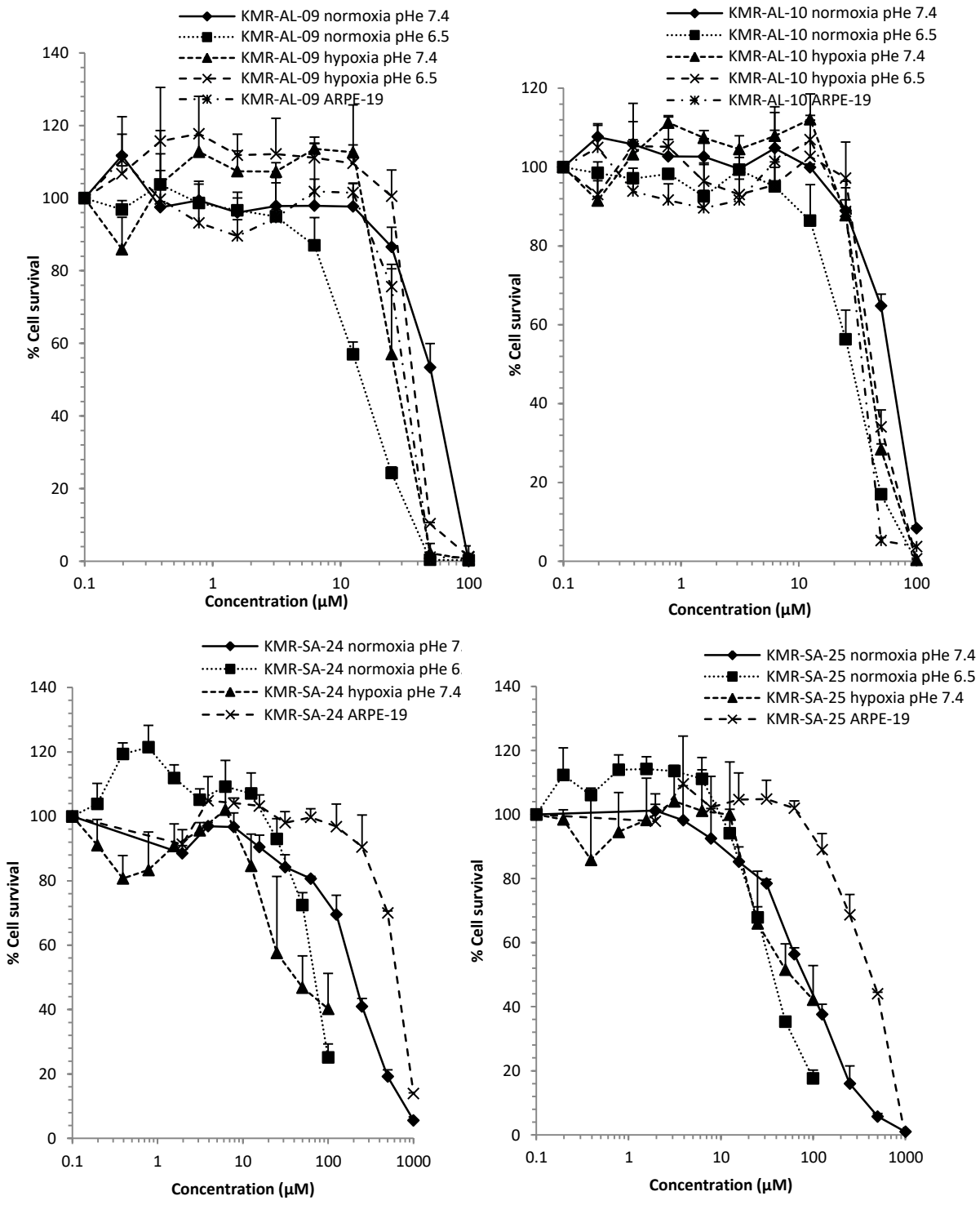


Figure 6.11 The response of HCT116 cells following a 96hour exposure to KMR-AL-09, KMR-AL-10, KMR-SA-24 and KMR-SA-25 under TME conditions. Each result represents the mean ± standard deviation of three independent experiments.

Compound	IC_{50} (μM) Normoxia pH 7.4	Normoxia pH 6.5	Hypoxia pH 7.4	Hypoxia pH 6.5	ARPE-19
		HCT116 cells			
KMR-AL-9	52.93 ± 4.81	15.09 ± 1.01	28.22 ± 6.35	38.94 ± 0.96	33.52 ± 1.24
KMR-AL-10	63.18 ± 1.91	28.58 ± 3.88	40.83 ± 0.69	43.66 ± 1.71	36.60 ± 1.07
KMR-SA-24	210.32 ± 3.44	73.69 ± 1.54	34.53 ± 13.86	N/A	678.55 ± 3.77
KMR-SA-25	84.20 ± 7.30	38.68 ± 1.68	39.67 ± 13.34	N/A	436.53 ± 22.12

Table 6.9 Response of HCT116 cells following a 96 hour exposure to KMR-AL-9, KMR-AL-10, KMR-SA-24 and KMR-SA-25 under normoxic and tumour microenvironment conditions. Each result represents the mean ± standard deviation of three independent experiments.

Chemosensitivity studies were carried out using the colorectal cancer cell line HCT116 cells. KMR-AL-9 and KMR-AL-10 both had an increase in activity under normoxic pHe 6.5 in comparison to pHe 7.4 as shown in figure 6.11 and table 6.9. Interestingly however, when KMR-AL-9 was evaluated under hypoxic and acidic pHe conditions, antagonistic effects were observed with the IC_{50} value increasing form 15.09 ± 1.01 μM under hypoxic conditions (pHe 7.4) to 38.94 ± 0.96 μM (table 6.9). Similar results were obtained for KMR-AL-10 albeit the magnitude of the effect was not as pronounced.

6.4.9 Summary of chemosensitivity testing of lead transition metal complexes under TME conditions

The activity of selected complexes increased significantly when evaluated under mild acidic conditions (pHe 6.5) under normoxic conditions with pHR was 3.50, 2.21, 2.85 and 2.29 for KMR-AL-9 and 10 and KMR-SA-24 and 25 respectively. Reducing the extracellular pH therefore significantly enhance the activity of these complexes, the magnitude of the pH enhancement being much greater than the chemotherapeutic complexes tested in the previous chapters.

Compound	pHR	HCR	TME ratio	SI	SI (TME)
Cisplatin	0.75	0.44	0.3	1.84	0.55
5-FU	0.55	0.40	0.27	2.10	0.57
Etoposide	0.76	0.61	0.57	0.59	0.34
Tirapazamine	0.61	9.55	n/a	2.33	22.46*
KMR-AL-09	3.50	1.87	1.44	0.63	0.86
KMR-AL10	2.21	1.54	1.82	0.57	0.83
KMR-SA-24	2.85	6.09	n/a	3.22	n/a
KMR-SA-25	2.29	2.12	n/a	5.18	n/a

Table 6.10 The effect of the TME on the response of cells to standard cytotoxic drug. Tirapazamine, KMR-AL-09-10 and KMR-SA-24-25. pHR, HCR and TME represent the pH ratio, the hypoxic cytotoxicity ratio and tumour microenvironment ratio as defined in the methods section (chapter 3 – 3.3.3). * refers to the SI value for the hypoxic data only and not the combo of hypoxia and acidic pHe.

Furthermore, all of the selected complexes displayed HAP like properties under hypoxia pHe 7.4 (particularly KMR-SA-24) which again was greater than the classic compounds tested earlier (table 6.10). Intriguingly however, when subjected to further testing of compounds under hypoxia combined with mildly acidic pHe of 6.5, both KMR-AL-9 and KMR-AL-10 compounds were not as active. Unfortunately, chemosensitivity studies on the 1, 8 napthyridine complexes KMR-SA-24 and KMR-SA-25 were not completed for the hypoxic combined with pHe 6.5 studies due to shortage of compound supplied. Nevertheless, only KMR-SA-24 and 25 displayed SI values greater than all of the classic chemotherapeutic complexes and based on this data in conjunction with its HAP-like properties, KMR-SA-24 was selected for further mechanistic studies by way of ϒH2AX phosphorylation induction.

6.4.10 Western blot analysis for the early signs of DNA damage by KMR-SA-24 under normoxic and hypoxic and pHe 7.4 conditions

To assess whether there are any early signs of DNA damage under hypoxic conditions, western blot analysis for ϒH2AX, a protein marker which is phosphorylated in the early stages of DNA damage was carried out. The results presented in figure 6.12A demonstrate that a 24 hour exposure to KMR-SA-24 at doses up to 50μM does not induce any noticeable change in cell morphology indicating that cell death has not occurred at this time point. By 96 hours however, cell kill is apparent under both normoxic and hypoxic conditions (figure 6B and C). Cells were therefore harvested for western blot analysis following a 24 hour drug exposure.

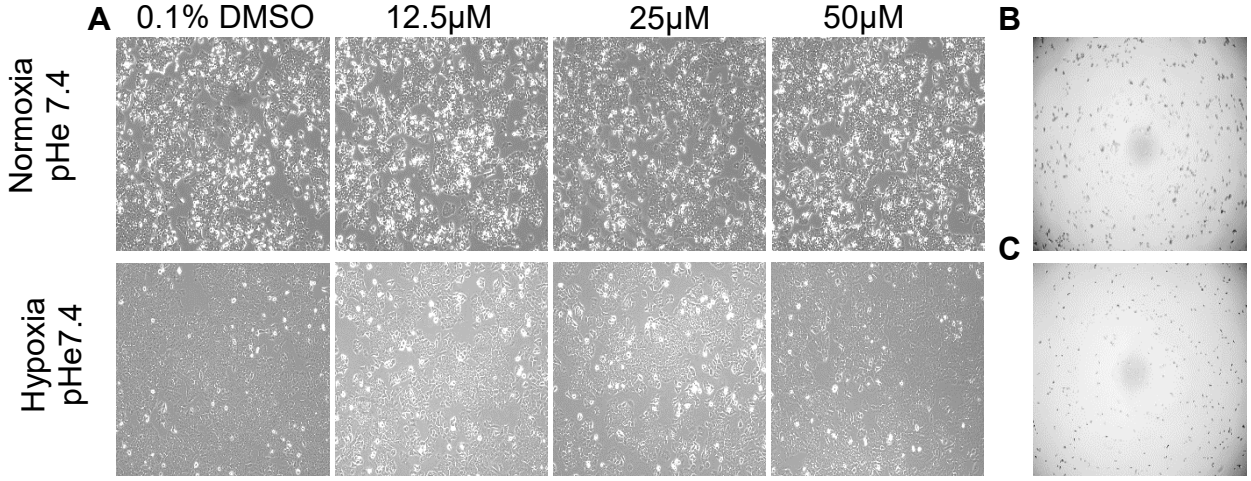


Figure 6.12 HCT116 cell images post 24h hour treatment with KMR-SA-24 under normoxic and hypoxic pHe 7.4. A. HCT116 cells were treated with KMR-SA-24 using a wide concentration range (12.5-50μM) for a duration of 24hours under the following conditions normoxia/pHe 7.4 and hypoxia/pHe 7.4. DMSO at 0.1% (v/v) was used as a control under each condition. B. HCT116 cells were treated with KMR-SA-24 (250μM) for a duration of 96 hours under normoxic pHe 7.4 conditions.

C. HCT116 cells were treated with KMR-SA-24 (100μM) for a duration of 96hours under hypoxic pHe 7.4 conditions.

HCT116 Cells were treated for 24 hours over a drug concentration range that fit approximated to the IC_{50} value for KMR-SA-24 under hypoxic conditions (34.53μM ± 13.86). Cells were immediately lysed and western blot analysis was carried out as described in the methods (see sections 2.9 to 13). As depicted in figure 6.13, western blot analysis, displayed a positive band for those HCT116 cells which were treated with KMR-SA-24 at 50μM under hypoxic conditions and pHe 7.4. These results suggest that KMR-SA-24 could be working as a HAP leading to DNA damage.

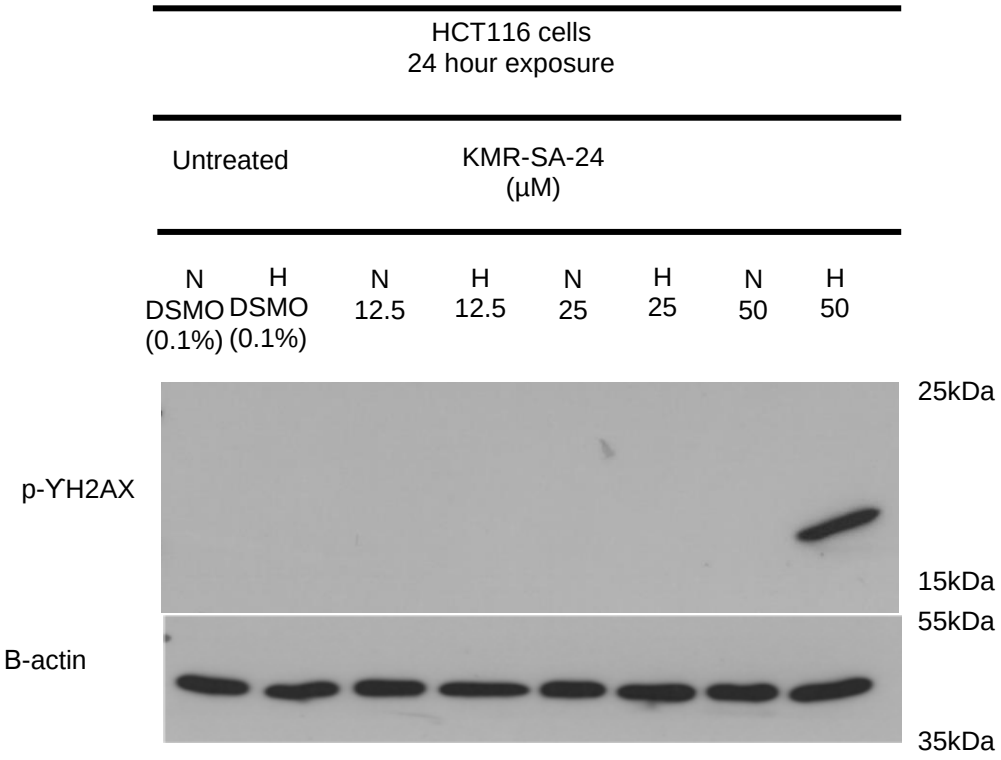


Figure 6.13 Influence of hypoxia and KMR-SA-24 on p-ϒH2AX. Colorectal cancer HCT116 cells were exposed to KMR-SA-24 for 24 hours under normoxic and hypoxic (0.1% O2) conditions. Cells were lysed and screened for γH2AX. B-actin was used as a loading control. N refers to normoxia and H refers to hypoxia (n=1).

6.5 Discussion

This chapter focuses on Professor Rao's compounds which have been synthesised at the Eastern Hill University, India. The compounds were categorised in accordance to their chemical 'type' and tested through the phenotypic screen for (i) their selective activity under TME conditions and (ii) their selectivity for cancer cells (HCT116, HT29, Mia-PaCa-2) as opposed to non-cancer ARPE-19 cells.

The first set of compounds tested were the thiourea derivatives, these compounds have the chemical formula R1R2N)(R3R4N)C=S and the addition of either ruthenium, iridium or rhodium as their core transition metal base. Compounds KMR-L1, KMR-L2 and KMR-L3 displayed no signs of activity towards those entire cell lines tested (table 6.1). Interestingly these 3 compounds do not contain a transition metal base and therefore demonstrates that the thiourea ligands alone do not induce cell death (IC_{50} > 100µM). This is further supported from a study carried out by Pommier *et al* (1983) who demonstrated that thiourea is a well-known and potent scavenger of the hydroxyl radical groups and managed to reduce the cytotoxic ability of DNA-intercalating agents in mouse leukaemia L1210 cells (Pommier et al., 1983). All of the remainder compounds (KMR-SA-1-9) did express some degree of activity towards the HCT116 and Mia-PaCa-2 cell lines. This is indicative that the combination of the metal base to the thiourea ligand is what causes the compound to behave like an anti-cancer agent. Compounds KMR-SA-1-3 and KMR-SA-7-9 were more active than compounds KMR-SA-4-6, which is interesting as complexes which consisted of rhodium were more active than those with ruthenium and iridium. Previous reports have stated that ruthenium is generally the most active out of these three metal complexes (Katsaros and Anagnostopoulou, 2002).

When compared to cisplatin, 5-FU or etoposide, none of these compounds were as potent, however their selectivity for cancer as opposed to non-cancer cell lines was much better than classic chemotherapeutic drugs tested. This provides the rationale for synthesising further thiourea compounds which can have greater cytotoxicity and selectivity towards cancer cells. Unfortunately these compounds were not tested under conditions that mimic the TME due to limited time constraints and limited drug supply. In this case therefore, the phenotypic screen was primarily based on identifying compounds with selective activity against cancer cells under standard physiological conditions (normoxia and pHe 7.4).

From all the 1,8 napthyridine compounds tested, it was apparent that none of these were as active as any of the three classic based drugs that are used in this study. 1,8 napthyridines have been extensively studied for their anti-cancer like properties which is demonstrated by Kumar *et al* (2009) who carried out *in vitro* based chemosensitivity studies by 1,8 napthyridine 3-carboxamide derivatives against a panel of 8 cancer cell lines (which also consisted of colon and pancreatic cancer cell lines). Kumar *et al* (2009) demonstrated good levels of cell toxicity towards the cancer cell lines which was similair to the chemotherapeutic drugs tested in this chapter but not the 1,8 napthyridines. However both sets of compounds displayed good levels of selectivity towards the cancer cell lines which was greater than the classic chemotherapeutic drugs tested in this chapter which shows some promise for these set of compounds.

The aroylthiourea compounds were not as active as any of the three classic chemotherapeutic drugs tested earlier in results chapter 3. Compound KMR-AL-11 was the most active with an IC_{50} of 35.17µM ± 1.17 but it had a better selectivity index in comparison to cisplatin. This indicates that this compound does show some

promise for further development. The remainder of the compounds interestingly were more active towards the normal human retinal epithelial ARPE-19 cell line in comparison to the colorectal HCT116 cancer cell line. To our knowledge this is the first time that the aroylthiourea ligand and ruthenium arene have been tested in its biological capacity for anti-cancer properties. Despite not showing much promise in our *in vitro* cancer models, thiourea and ruthenium have been shown to exert anti-cancer like properties in the past. Therefore it was hypothesised that using an aroylthiourea ligand to coordinate a wide range of metal ions (Lapasam et al., 2019) may make these set of drugs exert anti-cancer like properties, however this is not the case here. It may be of interest to test these set of compounds for their anti-inflammatory and anti-bacterial properties as thiourea has been previously shown to exert these properties (Lin et al., 2013; Mahajan et al., 2007).

In the following cancerous HCT116, HT29, Mia-PaCa-2 and normal ARPE-19 cell lines the benzylthiourea compounds displayed the best activity amongst the thiourea, 1-8, napthyridines and aroylthiourea drugs. Compounds L1-L3 were inactive with IC_{50} > 100µM across all the cell lines tested which is indicative that the arene/cp* alone is not active. The iridium based benzylthiourea complexes were found to be more active towards all the cell lines tested which is indicative that the metal base type also has a crucial role as well as the arene/cp* ring alone. Apart from being the most active set of compounds, these were also all selective towards the cancer lines tested.

A similar study carried out by Jeyalakshmi *et al* (2016) assessed the cytotoxicity of Ru-*p*-cymene complexes against the cancerous MCF7 (breast), A549 (lung) and the normal L929 (murine fibrosarcoma) cell lines. These set of complexes were selective

towards the cancer cell lines and is similar to our findings which is promising (Jeyalakshmi et al., 2016).

From all the compounds tested in this study under normoxic conditions it is evident that the majority of the compounds tested do not have a similar activity profile to cisplatin, 5-FU or etoposide with the exception of KMR-SA-32 (1.37µM ± 0.09). However the selectivity indexes of the majority of the compounds is more favourable than all of the classic chemotherapeutic standards in this study.

From all the drugs tested, only KMR-AL-9 and 10 and KMR-SA-24 and 25 displayed the best HAP like properties (tables 6.9 and 6.10) and were therefore subjected to further testing under mildly acidic pHe conditions. As the pHe is reduced under normoxic conditions there is an increase in activity amongst all the 4 complexes tested and this is confirmed by the pHR values (table 6.10). When tested under hypoxic conditions all four of the complexes displayed HAP like properties which was really striking. KMR-SA-24 displayed the best HAP like properties with a HCR of 6.09 as shown in table 6.10 which is almost similar to the classic HAP tirapazamine (HCR 9.55). The HCR of KMR-SA-24 is higher than all of the standard chemotherapy compounds which were also tested under these conditions (table 6.10).

Upon completion of phenotypic screening the 1,8 napthyridine KMR-SA-24 was chosen as a lead complex and preliminary studies were conducted to determine the mechanism of action. Specifically, it was tested to determine whether or not gamma H2AX phosphorylation was induced as a marker of DNA damage. The results in figure 6.13 clearly demonstrate that phosphorylation of **γ**H2AX in HCT116 cells by KMR-SA-24 occurs under hypoxic conditions (at 50 µM) only thereby suggesting that the complex is being activated to a DNA damaging species. This result is consistent

with the chemosensitivity studies of KMR-SA-24 against the HCT116 cell line where under 50μM there is activity under hypoxic pHe 7.4 compared to normoxic pHe 7.4 which was poor (figure 6.11 and table 6.9-10).

To conclude, the results of this study have shown that the phenotypic based screen has identified novel compounds that are both selective for cancer cells (as opposed to non-cancer cells) *in vitro* and have HAP like properties. KMR-AL-9, KMR-AL-10, KMR-SA-24 and KMR-SA-25 all had enhanced activity under mild acidic pHe under normoxic conditions and bio-reductive properties under hypoxia which is something that would have not been discovered had this screening test not been employed. Additional experiments were planned/ designed for KMR-SA-24 but due to time constraints this could not be achieved. Those experiments were to further validate the activity profile of KMR-SA-24 under pHe 6.5 and hypoxia and to expand the analysis of induction of γH2AX phosphorylation under these conditions. Whilst further studies are warranted to explore the mechanisms of action of these complexes, the main conclusion of this chapter is that the phenotypic screen has identified novel compounds that are selective and have HAP like properties. These would have remained unknown if they had been tested under conventional conditions *in vitro*.

Chapter 7: Performance of NHC-Ag (I) based complexes in the phenotypic screen

7.1 Introduction

In the search for new anti-cancer drugs, organometallic complexes have enjoyed a resurgence of interest in recent years. In particular, gold based N-heterocyclic carbenes (NHC) have been extensively evaluated with regards to their cytotoxic and cancer targeting properties. These complexes however, showed unfavourable toxicity *in vivo* which includes reproductive effects as well as oxidative damage to the heart in rats (Ciftci et al., 2011). An alternative to gold (NHC) complexes are silver (Ag) based compounds which were originally investigated for their antimicrobial activity and their ability to treat infected wounds and burns (Kalinowska-Lis et al., 2016). Over the years Ag compounds were disregarded in the first instance despite showcasing good levels of cytotoxicity in various cancer cell lines (Kalinowska-Lis et al., 2016). Recently however, a lot of interest has focused on their anti-cancer properties (Johnson et al., 2017). Numerous Ag (I) compounds have been developed which display cytotoxicity against various cancer cell lines and display low toxicity to non-cancer cells thereby exhibiting greater selectivity towards cancer cells than cisplatin (Kalinowska-Lis et al., 2016). NHC ligands and carboranes are an interesting class of organometallic compounds due to their low toxicity and stability (Allison et al., 2017; Holmes et al., 2019). Medvetz et al., 2008 were of the first researchers to report the anti-cancer properties of Ag (I) based NHC compounds. Cell lines such as OVCAR-3 (ovarian), MB157 (breast) and HeLa (cervical) cells were used for the basis of their *in vitro* study and the IC_{50} values of their Ag based compounds ranged between 20-25μM (Medvetz et al., 2008).

The mechanism(s) of action of these compounds is complex and there is now a considerable body of evidence demonstrating that their mechanism(s) are distinct from platinates (not alkylating agents) but involve multiple pathways involved in cancer biology. Whilst compounds that exhibit poly-pharmacological properties are now very attractive (in the context of tackling tumour heterogeneity), the challenge is to identify multi-targeted agents which show selectivity for cancer cells over normal cells (Reddy and Zhang, 2013). One Ag complex, which is commonly referred to as Ag8 ($Ag(NHC)_2AgBr_2$, has emerged as a promising lead compound based on its selective anti-cancer like abilities and several mechanism of actions including PARP1 inhibition, topoisomerase I and II inhibition, thioredoxin reductase and inhibition of glycolysis (Allison et al., 2017). In this study derivatives of Ag8 with the addition of carborane which has attracted a lot of attention in medicinal applications due to their high catabolic stability are evaluated in the phenotypic screen (Grimes, 2016; Valliant et al., 2002). Ag-NHC complexes have previously shown excellent potency in cancer cells but *in vivo* stability is a serious problem which needs to be addressed. The addition of the carborane to NHC ligands may increase solubility, stability and in essence increase the activity of these Ag complexes. In this study the cytotoxicity, selectivity and preferential activity of such compounds under physiological TME conditions are assessed using 4 genetically different strains of HCT116 cell lines. To explore the further potential of the phenotypic screening strategy employed, the HCT116 cell line panel was expanded to include HCT116 $p53^{-/-}$, $HCT116^{KRAS\ mutant}$ and $HCT116^{KRAS\ wildtype}$. As described in previous chapters, the ARPE-19 cell line was also used to determine selectivity.

7.2 Aims and objectives

The aim of this study is to evaluate whether or not a series of Ag (NHC) derivatives are cytotoxic towards the colon rectal cancer cell lines HCT116 $p53^{+/+}$, HCT116 $p53^{-/-}$, $HCT116^{KRAS\ mutant}$, $HCT116^{KRAS\ wildtype}$. To determine the selectivity, these compounds were also evaluated against the non-cancerous ARPE-19 cell line under conditions that reflect the 'normal conditions' found in healthy tissue (i.e. oxygenated and pHe 7.4). The Ag (NHC) compounds will also be subjected to hypoxia studies to determine preferential activity under (i) hypoxia (0.1% oxygen), (ii) acidic extracellular pH (pHe 6.5) or (iii) a combination of hypoxia and acidic pHe.

7.3 Material and methods

7.3.1 Compounds

All Ag complexes were kindly provided by Dr Charlotte Willans (University of Leeds) and these were dissolved in DMSO (100mM), aliquoted and stored at -20°C. The chemical structure and molecular weights of the complexes evaluated are presented in figure 7.1.

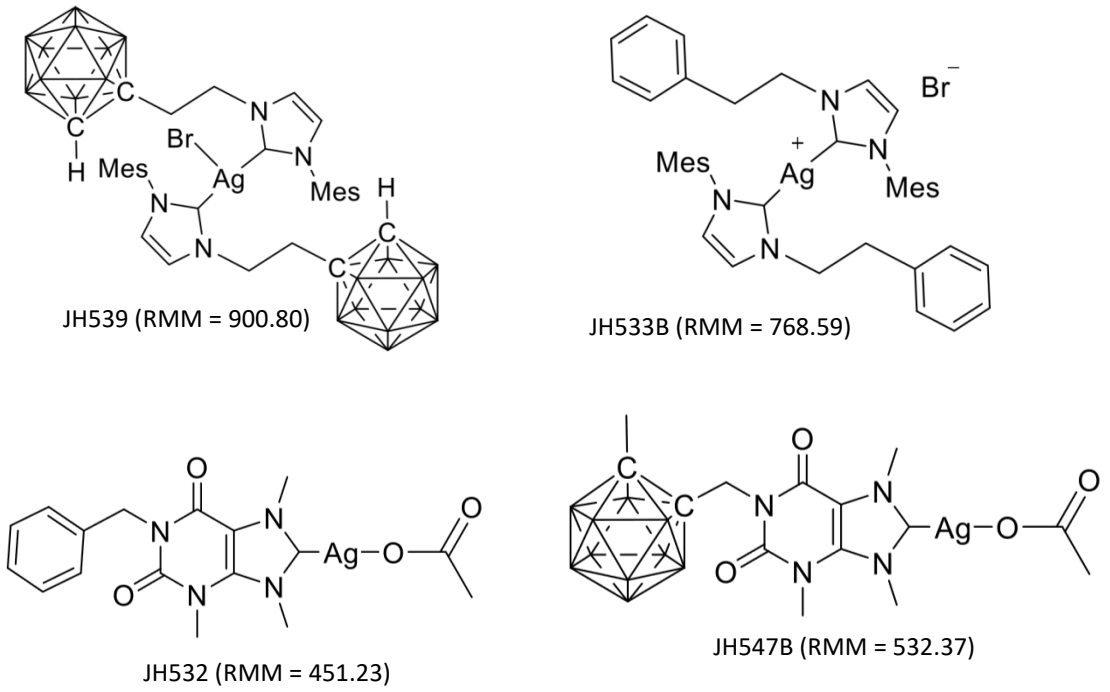


Figure 7.1 Chemical structure of Ag(NHC) based compounds and molecular weights (in parenthesis).

7.3.2 Cell culture and chemosensitivity

Cell culture, adjusting the pH of the media, drug dilutions and chemosensitivity studies under both normoxic and hypoxic conditions were carried out as previously described in methods chapter (see sections 2.6 to 2.8). The following cell lines were use for this study, HCT116 $p53^{+/+}$, HCT116 $p53^{-/-}$, HCT116$^{KRAS\ Wildtype}$ and

HCT116$^{KRAS\ Mutant}$. All the experiments were carried out using low or no light conditions as the silver complexes were light sensitive.

7.3.3 Statistical analysis

Statistical analysis was carried out using Microsoft Excel and Graph Prism. All experiments were done in triplicate (n=3), and results were presented as the mean ± SD. Statistical significance was determined by means of a two-tailed Student's t-test, or by One Way ANOVA where significance was only accepted when $p \leq 0.05$. The exact statistical analysis performed is mentioned in the figure legend.

7.4 Results

Ag (NHC) complexes JH532, JH547B, JH533B and JH539 were tested against the following colorectal cancer cell lines HCT116 $p53^{+/+}$, HCT116 $p53^{-/-}$, $HCT116^{KRAS\ Wildtype}$ and $HCT116^{KRAS\ Mutant}$ for their cytotoxicity and selectivity against normoxic and TME conditions. The non-cancer human retinal epithelial cell line APRE-19 was used to test for the cytotoxic activity of complexes and their selectivity for cancer cells. In this case, the activity of Ag complexes was performed under normoxic conditions only.

7.4.1 The effect of JH532 against the HCT116 $p53^{+/+}$ and ARPE-19 cell line under normoxia pHe 7.4 conditions

JH532 had IC_{50} values of, 11.63µM ± 4.61, 11.28µM ± 4.64, 11.18µM and 12.34µM ± 5.64 across all 4 different colorectal cancer cell lines tested (table 7.1) but it was its selectivity towards the ARPE-19 cell line which was the most striking as this was above 6 (table 7.2) for all colorectal cancer cell lines tested and much higher than the classic chemotherapy agents tested in chapter 3 (table 7.13).

Compound	**IC_{50} (µM)**				
	HCT116 $p53^{+/+}$	**HCT116 $p53^{-/-}$**	**HCT116 $^{KRAS\ Mut}$**	**$HCT116^{KRAS\ wt}$**	**ARPE-19**
JH532	11.63 ± 4.61	11.28 ± 4.64	11.18 ± 4.45	12.34 ± 5.64	76.87 ± 2.43

Table 7.1 The response of HCT116 cell lines following continuous exposure to JH532 under normoxic pHe 7.4 conditions. Each result represents the mean ± standard deviation of three independent experiments.

Compound	Selectivity index			
	HCT116 $p53^{+/+}$	HCT116 $p53^{-/-}$	HCT116 KRAS Mut	HCT116 KRAS wt
JH532	6.61	6.81	6.87	6.23

Table 7.2 The selective indices of JH532 for the HCT116 cell lines under normoxic pHe 7.4 conditions.

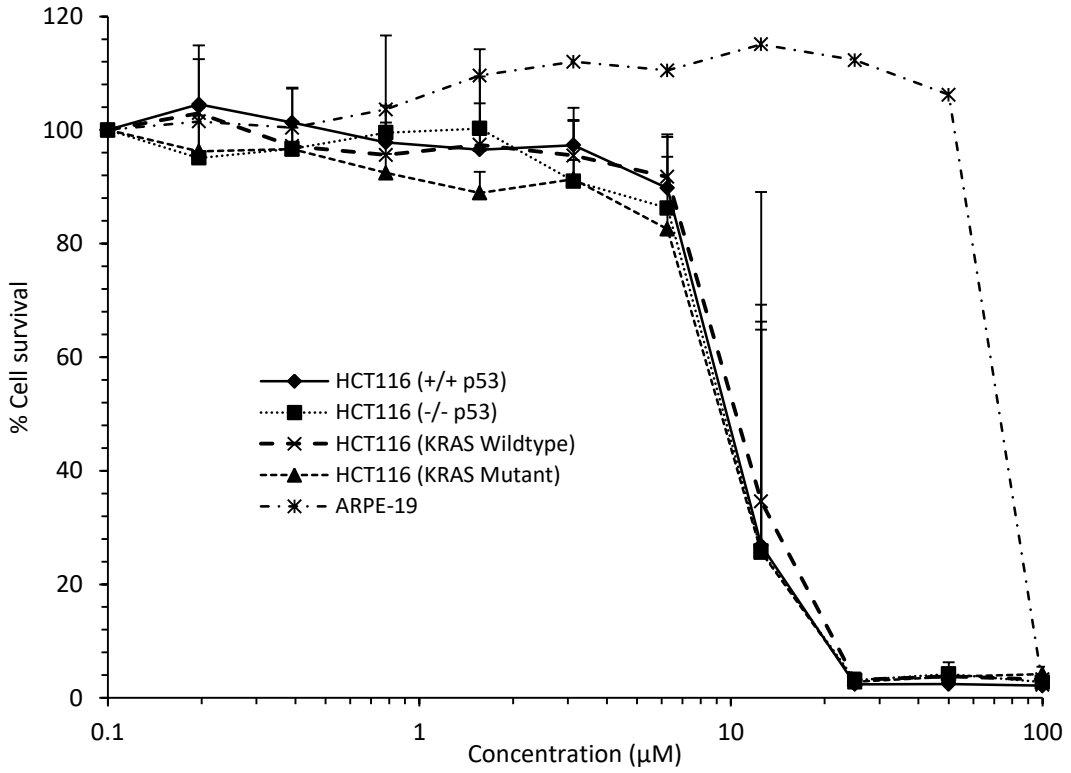


Figure 7.2 Response of HCT116 cell lines and ARPE-19 cells following a 96 hour exposure to JH532 under normoxia and pHe 7.4 conditions. Each result represents the mean ± standard deviation of three independent experiments.

7.4.2 The effect of JH547B against the HCT116 $p53^{+/+}$ and ARPE-19 cell line under normoxia pHe 7.4 conditions

JH547B was the least active of the Ag(NHC) compounds towards the different type of colorectal cancer cell lines tested in comparison to the different complexes from this study with IC_{50} values of 17.26μM ± 2.13, 20.24μM ± 4.95, 12.83μM ± 3.39 and 24.68μM ± 1.86 (table 7.3). However, there was a significant difference between ($p < 0.05$) the $HCT116^{KRAS\ Wildtype}$ and $HCT116^{KRAS\ Mutant}$ which may suggest that there is KRAS dependency for this complex (figure 7.3).

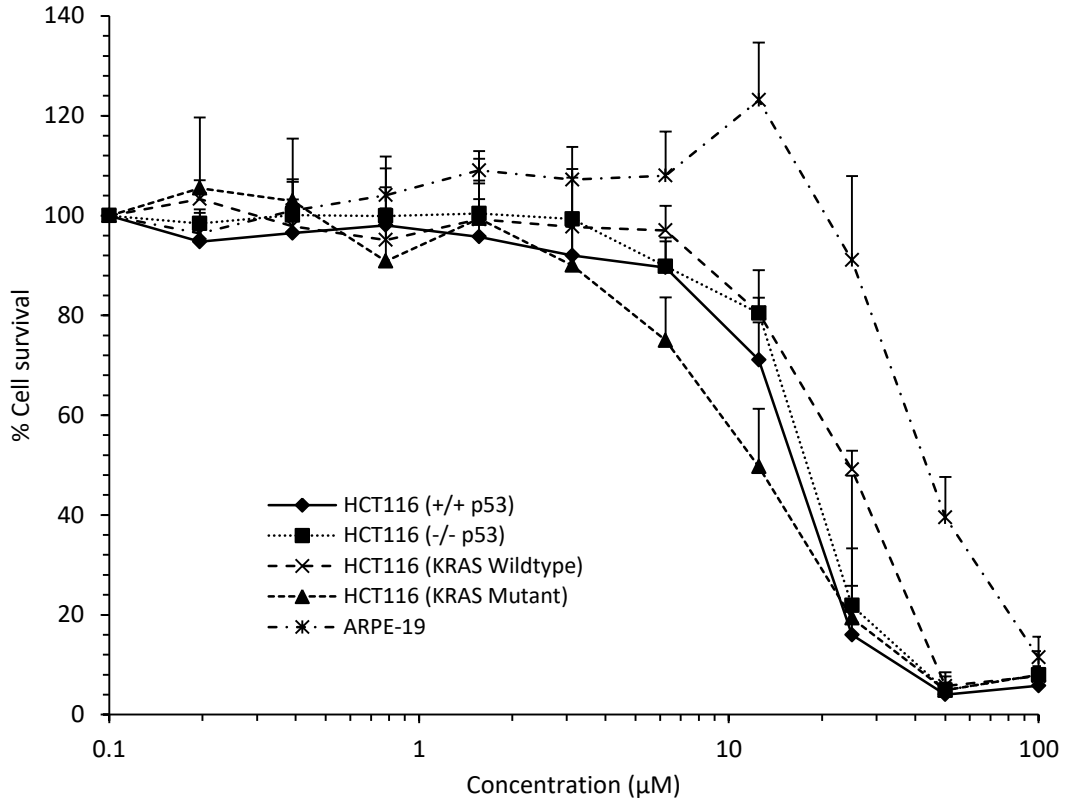


Figure 7.3 Response of HCT116 cell lines and ARPE-19 cells following a 96 hour exposure to JH547B under normoxia and pHe 7.4 conditions. Dose response curves to JH547B (0.1-100μM) under normoxic pHe 7.4 conditions. Each result represents the mean ± standard deviation of three independent experiments.

Compound	IC_{50} (μM)				
	HCT116 $p53^{+/+}$	HCT116 $p53^{-/-}$	HCT116 $^{KRAS\ Mut}$	HCT116$^{KRAS\ wt}$	ARPE-19
JH547B	17.62 ± 2.13	20.24 ± 4.95	12.83 ± 3.39	24.68 ± 1.86	43.87 ± 5.04

Table 7.3 The response of HCT116 cell lines following continuous exposure to JH547b under normoxic pHe 7.4 conditions. Each result represents the mean ± standard deviation of three independent experiments.

Compound	Selectivity index			
	HCT116 $p53^{+/+}$	HCT116 $p53^{-/-}$	HCT116 KRAS Mut	HCT116 KRAS wt
JH547B	2.49	2.17	3.42	1.77

Table 7.4 The selective indices of JH532 for the HCT116 cell lines under normoxic pHe 7.4 conditions.

7.4.3 The effect of JH533B against the HCT116 $p53^{+/+}$ and ARPE-19 cell line under normoxia pHe 7.4 conditions

JH533B was cytotoxic but did not display any significant difference between the different $p53^{+/+}$ or $p53^{-/-}$ or the $KRAS^{Wildtype}$ or $KRAS^{Mutant}$ cell lines (table 7.5). A moderate degree of selectivity to colorectal cancer cell line was observed (SI 2.85-4.86, table 7.6).

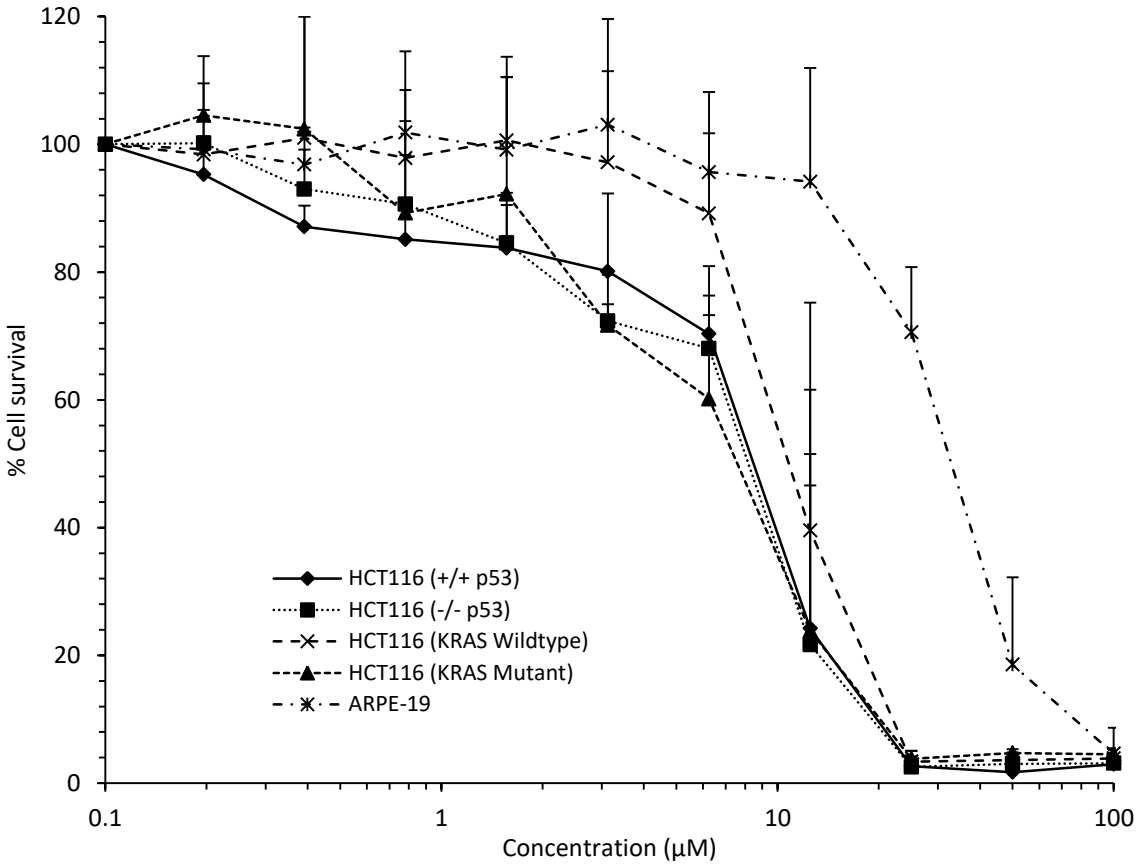


Figure 7.4 Response of HCT116 cell lines and ARPE-19 cells following a 96 hour exposure to JH533B under normoxia and pHe 7.4 conditions. Dose response curves to JH533B (0.1-100µM) under normoxic pHe 7.4 conditions. Each result represents the mean ± standard deviation of three independent experiments.

Compound	**IC_{50} (µM)**				
	HCT116 $p53^{+/+}$	**HCT116 $p53^{-/-}$**	**HCT116 $^{KRAS\ Mut}$**	**HCT116$^{KRAS\ wt}$**	**ARPE-19**
JH533B	10.46 ± 4.79	9.76 ± 3.64	8.51 ± 3.3	12.152 ± 4.36	34.59 ± 4.93

Table 7.5 The response of HCT116 cell lines following continuous exposure to JH533B under normoxic pHe 7.4 conditions. Each result represents the mean ± standard deviation of three independent experiments.

Compound	Selectivity index			
	HCT116 $p53^{+/+}$	HCT116 $p53^{-/-}$	HCT116 KRAS Mut	HCT116 KRAS wt
JH533B	3.31	3.54	4.06	2.85

Table 7.6 The selective indices of JH533b for the HCT116 cell lines under normoxic pHe 7.4 conditions.

7.4.4 The effect of JH539 against the HCT116 $p53^{+/+}$ and ARPE-19 cell line under normoxia pHe 7.4 conditions

JH539 displayed the highest level of cytotoxicity against all 4 different HCT116 cell lines with an IC_{50} of 1.273μM ± 0.24, 1.463 ± 0.38, 0.99μM ± 0.27 and 1.515μM ± 0.27 (table 7.7). JH539 also had moderate selectivity when tested against the normal cell line (table 7.8). However this complex was not further tested due to limited amount of drug available.

Compound	IC_{50} (μM)				
	HCT116 $p53^{+/+}$	HCT116 $p53^{-/-}$	HCT116 KRAS Mut	HCT116 KRAS wt	ARPE-19
JH539	1.273 ± 0.24	1.463 ± 0.38	0.99 ± 0.27	1.515 ± 0.27	3.69 ± 0.66

Table 7.7 The response of HCT116 cell lines following continuous exposure to JH539 under normoxic pHe 7.4 conditions. Each result represents the mean ± standard deviation of three independent experiments.

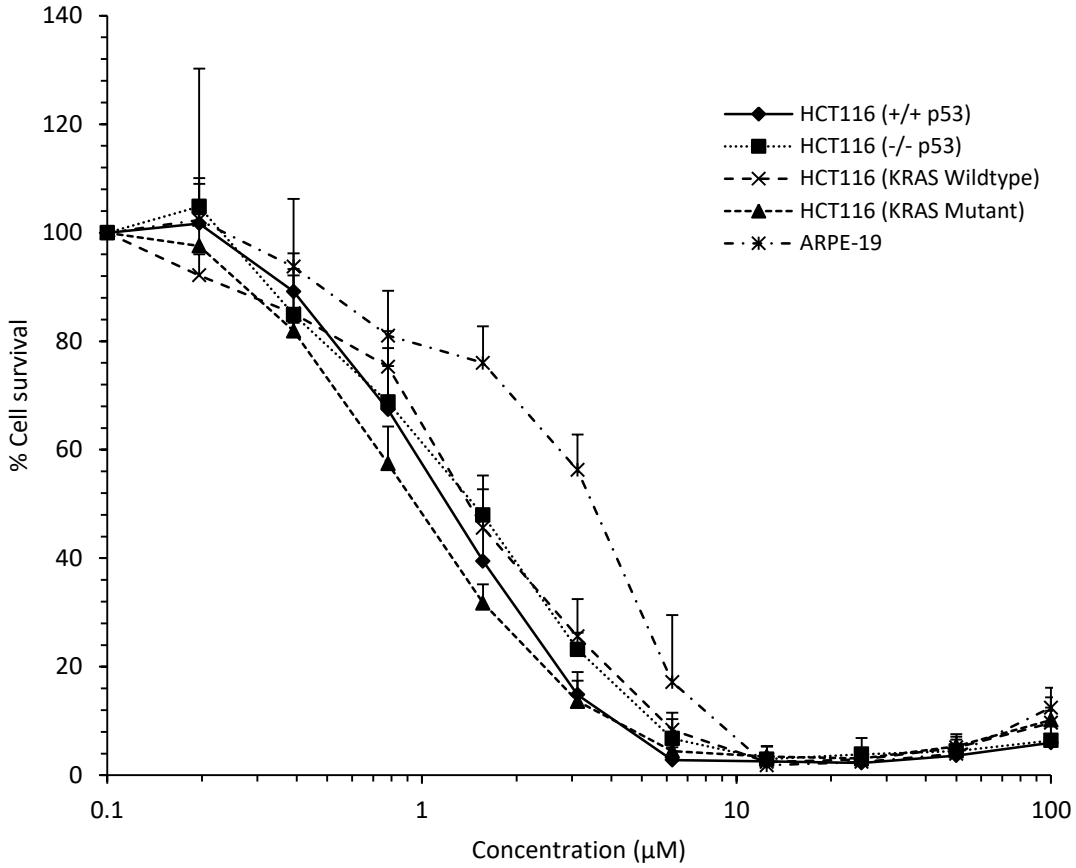


Figure 7.5 Response of HCT116 cell lines and ARPE-19 cells following a 96 hour exposure to JH539 under normoxia and pHe 7.4 conditions. Dose response curves to JH539 (0.1-100µM) under normoxic pHe 7.4 conditions. Each result represents the mean ± standard deviation of three independent experiments.

Compound	Selectivity index			
	HCT116 $p53^{+/+}$	**HCT116 $p53^{-/-}$**	**HCT116** KRAS Mut	**HCT116** KRAS wt
JH539	2.90	2.52	3.73	2.43

Table 7.8 The selective indices of JH539 for the HCT116 cell lines under normoxic pHe 7.4 conditions.

7.4.5 Summary of IC_{50} values for Ag complexes against HCT116 and ARPE-19 cell lines under normoxia pHe 7.4 conditions

All 4 Ag complexes displayed high levels of cytotoxicity against all 4 colorectal cancer cell lines tested (figure 7.6 and table 7.10). JH532, JH547B and JH533B displayed moderate levels of selectivity towards the colorectal cancer cell lines tested with SI ranging from 1.77 – 6.87 (table 7.10).

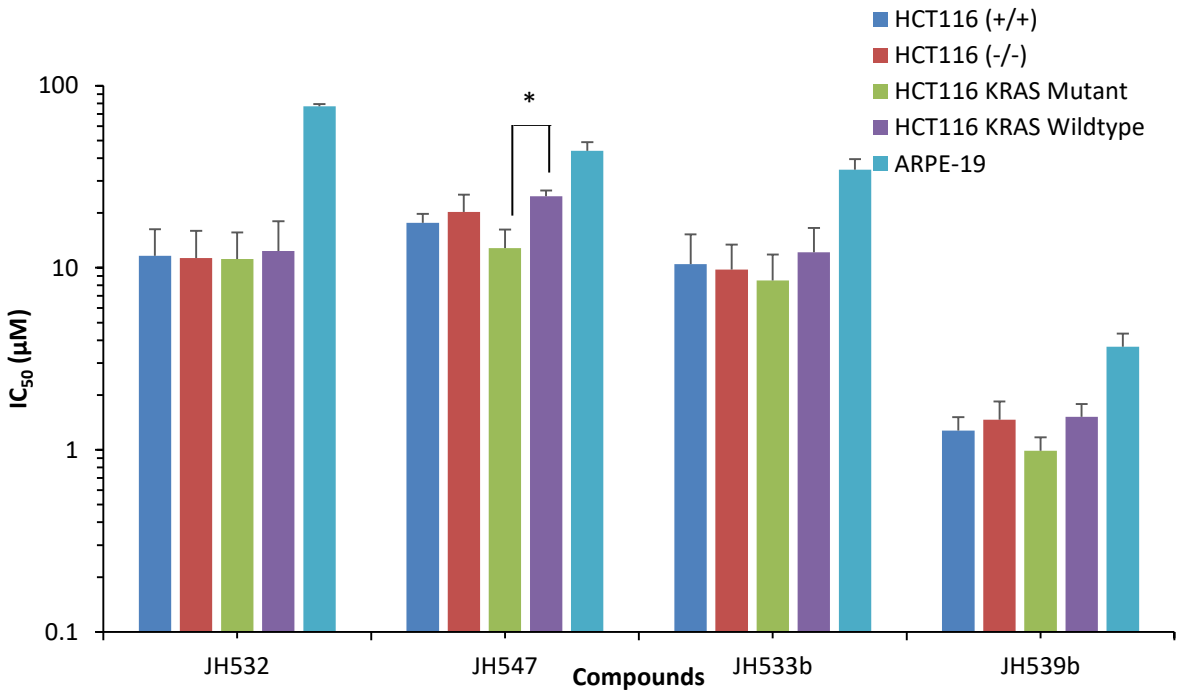


Figure 7.6 Summary of IC50 values following the continuous exposure of HCT116 $p53^{+/+}$, HCT116 $p53^{-/-}$, HCT116 $^{KRAS\ mutant}$, HCT116 $^{KRAS\ wildtype}$ and the normal Human retinal epithelial cells to JH532, JH547B, JH533B and JH539. A two-tail students t-test was applied where p values of ≤0.05 and ≤0.01 are represented as * and ** respectively.

Compound	IC_{50} (μM)				
	HCT116 $p53^{+/+}$	HCT116 $p53^{-/-}$	HCT116 $^{KRAS\ Mut}$	HCT116$^{KRAS\ wt}$	ARPE-19
JH532	11.63 ± 4.61	11.28 ± 4.64	11.18 ± 4.45	12.34 ± 5.64	76.87 ± 2.43
JH547B	17.62 ± 2.13	20.24 ± 4.95	12.83 ± 3.39	24.68 ± 1.86	43.87 ± 5.04
JH533B	10.46 ± 4.79	9.76 ± 3.64	8.51 ± 3.3	12.152 ± 4.36	34.59 ± 4.93
JH539	1.273 ± 0.24	1.463 ± 0.38	0.99 ± 0.27	1.515 ± 0.27	3.69 ± 0.66

Table 7.9 Summary of the response of HCT116 $p53^{+/+}$, HCT116 $p53^{-/-}$, HCT116$^{KRAS\ mutant}$, HCT116$^{KRAS\ wildtype}$ and the normal Human retinal epithelial cells under normoxic conditions against Ag complexes. Each result represents the mean ± standard deviation of three independent experiments.

Compound	Selectivity index			
	HCT116 p53$^{+/+}$	**HCT116 p53$^{-/-}$**	**HCT116** KRAS Mutant	**HCT116** KRAS Wildtype
JH532	6.61	6.81	6.87	6.23
JH547B	2.49	2.17	3.42	1.77
JH533B	3.31	3.54	4.06	2.85
JH539	2.90	2.52	3.73	2.43

Table 7.10 Summary of selectivity index values for Ag complexes towards the colorectal cancer cell lines. SI is defined as the ratio of IC_{50} values for ARPE-19 divided by HCT116 cell lines with values >1 indicating selectivity towards HCT116 cancer cells.

7.4.6 The effect of JH532, JH533B and JH547B against the HCT116 p53$^{+/+}$ cell line under hypoxia pHe 7.4 conditions

Complexes JH532, JH547B and JH533B were all subjected for further testing under hypoxic (pHe 7.4) conditions using the HCT116 p53$^{+/+}$ cell line to determine if any of these complexes exhibit HAP like properties.

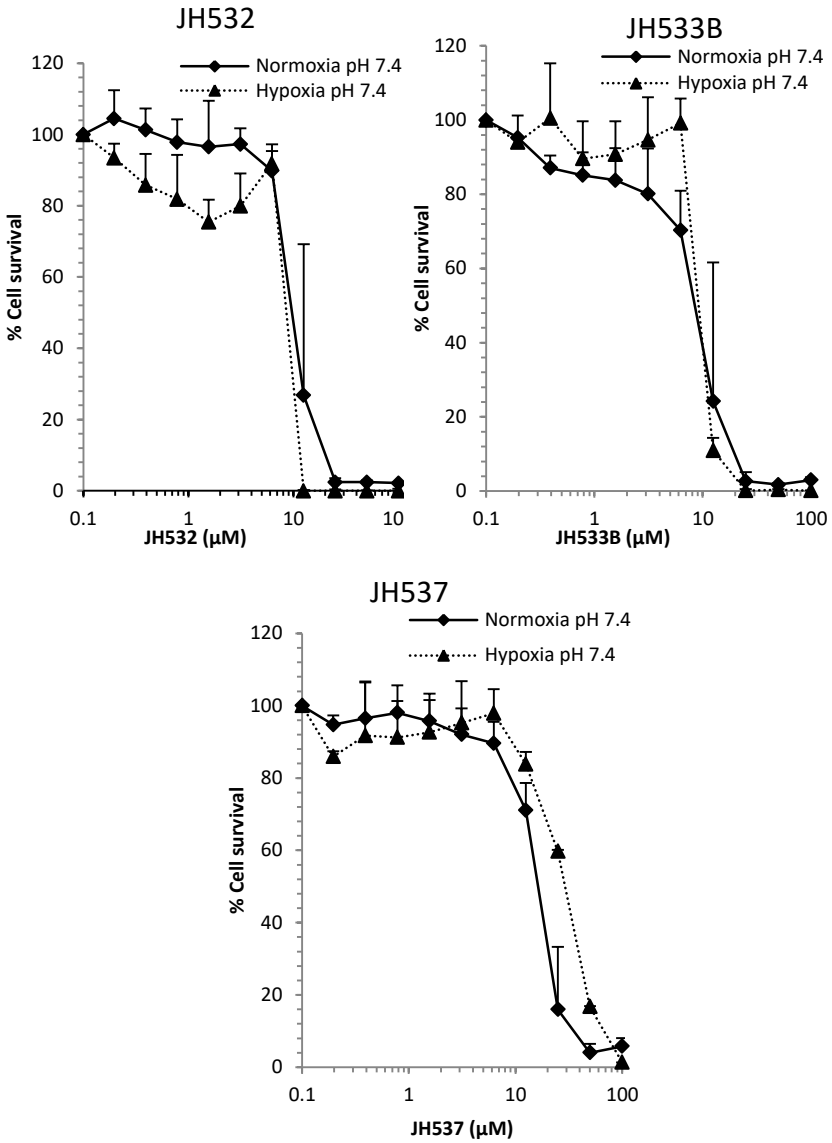


Figure 7.7 HCT116 p53$^{+/+}$ cell line was drug treated for 96hours with JH532, JH533B and JH547B under hypoxia pHe 7.4. Dose response curves to JH539 (0.1-100µM) under normoxic and hypoxic (pHe 7.4) conditions. Each result represents the mean ± standard deviation of three independent experiments.

JH547B displayed no HAP like properties with a HCR of 0.57 as shown in table 7.11. Complexes JH532 and JH533B did display minor HAP like properties but the

difference was not statistically significant. JH532 was considered to be the lead compound and was therefore subjected for further testing under the TME conditions.

Compound	HCT116 $p53^{+/+}$ Normoxic	HCT116 $p53^{+/+}$ Hypoxic	
	IC_{50} (μM)		HCR
JH532	11.633 ± 4.61	9.07 ± 0.21	1.28
JH547B	17.621 ± 2.13	30.70 ± 0.69	0.57
JH533B	10.462 ± 4.79	9.73 ± 0.31	1.08

Table 7.11 The HCR index of Ag complexes towards the colorectal cancer cell lines over a continuous 96 hour exposure. The HCR is defined as the ratio of IC_{50} values under normoxic conditions divided by the IC_{50} under hypoxic (0.1% oxygen) conditions. Values >1 indicate that compounds have selective toxicity towards cells under hypoxic conditions.

7.4.7 The effect of JH532 against the HCT116 p53$^{+/+}$ cell line under TME conditions

JH532 was chosen for further studies as this compound displayed the highest HCR and selectivity amongst all compounds tested. This was equitoxic under normoxia (pHe7.4 and 6.5) and hypoxia (pHe7.4 and 6.5) with IC_{50} values of 11.63, 13.96, 9.07 and 8.9µM respectively. Statistical analysis (one-way ANOVA) displayed no significant difference between all the different conditions tested and is graphically displayed below.

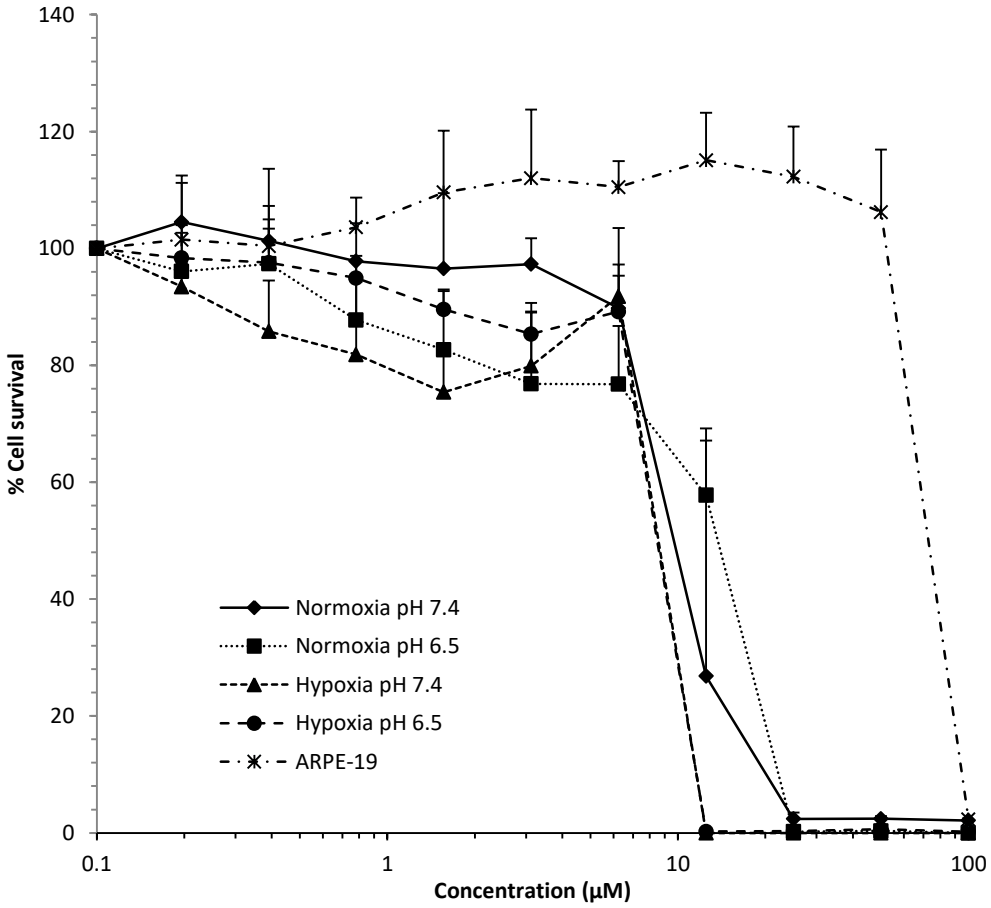


Figure 7.8 Dose response curve of HCT116 p53$^{+/+}$ cells to JH532 under normoxic and tumour microenvironmental conditions. Each result represents the mean ± standard deviation of three independent experiments.

	HCT116 $p53^{+/+}$				ARPE-19
Compound	Normoxic pHe 7.4	Normoxic pHe 6.5	Hypoxia pHe 7.4	Hypoxia pHe 6.5	Normoxic pHe 7.4
JH532	11.63 ± 4.61	13.96 ± 1.78	9.07 ± 0.21	8.9 ± 0.54	76.87 ± 2.43

Table 7.12 Summary of IC_{50} values for JH532 are presented in different combinations of oxygen concentration and pHe. Each value represents the mean IC_{50} ± SD for three independent experiments.

7.4.8 Summary on the effect of JH532 under TME conditions

The TME enhancement ratio for JH532 is 1.3 which is indicative that this complex is displaying some preferential activity towards the physiological TME conditions albeit minor. The TME ratios is also greater than the classic compounds that were tested earlier where resistance towards the physiological TME conditions were shown. The SI was also greater than all of the classic chemotherapeutic complexes tested. The SI (TME) enhancement ratio was 25 fold higher than etoposide and approximately 15 fold higher than cisplatin and 5-FU (table 7.13).

Compound	pHR	HCR	TME ratio	SI	SI (TME)
Cisplatin	0.75	0.44	0.3	1.84	0.55
5-FU	0.55	0.40	0.27	2.10	0.57
Etoposide	0.76	0.61	0.57	0.59	0.34
Tirapazamine	0.61	9.55	n/a	2.33	22.46*
JH532	0.83	1.28	1.3	6.61	8.63

Table 7.13 The effect of the TME on the response of cells to standard cytotoxic drugs, tirapazamine and JH532. pHR, HCR and TME represent the pH ratio, the hypoxic cytotoxicity ratio and tumour microenvironment ratio respectively as defined in the methods section 3.3.3. * refers to the SI value for the hypoxic data only and not the combination of hypoxia and acidic pHe.

7.5 Discussion

Structurally, Ag (NHC) complexes JH547B and JH532 are both similar. These are xanthine – derived imidazolium salts where JH547B has a carborane and the JH532 complex has a phenyl tail group which are distant to the Ag center. Both have similar IC_{50} values across the panel of HCT116 cell lines tested irrespective of their slight structural difference which displays the pivotal role of steric factors in the activity of these complexes (Holmes et al., 2019). Interestingly JH547B was more toxic towards $HCT116^{KRAS\ Wildtype}$ in comparison to $HCT116^{KRAS\ Mutant}$ cell line as shown in figure 7.3 and 7.6. For further confirmation of this, additional downstream signalling studies would need to be carried out in both the KRAS wildtype and mutant HCT116 cell line with JH547B. As KRAS plays a role in downstream signalling of cell growth and proliferation it may be worth carrying out the experiments to determine the effects of JH547B on the Ki67 protein which is a marker for cell proliferation (Stuart-Harris et al., 2008). JH533B and JH539 are both large planar Ag complexes where JH533B has 1 phenyl group attached to the head and tail of the complex whereas JH539 has 1 carborane attached to the head and tail of its complex. Both were cytotoxic towards all the HCT116 cell lines tested with JH539 being the most potent complex of all 4 tested. This could be due to the slower release of Ag over the 96 hour drug exposure due to the protection provided by the large carboranes and therefore maximising its cytotoxicity.

One of the major issues of chemotherapy drug failure is poor side effects and this is still an unmet challenge which is yet to be overcome (Barbuti and Chen, 2015). This has been demonstrated earlier in chapter 3 and is further displayed in table 7.13. All the Ag (NHC) complexes were screened for their cytotoxic and selective ability under both normoxic and TME conditions. The SI index under normoxic conditions was

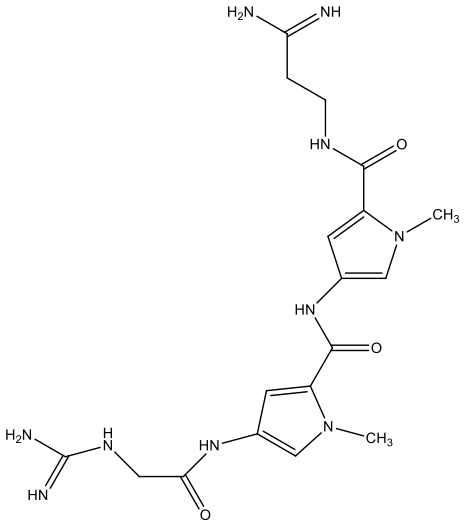
1.84, 2.1, 0.59 and 2.33 for cisplatin, 5-FU, etoposide and tirapazamine respectively. One of the key findings in this study is that all 4 Ag (NHC) complexes displayed SI ranging from 2.49 – 6.61 which demonstrates that even the poorest Ag (NHC) complex JH547B had a better SI against all the classic chemotherapy complexes tested. When these Ag (NHC) complexes were subjected for further testing under hypoxic conditions, it was apparent that none of these behaved like HAPs with a HCR below 1 for JH547B, 1.08 for JH533B and 1.28 for JH532 as shown in figure 7.7 and table 7.11.

The results of this study have demonstrated two key points. The first is that the compounds evaluated are selective for cancer cells as opposed to non-cancer cells *in vitro* under standard physiological conditions (normoxic and pHe7.4). In the case of JH532 where a SI value of 6.61 was obtained, this represents a substantial improvement on SI values for the established cytotoxic drugs tested here. In this context, these compounds are of interest and further studies are required to determine if this observation holds up in a broad panel of cancer cell lines and non-cancer cells. The second point is that these compounds do not posses any significant HAP like properties. Whilst these compounds do not show enhanced activity under TME conditions, it is important to state that their activity is not diminished by hypoxia or acidic pHe conditions which again marks them out as different from the established cytotoxic drugs. In the broader context of phenotypic screening, the results of this study have demonstrated that the screen can identify compounds that are not adversely affected by TME conditions and this in itself is potentially valuable. This is particularly the case when compounds demonstrate inherent selectivity for cancer cells under standard physiological conditions.

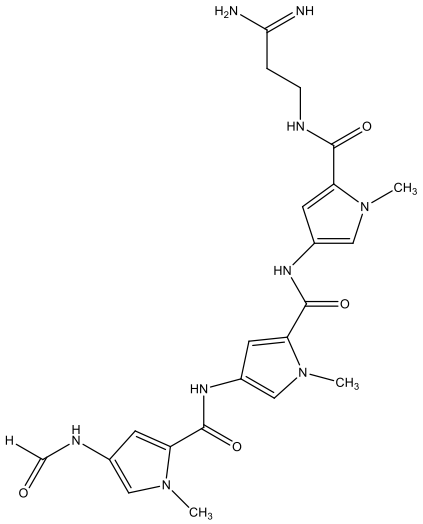
Chapter 8: Phenotypic evaluation of a series of DNA minor groove binders

8.1 Introduction

DNA minor groove binders (MGB) are small molecules which specifically bind to the minor groove of the DNA (Nanjunda and Wilson, 2012). MGBs are an interesting set of compounds which have been studied extensively in the field of infective, bacterial, fungal and cancer research (Scott et al., 2016). MGBs mechanistically work by a suggested method of inhibiting DNA topoisomerase I (Nanjunda and Wilson, 2012) which is further supported by *in vitro* evidence demonstrating that MGB with differing ligands can induce the formation of topoisomerase I mediated DNA breaks (Marchini et al., 2001). However previous reports have demonstrated that other mode of actions of the MGB may exist and these include sequence specific DNA alkylating damage, disruption to the cell cycle and interference with DNA repair (Zimmer et al., 1971). What also makes MGB of interest is their ability to inhibit DNA binding of transcription factors in a sequence dependent manner (Zimmer et al., 1971). Arguably the two most common antiviral antibiotics MGB that have been extensively researched are netropsin and distamycin (figure 8.1) as these were the first MGB compounds discovered which bind selectively to AT rich sequences in the minor groove (Bailly and Chaires, 1998). In this study 5 MGB which are based on the polyamide natural product of distamycin and netropsin are assessed for their cytotoxic, selective and HAP like abilities. These molecules were synthesised by Dr Fraser Scott at the University of Huddersfield and the results have been recently published (Nichol et al., 2019).



Netropsin



Distamycin

Figure 8.1 Chemical structure of netropsin and distamycin.

As described in previous chapters the selection of cell lines used in this chapter was based on pragmatic reasons and not on any biochemical rationale. In addition, to maintain consistency with other chapters in this thesis, HCT116 $p53^{+/+}$ and ARPE-19 cell lines were used.

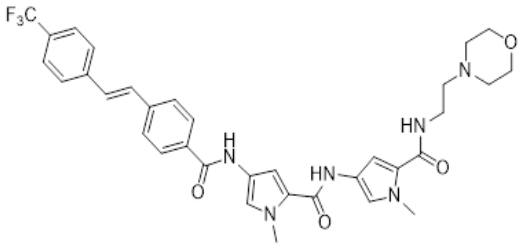
8.2 Aims

The aim of this study is to evaluate whether or not a series of distamycin and netropsin MGB derivatives are cytotoxic towards the colon rectal cancer cell lines HCT116 $p53^{+/+}$. To determine their selectivity for cancer cells, these compounds will also be evaluated against the non-cancerous ARPE-19 cell line under conditions that reflect the 'normal conditions' found in healthy tissue (i.e. oxygenated and pHe 7.4). The MGB compounds will also be subjected to hypoxia studies to determine preferential activity under (i) hypoxia (0.1% oxygen), (ii) acidic extracellular pH (pHe 6.5) or (iii) a combination of hypoxia and acidic pHe.

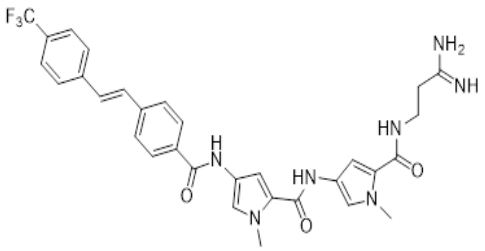
8.3 Material and methods

8.3.1 Compounds

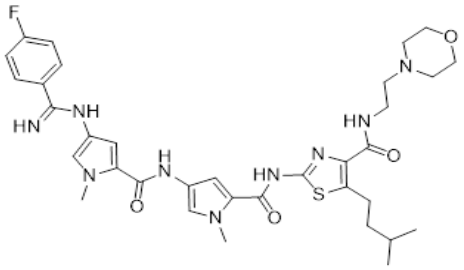
All MGB were kindly provided by Dr Fraser Scott (University of Huddersfield – now University of Strathclyde). These were prepared in dimethylsulphoxide (DMSO) at 10mM and aliquots (20µL) were stored at -20°C.



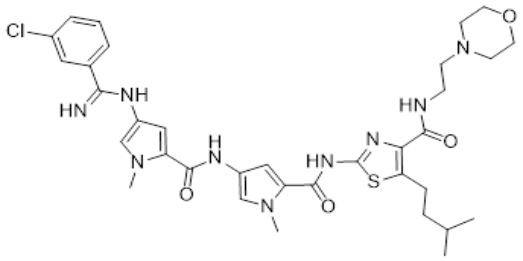
MGB 176 (RMM= 762.7)



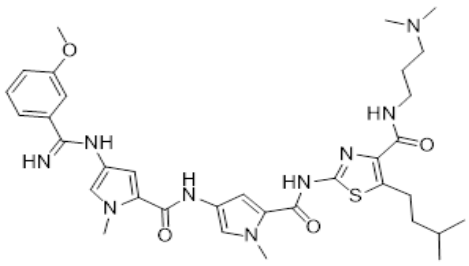
MGB 364 (RMM= 719.67)



MGB 372 (RMM= 919.86)



MGB 372 (RMM= 936.31



MGB 4 (RMM= 903.37)

Figure 8.2 Chemical structure of MGB based compounds and their molecular weights in parenthesis.

8.3.2 Cell culture and chemosensitivity

Cell culture, adjusting the pH of the media, drug dilutions and chemosensitivity studies were carried out as previously described in the general methods chapter (see sections 2.2, 2.6-2.8). The HCT116 $P53^{+/+}$ cell line was used in this chapter. Any variation from these methods are indicated in the figure legends and described in the results section.

8.3.3 Western blot analysis

Cells were treated with hit compounds for a duration of 24 hours at a drug concentration of 0.25, 0.5 and 1µM. These concentrations were chosen as it is a therapeutically relevant concentration (IC_{50} under normoxic conditions was 0.234 ± 0.005 µM and 4.418 ± 0.41 µM for MGB 4 and MGB 176 resepectively as shown in table 6.12). A 24 hour duration of drug exposure was selected as minimal cell death had occurred at this time point.

. Controls were treated with media alone or with DMSO (0.1% v/v) vehicle control (which has been previously reported not to be toxic to the cells). Doxorubicin was used as the positive control and these were carried out under the following tumour microenvironment conditions, normoxia pHe 7.4, normoxia pHe 6.5, hypoxia pHe 7.4 and hypoxia pHe 6.4. Upon completion of treatment, cells were harvested for western blot analysis for DNA damage induction (phosphorylated **γ**H2AX or p53). Details of western blot methodology and antibody dilutions are described in methods (see sections 2.12 and table 2.2).

8.3.4 Statistical analysis

Statistical analysis was carried out using Microsoft Excel and Graph Prism. All experiments were done in triplicate (n=3), and results were presented as the mean ±

SD. Statistical significance was determined by means of a two-tailed Student's t-test, or by One Way ANOVA where significance was only accepted when $p \leq 0.05$. The exact statistical analysis performed is mentioned in the figure legend.

8.4 Results

8.4.1 The effect of MGBs against the HCT116 p53$^{+/+}$ and ARPE-19 cell lines under normoxic pHe 7.4 conditions

The response of HCT116 p53+/+ and ARPE-19 cells to MGB compounds under normoxic and pHe 7.4 conditions is presented in figures 8.3A and B respectively. The second most potent MGB tested against the HCT116 cell line was MGB 373 with an IC_{50} of 1.286 ± 0.19 μM and a SI of 1.48 (table 8.1). The least potent MGB was the 364 which was inactive against the HCT116 p53$^{+/+}$ cell lines under normal physiological conditions (figure 8.1). The MGB 372 compound displayed potency with IC_{50} values of 2.953 ± 0.60 μM and moderate levels of selectivity towards the cancerous cell line with a SI of 2.41. MGB 176 displayed moderate level of activity towards the HCT116 p53$^{+/+}$ cell line with an IC_{50} value of 4.418 ± 0.41 μM.

	IC_{50} (μM)		
Compound	**HCT116 pHe 7.4 Normoxia**	**ARPE-19 pHe 7.4 Normoxia**	**SI**
MGB 364	IC_{50}>10	IC_{50}>10	-
MGB 176	4.418 ± 0.41	10.94 ± 1.19	2.47
MGB 372	2.953 ± 0.60	7.135 ± 0.79	2.41
MGB 373	1.286 ± 0.19	1.909 ± 0.07	1.48
MGB 4	0.234 ± 0.005	IC_{50} > 10	>42.73

Table 8.1 The IC_{50} values and SI for a series of MGBs against the HCT116 and ARPE-19 cell lines under normoxic pHe 7.4 conditions. Each result represents the mean ± standard deviation of three independent experiments.

The most potent MGB under these set of conditions was the MGB 4 compound with an IC_{50} value of 0.234 ± 0.005 μM which was similar to that of etoposide tested earlier in chapter 3. As well as being highly active, MGB 4 also has the highest SI index of 42.73 amongst all of the MGBs tested (table 8.1).

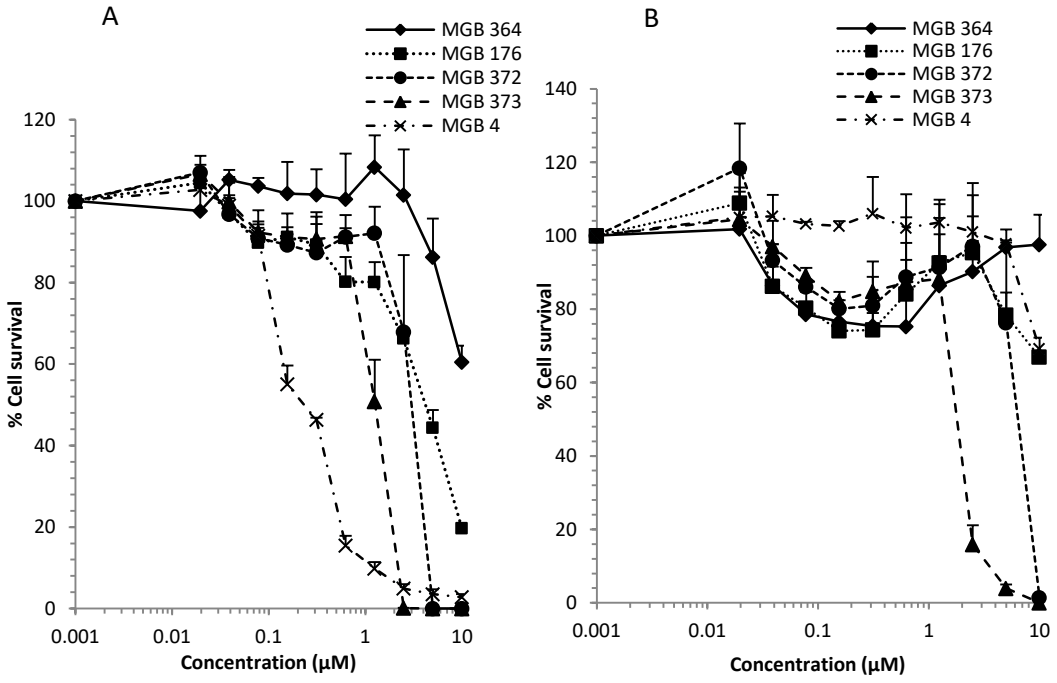


Figure 8.3 Response of HCT116 $p53^{+/+}$ (panel A) and ARPE-19 cells (panel B) following a 96 hour exposure to a series of MGBs under normoxic and pHe 7.4 conditions. Each result represents the mean ± standard deviation of three independent experiments.

8.4.2 The effect of MGBs against the HCT116 p53$^{+/+}$ cell line under normoxic and hypoxic pHe 7.4 conditions

HCT116 p53$^{+/+}$ cells were treated with a dose concentration range of MGBs over 96 hours under hypoxic pHe 7.4 conditions. The IC_{50} generated for MGB 364, 176, 372, 373 and 4 was 8.20 ± 0.1, 1.77 ± 0.02, 2.05 ± 0.01, 0.93 ± 0.02 and 7.65 ± 0.57 μM respectively (figure 8.4 and table 8.1). All of the MGBs apart from the MGB 4 displayed HAP like abilities (albeit minor) with HCR values of 1.22, 2.49, 1.44, 1.38 and 0.003 (MGB 364, 176, 372, 373 and 4) respectively and the data is shown in figure 8.4 and table 8.2.

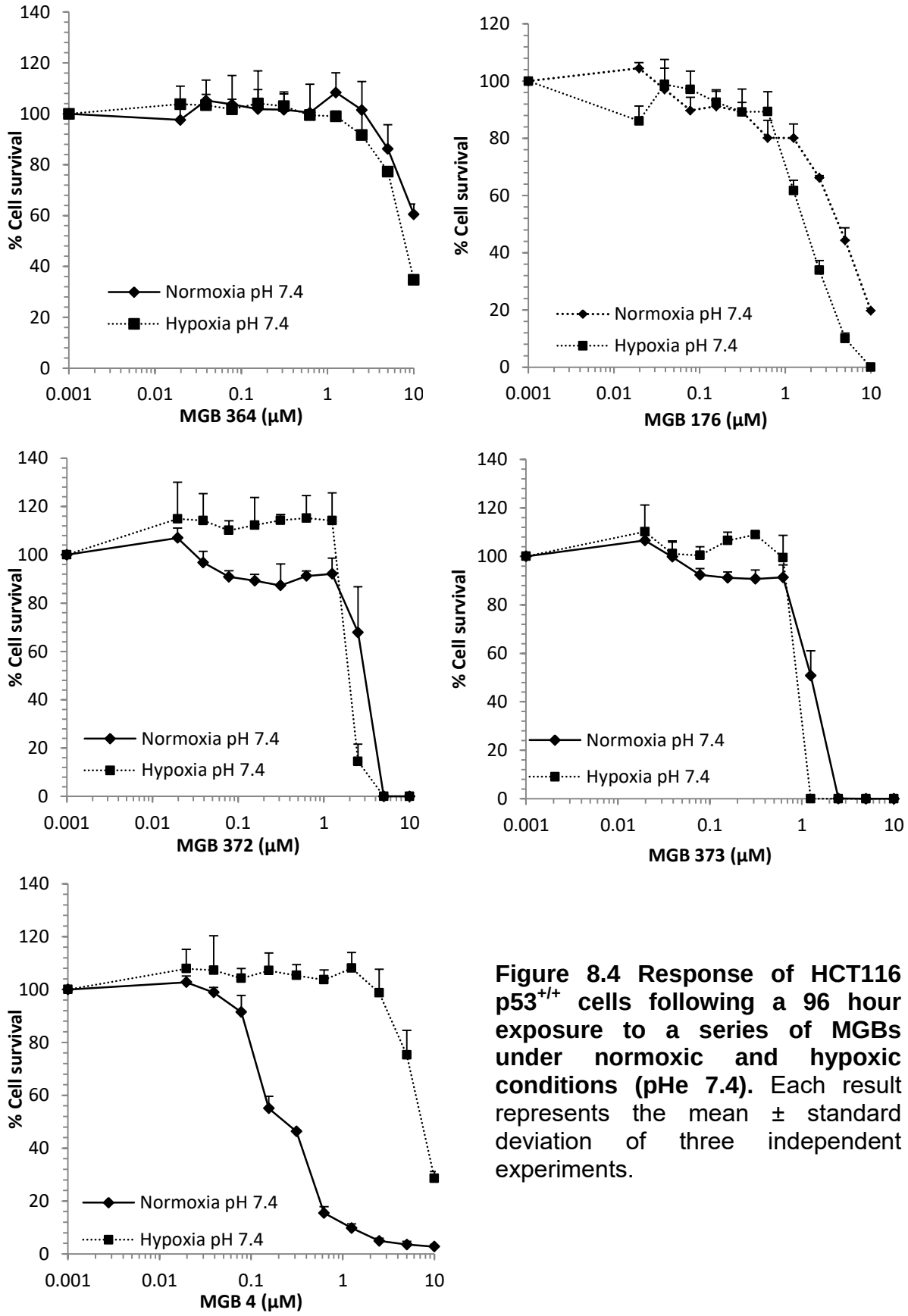


Figure 8.4 Response of HCT116 $p53^{+/+}$ cells following a 96 hour exposure to a series of MGBs under normoxic and hypoxic conditions (pHe 7.4). Each result represents the mean ± standard deviation of three independent experiments.

Compound	IC_{50} (μM) HCT116 pHe 7.4 Normoxia	HCT116 pHe 7.4 Hypoxia	HCR
MGB 364	IC_{50}>10	8.20 ± 0.1	>1.22
MGB 176	4.418 ± 0.41	1.77 ± 0.02	2.49
MGB 372	2.953 ± 0.60	2.05 ± 0.01	1.44
MGB 373	1.286 ± 0.19	0.93 ± 0.02	1.38
MGB 4	0.234 ± 0.005	7.65 ± 0.57	0.003

Table 8.2 The IC_{50} values and HCR for a series of MGBs against the HCT116 cell lines under normoxic and hypoxic pHe 7.4 conditions. Each result represents the mean ± standard deviation of three independent experiments.

8.4.3 The effect of MGBs against the HCT116 $p53^{+/+}$ cell line under normoxic pHe 7.4 and pHe 6.5 conditions

HCT116 $p53^{+/+}$ cells were treated with a dose concentration range of MGBs over 96 hours under normoxic pHe 6.5 conditions where only MGB 176 and 364 displayed preferential activity under pHe 6.5 conditions with a pHR of 2.06 and 1.04 respectively. The other MGBs displayed no preferential activity under normoxic pHe 6.5 conditions with a pHR all <1, 0.78, 0.69 and 0.02 (table 8.3) for MGBs 372, 373 and 4 respectively. The data is displayed below in figure 8.5.

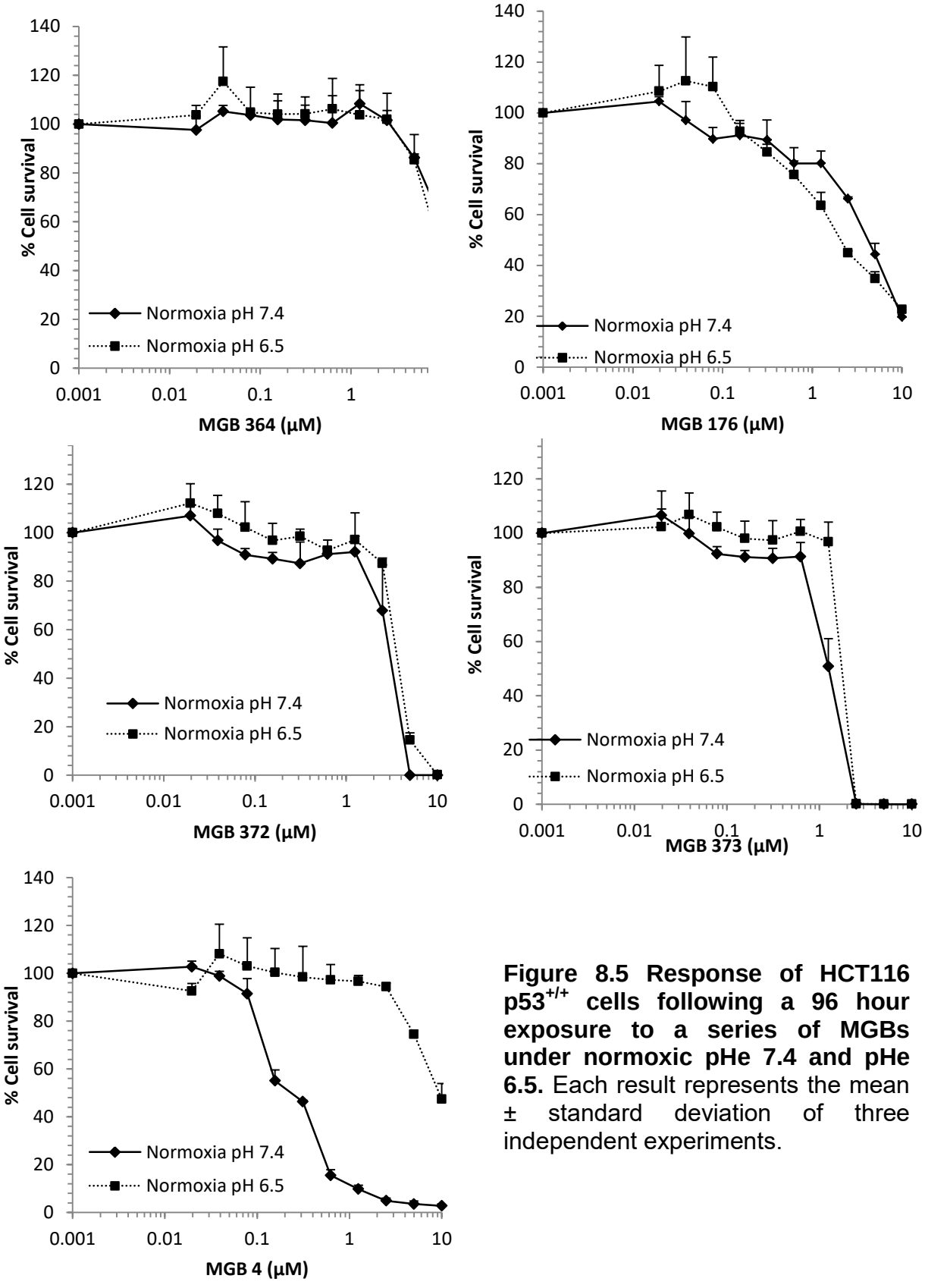


Figure 8.5 Response of HCT116 p53$^{+/+}$ cells following a 96 hour exposure to a series of MGBs under normoxic pHe 7.4 and pHe 6.5. Each result represents the mean ± standard deviation of three independent experiments.

Compound	IC_{50} (μM) HCT116 pHe 7.4 Normoxia	HCT116 pHe 6.5 Normoxia	pHR
MGB 364	IC_{50}>10	9.60 ± 0.55	>1.04
MGB 176	4.418 ± 0.41	2.14 ± 0.09	2.06
MGB 372	2.953 ± 0.60	3.78 ± 0.07	0.78
MGB 373	1.286 ± 0.19	1.85 ± 0.05	0.69
MGB 4	0.234 ± 0.005	9.72 ± 1.25	0.02

Table 8.3 The IC_{50} values and pHR for a series of MGBs against the HCT116 cell lines under normoxic pHe 7.4 and pHe 6.5 conditions. Each result represents the mean ± standard deviation of three independent experiments.

8.4.4 The effect of MGBs against the HCT116 $p53^{+/+}$ cell line under normoxic pHe 7.4 and hypoxic pHe 6.5 conditions

HCT116 $p53^{+/+}$ cells were treated with a dose concentration range of MGBs over 96 hours under hypoxic pHe 6.5 conditions. The IC_{50} generated for the compounds was 2.35 ± 0.02, 3.66 ± 0.16, 1.46 ± 0.05 μM and TME ratios were 1.88, 0.80, 0.88 for MGB 176, 372 and 373 respectively (figure 8.6 and table 8.4). There was no IC_{50} reached for MGB 364 and 4 which meant that a TME ratio could not be calculated for these.

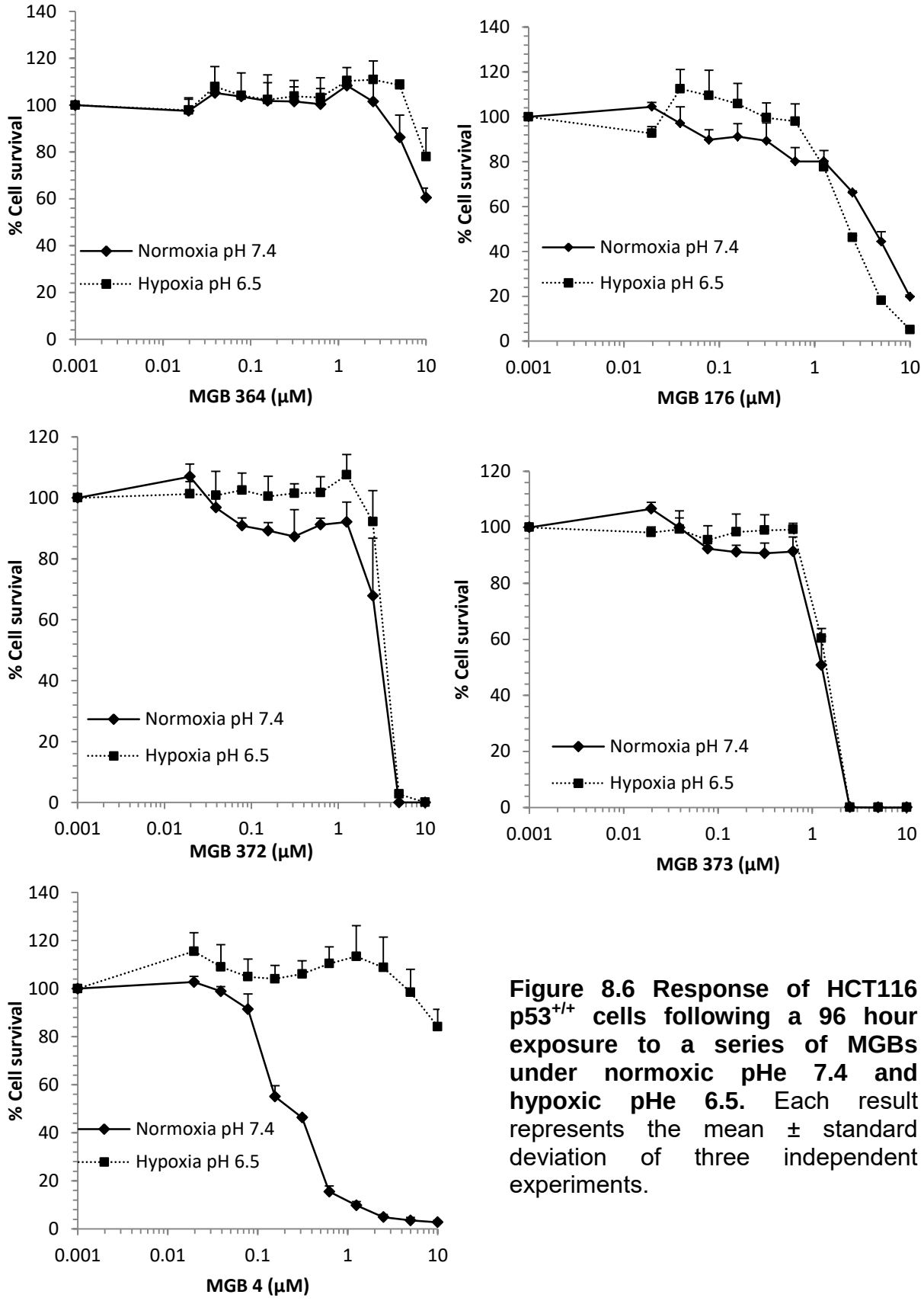


Figure 8.6 Response of HCT116 p53$^{+/+}$ cells following a 96 hour exposure to a series of MGBs under normoxic pHe 7.4 and hypoxic pHe 6.5. Each result represents the mean ± standard deviation of three independent experiments.

Compound	IC_{50} (μM) HCT116 pHe 7.4 Normoxia	HCT116 pHe 6.5 Hypoxia	TME (SI)
MGB 364	IC_{50}>10	IC_{50} > 10	-
MGB 176	4.418 ± 0.41	2.35 ± 0.02	4.65
MGB 372	2.953 ± 0.60	3.66 ± 0.16	1.94
MGB 373	1.286 ± 0.19	1.46 ± 0.05	1.29
MGB 4	0.234 ± 0.005	IC_{50} > 10	1.30*

Table 8.4 The IC_{50} values and TME (SI) for a series of MGBs against the HCT116 cell lines under normoxic pHe 7.4 and hypoxic pHe 6.5 conditions. Each result represents the mean ± standard deviation of three independent experiments. * refers to the SI value for the hypoxic data only and not the combo of hypoxia and acidic pHe.

8.4.5 Summary on the effect of MGBs against the HCT116 cell line under TME conditions

The results of the chemosensitivity studies performed in this chapter are summarised in tables 8.5 (IC_{50} values under different conditions) and 8.6 (various parameters derived from the IC_{50} values in comparison to the standard agents). Under normoxic conditions (pHe 7.4), a broad range of activity against HCT116 $p53^{+/+}$ cells was observed with MGB 364 being least active (IC_{50} > 10 μM) and MGB 4 being most potent (IC_{50} = 0.234 ± 0.005 μM). When evaluated under both hypoxic conditions, acidic pHe 6.5, and both hypoxic and acidic pHe conditions combined, the most significant finding was the almost complete loss of MGB 4 activity (table 8.5). This has a significant effect on pHR, HCR, TME and SI values as shown in table 8.6. Of

all the compounds evaluated, only MGB 176 showed HAP like activity although this was modest and below the values established for tirapazamine. This is nevertheless a promising lead compound to emerge from the screen which possesses modest HAP like activity and selectivity for cancer cells that is superior to the established anti-cancer drugs evaluated.

Based on the results above, MGB 4 displayed the high levels of activity which was on par with some of the classic compounds tested and selectivity towards the cancer cell lines which was much greater than the classic compounds. Furthermore, MGB 176 was the only compound evaluated here that showed HAP like activity. It was for this reason that both MGB 176 and MGB 4 were both chosen to be further tested in western blot analysis for the induction of γH2AX phosphorylation (a marker of DNA damage).

	IC_{50} (μM)				
Compound	**HCT116 pHe 7.4 Normoxia**	**HCT116 pHe 6.5 Normoxia**	**HCT116 pHe 7.4 Hypoxia**	**HCT116 pHe 6.5 Hypoxia**	**ARPE-19 cells**
MGB 364	IC_{50}>10	9.60 ± 0.55	8.20 ± 0.1	IC_{50} > 10	IC_{50}>10
MGB 176	4.418 ± 0.41	2.14 ± 0.09	1.77 ± 0.02	2.35 ± 0.02	10.94 ± 1.19
MGB 372	2.953 ± 0.60	3.78 ± 0.07	2.05 ± 0.01	3.66 ± 0.16	7.135 ± 0.79
MGB 373	1.286 ± 0.19	1.85 ± 0.05	0.93 ± 0.02	1.46 ± 0.05	1.909 ± 0.07
MGB 4	0.234 ± 0.005	9.72 ± 1.25	7.65 ± 0.57	IC_{50} > 10	IC_{50} > 10

Table 8.5 A summary of the response of HCT116 $p53^{+/+}$ and ARPE-19 cells following continuous exposure to MGB netropsin and distamycin derivatives under normoxic and tumour microenvironment conditions. Each result represents the mean ± standard deviation of three independent experiments.

Compound	pHR	HCR	TME ratio	SI	SI (TME)
Cisplatin	0.75	0.44	0.3	1.84	0.55
5-FU	0.55	0.40	0.27	2.10	0.57
Etoposide	0.76	0.61	0.57	0.59	0.34
Tirapazamine	0.61	9.55	n/a	2.33	22.46*
MGB 364	1.04	1.22	n/a	n/a	n/a
MGB 176	2.06	2.49	1.88	2.47	4.65
MGB 372	0.78	1.44	0.80	2.41	1.94
MGB 373	0.69	1.38	0.88	1.48	1.29
MGB 4	0.02	0.003	n/a	42.73	1.30*

Table 8.6 The effect of the TME on the response of cells to standard cytotoxic drugs and tirapazamine. pHR, HCR and TME represent the pH ratio, the hypoxic cytotoxicity ratio and tumour microenvironment ratio as defined in the methods section in results chapter (3.3.3). * refers to the SI value for the hypoxic data only and not the combination of hypoxia and acidic pHe.

8.4.6 Western blot analysis for the early signs of DNA damage by MGB 4 and MGB 176 under normoxic pHe 7.4

This was carried out by exposing HCT116 $p53^{+/+}$ cells to a range of concentrations of MGB 4 or MGB 176 as shown in figures 8.7 and 8.8. A positive control doxorubicin was used as this classic chemotherapy drug possesses several mechanisms of action, one of which being the inhibition of topoisomerase II (known to induce double stranded DNA breaks). For MGB 4 the level of the tumour suppressor protein p53 was also analysed. As shown in figure 8.7 the levels of γH2AX phosphorylation and p53 was increased substantially for doxorubicin but this was not the case for MGB 4

over a 24 hour time period where the levels of these two proteins were not higher than the 0.1% DMSO vehicle controls. Similar results were obtained for MGB 176 (figure 8.8).

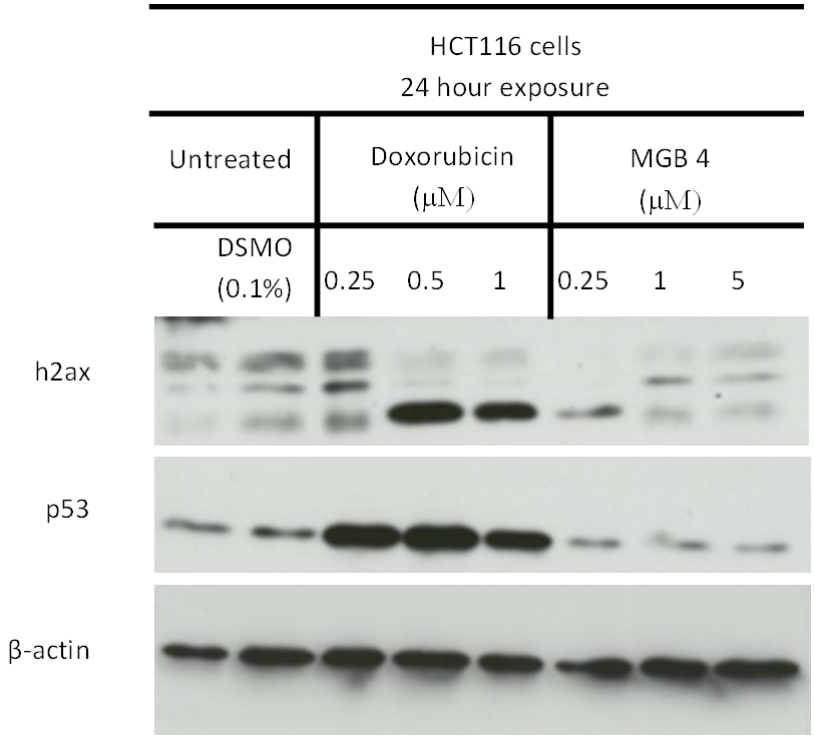


Figure 8.7 Analysis of γH2AX phosphorylation following the treatment of HCT116 p53$^{+/+}$ with MGB 4 (24 hour drug exposure (n=1)).

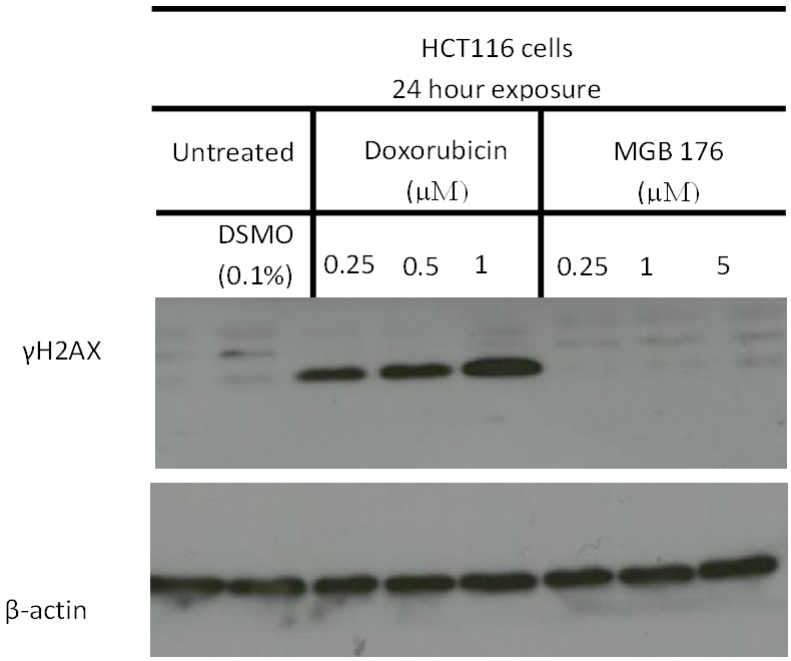


Figure 8.8 Analysis of γH2AX phosphorylation following the treatment of HCT116 p53$^{+/+}$ with MGB 176 (24 hour drug exposure (n=1)).

8.5 Discussion

The main objective of this study was to determine whether a group of MGB could possess more toxic and selective activity than those of classic chemotherapeutic agents tested in results chapter 3 and shown in table 8.6. Also of interest of this PhD study is to screen these compounds under those conditions which exist in the tumour microenvironment. The most potent compound from this study under normoxic (pHe 7.4) conditions was MGB 4 and it is hypothesised that this is due to its high lipophilicity which is generally correlated with high activity in medicinal chemistry (Nichol et al., 2019). MGB 4 was also the most selective (SI - 42.73) compound amongst all of the MGBs including the cytotoxic drugs evaluated in chapter 3 (table8.6) which is a positive result.

Despite being the most potent MGB 4 under normoxic pHe 7.4 conditions, this was not the case under those conditions favouring the TME (pHe and hypoxia) where the efficacy of this compound is significantly reduced. This is a major shift in chemosensitivity and whilst the mechanistic reasons for this are unknown, it does demonstrate the value of the phenotypic screen as it can identify compounds that are adversely affected by the TME conditions (table 8.6). MGB 176 displayed some HAP like activity (HCR - 2.49, table 8.6) which is modest at best but this is a potential lead for further development of these compounds as potential HAP-like agents.

MGB 176 and MBG 4 were both selected as lead compounds for further testing to determine if these are DNA damaging agents by γH2AX phosphorylation. This was carried out by western blotting and the data suggests that these compounds are not inducing DNA damage. This is however a preliminary conclusion as H2AX is not a universal marker of DNA damage and further assays are required to confirm this.

The MGBs were screened under various parameters, where MGB 4 initially displayed good activity and selectivity under normoxic pHe 7.4 conditions which is favourable to those classic chemotherapeutic drugs tested earlier in chapter 3. However MGB 4 was adversely affected by the TME conditions and although this is a negative outcome it identifies that the screen can also detect compounds that are resistant to the TME conditions. This tool allows the researcher to make an informed decision whether to develop or not to develop these compounds further only to find out later that they are completely ineffective against hypoxic cells that reside within an acidic tumour microenvironment.

Chapter 9: General conclusions

Drug discovery is a lengthy and complex process that typically follows a target orientated or phenotypic screening approach (Jaibu et al., 2018). Whilst often seen as lacking significant intellectual input, phenotypic screening has undergone a resurgence in interest largely because it is one of the major sources of 'first in class' drugs that target multiple pathways that are important in cancer biology. In this thesis, a phenotypic screen designed to identify compounds that are preferentially active in the tumour microenvironment (TME) has been combined with an assessment of selectivity for cancer cells. The aim of this is to identify novel compounds that are selectively more effective against cancer cells that reside within the TME, a region of tumours that is associated with chemotherapeutic limitations and poor clinical outcome in patients (Höckel and Vaupel, 2001; Kato et al., 2013).

To provide a baseline or yardstick, three classic chemotherapeutic drugs, cisplatin, etoposide and 5-FU were tested against the colorectal cancer cell line HCT116 $p53^{+/+}$ under physiological and TME conditions. These conditions were (i) normoxia / pHe 7.4 (ii) normoxia / pHe 6.5, (iii) hypoxia / pHe 7.4 and (iv) hypoxia / pHe 6.5. The results demonstrated that TME conditions adversely affected the activity of all three compounds (chapter 3). Statistical analysis using one-way ANOVA was significant in all cases where data from normoxic / pHe 7.4 was compared to hypoxia / pHe 6.5 ($p \geq 0.05$). The data generated under hypoxic conditions (pHe 6.5) for all three classic chemotherapeutic compounds adversely affected selectivity indices with tumour cells being less responsive than the non-cancer ARPE-19 cell line. These results are consistent with the issue of poor tumour selectivity and lack of efficacy against the hypoxic fraction of tumours associated with world leading chemotherapeutic drugs. The data generated from the phenotypic screen of the classic chemotherapeutic

compounds were used as a comparative baseline standard throughout this PhD. To further validate the screen, the HAP tirapazamine was also included and as expected, it demonstrated enhanced efficacy under hypoxic conditions (results chapter 3, figure 3.4). Further studies are required to assess the response of tirapazamine under hypoxic and acidic pHe conditions. To conclude, the results in this chapter effectively demonstrate that TME conditions (i) adversely affect the activity of established drugs and (ii) promote the activity of compounds designed to work better under TME conditions. The screen should therefore be capable of identifying compounds with preferential activity and compounds that are adversely affected by TME conditions.

Having established that the TME phenotypic screen is capable of identifying compounds that are preferentially active under TME conditions, novel compounds were then evaluated in this screen. Compounds were kindly provided from various collaborators across the world which were, (i) Ruthenium, Iridium and Rhodium based napthyridines, thiourea derivatives, benzylthiourea ligands and aroylthiourea complexes, (ii) Bromo-hexitol prodrugs, (iii) NHC-Ag based complexes, (iv) Minor groove binders and (v) Tyrosine kinase inhibitor (dasatinib) with the attachment of a 2-nitroimidazole bioreductive trigger. The compounds above are either novel compounds or designed as analogues of existing compounds (with unknown mechanisms) and can be broadly segregated into two main groups (i) those that were designed to have HAP-like properties (bromo-hexitol prodrugs and DAS-HAP) and (ii) completely unknown, novel compounds.

In total there were 72 (excluding the classis chemotherapeutic compounds) different compounds that were phenotypically screened under the TME conditions and from these, 40 compounds (55.5%) were selective towards the cancer cell line with a

selectivity index > 1. A further 8 compounds (11.1%) were further activated by pHe 6.5 alone under normoxic conditions. A total of 24 compounds (33.3%) displayed HAP like abilities with a HCR value > 1 and finally 3 compounds (4.1%) exhibited enhanced toxicity with the combination of pHe 6.5 with hypoxia. Furthermore, 19 compounds (26.3%) which were not designed as bio-reductive drugs were more active under hypoxic (pHe 7.4) conditions (HCR > 1) when compared to normoxia (pHe 7.4) and this is something that may have been missed if this phenotypic screening strategy was not employed. A prime example of this is the 1-8, napthyridine, KMR-SA-24 (not designed as a HAP) which displayed high selectivity towards the cancerous cell line and was also further activated under hypoxic (pHe 7.4) conditions. This compound shows much promise with some refinements to the as a potential HAP candidate. In comparison to the classic chemotherapeutic compounds, 15 (20.8%) of the compounds screened were more selective (SI > 2.10) than the established drugs. A major finding of this thesis therefore is that screening compounds under both acidic and hypoxic conditions has identified novel compounds that would not have been discovered otherwise. The phenotypic screen described in this study therefore represents a novel contribution to the field with the potential to identify lead compounds that have the ability to target the physiological TME of tumours.

Looking in more detail at the more specific highlights of this thesis, not all of the dibromohexitols exhibited HAP like properties or were influenced by all of the TME conditions apart from DS10 which displayed preferential activity under a combination of hypoxia and acidic pHe conditions. DAS-HAP although not as toxic as its counterpart dasatinib displayed HAP like abilities with superior HCR in comparison to dasatinib against the H460 cell line. This is striking as dasatinib has been previously

shown to not be as toxic against the H460 cell line under hypoxic conditions when compared with normoxic conditions. Here we have demonstrated for the first time that the addition of the N-OXIDE attachment to dasatinib makes this a very potent HAP. The organometallic compounds were not impressive with regards to toxicity alone regardless of their preferential selectivity towards the cancer cells. A major finding from this study was the KMR-SA-24 complex which despite not being as toxic as the other complexes or compounds in this study, it still possessed good HAP like properties. These compounds were not designed to work against the TME but this particular example demonstrates that the phenotypic screen can identify compounds that would not have been identified otherwise. Similarly, JH532 from the NHC Ag complexes displayed equitoxic activity under the TME conditions. All of the NHC Ag complexes however showcased moderate to good levels of selectivity towards the cancer cells which is also an important decision point for the selection of potential lead compounds. The MGBs were the last set of compounds that were tested in this study and from the 5 MGBs that were screened, MGB 4 displayed the most potent activity and selectivity towards the cancer cell line under normoxic conditions. MGB4 when screened further under the TME conditions did not perform as well. This screen therefore also demonstrates its ability to identify those compounds that may not be suitable under TME conditions. MGB4 is nevertheless an interesting compound because of its inherent selectivity for cancer cells under normoxic conditions. Therefore the following hit or lead compounds identified using this phenotypic screen were DS10, DAS-HAP, KMR-SA-24, JH532, MGB4 and MGB 176.

The approach described in this study is effectively a pilot study and there are numerous ways in which the screen could be improved or expanded. For example,

the phenotypic screen employed in this PhD was not tumour specific and the HCT116 cell line was mainly used as an initial model to test the proposed hypothesis. A potential improvement therefore would be to utilise different cell lines and if this is taken to the extreme, the NCI60 screening approach to screening could be implemented. There are obvious cost and time implications associated with expanding the number of cell lines and this needs to be carefully considered when designing a screen of this nature. This may however be favourable for compounds that hit specific targets such as DAS-HAP where high levels of Src have been previously reported in the colorectal cancer cell lines. Whilst DAS-HAP was specifically designed, it is conceivable that randomly selected compounds which target specific pathways could be missed if the cell lines used don't express the specific target of interest.

Another area that needs to be expanded is characterisation of existing approved anti-cancer drugs in the screen. Due to time constraints, only 3 classic chemotherapeutic drugs were tested against the physiological TME screen. By enhancing the library of classic chemotherapeutic compounds in this study under the physiological and TME conditions, the phenotypic screen may provide a more in depth overview as to how these classic compounds behave in the TME conditions.

A 2D monolayer model was used to screen the number of compounds under TME conditions (hypoxia and pHe 6,5) and this provided a quick and robust 'tool' to assess the effects of the TME on compounds. Whilst high throughput screening in drug discovery is also carried out in 2D cell culture models (which is cost and time effective), these models do not mimic the complexity of the TME in tumours (Langhans, 2018). An area where this approach could be improved therefore would be to use a 3D cell culture model such as spheroids which contain a much more

complex TME that closely mimics the features which are present *in vivo* (Ravi et al., 2015). The use of 3D models however adds further complexity to the conduct of experiments, particularly when large numbers of compounds are involved. The phenotypic screen employed throughout this PhD provides a platform for the rapid testing such compounds under conditions that mimic some aspects of the TME and once hit compounds have been identified, further testing using multi-cell layers or spheroids would be an appropriate next step prior to *in vivo* testing. The use of 3D models would address the issue of drug penetration which is essential if compounds that target the TME are to work effectively (Minchinton and Tannock, 2006).

In conclusion, the results of the work presented in this thesis have demonstrated that a phenotypic approach to the identification of compounds with preferential activity against the TME is both technically feasible and effective. Several compounds have been identified that are preferentially active under hypoxic, acidic pHe or a combination of the two conditions *in vitro*. These compounds require further evaluation in more complex 3D models but they demonstrate that the screen has identified new lead compounds that would not have been identified using conventional approaches. Furthermore, the screen also picks up compounds that are adversely affected (e.g. MGB 4) by the TME and this is equally valuable information for drug discovery programs. As with all screening approaches, the experimental models can be improved but there is a balance to be struck between increased complexity and the cost and efficiency of any changes. To conclude, the phenotypic screen described in this thesis is effective at identifying compounds with preferential activity against cells in the TME and further studies are required to expand upon this work.

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