THE TUMOUR MICROENVIRONMENT: MAPPING THE MICROREGIONAL EFFECTS OF DRUGS

by

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ABSTRACT

Rapid cell proliferation combined with a lack of adequate vascular supply can result in cells far from vasculature becoming oxygen and nutrient deprived. These hypoxic and quiescent cells can be resistant to radio- and chemotherapies and their location can result in sub-therapeutic exposures to drug. Insight into extra-vascular drug distribution and microenvironment changes in tumours could improve drug scheduling and delivery, as well as aid in the screening and rational design of new anti-cancer drugs.

The main objective of this research is to examine the tumour microenvironment after drug administration to determine which drugs penetrate effectively and to observe changes in vasculature, proliferation, and hypoxia. This is achieved by staining and imaging various markers in tumour cryosection and examining their locations relative to each other. For example, the fraction of dividing cells as a function of distance from vasculature can be used to evaluate the extent of drug effect. As well, analysis of the microenvironmental differences between tumours, after treatment, can provide insight into which characteristics lead to effective tumour control.

From examining the effects of pyrimidine analogues on proliferation in relation to vasculature we were able to show a differential effect on cells far from vasculature compared to cells proximal to vasculature. Additionally, we have shown the hypoxic cytotoxin tirapazamine unexpectedly caused central vascular dysfunction. This was characterized by a cessation of vascular perfusion in the central area of treated tumours, which became necrotic over time. By using similar tumour mapping techniques we were then able to show that increasing the vascular oxygen level, by carbogen breathing, does not completely eradicate the vascular targeting effect of tirapazamine. We further suggest the inhibition of NOS, by
tirapazamine, may lead to vessel constriction and thus increased hypoxia subsequently causing greater activation of tirapazamine.

During this research it became clear the response of proliferating tumour cells to drug is not necessarily uniform and additionally that drugs may exert unexpected mechanisms of action. The observations made by mapping the tumour microenvironment can be easily missed using traditional *in vitro* cell monolayer experiments or growth delay and clonogenic assays.
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<td>5-FU</td>
<td>5-Fluorouracil</td>
</tr>
<tr>
<td>AMT</td>
<td>2-amino-5,6-dihydro-6-methyl-1,3-thiazine</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the curve</td>
</tr>
<tr>
<td>BGS</td>
<td>bovine growth serum</td>
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<tr>
<td>BrdUrd</td>
<td>5-bromo-2-deoxy-uridine</td>
</tr>
<tr>
<td>BTZ</td>
<td>benzotriazine</td>
</tr>
<tr>
<td>CAIX</td>
<td>carbonic anhydrase IX</td>
</tr>
<tr>
<td>CA-4-P</td>
<td>combretastatin-A-4-phosphate</td>
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<tr>
<td>Carbogen</td>
<td>95% oxygen and 5% carbon dioxide</td>
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<td>DAB</td>
<td>diamino benzidine</td>
</tr>
<tr>
<td>DMSO</td>
<td>di-methyl sulfoxide</td>
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<td>DMXAA</td>
<td>5,6-dimethylxanthenone-4-acetic acid</td>
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<tr>
<td>DNA</td>
<td>deoxy-ribonucleic acid</td>
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<td>DPD</td>
<td>dihydropyrimidine dehydrogenase</td>
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<td>dThdPase</td>
<td>thymidine phosphorylase</td>
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<td>ECGS</td>
<td>endothelial cell growth supplement</td>
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<tr>
<td>ECM</td>
<td>extra-cellular matrix</td>
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<td>EtOH</td>
<td>ethanol</td>
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<tr>
<td>FAA</td>
<td>flavone acetic acid</td>
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<td>FBS</td>
<td>fetal bovine serum</td>
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<td>glucose transporter 1</td>
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<td>γH2AX</td>
<td>phosphorylated histone 2A variant X</td>
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<td>HCl</td>
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<td>hypoxic cytotoxicity ratio</td>
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<td>hypoxia inducible factor 1α</td>
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<td>HUVEC</td>
<td>human umbilical vein endothelial cell</td>
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<td>IL-1α</td>
<td>interleukin 1 alpha</td>
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<tr>
<td>LD₅₀</td>
<td>lethal dose 50 - Dose at which 50% of animals die</td>
</tr>
<tr>
<td>L</td>
<td>litre</td>
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<tr>
<td>L-NAME</td>
<td>N-omega-nitro-L-arginine methyl ester</td>
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<tr>
<td>L-NNA</td>
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</tr>
<tr>
<td>MCC</td>
<td>multilayered cell culture</td>
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<tr>
<td>MCL</td>
<td>multicellular layers</td>
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<td>MEM</td>
<td>minimal essential media</td>
</tr>
<tr>
<td>ml</td>
<td>millilitre</td>
</tr>
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<td>MRI</td>
<td>magnetic resonance imaging</td>
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<tr>
<td>MTD</td>
<td>maximum tolerated dose</td>
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<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
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<td>NaOH</td>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
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<tr>
<td>NOD/SCID</td>
<td>non-obese diabetic/sever combined immunodeficient</td>
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<td>------------</td>
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<td>octanol water coefficient</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PECAM (CD31)</td>
<td>platelet/endothelial cell adhesion marker</td>
</tr>
<tr>
<td>PET</td>
<td>positron emission tomography</td>
</tr>
<tr>
<td>pO₂</td>
<td>partial pressure of oxygen</td>
</tr>
<tr>
<td>RHC</td>
<td>relative hypoxic cytotoxicity</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumour necrosis factor alpha</td>
</tr>
<tr>
<td>Topo II</td>
<td>topoisomerase II</td>
</tr>
<tr>
<td>TPZ</td>
<td>tirapazamine</td>
</tr>
<tr>
<td>TS</td>
<td>thymidylate synthase</td>
</tr>
<tr>
<td>VE-cadherin</td>
<td>vascular endothelial cadherin</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VTA</td>
<td>vascular targeting agent</td>
</tr>
</tbody>
</table>
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1 INTRODUCTION

Cancer can arise due to uncontrolled, rapid cell proliferation, which results in cells far from functional vasculature that become oxygen and nutrient deprived. These hypoxic and quiescent cells (not in cycle) are less responsive to radiation and cell cycle targeting drugs. Additionally, a lack of adequate vascular supply can hinder the effective delivery of chemotherapeutic agents to all the cells within a tumour, resulting in sub-therapeutic exposures of drug to those cells distant from the tumour vessels. Inadequate drug exposure may contribute to repopulation or acquired resistance of the tumour.

The purpose of this research is to examine the tumour microenvironment after drug administration and evaluate the extent of drug effect. However, the assessment of drug penetration into tissue is hindered by the inability to directly visualize most drugs in tissue. Therefore, BrdUrd (5-bromo-2-deoxyuridine) incorporation was used as a marker for cell proliferation and thus a surrogate for the effect of anti-proliferative drugs.

Analysis of the relation between vascular architecture, proliferation and hypoxia in tumour sections can provide insight into which tumour characteristics lead to the effective treatment of tumours. For example, the relation between the type and amount of pre-treatment hypoxia in a tumour and survival outcome after radiation or treatment with a hypoxic cytotoxin, or the different characteristics between a tumour type that responds well to a particular drug as opposed to a tumour type that does not respond can be explored. Additionally, observing the tumour microenvironment can elucidate the target areas and effectiveness of directed therapies such as blood flow and oxygen modification, vascular targeting agents, and antibodies. Experimentally, these microenvironmental aspects can be missed by examining the tumour response using flow cytometry analysis, growth delay and clonogenic assays. Current drug administration, delivery, and screening can be improved by
understanding the tumour microenvironmental effects and the process of extra-vascular drug
distribution.

1.1 Tumour microenvironment

A tumour can form and continue to grow only in an environment of sufficient and
continually supplied oxygen and nutrients. For this reason cancerous cells secrete angiogenic
factors to encourage new blood vessel growth into the tumour region. Typically, the tumour
vasculature that is created has irregular branching, can be leaky and temporary occlusions of
vessels can occur preventing blood flow. Approximately 0.1-30% of cells in a tumour are in
S-phase (DNA synthesis phase) of the cell cycle at any particular time (Meyer and He 1993).
The clonogenic cells within the tumour can be well oxygenated if they are close to functional
blood vessels or poorly oxygenated, hypoxic, if they are far from vessels.

1.1.1 Tumour structure

Experimental solid tumours range between two distinct structure types from a well
corded to a non-corded morphology. A corded solid tumour, such as the HCT-116 colon
carcinoma, consists of perfused blood vessels surrounded by clonogenic tumour cells and
about 150 μm from the vasculature, beyond the diffusion limit of oxygen, cells become
chronically hypoxic. Necrosis forms in tumours where hypoxic cells have died due to a lack
of nutrients and oxygen. Figure 1.1 is an example of a composite image from a cryosection of
an HCT-116 tumour xenograft showing a corded structure. The blood vessels are in dark blue
surrounded by a perfusion marker shown in light blue, hypoxic cells are in green and the
tissue background is shown as greyscale with proliferating cells indicated in black.
Figure 1.1: A composite image of an HCT-116 tumour xenograft. The image shows CD31 staining of blood vessels in dark blue surrounded by a perfusion marker, carbocyanine, in light blue. The tissue background is a bright field image (greyscale) with proliferating cells indicated as black and the hypoxic cells labelled with pimonidazole are shown in green on the border of necrosis. Scale bar: 150 μm.
In contrast, a non-corded tumour structure has areas of perfused vessels surrounded by tumour tissue, but generally these tumours have a high vessel density and therefore less necrosis in the tissue (Figure 1.2). Areas of hypoxia occur but are transient in nature, due to acute vessel occlusion or collapse (red vessels), as opposed to chronically hypoxic. Cells are proliferating in the areas labelled with hypoxia likely due to the fact that hypoxia is short lived. Areas of necrosis are infrequent and may occur due to a lack of functional vasculature, rather than due to the diffusion distance of oxygen as with a corded tumour structure.

**Figure 1.2:** A composite tumour image of an SCCVII murine tumour. The image shows CD31 staining of blood vessels in dark blue surrounded by a perfusion marker, carbocyanine, in light blue. Vessels stained for CD31, but lacking perfusion are shown in red and indicate intermittent blood flow. Hypoxia (pimonidazole) is shown in green and the tissue background is greyscale with proliferating cells indicated as black. Scale bar: 150 μm.
Human tumours have similar characteristics to experimental tumours exhibiting avascular masses of tumour tissue growing around blood vessels in the stroma (Figure 1.3). As the tumour mass grows, cells become farther from the blood vessels and the centre of the mass becomes necrotic due to a lack of oxygen and nutrients. The structure is similar to a corded structure in that chronic hypoxic will occur and proliferation decreases as cells become hypoxic (Höckel and Vaupel 2001).

Figure 1.3: An H&E stained section of a human breast cancer. The structure shows an avascular tumour mass (Tumour cells, blue) with blood vessels (BV, red) in the stroma surrounding the tumour mass. As the tumour grows and cells are pushed further away from the blood vessels necrosis develops in the centre of the tumour mass.
1.1.2 Cell cycle

The duplication of a cell is a complex, regulated sequence of events known as the cell cycle. There are two major phases of the cell cycle; M phase (mitosis phase) where cell division occurs and interphase which consists of G₁ (gap 1), S-phase (synthesis phase), and G₂ (gap 2). During S-phase cells are actively replicating their DNA. Most chemotherapeutics such as the alkylating agents, antimetabolites, platinum compounds and antibiotics target actively dividing cells.

A cancerous cell is characterized by uncontrolled cell division and the ability to invade other tissues. Typically, tumours have a higher fraction of S-phase cells or proliferative index, a measure of the number of cells in a tumour that are dividing, compared with normal tissue. PCNA (proliferating cell nuclear antigen), Ki67, BrdUrd (5-bromo-2-deoxyuridine) and IdUrd (5-iodo-2-deoxyuridine) can be used as markers of dividing cells (Ohta and Ichimura 2000). PCNA and Ki67 are both endogenous markers, but PCNA labels cells that are in late G₁ or S-phase whereas Ki67 labels cells in all phases of the cell cycle except G₀ (resting phase) (Scholzen and Gerdes 2000). BrdUrd and IdUrd are exogenously administered and incorporate into the DNA of cells only during S-phase (Ohta and Ichimura 2000).

As cells reach distances far from the blood vessels they become hypoxic and the chronically hypoxic cells on the edge of necrosis can become quiescent (exit the cell cycle). In solid tumours the percentage of proliferating cells decreases with distance from vasculature even after high doses or continuous infusion of the proliferation markers (Tannock 1968; Hirst and Denekamp 1979; Rodriguez et al. 1994; Kyle et al. 2003).
1.1.3 Vasculature

Blood vessels play a vital role in the establishment and progression of tumour growth. In 1971 Folkman first proposed the concept that tumours will not grow or metastasize without angiogenesis, the process of new blood vessel formation (Folkman 1971). The development of angiogenesis occurs due to the secretion of angiogenic factors, such as vascular endothelial growth factor (VEGF), by tumour cells. An informative table of angiogenic factors can be found in a review by Folkman (Folkman 2003). The resulting tumour vasculature is irregular and disorganized often having uneven diameters and excessive branching, as well the tumour vasculature is highly permeable due to factors such as larger inter-endothelial junctions and discontinuous basement membrane (Brown and Giaccia 1998; Carmeliet and Jain 2000; Jain 2002). Temporary occlusions in the vasculature can also occur causing intermittent blood flow resulting in hypoxia (Figure 1.4). Increased vessel permeability leads to leaky tumour vasculature and when combined with the absence of functional lymphatics (Jain 1994; Leu et al. 2000; Padera et al. 2002) high interstitial fluid pressure (IFP) can be created leading to hindrance of chemotherapy diffusion (section 1.2.1).
Figure 1.4: A diagram of normal and tumour vasculature. (A) normal vasculature is structured and branched which maintains proper blood flow. (B) Tumour vasculature can be unstructured often with blind ends, occlusion, and irregular branching such as an arteriovenous (AV) shunt. Thus free flow of blood through vasculature is hindered. In areas where blood flow has temporarily ceased hypoxia can occur. Adapted from (Brown and Wilson 2004).

More recently, the tumour vasculature itself has become a target for cancer treatment. Anti-angiogenic agents were developed to target the growth of new vessels whereas vascular targeting agents are directed at the existing vasculature. Vascular targeting agents are further discussed in section 1.6. Briefly, the damage to existing tumour endothelial cells results in vessel collapse leading to central tumour necrosis. Anti-angiogenic therapies aim to prevent new blood vessel growth into tumours by targeting angiogenic signals such as VEGF or by inhibiting the VEGF receptor signalling thus preventing the growth of endothelial cells. For example Bevacizumab (Avastin, Genentech, San Francisco, CA) is a recombinant humanized monoclonal antibody targeting VEGF, which has been clinically successful. Small molecule compounds such as SU6668 and ZD6474 inhibit the VEGF growth receptor and prevent
signalling to the cell (Pandya *et al.* 2006). Additionally, a humanized VEGF receptor fusion protein, VEGF trap which binds and sequesters VEGF, is being tested in early clinical trials (Rudge *et al.* 2005).

1.1.4 Hypoxia

In 1955 a report by Thomlinson and Gray suggested tumour cells exist at distances far from vasculature (Thomlinson and Gray 1955) providing a basis for the existence of hypoxic cells in solid tumours. It is now widely accepted that experimental and human tumours contain cells at low oxygen tensions, hypoxic cells (Moulder and Rockwell 1987). There are two types of hypoxia, acute and chronic, both of which have been well characterized in experimental animal tumours (Chaplin *et al.* 1986; Chaplin and Acker 1987; Minchinton *et al.* 1990; Siemann *et al.* 1998). Acute hypoxia, also called perfusion dependent hypoxia, arises when cells are temporarily deprived of oxygen due to a collapsed or occluded vessel whereas chronic hypoxia, also called diffusion dependent hypoxic, occurs in cells that are far from vasculature, beyond the diffusion distance of oxygen.

In discussing hypoxic cells it becomes necessary to define what is oxic and what is hypoxic. The percentage of oxygen breathed in air is 21%, however the level of oxygen in normal tissue is well below 21% and is in fact closer to 5-8% (30-50 mmHg) (Brown and Wilson 2004). A review of hypoxic fractions, the fraction of hypoxic cells as a percentage of the total number of cells, in various tumour types suggests hypoxia varies significantly between tumour types (Moulder and Rockwell 1984) and median pO₂ measurements in several studies show overall oxygenation levels for tumours range between approximately 0.3-3% (2-20 mmHg) (Brown and Wilson 2004). Even the level of oxygen near vasculature in tumours can be quite low ranging between 2-5% oxygen (12-30 mmHg) (Dewhirst *et al.*
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Hypoxia can be defined by chemical markers of hypoxia such as pimonidazole, used in this thesis, which binds cells below 1% oxygen, (Raleigh et al. 1999). For the purpose of this thesis hypoxia will be defined as cells below 1% oxygen, intermediate oxygen tensions are defined as cells between 1-5% and well oxygenated or oxic cells are above 5%.

Hypoxic cells are problematic in the treatment of solid tumours because they are less responsive to radiotherapy than oxygenated cells and they can be insensitive to some chemotherapeutics (discussed in the next paragraph). In the early 1950’s Gray showed that the sensitivity of cells to radiation is dependent on oxygen whereby cells under nitrogen are 3 times less sensitive to radiotherapy compared with well oxygenated cells (Gray et al. 1953). Clinical studies of pO₂ measurements in tumours have correlated with survival outcome, where low oxygen measurements result in poor outcome, providing support for a decrease in the effectiveness of radiotherapy with reduced pO₂ in tumours (Höckel et al. 1993; Höckel et al. 1996; Nordsmark et al. 1996; Fyles et al. 1998; Nordsmark et al. 2005). Nicotinamide and carbogen (95% oxygen, 5% carbon dioxide) breathing have been used experimentally as a means of reducing tumour hypoxia to increase the effectiveness of radiotherapy (Dorie et al. 1994; Horsman et al. 1994a; Horsman et al. 1994b; El-Said et al. 1999).

Hypoxic cells can reduce the effectiveness of chemotherapy treatment, as shown in animal models, because these cells can be quiescent and therefore less responsive to cell cycle targeting drugs and they are far from vasculature, which may lead to sub-therapeutic drug exposure due to poor diffusion (Tannock et al. 2002; Huxham et al. 2004; Kyle et al. 2004). Chronically hypoxic cells play a role in cancer progression because they can adapt to a low oxygen environment by using glycolysis (Gatenby and Gillies 2004), by reducing cell
proliferation (Bedford and Mitchell 1974), by reducing overall protein synthesis (Vaupel et al. 2004) and by upregulating genes which may infer a greater resistance to cytotoxic drugs (Brown and Giaccia 1998). Once re-oxygenated these cells can return to cycle and repopulate tumours (Durand and Aquino-Parsons 2006).

Under hypoxic conditions the transcription factor hypoxia inducible factor-1α (HIF-1α) is stabilized and can upregulate genes involved in cell adaptation to hypoxia. For example, HIF-1α can upregulate glucose transporter 1 (GLUT-1) expression, which increases the transport of glucose into cells (Vaupel et al. 2004). Cells with low oxygen utilize glycolysis instead of oxidative phosphorylation for energy and an increase in glucose transport facilitates this change. As well, HIF-1α increases transcription of carbonic anhydrase IX (CAIX) which facilitates lactic acid transport and increases transcription of angiogenic factors such as VEGF (Vaupel et al. 2004). Additionally, tumour cells may adapt to the threat of acute hypoxia because it has been proposed that tumour cells will utilize glycolysis even under aerobic conditions as a survival measure (Gatenby and Gillies 2004).

Various exogenously administered nitroimidazole markers such as radiolabelled misonidazole (Hirst et al. 1985), pimonidazole (Azuma et al. 1997) and EF5 (Lord et al. 1993) have been used for well over ten years to detect hypoxic cells by flow cytometric analysis and in tumour sections. The latter two compounds are immunohistochemically detected and have been used in research both in cell monolayers, multicellular models and animal tumours as well as for detection of hypoxia in human tumours (Durand and Raleigh 1998; Fenton et al. 1999; Evans et al. 2001; Olive et al. 2001b; Kaanders et al. 2002; Bussink et al. 2003; Nordsmark et al. 2003; Dragowska et al. 2004; Hoskin et al. 2004; Janssen et al. 2004; Yaromina et al. 2005). More recently [18F]-misonidazole (Valk et al.
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1992) and [18F]-EF5 (Ziemer et al. 2003) have been developed for use in positron emission tomography (PET) for noninvasive hypoxia imaging.

The use of endogenous markers of hypoxia (CAIX, Glut-1, and HIF1-α), for the determination of hypoxia changes in relation to treatment response have recently been studied in comparison to the exogenous hypoxia marker pimonidazole. CAIX and Glut-1 can correlate with pimonidazole staining suggesting the potential use of HIF1-α regulated genes as possible indicators of hypoxia response to therapy (Olive et al. 2001a; Airley et al. 2003). More recently, CAIX and HIF1-α immunohistochemical staining of biopsies from 67 cervical biopsies have been compared with pimonidazole staining and concluded CAIX correlates well with hypoxia, however HIF1-α does not, which is possibly due to the short half-life of HIF1-α upon re-oxygenation (Jankovic et al. 2006).

1.2 Drug penetration

Most normal cells are within 30-50 μm (Krogh 1919) from a blood vessel, however due to the high proliferation rate of tumour cells compared to vasculature, tumour cells can be in excess of 100 μm from a blood vessel (Thomlinson and Gray 1955; Tannock and Hayashi 1972; Denekamp and Hobson 1982). The resulting consequence is a chemotherapeutic agent must travel from the blood vessel through stromal layers and then multiple cell layers (>10 to 15) yet still remain at a high enough concentration to exert a therapeutic effect on all cells.

A recent review of drug penetration through solid tumours outlines some key aspects regarding factors affecting drug diffusion and shows the current status of knowledge in the area (Minchinton and Tannock 2006). Inefficient delivery of drug from the blood vessels to the cells comprising the extra-vascular compartment of solid tumours could result in cells
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distant from vessels receiving sub-therapeutic drug exposures (Tannock et al. 2002; Huxham et al. 2004). As a result, some cells could survive, continue to proliferate or become drug resistant, permitting tumour re-growth.

1.2.1 Properties affecting drug penetration

Barriers to penetration in tumours include large inter-vascular distances, binding to extra-cellular components, and drug metabolism (Jain 1990; Jain 1998; Minchinton and Tannock 2006). The radial geometry of a tumour creates an impediment to penetration since the number of cells surrounding the vessel increases as a molecule diffuses out of the vasculature. Extra-cellular binding will decrease the amount of drug available to diffuse through tissue. Factors such as drug dose and pharmacokinetics will affect the concentration of a drug in blood vessels and therefore the supply of drug to cells. For example, a low concentration of drug in the blood and a short drug half-life will result in reduced exposure of cells to drug.

The charge, size and solubility of a molecule will also affect the amount of drug entering cells. A neutral, small drug with a “P” (octanol water partition coefficient) that is below 5 will exhibit better diffusion into cells but may conversely reduce drug penetration through tissue due to sequestration in cells (Lipinski et al. 2001).

Aspects of the tumour microenvironment such as the permeability of vasculature, interstitial fluid pressure (IFP), the extra-cellular matrix (ECM) and the density of cells in the tumour can effect drug penetration (Jang et al. 2003; Minchinton and Tannock 2006). High interstitial fluid pressure due to irregular leaky vasculature, as mentioned in section 1.1.3, can lead to poor diffusion of drugs. Negative pressure gradients in normal tissue allow water and molecules to flow out of vessels through the interstitial space and drain through the
lymphatic system (Heldin et al. 2004). However, in tumour tissue the high IFP (often due to poor lymph drainage) can hinder the flow of molecules out of vessels and contribute to poor drug diffusion (Young et al. 1950; Jain 1987b; Jain 1987a; Baxter and Jain 1989). The extracellular matrix can hinder molecule movement thereby causing resistance to drug transport (Jain 1987b; Davies Cde et al. 2002). For example, collagen is suggested to contribute to reduced drug distribution by increasing interstitial resistance (Netti et al. 2000; Ramanujan et al. 2002).

1.2.2 Methods used to examine drug penetration

Methods of determining drug penetration in vitro have involved the use of multicellular models such as spheroids, spherical cell aggregates grown in spinner culture flasks, and multilayered cell cultures (MCCs), discs of cell layers grown on a permeable membrane (Hicks et al. 1997; Minchinton et al. 1997). These methods have the advantage of using a 3-dimensional (3D) layered cell system that exhibits oxygen and nutrient gradients similar to those of a tumour and allows for drug distribution and examination without in vivo complications such as liver metabolism. Additionally, multilayer models of cells mimic an in vivo tumour better than a monolayer due to 3D cell-cell contacts and interactions.

1.2.2.1 Spheroids

Cells on the outer surface of spheroids are exposed to oxygen and nutrients in media with a diffusion gradient into the centre where chronically hypoxic cells and then necrosis occur (Sutherland and Durand 1976; Sutherland 1988). Spheroids can be used to directly assess drug penetration by visualizing fluorescent drugs such as anthracyclins in either cryosections or by disaggregating and using flow cytometry (Durand 1981; Kerr and Kaye 1987). Additionally, a cell survival assay can be done to determine the survival of cells
within different depths of the spheroid (Durand 1982). This is accomplished by administering Hoechst 33342 and allowing a gradient of penetration to develop through the spheroid. The spheroid is then disaggregated and single cells are run on a flow cytometer to sort for cells based on the different Hoechst 33342 intensities. The cells within each layer of the spheroid can then be plated and a clonogenic assay performed to determine the cell survival. Another method is to trypsinize the outer layer of the spheroids in succession to obtain cells from each layer and then perform a clonogenic assay (Durand 1975; Erlichman and Vidgen 1984).

1.2.2.2 Multilayered cell culture

MCCs, also called multicellular layers (MCLs), can be oriented to separate two reservoirs and allow for quantification of drug passage from the donating (drug added) reservoir through the MCC to the receiving reservoir. These flux assays can be used to determine drug diffusion by assaying the amount of drug in each reservoir at different times after drug exposure. The drug concentrations are assessed either by using radiolabelled drug (Tunggal et al. 1999; Tannock et al. 2002) or by HPLC quantification, as was used for tirapazamine (Hicks et al. 1998; Kyle and Minchinton 1999). An alternate method to flux assays is to examine cryosections of MCCs by detection of radiolabelled or fluorescent drug (Kyle et al. 2004; Grantab et al. 2006; Kyle et al. 2007). A novel application of MCCs uses an effect based assay to examine the percentage of dividing cells on the near and far side of cultures after exposure to drug from one side (Huxham et al. 2004; Kyle et al. 2004). Briefly MCCs are exposed to drug from one side while the other side is temporarily closed off to allow a drug gradient to form. The MCCs are then incubated with BrdUrd to label proliferating cells and an assessment is done on cryosection to relate the effect on the exposed side of the cultures to that on the closed off side.
1.2.2.3 Tumours

The diffusion of drugs within tumours grown *in vivo* has also been examined. Initially, information of drug distribution in tumour sections involved the use and detection of fluorescent drugs (Henneberry *et al.* 1987; Henneberry and Aherne 1992) or radiolabelled drugs (Kuh *et al.* 1999), but without direct relation to the location of blood vessels. Recent methods include the *in vivo* determination of drug distribution in tumour sections either by fluorescent microscopy (Primeau *et al.* 2005) or radiolabelled drug (Kyle *et al.* 2007) in relation to blood vessel location detected by immunohistochemistry on the same tumour section.

### 1.3 Pyrimidine analogues

Pyrimidines are one of the two types of bases that contribute to the structure of DNA. The pyrimidine analogues act as anticancer agents by mimicking the natural pyrimidines and incorporating into DNA and RNA and subsequently disrupting the normal functions. As well, the pyrimidine analogues can block enzymes thus preventing the synthesis of the natural pyrimidines. Experiments in this thesis studied four pyrimidine analogues, 5-fluorouracil (5-FU), capecitabine, cytarabine and gemcitabine.

#### 1.3.1 Structure

The structures of the pyrimidine analogues used in this thesis (5-FU, capecitabine, cytarabine and gemcitabine) are shown below (Figure 1.5). 5-FU is a uracil mimetic which is converted to a nucleotide in order to exert a cytotoxic effect. The basic structure is uracil where the 5' hydrogen has been replaced with a fluorine, which mimics the size of the original hydrogen, however the C-F bond is stronger than that of the C-H bond preventing methylation at the 5' position by thymidylate synthase (TS) and thus conversion to thymidine.
(Chabner et al. 2001). Capecitabine is a cytidine analogue, prodrug of 5-FU and has an N-4-pentoxy carbonyl group at the 6’ position of the pyrimidine ring. It is converted, through various steps, by the liver and tumour tissue to 5-FU, which then goes on to have a cytotoxic effect. In contrast, cytarabine and gemcitabine are cytidine analogues with alterations on the ribose moiety. Cytarabine has a 2’ hydroxyl group trans to the 3’ hydroxyl group of the sugar, which creates steric hindrance preventing the rotation of the base once incorporated into DNA. Gemcitabine is a synthetic, fluorinated analogue of cytidine structurally related to cytarabine with 2 fluorines at the 2’ position of the sugar. Incorporation of gemcitabine into DNA leads to termination of the DNA strand synthesis.

5-FU is a pyrimidine base and is thought to enter the cell either via passive diffusion or a nucleobase transporter (Chabner et al. 2001; Peters and Jansen 2001). The other three analogues, capecitabine, cytarabine and gemcitabine are hydrophilic nucleoside derivatives which would not be expected to readily permeate by diffusion and instead permeate the cell membrane via a family of nucleoside transporters (NT).
Figure 1.5: The structure of the pyrimidine analogues used in this work. 5-FU is a uracil mimic where the 5' hydrogen has been replaced with a fluorine. Capecitabine is an oral prodrug of 5-FU. Cytarabine and gemcitabine are cytosine analogues with alterations on the 2' position of the ribose moiety. Cytarabine has a 2' hydroxyl group trans to the 3' hydroxyl group of the sugar and gemcitabine has two fluorines in the 2' position. Adapted from (Chabner et al. 2001).

1.3.2 Properties, clinical uses and activity of pyrimidine analogues

Data outlining key information about all four pyrimidine analogues used in this work is summarized in Table 1.1. Details of the activity of the compounds are then summarized in the following paragraphs. Of the four analogues tested all are used in the treatment of solid tumours except cytarabine, which is used to treat acute myelogenous leukemia.
Table 1.1: Data for the pyrimidine analogues

<table>
<thead>
<tr>
<th>Compound</th>
<th>5-Fluorouracil</th>
<th>Capecitabine</th>
<th>Cytarabine</th>
<th>Gemcitabine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical name</td>
<td>5-Fluorouracil</td>
<td>N4-pentoxycarbonyl 5'-deoxy-5-fluorocytidine</td>
<td>1-b-D-arabinofuranosylcytosine</td>
<td>2',2'-Difluoro-deoxycytidine</td>
</tr>
<tr>
<td>Trade name</td>
<td>Adrucil</td>
<td>Xeloda</td>
<td>Cytosar, Ara-C</td>
<td>Gemzar</td>
</tr>
<tr>
<td>Molecular weight (g/mol)</td>
<td>130.08</td>
<td>359.35</td>
<td>243.22</td>
<td>299.66</td>
</tr>
<tr>
<td>Route of human administration</td>
<td>i.v. infusion</td>
<td>oral</td>
<td>continuous i.v. infusion</td>
<td>i.v. infusion (30 min)</td>
</tr>
<tr>
<td>Clinical toxicities</td>
<td>anorexia, diarrhea, myelosuppression</td>
<td>anorexia, diarrhea, myelosuppression</td>
<td>myelosuppression</td>
<td>myelosuppression</td>
</tr>
<tr>
<td>Primary uses</td>
<td>breast, colorectal, gastric, bladder, prostate, pancreatic, ovarian, head and neck</td>
<td>breast, colorectal</td>
<td>acute myelogenous leukemia, meningeal leukemia</td>
<td>pancreatic, non-small cell lung, bladder</td>
</tr>
<tr>
<td>Bound in plasma (%)</td>
<td>8-10</td>
<td>&lt;60</td>
<td>13</td>
<td>negligible</td>
</tr>
<tr>
<td>Half-life (minutes)</td>
<td>11 ± 4</td>
<td>78 (C)</td>
<td>43 (5-FU)</td>
<td>10</td>
</tr>
<tr>
<td>Human dose</td>
<td>750 mg/m² once a week for 6-8 weeks</td>
<td>1250 mg/m² 2 every day for 2 weeks</td>
<td>200 mg/m² daily for 5-7 days</td>
<td>1000-1200 mg/m² once every 7 days for three weeks</td>
</tr>
<tr>
<td>Peak plasma concentration</td>
<td>11.2 uM</td>
<td>18.3 uM ([Cl])</td>
<td>3.6 uM ([5-FU])</td>
<td>20 uM</td>
</tr>
<tr>
<td>Calculated AUC (µM/min)</td>
<td>178</td>
<td>223 (5-FU)</td>
<td>285</td>
<td>1100</td>
</tr>
<tr>
<td>Dose given for experiments (i.p.)</td>
<td>120 mg/kg</td>
<td>750 mg/kg</td>
<td>400 mg/kg</td>
<td>240 mg/kg</td>
</tr>
<tr>
<td>Maximum tolerated dose</td>
<td>100 mg/kg</td>
<td>752 mg/kg</td>
<td>250 mg/kg</td>
<td>&gt;240 mg/kg</td>
</tr>
</tbody>
</table>

a) Unless otherwise stated (Chabner et al. 2001)
f) weekly dose via i.p. administration (Peters et al. 1993a)
g) p.o. administration (Endo et al. 2003)
h) i.v. administration (Koga et al. 1995)
i) weekly dose via i.p. administration (Veerman et al. 1996)
1.3.2.1 5-FU

The cytotoxicity of 5-FU is confined to the S-phase of the cell cycle. 5-FU causes a \( G_1/S \) cell cycle block which can then lead to apoptosis if the cell damage is not repaired (Berg et al. 2001; De Angelis et al. 2006). In studies using resistant and sensitive murine colon tumour lines 5-FU was found to cause, approximately, a 3 and 5 day growth delay, respectively after i.p. administration of 100 mg/kg once a week for 4 weeks (Nadal et al. 1989).

5-FU is primarily used to treat solid tumours including breast, colorectal, gastric, bladder, prostate, pancreatic, ovarian, head and neck (Table 1.1). Administration is via i.v. infusion of 750 mg/m\(^2\) once a week for 6-8 weeks. The half-life of the drug is relatively short (~11 min) and conversion occurs in the liver and tumour tissue. The peak plasma concentration achieved in humans is 11.2 \( \mu \)M and 8-10% of the drug is plasma bound.

The conversion of 5-FU to its active form occurs in the liver and tumour tissue by various enzymes, which initiates with the conversion of the base to the nucleotide form 5-fluoro-uridine-5'-monophosphate (F-UMP). The nucleotide is then reduced by ribonucleotide diphosphate reductase to the deoxyuridine form (F-dUMP). F-dUMP then inhibits thymidylate synthase (TS) through covalent binding which requires the collate cofactor 5,10-methylenetetrahydrofolate. This inhibition leads to an inability to synthesize thymidine triphosphate. As well, F-dUMP can incorporate into DNA resulting in possible strand breaks and RNA resulting in errors in processing and functioning.

Low levels of the degradation enzyme dihydropyrimidine dehydrogenase (DPD) have been shown to correlate with poor response to 5-FU both experimentally and clinically.
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(Etienne et al. 1995; Fischel et al. 1997; Salonga et al. 2000). Inhibition of DPD using 5-ethynyluracil (Porter et al. 1992; Baccanari et al. 1993; Spector et al. 1993) and uracil (Takechi et al. 2002) leads to increased sensitivity of 5-FU. As well, the activity of 5-FU can be enhanced by the addition of folate in the form of 5-formyl-tetrahydrofolate (leucovorin), which increases the binding of 5-FdUMP to TS (Chabner et al. 2001).

1.3.2.2 Capecitabine

Capecitabine is used in the treatment of breast and colorectal cancers, similar to 5-FU, but since capecitabine is an orally administered prodrug of 5-FU the less invasive administration method is preferred (Table 1.1). The clinical half-life of capecitabine is longer than that for 5-FU, 1.3 hours versus 11 min, thus capecitabine is present for longer periods of time and increases the exposure time of cells to 5-FU. Capecitabine is able to achieve higher concentrations of 5-FU in tumour tissue than from direct administration of 5-FU (Ishikawa et al. 1998b; Ishitsuka 2000). Whereas 5-FU gives similar concentrations of drug in normal and tumour tissue, the drug levels after capecitabine administration are much higher in tumour tissue than in plasma and muscle tissue (Ishikawa et al. 1998b). Unlike 5-FU, capecitabine exhibits antimetastatic activity at low doses, which has been attributed to increased sensitivity of cells to capecitabine due to up-regulation of thymidine phosphorylase (dThdPase) by the metastatic factors IFNγ, TNFα and IL-1α (Ishikawa et al. 1998a).

Capecitabine is initially converted to 5-deoxy-5-fluorocytidine by carboxyl esterase in the liver and tumour tissue and then cytidine deaminase converts the cytidine base to uridine. Finally, conversion to 5-FU occurs by cleavage of the 5' deoxy sugar by dThdPase. 5-FU then exerts a cytotoxic effect on cells in the same method as mentioned above (section 1.3.2.1).
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1.3.2.3 Cytarabine

Cytarabine has a preferential effect on cells in S-phase and is effective against leukemia. Pre-clinical studies in mice using a L1210 ascites tumour model showed 50% cell kill of peritoneal ascites after a single dose of 200 mg/kg (unknown administration route) (Fietkau et al. 1984). The concentration of active metabolite of cytarabine is low due to a rapid half-life and elimination of the active metabolites (Peters et al. 1993b), which can be problematic since prolonged exposure of cells to cytotoxic levels of cytarabine is critical for its maximum activity (Hamada et al. 2002).

Cytarabine is used in the treatment of acute myelogenous leukemia and meningial leukemia (Table 1.1). The clinical schedule for cytarabine is 200 mg/m² daily for 5-7 days by continuous intravenous infusion or repetitive administration schedules to achieve high concentrations (Chabner et al. 2001; Hamada et al. 2002). After a bolus dose of 200 mg/m² a peak plasma concentration of 20 \( \mu \text{M} \) is achieved. Cytarabine has a rapid phase of disappearance of 10 min (half-life) and an elimination time of 2.5 hours. It has been shown that an inability to retain sufficient levels of cytarabine triphosphate, inhibitory to DNA, in cells is a problem associated with treatment (Peters et al. 1993b).

Cytarabine is actively transported into cells by nucleoside transporters used for the physiological nucleosides and is converted to the monophosphate nucleotide by deoxycytidine kinase. The di and tri phosphate nucleotides then compete with deoxycytidine triphosphate for incorporation into DNA. Incorporation blocks DNA polymerase activity and DNA repair due to improper base stacking from steric hindrance. The degree of DNA synthesis inhibition is related to the level of cytarabine incorporation. It has also been suggested that exposure of cells to cytarabine must occur for the duration of one cell cycle to expose cells during S-phase and effectively inhibit DNA synthesis (Chabner et al. 2001).
1.3.2.4 Gemcitabine

Unlike other pyrimidine analogues, the toxicity of gemcitabine, is not confined to cells in S-phase at the time of drug administration (Plunkett et al. 1996; Cappella et al. 2001; Chabner et al. 2001). Gemcitabine cause a dose dependent G1 block as shown by flow cytometry experiments, using BrdUrd to detect S-phase cells, where DNA synthesis resumes sooner after a lower dose (Milas et al. 1999). Tumour growth delays of 2.2 days occur after a single dose of 240 mg/kg gemcitabine and upon fractionated administration of 60 mg/kg every 3 days for 10 days this increases to 18 days (Cividalli et al. 2000). In vitro and in vivo studies have shown a substantial suppression of DNA synthesis after gemcitabine administration (Milas et al. 1999; Wexler et al. 2000; Cappella et al. 2001) and more recently the microregional effects relative to vasculature and the kinetics of proliferation have been reported by this lab (Huxham et al. 2004).

Gemcitabine was selected for clinical use based on its beneficial activity toward murine solid tumours and human xenografts (Hertel et al. 1990). It is primarily used in the treatment of solid tumours such as pancreatic, non-small cell lung and bladder (Table 1.1). The current clinical protocol for gemcitabine administration indicates a 30 min i.v. infusion of 33-40 mg/kg on days 1, 8 and 15 every 28 days giving a peak plasma concentration of 15-20 μM (Veerman et al. 1996; Chabner et al. 2001). Gemcitabine has a half-life of approximately 30-40 minutes that increases with age and a long terminal elimination of 14 hours relating to why gemcitabine is not S-phase specific.

Once inside the cell gemcitabine is phosphorylated by deoxycytidine kinase and further converted to the di- and triphosphate derivatives (Heinemann et al. 1988). Gemcitabine diphosphate interacts with ribonuclease reductase depleting the deoxyribonucleotid stores and the triphosphate form incorporates into DNA and RNA and
blocks DNA polymerase processing (Heinemann et al. 1990; Plunkett et al. 1995; Plunkett et al. 1996). The transport of gemcitabine into cells occurs via a family of nucleoside transporters (NT’s) and studies have shown that tumours are resistant to gemcitabine if these transporters are not expressed (Mackey et al. 1998). As well, blocking thymidylate synthase (TS) increases the number of equilibrative-sensitive NT’s expressed on the cell membrane which can lead to increased gemcitabine sensitivity (Rauchwerger et al. 2000). The activity of transported drugs, such as capecitabine, cytarabine and gemcitabine, could be affected if the levels of NT expression varies with distance from vasculature.

1.4 Radiosensitizers

The hypothesis that hypoxic cells in solid tumours hinder the effectiveness of radiation therapy led to the development of radiation sensitizers as a method to enhance the radiosensitivity of tumours (Adams and Dewey 1963; Adams et al. 1979; Overgaard 1994). The hope was that radiation sensitizers could be used to reach areas with chronically hypoxic cells, unlike oxygen that is diffusion limited, and sensitize cells by creating permanent DNA damage in the absence of oxygen.

After radiation treatment absorption of x-ray photons produces a secondary electron, which either has a direct effect on DNA or can interact with other molecules, such as water, to produce free radicals, which subsequently damage DNA (Hall 2000). Damage to DNA after radiation is “fixed” or made permanent by oxygen. This process involves radiation induced free radicals damaging DNA resulting in radical molecules in the DNA (DNA⁻) that oxygen (O₂) then reacts with producing the organic peroxide DNAO₂⁻ (Equation 1.1) (Koch 1998).
Equation 1.1: \[ \text{DNA}^* + \text{O}_2 \rightarrow \text{DNAO}_2^* \]

BrdUrd and IdUrd (iododeoxyuridine) act as oxygen non-specific radiosensitizers by enhancing the response of both oxic and hypoxic cells to radiation. The degree of radiosensitization depends on the amount of halogenated pyrimidine incorporated into DNA (Kaplan 1970). Compounds such as metronidazole, a 5-nitroimidazole, and misonidazole, a 2-nitroimidazole, were tested as radiosensitizers of which the 2-nitroimidazoles had the highest electron affinity, showed the most promise and advanced to clinical trials (Asquith et al. 1974; Denekamp and Harris 1975; Urtasun et al. 1977; Hall 2000). In general, these drugs failed because the neurotoxicity prevented the use of high doses, needed for effective radiosensitizing (Hall 2000). An alternate approach to radiosensitizers, using agents that are selectively toxic to cells at low oxygen tension, led to the discovery that tirapazamine is preferentially toxicity to hypoxic cells (Zeman et al. 1986).

1.5 Hypoxic cytotoxins

Hypoxic cytotoxins target the hypoxic cells in tumours that are refractory to radiation and chemotherapies. The first report of a hypoxic cytotoxin was in 1975 in a publication by Hall stating the radiosensitizer misonidazole (Ro 07-0582) was selectively cytotoxic to hypoxic cells (Hall and Roizin-Towle 1975) and shortly after in 1976 another report of selective toxicity to hypoxic cells by metronidazole (Foster et al. 1976). As well, Sutherland described preferential toxicity by metronidazole to non-cycling cells in the centre of spheroids, which he suggested could be due to hypoxia (Sutherland 1974). These nitroimidazoles compounds are reduced by reductases in hypoxic areas. Recently, RB 6145 (Jenkins et al. 1990), a prodrug of the bioreductively activated 2-nitroimidazole aziridine
analogue RSU-1069 (Adams et al. 1984) has showed promise as a hypoxic cytotoxin due to lower toxicity than RSU-1069. RB 6145 is further discussed in section 1.5.3. Interestingly, Brown has described that high doses of misonidazole (1200 mg/kg) administered i.p. to mice with EMT6 tumours causes central necrosis 24 hours after administration and a viable rim of tissue (0.2 mm) was left around the tumour periphery (Brown 1977).

Tirapazamine (3-amino-1, 2, 4-benzotriazine-1, 4-di-N-oxide) is the most clinically advanced hypoxic cytotoxin. Since the discovery of the initial benzotriazine compounds, many additional analogues have been synthesized and tested either with the notion of having increased cytotoxicity by attaching a DNA targeting agent (Hay et al. 2004) or by varying the side groups to change the solubility and reduction potentials to design a more successful agent (Kelson et al. 1998; Hay et al. 2003). A further discussion of tirapazamine and some analogues can be found in sections 1.5.1 and 1.5.2, respectively.

Quinone compounds were among the first tested hypoxic cytotoxins, but they were not clinically effective for this use (Lin et al. 1980). The quinone derivates are activated by one electron reduction by NADPH/cytochrome C(P450) reductase to a radical anion (Denny 2004). The two electron reduction of these compounds can occur via oxygen insensitive reductases making the compounds inactive, which may be related to poor clinical advancement (Beall and Winski 2000; Denny 2004).

Aliphatic N-oxides, of which the most interest has centred on the topoisomerase II (topo II) poison AQ4N, are another class of hypoxic cytotoxins (Denny 2004). The compounds are activated upon reduction of both N-oxides (Patterson 1993) and AQ4N has proceeded to clinical trials (Benghiat et al. 2004).
Other theories to target hypoxic cells include the development of cytotoxin releasing compounds or the use of anaerobic bacteria which grow selectively in the hypoxic environment to express genes encoding enzymes that can activate a prodrug (Denny 2004; Cerecetto et al. 2006).

1.5.1 Tirapazamine

Tirapazamine (SR 4233; 3-amino-1, 2, 4-benzotriazine-1, 4-di-N-oxide; WIN 59075; TPZ, Tirazone®), was initially synthesized in 1970 (Mason and Tennant 1970) for use as an antimicrobial (Seng et al. 1974). In 1986 tirapazamine was found to be preferentially toxic to hypoxic cells in culture (Zeman et al. 1986) and later shown to have the same effect in animals (Brown and Lemmon 1990; Kim and Brown 1994). The structure of tirapazamine (TPZ) and the overall scheme for tirapazamine reduction and subsequent activation leading to DNA strand breaks is shown in Figure 1.6. Tirapazamine is converted under conditions of low oxygen to a hydroxyl radical (Daniels and Gates 1994; Zagorevskii et al. 2003) and more recently proposed to form a benzotriazine radical upon conversion (Anderson et al. 2003). Both radicals are thought to cause DNA single and double strand breaks leading to cell damage and possibly induced apoptosis (Laderoute et al. 1988; Biedermann et al. 1991; Delahoussaye et al. 2001; Birincioglu et al. 2003). The DNA strand breaks are then stabilized in the absence of oxygen by tirapazamine or SR 4317, the two electron reduction product of tirapazamine (Jones and Weinfeld 1996). Tirapazamine has also been suggested to be a topo II poison, under hypoxic conditions, thus increasing the stability of topo II DNA double strand breaks (Peters and Brown 2002). The enzymatic reduction can occur in vitro via NADPH/cytochrome P450 reductases and cytochrome P450 (Walton and Workman 1990; Walton et al. 1992; Patterson et al. 1995), xanthine oxidase (Laderoute et al. 1988) and
DT-diaphorase (Patterson et al. 1994). Studies suggest that only the reduction of TPZ by various nuclear reductases leads to DNA damage (Evans et al. 1998; Delahoussaye et al. 2001). This is likely due to the short half-life of radical species and therefore a small distance within which they can cause damage. The one electron reduction potential is – 456 mV (Priyadarsini et al. 1996) and reports have shown that hypoxic cytotoxicity directly relates to the one electron reduction potential whereby a less negative (higher) reduction potential gave a higher relative hypoxic cytotoxicity ratio (Hay et al. 2003).
Figure 1.6: The conversion and activation of tirapazamine. Tirapazamine (TPZ) undergoes a 1 electron reduction in areas of low oxygen to form a tirapazamine radical (TPZ-) but under oxygenated conditions is oxidized back to its original form. The tirapazamine radical then forms either a benzotriazine radical (BTZ-) or hydroxyl radical (OH-) either of which can go on to damage DNA. The DNA radicals are “fixed” (stabilized) by either TPZ or SR 4317 the 2 electron reduction product, neither of which itself is toxic, leading to DNA single and double strand breaks. Adapted from (Siim et al. 2004).
Tirapazamine was the first hypoxic cytotoxin to be tested in clinical trials and is currently in active phase III clinical trials in combination with cisplatin and radiotherapy for the treatment of cervical cancer. Phase III trials for non-small cell lung and head and neck cancers are in various stages of completion or have been stopped. The most recent completed phase III studies have not been as promising as hoped, however tirapazamine was found to enhance the effects of cisplatin (von Pawel et al. 2000). Very few phase III trials have been completed and one issue with trials not showing the expected results could be due to the cohorts chosen and treated (Marcu and Olver 2006). The treatment outcome may depend on the hypoxic fraction of the tumours and the studies did not take the level of hypoxia in tumours into account. However, a study by the Trans-Tasman Radiation Oncology group examined the significance of pre-treatment tumour hypoxia on response showing that in patients with head and neck cancer 71% of tumours were hypoxic. In this study treatment with radiation and chemotherapy alone was less effective for patients with hypoxic tumours compared with the addition of tirapazamine to the treatment regime, which resulted in significantly less failures (Rischin et al. 2006). A comprehensive review of tirapazamine both clinically and preclinically has recently been published outlining the current status of tirapazamine (Marcu and Olver 2006).

The clinical dose limiting toxicity is most commonly muscle cramping. Other toxicities include nausea, neutropenia and reversible ototoxicity (Marcu and Olver 2006). Clinical reports suggest the maximum tolerated dose of tirapazamine in humans is 390 mg/m\(^2\) for which the \(C_{\text{max}}\) and AUC are 7.1 \(\mu g/ml\) and 1410 \(\mu g \cdot ml^{-1} \cdot \text{min}^{-1}\) in humans, respectively however a slightly lower dose of 330 mg/m\(^2\) is more commonly given (Graham et al. 1997). This is similar to the extrapolated \(C_{\text{max}}\) in C3H mice of 8.9 \(\mu g/ml\) for a dose of
11 mg/kg (0.06 mmol/kg) for which the AUC is much lower (192 μg·ml⁻¹·min⁻¹) as the half-life of tirapazamine is shorter in mice than in humans (15 min versus 56 min) (Minchinton et al. 1992; Graham et al. 1997). The half-life of tirapazamine is dose dependent and at the LD₅₀ (~ 80 mg/kg), close to the 60 mg/kg administered in this thesis work, the half-life in BALB/c mice is 42 minutes and the Cₘₐₓ is 56 μg/ml (Graham et al. 1997).

The initial experiments with tirapazamine examined cell kill in multiple animal and human cell lines and showed a large differential toxicity to hypoxic versus oxic cells (Zeman et al. 1986). Since then studies combining tirapazamine with radiation and chemotherapeutic agents have explored the synergy of the treatments. Studies combining tirapazamine with radiotherapy treatment showed an enhancement of tumour response attributed to the eradication of hypoxic, radiation resistant cells within tumours. (Zeman et al. 1986; Zeman et al. 1988; Dorie et al. 1994). One study in particular noted that while all three tumours studied showed enhanced response the tumour with the least response also had the lowest hypoxic fraction (Dorie et al. 1994).

Tirapazamine exerts a synergistic effect on tumour control when combined with cisplatin when it was given 2-3 hours before cisplatin, though the mechanism is not well understood (Dorie and Brown 1993; Siemann and Hinchman 1998). Another study contradicts this and suggests a synergistic effect was only seen when the agents were administered together (Lartigau and Guichard 1996). The chemosensitizing effect of tirapazamine on cisplatin may be due to the inability of cells to repair cross links caused by cisplatin if the DNA has been previously damaged by tirapazamine treatment (Dorie and Brown 1993; Kovacs et al. 1999). Further combination studies of TPZ with carboplatin, cyclophosphamide, doxorubicin, etoposide, 5-FU and taxol all showed an enhancement of
tumour control and all but 5-FU showed no or only a slight increase in the systemic cytotoxicity (Dorie and Brown 1997).

Koch showed the cytotoxicity of tirapazamine is oxygen dependent and cytotoxic radical formation can occur at levels of oxygen below 5% (Koch 1993). As well, experiments in vitro have shown tirapazamine is toxic at 100 μM under 2% oxygen and that at lower concentrations of drug a lower oxygen concentration is needed to cause cytotoxicity (Lartigau and Guichard 1995).

A lower than expected toxicity of tirapazamine on the central cells of multicellular spheroids (Durand and Olive 1992) and in the hypoxic regions of tumour xenografts (Durand and Olive 1997) was reported by Durand et al. suggesting limited penetration of tirapazamine due to bioreductive inactivation at intermediate levels of oxygen. Further in vitro experiments gave support to this theory by suggesting poor diffusion of tirapazamine through multilayered cell cultures may be due to consumption of drug at intermediate levels of oxygen (Hicks et al. 1998; Kyle and Minchinton 1999). Poor diffusion of tirapazamine would hinder the ability of the drug to reach its target cells, the chronically hypoxic cells far from vasculature.
1.5.2 Tirapazamine analogues

Extensive work synthesizing analogues of tirapazamine with varying side groups has been performed in order to develop compounds that are more soluble, and have higher one electron reduction potentials thus improving the biological activity (Kelson et al. 1998; Hay et al. 2003).

Three tirapazamine analogues from SRI International were used, which included SR 4754, 4482, and 4898 where the 3’ group is varied between the structures (Figure 1.7). Tirapazamine has an amino group in the 3’ position of the benzotriazine ring, which is substituted with a diethylaminoethylamine group, a hydrogen or a propyl group for SR 4754, 4482 or 4898, respectively. In theory, a 3’ alkyl will improve solubility of the compound and increase the electron affinity (Kelson et al. 1998). Removing H bond donors, such as hydrogen on the amine group, and replacing with alkyls (SR 4754) will increase solubility and diffusion. SR 4754 is three times more toxic to hypoxic cells than tirapazamine and has a higher hypoxic cytotoxicity ratio, unlike 4898 and 4482, which have a lower relative hypoxic cytotoxicity (RHC) compared to tirapazamine (Table 1.2) (Minchinton et al. 1992; Kelson et al. 1998). A higher RHC should translate into more cell killing and may relate to higher activity toward cells at intermediate oxygen tensions. None of the three analogues were found to be superior to tirapazamine in fractionated radiotherapy experiments in mice using a clonogenic assay for determination of effect (Kelson et al. 1998).
Tirapazamine (SR 4233)  \( \text{R} = \text{NH}_2 \)
SR 4754  \( \text{R} = \text{NH(CH}_2\text{)}_2\text{N(C}_2\text{H}_5\text{)}_2 \)
SR 4482  \( \text{R} = \text{H} \)
SR 4898  \( \text{R} = (\text{CH}_2\text{)}_2\text{CH}_3 \)

Figure 1.7: The structure of tirapazamine and the analogues studied in this thesis. Four benzotriazine analogues were studied in this work. The first, tirapazamine has an amino group in the 3' position of the benzotriazine whereas SR 4754 has a diethylaminoethylamine group, SR 4482 a hydrogen and SR 4898 a propyl group.

Table 1.2: Data for the benzotriazine analogues

<table>
<thead>
<tr>
<th>Compound</th>
<th>Tirapazamine (SR 4233)</th>
<th>SR 4754</th>
<th>SR 4482</th>
<th>SR 4898</th>
</tr>
</thead>
<tbody>
<tr>
<td>R group (Figure 1.7)</td>
<td>( \text{NH}_2 )</td>
<td>( \text{NH(CH}_2\text{)}_2\text{N(C}_2\text{H}_5\text{)}_2 )</td>
<td>( \text{H} )</td>
<td>( (\text{CH}_2\text{)}_2\text{CH}_3 )</td>
</tr>
<tr>
<td>Mol. weight (g/mol)</td>
<td>178.15</td>
<td>286.33</td>
<td>163.10</td>
<td>205.21</td>
</tr>
<tr>
<td>Aqueous solubility (mM)</td>
<td>13</td>
<td>36</td>
<td>18</td>
<td>12</td>
</tr>
<tr>
<td>HCR</td>
<td>77</td>
<td>114</td>
<td>64</td>
<td>112</td>
</tr>
<tr>
<td>E 1/2 (mV)</td>
<td>-332</td>
<td>-350</td>
<td>-190</td>
<td>-282</td>
</tr>
<tr>
<td>One electron reduction potential (mV)</td>
<td>-456(^b)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Half-life in mice (dose in mmol/kg) (^c)</td>
<td>15 min (0.06)</td>
<td>7 min (0.1)</td>
<td>2.1 min (0.2)</td>
<td>-</td>
</tr>
<tr>
<td>Dose mg/kg (mmol/kg)</td>
<td>60 (0.34)</td>
<td>80 (0.28)</td>
<td>100 (0.61)</td>
<td>90 (0.43)</td>
</tr>
<tr>
<td>% of LD(_{50})</td>
<td>77</td>
<td>70</td>
<td>68</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\) (Kelson et al. 1998)
\(^b\) (Priyadarshini et al. 1996)
\(^c\) (Minchinton et al. 1992)
1.5.3 RB 6145

RSU 1069 is selectively toxic to hypoxic cells, but the high clinical toxicity prevented further use of the compound (Adams et al. 1984). RSU 1069 is converted to an active form at levels of oxygen below 1% unlike tirapazamine which is converted at a higher level of oxygen (Koch 1993). RB 6145, a prodrug of RSU-1069, was later developed and found to have less toxicity (Jenkins et al. 1990). The structure of both compounds is shown in Figure 1.8. The maximum tolerated dose (MTD) for i.p. administration of RB 6145 to mice is 300 mg/kg and the drug has shown similar radiation enhancement as RSU-1069 in experimental tumours (Bremner 1993).

![Chemical structures of RSU 1069 and RB 6145]

**Figure 1.8: The structures of the 2-nitroimidazole drugs RSU-1069 and RB 6145.** RB 6145 is a brominated prodrug of RSU-1069 that cyclizes to form an aziridine ring under physiological conditions.
1.6 Vascular targeting agents

Anti-angiogenic agents, as discussed in section 1.1.3, are targeted towards growing tumour vasculature. A class of agents known as vascular disrupting or targeting agents (VTAs) selectively target the existing tumour vasculature. Typically these agents cause central necrosis in tumours within 24 hours of administration and leave a viable perfused rim of tissue around the outside of the tumour.

Initially, colchicine was discovered to cause vascular disruption and was the first report of a tubulin binding agent targeting vasculature (Seed et al. 1940; Ludford 1946; Ludford 1948). Later, reports were published showing the vinca alkaloids also caused vascular dysfunction. However, all of these compounds cause vascular disruption only at or near their maximum tolerated doses (Hill et al. 1993).

Two main groups of small molecule VTAs in clinical trials are the combretastatins, a class of tubulin binding agents, and DMXAA a non-tubulin binding agent that is thought to work via cytokine induction. The most widely studied VTAs are the combretastatins, a family of tubulin binding compounds derived from the South African Cape bushwillow tree, *Combretum caffrum*. Combretastatin A-4 (CA-4) was the most active compound, which was later made more soluble by producing CA-4 phosphate (CA-4-P) (Pettit et al. 1989; Pettit et al. 1995). CA-4-P causes microtubule dysfunction and reorganization of the cytoskeleton as well as disruption of the junction molecule vascular endothelial cadherin (Kanthou and Tozer 2002; Tozer et al. 2002; Vincent et al. 2005). Oxi 4503 (CA-1-P) has since been developed and shown increased toxicity compared to CA-4-P, but has not yet entered clinical trials (Pettit et al. 1987) (Hill et al. 2002). DMXAA (5,6-Dimethylxanthenone-4-acetic acid) a derivative of the less active FAA (flavone acetic acid) causes central vascular dysfunction,
similar to CA-4-P, but via a different mechanism that more likely involves induction of cytokines and subsequent disruption of the actin cytoskeleton (Hill et al. 1989; Baguley 2003; Tozer et al. 2005).

VTAs cause rapid shutdown of vasculature and it has been shown with CA-4-P (Tozer et al. 2001) and DMXAA (Zwi et al.) that within 20 and 15-30 minutes, respectively blood flow has ceased in affected areas of the tumour. Typically, tumours treated with CA-4-P then become necrotic within 24 hours, but a viable rim of tissue remains around the tumour periphery (Dark et al. 1997). The rim of remaining tumour tissue can become hypoxic within 1 hour after CA-4-P administration (El-Emir et al. 2005). DMXAA also causes the characteristic central necrosis with a remaining viable rim of tissue (Baguley 2003).

Another promising small molecule VTA, ZD6126 a colchicine analogue, shows the same central vascular necrosis in histological sections and ZD6126 has advanced to clinical trials (Blakey et al. 2002). Various other VTAs are being synthesized and tested such as AVE8062, a CA-4-P derivative, and a list of VTAs and their current clinical status can be found in a 2005 review by Tozer et al. (Tozer et al. 2005).

Other agents that are not small molecule inhibitors such as ligand based VTAs have been found to cause the same specificity for central tumour vasculature. These VTAs are targeted antibodies, peptides or growth factors that when administered cause vascular dysfunction (Thorpe 2004). For example, an antibody targeted to phosphatidyl serine, which is expressed on the outside of endothelial cells specifically in tumour vessels causes central necrosis after administration (Ran et al. 2005).

Photodynamic therapy also targets existing vasculature and 2 days after drug administration and subsequent light exposure (drug activation) central necrosis is seen in
tumours (Chen et al. 2006). The specificity is due to the location of the porphyrin compound, which stays in the vasculature for approximately 15 minutes after i.v. injection before extravasation into the tumour tissue. Therefore vascular targeting can be achieved by exposure of porphyrin to light a short time after injection.

As mentioned above, CA-4-P acts by disrupting the microtubule structure and therefore cell cytoskeleton, however DMXAA acts via disruption of the actin cytoskeleton via cytokine induction with no visible changes in microtubules (Tozer et al. 2005). The exact mechanism by which these compounds cause preferential toxicity to the central vasculature is unknown. It has been suggested that proliferating endothelial cells, changes in tubulin structure such as post translational modifications or variations in pericyte coverage may allow susceptibility to CA-4-P (Tozer et al. 2005). Whether these factors play a role in the susceptibility to other VTAs is not known. It is also possible the microenvironment can sensitize tumours to VTAs via such mechanisms as changes in hypoxia or increased vessel permeability (Tozer et al. 2005). However, it is important to note that all these various compounds are able to evoke toxicity specifically to central vessels through different mechanisms of targeting the vasculature.

1.7 NOS inhibition

Nitric oxide synthases (NOS) are a group of enzymes responsible for the synthesis of nitric oxide (NO) by the reaction below (Equation 1.2). NO is produced in the endothelial cells by endothelial NOS (eNOS) and signals to surrounding smooth muscle to relax thus dilating the blood vessels and increasing blood flow and oxygen delivery to tumours. Other NOS isoforms include neuronal NOS (nNOS) and inducible NOS (iNOS), which can be
expressed by tumour cells and elevated NOS expression has been found in several tumour types.

\textbf{Equation 1.2:} \quad \text{L-arginine} + \text{NADPH} + \text{O}_2 \leftrightarrow \text{citrulline} + \text{NO} + \text{NADP}(+) \\

The inhibition of NOS can decrease blood flow, red cell velocity and vessel diameter in tumours (Andrade \textit{et al.} 1992; Horsman \textit{et al.} 1996; Tozer \textit{et al.} 2001). Vessel perfusion 1 day after administration of NOS inhibitors decreases by up to 45\% depending on the NOS inhibitor (Davis \textit{et al.} 2002). As well, a small growth delay in SCCVII tumours has occurred after treatment with L-NNA (Korbelik \textit{et al.} 2000). The effects of NOS inhibition on tumours could be dependent on the levels of NOS expressed in the tumour and on the type of inhibitor used.

Non-specific NOS inhibitors such as L-NNA (N-omega-nitro-L-arginine), and L-NAME (N-omega-nitro-L-arginine methyl ester) have been used \textit{in vivo} combined with VTAs as potentiators of vascular dysfunction. NOS inhibition has been shown to increase the effects of small molecule VTAs such as CA-4-P (Parkins \textit{et al.} 2000) and ZD6126 (Wachsberger \textit{et al.} 2005; Cullis \textit{et al.} 2006) and of photodynamic therapy (Korbelik \textit{et al.} 2000). As well, in combination with NOS inhibition the hypoxic cytotoxin RB 6145 causes over 80\% tumour necrosis 1 day after treatment compared to 5-10\% with just RB 6145 alone. The increased effect of RB 6145 combined with a NOS inhibitor is thought to be due to vascular constriction from the decrease in NO and from the resulting hypoxia leading to increased activation of RB 6145.

Tirapazamine is an inhibitor of NOS and under hypoxic conditions tirapazamine is converted by NOS to a DNA damaging radical (TPZ\textsuperscript{-}, section 1.5.1) (Garner \textit{et al.} 1999).
Additionally, in cells transfected to overexpress eNOS an increase in tirapazamine cytotoxicity was seen (Chinje et al. 2003).

1.8 Thesis overview

The purpose of this research is to examine the microenvironment in entire tumour sections after drug administration and observe the interrelationships between vasculature, cell division, and hypoxia in response to the penetration of various chemotherapies. To determine the ability of a drug to penetrate through tumour tissue, focus will be placed on the effects of a drug by examining dividing cells in relation to their distance from blood vessels.

Understanding the process of extra-vascular drug distribution and microregional effects could improve current drug administration and delivery, as well as screening and rational design of new anti-cancer drugs. Observations of the tumour microenvironment, after administration of drug, allows for the regional determination of effect in relation to the entire tumour. For instance a lack of perfusion in the central area of a tumour can be realized, as is the case with tirapazamine (section 1.9.2).

In a typical experiment (Figure 2.1 in chapter 2 for an outline of the procedure) drug is administered to a mouse and following a period of time sufficient for the drug to take effect, the mouse will be given BrdUrd as a marker of S-phase cells and pimonidazole as a marker of hypoxia. Shortly before sacrifice, the fluorescent dye carbocyanine, which labels cells surrounding functioning vasculature, will be administered. The tumour is then excised, sectioned and the sections stained. Using a computerized image analysis system combined with a motorized microscope, developed in this lab, the microenvironment of entire tumour cryosections can be imaged for multiple markers. Initially the distribution of carbocyanine will be imaged to show which vessels were perfused after drug exposure and then the
sections will be stained for CD31 (an endothelial cell marker) and pimonidazole (a hypoxia marker) to indicate the position of all blood vessels (perfused and dysfunctional) and hypoxic areas. The same tumour section is then stained for incorporated BrdUrd, an indication of proliferating cells. The images are overlaid and analysed to find the distance between blood vessels and dividing cells as an indication of drug penetration. Variations on these experiments involved multiple doses of drug and changes in the lengths of time between drug exposure and tumour excision, as well as staining for other relevant markers.

The key aspects to these experiments are the abilities to revisit and stain the same tumour cryosection for various markers, to visualize the entire tumour section and to map the distribution of any marker as a function of distance to blood vessels.

1.8.1 Research objectives

One objective of this research is to establish which drugs penetrate well by visualizing the effect on cells with relation to vasculature. A second objective is to visualize tumour microenvironment changes in response to drug. From this, information regarding the regional effects of drugs in terms of where they have an effect and what changes they evoke can be gained. The central hypotheses are: (1) Chemotherapeutic agents will have a uniform effect on proliferating cells throughout a tumour. (2) A hypoxic cytotoxin will diffuse to hypoxic areas far from vasculature and exert an effect only on those cells. (3) If tirapazamine causes central vascular dysfunction via NOS inhibition then a NOS inhibitor will cause central vascular dysfunction in tumours.

1.9 Chapter summaries

The experiments in this thesis involved imaging markers of the tumour microenvironment in tumour cryosections after exposure to drug.
1.9.1 Overview of chapter 3 – Mapping the effects of antimetabolites

In this chapter the tumour microregional effects after pyrimidine analogue administration to mice were mapped to relate the location of proliferating cells relative to vasculature in human colon cancer xenografts (HCT-116). Gemcitabine showed a complete inhibition of proliferation throughout the tumour within 1 day after administration, however over time a re-emergence of dividing cells occurred in those cells far from vasculature. 5-FU showed an initial decrease in proliferation 1 day after treatment both near and far from vessels, although more of an effect on cells near vessels. With time the cells far from vessels returned to control levels of division but cells near vessels did not. Capecitabine, an oral prodrug of 5-FU, showed a decrease in division near vessels 1 day after treatment, but no effect on cells far from vasculature. However, proliferation in tumours treated with capecitabine remained below control levels suggesting a longer exposure to 5-FU after capecitabine administration. Cytarabine does not significantly change the overall proliferation of cells or have any observable effect in relation to distance from blood vessels.

We have concluded the differential effect on cell proliferation in relation to vasculature after gemcitabine administration is possibly due to limited penetration. 5-FU and capecitabine are more cell cycle phase specific than gemcitabine and both these compounds exhibited less effect on cells distal to vasculature possibly due to limited penetration. Additionally, the lack of effect with cytarabine in solid tumours could be due to sub-therapeutic exposure of all cells to the drug.

Clinically, gemcitabine is used for the treatment of solid tumours such as pancreatic lung and bladder and 5-FU and capecitabine are first line treatments for breast and colorectal cancers. Cytarabine, however is not successful for solid tumour treatment, but is used in the treatment of leukemia.
1.9.2 Overview of chapter 4 – Mapping the effects of hypoxic cytotoxins

The microregional effects of the hypoxic cytotoxin tirapazamine were determined by observing the changes in proliferation, blood vessels and hypoxia in tumour cryosections. Instead of a preferential effect on those cells far from vasculature, extensive central vascular dysfunction in HCT-116 and SiHa xenografts as well as SCCVII murine tumours was observed. Tirapazamine treated xenograft tumours had an absence of blood flow in the central area with a rim of functional vessels around the periphery of the tumour. The areas affected were replaced by necrosis over the following 1-2 days suggesting the disruption to vessels was permanent. The central area had no labelling for proliferation and hypoxia bleeding in from the surrounding outer rim of perfused tissue.

Other hypoxic cytotoxins were then examined, three of which are analogues of tirapazamine, SR 4754, 4482, 4898, and an unrelated 2-nitroimidazole prodrug RB 6145. SR 4754 caused central vascular dysfunction similar to tirapazamine exhibiting central areas that were non-perfused and then became necrotic 1-2 days later. 1 day after RB 6145 administration however, there was no effect on the central perfusion of tumours or on the levels of proliferation.

We found that tirapazamine and an analogue induce central vascular dysfunction in various tumour models and that this activity is likely related to toxicity towards blood vessels with intermediate oxygen tensions. This suggests tirapazamine exerts an effect on endothelial cells or perivascular cells and that the toxicity towards hypoxic cells far from vasculature may not be as important as previously believed.
Overview of chapter 5 - Determining the mechanism behind the vascular dysfunction caused by tirapazamine

In order to test whether the vascular toxicity by tirapazamine is due to toxicity toward blood vessels at intermediate levels of oxygen tirapazamine (60 mg/kg, i.p.) was administered to mice breathing carbogen. Tumour hypoxia can be decreased in mice breathing carbogen, a gas consisting of 95% oxygen and 5% carbon dioxide. Tirapazamine caused vascular dysfunction in 25% of the treated tumours, which was a decrease in response, however carbogen breathing did not prevent the vascular disruption caused by tirapazamine.

The vascular targeting effect of tirapazamine is similar to that of CA-4-P, a microtubule destabilizing agent that induced vascular dysfunction specifically in the centre of tumours. The ability of tirapazamine to destabilize microtubules and its effect on VE-cadherin was investigated to determine if the activity of tirapazamine occurs by the same path as CA-4-P. SiHa cells and HUVECs were exposed to tirapazamine (100µM) under 20% oxygen (oxic) and 0.1% oxygen (hypoxic) in glass slide chamber flasks. No effect on β-tubulin was seen after exposure to tirapazamine under hypoxic conditions, however under the same conditions an increase in DNA double strand breaks (γH2AX) was seen indicating tirapazamine was causing damage to cells. A fragmentation of the continuous membrane appearance of VE-cadherin, a membrane junction protein on endothelial cells, was seen after exposure to tirapazamine under hypoxic conditions.

Tirapazamine inhibits NOS, and under hypoxic conditions can be converted by NOS to an active radical form resulting in DNA strand breaks (Garner et al. 1999). We hypothesize the activity of tirapazamine could relate, in part, to NOS inhibition causing a constriction of blood flow and subsequent hypoxia which could potentiate the conversion of
tirapazamine to an active radical. To examine this possibility, we determined if NOS inhibition alone can cause central vascular dysfunction. The NOS inhibitor L-NNA was administered at increasing doses 20, 60 and 180 mg/kg and 1 day later central necrosis occurred in some treated tumours. Evidence of vascular dysfunction at early time points of 2, 6 and 12 hours after 60 mg/kg L-NNA, was seen only in small portions of some of the treated tumours.

The ability of tirapazamine to cause vascular dysfunction may involve more than one direct event. Inhibition of NOS may lead to increased vessel constriction resulting in a lower oxygen tension and subsequent increased conversion of tirapazamine to an active cytotoxin thus causing damage to endothelial cells.

1.10 Impact of the research

Early work in this thesis with the pyrimidine analogues showed a differential effect on cells far from vasculature compared to cells proximal to vasculature. This indicates that penetration of drug to all cells in a tumour can be hindered and should be considered in the treatment course planned for patients in terms of time between doses and optimal administration length. This work could provide vital information for planning of treatment regimes before new drugs enter clinical trials. As well, the knowledge of which cells are affected after treatment can allow for the better design of new agents targeted towards improving drug penetration through tissue.

During the course of this research it became apparent that regional changes to the microenvironment are created by drugs and observing the entire tumour section provided an advantage compared with the observation of selective portions of the tumour. By observing the changes in the microenvironment, as a whole in tumour sections, an unexpected effect of
the hypoxic cytotoxin tirapazamine was observed. Rather than seeing an effect on those cells far from vasculature that are chronically hypoxic, extensive vascular dysfunction in the centre of tumours was observed. These effects were characterized by an absence of perfusion in central vessels, a decrease or absence of cell proliferation and changes in the distribution of hypoxia. We were also able to show that the unaffected rim of tissue surrounding the dysfunctional tumour centre contains perfused tumour blood vessels.

It is important to consider that this effect can be missed by examining tumour response to drug using growth delay assays, clonogenic assays, flow cytometry analysis, or even immunohistochemical analysis of one marker in selected areas of a tumour section. The bulk of the literature on tirapazamine indicates a preferential effect of the drug on hypoxic cells and enhanced tumour control after combination with radio- and chemotherapies. However, the effect of tirapazamine in tumours may be due to vascular dysfunction, which would also remove the hypoxic cells in a tumour and contribute to synergistic effects with radio- and chemotherapies. Knowing an alternate activity of a drug like tirapazamine could provide more rational timing for administration and for combination with other treatments.

As mentioned above (section 1.6) there are many diverse agents that cause central vascular dysfunction. CA-4-P and DMXAA for example are both VTAs yet act via different mechanisms to target vasculature. Other agents such as antibodies to molecules expressed on the cell surface of endothelial cells and drugs that are activated while still in the vasculature (photodynamic therapy) cause similar vascular dysfunction and central necrosis.

The key aspect is that all these very different compounds cause the same end result to central vasculature and are unable to effectively target the vasculature surrounding the outside of the tumour. Understanding the specificity of these agents to the central vasculature
and why the vessels and tissue in the periphery are not affected to the same extent is important for understanding how to target these areas either with new drug targets or with combination therapy that will target the rim.

From this work it is clear the effects of drugs on the tumour environment are not uniform throughout the tumour tissue and do not necessarily occur through the expected mechanism. It is important to examine the microregional effects of drugs to determine their optimal administration and to provide a rationale for combination treatment and the design of new agents.
CHAPTER 2 – MATERIALS AND METHODS
2 MATERIALS AND METHODS

2.1 Cell culture

All cells were maintained in vitro at 37.5 °C with 5% CO₂/5% O₂ balance nitrogen. Unless otherwise specified cells were grown in minimum essential medium (MEM; Hyclone, Logan, UT, USA) supplemented with 10% foetal bovine serum (FBS; Gibco, Burlington, ON, Canada) or 10% bovine growth serum (BGS; Hyclone).

2.1.1 HCT-116

HCT-116, human colorectal carcinoma cells, were purchased from American Type Culture Collection. Cells were passaged every 3-5 days upon reaching 80-90% confluence. Cells grown for implantation were maintained according to procedure but supplemented with 50 units/ml penicillin/ 50 μg/ml streptomycin sulfate (Gibco) during expansion.

2.1.2 SiHa

SiHa, human cervical squamous carcinoma cells, were purchased from American Type Culture Collection. Cells were passaged every 3-5 days upon reaching ~ 80% confluence.

2.1.3 WiDr

WiDr, human colorectal adenocarcinoma cell suspensions for tumour implant were obtained from Dr. P. L. Olive at the BC Cancer Research Centre (BCCRC).

2.1.4 SCCVII

SCCVII, murine squamous carcinoma cells, were obtained in 1983 from Dr. M. Horsman (Stanford University). Cells were passaged every 3-5 days upon reaching 80-90% confluence.
2.1.5 HUVEC

Human umbilical vein endothelial cells (HUVEC) were a gift from Dr. A. Karsan (BCCRC). HUVECs were maintained in MCDB 131 medium (Gibco) supplemented with 10% FBS (HyClone), 5% BGS, 20 μg/ml endothelial cell growth supplement (ECGS; BD Biosciences, Mississauga, ON, Canada), 15 units/ml Heparin (Sigma-Aldrich, St. Louis, MO), 10 mM l-glutamine (Sigma-Aldrich), 50 units/ml penicillin/ 50 μg/ml streptomycin sulfate (Gibco). Cells were passaged every 7 days and medium was changed every 2-3 days. Cells were used between passages 1-4.

2.2 Tumour implantation

Female NOD/SCID and C3H/HeN mice were bred and maintained in our institutional animal facility in accordance with the Canadian Council on Animal Care guidelines. The experiments described in this thesis were approved by the Animal Care Committee of the University of British Columbia. Mice were allowed free access to standard laboratory rodent food and water.

2.2.1 HCT-116 xenografts

HCT-116 cells (50 μl of 1.6 x 10^8 cells/ml) were implanted from in vitro subcutaneously (s.c.) into the sacral region of female NOD/SCID mice. The mice used were between 8-20 weeks of age and ranged in weight from 20-28 g. The weight of the excised tumours was 84 ± 35 mg (mean ± std. dev.). Cells were used between passages 2-20 as tumours grown from cells with a later passage had a less corded structure.
2.2.2 SiHa xenografts

SiHa tumour xenografts were maintained in female NOD/SCID mice and cells (50 μl of 20 x 10⁶ cells/ml) were implanted s.c. into the sacral region of mice 11-13 weeks of age, weighing between 22-26 g. The mean weight of the excised tumours was 85 ± 31 mg.

2.2.3 WiDr xenografts

WiDr tumour xenografts were maintained in the hind legs of female NOD/SCID mice and cells (50 μl of a 40 x 10⁶ cells/ml) for experimental tumours were implanted s.c. into the sacral region of mice 14-16 weeks of age weighing between 19-26 g. The mean weight of the excised tumours was 303 ± 99 mg.

2.2.4 SCCVII

SCCVII tumours were maintained in female C3H/HeN mice and cells (50 μl of 4 x 10⁶ cells/ml) were implanted s.c. into the sacral region of mice 10 or 52-56 weeks of age, weighing between 26-46 g. The mean weight of the excised tumours was 121 ± 46 mg.

2.3 Tumour mapping - experimental procedure

The following scheme outlines the tumour mapping procedure (Figure 2.1). Tumours treated in the same experiment were all sectioned, stained and imaged together as a batch of slides.
Figure 2.1: Tumour mapping experimental procedure. Drug is administered to a tumour bearing mouse and hours to days later BrdUrd (marker of S-phase cells) and pimonidazole (marker of hypoxia) are given 2 hours prior to tumour excision. Five minutes prior to tumour excision carbocyanine is administered to mark vessel perfusion. Once the tumour is excised it is frozen on an aluminium block at -20 °C and then embedded in OCT. Tumour cryosections, 10 μm thick, are then cut and the tumours imaged for carbocyanine. The tumour sections are dried overnight before staining. The next day the sections are fixed and stained for CD31 (marker of vasculature) and hypoxia (pimonidazole). The sections are wet mounted and imaged. Once imaged the same tumour section is then stained for incorporated BrdUrd. Once the final staining is complete the tumours are mounted with permount and imaged.
2.4 Treatment

Mice were treated when their tumour volume reached 100 mm$^3$ as measured using calipers and the formula $V = \pi/6(lhxw)$. Typically between 4-6 tumours were used for each dose and time point.

All antimetabolites were obtained from the British Columbia Cancer Agency pharmacy. 5-Fluorouracil and cytarabine were from Faulding Canada Inc. Montreal QC Canada, gemcitabine was from Eli Lilly Canada. Inc. Toronto ON, Canada, and capecitabine Hoffmann-La Roche Ltd. Mississauga, ON, Canada. Tirapazamine and SR 4317 were synthesized as described in section 2.6. SR 4754, SR 4822, and SR 4898 were generously donated from SRI International. RB 6145 was donated from Dr Ian Stratford. L-NNA was purchased from Sigma-Aldrich Canada Ltd. Oakville, ON, Canada. All compounds were administered via intraperitoneal (i.p.) injection unless otherwise stated.

2.4.1 Untreated tumours

Controls were generally not administered any vehicle, but were weighed and the tumours measured at the same time as treated mice. Controls were given BrdUrd and pimonidazole 2 hours before sacrifice and carbocyanine 5 minutes before sacrifice. Due to the high volume of tirapazamine administered (1-1.2 ml) controls in some experiments with tirapazamine were given 1 ml of saline to control for the volume of injection.

2.4.2 Antimetabolites

Gemcitabine was administered at doses of 20, 40, 80, 160, and 360 mg/kg 24 hours prior to tumour excision, or at 240 mg/kg and the tumours excised 1, 2, 3, 4, and 6 days later, or 40 mg/kg on days 1, 4, 7, and 10 and the tumours excised on days 9, 12, 14, 16, and 18. 5-
Chapter 2 – Materials and methods

FU was administered at a dose of 120 mg/kg and the tumours excised 1, 2, 3, 4, 5 and 6 days later. Capecitabine, a prodrug of 5-FU, and cytarabine were given at doses of 750 and 400 mg/kg respectively and the tumours excised 1, 2, 3, 4, and 6 days later. The doses of drug given were near the MTD for each drug.

2.4.3 Hypoxic cytotoxins

Tirapazamine was administered at 60 mg/kg (0.34 mmolkg⁻¹, ~ 85% of the MTD) using a 1.25 mg/ml solution in saline. Tumours were excised on days 1-4 or 1, 2, 6, 8, and 24 hours after drug administration. A range of doses of tirapazamine were given including 30, 40, 48, and 55 mg/kg and the tumours excised 1 day later.

SR 4754 was administered at 75 mg/kg and the tumours excised 1 day later and at 80 mg/kg (dosage increased due to high MTD and incomplete response at 75 mg/kg) and the tumours excised 1, 2, 3 and 4 days later. SR 4754 was also given at 80 mg/kg via (i.v.) administration and the tumours excised 1 day later. SR 4482 and SR 4898 were administered at 100 and 90 mg/kg respectively and the tumours excised 1 day later. SR 4317 was administered at 162 mg/kg and the tumours excised 1 and 2 days later.

RB 6145 was administered at 250 mg/kg dissolved in cold saline (pH = 4) 10 minutes prior to injection and the tumours excised 1 day after administration.

2.4.4 5-Bromo-2-deoxyuridine

5-bromo-2-deoxyuridine (BrdUrd; Sigma-Aldrich) was administered at 1500 mg/kg 2 hours prior to tumour excision as a 30 mg/ml solution in saline. In cases where tumours were excised 2 hours after tirapazamine BrdUrd was co-administered with tirapazamine or in some experiments not given. When L-NNA was administered and the tumours excised 2 hours later BrdUrd was given 1 hour before excision.
2.4.5 Pimonidazole

Pimonidazole (HypoxyprobeTM-1 Kit, Chemicon International Inc., Temecula, CA, USA) was administered to mice at a dose of 60 mg/kg 2 hours prior to tumour excision.

2.4.6 Carbocyanine

Carbocyanine [3,3-diheptyloxacarbocyanine iodide, DiOC7(3)] (Molecular Probes, Eugene, Oregon, USA) was dissolved in 75% DMSO/25% H2O and ~75 µl was administered i.v. 5 minutes prior to sacrifice of the mouse.

2.4.7 Carbogen breathing

Carbogen (95% O2 and 5% CO2) was delivered to a modified cage through a water warming apparatus at 5 litres/min. Mice were placed in a cage with carbogen flow for 10 minutes before tirapazamine injection. After injection the mice were left breathing carbogen for 3 hours. The three hours after administration constitutes approximately 5 half-lives for the 60 mg/kg of tirapazamine (half-life ~ 35 minutes). Mice were then returned to air breathing for the remaining 21 hours before tumour excision.

2.4.8 L-NNA

L-NNA (N-omega-nitro-L-arginine) was dissolved in saline and administered at doses of 20, 60 and 180 mg/kg and the tumours excised 1 day later. As well, 60 mg/kg of L-NNA was given 2, 6 and 12 hours before tumour excision.

2.5 Tumour excision

Tumours were excised, weighed and frozen to −20 °C on an aluminium block, and then covered in embedding medium (O.C.T., Tissue-TEK, Torrance, CA, USA). Embedded tumours were kept at −20 °C for short periods until sectioned and stored at −80 °C.
2.6 Chemical synthesis

Both 3-amino-1,2,4-benzotriazine 1,4-dioxide (Tirapazamine; SR 4233) and 3-amino-1,2,4-benzotriazine 1-oxide (SR 4317) were synthesized using a modified method of Fuchs et al. (Fuchs et al. 2001).

Cyanamide, NaOH, and 2-nitroaniline were purchased from Sigma-Aldrich Canada Ltd and were of purity 98% or higher. HCl, 30% H₂O₂ and all other solvents were purchased from Fisher Scientific as A.C.S. grade or higher and used as supplied without further purification. DMSO-d₆ was purchased from Cambridge Isotope Laboratories Inc.

Solution ¹H NMR spectra were obtained using a Bruker AV-300 (300.13 MHz) FT-NMR spectrometer. ¹H chemical shifts are given as (ppm), with reference to the residual solvent peak as the internal standard, relative to tetramethylsilane. NMR multiplicity defined as: d = doublet, dd = double doublet, m = multiplet, td = triple doublet, bs = broad singlet.

2.6.1 Synthesis of SR 4317

Deviations from the methods of Fuchs et al. included the addition of acetone to the reaction vessel before the product was vacuum filtered. The yellow solid was then washed with acetone, followed by water and finally ethyl acetate. The product was recrystallized from 2-propanol. ¹H-NMR (DMSO-d₆) δ 8.12 (dd, 1H), 7.76 (td, 1H), 7.53 (dd, 1H), 7.30-7.35 (m, 3H).

2.6.2 Synthesis of tirapazamine

Tirapazamine was synthesized from SR 4317, the monoxide precursor of tirapazamine. Once oxidation of SR 4317 to tirapazamine was complete water was added to the reaction vessel and the solution was rotovaped to a volume of 5 ml. 2-Propanol was added and a bright orange solid precipitated. The solid was collected by vacuum filtration.
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and recrystallized from 2-propanol. $^1$H-NMR (DMSO-$d_6$) $\delta$ 8.20 (dd, 1H), 8.14 (dd, 1H), 8.03 (bs, 2H), 7.93 (td, 1H), 7.56 (td, 1H).

2.7 Immunohistochemistry

Tumour cryosections (10 $\mu$m thick) were cut with a cryostar HM560, air dried for 24 hours and then fixed in a 1:1 mixture of acetone-methanol for 10 minutes at room temperature and blocked with 3% H$_2$O$_2$. A 10 minute serum block (2% goat serum) was done before antibodies were added and all antibodies were made up in PBS with 2% goat serum.

2.7.1 Vasculature

Vasculature was stained using a PECAM/CD31 antibody (1:100 dilution; BD Pharmingen, San Jose, CA). An Alexa 546 goat anti-rat secondary (1:50 dilution; Molecular Probes, Eugene, OR) was used to detect CD31. The primary antibodies, CD31 and pimonidazole (section 2.7.2), were mixed together and the secondaries were applied separately.

2.7.2 Hypoxia

For tumour xenografts hypoxia was detected by staining for bound pimonidazole using HypoxyprobeTM-1Mab1 (1:50 HypoxyprobeTM-1 Kit, Chemicon International Inc.). An Alexa 488 goat anti-mouse secondary (1:100 dilution; Molecular Probes) was used to detect the primary. After the cryosections were stained for both CD31 and pimonidazole, slides were mounted with PBS and imaged (section 2.8). Due to the variability of pimonidazole intensity from the staining procedures, data for the percentage of hypoxia is not reported and instead visual observations of the images are used.
For mouse tumours bound pimonidazole was detected using a rabbit anti-pimonidazole antibody (1:1000 dilution, gift from Jim Raleigh) and an Alexa 488 goat anti-rabbit secondary (1:200 dilution; Molecular Probes).

2.7.3 S-phase cells

After the slides were imaged for vasculature and hypoxia they were rinsed in PBS, placed in distilled water for 10 minutes and then treated with 2 or 4 M HCl (depending on the iron content of the slides – the higher the iron content as judged by a green versus white coloured slide the higher the acid concentration) at room temperature for 1 hour followed by neutralization for 5 minutes in 0.1 M sodium borate. Slides were then washed in distilled water and transferred to a PBS bath. Subsequent steps were each followed by a 5 minute wash in PBS. S-phase cells were detected by staining for incorporated BrdUrd using a monoclonal mouse anti-BrdUrd (1:200 dilution; clone BU33, Sigma-Aldrich) followed by an anti-mouse peroxidase conjugate antibody (1:100 dilution; Sigma-Aldrich) and a metal enhanced DAB substrate (1:10 dilution; Pierce, Rockford, IL). Slides were then counterstained with hematoxylin, dehydrated and mounted using Permount (Fisher Scientific, Fair Lawn, NJ, USA) before imaging.

2.7.4 Cells in cycle

Some tumours were stained for Ki67 an endogenous marker of cell division, which labels all phases of the cell cycle. Ki67 was detected using a polyclonal rabbit anti-Ki67 antibody (1:200 dilution; Abcam Inc. Cambridge, MA, USA) and an Alexa 488 goat anti-rabbit secondary (1:150 dilution; Molecular Probes).
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2.7.5 Apoptosis

Apoptosis was detected using a rabbit anti-activated caspase 3 antibody (1:500 dilution; Pharmingen) and a biotinylated goat anti-rabbit secondary (1:50 dilution; Pharmingen). The secondary was amplified with extravadin HRP (1:100 dilution; Horse radish peroxidase; Sigma) and visualized with metal enhanced DAB substrate (1:10 dilution; Pierce).

2.7.6 Macrophages

Macrophages (pan, F4/80 antigen) were stained using a rat anti-mouse antibody (1:200 dilution; clone BM8, Cedarlane Laboratories Ltd.) and an Alexa 546 goat anti-rat secondary (1:200 dilution; Molecular Probes).

2.7.7 DNA double strand breaks

DNA double strand breaks were detected with a mouse anti phosphorylated H2AX (γH2AX) antibody (1:100 dilution; Upstate-Millipore, Mississauga, Ontario). An Alexa 488 goat anti-mouse secondary (1:100 dilution; Molecular Probes).

2.8 Image acquisition

Two microscope systems were used during the course of this work. System 1 is the first system used until early 2006 (the majority of the work utilized this system). The second system was developed and used from mid 2006 onwards.

2.8.1 System 1

The imaging system consisted of a fluorescence microscope (Zeiss III RS, Oberkochen, Germany), a cooled, monochrome CCD video camera (model 4922, Cohu, San Diego, CA, USA), frame grabber (Scion, Frederick, Maryland, USA), a custom built
motorized x-y stage and customized NIH-Image software (public domain 
age allowed for tiling of adjacent microscope fields of view. Using this system, images of 
etire tumour sections were captured, typically 1cm² in size at a resolution of 1 μm/pixel. 
Images of carbocyanine fluorescence within the sections were obtained prior to CD31 
immunostaining using a 450-480 nm excitation filter and a 525 nm long pass emission filter. 
Once immunostained for CD31 slides were imaged while wet mounted in PBS using a 510-
555 nm excitation filter and a 575-640 nm emission filter. Slides stained for pimonidazole 
were imaged under PBS using the same filter set as for carbocyanine. The slides were then 
immunostained for BrdUrd and bright field images of BrdUrd positive staining obtained.

2.8.2 System 2

The imaging system consisted of a robotic fluorescence microscope (Zeiss Imager Z1, 
Oberkochen, Germany), a cooled, monochrome CCD camera (Retiga 4000R, Q Imaging, 
Vancouver, BC, Canada), a motorized slide loader and x-y stage (Ludl Electronic Products, 
Hawthorne, NY, USA) and customized NIH-ImageJ software (public domain program 
developed at the U.S. National Institutes of Health, available at http://rsb.info.nih.gov/ij/)
running on a G5 Macintosh computer (Apple, Cupertino, CA, USA). The system allowed 
automated slide loading, focusing and tiling of adjacent microscope fields of view. Using this 
system, images of entire tumour cryosections were captured at a resolution of 0.75 μm/pixel.
Images were obtained similarly to System 1 (2.8.1).

2.9 Composite colour images

Once the individual images of cryosections were acquired the layers were stacked, the 
CD31 and carbocyanine layers were given a threshold and a composite colour image was
produced with the brightfield BrdUrd/hematoxylin layer (no threshold). The images show CD31 vessels that are perfused (dark blue) surrounded by regions of perfusion (light blue), unperfused vessels (red), and BrdUrd labelling (greyscale). An unperfused vessel (red) is defined as any CD31 positive area that is negative on the carbocyanine layer (has no light blue staining).

2.10 Image analysis

Using the NIH-Image software application and customized algorithms, images of carbocyanine fluorescence, CD31 fluorescence, pimonidazole and BrdUrd/tissue staining from each tumour section were overlaid and cropped to isolate the tumour area, and staining artifacts were removed (Figure 2.2A). The areas of necrosis were then removed by cropping (Figure 2.2B). For analysis, positive regions of staining were identified by selecting all pixels that were 2.5 standard deviations above background levels. CD31 or carbocyanine positive regions that were less than $5 \mu m^2$ in size were considered artifacts and removed from analysis. For the tirapazamine experiments where non-perfused tissue occurs the areas of non-perfused tissue were also cropped (Figure 2.2C). Percentage values are reported as the mean ± standard deviation unless otherwise stated. Analysis is performed on 1-2 cryosections per tumour from sections cut in the centre of the tumours.
Figure 2.2: Understanding the areas of necrosis and non-perfused tissue. The images A, B and C are of the same HCT-116 tumour, 2 days after tirapazamine treatment, which had areas of necrosis (N) and areas of non-perfused tissue (U) and are used to explain how the analysis of these areas was performed. (A) A tumour cropped to remove the outside areas beyond the tumour (indicated by the black line). (B) The same tumour cropped to remove the necrotic areas. (C) The same tumour cropped to remove the unperfused areas. The percent necrosis is calculated by dividing the number of pixels removed in B (A-B) by the number of pixels in A. The percent non-perfused tissue is calculated by dividing the number of pixels removed in C compared to B (B-C) divided by the number of pixels in A.

2.10.1 BrdUrd labelling in untreated HCT-116 xenografts

For the overall BrdUrd analysis, the fraction of BrdUrd positive tissue was taken as the number of BrdUrd positive pixels over the total number of pixels. Necrotic areas are visually identified and removed before analysis. For the tirapazamine experiments, where vascular dysfunction was involved and therefore areas did not have BrdUrd, the fraction of BrdUrd labelling was only analysed in the perfused regions where there was blood flow and BrdUrd labelling (Figure 2.2C).
Experiments were performed to validate the consistency of BrdUrd staining in untreated HCT-116 tumours on multiple sections from within the same tumour and between three different untreated tumours. Previous work in this lab has shown 1500 mg/kg to be a more than sufficient dose of BrdUrd to reach and label all cells in the tumour (Kyle et al. 2003). The overall fraction of BrdUrd labelling was consistent within sections from the same tumour (Figure 2.3A). The labelling between different untreated tumours was heterogeneous with the highest staining in tumour I (28 ± 1.9%) and the lowest in tumour II (13 ± 1.8%) with tumour III (18 ± 1.9%) falling in the middle (Figure 2.3A).

The data for the fraction of BrdUrd labelling with relation to vasculature, as with the above overall fraction of BrdUrd data, is consistent between sections of the same tumour and varies between the tumours (Figure 2.3B). Tumour I has a peak staining near vessels of between 35% and 45% and does not fall below 30% on the far side. Tumours II and III show the more typical staining where the fraction of BrdUrd falls to approximately half far from vessels. However the peak fraction of BrdUrd for tumour II (~ 30%) is lower than for tumour III (~ 40%), which is consistent with the overall BrdUrd staining data.

Due to the inter-tumour heterogeneity 5 untreated control tumours are a part of every experimental group. The untreated tumours are from the same batch of cells as the experimental tumours and are implanted into mice of the same age and size. Variability can also arise in the intensity of BrdUrd staining with each batch of staining therefore the experimental controls are always stained with the batch of slides from the same experiment. The corded structure of the cells would change depending on the passage number of the cells and tumours from cells passages 15-20 could be less corded than tumours from cells on an earlier passage.
Figure 2.3: Validation of BrdUrd staining in untreated HCT-116 tumour xenografts.
(A) The overall fraction of BrdUrd labelling in 3 different control tumours I, II, and III. Each bar represents data from a different section taken at 100 µm intervals through the centre of the tumour. (B) The fraction of BrdUrd labelling at each distance from vasculature for the same three tumours, respectively. Each line represents data from a different section corresponding to the bars in part (A).
2.10.2 Distance to vasculature

A marker, such as BrdUrd, can be spatially related to vasculature by measuring the distance from each point in the tissue to the nearest CD31 positive pixel (marker of vasculature) and noting if it were BrdUrd positive or negative. That is the algorithm will find each pixel in the BrdUrd layer and determine, based on a threshold, if it is positive or negative. Then it will calculate the distance to the nearest blood vessel by finding the nearest CD31 positive pixel based on a separate threshold for CD31. The data was tabulated to determine the fraction of BrdUrd positive pixels of the total number pixels found at each distance to a blood vessel. The fraction of BrdUrd positive tissue was then expressed as a fraction of the controls, where the averaged control level was 1, by dividing the treated values at each distance by the value for the average of all controls at that distance.

2.10.3 Fraction of perfused vessels

The fraction of perfused vessels was determined by comparing CD31 and carbocyanine labelling of vasculature. Raw images for the two stains were first given a threshold to identify positive staining. Then, on the CD31 images, positively stained objects were counted and classified as being perfused if they were co-labelled by carbocyanine (minimum 10% of pixels in each object).

2.10.4 Percentage of necrotic tissue

Images were cropped to remove areas of necrotic tissue (Figure 2.2B). The total number of pixels was calculated and the proportion of necrosis was calculated by dividing the number of pixels that were necrotic by the total number of pixels for each tumour. Necrotic areas show no immunohistochemical staining and appear cobwebbed (Figure 2.2A).
2.10.5 Percentage of non-perfused tissue

Images were cropped to remove areas that consisted of non-perfused tissue. Perfused regions are areas of viable tissue where both CD31 and carbocyanine are labelling vasculature, as well BrdUrd labelling occurs (Figure 2.2C). In contrast, non-perfused areas have positive CD31 staining and cells stain with hematoxylin, however these areas have no carbocyanine and no BrdUrd labelling. The total number of pixels for the non-perfused areas were obtained and the proportion of non-perfused tissue was calculated by dividing the number of pixels that were non-perfused by the total number of pixels for each tumour. For the SCCVII tumour, which has a high percentage of non-perfused vessels due to intermittent blood flow, the areas with non-perfused vessels have BrdUrd labelling and are not included in the calculation of areas considered to be non-perfused tissue caused by tirapazamine treatment.

2.11 Statistical analysis

Statistical analysis was performed using GraphPad Prism 4. Nonparametric equations were used in all instances and a $P$ value $<0.05$ was considered significant. A Kruskal-Wallis test was used to find significance within a group of samples and a Dunn's Multiple Comparison test was used to find the significance of a treated versus untreated sample in a group of samples. A Mann-Whitney U test was used to find the significance between two samples not in a group. Generally 4-6 mice per group were used and 1-2 sections per tumour were analysed. In some cases ($n=8$), repeat experiments are combined providing data for all tumours from the same treatment, but from different experimental batches.
2.12 Hypoxia chamber flask experiments

The following scheme outlines the experimental procedure for the in vitro hypoxia experiments performed using chamber flasks (Figure 2.4). Slides from flasks treated during the same experiment were all stained together.

**Figure 2.4: Hypoxia chamber flask experimental procedure.** Cells are seeded in chamber flasks with a removable plastic cover on a glass slide. The cells are grown for 1-4 days and then gassed with either 20 or 0.1% oxygen. Chambers were evacuated on high flow (1 L/min) for 10 minutes and left to equilibrate on low flow (5 ml/min) for 30 minutes. Tirapazamine is added and the cells exposed for 90 minutes. Pimonidazole is added 30 minutes later, if used. After treatment cells are rinsed, the glass slide is removed and the cells are immediately fixed for staining. Cells are then stained and imaged for various markers.
2.12.1 Cell seeding

Lab-Tek® chamber slide flasks with glass slides (Nalge Nunc Int., Rochester NY) were pre-treated with collagen (type I, calf skin, Sigma) in 70% EtOH for 30 minutes and rinsed with PBS before cells and medium were added. Cells were maintained in vitro as specified in section 2.1.

2.12.1.1 SiHa

2.5 x 10^5 SiHa cells were plated in a flask with 4 millilitres (ml) of medium and grown for 1-2 days before performing experiments.

2.12.1.2 HUVEC

2.0 x 10^5 HUVECs were plated in a flask with 4 ml of medium and grown for 1-4 days (depending on level of confluence desired). The medium was changed in the flasks every other day.

2.12.2 Gassing

Flasks containing cells were transferred to a non-humidified chamber at 37 °C to be gassed through tubing with either 20% O_2 / 5% CO_2 or 0.1% O_2 / 5% CO_2 (balance nitrogen) on high flow (1 L/min) for 10 minutes and left to equilibrate for half an hour with low flow (5 ml/min) through PEEK tubing.

For treatment, the flasks were injected either with PBS or drug dissolved in PBS, both of which had been gassed for 1 hour with stirring and bubbling of gas into the liquid.

2.12.3 Treatment

Tirapazamine was dissolved in PBS at a concentration of 2.7 μM and 150 μl was added to flask already containing 3.85 ml of medium to achieve a final concentration of 100
μM. The volume of medium decreased from 4 ml due to evaporation (130-150 μl) after 10 minutes under the high flow of gas. Vinorelbine was diluted and added to achieve a final concentration of 100 nM. Pimonidazole (100 μM) was added to some experiments half an hour after tirapazamine.

After gas equilibration (section 2.12.2) the chamber flasks were injected (via a gas tight syringe) either with gassed PBS or drug and incubated for 90 minutes under a low flow of gas. After 90 minutes the medium was removed and the cells rinsed with PBS and stained (section 2.12.4).

2.12.4 Immunohistochemistry

Cells were fixed for 10 minutes in a 1:1 mixture of acetone-methanol, except when staining for β-tubulin, and stained immediately following a 90 minute drug exposure. Cells were stained for β-tubulin followed by pimonidazole to ensure the cells were hypoxic during drug exposure. Experiments without pimonidazole were also done to ensure there was no effect of pimonidazole on the activity of tirapazamine.

2.12.4.1 Microtubules

Cells were fixed for 20 minutes in 3.7% formaldehyde prepared freshly on the day of staining and permeabilized with a 1:1 mixture of acetone-methanol. Microtubules were stained using a mouse anti β-tubulin antibody (1:200 dilution; Sigma) and an Alexa 488 goat anti-mouse secondary (1:100 dilution; Molecular Probes).
2.12.4.2 Hypoxia

Hypoxia was detected by staining for bound pimonidazole using a rabbit anti-pimonidazole antibody (1:1000, gift from Jim Raleigh) and an Alexa 546 goat anti-rabbit secondary (1:200 dilution; Molecular Probes).

2.12.4.3 DNA double strand breaks

DNA double strand breaks (γH2AX) were detected using the same method as used for tumours in section 2.7.7.

2.12.4.4 Endothelial cell junction protein

Vascular endothelial cadherin (VE-cadherin) was labelled, in HUVECs only, with a mouse anti-human VE-cadherin antibody (1:100 dilution; clone TEA1/31; Upstate-Millipore, Mississauga, Ontario). An Alexa 488 goat anti-mouse secondary (1:100 dilution; Molecular Probes) was used for detection.
CHAPTER 3 – MAPPING THE EFFECTS OF ANTIMETABOLITES
3 MAPPING THE EFFECTS OF ANTIMETABOLITES

3.1 Introduction

Insufficient drug delivery to cells far from blood vessels may result in sub-therapeutic drug exposures to these cells and eventual tumour re-growth or resistance (Tannock et al. 2002). The effectiveness of pyrimidine analogues could be dependent, in part, on the extent of penetration into the extra-vascular component of the tumour. In these studies the location of proliferating cells relative to vasculature was mapped in human colon cancer xenografts (HCT-116) to examine the tumour microregional effects after pyrimidine analogue administration to mice. Direct visualization of drug in tissue is not possible with this class of compounds therefore cell proliferation was used as a surrogate for the drug effect.

Pyrimidine analogues are a class of chemotherapeutic compounds known as antimetabolites, which interfere with nucleotide synthesis and DNA and RNA functions. In these studies four pyrimidine analogues were used: 5-Fluorouracil (5-FU), capecitabine, cytarabine, and gemcitabine. 5-FU is a uracil mimetic which must be converted in cells to its active nucleotide form in order to have a cytotoxic effect. The cytotoxicity of 5-FU is confined to the S-phase of the cell cycle and causes a G1/S cell cycle block (Berg et al. 2001; De Angelis et al. 2006). In studies using resistant and sensitive murine colon tumour lines 5-FU (100 mg/kg once a week for 4 weeks; i.p.) was found to cause a 3 day and 5.5 day growth delay, respectively (Nadal et al. 1989). Capecitabine is an oral prodrug of 5-FU converted by the liver and tumour tissue to 5-FU, which then goes on to exert its cytotoxic effect.

Cytarabine and gemcitabine are cytosine analogues with alterations on the ribose moiety. Cytarabine has a 2’ hydroxyl group trans to the 3’ hydroxyl group of the sugar and gemcitabine has 2 fluorines in the 2’ position of the sugar. All the studied analogues are used in the treatment of solid tumours except cytarabine, which is used in the treatment of
leukemia (Chabner et al. 2001). Cytarabine is preferentially toxic to cells in S-phase and causes approximately 50% cell kill of peritoneal L1210 ascites after a single dose of 200 mg/kg (Fietkau et al. 1984), but is relatively ineffective in solid tumours. Gemcitabine was selected for clinical use based on its activity toward murine solid tumours and human xenografts (Hertel et al. 1990) which may stem from the fact that the toxicity of gemcitabine is not confined to cells in S-phase at the time of drug administration (Plunkett et al. 1996; Cappella et al. 2001; Chabner et al. 2001). Studies in C3H mammary adenocarcinomas show gemcitabine administration causes tumour growth delays of 2.2 days and 18 days after a single dose of 240 mg/kg or 60 mg/kg on days 1, 4, 7 and 10, respectively (Cividalli et al. 2000). In vitro and in vivo studies have shown a substantial suppression of DNA synthesis after gemcitabine administration, but neither microregional effects relative to vasculature nor the kinetics of such an effect have been examined (Milas et al. 1999; Wexler et al. 2000; Cappella et al. 2001). The nucleoside derivatives gemcitabine, cytarabine and capecitabine, which are hydrophilic and would not be expected to readily permeate cells by diffusion, enter cells via a family of nucleoside transporters (NTs), whereas 5-FU is thought to enter cells either via passive diffusion or a nucleobase transporter (Chabner et al. 2001; Peters and Jansen 2001).

In these experiments the drugs were given via intraperitoneal injection as single dose administrations aside from studies with gemcitabine, which were more extensive. The HCT-116 tumour type was chosen because it is sensitive to fluoropyrimidines (Ishikawa et al. 1998b; Sawada et al. 1999; Ishitsuka 2000) and exhibits a corded structure with necrosis occurring ~150 µm from blood vessels, which facilitates assessment of microregional effects in relation to vasculature. The S-phase marker bromodeoxyuridine (BrdUrd) was used to
label dividing cells while vessel position and perfusion were assessed via staining for CD31 and i.v. injection of carbocyanine, respectively. Hypoxia was detected using pimonidazole. Images of the four markers were overlaid to reveal the spatial relationship between proliferation, vasculature and hypoxia, and examine the microregional effects.
3.2 Results

The following results show the overall proliferation and microregional effects for the four pyrimidine analogues (gemcitabine, 5-FU, capecitabine, cytarabine) studied using tumour mapping techniques. The doses of drug given were near the MTD for each drug. Details of the procedures are found in chapter 2, however relevant information for this chapter is briefly summarized here. Once the individual cryosection images were overlaid and the CD31 (vascular marker) and carbocyanine layers were given a threshold, a composite colour image was produced with the pimonidazole (green) image and greyscale BrdUrd/tissue layer. The images in this chapter show CD31 vessels that are perfused (dark blue) surrounded by regions of perfusion (light blue), hypoxia (green) and BrdUrd labelling (greyscale) (Figure 3.3). Unperfused vessels are shown in red and represent any CD31 positive area that is negative on the carbocyanine layer.

For the overall BrdUrd analysis positive regions were identified by a threshold value and the percentage of BrdUrd labelling was taken as the number of BrdUrd positive pixels over the total number of pixels. The percentage of BrdUrd labelling can exhibit variability for similarly treated groups of tumours due to individual tumour heterogeneity and this issue is discussed in detail in chapter 2. Microregional analysis was performed by measuring the distance from each point in the tissue to the nearest CD31 positive pixel and noting if it were BrdUrd positive or negative then determining the relation between proliferation and distance to the nearest blood vessel. The data were tabulated so as to determine the fraction of BrdUrd positive pixels of the total number pixels found at each distance to a blood vessel. The fraction of BrdUrd positive tissue was then expressed as a fraction of the controls, where the
averaged control level was 1, by dividing the treated values at each distance by the value for the average of all controls at that distance.

3.2.1 Gemcitabine

3.2.1.1 Gemcitabine causes a complete cessation of proliferation 1 day after administration

HCT-116 tumour bearing mice were treated with gemcitabine and the total fraction of BrdUrd labelling was assessed at various times after administration. In this tumour complete cessation of proliferation was evident 1 day after administration of gemcitabine at doses ranging from 20-360 mg/kg and quantitative analysis showed that overall BrdUrd labelling was reduced to less than 3% of the control levels irrespective of dose (data not shown). The kinetics of this suppression was examined in mice administered 240 mg/kg gemcitabine. The initial suppression of proliferation was followed by a gradual resumption in proliferation occurring over 6 days to 66% of the control levels (Figure 3.1) Multiple doses of gemcitabine, 40 mg/kg, administered on days 1, 4, 7 and 10, resulted in a similar initial decrease in proliferating cells followed by a return of labelling to 64% of the control levels 8 days after the end of dosing (Figure 3.2). The means for all groups treated with a single dose of 240 mg/kg were compared using the Kruskal-Wallis test for significance ($P < 0.0001$) and a Dunn's multiple comparison test showed days 1, 2 and 3 after treatment are significantly different from the controls ($P < 0.01$; for days 1 and 2, $P < 0.05$ for day 3).
Figure 3.1: Gemcitabine induces a complete cessation of proliferation after 1 day. BrdUrd labelling following a single dose of gemcitabine (240 mg/kg; i.p.) on day 0. Each bar represents data from an individual tumour. Horizontal lines show the mean values for each group.

Figure 3.2: Gemcitabine reduces proliferation after multiple dose administration. BrdUrd labelling on days 12, 14, 16 and 18 after 4 doses of gemcitabine (40 mg/kg on days 1, 4, 7, and 10; i.p.). Each bar represents data from an individual tumour. Horizontal lines show the mean values for each group.
3.2.1.2 The resumption of BrdUrd labelling initially occurs far from vasculature

The microregional effects of gemcitabine were analysed by examining composite tumour images and measuring the distance of BrdUrd labelled cells from the vasculature. Examples of small portions from entire composite tumour images and the corresponding analysis of tumours excised on various days after 240 mg/kg of gemcitabine, are shown in Figure 3.3. Consistent with our previous studies in SiHa xenografts, the analysis of untreated tumours showed division throughout the tumour with a labelling fraction decreasing from ~20% in cells proximal to vasculature to ~10% in cells on the border of necrosis distal to vasculature (Figure 3.3A) (Kyle et al. 2003). One day after treatment with gemcitabine, proliferation halts throughout the tumour, over the next 2-3 days cell proliferation returns in cells far from vasculature and 4-6 days later proliferation is returning throughout the tumour (Figure 3.3C). Quantitative analysis of complete tumour images confirms the fraction of BrdUrd labelled cells distal to vasculature returns to control levels at a faster rate than those cells proximal to the vasculature (Figure 3.3B). The level of BrdUrd labelling 2 days after treatment is below 20% of control levels for cells within 75 μm of a blood vessel and cells distal are ~50% of the control level. However by days 4-6 the levels of proliferation far from vasculature have reached control levels whilst those near vasculature are approximately 50% of control levels.
Figure 3.3: The resumption of proliferation is dependent on proximity to vasculature. (A) The fraction of BrdUrd positive tissue with distance to vasculature for untreated tumours. The corresponding image of an untreated tumour is shown in panel C - untreated. (B) The analysis of BrdUrd labelling relative to the controls 1, 2, 3, 4, and 6 days after treatment. Each line for the analysis graphs represents data from an individual tumour. (C) Composite tumour images for an untreated tumour and for days 1, 2, 3, 4, and 6 after i.p. treatment with 240 mg/kg of gemcitabine. The composite images are shown as a visual example and represent only 10% of the actual tumour cryosection used for analysis. Dark blue = vasculature, light blue = perfusion, black = dividing cells, red = unperfused vessels. Scale bar: 150 μm.
3.2.1.3 Multiple doses of gemcitabine cause similar microregional changes in BrdUrd labelling

Administering multiple doses of gemcitabine at 40 mg/kg shows a similar trend to a single dose both by analysis and visually, where cells far from vasculature are dividing 2 days after ending treatment and over time proliferation slowly returns near vasculature (Figure 3.4). 12 days from the first dose of gemcitabine the cells far from vasculature remain at control levels of division whilst cells near the vasculature have less then 25% labelling. Finally, 18 days after treatment began (8 days after the end of treatment) the tumours are returning to control levels of labelling and those cells at 75-150 μm from vasculature are labelling at control levels, whereas those cells near vasculature are at 50% of the control levels. Interestingly tumours excised on day 9, before the final dose of gemcitabine, are similar to those excised on day 12 and the analysis for both shows 25% of the labelling of controls near vasculature, however cells far from vasculature are labelling at control levels. Indicating that with multiple dosing where doses are 4 days apart cells are cycling distal to vasculature before the next dose of gemcitabine is administered.
Figure 3.4: Cell proliferation is evident distal to vasculature after multiple doses of gemcitabine. (A) The analysis of BrdUrd labelling relative to controls versus the distance to the nearest vessel on days 12, 14 and 18 after the first dose of gemcitabine (40 mg/kg on days 1, 4, 7, and 10; i.p.). Each line for the analysis graphs represents data from an individual tumour. (B) Portions of composite tumour images on days 12, 14, and 18 after the first day of treatment. Dark blue = vasculature, light blue = perfusion, black = dividing cells. Scale bar: 150 µm.

3.2.1.4 No visual change in tumour oxygenation occurred after treatment

In order to determine the effect of gemcitabine on tumour oxygenation pimonidazole was administered to a subset of mice. Composite images showed no apparent change in hypoxia between untreated and treated tumours, whereby hypoxia was detected on the edges of necrosis and in patches within the tumour distant from vasculature. The controls show less division far from vessels on the edge of the tumour cords and in the pimonidazole labelled
regions. 2-3 days after gemcitabine treatment cells in these regions are returning to cycle and can be seen in the regions labelled with pimonidazole (Figure 3.5). As well, vascular perfusion did not change between treated (73 ± 6.6 to 82 ± 5.6%) and untreated tumours (75 ± 10%) \((P = 0.48, \text{Kruskal-Wallis test})\) nor did we observe large areas where entire cords become hypoxic. Unperfused vessels in untreated HCT-116 tumours are not often observed and generally are only 1-3 vessels per entire cryosection.

**Figure 3.5: Hypoxia and vascular perfusion do not change.** Composite images of tumours treated with 240 mg/kg of gemcitabine and stained for hypoxia. Panels A, B, and C are untreated, 2 and 3 days after treatment, respectively. Arrows indicate proliferating regions on the edge of necrosis. Dark blue = vasculature, light blue = perfusion, black = dividing cells, green = hypoxia. Scale bar: 150 μm.

3.2.1.5 Cells located near vessels label with BrdUrd

An interesting observation from the data analysis 1 day after gemcitabine is that even though proliferation has ceased throughout the tumour the graph of fraction of BrdUrd versus distance from blood vessels shows a high level of BrdUrd labelling very near vessels (shown in Figure 3.6A and in Figure 3.3B). This indicates cells within approximately 5-10 μm of the
vessels are labelling with 50-70% of control levels while the rest of the tumour has ceased to proliferate. This appears to be a real effect as seen by the BrdUrd labelling on the same cells labelled with CD31 and no BrdUrd labelling in the rest of the tissue (Figure 3.6B).

Figure 3.6: Cells are dividing near blood vessels 1 day after treatment even though a complete cessation of proliferation is seen throughout the rest of the tumour. (A) The fraction of BrdUrd labelled cells relative to the controls at each distance from vasculature in a tumour 1 day after treatment with gemcitabine. Each line represents data from an individual tumour. (B) The corresponding image shows cells labelled with BrdUrd in black overlaid with CD31 positive staining (red) on the same cryosection. Scale bar: 150 μm.

3.2.1.6 No changes in macrophage percentage or distribution are seen

The overall percentage of macrophages in HCT-116 tumours does not change ($P = 0.17$, Kruskal-Wallis test) after treatment with gemcitabine suggesting the resumption of proliferation is not due to infiltration of macrophages (Figure 3.7A). The average percentage of macrophages in untreated tumours is $6.3 \pm 3.3\%$ showing significant heterogeneity between individual tumours. Composite images of cryosections stained for both BrdUrd and macrophages (red) show areas near necrosis where cells are returning to cycle 3 days after treatment do not have an increase in macrophages. Instead macrophages appear to be
consistently distributed throughout the control and treated tumours appearing mainly near vessels and on the edge of tumours with a few macrophages scattered throughout the tumour tissue (Figure 3.7B). Note, in these images vessels are detected only with carbocyanine, a perfusion marker, shown in dark blue. There is no CD31 labelling. The necrotic area in the controls is quite red suggesting macrophage staining in the necrotic areas, whereas the necrotic area in the treated tumour image is not. This staining in the necrotic area may be labelling macrophages, but could also be an artifact from background fluorescence in the necrotic areas.
Figure 3.7: No change in the percentage or distribution of macrophages is seen after treatment with gemcitabine. (A) A graph of percentage of macrophage positive pixels in untreated tumours and tumours 1, 2, 3, and 4 days after gemcitabine administration (240 mg/kg; i.p.). Each bar represents data from an individual tumour. Horizontal lines show the mean values for each group. (B) Representative images from cryosections light blue = vessel perfusion, red = macrophages, black = BrdUrd labelled cells and greyscale = the tissue background. Scale bar: 150 μm.
3.2.1.7 No change in activated caspase-3 was observed

No significant change in activated caspase-3 was observed in treated tumours suggesting no increase in apoptotic cells 1-6 days after treatment with a single dose of 240 mg/kg gemcitabine. A base level of 0.22 ± 0.16% activated caspase-3 occurs in untreated tumours and the treated tumour levels ranged from 0.11 ± 0.14 to 0.31 ± 0.25% (data not shown). The means for all groups were compared using the Kruskal-Wallis test for significance ($P = 0.17$).

3.2.1.8 A complete cessation of proliferation is not seen in WiDr tumour xenografts treated with gemcitabine

WiDr colon carcinoma tumour xenografts were analysed for overall changes in BrdUrd labelling after treatment with gemcitabine to examine inter-tumoural differences. We found no significant changes in the levels of BrdUrd within the first 3 days after treatment ($P = 0.10$, Kruskal-Wallis test). Four days after treatment the levels of BrdUrd labelling had dropped to 39% of control levels, but by 6 days the levels of BrdUrd labelling were back up to 68% of the control levels (Figure 3.8).
Figure 3.8: Gemcitabine does not have a large effect on BrdUrd labelling in WiDr tumour xenografts. BrdUrd labelling following a single dose of gemcitabine (240 mg/kg; i.p.) on day 0. Each bar represents data from an individual tumour. Horizontal lines show the mean values for each group.

3.2.2 5-Fluorouracil

3.2.2.1 BrdUrd labelling initially decreases after treatment with 5-FU

5-FU was administered to HCT-116 tumour bearing mice at a dose of 120 mg/kg and tumours were excised 1, 2, 3, 4 and 6 days later. The overall fraction of BrdUrd labelling was assessed at each excision time. One day after drug administration quantitative analysis showed the average level of BrdUrd had decreased to 2.4 ± 0.40% or 20% of the control levels (Figure 3.9). The means for all groups were compared using the Kruskal-Wallis test for significance ($P < 0.0005$) and a Dunn’s multiple comparison test showed 1 day and 2 days after treatment are significantly different from the controls ($P < 0.01$; for both treatments).
The initial suppression of proliferation was followed by a gradual resumption in proliferation occurring over 6 days to $8.0 \pm 0.78\%$ or $63\%$ of the control levels.

**Figure 3.9: An initial suppression of BrdUrd labelling occurs after 5-FU administration.**
BrdUrd labelling 1, 2, 3, 4, 5 and 6 days after a single dose of 5-FU (120 mg/kg; i.p.) on day 0. Each bar represents data from an individual tumour. Horizontal lines show the mean values for each group.

3.2.2.2 Cells near vessels stop dividing 1 day after treatment with 5-FU

The microregional effects of 5-FU were analysed by measuring the distance of BrdUrd labelled cells from the vasculature. Analysis showed that initially, 1-2 days after treatment, there is a decrease in proliferation in those cells close to vessels to less than $20\%$ of control levels but only a decrease of approximately $50\%$ of control levels far away from vessels (Figure 3.10A). At the later times (3-6 days later) a gradual resumption in proliferation occurs both near and far from vessels and 6 days after treatment cell
proliferation is nearing that of the controls throughout the tumour. By examining the composite tumour images a decrease in proliferation near vasculature was evident 1 and 2 days after administration as shown by a halo effect of no proliferation near vessels (Figure 3.10B). Areas of unlabelled cells surrounding the vessels can be seen particularly at the 2 day time point. Cells both near and far from vessels resume proliferation after 3 days and those cells far from vessels are close to control levels 4 days after treatment whereas cells near vessels take 6 days to return to near control levels of proliferation.
Figure 3.10: There is a differential effect on cell division with relation to distance from vasculature. (A) The analysis of BrdUrd labelling relative to controls versus the distance to the nearest vessel on days 1, 2, 3, 4 and 6 after 5-FU administration (120 mg/kg; i.p.). Each line for the analysis graphs represents data from an individual tumour. (B) Portions of composite tumour images on the corresponding days are also shown. Dark blue = vasculature, light blue = perfusion, black = dividing cells, red = unperfused. Scale bar: 150 μm.
3.2.3 Capecitabine

3.2.3.1 Capecitabine suppresses proliferation over 6 days

Capecitabine is an orally administered prodrug of 5-FU designed to allow for more convenient administration of the drug. Capecitabine was administered to HCT-116 tumour bearing mice at a dose of 750 mg/kg and tumours were excised 1, 2, 3, 4 and 6 days later. The overall fraction of BrdUrd labelling was assessed at each excision time. One day after drug administration quantitative analysis showed the average level of BrdUrd had decreased to 6.4 ± 2.5% or 32% of the control levels. The levels of BrdUrd remained low (37-62% of controls) over the next 2-4 days and after 6 days were only at 8.9 ± 1.6% or 45% of control levels (Figure 3.11). The means for all groups were compared using the Kruskal-Wallis test for significance ($P < 0.007$) and a Dunn's multiple comparison test showed 1 day and 4 days after treatment are significantly different from the controls ($P < 0.01$ and $P < 0.05$, respectively).
3.2.3.2 Cells far from vessels respond to capecitabine at later time points

Composite tumour images were used to examine the relation between changes in BrdUrd levels and distance to vasculature. Analysis showed that initially, 1 day after treatment, there is a decrease in proliferation in those cells close to vessels to approximately 25% of control levels but only a slight decrease in BrdUrd levels far from vasculature (Figure 3.12A). Cell division appears to increase over the next two days but remains below control levels at all distances from vasculature. However, 4 and 6 days later division seems to have decreased particularly 6 days later where levels near vessels are approximately 50% and far from vessels levels are below 50%. By examining the composite tumour images a decrease in
proliferation near and far from vessels is evident 1 day after treatment. BrdUrd labelling then appears to increase at 2 days far from vessels and by 3 days the tumours are reaching control levels. However 4 and 6 days later it is apparent that there is BrdUrd labelling directly around vessels yet very little labelling far from vessels (Figure 3.12B).
Figure 3.12: Proliferation is initially suppressed near vasculature. (A) Analysis of BrdUrd labelling relative to controls versus the distance to the nearest vessel on days 1, 2, 3, 4 and 6 after capecitabine administration (750 mg/kg; i.p.). Each line for the analysis graphs represents data from an individual tumour. (B) Portions of composite tumour images on the corresponding days. Dark blue = vasculature, light blue = perfusion, black = dividing cells, red = unperfused. Scale bar: 150 μm.
3.2.4 Cytarabine

3.2.4.1 Cytarabine does not decrease proliferation at any time point

Cytarabine was administered at a dose of 400 mg/kg. Tumours were excised and analysed on days 1, 2, 3, 4 and 6. The total percentage of BrdUrd labelling was determined for each time point and no change was seen in proliferation of HCT-116 tumours after treatment with cytarabine ($P > 0.05$, Kruskal-Wallis test). However, there is an observable (not statistically significant) increase in proliferation 1 day after treatment. The control tumours had an average of $12 \pm 3.2\%$ BrdUrd labelling and all treated groups ranged between $10 \pm 3.5$ and $17 \pm 2.3\%$ labelling (Figure 3.13). The largest decrease in overall proliferation was only $84\%$ of control values.
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Figure 3.13: No significant change in overall proliferation is seen at any time after treatment with cytarabine. BrdUrd labelling 1, 2, 3, 4, 5 and 6 days after a single dose of cytarabine (400 mg/kg; i.p.) on day 0. Each bar represents data from an individual tumour. Horizontal lines show the mean values for each group.

3.2.4.2 No microregional change in BrdUrd labelling is seen after cytarabine treatment

The effects of cytarabine on cell division in relation to distance from blood vessels were analysed and showed no microregional changes between treated groups and controls (Figure 3.14). There is slight variability between the data from each individual tumour, which can be expected due to tumour heterogeneity, however one tumour in the 3 day group is lower than the rest, which may suggest a decrease in BrdUrd in response to treatment. The corresponding composite images indicate that visually there is no apparent change in proliferation, which supports the data analysis.
Figure 3.14: **No change in BrdUrd labelling with relation to vasculature.** (A) The analysis of BrdUrd labelling relative to controls versus the distance to the nearest vessel on days 1, 2, 3, 4 and 6 after cytarabine administration (400 mg/kg; i.p.). Each line for the analysis graphs represents data from an individual tumour. (B) Portions of composite tumour images on the corresponding days. Dark blue = vasculature, light blue = perfusion, black = dividing cells, red = unperfused. Scale bar: 150 μm.
3.2.5 No changes in hypoxia were seen

Pimonidazole was administered to a subset of mice to determine if any changes in hypoxia or vessel perfusion occur 2 days after treatment. Composite images showed no apparent change in hypoxia, which was detected on the edges of necrosis and in patches within the tumour distant from vasculature similar to that seen with controls and after treatment with gemcitabine (Figure 3.15 and Figure 3.5). Vascular perfusion was examined and it was found that no significant changes in perfusion occurred in any of the treatment groups at any of the time points. The percentage of vessel perfusion after treatment with 5-FU (55 ± 8.6 to 77 ± 6.8%) did not differ from the untreated average (64 ± 8.9%). For capecitabine the range of values for treated tumours (63 ± 7.3 to 74 ± 6.7%) was not significantly different from that of the untreated percent perfusion (69 ± 5.0%). Lastly no change in perfusion was observed between the cytarabine treated (56 ± 15 to 72 ± 2.6%) and untreated tumours (54 ± 15%). The means for all groups were compared for each drug independently using the Kruskal-Wallis test for significance (P > 0.05 for all groups).
Figure 3.15: A comparison of the changes in hypoxia 2 days after pyrimidine analogue administration. Composite tumour images showing the location of BrdUrd labelled cells (black) in relation to vasculature (dark blue) and hypoxia (green) 2 days after treatment. N = necrosis. Light blue = perfusion. Scale bar: 150 μm.
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3.3 Discussion

Assessment of drug penetration into tissue is hindered by the inability to directly visualize drugs in tissue. In this study BrdUrd incorporation was used as a marker for cell proliferation and therefore a surrogate for the effect of anti-proliferative drugs on tissue. By quantitatively analyzing the location of affected cells relative to the position of the nearest blood vessel the extent of drug effect can be evaluated.

3.3.1 Gemcitabine

Tumours from mice treated with 240 mg/kg gemcitabine, near the weekly MTD in mice (Veerman et al. 1996), show a dramatic cessation of proliferation throughout the tumour, but over subsequent days cell proliferation resumed initially in areas distal to vasculature (Figure 3.3). If cells within the tumour were equally affected by gemcitabine one would expect a resumption in proliferation to occur first in cells close to blood vessels, which are rich in nutrients and oxygen. However, the opposite occurs suggesting all cells are exposed to gemcitabine but its effects are greatest on cells proximal to blood vessels. We hypothesize that a gradient of drug concentration could result in cells proximal to the vasculature being affected more than cells residing distal. This is consistent with observations in vitro showing cells exposed to gemcitabine for 1 hour at concentrations of 100 nM or less causes a G_1 block, the length of which is dose specific (Cappella et al. 2001) and multilayered cell culture experiments suggesting at concentrations <30 μM there is a gradient in effect related to drug exposure (Huxham et al. 2004).

Even though a complete cessation of division occurred throughout the tumour 1 day after treatment there were still cells dividing within 5-10 μms of the vasculature. These cells appear to be CD31 positive and labelling for BrdUrd so it is possible they are endothelial
cells that are less sensitive to gemcitabine, however further analysis using higher resolution images or flow cytometry would be needed to confirm co-staining. It could also be that clonogenic cells close to the vasculature are exposed to a lower concentration of drug due to drug washout within the first layer and therefore cells are not affected as strongly. This washout phenomenon was also observed in multilayered cell cultures whereby the first layer of cells on the edges of the cultures had dividing cells even though the rest of the culture had ceased proliferating (Huxham et al. 2004).

Cells located on the border of necrosis, which appear largely quiescent in untreated tumours, were proliferating 2-3 days after gemcitabine treatment (Figure 3.5). These quiescent cells may not be affected by gemcitabine either due to inadequate exposure to drug, their non-cycling status, or insensitivity due to down regulation of NTs on the cell membrane. If it is the quiescent cells returning to cycle one hypothesis is that an increased oxygen supply, resulting from the effects of gemcitabine on cells closer to blood vessels, may have allowed these cells to start proliferating. To test this the pattern of hypoxia in tumours was examined by administering pimonidazole. No change in hypoxia was observed discounting the possibility that cells return to division due to re-oxygenation. Furthermore, as hypoxia is mainly seen bordering necrosis in both treated and untreated tumours and vascular perfusion was not changed the effects of gemcitabine were not mediated by changes in vessel perfusion.

While the quiescent cells may be intrinsically less sensitive to gemcitabine and therefore able to divide after drug exposure they constitute only the last 2-3 layers of cells. Control levels of proliferation are reached at 75 to 150 μm from vessels 4-6 days after gemcitabine administration indicating most cells at these distances, despite being sensitive at
the time of exposure, were able to resume cycling. In addition, when multiple doses of
gemcitabine were administered, cells were dividing far from vasculature on day 9, before the
last dose, showing these distal cells were cycling and likely sensitive to gemcitabine when
the final dose was administered, yet cells still return to cycle 2 days after the last dose.

Our studies using a range of doses from 20-360 mg/kg of gemcitabine, to test the
concentration dependence on proliferation, show a dose-independent virtually complete
cessation of tumour cell proliferation 1 day after gemcitabine administration. This is not
unexpected as the toxicity of gemcitabine, unlike other pyrimidine analogues, is not confined
to cells in S-phase at the time of drug administration because of the long intracellular
retention time (Plunkett et al. 1996; Cappella et al. 2001; Chabner et al. 2001). The kinetics
of the proliferation resumption have not been explored at each dose but flow cytometry
experiments, using BrdUrd to detect S-phase cells, indicate DNA synthesis begins to recover
in SA-NH tumours administered 10 mg/kg gemcitabine after 12 hours whereas it takes 24-36
hours for DNA synthesis to recover after 400 mg/kg of gemcitabine (Milas et al. 1999).
Therefore beyond 1 day the resumption of proliferation throughout the HCT-116 tumour
could be faster for low doses of drug and take days for higher doses as is seen with
240 mg/kg of gemcitabine.

The current clinical protocol for gemcitabine administration commonly indicates a 30
min i.v. infusion of 33-40 mg/kg (1000-1200 mg/m²) on days 1, 8 and 15 for every 28 days
(Veerman et al. 1996; Chabner et al. 2001). In humans gemcitabine has a half-life of
approximately 30-40 minutes and a long terminal elimination half-life of approximately 14
hours (Abbruzzese et al. 1991). The peak plasma concentration for humans administered this
protocol is 15-20 µM and for mice administered a single dose of 240 mg/kg is >100 µM with
a plasma half-life of 15 minutes (Veerman et al. 1996; Chabner et al. 2001). Work with MCCs in this lab has shown a concentration dependent wave of proliferation inhibition 1 day after a 1 hour drug exposure where $\geq 30\ \mu M$ gemcitabine is required to halt division through 150 $\mu m$ of tissue (Huxham et al. 2004). Three days later these cells are returning to cycle throughout the MCCs indicating that even a 90 $\mu M$ exposure for 1 h does not prevent the cells from returning to division (Huxham et al. 2004). In vivo cells near the vasculature may be exposed to concentrations $>100\ \mu M$ and not return to cycle until over 6 days later, or undergo apoptosis. However, the cells far from vasculature, which receive lower exposures of drug, will return to cycling 2-3 days later. In the MCC experiments the discrete wave of proliferation inhibition observed is likely to be the result of the defined steady state exposure and the planar geometry of the culture. Tumour distribution is the result of a more complex drug exposure profile dictated by the in vivo pharmacokinetics and a more complex 3-dimensional geometry.

Mice administered multiple doses of gemcitabine 3 days apart show cells cycling far from vasculature 2-3 days after the final dose, similar to that seen after a single dose. Therefore multiple doses do not appear to enhance drug delivery since the resumption of proliferation follows similar kinetics as a single dose of gemcitabine. A study by Cividalli shows the tumour growth delay in C3H/TIF mammary adenocarcinomas is 18 days when gemcitabine is administered on days 1, 4, 7 and 10 and was reduced to 12.2 days if 5 days were left between doses (Cividalli et al. 2000) suggesting multiple doses given too far apart will allow cells to begin proliferating before another dose is given and therefore lead to a smaller tumour growth delay.
Tumour cryosections were stained for macrophages to determine whether the cells returning to cycle 2-3 days later on the edge of necrosis were macrophages (Figure 3.7). Conceivably the resumption in proliferation seen on the edge of necrosis could be due to invading macrophages from the necrotic area. However, no increase in the percentage of macrophages was seen nor was there any change in the distribution of macrophages. This shows there is no increase in macrophages near the edge of necrosis where cells resume proliferation and indicates that the cells returning to cycle are in fact clonogenic tumour cells.

As mentioned above cells near the vessels may be exposed to high enough concentrations of gemcitabine to cause apoptosis. Apoptosis was examined by staining for activated caspase-3 in tumour sections and determined no significant increase in apoptosis was seen from 1-6 days after drug administration. This lack of observed apoptosis could also be due to the timing of apoptosis, in that cells may undergo apoptosis at various times after exposure of drug instead of simultaneously (Brown and Attardi 2005). As well, the fast turn over of apoptotic cells could mean activated caspase-3 is not present at time points of 1 day or more (Hall 1999). There is some evidence that apoptosis is not the major response of cells in solid tumours to DNA damaging agents and a clonogenic assay could reflect cell death better than markers of apoptosis (Brown 1979; Steel 2001; Tannock and Lee 2001; Brown and Attardi 2005).

In contrast to the HCT-116 there was no change in BrdUrd labelling over the first 3 days after treatment with gemcitabine in WiDr colon carcinoma tumour xenografts. However the WiDr is refractory to fluoropyrimidines due to low thymidine phosphorylase (dThdPase) and has been shown to be relatively insensitive to gemcitabine, which could explain the lack of response (Ruiz van Haperen et al. 1994).
3.3.2 5-FU

Mice were treated with 120 mg/kg 5-FU and the tumours excised on days 1, 2, 3, 4, 5 and 6. An initial suppression (1 day after treatment) of BrdUrd to 20% of the control levels was observed and over time the level of BrdUrd labelling increased to near control levels. The microregional analysis of BrdUrd with relation to vasculature revealed that there was decreased cell division in cells near vessels and less of an effect on cells far from vessels (Figure 3.10). Suggesting a gradient of drug exposure to cells in relation to distance from vasculature. The fraction of dividing cells far from vasculature began to increase 2-3 days after treatment and had reached control levels by 4 days after treatment. However those cells near vasculature took 6 days to return to about 80% of control levels.

The cytotoxicity of 5-FU is confined to the S-phase of the cell cycle unlike the cytotoxicity of gemcitabine which, as mentioned above, is not confined to cells in S-phase at the time of administration (Plunkett et al. 1996; Cappella et al. 2001; Chabner et al. 2001). This may explain why fewer cells are affected overall by 5-FU than with gemcitabine. However this cannot explain the differential effect on cells in relation to vasculature and why there is a lack of response in those cells far from vasculature that were cycling at the time of drug administration. Control tumours have shown 10% cell division even at distance >100 μm from blood vessels yet after treatment cell division is only reduced to 50% of the control levels. 5-FU causes a G1/S cell cycle block which can then lead to apoptosis if the cell damage is not repaired (Berg et al. 2001; De Angelis et al. 2006). Cells far from vasculature may receive sub-therapeutic doses and be able to repair and return to cycling faster than those cells near vasculature, which receive a high dose. In contrast to gemcitabine 5-FU enters the cell via passive diffusion or a nucleobase transporter and therefore down regulation
of transporters far from vasculature is not an issue for the uptake of 5-FU in cells far from vasculature.

Clinically 5-FU has a short half-life (~11 min) after rapid intravenous infusion and is converted in the liver and tumour tissue by several possible mechanisms to the active nucleotide. The metabolites are formed quickly and consumed by cells, which could prevent efficient diffusion of 5-FU. Biochemical modification can enhance the activity of 5-FU and combination with leucovorin enhances the ability of the metabolite 5-FdUMP to bind to thymidylate synthase thus blocking the enzyme and depleting the stores of thymidine.

Studies with dihydropyrimidine dehydrogenase (DPD), the enzyme which inactivates 5-FU, suggest inhibition of DPD can lead to increased sensitivity of 5-FU (Baccanari et al. 1993; Takechi et al. 2002). Administering 5-FU with a DPD inhibitor could change the microregional effects of 5-FU possibly increasing the effect on those cells far from vasculature.

3.3.3 Capecitabine

Capecitabine is an oral prodrug of 5-FU that is converted in tumour tissue to 5-FU by thymidine phosphorylase. Capecitabine was administered to HCT-116 tumour bearing mice at a dose of 750 mg/kg and tumours were excised 1, 2, 3, 4 and 6 days later. One day after drug administration, quantitative analysis showed the average level of BrdUrd had decreased to 32% of the control levels. The levels of BrdUrd remained low even up to 6 days after treatment. Analysis with relation to vasculature showed that initially, 1 day after treatment, there is a decrease in proliferation in those cells close to vessels but only a slight decrease in BrdUrd levels far from vasculature (Figure 3.12). However cell division does not return to...
control levels by 6 days after treatment and the level of division near vessels is approximately 50% and far from vessels is below 50%.

Studies using HCT-116 tumour xenografts have shown that the levels of 5-FU in tumour tissue were higher after oral capecitabine administration than after 5-FU infusion, 110 versus 8 nmol/ml, respectively (Ishikawa et al. 1998b; Ishitsuka 2000). In addition, after capecitabine administration the levels of 5-FU in the tumour were 127 and 22 times higher than in the plasma and muscle, respectively whereas 5-FU administration resulted in similar levels in all three compartments (Ishikawa et al. 1998b).

Capecitabine unlike 5-FU has been shown to have antimetastatic activity at doses much lower than for primary tumour treatment (Ishikawa et al. 1998a). This is attributable to increased sensitivity of cells to capecitabine due to upregulation of dThdPase by the inflammatory cytokines IFNy, TNFα and IL-1α (Ishikawa et al. 1998a). The clinical half-life of capecitabine conversion is longer than that for 5-FU, 1.3 hours versus 11 min, and this causes capecitabine to be present for longer periods of time and thus 5-FU to be produced over a longer period. This increased drug exposure could explain why tumours treated with capecitabine have not returned to control levels even 6 days after treatment. The slower conversion of capecitabine in the plasma may contribute to a longer 5-FU exposure and further diffusion into tumour tissue.

3.3.4 Cytarabine

Cytarabine like gemcitabine is a cytosine analogue, however unlike gemcitabine, cytarabine has no effect on cell proliferation in HCT-116 solid tumour xenografts. A change in overall BrdUrd levels was not seen nor was there any difference in microregional effects over time (Figure 3.14). There was an observed increase in proliferation 1 day after treatment.
that was not statistically significant and could be due to the variability of BrdUrd labelling in untreated tumours. The average BrdUrd labelling can vary between untreated tumours as shown in chapter 2 (13-28%) and the BrdUrd labelling in the controls from the cytarabine group were on the low side at 12 ± 3.2%.

Interestingly cytarabine is used in the treatment of acute myelogenous leukemia and meningial leukemia whereas the other three pyrimidine analogues studied in this thesis are used in the treatment of solid tumours. Cytarabine is given clinically by continuous infusion or repetitive administration schedules to achieve high concentrations (Chabner et al. 2001; Hamada et al. 2002). The inability to retain sufficient levels of cytarabine triphosphate that are inhibitory to DNA in cells is a problem associated with treatment (Peters et al. 1993b). Therefore, achieving high, therapeutic intracellular concentrations of active metabolite even near vessels may be preventing cytarabine from having any anti-proliferative effect in solid tumours.

The cytotoxicity of cytarabine is confined to the S-phase of the cell cycle unlike gemcitabine (Chabner et al. 2001). Accumulation of the active metabolite of gemcitabine is higher than that of the active metabolite of cytarabine (Peters et al. 1993b) and the elimination of the active metabolites is more rapid for cytarabine than for gemcitabine the latter of which has a biphasic elimination with a very long terminal half-life. Therefore the exposure of cells to active metabolites of cytarabine is much less than for gemcitabine and it has been shown that prolonged exposure of cells to cytotoxic levels of cytarabine are critical for its maximum activity (Hamada et al. 2002). In this experiment a single dose of 400 mg/kg was administered and therefore prolonged therapeutic concentrations, even in cells near vessels, may never have been achieved.
3.3.5 Changes in hypoxia

The pattern of hypoxia in tumours did not appear to change after any of the drug administrations suggesting cells are not returning to division due to re-oxygenation. As well, vascular perfusion was not significantly altered in any of the treated group at any of the time points suggesting the effects of the drugs were not related to changes in vessel perfusion.

3.4 Summary

In summary, gemcitabine administration results in a complete cessation of proliferation in all cells throughout the tumour; however, over time cells distal from vasculature resume cycling and eventually the tumour approaches control levels of proliferation. Of all the pyrimidine analogues gemcitabine exhibited the broadest proliferation suppression 1 day after treatment, which may relate to a long exposure time of cells to the drug. 5-FU caused a decrease in proliferation in those cells close to vasculature but less of an effect on cells far from vessels. Over time cells resumed cycling and those cells far from vasculature returned to control levels sooner than those near vessels. Capecitabine, which is a prodrug of 5-FU, initially had less of an effect than 5-FU on cell proliferation near vessel but a similar effect on cells far from vessels. However, cell proliferation did not return to control levels even after 6 days suggesting better diffusion of capecitabine compared with that of 5-FU possibly due to a longer exposure time. Cytarabine showed no effect, at any time points, suggesting a single dose is not effective against solid tumours possibly because therapeutic concentrations cannot be achieved for prolonged periods.
CHAPTER 4 – MAPPING THE EFFECTS OF HYPOXIC CYTOTOXINS
4 MAPPING THE EFFECTS OF HYPOXIC CYTOTOXINS

4.1 Introduction

Solid tumours contain both chronic and acutely hypoxic cells that are less sensitive to the effects of ionizing radiation therapy (Gray et al. 1953; Thomlinson and Gray 1955; Raleigh et al. 1996). As well, chronically hypoxic cells are situated far from vasculature and can be quiescent therefore these cells may receive a sub-therapeutic dose of drug or they may not respond to chemotherapies that target cycling cells (Jain 1997; Jain 1998; Huxham et al. 2004; Kyle et al. 2004). Hypoxic cytotoxins were designed to exhibit preferential toxicity to cells at low oxygen tensions and work in combination with standard therapies (Brown and Lemmon 1991). Tirapazamine, the first hypoxic cytotoxin to enter clinical trials, is a prodrug activated in poorly oxygenated areas to a reactive free radical intermediate that causes DNA strand breaks (Laderoute et al. 1988; Peters and Brown 2002). Phase III clinical trials with tirapazamine in head and neck and non-small lung cancer are completed or closed and it is currently in active phase III trials in combination with cisplatin and radiotherapy for the treatment of cervical cancer. The primary target of hypoxic cytotoxins is the chronically hypoxic cells located approximately 150 μm from blood vessels; however, studies examining the tissue penetration of tirapazamine using multicellular models have suggested that tirapazamine exposure may be limited to cells close to blood vessels (Durand and Olive 1992; Hicks et al. 1998; Kyle and Minchinton 1999). Despite these studies tirapazamine has been shown to enhance the anti-tumour activity of radiation and chemotherapy in animal models (Zeman et al. 1988; Dorie and Brown 1993; Dorie et al. 1994; Dorie and Brown 1997; Siemann and Hinchman 1998).

The microregional effects of tirapazamine in vivo were examined to determine the location of drug effect by mapping proliferating cells in relation to markers such as blood
vessels and hypoxia in tumour cryosections. If tirapazamine were able to reach the intended target cells far from vessels that are hypoxic then a decrease in proliferation in these areas would be expected.

Instead of a preferential effect on those cells far from vasculature, extensive central vascular dysfunction in HCT-116 and SiHa xenografts as well as SCCVII murine tumours was observed. The disruption to vessels was permanent and areas affected appear to be replaced by necrosis over the following 1-2 days.

Due to the unexpected activity of tirapazamine other hypoxic cytotoxins were examined, three of which are analogues of tirapazamine, SR 4754, 4482, 4898, and an unrelated 2-nitroimidazole prodrug RB 6145.

The cytotoxicity of tirapazamine is oxygen dependent and cytotoxic radical formation can occur at levels of oxygen below 5% (Koch 1993). We propose the induction of central vascular dysfunction by tirapazamine is related to its activity toward blood vessels in the centre of tumours that reside at intermediate levels of oxygen (below 5%) or that have temporarily become hypoxic. Endothelial and perivascular cells at intermediate oxygen tensions would allow for the conversion of tirapazamine to a cytotoxic radical and result in damage directly to these cells.

Additionally, vessels may become acutely hypoxic due to intermittent blood flow causing tirapazamine induced damage to nearby cells thus resulting in disruption of blood flow through the entire vessel. We suggest the activity of tirapazamine in solid tumours administered >55 mg/kg is due to central vascular dysfunction and that its activity against hypoxic cells located distal to functional blood vessels may not be as important as previously believed.
4.2 Results

The following results show the overall proliferation and microregional effects of tirapazamine in HCT, SiHa and SCCVII tumours studied using tumour mapping techniques. A summary of the processes for composite image construction and BrdUrd image analysis can be read in chapter 3 (section 3.2) and the details for all procedures are found in chapter 2. Details on the analysis and definition of necrotic and non-perfused tissue areas are summarized below for clarification in the discussion of the data.

Perfused regions are defined as areas of viable tissue with both CD31 and carbocyanine labelling as well as BrdUrd labelling (proliferation) surrounding the vessels. In contrast, non-perfused areas have positive CD31 staining, but no carbocyanine and no BrdUrd labelling, however the cells do stain with hematoxylin and appear viable. Necrotic areas show no immunohistochemical staining and have a cobwebbed appearance.

In order to determine the degree of vascular dysfunction and necrosis in treated tumours the percentages of necrotic and non-perfused tissue were analysed. Images were cropped to remove areas of necrosis or areas that consisted of non-perfused tissue. The total number of pixels for each of these selections was obtained and the proportion of necrosis and of non-perfused tissue were calculated by dividing the number of pixels that were necrotic or non-perfused by the total number of pixels for each tumour.

4.2.1 The effects of tirapazamine in HCT-116 xenografts

The microregional effects of tirapazamine were determined by examining a range of times after treatment (1-24 hours, 1-4 days) and of drug doses from 30-60 mg/kg (43 – 85% of the MTD). Tumour cryosections were stained for markers of cell proliferation, hypoxia, blood vessels and blood flow to map the effects of tirapazamine. For some experiments
controls were administered 1 ml of saline to discount the effect of the high volume of liquid (1-1.2 ml) received by mice administered tirapazamine.

4.2.1.1 Tirapazamine causes central vascular dysfunction

The effect of tirapazamine on vessel perfusion 1 day after treatment is shown using composite tumour images of representative entire tumour cryosections (Figure 4.1). In the untreated, control tumour (Figure 4.1A) almost all vessels are perfused, indicated by the perfusion marker carbocyanine in light blue surrounding the CD31 labelled endothelial cells in dark blue. Non-perfused vessels (shown in red) are rare and proliferating cells (greyscale) are distributed around vessels throughout the tissue. One day (24 hours) after tirapazamine administration, large central regions of tumour tissue are unperfused (Figure 4.1B) compared with essentially none in the control tumours (Figure 4.1A). Perfused areas of the tumour are still present after treatment but are situated around the periphery of the tumours often creating a rim of perfused tissue surrounding the central non-perfused region. The areas of non-perfused tissue have little to no BrdUrd labelling and pimonidazole (hypoxia label) spreading in from the edges of the tumour.

Pimonidazole positive areas (green) in control tumours typically occur around isolated cords of cells and at the border between viable perfused tissue and necrosis (Figure 4.1A). In mice administered tirapazamine (Figure 4.1B) pimonidazole staining is localized to the border between the perfused and non-perfused regions. The intensity of the pimonidazole staining decreases moving into the non-perfused regions and the majority of the non-perfused regions show no pimonidazole staining.

The percentage of perfused vessels decreases 1 day after tirapazamine administration from a control level of 74 ± 6.3 down to 57 ± 21% (P = 0.023, Two-tailed Mann Whitney U
test). Perfused vessels are defined as vessels, which have greater than 10% overlap with carbocyanine (chapter 2). It is important to note that not all tumours respond to tirapazamine with vascular dysfunction (as discussed later in section 4.2.1.3) and this value is an average of the responding and non-responding tumours.

Figure 4.1: Tirapazamine causes central vascular dysfunction. Images of HCT-116 cryosections showing (A) a tumour from a control mouse and (B) a tumour from an animal 1 day after tirapazamine administration (60 mg/kg, i.p.) demonstrating large regions of tissue that contain blood vessels which are not perfused (red). Dark blue = vasculature, light blue = perfusion, black = dividing cells, red = unperfused vessels, green = hypoxia, greyscale = tissue background. The insets show details of tumour morphology including non-perfused tissue (U) and necrotic areas (N). Scale bar: 150 μm.
4.2.1.2 Central vascular dysfunction becomes necrotic

The amount of necrosis in tumours from untreated animals is variable, approximately 17 ± 9.0% of the tumour cryosection area, and typically confined to patches in the centre of tumours (Figure 4.3B and Figure 4.1A). The percentage of necrosis increases 2-3 days after tirapazamine treatment and appears to replace the areas of non-perfused tissue (Figure 4.2). After treatment the areas of necrosis encompass the entire central tumour area, rather than patches (Figure 4.1A), and are therefore considered to arise due to the previously non-perfused central areas. As the treated tumours become necrotic, tissue disintegration occurs and the CD31 staining of vessels that was in the non-perfused regions becomes faint or absent.
Figure 4.2: Central areas become necrotic 2-3 days after treatment with tirapazamine. Composite images of whole tumour sections 2 and 3 days after administration of tirapazamine (60 mg/kg, i.p.), (A) and (B) respectively. Large areas of necrosis have developed in the central regions of the tumours where areas of non-perfused tissue occurred. Dark blue = vasculature, light blue = perfusion, black = dividing cells, red = unperfused vessels, green = hypoxia, greyscale = tissue background. The insets show details of tumour morphology including non-perfused tissue (U) and necrotic areas (N). Scale bar: 150 μm.
4.2.1.3 The effect of central vascular dysfunction does not occur in all treated tumours

The central vascular dysfunction caused by tirapazamine does not occur in all tumours treated. Figure 4.3A shows the percentage of histologically non-perfused tissue in tumours from untreated (2.6 ± 2.5%) and tirapazamine (27 ± 18%) treated animals. Of note, the percentage of non-perfused tissue in treated tumours is an average of all tumours, both those that respond and those that do not. Controls have very little non-perfused tissue, typically only one to two vessels per tumour section. Some of the treated tumours show very little non-perfused tissue as well, whereas others show up to 60% of non-perfused tissue in a single tumour Figure 4.3A. The increase in necrosis 2-3 days after tirapazamine is also variable. The amount of necrosis can vary between untreated tumours due to inter-tumour heterogeneity and in this set of controls ranges from 5 to 30% of necrotic tissue, however after treatment the percentage of necrosis in responding tumours can be as high as 80%.

Figure 4.3: **Vascular dysfunction does not occur in all tumours treated.** (A) The percentage of non-perfused tumour tissue in HCT-116 tumour cryosections from mice prior to and 1 day after tirapazamine (60 mg/kg, i.p.). (B) The percentage of necrosis 1-4 days after treatment with tirapazamine indicating an increase after 2 days. Each bar represents data from an individual tumour. Horizontal lines indicate the mean values for each group.
4.2.1.4 Tirapazamine causes a reduction in cell proliferation

Tirapazamine reduced the fraction of BrdUrd labelled cells seen in perfused regions of tumours from an average of 10 ± 3.5% in the controls to 5.7 ± 2.0% 1 day after tirapazamine ($P = 0.012$, Two-tailed Mann-Whitney U test) (Figure 4.4). The minimum levels of BrdUrd labelling were seen 2 days after treatment (4.53 ± 2.0) and the levels remained low even 4 days after treatment.

![Graph showing BrdUrd incorporation over time](image)

**Figure 4.4: Proliferation decreases after treatment with tirapazamine.** BrdUrd incorporation was reduced within perfused regions of HCT-116 tumours from tirapazamine treated (60 mg/kg, i.p.) mice compared with BrdUrd levels in untreated animals. Each bar represents data from an individual tumour. Horizontal lines indicate the mean values within each group.
4.2.1.5 No preferential decrease in proliferation far from vasculature is seen after tirapazamine treatment

The fraction of BrdUrd labelled cells, relative to control values, was examined as a function of distance from vasculature in the tumours with vascular dysfunction and those without 1 day after tirapazamine treatment. The percentage of proliferation was calculated only in those areas that were perfused. In the 7 tumours with areas of vascular dysfunction only two showed a greater decrease in proliferation far from, as compared to near, vasculature (Figure 4.5 bold lines). The other 5 tumours had decreased proliferation both near and far from vasculature with the exception of one tumour, which appeared to have high levels of BrdUrd far from vasculature. It is important to note, the areas examined for proliferation can be small as they are only the perfused rim of tissue left after the central area becomes unperfused. In the 3 tumours that did not exhibit vascular dysfunction the entire perfused tumour area was analysed. None of the tumours showed a decrease in proliferation far from vasculature, but one tumour did show a decrease in proliferation near vasculature and another showed an apparent increase in proliferation compared to controls far from vasculature.
Figure 4.5: Tirapazamine does not preferentially decrease proliferation far from vasculature. (A) BrdUrd labelling relative to the controls as a function of distance from vasculature in tumours with central vascular dysfunction. Only two tumours (bold) show a greater decrease in proliferation far from vasculature as compared to near, the other 5 show fairly uniform decreases in proliferation with respect to distance from vasculature. (B) BrdUrd labelling relative to the controls as a function of distance from vasculature in tumours without central vascular dysfunction. Proliferation far from vasculature does not decrease. Tumours were analysed 1 day after treatment. Each line represents data from an individual tumour.
4.2.1.6 The degree of central vascular dysfunction is dose dependent

Tirapazamine was administered at a range of doses (30, 40, 48, 55, and 60 mg/kg) to HCT-116 tumour bearing mice to determine whether non-perfused areas are induced with lower doses of tirapazamine. A greater overall response of tumours to tirapazamine is seen with increasing dose (Figure 4.6 inset). As the dose increases a higher percentage of vascular dysfunction is observed in individual tumours that respond, but the response is still not in all tumours. The nonresponding tumours at the highest dose have small areas of non-perfused tissue but it is still higher than the percent of non-perfused tissue in unaffected tumours at lower doses (Figure 4.6 bars).

![Graph showing the response of tumours to tirapazamine](image)

**Figure 4.6:** The response of tumours to tirapazamine is dose dependent. Increasing doses of tirapazamine cause an increase in the average percentage of non-perfused tissue in tumours (inset). The inset shows the average ± standard error for n= 4-5. The bar graph shows an increase in the percentage of response for individual tumours but not a noticeable increase in the number of tumours responding. Each bar represents data from an individual tumour. Horizontal lines indicate the mean values for each group.
4.2.1.7 Tirapazamine causes central vascular dysfunction as early as 6 hours after treatment.

In order to determine how early vascular dysfunction occurs after treatment with tirapazamine tumours were examined 1, 2, 6, 8 and 24 hours after treatment. Two hours after treatment one tumour shows a small percentage of non-perfused tissue, but 6 hours after treatment all four treated tumours responded with some degree of vascular dysfunction (Figure 4.7). The later time points, 8 and 24 hours, continue to show tumours with areas that are non-perfused, although tumours at 8 hours do not show a large response. The average value of non-perfused tissue in the controls is $1.5 \pm 1.5$ which increases to an overall maximum percentage of $26 \pm 14$ after 6 hours. From this data it appears that tirapazamine starts to have an effect on vasculature between 2-6 hours after treatment.

**Figure 4.7:** Vascular dysfunction occurs within 6 hours after tirapazamine. (A) The percentage of non-perfused tumour tissue in HCT-116 tumour cryosections from untreated mice and 1, 2, 6, 8 and 24 hours (1 day) after tirapazamine administration (60 mg/kg, i.p.). Each bar represents data from an individual tumour. Horizontal lines indicate the mean values for each group.
4.2.1.8 Tirapazamine decreases BrdUrd incorporation 2 hours after treatment

The percent BrdUrd labelling in tumours was analysed, with the exception of the 1-hour time point, which did not have the usual 2 hours of BrdUrd exposure, to determine when a decrease in BrdUrd incorporation begins after treatment with tirapazamine. A decrease is seen within 2 hours from control levels of 14 ± 4.6% BrdUrd labelling to 8.4 ± 1.2% (Figure 4.8). A further decrease to 4.2 ± 1.0 and 5.9 ± 2.0% after 8 and 24 hours, respectively. The decrease is significant between the groups as shown by the Kruskal-Wallis test ($P = 0.0037$) and using the Dunn’s multiple comparison test the 8 and 24 hour group were shown to be significant ($P < 0.01$ and 0.05, respectively).

Figure 4.8: Proliferation begins to decreases 2 hours after treatment. BrdUrd incorporation was significantly reduced within perfused regions of HCT-116 tumours 8 to 24 hours after tirapazamine administration (60 mg/kg, i.p.). Each bar represents data from an individual tumour. Horizontal lines indicate the mean values within each group.
4.2.1.9 Proliferation decreases near vessels in some perfused areas

A few tirapazamine treated HCT-116 and SiHa tumours exhibited a decrease in BrdUrd labelling near vasculature. Two days after treatment unlabelled tissue around the blood vessels was observed in the perfused areas of the remaining tumour rim. A blood vessel that is perfused can be seen with no BrdUrd labelling directly surrounding the vessel, but at distances far from vasculature (>100 μm) cells with BrdUrd labelling are seen (Figure 4.9).

Figure 4.9: In a few tumours decreased proliferation is seen near some vessels. An image of a SiHa cryosection from a mouse tumour excised 2 days after tirapazamine administration (60 mg/kg, i.p.) demonstrating areas with rings of proliferation far from perfused vasculature and hardly any proliferation near the vessel (arrows). The inset shows a perfused vessel without proliferation in adjacent cells (arrow) next to a perfused vessel surrounded by proliferating cells. Dark blue = vasculature, light blue = perfusion, black = dividing cells, green = hypoxia, greyscale = tissue background. Necrotic tissue = N. Scale bar: 150 μm.
4.2.1.10 Labelling of γH2AX increases 1-2 hours after tirapazamine

In order to determine the extent and location of damage after treatment tumours were stained for γH2AX to label double-strand breaks caused by tirapazamine. γH2AX is phosphorylated at serine 139 in response to DNA double-strand breaks (DSBs) in order for recognition and repair of DNA damage. The tumours were analysed for the overall percent of γH2AX in the perfused regions. An increase occurred in γH2AX staining from control levels of 2.9 ± 2.2% to 11 ± 4.7 and 12 ± 5.6% 1 and 2 hours after treatment, respectively (Figure 4.10) ($P = 0.0001$, Kruskal-Wallis test and $P < 0.01$ and 0.001 for 1 and 2 hours, respectively using the Dunn’s multiple comparison test). γH2AX staining then decreases to control levels with the exception of one tumour at the eight-hour time point, which still had a high level of γH2AX staining.
Figure 4.10: $\gamma$H2AX increases within 1-2 hours after treatment with tirapazamine. The percentage of $\gamma$H2AX in perfused areas of HCT-116 tumour cryosections from mice prior to and 1, 2, 6, 8, and 24 hours after tirapazamine (60 mg/kg, i.p.). An increase in $\gamma$H2AX is seen shortly after tirapazamine administration and then staining returns to control levels 6 to 24 hours later. Each bar represents data from an individual HCT-116 tumour. Horizontal lines indicate the mean values for each group.
4.2.1.11 A larger increase in γH2AX is seen far from vasculature

At the 1 and 2 hour time points where γH2AX increased after tirapazamine treatment the fraction of positive γH2AX at each distance from vasculature was examined to show the relation between γH2AX levels and distance from vasculature. A larger increase in γH2AX occurs in cells farther from vasculature that would have a reduced oxygen tension (Figure 4.11). The fraction of labelling also increases near vasculature after treatment but not to the same extent as seen far from vasculature.

**Figure 4.11: A larger increase in γH2AX occurs in cells farther from vasculature.** The graphs show the fraction of γH2AX labelling in (A) untreated HCT-116 xenografts, and 1 (B) and 2 hours (C) after tirapazamine treatment (60 mg/kg, i.p). Controls have fairly consistent staining values both near and far from vasculature. After treatment the fraction of γH2AX increases to a larger extent far from vasculature. Each line represents data analysis from an individual tumour.
4.2.1.12 Labelling of γH2AX disappears in the non-perfused areas

Examination of composite tumour images revealed that at the time when vascular dysfunction occurs (6, 8 and 24 hours) there is a lack of γH2AX staining in the regions that are non-perfused (Figure 4.12). Representative images are shown from the 6-hour time point where one section shows the non-perfused areas (red vessels) and a lack of BrdUrd staining and a serial section shows the same area stained for γH2AX. The non-perfused areas have no γH2AX staining. In untreated tumours there is a low level of γH2AX throughout the tumour, which still occurs in the perfused regions of all treated tumours, but is completely absent in the non-perfused areas.
Figure 4.12: \(\gamma\text{H2AX} \) staining is absent in non-perfused areas. Images showing BrdUrd labelling (A and B) and \(\gamma\text{H2AX} \) labelling (C and D) in untreated (top) and treated (bottom) tumours. Serial sections were all stained for blood vessels (dark blue) and perfusion (light blue) then for both proliferating cells (greyscale), and hypoxia (green) or for \(\gamma\text{H2AX} \) (greyscale). Blood vessels that are not perfused appear red in all images. Scale bar: 150 \(\mu\text{m} \).
4.2.1.13  No significant increase in apoptosis is seen after treatment with tirapazamine

Changes in apoptosis were examined after treatment with tirapazamine. Analysis was performed on tumour cryosections stained for activated caspase-3 and the whole tumour, both the perfused and non-perfused areas, was analysed with the exception of necrosis. Tumours that exhibit vascular dysfunction have similar levels of activated caspase-3 to non-responsive tumours and to untreated tumours. No change in activated caspase-3 at anytime from 1-4 days after treatment was observed. The control values of activated caspase-3 were \(0.32 \pm 0.23\%\) and the treated values ranged from \(0.38 \pm 0.20\) to \(0.70 \pm 0.55\%\), which were not significantly different \((P >0.05, \text{Kruskal-Wallis test})\). One of the tumours, in the 3-day group, responded with an unusually high level of apoptosis (1.5%), which appeared in areas far from vasculature (Figure 4.13).

Figure 4.13: No overall increase in apoptosis was observed, however one tumour showed an increase in activated caspase-3 after treatment with tirapazamine. Images of HCT-116 tumour cryosections showing (A) an untreated tumour and (B) a tumour from a mouse 3 days after tirapazamine administration (60 mg/kg, i.p.). Demonstrating activated caspase-3 staining (apoptosis) far from vasculature after treatment. This is not a typical result most tumours did not have any significant increase in apoptosis. Dark blue = vasculature, light blue = perfusion, black = apoptosis, greyscale = tissue background. Scale bar: 150 \(\mu\)m.
4.2.1.14 Ki67 is present in areas of vascular dysfunction

Ki67 is an endogenous marker of cell proliferation that marks cells in all stages of the cell cycle. Unlike BrdUrd, which is exogenously administered, Ki67 is endogenous and does not need to diffuse from vasculature therefore its presence is not influenced by a lack of perfusion. Tumour cryosections were examined for Ki67 presence in treated and untreated tumours to determine if cells in non-perfused regions are still cycling. Ki67 is seen in non-perfused areas as seen by the labelling around red unperfused vessels (Figure 4.14). However some areas that have CD31 labelling do not have Ki67, but these areas may have already become necrotic.

Figure 4.14: Ki67 is present in central non-perfused areas. (A) An untreated tumour with Ki67 labelling surrounding blood vessels through out the tumour. (B) 1 day after tirapazamine (60 mg/kg, i.p.) Ki67 can be seen in areas with non-perfused regions. The inset shows details of tumour morphology including non-perfused tissue stained for Ki67 and necrotic areas (N). Dark blue = vasculature, light blue = perfusion, black = Ki67, red = unperfused vessels, greyscale = tissue background. Scale bar: 150 μm.
4.2.1.15 No change in Ki67 is seen in the perfused rim of tumours after tirapazamine treatment

No decrease in Ki67 labelling was seen in the perfused areas of tumours after treatment with tirapazamine. The average control value is $28 \pm 5.8\%$ compared with $33 \pm 6.5\%$ one day after treatment ($P = 0.16$, Mann-Whitney U test) (Figure 4.15). This is unlike BrdUrd, which showed a decrease 1 day after tirapazamine treatment (Section 4.2.1.4).

![Figure 4.15: The levels of Ki67 do not decrease after treatment with tirapazamine.](image)

The percentage of Ki67 in HCT-116 tumours from mice prior to and 1 day after tirapazamine administration (60 mg/kg, i.p.). Each bar represents data from an individual tumour. Horizontal lines indicate the mean values for each group.
4.2.2 The effects of tirapazamine in SiHa xenografts

The microregional effects of tirapazamine were determined by examining SiHa xenografts 1-4 days after treatment with tirapazamine (60 mg/kg, i.p.). Tumour cryosections were stained for markers of cell proliferation, hypoxia, blood vessels and blood flow to map the effects.

4.2.2.1 Tirapazamine causes central vascular dysfunction and leads to necrosis in SiHa tumour xenografts

A composite tumour image of a typical untreated SiHa (Figure 4.16A), shows mainly perfused blood vessels indicated by CD31 labelled endothelial cells in dark blue surrounded by the perfusion marker carbocyanine in light blue. Non-perfused vessels, which are CD31 positive only, are shown in red but are infrequent.

SiHa xenografts were examined 1, 2, 3 and 4 days after tirapazamine administration (60 mg/kg, i.p.). 1 day after treatment with tirapazamine the central regions of SiHa tumour xenografts are non-perfused similar to the effect seen in HCT-116 tumours (Figure 4.16B). A rim of viable, perfused tissue remains on the edges of the tumour. As described above (Section 4.2.1.1) non-perfused areas are characterized by CD31 staining with no perfusion (red vessels) and an absence of BrdUrd labelling, however the CD31 staining in the non-perfused areas of the SiHa tumours is not as prominent as with the HCT-116. Pimonidazole staining is on the edge of viable tissue areas and diffuses into the non-perfused regions as was previously seen. The non-perfused central areas are necrotic 2-3 days following treatment (Figure 4.16C).

The control level of vessel perfusion (75 ± 11%) in untreated SiHa xenografts was similar to that for HCT-116 tumours and 1 day after tirapazamine treatment it decreases to 61
± 17%. The results however were not significant possibly due to the sample size n=5 for both groups (\( P > 0.05 \), Two-tailed, Mann-Whitney U test). However, as noted above the CD31 staining in the non-perfused areas in SiHa tumours is less than that seen in the HCT-116. Suggesting the CD31 in non-perfused regions of SiHa tumours has already started to disappear 1 day after treatment. Therefore the calculation of perfused vessels will be skewed due to less CD31 staining.

Figure 4.16: Tirapazamine causes central vascular dysfunction in SiHa tumour xenografts. (A) A tumour from an untreated mouse. (B) A tumour from an animal 1 day after administration of tirapazamine (60 mg/kg, i.p.) demonstrating large regions of tissue that contain blood vessels which are not perfused. (C) A tumour 3 days after treatment showing a central necrotic core and a small rim of perfused tissue around the periphery. Dark blue = vasculature, light blue = perfusion, black = dividing cells, red = unperfused vessels, green = hypoxia, greyscale = tissue background. The insets show details of tumour morphology including non-perfused tissue (U) and necrotic areas (N). Scale bar: 150 μm.
4.2.2.2 The response does not occur in all tumours

As with the HCT-116 tumour the induction of non-perfused areas in SiHa tumours after treatment does not occur to the same degree in every tumour. Untreated tumours have 0.59 ± 0.36% of non-perfused tissue, which increases in treated tumours to 27 ± 16%, a value similar to that reported for the HCT-116 tumours (Section 4.2.1.3). In the case of the SiHa tumour 4 of the 5 treated tumours had greater than 24% non-perfused tissue and only one seemed to have a very low value. Necrosis increased over 2-3 days after treatment and some tumours had greater than 80% necrosis (Figure 4.17).

Figure 4.17: The percentage of non-perfused tissue and necrosis increases after treatment. (A) The percentage of non-perfused tumour tissue in SiHa tumour cryosections from mice prior to and 1 day after tirapazamine (60 mg/kg, i.p.). (B) The percentage of necrosis 1-4 days after treatment with tirapazamine. Each bar represents data from an individual HCT-116 tumour. Horizontal lines indicate the mean values for each group.
4.2.2.3 Tirapazamine decreases proliferation in SiHa xenografts

The percentage of BrdUrd labelling in the perfused areas of tissue after tirapazamine treatment was calculated. A slight reduction in BrdUrd incorporation occurs in the remaining perfused areas of the tumour 1 day after treatment with tirapazamine (Figure 4.18). However, 2 days later the level of BrdUrd drop from 23 ± 8.0 to 8.9 ± 3.1% and remains below 12% over the next 3-4 days ($P = 0.02$, Kruskal-Wallis test).

Figure 4.18: Proliferation decreases in SiHa xenografts after treatment with tirapazamine. BrdUrd incorporation was reduced in the perfused regions of SiHa tumours from tirapazamine treated (60 mg/kg, i.p.) mice compared with perfused regions of tumours from untreated animals. Each bar represents data from an individual tumour. The horizontal lines indicate the mean values within each group.
4.2.3 The effects of tirapazamine in SCCVII murine tumours

The microregional effects of tirapazamine were examined in the SCCVII murine tumour 1 and 2 days after treatment with tirapazamine (60 mg/kg, i.p.). Tumour cryosections were stained for markers of cell proliferation, hypoxia, blood vessels and blood flow to map the effects.

4.2.3.1 Tirapazamine causes vascular dysfunction in SCCVII tumours

Tirapazamine treatment causes areas of vasculature dysfunction in SCCVII tumours 1 day after 60 mg/kg of tirapazamine (Figure 4.19). These areas are characterized by the same general characteristics mentioned for defining non-perfused regions, which are CD31 positive vessels with no carbocyanine (blood flow) and an absence of BrdUrd labelling in surrounding cells. Untreated SCCVII tumours have a higher vessel density and less corded structure than either the HCT-116 and SiHa tumours which both have vessels surrounded by ~150 μm of tissue and then necrosis. As well, untreated SCCVII tumours have a high portion of temporarily unperfused vessels. The average percent of perfused vessels in untreated SCCVII tumours is 45 ± 13%, which is considerably lower than the 75% perfusion seen in both the HCT-116 and SiHa tumours. In composite images acute unperfused vessels tend to be a few vessels, which were unperfused at the time of carbocyanine and often have some pimonidazole staining indicating these vessels were hypoxic at some time during the 2-hour pimonidazole administration. This temporary vessel occlusion does not cause a lack of BrdUrd labelling because presumably BrdUrd is still able to diffuse to the tissue when the vessels is open during the two hour exposure. Therefore, in the case of SCCVII tumours, non-perfused regions, due to tirapazamine, are identified by areas of tumour cells that do not have BrdUrd labelled cells. An example of an untreated SCCVII is shown in Figure 4.19A.
indicating areas of unperfused vessels (red) surrounded by BrdUrd labelling. In contrast Figure 4.19B shows areas of vascular dysfunction after treatment, which again have unperfused vessels (red), hypoxia, but also have no BrdUrd labelling.

The overall percentage of vessel perfusion decreases in SCCVII tumours 1 day after treatment with tirapazamine from 45 ± 13 down to 26 ± 15% ($P = 0.0050$, Two-tailed Mann-Whitney U test).

Figure 4.19: Tirapazamine causes central vascular dysfunction in SCCVII murine tumours. (A) A stained SCCVII tumour cryosection from an untreated mouse. (B) An SCCVII tumour 1 day after administration of tirapazamine (60 mg/kg, i.p.) demonstrating regions of tissue that contain blood vessels which are not perfused and a lack of BrdUrd labelling. Dark blue = vasculature, light blue = perfusion, black = dividing cells, red = unperfused vessels, green = hypoxia, greyscale = tissue background. The insets show details of tumour morphology including temporarily unperfused vessels, in an untreated tumour, that have BrdUrd labelling (white arrow) and non-perfused tissue in a treated tumour (U). Scale bar: 150 µm.
4.2.3.2 Not all SCCVII tumours exhibit vascular dysfunction

Vascular dysfunction does not occur in all treated SCCVII tumours and on average the percentage of non-perfused tissue is $37 \pm 30\%$ compared with no areas in the control tumours. As mentioned non-perfused tissue is defined as tissue affected by tirapazamine, which exhibits no perfusion as well as no BrdUrd labelling, which is different from the non-perfused vessels that occur due to intermittent blood flow. Therefore in the SCCVII there is no non-perfused tissue in the controls even though there is intermittent blood flow because the areas that have intermittent blood flow are surrounded by BrdUrd labelling. The response of SCCVII tumours to tirapazamine ranges from less than 1% non-perfused tissue to greater than 95% non-perfused tissue (Figure 4.20A). Unlike with HCT-116 and SiHa xenografts most SCCVII tumours do not become necrotic over time. The percentage of necrotic tissue in control SCCVII is $0.37 \pm 0.63\%$ and this significantly increases 2 days after treatment to $11 \pm 18\%$ but only 3 of 8 tumours responded with over 10% of necrosis ($P = 0.013$, Mann-Whitney U test) (Figure 4.20B).
Figure 4.20: **Non-perfused tissue increases after treatment and necrosis increases in a few tumours.** (A) The percentage of non-perfused tumour tissue in SCCVII tumour cryosections from mice 1 day after tirapazamine (60 mg/kg, i.p.). Tumours from untreated mice have a value of 0. (B) Necrosis increases in a few tumours 2 days after treatment. Each bar represents data from an individual tumour. The horizontal lines indicate the mean value.

4.2.3.3 Proliferation decreases in regions not affected by tirapazamine

BrdUrd labelling decreases in SCCVII tumours 1 day after treatment with tirapazamine from control levels of $15 \pm 5\%$ down to $5.1 \pm 1.2\%$ ($P < 0.0001$, Mann-Whitney U test) (Figure 4.21). The data were analysed in the perfused portions of tumours to see what effect tirapazamine has in areas where vascular dysfunction does not occur.
4.2.4 The effects of tirapazamine analogues in HCT-116 xenografts

Three benzotriazine analogues of tirapazamine were administered to HCT-116 tumour bearing mice and the tumours were examined for indications of vascular dysfunction. The 3' group is varied between the structures where for tirapazamine it is an amino group, for SR 4754 a diethylaminoethylamine group, for SR 4482 a hydrogen and for 4898 a propyl group. The compounds were administered at approximately 70-80% of the LD50 values.

4.2.4.1 Only SR 4754 causes significant central vascular dysfunction

Tumours were examined 1 day after administration of each of the three tirapazamine analogues to assess the potential of each drug for central vascular dysfunction. Of all the three tirapazamine analogues tested only SR 4754 caused considerable observable central
vascular dysfunction as shown by the overall percent of non-perfused tissue (Figure 4.22). The average percentage of non-perfused tissue in untreated tumours is $1.8 \pm 1.0$ which increases to $34 \pm 25$ after treatment with SR 4754 (75 mg/kg, i.p.). There is a slight increase in non-perfused tissue after treatment with SR 4898 to $5.7 \pm 2.2$ and a slightly larger increase with SR 4482 to $9.2 \pm 6.6$. Statistical analysis using the Kruskal-Wallis test gave $P = 0.0093$ for all groups and groups were compared with the untreated tumours using a Dunn’s multiple comparison test where only the change caused by SR 4754 was significant ($P < 0.01$).

Figure 4.22: SR 4754 causes central vascular dysfunction. The percentage of non-perfused tumour tissue in HCT-116 tumour cryosections from untreated mice and mice 1 day after treatment with various tirapazamine analogues. An increase in non-perfused tissue is seen after SR 4754 (75 mg/kg, i.p.), however only a small increase in non-perfused tissue is seen in some tumours after treatment with SR 4898 (90 mg/kg, i.p.) and SR 4482 (100 mg/kg, i.p.). Each bar represents data from an individual tumour. Horizontal lines indicate the mean values for each group.
4.2.4.2 SR 4754 is the only analogue tested that causes a significant decrease in BrdUrd

A decrease in BrdUrd incorporation from untreated tumours with 13 ± 1.9% labelling to 4.2 ± 3.2% occurs after SR 4754 administration (Figure 4.23). By contrast SR 4898 and 4482 did not significantly decrease BrdUrd labelling (8.7 ± 2.8% and 8.9 ± 3.2%, respectively). Statistical analysis gave $P = 0.0019$ using the Kruskal-Wallis test for all groups and each group was compared with the untreated tumours using a Dunn’s multiple comparison test showing the only significant change was caused by SR 4754 ($P < 0.01$).

![Figure 4.23: Proliferation decreases after treatment with SR 4754.](image)

*Figure 4.23: Proliferation decreases after treatment with SR 4754.* The levels of BrdUrd incorporation were analysed in untreated mice and in mice 1 day after treatment with tirapazamine analogues. BrdUrd labelling in the perfused regions of HCT-116 tumours was significantly reduced 1 day after SR 4754 treatment (75 mg/kg, i.p.). Each bar represents data from an individual tumour. The horizontal lines indicate the mean values within each group.
4.2.4.3 Composite images show a typical pattern of vascular dysfunction after treatment with SR 4754

In preliminary experiments, which examined all three analogues, SR 4754 caused large central areas of non-perfused tissue in HCT-116 tumour xenografts 1 day after treatment. Due to the variable response of tumours at 75 mg/kg and the low toxicity seen in mice the dose of SR 4754 was increased to 80 mg/kg and tumours were examined 1, 2, 3, and 4 days after treatment. Composite images 1 day after SR 4754 administration show a typical pattern of perfused, viable tissue surrounding an unperfused central tumour area characterized by CD31 staining, no perfusion marker, a lack of BrdUrd and diffuse pimonidazole labelling (Figure 4.24B).
Figure 4.24: Images of tumours treated with SR 4754 show characteristic central vascular dysfunction. Images of HCT-116 cryosections showing (A) a tumour from a control mouse and (B) a tumour from a mouse 1 day after administration of SR 4754 (80 mg/kg; i.p.) demonstrating large regions of tissue that contain blood vessels which are not perfused (red). Dark blue = vasculature, light blue = perfusion, black = dividing cells, red = unperfused vessels, green = hypoxia, greyscale = tissue background. The insets show details of tumour morphology including non-perfused tissue (U) and necrotic areas (N). Scale bar: 150 μm.
4.2.4.4 Non-perfused regions become necrotic 2 days after treatment with SR 4754

All 5 of the tumours treated with SR 4754 (80 mg/kg, i.p.) respond to treatment, which suggests an increased effect from that seen when mice were treated with 75 mg/kg SR 4754 (Section 4.2.4.1). The percentage of non-perfused tissue increases from 0.82 ± 1.0% in untreated mice to 51 ± 5.8% 1 day after treatment ($P = 0.0079$, Two-tailed Mann-Whitney U test) (Figure 4.25A). 2 days after treatment an increase in necrosis is seen suggesting the non-perfused areas are becoming necrotic as is seen after tirapazamine treatment (Figure 4.25B).

Figure 4.25: Vascular dysfunction leads to necrosis after treatment with SR 4754. (A) The percentage of non-perfused tumour tissue in HCT-116 tumour cryosections from mice prior to and 1 day after SR 4754 (80 mg/kg, i.p.). (B) The percentage of necrosis 1-4 days after treatment with SR 4754. Each bar represents data from an individual tumour. Horizontal lines indicate the mean values for each group.
4.2.4.5 SR 4754 administered intravenously causes central vascular dysfunction

Tirapazamine, in saline, cannot be given intravenously at a dose of 60 mg/kg because due to the low solubility of the compound the volume of injection is too large to be administered intravenously. A lower dose (14 mg/kg) could be given, but as mentioned above (Section 4.2.1.6) the effect of tirapazamine is dose dependent and therefore central vascular dysfunction would likely not occur at a dose of 14 mg/kg.

However, tumours were treated intravenously with SR 4754 (80 mg/kg) and excised 1 day after treatment. Of the six tumours that received SR 4754 four responded with high levels of non-perfused tissue (>20%). The percent of non-perfused tissue was analysed and the average value was 25 ± 21 compared to 0.27 ± 0.18% in the controls (Figure 4.26). A rim of perfused tissue still remains around the edge of tumours.

![Figure 4.26: Intravenous SR 4754 causes central vascular dysfunction.](image)

The percentage of tumour tissue that is non-perfused in HCT-116 tumour cryosections from mice prior to and 1 day after SR 4754 administered i.v. (80 mg/kg). Each bar represents data from an individual tumour. Horizontal lines indicate the mean values for each group.
4.2.5 RB 6145, another class of hypoxic cytotoxin

4.2.5.1 RB 6145 does not induce vascular dysfunction

RB 6145, a prodrug of the bioreductive agent RSU 1069, was administered i.p. to HCT-116 tumour bearing mice at a dose of 250 mg/kg (80% of the MTD in mice) and the tumours excised 1 day after treatment. The data were compared with tumours treated with tirapazamine in the same experimental group as RB 6145 to ensure a positive control. RB 6145 did not cause central vascular dysfunction in HCT-116 tumours. The percent of non-perfused tissue in controls is 0.29 ± 0.48% which is not significantly different from 0.60 ± 0.19% of non-perfused tissue occurring after RB 6145 treatment (P = 0.19, Mann-Whitney U test) (Figure 4.27). Tirapazamine on the other hand showed an increase to 20 ± 23% of non-perfused tissue.

![Graph showing percentage of non-perfused tissue](image)

**Figure 4.27: RB 6145 does not cause central vascular dysfunction.** The graph shows the percentage of tumour tissue that is non-perfused in HCT-116 tumour cryosections from mice prior to and 1 day after tirapazamine (60 mg/kg, i.p.) and RB 6145 (250 mg/kg, i.p.). Each bar represents data from an individual tumour. Horizontal lines indicate the mean values for each group.
4.2.5.2 No change in proliferation occurs after RB 6145 administration

The percentage of BrdUrd incorporation was calculated for tumours treated with RB 6145. Control values of BrdUrd labelling are 15 ± 5.9% and no decrease in BrdUrd labelling occurs 1 day after RB 6145 administration (17 ± 4.7% BrdUrd labelling) \((P = 0.56, \text{ Mann-Whitney U test})\) (Figure 4.28). Tirapazamine showed a decrease 1 day after administration, as expected, to 7.7 ± 1.1% BrdUrd labelling.

![Graph showing BrdUrd labelling](image)

**Figure 4.28: No decrease in BrdUrd labelling occurs after RB 6145.** Compared to untreated tumour values no decrease in BrdUrd incorporation in the perfused regions of HCT-116 tumours was seen after RB 6145 administration (250 mg/kg i.p.). Each bar represents data from an individual tumour. The horizontal lines indicate the mean values within each group.
4.3 Discussion

The microregional effects of tirapazamine on cell proliferation were examined using our tumour mapping technique and unexpectedly found that tirapazamine causes vascular dysfunction in HCT-116 and SiHa tumour xenografts and SCCVII murine tumours. We then went on to determine if this is an isolated occurrence or whether non-perfused areas arise after the administration of other hypoxic cytotoxins and found SR 4754, an analogue of tirapazamine, also causes central vascular dysfunction, but 2 other analogues did not.

4.3.1 The effects of tirapazamine

Tirapazamine was designed to target hypoxic cells that are resistant to radiation. It has been shown to have preferential toxicity toward hypoxic cells \textit{in vitro} (Zeman et al. 1986) and in animal studies tirapazamine enhances the effects of both radiotherapy and chemotherapy (Zeman et al. 1988; Dorie and Brown 1993; Dorie et al. 1994; Dorie and Brown 1997; Siemann and Hinchman 1998). However, tirapazamine has a limited ability to penetrate through multiple layers of cells and therefore a diminished effect on the target cells far from vasculature (Hicks et al. 1998; Kyle and Minchinton 1999; Hicks et al. 2003).

4.3.1.1 Effect of tirapazamine in HCT-116 and SiHa xenografts

Examination of the microregional effects of tirapazamine in HCT-116 and SiHa tumour cryosections led to the discovery that tirapazamine causes extensive central vascular dysfunction 1 day after treatment followed by necrosis over the next 1-2 days (Figure 4.1 and Figure 4.16). In both of these tumour types the disruption of vasculature causes the typical tumour microenvironment to change.

Untreated HCT-116 and SiHa tumour xenografts exhibit similar characteristics whereby most tumour blood vessels are perfused and surrounded by ~15 layers of dividing
cells beyond which, 100 to 150 μm from the blood vessel, chronic hypoxia occurs. The areas of hypoxia tend to be located near necrosis in the central regions of tumours and less hypoxia is seen in tissue on the edge of tumours. Inter-tumour variability can lead to subtle differences in the percentages of hypoxia, proliferation, intermittent blood flow and degree of corded structure within tumours of the same cell type, however treatment with tirapazamine causes significant changes in these traits. Tirapazamine induced central vascular dysfunction is distinguished by an entire central area with blood vessels that stain for a vascular marker, yet have no perfusion marker, surrounded by cords of cells with no labelling of proliferation or hypoxia.

Hypoxia is detected using pimonidazole, which is an exogenously administered 2 hours prior to tumour excision. In untreated tumours, pimonidazole is able to diffuse from vasculature to the cells ~150 μm away as indicated by the labelling of cells on the edge of necrosis (Figure 4.1A). Tumours treated with tirapazamine exhibit a gradient of hypoxia from the border of the perfused areas towards the centre of non-perfused areas where there is no pimonidazole staining (Figure 4.1B). However, pimonidazole is administered 22 hours after tirapazamine and at this point vessels in the centre of the tumour would already be dysfunctional. As a result, pimonidazole would have to diffuse from the peripheral vasculature into the centre of the tumours. The lack of staining in the centre of tumours therefore suggests pimonidazole does not diffuse further than a few hundred microns through tissue and cannot reach the hypoxic cells located within the mass of tissue that has been affected by vascular dysfunction. As BrdUrd, a marker of proliferating cells, is also administered 2 hours prior to tumour excision the absence of BrdUrd labelling in the central non-perfused areas of tumours could similarly be due to lack of access. It is also possible the
non-perfused areas show no BrdUrd labelling due to a cessation of cell division. HCT-116 tumour were stained for Ki67, an endogenous marker present in all phases of the cell cycle except G0 (resting phase), to determine whether cells in the non-perfused areas are cycling. Ki67 is present in areas where central vascular dysfunction occurs, however this does not necessarily mean cells in the non-perfused regions are dividing as studies have shown cells in a cell cycle block that do not incorporate BrdUrd will stain for Ki67 (van Oijen et al. 1998; Scholzen and Gerdes 2000). As expected, a higher percentage of Ki67 than BrdUrd labelling is seen in untreated tumours as Ki67 labels all cells that are in cycle whereas BrdUrd labels only those cells in S-phase. In the HCT-116, no change in Ki67 labelling is seen in the perfused areas after treatment, but BrdUrd labelling decreases suggesting there is an effect on cell proliferation and again Ki67 may be labelling cells in a cell cycle block.

The levels of proliferation in the remaining perfused areas after central vascular dysfunction occurs (1 day after treatment) were examined to determine if a decrease in proliferation is preferentially seen far from vasculature. Only 2 of 7 tumours showed a larger decrease in proliferation at areas far from vasculature compared to proliferation near vessels. Tumours that did not exhibit vascular dysfunction did not have a decrease in proliferation far from vasculature and in fact one tumour showed a slight increase in proliferation far from vasculature as compared to the controls.

The effects of tirapazamine were examined at early time points from 1-24 hours after tirapazamine and found central vascular dysfunction initially occurs as early as 6 hours after treatment. Therefore the vessel perfusion does not cease immediately after tirapazamine treatment and the mechanism causing vessel dysfunction may involve a non-direct effect on vasculature or it may require time for an effect on endothelial cells to manifest.
In order to determine where the damage was occurring shortly after tirapazamine administration, tumours were stained for γH2AX, an immunohistochemical marker of double-strand breaks (DSB) (Banath and Olive 2003). HCT-116 tumours were stained for γH2AX 1, 2, 6, 8 and 24 hours after treatment. Phosphorylation of H2AX, to form γH2AX, is apparent within a minute of a DSB and reaches a maximum after 10 min. Dephosphorylation starts to occur after 30 min with a half-life of 2 hours (Redon et al. 2002). Therefore information from the time points greater than 6 hours after treatment may not be representative of damage. As expected, 1-2 hours after treatment an increase in DSBs occurred indicating tirapazamine causes DSBs in clonogenic cells before the onset of vascular dysfunction (Olive et al. 2004). As well, we show that the increase in γH2AX early on is higher far from vasculature, as has been shown by Olive et al., theoretically due to the decreased oxygen tension in these cells (Olive et al. 2004). It is questionable how important this early damage is as the non-perfused areas that occur after 6 hours and subsequent necrosis will inevitably kill these damaged cells. At the later time points the non-perfused areas have no γH2AX staining, levels below those of the controls, suggesting that a lack of energy in the non-perfused regions may render the ATM (ataxia telangiectasia mutated) protein kinase unable to phosphorylate H2AX (Shiloh 2003). Alternatively, since H2AX is phosphorylated preferentially in S-phase cells after tirapazamine administration the decrease in labelling may be due to an absence of S-phase cells in the non-perfused areas (Banath and Olive 2003). A report examining the levels of γH2AX in SiHa xenografts and SCCVII tumours 90 minutes after 60 mg/kg of tirapazamine (the same dose used in these experiments) shows γH2AX staining is about 50% lower than would be expected from survival data which could happen if non-perfused regions arise (Olive et al. 2004). However,
our data suggests large amounts of non-perfused tissue would not necessarily be present at this early time point.

Apoptosis was examined in tirapazamine treated HCT-116 tumours by staining for activated caspase-3 in tumour cryosections and determined no significant increase in apoptosis was seen from 1-4 days after tirapazamine administration. Apoptosis could increase in the non-perused areas if a cytotoxic effect on clonogenic cells occurred after treatment, however if the cytotoxic effect were only on endothelial cells an increase in apoptosis may be too small to detect in our system which analyses entire cryosections. An absence of apoptotic response is likely due to necrotic cell death instead, as suggested by the increased necrosis in the centre of tumours at later time points. It is also possible an increase in apoptosis is not observed due to the cell response time after treatment and the time points of 1-4 days at which tumours were analysed.

4.3.1.2 Effects of tirapazamine in SCCVII

Untreated SCCVII murine tumours differ from the above tumour types in that they have a higher vascular density than the corded xenograft tumours and acute hypoxia due to elevated intermittent blood flow (Figure 4.19A) (Chaplin et al. 1987; Durand and LePard 1995). Hypoxia occurs in patches throughout the SCCVII tumour including some areas where vessels are perfused, the later of which likely occurs over the 2 hours of pimonidazole exposure. BrdUrd labelling occurs throughout the tumour including those areas that have intermittent blood flow.

Administration of tirapazamine to mice bearing SCCVII murine tumours causes vascular dysfunction 1 day after treatment and the effects are characterized by non-perfused regions that label for hypoxia but do not have BrdUrd labelling (Figure 4.19B). The regions
of vascular dysfunction are for the most part in the centre of the tumour but the entire centre of the tumour is not dysfunctional as is seen in the corded tumours. The areas of non-perfused tissue in the SCCVII are in patches within the central region of the tumours and for this reason pimonidazole is likely able to diffuse from the nearest perfused vessel to the non-perfused regions and label hypoxia. Two days after treatment some tumours become necrotic but quite a few do not suggesting these tumours do not tend toward necrosis possibly due to the higher vessel density and patchy location of non-perfused areas. These factors would result in areas of vascular dysfunction within a few hundred microns of perfused vessels, which could provide nutrients and oxygen to the non-perfused areas. Interestingly the non-perfused areas do not have BrdUrd, which as mentioned above is either due to a cessation of cell division or to the inability of BrdUrd to diffuse to those cells (section 4.3.1.1). However, in the case of the SCCVII tumours the patchy occurrence of non-perfused areas and high vessel density suggests BrdUrd could diffuse to the non-perfused areas indicating a cessation of proliferation in these areas is more likely. Proliferation decreases in the perfused areas of SCCVII tumours, 1 day after treatment, compared with untreated tumours showing tirapazamine has an effect on cell proliferation in the remaining viable areas.

4.3.2 The effects of tirapazamine analogues

In order to determine whether hypoxic cytotoxins structurally similar to tirapazamine can cause central vascular dysfunction three analogues were examined, SR 4754, SR 4898 and SR 4482. All three are benzotriazine analogues with selective toxicity to hypoxic cells.

4.3.2.1 SR 4754 causes central vascular dysfunction

The effects of three tirapazamine analogues SR 4754, SR 4898, and SR 4482 were examined at doses of 75-80 mg/kg, 100 mg/kg and 90 mg/kg, respectively 1 day after
Chapter 4 – Mapping the effects of hypoxic cytotoxins

treatment. SR 4754 administered at 75 mg/kg caused central vascular dysfunction in HCT-116 tumour xenografts (Figure 4.22). The other two analogues did not cause central vascular dysfunction. A decrease in proliferation was seen 1 day after SR 4754 administration however neither SR 4898 or 4482 caused a decrease in proliferation. The effect of SR 4754 (80 mg/kg) 1-4 days after administration was then studied and 1 day after treatment all 5 of 5 tumours treated exhibited vascular dysfunction at this slightly higher dose and an increase in necrosis occurred two days after treatment. As with tirapazamine SR 4754 could be dose dependent and therefore the higher dose may have caused an increased response. SR 4754 is three times more toxic to hypoxic cells than tirapazamine and has a higher hypoxic cytotoxicity ratio (HCR) (Minchinton et al. 1992; Kelson et al. 1998). A higher HCR means more cytotoxicity under anoxia, which could relate to increased activation of SR 4754 compared to tirapazamine and therefore at intermediate levels of oxygenation increased cytotoxicity toward blood vessels. Both SR 4898 and 4482 have a lower relative hypoxic cytotoxicity compared to tirapazamine (Kelson et al. 1998). In fractionated radiotherapy experiments in mice SR 4754 was not found to be superior to tirapazamine and SR 4482 was less effective than tirapazamine (Kelson et al. 1998). Therefore SR 4754 may have acted similarly to tirapazamine causing vascular dysfunction, whereas SR 4482 did not. However, as these experiments were clonogenic assays it is possible the effects due to vascular disruption are not observed because the hypoxic cells in the centre of the tumour could be rescued by dissociating and plating them in media. Alternately, the cytotoxicity of the compounds could be reduced due to a lower drug exposure and SR 4482 has a much shorter half-life in mice (2.1 min) compared to either SR 4233 or SR 4754 (15 and 7 min, respectively) (Minchinton et al. 1992). Of note, SR 4317, the non-toxic, two electron
reduction product of tirapazamine, was examined and it was determined SR4317 does not cause vascular dysfunction in tumours (data not shown).

4.3.2.2 Intravenous administration of SR 4754 causes central vascular dysfunction

SR 4754 was designed to have improved aqueous solubility and is 3 times more soluble than tirapazamine. SR 4754 was intravenously injected into mice at a dose of 80 mg/kg (the same dose given i.p.) in order to introduce a higher concentration directly into the blood and increase the drug exposure to the endothelial cells. This cannot be done with tirapazamine as the aqueous solubility is too low. Central vascular dysfunction occurs when SR 4754 is given via intravenous (i.v.) administration with similar characteristics as seen after i.p. administration including an outside rim of unaffected perfused tissue (Figure 4.26). Compared with i.p. administration, no increased effect is seen and i.v. administration does not appear to be able to eradicate the healthy rim on the periphery of tumours.

4.3.3 The effects of RB 6145

Due to the positive result seen with SR 4754 an unrelated compound RB 6145, a prodrug of the hypoxic cytotoxin RSU 1069, was examined to determine if it causes vascular dysfunction.

4.3.3.1 RB 6145 does not cause central vascular dysfunction

The effects of RB 6145 (250 mg/kg, i.p.) in HCT-116 tumours was examined 1 day after tirapazamine and found no evidence of central vascular dysfunction (Figure 4.27). As well no change in proliferation was seen 1 day after treatment. The clonogenic surviving fraction, of excised tumours, after administration of RB 6145 at 0.75 mmol/kg (280 mg/kg) is ~ 0.7, which is much higher than the surviving fraction of 0.4 after administration of 30
mg/kg tirapazamine (Siemann 1995; Papadopoulou et al. 2001). Therefore, the dose of RB
6145 used in these experiments will not cause the same cell kill as the dose of tirapazamine
used, which probably relates to the different oxygen tensions required to activate the two
complexes. Additionally, the oxygen tension in blood vessel may not be low enough to
activate RSU 1069, which is converted at oxygen tensions below 1% (Koch 1993), resulting
in no effect on vasculature. This is further discussed in section (4.3.6).

4.3.4 Vascular dysfunction does not occur in all treated tumours

The ability of tirapazamine to induce non-perfused areas in all tumour types tested is
variable in terms of how many tumours respond and to what degree. Similarly the increase in
necrosis occurs in several, but not all tumours from tirapazamine treated animals. This
variability has not directly correlated with the size of tumours, age of mice, or growth rate
within the narrow range of experimental conditions used (chapter 2). Tirapazamine was
found to be dose dependent whereby the higher the dose the larger the percentage of non-
perfused tissue in tumours that respond. However, it does not appear that an increased dose
causes more tumours to respond with high levels of non-perfused tissue suggesting that a
dose above 60 mg/kg would not necessarily cause all the tumours to respond (Figure 4.6).

4.3.5 Vascular dysfunction caused by tirapazamine has not previously been reported

Tirapazamine induced vascular dysfunction has not been previously reported, which
may be due to the methods and timing used to determine the anti-tumour activity. Often
assays only look at vessel perfusion, cell damage or surviving fraction within a few hours
after treatment and for the most part excision assays, to quantify cell kill within tumours,
have been utilized (Durand and Olive 1997; Olive et al. 2004). Typically animals were
treated with tirapazamine and/or radiation or chemotherapy and up to 1 day later the tumour
was excised and surviving cells assessed using *in vitro* clonogenic assays. In these assays the tumour tissue is dissociated to a single cell suspension and the deprived cells in the non-perfused regions of the tumour could be rescued by being placed in nutrient and oxygen rich media for growth *in vitro*.

Indications that tirapazamine may do more than just kill hypoxic cells can be inferred from previous laboratory studies indicating tirapazamine treatment alone causes a growth delay of 2-3 days and a clonogenic surviving fraction of 0.2-0.3 in RIF-1 tumours (Dorie and Brown 1993). The extent of these effects are much greater than would be expected if only hypoxic cells in the tumour were eradicated (Moulder and Rockwell 1987).

Clinical reports suggest the maximum tolerated dose of tirapazamine in humans is $390 \text{ mg/m}^2$ for which the $C_{\text{max}}$ and AUC are $7.1 \text{ \mu g/ml}$ and $1410 \text{ \mu g ml}^{-1}\text{min}^{-1}$ in humans, respectively (Graham *et al.* 1997). This is similar to the extrapolated $C_{\text{max}}$ in C3H mice of $8.9 \text{ \mu g/ml}$ (AUC = $192 \text{ \mu g/ml}^{-1}\text{min}^{-1}$) for a dose of $0.06 \text{ mmol/kg (11 mg/kg)}$ (Minchinton *et al.* 1992). As the induction of non-perfused tissue by tirapazamine is dose dependent a concentration that is high enough to see the effect in humans may not be reached with a dose of $390 \text{ mg/m}^2$. This should be considered when assessing the effects of tirapazamine in clinical trials.

An observation of necrosis on the tail tip of mice administered tirapazamine was attributed to an effect on vasculature and could be relevant to our observation of vascular damage in tumours (Langmuir *et al.* 1994). Additionally a decrease in tumour blood flow was seen 10 minutes after i.p. administration of $60 \text{ mg/kg}$ of tirapazamine (Durand and Olive 1997).
4.3.6 Is the vascular dysfunction hypoxia related

One explanation for the observed central vascular dysfunction in tumours treated with tirapazamine is a direct effect on endothelial cells or perivascular cells at intermediate oxygen tensions resulting in damage to the vessel followed by oxygen and nutrient deprivation to cells fed by that vessel and subsequently necrosis. It is not common to see pimonidazole staining adjacent to blood vessels in untreated tumours, however the degree of hypoxia required to produce tirapazamine-induced damage may not be sufficient to cause pimonidazole staining. Pimonidazole binds below 1% oxygen, however the cytotoxicity of tirapazamine is oxygen dependent and radical formation begins to occur at levels of oxygen below 5% (Koch 1993; Raleigh et al. 1999). Tumour vasculature may very well reside between 2-5% oxygen as suggested by experiments in LS174T human colon xenografts where the measured oxygen partial pressure in cells close to blood vessels is 12-14 mmHg (about 2% oxygen) (Helmlinger et al. 1997). Another report examining rat mammary adenocarcinomas suggests a differential in the perivascular oxygen tension between the tumour periphery (4%) and the centre of the tumour (2%) (Dewhirst et al. 1992).

Alternatively a portion of a tumour blood vessel could become hypoxic, for example because the venous end of the vessel has a lower oxygen tension due to loss of oxygen to surrounding tissue as the blood travels through. In these later portions of the vessels the oxygen tension would be low and damage due to conversion of tirapazamine could prevent flow through the entire vessel.

Finally, blood vessels and thus endothelial cells in tumours can become severely hypoxic for short periods of time in areas of intermittent blood flow. In these regions tirapazamine could be present and activated to its toxic form thus damaging an area of the vessel and resulting in a cessation of perfusion through the vessel. Blood vessels in the centre
of tumours may be smaller or less stable than vessels in the rim of the tumour and thus more prone to collapse thereby creating vascular dysfunction selectively in the central regions of tumours (Tozer et al. 1999). Increasing interstitial fluid pressure (IFP) occurs from the edge into the centre of tumours and could contribute to increased intermittent blood flow in the centre of tumours (Boucher et al. 1990). In the xenografts tumours, which have very little intermittent blood flow, a small area of one vessel in the centre may collapse leading to damage and subsequent shutdown through that vessel, however this would not likely lead to the entire centre of the tumour becoming dysfunctional. Instead, this mechanism of selectivity may play a larger role in tumours, such as the SCCVII, which have a higher proportion of intermittent blood flow.

RB 6145 does not cause vascular dysfunction providing support for direct cell toxicity due to conversion of tirapazamine in blood vessels that have intermediate levels of hypoxia. As mentioned above (section 4.3.3.1), the conversion product of RB 6145, RSU 1069, has a lower Km than tirapazamine and is only converted to its toxic form at oxygen concentration below ~1% (Koch 1993). Therefore the oxygen tension in tumour blood vessels would not be low enough to activate RSU 1069 and no vascular dysfunction would be observed. Alternately, RB 6145 may not behave in the same manner as tirapazamine and therefore not be able to cause vascular dysfunction. The observation of a lack of BrdUrd labelling around vessels in the perfused areas of a few tumours (Figure 4.9) indicates tirapazamine is cytotoxic to cells near vessels, which could result if the vessels reside at a low oxygen tension.

Compression of the tumour vasculature due to clonogenic cell death and swelling is an alternate explanation for the vascular dysfunction seen, and although small areas of
reduced proliferation surrounding the vasculature suggest some clonogenic cells are affected (section 4.2.1.9) the lack of apoptosis implies these cells may be in a cell cycle block and not dying (section 4.2.1.13).

4.3.7 Other vascular disrupting agents show similar effects to tirapazamine

Tubulin binding agents such as colchicine and the vinca alkaloids can cause vascular dysfunction but at maximum tolerated doses (Seed et al. 1940; Ludford 1946; Ludford 1948; Hill et al. 1993). The most promising tumour vascular targeting agents are the combretastatins, a class of tubulin binding agents, which cause prolonged and extensive reduction in blood flow and induce vascular mediated tumour necrosis in animal models, effects similar to those seen after treatment with tirapazamine (Dark et al. 1997; Tozer et al. 2002). The exact mechanism by which vascular disruption occurs after treatment with combretastatin A-4 phosphate (CA-4-P) is unclear, however CA-4-P causes microtubule dysfunction and reorganization of the cytoskeleton as well as disruption of the junction molecule vascular endothelial cadherin (Kanthou and Tozer 2002; Tozer et al. 2002; Vincent et al. 2005). The kinetics of vascular shutdown differ between tirapazamine and CA-4-P where within 20 minutes after CA-4-P administration the central vasculature of tumours begins to shutdown and after 1 day the entire centre is necrotic (Dark et al. 1997; Tozer et al. 2001). Both CA-4-P and tirapazamine cause areas of central vascular disruption but have almost no effect on the tissue around the periphery of the tumour. It has been suggested the vasculature on the rim of tumours may have compromised blood flow and be hypoxic shortly after (within 1 hour) CA-4-P administration (El-Emir et al. 2005; Tozer et al. 2005). However, in the case of tirapazamine we have shown the vessel perfusion through the tumours is functional 1 hour after treatment (Figure 4.7) and from 6 hours to 24 hours after
treatment, when vessel dysfunction occurs, the vasculature in the rim is functional and these areas do not stain with pimonidazole suggesting that the cells in the rim are not critically hypoxic (Figure 4.12 and Figure 4.1). The reason for the remaining viable rim is not well understood and could be due in part to higher interstitial fluid pressure in the centre of tumours or differences between the regional vasculature, where the central vasculature is not as stable, established or dense so recovery from a disruptive event is more difficult (Tozer et al. 2005). Additionally, the central vasculature could have less pericyte coverage and be more susceptible to cytotoxic damage (Gee Michael et al. 2003). These factors may be a reason for the similar specificity of tirapazamine and the combretastatins or, as mention above (section 4.3.6), the central specificity of tirapazamine may primarily be due to the low oxygen tension in the central vasculature. The key interest here is that both compounds cause the same effect even though they may have different mechanisms of action. Elucidating the means by which these compounds affect the central vasculature may lead to a better understanding of how to target this area and the remaining perfused rim of tissue, and to the discovery of new drugs with better specificity.

4.3.8 NOS inhibition may be a mechanism for tirapazamine mediated vascular disruption

Tirapazamine can inhibit NOS (nitric oxide synthase) and be converted to an active radical under hypoxic conditions resulting in DNA single strand breaks and at high doses double strand breaks. (Garner et al. 1999). Additionally an increase in tirapazamine cytotoxicity was seen in cells transfected to overexpress NOSH which could suggest a mechanism of activity in endothelial cells (Chinje et al. 2003). Endothelial cells express eNOS (NOS II), which could be inhibited by tirapazamine thereby reducing the nitric oxide production in blood vessels causing them to constrict. This constriction will cause hypoxia in
the vessels and could further potentate the effect of tirapazamine. Enhancement of other vascular targeting agents has been seen when a NOS inhibitor is co-administered and the effect of RB 6145 was potentiated when combined with a NOS inhibitor, which was thought to be due to the increased hypoxia created after vasoconstriction (Butler et al. 1997; Davis et al. 2002). Experiments in chapter 5 further explore and discuss the concept of NOS inhibition leading to vascular dysfunction.

4.4 **Summary**

These findings contradict the widely held view that the activity of tirapazamine is due to selective toxicity to hypoxic tumour cells located distal to blood vessels. Instead our findings suggest that tumour vasculature, located in the centre of solid tumours, is disrupted and the resulting vessel dysfunction leads to widespread central necrosis. The hypoxic cells resistant to therapy are eradicated in this process, which could underlie the activity of tirapazamine in animal and human studies. Work is required to further characterize the mechanism of action behind the central vascular dysfunction and to determine whether this effect is operative in humans treated with tirapazamine.
Chapter 5 - Determining the mechanism behind the vascular dysfunction caused by tirapazamine

5 DETERMINING THE MECHANISM BEHIND THE VASCULAR DYSFUNCTION CAUSED BY TIRAPAZAMINE

5.1 Introduction

By mapping the tumour microenvironment in HCT-116 and SiHa tumour xenografts as well as the SCCVII murine tumour, we have shown that the hypoxic cytotoxin tirapazamine causes vascular dysfunction which progresses to necrosis in the xenograft models (chapter 4). The effect of tirapazamine is similar to that of CA-4-P, as both cause areas of central vascular disruption but have almost no effect on the tissue periphery. The mechanism by which vascular targeting agents (VTAs) evoke preferential toxicity toward the central vasculature is not well understood.

VTAs are compounds that selectively target the existing tumour vasculature. In general, VTAs all cause a cessation of perfusion in central blood vessels, which subsequently progresses to large areas of necrosis, within 1 day after administration. Additionally, a viable rim of tissue with perfused vasculature remains after treatment. Combretastatin A-4-phosphate (CA-4-P), the most studied VTA, is a microtubule destabilizing drug that also interferes with the endothelial cell-specific junctional molecule vascular endothelial-cadherin (VE-cadherin) resulting in central necrosis of the tumour (Dark et al. 1997; Tozer et al. 2002; Vincent et al. 2005). Other small molecule VTAs include ZD6126 and DMXAA, however while ZD6126 acts through tubulin binding DMXAA does not involve tubulin interaction and is instead thought to work via cytokine induction and disruption of the actin cytoskeleton (Blakey et al. 2002; Baguley 2003). As well as the small molecule VTAs other treatment strategies cause the same specificity for central tumour vasculature, such as antibodies targeted toward endothelial cells and photodynamic therapy (Thorpe 2004; Ran et al. 2005; Chen et al. 2006).
In chapter 4, it is proposed the induction of central vascular dysfunction by tirapazamine may be related to toxicity toward endothelial cells that are at intermediate levels of oxygen or that have temporarily become hypoxic. In order to test this theory tirapazamine was administered to mice breathing carbogen, a gas consisting of 95% O₂ and 5% CO₂, which can decrease tumour hypoxia (Raleigh et al. 1999).

Additionally, the activity of tirapazamine toward microtubules and VE-cadherin was investigated to determine if the initial cytotoxicity of tirapazamine was similar to that of CA-4-P. Human cervical squamous carcinoma cells (SiHa) and human umbilical vein endothelial cells (HUVEC) were exposed to tirapazamine (100μM) under 20% (oxic) and 0.1% oxygen (hypoxic) in glass slide chamber flasks.

Another possible mechanism of action for tirapazamine causing vascular constriction is the inhibition of nitric oxide synthases (NOS). NOS enzymes are responsible for the synthesis of nitric oxide (NO) which signals smooth muscle surrounding endothelial cells to relax thereby dilating blood vessels and increasing blood flow. Tirapazamine can inhibit NOS, and under hypoxic conditions can be converted by NOS to active radical form resulting in DNA strand breaks (Garner et al. 1999). Therefore the activity of tirapazamine could be a combination of NOS inhibition constricting blood flow and subsequent conversion of tirapazamine to an active radical. If NOS inhibition alone can cause the same characteristic central vascular dysfunction this would suggest the effect of tirapazamine could be solely due to NOS inhibition.

To examine the effects of NOS inhibition the NOS inhibitor L-NNA (N-omega-nitro-L-arginine) was administered and evidence of vascular dysfunction 1 day after treatment was explored.
5.2 Results

The results in this chapter pertain to experiments performed to provide insight into the mechanism behind the ability of tirapazamine to cause vascular dysfunction.

The effects of tirapazamine in mice breathing carbogen (95% O₂ and 5% CO₂) versus air or after administration of L-NNA were determined by analyzing the degree of non-perfused tissue and necrosis in treated tumours. The percentages of necrotic and non-perfused tissue were analysed by cropping images to remove areas of necrosis or areas that consisted of non-perfused tissue (chapter 4 section 4.2 for a summary of this procedure and chapter 2 for details of all analysis).

5.2.1 The effect of tirapazamine in mice breathing carbogen

Tirapazamine (60 mg/kg) was administered i.p. to mice breathing carbogen and the HCT-116 tumours excised 1 day later. For the experiments mice were placed in modified cages and breathed carbogen for 10 min before tirapazamine injection. After tirapazamine injection the mice breathed carbogen for 3 hours, which constitutes approximately 5 half-lives of tirapazamine in mouse blood. Mice were then returned to air breathing for the remaining 21 hours before sacrifice.

Tumours from mice breathing carbogen showed fewer tumours responding than in the air group (2/9 versus 5/8), however carbogen breathing did not completely prevent the occurrence of vascular dysfunction by tirapazamine (Figure 5.1). The two tumours that responded to tirapazamine treatment while exposed to carbogen had above 30% of tissue affected by vascular dysfunction. Overall, the levels of non-perfused tissue for untreated air breathing mice and untreated carbogen breathing mice were 1.8 ± 1.9 and 0.61 ± 1.3%, respectively. When treated, the vascular dysfunction in tumours from air breathing mice rose
to 12 ± 11% and in carbogen breathing mice rose to 8.5 ± 15%. For which, only the tirapazamine change is considered statistically significant ($P = 0.0109$, Kruskal-Wallis test; $P < 0.01$ and $>0.05$ for tirapazamine treatment under air and carbogen, respectively Dunn’s multiple comparison test). Pimonidazole was given 2 hours before sacrifice (22 hours after treatment) to indicate areas that had become hypoxic and to look for vascular dysfunction. Therefore, there is no indicator of the amount of hypoxia during the time of carbogen breathing.

![Figure 5.1: Tirapazamine causes vascular dysfunction in some tumours from mice breathing carbogen.](image)

The percentage of non-perfused tumour tissue in HCT-116 tumour cryosections from air breathing and carbogen breathing mice prior to and 1 day after tirapazamine (60 mg/kg, i.p.). Each bar represents data from an individual HCT-116 tumour. Horizontal lines indicate the mean values for each group.
5.2.2 The effect of tirapazamine on cells in vitro using a hypoxia chamber

A chamber flask system was developed to examine the effect of tirapazamine (100 μM) on cells in vitro under hypoxic conditions. Cells were seeded in a flaskette with a glass slide (chamber flask) and gassed with either 20 or 0.1% oxygen during exposure to tirapazamine for 90 minutes, after which slides were immediately removed, fixed and then stained. Details of this procedure can be found in chapter 2. In all chamber flask experiments four conditions were always tested: untreated oxygenated, treated oxygenated, untreated hypoxic and treated hypoxic.

The system was validated to ensure that DNA damage increased under hypoxic conditions in our chamber flask system after exposure to tirapazamine. The mechanism of action of tirapazamine causing vascular dysfunction could be related to tubulin binding as other vascular targeting agents, which are tubulin binding compounds, exert the same vascular disrupting effect via tubulin binding. The effects of tirapazamine on the vascular endothelial junction molecule were also examined to see whether disruption of VE-cadherin can be caused by the tirapazamine radical formed under hypoxic conditions.

5.2.2.1 Tirapazamine induces DNA damage in the hypoxic chamber flasks

In order to determine whether tirapazamine causes damage to cells, under hypoxic conditions, in our chamber flasks system, cells were stained for γH2AX to indicate DNA double strand breaks (DSBs) in SiHa and HUVECs (Figure 5.2). The nuclei were stained with hematoxylin (SiHa) or Hoechst 33342 (HUVEC) to indicate the location of cells. In the cells treated with tirapazamine under oxic conditions a low level of γH2AX is seen, however under hypoxia the γH2AX staining increases both in intensity and the number of cells
labelling. Therefore tirapazamine, which is preferentially toxic to hypoxic cells, causes an increase in DNA DSBs in our hypoxia chamber flask system.

![Figure 5.2: Under hypoxic conditions DNA double strand breaks increase after tirapazamine treatment.](image)

**Figure 5.2: Under hypoxic conditions DNA double strand breaks increase after tirapazamine treatment.** Images of $\gamma$H2AX staining (green) indicating DNA DSBs in both SiHa and HUVECs under oxic (20% O$_2$) or hypoxic (0.1% O$_2$) conditions after exposure to tirapazamine (100 $\mu$M). Nuclei are labelled with hematoxylin (bright field blue; SiHa) or Hoechst 33342 (fluorescent blue; HUVEC). Scale bars = 10 $\mu$m.

5.2.2.2 Effect on microtubule structure

To determine the mechanism of action underlying the central vascular dysfunction caused by tirapazamine microtubule structure was examined by staining for $\beta$-tubulin. Cells were placed under hypoxic conditions using the chamber flask and then exposed to tirapazamine.
A positive control to confirm the appearance of microtubule destabilization was performed by exposing SiHa cells to 100 nM vinorelbine, a known tubulin binding agent, for 90 minutes and immediately staining for β-tubulin. Untreated SiHa cells show a structured network of microtubules (microtubule fine structure) in the cell cytoplasm, however once this is destabilized by vinorelbine the network disappears and diffuse staining is seen in rounded up cells (Figure 5.3).

Cells exposed to tirapazamine (100µM) under hypoxic conditions did not exhibit a change in microtubule fine structure when compared with untreated cells under oxygenated conditions. Additionally, concurrent experiments were performed with untreated cells under hypoxic conditions or after drug exposure under oxygenated conditions (data not shown) as controls and showed no changes. Images of cells stained for β-tubulin show an intact
network of microtubules in both the SiHa and HUVEC cell lines after drug exposure (Figure 5.4). Cells were also administered pimonidazole and stained for hypoxia to confirm the cells were hypoxic during the time of drug exposure.

![Figure 5.4](image)

**Figure 5.4: Microtubule fine structure is not affected by tirapazamine treatment.** (A) and (C) are images of β-tubulin staining (white) showing microtubule fine structure in untreated SiHa and HUVECs, respectively. (B) and (D) show SiHa and HUVECs after exposure to tirapazamine (100 µM), respectively. Images on the left are under oxic conditions (20% O₂) and images on the right are under hypoxic conditions (0.1% O₂). Hypoxia is confirmed with pimonidazole staining (green) and Hoechst 33342 labelling (blue; HUVEC only) indicates cell nuclei. Scale bars = 10 µm. Note: a representative control image is shown as no change was seen under any control condition.
5.2.2.3 Effect on vascular endothelial cadherin

Untreated HUVECs under oxic conditions show a cobblestone appearance of the endothelial membrane junction protein VE-cadherin, which is expressed on the cell membrane (Figure 5.5A). As well, pseudopodia can be seen stretched between cell membranes and the nuclei are shown in blue by Hoechst 33342 labelling. After treatment with tirapazamine under hypoxic conditions (0.1% O₂) the uniform VE-cadherin staining is disrupted (Figure 5.5B). Pseudopodia are no longer seen stretching between cells and the membrane becomes discontinuous. No change in VE-cadherin was seen in concurrent control experiments with untreated cells under hypoxic conditions or after drug exposure under oxygenated conditions (data not shown).

**Figure 5.5: Disruption of VE-cadherin occurs after tirapazamine exposure under hypoxic conditions.** (A) VE-cadherin staining (red) in HUVECs showing a cobblestone appearance in untreated cells under oxic conditions (20% O₂). (B) After treatment with tirapazamine under hypoxic conditions (0.1% O₂) VE-cadherin membrane staining becomes discontinuous and pseudopodia are no longer present. Hoechst 33342 labelling (blue) indicates cell nuclei. Scale bars = 10 μm. Note: a representative control image is shown as no change was seen under any control condition.
5.2.3 NOS inhibition

Endothelial NOS (eNOS, NOS III), present in endothelial cells, can produce NO, which signals to surrounding smooth muscle to relax. The increase in NO dilates the blood vessels and increases blood flow. The NOS inhibitor L-NNA was used to determine if NOS inhibition and a subsequent decrease in NO could cause vascular dysfunction in tumours.

5.2.3.1 NOS inhibition causes central necrosis in some tumours

Mice bearing HCT-116 tumours were treated with 20, 60 and 180 mg/kg of L-NNA to determine if a high dose of NOS inhibitor could cause vascular dysfunction. 1 day after treatment with L-NNA an increase in necrosis occurred in treated tumours. The central areas of the tumours were necrotic and surrounded by areas of perfused tissue on the outside rim of the tumour (Figure 5.6). Some unperfused vessels were seen in the central necrotic areas, however the characteristic non-perfused tissue was only seen surrounding a few vessels, likely because these areas have already become necrotic.
Analysis of the treated tumours showed a small increase in non-perfused tissue 1 day after treatment in only the group treated with 180 mg/kg of L-NNA (Figure 5.7A). The percentage of non-perfused tissue increased from 0.24 ± 0.33 to 6.2 ± 3.6% after treatment with 180 mg/kg, however the rest of the central area is already necrotic at this point. The lower doses show almost no increase in non-perfused tissue but had large areas of necrosis. The percent of necrosis increased after all doses of L-NNA, however the response appeared to be variable, similar to that of tirapazamine, where 4 of 5 tumours at the lower doses (20
and 60 mg/kg) had an increase in necrosis (Figure 5.7B). All 5 tumours treated with 180 mg/kg had high percentages of necrosis. The average necrosis in the control tumours was 13 ± 11% which increased to 41 ± 18, 42 ± 22, and 48 ± 12% after 20, 60 and 180 mg/kg of L-NNA, respectively. However, none of the groups had a statistically significant increase in necrosis possibly due to the heterogeneity of necrosis in the control tumours and the small sample size (P > 0.05, Kruskal-Wallis test).

**Figure 5.7: Necrosis increases 1 day after treatment with L-NNA.** The percentage of non-perfused tumour tissue and necrosis in HCT-116 tumour cryosections from untreated mice and mice 1 day after treatment with 20, 60, and 180 mg/kg L-NNA (i.p). (A) Only small amounts of non-perfused tissue are observed likely due to the fact that most of the tissue is necrotic. (B) The percentage of necrosis increases 1 day after treatment of 60-180 mg/kg L-NNA. Each bar represents data from an individual tumour. Horizontal lines indicate the mean values for each group.

5.2.3.2 NOS inhibition causes small areas of vascular dysfunction within 12 hours after treatment

Given the increased necrosis seen in tumours after treatment with L-NNA earlier time points of 2, 6, and 12 hours were examined to see if central vascular dysfunction occurred before the necrosis. No large central area of the tumour exhibit vascular dysfunction at any
time point, however small areas in a portion of L-NNA treated tumours, were observed where vessels were unperfused and no BrdUrd was present (Figure 5.8). Of note, for the 2 hour group BrdUrd was given one hour after administration of L-NNA and therefore only present for one hour before the mouse was sacrificed. Pimonidazole was not given to the 2 hour group to avoid the possibility that non-perfused areas could be due to inhibition of NOS by pimonidazole.

Figure 5.8: Small areas of vascular dysfunction are seen in the centre of a tumour 2 hours after treatment with L-NNA. A composite HCT-116 tumour image 2 hours after of L-NNA (60 mg/kg, i.p.). A small central areas has non-perfused vessels with no proliferation labelling. Dark blue = vasculature, light blue = perfusion, black = dividing cells, red = unperfused vessels, greyscale = tissue background. The inset shows details of the effected area with unperfused vessels surrounded by non-perfused tissue. Scale bar: 150 μm.
Composite tumour images were analysed for non-perfused tissue and necrotic tissue 2, 6, and 12 hours after 60 mg/kg of L-NNA. There was no significant increase in non-perfused tissue at any time point suggesting no large effect on central vasculature (P > 0.05, Kruskal-Wallis test). Some small portions of a few tumours responded with areas of vascular dysfunction but this was not above 15% in any one tumour (Figure 5.9A). As well, no increase in necrosis was observed at any time point, however the longest time tested was 12 hours after treatment and this may not be enough time for extensive necrosis to occur (Figure 5.9B).

Figure 5.9: No central vascular shutdown is seen between 2-12 hours after treatment with L-NNA. The percentage of non-perfused tumour tissue and necrosis in HCT-116 tumour cryosections from untreated mice and mice 2, 6, and 12 hours after L-NNA (60 mg/kg, i.p.). No significant increase in non-perfused tissue (A) or the percentage of necrosis (B) occurs, however small areas of non-perfused vessels are seen in the centre of tumours. Each bar represents data from an individual tumour. Horizontal lines indicate the mean values for each group.
5.3 Discussion

In order to determine the mechanism of action behind the vascular dysfunction caused by tirapazamine various aspects related to the toxicity of tirapazamine were examined. The importance of hypoxia in tumour vessels was examined by subjecting mice to carbogen breathing. The effects of tirapazamine on microtubule and endothelial cell junction structure were examined in vitro under hypoxic conditions. Finally, we investigated whether tirapazamine may be causing vascular dysfunction via the inhibition of NOS.

5.3.1 The effect of carbogen breathing on the activity of tirapazamine

Previously we proposed the cytotoxicity of tirapazamine towards central vasculature is due to an effect on endothelial cells that are at intermediate levels of oxygen or that have temporarily become hypoxic (Huxham et al. 2006). HCT-116 colon carcinoma bearing mice breathing carbogen (95% O₂ and 5% CO₂) were administered tirapazamine (60 mg/kg, i.p.). Carbogen breathing did not prevent tirapazamine from causing central vascular dysfunction 1 day after treatment. Those tumours that responded with vascular dysfunction had 8.5 ± 15% non-perfused tissue. Only 2 of 9 tumours responded, however both responders had over 30% of tissue affected (Figure 5.1). Therefore an increase in oxygen tension by breathing carbogen was not able to prevent the vascular targeting effects of tirapazamine. The response rate of HCT-116 tumours to tirapazamine is approximately 60-70% and carbogen breathing did decrease this to 25%.

It has been shown that after 30 minutes of carbogen breathing the tumour oxygen tension can increase from ~1 to 20-30 mmHg, which is an increase to approximately 4% oxygen (Gallez et al. 1999). Therefore the increase in oxygen tension from carbogen breathing may not be enough to entirely prevent the activity of tirapazamine since conversion
of tirapazamine can occur, to some degree, below 5% oxygen (Koch 1993). Alternately, if the effect of tirapazamine is due in whole or in part to factors other than hypoxia such as NOS inhibition, discussed later on, or high IFP then a response while breathing carbogen could still be seen.

5.3.2 Development of a hypoxic chamber system

During the course of this work a chamber system was developed to gas cells with known amounts of oxygen, expose to drug and then incubate for the exposure duration. The system allows drug to be gassed and then injected through a needle port via a gas tight syringe into a chamber flask maintained at a chosen level of oxygen. A glass slide bottom allows for low levels of hypoxia to be achieved without the influence of oxygen leaching from plastic and also allows for ease of staining once the chamber has been removed. The cells can then be stained for various markers such as β-tubulin followed by pimonidazole, or VE-cadherin. The system was validated by testing for an increase in γH2AX labelling after exposure to tirapazamine under hypoxic compared with oxic conditions (Figure 5.2).

5.3.3 The effect of tirapazamine on microtubules and VE-cadherin

The vascular targeting agent CA-4-P is reported to cause vascular dysfunction via conformation changes in the cells from microtubule and VE-cadherin disruption (Tozer et al. 2002; Vincent et al. 2005). Therefore, we wanted to determine if the anti-vascular activity of tirapazamine occurs via the same process. Using the chamber flask system two cell lines (SiHa and HUVEC) were exposed to tirapazamine (100μM) under oxic and hypoxic conditions and then stained for β-tubulin and VE-cadherin. Microtubule fine structure (β-tubulin) was not disrupted, however a slight fragmentation of the normally continuous membrane staining of VE-cadherin was seen (Figure 5.4 and Figure 5.5). The vascular
targeting agent DMXAA does not cause microtubule destabilization but rather through the induction of cytokines a resulting disruption of the actin cytoskeleton occurs (Baguley 2003). It is possible that the action of tirapazamine toward vasculature is related to effects on other cytoskeletal proteins or related to an entirely different process. The vascular collapse from tirapazamine could be related to changes in vascular permeability or endothelial tube integrity due to VE-cadherin junction changes, however the disruption of VE-cadherin could be secondary to other induced cytoskeletal changes.

5.3.4 NOS inhibition may cause central necrosis

NOS produces nitric oxide (NO), which is a gaseous signalling molecule that can induce smooth muscle to relax. Therefore inhibition of NOS will cause a decrease in NO and subsequent constriction of the vasculature. As mentioned above, enhancement of vascular targeting agents such as CA-4-P, ZD6126, and photodynamic therapy occur when a NOS inhibitor is co-administered (Horsman et al. 1996; Butler et al. 1997; Korbelik et al. 2000; Parkins et al. 2000; Tozer et al. 2001; Davis et al. 2002; Wachsberger et al. 2005). The production of NO varies in mouse tumour types and tumours resistant to the effects of CA-4-P reportedly have high levels of NO production, which upon administration of a NOS inhibitor can be rendered sensitive to CA-4-P (Parkins et al. 1995; Parkins et al. 2000).

The inhibition of NOS by compounds, such as L-NNA and L-NAME, can decrease blood flow in tumours by up to 60% shortly after doses from 10-20 mg/kg (Andrade et al. 1992; Horsman et al. 1996) and cause a 50% decrease in red cell velocity and a decrease in vessel diameter (Tozer et al. 2001). A small, but non-significant growth delay was seen after administration of L-NNA (20 mg/kg, i.v.) to SCCVII tumours (Korbelik et al. 2000).
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It has been shown that tirapazamine is a NOS inhibitor and under hypoxic conditions NOS can convert tirapazamine to a cytotoxic radical (Garner et al. 1999). If the vascular constriction caused by tirapazamine is due solely to NOS inhibition then a NOS inhibitor could similarly cause vascular dysfunction in our system. The relation between NOS inhibition and central vascular dysfunction was investigated by using the non-specific NOS inhibitor L-NNA that inhibits endothelial, neuronal, and induced NOS.

Mice bearing HCT-116 colon tumour xenografts were analysed for non-perfused tissue and necrosis 1 day after 20, 60 and 180 mg/kg L-NNA and 2, 6 and 12 hours after 60 mg/kg L-NNA. Analysis of the treated tumours showed evidence of non-perfused tissue 1 day after all doses of L-NNA (Figure 5.7A). The highest dose of 180 mg/kg had the largest non-perfused area however it was still very little. The lower doses caused almost no increase in non-perfused tissue, but in all cases the absence of non-perfused tissue is probably due to the majority of the tumour already being necrotic at this time point. The percent of necrosis increased 1 day after all doses of L-NNA, however not all tumour responded to treatment and the increase was not significant likely due to variability and the small sample size (Figure 5.7B). When tumours were analysed within 12 hours after L-NNA extensive areas of central vasculature dysfunction were not seen (Figure 5.9A). However, small areas in the centre had characteristic unperfused vessels with no surrounding BrdUrd labelling (Figure 5.8). As well, no significant increase in necrosis was observed which is not surprising as it may take more than 12 hours for necrosis to occur (Figure 5.9B). Whether the small areas of non-perfused tissue seen up to 12 hours after treatment can lead to the extensive necrosis seen after 24 hours is not certain.
Chapter 5 – Determining the mechanism behind the vascular dysfunction caused by tirapazamine

Vessels perfusion 1 day after administration of NOS inhibitors has been assessed in tumour models (Davis et al. 2002). L-NNA administered at 10 mg/kg decreased perfusion by 15% and another class of NOS inhibitor, AMT (2-amino-5,6-dihydro-6-methyl-1,3-thiazine), decreased vessel perfusion by 45%. However, neither inhibitor caused an increase in necrosis at this time point. As a comparison, CA-4-P alone causes a 30% reduction in vessel perfusion (Davis et al. 2002). A higher dose of L-NNA (118 mg/kg) produced no observable effect, however this was in a SaS tumour reported to be resistant to vascular targeting due to high NO (Davis et al. 2002). The occurrence of necrosis by NOS inhibition may be dependent on both the inhibitor and the levels of NO produced by the tumour.

It appears the activity of tirapazamine could be, in part, related to NOS inhibition causing vessel constriction and increasing necrosis, however since a greater effect is seen with tirapazamine alone versus NOS inhibitor it is not likely NOS inhibition is the only mechanism by which tirapazamine causes vascular dysfunction. The conversion of tirapazamine to a cytotoxic radical in vessels with intermediate oxygenation could contribute to its activity as a NOS inhibitor. Further the increased hypoxia created by the constriction of vessels due to NOS inhibition by tirapazamine could potentiate the conversion of tirapazamine to a cytotoxic radical as has been suggested for the activity of RB 6145. The hypoxic cytotoxin RB 6145 exhibits vascular targeting when combined with NOS inhibition where over 80% of the tumour tissue becomes necrotic 1 day after combination treatment compared with 5% necrosis after RB 6145 alone (Butler et al. 1997). This increased effect is thought to be due to vessel constriction causing hypoxia and further potentiating the conversion of RB 6145 (Butler et al. 1997).
Tirapazamine is currently in clinical trials and the NOS inhibitor L-NNA has been administered in clinical trials at 4 mg/kg which was well tolerated (Sander et al. 1999). Combining tirapazamine treatment with NOS inhibition clinically may increase the efficacy of tirapazamine, however the addition of a NOS inhibitor would cause more hypoxia and be detrimental to radiotherapy which is given concurrently of after tirapazamine administration in trials (Doherty et al. 1994; Rischin et al. 2001; Rischin et al. 2005). Generally, tirapazamine is not given after radiotherapy due to possible reoxygenation of cells. A strategy such as radiotherapy administration to eradicate the oxic cells and then a NOS inhibitor to increase hypoxia before the administration of tirapazamine may increase tumour control.

5.4 Summary

Overall, it appears the mechanism by which tirapazamine causes vascular dysfunction may be more complicated than direct hypoxic cytotoxicity or NOS inhibition alone and may involve a combination of effects. The cytotoxic action does not occur through a similar path as that for CA-4-P, but it may involve changes in VE-cadherin. It appears tirapazamine could function partially as a NOS inhibitor thus inducing vessel constriction and increasing hypoxia, which would create an environment for cytotoxic radical formation by the reduction of tirapazamine. As well, the evidence for NOS inhibition causing central necrosis supports further work to determine to what extent the mechanism of action of tirapazamine is NOS related.
CHAPTER 6 – CONCLUSIONS AND FUTURE DIRECTIONS
CONCLUSIONS AND FUTURE DIRECTIONS

6.1 Research objectives

The purpose of this research was to map the changes in microenvironmental markers such as blood vessel location, blood flow, hypoxia and cell division in tumour sections after treatment with chemotherapeutics. From this we hoped to gain information about the penetration of drugs by examining dividing cells in relation to their distance from blood vessels. As well, observations of microenvironmental factors after administration of drug allowed regional determination of drug effects in relation to the entire tumour microenvironment. For instance, a lack of perfusion in the central area of a tumour can be observed as occurred with tirapazamine.

6.2 Pyrimidine analogues

A differential effect was seen on cells in relation to vasculature after administration of gemcitabine, 5-FU and capecitabine. In comparison, cytarabine showed little to no effect on cells at any distance to vasculature. 1 day after treatment with gemcitabine cessation of proliferation throughout the tumour was observed. This was the broadest effect seen from all the pyrimidine analogues tested. However, cells distal from vasculature resume cycling sooner than those proximal to vasculature. After 5-FU and capecitabine, cell proliferation decreased near vessels to a larger extent than it decreased far from vessels. Capecitabine was able to suppress proliferation longer than 5-FU. Clinically the most successful analogues for solid tumour treatment are gemcitabine, 5-FU and capecitabine. Cytarabine is not used for the treatment of solid tumours but is beneficial for the treatment of leukemia.

The pyrimidine analogue gemcitabine showed a differential effect on cells far from vasculature, which suggests the cells distal to vessels receive less drug. However, since
gemcitabine is actively taken up into cells via nucleoside transporters (NT) changes in the expression of these transporters could alter the effect of gemcitabine (Mackey et al. 1998). If the cells far from vasculature down regulate the NTs on the membrane then less effect from gemcitabine could occur. Staining for these transporters and relating their expression to distance from vasculature would give some insight into the involvement of the transporters, if any, in the decreased activity of the drug. An antibody to the equilibrative NT has been used to detect this particular transporter but it is not yet commercially available and there are various other NTs that would need to be detected.

Studies co-administering 5-FU modulators such as dihydropyrimidine dehydrogenase (DPD) inhibitors to prevent the deactivation of 5-FU would improve the availability of 5-FU and possibly show an increased effect on cells far from vasculature in our tumour mapping system (Baccanari et al. 1993; Takechi et al. 2002).

Purine analogues such as mercaptopurine and thioguanine are used in the treatment of leukemia and provide another class of antimetabolites with which to compare the pyrimidine analogues (Chabner et al. 2001). These agents may have results similar to cytarabine that is very little effect on any cells after a single dose or they may exhibit some differential effects and provide further insight into why these agents are successful in the treatment of leukemia but not solid tumours.

One method to determine if cells are in a cell cycle block or killed by drug would be to perform a clonogenic assay by sorting for cells at different distances from vasculature using Hoechst 33342 and then plating the fractions to determine the level of cell kill. This was not done using the HCT-116 as these tumours do not disaggregate into single cells due to cell clumping and in fact even the cell yield from in vivo transplant of this tumour is so low.
that the tumours must be implanted from *in vitro*. We tested the effects of gemcitabine on the WiDr tumour in hopes of performing a clonogenic assay to determine the clonogenic survival of cells at different distances from vasculature. However, the WiDr was relatively insensitive to gemcitabine (Ruiz van Haperen *et al.* 1994) and did not exhibit the same distribution of proliferation with relation to vasculature as was seen with the HCT-116. This assay would be useful to perform in a sensitive tumour that is amenable to disaggregation and plating.

### 6.3 Tirapazamine

Rather than observing an effect only on those cells far from vasculature by the hypoxic cytotoxin tirapazamine we found it caused central vascular dysfunction and subsequent necrosis in a portion of tumours. This indicates the activity of tirapazamine on hypoxic cells far from vasculature may not be as important as previously believed.

The activity of tirapazamine towards vasculature does not occur through tubulin interaction as seen with another vascular targeting agent, CA-4-P, but rather may involve conversion of tirapazamine to its active radical form in vessels with intermediate oxygen levels (<5%). As well, tirapazamine inhibits NOS, which could result in a decrease in nitric oxide (NO) production leading to vessel constriction. Both the increased vessel constriction and the resulting increase in hypoxia, which would reduce tirapazamine to an active radical, may lead to the observed central vascular dysfunction seen after tirapazamine treatment.

It would be interesting to compare the effects of tirapazamine and other VTAs, such as CA-4-P, DMXAA and ZD6126 in our tumour mapping system to evaluate the timing of vascular dysfunction and progression to necrosis, as well as the degree of tumour response and morphology changes. Further examination of the *in vivo* effects could also provide insight into the mechanism by which tirapazamine causes vascular dysfunction. For example,
we have examined the effects on the tubulin cytoskeleton after treatment with tirapazamine, however the actin cytoskeleton could alternately be involved as is the case with DMXAA (Baguley 2003).

One similarity between all the VTAs is the remaining viable rim of tissue after treatment, which is problematic because it can lead to repopulation of the tumour. Tumour mapping can be used to look at the success of combining tirapazamine with other chemotherapeutics and radiotherapy to target the perfused tumour rim. Elucidating the differences between the outer and inner vasculature could shed light on the mechanism of specificity of VTAs. The differences could include such aspects as a high IFP in the centre of tumours causing increased intermittent hypoxia (Boucher et al. 1990) or differences in the levels of NOS expression, smooth muscle coverage, or proliferation of endothelial cells between the centre and outside of tumours.

The evidence for NOS inhibition causing central necrosis supports further work examining more cells lines and different NOS inhibitors. Tumours can produce different levels of NO and tumours resistance to VTAs have been suggest to have high NO expression (Parkins et al. 2000). Examining the ability of NOS inhibitors to induce necrosis and vascular dysfunction in tumours with low to high NO expression would reveal the effectiveness of NOS inhibition.

Tumour response to tirapazamine can be variable, some exhibit vascular dysfunction and others do not. The pre-treatment IFP or hypoxia in tumours may determine whether a tumour is sensitive to vascular dysfunction. Measurements of IFP can be made before and after treatment and correlated with tumour response. Positron emission tomography (PET) can be used to examine the level of hypoxia, with $^{18}$F-EF5, in tumours before tirapazamine.
administration (Dolbier Jr et al. 2001). The same tumour can be examined before, during and after treatment for hypoxia changes.

It is important to determine if tirapazamine causes central vascular dysfunction in humans. The dose of tirapazamine used in mice is higher than that administered clinically therefore the concentration required to produce vascular dysfunction may not be achieved in humans. MRI or PET scans would allow for the investigation of reduced perfusion and necrosis in the centre of tumours in patients treated with tirapazamine (Beauregard et al. 2001; Beauregard et al. 2002; Anderson 2003). As well, this work suggests alternate clinical administrations and scheduling with radiotherapy or combination of tirapazamine with a NOS inhibitor may increase tumour response. For example, if tirapazamine can affect endothelial cells by direct cytotoxicity at intermediate oxygen tension then a low continuous dose (metronomic dosing) of tirapazamine could cause vascular damage in humans.

Additionally, the tumour mapping system developed in this work can be used to screen a host of current and new drugs to determine where they have an effect and how they alter the tumour microenvironment.
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