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Dissecting the role of NADPH:cytochrome P450 oxidoreductase in the activation of hypoxia-activated prodrugs using zinc finger nucleases


By

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Abstract

Tumour hypoxia results in aggressive tumours with increased metastatic potential and resistance to therapy. Exploitation of tumour hypoxia can be achieved using hypoxia-activated prodrugs (HAP), which are selectively activated by reductases in a process inhibited by oxygen. NADPH:cytochrome P450 oxidoreductase (POR) is widely considered to be the major enzyme responsible, but this conclusion is based on studies using purified POR or forced over-expression in cell lines. Thus the enzymology of HAP activation is still not fully understood. To examine the role of endogenous POR in HAP activation, POR knockout clones were generated by targeted mutation in exon 8 of POR using custom-designed zinc finger nucleases (ZFN) in HCT116 and SiHa cell lines. The POR knockout clones were validated for loss of POR expression by Western blotting, mutations were confirmed at the ZFN target site and a significant loss of POR enzyme activity was measured by cytochrome c assay and activation of a novel fluorogenic probe; FSL-61. The knockout models were compared to isogenic wild-type and POR over-expressing cells using metabolism and cytotoxicity assays across a panel of 12 HAP. POR over-expression significantly increased the sensitivity of HCT116 and SiHa cells to aromatic N-oxides (tirapazamine, SN29751, SN30000) and nitro compounds SN24349 and nitracrine under aerobic and anoxic conditions (p<0.05). Surprisingly, the effect of POR knockout on anoxic resistance to HAP was modest, with a statistically significant resistance to tirapazamine, SN30000, CB1954 and SN24349, in only one of the two HCT116 and SiHa POR knockout clones. Further interrogation with PR-104A and SN30000 showed trends to lower formation of anoxic metabolites in the POR knockouts compared to wild-type cells, but the decreases were not statistically significant. In addition, the POR knockout clones did not demonstrate increased resistance to these HAP by clonogenic assay. These observations confirm that most HAP are substrates for POR, but indicate that POR at endogenous levels of expression plays only a limited role in the activation of these prodrugs, with clearer signals of its contribution in SiHa than HCT116. This suggests the existence of redundant one-electron reductases that are able to affect prodrug activation in the absence of POR.
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Published papers containing data/materials from this thesis


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Part of the material in Chapter 4 is presented in a manuscript submitted to The Biochemical Journal. 'Su, J., Guise, C.P., & Wilson, W.R. (provisionally accepted) FSL-61 is a 6-nitroquinolone fluorogenic probe for one-electron reductases in hypoxic cells, Biochem J'.

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**Certification by Co-Authors**

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate’s contribution to this work, and the nature of the contribution of each of the co-authors; and
- in cases where the PhD candidate was the lead author of the work that the candidate wrote the text.

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<td>AKR1C3</td>
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<td>ECACC</td>
<td>European Collection of Cell Cultures</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray jet stream ionisation</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FDXR</td>
<td>NADPH:adrenodoxin reductase</td>
</tr>
<tr>
<td>FMN</td>
<td>Flavin mononucleotide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>FSL</td>
<td>Fluorescence substrate library</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>HAP</td>
<td>Hypoxia-activated prodrugs</td>
</tr>
<tr>
<td>HDR</td>
<td>Homology directed repair</td>
</tr>
<tr>
<td>HIF-1</td>
<td>Hypoxia inducible factor -1</td>
</tr>
<tr>
<td>HNSCC</td>
<td>Head and neck squamous cell carcinoma</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>Indels</td>
<td>Insertions/deletions</td>
</tr>
<tr>
<td>ISCN</td>
<td>International System of Cytogenetic Nomenclature</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo dalton</td>
</tr>
<tr>
<td>LC/MS</td>
<td>Liquid chromatography/mass spectrometry</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimal essential medium</td>
</tr>
<tr>
<td>MRM</td>
<td>Multiple reactions monitoring</td>
</tr>
<tr>
<td>MTRR</td>
<td>Methionine synthase reductase</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NDOR1</td>
<td>NADPH-dependent diflavin oxidoreductase 1</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Non-homologous end joining</td>
</tr>
<tr>
<td>NOS1</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NOS2</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>NOS3</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>NQO1 (2)</td>
<td>NAD(P)H:Quinone oxidoreductase 1 (2)</td>
</tr>
<tr>
<td>NZBI</td>
<td>New Zealand Bioinformatics Institute</td>
</tr>
<tr>
<td>NZGL</td>
<td>New Zealand Genomics Limited</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR (RT-PCR), PCR-ELISA</td>
<td>Polymerase chain reaction (reverse transcription), enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PHD</td>
<td>Prolyl hydroxylase domain</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>POR</td>
<td>NADPH:cytochrome P450 oxidoreductase</td>
</tr>
<tr>
<td>pVHL</td>
<td>von Hippel-Lindau protein</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>-------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>RIPA buffer</td>
<td>Radioimmunoprecipitation buffer</td>
</tr>
<tr>
<td>RNA/ siRNA</td>
<td>Ribonucleic acid (Small interfering)</td>
</tr>
<tr>
<td>RT</td>
<td>Retention time</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>TPZ</td>
<td>Tirapazamine</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>ZFN</td>
<td>Zinc finger nuclease</td>
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</table>
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1.1. Introduction

Cancer is one of the leading causes of mortality in New Zealand accounting for 28.9% of all deaths recorded in 2009 (Ministry of Health, 2012). One of the major hurdles in cancer therapy stems from the highly heterogeneous nature of this disease; recently illustrated by the identification of 183,916 somatic base pair mutations in a panel of 21 primary breast cancers (Nik-Zainal et al., 2012). It has been suggested that this high frequency of mutations reflects a mutator phenotype (Loeb et al., 2008), and that this genomic instability generates driver mutations responsible for the hallmarks of cancer (Hanahan & Weinberg, 2011). Adding to the complexity of treating cancer is the tumour microenvironment which is almost as important to tumourigenesis as mutational changes in the cells itself (Allen & Jones, 2011). One of the major components of the microenvironment is the extent of tumour oxygenation, with tumour hypoxia commonly accepted to be a major driver of tumour progression.

1.2. Tumour hypoxia

Tissue hypoxia occurs when the partial pressure of oxygen (pO₂) falls below a critical level (pO₂ ≤ 2.5 mmHg) and is a characteristic property of solid tumours (Vaupel et al., 2007). This oxygen deficit results from an imbalance between consumption and oxygen supply, and can be divided into chronic or acute hypoxia. Chronic hypoxia occurs in tumours when the distance between the blood supply and the tumour cells is beyond the diffusion distance of oxygen (100-200 μm) (Harris, 2002; Vaupel & Mayer, 2007). Acute or cycling hypoxia can result from changes in reperfusion and red blood cell flux due to functional and structural abnormalities in the tumour microvasculature (Dewhirst et al., 2008; Dewhirst, 2009). This phenomenon can be visualised by the administration of two different markers for hypoxia at two different times; with a subpopulation of tumour cells staining for one marker but not the other (Figure 1.1) (Ljungkvist et al., 2000; Russell et al., 2009). Nevertheless, numerous cellular mechanisms are present to assist cells in coping with the physiological stress of hypoxia.
1.2.1. Molecular responses to hypoxia

Changes in gene expression from the activation of the HIF-1 pathway, allow cells to survive under hypoxia. HIF-1 is a heterodimeric transcription factor consisting of two subunits, HIF-1α and HIF-1β (Wang & Semenza, 1995) which binds to promoter or enhancer regions of genes containing hypoxia response elements (Semenza & Wang, 1992; Wang & Semenza, 1993). Levels of HIF-1α are increased under hypoxia while HIF-1β is constitutively expressed. HIF-1α levels are tightly regulated via ubiquitin-dependent proteasomal degradation in the presence of oxygen following modification by the von Hippel-Lindau protein (pVHL), an E3 ubiquitin ligase (Maxwell et al., 1999). pVHL binding to the oxygen-dependent degradation domain of HIF-1α itself, is dependent on hydroxylation of proline residues P402 and P564 by prolyl hydroxylase domain (PHD) 1-3 (Ivan et al., 2001;
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Jaakkola et al., 2001). The PHDs display different affinities towards the different family members of the HIF-α proteins (Appelhoff et al., 2004) with PHD2 considered to be the primary mediator of HIF-1α degradation (Berra et al., 2003). In the absence of oxygen, the stabilisation of HIF-1α results in activation of the HIF-1 pathway, leading to activation of a wide range of genes that assist in cellular survival under hypoxia. These adaptive genes include genes for angiogenesis (VEGF), glucose metabolism (HK2, GLUT1), pH regulation (CA-IX), migration/invasion (c-Met, MMP2), cellular survival and autophagy (IGF2, MDM2, BNIP3) (Giaccia et al., 2003; Semenza, 2003; Bardos & Ashcroft, 2004; Ke & Costa, 2006).

Other molecular pathways are also activated under hypoxia, independent of the HIF-1 pathway. Hypoxia can induce a cellular response to DNA damage with the activation of ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and Rad3-related protein), enzymes important in DNA double-strand break signalling (Hammond et al., 2002; Bencokova et al., 2009). Hypoxia also leads to the rapid shutdown of DNA synthesis in all cell types, possibly due to the decreased supply of nucleotides from a decrease in ribonucleotide reductase activity (Pires et al., 2010). Other than effects on ATM and ATR, numerous studies have demonstrated the down-regulation of DNA repair pathways during hypoxia (reviewed in (Bristow & Hill, 2008; Hammond et al., 2011)) with genes in mismatch repair (MLH1) (Mihaylova et al., 2003), homology directed repair (HDR; Rad51 and BRCA2) (Chan et al., 2008) and non-homologous end joining (NHEJ; XRCC4 and DNA ligase IV) (Meng et al., 2005) affected by hypoxia. Taken together, these changes can lead to genetic instability and a means for the generation of additional mutations leading to malignant progression (Bristow & Hill, 2008). Hypoxia can also affect cellular pathways such as the unfolded protein response, which regulates protein homeostasis in the endoplasmic reticulum (Koumenis et al., 2002) and the mTOR pathway which regulates protein synthesis, autophagy and apoptosis (Arsham et al., 2003). Collectively, hypoxia leads to widespread changes in gene expression and cellular physiology through multiple oxygen-sensing mechanisms. These changes can act as a major force for tumour progression and hence, tumour hypoxia remains one of the more important targets of cancer therapy.

1.2.2. Tumour hypoxia as a therapeutic target

The presence of tumour hypoxia provides a potential therapeutic target for anti-cancer agents since hypoxic areas are more common and severe in tumours when compared to normal tissues (Brown & Wilson, 2004; Tatum et al., 2006). Measurements of tumour
oxygenation found regions of tumour hypoxia in most locally advanced solid tumours from the head and neck region, breast, uterine cervix, brain, prostate, pancreas and soft tissue sarcomas (Vaupel et al., 2007), and up to 50-60% of these tumours may show heterogeneous distribution of these hypoxic regions within the tumour mass (Vaupel & Mayer, 2007). Hypoxic regions in tumours typically correlate to more aggressive cancers with higher metastatic potentials (Zhang & Hill, 2004; Erler et al., 2006; Lunt et al., 2009) and the sensitivity of these tumours to radiation decreases under hypoxic conditions (Gray et al., 1953), due to decreased oxygen-dependent fixation of lethal DNA strand breaks (Alper & Howard-Flanders, 1956; Hodgkiss et al., 1987; Brown, 2007). Hypoxic cells are located in regions distant from a functional blood supply where it is difficult for most chemotherapeutic agents to reach by diffusion, thereby increasing resistance to these compounds (Minchinton & Tannock, 2006). In addition, tumour hypoxia also exerts a selective pressure for cells with increased resistance to apoptosis, leading to reduced effectiveness of chemotherapeutic agents (Graeber et al., 1996). These characteristics typically lead to poor patient outcome, illustrated by a lower overall survival rate in patients with more hypoxic head and neck squamous cell carcinomas (HNSCC) after radiation therapy (Figure 1.2) (Gatenby et al., 1988; Hockel et al., 1996; Nordsmark et al., 2005).

Strategies to exploit tumour hypoxia have therefore been an active area of research since the first evidence emerged for the presence of hypoxic regions in solid tumours many decades ago (Thomlinson & Gray, 1955). As oxygen is required for fixation of radiation-induced DNA damage, oxygen-mimetic radiosensitisers such as the nitroimidazoles (e.g. misonidazole and nimorazole), have been developed as an adjuvant to radiation therapy (Wardman, 2007). The usage of these radiosensitisers in conjunction with radiotherapy demonstrated improved loco-regional control of HNSCC but this effect was only observed in tumours that were classified as being more hypoxic based on a hypoxic gene signature (Toustrup et al., 2011). This indicates that the additive benefit of these radiosensitisers was restricted to patients with hypoxic tumours and the presence of hypoxic cells limits the therapeutic outcomes of radiotherapy in HNSCC. In addition to radiosensitisers, there is also considerable interest in targeting the HIF-1 pathway with a few inhibitors currently in clinical trials (Semenza, 2010; Rapisarda & Melillo, 2010). These therapies however, do not directly kill tumour cells and only modulate their cellular responses under hypoxic conditions. More relevant to this thesis is the development of bioreductive compounds that can be selectively activated under hypoxia to directly kill hypoxic tumour cells. This hypoxia-specific activation can potentially minimise adverse toxicity commonly associated with untargeted
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chemotherapeutic agents used in cancer therapy. In addition, diffusion of toxic effectors (from the activation of these compounds) in hypoxic regions to the surrounding tumour (the bystander effect) can increase the therapeutic efficacy of these compounds.

1.3. Exploiting tumour hypoxia with hypoxia-activated prodrugs

Hypoxia-activated prodrugs (HAP) are non-toxic prodrugs that are selectively activated by reduction under hypoxia, and are therefore often also known as bioreductive prodrugs. The moiety that is reduced enzymatically under hypoxia may be a nitro group, quinone, aromatic $N$-oxide, aliphatic $N$-oxide or transition metal (Chen & Hu, 2009; Wilson & Hay, 2011). This activation can occur either by an oxygen-sensitive one-electron reduction or a two-electron reduction step, the latter typically being oxygen-independent (Brown & Wilson, 2004; Wilson & Hay, 2011). A one-electron reductase capable of activating HAP under hypoxia, NADPH:cytochrome P450 oxidoreductase (POR; EC 1.6.2.4) is a major focus of this thesis.

Generally, the one-electron reduction of HAP by one-electron reductases generates a reactive radical intermediate which can be rapidly reoxidised by molecular oxygen, generating superoxide (Wardman & Clarke, 1976). This was demonstrated by early experimental observations showing that the oxidation of NADPH (Feller et al., 1971) and the consumption of oxygen during aerobic reduction of nitroaromatics with recombinant one-electron reductases can be partially reversed by superoxide dismutase (Mason & Holtzman, 1975). In the absence of oxygen, the intermediate prodrug radical can be reduced further to

Figure 1.2 Overall survival rate is reduced in patients with more hypoxic HNSCC treated with radiotherapy

Patients with less hypoxic tumours (hypoxic fraction, as defined by $pO_2$ <2.5 mmHg, of <19 %) have a significantly better (p=0.006) overall survival compared to patients with more hypoxic tumours. Reprinted (adapted) with permission from (Nordsmark et al., 2005). Copyright (2005) Elsevier Limited.
other downstream compounds (Figure 1.3). Since superoxide itself is a toxic free-radical (Keyer & Imlay, 1996), hypoxic-selectivity of these prodrugs is a function of the increased cytotoxicity of the prodrug radical or its downstream products compared to superoxide. Most of these HAP have reduction potentials between -0.5 V and -0.1 V, which reflects the minimum reduction potential for activation by flavoproteins (Wardman, 2001). Complicating the activation mechanisms of these HAP, are two-electron reductases such as NAD(P)H:quinone oxidoreductase-1 (NQO1 or DT-diaphorase, EC 1.6.5.2), capable of two-electron reduction of HAP straight to the active effector, bypassing the oxygen-sensitive step (in most cases). Understanding the enzymology of HAP activation is important to improve the tumour selectivity of these compounds and enable patient stratification during clinical trials in order to maximise the therapeutic benefits of these compounds.

Several other factors can determine the anti-tumour activity of HAP. Modelling studies have demonstrated the importance of moderately hypoxic cells (1-25 µM dissolved O₂) in the response of tumours to fractionated radiotherapy (Wouters & Brown, 1997). Hence for HAP to fully compliment other treatment modalities such as radiation therapy, the oxygen dependence of HAP needs to be quantified as this varies between different classes of bioreductive compounds (Koch, 1993; Hicks et al., 2004; Hicks et al., 2007). Therefore, to maximise cell killing by HAP when used in combination with other treatment modalities, two strategies can be used. Firstly, utilising HAP with a high K_O2 (oxygen concentration for 50% decrease in anoxic potency) to kill the moderately hypoxic cells (e.g. the aromatic-N-oxide tirapazamine (TPZ); K_O2 = 1.31µM). However, this increased activation could also lead to off-target effects from activation in physiologically hypoxic tissues such as the retina and bone marrow (Hicks et al., 2004). Alternatively, HAP with low K_O2 but with a bystander effect can minimise this off-target toxicity and kill the less hypoxic cells at the same time (e.g. PR-104A; K_O2 = 0.126µM) (Hicks et al., 2007; Wilson et al., 2007). In addition to the oxygen dependence and reductive activation of HAP, tumour cells can also be intrinsically more sensitive to HAP if the cells are deficient in DNA repair pathways. For example, cells deficient in homologous recombination repair enzymes (ERCC1, XPF and Rad51) are more sensitive to PR-104A, particularly in cells with high reductase activity (Gu et al., 2009). Therefore, these three major factors (hypoxia, reductase activity and intrinsic sensitivity to the active drug) need to be considered, in order to maximise the therapeutic benefit from the usage of HAP.
Some of the earliest studies on the metabolic activation of anticancer drugs were focused on quinones, which require bioreductive activation for DNA cross-linking activity (Schwartz et al., 1963; Iyer & Szybalski, 1964). The one-electron reduction of quinones results in the generation of a semiquinone radical anion (Iyanagi & Yamazaki, 1969), which can be further reduced to the hydroquinone metabolite via another one-electron reduction step in the absence of oxygen (Figure 1.4) (Iyanagi & Yamazaki, 1970; Wardman, 2001; Chen & Hu, 2009). In the presence oxygen, this semiquinone radical can be oxidised by oxygen back to the quinone form generating superoxide (Bachur et al., 1979). Two-electron reductases can directly reduce the quinone moiety to the hydroquinone species, bypassing this oxygen-sensitive step (Iyanagi & Yamazaki, 1970).
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Mitomycin C (Figure 1.5) was the earliest quinone-containing HAP used in clinical trials (Sartorelli, 1988). The reduction of the mitomycin C to its DNA cross-linking species requires the formation of the hydroquinone moiety, which activates the aziridine ring by the elimination of methanol from the C9-C9a position (Schwartz et al., 1963; Iyer & Szybalski, 1964). This DNA cross-linking species can then alkylate DNA either by forming guanine-N2 monoadducts, guanine-N7 adducts or guanine-N2 bifunctional adducts (Figure 1.6) (Tomasz et al., 1987). Dissection of the reductive activation pathways of mitomycin C demonstrated that the oxygen-dependent generation of semiquinone metabolites was catalysed by microsomal enzymes in a NADPH-dependent manner (Iyanagi & Yamazaki, 1969; Bachur et al., 1978). Subsequently, the one-electron reduction of mitomycin C was shown to be catalysed by purified rat POR (Komiyama et al., 1979; Bachur et al., 1979; Pan et al., 1984) and rabbit POR (Keyes et al., 1984) under hypoxia. In addition, cell lines over-expressing POR through viral promoters showed increased sensitivity to mitomycin C compared to isogenic Chinese Hamster Ovary (CHO) WT cells under oxic and hypoxic conditions (Belcourt et al., 1996b). POR over-expression was also capable of re-sensitising CHO cells previously resistant to mitomycin C (Baumann et al., 2001), and nuclear localisation of POR further increased sensitivity of cells to mitomycin C compared to endoplasmic reticulum localisation of POR (Seow et al., 2005). However contradictory observations have been reported, showing enhanced cytotoxicity of POR over-expressing cells to mitomycin C only occurs under aerobic conditions (Cowen et al., 2003), and a recent report demonstrating that over-expression of POR in CHO cells does not increase cytotoxicity to mitomycin C under oxic and hypoxic conditions (Wang et al., 2010).

Figure 1.4 Reductive activation of quinones by one- or two-electron reduction

![Diagram of quinone reductive activation](image)

Mitomycin C (Figure 1.5) was the earliest quinone-containing HAP used in clinical trials (Sartorelli, 1988). The reduction of the mitomycin C to its DNA cross-linking species requires the formation of the hydroquinone moiety, which activates the aziridine ring by the elimination of methanol from the C9-C9a position (Schwartz et al., 1963; Iyer & Szybalski, 1964). This DNA cross-linking species can then alkylate DNA either by forming guanine-N2 monoadducts, guanine-N7 adducts or guanine-N2 bifunctional adducts (Figure 1.6) (Tomasz et al., 1987). Dissection of the reductive activation pathways of mitomycin C demonstrated that the oxygen-dependent generation of semiquinone metabolites was catalysed by microsomal enzymes in a NADPH-dependent manner (Iyanagi & Yamazaki, 1969; Bachur et al., 1978). Subsequently, the one-electron reduction of mitomycin C was shown to be catalysed by purified rat POR (Komiyama et al., 1979; Bachur et al., 1979; Pan et al., 1984) and rabbit POR (Keyes et al., 1984) under hypoxia. In addition, cell lines over-expressing POR through viral promoters showed increased sensitivity to mitomycin C compared to isogenic Chinese Hamster Ovary (CHO) WT cells under oxic and hypoxic conditions (Belcourt et al., 1996b). POR over-expression was also capable of re-sensitising CHO cells previously resistant to mitomycin C (Baumann et al., 2001), and nuclear localisation of POR further increased sensitivity of cells to mitomycin C compared to endoplasmic reticulum localisation of POR (Seow et al., 2005). However contradictory observations have been reported, showing enhanced cytotoxicity of POR over-expressing cells to mitomycin C only occurs under aerobic conditions (Cowen et al., 2003), and a recent report demonstrating that over-expression of POR in CHO cells does not increase cytotoxicity to mitomycin C under oxic and hypoxic conditions (Wang et al., 2010).
Mitomycin C is reduced to the hydroquinone form either by two-sequential one-electron reduction steps or a two-electron reduction step. The hydroquinone is activated to the DNA cross-linking species by the loss of methanol at C9-9a position. This DNA cross-linking species can then alkylate DNA either by forming guanine-N2 monoadducts, guanine-N7 adducts or guanine-N2 bifunctional adducts.
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EO9 (Figure 1.5) is an indolequinone based HAP capable of forming DNA cross-links after reductive activation under hypoxia (Bailey et al., 1997). The reductive activation of EO9 to the DNA alkylating species is not yet fully understood but is suggested to occur via the release of hydroxyl groups at C2 and C3 of the hydroquinone of EO9 (Hargreaves et al., 2000). EO9 has shown excellent activity in several preclinical tumour models (Hendriks et al., 1993) and like mitomycin C was shown to be activated by recombinant rat POR (Bailey et al., 2001). In addition, POR over-expressing MDA-MB-231 cells showed increased sensitivity to this compound compared to isogenic WT cells under oxic and anoxic conditions (Saunders et al., 2000). The excellent activity of EO9 in preclinical tumour models however did not translate to success in Phase II clinical trials (Dirix et al., 1996; Pavlidis et al., 1996) with several reasons being attributed to the failure of EO9 in these trials. Firstly, the supply of EO9 to tumours was impaired by its rapid pharmacokinetic elimination and poor tumour penetration (Phillips et al., 1998). In addition, the reductase profile in patients and level of tumour hypoxia in patients was not utilised for patient stratification (Phillips et al., 2012). However, overcoming these important issues enabled improvements on the future designs of EO9 clinical trials, such as using intravesical therapy to directly deliver EO9 to superficial bladder cancers (van der Heijden et al., 2006). EO9 was subsequently evaluated for activity in non-invasive bladder cancer (due to its high NQO1 expression) in Phase III trials (www.clinicaltrials.gov identifier NCT00598806) but it did not meet its primary endpoint.

Several studies have implicated the role of NQO1 as a two-electron reductase for mitomycin C and EO9. NQO1 is an obligate two-electron reductase that utilises FAD as a co-factor and is thought to be physiologically involved in the detoxification of endogenous and exogenous quinone-containing compounds (Danson et al., 2004). NQO1 expression is regulated by the ARE-NRF2-Keap1 pathway (antioxidant response element - nuclear erythroid 2-related factor 2 – Kelch ECH associating protein 1) (Itoh et al., 1997; Nioi et al., 2003), which is involved in cellular protection against oxidative stress (Kensler et al., 2007). NQO1 is highly expressed in a wide range of normal tissues (Siegel & Ross, 2000), cancer cell lines and also many human malignant tumours such as non-small cell lung cancer, colon, breast and ovarian cancers (Siegel et al., 1998; Siegel et al., 2004). The increased expression of NQO1 in tumour cell lines (Siegel et al., 1990; Robertson et al., 1994; Plumb et al., 1994; Mikami et al., 1996; Fitzsimmons et al., 1996) and over-expression in CHO cells (Belcourt et al., 1996a; Seow et al., 2004) correlated to increased sensitivity to mitomycin C and EO9 under aerobic and hypoxic conditions, implicating the role of NQO1 in the activation of these quinones. In addition, purified NQO1 from rat livers and HT-29 colon carcinoma cell lines (van der Heijden et al., 2006) was shown to activate EO9 to its DNA alkylating species.
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were capable of reducing mitomycin C (Siegel et al., 1990) and EO9 (Walton et al., 1991). However the reductive activation of quinones by NQO1 can contribute to the dose-limiting toxicity of quinone compounds in the patients. For example the dose limiting toxicity of EO9 (Schellens et al., 1994) was possibly due to the high levels of NQO1 expression in the kidney (Schlager & Powis, 1990).

Although the studies listed above have suggested the involvement of POR and NQO1 in the activation of mitomycin C and EO9, other reductases have been shown to be capable of catalysing the reductive activation of these compounds. Purified recombinant NQO2 (EC: 1.10.99.2), a parologue of NQO1 that utilises NRH (dihydronicotinamide riboside) rather than NADPH as an electron donor (Wu et al., 1997) was capable of activation of mitomycin C (Jamieson et al., 2006). In addition, the over-expression of NQO2 in CHO cells increased sensitivity to mitomycin C compared to WT cells under aerobic conditions (Celli et al., 2006). Studies on recombinant rabbit NADH:cytochrome b5 reductase (CYB5R, EC 1.6.2.2) demonstrated its capability for the reductive activation of mitomycin C (Hodnick & Sartorelli, 1993) and the over-expression and localisation of CYB5R to the cytosol (soluble CYB5R) and nucleus increased sensitivity of CHO cells to mitomycin C under both aerobic and hypoxic conditions (Belcourt et al., 1998; Holtz et al., 2003). Purified enzyme studies have also demonstrated the ability of NADPH:adrenodoxin reductase (FDXR; EC: 1.18.1.2) (Jiang et al., 2001), endothelial nitric oxide synthase (NOS3, EC 1.14.13.39) (Jiang et al., 2000), xanthine oxidase (EC 1.17.3.2) (Pan et al., 1984) and xanthine dehydrogenase (EC 1.17.1.4) (Gustafson & Pritsos, 1992; Gustafson & Pritsos, 1993) to reduce mitomycin C under anoxic conditions.

1.3.2. Nitroaromatic HAP

Early observations that nitroimidazoles can act as oxygen-mimetic radiosensitisers (Chapman et al., 1972) led to the discovery that they can also kill hypoxic cells selectively in the absence of radiation (Sutherland, 1974; Hall & Roizin-Towle, 1975; Mohindra & Rauth, 1976). The hypoxic-selective toxicity of these compounds is a result of the hypoxic-selective reduction of the nitro moiety (Fouts & Brodie, 1957; Varghese et al., 1976; Taylor & Rauth, 1978). This led to the proposal that reduction of the nitro group could be used as an electronic switch to activate a cytotoxic moiety under hypoxia (Denny & Wilson, 1986). The nitro group is known to be one of the most electron withdrawing groups in an aromatic system with a Hammett substituent constant ($\sigma_p$) of 0.78 (Siim et al., 1997). Therefore the reduction
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of this nitro group to strong electron-donating groups such as the hydroxylamine ($\sigma_p = -0.34$) or amine groups ($\sigma_p = -0.66$), changes the electron density of the aromatic ring, allowing the selective activation of a toxic moiety under hypoxia (Figure 1.7) (Denny & Wilson, 1986; Siim et al., 1997). Various studies on nitroaromatic compounds such as simple nitrobenzyls (Teicher & Sartorelli, 1980), the 5-nitroimidazole metronidazole (Foster et al., 1976), 1-nitroacridines such as nitracrine (Wilson et al., 1984; Wilson et al., 1992a), 5-nitroquinolines such as SN24349 (Siim et al., 1994; Siim & Wilson, 1995) and dinitrobenzenes such as CB1954 (Palmer et al., 1992) and PR-104A (Patterson et al., 2007), have all shown increased hypoxia-selective cytotoxicity in various tumour models.

Figure 1.7 Mechanism of activation of dinitrobenzamide mustard prodrugs

The reduction of the 5-nitro group to the nitro radical occurs by one-electron reduction and in the presence of oxygen this nitro radical is oxidised back to the nitro compound. In the absence of oxygen, this radical can be further reduced to the nitroso, hydroxylamine and amine metabolites. This reduction changes the Hammett substituent constant (blue values) of the compound from an electron-withdrawing (shown by the upwards arrow) to an electron-donating (downwards arrow), activating the latent mustard group (red). Adapted with permission from (Denny & Wilson, 1986). Copyright (1986) American Chemical Society.
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PR-104 is a ‘pre-prodrug’ dinitrobenzamide mustard (DNBM) developed by the ACSRC and is converted systemically to the corresponding DNBM alcohol PR-104A (Patterson et al., 2007). PR-104A (Figure 1.8) can be reduced at the 5-nitro position to its active metabolites; hydroxylamine PR-104H and amine PR-104M (activation scheme based on Figure 1.7). This reduction acts as an electronic switch which activates the pre-positioned mustard moiety (shown in another DNBM, SN23862 (Siim et al., 1997; Helsby et al., 2003)), which allows PR-104H and PR-104M to form cytotoxic DNA inter-strand cross-links (Singleton et al., 2009). The cytotoxicity of PR-104A can be increased by the diffusion of its cytotoxic metabolites from the cells in which they are formed to the surrounding cells (the bystander effect) (Patterson et al., 2007). The bystander effect has been suggested to be one of the reasons for the excellent preclinical anti-tumour activity of PR-104 (Hicks et al., 2007; Patterson et al., 2007) and led PR-104 to phase I clinical trials in patients with solid tumours (Jameson et al., 2010; McKeage et al., 2012). Additionally, two-electron reduction of PR-104A can be catalysed by aldo-keto reductase 1C3 (AKR1C3, EC 1.1.1.188), a member of the aldo-keto reductase family of proteins (Guise et al., 2010); a surprising discovery considering that this family of enzymes was not previously known to be capable of activating HAP. The discovery that myeloid progenitor cells express high levels of AKR1C3 (Birtwistle et al., 2009) provides a possible explanation for the dose-limiting toxicity of PR-104 (severe myelosuppression) (Guise et al., 2010) and led to the new clinical pathway of this compound in acute myeloid leukaemia (recently closed; www.clinicaltrials.gov identifier NCT01037556).

Figure 1.8 Structures of nitroaromatic HAPs: PR-104A, CB1954 and TH-302
The enzymology of nitroaromatic activation is still not fully understood, however several enzymes that have been implicated in the reduction of quinone compounds are also involved in the reduction of nitroaromatic compounds. Studies with POR over-expressing SiHa cells demonstrated an increased hypoxic reduction of PR-104A, suggesting a role for POR in this setting (Guise et al., 2007; Guise et al., 2012). In addition, siRNA and anti-sense RNA studies to knockdown POR expression demonstrated that POR was an important one-electron reductase for PR-104A activation in SiHa cells but >40% of the residual hypoxic metabolism of POR was unaccounted for (Guise et al., 2007). POR over-expression models have also been used to demonstrate the involvement of POR in the hypoxic activation of other nitro-containing HAP including CB1954 (discussed below), TH-302 (discussed below), RSU1069 (Patterson et al., 1997) and nitro-chloromethylbenzindolines (nitroCBI) (Tercel et al., 2009).

Reductases other than POR can also catalyse the activation of nitroaromatic compounds. The hypoxic nitroreduction of compounds such as nitrofurazone, benznidazole and 2-nitrofluorene can be catalysed by the cytosolic molybdenum hydroxylases aldehyde oxidase (EC 1.2.3.1) (Wolpert et al., 1973; Tatsumi et al., 1986) and xanthine oxidase (Walton et al., 1989; Ueda et al., 2003). Thioredoxin reductase (EC: 1.8.1.9) has been demonstrated to be capable of catalysing either one or two electron reduction of nitroaromatic compounds under aerobic conditions (Cenas et al., 2006). A recent study using reductase over-expressing HCT116 cells demonstrated the capability of diflavin reductases methionine synthase reductase (MTRR, EC 1.16.1.8), inducible nitric oxide synthase (NOS2A) and NADPH-dependent diflavin oxidoreductase 1 (NDOR1, EC 1.6.2) to reduce PR-104A under hypoxia (Guise et al., 2012).

CB1954 (Figure 1.8) is a dinitrobenzamide compound first shown to have high activity against the Walker rat carcinoma model (Cobb et al., 1969). This was subsequently demonstrated to be caused by activation by rat NQO1, which was capable of reducing CB1954 at the 4-nitro group under aerobic conditions (Knox et al., 1988a; Knox et al., 1988b). However the human form of NQO1 was less capable of reducing CB1954 compared to rat NQO1 (Boland et al., 1991). Instead, CB1954 was more effectively reduced by its parologue NQO2 with NRH as a co-factor (Knox et al., 2000). Rat liver microsomal fractions and rat recombinant POR were also capable of reducing CB1954 to its amine metabolites under hypoxia and this reduction (as assayed by HPLC) was inhibited by 45-50 % when incubated with a POR antibody (Walton et al., 1989). CB1954 can also be activated by NOS2 and NOS3 under hypoxia (Chandor et al., 2008).
CB1954 can be more effectively reduced by *Escherichia coli* (*E coli*) nitroreductase (Knox et al., 1992), with this reaction being approximately 100 fold faster that rat NQO1 (Knox & Chen, 2004). The reduction of CB1954 by *E coli* nitroreductase generates either the 2- or 4-hydroxylamine metabolite (Boland et al., 1991; Helsby et al., 2004; Vass et al., 2009), with the 4-hydroxylamine metabolite capable of non-enzymatic activation by acetyl coenzyme A and other thioesters to form a DNA cross-linking 4-N-acetoxy species (Figure 1.9) (Knox et al., 1991). The 2-amine product of CB1954 reduction is equipotent to the 4-hydroxylamine metabolite in cancer cell lines and demonstrates a better bystander effect compared to the 4-hydroxylamine metabolite, suggesting that the 2-hydroxylamine reduction pathway is likely to be more relevant for *in vivo* cytotoxicity of CB1954 (Helsby et al., 2004). Since CB1954 is inefficiently reduced by human enzymes, CB1954 activation can be targeted to human tumours to minimise off-target toxicity. This strategy is the basis for *E coli* nitroreductase gene-directed enzyme prodrug therapy (GDEPT), where the nitroreductase is introduced to the tumour by a vector, minimising normal tissue activation. CB1954 was entered into phase I/II clinical trials using a replication-defective adenovirus armed with nitroreductase and the results for this trial were recently reported (Patel et al., 2009).

TH-302 (Figure 1.8) is currently developed by Threshold Pharmaceuticals and consists of a bromo-isophoramide mustard (Br-IPM) with a 2-nitroimidazole trigger (Duan et al., 2008). Reductive activation of TH-302 results in fragmentation of this 2-nitroimidazole trigger, releasing the DNA alkylating Br-IPM (Figure 1.10) (Meng et al., 2012). The reductive activation of 2-nitroimidazoles has been demonstrated using POR overexpressing models (Joseph et al., 1994), and POR over-expression increased sensitivity of A549 and SiHa cells to TH-302 compared to WT both under aerobic and hypoxic conditions (Meng et al., 2012). TH-302 demonstrated significant tumour growth inhibition in 8/11 human tumour xenograft models tested and the activation of TH-302 in those models showed good correlation to hypoxia (Sun et al., 2012). Based on the excellent preclinical results and Phase I/II clinical trials, TH-302 is currently the most clinically advanced HAP in clinical trials with ongoing Phase III trials in combination with gemcitabine for soft tissue sarcoma ([www.clinicaltrials.gov](http://www.clinicaltrials.gov) identifier NCT01440088) and in combination with gemcitabine for pancreatic cancer ([www.clinicaltrials.gov](http://www.clinicaltrials.gov) identifier NCT01746979).
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Figure 1.9 Activation mechanism of CB1954

CB1954 is reduced by 4-electron reduction to the 2- and 4-hydroxylamine metabolite, with the first one-electron reduction step inhibited by oxygen (Figure 1.7). The 4-hydroxylamine metabolite can be non-enzymatically activated by thioesters to form the 4-amino metabolite (major product) and N-acetoxy species (minor product). The putative DNA alkylating metabolite is the N-acetoxy species. Alternatively, the 2-hydroxylamine metabolite can be further reduced to the 2-amine metabolite and this was shown to be cytotoxic to cancer cell lines. However the mechanism of DNA alkylation for this metabolite remains unknown.

Figure 1.10 Reductive activation of TH-302

The reduction of TH-302 by one-electron reduction initiates the fragmentation of 2-nitroimidazole moiety, releasing the DNA alkylating species Br-IPM.
1.3.3. Aromatic N-oxides

Most studies on the reductive activation of aromatic-N-oxides are centred on the benzotriazene N-oxide tirapazamine (TPZ; previously known as SR4233) (Zeman et al., 1986). Reduction of TPZ results in the generation of an intermediate radical metabolite (Laderoute et al., 1988; Lloyd et al., 1991), which can then be further reduced to the two-electron reduction product (1-oxide molecule SR4317) in the absence of oxygen (Figure 1.11; (Laderoute & Rauth, 1986). Under aerobic conditions, this radical intermediate is back-oxidised to TPZ, generating superoxide, in a reaction similar to the redox cycling of quinones and nitroaromatic compounds. The 1-oxide metabolite and the four-electron reduction product of TPZ (nor-oxide molecule SR4330) are relatively non-toxic to hypoxic cells (Baker et al., 1988). This implies that the cytotoxicity of TPZ arises from reactions involving the intermediate TPZ radical, which leads to DNA double-strand breaks from stalled replication forks and the inhibition of topoisomerase II (Peters & Brown, 2002; Evans et al., 2008). The 1-oxide of TPZ (SR4317) can also potentiate the DNA damage caused by the free radical intermediates (Siim et al., 2004). Although the identity of the active radical(s) is still under investigation, it has been suggested that hypoxic cytotoxicity of TPZ is conferred by the hydroxyl radical, the benzotriazinyl radical or a combination of both (Shinde et al., 2009; Shinde et al., 2010; Yin et al., 2012a; Yin et al., 2012b).

TPZ was reported in numerous Phase I/II clinical studies (reviewed in (Marcu & Olver, 2006) with promising outcomes in HNSCC in combination with cisplatin (Phase II; (Rischin et al., 2005). However Phase III trials in this setting did not improve overall survival compared to patients treated with cisplatin alone (Rischin et al., 2010). It was suggested that this failure was due to poor quality radiotherapy at the trial centres, as patients receiving poor quality radiotherapy had a significant 20% decrement in overall survival compared to patients with good quality radiotherapy (Peters et al., 2010). In addition, a retrospective analysis of tumour hypoxia in a small proportion of the patients recruited to the Phase III trial demonstrated a significant improvement in TPZ treatment in hypoxic tumours, however patients were not stratified based on the level of tumour hypoxia in this trial (Rischin et al., 2006). Also, studies using three-dimensional cell culture models demonstrated that TPZ has limited hypoxic tissue penetration due to rapid metabolism, which possibly contributed to the clinical failure of TPZ (Hicks et al., 2003; Hicks et al., 2004; Hicks et al., 2007). Nevertheless, a new TPZ phase III clinical trial with cisplatin and radiation for treatment of cervical cancer is currently ongoing (www.clinicaltrials.gov identifier NCT00262821).
The reduction of TPZ (and other aromatic \(N\)-oxides) under hypoxic conditions can be catalysed by various reductases. Recombinant rat POR is capable of reducing TPZ to SR4317 (Walton et al., 1992; Fitzsimmons et al., 1994) and POR expression in a panel of six breast cancer cell lines correlated to sensitivity of those cell lines to TPZ (Patterson et al., 1995). Over-expression of POR in MDA-MB-231 cells, the cell line with the lowest expression of POR among the panel of breast cancer cell lines (Patterson et al., 1995), resulted in increased sensitivity to TPZ compared to isogenic WT cells (Patterson et al., 1997). Purified recombinant reductase domains of NOS1 (neuronal NOS), NOS2 and NOS3 were also capable of reducing TPZ (Garner et al., 1999) and the over-expression of NOS2 increased the cytotoxicity of MDA-MB-231 cells to TPZ under hypoxic conditions (Chinje et al., 2003). Other reductases that have been implicated in the reduction of TPZ include the molybdenum hydroxylases; xanthine oxidase and aldehyde oxidase (Walton & Workman, 1990) and the cytochrome P450s (CYPs) (Walton & Workman, 1990; Wang et al., 1993). TPZ can also undergo aerobic two-electron reduction to its 1-oxide SR4317 in a reaction catalysed by NQO1 (Walton & Workman, 1990; Riley & Workman, 1992). Unlike quinones or nitroaromatic compounds where toxic metabolites are formed by two-electron reduction, two-electron reduction of TPZ and its analogues (e.g. SN30000) produces non-toxic metabolites (Baker et al., 1988), providing an improved selectivity for hypoxic tissues.

More recently, an analogue of TPZ has been developed to overcome the problem with limited hypoxic tissue penetration of TPZ (Hicks et al., 2003). SN29751 and SN30000 (briefly known as CEN-209; structures in Figure 1.12) have been shown to have improved potency, tissue penetration and efficacy compared to TPZ (Hicks et al., 2010). The over-expression of POR in HCT116 cells increases metabolism of SN30000 to its one-electron reduced product by 3-4 fold compared to WT cells (Wang et al., 2012). In addition, SN30000 (\(R^2 = 0.64, p<0.001\)) and TPZ (\(R^2 = 0.65, p<0.001\)) metabolism had a significant correlation to the activation of the 2-nitroimidazole hypoxic probe EF5, suggesting that the one electron reductases of EF5 are shared by both these compounds (Wang et al., 2012). This correlation was stronger than the correlation between these compounds and POR activity as measured by cytochrome c assay (\(R^2 = 0.36, p = 0.023\) for SN30000 and \(R^2 = 0.34, p = 0.03\) for TPZ) suggesting that POR is not the only one-electron reductase for these compounds (discussed further in Chapter 3 and Chapter 5).
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1.3.4. Aliphatic N-oxides

The reduction of aliphatic (tertiary amine) N-oxides to the corresponding basic amines can be utilised as another mechanism for exploiting tumour hypoxia (Wilson et al., 1992b; Patterson, 1993). The oxygen-inhibited reduction of tertiary amines by two-electron reduction introduces a cationic charge that increases the affinity of these compounds for DNA.

Figure 1.11 Reductive activation of tirapazamine (TPZ)

TPZ is activated by one-electron reduction to the TPZ radical and this radical can be further reduced to the 1-oxide and nor-oxide metabolites in the absence of oxygen. In the presence of oxygen, this radical can be back-oxidised to TPZ, generating superoxide. The DNA alkylating species is postulated to be the benzotriazinyl (BTZ) radical and the hydroxyl radical, both formed from by reactions involving the TPZ radical.

Figure 1.12 Chemical structures of SN30000 and SN29751

1.3.4. Aliphatic N-oxides

The reduction of aliphatic (tertiary amine) N-oxides to the corresponding basic amines can be utilised as another mechanism for exploiting tumour hypoxia (Wilson et al., 1992b; Patterson, 1993). The oxygen-inhibited reduction of tertiary amines by two-electron reduction introduces a cationic charge that increases the affinity of these compounds for DNA.
processing enzymes such as topoisomerase II (Wilson et al., 1996; Smith et al., 1997). A few of these aliphatic N-oxides have been reported (e.g. nitracrine N-oxide and DACA-N-oxide) (Wilson et al., 1992b; Wilson et al., 1996) but this class is best represented by AQ4N. AQ4N contains two N-oxide moieties that are each reduced by two-electron reduction, first forming AQ4M (mono-N-oxide) as an obligate intermediate and then forming the cytotoxic AQ4 after another two-electron reduction step (Patterson, 2002). The two-electron reduction of AQ4N under hypoxia is catalysed by the CYP family of enzymes, most notably CYP3A4 (Raleigh et al., 1998) and the recently discovered CYP2S1 and CYP2W1 (Nishida et al., 2010). The inhibition of AQ4N reduction by oxygen was suggested to be the result of competition between oxygen and AQ4N for the binding to the haem active site of CYPs (Patterson & Murray, 2002). POR is unable to directly reduce AQ4N but can indirectly support AQ4N reduction through its activity via CYP activation (Patterson et al., 1999). Recent studies have demonstrated the ability of NOS2 to reduce AQ4N under anoxic conditions through its CYP-like haem domain (Nishida & Ortiz de Montellano, 2008) and tumour associated macrophages expressing NOS2 are capable of activating AQ4N to its toxic metabolite (Mehibel et al., 2009). AQ4N entered phase I/II clinical trials in glioblastoma and B-cell neoplasms but these trials were suspended due to clinical failure in this setting (Kling, 2012).

Figure 1.13 Reductive activation of AQ4N

AQ4N is reduced by two-electron reduction to AQ4M in a reaction inhibited by oxygen. The mono-N-oxide AQ4M can be further reduced to the DNA damaging agent AQ4 in another two-electron reduction step.
1.4. NADPH:cytochrome P450 oxidoreductase

1.4.1. Genetics and protein structure

Human NADPH:cytochrome P450 oxidoreductase (POR) (also known as cytochrome P450 reductase, TPNH-cytochrome c reductase, NADPH-hemoprotein oxidoreductase and NADPH-ferrihemoprotein oxidoreductase) is a one-electron reductase capable of activating HAP under hypoxia (discussed in the previous section). POR is an enzyme of 78 kDa consisting of 680 amino acid residues (Hart & Zhong, 2008; Miller et al., 2011). The gene encoding it, \( \text{POR} \), is located on chromosome 7q11.2 (Shephard et al., 1989) and consists of 15 protein coding exons, 14 introns (Figure 1.14A) and an untranslated exon (termed exon 1 U) located 5’ upstream of the first coding exon (Scott et al., 2007). The domain structures of POR were determined by x-ray crystallography of soluble rat POR at 2.6 Å (Wang et al., 1997), soluble yeast POR at 2.9 Å (Lamb et al., 2006) and soluble human POR at 1.75 Å (Xia et al., 2011). POR consists of five structural domains; a hydrophobic N-terminal domain, FMN, FAD and NADPH binding domains and the hinge domain (Figure 1.14B and C). The protein structure of human POR is similar to rat and yeast POR except for residue H621 in rat POR that is absent in human POR. The N-terminal membrane binding domain anchors POR mainly to the cytosolic face of the endoplasmic reticulum and also the outer membrane of the nuclear envelope (Kasper, 1971). The N-terminal anchor of POR is important to ensure proper spatial interaction between POR and CYPs for electron transfer, and the removal of this domain by trypsin-solubilisation retains the catalytic activity of POR to reduce cytochrome c but not CYPs (Black et al., 1979; Smith et al., 1994). The hinge domain is responsible for bringing the two flavin containing domains to within 4 Å and modulates electron transfer between the two flavin domains (Hubbard et al., 2001; Hamdane et al., 2009).

POR is a member of a group of diflavin reductases that utilise both FMN and FAD as electron transfer cofactors (Iyanagi & Mason, 1973; Wang et al., 1997). POR was suggested to be the result of an historical fusion of ancestral genes of flavodoxin (an FMN containing protein found in bacteria, blue-green algae and green algae) and ferrodoxin-NADP\(^+\) reductase (FAD containing protein from blue-green algae) (Porter & Kasper, 1986). Numerous studies demonstrate that the sequence of electron transfer starts from its electron donor NADPH, followed by FAD to FMN and finally to most electron acceptors including CYPs (Iyanagi et al., 1974; Vermilion & Coon, 1978; Iyanagi et al., 1981). Electrons from NADPH are
accepted by FAD in the form of a hydride ion transfer (Masters et al., 1965; Vermilion et al., 1981), modulated by residues W677 (Hubbard et al., 2001), S457, C630 and D675 (Shen et al., 1999). Although the removal of the FMN domain disrupts the electron transfer of POR towards the CYPs and cytochrome c (Vermilion et al., 1981; Smith et al., 1994), certain substrates (e.g. ferricyanide) are capable of being reduced by POR via the FAD domain in a FMN-independent manner (Vermilion & Coon, 1978; Smith et al., 1994). However, mutations in the FAD domain of POR result in a >98% reduction of catalytic activity of the enzyme (Kurzban & Strobel, 1986; Marohnic et al., 2006) indicating the importance of the FAD domain in the catalytic activity of POR.

Figure 1.14 Genetic and protein structure of POR

(A) Exonic structure and (B) domain structure of POR. POR contains consist of 16 exons and three co-factor binding domains; the NADPH, flavodoxin-like (FMN) and ferrodoxin reductase-type (FAD) domains (UniProt, http://www.uniprot.org/). Exon 1 is an untranslated exon. (C) The protein structure of POR at 1.75 Å as determined by x-ray crystallography showing the FMN binding domain (blue), the connecting domain (grey) and FAD and NADPH binding domains (green). Co-factors: FMN (blue) FAD (brown) and NADPH (red) are indicated with ball and stick models. Residues R457 and V492 are required for FAD binding while H621 from rat POR is missing in the human enzyme. Reprinted (adapted) with permission from (Xia et al., 2011). Copyright (2011) National Academy of Sciences, USA.
1.4.2. Physiological function and mutations in human disease

The CYP mixed-oxygenase system consists of phosphatidylcholine (Strobel et al., 1970), POR and the CYPs, which are haem-containing proteins in the endoplasmic reticulum membrane that catalyse monooxygenase reactions of a wide variety of substrates (Guengerich, 1992). POR is the major electron donor for this system, reducing the haem centre of CYPs in two sequential one-electron reduction steps (Guengerich, 2005). Cytochrome b₅ has been implicated in the CYP mixed-oxygenase system but the role it plays is still highly controversial (Schenkman & Jansson, 1999; Porter, 2002; Finn et al., 2008). Nevertheless, cytochrome b₅ has been postulated to be involved in modulating an allosteric effect on CYPC17 reduction (Auchus et al., 1998), directly donating electrons from NADH to the CYP during the second electron transfer step (Hildebrandt & Estabrook, 1971) and inhibiting the activity of CYP2B4 (Zhang et al., 2008).

The CYPs are divided into two major groups based on their optical difference spectra; whether the binding of the ligands is at residues close to the haem-binding site (Type I) or directly to the haem iron centre (Type II). There are 57 genes in the human genome encoding for CYPs, seven of which are classified as Type I mitochondrial CYPs and the other 50 are Type II microsomal CYPs (Guengerich, 2004; Guengerich, 2005). For mitochondrial CYPs, FDXR and adrenodoxin can serve the role of POR as an electron donor (Anandatheerthavarada et al., 1998). The cytochrome b₅/CYB5R system is capable of supporting CYP activity in yeast systems deficient in functional POR (Lamb et al., 1999), but this has not been observed in other eukaryotic systems.

The microsomal mixed-function oxygenase system plays an essential role in cells by catalysing numerous reactions including metabolism of exogenous compounds (e.g. therapeutic drugs, environmental toxins), vitamins and steroids and the biosynthesis of sterols, fatty acids, bile acids and eicosanoids (Guengerich, 2004; Guengerich, 2005; Hart & Zhong, 2008). In addition to being the activator of the microsomal CYPs, POR also acts as an electron donor to other physiologically important enzymes and molecules such as cytochrome b₅ (Enoch & Strittmatter, 1979), haem oxygenase (Schacter et al., 1972), squalene monooxygenase (Ono et al., 1982) and 7-dehydrocholesterol reductase (Nishino & Ishibashi, 2000). These enzymes are involved in a wide variety of functions from the degradation of haem to being the rate-limiting enzyme in sterol biosynthesis. POR has also been postulated to be important in regulating expression of hypoxia responsive genes such as VEGF and EPO by interacting with HIF-1α on the plasma membrane (Osada et al., 2002). However, a recent
study on POR expression in tumours from a tissue microarray containing 19 tumour types showed heterogeneous expression of POR in these tumours, with little evidence for expression in hypoxic tissue as assessed by carbonic anhydrase IX staining (Guise et al., 2012). However, these hypoxic regions may be under-represented due to a selection bias of non-necrotic tissue for evaluation. Nevertheless, with this myriad of important physiological functions, is not surprising that POR shows high sequence conservation from yeast to humans (Wang et al., 1997), is constitutively expressed in a variety of tissues and shows little inter-individual variation in hepatic POR expression (Shephard et al., 1992).

The importance of POR in direct or indirect regulation of the biosynthesis of sterols, fatty acids, bile acids and eicosanoids, which regulate processes such as reproduction, development and modulation of stress responses, means that disruption of POR can have detrimental physiological effects. Early work on mice expressing POR without the N-terminal membrane anchor domain showed embryonic lethality by day 13.5 (Shen et al., 2002) and the total loss of POR exacerbated this effect leading to a loss of viable embryos before day 10.5 (Otto et al., 2003). This suggests that the activation of the CYP mixed-function oxygenase system by POR is required during embryonic development. However, partial rescue of these phenotypes in POR knockout mice was achieved either by the additional loss of one retinaldehyde dehydrogenase 2 (Raldh2) allele (which is responsible for generating retinoic acid) or by growing the embryos in an in vitro culture devoid of retinoids (Ribes et al., 2007). Therefore POR through its maintenance of a functional microsomal cytochrome P450 system regulates retinoic acid homeostasis serving a vital function in early embryogenesis (Otto et al., 2003; Ribes et al., 2007).

In humans, mutations in POR result in a deficiency in CYP17A1 (17α-hydroxylase/17, 20 lyase) and CYP21A2 (steroid 21-hydroxylase), leading to a disorder called POR deficiency syndrome (Fluck et al., 2004). This disorder is characterised by ambiguous genitalia, congenital adrenal hyperplasia and Antley-Bixler syndrome (various skeletal malformations) with disordered steroidogenesis. The severity of clinical manifestations is dependent on the effect of the mutations on POR function (Fluck et al., 2007; Miller et al., 2011). Mutations in FGFR2 can also lead to Antley-Bixler syndrome (Reardon et al., 2000) but these patients do not present with disordered steroidogenesis, unlike POR deficient patients (Huang et al., 2005). The spectrum of clinical manifestations of this disorder, coupled with the knowledge that homozygous loss of function of POR is embryonic lethal, have led to numerous attempts to characterise effects of these mutations and polymorphisms (Miller et al., 2009; Miller et al., 2011) on the functions of POR (Arlt et al., 2004; Fluck et al., 2004; Ribes et al., 2007).
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2004; Huang et al., 2005; Agrawal et al., 2008; Huang et al., 2008). Generally mutations were mapped to functionally important regions of POR such as the cofactor binding domains, whereas polymorphisms were usually in less important locations (Figure 1.15). Therefore the dysregulation of POR enzymatic activity results in human disease, illustrating the essential role for POR in human physiology.

The importance of POR in human disease led to the development of various animal models to determine the effects of POR mutations on animal physiology. Since POR knockout models are embryonic lethal, conditional POR knockout models were generated to further study the functions of this protein. The first models were conditional hepatic POR knockout mice generated using the Cre/LoxP system with Cre recombinase under the control of a rat albumin promoter (Gu et al., 2003; Henderson et al., 2003). These mice were viable but their livers were enlarged with increased hepatic lipid levels and a decrease in bile acid volume, serum cholesterol and serum triglycerides. The mice also had an increase in CYP content compared to wild-type (WT) mice and displayed significant deficiencies in hepatic drug metabolism as a result of non-functioning hepatic CYPs (Gu et al., 2003; Henderson et al., 2003). These mice also showed multiple changes in gene expression in the liver, particularly in the CYP enzyme family (Weng et al., 2006). Another mouse model (POR$^{low}$ mice) carried an inverted neomycin gene ($neo$) in intron 15 of POR resulting in a global suppression (about a 74-95% reduction) of POR levels (Wu et al., 2005). Mice with POR deletions in other tissues such as lung (Weng et al., 2007), heart (Fang et al., 2008), intestine (Zhang et al., 2009), brain (Conroy et al., 2010) and mammary gland (Lin et al., 2012) have also been generated and reported in the literature.
1.5. Other mammalian diflavin oxidoreductases

POR is a member of a group of flavoproteins that utilises both FAD and FMN as cofactors. Other members of this family of reductases include methionine synthase reductase (MTRR), the three nitric oxide synthases (NOS) and NADPH-dependent diflavin oxidoreductase 1 (NDOR1) (Murataliev et al., 2004). The reductase domain of MTRR (Leclerc et al., 1998), the NOS isoforms (Bredt et al., 1991) and NDOR1 (Paine et al., 2000) have close homology with the reductase domain of POR (Figure 1.16). Like POR, these reductases have also been shown to be capable of reducing HAP (previously discussed).
1.5.1. Nitric oxide synthases

The most studied mammalian diflavin reductases (with the exception of POR) are the members of the NOS family of enzymes, which synthesise nitric oxide, an important cellular signalling molecule. There are three major isoforms of NOS, neuronal NOS (NOS1), inducible NOS (NOS2) and endothelial NOS (NOS3) (Bredt & Snyder, 1994). NOS functions as a homodimer with each subunit consisting of an N-terminal oxygenase domain and a C-terminal reductase domain. The C-terminal reductase domain contains binding sites for FAD, FMN and NADPH connected by a linker domain to the Ca$^{2+}$/calmodulin (CaM) binding site, allowing further regulation of enzymatic activity (Roman et al., 2002). Interflavin electron transfer is rate-limiting in the absence of CaM and binding of CaM increases the rate of this electron transfer (Matsuda & Iyanagi, 1999) so that the rate-limiting step becomes the reduction of FAD by NADPH, similar to the electron transfer process of POR (Masters, 2005). The two major intrinsic regulatory elements for NOS reductase activity are the auto-regulatory domain and the C-terminal ‘tail’. The auto-regulatory domain is found only in neuronal and endothelial NOS and acts as a competitive inhibitor for the CaM binding site, keeping the enzyme in an inactive state (Salerno et al., 1997). In the presence of CaM, this auto-regulatory domain is displaced and the enzyme can then subsequently be activated. The C-terminal tail consists of additional amino acid residues at the end of the C-terminus in all NOS isoforms and is postulated to modulate the distance between FAD and FMN (Roman et
al., 2000). The dominant regulator of activity of NOS enzymes in the presence of CaM is the auto-regulatory domain and in its absence the C-terminal tail becomes the dominant regulator (Roman et al., 2002).

1.5.2. Methionine synthase reductase

MTRR plays an indirect but important role in the synthesis of methionine where it reduces the cobalamin cofactor from an inactive cobalamin (II) to cobalamin (I), required for the enzymatic activity of methionine synthase (Banerjee, 1997). Deficiency of MTRR causes decreased activity of methionine synthase, leading to homocystinuria. MTRR is located on chromosome 5 (Leclerc et al., 1998) and shows similar reductase activity to NDOR1 but 100-fold less activity compared to POR as measured by the rate of cytochrome c reduction (Olteanu & Banerjee, 2001). The over-expression of MTRR in HCT116 tumour cell lines led to an increase in formation of cytotoxic metabolites of PR-104A (at a lower metabolism rate compared to POR) and increased sensitivity to PR-104A, indicating that it is capable of reductive activation of HAP (Guise et al., 2012).

1.5.3. NADPH-dependent diflavin oxidoreductase 1

NDOR1 (also known as novel reductase 1) is located on chromosome 9 and is capable of NADPH-dependent cytochrome c reduction albeit with a 100-fold lower catalytic activity compared to POR (Paine et al., 2000). It is capable of reduction of other compounds such as menadione and potassium ferricyanide, but also at 1-4% of the catalytic activity of POR (Paine et al., 2000). The biological role of this enzyme is still unknown, and it is unlikely to be involved in the CYP mixed-oxygenase system as it lacks an N-terminal membrane anchor. However NDOR1 is capable of reducing methionine synthase in the presence of cytochrome b5, suggesting possible redundancy of the activation of this enzymatic system (Olteanu & Banerjee, 2003). NDOR1 is also widely expressed in a number of cancer cell lines (HT29, Hela, HepG2 and MCF-7) and the over-expression of NDOR1 in HCT116 cells increases PR-104A metabolism compared to WT, suggesting a role for this reductase in HAP activation (Guise et al., 2012).

1.6. Zinc finger nucleases (ZFN)

One of the objectives for this thesis was to assess the role of POR, at endogenous levels of expression, in the activation of HAP in human tumour cell lines. One approach to study the
role of POR in this setting is by genetic knockout of POR. Previous studies by Guise and colleagues from the ACSRC used siRNA and anti-sense RNA methods to determine the contribution of POR to the activation of PR-104A (discussed further in Chapter 3) (Guise et al., 2007). However, residual expression of POR in these cell lines and possible off-target effects of these siRNA methods complicated the conclusions from that study. Alternatively, utilisation of rare-cutting endonucleases will minimise these off-target effects and ensure full knockout events occur by direct modification of DNA. Recent advances in these targeted molecular gene editing tools allows for the specific mutational inactivation of POR. One of these specific endonucleases is the zinc finger nuclease (ZFN). ZFNs are chimeric proteins consisting of a zinc finger domain linked with an endonuclease domain. The ZFN technology was utilised for this thesis and will be discussed further in the next section. In addition, a novel chimeric endonuclease, TALEN (or transcription activator-like effector nuclease) has been also reported in the literature will be discussed further in Chapter 6.

### 1.6.1. Zinc finger domain

Proteins that contain Zn$^{2+}$ ions in the DNA binding motif represent the largest group of transcription factors in the human genome (Luscombe et al., 2000). Zinc fingers, which typically consist of 30 amino acids, are a subtype of the zinc-coordinating proteins which are characterised by the coordination of Zn$^{2+}$ by conserved cysteine and histidine residues. The most common structural motif of zinc fingers comprises two cysteine and two histidine (C$_2$H$_2$) residues arranged as a ββα fold where the two cysteine ligands are near a turn in the anti-parallel β sheet while the histidines are in the C-terminal portion of the α helix (Lee et al., 1989). Together these residues form a globular domain known as a zinc finger domain that stabilises the central Zn$^{2+}$ ion. C$_2$H$_2$ zinc fingers were originally discovered as the DNA binding domain of transcription factor IIIA from *Xenopus laevis* (Diakun et al., 1986). Early studies on the zinc finger protein Zif268, (a protein consisting of three zinc finger units in tandem) demonstrated that zinc finger units bind to DNA in a base sequence-selective manner, with the α-helix fitting directly in the major groove with the N-terminal end closest to the DNA (Pavletich & Pabo, 1991). Each zinc finger unit was found to interact with a base pair triplet and this specificity was influenced by amino acid residues -1 to +6 where the ‘+’ and ‘–’ indicate the relative position from the start of the α-helix (Pavletich & Pabo, 1991; Elrod-Erickson et al., 1996). Therefore, modifying these residues while maintaining the
integrity of the zinc finger allows the specificity of zinc fingers to be tailored for specific recognition of most 3 bp combinations.

This characteristic of zinc fingers allows the design of novel zinc finger domains for ZFNs that can recognise multiple combinations of base pair triplets by modular assembly (Segal et al., 2003). Zinc finger units have been designed for most of the 64 triplet codons (Segal et al., 1999; Dreier et al., 2001; Dreier et al., 2005), allowing modification of the specificity of ZFNs to target any locus in the genome. Alternative methods have been described for the generation of zinc finger domains with high specificity and activity including the usage of phage display libraries to generate multi-finger domains by a bipartite method (Isalan et al., 2001), a directed domain shuffling and cell-based selection strategy (Hurt et al., 2003), the recently described library of validated two-finger units (Context-Dependent Assembly; CoDA) (Sander et al., 2011) or Oligomerised Pool ENgineering (OPEN) protocol, a publically available resource for engineering zinc finger arrays (Maeder et al., 2008; Maeder et al., 2009). These methods have been optimised to produce functional ZFNs for numerous target genes in different cell models (discussed later).

1.6.2. FokI restriction endonuclease domain

Type IIS (where ‘S’ stands for shifted cleavage) restriction enzymes typically recognise asymmetrical sequences and cleave DNA at a site a few nucleotides away from its recognition site (Durai et al., 2005). FokI, a 65.4 kDa (587 amino acids) restriction endonuclease from Flavobacterium okeanokoites, recognises a non-palindromic five nucleotide sequence 5’-GGATG-3’ and makes staggered cuts nine nucleotides downstream from the recognition site on the same strand and 13 nucleotides from the recognition site on the complementary strand (Sugisaki & Kanazawa, 1981; Bitinaite et al., 1998). Proteolytic fragment studies on the domain structure of FokI endonuclease revealed two distinct domains; the N-terminal DNA binding domain (41 kDa) and the C-terminal domain containing a non-specific nuclease domain (25 kDa) (Li et al., 1992). FokI was capable of binding to target DNA even with C-terminal domain mutations, indicating the modular nature of this enzyme (Li et al., 1993). The nuclease domain is sequestered by the DNA recognition domain through protein-protein interactions, and the presence of Mg$^{2+}$ promotes the switch towards the active confirmation of the enzyme (Wah et al., 1997). Crystallisation of FokI with (Wah et al., 1997) or without DNA (Wah et al., 1998), together with mutational studies (Waugh & Sauer, 1993) concluded that FokI has one catalytic site per molecule.
An advantage of FokI endonucleases is that modification by removal of its recognition site does not sacrifice its nuclease activity. This led to the generation of the first chimeric endonucleases combining *Drosophila melanogaster* Ubx homeodomain (recognition of 9-bp consensus DNA sites 5’ TTATT(G/T)(G/A)CC-3’) and Fok1 endonucleases (Kim & Chandrasegaran, 1994). However, the usage of zinc finger domains as the DNA recognition domain is an upgrade over the Ubx homeodomain due to the modular nature and malleability of the zinc finger domains (Kim et al., 1996). In addition, the specificity and binding affinity of the zinc finger DNA binding domain did not diminish after fusion to a nuclease domain, thereby ensuring that these chimeric endonucleases will remain selective for its target (Smith et al., 1999). These studies led to the generation of the first ZFNs (Figure 1.17).

ZFNs are specific endonucleases with their binding specificity determined by the zinc finger domain. The zinc finger domain is connected to the Fok1 endonuclease domain by an amino acid linker. The Fok1 nuclease was shown to bind to DNA as a monomer, but dimerisation with the other subunit in the presence of Mg$^{2+}$ activates its catalytic activity (Wah et al., 1997; Bitinaite et al., 1998). Dimerisation can occur through weak protein-protein interaction of the nuclease domain at high concentrations of Fok1 (Vanamee et al., 2001). Nevertheless the binding of two independent ZFN binding events are required to orientate the nuclease domain for optimal DNA double strand cleavage (Smith et al., 2000; Mani et al., 2005). Therefore, two different zinc finger nucleases have to bind to their target sequences within 35-40 bp in order for the nuclease domains to dimerise (Figure 1.17). Hence a pair of ZFN each containing three zinc finger modules will have an 18 bp recognition site (estimated to occur once every $4^{18} = 6.9 \times 10^{10}$ bp), which represents a unique site in the genome (Smith et al., 2000).
1.6.3. Specificity of ZFN

The requirement for dimerisation ensures high specificity in the ZFN-dependent double strand break at the nominal target site. However early ZFN constructs using modular assembly did not take into account the interactions between the zinc finger units; for example certain zinc finger units can have specificity for four base pairs, with the fourth base pair overlapping with the first base pair of the subsequent zinc finger unit (Isalan et al., 1997). These interactions contributed to the low efficiency of making functional ZFN pairs (Ramirez et al., 2008) and increased aberrant cleavage events from the lack of sequence specificity, leading to higher cytotoxicity (Cornu et al., 2008; Pruett-Miller et al., 2008). In addition, early ZFNs utilised WT FokI cleavage domains, which were not selective for the heterodimer species (Miller et al., 2007). The increased toxicity of ZFN was demonstrated by decreased survival of cells stably transfected with ZFN (Porteus & Baltimore, 2003) or when high concentrations of ZFNs were achieved using transient transfection (Beumer et al., 2006;...
Chapter 1. Literature review

Pruett-Miller et al., 2008) and also the observation that zinc finger domains with higher specificity and affinity for DNA had lower levels of ZFN-induced toxicity (Cornu et al., 2008).

Several studies on improving ZFN specificities to reduce excessive cleavage at off-target sites have been reported. Improvements to the zinc finger design can assist in improving specificity of these zinc finger units to their target site (discussed in Section 1.6.1). In addition, modification of FokI protein-protein interactions by mutagenesis at critical residues to ensure that the nuclease domains are only catalytically active as heterodimers improved specificity and reduced toxicity of ZFN pairs (Miller et al., 2007; Szczepak et al., 2007; Ramalingam et al., 2011; Doyon et al., 2011). Alternatively, the regulation of ZFN stability by the linkage of a destabilisation domain (e.g. FKBP12/Shield1 system) or an ubiquitin binding domain on the ZFN can also reduce the off-target cleavage events of these ZFN without compromising their gene targeting efficiency (Pruett-Miller et al., 2009). Optimisation of the length of the inter-domain linker (separates the zinc finger and nuclease domains) can minimise spacer length (distance between ZFN binding sites) and thereby improve specificity of ZFN pairs. Short inter-domain linkers of four (Handel et al., 2009) or six amino acids (Shimizu et al., 2009) typically demonstrate optimal activity with 6 bp spacers while longer linkers (8-16 amino acids) typically had a larger spectrum of activity with larger spacer lengths of 16 bp (Handel et al., 2009). Although shorter inter-domain linkers are more specific and have a more restricted activity profile, optimisation of the linker length needs conducted on a case by case basis as ZFN activity and cytotoxicity can be target or cell line dependent (Lombardo et al., 2007; Maeder et al., 2008; Handel et al., 2009; Shimizu et al., 2009; Gabriel et al., 2011). Maximising ZFN specificity can have a negative effect in decreasing frequency of successful gene targeting events (Pruett-Miller et al., 2008). However, recent studies have demonstrated the utilisation of modified FokI nuclease domain with improved catalytic activity whilst maintaining its specificity (Guo et al., 2010). While ZFNs clearly have the potential to be highly specific genome-editing tools, questions about their specificity remain and will be discussed further in Chapter 6.

1.6.4. ZFN-dependent gene modification events

The improvements in ZFN specificity have been accompanied by numerous studies showing successful gene modification events using ZFNs. These gene modification events are initiated by the DNA double-strand breaks induced by ZFN activity at the target site. The
double-strand breaks can be repaired either by HDR or NHEJ repair (Figure 1.18) (Pardo et al., 2009). HDR is a high fidelity DNA double strand repair pathway which maintains genomic integrity by utilising homologous DNA sequences to repair DNA double strand breaks (Moynahan & Jasin, 2010). The introduction of homologous donor DNA during ZFN transfections can stimulate gene targeting via HDR as the donor DNA can be utilised as a template for DNA repair, resulting in the introduction of specific mutations (including correction of existing mutations by reversion to the WT sequence) or gene addition events at the ZFN targeting site (Figure 1.18) (Urnov et al., 2010). Alternatively stimulation of NHEJ repair, a low fidelity repair pathway, can lead to more random changes in DNA sequence (Lieber, 2010) at the target site resulting in gene disruption events from missense or nonsense mutations.

Figure 1.18 Possible gene modification events from ZFN-induced double-strand breaks

The activation of ZFN catalytic activity after dimerisation results in DNA double-strand breaks which can be repaired by non-homologous end joining repair (NHEJ) or homology directed repair (HDR). The repair of these DNA lesions can generate multiple gene modification outcomes exploited to study the functions of known and novel genes. Reprinted (adapted) with permission from (Urnov et al., 2010). Copyright (2010) Nature Publishing Group.

The utilisation of ZFN for gene modification events represents an upgrade from low recombination frequencies of introduced DNA to homologous chromosomal targets in mammalian cells (Koller & Smithies, 1992) and the high risk of semi-random integration events using from viral gene delivery systems (Baum et al., 2004). Numerous studies have demonstrated the utility of ZFN in specific gene modification events by stimulation of HDR
in a variety of model organisms and cell lines. HDR induction from ZFN activity was first described in *Xenopus laevis* oocytes (Bibikova et al., 2001) and subsequently shown in *Drosophila melanogaster* where the *y* (Bibikova et al., 2003), *ry* and *bw* (Beumer et al., 2006) genes were replaced with mutant genes provided via the co-transfection of homologous donor DNA containing the mutations. Subsequent studies on other model organisms such as rodents (Cui et al., 2011b) and *Zea mays* (Shukla et al., 2009) demonstrate the application of this technology in different model organisms. Several studies have also reported ZFN-directed gene modification in human cells (Urnov et al., 2005; Moehle et al., 2007; Kandavelou et al., 2009; DeKelver et al., 2010), and embryonic stem cells (Hockemeyer et al., 2009; Zou et al., 2009). Certain studies have demonstrated excellent gene modification rates by ZFN-induced HDR in genes which are mutated in human disease such as *IL2Rγ* gene which has been implicated for X-linked SCID (Urnov et al., 2005; Lombardo et al., 2007) or *PIG-A* (mutated in patients with paroxysmal nocturnal haemoglobinuria (Zou et al., 2009)).

As with gene targeting using HDR, a number of studies have successfully shown ZFN-directed mutagenesis on a wide number of model organisms resulting in gene disruption events via stimulation of the NHEJ repair. This was first shown in drosophila where ZFN activity resulted in mostly small deletions and/or insertions (indels) mutations, many of which led to the loss of protein expression and activity (Bibikova et al., 2002; Beumer et al., 2006; Beumer et al., 2008). ZFN-directed gene disruption events have also been described in other model organisms such as *Caenorhabditis elegans* (Morton et al., 2006), zebrafish (Doyon et al., 2008; Meng et al., 2008), rodents (Geurts et al., 2009) and pigs (Hauschild et al., 2011); all of which have shown capability for these mutant alleles to be propagated to the next generation.

The first targeted gene knockout study in mammalian cell lines (CHO cells) demonstrated excellent efficacy of ZFN at the *DHFR* gene with about a third of the clones carrying biallelic mutations (Santiago et al., 2008). Subsequent studies have generated viable knockout CHO cells deficient in two genes (*BAX* and *BAK*) (Cost et al., 2010) and three genes (*DHFR*, *GS* and *FUT8*) (Liu et al., 2010). In addition, ZFN-induced mutations in the CCR5 receptor in CD4+ T helper cells (Perez et al., 2008) and CD34+ hematopoietic stem/progenitor cells (Holt et al., 2010) confer protection to HIV in a HIV-infection mice model. The utility of this approach is currently being investigated in phase I/II clinical trials ([www.clinicaltrials.gov](http://www.clinicaltrials.gov) identifier NCT01044654). Recently several studies have reported ZFN-directed knockout in several other mammalian cell models including pigs (Li et al., 2012), induced pluripotent stem cells (Soldner et al., 2011), hepatocellular carcinoma cell
lines (Inami et al., 2011), LS174T colon adenocarcinoma cell line (Le Floch et al., 2011),
A549 and K562 tumour cell lines (Gutschner et al., 2011). Taken together, these studies show
that ZFNs are robust molecular tools capable for targeted, multi-allelic knockout of genes
(including POR) in human tumour cell lines.

1.7. Objectives and organisation of this thesis

Understanding the enzymology of the activation of HAP is important as it represents
one of the major determinants of the efficacy of these compounds in tumours. As described in
Sections 1.3.1-1.3.3, POR has been suggested to be one of the major one-electron reductases
that activate HAP under hypoxia. However the role of POR and other one-electron reductases
involved in this activation step is not fully understood, as model systems utilised by these
studies (viral over-expression or purified recombinant protein studies) do not establish their
contribution at endogenous levels of expression in tumours. Therefore the generation of
model systems to further study the role of POR in the hypoxic activation of HAP is required.
This thesis will describe the use of ZFNs, custom-designed by Sigma-Aldrich from an
archive of pre-characterised two-finger modules each recognising an experimentally
validated 6 bp half-site (Isalan et al., 2001; Moore et al., 2001), using the methods outlined in
(Doyon et al., 2008). The ZFNs are used to introduce targeted mutations in the 8th exon of all
copies of the POR gene in two human tumour cell lines (HCT116 colon carcinoma and SiHa
cervical carcinoma). The latter cell lines were chosen in part because they have been widely
used in investigations of HAP metabolism and cytotoxicity (Guise et al., 2007; Singleton et
al., 2009; Guise et al., 2012; Meng et al., 2012). POR-null clones from these lines are
characterised in detail and used to test the hypotheses that:

(1) POR plays a role in HAP activation in the parental cell lines, and
(2) that other (redundant) one-electron reductases also contribute to HAP activation in these
cells.

Chapter 2 reports the Methods and Materials utilised throughout the thesis. Chapter 3
describes the generation and characterisation of the POR knockout clones, including
evaluation of POR expression by Western blotting and enzyme activity as NADPH-
dependent, cyanide-resistant cytochrome c reductase activity. The ZFN target site was
sequenced in the POR null clones to determine the nature of mutations and to predict
truncated protein products. POR over-expressing cells were also generated from the POR knockout clones to provide isogenic cell line pairs for evaluation of the activation of HAP and fluorogenic probes.

Chapter 4 focuses on a novel fluorogenic probe, FSL-61, which has recently been reported to be activated by the *E. coli* nitroreductase nfsB under aerobic conditions and by one-electron reductases in hypoxic tumour cells (Singleton, 2009; Smaill et al., 2010). The objectives of this study were to characterise the metabolites of FSL-61 in hypoxic cells, and to evaluate its utility as a probe for activity of POR and other one-electron reductases in intact cells.

Chapter 5 describes the use of the POR-knockout and POR-over-expressing lines to evaluate the role of POR in the activation of a panel of 12 HAP representing all major chemical classes of current interest for exploiting tumour hypoxia. This panel includes the compounds of currently or recently in clinical development (TPZ, mitomycin C, EO9, AQ4N, PR-104A, TH-302) and compounds from this laboratory in advanced preclinical development (SN30000, SN29428). The HAP, and some effector cytotoxins generated by their bioreductive metabolism (PR-104H, SN29932 and AQ4) were tested in aerobic and anoxic antiproliferative (IC$_{50}$) assays, using 4 h drug exposures. In addition clonogenic cell killing was tested for PR-104A and SN30000, and metabolic activation of these HAP was evaluated by quantifying their reduced metabolites using LC-MS/MS. The above studies demonstrated significant differences in HAP sensitivity between individual POR knockout clones. Therefore, initial steps towards understanding the basis for this clonal heterogeneity, and particularly whether ZFN off-target effects might contribute was conducted, using karyotyping and exome sequencing. Individual POR knockout HCT116 clones, and non-ZFN-treated clones isolated in a similar fashion from the parental HCT116 population, were evaluated, along with clones in which a different gene (ADPGK) had been knocked out with ZFNs by Dr Susan Richter (Richter, 2011).
Chapter 2. Materials and methods

2.1. Materials and compounds

2.1.1. General materials

Water was purified by filtering through ion exchange columns and a 0.22 μm filter (Milli-Q purification system, Millipore Corporation, MA, USA) unless stated otherwise. Reagents, chemicals and materials were purchased as listed in Table 2.1 unless stated otherwise.

Table 2.1 List of reagents, chemicals and materials

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<th>Source</th>
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<td>1 mL Titertube microtubes</td>
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<td>Restore Western Blot Stripping Buffer</td>
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<td>SOC medium</td>
<td>Invitrogen, Life Technologies Inc, CA, USA</td>
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### Chapter 2. Materials and methods

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<td>Sulforhodamine B</td>
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<td>Surveyor™ nuclease assay kit</td>
<td>Transgenomic Inc, NE, USA</td>
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<td>Invitrogen, Life Technologies Inc, CA, USA</td>
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<td>Mouse Anti-CYPOR monoclonal (Sc25263)</td>
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<td><strong>Secondary antibodies:</strong></td>
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<tr>
<td>Goat Anti-Mouse (Sc2055)</td>
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#### 2.1.2. Fluorescent probes

All compounds from the fluorescent substrate library were synthesised at the Auckland Cancer Society Research Centre (ACSRC, Table 4.1) and stock solutions in DMSO were stored at -80 °C. Fluorescence spectra of probes and their reduction products were determined at a concentration of 100 µM, using SpectraMax M2 microplate reader.
Chapter 2. Materials and methods

2.1.3. Bioreductive prodrug screen

All bioreductive prodrugs were synthesised at the ACSRC unless otherwise stated in Table 5.1. All compounds were dissolved in DMSO except for AQ4N [FB3 batch only, α-methanol], mitomycin C (50% ethanol in DMSO), EF5 (PBS) and AQ (50% ethanol in DMSO). Concentrations of compounds were determined by spectrometry where the compounds were diluted from stock solutions to 100 µM with 0.01 N HCl except for CB1954 (diluted in PBS), mitomycin C and EO9 (both diluted in methanol). The spectrophotometer was blanked with the solvent used for each compound and the compounds were added to a quartz cuvette (1 cm pathlength). The concentration was determined by the formula c = A/ε where A is absorbance at a fixed wavelength and ε is the molar extinction coefficient at that wavelength. Extinction coefficients are listed in Table 5.1.

2.1.4. PR-104A and metabolites

PR-104A was synthesised as previously described (Denny et al., 2005) and PR-104H was prepared by zinc dust reduction of PR-104A by Dr Kashyap Patel, ACSRC as previously described (Patterson et al., 2007) and were stored at -80 ºC. Internal standard for quantifying PR-104A metabolites was the tetradeuterated form of PR-104H (PR-104H.D4) which was synthesised similarly to PR-104A.D4 from PR-104A (Atwell & Denny, 2007).

2.1.5. SN30000 and metabolites

SN30000, SN30672 (1-oxide metabolite of SN30000) and SN33093 (nor-oxide metabolite of SN30000) were synthesised as previously described (Hay et al., 2006) and were stored at -80 ºC. Internal standards for quantifying SN30000 and its metabolites were the octadeuterated forms of the compounds themselves (SN30000.D8, SN30672.D8 and SN33093.D8) which were synthesised as previously described (Wang et al., 2012).

2.2. Culture medium

α-MEM was prepared by dissolving α-MEM powdered medium (Gibco, Life Technologies Inc, CA, USA) in water with 2.2 g/L NaHCO3. The pH of the medium was adjusted to 7.2 with 12 M HCl or 5 M NaOH. The medium was filter sterilised through 0.22 µm filters under positive pressure, after which 400 mL volumes were aliquoted into sterile
Chapter 2. Materials and methods

500 mL Duran bottles. Medium was stored at 4 °C and used within two months from the time of preparation. Culture medium was supplemented with 5 % FBS, which was heat inactivated at 56 °C for 40 minutes, stored at -20 °C and thawed prior to use. Antibiotics (100 units/mL penicillin, 100 μg/mL streptomycin; P/S, Sigma-Aldrich, MO, USA) were added to culture medium during certain experiments but not during passage of cell cultures. During certain experiments outlined below, 10 mM D-glucose and 200 μM 2′-deoxycytidine (IC\textsubscript{50} supplement) was added to the culture medium.

2.3. Cell lines

Cell lines (Table 2.2) were passaged (sub-cultured) in αMEM with 5 % FBS with the exception of Hep3B which was grown in Dulbecco’s modified eagle medium (DMEM, Gibco) culture medium with 10 % FBS. The cell lines were grown without antibiotics for <3 months from frozen stocks confirmed to be mycoplasma free by Mr Wayne Joseph (ACSRC) using PCR-ELISA (Roche Diagnostics, Mannheim, Germany). The cells were grown at 37 °C in 5.0 % CO\textsubscript{2} in humidified incubators and were passaged every 6-7 days or when the cells were 70-80 % confluent.

Generation of pools of HCT116 cells stably transfected with an F527.V5 plasmid for expression of reductases POR, AKR1C3, NQO1, NQO2, MTRR, NDOR1, NOS2A, CYB5R3 and FDXR with confirmation of expression using an inducible V5 tag, has been reported previously (Guise et al., 2010; Guise et al., 2012). SiHa cells stably transfected with the F527.V5 to over-express POR has also been reported previously (Guise et al., 2012). These transfected cell lines were grown in 5 % FBS with 3 μM puromycin (Sigma-Aldrich).

Table 2.2 Tumour cell lines used in this thesis

<table>
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<tr>
<th>Cell line</th>
<th>Tissue of origin</th>
<th>Source</th>
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<td>22Rv1</td>
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<td>A2780</td>
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<td>A431</td>
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<td>A549</td>
<td>Non-small cell lung carcinoma</td>
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<td>DU-145</td>
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<tr>
<td>Cell Line</td>
<td>Tumor Type</td>
<td>Source</td>
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<td>FaDu</td>
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<td>Cervical squamous cell carcinoma</td>
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<td>SKOV-3</td>
<td>Ovarian adenocarcinoma</td>
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2.4. Plasmids

Plasmid DNA was prepared by transformation into competent bacteria using the appropriate antibiotic selection for each plasmid and purified from bacterial cultures using PureLink® Quick Plasmid miniprep kit (Invitrogen, Life Technologies Inc, CA, USA). Plasmids used in this study are outlined in Figure 2.1. The F527.V5 plasmid backbone was
constructed by Dr Sophie Sydall as previously described (Syddall, 2010). In brief, the gateway cloning regions from pcDNA6.2V5DEST (Invitrogen) were cloned into a modified version of pIREs-P (Hobbs et al., 1998). Gateway cloning was used to recombine open reading frames encoding human oxidoreductases into F527.V5 as described previously (Guise et al., 2007; Guise et al., 2010; Guise et al., 2012). The F527.V5 plasmid is a bicistronic plasmid containing a puromycin resistance marker and a V5 Tag-on-demand system (Invitrogen). The addition of a Tag-on-demand suppressant supernatant allows the tagging of the protein with an inducible V5 polypeptide which allows confirmation of protein expression by Western blotting using a V5 antibody. The ZFN plasmids consist of a pair of plasmids encoding one ZFN and are denoted as pZFN.POR1 and pZFN.POR2 respectively.

Figure 2.1 Structure of the plasmids used in this study

(A) Plasmid structure of F527-ires.hrGFP (6386-bp, Dan Li). GFP expression in this plasmid is driven by an EF1α promoter and contains a puromycin resistance marker for selection. (B) pEGFP.N1 plasmid (GenBank Accession #U55762, Clontech Laboratories Inc, CA, USA) is a GFP plasmid driven by a CMV promoter (C) Plasmid structure of ZFN.POR plasmid (4032-bp, Sigma-Aldrich). (D) The F527.V5 POR plasmid (7422-bp) for POR overexpression and contains a puromycin resistance marker for selection.
2.5. Preparation of cell suspension

Culture medium (5% FBS) supporting the cells in log phase were aspirated and cells were washed with PBS (137 mM NaCl, 2.68 mM KCl, 1.47 mM KH2PO4, 8.16 mM Na2HPO4, pH 7.4). This PBS was then aspirated and cells were treated with 3 mL of 0.07% (w/v) trypsin in citrate buffered saline (4.4 g/L trisodium citrate, 10 g/L KCl, pH 7.6) for 5 minutes after which 7 mL of culture media with serum was added to stop the trypsinisation reaction. The cell density of this cell suspension was measured using a Z2 Coulter Particle Count and Size Analyser (Beckman Coulter Inc, FL, USA).

2.6. Transfection methods

2.6.1. Lipofectamine LTX (Invitrogen)

HCT116 and SiHa cells were plated into a 24-well plate at 7.5 x 10^4 and 1 x 10^5 cells/well respectively before the day of transfection. Prior to transfection, culture media (αMEM + 5% FCS) in each well was replaced with fresh media. Five hundred ng of DNA was diluted with 100 µL Opti-MEM (Invitrogen) ± 0.5 µL Plus reagent (optimisation as outlined in Section 3.4.3). The reagents were gently mixed and incubated for 5 min at room temperature. Two µL LTX reagent was added to the mixture and left to incubate for 30 min at room temperature before adding it to each well and mixing by gentle plate rocking.

2.6.2. FuGENE®6 (Roche Diagnostics)

The cells were prepared as above prior to transfection. The FuGENE® reagent was diluted with serum-free Opti-MEM culture medium (Invitrogen) to a final volume of 20 µL and left to incubate for 5 min at room temperature. The amount of FuGENE®6 reagent added was dependent on the DNA/well (Section 3.4.3). DNA was added and the mixture was incubated for 45 min before adding to the wells. The cells were transfected with a ratio of FuGENE®6 reagent: DNA of 3:1, 6:1 and 3:2 per well, starting at 0.2 µg plasmid DNA.

2.6.3. JetPEI (Polyplus-transfection SA, Illkirch, France)

The cells were prepared as above prior to transfection. 1 µg of DNA (HCT116 cells) and 0.5 µg of DNA (SiHa cells) was diluted in 150 mM NaCl to a final volume of 50 µL. Fifty µL of the jetPEI solution (1:25 dilution in 150 mM NaCl from stock solution) was
added to the DNA, vortexed and incubated for 30 min at room temperature before addition to cells.

2.6.4. Electroporation with an Amaxa Nucleofector®

10⁶ SiHa cells were centrifuged at 220 g for 5 min and the resulting cell pellet was resuspended with 100 µL of cGMP nucleofector solution V (Lonza Group Ltd, Basel, Switzerland). The cell suspension was then mixed with 2 µg of DNA and transferred to an Amaxa cuvette. After nucleofection with programme T30 (optimisation outlined in Section 3.4.3), Five hundred µL culture media (αMEM +5% FCS + 1% P/S) was added to the reaction and the cells transferred to a 6 well plate.

2.6.5. Fluorescence microscopy

Twenty four h post-transfection, the transfected cells were washed and submerged in 500 µL PBS and imaged using the Nikon Eclipse TE2000-E (Nikon Instruments Inc, NY, USA) at 450/490 nm (Excitation/Emission). Transfection efficiency was scored by manual counting of up to 200 cells in the brightfield image and fluorescence image. The average transfection efficiency was calculated for the total number of cells scored from 5 squares as outlined in Figure 3.4.

2.7. Transient transfection with pZFN.POR

Two x 10⁶ HCT116 were seeded overnight on 100 mm Petri dishes and transfected with the Lipofectamine LTX method (Section 2.6.1) after scaling to the larger dish size. A total of 10 µg of DNA was added to the reaction with equimolar pEGFP.N1 (4700 bp, 3.68 µg), pZFN.POR1 (4032 bp, 3.16 µg) and pZFN.POR2 (4032 bp, 3.16 µg). 10⁶ SiHa cells were transfected with 2 µg total DNA (0.736 µg pEGFP.N1, 0.632 µg each of pZFN.POR1 and pZFN.POR2) using Amaxa Nucleofection as above.

2.7.1. Fluorescence activated cell sorting (FACS) of ZFN treated cells

Twenty four h post transfection, transfected cells were trypsinised, centrifuged at 220 g for 5 min and resuspended in 3 mL of media (αMEM). Cells were sorted by GFP fluorescence using a Becton Dickinson FACSVantage Cell Sorter operated by Mr Stephen Edgar (University of Auckland). The GFP-positive cells were sorted into 5 mL tubes containing media (5% FBS + P/S) or directly into 96-well plates with one GFP-positive cell per well seeded.
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2.7.2. Limiting dilution cloning of ZFN treated cells

GFP-positive cells in 5 mL tubes (above) were diluted to 4 cells/mL and 200 µL was plated in 96-well plates. Wells were checked to ensure the presence of a single colony and were grown to confluence while wells with multiple colonies were discarded.

2.8. Generation of POR overexpressing cell lines

HCT116 and SiHa knockout cells were transfected with the F527.V5 plasmid for expression of POR using Lipofectamine LTX and Amaxa nucleofection respectively. After transfection, culture medium was replaced by media with increasing puromycin concentration (1 µM, 2 µM and 3 µM) for 3 days at each concentration to select for transfected cells. After the selection process the cells were maintained in 3 µM puromycin.

2.9. Western blotting

For preparation of total cell lysate, confluent 75 cm² tissue culture flasks were passaged as above and the cell pellet was washed with PBS, re-pelleted by centrifugation and resuspended with 300 µL of radioimmunoprecipitation (RIPA) buffer [50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Tergitol-type NP-40 and 0.25% (v/v) Na-deoxycholate with an added 1% (v/v) Sigma-Aldrich protease inhibitor cocktail]. Lysis was allowed to proceed for 15 min at 4 °C with regular agitation. The sample was then centrifuged at 220 g for 5 min and the supernatant stored at -20 °C.

The total protein concentration of the lysates was measured using the bicinchoninic acid protein (BCA) assay, modified from (Smith et al., 1985). Samples were diluted 1:4 in 0.1 M NaOH to a total volume of 50 µL. One hundred µL of BCA reagent (1:50 dilution of 4% (w/v) CuSO₄ in bicinchoninic acid (Sigma-Aldrich) was added to each sample and left on a plate shaker in the dark for 30 min at room-temperature. The absorbance of the wells was read at 562 nm using the EL808 Plate Reader (BioTek Instruments Inc, VT, USA). The protein concentration was calculated based on a standard curve prepared by a serial dilution of 2 mg/mL bovine serum albumin (Thermo Fisher Scientific, MA, USA) prepared in the same way as the samples.

Cell lysates containing 20-30 µg protein were mixed with NuPAGE® LDS sample buffer (4x) (Invitrogen) and 5% β-mercaptoethanol and heated at 95 °C for 5 min. The samples were loaded onto NuPAGE® 4-12 % Bis-Tris Gels (Invitrogen) and run for 40 min at
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200 V in NuPAGE® MES SDS running buffer (Invitrogen). The gel was blotted onto a nitrocellulose membrane (Bio-Rad Laboratories Inc, CA, USA) at 100 V for 1 h in transfer buffer [25 mM Tris (base) containing 200 mM glycine and 20% (v/v) methanol]. The membrane was then incubated in blocking buffer [PBS + 0.05% (v/v) Tween-20 + 5% (w/v) non-fat milk] for 1 h at room temperature. Primary antibody in blocking buffer was added to the membrane and left overnight at 4 °C with gentle agitation. Primary antibody was decanted and the membrane incubated with secondary antibody in blocking buffer for 1 h after 3 washes with PBS Tween-20. The membrane was imaged using Imagereader LAS-3000 (Fujifilm Holdings Corporation, Tokyo, Japan) with chemiluminescent methods using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Fischer Scientific). For certain blots where the expression of protein was very low (e.g. screening of POR knockout clones) a more sensitive chemiluminescent kit, Amersham ECL advance (GE Healthcare, Buckinghamshire UK) was used. Band densitometry analysis was done using ImageJ (version 1.42q).

2.10. PCR based assays

Genomic DNA samples were purified from cell lines using GenElute™ Mammalian Genomic DNA purification kit (Sigma-Aldrich) following the manufacturer’s protocols, resuspended in Tris-EDTA (10mM Tris, 0.5mM EDTA pH 9.0) and the concentration determined with a ND-1000 NanoDrop spectrophotometer (Thermo Fisher Scientific), using ND-1000 supporting software (V3.1.2). Samples were amplified by Eppendorf Mastercycler EP (Eppendorf, Hamburg, Germany) using cycling programmes appropriate to each experiment. Unless indicated otherwise, the reactions consisted of 12.5 µL ReadyMix™ Taq PCR Reaction mix with MgCl₂ (Sigma-Aldrich), 1 µL of forward and reverse primers at 5 pmol/µL and 100-150 ng DNA topped up to 25 µL with H₂O. The amplified PCR products were then run on a 1% (w/v) agarose gel in TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA) with 0.1 % (v/v) SYBR® safe DNA gel stain (Invitrogen) at 100 V for 60 min. The gel was then imaged by Gel Doc (Bio-Rad Laboratories) with supporting software (Quantity One Version 4.0.3). Primers were purchased from Invitrogen unless otherwise stated and are listed in (Table 2.3).
Table 2.3 Primers used in this thesis

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<td>POR-2R</td>
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<tr>
<td>Taqman primer R (Applied Biosystems)</td>
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</tr>
</tbody>
</table>

### 2.10.1. Copy number assay

Genomic DNA from WT cell lines were diluted to 10 ng/µL and amplified by qPCR using the qPOR-1 F/R primer pairs with Platinum® SYBR® Green qPCR Supermix (Invitrogen). The reactions consisted of 5 µL SYBR Green mix, 0.2 µL of each primer pair (10 µM) and 1 µL genomic DNA (10 µM) samples topped up to 10 µL with H₂O. An internal control (RNAseP F/R primer pair) was run with the same samples and all reactions were done in quadruplicates with duplicate no-template controls for each primer pair. The reactions were run on MicroAmp® 384-well plates (Applied Biosystems, Life Technologies Inc, CA, USA) with an ABI7900 real time PCR (Applied Biosystems) using the cycling program of 50 °C (2 min), 95 °C (10 min), [95 °C (15 s), 60 °C (30 s) 72 °C (30 s)] for 40 cycles and a dissociation step of 95 °C (15 s). Relative quantification of POR copy number was carried out with RQ manager v1.2 (Applied Biosystems) using blood from a healthy human subject as a comparison.

### 2.10.2. Surveyor™ mutation detection assay

Genomic DNA from ZFN treated cells was PCR-amplified using the POR-1F/R primer pair. PCR fragments were re-annealed with the cycling programme 95 °C (10 min),
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cooled to 85 °C at 2 °C/s, cooled to 25 °C at 0.1 °C/s and held at 4 °C. Surveryor™ Nuclease S and Surveyor™ Enhancer S (Transgenomic Inc, NE, USA) were added to the reaction and incubated at 42 °C for 45 min. The samples were run on a 2% (w/v) agarose with 0.1% (v/v) SYBR® safe DNA gel stain at 100 V for 40 min.

2.10.3. Taqman® assay

10⁶ cells were resuspended with 750 µL of TRIzol®, vortexed thoroughly and incubated for 5 min at room temperature. One hundred and fifty µL of chloroform was added and the samples were vortexed for 15s, incubated at room temperature for 3 min before centrifuging at 13500 g for 5 min at 4 °C. The aqueous layer was transferred to a new tube and an equal volume of 70 % ethanol in DEPC-treated water was added. RNA was extracted from these samples using the Qiagen Mini RNeasy Kit and Spin (Qiagen, Hilden, Germany) following the manufacturer’s protocols and the concentration determined by ND-1000 NanoDrop spectrophotometer. The conversion of this RNA to cDNA was done using Superscript III First-Strand Synthesis Supermix (Invitrogen) according to the manufacturer’s protocols.

Five hundred ng of cDNA from each cell line was used and amplified by qPCR using the cycling programme 50 °C (2 min), 95 °C (10 min), [95 °C (15 s), 60 °C (60 s)] for 40 cycles using Taqman® primers F/R for the POR gene. The fluorescent Taqman® probe (Applied Biosystems) binds between these primers (sequence = 5’GGCCAAGGTGTACATGG) but since there were more G’s than C’s in that sequence, the complement of that sequence was used (Probe = 5’CCATGTACACCTTGGCC3’). Transcript levels of POR were estimated as relative quantification compared to an endogenous control gene, 18S rRNA (Assay iD = Hs99999901_s1; Applied Biosystems).

2.10.4. Sequencing of the ZFN cut site

POR-1F and POR-1R primers flanking the ZFN cut site were modified by attaching flanking Gateway® regions (attB1 to the forward primer = GGGGACAAAGTTTGTACAAAAAAGCAGGCACC and attB2 to the reverse primer = GGGGACCACTTTGTACAAAGGAAGCCTGGGTTTC). Genomic DNA from cell lines was amplified by PCR using the cycling programme 95 °C (3 min), [95 °C (30 s), 62 °C (15 s), 72 °C (60 s)] for 30 cycles, 72 °C (5 min) and 4 °C (hold).
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The PCR products were purified using a DNA Clean and Concentrator kit (Zymo Research Corporation, CA, USA) following the manufacturer’s protocols and added to a BP clonase recombination reaction along with the donor vector pDONR221 (Invitrogen). A molar ratio of 1:1 of PCR product (671 bp) to pDONR221 (4762 bp) was required which therefore meant a 1:7 concentration ratio with respect to bp was used. The BP clonase reaction consisted of 150 ng pDONR221 vector (Invitrogen), 21.4 ng PCR product and 2 µL of BP clonase II reaction buffer (5x) topped up to 10 µL with MilliQ H₂O. This reaction was incubated at 25 °C for 1 h and then terminated by addition of 1 µL of proteinase K, followed by incubation at 37 °C for 10 min. Two µL of the BP clonase reaction was used to transform One Shot® TOP 10 competent E.coli cells (Invitrogen) according to the manufacturer’s protocol, and the transformed cells were plated on 32 g/L Lennox L agar (Invitrogen) plates containing 20 µg/mL kanamycin.

Colonies were picked and grown overnight at 37 °C in 5 mL 25 g/L Miller’s LB Broth Base (Invitrogen) containing 20 µg/mL kanamycin. The entry vector was extracted and purified using the Purelink® Quick Plasmid Miniprep Kit (Invitrogen) according to the manufacturer’s instructions. The entry vector containing the ZFN site was amplified by PCR using Applied Biosystems BigDye® version 3.1 terminator chemistry (Applied Biosystems) with the M13 forward primer using the cycling program 96 ºC (60 s), [96 ºC (10 s), 50 ºC (5 s), 60 ºC (4 min)] for 25 cycles and 4 ºC (hold). The PCR reaction consisted of 1 µL M13 primer, 2 µL V3 mix and 3 µL 5x buffer topped up to 20 µL with MilliQ H₂O. The V3 mix consists of dideoxyribonucleotides (ddNTPs) conjugated to a fluorescent dye, each with a different excitation and emission wavelength. The PCR products were purified by ethanol precipitation. The sequencing reaction was run on Applied Biosystems 9700 Gold Block thermal cyclers by Ms Kristen Boxen at the School of Biological Sciences, University of Auckland.

2.11. Growth Curves and doubling time calculation

Fifty thousand cells/well were seeded into a 24-well plate. At each time point, triplicate wells were harvested by trypsinisation with 100 µL trypsin for 5 min before the addition of 100 µL media. This was added to 9.8 mL of 0.9% (w/v) saline and the mixture was added to the wells as a backwash step to recover the remaining cells which were counted using the Coulter Particle Count and Size Analyser. Calculations for doubling time were also
made during passaging of sub-confluent cultures using the formula: Doubling time = $T / \log_2 \left( \frac{\text{cell density at time } T}{\text{cell density at } T = 0} \right)$ where $T$ is time since seeding.

### 2.12. Treatment of cell lines to eliminate mycoplasma

For Plasmocin™ (Invivogen, CA, USA) treatment, cells were thawed from liquid nitrogen stocks and grown in culture to confluence. The cells were washed three times with fresh media and trypsinised to obtain a cell suspension which was then centrifuged at 220 g for 5 min. The cell pellet was washed twice with PBS by centrifugation before the cells were seeded in 25 cm$^2$ flasks at 1000 cells/flask in aMEM + 5% FBS + 25 µg/mL Plasmocin™. The cells were grown and passaged in media with Plasmocin™ for two weeks, changing the media every 4-5 days, after which the cells were grown in media without Plasmocin™ for two weeks. For Plasmocure™ (Invivogen) treatment, cells were thawed from liquid nitrogen stocks and treated as with Plasmocin™ except using Plasmocure™ (100 µg/mL). After treatment, the cells were tested for mycoplasma to confirm mycoplasma status. For transfected cells carrying the F527.V5 or F279.V5 plasmids, 3 µM puromycin was also added to the media during this treatment.

### 2.13. Cell cycle analysis by flow cytometry

$10^6$ cells in exponential phase growth were resuspended in 0.3 mL PBS and vortexed gently while 0.7 mL of cold 100% ethanol was added dropwise. The cells were left in the 70% ethanol solution for at least 2 h at 4 °C and stored at -20 °C for up to two days. The cells were centrifuged at 220 g for 8 min, the supernatant was aspirated and the cell pellet resuspended in PBS. The wash step was repeated before the cell pellet was resuspended in 1mL propidium iodide staining solution consisting of 0.1% (v/v) Triton X-100 (Serva Electrophoresis GmbH, Heidelberg, Germany), 10 µg/mL propidium iodide (Invitrogen) and 100 µg/mL DNAse-free RNase A (Sigma-Aldrich) in PBS. The cells were kept in the dark at room temperature for 30 min followed by flow cytometer analysis by BD FACSDiva software using the BD LSR II flow cytometer (Becton, Dickson and Company, NJ, USA). The samples were excited with a 488 nm laser and detected at an emission of 617 nm. Cell cycle analysis was conducted using Flowjo (version 7.6.5).
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2.14. Cyanide-resistant NADPH cytochrome c reductase assay

Approximately $4 \times 10^7$ cells or two 175 cm$^2$ tissue culture flasks at 70-80% confluence were washed with ice-cold PBS twice, harvested with a sterile cell scraper and centrifuged at 220 g for 5 min. The cell pellet was homogenised with ice-cold nuclear buffer A (10 mM HEPES pH7.4, 1.5 mM MgCl$_2$, 10 mM KCl, 0.05 mM dithiothreitol) and allowed to stand for 10 min at 4°C. The cells were then sonicated using Sonicator Ultrasonic Processor for 3 x 5s (oscillation amplitude between 5-10µm) or SONOPLUS HD 2070 (Bandelin, Berlin, Germany) with the samples placed on ice after each sonication. The suspensions were left to stand on ice for another 10 min, centrifuged at 600 g for 5 min followed by centrifugation at 9000 g for 15 min at 4 °C. The supernatant (S9 fraction) was aliquoted and protein content measured by BCA assay.

The protocol was modified from (Guengerich et al., 2009) and is as follows. 50-100 µg of S9 fractions from the cell lines were added to cuvettes containing 1 mM KCN and 40 µM cytochrome c. The final volume of the cuvette was brought to 990 µL with 0.3 M phosphate buffer (KH$_2$PO$_4$ 13.6 g/L, K$_2$HPO$_4$ 17.4 g/L; pH 7.6); with the final concentration of phosphate buffer about 0.23 ± 0.02 M after taking into account the different volumes of S9 added to the solution. The cuvette was allowed to equilibrate at 37 °C for 5 min before the changes in absorbance at 550 mm were recorded for 150s (background rate) with an Agilent 8453 diode array spectrophotometer (Agilent Technologies Inc, CA, USA). After 150 s of recording, the reaction was initiated with the addition of NADPH (100 µM) and the initial rate of absorbance change at 550 mm was calculated with the background activity subtracted (pre-NADPH addition). The activity was calculated as nmol/min/mg protein, using the extinction coefficient of reduced cytochrome c (0.021 mM$^{-1}$cm$^{-1}$) (Williams Jr & Kamin, 1962).

2.15. Fluorogenic probe plate reader screen

Cells were seeded (50000 cells/well) in black 96-well plates (Greiner Bio-One Ltd, Frickenhausen, Germany) in 100 µL αMEM without FBS and IC$_{50}$ supplement and incubated for 2 h at 37 °C. Candidate fluorogenic probes were added to each well to a final concentration of 50 µM and left in 37 °C under oxic and anoxic conditions for 2 h. Anoxic incubations were done with a 5% H$_2$/palladium catalyst scrubbed anaerobic chamber (Coy Laboratories, MI USA) with culture medium and plasticware equilibrated to anoxic conditions at least three days before experiments. The flavoprotein inhibitor
diphenyleneiodonium chloride (DPI; Sigma-Aldrich) was added to a final concentration of 100 µM, or an equal volume of culture medium was added to controls. The plates were analysed by a SpectraMax M2 microplate reader with the following Ex/Em: FSL-41, FSL-59 (350/455 nm), FSL-61, FSL-67, FSL-111, FSL-141 (355/460 nm), FSL-76 (405/585 nm) and FSL-95 (350/475 nm). The Ex/Em maxima for the fluorescent probes were determined previously (Singleton, 2009).

### 2.16. Chemical or enzymatic reduction of FSL-61

Chemical reduction of FSL-61 and the corresponding free acid was achieved by zinc dust reduction in anoxic conditions. The reaction was initiated by addition of ammonium acetate (30 mg) to the FSL-61 solution (7 mg/mL in acetonitrile) followed by 30 mg zinc powder. After mixing by inversion for 5 min the reaction was terminated by centrifugation (220 g x 5 min) and the supernatant stored immediately at -80 °C. Enzymatic reduction of FSL-61 was conducted under anoxic conditions in the same anaerobic chamber. 5.3 µg human recombinant POR (20-55 U/mg, Abcam PLC, Cambridge, UK), 1 mM FSL-61 and 5 mM NADPH in 100 µL 0.3 M phosphate buffer were incubated for 1 h at room temperature and the reaction was terminated by freezing at -80 °C. Samples were diluted 100-fold (chemical reduction) or 10-fold (enzymatic reduction) in mobile phase for analysis by HPLC and LC/MS.

### 2.17. Analysis of fluorescence of POR probes by flow cytometry

Optimisation of this method will be outlined in Chapter 4. The optimised protocols are as follows. $10^6$ cells were seeded on non-tissue culture treated 24 well plates in 500 µL phenol red free αMEM + 5% FBS. Fluorogenic probes were added at concentrations indicated in Chapter 4 and cultures were incubated at 37 ºC under oxic and anoxic conditions (as above) for up to 3 h. After incubation, the contents of each well were collected and the cells were resuspended in the same medium which they were exposed to the compounds. The cells were kept in the dark on ice and analysed by flow cytometry within 2 h using a BD LSRII flow cytometer and analysed with BD FACSDiva software (Becton, Dickson and Company). The excitation wavelength was 355 nm, with emission at 425-475 nm for FSL-41, FSL-61 and FSL-76 and 515-545 nm for FSL-95. Gating for single cells used forward-scatter and side-scatter with a 488 nm laser.
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For optimisation steps after cell harvest, cells were either harvested in the medium which they were exposed to the compounds and analysed by flow cytometry immediately or centrifuged for 5 min and resuspended in ice-cold fresh medium or PBS. For cells that were fixed before analysis on the flow cytometer, the cells were centrifuged for (220 g x 5 min) and resuspended in 100 µL 4% paraformaldehyde (PFA) and left for 60 min on ice. The solution was then diluted with 900 µL of PBS and analysed by flow cytometry. The cells were also stored for 24-48 h in the dark with the same solution at 4 ºC.

2.18. FSL-61 metabolite analysis by HPLC with absorbance, fluorescence and mass spectrometry detection

Cells were seeded in 24 well plates (10^6 cells/well) as for flow cytometry, incubated with FSL-61 (300 µM) at 37ºC under oxic and anoxic conditions for 0.5 or 3 h. The cells were collected by centrifugation and the cell pellet was then resuspended in 10 µL H_2O followed by addition of 50 µL ice-cold acetonitrile which was added dropwise while vortexing. A sample of the supernatant was processed similarly (20 µL of supernatant mixed with 10 µL H_2O, then 50 µL acetonitrile). For the optimisation experiments, the metabolites were extracted by the addition of 1 mL acetonitrile and the supernatant was collected by centrifugation at 4ºC (15000 g at 5 min). The samples were stored in glass LC/MS vials at -80ºC, and subsequently analysed by High Performance Liquid Chromatography (HPLC) with photodiode array absorbance and fluorescence detectors (Agilent 1100 LC; Agilent Technologies Inc). Retention times were corrected for the offset between detectors.

Optimisation of acetonitrile gradient conditions will be outlined in Chapter 4. The optimised method was as follows: The samples were diluted 1 in 2 with mobile phase (0.01% (v/v) formic acid in water) and 10 µL was injected for separation on a Zorbax SB C18 capillary column (3.0 x 150 mm, 5 µm; Agilent Technologies Inc) at 45 ºC with a flow rate of 0.5 mL/min. The mobile phase was a linear gradient of acetonitrile (10-50 % over 25 min) in 0.01% formic acid in water. UV absorbance spectra (230 nm to 900 nm) were collected at 254 nm (bandwidth 50 nm) with a reference of 550 nm (bandwidth 50 nm). Fluorescence excitation was at 280 nm and emission was monitored at 460 nm. Mass spectra were collected using an Agilent LC/MS (Agilent 6150) with electrospray jet stream ionisation (ESI) using alternating positive and negative ionisation. The mass/charge (m/z) ratio was scanned from 100 to 800 with a fragmentation voltage of 135 V. An Agilent LC/tandem MS (MS/MS; model 6460) was also utilised for the analysis of metabolites. FSL-61 and its
Chapter 2. Materials and methods

corresponding nitro-acid (SN24642, Section 4.4.5) were quantified against standard curves using UV absorbance peak areas.

2.19. FSL-61 fluorescence microscopy

10^5 cells SiHa cells were seeded per well into a 96-well plate in 100 µL phenol red free αMEM + 5% FBS and left for 2 h at 37 ºC under oxic or anoxic conditions. Culture medium was aspirated from the cells and FSL-61 was added to each well at a final concentration of 300 µM for 3 h. The medium was aspirated and the wells washed with PBS twice. 150 µL of PBS was added to the wells and imaged using the Nikon Eclipse TE2000-E inverted microscope using the DAPI filter (Ex/Em = 345/455 nm) with the 20x objective lens and an exposure of 1/3 s. The brightfield images were taken with an exposure time of 1/40 s.

For confocal images, 10^6 HCT116 cells were seeded in tissue culture 35 mm Petri dishes in 2 mL phenol red-free αMEM with 5% FBS and left for 2 h at 37ºC under oxic or anoxic conditions. FSL-61 was added to a final concentration of 300 µM and the cells were left to incubate for 3 h under oxic and anoxic conditions. After incubation, the medium was aspirated and the cells were washed once with fresh medium and imaged on a Leica TCS SP2 confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany) with a dual line Argon laser (351 and 364 nm) and a detection bandwidth of 400-550 nm.

2.20. FSL-61 oxidoreductase screen

Each of the reductase-overexpressing HCT116 lines (Section 2.3) was seeded in 24 well plates as for flow cytometry. DPI incubations were carried out for 1 h at 37 ºC under oxic or anoxic conditions (as above). FSL-61 was then added to the cells to 300 µM and 3 h later cells were harvested for flow cytometry, as above.

2.21. Proliferation (IC_{50}) assay

Four hundred HCT116 cells or 1500 SiHa cells per well were seeded into 96 well-plates in duplicate wells for each compound with 100 µL culture media (10 % FBS + 1 % P/S + IC_{50} supplement; IC_{50} media) for HCT116 and SiHa cells respectively and left for 2 h at 37 ºC. The culture media from the top wells (wells for the addition of the highest drug concentration) were aspirated and 150 µL of compounds in culture medium was added to the wells and serial 3-fold dilutions made by transfer of 50 µL samples. The cells were incubated
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with the compounds for 4 h under oxic or anoxic conditions. The plates were washed by aspirating drug solution from the wells and adding 150 μL of culture medium (5% FBS + 1% P/S) to each well. This was step was repeated 2 times with the last step being the addition of 200 μL of culture media (5% FBS + 1% P/S) to each well.

After 5 days, the plates were fixed by addition of cold trichloroacetic acid at a final concentration of 10 % (w/v) and the plates were incubated at 4 ºC for 1 h. The wells were rinsed with tap water and the cells stained by addition of 50 μL 0.4 % sulforhodamine B in 1 % acetic acid for 30 min. Unbound stain was removed by sequentially rinsing in three 20 L containers with 0.1 % acetic acid and the plates were air-dried. 100 μL of 10 mM unbuffered Tris base (pH 10) was added to each well and mixed on a plate shaker and for 2 h in the dark. Absorbance was read at 490 nm using the EL808 Plate Reader and the data generated by GEN 5 (version 1.04.5). The IC50 (drug concentration reducing staining to 50 % of controls on the same plate) was determined by interpolation with a 4-parameter logistic regression model.

2.22. PR-104A metabolism assay

Five x 10^5 cells were plated per well into 24-well plates in 350 μL IC50 media and left for 2 h at 37 ºC under oxic or anoxic conditions. One hundred μM DPI incubations were conducted as above. 50 μL of culture media was added to the wells for 1 h (a final concentration of 100 μM PR-104A, 3 replicates per condition), after which the media from the wells was transferred into an ice-cold Eppendorf tube. Metabolites from the attached cells were extracted with ice cold methanol spiked with PR-104H.D4 (final concentration 0.3 µM) by addition of 800 μL into the wells. This was combined with the culture medium recovered previously and vortexed briefly before storage at -80 ºC.

A PR-104H calibration curve was prepared at the same time by adding PR-104H solutions of differing concentrations to untreated wells. A 1.5 µM PR-104H solution was prepared and a dilution series made. As above, media from the untreated cells were added to ice-cold Eppendorf tubes and a solution of PR-104H from the dilution series was added to the well. The standard curve provided final concentrations of PR-104H standards of 1 µM, 0.5 µM, 0.1 µM, 0.05 µM, 0.01 µM, 0.005 µM and 0.001 µM. The solutions were then vortexed briefly before storage at -80 ºC.
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2.22.1. Analysis by LC-MS/MS

Prior to analysis by LC-MS/MS, the samples were centrifuged at 15000 g for 5 min at 4 ºC and 75 µL of sample was diluted with 25 µL of water. 25 µL of sample was injected by an autosampler kept at 4 ºC and chromatographic separation was performed on a Zorbax SB-C18 column (50 x 3.0 mm, 1.8 µm; Agilent Technologies Inc) with a flow rate of 0.8 mL/min maintained at 25 ºC. The mobile phase was an acetonitrile gradient constructed using 0.01 % formic acid in 80 % acetonitrile (A) and 0.01% formic acid in water (B) with 20 % of A increasing linearly over 3 min, with a further increase to 80 % over 1.5 min, held for 1 min and returned to the 20 % of A over 1.5 min and maintained for 2 min before the next injection. UV absorbance detection was at 254 nm (bandwidth, 4 nm). The eluent was led into an Agilent LC/tandem MS (LC-MS/MS; model 6410) mass spectrometer starting 0.5 min after injection by switching the MS inlet valve. The MS interface was a combined electrospray-atmospheric pressure chemical ionisation (multimode) source set at positive ionisation mode. The mass/charge (m/z) ratio was scanned from 100 to 1000 with a fragmentation voltage of 135 V, capillary voltage of 2500 V, charging voltage of 1500 V, a gas flow of 6 L/min, nebuliser pressure of 50 psi, gas temperature of 350 ºC and vaporizer temperature of 225 ºC. Metabolites were quantified by multiple reactions monitoring (MRM) using the following ion transitions: PR-104H (485 > 389), PR-104H.D4 (491 > 395) and PR-104M (469 > 373). Data were acquired and analysed with Agilent MassHunter Workstation Software (Agilent Technologies Inc, B.01.04). Quantification of PR-104H was calculated using the ratio between the ion counts of the MRM transitions for each metabolite to the ion counts with the D4 deuterated internal standards PR-104H.D4 (m/z 491 [M+H⁺]) using a linear standard curve forced to zero. PR-104M was estimated by the ratio of PR-104M/PR-104H.D4 after applying a correction factor of 1.73 for the higher detection efficiency of PR-104M compared to PR-104H under these conditions (Singleton et al., 2009). The structures of these D4 compounds have been previously reported by Dr Yongchuan Gu for his PhD thesis (Gu, 2010).

2.23. SN30000 metabolism assay

10^5 cells were plated per well into 96-well plates in 90 µL phenol red free IC₅₀ media with 10 µL of SN30000 for a final concentration of 30 µM and 10 µM with three replicates per measurement. The samples were incubated for 3 h under oxic or anoxic conditions at 37 ºC. After incubation, all media from the wells were transferred into 1 mL Titertube
microtubes (Bio-Rad Laboratories). 200 µL cold methanol spiked with internal standard (SN30000.D8, SN30672.D8 and SN33093.D8 at 0.1 µM) was mixed into each well and added to the Titertubes containing the media collected earlier. The tubes were vortexed at 600 rpm for 30 s on a Gilson orbital shaker (Gilson Inc, Middleton, WI, USA) and left at -80 °C for at least 1 h. The tubes were then centrifuged at 15000 g for 10 min at 4 °C and 100 µL of the supernatant was added to a new 96-well plate, covered with a Beckman Coulter Seal and Sample aluminium foil lid (Beckman Coulter Inc) and stored at -80 °C until needed for LC-MS/MS analysis. A calibration curve was set up by a 1 in 10 dilution of a mixed standard stock (1 mM solution of SN30000, 500µM SN30672 and 100µM SN33093 in DMSO) using culture medium. A dilution series for the mixed standard stock was set up as illustrated in Table 2.4 (example of dilution series for SN30000 only). Standard curve samples were in triplicates and processed as per other samples.

Table 2.4 SN30000 calibration curve dilution series

<table>
<thead>
<tr>
<th>Stock concentration (µM)</th>
<th>Volume (µL)</th>
<th>Culture media (µL)</th>
<th>Final concentration (µL)</th>
</tr>
</thead>
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</table>
Chapter 2. Materials and methods

2.23.1. Analysis by LC-MS/MS

Prior to analysis by LC-MS/MS, 45 µL of the samples was diluted with 155 µL 0.01% formic acid water and added to a 96-well plate (Agilent Technologies Inc) with a pre-split silicon cap (Agilent Technologies Inc). 20 µL of sample was injected by an autosampler maintained at 4 ºC and chromatographic separation was performed on a Zorbax SB-C18 column (50 x 3.0 mm, 1.8 µm; Agilent Technologies Inc) with a flow rate of 0.8 mL/min maintained at 35 ºC. The mobile phase was an acetonitrile gradient constructed using 0.1% formic acid in 80% acetonitrile (A) and 0.1% formic acid in water (B) with 15% of A for 2 min, increasing linearly to 80% over 2 min, held for 0.3 min and returned to the 15% of A over 0.2 min and maintained for 1.5 min before the next injection. UV absorbance detection was at 252 nm (bandwidth, 4 nm). The eluent flow was led into an Agilent LC/tandem MS (MS/MS; model 6460) mass spectrometer starting 0.1 min after injection by switching the MS inlet valve. The MS interface was a Jet Stream electrospray ionisation source set at positive ionisation mode. The mass/charge (m/z) ratio was scanned from 100 to 1000 with a fragmentation voltage of 135 V, capillary voltage of 3000 V, a gas flow of 10 L/min, nebuliser pressure of 40 psi and a gas temperature of 320 ºC. Metabolites were quantified by multiple reactions monitoring (MRM) using the following ion transitions: SN30000 (331 > 100), SN30000.D8 (339.2 > 108), SN30672 (315 > 228), SN30672.D8 (323 > 228), SN33093 (229 > 212), SN33093.D8 (307 > 212) (Wang et al., 2012). Data were acquired and analysed with Agilent MassHunter Workstation Software (Agilent, B.01.04). Quantification of SN30000 metabolites was calculated by the ratio of ion counts (obtained from specific MRM transitions) to the ion counts of D8 octadecuterated internal standards. SN30000 consumption was measured using SN30000.D8 while formation of SN30672 and SN33093 metabolite was measured using SN30672.D8 and SN33093.D8 respectively. Quantitation of metabolites was done with a standard curve fitted with a quadratic fit using a 1/X weighting and the average metabolites were taken from triplicate wells at two different SN30000 concentrations.

2.24. Clonogenic assay

Cells were seeded at 3 x 10^5 cells/well and 3 x 10^4 cells/well for HCT116 and SiHa cells respectively with 150 µL of IC50 media in 96-well plates and incubated for 2 h in 37 ºC under anoxic conditions. The compounds were diluted serially (1 in 2 dilution) and 150 µL were added to the wells and left for 4 h, after which the media from the wells were aspirated and 100 µL of PBS was added to each well. This PBS was then transferred into 1 mL
Chapter 2. Materials and methods

Titertubes and 100 µL trypsin was added to each well and incubated at 37 °C for 5 min. One hundred µL of culture media (5% FBS + 1% P/S) was added to each well and the 200 µL was transferred into the same 1 mL Titertube. One hundred µL of fresh culture media was then added to the wells as an additional wash step and this was also added to the Titertubes. Two hundred µL of the cell suspension in the Titertubes were then added to 400 µL of media (1 in 3 dilution), followed by three subsequent 1 in 10 dilutions (60 µL into 540 µL). The cells were plated at $10^5$ (HCT116 only), $10^4$, $10^3$ and $10^2$ cells/plate in 60 mm Petri dishes and incubated for 10 (HCT116 cells) or 14 (SiHa cells) days. Colonies were stained with 2 g/L methylene blue in 50 % aqueous alcohol for 30 min and colonies of >50 cells were scored.

2.25. Karyotyping

Subconfluent 25 cm$^2$ tissue culture flasks were sent to LabPLUS, Auckland for G-banding karyotyping (conducted by Mrs Violeta Velkoska-Ivanova, LabPLUS). The cultures (overnight cultures or cultures grown for 24 h or 48 h) were treated with colcemid (50 ng/mL for 1 h) and metaphases were prepared for Typsin-Giemsa staining as detailed previously (Seabright, 1972; MacLeod et al., 2007). Twenty metaphases were counted and analysed for each cell line.

2.26. Exome sequencing

Subconfluent 25 cm$^2$ tissue culture flasks were harvested for genomic DNA isolation (as above, Section 2.10). Genomic DNA was quantified using Qubit® Fluorometer (Invitrogen) using the dsDNA BR (Broad Range) assay kit (Invitrogen) with the 260/280 and 260/230 ratios quantified using the ND-1000 NanoDrop spectrophotometer. Samples were run on a 0.8% (w/v) agarose gel for quality control for DNA degradation before being sent to New Zealand Genomics Limited (NZGL) for sequencing using an Illumina HiSeq 2000 (Illumina Inc, CA, USA). 12 TruSeq DNA libraries were prepared from 11 samples (Section 5.4.8) with two HCT116 WT DNA libraries prepared. The libraries were run in two separate lanes (2 x 100-bp paired end reads) with the HCT116 WT sample repeated in both as a reference. Initial bioinformatic analysis (variant calling and reference mapping) was conducted by Mr Peter Tsai from the New Zealand Bioinformatics Institute (NZBI). The data were mapped to human genome version 37 using BWA (Burrows-Wheeler Aligner) version 0.6.1-r104. Variant calling was made using Samtools 0.1.17 (http://samtools.sourceforge.net/) and VarScan 2.3.3 (http://varscan.sourceforge.net/).
2.27. Graphing and statistical analysis

Graphs were drawn with Sigmaplot (version 11.0) with statistical analysis conducted using one-way/two-way ANOVA with Holm-Sidak test for post-hoc pairwise analysis and Student’s t-test as indicated. Statistical significance was set at p<0.05.
Chapter 3. Generation and characterisation of POR knockout clones

3.1. Summary

NADPH-cytochrome P450 oxidoreductase (POR) is a one-electron reductase essential for the activation of the CYP mixed-oxygenase system and has been implicated in the activation of HAPs. However, model systems utilised by these studies are either cell-free systems or cells with over-expression of POR. A recent study utilised siRNA and anti-sense RNA methods to knockdown POR expression in tumour cells, but residual POR was still present (Guise et al., 2007). Hence, the creation of cellular models with specific full knockout of POR was undertaken to elucidate its role in bioreductive prodrug activation. Human tumour cell lines (HCT116 and SiHa) were transiently transfected with plasmids encoding customised zinc finger nucleases targeting \textit{POR}, cloned to ensure homogeneity of the POR genotype and screened for POR expression by Western blotting. The selected POR negative clones were characterised in further detail by sequencing the ZFN cut site, revealing multiple mutations at the target site that are predicted to lead to loss of POR expression and enzymatic activity. POR enzymatic activity of the knockout clones as measured by cyanide-insensitive NADPH-dependent cytochrome c reduction was found to be strongly reduced compared to WT cells, but activity was not completely ablated in the knockouts suggesting that the assay is not completely specific for POR. The morphology and growth of the POR knockout clones was similar to WT cells. These results demonstrated that ZFN activity resulted in multi-allelic mutations at the ZFN target site, leading to the loss of POR expression and activity in these tumour cell lines.
3.2. Introduction

POR is a one-electron reductase widely considered to be a major enzyme that activates bioreductive prodrugs under hypoxia (reviewed in Chapter 1). Numerous studies have shown that purified POR is capable of metabolising these bioreductive compounds (Keyes et al., 1984; Walton et al., 1989; Fitzsimmons et al., 1994; Bailey et al., 2001). These purified enzyme studies provide the most direct evidence of the ability of POR to reductively activate these compounds; however this model is an isolated system and does not provide information about the role of this enzyme intracellularly. Cell culture studies using POR over-expressing cell lines have also shown increased metabolism and sensitivity to bioreductive prodrugs (Belcourt et al., 1996b; Patterson et al., 1997; Saunders et al., 2000; Guise et al., 2007; Meng et al., 2012). However these cell lines also do not represent POR activity as observed in tumour cell lines, as artificial expression of POR attained by viral promoters typically exceeds levels of expression by endogenous promoters. Currently there is a lack of cellular models that enables the interrogation of the role of POR, at endogenous levels of expression, in bioreductive prodrug activation by tumour cell lines.

Recently, Guise and colleagues developed a cellular model to interrogate the role of endogenous POR in the activation of PR-104A (Guise et al., 2007). They used a SiHa cell line stably transfected with an antisense-POR construct which showed a 67% decrease in POR/GAPDH ratio relative to the parental cell line by Western blotting. Subsequently this cell line was treated with siRNA which further suppressed POR expression to 11.5 ± 5.5% of WT levels. This suppression led to an average change in C₁₀ (concentration for 10% survival) from 11.5 µM for WT cells to 21.5 ± 4.9 µM representing a 45 ± 12% decrease in hypoxic cell sensitivity. A corresponding 61.5 ± 0.7% decrease in PR-104A metabolism in these cell lines compared to WT cells was also observed. The authors extrapolated their results to a zero POR/GAPDH ratio (western blotting) and estimated a 44% decrease in PR-104A cytotoxic potency and a reductive metabolism rate of 28.2% of WT levels. This suggested that POR was important in the activation of PR-104A but that other enzymes were also involved. However, residual expression stemming from the leakiness of siRNA methods and potential off-target effects by partial inhibition of other transcripts with homology to POR (Jackson et al., 2003; Jackson & Linsley, 2004) meant that the role of POR in HAP activation at endogenous expression levels was still not well defined.

ZFN are novel chimeric endonucleases that can be utilised as highly specific gene modification tools (reviewed in Chapter 1). The specificity of ZFN is controlled by the zinc
finger domain which recognises three bp of DNA per zinc finger module. In addition, endonuclease activity of the Fok1 nuclease domain depends on dimerisation of a pair of ZFNs, resulting in an extended recognition site (typically two sequences of 9-18 bp each) separated by a 5-6 bp spacer at which the Fok1 domain localises. The activation of catalytic activity results in DNA double strand breaks at the recognition site that are repaired by endogenous DNA repair pathways. Repair by NHEJ results in base substitution and indels at the target site; many of which will result in targeted gene knockout. The modular nature of the zinc fingers allows modification to their specificity, thereby allowing gene targeting at almost any locus in the genome.

For the interrogation of the role of endogenous POR in bioreductive prodrug activation, POR knockouts were generated in the HCT116 colon carcinoma and SiHa cervical carcinoma human tumour cell lines. Tumour cell lines were transiently transfected with a custom-designed pair of ZFN targeting a genomically unique 33 bp sequence in the 8th exon of POR (Section 3.4.4), since transient expression is preferred as prolonged expression of ZFN can result in cellular toxicity (Pruett-Miller et al., 2009; Pattanayak et al., 2011). ZFN activity in these transfected cells was shown by the Surveyor™ nuclease assay which is a CEL-III nuclease, a 39kDa neutral pH endonuclease isolated from celery (Oleykowski et al., 1998; Yang et al., 2000). This enzyme cuts mismatch bubbles arising from mutations in one of the strands of DNA due to imperfect alignment of mutated and WT templates during DNA heteroduplex annealing. The amplification of the ZFN target site by PCR reveals the presence of mutations based on the formation of these cleavage products. POR knockout clones were detected by a negative POR Western blotting result and a few clones were chosen for further characterisation. Sequencing of the ZFN target site was done using the Gateway® cloning system, which allows the amplification of a single allele from each clone through creation of a destination vector with the use of site specific recombination (Hartley et al., 2000; Walhout et al., 2000). NADPH-dependent cytochrome c reductase activity was used as an assay for POR enzymatic activity (Guengerich et al., 2009) to determine the effect of these mutations on POR function and to validate the knockout status of these cell lines. The addition of cyanide to this assay inhibits the activity of cytochrome c oxidase (Jensen et al., 1984), which catalyses the oxidation of cytochrome c under the same experimental conditions.
3.3. Aims of this chapter

The aims of this chapter are:

1. To generate multi-allelic knockout models of POR (null clones) from two human tumour cell lines (HCT116 and SiHa) using a pair of ZFNs specific for a 33 bp sequence in the 8th exon of POR.
2. To characterise the resulting POR-null clones in terms of loss of protein expression and enzyme activity, mutation at ZFN cut site, growth and morphology.

The generation and characterisation of these cell lines will enable the determination of the role of POR in the activation of bioreductive prodrugs in the parental cell lines (Chapter 5).

3.4. Results

3.4.1. POR expression and activity in tumour cell lines

POR expression and activity in a panel of 23 tumour cell lines was measured by Western blotting and the cytochrome c assay. Western blotting of the total cell lysates demonstrated a heterogeneous level of POR expression (Figure 3.1A), with certain cell lines showing high POR expression (e.g. HepG2 and Hep3B) while others demonstrated low expression of POR (e.g. FaDu and PC3). Similarly, the rate of NADPH-dependent cyanide inhibited cytochrome c reduction in these cells was highly heterogeneous (Figure 3.1B) and the relationship between POR expression, as calculated by the POR/actin ratio and cytochrome c reductase was highly significant ($R^2 = 0.46$, $p < 0.001$).

Prodrug metabolism studies on PR-104A (Guise et al., 2012), SN30000 and EF5 (Wang et al., 2012) on the same panel of cell lines have been previously reported. To determine whether the metabolism of these compounds was associated with POR activity, the anoxic PR-104A, SN30000 and EF5 metabolism in these cells was compared to the cytochrome c reductase activity. Weak but significant correlations were observed between POR activity and the reduction of PR-104A ($R^2 = 0.23$, $p = 0.03$, Figure 3.2A), SN30000 ($R^2 = 0.36$, $p = 0.023$, Figure 3.2B) and EF5 ($R^2 = 0.33$, $p = 0.03$, Figure 3.2C) in these cell lines. These correlations suggest that POR is required for the anoxic metabolism of these compounds but that it may play only a minor role in this setting.
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Figure 3.1 POR expression and activity in a panel of 23 tumour cell lines

(A) A representative western blotting image of POR expression from a panel of 23 human tumour cell lines. POR and actin (3 min exposure) were imaged after incubation with SuperSignal West Pico Chemiluminescent substrate. POR mouse monoclonal (Santa Cruz, Sc-25263) 1:2500, Actin (Milipore, MAB1501R) 1:10000, secondary antibody (Santa Cruz Biotech, Sc-2055) 1:10000. POR: actin ratio was calculated by band densitometry using ImageJ. (B) POR activity as measured by cyanide inhibited NADPH-dependent cytochrome c reduction assay of S9 fractions of the cell lines. Errors are the standard error of the mean from three repeated measurements of the same biological sample.

Figure 3.2 Relationship between POR activity and bioreductive prodrug activation in a panel of human tumour cell lines

(A) PR-104A. (B) SN30000 or (C) [14C] EF5. PR-104A anoxic metabolism data was reported by (Guise et al., 2012) while SN30000 and [14C] EF5 binding were reported by (Wang et al., 2012). Cytochrome c assay data replotted from Figure 3.1.
3.4.2. POR copy number in tumour cell lines

Tumour cell lines often have high levels of genomic instability leading to aberrations in chromosome number (Lengauer et al., 1998), which result in changes to gene copy number. These amplification events might be expected to compromise the efficiency to which ZFN treatment will generate multi-allelic knockout clones. Therefore a database search was conducted on the SNP6.0 tumour sample databases from the Cancer Genome Project at the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/cgi-bin/genetics/CGP/cghviewer/CghHome.cgi) to assess copy number of POR genes in a panel of tumour cell lines where their capacity of metabolism of bioreductive prodrugs has been evaluated at the ACSRC (Table 3.1). This estimation was conducted by determining the total number of gene copies at the genomic locus of POR (chromosome 7q11.2, base pairs = 75,544,420 to 75,616,172). This analysis showed that POR copy number was highly variable in these tumour cell lines, ranging from two to six copies (Table 3.1).

From these lines, HCT116 was selected as an example with a diploid POR copy number; primarily because this cell line has favourable growth characteristics, has a moderate rate of bioreductive prodrug metabolism (Guise et al., 2012), has very low activity of the aerobic PR-104A reductase AKR1C3 (Guise et al., 2010), is readily transfectable, and is the parental line for a panel of widely studied cell lines that over-express specific candidate one-electron reductases including POR (Guise et al., 2012). SiHa was also selected for knockout of POR despite a high apparent copy number of four, because this cell line has a high rate of bioreductive prodrug activation under hypoxia e.g. PR-104A (Guise et al., 2012), SN30000 and EF5 (Wang et al., 2012) and nitroCBI (Jagdish Jaiswal and Frederik Pruijn, unpublished results). The POR copy number in these lines was estimated experimentally using relative quantitation by qPCR (Figure 3.3) and the copy number of POR relative to normal human leukocytes in HCT116 and SiHa was 2.39 ± 0.04 and 3.83 ± 0.51 respectively, which was broadly consistent with the values from the Cancer Genome Project.
Table 3.1 Panel of 23 tumour cell lines

*POR* copy number was evaluated using the Wellcome Trust Sanger Institute’s Cancer Genome Project database by comparing the region of the chromosome 7 where *POR* was located (Chromosome 7q11.2: 75,544,420-75,616,172 bp). In certain cell lines, multiple values are listed for the *POR* copy number as *POR* was located at the junction where multiple estimations of copy number were made. *POR*: actin ratio was calculated from one representative gel (Figure 3.1A). Cytochrome c reduction values are from Figure 3.1. NA represents cell lines that were not present in the Wellcome Trust Sanger Institute’s database. POR activity for 22Rv1 was not determined (ND).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>POR:actin ratio</th>
<th>NADPH-dependent cytochrome c reduction (nmol/min/mg ± SEM)</th>
<th><em>POR</em> copy number</th>
</tr>
</thead>
<tbody>
<tr>
<td>22Rv1</td>
<td>0.13</td>
<td>ND</td>
<td>2</td>
</tr>
<tr>
<td>A2780</td>
<td>0.11</td>
<td>11.9 ± 0.7</td>
<td>2</td>
</tr>
<tr>
<td>A431</td>
<td>0.09</td>
<td>8.15 ± 0.24</td>
<td>3</td>
</tr>
<tr>
<td>A549</td>
<td>0.10</td>
<td>9.73 ± 0.56</td>
<td>3</td>
</tr>
<tr>
<td>C-33 A</td>
<td>0.20</td>
<td>11.5 ± 0.4</td>
<td>NA</td>
</tr>
<tr>
<td>DU145</td>
<td>0.03</td>
<td>12.0 ± 0.5</td>
<td>3</td>
</tr>
<tr>
<td>FaDu</td>
<td>0.03</td>
<td>6.05 ± 0.64</td>
<td>3</td>
</tr>
<tr>
<td>H1299</td>
<td>0.07</td>
<td>16.3 ± 1.6</td>
<td>6</td>
</tr>
<tr>
<td>H460</td>
<td>0.10</td>
<td>19.0 ± 1.2</td>
<td>3</td>
</tr>
<tr>
<td>H522</td>
<td>0.12</td>
<td>6.19 ± 1.51</td>
<td>3</td>
</tr>
<tr>
<td>H69</td>
<td>0.07</td>
<td>13.6 ± 0.4</td>
<td>2</td>
</tr>
<tr>
<td>H82</td>
<td>0.07</td>
<td>7.29 ± 0.39</td>
<td>3</td>
</tr>
<tr>
<td>HCT116</td>
<td>0.05</td>
<td>20.6 ± 1.0</td>
<td>2</td>
</tr>
<tr>
<td>HCT8-Sa</td>
<td>0.29</td>
<td>18.0 ± 1.9</td>
<td>NA</td>
</tr>
<tr>
<td>Hep3B</td>
<td>0.25</td>
<td>8.46 ± 0.96</td>
<td>NA</td>
</tr>
<tr>
<td>HepG2</td>
<td>0.42</td>
<td>60.3 ± 2.1</td>
<td>NA</td>
</tr>
<tr>
<td>HT-29</td>
<td>0.18</td>
<td>10.9 ± 0.9</td>
<td>4</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>0.05</td>
<td>6.40 ± 1.01</td>
<td>3</td>
</tr>
<tr>
<td>MIA Paca-2</td>
<td>0.03</td>
<td>2.41 ± 0.65</td>
<td>3 or 4</td>
</tr>
<tr>
<td>Panc-1</td>
<td>0.12</td>
<td>22.6 ± 0.5</td>
<td>NA</td>
</tr>
<tr>
<td>PC-3</td>
<td>0.01</td>
<td>4.11 ± 1.53</td>
<td>5</td>
</tr>
<tr>
<td>SiHa</td>
<td>0.06</td>
<td>18.9 ± 0.6</td>
<td>4</td>
</tr>
<tr>
<td>SKOV-3</td>
<td>0.05</td>
<td>11.8 ± 1.0</td>
<td>2 or 3</td>
</tr>
</tbody>
</table>
3.4.3. Optimisation of transfection efficiency in HCT116 and SiHa

Gene knockout with CompoZr® ZFNs requires simultaneous expression from two plasmids and is considered by the manufacturer to require transfection efficiencies of >80%. Hence optimisation of the transfection protocols was essential to ensure sufficient ZFN activity in the cells. The first step of optimisation was to compare transfection efficiencies of three different transfection reagents; FuGENE®6, Lipofectamine LTX and JetPEI on HCT116 and SiHa cells using a plasmid encoding for humanised renilla GFP (F527-ires.hrGFP; Figure 2.1). Transfection efficiency was scored as the average of the GFP-positive cells divided by the cells in the brightfield image for each of the five squares outlined in Figure 3.4A. Lipofectamine LTX with Plus reagent was found to be the most effective transfection reagent for HCT116 cells with 30.5 ± 8.8% of cells expressing GFP (Figure 3.4A-C). Lipofectamine LTX without the Plus reagent and JetPEI were about half as efficient (13.5 ± 4.8% and 14.1 ± 2.6% respectively) while FuGENE®6 was much less effective (1.55 ± 0.87%). None of these reagents resulted in transfection efficiencies of >5% in SiHa cells (Figure 3.4C).

To determine whether the low percentage of GFP positive cells was due to poor expression of GFP rather than poor transfection efficiency, a comparison between GFP plasmids driven by two different viral promoters was made in HCT116 using the optimised transfection reagent (Lipofectamine LTX + Plus reagent). Transfection of HCT116 cells with the pEGFP.N1 (CMV promoter) plasmid gave a 2.4-2.5 fold higher transfection efficiency compared to F527-ires.hrGFP (EF1α promoter) at 250 ng and 500 ng of DNA respectively, with 500 ng of DNA resulting in slightly higher transfection efficiencies (83.1 ± 11.6%, Figure 3.5A and B). Hence the optimal transfection method for HCT116 was determined as 500 ng of DNA using Lipofectamine LTX with Plus reagent. There was no improvement...
between the plasmids in SiHa cells suggesting a low transfection efficiency rather than poor expression of GFP (data not shown), so alternative transfection methods were sought. The Amaxa nucleofector (an electroporation method) was trialled for SiHa cells and six different programs (A20, D23, L29, T20, T30 and X01) from kit V were tested. Nucleofector programme T30 demonstrated the best transfection efficiencies for pEGFP.N1 24 h after transfection with $79.1 \pm 6.6\%$ GFP-positive cells scored indicating a marked improvement over the transfection reagents described previously (Figure 3.6A and B). Programme T30 was utilised for subsequent ZFN transfection of SiHa cells.

Figure 3.4 Transfection of HCT116 and SiHa cells with three transfection reagents
Fluorescent images at 10x magnification of (A) HCT116 and (B) SiHa WT cells transfected with F527-ires.GFP using FuGENE®6, Lipofectamine LTX (± Plus reagent) and JetPEI. (C) Average transfection efficiency of the transfection reagents in HCT116 and SiHa cells was derived from five squares indicated in the top left fluorescent image. Errors are standard deviation of the mean of cell counts from five squares from one experiment.
Figure 3.5 GFP transfection of HCT116 using Lipofectamine LTX (A) Fluorescent images and (B) average transfection efficiencies for HCT116 transfected with F527-ires.hrGFP or pEGFP.N1 with three different amounts of DNA using Lipofectamine LTX + plus reagent. Images are at 10x magnification. Average transfection efficiency was calculated from the average of the five squares in Figure 3.4. Errors are standard deviation of the mean of cell counts from five squares from one experiment.
Figure 3.6 Amaxa nucleofection on SiHa cells

(A) Fluorescent images and (B) average transfection efficiency of SiHa cells transfected with pEGFP.N1 using Nucleofector solution V with six different programs recommended by the manufacturer (A20, D23, L29, T20, T30 and X01). Images are at 10x magnification. Average transfection efficiency was calculated from five squares indicated in Figure 3.4. Errors are standard deviation of the mean of cell counts from five squares from one experiment.
3.4.4. pZFN.POR transfection of HCT116 and screening of clones

HCT116 cells were transfected with pZFN.POR using the optimised transfection protocols described above. However to maximise the probability of cloning multi-allelic knockouts, co-transfection with a GFP plasmid with enrichment for transfectants by fluorescence activated cell sorting (FACS) was conducted, with the transfected cells sorted based on GFP fluorescence (Figure 3.7). The targeting sequence of POR was confirmed to be unique to the human POR gene by a basic local alignment search tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi, Figure 3.8A) and mutational analysis was conducted using the Surveyor™ nuclease assay with the POR-1 primer pair which amplifies a 665 bp region of the ZFN cut site. FACS enrichment after co–transfection with the GFP plasmid resulted in a positive Surveyor™ nuclease assay (lane 3) with characteristic 417 bp and 248 bp bands from cleavage of the 665 bp PCR product while a negative result was observed without prior FACS enrichment (Figure 3.8B).

To assess whether POR mutations are retained in the population (i.e. are non-lethal), the frequency of mutations was evaluated in ZFN-treated HCT116 pools at multiple times after transfection (Figure 3.9). This experiment was done after isolation of the POR knockout Hko3 (see Figure 3.10), which was used as a positive control. Hko3 has two different mutant alleles (see Table 3.2). Thus, 50 % of re-annealed PCR amplicons are expected to be heteroduplexes containing mismatch bubbles, resulting in strong 417 bp and 248 bp bands (Figure 3.9). In the pools, the ~417 bp and ~248 bp bands were strongest 24 h after FACS sorting but were still present up to 14 days (cells were sub-cultured to maintain culture in log phase during the experiment). These bands were not present in samples that had not undergone FACS sorting (data not shown), consistent with Figure 3.8B. This was also mirrored in another ZFN pair targeting ADGPK in HCT116 cells, which showed a similar pattern (reported in (Richter et al., 2013)), and suggests that the progressive loss of cells carrying ZFN-induced mutations reflects the compromised loss of viability due to high ZFN plasmid copy number in highly GFP-enriched cells rather than an effect of knockout of these genes.
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Figure 3.7 Example of FACS profiles 24 h after ZFN co-transfection with pEGFP.N1

(A) Outline of flow cytometer gate settings from which (B) cells with high GFP fluorescence (arrow) were sorted.

Figure 3.8 POR ZFN target site and Surveyor™ mutation assay

(A) POR-1 primer pair (bold) binding location in POR with the ZFN binding sites indicated in lower case and in red. The cleavage of the PCR amplicon from this primer pair (665 bp) using the Surveyor™ nuclease is predicted to generate 417 bp and 248 bp products. (B) Surveyor™ mutation assay on ZFN transfections with (right) and without (left) FACS enrichment showing the appearance of the characteristic bands as a result of ZFN activity. Control = DNA from K562 cells treated with POR ZFN (provided by Sigma-Aldrich). WT = HCT116 WT DNA, ZFN = HCT116 co-transfected with ZFN.
Additional transfections with pZFN.POR and pEGFP.N1 plasmids were conducted for isolation of POR knockout cells and the FACS-enriched cells were cloned by limiting dilution. Wells with a single clone were expanded and screened for POR expression by Western blotting. After two independent co-transfections of pZFN with pEGFP.N1 and enrichment of transfected HCT116 cells by FACS, 14 clones were isolated. Total cell lysates made from each clone were tested for POR expression by Western blotting and three clones were found to be negative for POR (Hko1, Hko2 and Hko3, Figure 3.10A). An HCT116 WT cell line stably transfected with F527.V5 plasmid to over-express POR (HCT116/POR) (Guise et al., 2012) was included as a positive control and this cell line demonstrated a large increase in POR expression compared to WT cells.

To determine whether the loss of POR protein in these cells also resulted in the loss of POR enzymatic activity, the cyanide-resistant NADPH-dependent cytochrome c reduction assay was conducted. HCT116 WT cells had a mean cytochrome c reduction activity of 20.9 ± 1.1 nmol/min/mg and over-expression of POR in HCT116 cells (HCT116/POR) resulted in a highly significant increase in the rate of cytochrome c reduction compared to WT (8.5 fold, p<0.001, Figure 3.10B). POR negative clones showed a significantly decreased rate of cytochrome c reduction with a 4.4 fold and 17 fold (p<0.001) reduction in HCT116 clones.
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Hko1 and Hko2 respectively. The loss of enzymatic activity in Hko3 was less marked than the other knockout clones but this decrease was still significant compared to WT (2.3 fold, p<0.001).

3.4.5. pZFN.POR transfection of SiHa and screening of clones

Transfection of SiHa with pZFN.POR and pEGFP.N1 resulted in the isolation of 46 clones after FACS enrichment for high GFP expressing cells. None of these clones were negative for POR when screened by Western blotting. However when probed using the more sensitive chemiluminescent substrate ECL advance, some of these clones had a decreased POR expression compared to WT (Figure 3.11A). Further characterisation of some of these candidate ‘partial’ knockout clones was conducted with the cyanide-inhibited NADPH-dependent cytochrome c assay, with all clones showing significant decreases in POR activity (Figure 3.11B). Clones S2 and S3 displayed the biggest loss of POR activity compared to WT.
cells (3.5 and 7 fold respectively, p<0.001) and therefore were chosen for re-transfection with the pZFN.POR and pEGFP.N1 plasmids.

From four secondary transfections followed by FACS enrichment, a total of 121 clones were isolated and screened for POR expression by Western blotting. Of the 56 clones derived from the candidate partial knockout S2, one (S2ko1) was found to be POR negative (Figure 3.12A). Of the 65 clones derived from the candidate partial knockout clone S3, two POR-negative clones (S3ko1 and S3ko2) were isolated (Figure 3.12B). Hence in total, two transfections in series were required to generate full multi-allelic knockout of POR in SiHa cells, (Figure 3.12C) compared to one transfection in HCT116. The POR over-expressing SiHa/POR line (Guise et al., 2007) was included as a positive control. Cyanide-resistant NADPH-dependent cytochrome c reductase activity was 8.5 fold higher for the SiHa/POR...
line than WT, while the candidate POR knockouts showed significant decreases in POR activity compared to WT (all \( p<0.001 \), Figure 3.12D).

![Figure 3.12 POR protein and enzyme activity in SiHa clones following primary and secondary transfections with ZFN and pEGFP.N1 plasmids](image)

Western blotting for POR expression in (A) S2ko1 and (B) S3ko1 and S3ko2 following re-transfection with ZFN of the primary clones S2 and S3 respectively. 30 µg of protein was loaded for each lane. POR was exposed for 10 s with ECL advance and actin was exposed for 5 min with SuperSignal West Pico chemiluminescent substrate. Primary and secondary antibodies are indicated in Figure 3.1. (C) The samples were re-run to enable direct comparison between the presumed partial knockouts from the first transfection (S2 and S3) and the candidate full knockouts from the second. (D) NADPH dependent cytochrome c assay using S9 fractions prepared from SiHa WT, full knockout clones and a POR over-expressing clone. Errors are the standard error of the mean from >3 repeated measurements of the same biological sample. ‡ \( p<0.001 \) compared to WT as measured by student’s t-test.

3.4.6. Genotyping of POR knockout clones at ZFN cut site

To confirm that loss of POR expression and activity in these clones is a result of ZFN-induced mutation at the target site, Sanger sequencing was undertaken to determine the nature of these mutations. The POR-1 primer pair, modified by attachment of flanking Gateway®
regions (Invitrogen; Section 2.10.4) was used to amplify a region of POR. This PCR product was then cloned into a Gateway® compatible vector which was transformed into E. coli. Multiple bacterial colonies, each carrying a fragment of POR amplified by the modified POR-1 primer pair (Figure 3.8A), were cloned and sequenced. Ten bacterial colonies were sequenced for the HCT116 POR null clones and 20 were sequenced for the SiHa POR null clones due to a higher POR copy number in SiHa (Figure 3.3 and Table 3.1). Sanger sequencing of Hko1 revealed two mutations, one resulting in a frameshift (Δ26 bp) and the other (Δ15 bp) corresponding to a 5 amino acid deletion (YMGEM, Table 3.2). Hko2 sequencing revealed a single frameshift mutation (4 bp insertion), while Hko3 contained a frameshift mutation (1 bp insertion) with a non-synonymous A>T substitution in the other allele resulting in an amino acid change (M263L). In SiHa POR knockout clones, frameshift mutations were found in both S3ko1 (Δ2 bp) and S3ko2 (Δ5 bp and Δ2 bp). Two mutations were found in both S3ko1 and S3ko2; a small (6 bp) deletion mutation corresponding to a deletion in two amino acids Y262 and M263 and the 2 bp deletion. This suggests that these alleles were present in the parental clone S3 as it is present in both knockout clones derived from this cell line. A 4th allele was not detected in any of the SiHa knockouts. However the increased frequency of PCR products sequenced carrying the same mutation (Table 3.2) inferred that duplication of certain alleles occurred during DNA double-strand break repair (discussed in Section 3.5).

The only sequence recovered for S2ko1 was a WT sequence, which was puzzling given the negative POR Western blot and significant decrease in POR activity compared to WT cells. To determine whether the mutations in S2ko1 resulted in the ablation of the binding site of one of the POR-1 primer pair, another primer pair (POR-2, Figure 3.13A) was used to capture the mutations. This primer pair amplifies a larger region of POR (1.9 kb) and therefore enables sequencing of larger mutations. The resulting analysis revealed that S2ko1 carried alleles with ~150 bp (Δ150 bp) and ~300 bp (Δ300 bp) deletions (Figure 3.13B). This analysis was carried out on all the other knockout clones and it subsequently revealed that Hko2 carried an allele with a ~300 bp insertion not detected with the POR-1 primers. Thus, this sequencing study revealed the presence of multi-allelic mutations caused by ZFN activity, which led to loss of POR expression and activity in these POR knockout clones.
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Table 3.2 Summary of sequencing results for the ZFN target site in HCT116 WT, SiHa WT and their respective POR knockout clones

Individual PCR fragments containing the ZFN cut site were amplified and inserted into an entry vector and transformed into competent bacteria using the Gateway® cloning system. Individual bacterial clones were selected using kanamycin. Underlined are the ZFN binding sites. Insertion mutations are indicated in bold and substitution mutations indicated in lower case.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Sequence at ZFN target Site</th>
<th>Prop(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT116 WT</td>
<td>CGACATAGATGCGGCCAAGGTGTACATGGGGAGATGGGCGGCTG</td>
<td>5/5</td>
</tr>
<tr>
<td>Hko1</td>
<td>CGACATAGATG……………………………………..GGCCGGCTG (Δ26 bp)</td>
<td>2/6</td>
</tr>
<tr>
<td></td>
<td>CGACATAGATGCGGCCAAGGT………………..GGCCGGCTG (Δ15 bp)</td>
<td>4/6</td>
</tr>
<tr>
<td>Hko2</td>
<td>CGACATAGATGCGGCCAAGGTGTACATACATGGGGGAGATGGGCGGCTG (4 bp ins) [~300-bp ins]</td>
<td>6/6</td>
</tr>
<tr>
<td></td>
<td>Hko3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CGACATAGATGCGGCCAAGGTGTACATGGGGAGATGGGCGGCTG (A→T, M263L)</td>
<td>6/8</td>
</tr>
<tr>
<td></td>
<td>CGACATAGATGCGGCCAAGGTGTACATGGGGAGATGGGCGGCTG (1 bp ins)</td>
<td>2/8</td>
</tr>
<tr>
<td>SiHa WT</td>
<td>CGACATAGATGCGGCCAAGGTGTACATGGGGAGATGGGCGGCTG</td>
<td>NA</td>
</tr>
<tr>
<td>S2ko1</td>
<td>CGACATAGATGCGGCCAAGGTGTACATGGGGAGATGGGCGGCTG (WT)</td>
<td>6/6</td>
</tr>
<tr>
<td></td>
<td>[~Δ150 bp, including ZFN target site] and [~Δ300 bp, including ZFN target site]</td>
<td></td>
</tr>
<tr>
<td>S3ko1</td>
<td>CGACATAGATGCGGCCAAGGTG…………………………GAGATGGGCGGCTG (Δ6 bp)</td>
<td>9/20</td>
</tr>
<tr>
<td></td>
<td>CGACATAGATGCGGCCAAGGTGTCATGGGGAGATGGGCGGCTG (Δ2 bp)</td>
<td>6/20</td>
</tr>
<tr>
<td></td>
<td>[Δ-81 bp (75,611,582-75,611,663), including ZFN target site]</td>
<td>5/20</td>
</tr>
<tr>
<td>S3ko2</td>
<td>CGACATAGATGCGGCCAAGGTG…………………………GGAGATGGGCGGCTG (Δ6 bp)</td>
<td>5/20</td>
</tr>
<tr>
<td></td>
<td>CGACATAGATGCGGCCAAGGTGTCATGGGGAGATGGGCGGCTG (Δ2 bp)</td>
<td>5/20</td>
</tr>
<tr>
<td></td>
<td>CGACATAGATGCGGCCAAGGTGTCATGGGGAGATGGGCGGCTG (Δ5 bp)</td>
<td>10/20</td>
</tr>
</tbody>
</table>
3.4.7. Transcript levels in POR knockout cells by RT-qPCR

The identification of a WT sequence at the ZFN target site of S2ko1 was surprising considering POR protein was not detected by Western blotting and POR activity was significantly decreased compared to WT cells. To determine whether the WT POR allele in S2ko1 is poorly transcribed (e.g. due to epigenetic silencing), cDNA from RNA isolated S2ko1 was analysed using a custom Taqman® probe by reverse transcription-qPCR (RT-qPCR, Figure 3.14A) and levels of POR transcript were compared against WT cells. A fluorogenic probe was attached to a primer sequence designed to bind to a 17 bp region within the ZFN target site of POR. This Taqman® assay, revealed amplification from all POR knockout clones except Hko1, which is consistent with the genomic sequence data in that the Taqman probe sequence is deleted in both alleles in Hko1 while all other clones have at least
one allele that retains this sequence (Figure 3.14B). For S2ko1, the threshold cycle was increased compared to WT (29.2 ± 1.0 vs 24.2 ± 1.0 respectively) indicating lower transcript abundance. Quantification of transcript levels relative to WT showed that POR RNA levels of clone S2ko1 were 3.2 ± 0.2 % of WT levels, suggesting a poorly expressing WT allele (Figure 3.14C). Expanding this analysis to the other POR knockout clones showed decreases in POR transcript to less than 5 % of WT transcript levels with the exception of Hko3 (22.1 ± 7.1 %). This was consistent with the Sanger sequencing of the ZFN cut site where some alleles that carry deletions (e.g. the Δ6 bp allele in S3ko2) removes a large segment of the Taqman® probe binding site thus severely disrupting probe binding.

Figure 3.14 RT-qPCR analysis of RNA transcripts from POR knockout clones
(A) Location of the Taqman® fluorescent probe binding site in exon 8 of POR. The amplicon containing the ZFN binding site (black line) was amplified by the forward and reverse Taqman® primers (blue). The Taqman® probe binding site is indicated in red. (B) Example of amplification plots from one experiment showing threshold cycles (green line) of HCT116 and SiHa cell lines. (C) Average POR transcript relative to WT HCT116 (top) and SiHa (bottom) cells. Errors are the standard error of the mean from ≥ 2 qPCR experiments run with RNA isolated from two separate cell cultures for each cell line.
3.4.8. Growth, morphology and mycoplasma treatment of POR KO clones

Mutations at the ZFN target site in the POR knockout clones resulted in the loss of POR expression and enzymatic activity in those cell lines. Since sensitivity to DNA-damaging cytotoxins from the activation of HAPs is primarily measured by proliferation assays, growth and morphological characteristics of these cell lines was determined to ensure that there were no adverse effects on cellular proliferation either from the loss of POR or from clonal variation. Morphologically, both HCT116 and SiHa POR knockout clones were identical to their parental WT cells (Figure 3.15) with similar growth kinetics as measured by growth curves (Figure 3.15C and D), except for Hko3 which grew slightly slower than HCT116 WT cells. When cell number increases were monitored over a series of passages (Table 3.3), the estimated doubling time of Hko3 was again significantly longer than HCT116 WT cells (p<0.001) confirming the slower growth rate in Figure 3.15C. The growth rate of the other POR knockout clones was not significantly different from the parental line in agreement with the growth curves. In addition, the mean cell diameter of POR knockout clones (measured by the Countess automated cell counter) was similar in size to WT cells (Table 3.3). Cell cycle analysis was undertaken for the POR knockout cell lines using propidium iodide staining of DNA and analysis by flow cytometry on sub-confluent cultures. The cell cycle distributions of HCT116 and SiHa POR knockout cells were similar to the parental WT cells with some exceptions (Figure 3.16 and Table 3.4). SiHa POR knockout clone S3ko2 had an atypical DNA content distribution and showed mixed ploidy compared to SiHa WT cell lines (Figure 3.16).

At a later date, it was found that SiHa clones S2ko1 and S3ko2 were mycoplasma-positive and were treated with Plasmocin™ as described in Methods (Section 2.12). Two weeks after treatment with Plasmocin™, the cells were negative for mycoplasma by PCR-ELISA. However after subsequent retesting of frozen stocks approximately four weeks after cessation of Plasmocin™, both these cell lines were found to be mycoplasma positive again. Therefore a new reagent, Plasmocure™, was used to treat these cultures and subsequent repeated retesting indicated that they remained negative for mycoplasma (data not shown). Unfortunately, the detection of mycoplasma in these cultures occurred after some experiments had been completed (e.g. IC_{50} assays, Chapter 5), the implications of which will be discussed in Chapter 5. However, all other experiments were done with mycoplasma negative cell lines unless stated otherwise.
Figure 3.15 Morphology and growth characteristics of POR knockout clones

Phase contrast photomicrographs of the knockout clones and their parental WT cells for (A) HCT116 and (B) SiHa. Growth curves for (C) HCT116 and (D) SiHa WT and POR knockout cells. Cells were seeded in 24-well plates at 50000 cells/well, harvested at five different time points and counts were made using a Coulter counter as outlined in the methods. Errors are the standard deviation of the mean from triplicate wells from one experiment.
Chapter 3 Generation and characterisation of POR knockout clones

Table 3.3 Doubling time and a cell size of WT and knockout clones

Average doubling time and average cell size were calculated based on cell counts using the Countess automated cell counter. Cells were taken from tissue culture flasks that were 60-70% confluent. Errors are the standard error of the mean from ≥4 cell passages. Statistical significance was measured by one-way ANOVA with Holm-Sidak post-hoc analysis compared to isogenic WT cells. NS = not significant (p>0.05).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Average doubling time (h)</th>
<th>SEM</th>
<th>p-value</th>
<th>Average cell diameter (µm)</th>
<th>SEM</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT116</td>
<td>16.4</td>
<td>0.1</td>
<td>-</td>
<td>12.6</td>
<td>0.4</td>
<td>-</td>
</tr>
<tr>
<td>Hko1</td>
<td>17.1</td>
<td>0.5</td>
<td>NS</td>
<td>13.3</td>
<td>0.7</td>
<td>NS</td>
</tr>
<tr>
<td>Hko2</td>
<td>17.1</td>
<td>0.5</td>
<td>NS</td>
<td>12.8</td>
<td>0.4</td>
<td>NS</td>
</tr>
<tr>
<td>Hko3</td>
<td>20.8</td>
<td>1.0</td>
<td>&lt;0.001</td>
<td>14.1</td>
<td>0.8</td>
<td>NS</td>
</tr>
<tr>
<td>SiHa</td>
<td>27.2</td>
<td>0.6</td>
<td>-</td>
<td>12.7</td>
<td>0.2</td>
<td>-</td>
</tr>
<tr>
<td>S2ko1</td>
<td>29.5</td>
<td>1.0</td>
<td>NS</td>
<td>12.9</td>
<td>0.3</td>
<td>NS</td>
</tr>
<tr>
<td>S3ko1</td>
<td>27.2</td>
<td>0.8</td>
<td>NS</td>
<td>11.6</td>
<td>0.2</td>
<td>NS</td>
</tr>
<tr>
<td>S3ko2</td>
<td>27.8</td>
<td>0.8</td>
<td>NS</td>
<td>13.1</td>
<td>0.2</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 3.4 Cell cycle analysis of HCT116 and SiHa WT and POR knockout clones

SiHa clone S3ko2 was excluded from this table as G1, S and G2 statistics cannot be calculated. Cell cycle statistics from one cell culture were calculated using Flowjo.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G1%</td>
</tr>
<tr>
<td>HCT116 WT</td>
<td>57.3</td>
</tr>
<tr>
<td>Hko1</td>
<td>37.4</td>
</tr>
<tr>
<td>Hko2</td>
<td>54.4</td>
</tr>
<tr>
<td>Hko3</td>
<td>47.5</td>
</tr>
<tr>
<td>SiHa WT</td>
<td>55.3</td>
</tr>
<tr>
<td>S2</td>
<td>63.8</td>
</tr>
<tr>
<td>S3</td>
<td>53.5</td>
</tr>
<tr>
<td>S2ko1</td>
<td>62.3</td>
</tr>
<tr>
<td>S3ko1</td>
<td>65.8</td>
</tr>
</tbody>
</table>
Figure 3.16 Cell cycle analysis of HCT116 and SiHa WT and POR knockout clones

Representative cell cycle distributions from one sub-confluent tissue culture flask. 10^6 cells were fixed with 70 % ethanol, stained with propidium iodide and analysed by flow cytometry. Cell cycle distributions were plotted using Flowjo.
3.4.9. POR re-expression in POR knockout cells

The final step in the development of the cell line models was to generate isogenic cell lines that express POR in the knockout background. POR knockout clones were stably transfected with the F527.V5 plasmid (Figure 2.1), the same plasmid used in the generation of the HCT116/POR (Figure 3.10) and SiHa/POR (Figure 3.12) lines from WT pools. The same transfection method was used as described previously (Section 2.8). POR expression and activity was restored to the Hko1 and S3ko2 POR knockout lines and their POR expression was enhanced to levels similar to WT cells over-expressing POR (Hko1/POR = 6.1 fold relative to WT, S3ko2/POR = 11.0 fold: Figure 3.17A and B; HCT116/POR = 8.5 fold: Figure 3.10; SiHa/POR = 8.5 fold: Figure 3.12). Surprisingly, Hko2/POR did not express POR protein or enzyme activity. S2ko1/POR showed a significant increase in POR, but to lower levels than SiHa/POR. Interestingly, although the POR expression and activity in these knockout-derived cell lines was lower than the WT-derived over-expressing cell lines, they were still resistant to puromycin which implied that they were expressing POR from the F527.V5 plasmid.
Figure 3.17 POR expression and activity in POR knockout clones stably transfected with F527.V5 plasmid

Western blotting and cytochrome c assay of POR over-expressing cells derived from (A) HCT116 and (B) SiHa POR knockout cells. 20 µg and 30 µg of total protein lysate were loaded for HCT116 and SiHa gel respectively. Primary and secondary antibodies are indicated in Figure 3.1. The cytochrome c assay was conducted with cellular S9 fractions. Errors are the standard error of the mean from >3 repeated measurements of the same biological sample. ‡ p<0.001 compared to WT as measured by Student’s t-test.
Chapter 3 Generation and characterisation of POR knockout clones

3.5. Discussion

This chapter describes the generation and characterisation of POR knockout cell lines using ZFNs. The generation of POR knockout cell lines required optimisation of transfection efficiency to maximise the probability of expressing both members of the pair of ZFN plasmids at levels sufficient to mutate multiple alleles of the target gene. The inclusion of a co-transfected GFP plasmid (pEGFP.N1) allowed for the enrichment of transfected cells by FACS, further increasing the frequency of cloning full knockout clones. This was reflected in the absence of surveyor nuclease activity without FACS enrichment (Figure 3.8B), where the frequency of cells in the transfected population carrying ZFN-modified POR alleles was lower than the detection limit of the assay (considered to be approximately 1 in 32 heteroduplexes to homoduplexes) (Qiu et al., 2004).

The transient expression of ZFN in HCT116 and SiHa resulted in the loss of detectable POR expression and marked reduction in cytochrome c reductase activity (Figure 3.10 and Figure 3.12) in the full knockout clones. ZFN activity in these clones was expected to create double strand breaks at the target site which can then be acted on by cellular DNA repair mechanisms (Urnov et al., 2010). Error-prone DNA repair by NHEJ was evident in the variety of mutations sequenced that consisted of mostly small insertion/deletion events (Table 3.2). This was consistent with the observation that NHEJ repair of double strand breaks favours the introduction of small deletions (< 60-bp) at the site of lesion (Honma et al., 2007) and with previous ZFN-dependent gene modification studies (Morton et al., 2006; Santiago et al., 2008; Cost et al., 2010).

The loss of cells carrying mutations at the ZFN target site over time (Figure 3.9B) might suggest that ablation of POR activity results in a growth disadvantage, leading to dilution of mutants from the pool of transfected cells. However isolated clones from this pool of transfected cells had no growth or morphological defects compared to WT cells. In addition, most of the mutations detected with the Surveyor™ assay in the transfected pools were expected to be mono-allelic, and loss of one copy of POR is unlikely to elicit a haploinsufficiency phenotype. Hence a more probable explanation for the dilution of ZFN mutations is increased toxicity due to high ZFN activity in the FACS enriched cells with high plasmid copy number (Beumer et al., 2006; Pruett-Miller et al., 2008; Pruett-Miller et al., 2009; Pattanayak et al., 2011), leading to the loss of these mutants from the population. The transient incubation of ZFN transfected cells at 30 °C has been demonstrated to be an alternative method for increasing mutation efficiency (Doyon et al., 2010), which may be a
useful alternative to FACS enrichment. However this finding was published after the generation of the POR knockout clones and this hypothermic method was not evaluated in this thesis.

The total number of sequenced mutations for most of the clones (e.g. S2ko1) was found to be less than the POR copy number in those cell lines (Figure 3.3 and Table 3.1). Although the low number of bacterial clones sequenced means that there could be some statistical fluctuations, the results strongly suggest gene conversion is occurring. ZFN-induced mutations can be repaired via homology-directed repair (HDR) with a pre-existing allele (whether it is a WT or a mutant allele) as a template (Liu et al., 2010; Hauschild et al., 2011). The combination of NHEJ and HDR repair pathways can therefore plausibly generate the genotypes of clones S2 and S2ko1 as shown in Figure 3.18. In this model the first ZFN transfection in SiHa resulted in double-strand breaks in two alleles which were repaired by NHEJ, generating the Δ150 bp and Δ300 bp alleles. The second ZFN transfection results in a double-strand break in a third allele that is repaired by HDR with one of the mutated alleles as a template. This allele was inferred to be the Δ300 bp allele for S2ko1, due to a higher band intensity compared to the other alleles (Figure 3.13). Similarly, genotypes for clone S3 and the derived full knockout clones S3ko1 and S3ko2 can also be predicted using the sequencing data. DNA double strand breaks from the first transfection with ZFN were repaired by NHEJ repair resulting in the formation of the Δ2 bp and Δ6 bp alleles in the S3 clone (Figure 3.19A). After the second ZFN transfection, ZFN-induced double-strand breaks on one of the WT alleles of S3ko1 were repaired by HDR using the Δ6 bp allele as a template, while the other allele was repaired by NHEJ resulting in the formation of the Δ81 bp allele (Figure 3.19B). In S3ko2, a mutant allele (Δ5 bp allele) was created via NHEJ repair and was subsequently duplicated by HDR (Figure 3.19C) using the Δ5 bp allele as a repair template.
Figure 3.18 Model of the formation of POR alleles in S2 and S2ko1 after two serial transfections with ZFN.

The model describes the formation of the Δ150 bp and Δ300 bp alleles after transfection with ZFN. NHEJ repair (red lines) and HDR repair (blue lines) on DNA double strand breaks (lightning bolt) are both involved to repair the DNA lesions. The repair of the double strand breaks by NHEJ leads to the generation of Δ150 bp and Δ300 bp after the first transfection step. The duplication of the Δ300 bp allele occurs during the second transfection step, where the double strand breaks are repaired by HDR using the existing Δ300 bp allele as a template.
Chapter 3 Generation and characterisation of POR knockout clones

Figure 3.19 Model of the formation of POR alleles in S3 and derived POR knockout clones

(A) Model for the formation of mutations in clone S3 after transfection of SiHa WT cells with ZFN. Both NHEJ and HDR repair are predicted to give rise to the Δ2 bp and Δ6 bp alleles. (B) The formation of S3ko1 alleles from S3 required HDR repair for the duplication of the Δ6 bp allele and NHEJ for the formation of the Δ81 bp allele. (C) The formation of the Δ5 bp allele required NHEJ repair of the DNA double strand break. This was subsequently utilised as a template for HDR repair for another allele resulting the duplication of this allele in S3ko2.
The frequency of clones in HCT116 that were POR-negative by Western blotting (3/14 or ~0.21) was higher than in SiHa, which gave no null clones after the first transfection and 3/121 (0.024) after a second ZFN transfection. This could be due to the higher POR copy number in SiHa, since the probability of knockout events in four copies of SiHa is smaller compared to two copies in HCT116. This observation has been demonstrated in previous studies showing a decreasing frequency of generating full knockout clones with increasing copy number; the isolation of 11/53 clones defective for functional BAX in CHO (haploid gene), 2/552 clones defective for BAK (diploid) (Cost et al., 2010) and 0/225 clones for full CCR5 knockout (with one biallelic mutant) in HEK293 cells (triploid) (Kim et al., 2009). The models for the genotype of the SiHa clones S2 and S3 described above (Figure 3.18 and Figure 3.19) estimate that two alleles were altered per ZFN transfection, necessitating the need for two serial transfections for SiHa (four POR alleles). However, this estimate can be complicated by the contribution of the competing HDR pathways as noted above. Other factors, including differences in chromatin structure or epigenetic modification in that locus and differences in efficacy of a particular ZFN can also influence the activity of ZFN in certain cell lines (Maeder et al., 2008; Pattanayak et al., 2011). These factors lead to observed differences in the frequency of multi-allelic gene disruptions for different genes in the same cell line, with an example of studies with CHO cells showing biallelic disruption in 2/552 clones screened for BAK (Cost et al., 2010), 2/68 for DHFR (Santiago et al., 2008) and 10/54 for GS, 4/200 for DHFR and 4/200 for FUT8 (frequency of DHFR and FUT8 biallelic knockouts were in the context of a GS knockout and GS-DHFR double knockout cell lines respectively) (Liu et al., 2010).

The sequencing study for the POR knockout clones provided valuable information about the mutations at the ZFN target site and allows for the prediction of POR protein fragments resulting from the generation of premature termination codons (TAG, TAA or TGA; Figure 3.20). Transcripts containing frameshift mutations that lead to premature termination codons are degraded by nonsense-mediated decay (Holbrook et al., 2004). Assuming these truncated protein fragments of POR are not degraded, they will be also catalytically inactive, since mutations that disrupt the FAD binding domain results in a >98% reduction of catalytic activity of POR (Kurzban & Strobel, 1986; Marohnic et al., 2006). However four POR knockout clones did not carry allelic mutations resulting in nonsense mediated decay of POR, with Hko2 carrying a Δ15 bp leading to a 5 amino acid (YMGEM) deletion, Hko3 carrying an allele with a non-synonymous A>T substitution leading to a M263L, and S3ko1 and S3ko2 carrying an allele with a Δ6 bp leading to ΔY262 and ΔM263.
These clones were negative for POR expression by Western blotting and demonstrated a decreased cytochrome c reductase activity compared to WT cells, suggesting that M263 is an important residue for the POR antibody epitope and/or stability of the protein. A mutation in M263 (an M263V mutation) was previously reported to seriously impair POR activity (decreased to 9% of WT levels) as measured by CYP17A1 activity, but this mutation did not severely affect cytochrome c reduction, with a measured decrease to 76% of WT levels (Huang et al., 2005). Therefore the high residual POR activity measured in Hko3 could be due to the residual cytochrome c reductase activity of POR from M263L allele given that leucine and valine are similar in structure. Nevertheless, even after taking these mutations into account, the POR knockout clones still demonstrated residual NADPH-dependent and cyanide-inhibited reduction of cytochrome c (at levels of ~3 nmol/min/mg). Therefore this suggests that the cytochrome c assay is not completely POR specific; consistent with studies showing the activity of other flavoproteins such as MTRR (Olteanu & Banerjee, 2001), NOS2 (Newton et al., 1998) and NDOR1 (Paine et al., 2000) in this assay.

The sequencing study also detected a WT allele from SiHa clone S2ko1. If the transcripts from the other alleles in S2ko1 (Δ150 bp and Δ300 bp) are degraded by nonsense mediated decay, transcript abundance measured from S2ko1 should be ~25% assuming the four WT alleles in SiHa cells are equivalent. However the RT-qPCR assay of POR transcript levels showed that S2ko1 POR transcripts were 3.2 ± 0.2 % of that in WT cells, much lower than the expected value (Figure 3.14). This suggests that this allele could be either epigenetically silenced or be an incomplete sequence arising during the amplification event(s) that generated the additional two copies of POR. Expanding this assay to the other knockout clones also showed reduced levels of POR transcripts compared to WT cells, which is broadly consistent to what would be expected from nonsense mediated decay of mRNA from the mutated alleles.

Cell cycle analysis of the POR knockout cell lines showed an atypical result for S3ko2 where two populations of cells that differ in total DNA content were found (Figure 3.16). The shift in G1 peak in this second population of cells infers that the population is pseudodiploid compared to the hypertriploid state of WT SiHa cells (Friedl et al., 1970; Szuhai et al., 2000). The total DNA content of S3ko2 can only be compared to WT SiHa cells since the cytometer settings were calibrated to each WT G1 peak (i.e. 402 V for HCT116 and 386 V for SiHa). The pseudodiploid population of S3ko2 could have arisen during the prolonged culture period (4-5 weeks) required to eliminate the mycoplasma contamination, as the parental cell line S3 and S3ko2/POR (both of which were generated before mycoplasma
testing) showed total DNA content similar to WT cells. One possibility for the origin of the pseudodiploid population was an unequal cell division, leading to two distinct populations of cells with different chromosome numbers (Neumuller & Knoblich, 2009).

The generation and characterisation of POR knockout cell lines outlined in this chapter will be a valuable tool for evaluating the role of POR in bioreductive prodrug activation. All POR knockout clones displayed lower POR expression and activity compared to WT cells but some clones (Hko3 and S3ko2) displayed phenotypic differences in terms of growth and chromosome number respectively. In addition, the generation of isogenic POR over-expressing cell lines from the knockout cell lines provides an additional point of comparison for the role of POR in the activation of bioreductive prodrugs (Chapter 5). Further characterisation of the reductase activity in these knockout clones will be carried out using the fluorogenic probe outlined in Chapter 4. From these characterisations, two clones were selected from HCT116 and SiHa cell lines for detailed investigation of bioreductive prodrug cytotoxicity and metabolic activation in Chapter 5.
Figure 3.20 POR protein domain structure and predicted protein fragments expressed in POR knockout cells

(A) Redrawn from Figure 1.14. ZFN target site is located between the flavodoxin-like and FAD binding ferrodoxin reductase-type domains. The target site consists of amino acids 255-268 corresponding to amino acid sequence IDAAKVYMGEMGR. (B) Predicted protein fragments of the POR knockout clones. The predicted mRNA sequence from the POR knockout clones were translated to an amino acid sequence using an online sequence converter (http://bioinformatics.picr.man.ac.uk/research/software/tools/sequenceconverter.html)
Chapter 4 Characterisation of FSL-61: A fluorogenic probe for one-electron reductase activity

Content from this Chapter is reported in Su, J., Guise, C.P., & Wilson, W.R. (2013) FSL-61 is a 6-nitroquinolone fluorogenic probe for one-electron reductases in hypoxic cells. Biochem J, 452, 79-86.

4.1. Summary

Mutations in the POR gene abolishing detectable expression of the protein did not appear to completely abolish NADPH-dependent, cyanide-resistant cytochrome c reductase activity in post-mitochondrial (S9) supernatants from the POR knockout cells (Chapter 3). This residual reductase activity varied between knockout clones. To characterise one-electron reductase activity further in these cells, a fluorogenic probe suitable for detecting one-electron reduction in individual intact cells was sought. Previously reported probes tend to have only modest selectivity for hypoxia, and the reductases responsible for their activation (reductase profiles) are poorly understood. This Chapter describes the characterisation of a 6-nitroquinolone ester (FSL-61) as a fluorogenic probe for POR and other one-electron reductases under hypoxia, and demonstrates its suitability for monitoring POR by flow cytometry. Reduction of FSL-61 by purified human recombinant POR generated the corresponding hydroxylamine, which was reduced further to the much more intensely fluorescent amine in cells. Hydrolysis of the ester side-chain facilitated cellular entrapment, which enabled its use to detect heterogeneous POR expression in mixed populations of cells. In addition to POR, forced expression of three other diflavin reductases (MTRR, NDOR1 and NOS2A) and of FDXR in HCT116 cells significantly increased hypoxic activation of FSL-61. The activation of FSL-61 to its fluorescent product correlated with the metabolism of PR-104A under hypoxia in a panel of 22 human tumour cell lines ($R^2 = 0.259$; $p = 0.016$). The activation of FSL-61 in the POR knockout clones was significantly reduced compared to WT cells, confirming the POR knockout status of these cells. Thus, this study demonstrates the utility of FSL-61 for rapid and non-destructive interrogation of the activity of one-electron reductases in hypoxic cells at the single cell level.
Chapter 4 Characterisation of FSL-61: A fluorogenic probe for one-electron reductase activity

4.2. Introduction

Tumour hypoxia has emerged as an important target for cancer therapy. As a consequence, the development in techniques for detecting hypoxia in tumours is of ongoing interest (Tatum et al., 2006; Vaupel et al., 2007; Wilson & Hay, 2011). The latter include the use of antibodies for identification of protein adducts formed by nitroimidazoles (Raleigh et al., 1987; Lord et al., 1993; Koch et al., 1995), monitoring HIF-1 markers such as CAIX (Wykoff et al., 2000; Dubois et al., 2009; Hoogsteen et al., 2009), fluorinated nitroimidazoles such as $^{18}$F EF5 for PET imaging (Komar et al., 2008) and fluorogenic probes that generate fluorescent metabolites via enzymatic reduction.

The fluorogenic probe approach has been most thoroughly studied for nitroaromatic compounds in which the fluorescence-quenching electron-withdrawing nitro group can be reduced by one-electron reductases, via a nitro radical, to an electron-donating hydroxylamine or amine with high fluorescence quantum yield. This nitro radical intermediate is re-oxidised to the parent compound by oxygen to suppress fluorescence in oxic cells (Wardman et al., 1984; Stratford et al., 1984; Rajapakse & Gates, 2012). Using this strategy, a number of fluorescent compounds activated by nitroreduction have been reported in the literature as hypoxia probes such as nitrofurans (Olive & Durand, 1983), nitroacridines and nitronaphthalimides (Begg et al., 1985; Hodgkiss et al., 1991) and nitrobenzyls (Cui et al., 2011a). Notably, this same biochemistry is the basis for the hypoxia-selective cytotoxicity of nitroaromatic prodrugs, including agents currently in clinical trials such as the PR-104A (Patterson et al., 2007; Guise et al., 2012) and TH-302 (Meng et al., 2012; Sun et al., 2012). Fluorescent probes other than nitroaromatic compounds have also been reported, such as the azobenzene probe QCys (Kiyose et al., 2010), indolequinone conjugated to coumarin (Tanabe et al., 2008) and aliphatic N-oxides of naphthalimides (Yin et al., 2011).

The utility of bioreductive prodrugs for targeting hypoxia depends not only on the presence of hypoxia, but also the activity of one-electron reductases in those cells. It is reasonable to expect that a close chemical and mechanistic match between a fluorogenic probe and its cognate bioreductive prodrug will make it more likely that both are activated by the same reductases, and thus that the probe can interrogate reductase activity as well as hypoxia. However, the one-electron reductases in human tumour cells are poorly characterised and a wide range of reductases such POR, MTRR, NOS isoforms or xanthine oxidase (reviewed in Chapter 1) are capable of catalysing the one-electron reduction of nitro
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compounds. In addition, there is little information on the contribution these or other enzymes make, at physiological levels of expression, to bioreductive prodrug activation in tumours.

In the previous Chapter, ZFN-induced mutations in POR resulted in the ablation of POR expression. However, these cell lines are still capable of catalysing NADPH-dependent cytochrome c reduction, with residual activity varying between cell lines. Therefore to further characterise one-electron reductase activity in these cell lines, a fluorogenic probe for one-electron reduction suitable for detecting activity in individual intact cells was sought. This Chapter outlines the identification and characterisation of a novel fluorogenic probe for the one-electron reductase activity in hypoxic cells. This probe was selected from a library of nitroaromatic compounds (fluorescent substrate library, FSL), which was previously screened under oxic and hypoxic conditions using MDA-MB-231 WT cells (low expression of POR) (Patterson et al., 1995; Guise et al., 2012) and a stable transfectant over-expressing POR [Figure 4.1, work conducted by Dr Dean Singleton (Singleton, 2009)].

Figure 4.1 FSL compound screen

A) MDA-MB-231 WT or (B) POR over-expressing (MDA-MB-231/POR) cells was incubated with a library of fluorogenic compounds for 2 h under oxic or anoxic conditions. The fluorescence of these compounds were measured using optimised Ex/Em settings (Singleton, 2009).
Table 4.1 FSL compounds evaluated in this thesis and their corresponding amines

The structures of the FSL compounds with their excitation and emission wavelength maxima as determined by Dr Dean Singleton (Singleton, 2009).

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<th>Structure</th>
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<th>Em max (nm)</th>
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Chapter 4 Characterisation of FSL-61: A fluorogenic probe for one-electron reductase activity

4.3. Aims of this Chapter

The aims of this Chapter are:

1. To identify a fluorogenic probe suitable for detecting one-electron reductase activity in individual living cells.
2. To characterise the metabolic pathway of activation for the preferred compound (FSL-61), including identification of its fluorescent metabolites.
3. To assess the reductase profile of FSL-61.
4. To determine the relationship between FSL-61 and HAP (e.g. PR-104A) activation.
5. To utilise FSL-61 to evaluate one-electron reductase activity in POR knockout cell lines isolated in Chapter 3.

4.4. Results

4.4.1. FSL-61 as a candidate fluorogenic probe for flow cytometry assay

Eight FSL compounds (FSL-41, FSL-59, FSL-61, FSL-67, FSL-76, FSL-95, FSL-111 and FSL-141) with the highest hypoxic selectivity and greatest differential activation in the POR over-expressing line were identified through a screen in MDA-MB-231 cells (Figure 4.1). The structures and optimised excitation/emission maxima for these eight compounds are indicated in Table 4.1. These compounds were selected for further evaluation in HCT116 and HCT116/POR over-expressing cells, demonstrating an increase in anoxia-dependent fluorescence after a plate-reader assay screen (Figure 4.2A and B). Four compounds (6-nitroquinolones FSL-41 and FSL-61, 2-nitroacridone FSL-76 and 6-nitroquinoxaline FSL-95) were found to have the highest anoxic-selectivity and greatest differential activation in the POR over-expressing cells. FSL-59 and FSL-111, although meeting these criteria, were not investigated further as they are close structural analogues of FSL-41 and FSL-61. The corresponding amines for FSL-41, FSL-76 and FSL-95 (FSL-70, FSL-158 and FSL-96 respectively, Table 4.1) were diluted to 100 µM in PBS and the excitation and emission spectra was measured to enable selection of flow cytometry lasers and filters. Chemical reduction of FSL-61 by zinc dust reduction in acetonitrile under anoxia was conducted as the amine for FSL-61 was not available. FSL-70, FSL-96 and the zinc dust reduction mixture of FSL-61 had similar excitation (Figure 4.3) and emission (Figure 4.4) spectra with an Ex/Em maxima of 360/460 nm, while FSL-158 had a longer Ex/Em maxima of 405/540 nm. Hence a
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355 nm laser was chosen for the flow cytometry study and for the three compounds with the same emission spectrum; detection by flow cytometry was done in their emission range 425-475 nm. FSL-158 was detected in the range of 515-545 nm.

Figure 4.2 The activation of fluorogenic probes in HCT116 and HCT116/POR cells. (A) HCT116 and (B) HCT116/POR cells were seeded into a 96-well plate at 50000 cells/well and 50 µM of compound was added for 2 h under oxic and anoxic conditions. The plates were analysed using excitation and emission maxima as indicated in Table 4.1. Errors are the standard error of the mean from ≥3 independent experiments.
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Excitation spectra FSL compounds chosen for the flow cytometry study

Excitation spectra of (A) FSL-70 (B) chemically reduced FSL-61 (C) FSL-158 and (D) FSL-96. The compounds were diluted to 100 µM in PBS and an excitation scan was conducted for each of the compounds at emission maxima indicated in Table 4.1.
The suitability of these compounds for flow cytometry was then tested using HCT116/POR cells. Cells were seeded in non-tissue culture treated plates to avoid the need for trypsinisation, which might affect cellular entrapment of the reduced fluorogenic probes. After exposure to the probes at 50 µM for 2 h, FSL-95 gave the highest median fluorescence intensity in anoxic HCT116/POR cells but this was incompletely inhibited by oxygen, with only a 3.5 fold difference compared to oxic conditions (Figure 4.5A). FSL-76 showed an even weaker median fluorescence differential for anoxic cells (1.16 fold), while FSL-41 gave little signal in anoxic cells. The best balance of anoxic signal intensity and anoxic selectivity was achieved with FSL-61, which showed no detectable fluorescence under oxic conditions. FSL-61 activation was POR-dependent in these cells, with similar median fluorescence intensity in parental HCT116 cells at 300 µM and HCT116/POR at 33 µM (Figure 4.5B).
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marked anoxic selectivity of FSL-61 in HCT116/POR cells was confirmed over a wider range of concentrations, with a linear FSL-61 concentration dependence at ≤300 µM (Figure 4.5C). The linear fit over-predicted fluorescence at 1000 µM suggesting possible saturation kinetics at very high FSL-61 concentrations. By confocal microscopy, fluorescence was highly selective for anoxia and appeared to be cytosolic with no evidence of subcellular localisation (Figure 4.5D). These characteristics identified FSL-61 as the preferred fluorogenic probe for this study.

4.4.1.1. Optimising the flow cytometry assay

In the pilot assays, cells were harvested and centrifuged to wash away excess FSL-61 in the medium after 3 h of FSL-61 incubation. To determine whether this additional step led to the leakage of fluorescent metabolites, FSL-61 treated cells were analysed by flow cytometry between successive centrifugation wash steps in ice-cold medium. Cellular fluorescence was stably retained after these wash steps indicating no leakage of fluorescent metabolites (Figure 4.6A). In addition, re-suspension in ice-cold PBS or culture media after washing had no effect on sample fluorescence (Figure 4.6B). However, since the flow cytometer gating ensures that intracellular fluorescence of individual cells was measured, the centrifugation wash steps were redundant and were subsequently removed. The short-term storage of the samples in the dark on ice was essential to maintain intracellular fluorescence of the compounds (Figure 4.6C). Also, fluorescence intensity was unchanged when samples were held in ice-cold medium for up to 2 h before flow cytometry analysis (Figure 4.6D).

To provide information about the cellular heterogeneity of reductase expression, an important question is whether the fluorescent metabolite(s) can be sufficiently well retained in cells during FSL-61 exposure at 37 °C. Exposure of mixed populations of HCT116/POR and parental HCT116 cells to FSL-61 demonstrated that the low and high fluorescence sub-populations could be readily distinguished, even after an incubation time of 3 h, with the proportion of the two peaks in good agreement with the input proportions of WT and POR cells (Figure 4.7). However, an increase in the median fluorescence intensity of the low-reductase population was evident in the presence of an excess of POR over-expressing cells indicating that some metabolite transfer does occur under these conditions.
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Figure 4.5 Flow cytometric detection of hypoxia- and POR-dependent activation of fluorogenic probes
(A) Median fluorescent intensity of POR over-expressing HCT116 cells (2 x 10^6 cells/mL) incubated with compounds at 50 µM for 2 h under oxic and anoxic conditions. Excitation was at 355 nm, and emission detected at 425-475 nm (FSL-41, FSL-61 and FSL-95) or 515-545 nm (FSL-76). Single cells were gated using a 488 nm laser for the forward-scatter and side-scatter signals. Median fluorescence was calculated from ~30000 single-cell events from one experiment. (B) Comparison of WT and POR over-expressing HCT116 cells incubated with 33.3 µM and 300 µM FSL-61 for 3 h under anoxic conditions. (C) Median fluorescence of HCT116/POR cells incubated with FSL-61 at a range of concentrations under oxic and anoxic conditions for 3 h. Errors are the range from duplicate wells from 1 experiment (D) Confocal images of HCT116/POR cells in brightfield (left) or fluorescence (right) mode (excitation with the 351 and 364 nm lines of an Argon laser, emission 400-550 nm) after incubation with FSL-61 at 300 µM for 3 h and washing with fresh medium.
Figure 4.6 Optimisation of flow cytometry method

Fluorescence distribution histograms showing $10^6$ HCT116/POR cells treated with 300 µM FSL-61 for 3 h under anoxia before analysis by flow cytometry. (A) The samples were harvested after FSL-61 incubation and analysed immediately on the flow cytometer (no wash). The same sample was washed by centrifugation once (wash 1) and twice (wash 2) before re-suspension in cold phenol red-free αMEM + 5 % FBS and analysed by flow cytometry. ‘Untreated’ samples are FSL-61-free samples. (B) Comparison of samples that were re-suspended in ice cold PBS or culture media after the centrifugation wash step. (C) Comparison of samples kept in the dark at room temperature and on ice after FSL-61 incubation. (D) Samples were harvested and analysed immediately on the flow cytometer (0 h). The same sample was kept in the dark on ice for 1 h and 2 h before subsequent analysis.
To determine whether fluorescence can be retained in cells for a prolonged period of time, FSL-61 treated cells were stored overnight at 4 °C in the dark. Another sample of cells was fixed with 4 % paraformaldehyde (PFA) for 1 h on ice. Fixation of cells with 4 % PFA changed the cell scatter properties but these were stable with time, while forward scatter decreased in unfixed cells during storage (Figure 4.8). Cellular fixation also changed cellular auto-fluorescence, which was compensated by increasing laser voltage from 216 V to 259 V (Figure 4.9A) leading to a corresponding increase in the fluorescence of fixed cells treated with FSL-61 after 24 h (Figure 4.9B). Fixation enabled retention of cellular fluorescence at 24 ± 4 % and 25 ± 2 % of 1 h samples for the 24 h and 1 week samples respectively (Figure 4.9C). In contrast cells that were not fixed demonstrated fluorescence levels of 3.2 ± 0.1 % and 3.0 ± 0.1 % of 1 h samples after 24 h and 1 week respectively (Figure 4.9D). This decrease in fluorescence was most likely due to changes in cell scatter associated with cell death (Figure 4.8). Therefore, maximal FSL-61 fluorescence was measured in cells analysed within 2 h of FSL-61 incubation and prolonged incubation (>2 h post-FSL-61 incubation) results in a >75 % decrease of cellular FSL-61 fluorescence, irrespective of whether the cells were fixed or not.
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Figure 4.8 Cell scatter properties of cells in media and fixed with 4% paraformaldehyde

Representative cell scatter properties from one experiment where HCT116/POR cells were analysed on the flow cytometer within 1 h and 24 h post-FSL-61 incubation (300 µM, 3 h anoxic conditions). The percentage of cells that fall within the gating for live cells are noted next to the gates.
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Figure 4.9 Cellular fixation of FSL-61 treated cells

Representative fluorescence distribution histograms showing $10^6$ HCT116/POR cells treated with 300 µM FSL-61 for 3 h under anoxia before analysis by flow cytometry. (A) Changes in FSL-61 fluorescence after fixation with 4% PFA on ice. Laser voltage was changed to 259 V to restore cellular auto-fluorescence to levels of ‘unfixed cells’ at 216 V. (B) Differences between cellular fluorescence after 24 h post-FSL-61 treatment analysed at 216 V and 259 V. (C) Cells were fixed with 4% PFA and analysed by flow cytometry at 259 V after 1 h. The cells were then stored in the dark at 4 °C before re-analysis at 24 h and 1 week. (D) Cells were kept in αMEM + 5% FCS and re-analysed after 24 h and 1 week at 216 V (as in C). The numbers above the bars represent the percentage of fluorescence of the treated samples at 24 h and 1 week compared to the 1 h treated samples. Errors are the range of two experiments and median fluorescence was calculated from ~30000 single-cell events.
4.4.2. Optimising HPLC conditions to determine fluorescent metabolite of FSL-61

The first step to the optimisation of the FSL-61 HPLC method was to determine the excitation and emission maxima suitable for HPLC. This optimisation was conducted using FSL-70, the amine of FSL-41 which is a close analogue of FSL-61. HPLC excitation spectra using an emission of 460 nm resulted in an excitation maximum of 280 nm (Figure 4.10A), a change from the 360 nm aqueous spectrum (Figure 4.3). The emission spectrum for FSL-70 (Figure 4.10B) however was similar to its aqueous spectra (Figure 4.4). Therefore all subsequent fluorescence analysis on the HPLC used an excitation maximum of 280 nm.

Initial optimisation of the HPLC method to determine the fluorescent metabolite of FSL-61 was conducted using HCT116/POR samples that had been treated with FSL-61. These cells were incubated with FSL-61 similarly to the flow cytometry assay except for the extraction of metabolites from whole cell culture with the addition of 1 mL acetonitrile after FSL-61 incubations (as outlined in Section 2.18). The initial gradient, run on the Agilent 1100 LC (Gradient 1; Table 4.2) was a linear gradient of acetonitrile (2-40 % over 6 min) in 0.01% formic acid in water. This chromatogram showed a major fluorescent peak at 2.1 min and 2 major UV peaks at 2.1 min and 14 min (Figure 4.10C). However the major fluorescent peak occurred at 2 min, close to the void peak. Therefore the gradient was modified by removal of the initial step and increasing the gradient to 10-40 % over 6 min to increase the retention of this peak (Gradient 2; Table 4.2). This gradient, run on the Agilent LC/tandem MS (MS/MS; model 6460) resulted in a change in retention time (RT) of the first major UV peak to 2.4 min from 2 min and the second major peak to 6 min from 14 min (Figure 4.11).

Further modification of the gradient was made to create a composite of two gradients, firstly a linear acetonitrile gradient of 10-55 % over 6 min in 0.01% formic acid in water to 55-80 % over the next 8 min (Gradient 3; Table 4.2). This further delayed the major metabolite and fluorescent peaks by shifting their RT to 4 min from 2.4 min (Figure 4.12A). When this method was run on the Agilent 6460 LC/tandem MS to match the MS to the UV absorbance peaks, the UV peaks between the two systems were different (Figure 4.12B), meaning that determination of the fluorescent metabolites cannot be done accurately. However the UV chromatogram from the same method on a different LC system, an Agilent 6150 LC/MS, was comparable to the Agilent 1100 LC (Figure 4.12C). This method was validated by the resolution of a sample of chemically reduced FSL-61 into the two major peaks on the Agilent 6150 system from (Figure 4.12D), corresponding to the two peaks.
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observed previously in Figure 4.10 and Figure 4.11. Since there appears to be two different UV peaks eluting very close together in the first major UV peak, the method was modified for a last time by decreasing the acetonitrile gradient and increasing the column temperature to 45 °C (optimised gradient; Table 4.2 and outlined in Section 2.18) as an attempt to resolve this peak.

Table 4.2 Summary of LC-MS gradients utilised for chromatographic separation of FSL-61 and its metabolites

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<th>Time (min)</th>
<th>100% acetonitrile (%)</th>
<th>0.01% formic acid in water (%)</th>
<th>Time (min)</th>
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Figure 4.10 Optimisation of method for HPLC analysis of FSL-61 metabolites

(A) Excitation and (B) emission spectra obtained from a sample of FSL-70 which was used to set up the fluorescence detector on Agilent 1100 LC system at Ex/Em of 280/460 nm. (C) Chromatograms obtained from a sample of $10^6$ HCT116/POR cells treated with 300 µM FSL-61 for 3 h under anoxia. The gradient was an acetonitrile gradient with a flow rate of 0.4 mL/min constructed using 100 % acetonitrile (A) and 0.01 % formic acid in water (B) as indicated in the text. FSL-61 UV absorbance was detected at 254 nm (bandwidth 50 nm) and fluorescence was measured at Ex/Em = 280/460 nm.
UV and total ion chromatograms obtained from a sample of HCT116/POR treated with 300 µM FSL61 for 3 h under anoxia. The gradient was run on the Agilent 6460 LC/tandem MS and modified by removal of the initial step and increasing the initial composition to 10% of acetonitrile. Arrows in the figures indicated the two major peaks from this gradient that was absent in the cells only sample (FSL-61-free). FSL-61 UV absorbance was detected at 254 nm (bandwidth 50 nm).
4.4.3. Fluorescence properties of chemical reduction products of FSL-61

Reverse phase HPLC of the chemically reduced FSL-61 sample initially demonstrated a single polar UV-absorbing product (Figure 4.12), but mass spectrometry evidence for both the hydroxylamine (m/z 249) and amine (m/z 233) in this peak, led to the optimisation of the chromatography. This resulted in the partial resolution of two peaks (Figure 4.13A) at retention times of 3.7 min (cmpd 1) and 3.9 min (cmpd 2). Their identities were assigned (structures in Figure 4.13B) as the hydroxylamine and amine based on their distinctive UV spectra (Figure 4.14A-C); for cmpd 2 the spectrum was identical to an authentic sample of
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the amine analogue of FSL-41 (FSL-70; Figure 4.15). This assignment was supported by the ESI mass spectra (Figure 4.14D-F) which gave base peaks corresponding to [M+H]⁺ at m/z 249 and 233 for cmpd 1 and 2 respectively cf m/z 263 for FSL-61. On-line fluorescence peaks were overlaid with the UV chromatogram after correction for the time lag of 0.08 min between detectors and identified one major fluorescence peak, corresponding to the amine (cmpd 2). The hydroxylamine (cmpd 1) and FSL-61 were not fluorescent. Reduction of an earlier batch of FSL-61 revealed the presence of the corresponding ethyl ester [cmpd 5, m/z 277 ([M+H]⁺), RT 14.1 min] and its reduced metabolites (Figure 4.16A; structures shown in Figure 4.16B). The UV spectra for those compounds were consistent with the chemically reduced products of FSL-61 (Figure 4.17A-C). In addition, the identity of the hydroxylamine [cmpd 3, m/z 263 ([M+H]⁺)] and amine [cmpd 4, m/z 247 ([M+H]⁺)] which eluted at RT 5.7 min and 6.0 min respectively were confirmed by mass spectrometry (Figure 4.17D-F).

Figure 4.13 Products of chemical reduction of FSL-61

(A) HPLC analysis of products from the reduction of FSL-61 with zinc dust under anoxia with structures indicated in (B). The fluorescence chromatogram was adjusted by -0.08 min to account for the delay in UV and fluorescence detection on the HPLC. FSL-61 UV absorbance was detected at 254 nm (bandwidth 50 nm) and fluorescence was measured at Ex/Em = 280/460 nm.
Figure 4.14 UV and ESI mass spectra for FSL-61 and reduced metabolites

The UV spectra for (A) cmpd 1, (B) cmpd 2 and (C) FSL-61 with the mass spectra (D-F) for the same compounds (bottom row) corresponding to the positive mode ESI mass spectra. Numbers represent the $m/z$ values of the [M+H]$^+$ ion. FSL-61 UV absorbance was detected at 254 nm (bandwidth 50 nm).
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Figure 4.16 Products of chemical reduction of an earlier batch of FSL-61

(A) HPLC chromatogram of the reduced sample of an earlier batch of FSL-61 with the ethyl-ester cmpd 5 as the major contaminant with structures of reduced metabolites (cmpd 3 and 4, structures indicated in B). The fluorescence chromatogram was adjusted by -0.08 min to account for the delay in UV and fluorescence detection on the HPLC. FSL-61 UV absorbance was detected at 254 nm (bandwidth 50 nm) and fluorescence was measured at Ex/Em = 280/460 nm.
Figure 4.17 UV and ESI mass spectra of products from chemical reduction an earlier batch of FSL-61

The UV spectra for (A) cmpd 3, (B) cmpd 4 and (C) ethyl ester cmpd 5 with the mass spectra (D-F) for the same compounds (bottom row) corresponding to the positive mode ESI mass spectra. Numbers represent the m/z values of the [M+H]^+ ion. FSL-61 UV absorbance was detected at 254 nm (bandwidth 50 nm).
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4.4.4. Fluorescence properties of enzymatic reduction products of FSL-61

Enzymatic reduction of FSL-61 with purified human recombinant POR under anoxic conditions caused ~78% loss of FSL-61 (Figure 4.18A) resulting in a single UV peak. This peak corresponded to the hydroxylamine (confirmed by UV and mass spectra, Figure 4.18B and C), with a very small fluorescent peak at a slightly longer retention time than the amine. Based on the ratio of extracted ion counts of both products eluting at 3-4 min, and assuming the hydroxylamine and amine to have similar detection efficiencies by ESI, the hydroxylamine/amine ratio was estimated to be ~90 under these conditions.

Figure 4.18 Products of enzymatic reduction of FSL-61 by human recombinant POR

(A) HPLC analysis of products from enzymatic reduction of FSL-61 by 5.3 µg human recombinant POR (20-55 U/mg, Abcam PLC). Enzymatic reduction was conducted with 1 mM FSL-61 and 5 mM NADPH in 100 µL 0.3 M phosphate buffer for 1 h at room temperature under anoxic conditions. (B) UV and (C) MS spectra of the major metabolite which was identified as cmpd I. The fluorescence chromatogram was adjusted by -0.08 min to account for the delay in UV and fluorescence detection on the HPLC. FSL-61 UV absorbance was detected at 254 nm (bandwidth 50 nm) and fluorescence was measured at Ex/Em = 280/460 nm.
4.4.5. Characterisation of FSL-61 metabolites in anoxic HCT116/POR cultures

To characterise the cellular metabolism of FSL-61, HCT116/POR cells were treated with FSL-61 under oxic and anoxic conditions for 0.5 h and 3 h. Cell pellets (collected rapidly without trypsinisation) and extracellular medium were analysed to assess the extent of intracellular entrapment of metabolites. UV chromatograms of metabolites extracted from cell pellets revealed a peak at 3.1 min (cmpd 6, Figure 4.19A), which was present at lower levels in the extracellular medium (Figure 4.19B) and not observed as a chemical reduction product (Figure 4.13A). Its ESI mass spectum showed a base peak at m/z 219 (Figure 4.20A), consistent with the [M+H]^+ ion of the free acid resulting from hydrolysis of cmpd 2. Its UV spectrum (Figure 4.20A) was similar to cmpd 2, and thus consistent with this assignment. To confirm its identity, the acid analogue of FSL-61, SN24642, was chemically reduced by zinc dust reduction. This provided the same product by retention time, UV and mass spectrum (Figure 4.20A), confirming the identity of cmpd 6 as the 6-amino acid (Figure 4.19A). A minor peak was also detected in cells at retention time ~15.5 min which corresponded to the nitro acid SN24642 (Figure 4.19B, 13.2 min in Figure 4.20B), the assignment of which was confirmed by UV and mass spectra ([M+H]^+, m/z 249).

Figure 4.19 Determination of the cell-entrapped fluorescent metabolite of FSL-61

$10^6$ HCT116/POR cells were treated with 300 µM FSL-61 for 3 h under anoxia and the cell pellet (A) and extracellular medium (B) was analysed by HPLC. The identities of the compounds were determined by UV and MS spectrometry. FSL-61 UV absorbance was detected at 254 nm (bandwidth 50 nm) and fluorescence was measured at Ex/Em = 280/460 nm.
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Figure 4.20 Analysis of the chemical reduction of SN24642 by HPLC
A representative UV chromatogram, UV spectra and mass spectra of (A) a sample of chemically reduced SN24642 (cmpd 6) and (B) SN24642. Cmpd 6 is the amine-acid of FSL-61 (cmpd 2) and SN24642 is the nitro-acid of FSL-61 (Figure 4.27). FSL-61 UV absorbance was detected at 254 nm (bandwidth 50 nm). Mass spectra are of M+H⁺ ions.

Time-dependent changes in the relative concentration of these species show that FSL-61 hydrolyses progressively to the non-fluorescent nitro-acid (SN24642), which accumulates between 0.5 h and 3 h in both cells and medium (Figure 4.21A). In contrast the initial fluorescent product, the 6-amino ester (cmpd 2), is seen at higher concentrations intracellularly than extracellularly at 0.5 h but clearly diffuses into the extracellular medium by 3 h (Figure 4.21B). The corresponding 6-amino acid (cmpd 6) is more highly retained in cells, as expected (Figure 4.21C). However, even for this second fluorescent product, some efflux into medium is evident by 3 h. The 6-hydroxylamine ester (cmpd 1) was also seen at higher concentrations after 0.5 h but it decreases after 3 h (Figure 4.21D). The inferred metabolic scheme (Figure 4.22) is consistent with the time-dependent changes in the concentration of the metabolites (e.g. cmpd 1 concentration is highest at 0.5 h, cmpd 2 similar
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at both timepoints, and Cmpd 6 strongly elevated by 3 h), and is also consistent with the slow transfer of fluorescent metabolites from high POR-expressing cells as seen by flow cytometry (Figure 4.7). However the possibility that the reduction of the nitro-acid (SN24642) also contributes to the formation of cmpd 6 cannot be excluded, although the putative hydroxylamine-acid intermediate was not detected. This metabolic scheme only occurs under anoxic conditions and none of the fluorescent metabolites were present in the oxic samples (data not shown).

Figure 4.21 Quantitation of cell entrapped FSL-61 metabolites

Concentrations of the nitro acid SN24642 (A) were determined by LC-MS using standard curves prepared with authentic compound. Concentrations of the other identified metabolites (B - D) were estimated based on LC-MS ion counts by assuming efficiency of detection of cmpd 1 and 2 were the same as for FSL-61, and cmpd 6 the same as SN24642. Error bars are the range of two independent experiments.
Figure 4.22 Metabolic scheme for FSL-61 reduction in tumour cell lines
Proposed major pathways of metabolic reduction of FSL-61 under hypoxia. Hydrolysis to the corresponding acid assists with entrapment of the reduced metabolites in cells. Compounds in blue are the fluorescent metabolites.
4.4.6. Reductase profile of FSL-61

As a nitroaromatic compound, FSL-61 is potentially a substrate for reduction by oxidoreductases other than POR. To assess its reductase profile, flow cytometry was used to evaluate the oxic and anoxic activation of FSL-61 in a panel of HCT116 cell lines over-expressing various enzymes known to be capable of nitroreduction (Figure 4.23A). These cell lines were stably transfected with the F527.V5 plasmid, with reductase expression confirmed using an inducible V5 tag [reported previously by (Guise et al., 2010; Guise et al., 2012)]. The members of the diflavin reductase family (MTRR, NDOR1 and NOS2a, as well as POR) showed a highly significant increase (p<0.001) in reduction of FSL-61 to its fluorescent metabolites compared to HCT116 WT cells. FDXR, an FAD containing oxidoreductase previously shown to be capable of reducing mitomycin C (Jiang et al., 2001), showed a smaller but statistically significant increase (p = 0.023) in FSL-61 median fluorescence compared to WT cells. In each of these cases FSL-61 reduction was specific for anoxia, and the FSL-61 signal indicated a single population of reductase-expressing cells (Figure 4.23B). The two-electron reductases NQO1, NQO2 and AKR1C3, and the NADH-dependent reductase CYB5R3, did not increase median fluorescence compared to WT cells under either oxic or anoxic conditions. Incubation with 100 µM of the flavoprotein inhibitor diphenyleneiodonium (DPI) (Riganti et al., 2004) for 1 h before and during FSL-61 exposure resulted in the complete ablation of FSL-61 metabolism in all of the reductase over-expressing lines. DPI also completely inhibited FSL-61 activation in WT cells, suggesting that the bioreductive metabolism of this compound is mediated exclusively by flavoproteins.
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Figure 4.23 Reductase profile of FSL-61

(A) Median fluorescence of HCT116 WT and a panel of oxidoreductase over-expressing HCT116 cell lines after 3 h oxic and anoxic exposure to 300 µM FSL-61. Cellular auto-fluorescence is indicated as ‘untreated’. Statistical analysis conducted by student’s t-test compared to WT cells (* = p<0.01, ‡ = p<0.001 for differences from HCT116 WT). Errors are the standard error of the mean from three independent experiments. (B) Representative fluorescence histograms for the oxidoreductase over-expressing HCT116 cells after 3 h treatment with FSL-61 under anoxia.
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4.4.7. FSL-61 as a predictive marker for hypoxic metabolism of HAPs

The reductase profile of FSL-61 under anoxic conditions was very similar to that reported for the bioreductive prodrug PR-104A in the same panel of reductase over-expressing HCT116 cell lines under anoxia (POR > MTRR > NOS2a ≥ NDOR1 > other reductases) (Guise et al., 2012). This suggested that FSL-61 activation might be a predictive biomarker for the ability of human tumour cells to activate PR-104A to its cytotoxic metabolites (hydroxylamine PR-104H and amine PR-104M). To test this, FSL-61 activation (as measured by flow cytometry) was compared with published data on the formation of PR-104A metabolites (Guise et al., 2012) in the same set of 22 human tumour cell lines, determined under the same conditions in this laboratory (Figure 4.24A). The formation of PR-104A metabolites under oxic conditions was subtracted from the anoxic metabolites to exclude oxygen-insensitive activation of PR-104A by AKR1C3 (Guise et al., 2010), given that AKR1C3 does not activate FSL-61 (Figure 4.22). The five cell lines showing the highest activation of FSL-61 (median fluorescence >600; H522, Panc-1, H1299, SiHa and Hep3B) were also fast metabolisers of PR-104A by anoxic one-electron reduction, while the lines with less capability for FSL-61 activation were slow metabolisers of PR-104A. By linear regression, the correlation between FSL-61 and PR-104A activation was statistically significant (p=0.009) although weak (R² = 0.293). This correlation was stronger (R² = 0.586) and highly significant (p<0.001) when HCT8-Sa was excluded. In addition, strongly significant correlations were observed between FSL-61 activation in a panel of 13 cell lines to anoxic bioreductive metabolism of SN30000 (R² = 0.48, p = 0.008, Figure 4.24B) and EF5 (R² = 0.50, p = 0.006, Figure 4.24C), using published datasets for these compounds from this laboratory (Wang et al., 2012). These results suggest the potential use of FSL-61 as a predictive marker for HAP activation in tumour cell lines. The usefulness of FSL-61 as an assay for one-electron reductase activity was compared to the classical cyanide-resistant NADPH-dependent cytochrome c reduction assay. A strongly significant correlation (R² = 0.79, p < 0.0001) between FSL-61 activation and the cytochrome c assay was observed in this panel of 22 cell lines but this was highly dependent on HepG2, with its exclusion abolishing the correlation to R² = 0.075, p = 0.229 (Figure 4.24D).
Figure 4.24 Correlation between reduction of FSL-61 and HAPs and cytochrome c in tumour cell lines

The activation of FSL-61 (300 µM for 3 h, assayed by flow cytometry) under anoxia was compared to (A) PR-104A metabolism as measured by the formation of PR-104H and PR-104M [data from (Guise et al., 2007)], (B) SN30000 metabolism as measured by the formation of 1-oxide and nor-oxide metabolites [data from (Wang et al., 2012)], (C) EF5 binding data [data from (Wang et al., 2012)] and (D) NADPH-cytochrome c reduction assay in a panel of tumour cell lines. Data for NAPDH-cytochrome c reductase assay is redrawn from Figure 3.1. Errors for the FSL-61 fluorescence are from three independent experiments. Median fluorescence is the net fluorescence of the cells after subtraction of cellular auto-fluorescence.
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To determine whether the low activation of FSL-61 relative to PR-104A metabolism by anoxic HCT-8Sa cells reflects a difference in the FSL-61 metabolic pathway in this cell line [e.g. failure to reduce the hydroxylamine (cmpd 1) to amine (cmpd 2), or failure of esterase activity to generate the cell-entrapped acid metabolites], the metabolite profile of FSL-61 in HCT8-Sa was compared to HCT116. The metabolite profiles in HCT-8Sa and HCT116 cells were not significantly different, with similar production of cmpd 2, cmpd 6 and SN24642 in both cell lines (Figure 4.25). Given that HCT-8Sa is not an obvious outlier in the correlations between FSL-61 and SN30000 or EF5 metabolism (Figure 4.24), it is more likely that PR-104A rather than FSL-61 metabolism is anomalous in this line. Global gene expression arrays for these cell lines [Array Express ID E-TABM-767; previously reported by (Guise et al., 2010)], were interrogated to identify oxidoreductases with high expression in HCT8-Sa. Aldo-keto reductase 1B10 (AKR1B10) transcript abundance was strikingly elevated in HCT8-Sa (114 fold higher than the median for the other 10 tumour cell lines) but over-expression of AKR1B10 in HCT116 cells did not increase metabolic reduction of PR-104A under anoxia (unpublished data from Dr Chris Guise, ACSRC). Thus the reason for very high rates of PR-104A metabolism in anoxic HCT-8Sa cells is not yet clear.
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**Figure 4.25 Comparison of FSL-61 metabolites in HCT116 and HCT8-Sa**

$10^6$ HCT116 and HCT8-Sa cells were treated with 300 µM FSL-61 for 3 h under anoxia. Intracellular metabolites were extracted from the cell pellets and extracellular metabolites were extracted from the incubation culture medium. The measured concentration of (A) FSL-61 and (B) SN24642 were calculated from standard curves using authentic compound. The concentrations of (C) cmpd 2 and (D) cmpd 6 were estimated using the standard curve of (A) and (B) respectively. Cmpd 6 was not detected in the extracellular component. Errors are the range of two experiments.
4.4.8. FSL-61 activation in POR knockout cell lines

In Chapter 3, POR enzymatic activity in the POR knockout cells as measured by the cytochrome c assay showed some residual activity that was variable between the cell lines. Since the FSL-61 flow cytometry assay was shown to be capable of discerning reductase-expressing cells at a single cell level and it is activated at least in part by POR (Figure 4.23), POR activity in the cell lines generated in Chapter 3 was interrogated using the FSL-61 flow cytometry assay. The assay confirmed the high POR activity in the POR over-expressing cell lines (HCT116/POR, Hko1/POR, SiHa/POR and S3ko2/POR) with a large significant increase in FSL-61 median fluorescence compared to HCT116 and SiHa WT cells (p<0.001; Figure 4.26A and B). The flow cytometry assay also provided information about the heterogeneity of POR activity in these cell lines, revealing a large proportion of cells in the Hko2/POR and S2ko1/POR cell populations that have lost high level POR expression as measured by FSL-61 metabolism (Figure 4.27 A-C).

The HCT116 and SiHa POR knockout clones displayed varying but low levels of FSL-61 activation when assayed by flow cytometry (Figure 4.26A and B), and FSL-61 fluorescence was not detected by microscopy (Figure 4.28). For HCT116 POR knockout cells, activation of FSL-61 was significantly decreased in Hko1 (p<0.01) and Hko2 (p<0.001) compared to WT cells while a non-significant decrease in FSL-61 fluorescence was observed in Hko3. FSL-61 median fluorescence in the SiHa partial knockout clone S2 and its derived full knockout S2ko1 was significantly lower compared to WT cells (decrease to >50 % of WT fluorescence, p<0.001). Surprisingly, the other partial knockout clone S3 and its derived clone S3ko1 demonstrated a small significant increase compared to WT cells (p<0.01). For the other derived S3 full knockout clone, S3ko2, the change in median fluorescence compared to SiHa WT cells was not significant. Thus substantial FSL-61 activation was observed in all the POR knockout clones, similar to the reduction of cytochrome c (Figure 3.10 and Figure 3.12). However 100 µM DPI treatment completely ablated FSL-61 activation in the POR knockout clones (Figure 4.29A and B), suggesting that this residual activity was mediated by flavoprotein activity.
Figure 4.26 FSL-61 activation in HCT116 and SiHa WT, POR knockout and POR over-expressing clones

Average median fluorescence of (A) HCT116 and (B) SiHa WT, POR knockout and POR over-expressing cells from three independent experiments after 3 h anoxic incubation with 300 µM FSL-61. † p<0.01 and ‡ p<0.001 compared to WT as measured by one-way ANOVA with Holm-Sidak post-hoc pairwise analysis. Median fluorescence is the net fluorescence of the cells after subtraction of cellular auto-fluorescence.
Figure 4.27 Flow cytometry histograms of FSL-61 treated HCT116 and SiHa cells

Representative histograms of fluorescent POR over-expressing cells derived from a representative sample of (A) HCT116 and (B and C) SiHa WT, POR knockout and POR over-expressing cells. 10^6 cells were treated with 300 µM FSL-61 for 3 h under anoxia. The ‘untreated’ sample is the auto-fluorescence of sample of HCT116 WT (cellular auto-fluorescence for all cell lines was the same)
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Figure 4.28 Fluorescence microscopy of FSL-61 in WT, POR knockout and over-expressing cells

$10^5$ SiHa (left) and $10^6$ HCT116 (right) cells were seeded in 96-well plates and 35 mm Petri dishes respectively. The cells were treated with 300 µM FSL-61 for 3 h under anoxic conditions. The cells were imaged by the Nikon Eclipse TE2000-E fluorescence microscope and Leica TCS SP2 confocal microscope respectively.

Figure 4.29 Effect of DPI on FSL-61 activation in POR knockout clones

(A) HCT116 and (B) SiHa POR knockout clones were pre-incubated with 100 µM DPI for 1 h before addition of 300 µM FSL-61. The cells were also incubated with DPI for the duration of FSL-61 incubation (3 h). The ‘untreated’ sample is the auto-fluorescence of sample of HCT116 WT (cellular auto-fluorescence for all cell lines was the same)
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4.5. Discussion

In this Chapter a simple 6-nitroquinolone carboxylic acid ester, FSL-61, was identified as a fluorogenic probe for monitoring the enzymatic activity of POR and related one-electron reductases at the single cell level. The compound was identified from a collection of nitroheteroaromatic compounds (Figure 4.1) by screening for anoxia-selective metabolism in cell lines (Figure 4.2), and then secondarily for detection of hypoxia-dependent POR activity by flow cytometry (Figure 4.5). FSL-61 displayed high hypoxic selectivity with no detectable activation under aerobic conditions. After subtracting the auto-fluorescence control, the data of Figure 4.5C indicate an anoxic/oxic differential of ~400-500 fold at 300-1000 µM FSL-61, which compares very favourably with other reported compounds using flow cytometry methods (Olive, 1984; Begg et al., 1985; Olive & Chaplin, 1986; Hodgkiss et al., 1991) or plate reader assays (Liu et al., 2006; Dai et al., 2008; Zhu et al., 2008; Cui et al., 2011a).

Once the utility of the flow cytometry assay had been demonstrated, a detailed characterisation of the metabolites and reductase specificity of FSL-61 was conducted. Chemical reduction of FSL-61 generated the 6-hydroxylamine (cmpd 1) and 6-amine (cmpd 2). Partial chromatographic resolution showed the amine to be much more intensely fluorescent than the hydroxylamine (Figure 4.13), reflecting the weaker electron donating character of the hydroxylamino compared to the amine moiety and is consistent with earlier observations suggesting that hydroxylamines from nitroacridines and nitronaphthalimides are much less fluorescent that the corresponding amines (Stratford et al., 1984; Wardman et al., 1984). Enzymatic reduction with purified human POR generated the 6-hydroxylamine, with only a trace of the fluorescent amine (Figure 4.18); reduction only to the hydroxylamine by POR is also seen with other nitroaromatics (Zhu et al., 2008; Penketh et al., 2012). In contrast, a recent evaluation of the reduction of 6-nitroquinoline by POR (Rajapakse & Gates, 2012) identified an azoxy-helicene derivative as the major fluorescent product. However no evidence of the formation of the corresponding helicene (or other azoxy derivatives) species from FSL-61 was found by LC-MS.

The metabolite profile from FSL-61 in POR over-expressing HCT116 cells was distinctly different from that for purified POR, and was consistent with the metabolic scheme shown in Figure 4.22. Intracellular hydroxylamine (cmpd 1) was detectable as an intermediate by MS, but the major reduced metabolites were the amine-ester (cmpd 2) and the corresponding free acid (cmpd 6), both of which were highly fluorescent. Thus the initial
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POR reduction product, the hydroxylamine-ester 1, is presumably a substrate for further reduction in HCT116 cells by other enzymes capable of reduction of hydroxylamines such as the NADH-dependent cytochrome b₅ reductase in concert with cytochrome b₅ (Kurian et al., 2004). The hydrolysis product of unreduced FSL-61 was also detected (nitro-acid SN24642), but the hydroxylamine-acid was not detected by MS, suggesting that cmpd 6 derives primarily from hydrolysis of cmpd 2 rather than reduction of SN24642. Evaluation of the metabolite profile in cells and extracellular medium after 0.5 h and 3 h (Figure 4.21) showed that the amine-ester 2 was less well entrapped in the cells than the amine-acid 6. Hence the presence of the acid side-chain increased cellular entrapment of the amine-acid 6 compound, consistent to the comparison between FSL-41 and FSL-61 (Figure 4.5A). Thus the transfer of fluorescent metabolites from cells with high POR activity to cells with low activity (Figure 4.7) is likely mediated primarily via diffusion of amine-ester 2, and through hydroxylamine-ester 1 which would be reduced further in low-POR cells, with lesser contributions from the acid metabolites.

While FSL-61 is clearly a substrate for POR, it is also important to establish whether it is specific for POR or might act as a more generic reporter of one-electron reductase activity. Using a previously characterised panel of HCT116 cell lines that over-express specific oxidoreductases (Guise et al., 2012), FSL-61 was shown to also be activated under anoxia by diflavin reductases orthologous to POR (MTRR, NDOR1, NOS2A), as well as to the unrelated (Jiang et al., 2001) flavoenzyme FDXR (Figure 4.23). Given that four of five candidate one-electron reductases tested significantly activated FSL-61, it is unlikely that all the FSL-61 reductases have been identified in this small panel. Importantly for use of FSL-61 as a hypoxia-specific probe, the known human oxygen-insensitive nitroreductases (NQO1, NQO2 and AKR1C3) did not activate FSL-61 (Figure 4.23A). The ablation of fluorescence in these over-expressing cells after pre-incubation with the flavoprotein inhibitor DPI suggests that reduction of FSL-61 is solely dependent on flavoproteins.

The one-electron reductase profile for FSL-61 was very similar to that for PR-104A in the same cell lines (Guise et al., 2012), which raised the question of whether FSL-61 reduction would correlate with PR-104A reduction (or other HAP for that matter) in a diverse panel of human tumour cell lines. This relationship was investigated in a panel of 22 cell lines for which PR-104A reduction has recently been reported (Guise et al 2012); changes in flow cytometry scatter parameters indicated FSL-61 was toxic in one of these lines (HepG2), which was excluded from further analysis. The anomalously high PR-104A reduction in HCT-8Sa relative to FSL-61 metabolism could reflect differences in the one-electron
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reduction profile of FSL-61 and PR-104A (beyond the limited number of reductases investigated in the present study). Further investigation will be needed to clarify the apparent difference between PR-104A and FSL-61 metabolism. The poor correlation between the activation of FSL-61 and the reduction of cytochrome c (Figure 4.24D) suggests that the FSL-61 assay may interrogate the activity of a different set of reductases compared to cytochrome c. A strong significant correlation between FSL-61 and SN30000 or EF5 reduction in a panel of 13 cell lines, coupled with the significant correlation to PR-104A after the exclusion HepG2 suggests that these compounds are reduced by the same reductases. These results indicate that FSL-61 may be a useful biomarker for the activation of these hypoxia-activated compounds.

The one-electron reductase activity measured by FSL-61 was significantly lower in three of the POR knockout clones (Hko1, Hko2 and S2ko1), but was not significantly decreased in the others (Hko3, S3ko1 and S3ko2; Figure 4.26). These results are consistent with the above evidence that FSL-61 is a substrate for POR and is consistent with the loss of POR activity in these clones. However the substantial residual activity in all the clones strongly suggests that reductases other than POR play an important role in the reduction of FSL-61 in these cell lines. The FSL-61 assay also demonstrated heterogeneous POR activity in Hko2 and S2ko1 POR over-expressing cells (Figure 4.27) which is consistent with the loss of POR activity as measured by NADPH:cytochrome c assay in those cell lines (Figure 3.10 and Figure 3.12). However it is not known when the loss of POR expression from Hko2/POR and S2ko1/POR occurred or how quickly this loss occurred over time. Therefore characteristics such as a good correlation to HAP metabolism and the capability to monitor population changes in reductase expression over time at the single cell level (e.g. change in reductase expression due to differences in growth between low and high reductase expressers) provides an advantage for the FSL-61 assay over the cytochrome c assay to determine reductase activity in tumour cell lines.

This Chapter outlines the characterisation of a hypoxic specific fluorogenic probe FSL-61. FSL-61 demonstrates high hypoxic selectivity, its fluorescent metabolites are well characterised and the use of this assay is a rapid and convenient method for the interrogation of one-electron reductase activity in hypoxic cells. FSL-61 also demonstrates sufficient cell entrapment for flow cytometry and microscopy methods, allowing the determination of reductase heterogeneity in tumour cell populations. The partial characterisation of its reductase profile suggests that it is a useful probe for the reductases that activate HAPs from different chemical classes, as well as the 2-nitroimidazole probe EF5.
Chapter 5. Role of POR in the activation of hypoxia-activated prodrugs

5.1. Summary

The POR knockout and POR over-expressing cell lines characterised in Chapters 3 and 4 were used to evaluate the role of POR in the activation of a panel of 12 hypoxia activated prodrugs (HAPs) using a cellular proliferation (IC\textsubscript{50}) assay following 4 h exposure to the compounds. POR over-expression increased the sensitivity of HCT116 and SiHa cells to aromatic N-oxides (TPZ, SN29751 and SN30000), and the nitro compounds SN24349 and nitracrine (>5 fold compared to WT, p<0.05) under aerobic conditions. Under anoxic conditions, POR over-expression increased sensitivity to a larger number of these HAPs, with essentially all N-oxides and nitro compounds showing significant hypersensitivity in at least one of the two POR over-expressing cell lines. The knockout of POR in HCT116 had little impact on the sensitivity to HAPs under aerobic conditions. However an increased resistance was observed more commonly for the SiHa clones, with significant resistance to aromatic N-oxides and the nitro compounds SN24349 and nitracrine in S2ko1 under aerobic conditions. Surprisingly, the effect of POR knockout on anoxic sensitivity to HAPs was modest, with statistically significant resistance to TPZ, SN30000, CB1954 and SN24349 seen in only one of the two HCT116 and SiHa clones (Hko2 and S2ko1). Further interrogation of two prodrugs, PR-104A and SN30000, in the POR knockout clones Hko1 and S2ko1 showed a non-significant decrease in bioreductive metabolite formation under anoxia compared to WT. In addition, the POR knockout clones did not demonstrate a decrease in sensitivity to PR-104A or SN30000 in clonogenic survival assays compared to WT cells. These observations confirm that most HAPs are substrates for POR, but suggests that POR at endogenous expression levels plays a limited role in the activation of these prodrugs in human tumour cell lines, with clearer signals of its contribution in SiHa than in HCT116. This suggests the existence of redundant one-electron reductases that are able to effect prodrug activation in the absence of POR.
Chapter 5 Role of POR in the activation of hypoxia-activated prodrugs

5.2. Introduction

The activation of most hypoxia-selective bioreductive prodrugs to their toxic effectors requires the one-electron reduction of the compound in the absence of oxygen. One of these reductases, POR, has been suggested to play a role as a one-electron reductase responsible for this activation step in a variety of bioreductive prodrug classes (Chapter 1). The generation and subsequent validation of POR knockout clones and POR-over-expressing lines from HCT116 and SiHa cells in Chapters 3 and 4 allows for the interrogation of the role of POR in the activation of these compounds.

This Chapter is organised into two major parts; starting with a cell line screen of POR knockout clones with a panel of bioreductive prodrugs using a proliferative (IC_{50}) assay. The panel of bioreductive prodrugs was chosen to represent compounds from four major classes of bioreductive triggers: aromatic N-oxides (tirapazamine, SN29751 and SN30000), aliphatic-N-oxides (AQ4N), quinones (mitomycin C and EO9) and nitroaromatic compounds (PR-104A, CB1954, the 5-nitroquinoline SN24349, nitracrine, TH-302 and the nitroCBI SN29428). Most of these compounds have been or are currently in clinical trials for a variety of indications (reviewed in Chapter 1). In addition to the 12 bioreductive prodrugs, three toxic effectors (PR-104H, AQ4 and SN29932) of the prodrugs PR-104A, AQ4N and SN29428 respectively were included in the panel of compounds as controls for bioreductive activation.

The second part of this Chapter interrogates the effects of POR knockout on the metabolism and clonogenicity of two bioreductive prodrugs developed by the ACSRC; PR-104A and SN30000. POR over-expressing cells have been shown to increase the activation of both these compounds under anoxia (Guise et al., 2007; Wang et al., 2012). In addition, a large amount of technical experience and historical data exists for these compounds. The formation of PR-104A and SN30000 metabolites will be measured using optimised LC-MS/MS methods developed by the ACSRC for PR-104A (Gu & Wilson, 2009) and SN30000 (Wang et al., 2012). Clonogenic assays were also carried out to determine the effects of POR knockout on clonogenic survival after incubation with these compounds.
5.3. Aims of this Chapter

The objective of this Chapter was to test the hypothesis that POR plays a major role in the activation of bioreductive prodrugs using the POR knockout and POR-over-expressing cells generated in Chapter 3. With this broad objective, the specific aims of this Chapter were:

1. To screen a panel of bioreductive prodrugs in HCT116 and SiHa cells in IC\textsubscript{50} assays to determine POR dependence of sensitivity to these compounds.
2. To investigate the POR dependence of PR-104A and SN30000 cytotoxicity in these same cell lines in further detail using clonogenic assays.
3. To evaluate the role of POR in the bioreductive metabolism of PR-104A and SN30000 in these cell lines using LC-MS/MS assays.

5.4. Results and Discussion

5.4.1. IC\textsubscript{50} of HAPs in POR over-expressing cells

To determine the role of POR in the activation of HAPs, IC\textsubscript{50} values for POR over-expressing HCT116 (HCT116/POR) and SiHa (SiHa/POR) cells were compared against WT cells using a panel of 12 HAPs and three toxic effectors (Table 5.1 and structures in Figure 5.1). The three effector compounds were included in this assay as controls to help isolate the effect of POR on bioactivation as distinct from any secondary effects of POR over-expression on sensitivity. The concentration-dependent proliferation curves for a typical HAP such as PR-104A (Figure 5.2A) allows the calculation of the IC\textsubscript{50} of the compound, which is the concentration of the compound that inhibits proliferation to the extent that it reduces total cell mass by 50% relative to controls. Under anoxic conditions, HCT116 POR over-expressing cells were 16.5 fold (p = <0.001) more sensitive than WT cells to PR-104A as shown by IC\textsubscript{50} values of 0.195 ± 0.021 µM and 3.21 ± 0.70 µM respectively (values and errors are from 9 replicate experiments for HCT116 and 4 for HCT116/POR). PR-104A was much less potent under oxic conditions, with no significant increase in cytotoxicity when POR was over-expressed (57.2 ± 6.6 µM for WT and 44.8 ± 5.8 µM for POR; Figure 5.2A). In contrast to PR-104A, the cytotoxicity of PR-104H was similar under oxic or anoxic conditions in HCT116 WT or POR over-expressing cells (Figure 5.2B). The data confirms previous studies showing that PR-104A is hypoxia-selective (Patterson et al., 2007; Hicks et al., 2007; Singleton et al., 2009) and that is a substrate for POR (Guise et al., 2007).
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Figure 5.1 Structures of HAPs and toxic effectors used in the proliferative (IC$_{50}$) assay

Figure 5.2 Concentration-dependent proliferation curves of a HAP and its toxic effector

A representative IC$_{50}$ curve for HCT116 WT (black) and POR over-expressing (red) cells after 4 h treatment with (A) PR-104A and (B) PR-104H under oxic (solid lines) and anoxic (dotted lines) conditions. IC$_{50}$ curves were fitted using a logistic four-parameter regression. Errors are the range of duplicate wells from one representative experiment.
Table 5.1 Panel of bioreductive prodrugs

All compounds were synthesised in the ACSRC except mitomycin C which was purchased from Sigma-Aldrich. The molar extinction coefficients (determined by the ACSRC) in 0.01N HCl (except as specified in the footnotes) were used to determine compound concentration by spectrometry as outlined in Section 2.1.3.

<table>
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<tr>
<th>Name</th>
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<td>480</td>
<td>6418</td>
</tr>
<tr>
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<td>(Hicks et al., 2010)</td>
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<td></td>
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<tr>
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<td>(Wilson et al., 1984)</td>
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<td>38306</td>
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<td>TH-302</td>
<td>(Duan et al., 2008)</td>
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<td>NA</td>
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<td>SN29428</td>
<td>(Tercel et al., 2009)</td>
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<td>(Tercel et al., 2009)</td>
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<td>PR-104H</td>
<td>(Patterson et al., 2007)</td>
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⁹ Methanol used as a solvent
⁹⁹ PBS used as a solvent
Chapter 5 Role of POR in the activation of hypoxia-activated prodrugs

The IC$_{50}$ values for all compounds and cell lines are compiled in Table 5.2 (HCT116 cell lines, oxic exposure) and Table 5.3 (SiHa cell lines, oxic exposure) and Table 5.4 (HCT116 cell lines, anoxic exposure) and Table 5.5 (SiHa cell lines, anoxic exposure), showing means and standard error of the means (SEM) for n $\geq$ 3 independent experiments (unless stated otherwise) for each compound. Out of range values were excluded from calculation of means. The same data are presented in the following figures. Ratios were calculated intra-experiment, and unpaired values (e.g. on scale oxic IC$_{50}$ but off-scale anoxic IC$_{50}$) were discarded for this purpose. Two-way ANOVA (with cell lines and experimental repeats as the two factors) with the Holm-Sidak post-hoc pairwise analysis was conducted on the ratios to determine statistical significance. Results for AQ4N and AQ4 are presented separately in Section 5.4.3 due to inconsistencies in IC$_{50}$ values (discussed later).

POR over-expression increased the sensitivity of HCT116 (Figure 5.3A) and SiHa (Figure 5.3B) cells compared to WT cells for several HAP under oxic conditions. This increased cytotoxicity, as measured by the hypersensitivity ratio (IC$_{50}$ of the POR over-expressing cell line divided by the IC$_{50}$ of the WT cell line), was evident in the aromatic N-oxides (tirapazamine, SN29751 and SN30000), and the nitro compounds SN24349 and nitracrine, for which a significant ($p<0.05$) and typically substantial (>5 fold) increase in sensitivity was observed (Figure 5.3C). This increased toxicity could be the result of increased redox cycling leading to the formation of superoxide, in a reaction catalysed by the increased POR activity in these cells. This result is in agreement with previous studies demonstrating redox cycling of aromatic N-oxides (Silva & O'Brien, 1993; Hunter et al., 2012) and the 5-nitroquinoline SN24349 (Siim & Wilson, 1995) in aerobic cells. An increase in oxic sensitivity of POR over-expressing cells to TH-302 was also observed in HCT116, as previously reported in a CHO cell model (Hunter et al., 2012), while POR over-expression did not increase oxic sensitivity of SiHa to TH-302 which is inconsistent with a recent report with the same cells after incubation with TH-302 (only a 2 h incubation in this study) (Meng et al., 2012). Other small (<2 fold) but significant increases in cytotoxicity was observed in EO9 (SiHa/POR), SN29428 (HCT116/POR) and CB1954 (HCT116/POR and SiHa/POR).
Chapter 5 Role of POR in the activation of hypoxia-activated prodrugs

Table 5.2 Summary of oxic IC$_{50}$ values for HCT116 cell lines

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<tr>
<th>Compound</th>
<th>HCT116 cell lines</th>
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<td>WT</td>
<td>Hko1</td>
<td>Hko2</td>
<td>HCT116/POR</td>
</tr>
<tr>
<td></td>
<td>IC50 (µM)</td>
<td>SEM</td>
<td>IC50 (µM)</td>
<td>SEM</td>
<td>IC50 (µM)</td>
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<td>121</td>
<td>43</td>
<td>148</td>
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<tr>
<td>SN30000</td>
<td>125</td>
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<td>10.7</td>
<td>109</td>
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<tr>
<td>Mitomycin C</td>
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<td>0.199</td>
<td>0.034</td>
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<td>0.0275</td>
<td>0.0026</td>
<td>0.0218</td>
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<tr>
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<td>39.3</td>
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<td>SN24349</td>
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<td>0.84</td>
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*The errors are standard error of the mean from ≥ 3 independent experiments.*
## Table 5.3 Summary of oxic IC₅₀ values for SiHa cell lines

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<th>Compound</th>
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<th>SiHa cell lines</th>
<th>SiHa cell lines</th>
<th>SiHa/POR</th>
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<td>Tirapazamine</td>
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<td>632 56</td>
<td>1380 156</td>
<td>1090 172</td>
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<td>580 23</td>
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<td>75.1 9.1</td>
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<td>41.3 7.7</td>
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<td>2.89x10⁻³ 6.6x10⁻⁴</td>
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<td>28.7 5.4</td>
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*The errors are standard error of the mean from ≥ 3 independent experiments.*
Table 5.4 Summary of anoxic IC$_{50}$ values for HCT116 cell lines

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<td>Hko2</td>
<td>HCT116/POR</td>
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<td>SEM a</td>
<td>IC$_{50}$ (µM)</td>
<td>SEM</td>
<td>IC$_{50}$ (µM)</td>
<td>SEM</td>
<td>IC$_{50}$ (µM)</td>
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*a The errors are standard error of the mean from $\geq 3$ independent experiments.
Table 5.5 Summary of anoxic $IC_{50}$ values for SiHa cell lines

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<td>SEM</td>
<td>IC50 (µM)</td>
<td>SEM</td>
<td>IC50 (µM)</td>
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<td>CB1954</td>
<td>51.0</td>
<td>4.0</td>
<td>66.2</td>
<td>8.1</td>
<td>187</td>
<td>41</td>
<td>5.40</td>
</tr>
<tr>
<td>SN24349</td>
<td>0.822</td>
<td>0.127</td>
<td>2.25</td>
<td>0.75</td>
<td>1.31</td>
<td>0.27</td>
<td>0.175</td>
</tr>
<tr>
<td>Nitracrine</td>
<td>3.61x10^{-4}</td>
<td>4.3x10^{-5}</td>
<td>6.18x10^{-4}</td>
<td>8.7x10^{-5}</td>
<td>7.68x10^{-4}</td>
<td>1.01x10^{-4}</td>
<td>1.72x10^{-4}</td>
</tr>
<tr>
<td>TH-302</td>
<td>0.0536</td>
<td>0.0079</td>
<td>0.0458</td>
<td>0.0063</td>
<td>8.89x10^{-2}</td>
<td>1.67x10^{-2}</td>
<td>0.0343</td>
</tr>
<tr>
<td>SN29428</td>
<td>0.146</td>
<td>0.035</td>
<td>0.181</td>
<td>0.042</td>
<td>0.265</td>
<td>0.030</td>
<td>0.0232</td>
</tr>
<tr>
<td>SN29932</td>
<td>6.56x10^{-3}</td>
<td>6.3x10^{-4}</td>
<td>5.85x10^{-3}</td>
<td>9.6x10^{-4}</td>
<td>0.0172</td>
<td>0.0019</td>
<td>5.57x10^{-3}</td>
</tr>
<tr>
<td>PR-104H</td>
<td>29.1</td>
<td>4.3</td>
<td>39.0</td>
<td>4.5</td>
<td>39.6</td>
<td>3.0</td>
<td>18.8</td>
</tr>
</tbody>
</table>

$^a$ The errors are standard error of the mean from $\geq 3$ independent experiments.
Figure 5.3 Oxic IC$_{50}$ for WT and POR over-expressing cell lines

IC$_{50}$ values for (A) HCT116 and (B) SiHa WT and POR over-expressing cell lines after 4 h incubation with compounds under oxic conditions. (C) Hypersensitivity ratios for POR over-expressing cells (IC$_{50}$ WT/IC$_{50}$ of POR over-expressing cells) were calculated from paired IC$_{50}$ determinations (intra-experimental ratios). * p<0.05, † p<0.01 and ‡ p<0.001 compared to WT as measured by two-way ANOVA with Holm-Sidak post-hoc pairwise analysis. Errors are the standard error of the mean from ≥3 independent experiments.
Under anoxic conditions, the compounds again showed a wide range of cytotoxic potencies in the pairs of WT and POR over-expressing HCT116 (Figure 5.4A) and SiHa lines (Figure 5.4B). Under anoxia, a larger number of the HAPs showed increases in activity when POR was over-expressed than was the case under oxic conditions, with essentially all N-oxides and nitro compounds showing significant hypersensitivity in at least one of the two POR lines (Figure 5.4C). Additional >5 fold increases in anoxic hypersensitivity in the POR over-expressing cells were observed for SN29428 (both cell lines), nitracrine (HCT116/POR) and PR-104A (SiHa/POR), but these results were not statistically significant. The lack of hypersensitivity of POR over-expressing lines to the cytotoxic effectors SN29932 and PR-104H under anoxia indicates that forced expression of POR has not altered the sensitivity of the lines to these alkylating agents.

Examining the same IC50 data from the perspective of hypoxic selectivity showed a wide range in hypoxia cytotoxicity ratios (HCR), varying from unity for mitomycin C in the SiHa lines to ~1000 fold for TH-302 (Figure 5.5A and B). The results are consistent with previous studies showing low hypoxic selectivity for mitomycin C (Marshall & Rauth, 1986; Keohane et al., 1990; Belcourt et al., 1996b; Wang et al., 2010), and very high selectivity for TH-302 (Meng et al., 2012; Hunter et al., 2012). As expected, the effectors showed no hypoxic differentials. Over-expression of POR increased HCR values in most cases, although this increase was statistically significant (p<0.05) in both backgrounds only for PR-104A and in one of the two lines for CB1954 and TH-302. The increase in HCR of PR-104A when POR is over-expressed is consistent with previous studies demonstrating that its hypoxic selectivity is compromised by a non-bioreductive mechanism of cytotoxicity in cells with low one-electron reductase activity (Singleton et al., 2009; Gu et al., 2009).
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Figure 5.4 Anoxic IC$_{50}$ for WT and POR over-expressing cell lines

Anoxic IC$_{50}$ values for (A) HCT116 and (B) SiHa WT and POR over-expressing cell lines after 4 h incubation with compounds under anoxia. (C) Hypersensitivity ratios for POR over-expressing cells were calculated from paired IC$_{50}$ determinations (intra-experimental ratios). * $p<0.05$, † $p<0.01$ and ‡ $p<0.001$ compared to WT as measured by two-way ANOVA with Holm-Sidak post-hoc pairwise analysis. Errors are the standard error of the mean from $\geq$3 independent experiments.
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Figure 5.5 Hypoxia cytotoxicity ratio (HCR) for WT and POR over-expressing cell lines HCR of HAPs in (A) HCT116 and (B) SiHa WT and POR over-expressing cells. HCR was measured as the ratio of oxic to anoxic IC\(_{50}\) and was calculated from from paired IC\(_{50}\) determinations (intra-experimental ratios) after 4 h incubations with compound. * p<0.05 and † p<0.01 compared to WT as measured by two-way ANOVA with Holm-Sidak post-hoc pairwise analysis. Errors are the standard error of the mean from ≥3 independent experiments.
Overall, these results show that most of the HAPs demonstrated a clear increase in sensitivity when the activity of POR is increased, indicating that most of these compounds are substrates for POR. The results are consistent with previous observations in POR over-expressing cells, where increases in anoxic toxicity of aromatic $N$-oxides (Patterson et al., 1995; Patterson et al., 1997; Wang et al., 2012), CB1954 (Wilson et al., 2007), PR-104A (Guise et al., 2007), SN29428 (Tercel et al., 2009) and TH-302 (Hunter et al., 2012; Meng et al., 2012) have been reported. Also, most of these compounds belong to chemical classes that have one-electron reduction potentials between -0.5 and -0.1 V, which are within the range typical of substrates for POR (Wardman, 2001). The hypersensitivity ratios of the quinones mitomycin C and EO9 (ratio of 0.96-1.8) were around the same range as for the toxic effector compounds (SN29932 and PR-104H). This lack of a large increase in the hypersensitivity ratios compared to the other classes of HAPs was inconsistent with previous studies showing increased hypoxia-dependent cytotoxicity of mitomycin C (Belcourt et al., 1996b; Baumann et al., 2001; Cowen et al., 2003; Martinez et al., 2008) and EO9 (Saunders et al., 2000; Bailey et al., 2001) in POR over-expressing cells compared to WT cells. However some other studies have also shown that POR over-expression did not increase mitomycin C cytotoxicity under either aerobic or anoxic conditions (Cowen et al., 2003; Wang et al., 2010).

### 5.4.2. IC$_{50}$ of HAPs in POR knockout cells

Since POR was shown to be capable of activating most HAPs, the ablation of POR activity would be expected to result in decreased cytotoxicity if endogenous levels of this enzyme makes a significant contribution to overall one-electron bioreductive activation. The IC$_{50}$ values in four POR knockout clones are summarised in Table 5.2-Table 5.5, and the resistance factors (IC$_{50}$ of knockouts/IC$_{50}$ of WT cells) are displayed in Figure 5.6. Under oxic conditions, knockout of POR had little impact on HAP sensitivity in HCT116 (Figure 5.6A); a significant increase in resistance was seen only for nitracrine, and then only in one of the two clones (Hko2, $p=0.015$). Increased resistance under oxic conditions was seen more commonly for the SiHa clones. Although this increased resistance was more marked with S3ko2, the latter clone also showed resistance to the effectors SN29932 and PR-104H (Figure 5.6B). This indicates altered intrinsic sensitivity of this cell line to alkylating agents, which may be related to the subsequent finding that S3ko2 has mixed ploidy (see Section 3.4.8). Concentrating on the S2ko1 clone, the largest and most statistically significant effects on oxic sensitivity to the HAPs were seen with the aromatic $N$-oxides and with nitro compounds.
nitracrine and SN24349, consistent with the enhanced oxic cytotoxicity of these same five compounds in the POR-over-expressing cell lines (Figure 5.3C). The 1.5-2-fold increase in resistance to these compounds in S2ko1 implies that endogenous levels of POR account for up to ~50% of the oxic cytotoxicity of these POR substrates in SiHa cells. Given that the cytotoxicity of the same HAPs were similarly increased by POR over-expression in aerobic HCT116 cells (Figure 5.3C), with little or no evidence for resistance in Hko1 or Hko2 (Figure 5.6A), the same reasoning would suggest that POR makes a lesser contribution to aerobic cytotoxicity of HAPs in HCT116 than SiHa.

Under anoxia, the resistance caused by knockout of POR in HCT116 (Figure 5.6C) and SiHa (Figure 5.6D) was also surprisingly modest. For HCT116, statistically significant resistance was seen only with one of the two clones (Hko2), except for a significant (p<0.05) 2-fold change in Hko1 for CB1954. In addition, differences between the two POR knockout clones are consistent with the differences between POR activity (higher in Hko1) and the possible expression of mutant protein with residual activity in Hko1. However, interpretation of the Hko1 data is complicated by an apparent increase in sensitivity of this clone to the effectors SN29932 and PR-104H, although these increases in sensitivity were not statistically significant. For Hko2, large (~4 fold) increases in resistance were seen with CB1954 and SN24349, suggesting that POR may play a major role in activation of these two HAPs in HCT116. In the SiHa knockouts, resistance under anoxia (Figure 5.6D) was broadly similar in magnitude to that under oxic conditions (Figure 5.6B). The mixed ploidy line S3ko2 was resistant to the effector SN29932 (as also seen under aerobic conditions), so the apparent resistance to its prodrug SN29428 cannot be ascribed to lack of metabolic activation. The perhaps more reliable S2ko1 clone showed a trend towards resistance (relative to SiHa WT) for all the validated POR substrates (aromatic N-oxides and nitro compounds) with the exception of TH-302, but these changes reached statistical significance only for TPZ, SN30000 and SN24349. As for the results under oxia, the anoxic data implies a modest role for POR at endogenous levels in the activation of HAP with clearer signals of its contribution in SiHa than in HCT116.

Interrogating the same data a different way, the hypoxic selectivity (HCR values) of the HAPs were little affected by knockout of POR (Figure 5.7), reflecting the similar small effects of loss of POR on aerobic and anoxic sensitivity. None of the HCT116 POR knockout clones showed a significant loss of anoxic selectivity (Figure 5.7A). POR knockout in the SiHa clones also had little effect on anoxic selectivity (Figure 5.7B), with only SN29751, CB1954 and nitracrine demonstrating a significant change in anoxic selectivity (p<0.05).
The above IC_{50} experiments were conducted on the SiHa knockout cell lines before they were found to be positive for mycoplasma infection. To determine whether mycoplasma infection affected sensitivity of the SiHa knockout cell lines to the compounds, an additional experiment was conducted after the cell lines were cured, using anoxic drug exposures only (Table 5.6). No large changes in sensitivity, relative to the previous determinations, were evident. Statistical comparison for the individual compounds was not possible because only a
single experiment was conducted with the mycoplasma-free lines. However, the overall impact on sensitivity was assessed by comparing the ratios of $IC_{50}$ values before and after elimination of mycoplasma. The average ratio (post/pre) for all compounds was 1.06 (95% confidence interval 0.83-1.29) for S2ko1 which was similar to the post/pre ratio for never-contaminated SiHa WT cells (0.91, 95% confidence interval 0.77-1.05) assayed at the same time (Table 5.6). This suggests that mycoplasma infection in S2ko1 did not have a major effect on sensitivity to HAPs under anoxia. On average, S3ko2 (0.78, 95% confidence interval 0.61-0.95) did appear to be slightly more sensitive to HAPs under anoxia after elimination of mycoplasma. However, as previously mentioned, increased ploidy in S3ko2 compared to WT cells that occurred during the course of mycoplasma elimination (discussed in Section 3.4.8) could have been responsible for this increased sensitivity to these HAPs. Therefore it cannot be certain whether mycoplasma infection or the change in ploidy affected sensitivity of S3ko2 to the HAPs. Overall, the impact of mycoplasma contamination did not appear to be sufficient to warrant further testing.

Figure 5.7 HCR for POR knockout cell lines

HCRs of HAPs in (A) HCT116 and (B) SiHa POR knockout cells calculated from paired $IC_{50}$ determinations after 4 h incubation with compounds. Errors are the standard error of the means from $\geq$3 independent experiments. Data from HCT116 and SiHa WT were redrawn from Figure 5.5. * $p<0.05$ and † $P<0.01$ compared to WT as measured by two-way ANOVA with Holm-Sidak post-hoc pairwise analysis.
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Table 5.6 Anoxic IC$_{50}$ of SiHa cell lines ‘pre-’ and ‘post-’ curing of mycoplasma infection

<table>
<thead>
<tr>
<th>Compound</th>
<th>SiHa WT</th>
<th>S2ko1</th>
<th>S3ko2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre- a</td>
<td>SEM</td>
<td>Post- b</td>
</tr>
<tr>
<td>Tirapazamine</td>
<td>2.06</td>
<td>0.17</td>
<td>1.78</td>
</tr>
<tr>
<td>SN29751</td>
<td>6.99</td>
<td>0.43</td>
<td>5.13</td>
</tr>
<tr>
<td>SN30000</td>
<td>2.47</td>
<td>0.40</td>
<td>1.95</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>0.172</td>
<td>0.021</td>
<td>0.192</td>
</tr>
<tr>
<td>EO9</td>
<td>1.95x10$^{-3}$</td>
<td>3.4x10$^{-4}$</td>
<td>1.20x10$^{-3}$</td>
</tr>
<tr>
<td>PR-104A</td>
<td>4.68</td>
<td>0.34</td>
<td>2.88</td>
</tr>
<tr>
<td>CB1954</td>
<td>58.1</td>
<td>9.9</td>
<td>49.0</td>
</tr>
<tr>
<td>SN24349</td>
<td>0.908</td>
<td>0.175</td>
<td>0.461</td>
</tr>
<tr>
<td>Nitracrine</td>
<td>3.85x10$^{-4}$</td>
<td>5.9x10$^{-5}$</td>
<td>3.43x10$^{-4}$</td>
</tr>
<tr>
<td>TH-302</td>
<td>0.0472</td>
<td>0.0096</td>
<td>0.0738</td>
</tr>
<tr>
<td>SN29428</td>
<td>0.0414</td>
<td>0.0035</td>
<td>0.0419</td>
</tr>
<tr>
<td>SN29932</td>
<td>7.16x10$^{-3}$</td>
<td>7.8x10$^{-4}$</td>
<td>8.15x10$^{-3}$</td>
</tr>
<tr>
<td>AQ4</td>
<td>0.0106</td>
<td>0.0004</td>
<td>0.0118</td>
</tr>
<tr>
<td>PR-104H</td>
<td>37.3</td>
<td>0.8</td>
<td>37.7</td>
</tr>
</tbody>
</table>

| Average ratio d | 0.91 | 1.06 | 0.78 |
| 95% CI e         | 0.14 | 0.23 | 0.17 |

a Average IC$_{50}$ values of n = 4 independent experiments prior to curing of mycoplasma infection in S2ko1 and S3ko2 (part of the data reported in Table 5.5).

b IC$_{50}$ values determined from duplicate wells from one experiment post-curing of mycoplasma infection in S2ko1 and S3ko2 (part of the data reported in Table 5.5).

c Paired post-/pre- IC$_{50}$ values for each compound.

d Average of post-/pre- IC$_{50}$ ratios for all compounds.

e 95% confidence interval for average of post-/pre- IC$_{50}$ ratios for all compounds.
5.4.3. Comparisons between AQ4N stock solutions

The effect of POR knockout on sensitivity to AQ4N was of particular interest given that this HAP is activated by CYPs in a two-electron reduction step (Raleigh et al., 1998; Nishida et al., 2010) and the activity of the CYPs is dependent on electron transfer from POR (Lu & Coon, 1968; Black et al., 1979). AQ4N was therefore included in the original panel of HAPs, but the results were not shown in the analysis above as they were highly variable between experiments. These studies included two batches of AQ4N (free base FB3 and hydrochloride salt Cl2); under oxic conditions batch Cl2 was ~10-fold more toxic than FB3 against all cell lines tested (Figure 5.8A). This effect was not as pronounced under anoxic conditions (Figure 5.8B). Unpublished historical data from the ACSRC using similar AQ4N batches also showed similar discrepancies, suggesting a potential contamination by AQ4 (or its mono-oxide AQ4M) in the AQ4N batches. Comparison of the two batches by HPLC did not detect either AQ4 or AQ4M (data not shown), although the method would not have been sensitive enough to detect contaminants at a level required to explain this difference in oxic sensitivity. When the IC_{50} data for the two batches were analysed separately, POR over-expression increased anoxic sensitivity to AQ4N in HCT116 and SiHa compared to WT cells (although the differences were not significant due to only two on-scale determinations). No evidence of increased anoxic resistance was observed in the POR knockout clones for either of the FB3 or Cl2 batches compared to WT cells except for S3ko2 (FB3, p<0.05). Statistical analysis cannot be conducted on the SiHa POR knockout cells for the Cl2 batch due to insufficient on-scale determinations (n=1). Nevertheless, as this compound was only 1 of 12 compounds assayed in the HAP screen, its inclusion or exclusion does not severely impact the major conclusions obtained from the IC_{50} assays as previously discussed.
5.4.4. PR-104A metabolism

The small magnitude of effects of POR knockout on the anti-proliferative activity of the bioreductive prodrugs led to further investigations (prodrug metabolism and cytotoxicity in clonogenic assays) to confirm this apparent weak dependence on endogenous POR. Chromatographic separation and quantification of PR-104A and its downstream metabolites (Figure 5.9A) were conducted using a previously validated LC-MS/MS method (outlined in Section 2.22). Good separation of metabolites was achieved, with PR-104A and its major metabolites (the hydroxylamine PR-104H and the amine PR-104M) eluting at 3.3 min, 2.5 min and 2.6 min respectively (Figure 5.9B). The metabolites were quantified using the MS/MS transitions shown in Figure 5.9C and D. PR-104H was quantified using a deuterated isotope internal standard and PR-104M was quantified using the deuterated PR-104H as an analogue internal standard (outlined in Section 2.22).
Figure 5.9 Quantitation of PR-104A metabolites by LC-MS/MS

(A) Structures of PR-104A, PR-104H and PR-104M. The positions of the four deuteriums of the PR-104H.D4 internal standard compound are indicated with asterisks. (B) Representative total ion chromatogram of a sample of Hko1/POR treated with 100 µM PR-104A for 1 h under anoxia, showing peaks representing PR-104H, PR-104M and PR-104A. (C and D) The extracted ion chromatograms for PR-104H and PR-104M at the indicated MRM ion transitions for the same sample as in panel B.
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The major reduced metabolites of PR-104A (PR-104H and PR-104M), were found to be significantly increased in POR over-expressing cell lines under anoxia (Figure 5.10A), with a 7.4 fold and 6.7 fold increase in the sum of the two active metabolites in HCT116/POR (p < 0.001) and Hko1/POR cells (p = 0.001) respectively relative to their parental cell lines. This increase in metabolite formation was broadly consistent with the 8.5 fold (HCT116/POR; Figure 3.10) increase in cytochrome c reductase activity in these cells compared to WT. However, the 6.7 fold increase in Hko1/POR was lower than the increase in cytochrome c reductase activity (26.7 fold) compared to Hko1 (Figure 3.17). This was possibly due to the expression of PR-104A reductases in Hko1 that are incapable of reducing cytochrome c. Also, measured PR-104A metabolites in Hko2/POR were similar to WT levels (results not shown), which was consistent with the POR expression and POR activity results from cytochrome c assays (Figure 3.17) and FSL-61 flow cytometry assays (Figure 4.26). Similarly a significant increase in PR-104A metabolites was observed in SiHa POR over-expressing cells with a 2.6 fold increase in SiHa/POR (p<0.001) compared to WT cells (Figure 5.10B). These results confirm the activity of POR as a one-electron reductase for PR-104A under anoxia, consistent with the above. It is noteworthy that the increases in the SiHa-derived POR over-expressing lines were lower than the increases in cytochrome c reductase activity compared to WT SiHa cells (SiHa/POR = 8.5 fold, S3ko2/POR = 11.6 fold). This is consistent with the presence of substantial non-POR PR-104A reductase activity in SiHa cells, such that increasing POR activity results in a relatively small increase in total PR-104A reduction.

PR-104A metabolite formation in the HCT116 (Hko1 and Hko2) and SiHa (S2ko1) POR knockout cells in both were slightly decreased compared to WT cells (16 %, 37 % and 27 % inhibition respectively), consistent with a minor role for endogenous POR in PR-104A activation under anoxia. Two-way ANOVA demonstrated a significant difference between the experiments, accounting for the large standard errors in Figure 5.10. However, after factoring in these inter-experimental variations, the difference between the POR knockout and parental cell lines was still not statistically significant for any of the clones (p>0.05; Figure 5.10A and B). In conclusion, the PR-104A metabolism data was broadly consistent with the IC_{50} data in demonstrating a trend to decreased activation in all the POR knockouts, but any such effect was too small to quantify robustly.
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Figure 5.10 Summary of anoxic PR-104A metabolism

Stacked bar histograms of PR-104A metabolites PR-104H and PR-104M of $5 \times 10^5$ (A) HCT116 and (B) SiHa WT, POR knockout and POR over-expressing whole cell cultures after 1 h incubation with 100 µM PR-104A under anoxia. PR-104H is indicated by black bars while PR-104M is in grey. † $p<0.01$ and ‡ $p<0.001$ compared to parental cell lines as measured by two-way ANOVA with Holm-Sidak post-hoc pairwise analysis. Errors are the standard error of the mean for the sum of both PR-104A metabolites from ≥3 independent experiments.
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5.4.5. PR-104A clonogenic assays

The above results indicate little difference in the anti-proliferative activity of PR-104A, or its reductive metabolism, between POR knockout and WT cell lines under anoxia. To further test whether cytotoxic activity is altered by POR knockout, clonogenic assays were used to compare PR-104A sensitivity of WT, POR knockout and POR over-expressing cell lines. These experiments (and SN30000 experiments in the next two sections) were performed using Hko1 the candidate POR knockout clone. The selection of this clone was conducted prior the knowledge that this cell line might still express an active POR. Average plating efficiency was lower in POR over-expressing cells compared to WT (this was statistically significant for SiHa/POR), while POR knockout cells had similar plating efficiencies to WT cells (Figure 5.11A). The lower plating efficiency for the POR over-expressing cells may reflect increased production of reactive oxygen species from POR (Heine et al., 2006). The concentration-dependent survival curves were fitted by four-parameter logistic regression, as for the IC_{50} study, to estimate the C_{37} which is the concentration that kills 63% of the cells. The errors are the standard error of the mean from three independent C_{37} estimations. Cells over-expressing POR were significantly more sensitive (C_{37} = 0.31 ± 0.08 µM, p = 0.004) to PR-104A compared to WT cells (C_{37} = 2.06 ± 0.29 µM) (Figure 5.11B). This 6.6 fold increase in sensitivity for Hko1/POR was consistent with the 5.6 fold increase in formation of PR-104A metabolites seen in this line compared to WT. POR knockout cells Hko1 had similar sensitivity to PR-104A (C_{37} = 1.96 ± 0.10 µM, p = 0.73) as WT cells.

Clonogenic assays were also conducted using SiHa cells and the starting seeding cell density was set 10 fold lower than that of HCT116 to minimise any bystander killing which might contribute at this cell density, in these more rapidly PR-104A metabolising lines. The plating efficiencies of the SiHa lines showed similar trends to HCT116, where the POR over-expressing cells had lower plating efficiencies compared to the other two cell lines (Figure 5.11C). POR over-expressing cells (C_{37} = 0.91 ± 0.14 µM) were significantly more sensitive to PR-104A compared to WT cells (C_{37} = 3.22 ± 0.20 µM, p = 0.009), while the POR knockout S2ko1 (C_{37} = 3.18 ± 0.54 µM, p = 0.94) was no more resistant to PR-104A (Figure 5.11D). The clonogenic survival curves for the HCT116 and SiHa cell lines were thus broadly consistent with the metabolism assays and the IC_{50} assay for PR-104A, where POR over-expression increases activation but POR knockout did not have a major impact on clonogenic survival after PR-104A incubation.
Figure 5.11 PR-104A clonogenic assay

(A) Average plating efficiency and (B) clonogenic survival curves for HCT116 cell lines after 4 h anoxic incubation with PR-104A at $3 \times 10^5$ cells/well. (C) Average plating efficiency and (D) clonogenic survival curves for SiHa cell lines at a seeding density of $3 \times 10^4$ cells/well. WT cells are indicated in black (solid line), POR knockout cells in red (solid line) and POR over-expressing cells in grey (dotted lines). * p<0.05 compared to WT as measured by two-way ANOVA with Holm-Sidak post-hoc pairwise analysis for average plating efficiency. Errors are the standard error of the mean from ≥3 independent experiments.
5.4.6. SN30000 metabolism

In addition to the PR-104A study, SN30000 metabolism and clonogenic assays were conducted to test the effect of POR knockout on the activation of this compound. Chromatographic separation and quantification of SN30000 and its downstream reduced metabolites, 1-oxide SN30672 and nor-oxide SN33093 (Figure 5.12A), was conducted using a validated LC-MS/MS method (Wang et al., 2012). Good separation of SN30000 and its metabolites was achieved, with a retention time of 1.6 min for SN30000, 3.2 min for SN30627 and 3.4 min for SN33093 (Figure 5.12B). The analytes were quantified from the MS/MS transitions shown in Figure 5.12C-E, using deuterated isotope internal standards for each metabolite (outlined in Section 2.23). Concentrations of SN30000 in HCT116 and SiHa whole cell cultures after 3 h oxic incubations were similar to the concentration of added SN30000 in cell-free controls, indicating lack of detectable SN30000 loss due to metabolism under oxic conditions (Figure 5.13A and B). However under anoxia, SN30000 concentration in the POR over-expressing cell lines was 25% of control (medium only) for Hko1/POR and 7-8% of control in SiHa/POR (Figure 5.13C and D), illustrating a significant increase in SN30000 consumption in these cells (p<0.001). A small but significant loss of SN30000 was also detectable in HCT116 and SiHa WT cells (p<0.01) and POR knockout cell lines Hko1 (p<0.05) and S2ko1 (p<0.01) compared to control samples.

The two reduced SN30000 metabolites (SN30672 and SN33093) were present at low concentrations in aerobic cultures (Figure 5.14A and B), with a ~2 fold increase in SiHa/POR relative to SiHa WT (P<0.01) and no difference between SiHa and S2ko1. However, the large errors on the determination for HCT116 WT made it less clear whether there were differences between the HCT116 lines. As expected, in the absence of oxygen there was a large increase in the formation of SN30000 metabolites in all cell lines (Figure 5.14C and D), consistent with the large measured consumption of SN30000 in Figure 5.13. POR over-expressing cell lines showed the highest metabolite formation, with a significant 2.8-3.2 fold increase in Hko1/POR (p<0.001) and 1.8-2 fold increase in SiHa/POR (p<0.05) compared to their parental cells. SN30000 metabolite formation in POR knockout clones was slightly decreased compared to WT but this was not significant.
Figure 5.12 Quantitation of SN30000 metabolites by LC-MS/MS

(A) Structures of SN30000, 1-oxide SN30672 and nor-oxide SN33093. The positions of the deuteriums in the D8 internal standard compounds are indicated with asterisks. (B) Representative total ion chromatogram of a sample of Hko1/POR treated with 30 µM SN30000 for 3 h under anoxia, showing peaks representing SN30000, SN33093 and SN30672. (C, D and E) The extracted ion chromatograms for SN30000, SN33093 and SN30672 at the indicated MRM ion transitions for the same sample as in panel B.
Figure 5.13 SN30000 concentration in HCT116 and SiHa cells

Measured SN30000 concentration from whole cell culture in $10^5$ HCT116 and SiHa WT, POR knockout and POR over-expressing cells after 3 h (A and B) oxic and (C and D) anoxic incubation with 10 µM (black bars) and 30 µM (grey bars) SN30000. * p<0.05, † p<0.01 and ‡ p<0.001 compared to media only controls as measured by two-way ANOVA with Holm-Sidak post-hoc pairwise analysis. Errors are the standard error of the mean from three independent experiments.
Figure 5.14 Total SN30000 metabolites in HCT116 and SiHa cells

Stacked bar histograms of total SN30000 metabolites from whole cell culture in $10^5$ HCT116 and SiHa WT, POR knockout and POR over-expressing cells after 3 h (A and B) oxic and (C and D) anoxic incubations with 10 µM (solid bars) and 30 µM (shaded bars) SN30000. The metabolites, 1-oxide SN30672 and nor-oxide SN33093 are indicated as grey and white bars respectively. * p<0.05, and † p<0.01 compared to parental cell lines (WT vs Hko1 or S2ko1, Hko1 vs Hko1/POR, SiHa WT vs SiHa/POR) as measured by two-way ANOVA with Holm-Sidak post-hoc pairwise analysis. Errors are the standard error of the mean of the sum of both metabolites from three independent experiments.
The data from these experiments was aggregated by normalising for the two different input concentrations of SN30000. The rate of media-subtracted SN30000 cellular consumption was calculated by subtraction of the measured SN30000 in cell-free controls and in aerobic conditions. This showed a significant loss of SN30000 in POR over-expressing cells (Hko1/POR p<0.01; SiHa/POR p<0.01) compared to WT cells and POR knockout cells (Figure 5.15A and B). Although a slight decrease in the rate of SN30000 consumption was observed in the POR knockout cells compared to WT (1.5 fold in Hko1 and 1.2 fold in S2ko1), this was not significant (p>0.05). The subtraction of aerobic metabolism to calculate the rate of total SN30000 metabolite formation (SN30672 + SN33093) was conducted to minimise the contribution of two-electron reduction in the formation of the non-toxic metabolites SN30672 and SN33093 (reviewed in Chapter 1). Hence if there was a significant two-electron reduction in these cells, this would not relevant to the cytotoxic activation of SN30000 since it bypasses the formation of the cytotoxic radical. The rate of total SN30000 metabolite formation demonstrated a significant increase of SN30000 metabolism in POR over-expressing cells (Hko1/POR p<0.05; SiHa/POR p<0.05) compared to WT and POR knockout cells (Figure 5.15C and D), consistent with the ability of POR to act as an SN30000 reductase. In addition, the formation of SN30000 metabolites was also slightly decreased in Hko1 (1.4 fold) and S2ko1 (1.1 fold) compared to WT, but these changes did not reach statistical significance (p>0.05). These results suggest that POR at endogenous levels of expression does not play a major role in the metabolism of SN30000, similar to the conclusions from the PR-104A assay.
Figure 5.15 Summary of anoxic SN30000 metabolism

The rate of SN30000 consumption by (A) HCT116 and (B) SiHa cell lines after 3 h anoxic incubation with SN30000. (C and D) Stacked bar histograms showing the net rate of SN30000 metabolite formation (oxic subtracted) and the formation of SN30672 (black bars) and SN33093 (grey bars) in (C) HCT116 and (D) SiHa cells. SN30000, SN30672 and SN33093 concentrations are calculated from the average of whole cell cultures after treatment with 10 µM and 30 µM SN30000. * p<0.05 and † p<0.01 compared to parental cell lines (WT vs Hko1 or S2ko1, Hko1 vs Hko1/POR, SiHa WT vs SiHa/POR) as measured by two-way ANOVA with Holm-Sidak post-hoc pairwise analysis. Errors are the standard error of the mean for the sum of both SN30000 metabolites. Data are from the same experiments as Figure 5.13 and Figure 5.14.
5.4.7. SN30000 clonogenic assay

As with PR-104A, clonogenic assays were conducted to further evaluate the role of POR in SN30000 activation. Control plating efficiencies in these experiments (Figure 5.16A and C) were broadly similar to those in the PR-104A experiments, with a lower plating efficiency in the POR over-expressing lines. However in this series of experiments, the Hko1 line also appeared to have a significantly lower control plating efficiency compared to WT (p<0.05). The over-expression of POR in Hko1 cells (Hko1/POR; C_{37} = 1.70 ± 0.45 µM) and SiHa WT cells (SiHa/POR; C_{37} = 0.61 ± 0.13 µM) significantly increased the cytotoxicity of SN30000 under anoxia compared to WT cells (HCT116 WT: C_{37} = 3.49 ± 0.28 µM, p = 0.028, Figure 5.16B, SiHa WT: C_{37} = 2.11 ± 0.62 µM, Figure 5.16D). However, only two on-scale C_{37} determinations were obtained for the SiHa/POR cell line, hence the statistical significance of this difference to WT cells was not established. In the POR knockout clones, Hko1 (C_{37} = 3.44 ± 0.2 µM, p>0.05) had a similar clonogenic sensitivity to WT after treatment with SN30000, while S2ko1 was slightly more resistant (C_{37} = 2.57 ± 0.44 µM) than WT although this difference was not significant. However the trend towards increased resistance is consistent with the small but significant change in the proliferative IC_{50} assays for the same cell lines. Collectively, these results suggest that the knockout of POR results in a minor effect on metabolism and cytotoxicity of SN30000 in HCT116 and SiHa cells under anoxia.
5.4.8. Dissecting clonal variations in HCT116 knockouts

The results from the prodrug screening assays in HCT116 and SiHa demonstrated that clonal differences between the POR knockouts in sensitivity to HAPs, despite the expectation that ZFN treatment would result in very specific mutations and that the resulting knockouts would be strictly isogenic. Thus Hko2 and S3ko2 were generally more resistant to the HAPs under anoxia compared to Hko1 and S2ko1 respectively (Figure 5.6). Altered ploidy in S3ko2 and possible expression of an almost full length protein with residual enzymatic activity in Hko1 may have contributed to its difference from S2ko1 and Hko2 respectively.
Chapter 5 Role of POR in the activation of hypoxia-activated prodrugs

Since SiHa cells have a more complex genomic profile, the HCT116 lines were therefore investigated further to assess whether other stochastic differences as a result of genomic instability, or off-target effects of the ZFNs, might be present.

The HCT-116 POR knockout clones Hko1, Hko2 and Hko3 were compared with the WT population by karyotyping to assess whether there is obvious gross chromosomal variation between the clones. This study included two clonal knockouts of the ADPGK gene in HCT116, obtained using ZFNs with the same general methodology as for POR by Dr Susan Richter (Richter et al., 2013). In addition, five clones were picked from the parental HCT116 population to assess stochastic clonal variation. The relationship between the analysed cell populations is shown in Figure 5.17. DNA content analysis by flow cytometry on these WT clones demonstrated no gross change in ploidy in these WT clones (Figure 5.18).

Figure 5.17 Relationship between cell populations for the exome sequencing study

HCT116 cells were expanded in culture (solid lines) before the ZFN transfection to generate POR and ADPGK knockout clones (Ako1 and Ako2). These cells were cloned and expanded in culture (dotted lines) before frozen stocks were stored in liquid nitrogen for use in experiments reported in this thesis. Five WT clones were picked from the WT population and expanded in culture for approximately the same time period as the ZFN-treated clones to assess for stochastic clonal variation.
Chapter 5 Role of POR in the activation of hypoxia-activated prodrugs

Figure 5.18 Cell cycle analysis of WT clones

Log phase cultures of HCT116 WT derived clones clones harvested, treated with propidium iodide and analysed by flow cytometry. Voltage for PI was set at 414 V to align the G1 peak to 200 fluorescence units.
The G-banding karyotypes of colcemid-arrested cultures of each of these lines were established with the assistance of Mrs Violeta Velkoska-Ivanova at LabPLUS, Auckland District Health Board. Metaphase spreads were analysed from one type of colcemid culture from each cell line unless a particular cell line displayed multiple karyotypes. These cultures were either a direct colcemid treatment of the culture for karyotyping analysis or a culture grown for 24 h or 48 h prior to colcemid treatment for karyotyping analysis. According to the International System of Cytogenetic Nomenclature (ISCN), a ‘clone’ is characterised as the loss of the same chromosome observed in 3/20 metaphases in at least two types of colcemid cultures, or the gain of a marker chromosome with identical morphology in at least 2/20 metaphases analysed (Shaffer & Tommerup, 2005).

The cytogenetic analysis of 20 metaphase cells in HCT116 WT revealed three different clones compared to the reference human karyotype (summary of karyotypes are in Table 5.7 and Table 5.8). The dominant clone (14/20 metaphases) denoted karyotype A, was 45,X,-Y,der(10)t(5;10)(q31;q26),der(16)t(8;16)(q13;p13.3),add(18)(p11.3). This shows the loss of the Y chromosome and the formation of two derivative chromosomes from unbalanced translocations (Figure 5.19). These are derivative chromosome 10 from the translocation of the long arms of chromosome 5 (q31) to chromosome 10 (q26) and derivative chromosome 16 from the translocation of the long arm of chromosome 8 (q13) to the short arm of chromosome 16 (p13.3). In addition, some chromosomal material of unknown origin was found on the short arm (p11.3) of chromosome 18. A small proportion of the WT cell line (4/20 metaphases), denoted karyotype B, showed the same karyotype as A but with retention of the Y chromosome (Figure 5.20) and 2/20 metaphases showed a tetraploid form of karyotype A which was termed karyotype C (Figure 5.21). The presence of the tetraploid clone (karyotype C) in the WT cells provides a possible explanation for the discrepancy in the POR copy number in HCT116 (estimated to be 2.34 ± 0.04; Figure 3.3). This karyotyping profile was consistent with the cytogenetic analysis of HCT116 cells as stated by ATCC (http://www.atcc.org/) and reported by previous studies (Masramon et al., 2000; Kleivi et al., 2004). A separate cytogenetic analysis by comparative genomic hybridisation and whole chromosome painting demonstrated that the der(10)t(5;10) and add(18)(p11.3) chromosomes detected by G-banding were actually dup(10)(q24q26) and der(18)t(17;18)(q21;p11.3) respectively (Masramon et al., 2000; Kleivi et al., 2004).
Table 5.7 Summary of karyotypes for HCT116 WT and POR knockout clones

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Number of metaphases</th>
<th>Karyotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT116 WT</td>
<td>14/20</td>
<td>A: 45,X,-Y,der(10)t(5;10)(q31;q26),der(16)t(8;16)(q13:p13.3),add(18)(p11.3)</td>
</tr>
<tr>
<td></td>
<td>4/20</td>
<td>B: 46,X,Y,der(10)t(5;10)(q31;q26),der(16)t(8;16)(q13:p13.3),add(18)(p11.3)</td>
</tr>
<tr>
<td></td>
<td>2/20</td>
<td>C: tetraploid form of karyotype A</td>
</tr>
<tr>
<td>Hko1</td>
<td>15/20</td>
<td>D: 45,X,-Y,der(10)t(5;10)(q31;q26),der(16)t(8;16)(q13:p13.3),add(18)(p11.3),add(17)(q22)</td>
</tr>
<tr>
<td></td>
<td>5/20</td>
<td>Karyotype A</td>
</tr>
<tr>
<td>Hko2</td>
<td>15/20</td>
<td>Karyotype A</td>
</tr>
<tr>
<td></td>
<td>5/20</td>
<td>E*: 44~45,X,-Y,der(10)t(5;10)(q31;q26),der(16)t(8;16)(q13:p13.3),add(18)(p11.3),-21,+mar</td>
</tr>
<tr>
<td>Hko3</td>
<td>12/20</td>
<td>Karyotype A</td>
</tr>
<tr>
<td></td>
<td>8/20</td>
<td>E*: 43~45,X,-Y,der(10)t(5;10)(q31;q26),der(16)t(8;16)(q13:p13.3),add(18)(p11.3),-21,+mar</td>
</tr>
</tbody>
</table>

* Chromosome number for karyotype E in Hko2 and Hko3 is different
Chapter 5 Role of POR in the activation of hypoxia-activated prodrugs

Table 5.8 Summary of karyotypes for HCT116 WT derived clones and ADPGK knockout clones

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Number of metaphases</th>
<th>Karyotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ako1</td>
<td>20/20</td>
<td>Karyotype A</td>
</tr>
<tr>
<td>Ako2</td>
<td>20/20</td>
<td>Karyotype A</td>
</tr>
<tr>
<td>WT clone 1 (1C7)</td>
<td>20/20</td>
<td>Karyotype A</td>
</tr>
<tr>
<td>WT clone 2 (5B2)</td>
<td>22/27</td>
<td>Karyotype B</td>
</tr>
<tr>
<td></td>
<td>3/27</td>
<td>F: 47, Karyotype B +mar1</td>
</tr>
<tr>
<td></td>
<td>2/27</td>
<td>G: 47, Karyotype B +mar2</td>
</tr>
<tr>
<td>WT clone 3 (5H4)</td>
<td>20/20</td>
<td>Karyotype B</td>
</tr>
<tr>
<td>WT clone 4 (1H2)</td>
<td>20/20</td>
<td>Karyotype A</td>
</tr>
<tr>
<td>WT clone 5 (5B9)</td>
<td>20/20</td>
<td>Karyotype A</td>
</tr>
</tbody>
</table>
Figure 5.19 Representative metaphase spread for karyotype A
Arrows indicate changes to the chromosomes compared to a reference human karyotype.

Figure 5.20 Representative metaphase spread for karyotype B
Cytogenetic analysis of the POR knockout clones revealed a mixed karyotype based on karyotype A, indicating no extensive chromosomal changes as a result of ZFN activity in the three clones. This observation is consistent to previous studies showing no gross chromosomal changes after ZFN activity in CHO, human ES and iPS cells (Santiago et al., 2008; Hockemeyer et al., 2009). The dominant clone for Hko1 (15/20 metaphases) displayed karyotype D, a derivative of karyotype A with additional extra chromosomal material of unknown origin on the q22 region of chromosome 17 while the minor clone (5/20 metaphases) displayed karyotype A. The dominant clones for Hko2 (15/20 metaphases) and Hko3 (12/20 metaphases) were karyotype A. However the minor clone in Hko2 (5/20) and Hko3 (8/20) displayed karyotype E, a derivative karyotype A with the loss of one copy of chromosome 21 and a marker chromosome of unknown origin. In Hko2, 2/5 metaphases presented with karyotype E with the loss of both copies of chromosome 21 (instead of one) in only one of the colcemid culture assayed. According to the ISCN this was not considered to be a clone as 3/20 metaphases are required to present with the same karyotype. Therefore the karyotype for Hko2 was described as a composite karyotype with 44 or 45 total chromosomes showing the loss of one chromosome 21. None of this heterogeneity was observed for the
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ADPGK knockout clones Ako1 and Ako2, with all 20 metaphases scored were of karyotype A. The karyotype analysis of the WT clones revealed a mixture of karyotypes based on both karyotypes A (clones 1, 4 and 5) and karyotype B (clone 2 and 3). Clone 2 displayed a mixed karyotype with the minor clones showing karyotype B with an additional marker chromosome that was different in both karyotypes F (3/27 metaphases) and G (2/27 metaphases). Thus, this karyotyping study demonstrated some karyotypic variation in the POR knockout clones that was not present in the ADPGK knockout clones and 4/5 of the non-ZFN treated clones.

To provide a higher resolution understanding of this clonal variation, genomic DNA and RNA was prepared from cultures of each clone as shown in Figure 5.17 for molecular studies. Exome sequencing using an Illumina HiSeq 2000 has recently been completed, but analysis of these data to establish mutational differences (single nucleotide variation) from the WT pool has not yet been undertaken. A preliminary analysis of exome sequences for the POR gene has however, confirmed the presence of the mutations previously identified in Hko1, Hko2 and Hko3 by Sanger sequencing (see Section 3.4.6), with the wild-type sequence in all other clones. Mutations at the ZFN target site of the POR knockout clones showed deletions of 16-bp and 26-bp in Hko1 (indicated by the horizontal lines), insertions of 4-bp in Hko2 (indicated by the purple line) and an A>T substitution (indicated by the red T) and an A insertion (indicated by the purple line) in Hko3 (illustrated in Figure 5.22). Future analysis of the exome sequence data will seek to evaluate whether ZFN treatment has generated any global changes in genomic stability or off-target mutations in sequences related to the target DNA sequences in POR and ADPGK. This and other molecular analyses may generate additional insights into the reasons for the differences in HAP sensitivity in Hko1 and Hko2.
5.5. Conclusion

As discussed in Chapters 1 and 3, the model systems utilised by several studies in the literature has implicated POR in the activation of HAPs under anoxia. However, these studies were conducted with artificial model systems that do not reflect levels of endogenous POR expression and activity in human tumours and human tumour cell lines. The observations in this study with POR-over-expressing cell lines confirm that aromatic N-oxide and nitroaromatic HAPs are substrates for POR, but indicate that POR at endogenous levels of expression plays only a limited role in the activation of these prodrugs in HCT116 and SiHa cells.
Chapter 6. Concluding discussion

Hypoxia activated prodrugs (HAPs) were designed to exploit one of the most important targets in cancer therapy, tumour hypoxia. The efficacy of these compounds is dependent on the level of tissue oxygenation, cellular expression of reductases and intrinsic cellular sensitivity to these compounds (Figure 6.1). Therefore the understanding of the interplay between these determinants and their relative contribution towards HAP efficacy is important for maximising patient outcomes based on optimal patient selection (e.g. stratification based on reductase expression or level of tumour hypoxia). This thesis examined one aspect of these determinants by dissecting the role of POR in the activation of these HAPs under anoxia.

Figure 6.1 Determinants of the efficacy of hypoxia-activated prodrugs

The efficacy of hypoxia-activated prodrugs depends on the interplay between tumour hypoxia, cellular reductases and intrinsic sensitivity to the compounds.
Chapter 6. Concluding discussion

6.1. ZFN as a molecular gene editing tool

To understand the role of endogenous POR in HAP activation in tumour cell lines, POR expression and activity was knocked out by targeted gene editing using custom-made zinc finger nucleases. Our laboratory was the first commercial client for Sigma-Aldrich’s CompoZr ZFN technology. Although high efficiency gene modification using ZFN was expected based on pioneering studies (Bibikova et al., 2002; Beumer et al., 2006; Santiago et al., 2008), the efficiency of generating full POR-null cell lines was lower than expected (especially in SiHa). This was partly due to the fact that most of the reported studies were conducted in model cell lines or against target genes that were either haploidal [e.g. BAX in CHO cell lines (Cost et al., 2010)] or diploidal in nature [e.g. BIM in K562 cells (Ng et al., 2012) or BAK (Cost et al., 2010) and DHFR in CHO cells (Santiago et al., 2008; Liu et al., 2010)]. Only one study attempted the targeting of a gene with more than two copies (three copies of CCR5 in HEK293 cells), but the generation of full CCR5 knockout cell lines in this study was not achieved (0/225 clones for full CCR5 knockout and 1/225 biallelic mutant clone isolated) (Kim et al., 2009). Therefore the generation of full POR knockouts in tumour cell lines with >2 allelic copies of the target gene (four copies of POR for SiHa) was a unique challenge to overcome and probably contributed to the lower efficiency of full POR-null cell lines.

Studies on the specificity of ZFNs have been reported ever since the utility of these chimeric proteins as a mammalian gene modification tool became apparent. Improvements in the design of the zinc finger domains (Maeder et al., 2008; Maeder et al., 2009), dimerisation of the FokI nuclease domain (Miller et al., 2007; Szczepak et al., 2007; Doyon et al., 2011) and regulation of ZFN half-life (Pruett-Miller et al., 2009), have increased the specificity of ZFN and decreased the occurrence of off-target cleavage events. However even with these modifications, off-target cleavage events have been detected from ‘highly specific’ ZFNs (Gabriel et al., 2011). The consensus sequence sites for those off-target cleavage sites often share high sequence homology to the putative ZFN target site. These sites can tolerate a change from the ZFN binding site of up to 3 bp and these off-target events can be exacerbated by higher intracellular ZFN concentrations (Pruett-Miller et al., 2009; Pattanayak et al., 2011). This suggests that ZFN-dependent off-target cleavage events can produce unwanted side-effects when generating knockout cell models, and that the resulting cell lines may not be strictly isogenic.
Chapter 6. Concluding discussion

The potential for ZFN-dependent off-target cleavage events was a concern in this thesis since high transfection efficiency was required in order to observe ZFN activity by the Surveyor™ nuclease assay. The optimised transfection protocols led to high intracellular plasmid concentrations which likely caused the loss of cells with POR mutations over time as measured by the Surveyor™ nuclease assay (Figure 3.9). This loss was likely due to a combination of ZFN off-target effects, GFP toxicity or high intracellular plasmid concentrations (ZFN and/or GFP). A possible strategy for avoiding selection of cells with high plasmid copy number would be to utilise a POR-dependent HAP (such as SN24349 under anoxia) to select cells with decreased POR activity, although the redundancy in HAP reductases suggests this would be an inefficient screen and the problems of genetic instability in tumour cell lines would remain. In hindsight, to maximise efficiency of cloning null mutants, tumour cell lines should be screened for the allelic copy number of the target gene to select for tumour cell lines that are diploid at the ZFN target site. Nevertheless, Chapter 3 outlined the successful targeting and modification of POR in tumour cell lines, resulting in the generation of three POR knockout clones each from HCT116 and SiHa WT cell lines.

6.1.1. Alternatives to ZFN technology

The emergence of ZFN technology in the past few years for targeted genome editing has increased our understanding on the function of certain genes (e.g. POR in this study). This technology improves on the early discovery of rare-cutting endonucleases such as I-Sce I (intron-encoded endonuclease 1 from Saccharomyces cerevisiae) which recognises a specific DNA sequence (18 bp) that is unique to the yeast genome (Colleaux et al., 1988). However the utility for these rare cutters in targeted genome editing is limited, since their target specificity cannot be modified as easily as ZFN. A novel chimeric endonuclease with malleable target specificity (like ZFNs) has been recently described. These so called TALENs (transcription activator-like effector nucleases) utilises a highly conserved 33-35 amino acid repeats encoded by naturally occurring TALEs from Xanthomonas spp. proteobacteria as their DNA recognition domain (Joung & Sander, 2013). Individual TALE repeats are capable of recognising a single base of DNA, with its specificity determined by two hypervariable residues at positions 12 and 13 of this repeat (Boch et al., 2009). These TALENs also utilise the FokI endonuclease domain, meaning their mechanism of gene editing is similar to ZFNs (Li et al., 2011). The modular organisation of these TALENs increases their targeting specificity, allowing targeting of any unique region of the genome.
Chapter 6. Concluding discussion

The usage of TALENs can potentially overcome some limitations associated with ZFNs. One of the advantages of the utilisation of TALENs over ZFN is the 1:1 correspondence of the hypervariable residues with the single base pairs; allowing simple modular assembly of these units (Carlson et al., 2012; Joung & Sander, 2013). In addition, the problem with context dependence in ZFN caused by protein-protein interactions such as overlapping base pair specificity between the zinc finger units (Isalan et al., 1997), is non-existent in TALENs. Also, the cost and time for design of custom-designed TALENs is considerably less than ZFNs (DeFrancesco, 2011). With a potentially large investment in time and money and the potential issues surrounding the specificity of ZFNs to target one gene, TALENs could potentially be a more attractive technology for gene editing studies in the future.

6.2. Clonal heterogeneity in the POR knockout clones

The generation of POR knockout clones was important to determine the role of POR in the activation of HAPs under hypoxia. Three POR knockout clones each were generated in HCT116 and SiHa using ZFN. All six clones demonstrated the loss of POR expression and POR mRNA transcripts as measured by Western blotting and RT-qPCR respectively. Sanger sequencing of the ZFN target site confirmed the presence of ZFN-induced mutations, which in most cases led to the premature truncation of POR protein in these clones. However differences in the nature of these mutations and clonal heterogeneity of reductase expression (other than POR) in these clones led to differences in one-electron reductase activity as measured by cytochrome c reduction and FSL-61 activation. Two clones (Hko2 and S2ko1) emerged as the most useful clones for the interrogation of the role of POR in the activation of HAPs. The POR knockout status of these clones (and Hko1) was well established and is summarised in Table 6.1. In particular these clones showed the absence of immunodetectable POR protein by Western blot, dramatic reduction in POR mRNA transcript levels, identified frameshift mutations at the ZFN target site and a significant decrease in one-electron reductase activity as measured by the reduction of cytochrome c, FSL-61 and HAPs previously shown to be substrates for POR (PR-104A and SN30000).

The evidence for the POR knockout status of the other clones (Hko1, Hko3, S3ko1 and S3ko2) was less reliable. Although HCT116 clones Hko1 and Hko3 was negative for POR by Western blotting, these clones may express a full length protein [5 amino acid (YMGEM) deletion and missense mutation, (M263L) respectively] with residual enzymatic
activity, possibly contributing to the high measured residual cytochrome c reduction and FSL-61 activation compared to Hko2. Sanger sequencing of the SiHa clones generated from the secondary ZFN transfection of clone S3, S3ko1 and S3ko2, demonstrated the possibility of the expression of a full length protein with residual enzymatic activity (derived from the ΔY262 and ΔM263 allele). This allele could partly contribute to the surprisingly high FSL-61 activation (similar to WT levels) in these clones. A puzzling observation from the POR activity assays is the disparity between the low cytochrome c reduction in S3ko1 and S3ko2 and the high FSL-61 activation in these clones. The disparity between these two assays can be possibly explained by the lack of specificity of these assays for POR and that both these assays interrogate the activity of a different set of reductases. Therefore this limitation in the POR assays in terms of redundant one-electron reductase activation makes it difficult to fully characterise the POR activity of the POR knockout clones. However, results from numerous validation experiments in Hko2 and S2ko1 were consistent to the loss of POR expression and activity in these cell lines.

One of the issues from the screen of a panel of HAPs was the phenotypic variations in HAP sensitivity between the POR knockout clones. Although these differences were evident between the clones generated from both HCT116 and SiHa, the interpretation of SiHa clonal variation was complicated with the difference in ploidy of S3ko2. In the HCT116 clones, Hko2 was more resistant to the HAPs, in particular all of the aromatic N-oxides and some nitroaromatic compounds screened in this panel of bioreductive compounds (some examples shown in Table 6.1). One of these nitroaromatic compounds is TH-302, which is currently the most clinically advanced HAP in clinical trials with ongoing Phase III trials (reviewed in Chapter 1). The low anoxic resistance factor scores of TH-302 in the POR knockout clones suggests that this compound was activated by one-electron reductases other than POR (resistance factors: Hko1 = 0.89 ± 0.17; Hko2 = 0.97 ± 0.03), despite previous reports that it is a POR substrate (Meng et al., 2012) (also shown in this thesis; Figure 5.4). The differences in HAP sensitivity between Hko1 and Hko2 were consistent with the higher capability of Hko1 to reduce cytochrome c and FSL-61, suggesting the expression of functional mutant POR in Hko1. Alternatively, this might also suggest a higher expression of one-electron reductases other than POR that can activate HAPs in Hko1.

A certain level of clonal heterogeneity was expected considering the genomic instability of many tumour cell lines and therefore certain steps, such as the selection of multiple full knockout clones (three clones each for HCT116 and SiHa, with the SiHa knockouts derived from two different partial knockout clones), were taken to minimise the
impact of clonal heterogeneity. However these steps were not sufficient to unambiguously associate phenotypes with genotypes. The cloning of WT populations prior to the ZFN transfection and increasing the number of isolated ZFN-treated clones from each tumour cell line, could be utilised to decrease the impact of clonal heterogeneity. In addition, a larger number of WT cell lines (with a pre-screen for copy number of the ZFN target site) would be useful in order to determine the effect of the knockout of a gene in a wider range of genetic backgrounds. There would be merit in using a smaller range of HAPs with a larger set of cell lines, and many clones from each, in order to clarify the role of POR more generally, although the low efficiency of recovery of null mutants without using very high plasmid loadings would make this a major undertaking with ZFNs.

6.3. The role of POR in the activation of HAPs

The importance of understanding the enzymology of HAPs is to enable the use of more targeted approaches (i.e. compounds activated by a defined set of reductases in specific tumours) and prevent activation by reductases in non-tumour tissue at the same time. For example the inhibition of mitomycin C induced myelotoxicity in NOQ1 knockout mice (Adikesavan et al., 2007) or the dose limiting toxicity of EO9 [proteinuria; (Schellens et al., 1994)] possibly as a result of high NQO1 expression in the kidney (Schlager & Powis, 1990). The one-electron reductase profile of bioreductive prodrugs is still not well understood but this thesis has made some progress in clarifying the role of POR in two widely studied human tumour cell lines, despite the complicating effects of clonal variation. One of the main findings from this thesis was the modest effect on the sensitivity of tumour cell lines to HAPs after the knockout of POR. This was a surprise considering most of the previously published studies have demonstrated an important role for POR as a one-electron reductase for these compounds. Moreover, the results from these studies was confirmed in this thesis by using similar model systems (i.e. POR over-expressing cell lines) and showing the increased cytotoxicity and metabolism of most HAPs by these cell lines. This thesis therefore extends the work done by these studies and also the work by Guise and colleagues who studied the effects of POR knockdown by RNAi technology in the activation of HAP (Guise et al., 2007). In addition, the POR knockout tumour cell lines generated in this thesis presented a novel opportunity to examine the role of POR in the activation of HAPs at endogenous expression levels with a fresh approach. However in hindsight, the selection of Hko2 as the candidate POR knockout cell line for HCT116 would have been preferred considering the evidence for
expression of a functional mutant POR in Hko1 based on the POR enzymatic assays (cytochrome c reduction and FSL-61 activation). This residual POR could still be capable of metabolising PR-104A and SN30000, thereby adding some uncertainty in the findings for HCT116. Nevertheless, the results still implicate a minor role for POR in the activation of HAP.

The lack of a striking phenotype after the knockout of POR in both these tumour cell lines suggested that other reductases that can also reduce these compounds. In addition, the residual activity from the cytochrome c assay and the FSL-61 assay demonstrated the activity of other reductases in the POR knockout clones. This redundancy in the reductase pathway may be an evolutionary advantage in some settings by acting as a compensatory mechanism that maintains cellular survival in the aftermath of the loss of an otherwise essential reductase (such as POR). In the context of use of HAPs in cancer therapy, such redundancy is also potentially valuable if it lessens the selective pressure for emergence of resistance through loss of the activating reductase. The identification and characterisation of these redundant reductases may be feasible if the number of reductases is small. However if the number of these reductases is large, then the use of molecular probes with a similar broad substrate specificity to HAPs (e.g. FSL-61 to PR-104A or EF5 to SN30000; discussed in the next section) may be the best strategy to predict the activation of bioreductive prodrugs.

An alternative possibility to the lack of a phenotype after POR knockout is the compensatory gene expression changes in these clones. An attempt was made to determine these compensatory changes in the POR knockout clones by examining and comparing their global gene expression signature. Although the results were not reported in this thesis due to insufficient replicates and controls, preliminary results from this small study showed no gross differences in reductase expression in these clones compared to WT (and also to each other). However further studies need to be conducted with better replicates and controls in order to fully determine the gene expression changes in these cells after the knockout of POR, whether a direct consequence of loss of POR or stochastic changes in the clones.
Table 6.1 Summary of three best POR knockout clones generated in Chapter 3
Data taken from Chapters 3-5. Anoxic resistance factors for three aromatic N-oxides (TPZ, SN29751 and SN30000) and three nitroaromatic compounds (PR-104A, CB1954 and SN24349) are listed.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Genotype</th>
<th>Taqman (POR transcript abundance relative to WT, %)</th>
<th>Cytochrome c reduction (nmol/min/mg)</th>
<th>Anoxic FSL-61 activation (median fluorescence)</th>
<th>Anoxic resistance factor (IC(<em>{50}) KO/IC(</em>{50}) WT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT116</td>
<td>WT</td>
<td>---</td>
<td>20.9 ± 1.1</td>
<td>211 ± 22</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hko1</td>
<td>Δ15 bp</td>
<td>0.04 ± 0.04</td>
<td>4.74 ± 0.77*</td>
<td>91.5 ± 7.4*</td>
<td>1.08 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>Δ26 bp</td>
<td></td>
<td></td>
<td></td>
<td>0.889 ± 0.075</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.978 ± 0.126</td>
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<td></td>
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<td></td>
<td></td>
<td>0.832 ± 0.266</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.96 ± 0.41*</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>1.44 ± 0.23</td>
</tr>
<tr>
<td>Hko2</td>
<td>ins 4 bp</td>
<td>2.51 ± 0.08</td>
<td>1.16 ± 0.43*</td>
<td>56.5 ± 1.7*</td>
<td>1.33 ± 0.10*</td>
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<tr>
<td></td>
<td>ins ~300 bp</td>
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<td></td>
<td></td>
<td>1.67 ± 0.38</td>
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<td></td>
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<td></td>
<td></td>
<td>1.79 ± 0.35*</td>
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<td>1.08 ± 0.09</td>
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<td></td>
<td></td>
<td>4.49 ± 0.63*</td>
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<td></td>
<td></td>
<td>4.23 ± 0.29*</td>
</tr>
<tr>
<td>SiHa</td>
<td>WT</td>
<td>3.18 ± 0.19</td>
<td>0.481 ± 0.157*</td>
<td>168 ± 18*</td>
<td>1.47 ± 0.15*</td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td></td>
<td></td>
<td></td>
<td>1.48 ± 0.34</td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td></td>
<td></td>
<td></td>
<td>2.00 ± 0.24*</td>
</tr>
<tr>
<td></td>
<td>WT</td>
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<td></td>
<td></td>
<td>1.21 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td></td>
<td></td>
<td></td>
<td>1.46 ± 0.37</td>
</tr>
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<td>WT</td>
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<td>2.68 ± 0.18*</td>
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<tr>
<td>S2ko1</td>
<td>~Δ150 bp</td>
<td>3.18 ± 0.19</td>
<td>0.481 ± 0.157*</td>
<td>168 ± 18*</td>
<td>1.47 ± 0.15*</td>
</tr>
<tr>
<td></td>
<td>~Δ300 bp</td>
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<td></td>
<td>1.48 ± 0.34</td>
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<tr>
<td></td>
<td>~Δ300 bp</td>
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<td>2.00 ± 0.24*</td>
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<td></td>
<td>~Δ300 bp</td>
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<td></td>
<td></td>
<td>2.68 ± 0.18*</td>
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* denotes a significant change compared to parental WT cells (p<0.05)
6.4. FSL-61 as a mechanistic match for HAP activation

In this thesis, FSL-61 was reported as a fluorogenic probe with excellent selectivity for hypoxic activation and good cell retention of fluorescent metabolites. In addition, the FSL-61 assay is a rapid and convenient method for the interrogation of one-electron reductases activity in hypoxic cells and was capable of determining heterogeneity of reductase activity in tumour cells. An added advantage of FSL-61, compared to conventional immunological approaches to detect tumour hypoxia (e.g. antibodies to 2-nitroimidazole adducts) was the elimination of antibody binding steps which can introduce cross-species reactivity problems in certain cases. These characteristics allow FSL-61 to be a potential molecular tool for monitoring hypoxia in tissue culture studies or characterising reductase manipulation such as over-expression or knockout of reductases in cell culture models. Another important finding from this thesis was that the reductase profile FSL-61, although not defined in detail, appears to match that of the DNBM PR-104A, with the anoxic activation of both compounds showing the same trend in HCT116 cells over-expressing candidate reductases (POR >> MTRR > NOS2a ≥ NDOR1 > other reductases). In addition, the correlation between anoxic FSL-61 and PR-104A activation in a set of 22 human tumour cell lines was highly significant after the exclusion of an outlier ($R^2 = 0.586; p<0.001$). FSL-61 activation was also correlated with the activation of the aromatic N-oxide SN30000 ($R^2 = 0.48, p = 0.008$) in a panel of 13 tumour cell lines. Both these correlations suggest the utility of FSL-61 as a predictive biomarker to determine the activation and efficacy of PR-104A and SN30000 (and potentially other HAPs) in tumour models.

The usage of molecular probes as predictive biomarkers for HAP activation is potentially a more effective way to predict HAP activation than the characterisation of expression of individual reductases and tumour hypoxia required to activate HAPs in tumours. This idea has been expressed in a recent publication by Wang and colleagues showing the mechanistic match between the 2-nitroimidazole EF5 and SN30000 (Wang et al., 2012). The good correlation between EF5 binding and SN3000 activation in a panel of tumour cell lines demonstrates the capability of EF5 to interrogate the enzymatic activity of cell lines without identification of all potential SN30000 reductases. FSL-61 might have a similar application in the context of PR-104 in leukaemias, due to evidence for hypoxia in bone marrow (Parmar et al., 2007; Benito et al., 2011) and the fact that biopsies providing single cells suitable for flow cytometry applications are routine in leukaemias. Although high AKR1C3 expression in these leukaemias cannot be interrogated by FSL-61 (see Figure 4.23), other assays are in
development for this purpose and, FSL-61 can still be utilised to interrogate the hypoxic reductases for PR-104A in this setting.

6.5. Future directions

Understanding the role of each of the three determinants of HAP activation is essential to maximise the therapeutic benefits from this class of compounds. The activation of HAPs in the absence of oxygen is an essential component of this relationship, with evidence that patient stratification based on the level of tumour hypoxia identifies a subset of tumours with improved responsiveness to HAPs (Rischin et al., 2006). Several studies have demonstrated that bioreductive prodrug activation changes at different oxygen levels, with some prodrugs demonstrating a higher $K_{O2}$ than others (e.g. TPZ compared to PR-104A) (Hicks et al., 2004; Hicks et al., 2007). Although the terms hypoxia and anoxia were used interchangeably in this thesis, the prodrug screening assays reported in this thesis were limited to anoxic incubations (i.e. $pO_2 = 0 \, \mu M$). However this was sufficient to determine the contribution of POR in the activation of these compounds, since all these compounds were activated in the absence of oxygen. Nevertheless, the oxygen tension of $pO_2 = 1-25 \, \mu M$ was not interrogated in this study. Tumour cells in these moderate levels of hypoxia are arguably more important than anoxic tumour cells since they contribute to resistance to fractionated radiotherapy (Wouters & Brown, 1997). In addition, gene expression changes from hypoxia-activated pathways differ with the severity of $O_2$ deprivation (e.g. HIF-1 activation at $PO_2 = 10 \, \mu M$ vs the unfolded protein response at $PO_2 = 0.1 \, \mu M$) (Tuttle et al., 2007; Rzymski & Harris, 2007). Therefore, future studies should determine whether the role of POR in the activation of HAP changes at these intermediate levels oxygen.

Residual reductase activity in the POR knockout clones as measured by the cytochrome c assay and FSL-61 assay points towards a redundancy of the cellular reductase pathways. Moreover it also suggests that these assays are not fully specific for one reductase. A new assay currently developed by Yongchuan Gu and Frederik Pruijn (ACSRC) is capable of directly measuring the presence of a protein in a cell by quantifying unique tryptic peptides (proteotypic peptides) using a mass spectrometry assay. Strengths of this approach include its applicability to any reductase (or protein of interest), high sensitivity and potential for multiplexing. This assay also improves on the limitations of current immunoblot techniques such as the reliance of antibody specificity and the semi-quantitative nature of Western blotting. Proteotypic peptide mass spectrometry assays also have potential for characterising
Chapter 6. Concluding discussion

the expression of mutant truncated proteins of POR in the knockout clones (Figure 3.20), by quantitation of peptide fragments located upstream and downstream of the ZFN cut site. Detection of N-terminal fragments is unlikely with nonsense mutations since premature protein truncation typically results in nonsense-mediated decay. However some of these clones (e.g. Hko1, Hko3, S3ko1 and S3ko2) still carry alleles with mutations that would not result in premature protein truncation, meaning that POR proteotypic fragments from these cell lines might be still be detected. Since the POR activity assays reported in this thesis were not fully specific for POR, this study will be important to determine whether these POR knockout clones are fully negative for expression of POR-related proteins with electron transfer activity.

The determination of the role of POR in the activation of bioreductive compounds using POR knockout clones has increased our understanding of the reductase profiles of some HAPs. However the observed variations between the POR knockout clones in their response to HAPs raised a couple of important questions, particularly regarding the identity of the other reductases that can also contribute to the activation of HAPs. Several approaches are currently being undertaken by this laboratory in collaborations with other groups. One is to explore correlations between anoxic HAP metabolism and expression of flavoprotein genes at the mRNA level, essentially as reported for aerobic activation of PR-104A which led to identification of AKR1C3 as a PR-104A reductase (Guise et al., 2010). In addition, the POR knockout clones (in particular Hko2) are being utilised as a starting point for whole genome shRNA (small-hairpin RNA) screens for HAP sensitivity genes. A further approach being explored is the use of a transposon insertional mutagenesis screen, which utilises the random integration of properties of transposons (Kong et al., 2010). Transfected tumour cell lines expressing these transposons can be treated with a bioreductive compound and resistant clones selected for analysis to determine the identity of the disrupted gene. These studies provide relatively unbiased methods for the interrogation of the human genome for novel determinants of bioreductive prodrug sensitivity (e.g. reductases, DNA repair enzymes, drug transporter proteins), which may lead to the discovery of new biomarkers for use in patient selection during cancer therapy.
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