EVALUATING THE EXTRAVASCULAR PENETRATION AND DISTRIBUTION OF ANTI-CANCER DRUGS USING A NOVEL APPLICATION OF THE MULTILAYERED CELL CULTURE MODEL

by

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The inefficient distribution of anti-cancer drugs in the tumour extravascular compartment may be an important mechanism of drug resistance in solid tumours. However, gaining an understanding of diffusion limitations of existing drugs and developing new drugs with improved tumour penetration is limited by a lack of techniques with which to evaluate drugs. The purpose of this research project was to assess the extravascular penetration and distribution of anti-cancer drugs using a novel application of the multilayered cell culture (MCC) model. MCC is a planar analogue of spheroidal cell culture, in which tumour cells are instead grown into discs of tissue. Due to their 3-D conformation they model many characteristics of the tumour extravascular compartment. A unique property of MCC is that it possesses two populations of rapidly proliferating cells, one on each side of the culture, that are separated by a known thickness of tissue. In tumours and spheroids proliferation status falls off with distance from the vasculature (tumours) or tissue edge (spheroids), which in turn modifies cellular response to drugs. Hence, visualizing the distribution of a drug’s effect within these tissues cannot be used to directly determine its actual distribution. The hypothesis of this project was that the symmetrical proliferation seen in MCCs could be applied to measure the ability of anti-cancer drugs to penetrate and distribute within tissue using a biological endpoint. Based on this hypothesis an effect-based assay was devised and applied to assess the tissue distribution of a selection of anti-cancer drugs from several distinct classes. Using the assay, drug exposure to cells 150 μm into tissue (a diffusion distance commonly observed in solid tumours) was estimated to vary from as low as 3-10% of the reservoir exposure (anthracyclines) to cases where no reduction in exposure could be detected (5-FU and cisplatin). The MCC-based assay was validated by comparing the predicted drug distributions with direct visualization of the drugs themselves. Results from this study suggest that an MCC effect-based assay could be used as a simple drug penetration screen in the development of new anti-cancer drugs.
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Plan of work

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Antimetabolites
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<th>Definition</th>
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<tbody>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the curve</td>
</tr>
<tr>
<td>BGSTM</td>
<td>bovine growth serum (FBS replacement)</td>
</tr>
<tr>
<td>BrdUrd</td>
<td>5-bromo-2-deoxy-uridine</td>
</tr>
<tr>
<td>Cremophor® El</td>
<td>Polyoxyl 35 Castor Oil (vehicle used in paclitaxel formulation)</td>
</tr>
<tr>
<td>DiOC(_3)(3)</td>
<td>a carbocyanine, fluorescent molecule used as blood perfusion marker</td>
</tr>
<tr>
<td>DOX</td>
<td>doxorubicin (adriamycin)</td>
</tr>
<tr>
<td>DAU</td>
<td>daunorubicin</td>
</tr>
<tr>
<td>EPI</td>
<td>epirubicin</td>
</tr>
<tr>
<td>FBS</td>
<td>foetal bovine serum</td>
</tr>
<tr>
<td>3H</td>
<td>tritium</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>tissue having low oxygen status</td>
</tr>
<tr>
<td>IC(_{50})</td>
<td>drug concentration that produces a 50% decrease in proliferation</td>
</tr>
<tr>
<td>MCC</td>
<td>multilayered cell culture - cells grown as discs of tissue</td>
</tr>
<tr>
<td>MIT</td>
<td>mitoxantrone</td>
</tr>
<tr>
<td>PTFE</td>
<td>polytetrafluoroethylene (Teflon®)</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SE</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>Spheroids</td>
<td>multicellular spheroids - cells grown as spherical aggregates</td>
</tr>
<tr>
<td>Xenografts</td>
<td>tumour cells of non-mouse origin grown in mice</td>
</tr>
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Chapter 1 - Introduction

1. Introduction

The role played by the tumour microenvironment in limiting the extravascular distribution of anti-cancer drugs has been largely ignored in the development of most new and existing agents (Minchinton and Tannock 2006). The most commonly measured parameter characterising drug kinetics within solid tumours is net tumour drug accumulation. However, critically, this parameter does not differentiate between drugs that distribute throughout the tissue versus those that are confined to the first few cells surrounding tumour vasculature. Through studies in solid tumours and multicellular spheroids it has become clear that the tumour microenvironment plays a critical role in modifying the efficacy of anti-cancer drugs (see sections 1.1 TUMOUR MICROENVIRONMENT and 1.2 DRUG PENETRATION). Unregulated proliferation and survival of tumour cells result in increased distances between tumour blood vessels producing gradients in drugs, oxygen, nutrients, proliferation and acidity away from blood vessels, all of which can modify the success of anti-cancer drugs that are generally selected from monolayer-centric studies. Multicellular spheroids are now often applied to integrate these factors and determine the activity of new anti-cancer drugs in an environment that is more representative of solid tumours than monolayer cell culture (see section 1.3 - IN VITRO TUMOUR MODELS). However, when a drug fails in spheroids it is often difficult to ascertain what role tissue penetration may have played in limiting its efficacy.

The most commonly used methods of assessing the tissue penetration of a drug are either through visualisation of the drug itself within tissue cryosections (see section 1.2 - direct imaging of drugs) or through characterisation of drug flux through slabs of tissue followed by analysis and mathematical modelling to predict tissue pharmacokinetics (see section 1.3 - simulating drug distribution). However both these approaches have certain drawbacks. Imaging techniques (e.g. autoradiography, drug derived fluorescence or immunodetection) often can’t differentiate between parent drug and inactive metabolites that may be smaller and diffuse more easily and drug derived fluorescence can be modified by cell interactions (e.g. binding, acidic sequestration). Immunodetection also requires the drug to be immobilised to avoid redistribution and washout during the immunostaining process. Additionally, direct imaging produces a discrete snapshot in time and to determine overall exposure to drug requires a sequence of snapshots spanning the entire process of drug build-up and subsequent washout. Flux-based studies coupled with mathematical modelling can in theory solve many of these issues. However, successful application of flux data to predict the tissue pharmacokinetics of a drug requires the full characterisation of all relevant drug-tissue interactions that may contribute in determining its tissue penetration. The goal of this project was to develop an assay that would be able to assess drug penetration using a biological endpoint. Potential benefits from such an approach would be that it could be fast and simple, not requiring the drug to be visualizable, and also be insensitive to inactive
metabolites. It might also be able to integrate drug exposure over the entire build-up and wash out process and could potentially produce meaningful results without requiring the full characterization of all relevant drug-cell interactions. Such an assay could thereby allow for the tissue penetration-based screening of new drugs for which physicochemical parameters had not been completely assessed.

The purpose of this research was to investigate the use of a novel application of multilayered cell culture (MCC) in the assessment of the extravascular penetration and distribution of anti-cancer drugs using a biological endpoint. MCC is the planar analogue of spheroidal cell culture, in which tumour cells are grown into discs of tissue rather than spherical aggregates. Due to their growth in a 3-D conformation spheroids and MCCs can model many characteristics of the tumour extravascular compartment (see section 1.3 - IN VITRO TUMOUR MODELS). MCC was originally developed to allow measurement of the flux of drugs through tumour-like tissue (see section 1.3 - MULTILAYERED CELL CULTURE). This project investigates a novel application of MCC to directly assess a drug's ability to distribute within tissue that does not require detection of the drug itself by making use of a unique property of MCC, that it possesses two populations of rapidly proliferating cells, one on each edge of the culture, that are separated by a known thickness (see section 1.4). In tumours and spheroids, proliferation falls off with distance from the vasculature/depth into tissue, which in turn affects cellular response to drugs (see section 1.1 and 1.3 - SPHEROIDAL CELL CULTURE). Hence, visualizing the distribution of a drug's effect within these tissues cannot be used to directly determine its actual distribution. The hypothesis of this project was that the symmetrical proliferation seen in MCC could be used as a simple means of measuring the ability of a drug to penetrate and distribute in tissue. That is, exposing just one side of an MCC to a drug and then comparing the effect on the two proliferating populations will allow for evaluation of drug penetration without the need for direct visualization or mathematical modeling of tissue pharmacokinetics based on MCC-derived flux-data.

1.1 TUMOUR MICROENVIRONMENT

While cancer cells grown in a petri dish can be selectively targeted and killed, when grown as solid tumours achieving cure often becomes an intractable problem. One of the reasons for this is the unusual environment that is created by the combination of sustained expansion of the tumour extravascular compartment and subsequent imperfect neovascularization. Deregulated proliferation and survival of tumour cells result in increased separation of blood vessels (Thomlinson and Gray 1955; Rubin and Casarett 1966) and unstable perfusion (Brown 1979; Chaplin et al. 1986; Minchinton et al. 1990; Durand and LePard 1995), in turn leading to a reduction in the distribution and removal of molecules supplied by the blood (Jain 1987; Jain 1997). Tumour cells can be located up to 15-20 cells away from the nearest blood vessel (more in some cases) while in most normal tissue cells are within a few cell layers of a vessel (Krogh 1919). In addition to this, the process of tumour neovascularization often produces aberrant or leaky vessels which further
complicates the situation (Peterson and Appelgren 1977; Dvorak et al. 1988; Hashizume et al. 2000; Dvorak 2002). It is these limitations in diffusion and perfusion within solid tumours and resultant variable tissue oxygenation and acidification that define the tumour microenvironment (Sutherland 1988; Jain 1994; Tannock 2001; Minchinton and Tannock 2006).

**ARCHITECTURE**

Examples of two tumour architectures commonly observed in experimental tumours grown in mice are shown in FIGURE 1-1. Specimens were multiply immunostained to reveal the relation between vasculature, proliferation, hypoxia and necrosis. In both examples the tumours are fast growing, subcutaneous tumours in which the majority of tissue is comprised of tumour cells. **Panel A** shows a sparsely vascularized HCT-116 human tumour xenograft in which cords of tumour cells can be seen to surround vessels. In this example clear gradients in tissue oxygenation and proliferation are seen in relation to vasculature. Most cells are within ~150 μm of a perfused vessel beyond which hypoxia is seen to occur, shown in green, followed by necrosis. **Panel B** shows an example of the experimental SCCVII mouse tumour which exhibits a dense vasculature, where most tumour tissue is within ~50 μm of a vessel. In this case the hypoxic areas do not appear to be the product of diffusion limited oxygen and more likely arise from intermittent blood flow (Chaplin et al. 1987). Proliferating cells can be seen in both the green hypoxic areas and in the vicinity of unperfused blood vessels, consistent with the regular opening and closing of vessels.
FIGURE 1-1 Examples of two tumour architectures commonly observed in experimental tumours grown subcutaneously in mice. A | A human HCT-116 tumour xenograft, which exhibits a sparse vasculature where cords of tumour cells are often seen to surround individual vessels. B | An SCCVII mouse tumour, which exhibits a dense vasculature and significant intermittent flow. Source: Minchinton Lab.
An example of a clinical human breast cancer is shown in FIGURE 1-2. The section, stained using haematoxylin and eosin, shows nests of tumour cells existing within the normal tissue stroma. Blood vessels, observed by the presence of red haemoglobin, are seen in stromal areas but not within the tumour regions. In this case the relation between vasculature, oxygenation and proliferation will be more complex with perhaps even greater diffusion distances involved.

FIGURE 1-2 Example of a clinical human breast cancer. Three distinct regions are seen: i) vascularized stroma, arrows indicated visible haemoglobin, ii) unvascularized tumour cell nest and iii) areas of necrosis. Photomicrograph of core biopsy courtesy of Dr Malcom Hayes, pathology department BC Cancer Agency.

FACTORS THAT DEFINE THE TUMOUR MICROENVIRONMENT

Diffusion limitations

The sparse or aberrant vasculature that is often observed in solid tumours leads to diffusion limited gradients of potentially any molecule supplied and removed by the blood. Early evidence of a diffusion limit for oxygen supplied from the blood was obtained from observations that their often appeared to be a cut off, in the range of ~150 μm, to which viable tumour cells were observed, beyond which occurred necrosis (Thomlinson and Gray 1955). The hypothesis of the existence of oxygen gradients in tumour tissue was later confirmed using radiolabelled misonidazole, which is reduced and forms adducts under conditions of low oxygenation (Chapman et al. 1981; Urtasun et al. 1986). Oxygen gradients are now easily detected using antibodies against several molecules (e.g. pimonidazole, EF5), which are reduced and bound under conditions of low tissue oxygenation (Raleigh et al. 1987). Limited diffusion also applies to other important
molecules such as glucose (Groebe et al. 1994; Venkatasubramanian et al. 2006) and the metabolic waste product lactic acid, build-up of which has been shown to be responsible for increasing the acidity of tumours (Tannock and Rotin 1989; Gatenby and Gillies 2004). All three of these factors, oxygen, glucose and lactate can on their own effect a reduction in proliferation of tumour cells. Hence it is not surprising that there is also a reduction in proliferation away from blood vessels (Tannock 1968; Hirst and Denekamp 1979).

Variable vessel perfusion

Variable blood vessel perfusion was originally demonstrated in experimental mouse tumours grown using SCCVII cells, where it was found that at any given time on the order of 20% of the vessels exhibited intermittent flow (Chaplin et al. 1987). Redundancy of vessels and short periods of closure may explain why tumour cells are able to maintain division under these conditions, see FIGURE 1-1B. In the original study, evaluation of blood flow status was performed through mismatch experiments in which two different fluorescent markers of vessel perfusion were injected intra-venously at 20-minute intervals (Chaplin et al. 1987). Mismatch of the two dyes seen in histological tissue sections was then used to determine the fraction of intermittently perfused vessels. Evaluation of the duration of closure and re-opening of vessels has been more recently examined using a tumour window model (Kimura et al. 1996). In this assay, a small glass window is attached to the skin of mice and tumours are grown against the window. The passage of blood cells through the vessels adjacent to the window can then be recorded via video microscopy. Results from such experiments have suggested closure and opening can occur repeatedly with a cycle time as short as tens of minutes (Dewhirst et al. 1998).

Harsh conditions & aggressive phenotypes

Diffusion limited regions of chronic reduced oxygen and glucose and elevated acidity that exist in tumours are compounded by irregular flow which can lead to cyclical re-oxygenation in some tumour regions, potentially creating an environment of repeated oxidative stress leading to DNA damage and mutation (Kimura et al. 1996). In addition, leaky vessels in tumours allow in molecules that would not normally have regular access to extravascular space (Peterson and Appelgren 1977; Dvorak et al. 1988; Hashizume et al. 2000; Dvorak 2002). All this creates a wide range of environmental conditions which, when linked with genomic instability, may be responsible for producing aggressive phenotypes seen in later stages of tumour progression (Harris 2002). In fact, the gradients in physiological parameters such as oxygenation, acidity and proliferation that exist away from vasculature that are observed in solid tumours could be considered the ideal system for production and selection of cell variants that will ultimately lead to tumour progression (Gatenby and Gillies 2004; Gatenby et al. 2006; Smallbone et al. 2006).
MODULATION OF THERAPY

The tumour microenvironment, its modulation of anti-cancer therapy and its effect on tumour cell progression towards malignancy are topics currently under intense research. Perhaps the earliest observation of a potential for modulation of cancer therapy by the tumour microenvironment was made by Thomlinson and Gray in 1955. Upon observing sparse tumour vasculatures, as depicted in FIGURE 1-2, in which blood vessels feeding tumour cells were confined to stromal tissue, they hypothesised that reduced tissue oxygenation might exist in the tumour regions most distant from vasculature, which would result in the existence of radiation resistant subpopulations (Thomlinson and Gray 1955).

<table>
<thead>
<tr>
<th>Oxygen</th>
<th>gradient</th>
<th>physiological effect</th>
<th>effect on therapy</th>
<th>counter measure</th>
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<tbody>
<tr>
<td></td>
<td>~5% O₂ near vessels down to &lt;0.5% far from vessels</td>
<td>reduced proliferation, increased glucose reliance, increased lactate production</td>
<td>repeated treatments, radio sensitizers (e.g. nimorazole), hypoxic cytotoxins (e.g. tirapazamine)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>reduced radiosensitivity, reduced effect of some drugs, e.g. bleomycin, etoposide</td>
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<table>
<thead>
<tr>
<th>Proliferation</th>
<th>gradient</th>
<th>effect on therapy</th>
<th>counter measure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>up to 30% cells in S-phase near vessels, down to 0% far from vessels</td>
<td>reduced effect of cell cycle dependent drugs</td>
<td>repeated treatments, continuous infusions, use of drug combinations</td>
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<tr>
<th>Acidification</th>
<th>gradient</th>
<th>physiological effect</th>
<th>effect on therapy</th>
<th>counter measure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>gradient not known, extracellular pH is likely ~7.5 near vessels down to ~6.8 far from vessels</td>
<td>degradation of extracellular matrix, increased invasion, mutagenic</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>modified cellular uptake of many drugs (e.g. doxorubicin), radioresistance</td>
<td>repeated treatments, use of drug combinations</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Drugs</th>
<th>gradient</th>
<th>physiological effect</th>
<th>effect on therapy</th>
<th>counter measure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>free plasma levels near vessels down to potentially negligible levels far from vessels</td>
<td>potential for cultivation of drug resistance mechanisms</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>reduced exposure, increased cell survival</td>
<td>repeated treatments, increase exposure</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE 1-1 Diffusion/perfusion limited gradients in solid tumours and their effect on therapy.**

a(Thomlinson and Gray 1955) (Raleigh et al. 1987), b(Tannock 1968; Hirst and Denekamp 1979), c(Tannock and Rotin 1989; Gatenby and Gillies 2004)

Successful strategies for anti-cancer therapy must be able to address a wide range of environmental factors that will modulate treatment efficacy. TABLE 1-1 summarizes gradients that are commonly observed in solid tumours. Gradients in tissue oxygenation likely reduce the efficacy of radiation (Thomlinson and Gray 1955; Moulder and Rockwell 1987; Janssen et al. 2005) as well as drugs such as bleomycin and etoposide (Yamauchi et al. 1987). To attempt to counter this, oxygen mimetic radiosensitizers, which substitute for oxygen to prevent reconnecting of radiation induced DNA strand breaks, were developed e.g. misonidazole and nimorazole (Overgaard et al. 1989; Overgaard et al. 1998). Hypoxic cytotoxins, which are enzymatically converted to cytotoxic species under conditions of low oxygen, were later developed in an attempt to exploit
AQ4N (Brown 1993; Patterson and McKeown 2000). Increased tumour acidity modifies the activity of many anticancer agents (Tannock and Rotin 1989), for example, reducing the cellular uptake and efficacy of weak bases such as the anthracyclines (Mahoney et al. 2003). Most anticancer drugs in current use were developed to exploit the increased proliferation status of tumour cells, hence reduced cellular proliferation modulates the efficacy of the majority of anticancer drugs in existence. Finally, diffusion limited penetration can result in lower drug exposure in the tumour regions that are distant from vasculature, resulting in reduced efficacy. The repeated exposure to sub-toxic drug levels is perhaps the simplest way of cultivating a drug resistant phenotype. In theory the gradients in oxygen, acidity and drugs that can occur in solid tumours will effect changes in treatment response. However direct causal evidence linking these problems with clinical management of cancer is often lacking.

1.2 Drug Penetration

In contrast to most normal tissues, with relatively high microvessel density, the extravascular compartment of solid tumours may pose a significant barrier to the penetration of molecules supplied by the blood. However, little is known of the ability of many of the most commonly used anti-cancer drugs to distribute within solid tumours (Tannock et al. 2002). Measurement of gross tumour drug accumulation is a routine component of drug development but it tells little about extravascular drug distribution. This is especially true in the case of drugs that are highly reactive in tissue, which may produce high tumour accumulation that is confined to the cells immediately surrounding active vessels. In principle, visualization of a drug using radiolabelled or immunohistochemical-based assays is the most direct way to evaluate drug penetration. However, this approach is not always possible and has limited applicability as a fast, generic screening assay for newly synthesised drugs, especially when stability issues/metabolites are not known.

Factors Influencing Tumour Drug Distribution: Supply and Demand

Like the gradients in oxygen that were first postulated by Thomlinson and Gray (Thomlinson and Gray 1955), the tumour distribution of any molecule supplied from the blood will be subject to the constraints of supply and demand. Table 1-2 lists factors that can play important roles in determining the tumour distribution of a drug. Supply factors are controlled by pharmacokinetic variables such as dose, metabolism, excretion and blood binding. Flux of drugs within tumour tissue is controlled by factors such as MW, lipophilicity, charge etc. Factors that determine demand of the drugs by tissue can include cellular metabolism, binding and sequestration.
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**Pharmacokinetics**

- dose: determines $C_{\text{max}}$, AUC, $T_{1/2}$
- metabolism and excretion: will limit free drug available for diffusion, may serve as a reservoir of drug that is slowly released while protected from metabolism in liver
- blood binding: protects drug from metabolism in liver

**Tissue Characteristics**

- vascular density: often low in tumours, increases gradients of diffusion limited molecules
  - typical max. distance to a vessel: normal tissue 3-5 cell layers, tumours up to 15-20 cell layers
- vascular integrity: often compromised in tumours, increases access to extravascular space
- interstitial pressure: often elevated in tumours may lead to intermittent blood flow
  - will determine traction or available volume for diffusion for drugs that enter cells slowly
  - typical extracellular fraction observed in tumours: 35-60%
- extracellular fraction: charged matrix that may modify the diffusion of some drugs
- extracellular matrix: negatively charged extracellular matrix components as well as membrane and protein binding

**Drug Characteristics**

- MW: inversely proportional to a molecule's diffusion coefficient in water
- lipophilicity: will control access of drugs to cellular compartment, easy access means more flux through tissue but also possibly more cellular consumption

**Drug-cell interactions**

- metabolism: consumption of a drug may limit its tissue penetration
- binding: leads to accumulation of drugs, may limit tissue penetration, may serve as a reservoir that is slowly released after drug is cleared from plasma
- Egp status: will reduce access to cellular compartment, may increase tissue penetration

**TABLE 1-2 Factors which will influence a drug’s ability to penetrate and distribute within solid tumours.**

<table>
<thead>
<tr>
<th>Factor</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Krogh 1919), (Thomlinson and Gray 1955),</td>
<td>(Peterson and Appelgren 1977;</td>
</tr>
<tr>
<td>(Dvorak et al. 1988; Hashizume et al. 2000;</td>
<td>Dvorak 2002), (Chaplin et al. 1987)</td>
</tr>
<tr>
<td>(Kimura et al. 1996) (Jain 1987; Dewhirst</td>
<td></td>
</tr>
<tr>
<td>et al. 1998), (Gain 1987).</td>
<td></td>
</tr>
</tbody>
</table>

Upon leaving the vasculature and entering the extravascular tumour compartment a drug’s ability to penetrate and distribute within tissue will depend on the balance between its ability to diffuse into tissue versus its rate of sequestration/consumption by the tissue. FIGURE 1-3 illustrates potential drug-tissue interactions that may limit tissue penetration for a hypothetical anthracycline-like drug. In panel A, the drug is seen to either be confined predominantly to the extracellular space, with occasional entry and binding within cells (upper path) or to have easy access to the cellular compartment (lower path). In the second case the increased sequestration/consumption of the drug due to its greater access to the cellular compartment may or may not limit its tissue penetration. Panel B illustrates the known drug-tissue interactions of doxorubicin. Interactions include DNA intercalation (Zunino et al. 1980), sequestration in acidic lysosomes (Coley et al. 1993; Hurwitz et al. 1997), passive uptake in proportion to lipophilicity and uncharged fraction and active efflux (Frezard and Garnier-Suillerot 1991) and membrane binding (de Wolf et al. 1993; Demant and Friche 1998a). Potential extracellular interactions include negatively charged extracellular matrix components as well as membrane and protein binding (Chassany et al. 1994). The mathematical modelling of any drug’s tissue penetration will require detailed knowledge of its physicochemical properties and all significant cellular interactions.
TUMOUR MAPPING

Recent advances in immunohistochemistry and digital imaging mean that many drugs can now be visualized and mapped in relation to tumour vasculature. Key innovations were the development of antibodies to detect vasculature, e.g. anti-CD31, Von Willebrand factor VIII, improvements in quantitative digital imaging, robotic microscopy and computer processing. For the last 5-10 years it has been possible to capture images of entire tumour sections at high enough resolution to map drug levels in tissue relative to tumour vasculature. Reports are now beginning to appear that examine radiation and drug effects in relation to immunodetected tumour vasculature. Examples include doxorubicin as detected by fluorescence (Primeau et al. 2005), radiation via effects on proliferation (Ljungkvist et al. 2006) and gemcitabine via effects on proliferation (Huxham et al. 2004).

Direct imaging of drugs and their adducts

Studies examining drug distribution without immunodetected tumour vasculature information have looked at radiolabelled and fluorescent drugs and dyes. The earliest studies carried out looked at the diffusion of lissamine green and other dyes in tumours (Goldacre and Sylven 1962). Doxorubicin is perhaps the most studied anti-cancer drug due to its fluorescence (Ozols et al. 1979; Lankelma et al. 1999), though in general no attempt has been made to differentiate it from its less active but fluorescent metabolite doxorubicinol or between free and partially quenched bound drug. Radiolabelled drugs studied include paclitaxel (Li et al. 2000) and immunotoxins (Sung et al. 1993). In addition to classical detection via radiolabelling or direct fluorescence there now exist a number of drug adducts which can be detected via antibody recognition. Examples of drugs that can be detected by antibodies include cisplatin-DNA adducts (Terheggen et al. 1987; Tilby et al. 1991; Liedert et al. 2006). The thymidine analogue, Bromodeoxyuridine (BrdUrd),
originally developed as a radiation sensitizer is incorporated into DNA during replication and can be detected via antibodies raised against DNA-BrdUrd adducts (Gratzner 1982). Doxorubicin has also been reported to have been detected immunohistochemically (Henneberry and Aherne 1992). Other examples include immunodetection of adducts formed by the 2-nitroimidazoles such as EF5 and pimonidazole binding to thiol groups in proteins and amino acids under hypoxic conditions (Raleigh et al. 1987; Lord et al. 1993; Azuma et al. 1997).

Mapping drug effect in tumours
Markers for proliferation (e.g. BrdUrd, Ki67, PCNA), apoptosis (e.g. Tunel, caspase 3) and double strand breaks (e.g. γH2AX) can also be used in an attempt to map drug distributions via their effect on cells in tumours relative to vasculature. However, the relation between drug effect and drug distribution is generally obscured by changes in the intrinsic sensitivity of cells to drug with depth into tissue. Hence these studies address more general questions of drug efficacy in solid tumours rather than questions specific to drug penetration. The earliest mapping studies were done in spheroids and tumours using either sequential trypsinization or cell-sorting techniques with cell survival as the endpoint (Giesbrecht et al. 1981; Durand 1982; Olive et al. 1985). For the cell-sorting method, treated cells were dissociated and sorted based on the strength of Hoechst 33342 fluorescence, which was allowed to diffuse into the tissue and used as an indicator of the distance of each cell from a perfused vessel/tissue edge. Work was first carried out with radiation and doxorubicin (Chaplin et al. 1985; Durand 1990) and later other anti-cancer agents (Olive and Durand 1994). New approaches involve imaging of tissue sections for markers of vasculature and drug effect. Using the tissue section-based techniques, the effects of radiation (Ljungkvist et al. 2006) and drugs such as gemcitabine (Huxham et al. 2004) and tirapazamine (Huxham et al. 2006) have been evaluated in relation to tumour vasculature.

Limitations of tumour mapping for evaluation of drug penetration
While histological based tumour mapping represents a relatively new and unexplored area of research, numerous limitations exist in terms of employing it in assessing drug penetration. With regards to direct imaging, radiolabelled drugs are not always available and are generally expensive. Autoradiography-based experiments are labour intensive and getting results can entail waiting weeks or months while film is being exposed. In addition, the potential for presence of radiolabelled drug metabolites means that data will often be inconclusive. Assays involving biological endpoints are prone to changes in intrinsic sensitivity of cells to drugs with changing proliferation status, oxygenation etc. (see Table 1-1). In addition, indirect drug-derived host-toxicity may result in changes in tumour proliferation and even apoptosis via a reduction in oxygen and nutrients supplied from the blood.
1.3 *In Vitro Tumour Models*

There exist several *in vitro* models for evaluating tumour drug diffusion, the best characterized and most widely used being multicellular spheroids (Sutherland 1988). Other models include the one used here, multilayered cell culture (Cowan *et al.* 1996; Minchinton *et al.* 1997). In addition, chunks and slabs of tissue excised from solid tumours and cellular aggregates grown on tissue culture plates or embedded in collagen or sponge are also used (Leighton 1951; Swabb *et al.* 1974; Yang *et al.* 1979; Vescio *et al.* 1987).

**Spheroidal Cell Culture**

Multicellular spheroidal cell culture is the mainstay of *in vitro* 3-dimensional tumour modelling. Developed over 30 years ago in the Sutherland Lab at the University of Western Ontario, spheroids have been shown to reproduce many of the features of the tumour microenvironment. Diffusion limited penetration of molecules into the cultures means they can reproduce the oxygen and nutrient gradients and gradients in proliferation seen in solid tumours. Thicker spheroids exhibit acidified central regions, due to lactate build-up, and if large enough they form a central necrotic region. In addition they have been shown to exhibit a 3-D culture cell contact effect which modifies their response to radiation and drugs. Because they are able to integrate a number of characteristics of the tumour microenvironment they are an ideal system for modelling the *in vivo* response to anti-cancer agents.

**Modelling solid tumours**

Spheroids have been applied to study relations between oxygen, glucose, serum and proliferation/necrosis (Freyer and Sutherland 1983; Freyer *et al.* 1984; Casciari *et al.* 1988; Acker *et al.* 1992; Groebe and Mueller-Klieser 1996). Diffusion limited penetration of molecules into the cultures means that as well as producing oxygen and nutrient gradients they can also develop an acidic central region due to lactate accumulation (Walenta *et al.* 2000) and have been used to study relations between central pH and medium buffering capacity (Acker *et al.* 1987). They have been grown using a number of cell types including normal and drug resistant variants (Martin *et al.* 2003) and have been shown to create an extracellular matrix (Glimelius *et al.* 1988).

**Effect of tumour microenvironment on response.**

Cells located on the outer versus inner layers of spheroids have been shown to exhibit differential sensitivity to a number of drugs and also to radiation (Sutherland *et al.* 1970; Durand and Sutherland 1972; Sutherland and Durand 1976; Durand 1980; Olive and Durand 1994). Changes in sensitivity of cells have been attributed to a number a factors including cell cycle, inter-cellular communication and drug penetration.
Drug penetration

The tissue penetration of drugs from several classes has been studied using spheroidal cell culture. Direct visualization of the anthracycline doxorubicin (Kerr et al. 1988) and radiolabelled vinblastine (Nederman et al. 1981), methotrexate (West et al. 1980) and other biological agents (Nederman et al. 1988) have been investigated using spheroids. Indirect effect-based detection has been used to study several drugs including doxorubicin, cisplatin and others (Durand 1990; Olive and Durand 1994). However, in these studies it was shown that the differential response of inner versus outer cells in spheroids depended on a number of factors.

MULTILAYERED CELL CULTURE

Multilayered cell culture (MCC) is a variation on spheroidal cell culture in which cells are grown as discs of tissue. Cultures are grown starting from a single cell suspension which is added to a tissue culture insert and allowed to attach overnight to form a confluent layer. Inserts are then transferred to stirred reservoirs and grown for 2-5 days to form planar cultures typically 15-30 cell layers thick, see FIGURE 1-4.

![FIGURE 1-4 Multilayered cell culture (MCC) growth apparatus and histology. Cultures are grown using standard tissue culture inserts (Millipore CM). A | Cells are first seeded and allowed to attach to the porous PTFE membranes pre-coated with collagen. B | Cultures are then immersed in stirred growth medium where they have access to oxygen and nutrients from both sides. C | Haematoxylin stained MCC cryosection showing ~15-20 cell layers resting on the PTFE membrane.

MCC-based flux assay

MCC was developed to allow measurement of flux of drugs through tumour-like tissue in a controlled environment. There are now numerous studies published using this application for a variety of anti-cancer drugs including radiosensitizers (Cowan et al. 1996; Kyle 1999), hypoxic cytotoxins (Hicks et al. 1998; Phillips et al. 1998; Kyle and Minchinton 1999), DNA-intercalators (Hicks et al. 1997) and other agents (Tannock et al. 2002). FIGURE 1-5 shows schematics of single
and a dual-reservoir conformation that are often used for measurement of drug flux. The dual-reservoir configuration allows drug concentration from both donating and receiving reservoirs to be monitored and allow modelling of mass balance. **FIGURE 1-5** C-D show typical data produced using the dual-reservoir configuration. Fitting both data sets simultaneously allows for maintenance of mass balance between reservoirs and monitoring of net drug sequestration and consumption in the culture. **FIGURE 1-6** illustrates the basic process of drug build-up within the cultures that occurs during flux experiments.

**FIGURE 1-5** Schematics of two types of apparatus used for MCC-based flux experiments and typical data produced by the assay. A | Single reservoir with floating MCC and B | dual reservoir configuration. In A, the unstirred MCC is filled with agar, premixed with the drug to prevent convection; samples are then taken from the stirred reservoir over time. In B, drug is added to one side and the flux through the culture and into the second reservoir is monitored over time. C-D | Typical data from donating and receiving reservoirs using the dual-reservoir configuration. In this case drug diffuses through a tissue culture insert membrane with no cells. In C, the drug misonidazole diffuses without loss and drug disappearance from the donating reservoir is matched by its appearance in the receiving reservoir. In D, doxorubicin is lost through instability in the growth medium and the change in the donating reservoir is not matched by appearance in the receiving reservoir. Adapted from (Kyle 1999).

Results from flux experiments can be used for direct comparison of relative flux between two or more drugs or experimental conditions or used to estimate rates of diffusion and sequestration/consumption within the tissue. On their own, raw data from one or both reservoirs can be used to compare the relative flux of two or more drugs. It is also possible to examine the effect of
altering some experimental parameter, often the inhibition of a specific consumptive process. Examples of this include variation of tissue oxygenation to inhibit the enzymatic conversion of the hypoxic cytotoxin tirapazamine (Hicks et al. 1998; Phillips et al. 1998; Kyle and Minchinton 1999) and over-expression of Pgp to reduce cellular accumulation of doxorubicin (Tunggal et al. 2000). Data from flux experiments can also be fitted to differential equations to obtain estimates of parameters representing diffusion and sequestration/consumption processes within the cultures. Examples include analysis of the DNA intercalator DAPA (Hicks et al. 1997), the hypoxic cytotoxin tirapazamine (Hicks et al. 1998; Kyle and Minchinton 1999) and recently a panel of tirapazamine analogues (Hicks et al. 2006).

**FIGURE 1-6** Schematic of the process of drug build-up within an MCC during flux experiments using the dual-reservoir configuration. Following addition of drug to one reservoir, a phase of transient build-up occurs within the culture. During the subsequent steady-state phase, for drugs that are not sequestered/consumed in the tissue a linear gradient will form within the tissue and the amount of drug entering the culture from the donating reservoir will be matched by drug exiting the culture to the receiving reservoir. If loss does occur within the tissue, a non-linear gradient will link concentrations in the two reservoirs and less drug will exit the MCC than enter it (Crank 1975).
Simulating drug distribution

In cases where the key drug-tissue interactions are understood and have been quantified it may be possible to mathematically model the tissue pharmacokinetics of a drug. Once the initial characterization work has been carried out this approach provides flexibility in terms of exploring the effect of variations in exposure conditions such as plasma pharmacokinetics or tumour architecture on drug distribution (Kyle and Minchinton 1999; Hicks et al. 2006). However, problems may be encountered if this approach is used to model an under characterized system or through oversimplification of complex drug-tissue interactions, potentially leading to false predictions.

**FIGURE 1-7 Apparatus used to expose MCC to drug from just one side.** A | MCCs are placed in a stirred reservoir with the membrane side of each tissue culture insert clamped against the reservoir wall. The culture then has access to growth medium and drug from one side only, thereby allowing build-up of drug to occur within the tissue. B | Following drug penetration experiments cultures, are embedded in cryosectioning gel (O.C.T.) and frozen with the bottom of the tissue culture insert in contact with an aluminium block.

**Visualizing drug fluorescence in MCC**

An alternative application of MCC to assess the distribution of drugs is to directly examine drug within the tissue. One way of doing this is to close-off one side of the MCCs and allow drug to build-up from the other side, as shown in **FIGURE 1-7**.

Examples of the possible range of variation in drug profiles within 150 μm thick cultures following a 1-hour drug exposure to one side are shown in **FIGURE 1-8**. Panels show drug-derived fluorescence visualized in tissue cryosections. Fluorescent profiles indicate that in some cases drug can be limited to just the first few cell layers, while in others, drugs can exhibit almost uniform distribution over the full width of the tissue. Of the examples shown here the fluorescent-tagged goat IgG, with a molecular weight over 100 times greater than the other molecules, not surprisingly exhibits the poorest distribution after the 1-hour exposure. However, ranking of the other agents doesn’t strictly follow molecular weight. Factors that can modify each drug’s tissue penetration will include their level of access to the cellular compartment and degree of sequestration/consumption within the tissue.

Comparison of two the Hoechst drugs examined here indicates that 33258 exhibits a more uniform tissue distribution than 33342 despite 33342 being more lipophilic and entering cells.
more easily than 33258. Both drugs accumulate in cells through binding to DNA. In the case where a drug's accumulation is negligible in comparison with its rate of flux through tissue, a more lipophilic drug will have better access to the intra-cellular compartment of the tissue and hence exhibit faster flux. However, if access to the intra-cellular compartment leads to significant accumulation then the opposite may occur and the less lipophilic drug, confined to the extra-cellular compartment, will exhibit a more uniform distribution. In this case it is 33258, the less lipophilic drug, which exhibits the more uniform distribution, suggesting that drug sequestration via DNA binding may be high enough to limit its tissue penetration.

Fluorescence-based evaluation of drug penetration.

![Graph showing drug penetration profiles](image)

**FIGURE 1-8** Drug derived fluorescent profiles observed in HCT-116 MCCs following 1-hour exposure using the closed-off apparatus. Drug profiles are not seen to rank solely by MW. Following drug exposure cultures were embedded in cryosectioning medium and immediately frozen. Panels show fluorescence as a function of depth into tissue as determined from epifluorescence imaging of unmounted dry-cryosections. Hoechst 33342, hoechst 33258 (excitation 365, emission 460 nm); irinotecan, topotecan, tetracycline (ex 425 nm em 540 nm); DiOC$_3$(3), fluorescein (ex 480 nm, em 535 nm); goat IgG –Alexa 546, rhodamine 6G (ex 540 nm, em 605 nm). Source: Kyle unpublished.
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Fluorescein and topotecan, which appear to distribute the most efficiently of the agents studied, both exhibit oddly shaped profiles. This is likely due to an unusually fast washout during the freezing process, consistent with a molecule that exhibits rapid diffusion and negligible retention through cellular accumulation or binding. During freezing, the edges of the cultures come into contact with embedding gel for 1-2 min before it solidifies, Figure 1-7. Freezing occurs from the bottom/far edge of the cultures, which likely explains why there appears to be less washout from that side.

1.4 Project Outline: Developing a Novel MCC-based Assay for Assessing Drug Distribution Using a Biological Endpoint

The objective of this research project was to develop and evaluate a novel MCC-based assay that would measure the penetration and distribution of drugs in tissue using a biological endpoint. A unique property of the disc geometry of MCC is that it presents a barrier to penetration in which there exist two populations of rapidly proliferating cells, one on each edge of the culture, that are separated by a known thickness. This symmetry presents an opportunity to use the effect of the drugs on proliferating cells as an indication of their penetration, in effect using the cells themselves as the drug detection end-point. In tumours and spheroids this technique is not possible because proliferation and cellular response fall off with depth into tissue and hence drug penetration is only one of several factors that will determine cellular response.

Effect-based Drug Distribution Assay

Like spheroids, MCCs exhibit a gradient in proliferation but since they grow as planar discs, this gradient forms as a mirror image from either side towards the middle, Figure 1-9. In this study, we exploit this symmetry by exposing cultures to drugs from one side and then comparing their effect on the cells located on the near (exposed) side versus the far side of the cultures.

Figure 1-9 Symmetrical S-phase cell distribution seen in an HCT-116 MCC as detected by immunohistochemical staining of BrdUrd incorporated into DNA. Image of MCC cryosection, 10 μm thick, showing immunodetected BrdUrd and counterstained with haematoxylin. BrdUrd exposure and staining were carried out as described in section 2.3.

Choice of boundary conditions

Drug exposure can be made with the far side of the cultures either open or closed-off to growth medium, the in vivo analogue of each of these configurations is depicted in Figure 1-10. Exposures made with the far side of the cultures closed-off, Figure 1-7, represent the best-case scenario.
in terms of allowing drug to build-up within tissue and are most closely related to the *in vivo* situation in which there exist multiple blood vessels feeding a region of viable tissue, as depicted in Figure 1-10A. A second "open" situation, where an MCC is placed between two reservoirs, of which one contains drug, would represent a greater barrier to achieving a uniform distribution of drug, and most closely resembles, but may exceed, the *in vivo* situation where viable tissue is interspersed with necrosis, Figure 1-10B.

**Figure 1-10 Examples of two possible boundary situations: closed-off versus open.**

A | Symmetrical diffusion outwards from two blood vessels leads to build-up of drug at the midpoint. This configuration, approximated by the closed-off MCC, represents the best-case scenario in terms of allowing drug to build-up within the tissue.

B | Diffusion out from a single vessel beyond which occurs necrosis. In this case drug is not permitted to build-up at the tissue boundary but washes out into the necrotic region. This situation most closely resembles the open MCC configuration and poses a greater barrier to achieving a uniform distribution of drug within the tissue.

**Procedure**

An effect-based assay that could be used to determine a drug's distribution is outlined in Figure 1-11. After cultures are grown to their desired thickness they are placed in the drug exposure apparatus. The "closed-off" configuration was chosen for performing drug exposures to allow for the best-case scenario in terms of achieving a uniform distribution of drug. Using this configuration the maximum period of drug exposure will be limited to the duration that the cultures can be closed-off and still maintain symmetrical proliferation, likely of the order of hours. Following drug exposure, cultures will then be returned to the growth chambers and incubated for a period of time, of the order of days, to allow drug effect to manifest itself. During this incubation period drug may also redistribute and penetrate further into the culture. Boundary conditions during the incubation period match the "open" configuration to ensure that symmetrical proliferation is sustained. The incubation period must be long enough to allow manifestation of drug effect but short enough to avoid substantial re-growth of the cultures that would further complicate interpretation of the data. Following the incubation period the S-phase cell marker BrdUrd will be used to detect drug effect.

For drugs that diffuse in and wash out of tissue quickly, it may be critical that the proliferation status of the cells on the far side of the cultures is not perturbed during the exposure period.
However in other cases the proliferation status on the far side of the cultures during drug exposure may not be as important. For example if the drug is significantly retained following the exposure period, then it may only be important that the cells are in proliferation during the incubation period. Alternatively, for drugs that accumulate and are retained in the first few layers of cells and later diffuse slowly into the tissue, significant exposure to the far side of the cultures may not actually occur until the incubation period, when the drug that accumulated in the first few layers of cells slowly redistributes within the tissue. In this case, it might not even be necessary to close off the cultures during the exposure period.

**Effect-based drug penetration screening assay**

![Diagram](image)

**FIGURE 1-11** Outline of the assay proposed to evaluate drug distribution using the symmetrical proliferation observed in MCC. Once cultures are grown to the desired thickness they would be transferred temporarily to a penetration apparatus where one side would be closed-off and drug exposure take place. Cultures would then be returned to the growth apparatus and incubated for a period of time to allow for the manifestation of drug effect. Following this incubation period, cultures would then be assayed to detect differential effect to either side, in effect using the cells themselves as the drug detection device.

Interpreting results

Comparison of dose response curves from the exposed versus far sides of treated MCCs should allow for evaluation of drug distribution within the tissue. **FIGURE 1-12** shows examples of some of the potential situations that may arise when the assay is applied to assess drug penetration. In the simplest case, **FIGURE 1-12B**, the drug exposure to the far side of MCCs will be proportional to the exposed side. In this case the relative distribution of drug will be concentration independent and the reduction in exposure to the far side can be determined by measuring the shift between the two dose response curves. In the case where drug distribution is concentration dependent, **FIGURE 1-12C**, as might occur when a saturable process is involved in limiting drug penetration, the gap between dose response curves may become smaller as the driving concentration is increased. Another situation may arise for drugs that transiently increase the fraction of proliferating cells in the tissue or induce immediate cell loss at high enough exposures. Both these cases may actually produce an increase S-phase labelling at high drug exposures, **FIGURE 1-12D.** In the case of drug induced cell loss, the apparent increase in proliferation that might be observed would be because the cells located on the exposed edge at the end of the experiment would
actually have been located deeper into the tissue at time of drug exposure and hence shielded from the drug.

FIGURE 1-12 Estimating drug distribution based on comparison of dose response curves on exposed versus far sides of MCCs. A | Proliferation seen in an MCC following drug exposure from one side. Hypothetical dose response curves obtained from analysis of proliferation on exposed versus far sides of MCC following treatment for the following cases: B | drug distribution profile is concentration independent, C | drug distribution is concentration dependent and D | cell loss or cell cycle perturbation occurs at higher concentrations.

PROJECT HYPOTHESIS

The symmetrical proliferation seen in MCCs can be applied to measure the ability of anti-cancer drugs to penetrate and distribute within tissue using a biological endpoint.

DEVELOPMENT AND VALIDATION OF THE MCC EFFECT-BASED ASSAY

The development and validation of the assay will need to address the following questions:

-What effect does temporarily shutting-off one side of the culture have? Can symmetrical proliferation be maintained during the temporary closure period during drug exposure.

-What control assays can be performed to detect non-diffusion related differences in drug effect to the two sides? For instance, effects related to reduced oxygen and pH gradients that may form during the closure period.

-Does the assay correlate with direct visualization of drug in MCCs?

-How do results from MCC tissue correlate with tumour xenograft based work?

-Is the S-phase cell marker BrdUrd a good end-point for the MCC based work? Can a reliable BrdUrd-based assay be developed for detection of drug effect that can be used in MCC and also in tumour xenografts grown in mice.
-What classes of drugs can be tested using the assay?
-What are the limitations of the assay?

1.5 OUTLINE OF PRESENTED WORK

SUITABILITY OF THE S-PHASE MARKER BrdUrd AS BIOLOGICAL END-POINT

An initial decision was made to assess the S-phase marker BrdUrd as the end-point used to evaluate drug effect. Choices were between markers of cell death/apoptosis (TUNEL, activated caspase-3) and markers of proliferation (BrdUrd, Ki-67, PCNA). We chose to start by trying a marker of DNA synthesis due to the large existing body of knowledge of the cell cycle effects of most anticancer drugs derived from tritiated thymidine based work and more recently BrdUrd and flow cytometry work.

Bromodeoxyuridine (BrdUrd) is a thymidine analogue that is incorporated into DNA during the S-phase of the cell cycle. Originally investigated as a radiation sensitizer (Djordjevic and Szybaliski 1960; Kinsella et al. 1984b; Phillips et al. 1991; Ensminger et al. 1994), it has found use as a marker of cell proliferation particularly in cancer (Dolbeare et al. 1983; Begg et al. 1985; Wilson et al. 1985; Rew et al. 1992), where immunohistochemical detection of BrdUrd-DNA adducts (Gratzner 1982) makes it a simpler alternative to autoradiographic techniques using radiolabelled thymidine. Unlike endogenous markers for cellular proliferation such as PCNA or Ki-67, BrdUrd is exogenously administered and must reach proliferating cells via the vasculature. Upon leaving the vascular compartment, it then diffuses through cellular and extracellular compartments. In order for BrdUrd to act as an effective marker of cellular proliferation it must be able to distribute within the extravascular compartment with enough uniformity or at high enough concentration so that proliferating cells at any distance from a blood vessel are uniformly labelled.

Plan of work

To evaluate the feasibility of using BrdUrd as the effect-based assay end-point we set out to compare the ability of BrdUrd to penetrate and label proliferating cells in tumour xenografts and in MCCs. We began by looking at the dose dependence of BrdUrd labelled cells in relation to blood vessels in tumour xenografts and then carried out parallel experiments in MCCs. For the tumour based work the key objective was to determine if tissue penetration played a role in limiting BrdUrd labelling in cells located away from blood vessels and if so then what dose was required to avoid this phenomena. For the MCC-based work, in addition to determining the BrdUrd exposure required to label cells within the tissue, we also wanted to determine if proliferation could be sustained on the far side of the cultures during a short period of closure. Examination of BrdUrd exposure requirements in tumours and MCCs also provided an opportunity to compare the barrier to diffusion presented by the cells grown as tumour xenografts versus MCCs.
Chapter 1 - Introduction

ANTHRACYCLINE DISTRIBUTION IN MCC: EFFECT-BASED ASSAY VERSUS DIRECT VISUALIZATION

The anthracyclines, doxorubicin (DOX), epirubicin (EPI), daunorubicin (DAU) and the related compound mitoxantrone (MIT) were chosen as the first test group for the effect-based assay. A considerable body of knowledge surrounds their cellular interactions and there exist numerous reports on the limited ability of doxorubicin in particular to distribute within tissue. In spheroids, early work showed that doxorubicin exhibited limited tissue penetration (Sutherland and Durand 1976; Kerr et al. 1988). The potential for redistribution of the drug was shown in later studies where it was found that the initial distribution of cell kill following a 2-hour exposure could be improved if spheroids were then left intact for an additional 24 hours (Durand 1990). Work in experimental and human tumours has found that doxorubicin can exhibit gradients in relation to vasculature (Lankelma et al. 1999; Primeau et al. 2005). In addition to poor tissue distribution, further motivation for studying the anthracyclines was that the first three of these agents fluoresce hence allowing for a comparison to be made between the effect-based assay and direct visualization of drug fluorescence. While the drugs possess similar mechanisms of action their differing physicochemical properties and clinical activities suggest they may exhibit different tissue distributions.

The agents are weak bases with pKa values of ~8.3 for DOX, DAU and MIT and ~8.1 for EPI (Di Marco et al. 1977; Mahoney et al. 2003) and at pH 7.4 are between 85-90% charged. Entry into cells is believed to occur via passive uptake of the neutral species for all four drugs. Of the three, DAU is the least hydrophilic, entering cells 3-10 times more quickly than DOX (Frezard and Garnier-Suillerot 1991; Demant and Friche 1998b), while EPI exhibits an uptake rate approximately twice that of DOX (Cantoni et al. 1990). Log octanol-water partition coefficients at pH 7.4 are DOX ~0.3, EPI ~0.6, DAU ~0.9 and MIT ~0.8 (Burns et al. 1988; Mulder et al. 1995; Demant and Friche 1998a). All bind readily to DNA and accumulate in cells (Frezard and Garnier-Suillerot 1990). From previous experience with these drugs we anticipate that the more rapid rate of cellular uptake of the more lipophilic drugs may compromise their ability to penetrate into tissue.

Plan of work

The work outline for this section was first to determine if doxorubicin produced a differential effect on proliferation on the two edges of the cultures following exposure to one side. As the first drug to be tested it was also necessary to determine conditions required to maintain proliferation on the far side of the cultures during drug exposure. Exposure duration, wait period to the BrdUrd end-point and method for data analysis needed to be determined. Assuming success with doxorubicin, the other three agents would be tested.

Results from the effect-based assay would then be compared with direct visualization of the fluorescent agents under comparable conditions. As an additional benchmark, the rate of flux of the four agents through MCC was determined using the flux apparatus outlined in FIGURE 1-5B.
**Chapter 1 - Introduction**

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**Figure 1-13** Chemical structures of the anthracyclines to be evaluated using the effect-based assay. Adapted from (Goodman et al. 2006)

**Taxane Distribution in MCC & Tumours: Effect-Based Assay & Direct Visualization**

As a second test group, we compared the tissue distribution of the taxanes, paclitaxel and docetaxel. Motivation for choosing the taxanes was based on published reports suggesting poor tissue penetration of paclitaxel and the commercial availability of radiolabelled versions of both agents, which enabled comparison of effect-based results from MCC with direct visualization in MCC and tumour xenografts. In addition it was hypothesised that the considerable differences in the physicochemical parameters between the two drugs might lead to differences in their tissue penetration.

The tissue distribution of paclitaxel has been studied previously via detection of radiolabelled drug in whole tumour sections (Li et al. 2000), but without vasculature information, and also in tumour histocultures (Kuh et al. 1999). These studies suggested poor penetration of paclitaxel though no analysis was performed. Paclitaxel tissue distribution has been studied quantitatively, via HPLC detection, in bladder tissue that was sequentially sectioned in relation to the urothelium (Song et al. 1997; Knemeyer et al. 1999). The ability of paclitaxel to distribute within tissue has also been studied indirectly using average cell survival in multicellular spheroids (Nicholson et al. 1997) and via flux through multilayered cell cultures (Nicholson et al. 1997; Tannock et al. 2002; Grantab et al. 2006), though without subsequent mathematical modelling to predict the tissue pharmacokinetics.
Both of the taxanes studied exert their anti-mitotic effects through binding and stabilization of microtubules leading to cell cycle arrest (Garcia et al. 1994). In the human colorectal carcinoma cell line used here, HCT-116, both drugs exhibit a similar IC$_{50}$ (Bissery 1995), though in general docetaxel exhibits a lower IC$_{50}$ than paclitaxel. There are numerous differences between them which might affect their tissue penetration. Docetaxel has been reported to have a higher binding affinity to microtubules as well as faster accumulation and longer retention than paclitaxel (Kelland and Abel 1992; Lavelle et al. 1995) which might limit its ability to distribute within tissue. In addition, in vivo, both drugs are highly blood bound, paclitaxel 88-98% and docetaxel ~94% (Song et al. 1996; Sparreboom et al. 1998; Goodman et al. 2006). Assuming blood bound drug does not contribute to tissue build-up then the extent and speed at which binding occurs and the degree of reversibility for each drug could play an important role in determining the amount of free drug available for diffusion into tissue. Paclitaxel has the added factor of its formulation vehicle, Cremophor EL, which through micelle formation is thought to reduce the free drug available for diffusion but increase the duration of exposure (Gelderblom et al. 2002; ten Tije et al. 2003).

Plan of work

A - Initial work was carried out to determine conditions for drug exposure and end-point for measurement of drug distribution using the effect-based assay. Once it was determined that the assay could be implemented for both drugs, work was carried out to evaluate their ability to penetrate and distribute within tissue.

B - Based on the significant differences between the two agents observed in part A we attempted to determine the distribution of drug effect on proliferation in tumour xenografts grown from the same cell line. Work was carried out where immunodetected BrdUrd labelled cells were mapped in relation to tumour vasculature. We then looked at the proliferation profile relative to tumour vasculature following drug treatment over a range of doses.

C - We subsequently carried out radiolabelled based work to directly visualize drug profiles within MCCs and tumour xenografts, where the effect of vehicle Cremophor EL was also evaluated. The tissue distribution of $^3$H-paclitaxel and $^3$H-docetaxel was mapped in relation to tumour vasculature by combining a film-based dry-autoradiography technique with subsequent immunohistochemical detection of tumour vasculature. Superimposition and alignment of tumour vasculature and radiolabelled drug was made possible by capturing high-resolution images of entire tumour cryosections using a robotic tiling microscope. Following automated analysis of whole tumour cryosections, drug distribution was determined as a function of distance to the nearest visible blood vessel. While mapping radiolabelled taxane distributions relative to tumour vasculature will yield information about in vivo taxane distribution, it has the drawback of incorporating the contribution of $^3$H-labelled liver metabolites, which might diffuse more easily.
A second drawback of the technique is that drug penetration will likely appear to be greater than it actually is due to contributions from vessels that lie just outside of the tissue section, the occurrence of which will increase in likelihood with increasing distance from an observed vessel. These two factors mean that the in vivo based detection of drug penetration will likely over predict taxane tissue penetration.

ANTI-CANCER DRUG SURVEY: EFFECT-BASED ASSAY

In addition to the data presented on anthracyclines and taxanes, the tissue distribution of a range of other anti-cancer agents was surveyed using the effect-based assay. The objective was to evaluate the applicability of the effect-based assay for some of the more widely used anti-cancer agents. In addition to determining the usefulness and limitations of the assay, it was hoped that the survey would produce comparative estimates of the tissue distribution of some of the most commonly used anti-cancer drugs using a standardised assay. Drugs examined included representative agents from vinca alkaloids, antimetabolites, antibiotics, platinum agents, epipodophyllotoxins and alkylating agents.

Vinca alkaloids

The vinca alkaloids are a class of tubulin binding drugs of which several agents have been developed for clinical use. As such they present another opportunity to compare tissue distribution of a group of related compounds possessing differing physicochemical properties. Due to their high level of cellular accumulation, up to 100-fold over extracellular levels (Ferguson and Cass 1985), and differing rates of cellular uptake it was hypothesised that they might exhibit different tissue penetration. Using the effect-based assay the tissue distribution of vincristine, vinblastine and vinorelbine was compared. Of the three, vincristine is the least lipophilic (Owellen et al. 1977) and has been shown to enter cells more slowly in comparison to vinblastine but is also retained longer (Ferguson and Cass 1985). Vinorelbine, which is the most lipophilic of the three (Zhou et al. 1994), might benefit from an increase in access to the intracellular compartment if cellular sequestration or consumption do not play limiting roles in determining its tissue penetration.

Antimetabolites

The tissue distribution of three antimetabolites, gemcitabine, 5-fluorouracil (5-FU) and cytarabine was examined using the effect-based assay. All three drugs inhibit DNA and RNA function through their similarity with thymidine, in the case of 5-FU, and cytidine as with cytarabine and gemcitabine. While all three target cells during S-phase, the activity of gemcitabine, unlike cytarabine and 5-FU, is not solely confined to that portion of the cell cycle (Goodman et al. 2006). Additionally, cytarabine and 5-FU are commonly administered as continuous infusions and hence represent drugs that may not perform well using the short drug exposure required for the closed-off assay.
Others

In addition to the drugs listed above a selection of other clinically used agents were tested using the assay including cisplatin, mitomycin C, bleomycin, etoposide, BCNA and thiotepa. Of these drugs mitomycin C, bleomycin and etoposide have all been reported to exhibit oxygen dependent toxicity and may exhibit gradients in effect which do not arise from limited diffusion and hence present test cases for the assay.

Plan of work

The work outline for this section was to follow a similar approach as was used for the anthracyclines and taxanes. Initial work was carried out to determine conditions for drug exposure and end-point for measurement of drug distribution using the effect-based assay. Once it was determined that the assay could be implemented, work was carried out to evaluate each of the drugs.
2. MATERIALS & METHODS

2.1 CELL CULTURE

**MONOLAYER CULTURE**

HCT-116 (human colorectal carcinoma) and SiHa (human cervix squamous carcinoma) cells were purchased from American Type Culture Collection. Cells were grown as monolayers using minimum essential medium (GIBCO/BRL, Burlington, ON, Canada) supplemented with 10% foetal bovine serum\textsuperscript{ab} (GIBCO/BRL) or 5% bovine growth serum\textsuperscript{cd} (HyClone, Logan, Utah) and passaged every 3-5 days for up to 4 months before being discarded.

**MULTILAYERED CELL CULTURE**

Standard tissue culture inserts (CM 12 mm, pore size 0.4 μm, Millipore, Nepean, ON, Canada) were coated with 150 μl collagen (rat tail type I\textsuperscript{ab} and calf skin type III\textsuperscript{cd} Sigma), dissolved in 0.01 M HCl and diluted 1:4 with 60% Ethanol to 0.75 mg/ml, and allowed to dry overnight. HCT-116 (7.5 x 10^6 cells) or SiHa (5x10^6 cells) in 0.5 ml growth medium, were then added to the inserts and incubated for 15-18 hours to allow the cells to attach. The cultures were then incubated for 2-3 days in custom-built growth vessels, **FIGURE 2-1A**, to form MCCs ~150 μm in thickness. Each growth vessel contained a frame that held up to 8 inserts completely immersed in 130 ml of stirred growth medium (700 rpm, 25 mm stir bar) under continual gassing (5% CO\textsubscript{2}, balance air) at 37°C.

**FIGURE 2-1 Schematic of growth vessel and drug penetration apparatus used for MCC-based work.** A | Configuration used during MCC growth, up to 8 MCCs could be held suspended in stirred growth medium. B | Apparatus used during drug exposure to one side, the membrane side of each MCC was clamped sideways against a polyacrylate block, with a layer of parafilm sandwiched in between to ensure a complete seal. MCC sides were defined as near (exposed) and far (closed-off). C | Apparatus used to conduct drug flux experiments and also for control experiments, where MCCs were exposed to drug from both sides but under different oxygenation and glucose/lactate conditions.

\textsuperscript{a}BrdUrd work presented in chapter 3, \textsuperscript{b}anthracycline work presented in chapter 4, \textsuperscript{c}taxane work of chapter 5 and \textsuperscript{d}miscellaneous work from chapter 6.
2.2 BrdUrd-penetration in MCC and xenografts

MICE AND TUMORS

Female nod/SCID mice were bred and maintained in our institutional animal facility in accordance with the Canadian Council on Animal Care guidelines. The experiments described here 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. Human cervical carcinoma (SiHa) cells (50 μl of 2 x 10⁶ cells/ml) were implanted sub-cutaneously into the sacral region of the mice. The mice were used at 12-16 weeks of age, and ranged in weight from 20-26 g. The weight of the excised tumours was 70 ± 30 mg (mean ± std. dev.).

TUMOUR PENETRATION ASSAY

Tumour bearing mice were administered 5-bromo-2-deoxyuridine, BrdUrd (Sigma Chemical, Oakville, ON, Canada), i.p. at 25, 50, 100, 400, 1000 and 2000 mg/kg 2 hours prior to sacrifice. BrdUrd was administered as a 30-mg/ml solution in saline. As a marker of blood perfusion, mice were intravenously administered 75 μl of 0.6 mg/ml DiOC₇(3) (Molecular Probes, Eugene, Oregon, USA) dissolved in 75% DMSO, 5 minutes prior to sacrifice. After excision, tumours were cooled to -20° C on an aluminium block, embedded in O.C.T. medium (Tissue-TEK, Torrance, CA, USA) and stored at -20° C until sectioning. An outline of the experimental plan for this section is shown in FIGURE 2-2.

MCC-BASED BrdUrd PENETRATION ASSAY

MCCs were exposed to BrdUrd from one side using a polyacrylate jig, FIGURE 2-1B, designed such that the bottom of each insert was clamped against a flat block, with a layer of Parafilm (American National Can, Chicago, IL, USA) sandwiched in between to ensure a complete seal. Each MCC was exposed to a 7.5 ml stirred reservoir kept under controlled gassing and temperature. A silicone o-ring was used to seal the gap between the insert and the orifice in the reservoir.
During the penetration assay growth medium was supplemented with excess glucose (10 g/1 d-glucose, Sigma) in attempt to sustain proliferation on the far side of the cultures (Mueller-Klieser et al. 1986). Once placed in the jig, MCCs were allowed to equilibrate for 45 minutes and then BrdUrd was added to the growth medium at concentrations of 1, 5, 25 and 100 μM. Control MCCs were exposed to 25 μM BrdUrd from both sides under otherwise similar conditions. After an exposure of 1 hour the cultures were removed, rinsed in PBS (phosphate buffered saline), embedded in O.C.T. and stored at -20° C until sectioning. An outline of the experimental plan is shown in FIGURE 2-3.

**PHARMACOKINETIC ASSAY**

Bromodeoxyuridine was administered i.p. at a dose of 100 mg/kg to six SCID mice. Blood samples of approximately 20 μl were taken from the tail vein of the mice at 5, 10, 20, 30, 60 and 120 minutes after administration. For the 5-30 minute time points the mice were divided into two groups and sampling was alternated between groups at each time point. All mice were sampled for the 60 and 120-minute time points. Following each time point, blood samples were weighed, diluted with 500 μl methanol, vortexed for 20 seconds and stored at 4° C. Upon completion of all time points, samples were centrifuged at 2500 g for 10 minutes and 450 μl of the supernatant was removed and evaporated to dryness using a centrifugal evaporator (1 hour at 40° C, Labconco, Kansas City, MO, USA). Samples were then reconstituted in 50 μl of 0.1 M ammonium acetate pH adjusted to 3.5 and then assayed via HPLC on the same day.

**HIGH PRESSURE LIQUID CHROMATOGRAPHY**

Chromatographic analysis was carried out with Waters equipment (Mississauga, ON, Canada), including a model 510 pump, model 712 WISP injector and model 996 photodiode array detector. A Symmetry C18 column (3.9 x 150 mm) was used for sample separation. With a mobile phase
consisting of a mixture of 25% acetonitrile and 0.1 M ammonium acetate, pH 3.5 (0.15:0.85) flowing at 1.0 ml/min whereby BrdUrd eluted after 9.2 minutes. Samples of 30 µl were injected and absorbance detection was carried out at 280 nm. The assay linearity for BrdUrd in water was verified over the range of 0.2-200 µM and BrdUrd recovery from spiked blood was greater than 90% for 1-200 µM range. BrdUrd stability to within greater than 3% in the processed samples was confirmed over a 24 hour time period.

BrdUrd Immunohistochemistry

MCC cryosections (10 µm) were air dried for 24 hours and then fixed in a 1:1 mixture of acetone-methanol for 10 minutes at room temperature. Slides were immediately transferred to distilled water for 10 min and then treated with 2 M HCl at room temperature for 1 hour followed by neutralization for 5 min in 0.1 M sodium borate. Slides were then washed in distilled water and transferred to a PBS (phosphate buffered saline) bath. Subsequent steps were each followed by a 5 min wash in PBS. BrdUrd incorporated into DNA was detected using a 1:200 dilution of monoclonal mouse anti-BrdUrd (clone BU33, Sigma) followed by 1:100 dilution of anti-mouse peroxidase conjugate antibody (Sigma) and 1:10 dilution of metal enhanced DAB substrate (Pierce, Rockford, IL). Slides were then counterstained with haematoxylin, dehydrated and mounted using Permount (Fisher Scientific, Fair Lawn, NJ, USA).

Image Acquisition

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 program developed at the U.S. National Institutes of Health, available at http://rsb.info.nih.gov/nih-image/) running on a G4 Macintosh computer. The motorized stage allowed for tiling of adjacent microscope fields of view. Using the image acquisition system, images of entire tumour sections were captured, typically 25-50 mm² in size at a resolution of 1 pixel/µm². Two cryosections, typically 8 mm in length each, were imaged for each MCC, making a ~2 mm² imaged tissue area. For tumour cryosections, images of DiOC₇(3) fluorescence within the sections were obtained prior to BrdUrd immunostaining using a 450-480 nm excitation filter and a 525 nm long pass emission filter. Once immunostained and mounted, the slides were then revisited and bright field images of BrdUrd positive staining obtained.

Image Analysis: Tumours

Using the NIH-Image software application and user supplied algorithms, images of DiOC₇(3) fluorescence and BrdUrd/tissue staining from each tumour section were overlaid and areas of necrosis and staining artefacts were removed. On the fluorescence image DiOC₇(3) positive regions were then identified by selecting all pixels that were 20% above the maximum tissue background intensity. DiOC₇(3) positive regions that were less than 20 µm² in size were consid-
ered artefacts and removed from the analysis. On the bright field images, BrdUrd positive staining was identified by selecting pixels that were two and a half standard deviations above tissue background levels. Measuring the distance from each point in the tissue to the nearest DiOC\(_7\)(3) positive pixel and noting if it were BrdUrd positive or negative then determined 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.

**IMAGE ANALYSIS: MCCs**

BrdUrd positive staining was identified as above. For the MCCs that were sealed off from one side during exposure to BrdUrd, the edges of the MCCs were traced out and then the position of each point in the tissue was measured relative to the two edges (expressed as a fraction between 0 and 1) and tabulated along with whether or not the point was BrdUrd positive. The fraction of tissue positive for BrdUrd of the total tissue found at each position within the MCC, divided into 30 steps, was then calculated. For MCCs that were exposed to BrdUrd from both sides, the fraction of BrdUrd positive pixels at each distance away from the nearest edge was determined.

**FIGURE 2-4 Outline of the proposed effect-based assay for evaluation of drug distribution.** Once cultures were grown to the desired thickness, they would be temporarily transferred to the penetration apparatus where drug exposure would occur. Following exposure and wash out, cultures were returned to the growth apparatus and incubated for a period of 1-3 days before labelling S-phase cells.

**2.3 EFFECT-BASED MCC DRUG DISTRIBUTION ASSAY**

**FIGURE 2-4** outlines the proposed effect-based assay for evaluation of drug distribution using a biological endpoint. Once grown, MCCs were exposed to drugs from one side using a polyacrylate jig, **FIGURE 2-1B**, designed such that the bottom of each insert was clamped sideways against a flat block, with a layer of Parafilm (American National Can, Chicago, II, USA) sandwiched in between to ensure a complete seal. Each MCC was exposed to a 2.5 cm\(^2\) or 7.5 cm\(^2\)-ml stirred reservoir.

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\(^a\)BrdUrd work presented in chapter 3, \(^b\)anthracycline work presented in chapter 4, \(^c\)taxane work of chapter 5 and \(^d\)miscellaneous work from chapter 6.
kept under controlled gassing and temperature. A silicone o-ring was used to seal the gap between the insert and the orifice in the reservoir. Once placed in the jig, MCCs were allowed to equilibrate for 45 minutes and then drug was then added to the growth medium. After 1-2 h drug exposure, the reservoirs were rinsed twice with fresh medium. MCCs were then incubated for 1 hour prior to removal from the jig to allow initial drug wash out from the exposed-side only. For some experiments growth medium was changed halfway through the 1-hour wash-out period\textsuperscript{c,d}. MCCs were then removed, rinsed in fresh medium three times for 15 seconds and placed in a growth jar containing 2 untreated control MCCs, that were not subjected to closure. Separate control experiments were carried out to verify that closure of the MCCs did not, on its own, affect proliferation 1 or 3 days later. To reduce the build-up of washed out drug, medium was replaced at 1 and 3 hours following return to the growth jar and the incubation with control MCCs allowed for detection of potential drug effects to unexposed edges that were derived from drug wash out from the exposed sides. MCCs were left for 1-3 days from the time of beginning drug exposure to allow manifestation of drug effect, following which 100 \textmu M BrdUrd (5-bromo-2-deoxyuridine, Sigma Chemical, Oakville, ON, Canada) was added to the growth medium and cultures incubated for 4 hours to label S-phase cells. MCCs were then removed, frozen in O.C.T. medium (Tissue-TEK, Torrance, CA, USA) and stored at -80° C until sectioning.

**MCC CONTROL ASSAY**

Control experiments were carried out using a dual-reservoir apparatus, FIGURE 2-1C, in which equal drug exposure was made to both sides of MCCs but under 20% oxygen, 1 g/L glucose in the first reservoir versus 0% oxygen, 0.1 g/L glucose and 1 mM lactate\textsuperscript{c} in the second reservoir. The low oxygen growth medium was gassed overnight at 0% O\textsubscript{2} and then re-equilibrated for 1-hour upon insertion of the MCCs within the dual-reservoir apparatus. For these experiments, the medium used was glucose free DMEM GIBCO/BRL, Burlington, ON, Canada) supplemented with 10% foetal bovine serum\textsuperscript{a,b} (GIBCO/BRL) or 5% bovine growth serum\textsuperscript{c,d}, (HyClone, Logan, Utah). Sodium bicarbonate levels were set to produce pH 7.4 under 5% carbon dioxide. Glucose was then added to the oxygenated side to match physiological levels, 1 g/L. On the anoxic side, the glucose was derived from the growth serum. Drug exposure and wash out were carried out in the manner described in the previous section.

**BrdURd IMMUNOHISTOCHEMISTRY**

BrdURd immunostaining was carried out as described in section 2.2.

\textsuperscript{a}BrdUrd work presented in chapter 3, \textsuperscript{b}anthracycline work presented in chapter 4, \textsuperscript{c}taxane work of chapter 5 and \textsuperscript{d}miscellaneous work from chapter 6.
Chapter 2 – Materials & Methods

IMAGE ACQUISITION

The imaging system was the same as described in section 2.2. Images of MCC sections, 8 mm in length, were captured at a resolution of 1 pixel/\(\mu \text{m}^2\). Two cryosections were imaged from each MCC. For MCCs immunostained for BrdUrd incorporation, bright field images were captured. For drug fluorescence visualization, MCCs were imaged using unmounted cryosections 1-hour after sectioning using a 510-555 nm excitation filter and a 575-640 nm emission filter.

IMAGE ACQUISITION

The imaging system consisted of a robotic fluorescence microscope (Zeiss Imager Z1, Oberkochen, Germany), a cooled, monochrome CCD camera (Retiga 4000R, QImaging, Vancouver, BC, Canada), a motorized slide loader and x-y stage (Ludl Electronic Products, Hawthorne, NY, USA) and customized 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 tiling of adjacent microscope fields of view. Using this system, images of entire tumour cryosections up to 1 cm\(^2\) and MCC sections, 0.8 cm in length, were captured at a resolution of 0.75 \(\mu \text{m}/\text{pixel}\).

IMAGE ANALYSIS

Using the NIH-Image software application and user supplied algorithms, digital images of BrdUrd staining and drug fluorescence within MCC cryosections were analyzed. Pixels making up the cryosection were first sorted based on their distance relative to either edge of the tissue. For BrdUrd images, the fraction of positively stained pixels at each position relative to the two edges was then calculated. Pixels two and a half standard deviations above tissue background levels were classified as BrdUrd positive. For fluorescence images, the average intensity of pixels from each group relative to the two edges was calculated. Detection of tissue edges was facilitated by capturing high-contrast, bright-field images of the MCCs (prior to staining and mounting the slides in the case of the BrdUrd sections).

2.4 ANTHRACYCLINE FLUX EXPERIMENTS

FLUX ASSAY

Flux experiments were performed using the dual reservoir diffusion apparatus shown in FIGURE 2-1C. Prior to the experiment cultures were selected and visually assessed for uniformity. The selected cultures were then placed in the apparatus with 7 ml medium per reservoir. Each reservoir was individually gassed, 10 ml/min, and maintained at 37°C via forced air heating.

*BrdUrd work presented in chapter 3, anthracycline work presented in chapter 4, taxane work of chapter 5 and miscellaneous work from chapter 6.
Chapter 2 - Materials & Methods

Each reservoir was stirred using a magnetic stir bar (15 mm x 4 mm) at 450 r.p.m.. Following a 45 min equilibration 100 µM drug was added and growth medium reservoirs were sampled, 100 µl, periodically over a 3-hour period. Following experiments, MCCs were frozen and sectioned to determine culture thickness.

HIGH PRESSURE LIQUID CHROMATOGRAPHY

HPLC analysis of samples taken from the flux apparatus reservoirs was generally begun midway through drug flux experiments. Analysis used a Waters HPLC system (Mississauga, ON) which included a model 510 pump, model 712 WISP Injector, a model 996 Photo-Diode Array (PDA) Detector and a 474 Scanning Fluorescence Detector. All eluents were made using HPLC grade products and distilled and de-ionised water was used for all dilutions. Before running sample analysis, HPLC eluents were degassed via vacuum filtration (0.5 µm FHLP filter, Millipore, Mississauga, ON).

Sample Preparation

Reservoir medium samples were prepared for HPLC analysis immediately following their acquisition. Samples were de-proteinated by adding an aliquot of a 0.1-M citrate buffer pH 3.0 (volume equal to 10% of sample volume) followed by methanol (5x sample volume). After shaking, the protein precipitate was pelleted by centrifugation for 10 minutes at 7 x 10^3 r.p.m. (radial distance of ~5 cm). Samples were assayed via HPLC on the same day of each flux experiment.

Analysis

A Symmetry C18 column (3.9 x 150 mm, Waters, Mississauga, ON) was used for sample separation. Samples of 30 µl were injected using a mobile phase of 90% acetonitrile/buffered H_2O mixture (0.3:0.7) flowing at 1 ml/min, whereby drugs eluted after ~4 minutes. The buffered H_2O eluent consisted of 0.1 M ammonium acetate, pH adjusted to 4 with HCl. Fluorescence detection was carried out using the 474 Scanning Fluorescence Detector using an excitation wavelength of 480nm and an emission wavelength of 560 nm (bandwidth 40 nm). For mitoxantrone, absorbance at 640 nm was used for detection. The assay linearity for the anthracyclines in spiked growth media was verified over the range of 0.01-100 µM for DOX, EPI and DAU and 0.025-100 µM for MIT. Stability in the processed samples to within 5% was confirmed over a 24 hour time period.

2.5 \(^3\)H-TAXANE AND TAXANE-TUMOUR XENOGRAFT WORK

REAGENTS

Stock solutions were prepared of paclitaxel (Sigma-Aldrich, Oakville, ON, CA) in Tween 80 (Sigma-Aldrich) at 12 mg/ml and Cremophor EL (Sigma-Aldrich) at 6 mg/ml and docetaxel (Sigma-Aldrich) in Tween 80 at 40 mg/ml. For tritium based experiments, \(^3\)H-paclitaxel (2-benzoyl ring-\(^3\)H, ART-1195, American Radiolabelled Chemicals inc. St Louis, MO, USA) in EtOAc
and $^3$H-docetaxel (2-benzoyl ring-$^3$H, ART-1267, American Radiolabelled Chemicals inc.) in EtOH were centrifugally evaporated at 30° C and mixed with cold drug stock at a ratio of 60 μCi per mg cold drug immediately prior to diluting for injection. For paclitaxel in Cremophor EL, stock was then diluted 1:1 in saline before injection for a final concentration of 3 mg/ml. For paclitaxel in Tween 80, stock was diluted 1:1 in EtOH and then 1:1 again in saline (Sparreboom et al. 1996) for a final concentration of 3 mg/ml. For docetaxel, stock was diluted 1:3 in 13% EtOH and then 1:25 in saline, final concentration 1.38 mg/ml.

**FIGURE 2-5 Radiolabelled taxane tumour mapping protocol.** HCT-116 tumours xenografts grown in NOD/SCID mice were treated with $^3$H-paclitaxel and docetaxel i.v. and at 2 or 8 hours following treatment tumours were excised, frozen and cryosectioned. Following imaging of the blood flow marker DiOC$_7$(3), tumour sections were clamped against tritium sensitive film and left for 80 days. Film was then developed and slides immunostained for the endothelial cell marker CD31. Imaging of entire tumour cryosections allowed subsequent alignment of film and tissue images.

**MICE AND TUMOURS**

Female NOD/SCID mice were bred and maintained in our institutional animal facility in accordance with the Canadian Council on Animal Care guidelines. Experiments 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 and were used between 8-11 weeks of age, weighing between 20 to 28 g. For radiolabelled-based work HCT-116 cells (50 μl of $1.6 \times 10^6$ cells/ml) were implanted sub-cutaneously into the left and right sacral regions, with 2 mice for a total of four tumours used for each data point. An outline of the experimental plan for radiolabelled work is shown in **FIGURE 2-5**. For BrdUrd-based work, outlined in **FIGURE 2-6**, only one tumour was implanted per mouse, with five mice per data point. Mice were administered drug when one or both tumours reached 100-150 mm$^3$ as calculated from calliper measurement of three orthogonal diameters (a,b,c) using the formula volume = $\pi/6$abc. The mean weight (± SD) of the excised tumours was 60 ± 24 mg. For radiolabelled-based work, drugs were administered i.v.. For BrdUrd-based work, paclitaxel in Cremophor EL and docetaxel in Tween 80 were administered i.p. and mice were administered 1500 mg/kg BrdUrd and 60 mg/kg pimonidazole (Hy-
poxyprobe-1 Kit, Chemicon International Inc., Temecula, CA, USA) 2 hours prior to sacrifice, as previously described (Huxham et al. 2004).

HCT-116 tumours were grown to ~150 mm³

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BrdUrd i.p. &amp; pimonidazole</th>
<th>excise &amp; freeze tumours</th>
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<tbody>
<tr>
<td>24-144 hrs</td>
<td>2 hrs</td>
<td>5 min</td>
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**FIGURE 2-6 Protocol for mapping the effect of taxanes on S-phase cell distribution tumours.**

HCT-116 tumour xenografts grown in NOD/SCID mice were treated with ³H-paclitaxel and docetaxel i.p. and at 24-144 hours following treatment tumours were excised, frozen and cryosectioned. Following imaging of the blood flow marker DiOC₆(3), tumour sections were immunostained for the endothelial cell marker CD31 and hypoxia, via detection of pimonidazole adducts. Slides were then immunostained for S-phase cells via detection of incorporated BrdUrd. Imaging of entire tumour cryosections allowed subsequent alignment of the images.

**RADIOLABELLED TAXANES IN MCCS**

MCC drug penetration was performed using the apparatus shown in **FIGURE 2-1B** as described above but with cultures frozen immediately following the 1 or 2-hour drug exposures with no rinsing. An outline of the experimental plan is shown in **FIGURE 2-7**. 10 µm thick tumour xenograft and MCCs cryosections were placed in triplicate on glass slides, allowed to dry overnight, and then clamped against tritium sensitive film (Hyperfilm-³H, GE Healthcare, England) under GBX filter safelight conditions (Kodak, Rochester, NY, USA) and stored in darkness for a period of 80 days. Film was developed for 1 minute using a 1:1 dilution of D-19 developer (Kodak). Calibration of film was performed using a tritium standard slide, range 0-489 µCi/g (American Radiolabelled Chemicals inc., St Louis, MO, USA).

**FIGURE 2-7 Radiolabelled taxane MCC mapping protocol.** Following growth to 150 µm cultures were transferred to penetration apparatus and equilibrated for 45 min prior to the 1&2-h exposures to ³H-paclitaxel and docetaxel from one side. Cultures were subsequently frozen and cryosectioned. Slides were then clamped against tritium sensitive film and left for 80 days after which film was then developed. Imaging of entire cryosections allowed subsequent alignment of film and tissue images.
Chapter 2 – Materials & Methods

IMMUNOHISTOCHEMISTRY

Cryosections were fixed in acetone-methanol for 10 minutes at room temperature. Vasculature was stained using an antibody to PECAM/CD31 (BD PharMingen, San Diego, CA, USA) and fluorescent Alexa 546 anti-rat secondary (Invitrogen, Burlington, ON, CA). Hypoxia was detected via bound pimonidazole adducts using Hypoxyprobe Mab1 (Chemicon) and an Alexa 488 anti-mouse secondary. Slides were subsequently stained for incorporated BrdUrd following the method described in section 2.2 (proceeding directly to the first distilled water wash).

IMAGE ANALYSIS

Using the NIH-Image software application and user supplied algorithms, digital images of $^3$H-taxane distributions were analyzed. For tumours, digital images of tritium sensitive film and CD31 immunostained tumour cryosections were superimposed and aligned. Then each pixel making up the film image was sorted based on its distance relative to CD31 positive vasculature. Finally, the fraction of pixels from the film that were positive was tabulated as a function of distance from vasculature. Data were scaled using results for fraction positive pixels versus radioactivity ($\mu$Ci/g) using a tritium standard slide. For MCCs, digital images of tritium sensitive film were aligned with bright field images of unstained tissue cryosections. Pixels making up the tissue were then sorted based on distance from each edge of the section. For BrdUrd-based work, analysis was carried out as previously described (Huxham et al. 2004).
3. BROMODEOXYURIDINE

3.1 SUMMARY

Bromodeoxyuridine (BrdUrd) is used extensively to measure the fraction of proliferating cells in tumours. Unlike endogenous markers of proliferation, such as proliferating cell nuclear antigen (PCNA) and Ki-67, BrdUrd is exogenously administered and reaches the tumour via vasculature where it must then diffuse throughout the tissue in order to label S-phase cells. In this study we examine the dose dependence of BrdUrd on the tumour distribution of labelled cells in histological sections. Analysis of the distribution of labelled cells in SiHa tumour xenografts showed that a dose between 400 and 1000 mg/kg was required to label S-phase cells 150 μm from blood vessels, approaching the border of necrosis. Lower doses resulted in only the cells close to blood vessels being labelled. Cells residing 150 μm from blood vessels were found to label at half the level of those situated in close proximity to the tumour vasculature. Results were compared with the penetration of BrdUrd seen in vitro using multilayered cell culture (MCC). Using MCC, an exposure of 100 μM BrdUrd for 1 hour was required for labelling of S-phase cells 150 μm into the tissue, while cells adjacent to the edge of the tissue could be adequately labelled with just 5 μM BrdUrd for 1 hour. The plasma AUC for a 100-mg/kg BrdUrd dose in mice was found to be ~30 μMxh.

3.2 RESULTS

BrdUrd penetration into tumor xenografts

The distribution of BrdUrd labelled S-phase cells around tumour micro-vessels was assessed via simultaneous detection of BrdUrd-DNA adducts and DiOC₇(3) stained blood vessels in tumour cryosections. BrdUrd was injected i.p. at four doses 100, 400, 1000, 2000 mg/kg to multiple mice 2 hours prior to sacrifice; single mice were dosed at 25 and 50 mg/kg. Images of whole tumour cryosections were captured for both markers and then overlaid. Panels A-D of FIGURE 3-1 show sample areas from images of the 100-2000 mg/kg dosed mice. Results of analyses to determine the distribution of BrdUrd labelling relative to tumour vasculature from images of entire cryosections are shown in panels E-H. At all doses a trend of decreasing BrdUrd staining with distance away from vasculature was seen. As dose increased, an increase was seen in the intensity of staining and the number of cells exhibiting staining. This was interpreted as being due to the higher plasma BrdUrd concentrations and the longer period over which labelling could be expected to occur at the higher doses.
FIGURE 3-1 SiHa tumour cryosections showing S-phase labelled cells (non-thresholded, greyscale) relative to vasculature (thresholded, magenta) as a function of BrdUrd dose. A | 100, B | 400, C | 1000 and D | 2000 mg/kg BrdUrd. Fluorescence images of DiOC₇(3) demarcated vasculature were thresholded and overlaid on subsequent bright-field images of cryosections immunostained to show BrdUrd labelled S-phase cells counterstained with haematoxylin (non-thresholded). Inlays show magnified regions. Scale bars 150 μm. Line graphs show the distribution of BrdUrd labelling (fraction tissue identified as being BrdUrd positive, see section 2.2) as a function of distance to nearest visible blood vessel at the four doses, E | 100, F | 400, G | 1000 and H | 2000 mg/kg BrdUrd. Each line represents data from an individual tumour.
An increase was also seen in the penetration of BrdUrd away from the vasculature with increasing dose. From the distribution plots, the depth at which proliferation appeared to drop to half-maximum was determined as 60 ± 10 μm (n=2), 120 ± 10 μm (n=2), 135 ± 25 μm (n=4) and 110 ± 20 μm (n=4) (mean ± max. deviation) for the 100, 400, 1000 and 2000-mg/kg doses respectively; the 400 and 1000 mg/kg groups each contained a tumour that did not follow the general trend and was excluded from the half-maximum calculations.

The half-maximum values from the highest three doses were within experimental error of each other, however peak staining levels were significantly higher for the 1000 and 2000-mg/kg doses. Single mice dosed with 25 and 50 mg/kg continued the trend towards reduced staining and penetration (data not shown). Results indicated that a dose between 400 and 1000 mg/kg was required for maximal immunohistochemical staining of S-phase cells located far from blood vessels in this tumour model.

**BrdUrd Pharmacokinetics in SCID Mice**

The plasma concentration of BrdUrd in SCID mice was measured at several times after i.p. administration of 100 mg/kg. BrdUrd blood concentration versus time is shown in FIGURE 3-2. The rate of disappearance was found to be exponential over the first 2 hours with a half-life of 19 ± 1 (SE) minutes. The peak level was determined as 58 ± 12 (SE) μM and the area under the curve for the first 2 hours was ~30 μMxh. A well defined absorption phase was not observed from these data.

**FIGURE 3-2 BrdUrd plasma kinetics in SCID mice after intraperitoneal administration of 100 mg/kg i.p..** Plasma levels follow an exponential decay over the first 2 hours. The half-life was determined to be 19 ± 1 (SE) minutes, peak plasma levels were 58 ± 12 (SE) μM and the area under the curve for the first 2 hours was ~30 μMxh. Points show mean ± SE (n= 3-6).
BRDUrd penetration into MCC

The penetration of BRDUrd into MCC was assessed via immunohistochemical detection of BRDUrd-DNA adducts in cultures exposed to BRDUrd at varying concentrations. Exposure to BRDUrd was made from one side of the cultures in order to observe penetration of BRDUrd through the entire width of the culture and enable comparison of BRDUrd staining on either edge, where cells were expected to display similar rates of proliferation. FIGURE 3-3 shows sample areas of MCCs exposed to 1, 5, 25 and 100 μM BRDUrd for 1 hour and an additional MCC was exposed to 25 μM from both sides. The images show that 100 μM BRDUrd is required to label S-phase cells on the far edge of a 150-μm thick culture. Results from analysis of the relation between BRDUrd labelling and position within the cultures, performed on images of the entire cryosections, are also shown. Data show that at 100 μM BRDUrd, labelled cells are uniformly distributed within the culture and match the labelling seen after exposure to 25 μM BRDUrd from both sides.

FIGURE 3-3 Greyscale images of 3-day-old MCC showing the distribution of S-phase labelled cells as a function of BRDUrd concentration. 1 h exposures to 1-100 μM BRDUrd were made from the top-side of the cultures and additional cultures were exposed to 25 μM BRDUrd from both sides. S-phase cells were detected via immunohistochemical staining of BRDUrd-DNA adducts. Cryosections were counterstained with haematoxylin. Scale bars 150 μm. Line graphs show the distribution of BRDUrd labelling for corresponding cultures. Data are expressed as a function of position relative to each edge of the cultures, 0 indicating cells adjacent the top (exposed) edge and 1 indicating the cells adjacent the bottom (far) edge of the cultures. Each line represents data from an individual cryosection.
In order to observe the effect of exposure time on the penetration of BrdUrd at low concentrations, additional cultures were exposed to BrdUrd for 24 hours. In this case, the cultures were exposed to BrdUrd from both sides in order to avoid loss of proliferation on the far edge due to closing off the culture over the extended period. MCCs were exposed to 1 μM BrdUrd for 2 hours, 1 μM BrdUrd for 24 hours and 1 μM BrdUrd for 24 hours followed by 200 μM BrdUrd for 1-2 hours to detect S-phase cells not labelled by the 24-hour exposure at the low concentration. During the 24-hour exposure BrdUrd concentrations were monitored by HPLC and maintained at 1 ± 0.5 μM (BrdUrd fell to ~0.4 μM after 12 hours at which point it was supplemented to 1.5 μM). Figure 3-4 shows sample areas of the immunostained MCC cryosections. Due to the additional day of growth the MCCs are thicker than those from the 1-hour exposures and a loss of proliferation occurs towards their centres. Results indicate a marked increase in BrdUrd penetration after exposure to 1 μM BrdUrd for 24 hours versus 2 hours. However, labelling in the central region of the MCCs was seen to increase after exposure to 200 μM BrdUrd for 2 hours suggesting inadequate penetration of 1 μM BrdUrd even after 24 hours. Analysis of the distribution of BrdUrd labelling as a function of distance to the nearest edge of the tissue is shown in the lower panel of Figure 3-4. A marked increase in labelling is seen throughout the MCCs after exposure to BrdUrd for 24 hours in comparison to the 2-hour data and an additional increase in labelling at a depth of 150 μm is seen upon subsequent exposure to 200 μM BrdUrd.
FIGURE 3-4 Greyscale images of 4-day-old MCC showing distribution of BrdUrd labelled S-phase cells after extended exposure from both sides under various conditions: 1 μM BrdUrd for 2 hours, 1 μM BrdUrd for 24 hours, 1 μM BrdUrd for 24 hours followed by 200 μM BrdUrd for 1 hour and 1 μM BrdUrd for 24 hours followed by 200 μM BrdUrd for 2 hours. Cryosections were counterstained with haematoxylin. Scale bars 150 μm. Lower panel shows analysis of the distribution of BrdUrd labelling as a function of distance to nearest edge of the cultures. Error bars show standard deviation.
3.3 DISCUSSION

The distribution of BrdUrd labelled S-phase cells following BrdUrd administered by intraperitoneal injection over a range of doses was evaluated using a human tumour xenograft grown in mice. Results indicated that a dose of between 400 and 1000 mg/kg BrdUrd was required to adequately label S-phase cells located distal to vasculature. We compared the results from tumour cryosections with data from multilayered cell culture (MCC) experiments using a new method which circumvented the existence of gradients in proliferation seen in vivo. MCCs exposed to increasing concentrations of BrdUrd for 1 hour showed a trend in tissue distribution versus exposure that was consistent with that seen from the tumour data. MCC results indicated that a BrdUrd exposure of approximately 100 μM for 1 hour was required to label S-phase cells residing 150 μm from the site of exposure. It appeared that at the highest two doses, both in the tumour and MCC studies, the maximal rate of BrdUrd incorporation into DNA was being achieved throughout the tissue for the given proliferation status. At these doses the actual distribution of free BrdUrd was unknown and a gradient may still have existed within the tissue.

A relationship between increasing dose of BrdUrd and increasing labelling has previously been shown (Peschke et al. 1999) and has generally been attributed to increased staining intensity and increased number of cells entering S-phase during the longer exposure to relevant BrdUrd levels. This study shows that there is an additional tissue penetration component, characterized by an increase in the labelling of cells far from blood vessels with increasing BrdUrd dose.

The finding that a dose of BrdUrd between 400 and 1000 mg/kg was required to label S-phase cells furthest from tumour vasculature in cryosections was initially surprising since most flow cytometry studies are performed with doses of 100 mg/kg or lower. Flow cytometry can have a higher signal to noise ratio compared to immunohistochemical studies as is illustrated by data from the literature for SiHa xenografts showing a labelling index of approximately 25% for a 90 mg/kg dose of BrdUrd (Sham and Durand 1999), which is comparable to results shown here for the 1000-mg/kg dose. However, a BrdUrd-unlabeled population of cells containing S-phase levels of DNA is commonly observed in flow cytometric studies (Begg et al. 1985; Wilson et al. 1985; Rew et al. 1992) examining tumour cell proliferation. The presence of the unlabelled cells has generally been interpreted as indicating non-cycling cells with intermediate levels of DNA rather than unlabeled and proliferating cells. BrdUrd labelling of tumour tissue in vitro has been shown in some instances to increase the labelling index detected via flow cytometry (Wilson et al. 1985). Also, a study by Rodriguez using iododeoxyuridine in the HCT-116 xenograft, which exhibits similar vascular density to SiHa xenografts, found that after continuous infusion for 5 days at 100 mg/kg/day, immunohistochemical staining clearly showed incomplete tissue staining while flow cytometric data indicated close to a 90% labelling index (Rodriguez et al. 1994). Human studies involving BrdUrd as a diagnostic tool have typically used much lower doses of the agent, generally 100-250 mg/patient, (approx. 1-3 mg/kg) (Wilson et al. 1985; Tsang...
et al. 2000; Zackrisson et al. 2002) compared with the 400-1000 mg/kg needed in our studies in mice. Based on the findings presented here it seems likely that these low doses may underestimate the fraction and distribution of S-phase cells in solid tumours.

Using MCCs, a 1-hour exposure time to BrdUrd was chosen to match the exposure for typical bolus injections used in mice. Results indicate that BrdUrd concentration must be kept above 100 μM for 1 hour in order to adequately label S-phase cells 150 μm from the site of exposure. The reason for poor penetration at lower concentrations is interpreted as being due to consumption of BrdUrd by the tissue. Extended exposure to 1 μM BrdUrd, FIGURE 3-4, improved its tissue penetration relative to the 1-hour data, but was still unable to produce maximal labelling in the cells furthest removed from the site of exposure. The MCC data showed that BrdUrd penetration, and not solely the reduction in proliferation away from blood vessels, could be involved in determining the labelling pattern seen in the tumour xenografts.

The clinical testing of BrdUrd as a radiation sensitizer in the 1980-90's generally utilized continuous infusion over several days, with doses of ~1000 mg/m²/day (equivalent to approx. 25 mg/kg/day). At these dose levels, BrdUrd reached steady state plasma concentration of ~1 μM (Kinsella et al. 1984a). In the MCC system a 24-hour exposure to 1 μM BrdUrd did not produce maximal labelling of proliferating cells distant from the site of exposure, indicating sub-optimal exposure due either to limited tissue penetration or cellular uptake of drug. It is possible that an extended exposure to 1 μM BrdUrd may be insufficient to fully sensitize proliferating cells located distant from vasculature.

MCCs have in the past been used to evaluate drug penetration via measurement of flux through the cultures (Hicks et al. 1998; Kyle and Minchinton 1999; Tunggal et al. 2000; Hicks et al. 2001; Tannock et al. 2002). The data presented here represents a new application of MCCs in the evaluation of drug penetration via immunodetection of drug-cell interactions within histological sections. Evaluating a drug's distribution via its effect is usually confounded by intrinsic changes in cells with depth into tissue (e.g. reduced proliferation, drug sensitivity, hypoxia, necrosis) (Sutherland 1988; Olive and Durand 1994). In this study, this problem was circumvented by temporarily closing off one side of the culture during exposure from the other side and then examining the cell layers adjacent to each surface. The purpose of this approach was to attempt to allow comparison of BrdUrd labelling in two cell populations with similar rates of proliferation. This approach could be used to evaluate the distribution of anticancer agents based on their effect on cells located at different depths in tissue using immunohistochemical assays for proliferation or cell death.
4. ANTHRACYCLINES

4.1 SUMMARY

The failure of many anticancer drugs to control growth of solid cancers may stem in part from inadequate delivery to tumour regions distant from vasculature. While the identification of new anticancer drug targets has led to the development of many new drug candidates, there is currently a lack of methodology for studying the role played by drug penetration in determining their activity. In this chapter the feasibility of using multilayered cell culture (MCC) in conjunction with an effect-based assay to evaluate the ability of drugs to penetrate and distribute within tissue is explored. MCCs were grown to ∼150 µm in thickness and then exposed to anthracyclines with one side temporarily closed off. Penetration was then assessed 1 to 3 days later by looking at the differential effect on the exposed and far sides of the cultures of the drugs via BrdUrd-based detection of S-phase cells. We chose to study the relative distribution of four anthracyclines, doxorubicin (DOX), epirubicin (EPI), daunorubicin (DAU) and the related compound mitoxantrone (MIT).

Initial work was conducted to evaluate the effect of a 1-4 hour closure of the MCCs on proliferation status within the cultures. Following this, a pilot study was carried out with DOX alone to determine appropriate concentrations and waiting periods to produce detectable effects using the assay. Upon optimisation of the assay the tissue distribution of the selected anthracycline analogues was assessed. At clinically relevant exposures, none of the agents was able to affect cells on the far side of the culture at levels approaching that seen on the near (exposed) side. DOX and EPI exhibited ∼10-fold decreases in the drug exposure seen by the cells on the far side relative to those on the near side of the cultures, while for DAU and MIT ∼30-fold and >30-fold decreases were observed respectively. Results from the effect based assay were then compared with direct imaging of drug-derived fluorescence as well as ranking of drugs based on their flux through MCCs. Results from drug-derived fluorescence of DOX, EPI and DAU were found to be consistent with effect-based data. Flux experiments, which measured the relative flux of the four anthracyclines through MCCs, were found to rank the anthracyclines in order of their observed distributions. Additional work was carried out to examine the effect of inhibition of drug sequestration in acidic lysosomes/endosomes. Results showed that while inhibition of sequestration reduced net accumulation of the drug it did not appear to increase tissue penetration.

4.2 RESULTS

PRELIMINARY FINDINGS: FLUX-BASED EVALUATION OF ANTHRACYCLINE DIFFUSION THROUGH MCCs

Prior to conducting BrdUrd-based MCC experiments, the flux through MCCs of the four anthracyclines was measured. This initial work was carried out using MCC grown with SiHa (human squamous carcinoma) cells rather than HCT-116 cells, which were chosen for the subsequent
BrdUrd-based work due to their more consistent growth for both MCC and tumour based work. FIGURE 4-1 summarizes results from a series of experiments in which anthracycline flux through MCC was measured. From the data, doxorubicin, followed by epirubicin, were seen to exhibit a greater rate of flux through tissue than daunorubicin and mitoxantrone. In the graphs the rate of appearance of drug in the receiving reservoir is shown as percent of the driving concentration in the donating reservoir. When the lysosomotropic base NH₄Cl was added to both reservoirs, under which the process of acidic sequestration of the drugs is meant to be inhibited, the flux of all four drugs was seen to increase dramatically. This increase in drug flux suggests that cellular accumulation via lysosomal sequestration could be one factor that limits anthracycline tissue penetration. However this work was carried out at relatively high concentrations, 100 μM drug (discussed below), and work shown later in this chapter at 10 μM drug did not confirm these results. Net metabolites refers to fluorescent peaks that appeared in HPLC chromatograms that were not present at time zero. For interest sake, the area of these peaks were summed and normalised to the peak area of the driving concentration in the same manner of the parent drug.

Separate experiments using inserts without cells found all four drugs reached ~18% of the driving concentration after 180 min (DOX 19.7±1%, EPI 18±1%, DAU 17.5±1%, MIT 18±1%). Drug loss through hydrolysis was determined in separate experiments to be ~5% over the 180 minute period. Concentrations following incubation of 100 μM drug in the growth medium at 37°C for 180 min were DOX 97±5 μM, EPI 94±5 μM, DAU 94±5 μM, MIT 100±5 μM. Loss in the donating reservoirs that could be attributed to flux into the cultures was below the reproducibility of the assay.
Flux assay: measure appearance of drug in receiving reservoir

normal conditions  + 50 mM NH₄Cl

<table>
<thead>
<tr>
<th>parent drug</th>
<th>% donating reservoir conc.</th>
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<tbody>
<tr>
<td>dox</td>
<td>0.75</td>
</tr>
<tr>
<td>epi</td>
<td>0.50</td>
</tr>
<tr>
<td>dau</td>
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<tr>
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<table>
<thead>
<tr>
<th>net metabolites</th>
<th>% donating reservoir conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.15</td>
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<td></td>
<td>0.10</td>
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<td>0.05</td>
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**FIGURE 4-1** Flux data showing the rate of appearance of anthracyclines and their metabolites in the receiving reservoir following diffusion through SiHa MCCs. All experiments were carried out using 100 μM drug in the donating reservoir, with results plotted as percent of the starting concentration. Data shown are averages from several experiments. Points show mean ± SE (n=5-8). (DOX: MCC thickness 180 ± 15 μm, n=7; EPI: 165 ± 15 μm, n=8; DAU: 165 ± 15 μm, n=7; MIT: 175 ± 5 μm).

Images of drug-derived fluorescence in cryosections taken from the cultures used for the flux assay are shown in **FIGURE 4-2**. Results indicate that the rate of flux through the cultures ranks inversely to the amount of fluorescence seen in the cryosections. Having the culture open to stirred reservoirs on both sides will in theory reduce build-up within the culture because the far side should follow the concentration seen in the receiving reservoir. For example at the end of the doxorubicin flux assay the receiving reservoir is only at ~0.25% of the 100-μM driving concentration (0.25 μM).

Under conditions of 50 mM NH₄Cl, anthracycline accumulation is seen to be reduced, which is consistent with the increased flux measured through the tissue shown in **FIGURE 4-1** and the overall expectation of reduced sequestration of the drugs. However, the cultures also appear to be less intact at the end of the experiment as seen from examination of the haematoxylin images shown in **FIGURE 4-2**. Hence, a compromised barrier to diffusion may be responsible for the increased flux, this second explanation is consistent with data conducted at 10 μM drug presented later in this chapter (see **FIGURE 4-9**). The far edge of the daunorubicin culture under NH₄Cl does not appear to follow the receiving reservoir concentration, which reaches only ~0.2 μM at the end of the experiment versus driving concentration on the other side of 100 μM.
FIGURE 4-2 Anthracycline fluorescence in 10 μm cryosections taken from SiHa MCCs exposed to 100 μM drug for 3 hours under conditions used for flux experiments. Colour images on left show haematoxylin staining of the tissue carried out after fluorescence imaging. Traces on the far right show fluorescence intensity as a function of position within each MCC.

EFFECT BASED EVALUATION OF ANTHRACYCLINE PENETRATION IN MCCs

The effect of the anthracyclines on S-phase labelling on either edge of MCCs was initially examined over a range of concentrations (0 to 100 μM), drug exposures (1-3 hours), incubation periods following exposures (1-3 days) and growth conditions before a limited range of each parameter was selected for the study. Drug exposures were made to one side of the MCCs using the apparatus shown in FIGURE 2-1B; the two sides of the MCCs were defined as the near side (exposed) and far side (closed-off). Exposure from one side enabled a comparison of drug effect on cells that displayed similar rates of proliferation that were either directly exposed to drug in medium or separated from the drug by the thickness of the culture itself. Following drug expo-
sures, cultures were returned to their growth vessels and incubated in fresh medium to allow manifestation of drug effect within the tissue. Results from preliminary experiments to show proof of principle for the assay are shown in FIGURE 4-3.

![FIGURE 4-3 Proof of principle of BrdUrd-based drug penetration assay. HCT-116 MCC grown to ~175 μm thick were exposed to increasing concentrations of doxorubicin for 1-hour and then incubated for 1-day prior to detecting S-phase cell distribution within the cultures. Results of analysis, right-hand panels, show that proliferation is halted on the exposed side of the cultures with 5 μM doxorubicin but only at 100 μM drug were cells on the far side of the cultures affected.

Initial experiments found that a cell cycle block occurred within 24h of drug exposure. Cultures left for longer periods exhibited continued growth from the far side of the cultures, which complicated the interpretation of data. Based on these findings the 24-hour time point was chosen as the period of incubation following exposure. Growth conditions were modified from the initial conditions used for experiments shown in FIGURE 4-3 to produce cultures which exhibited a higher level of proliferation towards the centre of the cultures and more symmetrical growth, allowing for more reliable analysis of proliferation. This was achieved by modifying the growth apparatus to allow higher and more uniform stirring and by switching oxygen levels of the atmosphere within the vessels from 5% to 20%. A target thickness of 150 μm, similar to the typical maximum thickness of tumour cords observed in vivo, was chosen for MCC thickness at time of
drug exposure. The concentration range of the four anthracyclines was narrowed to 0.3-10 \( \mu \text{M} \), which was found to span the range of drug effect as was detectable via the BrdUrd-based end point. S-phase cell labelling conditions that produced a strong signal were chosen based on findings of the previous chapter (4 hour exposure at 100 \( \mu \text{M} \) BrdUrd).

A requirement of using drug effect within closed-off MCCs as an assay for evaluation of drug penetration is that cellular proliferation must be sustained on the far side of the cultures during the period of drug exposure. FIGURE 4-4 shows that proliferation is sustained after up to a 4 hour period of closure of the cultures under the conditions chosen for the drug penetration studies. Duration of drug exposure was conservatively set to one hour to allow additional time for equilibration and wash-out procedures. No reduction in proliferation was observed 24 hours following a 2-h period of closure (data not shown).

**FIGURE 4-4** Comparison of BrdUrd labelling on far-side relative near side of MCCs following 45 min and 4 h closures. Control cultures, not closed-off, were exposed to 200 \( \mu \text{M} \) BrdUrd for 1 h from both sides. Closed-off cultures were equilibrated for 45 min or 4 hours and then exposed to 200 \( \mu \text{M} \) BrdUrd from one-side for 1 hour. Data shows mean ± standard deviation (n=6).

Typical results based on the finalised parameters, where MCCs were exposed for 1 hour and then incubated for 1 day to allow time for manifestation of drug effect are shown for EPI in **FIGURE 4-5**. Data show a gradual increase in depth of the effect of EPI when concentration is increased from 0.3 to 10 \( \mu \text{M} \). Only at the 10-\( \mu \text{M} \) exposure is EPI found to exert an equal effect to either edge of the cultures. Cells in the central region of the MCCs show less inhibition of labelling. The MCC exposed to 10 \( \mu \text{M} \) EPI is approximately the same thickness it was at the time of treatment while the other cultures, exposed to lower concentrations, are seen to have grown increasingly thicker during the day following treatment.
FIGURE 4-5. Distribution of S-phase cells in MCCs 1 day after a 1-h exposure to increasing concentrations of EPI (0.3 to 10 μM) from one side. One day following the exposure, cultures were exposed to 100 μM BrdUrd for 4 hours from both sides to label S-phase cells. Cryosections counter stained with haematoxylin. Scale bars, 150 μm, show approximate thickness of culture at time of drug exposure.

FIGURE 4-6 shows a summary graph comparing the effect of the four anthracyclines examined on the near and the far side of MCCs 1 day following a 1-h drug exposure. BrdUrd labelling in the first 30 μm of tissue on either side of the MCCs is expressed as the fraction of labelling seen on the respective sides of untreated MCCs from each experiment. For comparison, shaded regions on each panel indicate typical drug exposures achieved in humans for each drug (Rentsch et al. 1998; Pea et al. 2000; Danesi et al. 2002; Swenson et al. 2003). In all cases the relevant human exposures are not high enough to achieve an equal effect on both sides of the cultures. For human exposures, typically 70-80% of the total drug will be blood bound (Chassany et al. 1994; Chassany et al. 1996) while for the MCC work, carried out with 10% FBS, serum binding is estimated at 20-30% of total drug (Chassany et al. 1994; Chassany et al. 1996). Hence, if data were plotted versus free drug, the estimated human exposures would likely be ~3-fold lower relative to the MCC data shown in FIGURE 4-6 (assuming serum bound drug does not contribute to significantly to the tissue penetration of the drugs). For DOX, EPI and DAU, data from both near and far sides
exhibit a general trend of decreasing labelling with increasing drug concentration. However, the MIT data show an increase in labelling on the near side with increasing drug concentration. Since MIT already exhibits a large effect at the lowest concentration, this is likely due either to non-proliferation related BrdUrd labelling or to cell loss on the near side at the higher concentrations. From the data, estimates of the decrease in drug exposure seen by cells on the far side relative to the near side were ~12-fold for DOX, ~10-fold for EPI, ~30-fold for DAU and greater than 30-fold for MIT. These values were arrived at through determination of the drug concentration which produced an effect on the near side that matched that seen at 10 μM drug on the far side. The average fraction of BrdUrd positive tissue in untreated MCCs was 0.40 ± 0.06 (SD) on near side, versus 0.46 ± 0.04 (SD) on far sides. This difference is thought to be mainly due to differences in edge detection at the tissue to growth medium transition on the near side in comparison with the more well defined tissue to membrane transition on the far side. MCC data shown in FIGURE 4-6 are expressed relative to untreated MCCs from respective experiments rather than the overall averages. Middle regions of the MCCs exhibited a significantly lower average BrdUrd stained fraction, 0.28 ± 0.05 (SD).

FIGURE 4-6. Comparison of the effect of the four anthracyclines on the first 30 μm of tissue on the near side (●) and far side (○) of MCCs 1 day after a 1-h exposure. Control experiments under normal (▼) versus deprived conditions, low oxygen and glucose, (▼) are also shown. Data show BrdUrd immunostaining expressed as the fraction of control levels seen in untreated MCCs from the same experiments. Points show mean ± SD (n = 4 - 6). Shaded boxes indicate typical clinical exposures (the concentration over a 1 h exposure that matched typical plasma AUC). Differences in the effects of the drugs on the far sides of the cultures were found to be statistically indistinguishable, P>0.05, except at the 10-μM x h exposure, P<0.005 (ANOVA), where the effect of EPI was significantly greater than DOX. Bottom panels show the relative thickness of MCCs at the end of the experiment. Data are expressed relative to MCC thickness at time of exposure, 160 ± 20 (SD) μm, Data points show mean ± SD.
Control experiments were carried out using a dual-reservoir apparatus, FIGURE 2-1C, in which both sides of MCCs were exposed to drug but with normal conditions on one side versus low oxygen and glucose on the other (see material and methods section), results also shown in FIGURE 4-6. The low oxygen and glucose levels were chosen to simulate levels seen on the far side of the MCCs during the closed-off experiments. Results were used to estimate what effect an equal drug exposure would have had on the two sides of the MCCs during the closed-off experiments. No significant difference in drug effect between the two sides was observed. These experiments were carried out separately from previous work and performed in different growth medium, see materials and methods, which may explain their slightly lower BrdUrd labelling values as compared with the other data presented in FIGURE 4-6.

The effect of the 1-h drug treatment on MCC thickness 1 day following exposure is summarized in the lower panels of FIGURE 4-6. Data are expressed relative to the average thickness of control MCCs at the time of exposure, 160 ± 20 μm (SD). Untreated cultures grown for 1 day from time of treatment reached 225 ± 30 μm (SD) in thickness. The thickness of drug treated cultures was found to lie within this range with MCCs exposed at the higher drug concentrations exhibiting the least amount of growth. Based on the relatively low amount of proliferation observed at the highest exposures, cell loss on the exposed side of cultures is likely no more than a few layers. In separate experiments, MCCs exposed to 100 μM DOX were only 1-2 cell layers thinner than MCCs exposed to 10 μM DOX, indicating only a modest increase in net cell loss.

MCCs cultured for 3 days following clinically relevant exposures showed a similar distribution of S-phase cells as seen after 1 day, FIGURE 4-7. (A) 3 μM x h DOX, (B) 3 μM x h EPI, (C) 1 μM x h DAU and (D) 1 μM x h MIT. Cultures continued to increase in thickness and in all cases there was an increase in proliferation towards the far sides indicating that penetration limited each drug's effectiveness.

FIGURE 4-7. Distribution of S-phase cells in MCCs 3 days after a 1-h exposure from one side to the four anthracyclines at their peak human plasma levels; 3 μM DOX, 3 μM EPI, 1 μM DAU and 1 μM MIT. Three days after drug exposure, cultures were exposed to 100 μM BrdUrd for 4 hours from both sides. Cryosections were counter stained with haematoxylin. Scale bars, 150 μm, show approximate thickness of the cultures at the time of drug exposure.
FLUORESCENCE BASED EVALUATION OF ANTHRACYCLINE DISTRIBUTION IN MCCs

The profile of anthracycline-derived fluorescence within cultures was examined using the closed-off MCC apparatus immediately after the end of a 1-h drug exposure at 10 μM and 1 day following incubation in fresh medium. Only DOX, EPI and DAU, which all possess the same fluorophore, could be examined. Emission intensities of the three drugs in aqueous solution were within 10% of each other, however tissue fluorescence will be derived from a combination of emissions from free and partially quenched bound drug. Figure 4-8 shows average tissue fluorescence as a function of distance into the cultures from the near sides. DAU shows a much steeper gradient than DOX or EPI consistent with the effect-based data. Of the three drugs, DOX derived fluorescence accumulates the least over the 1-h exposure with EPI and DAU reaching ~1.5 and ~7 times higher levels respectively on the near sides, panels A-C. While a higher tissue accumulation of DAU was expected due to its higher rate of entry into cells a direct comparison of absolute fluorescence intensity is problematic due to changes to quantum efficiencies of drug fluorescence upon binding. Interestingly, the drugs show similar cellular accumulation from the middle regions towards the far sides of each culture, see panels D-F, which show the data on the same scale. Position within MCCs is presented as a fraction between 0 and 1 to allow comparison between MCCs taken immediately after drug exposure, which measured 140 ± 15 μm in thickness, and MCCs incubated for the additional day, which were 185 ± 20 μm in thick.

**FIGURE 4-8. Anthracycline fluorescence in MCCs following a 1-h exposure at 10 μM drug from one side.** Red lines show data for MCCs frozen immediately after exposure and black lines show data for MCCs that were incubated for an additional day in drug free medium. Panels are scaled differently and for reference the dotted horizontal lines in each panel show the averaged peak DOX levels. Panels on the right-hand side show data on a magnified scale. Position within the cultures is scaled between 0 and 1, with 0 being the near side and 1 the far side of the MCCs. MCCs frozen immediately following exposure measured 140 ± 15 (SD) μm in thickness while MCCs incubated for an additional day were 185 ± 20 (SD) μm in thickness.
A comparison of effect-based evaluation of drug distribution versus drug-derived fluorescence is shown in TABLE 4-1. Results from the two methods indicated that the effect-based data predictions are consistent with drug distribution as seen from fluorescence data. Effect based results were estimated from data in FIGURE 4-6. The decrease in fluorescence on the far side relative to the near side was calculated by averaging fluorescence over the first 30 μm of tissue on each side of the MCCs shown in FIGURE 4-8.

<table>
<thead>
<tr>
<th>Ratio of near to far side drug exposure</th>
<th>Effect-Based assay</th>
<th>Drug fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOX</td>
<td>~12</td>
<td>11 ± 5 (SD)</td>
</tr>
<tr>
<td>EPI</td>
<td>~10</td>
<td>12 ± 5 (SD)</td>
</tr>
<tr>
<td>DAU</td>
<td>~30</td>
<td>50 ± 5 (SD)</td>
</tr>
<tr>
<td>MIT</td>
<td>&gt;30</td>
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TABLE 4-1. Drug exposure to the near side relative to the far side of MCCs as estimated from the effect-based assay or drug derived fluorescence. Effect-based results are estimated from data shown in FIGURE 4-6, by determining the drug concentration in the medium that produced an effect on the near side that matched the effect seen on the far side at 10 μM drug, see text. The ratio of fluorescence on each side is calculated from data shown in FIGURE 4-8 using the MCCs frozen immediately following exposure to 10 μM drug over 1 h.

**INHIBITION OF ACIDIC SEQUESTRATION**

We decided to re-examine the effect in inhibition of acidic sequestration that was seen in the preliminary data of FIGURE 4-1 and FIGURE 4-2, in which ammonium chloride was shown to increase drug flux through cultures and reduce drug-derived tissue fluorescence but also possibly compromise the integrity of the MCCs themselves. The earlier work was performed using the flow-through flux apparatus, which did not allow for drug to build-up on the far side of the cultures. In addition to switching to the closed-off apparatus, the high drug concentration and long exposure period, 3-hours at 100 μM, which were thought to be responsible for histological changes in the cultures seen after daunorubicin were also reduced.

Results for drug-derived fluorescence seen in the cultures immediately following 1 hour drug exposures with and without ammonium chloride are shown in FIGURE 4-9. Surprisingly, while peak tissue fluorescence is again seen to be reduced, tissue accumulation on the far sides is not seen to be increased, suggesting that inhibition of acidic sequestration of the drugs reduces accumulation but does not limit drug penetration through the tissue. It does however, improve drug distribution but achieves it mostly through the reduction in accumulation on the exposed side of the tissue rather than improved penetration to the far side.
Chapter 4 - Anthracyclines

**Effect of Ammonium Chloride in HCT-116 MCCs**

1-h exposure, 10 μM drug

| 1-h doxorubicin | 1-h epirubicin | 1-h daunorubicin |

**FIGURE 4-9** Effect of inhibition of acid sequestration via ammonium chloride on the tissue distribution of drug derived fluorescence in HCT-116 MCCs. Cultures were frozen immediately following a 1-hour exposure to 10 μM doxorubicin, epirubicin or daunorubicin with or without 50 mM ammonium chloride. Results show fluorescence profiles observed from 10 μm cryosections.

The effect in inhibition of acidic sequestration on proliferation following exposure to 1 μM DAU is shown in **FIGURE 4-10**. Results show no improvement in the ability of daunorubicin to exert an effect on the far side of the MCC following inhibition of acidic sequestration. These results are consistent with published data in which inhibition of acidic sequestration by omeprazole was found to reduce cellular accumulation of doxorubicin by up to 3 fold but only produce a marginal increase in flux out of cultures (Lee and Tannock 2006).

**FIGURE 4-10** Effect of inhibition of acidic sequestration on BrdUrd labelling in HCT-116 MCC after exposure to 1 μM daunorubicin for 1-hour from one side. 150 μm thick MCCs were exposed to 1 μM daunorubicin ± 50 mM NH₄Cl for 1 hour using the closed-off assay. Results show BrdUrd incorporation 1-day after drug exposure. Data points show mean ± SD (n=3).

### 4.3 DISCUSSION

In this study MCCs were used in a novel configuration to isolate the role of drug penetration from other factors that determine drug efficacy with depth into tissue. By temporarily closing off
one side of the MCCs during drug exposure a comparison could be made between a drug’s effect on proliferating cells that were directly exposed to drug and those that lay on the far side of the MCCs. This technique differs from multicellular spheroid based cell survival experiments which predict overall drug toxicity with depth into tissue and combine changes in intrinsic sensitivity with penetration limited drug exposure (Durand 1990; Olive and Durand 1994). Results shown here found that limited tissue penetration of the drugs resulted in an estimated 10-30 fold difference in drug exposure between cells on the near versus far side of the cultures. None of the drugs were able to exert an equal effect on both sides of the cultures after a 1-h exposure matching their typical plasma AUC seen in humans, indicating penetration may limit their effectiveness. Surprisingly, in both the effect based assay and direct fluorescence assay, the drugs performed similarly on the far sides of the MCCs regardless of their differing lipophilicity and intrinsic potency and it was only on the near sides that their effects could be differentiated. This suggested that the increase in penetrative potential of the more lipophilic drugs was being countered by increased binding and sequestration within cells.

Due to the sustained growth of the cultures after treatment, especially on the far side, it was not feasible to perform an in-situ cell survival assay by leaving the cultures for longer periods. However, the distribution of BrdUrd labelling after 3 days was similar to that seen 1 day after drug exposure and results were generally consistent with published work with V79 multicellular spheroids, in which cell survival assays were performed. Following DOX exposure of ~3.5 $\mu$M x h, spheroids that were incubated in drug free medium for an additional 24 hours prior to disaggregation exhibited a surviving fraction of ~0.2 on the exposed outer layers versus ~0.6, 130 $\mu$m into the tissue (Durand 1990). In the spheroid study, reduced intrinsic sensitivity of cells in the central area of the spheroids and time of disaggregation were shown to play key roles in determining drug toxicity. In comparison with spheroids, MCCs pose a greater barrier to penetration due to their planar rather than spherical geometry.

A main concern of closing off the MCCs during drug exposure was that deprivation of oxygen and glucose and build-up of lactate on the far (closed-off) side could potentially lead to a change in the intrinsic sensitivity of the cells to the drugs. In this study, we simulated the effect of oxygen and nutrient deprivation on the far side of the cultures in control experiments where equal drug exposure was made to both sides of MCCs but with one side under normal oxygen and glucose and the other under low oxygen and glucose. Comparison of drug effect on the two sides was found to be within experimental error for all four drugs.

Results from the flux-based assay and also from direct fluorescence-based detection of the distribution of DOX, EPI and DAU in MCCs immediately after exposure were consistent with results from the effect-based assay. The tissue distribution, as determined from the effect-based assay, was found to rank in order of the rate of flux out of the drugs out from cultures. Results were also consistent with tissue distribution as observed via direct visualization of the drugs. In
this case, the effect of fluorescent metabolites of parent drugs was expected to play only a minor role on overall tissue fluorescence because of the short period of exposure. Fluorescence in the near edge of the MCCs matched the expected ranking of drug uptake rates from reported cell suspension data (determined mostly by lipophilicity). As previously discussed, drug fluorescence seen on the far sides of the cultures was not significantly different for the three drugs. Following 1 day of wash out, drug fluorescence was much reduced on the exposed edge of the cultures but still present towards the central region of the cultures.

In this study it was found that despite their differing lipophilicity and rate of entry into cells, none of the drugs performed better either in penetration to or effect on the far sides of the cultures. Results indicated that the increase in diffusion potential of the more lipophilic drugs, was tempered by the competing effects of more rapid DNA intercalation and other forms of sequestration once in the cells, as indicated by the higher level of drug-derived fluorescence and degree of drug effect on the near sides. Despite the similarities in drug effect and fluorescence seen on the far sides of the cultures, DOX and EPI did exhibit a more uniform distribution due to their lower of accumulation on the exposed edges. In general, a drug that does not distribute uniformly will require an increased plasma exposure in order to exert an effect far into tissue which will erode its tumour to normal tissue specificity. In the future, this assay could be used to identify analogues that balanced these competing effects and exhibit improved penetrative characteristics.
5. Taxanes

5.1 Summary

Taxanes represent a class of drugs that are currently undergoing a new round of development but with little known of their ability to penetrate and distribute relative to blood vessels within solid tumours. Based on the shared mechanism of action but differing physicochemical properties, we chose to compare the tissue penetration of the taxanes paclitaxel and docetaxel. Using the MCC effect-based assay, initial work was conducted to determine concentration range and waiting period required to produce an effect using the BrdUrd end-point. Experiments were then carried out to determine the dose response curves of paclitaxel and docetaxel on near and far MCC edges. Analysis of MCC effect-based data indicated that there was a 5-fold reduction for paclitaxel and 10-fold reduction for docetaxel in exposure to cells on the far sides of the cultures, indicating that both drugs exhibited poor tissue distribution. Effect-based results were compared with direct visualization of \(^3\)H-paclitaxel and \(^3\)H-docetaxel in MCC as well as tumour xenografts. In xenografts, taxanes were mapped relative to blood vessels to obtain drug profiles as a function of distance from vasculature. For MCC, cultures were exposed to stirred drug reservoirs and taxanes measured as a function of depth into tissue. Both taxanes exhibited limited penetration, with little drug reaching further than 100 \(\mu\)m into tissue. Of the two, paclitaxel exhibited up to 2-fold better penetration than docetaxel. In xenografts, proliferation status following treatment was also assessed in relation to vasculature. Up to a 75\% reduction in S-phase cells was observed in cells nearest vessels but only 50\% reduction was observed in the tissue 150 \(\mu\)m away, though in this case a reduced drug exposure to the cells far from vasculature would be expected to be only one of several factors influencing response to treatment.

5.2 Results

Effect-based evaluation of taxane tissue distribution in MCCs.

FIGURE 5-1 shows the effect of paclitaxel and docetaxel on proliferating cells in MCCs 2 days after a 1-hour exposure using the penetration apparatus shown in FIGURE 2-1B. MCC cryosections were immunostained for incorporated BrdUrd to show S-phase cells. Scale bars, 150 \(\mu\)m, indicate the approximate thickness of the cultures at time of exposure. While the untreated cultures are seen to double in thickness over the two days from time of exposure, treated cultures show an increasing reduction in growth as a function of drug concentration. To estimate drug distribution, proliferation was compared on either edge of the cultures, where in untreated cultures similar proliferating fractions were observed. For both drugs at the intermediate concentration of 0.3 \(\mu\)M, the number of S-phase cells is clearly reduced on the exposed side of the culture while only at the 3-\(\mu\)M drug exposure is the S-phase fraction on the far side of the cultures seen to be reduced.
FIGURE 5-1 S-phase cells in HCT-116 MCCs 2 days after a 1-hour drug exposure from one side. Images show cryosections that were immunostained for incorporated BrdUrd. Arrows show direction of drug exposure. Scale bars, 150 μm, show approximate thickness of cultures at time of drug exposure.
FIGURE 5-2 Analysis of drug effect on the rapidly proliferating cells of the first 30 μm of tissue on the near (●) and far (○) side of MCCs for paclitaxel and docetaxel. Data show BrdUrd immunostaining expressed as the fraction of levels seen in untreated MCCs. Results from control experiments under conditions of normal (▲) versus energy deprived conditions (low oxygen/glucose and high lactate) (△) are also shown. Bottom panels show MCC thickness 2 days after treatment plotted relative to thickness at time of treatment. Points show mean ± SE (n=4-6). "*" indicates data points where the drug effect on the far side was significantly less than on the near side, p<0.005 (ANOVA). The effect of paclitaxel versus docetaxel on culture thickness was comparable for all concentrations, p>0.05 (ANOVA).

FIGURE 5-2 shows results from analysis of the effect-based taxane penetration experiments. Top panels show the BrdUrd fraction in the first 30 μm of tissue on the exposed versus far sides of the cultures for paclitaxel and docetaxel as a function of drug exposure. For both drugs a significantly greater reduction in proliferation is seen on the exposed side of the cultures. Comparison of the drug concentration required to produce a reduction in proliferation on the exposed side of the cultures as produced on the far side of cultures by the 3-μM exposure indicates an approximate 5-fold decrease in drug exposure on the far side of the culture for paclitaxel and a ~10-fold decrease for docetaxel. Bottom panels show that despite paclitaxel exerting a more uniform effect within the cultures both drugs were similar in their ability to control culture growth, as reflected by their thickness. Control experiments were carried out to determine the effect that closing-off the culture during drug exposure had on response of cells on the far side. Conditions encoun-
tered on the far side of the cultures during drug exposure were simulated using the apparatus shown in FIGURE 2-1C, in which both sides of the cultures were exposed to 0.1 μM drug but under normal conditions on the near side versus oxygen/glucose deprived conditions on the far side. Results, also shown in the top panels of FIGURE 5-2, indicate that the temporary deprivation of oxygen and nutrients on the far side of cultures during drug exposure did not affect response to the drugs.

$^{3}$H-TAXANE DISTRIBUTION IN MCCs.

To assess results from the MCC effect-based penetration assay, direct visualization of radiolabelled taxanes was carried out in MCC and tumour xenografts. Experiments were carried out using a film-based dry-autoradiographic technique described in section 2-5. Typical MCC-based results produced from the experiments are shown in FIGURE 5-3. To allow analysis, images of radiolabelled drug distribution from entire MCC cryosections were superimposed on images of the actual tissue sections, where MCC edges were clearly demarcated (see panels A-C). This permitted the determination of radioactivity as a function of depth into tissue.

![1-h $^{3}$H-paclitaxel](image)

**FIGURE 5-3** Image of $^{3}$H-paclitaxel distribution in HCT-116 MCC following a 1-hour exposure to 3 μM drug from one side. Top two panels show images of A | the tritium sensitive film and B | the unstained tissue cryosection. C | MCC edges, as detected from the tissue section were overlaid onto the film image to allow computer analysis of grain density as a function of distance into the MCC. Scratches on the film layer were deleted from the image prior to analysis.
FIGURE 5-4 shows the distribution of tritium labelled paclitaxel and docetaxel in MCCs following 1 & 2 hour exposures at 0.3 & 3 μM. In all cases, data show moderately lower accumulation and better penetration for paclitaxel relative to docetaxel. Increasing the drug concentration 10-fold from 0.3 μM to 3 μM resulted in only a ~3-fold increase in peak tissue levels indicating partial saturation of binding. At the higher concentration both drugs show improved tissue penetration, indicating that accumulation of the drugs by tissue played a role in limiting their penetration. The longer 2 h exposure time was seen to improve tissue distributions but peak tissue levels were only seen to increase for 0.3 μM docetaxel. Peak radioactivity levels observed at a depth of ~20 μm into tissue was likely due to averaging over gaps in the tissue on the edge of the cultures and alignment of autoradiography images which blurred slightly on the edges.

FIGURE 5-4 ³H-taxanes in MCC

³H-taxane distributions in HCT-116 MCCs following 0.3 & 3 μM exposures for 1 & 2 hours to paclitaxel (○) and docetaxel (●). MCCs, discs of tissue grown to 150 μm, were exposed to drug on one side with the other side closed-off to allow drug to build-up. Results are shown as a function of depth into tissue relative to the exposed side of the cultures. Points show mean ± SE (n=9).

³H-TAXANE DISTRIBUTION IN TUMOUR XENOGRAPHTS.

Typical data from the tumour-based ³H-taxane experiments are shown in FIGURE 5-5. Experiments were carried out using a film-based dry-autoradiographic technique described in section 2.5, which produced an image of radiolabelled drug on tritium sensitive film and a separate image of immunohistochemical detected tumour vasculature. The critical alignment of radioactive drug distribution and tissue vasculature was only possible because images of entire cryosections were captured, allowing tumour edges to be lined up on all sides.
**3H-paclitaxel (Tween-80), 2-h post injection, 10 mg/kg i.v.**

**FIGURE 5-5** Composite image of 3H-paclitaxel distribution (black) and CD31 positive vasculature (red) in an HCT-116 tumour xenograft 2 hours post i.v. injection of 10 mg/kg 3H-paclitaxel. Images were obtained from tritium sensitive film clamped against 10 µm tumour cryosection for 80 days, followed by immunohistochemical staining of CD31 positive tumour vasculature on the same section. Subsequent computer analysis of taxane distribution relative to vasculature was carried out with non-tumour areas and scratches in x-ray film cropped out.
Chapter 5 - Taxanes

**Figure 5-6** shows results from analysis of tritium labelled paclitaxel and docetaxel as a function of distance to nearest blood vessel. Data show that drug accumulation is limited to the first ~100 μm of tissue, with little drug reaching 100-150 μm away from vessels for both drugs. The vehicle Cremophor EL, which was expected to reduce plasma levels of free paclitaxel and increase its duration of exposure, reduced tissue drug levels at the 2-hour time point relative to Tween 80 by more than 2-fold, **Figure 5-6**. However, at the 8-hour time point paclitaxel in vehicle Tween 80 is seen to have undergone substantial washout while paclitaxel in the Cremophor EL vehicle shows increased tissue accumulation. For both vehicles the profile for paclitaxel at the 8-hour time point was found to be similar. Docetaxel in comparison is seen to fall off more sharply though in contrast to paclitaxel in Tween 80 docetaxel, also in Tween 80, showed increased accumulation at 8 hours.

**Figure 5-6** ³H-taxane distribution relative to nearest blood vessel in HCT-116 tumour xenografts. Data show distributions observed 2 and 8 hours following tail vein injection; 10 mg/kg paclitaxel with vehicle A | Tween 80 or B | Cremophor EL and 5 mg/kg docetaxel with vehicle C | Tween 80. Points show mean ± SE (n=9).

**Comparison of xenograft and MCC-based ³H-taxane data.**

**Figure 5-7** shows a comparison of tissue accumulation and depth of penetration of the taxanes in MCC and tumour xenografts. Peak tissue levels in the tumour xenografts were ~3-fold lower for paclitaxel and ~10-fold lower for docetaxel than the 2-hour, 0.3 μM MCC results, suggesting significantly lower free drug levels in the plasma (see Discussion). In MCCs, peak levels do not increase when going from a 1 to 2-hour exposure (p>0.05 ANOVA except for the 0.3-μM docetaxel). In MCCs, the depth of penetration was seen to increase with exposure. Due to the increased tissue distribution after 2 hours, total tissue radioactivity in MCCs increased moderately when going from 1 to 2 hours (significant differences, p<0.005 ANOVA, for 1 versus 2 hour exposures in all cases except for 0.3 μM paclitaxel). The depth of penetration was also seen to increase with reservoir drug concentrations. Given this, the xenograft-based results exhibited surprisingly large penetration half-depths in comparison with the MCC-based data given their relatively low peak tissue levels and hence free-drug concentrations.
FIGURE 5-7 Summary of $^3$H-taxane peak tissue levels, total radioactivity and depth into tissue where radioactivity falls to half-maximum: paclitaxel (■), docetaxel (■) and for tumour data paclitaxel in Cremophor EL (■). Data show mean ± SE (n=9).
EFFECT OF TAXANES ON S-PHASE CELL DISTRIBUTION IN TUMOUR XENOGRAPHS.

S-phase cell distributions were mapped in tumour xenografts following drug treatment. In these experiments S-phase cells were mapped relative to tumour vasculature as a function taxane dose and time following treatment. Typical data are shown in FIGURE 5-8. The protocol for staining for the multiple markers, BrdUrd, CD31 and pimonidazole is described in section 2-5 and FIGURE 2-6. These experiments were conducted prior to the isotope studies and drugs were injected i.p. rather than i.v.. While images of entire tumour sections were captured, only small areas are shown in the figure. The areas show typical response to the drugs at 24-hours, both of which showed a reduction in proliferation near vessels the degree of which varied from vessel to vessel.

**S-phase cells 72 h following taxane treatment**

![Staining Key]

FIGURE 5-8 S-phase cells seen in HCT-116 tumour xenografts 72 hours following i.p. administration of paclitaxel and docetaxel at approximately their maximum tolerated doses. A general reduction in S-phase cells near vasculature was seen relative to controls, though the degree of response varied greatly between vessels.

Results from analysis of entire tumour cryosections are summarized in FIGURE 5-9. The time course of paclitaxel effect on S-phase cells 1, 3 and 6 days following treatment is shown in panel A, with raw data in the top panel and data normalized to the untreated controls in the bottom panel. Data show there is a decrease in S-phase cells over the first three days following treatment. From the normalized data, paclitaxel is seen to produce up to a 75% reduction in proliferation near vessels but only a 50% reduction in cells 150 μm away. Further work looking at drug effect at the 3-day time point as a function of dose is shown in panel B for paclitaxel and panel C for docetaxel. Drug doses were chosen to span the range typically administered to mice and produce equitoxic effects for the two drugs (Nicoletti et al. 1994). From normalized data, bottom panels of B and C, a preferential effect is seen on the cells in the first ~50 μm of tissue away from vessels, where proliferation and drug exposures are highest.
Chapter 5 - Taxanes

5.3 DISCUSSION

Data from both radiolabelled drug imaging and drug effect on cell proliferation suggested poor distribution of the taxanes, with most drug limited to the first 100 μm of tissue. Paclitaxel penetrated further into tissue than docetaxel, consistent with previous reports of increased cellular accumulation of docetaxel (Lavelle et al. 1995). The greatest difference between the two drugs was seen in the MCC effect-based assay where paclitaxel showed a 2-fold increase in penetration relative to docetaxel. MCC based results for both drugs showed a partial saturation of tissue binding and improved penetration at higher concentrations.

The formulation vehicle Cremophor EL had the expected effect of reducing initial peak levels of paclitaxel in tumours and increasing its duration of exposure. It was interesting that at the 8-hour time point both paclitaxel formulations produced similar drug profiles, however, the tissue AUC for 0-8 hours would still be higher for the Tween 80 data. Later time points might eventually result in equal or greater accumulation for the Cremophor EL data.

Drug exposures for the MCC-based radiolabelled studies were chosen to bracket the expected mouse plasma levels. However, peak tumour levels for paclitaxel and docetaxel were respectively ~3 and 10-fold lower than those seen in MCC following the 0.3-μM exposure. This was

FIGURE 5-9 Analysis of S-phase cell distribution HCT-116 tumour xenografts following i.p. administration of paclitaxel and docetaxel. A | Time course for action of 30 mg/kg paclitaxel 1, 3 and 6 days following treatment and B-C | dose response 3 days following treatment for paclitaxel, 10, 15 & 30 mg/kg, and docetaxel, 5, 10 & 20 mg/kg. Results are shown for raw data (top panels) and data normalized to controls (bottom panels), where a value of 1 indicates no change relative to the untreated controls. Data was obtained from analysis of whole tumour cryosections containing 50-100 vessels, points show mean ± SE (n=3-5).
despite reported plasma AUCs in mice in the range of 35 and 5 \( \mu \text{M}\)-hours and peak levels of 40 \( \mu \text{M}\) and 10 \( \mu \text{M}\) for paclitaxel and docetaxel respectively (Sparreboom et al. 1996; Sparreboom et al. 1998). The relatively low peak tissue accumulation seen here indicates that blood binding of the drugs plays a significant role in limiting their tissue distribution and that only the free drug is likely to contribute to tissue penetration. MCC work, which was carried out in 5% serum, likely resulted in ~50% unbound drug (Song et al. 1996), compared to 2-10% unbound drug expected in vivo (Sparreboom et al. 1998; Goodman et al. 2006). Based on these findings, future work could be carried out to look at taxane penetration in MCCs at lower drug concentrations, matching the levels detected from the tumour-based work. Under these conditions, the taxanes might exhibit an even greater reduction in penetration or perhaps behave in a similar way to the 0.3-\( \mu \text{M}\) data. It would also be interesting to use the assay to examine the relation between exposure duration and drug concentration on tissue distribution at lower concentrations and longer exposures.

The apparent depth of penetration of the drugs in tumours as compared to MCCs was greater than expected given the marked concentration dependence of tissue penetration seen there. In addition, the radial geometry observed in the HCT-116 tumour type, in which cords of tumour cells surround individual vessels (Kyle et al. 2003), should actually pose a greater barrier to penetration than does the planar disc geometry of MCCs. There are two factors which might explain the increased penetration seen in the tumour data. Firstly, there will exist unobserved vessels that lie just above or below the 10-\( \mu \text{m}\) tissue sections cut from the tumours. The likelihood of occurrence of unobserved vessels will increase with distance from observed ones and hence they may increase the measured penetration half-depth of a drug. Another factor that might increase the apparent penetration of the taxanes in vivo is the contribution of \( ^3\text{H} \)-labeled liver metabolites, which could diffuse more easily than the parent drug.

A unique property of the disc geometry of MCCs is that it presents a barrier to penetration in which there exist rapidly proliferating cells at two positions in the tissue (either edge). This symmetry made it possible to use the effect of the taxanes as an indicator of their distribution, in effect using the cells themselves as the drug measurement end-point. Results from the MCC effect-based assay were interesting in that the differential in drug effect on the two edges was not as great as might be expected from the steep drug gradients observed from the autoradiography-based data. However, the radiolabelled data produced discrete snapshots of drug distribution at two time points during drug build-up, while the effect-based assay integrated exposure over the entire process of build-up and washout. Redistribution of the drugs during washout and differences in cellular retention of the drugs would affect overall response. Hence the effect-based assay probably produces a more realistic evaluation of drug penetration.

The proliferation-based tumour xenograft data showed that both drugs were less effective on the cells furthest from tumour vasculature. However, it was difficult to interpret this data on its own, in terms of in vivo evidence of limited tissue penetration of the drugs because, unlike the MCC-
based BrdUrd work, the cells furthest into the tissue exhibited only a fraction of the proliferation seen in those cells near vessels. Hence, the tissue furthest from vasculature will exhibit reduced sensitivity to the drugs and also experience a different lag time between drug exposure and progression through the cell cycle to G2/M block (Lopes et al. 1993). Additional factors that may influence cellular response to the drugs include indirect host toxicity, which might conceivably reduce proliferation status throughout the tumour.

Based on the results of this study, the effect-based MCC assay could be used as a screening method for selecting taxane analogues for improved ability to distribute within tissue. In the development of new taxanes with improved anti-tumour capabilities, tissue penetration would be one of many parameters that could be modified. However, knowledge of tissue penetration could provide additional criteria for selecting potential analogues to further develop. Because drugs that distribute poorly will require higher driving concentrations from the blood to reach and kill the cells situated furthest from blood vessels, improving drug penetration could reduce systemic toxicity by reducing the plasma levels required to achieve maximum tumour response. In addition to increasing the concentration of drug seen by the cells furthest from blood vessels, improved drug penetration might also increase the window of exposure of these cells to therapeutic levels of drug, which might counter the effects of the lower S-phase fraction observed in this population.
6. SURVEY OF THE PENETRATION AND DISTRIBUTION OF SOME COMMON ANTI-CANCER DRUGS

6.1 SUMMARY

To survey the range of applicability of the MCC effect-based assay, we attempted to assess the tissue distribution of a selection of anticancer drugs from several distinct classes by comparing their effect to either side of MCCs. Drugs examined included representative agents from vinca alkaloids, antimetabolites, antibiotics, platinum agents, epipodophyllotoxins and alkylating agents. Additional data presented at the end of the chapter examines the tissue oxygenation status within MCCs using the hypoxia marker pimonidazole.

6.2 RESULTS

Work presented in this chapter involves the assessment of drug distribution using the MCC effect-based assay. Cultures, 150 μm thick, were grown using HCT-116 cells and penetration experiments were carried out using the apparatus described in FIGURE 2-1B in which cultures were exposed to drug for 1 h from one side. Drug exposure was followed by a 1-h wash out period after which the cultures were then returned to the growth apparatus and incubated in drug free medium for 1-3 days. Drug distribution was then inferred from the differential effect to the cells on either side of the cultures as detected using the BrdUrd based S-phase cell endpoint.

VINCA ALKALOIDS

FIGURE 6-1 shows the effect of vincristine, vinblastine and vinorelbine on proliferation in MCCs 1 day after a 1-hour exposure to one side. MCC cryosections were immunostained for incorporated BrdUrd to show S-phase cells. The cultures showed sustained proliferation in central areas even at the highest exposure, notably for vincristine and vinorelbine indicating differential response of non-cycling cells. Vincristine showed the most distinct difference between effect on the near versus far edge, while vinblastine and vinorelbine showing only marginal differences.

FIGURE 6-2 shows results from analysis of the BrdUrd data from 1 day following exposure to the vinca alkaloids. Top panels show the BrdUrd fraction in the first 30 μm of tissue on the near (exposed) versus far sides of the cultures as a function of drug exposure. Of the three drugs, vincristine is the only one which consistently showed a differential effect on near versus far sides, with results indicating a ~3-fold drug gradient across the culture.
FIGURE 6-1 Vinca alkaloid data: S-phase cells 1 day after exposure to vincristine, vinblastine or vinorelbine for 1 hour to one side. Images show BrdUrd immunostained cryosections from HCT-116 MCCs. Scale bars, 150 μm, show approximate thickness of cultures at time of drug exposure, arrows show direction of drug diffusion.

Vinblastine exhibited a greater effect on the far side than the exposed side at the two highest exposures. One possible explanation for this is that the drug was washed out from the exposed side quickly enough to rescue the cells while the cells on the far side, being closed off during the 1-hour wash out period, received a longer exposure. This explanation is consistent with the reported low retention and fast wash out of vinblastine in comparison with vincristine (Owellen et al. 1977). Another possible explanation for this reversal would be that cell loss on the exposed side at the higher exposures is giving the impression of reduced drug effect. In that case it would still be possible to estimate the drug gradient within the tissue by comparing the concentration at which their first appears to be an effect on the exposed side with the concentration required to produce a similar effect on the far side. In this case an ~3-fold gradient would be estimated. Vinorelbine, which is the most lipophilic (Rahmani-Jourdheuil et al. 1994) of the three showed no evidence for a differential effect between near and far sides. Interestingly the main difference between the drugs appears to be the effect they exert on the exposed sides of the cultures rather than the far side, where they appear quite similar. This suggests that retention/wash out of the drugs on the exposed side might be a more important difference between them than drug
penetration. Control experiments under deprived conditions (see Materials & Methods), carried out to determine what effect closing-off the culture during drug exposure had on response of cells on the far side, showed equal response. Indicating that temporary deprivation of oxygen and nutrients on the far side of cultures during drug exposure did not affect the findings.

**FIGURE 6-2 Vinca alkaloids analysis:** Analysis of drug effect on the first 30 μm of tissue on the near (●) and far (○) side of MCCs 1 day following a 1-hour exposure to vincristine, vinblastine or vinorelbine. Data show BrdUrd immunostaining expressed as the fraction of levels seen in untreated MCCs. Results from control experiments under conditions of normal (▲) versus energy deprived conditions (low oxygen and glucose) (△) are also shown. Bottom panels show MCC thickness 1 day after treatment plotted relative to thickness at time of treatment. Points show mean ± SD (n=3-4).
ANTIMETABOLITES

FIGURE 6-3 shows the effect of the pyrimidine analogue gemcitabine on proliferation in MCCs 1 and 3 days after a 1-hour exposure to one side. MCC cryosections were immunostained for incorporated BrdUrd to show S-phase cells. An unusually distinct transition in proliferation status due to an increase of BrdUrd labelling in the cells adjacent to the ones forced into a block is seen at the 1-day time point. Also, the first layer of cells on each edge behave differently, showing sustained labelling even at the highest exposure, than the adjacent cells 2-3 layers in. It might be that the 1-h drug exposure is short enough that the wash out process is able rescue these cells. The 3-day data are consistent with results seen 1 day after exposure with the exception that the cultures at the lower exposures exhibit increased thickness due to sustained proliferation on the far side.

![Figure 6-3](image)

FIGURE 6-3 Gemcitabine data: S-phase cells 1 & 3 days after exposure to 3-90 μM gemcitabine for 1 hour from one side. Images show BrdUrd immunostained cryosections from HCT-116 MCC. Scale bars, 150 μm, show approximate thickness of cultures at time of drug exposure, arrows show direction of drug diffusion.
FIGURE 6-4 shows results from analysis of the gemcitabine data for 1 day after exposure. Top panels show the BrdUrd fraction in the first 30 μm of tissue on the near (exposed) versus far sides of the cultures as a function of drug exposure. A significantly greater reduction in proliferation is seen on the exposed side of the cultures. Comparison of the drug concentration required to produce a reduction in proliferation on the exposed side of the cultures as was produced on the far side of cultures by the 30-μM exposure indicates a >10-fold decrease in drug exposure on the far side of the culture. Bottom panel shows the effect of gemcitabine on culture thickness. Results show that the 3-μM exposure reduces culture growth to half that seen in untreated cultures, which is consistent with the BrdUrd profile shown in FIGURE 6-3.

![Graph showing BrdUrd-based penetration assay and control assay](image)

**FIGURE 6-4 Gemcitabine analysis:** Analysis of drug effect on the first 30 μm of tissue on the near (●) and far (○) side of MCCs 1 day following a 1-hour exposure to gemcitabine. Data show BrdUrd immunostaining expressed as the fraction of levels seen in untreated MCCs. Results from control experiments under conditions of normal (▲) versus energy deprived conditions (low oxygen and glucose) (△) are also shown. Bottom panels show MCC thickness 1 day after treatment plotted relative to thickness at time of treatment. Points show mean ± SD (n=3-4).
Control experiments under normal versus deprived conditions were carried out to determine the effect of closing-off the culture during drug exposure on response of cells on the far side. Results, also shown in Figure 6-4, showed equal response to the drug indicating that temporary deprivation of oxygen and nutrients on the far side of cultures during drug exposure did not affect the findings. The MCC-based work was seen to be consistent with tumour xenograft-based data from our lab also grown using HCT-116 cells (Huxham et al. 2004).

Further work looking at two other pyrimidine analogues, 5-fluorouracil and cytarabine, required concentrations significantly higher than those used clinically to achieve a detectable effect. Unlike gemcitabine, both these agents are selective for S-phase cells and are commonly administered as continuous infusions over 24 hours (Goodman et al. 2006). At the concentrations used here 5-flurouracil exhibited a uniform effect on both sides and cytarabine showed a slight decrease in effect on the far side, see data Figure 6-5. Due to the unusually high concentrations required to produce an effect after the 1-h exposures, further work was not pursued for these two agents.

**FIGURE 6-5** 5-FU & cytarabine data: S-phase cells 1 day after exposure to 5-FU or cytarabine for 1 hour to one side. Images show BrdUrd immunostained cryosections from HCT-116 MCC. Scale bars, 150 µm, show approximate thickness of cultures at time of drug exposure, arrows show direction of drug diffusion.
FIGURE 6-6 shows the effect of cisplatin on proliferation in MCCs 1 & 3 days after a 1-hour exposure to one side. MCC cryosections were immunostained for incorporated BrdUrd to show S-phase cells. At the 1-day time point, cisplatin exhibits increasing control of MCC thickness with concentration but does not produce a cessation of BrdUrd labelling. Only after 3 days is labelling partially reduced at the higher exposures. No difference in drug effect on near versus far sides was detected.

FIGURE 6-6 Cisplatin data: S-phase cells 1 & 3 days after exposure to 10-300 μM cisplatin for 1 hour from one side. Images show BrdUrd immunostained cryosections from HCT-116 MCC. Scale bars, 150 μm, show approximate thickness of cultures at time of drug exposure, arrows show direction of drug diffusion.

FIGURE 6-7 shows results from analysis of the BrdUrd data from 3 days following cisplatin exposure. Top panels show the BrdUrd fraction in the first 30 μm of tissue on the near/exposed versus far sides of the cultures as a function of drug exposure. No significant decrease in effect on
the far side was observed. However, concentrations required to achieve a detectable reduction in BrdUrd labelling were significantly higher than what is typically encountered during human exposures, suggesting that the 1-hour exposure used here was not appropriate and that a longer exposure would likely have permitted work to be carried out at lower concentrations. Control experiments under deprived conditions were not performed for cisplatin.

**FIGURE 6-7 Cisplatin analysis:** Analysis of drug effect on the first 30 μm of tissue on the near (●) and far (○) side of MCCs 3 days following a 1-hour exposure to cisplatin. Data show BrdUrd immunostaining expressed as the fraction of levels seen in untreated MCCs. Bottom panels show MCC thickness 3 days after treatment plotted relative to thickness at time of treatment. Points show mean ± SD (n=3-4).
BLEOMYCIN

FIGURE 6-8 shows the effect of bleomycin on proliferation in MCCs 1 day after a 1-hour exposure to one side. MCC cryosections were immunostained for incorporated BrdUrd to show S-phase cells. At all but the lowest exposure, bleomycin can be seen to exhibit a greater effect on the near sides of the cultures. However, bleomycin requires oxygen to exert cytotoxicity hence even with a uniform distribution it would be expected to exhibit less effect on the far side of the culture (oxygen profiles in MCCs are shown in FIGURE 6-15).

FIGURE 6-8 Bleomycin data: S-phase cells 1 day after exposure to bleomycin for 1 hour from one side. Images show BrdUrd immunostained cryosections from HCT-116 MCC. Scale bars, 150 μm, arrows show direction of drug diffusion.

FIGURE 6-9 shows results from analysis of the BrdUrd data for 1 day following exposure to bleomycin for normal cultures, 140 μm, or thick cultures, 190 μm. Top panels show the BrdUrd fraction in the first 30 μm of tissue on the near (exposed) versus far sides of the cultures as a function of drug exposure. Initial comparison of the effect on the near versus far sides of the normal cultures suggests a modest differential in drug exposure. However, results from the
control/energy deprived experiments indicate that oxygen deprivacion on the far sides of the cultures may be completely responsible for the differential effect. For the thick cultures the difference appears to be due to a combination of drug gradient and oxygen effect.

**FIGURE 6-9 Bleomycin analysis:** Analysis of drug effect on the first 30 μm of tissue on the near (●) and far (○) side of thin or thick MCCs 1 day following a 1-hour exposure to bleomycin. Data show BrdUrd immunostaining expressed as the fraction of levels seen in untreated MCCs. Results from control experiments under conditions of normal (▲) versus energy deprived conditions (low oxygen and glucose) (△) are also shown. Bottom panels show MCC thickness 1 day after treatment plotted relative to thickness at time of treatment. Points show mean ± SD (n=2-3).
ETOPOSIDE

FIGURE 6-10 shows the effect of etoposide on proliferation in MCCs 1 day after a 1-hour exposure to one side. MCC cryosections were immunostained for incorporated BrdUrd to show S-phase cells. At 3 and 10 μM, etoposide can be seen to exhibit a greater effect on the near sides of the cultures and at the highest exposure, 10 μM, etoposide exhibits a greater effect on the rapidly proliferating cells on the far side than it does in the middle region of the culture which exhibits a lower proliferating fraction.

FIGURE 6-11 shows results from analysis of the BrdUrd data for 1 day following exposure to etoposide. Top panels show the BrdUrd fraction in the first 30 μm of tissue on the near (exposed) versus far sides of the cultures as a function of drug exposure. Initial comparison of the effect on the near versus far sides of the cultures suggest approximately a 5-fold differential in drug diffusion.
exposure. However, results from the control/energy deprived experiments indicate that drug penetration may be only partially responsible for the difference in effect between the two sides. Based on the control studies, the gradient in drug exposure to the two sides is likely limited to ~2-fold. Bottom panel shows the effect of etoposide on culture thickness. Results show that only at 100 μM drug is there a large decrease in culture thickness relative to untreated controls. This is despite significant decreases in proliferation on both edges at lower doses. These results suggest that proliferation in the central areas of the cultures may be responsible for their sustained growth.

**FIGURE 6-11 Etoposide analysis:** Analysis of drug effect on the first 30 μm of tissue on the near (●) and far (●) side of MCCs 1 day following a 1-hour exposure to etoposide. Data show BrdUrd immunostaining expressed as the fraction of levels seen in untreated MCCs. Results from control experiments under conditions of normal (▲) versus energy deprived conditions (low oxygen and glucose and high lactate) (△) are also shown. Bottom panels show MCC thickness 1 day after treatment plotted relative to thickness at time of treatment. Points show mean ± SD (n=2-4).
Mitomycin C

**Figure 6-12** shows the effect of mitomycin C on proliferation in MCCs 1 and 3 days after a 1-hour exposure to one side. MCC cryosections were immunostained for incorporated BrdUrd to show S-phase cells. At the 1-day time point, mitomycin C exhibits partial control of MCC thickness but appears to stimulate BrdUrd labelling, more so in the central region of the cultures. After 3 days BrdUrd labelling is seen to be reduced throughout the cultures.

![Mitomycin C data: S-phase cells 1 & 3 days after exposure to mitomycin C for 1 hour from one side. Images show BrdUrd immunostained cryosections from HCT-116 MCC. Scale bars, 150 μm, show approximate thickness of cultures at time of drug exposure, arrows show direction of drug diffusion.](image)

**Figure 6-13** shows results from analysis of the BrdUrd data for 3 days following mitomycin C exposure. Top panels show the BrdUrd fraction in the first 30 μm of tissue on the near (exposed)
versus far sides of the cultures as a function of drug exposure. From the data, only a modest difference in drug effect is observed between the two sides. Control experiments under normal versus deprived conditions (low oxygen and glucose) showed equal drug effect. Mitomycin C did not show a differential effect in the control assay under conditions of normal versus low oxygen. In some instances mitomycin C has been reported to exhibit increased toxicity under conditions of low oxygen (Gupta and Costanzi 1987; Pan et al. 1993). One possible, though perhaps unlikely, explanation for the lack of an oxygen effect observed here would be if the drug were retained in the tissue long enough such that a significant portion of its activity occurred during the 3-day incubation period subsequent to the 1-h drug exposure, during which the tissue oxygenation on the far sides of cultures would be at normal levels. In this case, the effect-based estimate of drug distribution would remain valid.

**FIGURE 6-13 Mitomycin C analysis:** Analysis of drug effect on the first 30 μm of tissue on the near (●) and far (○) side of MCCs 3 days following a 1-hour exposure to mitomycin C. Data show BrdUrd immunostaining expressed as the fraction of levels seen in untreated MCCs. Results from control experiments under conditions of normal (▲) versus energy deprived conditions (low oxygen and glucose) (△) are also shown. Bottom panels show MCC thickness 3 days after treatment plotted relative to thickness at time of exposure. Points show mean ± SD (n=3-4).
**ALKYLATING AGENTS**

**FIGURE 6-14** shows the effect of BCNU and thiotepa on proliferation in MCCs 3 days after a 1-hour exposure to one side. MCC cryosections were immunostained for incorporated BrdUrd to show S-phase cells. Both agents exhibited little or no effect on BrdUrd labelling or culture thickness despite exposures well above typical blood plasma levels achieved clinically. Based on the lack of detectable effect or difference in effect on either edge of the cultures further work was not pursued.

**FIGURE 6-14** Alkylating agents data: S-phase cells 3 days after exposure to bcnu or thiotepa for 1 hour to one side. Images show BrdUrd immunostained cryosections from HCT-116 MCC. Scale bars, 150 μm, show approximate thickness of cultures at time of drug exposure, arrows show direction of drug diffusion.
HYPOXIC PROFILE

The hypoxia marker pimonidazole was used to define regions of low oxygen within the cultures under different gassing conditions. Pimonidazole is a 2-nitroimidazole that is reduced under conditions of low oxygen, half-maximum binding occurring at ~0.5% O₂, and forms adducts with thiol groups in proteins, peptides and amino acids within cells (Raleigh et al. 1987; Lord et al. 1993; Azuma et al. 1997). Subsequent immunodetection of pimonidazole-thiol adducts allows visualization of regions of low oxygen within tissue. Figure 6-15 shows pimonidazole immunostaining following a 1-hour labelling period under gassing conditions of 20, 5 and 0% O₂. Results indicate that under 20% gassing, 150 μm thick cultures exhibit a small region of low oxygen along the far edge of the cultures. The hypoxic area is roughly half the culture width under 5% O₂ gassing and the entire culture is seen to stain for pimonidazole under 0% O₂.

FIGURE 6-15 Hypoxic profile: Pimonidazole immunostaining profile in 150 μm thick HCT-116 MCC. Cultures were equilibrated for 1h hour under 20, 5 or 0% O₂ and then exposed to 200 μM pimonidazole for 1 hour from one side. Scale bars, 150 μm, show approximate thickness of cultures at time of drug exposure, arrows show direction of pimonidazole diffusion.
Results for analysis of a series of experiments looking at the distribution of pimonidazole staining in 150 μm thick cultures under various conditions of gassing and pimonidazole concentration are shown in **FIGURE 6-16**. In addition to characterizing the oxygenation status of cultures during a typical drug penetration experiment, we also wanted to examine the effect of consumption of pimonidazole through tissue binding on its penetration. It was hypothesized that under conditions of 0% O₂ gassing, fully hypoxic cultures might consume enough pimonidazole on the exposed side of the cultures to limit its ability to label tissue on the far side of the cultures. However, this phenomenon was not observed under the range of conditions shown in **FIGURE 6-16**. Staining intensity on either edge was found to be quite variable, likely due to poor control of O₂ status within the apparatus when under 0% O₂ gassing.

**FIGURE 6-16 Hypoxic profile analysis**: Pimonidazole immunostaining profile following exposure to 50, 200 or 400 μM pimonidazole for 1 hour under 20, 5 or 0% O₂. Points show mean ± SE (n=4).
6.3 DISCUSSION

The survey conducted here gives insight into the range of applicability and limitations in applying the MCC-effect based assay to evaluate the ability of anti-cancer drugs to distribute within tissue. Overall, the 1-h exposure period followed by a 1 day incubation period and BrdUrd-based endpoint were found to work best for the vinca alkaloids, gemcitabine and etoposide. Other drugs, such as mitomycin C and cisplatin, required longer incubation periods or higher drug exposures to reduce BrdUrd labelling. The limitation in the maximum duration of sustained exposure may limit the applicability of the assay for drugs which require extended exposures to manifest activity (e.g. cytarabine and 5-FU). Other limitations encountered here include the reduction in oxygenation on the far sides of cultures that occurs during drug exposure, which was shown through control experiments to modify the toxicity of bleomycin.

While overall the BrdUrd based end point was found to yield useful results in terms of detecting drug effect, it may not be the optimal end-point for many drugs. For example cisplatin and mitomycin C required extending the delay period following drug exposure to detect an effect. Other drugs showed little effect following exposure (e.g. BCNA and thiotepa). An apoptosis based endpoint, perhaps using the TUNEL assay, might prove more effective in some cases or possibly direct detection of drug induced DNA damage using a maker such as γH2AX.
7. SUMMARY AND FUTURE DIRECTIONS

The overall aim of this research project was to develop and validate a novel application of MCC in the assessment of the extravascular penetration and distribution of anti-cancer drugs. The original hypothesis of the study was that the symmetrical proliferation that occurs in MCC could be used as a simple means of measuring drug distribution using a biological endpoint. While there already exist several methods for evaluating drug penetration such as direct visualization of radiolabelled or fluorescent molecules and indirect flux-based experiments, none of these methods are particularly amenable as a generalised screen that could be used at an early phase in the development of new anticancer drugs. Results from this study suggest that an MCC effect-based assay could be fast and simple enough to be used to test newly synthesised drugs with little knowledge required of physicochemical parameters or drug-tissue interactions. On the most basic level the assay might be useful in determining when a drug's cellular interactions, e.g. binding, metabolism or accumulation, are significant enough to affect its ability to distribute within tissue. For example to determine what is "too much" in terms of a drug's binding site affinity. In this sense the assay could be useful in categorizing a family of related drug analogues with differing physicochemical parameters. While ideally the assay would be able to select the analogues most likely to succeed in vivo, at the very least it could provide another level of information in terms of interpreting differences in the observed anti-tumour efficacy of the drugs. An effective strategy for making use of information provided by the assay might be as a quick way of sorting related analogues so that representative compounds spanning the range of possible behaviour could be selected for in vivo testing.

A summary of the MCC-based tissue distribution data presented in this report is shown in Table 7-1. Each drug's ability to distribute within tissue was estimated from the differential effect observed on the two sides following exposure to one side only. Drug exposure to the far sides of the cultures was seen to vary from as low as 3-10% of reservoir exposure for the anthracyclines to cases where no reduction in exposure could be detected on far sides, as was seen for 5-FU and cisplatin. In between were the taxanes, where far sides received 10-20% of reservoir exposures. Several drugs were observed to exhibit marginal reduction in exposure to the far side such as the vinca alkaloids, 33-100% of reservoir exposure. Cases where the assay was observed to fail included situations where 1 h drug exposures produced no detectable effect on the BrdUrd endpoint, e.g. cytarabine and thiotepa both of which are normally administered as continuous infusions. Other examples of assay failure included drugs for which control experiments under conditions of reduced oxygen and glucose showed response on the far side to vary for reasons other than drug penetration, e.g. bleomycin and etoposide which exhibit oxygen dependent toxicity. Pertinent drug-tissue interactions and their cell cycle effects, Table 7-2, as well as human
pharmacokinetic data and some physicochemical parameters, TABLE 7-3, have also been summarized.

The MCC effect-based assay was found to compare reasonably well with the direct visualization of drugs in MCCs. Anthracycline gradients as predicted by the effect-based assay were found to agree with direct visualization of the drugs immediately following the 1-h exposure. Ranking of the ability of the anthracyclines to distribute within tissue by the effect-based assay was also consistent with ranking derived from flux-based data. For the taxanes, while ranking via the effect-based assay was consistent with direct visualization of the drugs, a comparison between the methods was difficult to make and it was hard to imagine how the poor drug distribution observed from radiolabelled assays would produce any effect at all to the far sides of the cultures. This apparent problem likely originates from the inherent difference in the two assays; while the effect-based assay integrates the original 1-h drug exposure as well as subsequent redistribution of drug over the following 48 h, the radiolabelled assay produces a discrete snapshot in time of drug levels.

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<tr>
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<tr>
<td>215-215</td>
<td>10-90</td>
<td>3</td>
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<td>189-189</td>
<td>3-30</td>
<td>3</td>
<td>-</td>
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</tr>
<tr>
<td>1-100</td>
<td>1-100</td>
<td>1</td>
<td>100</td>
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<tr>
<td>50-400</td>
<td>50-400</td>
<td>1</td>
<td>100</td>
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</tbody>
</table>

TABLE 7-1 Summary of results obtained from the MCC effect-based assay for evaluation of drug distribution. Gradient refers to the fold reduction in exposure estimated on the far sides of MCCs following 1h drug using the closed-off assay and % exposure is calculated from this. Concentration refers to the concentration range that was studied and wait refers to the incubation period following completion of drug exposure before evaluating drug effect. !(Kelland and Abel 1992; Lavelle et al. 1995), *(Goodman et al. 2006), *(Ferguson and Cass 1985), *(Rahmani-Jourdheuil et al. 1994), *(Yamauchi et al. 1987).
the drug distribution as it existed immediately following the 1-h exposure. This same limitation also exists when comparing the effect-based anthracycline results with direct visualization data and it is perhaps surprising that they compared so well.

A comparison between the tumour and MCC results could be made from the BrdUrd penetration work and the radiolabelled taxane work. While the distribution of BrdUrd labelled S-phase cells does not reflect actual free BrdUrd levels that existed within cells, due to the saturation in the level of BrdUrd incorporated into DNA, some insight in BrdUrd penetration can still be obtained from comparing the tumour and MCC data. MCC results found that a sustained exposure to 100 μM BrdUrd for 1 hour was required to label S-phase cells 150 μm into the tissue while in tumours the 100-mg/kg BrdUrd dose did not adequately label cells 100-150 μm away from vessels and was found to produce only a 30-μMxh AUC, which was consistent with the MCC prediction. The distribution of radiolabelled taxanes was found to rank similarly between MCC and tumours. The observed differences in depth of penetration of the taxanes, though reasonably

<table>
<thead>
<tr>
<th>DNA effects</th>
<th>microtubules</th>
<th>cell cycle</th>
<th>induced block</th>
</tr>
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<tbody>
<tr>
<td>interaction</td>
<td>topo II</td>
<td>breaks</td>
<td>specific</td>
</tr>
<tr>
<td>doxorubicin</td>
<td>binds</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>epirubicin</td>
<td>binds</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>daunorubicin</td>
<td>binds</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>mitoxantrone</td>
<td>binds</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>paclitaxel</td>
<td>stabilizes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>docetaxel</td>
<td>stabilizes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gemcitabine</td>
<td>incorporates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-fluorouracil</td>
<td>incorporates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cytarabine</td>
<td>incorporates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>vincristine</td>
<td>inhibits</td>
<td>G2/M</td>
<td>e</td>
</tr>
<tr>
<td>vinblastine</td>
<td>inhibits</td>
<td>G2/M</td>
<td>e</td>
</tr>
<tr>
<td>vinorelbine</td>
<td>inhibits</td>
<td>G2/M</td>
<td>e</td>
</tr>
<tr>
<td>mitomycin C</td>
<td>cross links</td>
<td>yes</td>
<td></td>
</tr>
<tr>
<td>bleomycin</td>
<td>scission</td>
<td>yes</td>
<td></td>
</tr>
<tr>
<td>cisplatin</td>
<td>cross links</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>etoposide</td>
<td>cross links</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>BCNU</td>
<td>cross links</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>thiopeta</td>
<td>incorporates</td>
<td>yes</td>
<td></td>
</tr>
<tr>
<td>BrdUrd</td>
<td>incorporates</td>
<td>yes</td>
<td></td>
</tr>
<tr>
<td>pimonidazole</td>
<td>incorporates</td>
<td>yes</td>
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</tbody>
</table>

similar, were more likely due to experimental artefacts rather than differences in diffusion in the two models.

The control assay, in which both edges received equal drug exposure but under different oxygen and glucose conditions, was found to work reasonably well in detecting non-diffusion related effects. Key examples were the bleomycin and etoposide studies, in which low oxygen was known to modify drug effect. It is not clear how useful this assay was in detecting acidity related effects that might occur due to decreasing pH with depth into tissue. In MCC, a drop in pH of -0.3 likely occurs at a depth of 150 μm into tissue in relation to the edge, based on measurements in large, 650-μm-thick spheroids where a drop of -0.25 occurred at 150 μm into the tissue (Acker et al. 1987). Hence, for experiments conducted here at pH 7.4, the far edge of the cultures likely resides at -7.1. This would be expected to produce a -25% reduction in anthracycline efficacy (Born and Eichholtz-Wirth 1981; Mahoney et al. 2003) and perhaps a 10%-15% reduction for vinca

<table>
<thead>
<tr>
<th>Peak plasma, administration (μM)</th>
<th>Ref</th>
<th>Blood binding</th>
<th>Ref</th>
<th>logP</th>
<th>Ref</th>
<th>pKa</th>
<th>Ref</th>
<th>Active uptake ref</th>
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<tbody>
<tr>
<td>doxorubicin 8 mg/m², bolus a</td>
<td>76%</td>
<td>c</td>
<td>0.3 j</td>
<td>8.3 n</td>
<td></td>
<td></td>
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<tr>
<td>epirubicin 7 mg/90 mg², bolus a</td>
<td>50-60% i</td>
<td>0.6 j 8.1 n</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>daunorubicin 0.5 mg/m²</td>
<td>45 mg/m² b</td>
<td>77% i</td>
<td>0.9 j 8.3 n</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>mitoxantrone 0.7 mg/m², 0.5-h infusion c</td>
<td>97% c</td>
<td>0.8 k 8.1 o</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>paclitaxel 4.25 mg/m² 3h inf d</td>
<td>90% c</td>
<td>5.0 l</td>
<td></td>
<td></td>
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<tr>
<td>docetaxel 2.5 mg/m², 1.6-h infusion c</td>
<td>94% c</td>
<td>4.2 l</td>
<td></td>
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<td>gemcitabine 27 mg/m² 2h inf c</td>
<td>negligible c</td>
<td>-1.3 l 3.6 l yes c</td>
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<tr>
<td>5-fluorouracil 10 mg/m², continuous infusion c</td>
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<td>-0.8 l 7.6 p yes c</td>
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<tr>
<td>cytarabine 0.4 mg/m², continuous infusion c</td>
<td>13% c</td>
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<tr>
<td>vincristine 0.3 mg, bolus c</td>
<td>low, 75% i</td>
<td>2.6 m 7.4 m</td>
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<td>vinblastine 0.4 mg/m², bolus e</td>
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<td>vinorelbine 1 mg, 15-min infusion c</td>
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<td>5.8 l</td>
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<tr>
<td>mitomycin C 0.6 mg/m²</td>
<td>c</td>
<td>-2.3 l 7.4 o</td>
<td></td>
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<td>bleomycin 0.85 U/L, 15 U/m², bolus d</td>
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<td>-2.2 l</td>
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<tr>
<td>cisplatin 10 mg/m² 2h inf c</td>
<td>90% c</td>
<td>1.2 l</td>
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<tr>
<td>etoposide 7 mg/m², oral 50 mg/m² f</td>
<td>96% c</td>
<td>1.3 l</td>
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<td></td>
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</tr>
<tr>
<td>BCNU 5 mg/m², 2h inf g</td>
<td>77% g</td>
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<td></td>
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<tr>
<td>thiotepa 4 mg/m², 2h inf h</td>
<td>low h</td>
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<td>BrdUrd 100-250 mg</td>
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</tr>
<tr>
<td>pimonidazole 500 mg/m²</td>
<td>q</td>
<td>8.7 r</td>
<td></td>
<td></td>
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</table>

alkaloids (Ferguson et al. 1984). In both cases these changes are likely too small to be detected using the assay.

An underlying assumption that motivated this project was the expectation that drugs which exhibited higher levels of cellular “consumption”, either through binding, sequestration or metabolism, were likely to experience more limited tissue penetration. It was therefore a surprise to find that despite the less hydrophilic anthracycline daunorubicin exhibiting significantly greater effect and higher tissue fluorescence on the exposed side of MCCs, it was still able to match the other more hydrophilic anthracyclines in effect and fluorescence observed on the far sides of the cultures. However, having said this, it still exhibited the poorest relative distribution due to its higher accumulation on the exposed edge. A similar situation may have occurred with the vinca alkaloids. The vinca alkaloids all appeared to exert a similar effect on the far sides of the cultures suggesting similar penetration but exhibited different effects on the exposed edges, likely related to differences in their accumulation and retention within cells. The taxanes on the other hand did follow the original assumption with docetaxel exhibiting poorer tissue penetration to the far side of cultures in comparison with paclitaxel.

While the tissue penetration of most anticancer drugs in clinical use has not been fully characterized, it is often argued that repeated dosing regimes used in the clinic, in addition to addressing cell cycle issues also address those related to inefficient drug distribution. Under such regimes, a drug that exhibits inefficient distribution might kill tumour cells within a certain distance of the vasculature with each treatment, and work in a manner similar to peeling away layers of an onion. However, such regimes might actually provide an ideal environment for the cultivation of drug resistant phenotypes, as the cells residing away from tumour vasculature would receive repeated sub-lethal exposures the drug. Other drugs in clinical use may reach high enough levels in the tissue furthest away to exert activity despite exhibiting significant gradients away from vessels. In these cases, poor tissue penetration is actually forcing higher systemic drug exposure and eroding the original tumour to normal tissue specificity of the drug. Developing drug analogues which exhibit more uniform tissue distribution might lower the plasma levels required to achieve an effect in the tissue furthest from vessels and help preserve tumour specificity.
REFERENCES:


References


References


References


References


