Distribution of anti-cancer drugs within solid tumours and normal tissues and its potential for modification to improve therapeutic index

by

Krupa J. Patel

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Medical Biophysics
University of Toronto

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Limited drug distribution is an important cause of drug resistance. Anti-cancer drugs gain access to solid tumors via the blood, and must penetrate tissue to reach all viable cancer cells. This thesis aims to compare the distribution of anticancer drugs in normal tissues and tumours, to examine whether drug distribution is modifiable and quantifiable in solid tumours, and to determine whether extracellular drug distribution can be improved by modifying intracellular drug distribution.

The time-dependent spatial distribution of three anticancer drugs, doxorubicin, mitoxantrone and topotecan, were studied in normal tissues and tumours. Ten minutes after drug administration, there was fairly uniform distribution in the heart, kidney and liver whereas drug distribution within tumours was limited to perivascular regions.

Doxorubicin distribution in P-glycoprotein (PgP) over-expressing tumours was compared to that in wild-type tumours and changes in distribution were evaluated with the use of PgP inhibitors. There was better doxorubicin distribution in PgP-over-expressing tumours compared to wild-type tumours, and pretreatment of PgP-over-expressing tumours with PgP inhibitors decreased doxorubicin distribution. These data suggest that reduced uptake of drug into cells may enhance extracellular drug distribution, and the dual effects of PgP inhibitors (increased drug uptake in proximal
cells, but poorer drug distribution) may explain, in part, why these agents have not provided clinical benefit.

The effect of the proton pump inhibitor pantoprazole on intracellular and extracellular drug distribution was determined. Pantoprazole increased endosomal pH in cells, leading to less sequestration of doxorubicin within them, and increased the toxicity of doxorubicin for cultured cells. In wild-type MCF7 tumours, pretreatment with pantoprazole enhanced doxorubicin distribution and tumour growth delay without apparent increase in toxicity. These studies have led to initiation of a phase I clinical trial of pantoprazole and doxorubicin for patients with solid tumours.

The work completed in this thesis has demonstrated that limited drug distribution in solid tumours is markedly different compared to normal tissues and this is likely due to features of the tumour microenvironment. The data show that drug distribution can be modified and that these changes can be quantified, and may correlate with improved anti-tumour effects. Improving drug distribution through the use of proton pump inhibitors may be an effective strategy to improve chemotherapeutic efficacy.
I dedicate my thesis to Mummy and Papa –
Your love and sacrifice are my strength and motivation.
I also dedicate my thesis to Ritaben and Poonam –
With you both standing on either side of me holding my hands, I will never fall.
ACKNOWLEDGMENTS

First and foremost I would like to thank God because without Him, nothing is possible and with Him, nothing is impossible. It is because of Him that I have people in my life who make me who I am today and continue to help me to grow and progress in my journey of life.

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I would like to thank my committee members Dr. David Hedley and Dr. Lothar Lilge for keeping me in check every once in a while through regular committee meetings. Thank you Dr. Lilge for taking time between committee meetings to help me strengthen parts of my project and my understanding of it.

I am grateful for having wonderful lab mates throughout the years who have made the day-to-day routine of grad school all the more enjoyable. Carol Lee, thanks for all your help and guidance over the years. I inherited my project from the hands of Andy Primeau, and I thank him for being so helpful and accommodating while I learned the ropes! Thank you to Olivier, Rama, Vithika, Alaina, Jas, Susie and Man for being wonderful labmates with whom I have had many helpful and enjoyable discussions and conversations – I wish each of you all the best in your future endeavours.

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I have received so much love from my Swadhyay family. To all my brothers and sisters, uncles and aunties, your love has meant so much to me. To the one person who has accepted me as I am and awaken in me the belief that “I can”, Dadaji, you are the source of the strength and love I feel in my heart.

In the last year of my thesis, when I needed someone to hold my hand, I met you Jay. Your confidence in me has strengthened mine. It is truly a blessing to be able walk the last steps of this chapter together as we embark on many more to come. Nakupenda wewe sana, just the way you are.

And lastly, I would like to thank four important people in my life. My sisters, Ritaben and Poonam, I have only been able to walk down this path because you have stood by me in every decision I have made. Your faith in me has given me the courage and confidence to truly believe in myself. You both work extremely hard and have complete dedication in all that you do. It is inspiring to see how selfless and committed you both are to your patients, to your friends and to your family. I love you both so much and I want to thank you for being amazing sisters and my best friends.

To my parents, Mummy and Papa, it is only because of everything you have sacrificed and your unconditional love that I have been able to accomplish this chapter of my life. Bapuji and Dada have always given importance and value to education, and you have instilled that into all of us. You are my role models of patience, hard work and success. You have taught me to always aim for the best and take the lead, and I will but, your footsteps are the ones I will always follow. I love you.

Tvamevamata cha pita tvameva
Tvamevabandhus cha sakhatvameva
Tvamevavidhyadravinamtvameva
Tvamevasarvam mama deva deva

(You are my mother, you are my father
You are my friend
You are my source of knowledge, wealth and wisdom
You are everything to me)
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>γH2AX</td>
<td>gamma histone 2AX</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>α-MEM</td>
<td>Alpha minimal essential medium</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP binding cassette</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
</tr>
<tr>
<td>AOI</td>
<td>Area of interest</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>DiOC7</td>
<td>3,3’-diheptyloxy carbocyanine</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DOX</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-related kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione-S-Transferase</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
</tbody>
</table>
HIF Hypoxia inducible factor
HPLC High performance liquid chromatography
IFP Interstitial fluid pressure
IP Intraperitoneal
IV Intravenous
LPZ Lansoprazole
mAb Monoclonal antibody
MAPK Mitogen activated protein kinase
MCC Multilayered cell cultures
MDR Multidrug resistance
MITOX Mitoxantrone
MRP1 Multidrug resistance protein 1
MTD Maximum tolerated dose
OCT Optimal cutting temperature
PBS Phosphate-buffered saline
PECAM Platelet endothelial cell adhesion molecule
PgP P-glycoprotein
PPI Proton pump inhibitor
PTP Pantoprazole
RNA Ribonucleic acid
RPMI Roswell Park memorial institute medium
TGF Transforming growth factor
TMX Tamoxifen
TOPO Topotecan

V-\(H^+\)-ATPase Vacuolar- \(H^+\)-ATPase

VEGF Vascular endothelial growth factor

VEGFR Vascular endothelial growth factor receptor
CHAPTER 1

INTRODUCTION
1.1 OVERVIEW

In 2010, Canada will continue to see an increase in the number of individuals diagnosed with and dying from cancer. Every hour of every day, an average of 20 people will be diagnosed with some type of cancer and 8 people will die from cancer. Although awareness of cancer has increased in modern times, it is not a modern disease. From the ancient civilizations, through the Middle Ages, cancer occurrences have been described in various ways. Through the ages, various methods to treat cancer have been explored. The use of chemotherapy in cancer came from studies during World War II of the effects of sulphur and nitrogen mustard to cause death of proliferating cells. These studies eventually led to the application of nitrogen mustard for the treatment of leukemia and lymphoma. Since the 1950’s, chemotherapy has been used with modest success to treat solid tumours. In the past 5 decades major advances have been made in the development of a variety of cytotoxic agents and there are currently over 500 drugs in clinical trials for cancer, 300 or which are directed against specific molecular targets.

Chemotherapy is used widely to prevent disease recurrence after local treatment (adjuvant therapy) by eradicating micro-metastases and improving local control of the primary tumour. Chemotherapy may also be administered prior to local treatment (neoadjuvant therapy) in order to reduce tumour size and decrease probability of metastasis. Chemotherapy is used commonly as palliative treatment for many types of cancer in advanced phases to reduce the volume of established metastases.
1.2 CANCER CHEMOTHERAPY

Current cancer therapies, including chemotherapy, radiation, immunotherapy and gene therapy, are designed to induce sufficient damage to tumour cells to cause them to either lose their proliferating capacity or cause cell death, while having minimal toxicity to normal tissue. Many anticancer drugs are selectively toxic to proliferating cells, and therefore have toxicity for cells in the bone marrow (precursors of mature granulocytes) and intestinal mucosa. As a result, most cytotoxic treatments are unable to reach a high level of selectivity for cancer cells and also cause toxicity to normal tissues that limits both dose and frequency of drug administration. Chemotherapy is administered at intervals to permit recovery of blood counts and prevent infection and bleeding.

Effective anticancer therapy for many tumours requires a combination of multiple drugs that have different mechanisms of action and different dose-limiting toxicities to produce larger additive effects against a tumour while reducing toxicity to normal tissues. Heterogeneous tumour cell populations increase the number and diversity of potential target sites. As well, heterogeneity within a single tumour can lead to varying sensitivity to drugs among cell subpopulations and this can lead to the selection or induction of drug-resistant subpopulations. Combining multiple drugs can improve therapeutic efficacy by having a greater number of targets and reducing cross-resistance (1). Mechanisms by which different agents may give therapeutic benefit when used in combination can be classified as follows: (1) independent toxicity, which may allow for the potential use of anticancer drugs at full dosage; (2) spatial cooperation, where subpopulations missed by one agent may be treated by another; (3) protection of normal tissues; and (4) enhancement of tumour response (2).
1.3 PROPERTIES OF ANTI-CANCER DRUGS

In order for anticancer drugs to be efficacious, they must reach targeted areas in the body. The distribution of anticancer drugs within the body is governed by various factors such as blood flow to different organs, diffusion, protein and tissue binding, and lipid solubility. Response to chemotherapeutic agents once they are delivered to tissues and tumour via the bloodstream will depend on several processes including drug uptake into cells, intracellular metabolism, binding to molecular targets and cellular mechanisms for overcoming drug-induced damage. Drug effects inside the cells and the mechanisms that the cell uses to try to circumvent or repair damage vary widely among different types of drugs and this variability in these processes will influence response. Pharmacokinetic and pharmacodynamic modeling examines the relationship between a clinical endpoint, such as toxicity or response and the time course of the drug in the body. This type of modeling is useful for identifying clinical parameters that will guide dose and frequency of administration. Most chemotherapeutic drugs have a narrow therapeutic index and are given at close to the maximum tolerated dose (MTD).

Although metabolism of chemotherapeutic agents is drug specific, many chemotherapeutic agents are metabolized primarily in the liver where oxidative and reductive reactions take place via the cytochrome P-450 system, known as phase I. Phase II is conjugation where reactions lead to inactive metabolites that can be eliminated from the body by biliary or renal excretions. During phase I reactions, metabolites can be produced that retain therapeutic activity (e.g. doxorubicin) for some drugs, whereas for others (e.g. cyclophosphamide) metabolism is required for activation. Many anticancer drugs have active metabolites, and this introduces additional complexity into
understanding the relationship between drug pharmacokinetics and effect. Most drugs are eventually eliminated from the body by the kidney or the biliary tract. Renal excretion can either be of the active drug (e.g. carboplatin) or of metabolites that have undergone phase II metabolism (e.g. doxorubicin).

1.4 CLASSIFICATION OF ANTI-CANCER DRUGS

The majority of anti-cancer drugs used today are cytotoxic agents with activity against proliferating cells, however they have little specificity against neoplastic cells.

1.4.1 Alkylating agents

Alkylating agents were among the first group of drugs used for the systemic therapy of cancer, identified by their ability to cause DNA damage. Nitrogen mustard was developed as a by-product from a study on poisonous war substances and was one of the first agents used clinically. Derivatives of nitrogen mustards include cyclophosphamide, melphalan and chlorambucil. They are chemically diverse agents that act through the covalent bonding of alkyl groups (e.g. –CH₂Cl) to intracellular macromolecules. Alkylation of pyrimidine and purine bases in DNA is likely to be the major cause of lethal toxicity, as it has been observed that there is a quantitative relationship between the concentration of drug that causes toxicity to the cells and the production of lesions in DNA, such as single-strand breaks and cross-links (3, 4). Inter-strand DNA cross-links subsequently inhibit DNA separation or cause abnormal DNA separation preventing cell division from occurring. Alkylating agents bind directly to DNA, and have limited cell cycle specificity. Although the nitrogen mustards were
originally used for the treatment of lymphomas, they are now widely used clinically as part of treatment protocols for breast, lymphatic, gynecological and pediatric tumours, in high-dose chemotherapy regimens and for a number of autoimmune diseases. Toxicities common to all agents of this class are myelosuppression, immunosuppression, hair loss, nausea and vomiting. Many alkylating agents have longer-term effects such as infertility and carcinogenesis due to long-lasting DNA damage.

1.4.2 Platinating agents

Platinating agents came about from an observation that an electric current delivered to bacterial culture via platinum electrodes leads to inhibition of bacterial growth (5, 6). The active compound was cisplatin (cis-diaminedichloroplatinum II). Cisplatin was found to have substantial toxicities including severe renal, neurologic, and emetogenic effects. Carboplatin (cis-diammine(1,1-cyclobutanedicarboxylato) platinum(II)), an analog of cisplatin, was introduced into clinical trials in 1981 to help circumvent some of the toxicities of cisplatin (7). Both cisplatin and carboplatin are platinum (II) complexes with two ammonia groups in the cis position. While cisplatin has two chloride “leaving” groups, carboplatin has a cyclobutane moiety. The major cytotoxic target of these two analogs is DNA. The agents bind to DNA at two sites and produce inter-strand cross-linkages and lead to the formation of intra-strand bifunctional adducts (8, 9). Although this is known, the type of DNA lesions responsible for cytotoxicity and the mechanism that leads to cell death remain unclear. Platinating agents are commonly used as part of first-line therapy for testicular, urothelial, lung, gynecological, and other cancers. Carboplatin and its derivatives are comparable in efficacy to cisplatin, for example for ovarian cancer the two have equal efficacy, however
for gastrointestinal cancer, oxaliplatin is more active (10, 11). Common toxicities include myelosuppression, nephrotoxicity and neurotoxicity.

1.4.3 Antimetabolites

Antimetabolites are synthetic drugs that act as inhibitors of critical biochemical pathways in the formation of DNA, or as nucleotide analogs in DNA or RNA that result in the formation of abnormal nucleic acids. The metabolic processes of the cell are complex and involve many enzymes. Two major pathways give rise to the synthesis of purines and pyrimidines. Purine analogs (e.g. 6-thioguanine and 2-chlorodeoxy adenosine) and pyrimidine analogs (e.g. 5-fluorouracil, cytosine arabinoside and gemcitibine) act by either inhibiting the formation of the normal nucleotides or interacting with DNA and preventing further extension of the new DNA strand, leading to the inhibition of cell division. Folate-derived co-factors are also involved in these processes and are essential for certain reactions, such as the conversion of deoxyruridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) (12). Antifolates are not nucleoside analogs but rather they prevent the formation of reduced folates, which are required for DNA synthesis. Methotrexate is an antifolate that acts as a competitive inhibitor of dihydrofolate reductase (DHFR) (13). Most antimetabolites are cell cycle specific and effective against rapidly dividing cells. Toxicity of antimetabolites reflects their effects on proliferating cells; therefore mucositis, myelosuppression and thrombocytopenia are common.
<table>
<thead>
<tr>
<th>Drug Category</th>
<th>Chemotherapeutic agent or molecular targeting agent</th>
<th>Commonly occurring toxicities</th>
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<tr>
<td>Alkylating agents</td>
<td>Nitrogen Mustards</td>
<td>myelosuppression, immunosuppression, hair loss, nausea and vomiting</td>
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<tr>
<td></td>
<td>- cyclophosphamide</td>
<td></td>
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<td></td>
<td>- melphalan</td>
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<td></td>
<td>- chlorambucil</td>
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<td></td>
<td>Nitrosoureas</td>
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</tr>
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<td></td>
<td>- chloroethylnitrosoureas</td>
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<tr>
<td>Platinating agents</td>
<td>Cisplatin</td>
<td>myelosuppression, nephrotoxicity and neurotoxicity</td>
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<tr>
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<td>Antimetabolites</td>
<td>Methotrexate</td>
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<td>- cytosine arabinoside</td>
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1.4.4 Topoisomerase inhibitors

Topoisomerase inhibitors are widely used as anti-tumour drugs. DNA topoisomerases are ubiquitous nuclear enzymes that relax supercoiled double-stranded DNA to allow DNA replication and RNA transcription. Topoisomerases bind to DNA creating a DNA-topoisomerase cleavable complex. This subsequently leads to the formation of either a single strand nick (topoisomerase I) or a double-strand nick (topoisomerase II) which allow the DNA to swivel to relieve torsional strain and to religate (14-16). Thus, topoisomerases are known to be involved in many important DNA metabolism reactions including replication, recombination, transcription and chromosome segregation during mitosis (17). Topoisomerase inhibitors prevent DNA religation by binding to and stabilizing the cleavable complex. Stabilized DNA intercalation and the formation of a cleavable complex may lead to interaction with the replication machinery and this interaction may be responsible for DNA synthesis inhibition. This interaction between the replication machinery and the reversible cleavable complex leads to cell death and G2 arrest of the cell cycle (18, 19). Despite the fact that most of the inhibitors in this class share the same target, they have different experimental and clinical antitumour properties which could be due to different modes of action or to specific sites of interactions (20, 21).

Topoisomerase I inhibitors include camptothecin and its derivatives topotecan and irinotecan. DNA topoisomerase I is expressed continuously during the cell cycle and in quiescent cells, whereas topoisomerase II expression increases during the S phase of the cell cycle and is almost absent in quiescent cells (22, 23). Thus, DNA topoisomerase I can be targeted in slow growing tumours (24). Camptothecins have shown promise in the
treatment of ovarian cancer and small cell lung cancer and are now used as part of first
line therapy in metastatic colorectal cancer where they have a modest effect to improve
survival (25-28).

Topoisomerase II inhibitors comprise several classes of drugs including
anthracyclines, anthracenediones, amsacrine, ellipticines (DNA intercalators), and non-
intercalating etoposides (16). It has been found that the high levels of topoisomerase II in
tumour cells parallel the high proliferative potential of these cells (29, 30).

Topoisomerase II inhibitors are used in the treatment of a wide range of cancers such as
leukemia, lymphoma, breast cancer and other solid tumours. Although dose-limiting
toxicities are specific to groups of drugs, some of these toxicities include
myelosupression, cardiac toxicity, mucositis and loss of hair.

1.4.5 Antimicrotubular agents

Antimicrotubular agents include naturally occurring compounds such as vinca
alkaloids and plant-derived taxanes. The vinca alkaloids, vinblastine and vincristine, act
by binding to the protein tubulin and inhibit its polymerization to form microtubules (31).
Cells damaged with these agents may lead to lethality by entering an abortive metaphase
followed by cell lysis. These drugs are used in the treatment of testicular cancer and
leukemia, and in combination with other cytotoxic agents to treat lymphomas or various
solid tumours. Myelosuppression and neurotoxicity are commonly associated with the
use of these agents in the clinic. The taxanes, paclitaxel and docetaxel, are also anti-
microtubular agents and bind to tubulin, but at a site that is different from that of vinca
alkaloids. Taxanes have been proposed to act by inhibiting microtubular disassembly,
preventing the normal growth and breakdown of microtubules that is required for cell
division (32). This class of drugs has been observed to have activity against ovarian, breast, lung and breast tumours. Dose-limiting toxicities are myelosuppression and neurotoxicity (1).

1.4.6 Molecular-targeted agents

An emerging group of antineoplastic agents are molecular targeted agents. Cancer occurs through a series of steps including the accumulation of molecular changes that allow histological premalignant lesions to develop into invasive tumours (33). Targeted therapies that are directed to these specific molecular changes/targets that are selectively expressed in cancer cells show promise. These agents have potential for increasing specificity. A large research effort has focused on the development of therapeutic antibodies. Monoclonal antibodies can have various modes of actions. They can bind to the respective antigen and interfere with its activity and interact with binding partners. The antigen can be a soluble ligand and examples of such antibodies include infliximab, adalimumab, and certolizumab (anti-TNFα) or bevacizumab (anti-vascular endothelial growth factor). Alternatively, the antibody may target a receptor expressed at the cell surface, block its interaction with a ligand, interfere with a multimerization process or trigger internalization of receptors or apoptosis of targeted cells. Examples of such antibodies include cetuximab and panitumumab [(anti-epidermal growth factor receptor) or HER1 (human epidermal growth factor receptor)] and trastuzumab (anti-HER2) (34). Other targeted agents include small molecules that interact with various cell signalling molecules. Examples of these agents include imatinib, erlotinib, gefitinib and tipifarnib. By targeting specific gene products such as those involved in signal transduction
pathways, angiogenesis, cell cycle control, apoptosis and inflammation, monoclonal antibodies and small molecules show much promise for anticancer treatment.

1.5 RESISTANCE TO CHEMOTHERAPY

The treatment of disseminated cancer has become increasingly aimed at molecular targets derived from studies of oncogenes and tumour suppressors known to be involved in the development of the disease (35). Cancer treatment has transformed from the use of general cytotoxic agents such as nitrogen mustards to the development of natural product anticancer drugs which are more cytotoxic to cancer cells than normal cells, to the use of specific monoclonal antibodies and immunotoxins targeted to cell surface receptors (36, 37) and specific agents that inactivate kinases in growth-promoting pathways (38). While these targeted agents hold promise, in clinic, over 95% of systemic treatment is with chemotherapeutic agents.

Despite its widespread use, improved response rates and reduced side effects, many human cancers either do not respond to chemotherapy, or acquire resistance during the course of therapy. The mechanisms that contribute to drug resistance are complex and multifactorial. They occur at the molecular, cellular, and physiological level of the tumour. A large research effort has been devoted to studying the various underlying mechanisms of drug resistance. Mechanisms of drug resistance can be summarized as those that operate at the level of the cell membrane, cytoplasm and nucleus (Figure 1.1).
Figure 1.1 Schematic of mechanism of drug resistance. Illustrates the basic molecular mechanisms of drug resistance such as efflux pumps (eg. P-glycoprotein), decreased drug activation and increased drug inactivation (eg. cyclophosphamide resistance decrease in cellular uptake (eg. methotrexate resistance), alterations in drug targets (topoisomerase II and doxorubicin resistance), sequestration/compartmentalization within acidic endosomes and organelles (eg. doxorubicin resistance), and increased DNA repair ability (eg. alklating agents). (Adapted from Gottesman, 2002; (39)).
1.6 CELLULAR AND MOLECULAR CAUSES OF DRUG RESISTANCE

Cellular and molecular causes of drug resistance include up-regulation of target enzymes, increased drug metabolism, multidrug resistance via drug export transporters such as p-glycoprotein and multidrug resistance protein, and in the case of haematological malignancies, enhanced survival due to defects in the apoptotic pathway.

1.6.1 Decreased Drug Uptake

Accumulation of a drug within a cell results from a balance between drug entry and exit mechanisms. Drugs enter cells through three different routes: (1) passive diffusion across the plasma membrane, (2) facilitated diffusion in which the drug enters through a receptor or transporter and (3) active transport by a carrier-mediated process such as endocytosis. The latter two mechanisms of entry have pharmacological significance that is apparent through the observation of the existence of resistant mutants with defects in these pathways (39).

For drugs that enter cells via receptors or transporters, selection of drug-resistant cells can demonstrate mutations that eliminate or modify these cell surface molecules. An example of this is resistance to toxic folate analogs such as methotrexate that commonly occurs by mutation of one or both of the folate transporters (folate binding protein and /or the reduced folate transporter) (40).

A defective drug uptake system that results in altered intracellular drug content has been observed in cisplatin-resistant cells (41-43). Members of the Ctr transporter family have been shown to be involved in mediating cisplatin transport across the plasma
membrane (44). Mice with a mutant *ctr* gene have showed a substantial increase in cisplatin resistance and decrease in cellular cisplatin accumulation (45).

Immunotoxins are among the types of drugs that bind to cell surface receptors and are generally internalized via receptor-mediated endocytosis. Cancer cell mutants that have defective endocytosis are resistant to both toxins and immunotoxins (39, 46).

### 1.6.2 Multidrug resistance and altered drug efflux

Multidrug resistance is a term used to describe the phenomenon characterized by the ability of drug resistant tumours to exhibit simultaneous resistance to a number of structurally and functionally unrelated chemotherapeutic agents. The ATP-binding cassette (ABC) family of membrane transport ATPases have significant importance in the clinic. The family of proteins is phylogenetically ancient and their normal function in eukaryotic cells remain to be fully characterized. The general structure of these ATPases is composed of four structural domains, two that span the membrane (each containing several transmembrane segments) and two that remain in the cytoplasm. The last two units, called nucleotide domains, play a role in cleaving ATP (hydrolysis) to derive energy necessary for transporting cell nutrients such as sugars, amino acids, ions and small peptides across membranes (47). The cytotoxic drugs that are most frequently associated with MDR are hydrophobic, natural products such as taxanes (paclitaxel, docetaxel), vinca alkaloids (vinorelbine, vincristine, vinblastine), anthracyclines (doxorubicin, daunorubicin, epirubicin), epipodophyllotoxins (etoposide, teniposide), topotecan, dactiniomycin and mitomycin C (47, 48). Among the ABC transporters involved in MDR are multidrug resistance associated protein (MRP) and p-glycoprotein (PgP). Drug resistance develops in cancer cells often as a result of over-expression of
these transporters, which cause an increased efflux of cytotoxic drugs from the cancer cells leading to insufficient intracellular levels of the drug necessary for effective therapy. In addition, MDR can occur intrinsically in some cancers without previous exposure to chemotherapy agents (47). This type of gene amplification is probably due in part to selection and in part to induction during chemotherapy.

1.6.2.1 P-glycoprotein

P-glycoprotein (PgP) is also a member of a super-family of ATP-dependent membrane transport proteins that is predominantly found in the plasma membrane. PgP has been shown to pump substrates out of tumour cells through an ATP-dependent mechanism in a unidirectional manner. PgP is a 170-kD protein that contains two homologous halves, each comprised of six putative trans-membrane segments and a cytosolic ATP-binding site. PgP confers resistance to a broad range of complex heterocyclic hydrophobic, natural product antineoplastic drugs that include anthracycline antibiotics, the Vinca alkaloids, and the taxanes. In addition to its expression on the plasma membrane PgP is also present on the luminal side of the Golgi stack membranes in different MDR-resistant cells. However, it is absent from endocytic vesicles and lysosomes and present in only small amounts in the endoplasmic reticulum (49). PgP is not only expressed in tumour cells, but also on the luminal side of epithelial cells of several normal tissues, suggesting that it may have a physiological role in the elimination of xenobiotics or endogenous metabolites. Expression of PgP in human normal tissue is quite variable with the highest levels found in the apical membranes of the blood-brain barrier, intestines, liver and kidney (50). In cancerous tissue, the expression of PgP is usually highest in tumours that are derived from tissues that normally express PgP, such
as epithelial cells of the colon, kidney, adrenal, pancreas and liver, resulting in the potential for resistance to some cytotoxic agents before chemotherapy is first administered. In other tumours, the expression of PgP may be low at the time of diagnosis but increases after exposure to chemotherapy agents, thereby resulting in the development of MDR in those cells (51).

1.6.2.2 Multidrug resistance associated protein (MRP)

MRP is a 190-kd member of the ABC super-family of transmembrane transporter proteins encoded by the *MRP1* gene on chromosome 16 (1). Depending on the cell type, MRP may be located in either the plasma membrane or in intracellular membranes (52, 53). This protein has seventeen transmembrane segments (Figure 1.2). PgP and MRP1 confer resistance to a similar but not identical spectrum of anticancer agents. The transport kinetics of anthracyclines by PgP and MRP are very similar (54). However, unlike PgP that targets and transports hydrophobic drugs, MRP proteins can transport hydrophilic molecules and even organic anions. They also transport neutral drugs conjugated with glutathione (GSH, L-γ-glutamyl-L-cysteinyl-glycine), glucoronide, or sulfate and anticancer agents that are not metabolized to glutathione conjugates by co-transport with free GSH (55, 56). The emergence of resistance of tumour cells to chemotherapy and correlation of resistance to over-expression of transport proteins have led to considerable efforts to develop agents able to inhibit MRP-1 mediated transport. While several PgP inhibitors have entered clinical trials, the development of specific MRP inhibitors is still ongoing. Among the recent advances in development of MRP inhibitors are agosterol A and its analogs, verapamil derivatives (57), the flavonoids
(genistein, flavopiridol) (58), raloxifene-based inhibitors (59, 60), isoxazole-based compounds and quinoline derivatives (57).

1.6.2.3 Reversal of multidrug resistance

Over the last two decades a large research effort has been dedicated to studying methods of inhibiting PgP as a way of reversing MDR. The field of MDR modulators in the clinic became increasingly more popular after studies that showed that the expression of PgP was a significant prognostic marker in certain childhood malignancies (61, 62). By blocking the action of the pump, through a variety of mechanisms, chemotherapeutic agents can be retained within the cell and allow for intracellular accumulation of drug to ultimately cause cytotoxicity (Figure 1.3). In order for reversal agents to be successful in the clinic, several conditions have to be considered: (1) MDR must be a major mechanism of resistance to chemotherapy (2) the inhibition of PgP or another pump should be feasible in tumour cells in vivo without deleterious effects in normal tissues expressing the pump, (3) compounds should not have an intrinsic toxicity preventing their safe usage. Most reversal agents have not been successful in clinical trials because they have failed to meet these conditions. Through a succession of steps, three generations of compounds were developed. The first generation of compounds were already in clinic for other therapeutic applications. In the case of verapamil, cyclosporine A, and quinidine, they were unsuccessful because their low binding affinities necessitated the use of high doses, resulting in unacceptable toxicities (47, 63).
Figure 1.2  The trans-membrane domains and ATP binding sites of P-glycoprotein and Multidrug Resistance Protein 1. P-glycoprotein comprises of six putative trans-membrane domains and MRP1 has seven transmembrane domains. (Adapted from Morrow and Cowan, 2000; (64))
Many of the second generation of PgP inhibitors were analogues of the previous generation but they were designed to be more potent and less toxic. Second generation modulators include dexverapamil, dexniguldine, valsodar (PSC 833) and biricodar (VX-710). Of these, the best characterized and most studied is PSC 833, which is a non-immunosuppressive derivative of cyclosporine A. Although PSC 833 has been studied in numerous clinical trials in combination with cytotoxic agents, results have been disappointing (65-70).

Although second generation inhibitors have better pharmacogenic profiles than their predecessors, these compounds significantly inhibit the metabolism and excretion of cytotoxic agents. Cytochrome P450 enzymes are involved in the metabolism of cytotoxic agents and are often induced in response to chemotherapy. Many cytotoxic agents are substrates for both PgP and for the cytochrome P450 isoenzyme 3A4, including several of the second-generation PgP inhibitors. Due to the competition between cytotoxic agents and these PgP inhibitors for cytochrome P450 3A4 activity, there have been unpredictable pharmacokinetic interactions (71). For example, the inhibition of the cytochrome P450 3A4-mediated metabolism of paclitaxel and vinblastine by valsodar has been observed to result in increased serum concentrations of the chemotherapy agents and increased risk of drug overexposure to the patients (72). Use of second-generation inhibitors has thus been limited due to the unpredictability of interactions that could result in under- or over-dosing patients. As well, the ability of some second generation compounds to affect other ABC transporters such as MRP1 may lead to greater adverse effects of anticancer drugs (73).
Third generation inhibitors have been designed to specifically and potently inhibit PgP without affecting cytochrome P450 3A4 (74). This specificity in preclinical studies have translated to clinical trials where minimal pharmacokinetic interactions have not necessitated dose reductions. Third generation inhibitors currently in clinical development include tariquidar (XR9576), zosuquidar (LY335979), laniquidar (R101933) and ONT-093 (71). Of these the most promising inhibitor is tariquidar, which binds with high affinity to the PgP transporter and potently inhibits its activity (75). However, phase II studies show that tariquidar in combination with chemotherapeutic agents has only modest effects in patients who have already acquired drug resistance to these cytotoxic agents (76).

From this section it is evident that resistance to chemotherapy is complex and intricate and drug efflux pumps are just one of the multitude of mechanisms that can simultaneously lead to drug resistance. Other in vitro studies have suggested that drug efflux pumps such as PgP are likely to influence the distribution of anticancer drugs and that reversal agents may alter these distribution patterns making the drugs less efficacious (77). Drug efflux pumps are likely to have important effects on drug distribution in solid tumours, this concept is important in the context of the work shown in this thesis and will be examined in detail in later sections.
Figure 1.3 A schematic showing P-glycoprotein and its inhibitors. P-glycoprotein pumps within the cell membrane prevents the accumulation of most anticancer drugs within a cell. Inhibitors of P-glycoprotein may block the action and increase intracellular concentration of drug.
1.6.3 Decreased drug activation and increased drug inactivation

Altered activity of specific enzymes systems (such as glutathione S-transferase (GST) and topoisomerase) can be cause for cellular based resistance. Despite unaltered intracellular drug concentrations, this mechanism of drug resistance can decrease the cytotoxic activity of drugs.

Glutathione is an enzyme system involved in drug and xenobiotic detoxification where biotransformation processes are catalyzed by GST to conjugate organic molecules with glutathione (GSH) to form excretable polar molecules (78). The GSTs are extensively involved in the metabolic biotransformation of many anticancer drugs, such as the nitrogen mustards, in particular cyclophosphamide. Several resistant cell lines have been shown to overexpress GST (79, 80). The GST-\(\pi\) isozyme has been shown to be over-expressed in MCF-7/ADR cells, which also express elevated levels of P-glycoprotein (PgP), and exhibit increased peroxidase activity (81). Similar increases in GST-\(\pi\) levels have been reported in other multidrug resistant (MDR) cell lines (82, 83).

Although transfected GST-\(\pi\) has been shown to increase resistance to drugs such as chlorambucil and melphalan (84, 85), evidence suggests that GST- \(\pi\) does not confer resistance for doxorubicin (86). Glutathione also appears to play a key role in detoxification and cellular repair following damaging effects from anticancer drugs. Mechanisms to overcome this form of resistance involve reducing intracellular GSH levels using agents such as buthionine sulfoximine (BSO). BSO has been observed to selectively inhibit \(\gamma\)-glutamylcysteine synthetase, the rate-limiting step in the synthesis of GSH (87). However, studies showing the use of BSO in combination with chemotherapy may have minimal or limited effects on improving drug efficacy (88).
The enzymes topoisomerase I and topoisomerase II constitute effective therapeutic targets for anticancer drugs in rapidly dividing tumour cells. For example, doxorubicin and etoposide specifically target topoisomerase II (89, 90), while camptothecin analogs target topoisomerase I (91). Cells can become resistant to topoisomerase II inhibitors due to either under-expression of topoisomerase II or topoisomerase II gene mutations. This may occur by reduced activity of topoisomerase II activity (92), as well as reductions in topoisomerase II mRNA levels. Evidence suggests a compensatory mechanisms of over-expression of topoisomerase I in cells resistant to topoisomerase II due to reduced expression of topoisomerase II (93) and over-expression of topoisomerase II in camptothecin-resistant cells (94). One approach to circumvent topoisomerase I and topoisomerase II resistance is to target both enzyme classes in sequential combination. This method has been tested in phase I trials and shows some potential as an effective treatment strategy (95).

1.6.4 Alteration of drug targets

In order for antimetabolites to cause cytotoxicity, these agents must interact with their intracellular protein targets. Resistance to these agents can occur in two ways: the levels of the target protein may be increased requiring increased concentrations of drug, or the levels of the target protein may remain the same but the gene encoding the protein can acquire a mutation that maintains near normal metabolic activity but leads to lower affinity for the drug. An example of the latter type of resistance has been observed for 5-fluorouracil (5-FU) with the acquisition of mutations in its target enzyme, thymidylate synthase (96).
Although taxanes have recently emerged as effective anticancer drugs with broad ranging anticancer activity, some cancer cells have acquired the ability to confer resistance to these microtubule-targeting drugs. Several mechanisms of resistance to paclitaxel involving alterations in tubulin have been described. Such alterations include: (1) altered expression of β-tubulin isotypes in paclitaxel-resistant cells (97, 98); (2) increased microtubule dynamics in paclitaxel-resistant cancer cells (99), and (3) the presence of β-tubulin mutations in paclitaxel-resistant cells (100-102). The naturally occurring epothilones and their analogs are a new class of antineoplastics that may be a promising strategy to circumvent paclitaxel-resistant cells by inducing tumour cell apoptosis by stabilizing microtubules involved in mitosis (103, 104).

### 1.6.5 Defects in DNA repair

When cancer cells are treated with cytotoxic agents that cause DNA damage they will die unless the damage is repaired. Cells can become resistant by acquiring more efficient processes to repair DNA damage. While numerous DNA repair pathways exist, the major ones include direct (O\(^6\)-alkylguanine DNA methyltransferase, AGT), base excision (BER), nucleotide excision (NER), mismatch (MMR), homologous (HR), and non-homologous end-joining (NHEJ) repair. Inhibiting enzymes in these pathways to prevent DNA repair has been researched as a potentially effective method to circumvent this type of drug resistance. However normal tissues may also become more sensitive to drugs. The direct and base excision repair pathways show evidence of being the most clinically relevant targets (105). The direct repair pathway is typified by O\(^6\)-alkylguanine DNA methyltransferase (AGT), which plays an important biological role in directly
repairing O₆-alkylguanine in DNA. Elevated levels of AGT in tumours correlate to resistance and make AGT a potential DNA repair target for cancer therapeutics (106-108). The chemotherapeutic agent BCNU (1,3-bis (2-chloroethyl)-1-nitrosourea) generates chloroethyl modifications at O₆, which can rearrange and lead to an inter-strand crosslink that is particularly toxic because it blocks DNA replication (109). Selective targeting of such DNA repair enzymes may have the potential to enhance and augment the currently used chemotherapeutic agents and radiation and overcome drug resistance. Although attention to DNA repair pathways as potential targets has not received too much attention, this field shows encouraging promise in translational studies.

1.6.6 Defects in apoptosis / Altered regulation of apoptosis

Chemotherapeutic agents can result in the induction cellular apoptotic pathways. Hence, alterations that inhibit apoptotic pathways might contribute to drug resistance. Apoptosis is programmed cell death characterized by cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation caused by endonucleolytic cleavage of genomic DNA. Whether a cell continues through the cell cycle or undergoes apoptosis, is dependent upon a complex interplay of a team of genes and proteins that exert a regulatory role in cellular events (47). Chemotherapy induced apoptosis may occur via the intrinsic pathway mediated by the Bcl-2 family of proteins, or by the extrinsic pathway regulated by members of the tumour necrosis factor (TNF) family of receptors (110). The Bcl-2 family consists of more than 30 anti and pro-apoptotic molecules and there is evidence to support the role of Bcl-2 family members in resistance to chemotherapy (110, 111). Clinical studies show a correlation between elevated anti-
apoptotic Bcl-2 expression and poor prognosis in a number of neoplasms including AML, lymphocytic leukemia and prostate cancer (112-114). Over-expression of Bcl-2 in tissue culture has been observed to prevent apoptosis upon treatments with the majority of cancer chemotherapeutic agents (115, 116).

In addition to its key role in inducing cell cycle arrest in G1 and apoptosis following DNA damage cause by anticancer drugs, it has been suggested that the tumour suppressor gene p53 also plays an important role in the regulation of expression of the downstream proteins bcl-2 and bax (117, 118). Numerous studies support the importance of p53 in therapeutic response and the vast majority of chemotherapeutic agents have shown greater efficacy in killing human tumours with wild type as compared with mutant p53 (119). The role of p53 and other apoptotic proteins in the development of drug resistance in solid tumours has been challenged by a number of studies. A number of in vitro and in vivo experiments have found that p53 status does not correlate with chemotherapeutic sensitivity and in some cases disruption of p53 function has been shown to enhance chemosensitivity (120, 121). These studies suggest that assays used to assess the extent of cell kill by anticancer agents tend to examine the rate rather than the overall cell kill. Colony forming assays have shown that changes in apoptosis do not lead to any changes in eventual cell killing and p53 status does not affect sensitivity of DNA damaging agents (122). There has been little affect on clonogenic survival after drug treatment in cell lines transfected with Bax and Bcl-2 (121). Clinical reports have also shown no direct correlation between Bcl-expression and therapeutic response (123).
1.7 MECHANISMS OF DRUG RESISTANCE THAT RELATE TO THE TUMOUR MICROENVIRONMENT

Although genetically determined factors contribute to drug resistance, mechanisms related to the microenvironment of solid tumours may also have a profound effect on the sensitivity of tumours to chemotherapeutic agents (39, 124). Solid tumours are societies of cells that reside within complex physiological microenvironments that can influence their response to chemotherapy. Microenvironmental factors that may influence the sensitivity of tumours to anti-cancer drugs include: the requirement for drugs to penetrate tumour tissue from blood vessels and distribute widely enough to reach target cancer cells, the extracellular matrix and cell-cell adhesion, the variation in vascular density and blood flow leading to decreased and varying delivery of drugs, the altered interstitial fluid pressure, the presence of hypoxia, the low extracellular pH of the tumour microenvironment, and the proliferation of surviving cells between courses of chemotherapy (repopulation) (124). There is a strong interplay between various microenvironmental factors that is likely to be conducive to a drug resistant phenotype.

1.7.1 Drug distribution

The ability of pharmacological agents to reach the target cells in living systems determines the effectiveness of these substances. Most of the cells in the human body are within a few cell diameters of blood vessels. This high level of organization facilitates the delivery of oxygen and nutrients to the cells that form the tissues of the body. It also enables the efficient delivery of most medicines.
Many drugs are administered via the bloodstream. Once the drug has entered the circulatory system there are several limitations to transport before the substance can reach the cells of normal or neoplastic tissue. A tissue may be represented as three well-defined regions: (1) a vascular space through which blood flows; (2) an interstitial space, which provides the support structure of the tissue; and (3) the cellular space. A drug must first permeate the vessel wall from the bloodstream, then move through the interstitial space by convection and diffusion, and once in contact with a cell, it must cross the cellular wall and then transport through the intracellular space to a specific action site. At this cellular level, the majority of the therapeutic effect takes place. Any one of these transport processes: vascular distribution, capillary permeation, interstitial transport and cellular uptake or cellular metabolism, could be the rate-limiting step in the uptake of a drug in a tissue.

Drugs leave blood vessels and penetrate into tissues by the processes of convection and/or diffusion. Convection depends on gradients of pressure (both hydrostatic and osmotic) between the vascular space and the interstitial space; vessel permeability and the surface area for exchange; and the volume and structure of the extracellular matrix. Drug diffusion is determined by concentration gradients and the size and charge of the molecules.

The homeostatic regulation of tissue and the growth of blood vessels is vastly different between normal tissues in the body and tumour tissue leading to drastic differences in drug distribution between the two.
1.7.1.1 Drug distribution in normal tissues

The circulatory system is used to distribute anticancer drugs to the body. Therefore highly perfused normal tissues such as the liver and kidney generally have the largest uptake of anticancer drugs when compared to poorly perfused tissues such as most solid tumours. In addition to being well-perfused, normal tissue vasculature is highly organized and large pressure gradients across the endothelial membrane increase the efficiency of drug delivery compared to that in tumour tissue. The toxicity of most anticancer drugs to many normal cells is a major disadvantage of chemotherapy. For example, one of the major dose-limiting factors of doxorubicin is cardiac toxicity. Large concentrations of drug are also often found in normal tissues such as muscle and liver (125). The brain on the other hand has been reported to have limited accumulation of drug due to the blood-brain barrier (BBB) (126). The key cellular elements of the brain microvasculature that compose the BBB include tight junctions between cerebral endothelial cells and pericytes embedded within the basement membrane (127). As well, ABC transporters such as P-glycoprotein expressed and polarized on the luminal side of the BBB play a critical role in keeping drugs and neurotoxic substances from entering the brain and in transporting toxic metabolites out of the brain (128).

1.7.1.2 Drug distribution in solid tumours

Limited delivery of anticancer drugs is the result of large distances (up to 100µm or more) between some proliferating tumour cells and functional blood vessels (129). Large intercapillary distances are caused by rapid proliferation of tumour cells that forces blood vessels apart and reduces vascular density. This process is exacerbated by a poorly organized vascular architecture (130, 131), irregular blood flow (132, 133), and
compression of blood and lymphatic vessels by cancer cells (134). Some reasons why cells that are distant from blood vessels might be resistant to conventional chemotherapy include: selective toxicity of most anticancer drugs for cycling cells so that non-proliferating or slowly proliferating cells distant from blood vessels are resistant (135), decreased activity of some drugs in hypoxic, acidic or nutrient-deprived microenvironments (130, 136), and restricted exposure to drug in sufficient concentrations to cause cell death due to limited drug distribution.

The effective treatment of solid tumours requires constituent cells be sensitive to the drug and that the drug be able to achieve a concentration within the cells sufficient to cause cytotoxicity. Anticancer drugs need to gain access to all of the cells within a tumour; if they are unable to do so, then their effectiveness will be compromised regardless of their mode of action or potency. If the drugs are unable to reach tumour cells that are responsible for regenerating the tumour (i.e. clonogenic cells or tumour stem cells), then regardless of the novelty of even new molecular medicines, tumour recurrence is highly likely (137). A curative cancer treatment is one that accesses all crucial tumour cells and kills them; the survival of one cell could form the focus of tumour recurrence. Therefore even if large distances between tumour cells and non-functional blood vessels occur only in a small region of a tumour, they can pose an important barrier to a positive therapeutic outcome.

**1.7.1.3 Factors that affect drug distribution in solid tumours**

Distribution of anticancer drugs in tumour tissue is influenced by properties such as supply, flux and consumption of drug. The supply of a drug to the tissue will depend on its dose and pharmacokinetics. Flux of the drug through tissue refers to movement of
the drug after leaving the vasculature. Physicochemical properties of drugs such as molecular weight, shape, charge and solubility determine the flux and the rate of diffusion of the drug through tissue. Consumption of drug involves properties such as binding, metabolism and sequestration of drug. Cellular metabolism will reduce drug penetration and build-up within the tissue, and binding and sequestration can increase net levels of a drug but limit its penetration (Figure 1.4).

In addition to characteristics of the drug that can influence its distribution, cellular properties and properties of the tumour microenvironment can also have a profound impact on the ability of anti-cancer drugs to not only reach their targets but to elicit their effects. One of the major cellular mechanisms of drug resistance is the presence of drug efflux pumps that are often over-expressed in solid tumours. This mechanism of resistance was discussed earlier in the context of their effects of reducing drug concentrations at drug targets. However, there is strong evidence to suggest that drug efflux pumps such as P-glycoprotein may also have important and significant effects on drug distribution.

Factors within the microenvironment that may alter drug distribution include the extracellular matrix (ECM), cell-cell adhesion, cellular packing density, tumour vasculature and interstitial fluid pressure, the presence of tumour hypoxia, tumour acidity, and repopulation of surviving to tumour cells between courses of chemotherapy. With increasing knowledge of these factors, strategies to modify tumour physiology and improve drug distribution can be developed.
Figure 1.4  Factors that influence drug distribution are supply, flux and consumption. Cells close to blood vessels have a high rate of cell proliferation whereas cells distal from blood vessels have a low rate of cell proliferation. (Adapted from Tredan et al., 2007; (138)).
1.7.1.3.1 P-glycoprotein expression and drug distribution

Drug resistance develops in cancer cells often as a result of over-expression of drug efflux pumps such as PgP, which by actively removing drug from the cytoplasm of tumour cells into the interstitial space, prevents the accumulation of intracellular drug concentrations sufficient for cell death (77).

Drug resistance develops from limited drug distribution because while cells close to blood vessels retain large quantities of drugs, cells that are more distal are exposed to minimal concentrations of drugs. Drug efflux pumps expressed in tumour cells prevent intracellular accumulation of drugs. By actively pumping out drugs in cells that are close to blood vessels, more drug may be available to reach distal cells. It has been proposed that drug efflux pumps may alter drug distribution in this way. Studies using in vitro models have suggested that tumour cell lines which over-express PgP have improved drug penetration in cells distal from the source of drug compared to their wild-type counterparts (77). Improved drug distribution due to PgP over-expression is not necessarily beneficial for therapeutic outcome because sufficient intracellular concentrations of drug are required in all tumour cells both close to and distal from blood vessels.

Inhibitors of PgP, through various mechanisms, block the action of the pump, and allow for drug to be retained within the cells. Since PgP over-expression improves drug distribution in cells distal from the drug source, then PgP inhibitors are likely to counter this effect and show penetration profiles similar to those of wild-type cells (i.e. of limited drug penetration restricted to cells peripheral to blood vessels), this effect has been observed in in vitro models (77). Therefore expression of drug efflux pumps is likely to
have an important influence on drug distribution within the solid tumour microenvironment.

1.7.2 The extracellular matrix and cell-cell adhesion

The extracellular matrix (ECM) is produced by mesenchymal cells and contains fibrillar collagen, fibronectin, hyaluronic acid and proteoglycans. The ECM plays a role in regulating processes such as growth, differentiation, death of cells, transport of molecules and gene expression by stabilizing the microenvironment through interaction with various cell surface receptors and soluble molecules such as cytokines and growth factors (139). The ECM maintains tissue integrity and 3-dimensional architecture and provides structural and mechanical support to cells and surrounding tissue. The ECM is remodelled to sustain tumour growth, angiogenesis and invasion. Increased synthesis of many matrix components has been reported in tumour tissue and has been correlated with poor prognosis and resistance to chemotherapeutic agents (140, 141).

In addition to interacting with components of the ECM, cells within the tumour microenvironment also interact with neighbouring cells. These interactions between tumour cells and their environment have been shown to play a critical role in cell adhesion-mediated drug resistance. Cell-cell adherens junctions are the most common type of intracellular adhesions and are important for maintaining tissue architecture and cell polarity. Disruption of normal cell-cell adhesion in transformed cells may contribute to tumour cells having enhanced migration and proliferation, leading to invasion and metastasis (142). Cellular adhesion is mediated by the interaction of several families of molecules including integrins, cadherins, selectins and the immunoglobulin super-family
of cell adhesion molecules that bind to receptors on the cell surface (143). The interactions of tumour cells with various components of the ECM is mediated by integrins, and those interactions have been shown to protect them from cell death induced by various cytotoxic agents (143).

1.7.2.1 The influence of the ECM on drug distribution

The composition and structure of tumour ECM have been shown to influence drug transport in solid tumours. Cellular adhesion to other cells or the ECM can also cause drug resistance by limiting penetration of anticancer drugs into solid tumour tissue. Modification of the tumour extracellular matrix might facilitate the penetration of drugs into tumours. Treatment of tumours with collagenase has been shown to enhance the interstitial diffusion rate and the intratumoural delivery of macromolecules such as monoclonal antibodies (144). Previous studies have shown poor drug penetration into solid tumours with high packing density, and drug penetration was shown to improve upon administration of agents that induced apoptosis and reduction in cell density (145-148). The clinical applicability of such agents, however, remains unclear because modification of the extracellular matrix or its interactions with tumour cells might increase the probability of metastatic spread.

1.7.3 Tumour vasculature

One of the hallmarks of cancer is the acquired capability of tumours to sustain angiogenesis, which is the process of new vessel formation from the endothelium of existing vasculature. The oxygen and nutrients supplied by the vasculature are crucial for cell function and survival. This creates a requirement for all cells within a tissue to reside within 100-200μm of a capillary blood vessel (149). The formation of new vessels in
normal tissues is governed by the net balance between pro- (e.g. vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8)) and anti-angiogenic factors (e.g. endostatin and angiostatin). This process is highly regulated so that angiogenesis is “on” or “off” depending on the conditions (e.g. “on” during wound-healing). However, during neoplastic transformation and tumour progression, a disruption in the balance between pro- and anti-angiogenic molecules occurs (150). This is sometimes referred to as the angiogenic switch and allows for continued proliferation and growth of tumour cells (151, 152) (Figure 1.5). A number of factors appear to contribute to a change in the balance of angiogenic signals during tumour development. These factors include oncogene-driven production of growth factors by tumour cells, changes in the tumour microenvironment, the recruitment of progenitor endothelial cells from bone marrow, and the down-regulation of natural inhibitors of angiogenesis. Hypoxia, (by deregulation of HIF-1α), glucose deprivation, low pH, and inducers of reactive oxygen species (ROS) are among the microenvironmental alterations that stimulate angiogenic signals (153-155). Although the exact molecular mechanisms that underlie the formation of abnormal vasculature are unknown, it is likely that uncontrolled VEGF signalling plays a key role (156).
Figure 1.5 Angiogenesis is driven by a balance between various pro and anti-angiogenic factors. In normal tissues (A) factors remain in balance, whereas in tumour tissue (B), this balance is offset leading to abnormal vessel formation. (Adapted from Tredan et al., 2007; (138))
In normal vasculature, the angiogenic process involves activation followed by the binding of angiogenic factors to their cognate receptors on endothelial cells (EC) and the subsequent migration, proliferation, and differentiation of ECs into newly formed capillaries (157). Newly formed vessels are stabilized in the resolution phase with the establishment of a basement membrane, formation of junctional complexes and lining of vessels with pericytes, resulting in the formation of well-organized, dichotomously branched, even-diameter vessels.

Vascular resolution is incomplete in tumour angiogenesis leading to the formation of irregular, dilated, tortuous and disorganized microvessels with partial endothelial lining, fragmentary basement membrane, high permeability, inconsistent diameter, highly branched structures and lack of differentiation (158, 159). Fenestrations within tumour vessels make them leaky and highly permeable. In addition, the chaotic architecture of tumour vessels are unevenly distributed within tumours leading to areas of high vascular concentration (vascular hotspots) that usually localized at the periphery of the tumour and areas of sparse vascular density that are often found in the tumour core (160). Although tumour vessel formation is disorganized and unregulated, the ability to hastily form microvessels is highly conducive to rapid tumour growth.

As a consequence of a disorganized vascular network and the absence of functional lymphatics (161, 162), tumour cells have limited access to oxygen and nutrients, and there is increased interstitial fluid pressure (163, 164). Interstitial fluid pressure (IFP) in most normal non-diseased tissues is tightly regulated and remains close to atmospheric levels (-3 to +3 mmHg). In solid malignant tumours, however, IFP is often elevated. Measurements of IFP in a variety of human tumours including head and
neck, cervix and breast cancers, are typically in the range of 10-40 mmHg (164-166).

Although the causes of elevated IFP levels in tumours are not fully understood, they likely involve blood vessel leakiness, the lack of functional lymphatics, interstitial fibrosis and a contraction of the interstitial space mediated by stromal fibroblasts (167). Poorly-formed tumour vasculature is associated with raised interstitial fluid pressure and poor drug distribution, which markedly affect therapeutic response and lead to resistance.

1.7.3.1 The influence of tumour vasculature and increased interstitial fluid pressure on drug distribution

Because blood flow within tumours is slow and intermittent, the delivery of therapeutics is inefficient. Many high-molecular weight anti-cancer drugs are transported from the circulatory system through the interstitial space by convection rather than by diffusion (168). Increased IFP contributes to decreased transcapillary transport in tumours, and decreased uptake of drugs or therapeutic antibodies into the tumour. High IFP is a major impeding factor on drug distribution preventing cancer cells from acquiring effective drug concentrations and reducing overall therapeutic efficacy.

Approaches to improve blood flow, and thereby reduce IFP and overcome this form of drug resistance, involve the use of VEGF antagonists, such as bevacizumab (169), platelet-derived growth factors (PDGF) inhibitors (170), TGFβ antagonists (171), hyaluronidase (172), and prostaglandin E₁ (PGE₁) (173). Inhibitors of VEGF and other anti-angiogenic agents used in combination with chemotherapy in experimental and clinical settings aim to eradicate two tumour subpopulations: tumour endothelial cells to prevent the formation of new vessels, and tumour cells. Cytotoxic agents kill cancer cells directly and anti-angiogenic agents kill cancer cells indirectly by depriving them of nutrients. Some studies have shown, however, that by destroying vasculature, delivery of
oxygen and therapeutics to the tumour is severely compromised, decreasing the effectiveness of chemotherapy agents as well as radiation (174-176). It has been proposed that the judicious application of anti-VEGF agents can prune immature and ineffective blood vessels, while sparing the more effective vessels. This effect of anti-VEGF agents has been shown to decrease vessel permeability and lower IFP. In this way, anti-VEGF agents might temporarily improve drug penetration in solid tumours (177, 178). This process is referred to as vessel normalization and provides the rationale for the use of antiangiogenic agents to enhance drug distribution and chemotherapeutic efficacy. The theory of vascular normalization seems to be model dependent as other studies have not been able to find such an effect (179).

PDGF controls the IFP of normal connective tissue by promoting interactions between integrins of stromal fibroblasts with ECM molecules and by stimulating contraction of these cells. PDGF inhibitors such as imatinib have been shown to lower IFP and increase uptake of chemotherapeutic drugs by blocking these interactions (170). It has been observed that the collagen-fiber network can affect IFP, and since TGFβ promotes ECM formation, its antagonists might cause a decrease in tumour IFP by reducing relative fibrosis in tumours (171). TGFβ may also have an effect of normalizing blood vessels. Hyaluronidase acts by degrading hyaluronan, which is a high-molecular weight polysaccharide that is an important constituent of the ECM to form large networks with proteoglycans. Hyaluronan attracts water and causes tissue swelling, and so degrading it is likely to alter the ECM and reducing the swelling and IFP (172). PGE₁ was observed to lower IFP by decreasing the contractility of stromal fibroblasts (173).
Although many strategies to affect tumour vasculature and IFP have been proposed, it is not possible to determine which approach has the best clinical potential. Some methods exert their effects immediately, while others take longer, some may have toxic effects on normal tissues, and some may actually promote tumour growth if not used in combination with other therapeutics. While, abnormal tumour vasculature and high interstitial fluid pressure are important modulators of drug distribution, mechanisms to overcome this form of resistance have yet to be validated in the clinic.

1.7.4 Tumour Hypoxia

Due to the inconsistency in vessel formation and morphology, the rates of blood flow may vary with location and time within a tumour. Along with abnormal vasculature, there is compression of blood vessels by cancer cells, which increases resistance to blood flow and impairs blood supply to the tumour (134). The limited vasculature and the large distances between tumour cells and capillaries results in a reduction of delivery of oxygen, leading to regions within tumours where oxygen gradients fall to zero (180). Cells in hypoxic regions may be viable, but they are often adjacent to regions of necrosis. Transient hypoxia is also common in tumours and results from the temporary shutdown of blood vessels (132, 181).

Tumour hypoxia is an important mechanism of drug resistance. Resistance to radiation is a well-known effect of hypoxia in solid tumours, but hypoxic cells may also be resistant to most anticancer drugs for several reasons: first, hypoxic cells are distant from blood vessels and, as a result, are not adequately exposed to some anticancer drugs (182, 183); second, sustained hypoxia, together with low concentrations of nutrient metabolites in hypoxic regions leads to inhibition of proliferation, and non-proliferating
cells are resistant to many anticancer drugs (184); third, hypoxia selects for cells that have lost sensitivity to p53-mediated apoptosis, which might lessen sensitivity to some anticancer agents; fourth, the action of some anticancer drugs resembles that of radiation in that oxygen increases the cytotoxicity of DNA lesions they cause (185); fifth, hypoxia up-regulates genes involved in drug resistance, including genes encoding PgP (186). Thus, cells in chronically hypoxic regions are likely to be resistant to most anti-cancer drugs.

1.7.4.1 Hypoxia as it relates to drug distribution

Fluctuations in blood flow that lead to transient hypoxia also lead to interruptions in the delivery of drugs through the same blood vessels. As a result, the effect on overall tumour sensitivity is likely to depend on the duration of the interruptions in blood flow relative to the duration for which effective concentrations of the anticancer drug are maintained in tumour blood vessels.

Some anticancer drugs generate free radicals that damage DNA. These drugs accept electrons from sources within the cell and then transfer the electrons to oxygen (187). Doxorubicin, for example, undergoes a chemical reduction to a semiquinone radical which in turn reduces oxygen to a superoxide that may contribute to cytotoxicity (188). Therefore drugs with limited distribution profiles such as doxorubicin are already in low concentrations in regions of hypoxia, and with low oxygen concentrations their activity is further reduced. An increasingly growing area of research is focussed on developing agents that are activated under hypoxic conditions. Nontoxic pro-drugs can be activated in a hypoxia-dependent manner. Essentially, hypoxia-selective cytotoxicity requires one-electron reduction of a relatively non-toxic pro-drug to a radical that then
becomes a substrate for back-oxidation by oxygen to the original form. If the so-formed radical or downstream products of the radical are much more toxic than the superoxide generated by redox cycling in oxic cells, hypoxia-dependent cytotoxicity arises. These pro-drugs can improve therapeutic index by complementing the selective activities of radiotherapy for well-oxygenated cells and of chemotherapy for cells closer to blood vessels. Tirapazamine was one of the first drugs to be developed specifically as a hypoxic cytotoxin (189). Clinical trial results have shown conflicting results that may be attributed to dose-limiting toxicities and the limited capacity of tirapazamine to penetrate tumour tissue to reach the sensitive tumour cells (190-193). Other hypoxia-activated agents such as AQ4N, PR-4, and TH-302 might have greater clinical potential because of their ability to better penetrate into tissues. Drug distribution studies suggest that AQ4N penetrates deep within experimental tumour tissue and selectively accumulates in hypoxic tumour cells. Combination treatment with mitoxantrone which distributes effectively in well-oxygenated regions of tumour (i.e. proximal to blood vessels), and AQ4N to hypoxic regions results in effective drug distribution over the entire tumour region (194). Thus agents that improve drug delivery or activity by targeting the tumour microenvironment, especially in hypoxic regions of tumours, are likely to show more promise in the clinical setting.

1.7.5 Tumour Acidity

Compared to normal tissues, the microenvironment of most solid tumours is acidic. Mechanisms leading to the acidic pH in tumours include environmental factors that occur during tumour growth, such as: deficiencies in tumour perfusion, due to the abnormal tumour vasculature, hypoxia and metabolic abnormalities associated with
transformation and uncontrolled cell growth, and possible increased capacity for transmembrane pH regulation. Also, tumours may use glycolysis with the production of lactate even under aerobic conditions, this is known as the Warburg hypothesis. Low blood supply and hypoxia have been observed to translate to altered pH gradients between the extracellular environment and the cytoplasm and between the cytoplasm and the endolysosomal vesicles (195-197). As well, there is evidence that indicates that hypoxia upregulates both the expression and activity of carbonic anhydrase in order to enhance the extracellular acidification (198). An important mechanism of tumour acidity is also through the production of lactate that occurs via glycolysis during anaerobic metabolism. Through the upregulation of hypoxia-inducible factor 1-alpha (HIF1-α) and the adaptation of a glycolytic phenotype with generation of lactate, tumour cells are selected to be able to survive in a hypoxic-anoxic environment (199). These environmental conditions favour the selection of highly malignant tumour cells that both contribute to tumour progression and are increasingly resistant to a wide range of chemotherapeutics.

The pH in the tumour microenvironment can also influence the cytotoxicity of anticancer drugs. Low extracellular pH has the effect of decreasing the ionization of weak acids and increasing the ionization of weak bases. Diffusion of drugs across the cell membrane usually occurs in the uncharged form, so that low extracellular pH leads to increased cellular uptake of weak acids (such as melphalan) and decreased cellular uptake and activity of weak bases (such as doxorubicin) (136, 200).

As tumours have an acidic pH compared with normal tissues, alkalinization has a greater effect on tumours compared with normal tissues and may be an area worth
exploiting for novel therapeutics. Alkalinizing treatment alone has no effect on growth rates of tumours. This suggests that metabolic alkalosis may result in the enhancement of antitumour activity for weakly basic chemotherapeutic drugs, while not enhancing the toxicity to the host. In vitro alkalinisation may alter the homeostasis of human tumour cells and this effect is consistent with in vivo sensitization of human tumours to cytotoxic agents (197).

**1.7.5.1 The effects of tumour acidity on drug distribution**

The pH gradient between the cytoplasm and the intracellular organelles may also be involved in resistance to anticancer drugs. The extracellular pH is often low in tumours and the intracellular pH is more alkaline, and there is a strong pH gradient across vesicular membranes of acidic organelles such as endosomes and lysosomes. Many chemotherapeutic agents, such as the anthracyclines doxorubicin and daunorubicin, vincristine, vinblastine, and mitoxantrone, are weak lipophilic bases. Since a substantial fraction of these molecules are uncharged at normal intracellular pH, they are able to freely penetrate the membranes of cytoplasmic organelles. When the drug encounters an acidic environment, such as the interior of acidic vesicles, it is converted into a charged form that is unable to cross internal membranes. This results in the sequestering and accumulation of such anticancer drugs in these organelles. Tumour cells closest to blood vessels receive the largest quantities of anticancer drugs. When drugs accumulate in the organelles of tumour cells they are ineffective and result in less drug being available to enter the nucleus to exert cytotoxic effects. Intracellular distribution is altered because of drug sequestration in acidic vesicles such as endosomes. Due to the accumulation of drug in tumour cells proximal to blood vessels, it is likely that less drug is available to
penetrate to cells distal from blood vessels in sufficient nuclear concentrations to cause cell death (Figure 1.6). Thus, altered pH gradients in tumour cells are likely to be an important factor in limiting drug distribution in solid tumours.

Agents that disrupt pH gradients in tumours may provide a strategy for overcoming this type of drug resistance. One approach may be to inhibit the function of the pumps that establish pH gradients. Vacuolar-\(H^+\)-ATPases (\(V-H^+\)-ATPases) represent a major mechanism in the regulation of pH (201). \(V-H^+\)-ATPases pump protons across the membranes of intracellular compartments. Some human tumour cells, particularly those exhibiting multidrug resistance, show enhanced \(V-H^+\)-ATPase activity, and increased acidity in intracellular vesicles (202, 203). Molecules that inhibit \(V-H^+\)-ATPases, such as proton pump inhibitors like omeprazole and pantoprazole, may reverse tumour resistance to cytotoxic drugs by alkalinizing acidic vesicles and preventing drug sequestration in these compartments (197, 204, 205). This approach may improve intracellular and extracellular drug distribution in solid tumours and holds promise as an effective method of overcoming drug resistance in some cancer patients (Figure 6). Other agents such as chloroquine can neutralize acidic organelles by competing as a weak base and potentially preventing drug sequestration.
Figure 1.6 Altered pH within tumour cells influences the intracellular distribution of basic anticancer drugs. When some drugs enter tumour cells proximal to blood vessels they are taken up into the nucleus as well as in acidic organelles such as endosomes (A). Drug molecules that are sequestered within endosomes are neither available to enter the nucleus to cause cell death nor are they available to distribute to cells distal from blood vessels (B).
1.7.6 Repopulation

An additional cause of drug resistance that relates to the tumour microenvironment is the repopulation of surviving tumour cells between courses of chemotherapy. Chemotherapy schedules allow for the recovery and repopulation of surviving cells in normal tissues (e.g., bone-marrow stem cells). However, during this period, repopulation of tumour cells also occurs, thereby increasing the number of tumour cells that must be eradicated, and limiting the effectiveness of chemotherapy. Studies have shown higher rates of proliferation of surviving tumour cells in chemotherapy treated tumours compared to untreated controls (206). Models of repopulation during courses of chemotherapy show that the rate of repopulation may increase in the intervals between successive courses of treatment (207).

1.7.6.1 Repopulation and drug distribution

Studies investigating the spatial origins of cells that participate in accelerated repopulation after treatment of multicellular tumour spheroids by chemotherapy show that there is a gradient of decreasing cell proliferation with increasing distance from the surface of spheroids, similar to that from tumour blood vessels (208-210). Most anticancer drugs are preferentially toxic to proliferating cells that are usually located near the tumour periphery or proximal to blood vessels. Therefore following chemotherapy, tumour cells close to blood vessels are likely to be killed because of their higher rate of proliferation, and better drug access. However, distal cells have the capacity to proliferate and repopulate the tumour. Therefore it is important for distal cells to receive cytotoxic concentrations of drug with each course of chemotherapy. The problem of repopulation is therefore exacerbated by limited drug distribution.
An approach to inhibiting repopulation in tumours may involve the use of cytostatic molecular-targeted agents. Repopulation probably depends on the activation of signalling pathways that stimulate the proliferation of tumour cells, and many molecular-targeted agents have been developed that inhibit these pathways. One such pathway involves the epidermal growth factor (EGFR). EGFR inhibitors such as gefinitib, erlotinib and the monoclonal antibody cetuximab are examples of agents that have shown some promise in the clinical setting. However, inhibition of repopulation by cytostatic agents between cycles of chemotherapy that are more active against cycling cells is complex and the outcome may greatly depend on treatment schedule. Combined treatment of cytostatic and cytotoxic agents with the appropriate scheduling may effectively sensitize tumours and circumvent this form of drug resistance (211).

1.8 METHODS FOR STUDYING DRUG DISTRIBUTION

1.8.1 In vitro models

*In vitro* multicellular models can be used to assess drug distribution, and they provide the 3-dimensional morphology of solid tumours that is lacking in monolayer cultures. In monolayer cultures, drug exposure is homogenous to each cell and this is quite different to the heterogeneity in drug concentration observed in solid tumours. These 3-dimensional models do lack some of the features of solid tumours such as extensive stroma, presence of vasculature and raised interstitial fluid pressure, and some of them do closely model intracellular interactions and the tumour metabolic environment. Solid tumours have large intercapillary distances and *in vitro* models can mimic avascular regions of tumours and assess drug distribution through solid tumour
tissue. Although factors such as pharmacokinetics and hepatic metabolism are not modelled by \textit{in vitro} techniques, this may be advantageous to study drug distribution for drug discovery and development without having to consider these complicating factors in initial stages.

\subsection*{1.8.1.1 Spheroids}

One approach to studying drug distribution of tumour microregions is to culture cancer cells in the form of 3-dimensional multicellular spheroids that simulate micrometastases and microregions of larger tumours (212-214). They are spherical aggregates of tumour cells that grow in contact to form nodules in suspension culture (Figure 1.7). This tumour model is intermediate in complexity between standard 2-dimensional monolayer cultures \textit{in vitro} and solid tumours \textit{in vivo}. Similar to solid tumours, spheroids show properties such as development of an ECM, tight junctions between epithelial cells and gradients of nutrient concentration and cell proliferation from the exterior to the centre (212). Larger spheroids show heterogeneous cell populations with a necrotic core surrounded by a viable yet quiescent rim of cells and proliferating cells in the outer 3-5 cell layers (213, 215, 216). The distribution of fluorescent or radiolabelled drugs has been studied in spheroids (217-220). Penetration of a drug through tissue can be measured by adding the drug to the media and assessing the penetration of the drug through the spheroid using fluorescent imaging techniques. Investigations using this method have indicated poor penetration within tumour tissue of several drugs including doxorubicin and methotrexate (212, 221). These studies showed that poor distribution of such drugs was due to rapid binding by cells in the outer layer of spheroids.
1.8.1.2 Multilayered Cell Cultures

Quantitative assessment of drug distribution is limited in spheroids. A conceptually simple technique was established by Wilson et al., that allows the direct assessment of tissue penetration by anticancer drugs (222). Tumour cells are grown on a collagen-coated microporous Teflon membrane as a multilayered cell culture (MCC) that has similar characteristics to tumour tissue in vivo. MCCs typically achieve a thickness of ~200µm, similar to the maximum distance between blood vessels and necrosis in human tumours (223). Similar to spheroids, MCCs have been shown to reflect many of the properties of solid tumours, including the generation of an ECM, gradients of nutrient concentration and cell proliferation and regions of hypoxia and necrosis in thicker layers (224-226). To examine the penetration of an index drug, the compound is added on one side of the MCC, and its appearance on the other side of the MCC is measured by appropriate analytical methods (Figure 7). Since drugs must move through the cell layers and into the receiving reservoir, this model permits direct assessment of penetration of drugs through tumour tissue as a function of time (227). MCCs can also be sectioned and the distribution of fluorescent drugs can be visualized directly. Previous studies have shown poor penetration of many commonly used anticancer drugs, such as doxorubicin and 5-fluorouracil, through MCCs generated from several human and murine cell lines (224, 228). Using this model system, it has been established that cells close to the source of drug are exposed to high concentrations, whereas distal cells are likely to experience only low concentrations of drug. Cells distal from blood vessels are likely to have minimal drug available for uptake.
Figure 7. *In vitro* models to study drug distribution include multicellular spheroids (A), and multilayered cell cultures (MCCs) (B). The set up of MCCs allows for quantitative measurements of drug penetration as a function of time (C).
1.8.2 *In vivo models*

Pharmacokinetic studies attempt to quantify total drug concentrations in tumours using high performance liquid chromatography (HPLC) or mass spectrometry. These analytical methods are able to measure mean drug concentration within solid tumours as a function of time, and to distinguish between the parent compound and its metabolic products. However, these methods do not assess drug distribution within tumours because they do not provide a spatial representation of drug distribution. Large drug concentrations in cells proximal to blood vessels with minimal to no drug in distal cells may average to adequate levels in the tumour as a whole. However, it is important for adequate concentrations of drug to reach all viable cells.

Initial studies of drug distribution in solid tumours used the auto-fluorescent drug doxorubicin (229). The spatial distribution of doxorubicin relative to blood vessels was first assessed in breast tumour biopsies by overlaying digital images of doxorubicin with immunostained images of endothelial cells (stained with CD31 antibody) using fluorescence microscopy (230). Studies in our laboratory have used this method to generate gradients of drug distribution in relation to blood vessels in solid tumours extracted from subcutaneous mouse models. Drug distribution profiles are overlaid with blood vessels and these composite images are quantified using sophisticated algorithms. In our laboratory, this algorithm calculates the intensity of every pixel, its distance from the nearest blood vessel and then sums these over defined regions of interest in a tumour section. Markers of hypoxia, such as EF5, can also be injected to enable the recognition of hypoxic regions using a fluorescent-labelled antibody applied to the same sections. As well, markers of blood flow such as Hoescht 33342, lectins, or DiOC7, can be
injected into mice just prior to tumour extraction to visualize functional blood vessels. Studies in our laboratory have used this method to quantify doxorubicin distribution in relation to blood vessels in xenografts and have shown that doxorubicin intensity decreases to half at approximately 40-50μm from the nearest blood vessels such that many viable cells are not exposed to detectable concentrations of drug after a single injection (231). Using this method of overlaying drug fluorescence with blood vessels and hypoxic regions, photomicrographs can also be generated to qualitatively assess drug distribution in solid tumours (Figure 1.8).
Figure 1.8  Photomicrograph of doxorubicin distribution in a 16C mouse mammary tumour xenograft. Ten minutes after drug administration, doxorubicin fluorescence (blue) is observed mostly in regions proximal to blood vessels (red). There are large regions of viable cells between blood vessels and hypoxic regions (green) that show little to no drug. (Adapted from Primeau et al., 2005; (231)).
1.9 RATIONALE

A large research effort has been devoted to understanding the multifactorial causes of drug resistance. Most of the literature has emphasized cellular and molecular causes of resistance, and although understanding these factors is essential for the development of novel treatment strategies, mechanisms related to the microenvironment of solid tumours may also have a profound effect on the sensitivity of tumours to chemotherapeutic agents. Drug distribution is likely to be an important cause of drug resistance since large numbers of tumour cells receive little to no drug. Vascular structure and function in tumours is different to that in normal tissue; tumour vessels are observed to be leaky, often tortuous and highly permeable (137). These abnormalities result in irregular blood flow and high interstitial fluid pressure within the tumour, which can impair the delivery of oxygen (a known radiation sensitizer) and drugs to the tumour. Cellular uptake of anticancer drugs in cells proximal to blood vessels, leaves less drug available for distribution to more distal cells. Drug efflux pumps such as P-glycoprotein prevent cellular accumulation of drugs by their active removal from cells, and have been shown to alter drug penetration in MCCs (232). However, it is unclear whether this applies to solid tumours and whether quantitative fluorescence microscopy of solid tumour sections is sufficiently sensitive to detect changes in drug distribution. Increased cellular uptake of basic drugs may be due to their sequestration in acidic compartments such as endosomes, where they are not thought to exert cytotoxic effects (204, 233, 234). Such uptake and sequestration may limit the amount of drug that is available to distal cells. Obtaining information about the factors that affect drug distribution is important because from these studies a potential strategy to improve drug distribution may be
found. Since vascular access is far more limited in tumours than in normal tissues, strategies to improve drug distribution are likely to have a much greater effect on tumour growth delay than on toxicity to normal tissues, and may be expected to improve the therapeutic index. One potential strategy to improve drug distribution in solid tumour tissue involves the inhibition of the sequestration of basic anti-cancer drugs in acidic compartments.

In this thesis, I have undertaken studies to gain insight as to how drug distribution differs between normal and tumour tissue, whether drug distribution within solid tumours is modificable, and whether there are agents that can overcome specific limiting factors, enhance drug distribution, and improve therapeutic efficacy of the anticancer drug doxorubicin.

1.10 HYPOTHESES

1. The distribution of anticancer drugs from blood vessels is better in normal tissues, such as the heart and liver compared to the distribution in solid tumours.

2. The distribution of doxorubicin in solid tumours is increased if tumour cells express high levels PgP, and decreased by agents that inhibit the function of PgP.

3. The distribution of doxorubicin in solid tumours is improved by pre-treatment with the agent pantoprazole, which inhibits sequestration of basic drugs in acidic endosomes of cells.
1.11 OBJECTIVES & SPECIFIC AIMS

The aims of this thesis are to assess the distribution of anticancer drugs in normal tissues and tumour tissue, determine the effect of altered cellular uptake due to P-glycoprotein on drug distribution in tumour tissue and to evaluate whether inhibition of drug sequestration can improve drug distribution in solid tumours and enhance tumour growth delay.

Objectives:

I. To qualitatively and quantitatively compare the distribution of three anticancer drugs in normal tissue versus tumour tissue over time

II. To determine whether PgP overexpression alters drug distribution in solid tumours and establish whether changes in drug distribution can be quantified in experimental tumours

III. To evaluate the effect of pantoprazole pre-treatment on distribution and activity of doxorubicin in solid tumours
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CHAPTER 2

DISTRIBUTION OF ANTICANCER DRUGS IN TUMOURS AND NORMAL TISSUES

Krupa J. Patel and Ian F. Tannock
2.1 ABSTRACT

**Background:** The effectiveness of chemotherapy is limited by drug resistance and by toxicity to normal tissues. While pharmacokinetic analyses can estimate the mean concentration of drug within a given tissue, they do not give information about the spatial distribution of drugs. Here, we compare the time-dependent spatial distribution of three different anticancer drugs within tumours and normal tissues.

**Methods:** Mice bearing various human tumour xenografts were treated with doxorubicin, mitoxantrone or topotecan, all of which are autofluorescent, thus allowing their quantification in tissue sections by immunohistochemistry. After 10 mins, 3 hours or 24 hours, tumours and samples of heart, kidney, liver and brain were excised and frozen. Tissue sections were analyzed for drug fluorescence in relation to distance from the nearest blood vessel. Hypoxic regions were also identified within tumour tissue by uptake of EF5.

**Results:** The distribution of doxorubicin within solid tumours was limited to perivascular regions but was relatively uniform in the heart, kidney and liver. Little to no drug fluorescence was observed in the brain. Gradients of drug distribution in tumours were steep at 10 minutes after drug administration and become shallower over time, with a decrease in overall fluorescence. All three drugs led to increased regions of hypoxia in tumours at 3 hours after treatment, but the extent of hypoxia decreased after 24 hours. Drug-associated fluorescence decreased with time in the heart, kidney and liver.

**Conclusions:** Spatial distributions of anticancer drugs can provide important information about limitations of drug delivery to tumours and about toxicity within normal tissue.
2.2 INTRODUCTION

Effective treatment of solid tumours with anticancer drugs requires both that constituent cells are sensitive to the drug and that the drug achieves a concentration within the tumour cells sufficient to cause cytotoxicity. The ability of anti-cancer drugs to gain access to solid tumours depends on efficient delivery of drugs through the vascular system of the tumour, and penetration of drugs to tumour cells that are distant from blood vessels.

Toxicity to normal tissue limits the dose of a drug that can be administered. Drugs are distributed in the blood, which is likely to provide greater access to well-perfused organs such as the heart, liver and kidney as compared to poorly perfused tumours.

The distribution of a drug within the body is governed by factors such as blood flow to different organs, diffusion, protein and tissue binding, and lipid solubility. In general, drugs with extensive binding to tissues (e.g. doxorubicin) or with high lipid solubility will tend to exhibit prolonged elimination phases because there is slow release from tissues. Metabolism of drugs takes place primarily in the liver and consists of oxidative and reductive reactions via the cytochrome P-450 system. Most drugs are eventually eliminated from the body by the kidney or through the biliary tract.

Doxorubicin is a naturally occurring anthracycline that has cytotoxic effects attributed to DNA intercalation and alkylation, generation of reactive oxygen species, interference with RNA and DNA polymerase and inhibition of topoisomerase II (1, 2). Doxorubicin has been used for many years as part of combination chemotherapy regimens in the treatment of lymphomas, leukemias and solid tumours (3). The initial
distribution half-life ($t_{\alpha}$) of doxorubicin in mice is approximately 4-5 minutes (4), and in humans approximately 11 minutes (5). Dose-limiting toxicities are myelosuppression and dose-dependent cardiac toxicity (3, 6-8). Mitoxantrone is an anthracenedione that differs from the anthracyclines in lacking the sugar and the tetracyclic ring. It is a synthetic drug with three planar rings that intercalates into DNA and inhibits topoisomerase II. Mitoxantrone has been used in chemotherapy regimens for leukemia, lymphoma, breast and prostate cancer, and to treat multiple sclerosis (9-11). The mean $t_{\alpha}$ of mitoxantrone ranges from 6-12 minutes in mice and 6 minutes in humans (12). Myelosuppression and dose-dependent cardiomyopathy are major toxicities (13).

Topotecan is a semisynthetic, water-soluble derivative of camptothecin and is a potent inhibitor of topoisomerase I (14, 15). It is primarily used for the treatment of ovarian cancer and small cell lung cancer (16-19). The $t_{\alpha}$ of topotecan in mice is approximately 30 minutes (20) and 6 minutes in humans (21) and the major toxicity is myelosuppression (22).

In various pharmacokinetic studies, doxorubicin, mitoxantrone and topotecan have attained quite high concentration in normal tissues such as the liver, kidney and heart (23-25). These studies utilized techniques such as high performance liquid chromatography (HPLC) or liquid scintillation to determine mean concentration of drug within normal and tumour tissues as a function of time after drug administration. However, these techniques do not give information about the distribution of drug within that tissue. Studies examining the distribution of various anti-cancer drugs in solid tumours have shown limited penetration in relation to blood vessels (26-31). Compared to tumours, drug delivery to normal tissues, such as the liver, kidney and heart, is likely
to be more efficient due to a highly organized vascular structure and large pressure gradients across the endothelial membrane. In contrast, the brain has been reported to have limited accumulation of drug due to various mechanisms utilized by the blood-brain barrier (BBB) to exclude toxic substances (32).

In the present study we examine the time-dependent distribution of three anti-cancer drugs, doxorubicin, mitoxantrone and topotecan, in a human tumour xenograft model, and compare drug distribution in tumours with that in the liver, kidney, heart and brain.

2.3 MATERIALS AND METHODS

2.3.1 Drugs and reagents

Doxorubicin (Pharmacia, Mississauga, Canada) was purchased from the hospital pharmacy as a solution diluted in 0.9% sodium chloride to a concentration of 2 mg/mL. Mitoxantrone (Mayne Pharma) was obtained from the hospital pharmacy as a solution diluted in 0.8% sodium chloride at a concentration of 2mg/mL. Topotecan was also obtained from the hospital pharmacy as a powder and was dissolved in phosphate-buffered saline (PBS) to a concentration of 1mg/mL. Purified rat anti-mouse CD31 (platelet/endothelial adhesion molecule 1) monoclonal antibody was purchased from BD PharmMingen (Mississauga, Canada), and Cy3-conjugated goat anti-rat IgG secondary antibody was purchased from Jackson Immuno Research Laboratories, Inc. (Pennsylvania, USA). The hypoxia-selective agent EF5 and Cy5-conjugated anti-EF5 antibody were provided by Dr. C.J. Koch (University of Pennsylvania).
2.3.2 Cell lines

The human breast cancer cells MDA-MB-231 and MCF-7, the human vulvar epidermoid carcinoma cell line A431 and the prostate cancer cells PC-3 and DU145 were purchased from the American Type Culture Collection (ATCC; Virginia, USA). MDA-MB-231, MCF-7 and DU145 cells were maintained in α-MEM (Life Technologies, Inc), A431 cells were maintained in Dulbecco’s Modified Eagle’s Medium (Life Technologies, Inc), and PC-3 cells were maintained in Ham's F-12K medium (Life Technologies, Inc.). All media were supplemented with 10% fetal bovine serum (FBS; Hyclone) at 37°C in a humidified atmosphere of 95% air plus 5% CO₂.

2.3.4 Mice

Athymic nude female mice were purchased from Harlan Sprague-Dawley Laboratory Center at about 6 weeks of age. Mice were housed five per cage and allowed 1 week to acclimatize in our animal colony before experimentation. The animals received filtered sterilized water and sterile rodent food ad libitum. Tumours were generated by subcutaneous injection of 1 x 10⁶ exponentially growing cells into the left and right flank regions. Drug distribution studies were initiated when tumour diameters reached approximately 6-8 mm. All procedures were carried out following approval of the Institutional Animal Care Committee in accordance with the Canadian Council on Animal Care guidelines (Animal Protocol # 12329).

2.3.5 Evaluation of drug distribution

Tumour-bearing mice were treated intravenously with 25 mg/kg of doxorubicin, mitoxantrone or topotecan, with the exception of PC-3 and DU145 tumour-bearing mice which were treated with 40mg/kg of mitoxantrone to facilitate detection and
quantification of drug auto-fluorescence. Mice in the control group were treated with phosphate-buffered saline. Mice also received 0.2mL of a 10 mM solution of EF5 intraperitoneally 2-3 hours prior to being killed to identify hypoxic tumour regions. Animals were killed at 10 minutes, 3 or 24 hours after drug injection. The tumours, liver, kidney, heart and brain were excised and the tissues were embedded immediately in OCT compound, frozen in liquid nitrogen, and stored at -70°C prior to tissue sectioning and immunohistochemical staining. From each tissue cryostat sections 10 µm thick were cut at 3 levels approximately 100 µm apart, mounted on glass slides and allowed to air dry.

2.3.6 Fluorescence imaging

Fluorescence was quantified utilizing an Olympus Upright BX50 microscope with a 100W HBO mercury light source using the Olympus UPlanSApo 10X/0.40 objective lenses. Tissue sections were imaged with a Photometrics CoolSNAP HQ2 (monochrome for fluorescence imaging) camera, keeping exposure settings the consistent, and tiled using a motorized stage so that the distribution of drug was measured across the entire tissue section. Doxorubicin was imaged using the Cy3 (530-560 nm excitation/573-647 nm emission) filter set, mitoxantrone and topotecan were imaged using the FITC (490 nm excitation/ 525 nm emission) filter set. All images were captured at 8-bit signal depth and subsequently pseudo-coloured. Sections were then stained for blood vessels using antibodies specific for the endothelial cell marker CD31 [rat anti-CD31 primary antibody (1:100); BD Biosciences, and C73-conjugated goat anti-rat IgG secondary antibody (1:400)], hypoxic regions were identified using a Cy5-conjugated4d mouse anti-EF5 antibody (1:50). Tumour sections were imaged for CD31
using the Cy3 (530-560 nm excitation/573-647 nm emission) filter set, and EF5 using the Cy5 far-red filter set.

2.3.7 Image analysis

Composite images of drug and CD31 were generated utilizing Media Cybernetics Image Pro PLUS software (version 5.0). Images displaying anti-CD31 staining were converted to a black and white binary image, and small white objects were removed as artifacts based on conservative estimation of minimal capillary diameter (33). The resultant image was overlaid with the corresponding field of view displaying drug fluorescence resulting in an 8 bit black and white image with blood vessels identified by pixels with intensities between 250 and 255 (white) and fluorescence representing drug concentration ranging from 0-249. Several regions, 1.6 mm$^2$ in area, with moderate blood vessel density were selected from each tissue section. Areas of necrosis and staining artifact were excluded. To minimize noise from tissue auto-fluorescence a minimum signal level just below threshold for detection of drug was set for each tissue section; this was based on an average background reading from regions without nuclear fluorescence/staining. The pixel intensity (the area of each pixel was 0.4 μm$^2$) and distance to the nearest vessel for all pixels within the selected region of interest above threshold were measured with a customized algorithm. Drug intensity (I) was averaged over all pixels at a given distance (x) from the nearest vessel and plotted as a function of distance to the nearest vessel. Linear regression was performed to correlate the average drug fluorescence intensity with distance from the nearest blood vessel. The gradient was determined from the slope of the linear regression. The fluorescence intensity in regions closest to blood vessels was determined from the y-intercept of the linear regressions.
The area under the curve was calculated for tumour tissue with a maximum distance from the nearest vessel in the section of 120μm and for normal tissue with a maximum distance of 60μm because of noise of close-by adjacent vessels.

The concentration of blood vessels per tumour area was determined by counting the number of “objects” that fell within the 250-255 pixel intensity range. “Objects” are made up of adjacent pixels that have the same range in intensity, and those with an area below 62 μm² were gated out using ImagePro Plus Software. The number of “objects” (blood vessels) was divided by the area of the tumour region being quantified. To quantify hypoxic regions, a threshold range between 30-50 intensity units was set for images with EF5 staining. The area of positive staining within this intensity was calculated and divided by the tumour area.

The slopes of the gradient, y-intercept, area under the curve, quantity of blood vessels per tumour area and hypoxic regions per tumour area were tested for statistical significance using one-way ANOVA tests (assuming unequal variances between groups), and subsequent t-tests.

Coloured photomicrographs were generated using ImagePro Plus software. Grayscale images were converted to RGB 24 bit images and pseudo-coloured.

2.4 RESULTS

2.4.1 Distribution of drugs in tumours

Photomicrographs of various tumours indicate limited distribution of doxorubicin and mitoxantrone such that the majority of fluorescence due to the drug is localized near blood vessels and areas away from vessels show little or no drug (Figure 2.1a-d). There
are steep gradients of drug distribution with decreasing fluorescence intensity at increasing distance from the nearest blood vessel. The distances at which drug fluorescence falls to half are within a small range among the four tumour lines (Table 2.1).

**Table 2.1**: Drug distribution gradients and distance at half intensity in PC3 and DU145 tumours treated with mitoxantrone and A431 and MCF-7 tumours treated with doxorubicin

<table>
<thead>
<tr>
<th>Tumour Type - Drug</th>
<th>Regression Coefficient</th>
<th>Slope of Gradient</th>
<th>Distance at $I_{\alpha}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC3 – MITOX</td>
<td>0.95</td>
<td>-0.22</td>
<td>50</td>
</tr>
<tr>
<td>DU145 – MITOX</td>
<td>0.99</td>
<td>-0.23</td>
<td>50</td>
</tr>
<tr>
<td>A431 – DOX</td>
<td>0.99</td>
<td>-0.089</td>
<td>50</td>
</tr>
<tr>
<td>MCF-7 – DOX</td>
<td>0.91</td>
<td>-0.13</td>
<td>59</td>
</tr>
</tbody>
</table>

$I_{\alpha}$ – distance (µm) at which fluorescence intensity falls to half

Images showing the time-dependent distribution of doxorubicin, mitoxantrone and topotecan in MDA-MB-231 xenografts after injection of equal doses into mice are shown in Figure 2.2. These images demonstrate limited penetration of these anticancer drugs to tumour regions far from blood vessels. At ten minutes after injection, mitoxantrone had a gradient of decreasing fluorescence in relation to distance to the nearest blood vessel that was much steeper than for either doxorubicin or topotecan. The total fluorescence intensity of mitoxantrone in relation to blood vessels (area under the curve) was higher than that of doxorubicin by 5-fold ($p<0.01$), and topotecan by 3-fold ($p<0.01$) (Figure 2.3a). Similarly, 3 hours after drug administration, mitoxantrone had steeper gradients of decreasing fluorescence with distance from blood vessels, and higher total fluorescence compared to doxorubicin and topotecan ($p<0.05$) (Figure 2.3b). After 24 hours, all three drugs show similar gradients of decreasing fluorescence, but total
mitoxantrone fluorescence in relation to blood vessels remained consistently higher than that of doxorubicin (p<0.05) (Figure 2.3c).

For all three drugs, fluorescence in regions adjacent to blood vessels was maximal at 10 mins, and decreased thereafter (Table 2.2). Total fluorescence of each drug in tumours decreased significantly between 10 minutes and 3 hours (p<0.01) and for mitoxantrone from 3 hours to 24 hours as well (p<0.05). Among the three drugs, doxorubicin showed a 4-fold shallower fluorescence gradient at 24 hours compared to at 3 hours (p<0.05), and subtle decreases in fluorescence in regions adjacent to blood vessels over time (Table 2.2). In contrast, mitoxantrone showed an approximately 10-fold shallower distribution gradient between 10 mins and 3 hours and a 6-fold decrease between 3 hours and 24 hours (p<0.05 for both).

Table 2.2 Characteristics of drug distribution gradients in relation to distance to the nearest blood vessel

<table>
<thead>
<tr>
<th>Drug</th>
<th>Time After Injection</th>
<th>Slope of Gradient</th>
<th>Fluorescence Adjacent to Blood Vessels</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Doxorubicin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mins</td>
<td>-0.013 ± 0.013</td>
<td>6.9 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>3 hrs</td>
<td>-0.012 ± 0.003</td>
<td>5.2 ± 0.51</td>
<td></td>
</tr>
<tr>
<td>24 hrs</td>
<td>-0.003 ± 0.003</td>
<td>4.0 ± 0.48</td>
<td></td>
</tr>
<tr>
<td><strong>Mitoxantrone</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mins</td>
<td>-0.42 ± 0.070</td>
<td>61 ± 9.6c</td>
<td></td>
</tr>
<tr>
<td>3 hrs</td>
<td>-0.04 ± 0.010</td>
<td>9.0 ± 0.46c</td>
<td></td>
</tr>
<tr>
<td>24 hrs</td>
<td>-0.007 ± 0.002</td>
<td>6.2 ± 0.07</td>
<td></td>
</tr>
<tr>
<td><strong>Topotecan</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mins</td>
<td>-0.024 ± 0.0004</td>
<td>14 ± 1.9d</td>
<td></td>
</tr>
<tr>
<td>3 hrs</td>
<td>0.003 ± 0.004b</td>
<td>4.3 ± 0.39d</td>
<td></td>
</tr>
<tr>
<td>24 hrs</td>
<td>0.002 ± 0.002b</td>
<td>4.4 ± 0.19</td>
<td></td>
</tr>
</tbody>
</table>

a,b,c,d indicate statistical significance between the pairs of means (p< 0.05)

2.4.2 Change in tumour vasculature and hypoxia following drug treatment

The concentration of blood vessels was compared with the 10 minute time point, since this reflects the control value for untreated tumours; we did not evaluate blood flow
which might have changed within this short interval. Twenty-four hours after doxorubicin
treatment, there was a decrease in the number of total blood vessels per tumour area
(p<0.05) compared to 3 hours post-treatment. After mitoxantrone and topotecan
treatment there was no significant change in the number of blood vessels over time
(Figure 2.4a).

Large regions of hypoxia were visible in tumour tissue at all three time points,
and there was a consistent trend of increased hypoxia at 3 hours compared to 10 minutes
after administration of for all three drugs (p=0.07 for doxorubicin and topotecan).
Twenty-four hours after any of the three drug treatments there were substantially fewer
regions of hypoxia (p<0.01), although there remained more hypoxia in doxorubicin
treated tumours compared to those treated with either mitoxantrone or topotecan
(p<0.001) (Figure 2.4b).

2.4.3 Drug distribution in heart, kidney and liver

In contrast to tumour tissue, doxorubicin fluorescence was observed throughout
heart, kidney and liver tissue (Figure 2.5a-d). Perivascular fluorescence of doxorubicin
was much higher in the normal tissues than in the tumour at 10 minutes after injection
(Figure 2.6a). There were steep gradients of decreasing fluorescence with increasing
distance from blood vessels in the heart, kidney and liver at 10 minutes after injection of
doxorubicin to a maximum of 60μm. At distances from blood vessels greater than 60μm,
fluorescence signal from nearby vessels created noise in the fluorescent intensity gradient
(Figure 2.5e).
The concentration of blood vessels in liver tissue was less than in the kidney and tumour (p<0.05) with the concentration in the heart being intermediate (Figure 2.6b). The much lower perivascular concentration of drug in the tumour therefore implies much reduced net delivery of drug to the tumour.

Photomicrographs of doxorubicin distribution in the heart, kidney and liver, 10 minutes, 3 hours and 24 hours after administration show decreasing fluorescence over time (Figure 2.7). Ten minutes after doxorubicin is given, fluorescence intensity is observed throughout the normal tissues. After 3 hours doxorubicin fluorescence intensity is decreased slightly and after 24 hours it is substantially lower (Figure 2.7).

Mitoxantrone fluorescence was observed throughout the heart, kidney and liver 3 and 24 hours after intravenous administration (Figure 2.8). Similarly, topotecan fluorescence was observed in these tissues at 10 minutes, 3 and 24 hours after intravenous administration (Figure 2.9). As in the tumours, doxorubicin and mitoxantrone staining within the liver and heart is localized within the nucleus, whereas in the kidney, the staining appears more diffuse throughout the cell. Topotecan fluorescence is not confined to the nucleus and has more diffuse staining in the heart, kidney and liver.

In all normal tissues at 10 minutes after drug administration, the steepest gradients of decreasing fluorescence occurred within the first 30μm from blood vessels. At greater distances, there was a substantially shallower gradient of decreasing fluorescence intensity. Similar patterns were observed at 3 hours and 24 hours within the first 30μm, but the gradients were much shallower than at 10 minutes. Gradients for the heart and kidney were not evident at distances greater than 60μm due to fluorescence noise from neighbouring vessels. This effect was less evident in liver, probably because of the
organization of blood vessels into portal triads and there were fluorescence signals up to 100μm away from blood vessels. Twenty-four hours after drug administration, fluorescence distributions in normal tissues are fairly uniform, with the exception of mitoxantrone in the liver (Figure 2.10).

Comparing total drug fluorescence in relation to blood vessels over time, topotecan was observed to have the lowest levels of drug after all three time points in the heart and kidney and the highest levels in the liver. Mitoxantrone showed a decrease in total drug fluorescence over time in the kidney and the liver and not in the heart. All three drugs had the highest total fluorescence in the liver compared to the heart and kidney, probably reflecting their clearance through the biliary tract (Figure 2.11).

The contrast in drug distribution between liver and metastatic tumour in the liver is illustrated for mitoxantrone in Figure 2.12. Regions of tumour have markedly less fluorescence than regions of normal liver tissue despite the presence of blood vessels in both. In the central area of the tumour no blood vessels are visible and this area is likely necrotic.

2.4.4 Drug distribution in the brain

Blood vessels within the brain show a high level of organization, consistent thickness and structured branching patterns. Drug distribution within the brain however, is very limited. While some drug fluorescence was observed within blood vessels, little to no doxorubicin, mitoxantrone or topotecan fluorescence was observed in cells of the brain 10 minutes, 3 hours or 24 hours after administration (Figure 2.13).
2.5 DISCUSSION

Drug delivery to tissues involves three processes: distribution through the vascular space, transport across microvessel walls, and diffusion and/or convection through tissue (34). Tumour vasculature has distinct characteristics compared to normal blood vessels that lead to unique drug distribution profiles within tumour tissue. Blood vessels in tumours are often dilated, convoluted and highly branched (35), flow through them is irregular and frequently static, and the vessel walls may have fenestrations, inconsistent basement membranes, and few pericytes (36, 37). These abnormalities tend to result in leaky tumour vessels that create variable permeability within tumours (38). In normal tissues, fluid is removed through a network of lymphatic vessels and through veins. Solid tumours often lack functional lymph vessels which may lead to increased interstitial fluid pressure, leading to inhibition of the distribution of larger molecules by convection (39, 40). The penetration of most small molecule drugs relies on diffusion, which is dependent on molecular properties such as size, charge and solubility, and on avidity of binding in tissue. Poor diffusion, particularly for agents that are bound to or within proximal cells is likely to lead to poor drug delivery to cells distal from blood vessels. Our studies and others have shown limited drug distribution in various solid tumour models such that much of the drug that is delivered to the tumour remains localized in areas closest to blood vessels (28, 29, 41, 42). The predominant perivascular localization of drugs after only 10 minutes may be due to their binding properties. Studies using tumour cell spheroids show that drug binding affects drug penetration such that drugs that do not bind to cellular macromolecules (such as 5-fluorouracil, cisplatin and monoclonal antibodies) readily penetrate spheroids (43). In contrast, drugs with high
binding affinities, such as methotrexate, vinblastine, paclitaxel and the ones used in our study, remain localized in the periphery of spheroids (43, 44). Interestingly, measures of average drug concentration in spheroids indicate that high-binding drugs show higher concentrations than low-binding drugs. This implies that average tumour concentration is not a good indicator of drug distribution within tumour tissue and studies of spatial distribution are necessary.

In our studies the gradients of decreasing drug fluorescence in relation to distance to nearest blood vessels are likely to be even steeper than we demonstrate due to (i) some non-perfused blood vessels within the tumour tissue being averaged with the perfused vessels, and (ii) the presence of neighbouring blood vessels outside of the tissue section. Both of these effects will lead to flattening of the apparent gradient of distribution within tumour tissue.

Multiple factors affect drug distribution. In addition to physicochemical properties of the drug/macromolecule such as binding affinity and diffusion coefficients, biological properties of a tumour are also important. These include blood flow, interstitial fluid pressure, regional vessel distribution, cell density, and the extracellular matrix. Some of these properties of tumours are dynamic and change with time, and drug distribution is also therefore a dynamic process that may change with time after drug administration. The intensity of drug fluorescence may be strongly time-dependent due to the changing concentration of the drug in circulation over time. Our studies show a dramatic decrease in total fluorescence due to doxorubicin, mitoxantrone or topotecan in solid tumours between 10 minutes and 3 hours after each drug is given. The gradient of drug concentration in relation to blood vessels also decreases with time for all three drugs.
and most sharply for mitoxantrone. Twenty-four hours after drug treatment, shallow
gradients imply more even distribution than at earlier time points, but the total amount of
drug fluorescence shows substantially less drug within the tumours compared to the
amount of drug fluorescence at 10 minutes.

Our results indicate an increase in hypoxia, 3 hours after treatment for all three
drugs. Doxorubicin has been previously reported to alter tumour blood flow and
subsequently lead to an increase in acute hypoxia (45). Doxorubicin also caused a
reduction in the concentration of total blood vessels, but use of perfusion markers such
Hoescht 33342 and DiOC7 would be needed to demonstrate alterations in blood flow over
time. The anti-angiogenic effects of chemotherapeutic agents have been reported using
both in vitro and in vivo models (46, 47) and are likely to influence levels of hypoxia.
Varying blood flow and hypoxic levels in tumours have major implications for
effectiveness of treatment with both chemotherapy and radiotherapy.

In normal tissue the vascular system is more orderly and functional leading to
more homogeneous drug distribution. We found that the distribution of drugs in normal
tissue was more uniform than in the tumour with a higher level of tissue exposure.
Although there are steep gradients of decreasing drug concentration in relation to blood
vessels at 10 minutes after injection, especially for doxorubicin, by 3 hours the drugs are
fairly evenly distributed. While drug concentration decreases over time, there are still
large quantities of drug within normal tissues even at 24 hours. The plasma kinetics of
doxorubicin following a bolus injection have been shown to exhibit a rapid initial decline
followed by a slow decline in plasma concentration which has been ascribed to the ability
of tissues to rapidly accumulate the drug intracellularly followed by a slow release of the
drug from tissue stores as plasma levels decline due to drug elimination (48). Although there are many routes of elimination, the primary clearance of doxorubicin is through biliary elimination (49), clearance of mitoxantrone is biliary and fecal and of topotecan is through biliary and urinary elimination (50).

Drug delivery in the brain is unlike that of other normal tissues or tumour tissue due to the presence of the blood brain barrier (BBB). The function of the BBB is to maintain a constant internal environment inside the brain by strictly regulating the extracellular fluid composition and to protect the brain against potentially toxic substances. Endothelial cells of the capillaries in the brain form the BBB that is impermeable due to the presence of tight junctions between endothelial cells and the absence of fenestrations (57). Due to limited mechanisms of transport through the BBB, such as passive diffusion of small molecules, endocytosis and carrier-mediated efflux (e.g. P-glycoprotein), most anticancer drugs are unable to gain access to the brain (58). Topotecan, however has been reported to be present in the extracellular fluid of the brain in pharmacokinetic studies using high performance liquid chromatography (59). Our results complement this study by showing that although topotecan may cross the BBB, it achieves only low concentration and its distribution within the brain is limited.

One of the limitations of this study is the doses of drugs that were used. In order to visualize the fluorescence signal of each drug, high doses of each drug were used and in order to compare fluorescence distribution patterns, equal doses of each drug were used. In fact, in the clinical setting, there is quite a range of doses among the three drugs. Doxorubicin is typically given at 60-75mg/m² (48), mitoxantrone at 12mg/m²

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(12) and topotecan at 2mg/m². Although our doses do not reflect the clinical doses, the fluorescence distributions in tumour compared to normal tissue over time are relevant and important. As well, during tissue processing (e.g. primary and secondary antibody staining), the autofluorescence of the drug may be affected and this may depend on drug properties such as binding and stability. This could also partially account for differences in cellular fluorescence between drugs.

The present study shows marked difference in the spatial distributions of three anticancer drugs within tumour tissue and normal tissues over time, with greater exposure to most normal tissues and limited drug distribution to many cells in tumours. The tumour microenvironment is dynamic and efforts to improve therapeutic efficacy might be achieved by modifying these dynamic processes to enhance drug delivery. Studies of the spatial distribution of drugs are required to complement pharmacokinetic data in order to better understand and predict drug effects and toxicities.

Acknowledgements
Mouse experiments using mitoxantrone and topotecan were done in collaboration with Dr. Olivier Tredan.
2.6 FIGURES
Figure 2.1 Drug distribution in tumour sections at 10 minutes after intravenous administration. Mice bearing (A) A431 and (B) MCF-7 tumours were treated with doxorubicin and mice bearing (C)PC-3, and (D) DU145 tumours were treated with mitoxantrone. Drug (blue), blood vessels (red). (Scale bar represents 100µm). (E) Gradients of fluorescence intensity due to the drugs are plotted in relation to distance from the nearest blood vessel.
Figure 2.2 Time-dependent drug distribution in tumour tissue. MDA-MB-231 tumour-bearing mice were treated intravenously with either doxorubicin, mitoxantrone or topotecan. After 10 minutes, 3 hours or 24 hours, tumours were excised, sectioned and imaged for drug (blue = DOX; cyan = MITOX; yellow = TOPO), blood vessels (red) and hypoxia (green). (Scale bar represents 100µm).
Figure 2.3 Drug distribution in MDA-MB-231 tumour tissue in relation to distance to the nearest blood vessel. Fluorescence intensity gradients of (A) doxorubicin, (B) mitoxantrone and (C) topotecan in relation to distance to the nearest blood vessel are shown in left panels at 10 minutes, 3 hours and 24 hours after intravenous administration. A measure of total drug concentration in the tumour (AUC) is shown in right panels. (Data represent mean ± SE for 3-10 tumours; * indicate p<0.05).
Figure 2.4 Time-dependent effects of the anticancer drugs on blood vessels and hypoxia in MDA-MB-231 tumours. (A) Blood vessels and (B) hypoxic regions per tumour area were quantified. Within each drug the first bar is after 10 minutes, second bar after 3 hours and third bar after 24 hours. (Data represents mean ± SE for 3-8 tumours).
Figure 2.5  Doxorubicin distribution in tumour and normal tissue in MDA-MB-231 tumour bearing mice at 10 minutes after injection. Photomicrographs of heart (A), kidney (B), liver (C) and tumour (D) tissues are shown. Doxorubicin (blue), blood vessels (red) and hypoxic regions (green). Fluorescence intensity gradients are shown in relation to distance to the nearest blood vessel for all tissues (E) and on an expanded scale for tumours (F). (Scale bar represents 100µm).
Figure 2.6 Perivascular doxorubicin distribution and blood vessel density in normal and tumour tissue at 10 minutes after injection. (A) Quantification of doxorubicin fluorescence the regions closest to blood vessels and (B) total number of blood vessels (CD31) per tissue area. (Data represents mean ± SE; for 3-7 normal and tumour tissues).
Figure 2.7 Time-dependent doxorubicin distribution in normal tissues. Ten minutes, 3 hours or 24 hours after intravenous administration, tissues were excised, sectioned and imaged for drug (blue) and blood vessels (red). Photomicrographs at 3 hours show a larger tissue area to observe drug fluorescence within the architecture of the different tissues. (Scale bar represents 100µm).
Figure 2.8 Time-dependent mitoxantrone distribution in normal tissues. Three hours and 24 hours after intravenous administration, tissues were excised, sectioned and imaged for drug (cyan) and blood vessels (red). Photomicrographs at 3 hours show a larger tissue area to observe drug fluorescence within the architecture of the different tissues (Scale bar represents 100µm).
Figure 2.9 Time-dependent topotecan distribution in normal tissues. Ten minutes, 3 hours or 24 hours after intravenous administration, tissues were excised, sectioned and imaged for drug (yellow) and blood vessels (red). Photomicrographs at 3 hours show a larger tissue area to observe drug fluorescence within the architecture of the different tissues (Scale bar represents 100µm).
Figure 2.10 Drug distribution in normal tissues in relation to distance to the nearest blood vessel. Fluorescence intensity gradients of the drugs in relation to distance to the nearest blood vessel are shown 10 minutes, 3 hours and 24 hours after intravenous administration. (Data represents mean ± SE; n = 3 (for each drug and each normal tissue)).
Figure 2.11 Time dependent drug concentrations in normal tissue. A measure of total (A) doxorubicin (B) mitoxantrone and (C) toptecan concentration 10 minutes, 3 hours and 24 hours after drug treatment. (Data represents mean ± SE for 3 samples per drug per tissue).
Figure 2.12 Mitoxantrone distribution in mouse liver with a secondary tumour. Drug distribution 3 hours after treatment is observed in regions of normal liver tissue compared to regions of tumour tissue. Drug is shown in cyan and blood vessels are shown in red. Two sections that were either predominantly liver (A) and predominantly tumour (B) were quantified for fluorescence distribution in relation to the nearest blood vessel (C), and the number of blood vessels per tissue area (D). (Scale bar represents 100µm).
Figure 2.13 Drug distribution in the brain. Nude mice were treated intravenously with either doxorubicin, mitoxantrone or topotecan. Photomicrographs represent drug (doxorubicin (blue), mitoxantrone (cyan), topotecan (yellow)) distribution in the brain 3 hours after treatment. (Scale bar represents 100µm).
2.7 REFERENCES


CHAPTER 3

THE INFLUENCE OF P-GLYCOPROTEIN EXPRESSION AND ITS INHIBITORS ON THE DISTRIBUTION OF DOXORUBICIN IN BREAST TUMOURS

Krupa J. Patel1 and Ian F. Tannock1,2

1 Department of Medical Biophysics, University of Toronto, Toronto, ON, Canada

2 Medical Oncology and Hematology, Princess Margaret Hospital and University of Toronto, Toronto, ON, Canada

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3.1 ABSTRACT

**Background:** Anti-cancer drugs access solid tumours via blood vessels, and must penetrate tumour tissue to reach all cancer cells. Previous studies have demonstrated steep gradients of decreasing doxorubicin fluorescence with increasing distance from blood vessels, such that many tumour cells are not exposed to drug. Studies using multilayered cell cultures show that increased P-glycoprotein (PgP) is associated with better penetration of doxorubicin, while PgP inhibitors decrease drug penetration in tumour tissue. Here we evaluate the effect of PgP expression on doxorubicin distribution *in vivo.*

**Methods:** Mice bearing tumour sublines with either high or low expression of PgP were treated with doxorubicin, with or without pre-treatment with the PgP inhibitors verapamil or PSC 833. The distribution of doxorubicin in relation to tumour blood vessels was quantified using immunofluorescence.

**Results:** Our results indicate greater uptake of doxorubicin by cells near blood vessels in wild type as compared to PgP-overexpressing tumours, and pre-treatment with verapamil or PSC 833 increased uptake in PgP-overexpressing tumours. However, there were steeper gradients of decreasing doxorubicin fluorescence in wild-type tumours compared to PgP overexpressing tumours, and treatment of PgP overexpressing tumours with PgP inhibitors led to steeper gradients and greater heterogeneity in the distribution of doxorubicin.

**Conclusions:** PgP inhibitors increase uptake of doxorubicin in cells close to blood vessels, have little effect on drug uptake into cells at intermediate distances, and might have a paradoxical effect to decrease doxorubicin uptake into distal cells. This effect probably contributes to the limited success of PgP inhibitors in clinical trials.
3.2 INTRODUCTION

Effective systemic treatment of solid tumours requires that constituent cells be sensitive to the drug(s) used and that the drug(s) be able to achieve a concentration within the cells sufficient to cause cytotoxicity. The ability of anti-cancer drugs to gain access to all of the viable cells in solid tumours depends on efficient delivery of drugs through the vascular system, and on penetration of drugs to tumour cells that are distant from blood vessels. Solid tumours have an inefficient bloody supply with tortuous and leaky vessels, large intercapillary distances and intermittent blood flow. There is evidence that several anticancer drugs have poor penetration of tumour tissue from blood vessels [1-5]. In particular there are steep gradients of decreasing doxorubicin fluorescence with increasing distance from blood vessels in tumours grown in mice and in human breast cancer, suggesting that limited drug penetration may be an important cause of resistance to treatment [6, 7].

Many of the anticancer drugs in clinical use are natural products, or derivatives of natural products (e.g. anthracyclines such as doxorubicin, vinca alkaloids such as vincristine and taxanes) and they share common mechanisms of resistance. Many of these drugs are high affinity substrates for energy-dependent membrane transporter proteins that act to pump drugs out of cells. The best characterized of these drug efflux pumps is P-glycoprotein (PgP) [8-10]. P-glycoprotein is widely expressed in many human cancers including those of the liver, pancreas, kidney, ovary and breast. PgP is encoded by the multidrug resistance (MDR1) gene in humans and is a member of a large family of ATP-dependent transporters known as the ATP-binding cassette (ABC) family. Levels of PgP correlate with drug resistance in several different cancers [11, 12]. This has led to the development of agents that reverse resistance to PgP substrates by inhibiting the action of the pump. However, while phase II studies have suggested that PgP
inhibitors might increase drug sensitivity [13, 14], the majority of randomized controlled trials evaluating PgP substrate drugs used in combination with or without PgP modulators, have not shown significant improvements in outcome [15]. Various factors have been attributed to the failure of PgP modulators in clinical trials such as high levels of toxicity and pharmacokinetic interactions with the anticancer drugs [15, 16]. In the last decade novel techniques using liposome-encapsulated doxorubicin in combination with PgP inhibitors such as verapamil and PSC 833 have been developed to increase intracellular drug concentration and minimize toxicity [17-19], however clinical data showing significant improvements in outcome have yet to be reported. Thus, other factors may also contribute in explaining the limited effectiveness of PgP inhibitors in vivo.

Studies have revealed differences in drug distribution within ex vivo tumour tissue models containing cells expressing low levels of PgP and those with high PgP expression, and these distributions can be modified by PgP inhibitors [20, 21]. The role of PgP and its inhibitors to affect drug distribution within the microenvironment of solid tumours has not been studied. In tumours that express low levels of PgP, it has been demonstrated that large quantities of drug remain in the cell layers closest to blood vessels, while distal cells acquire little to no drug [7, 22]. We hypothesized that PgP, by preventing cellular accumulation of drug in cells close to blood vessels would allow greater quantities of drug to be available to distal cells. Studies of drug penetration through multilayered cell cultures (MCC) support this hypothesis [20]. Although cellular accumulation of drug is important for cytotoxicity, if it is localized to perivascular regions, then there is likely to be a limited overall therapeutic effect on the tumour. Here we examine in solid tumour models the trade-off between drug uptake and drug distribution that is presented by PgP overexpression and PgP inhibitors.
3.3 MATERIALS AND METHODS

3.3.1 Drugs and reagents

Doxorubicin (Pharmacia, Mississauga, Canada) was purchased from the hospital pharmacy as a solution at a concentration of 2 mg/mL. Purified rat anti-mouse CD31 (platelet/endothelial adhesion molecule 1) monoclonal antibody was purchased from BD PharmMingen (Mississauga, Canada), and Cy3-conjugated goat anti-rat IgG secondary antibody was purchased from Jackson ImmunoResearch Laboratories, Inc. (Pennsylvania, USA). The first-generation PgP inhibitor, verapamil was purchased from Sigma Laboratories (Oakville, Canada). The second-generation inhibitor, PSC 833 or valsodar, was generously provided by Novartis (Basel, Switzerland).

3.3.2 Tumour model

The mouse mammary sarcoma EMT6, and its PgP-upregulated variant AR1, were provided originally by Dr Peter Twentyman, Cambridge UK. The human breast cancer cell line MCF-7 was obtained from the American Type Culture Collection (ATCC; Virginia, USA), and its PgP upregulated variants BC19 and MCF-ADR were provided by Dr. Marilyn Morris (Buffalo, NY), and Dr. Kenneth Cowan (Nebraska).

EMT6 and MCF-7 cells were maintained as monolayers in α-MEM and RPMI media respectively, supplemented with 10% FCS at 37°C in a humidified atmosphere of 95% air plus 5% CO2. The AR1, BC19 and MCF-7/ADR cell lines were maintained in similar conditions to the parental lines with the exception that the media contained 10µg/mL doxorubicin.

Tumours were generated by subcutaneous injection of 1 – 5 x 10^6 exponentially growing cells into the left and right flank regions of 6-8 week old Balb/C (EMT6 and AR1) or athymic nude (MCF-7, BC19 and MCF-7/ADR) female mice. Mice were housed five per cage in our
animal colony. Sterile water and food were given ad libitum. All procedures were carried out following approval of the Institutional Animal Care Committee.

3.3.3 Evaluation of doxorubicin distribution

Tumour-bearing mice were divided randomly into groups of five and were treated when the mean tumour diameter was in the range of 8-12 mm. Animals were treated with doxorubicin, doxorubicin and a PgP inhibitor, or phosphate-buffered saline (CaCl$_2$-MgCl$_2$). Doxorubicin was given intravenously at a dose of 25mg/kg to facilitate detection and quantification of drug auto-fluorescence. Each PgP inhibitor was administered intraperitoneally two hours prior to doxorubicin treatment at a dose of 25 mg/kg. Animals were killed 10 minutes after doxorubicin injection and the tumours were excised. The tissues were embedded immediately in optimal cutting temperature (OCT) compound, frozen in liquid nitrogen, and stored at -70°C prior to tissue sectioning and immunohistochemical staining. Cryostat sections 10 µm thick were cut at 3 levels approximately 100 µm apart from each tumour, mounted on glass slides and allowed to air dry.

3.3.4 Fluorescence imaging

Doxorubicin fluorescence was detected utilizing an Olympus Upright BX50 microscope with a 100W HBO mercury light source equipped with 530 to 560 nm excitation and 573 to 647 nm emission filter sets. Tissue sections were imaged with a Photometrics CoolSNAP HQ2 (monochrome for fluorescence imaging) camera and tiled using a motorized stage so that the distribution of doxorubicin was obtained for the entire tissue section. All images were captured in 8-bit signal depth and subsequently pseudo-colored.

Blood vessels in tissue sections were recognized by the expression of CD31 on endothelial cells. Subsequent to imaging of doxorubicin, tissue sections were stained with a rat
anti-CD31 antibody (1/100) followed by a Cy3-conjugated goat anti-rat IgG secondary antibody (1/400). Tissue sections were re-imaged in an identical way to that used to capture doxorubicin fluorescence.

### 3.3.5 Image analysis

Composite images of doxorubicin and CD31 were generated utilizing Media Cybernetics Image Pro PLUS software (version 5.0). Images displaying anti-CD31 staining were converted to a black and white binary image, and small white objects were removed as artifacts based on conservative estimation of minimal capillary diameter [23]. The resultant image was overlaid with the corresponding field of view displaying doxorubicin fluorescence resulting in an 8 bit black and white image with blood vessels identified by pixels with intensities between 250-255 (white) and doxorubicin ranging from 0-249. Several regions, 1.6 mm² in area, with moderate blood vessel density were selected from each tissue section. Blood vessel density was determined by quantifying the number of pixels with intensities between 250-255 as a percent of the number of pixels with intensities from 1-249 using Image J software. Areas of necrosis and staining artifact were excluded. To minimize noise from tissue auto-fluorescence a minimum signal level just below threshold for detection of doxorubicin was set for each tissue section; this was based on an average background reading from regions without nuclear fluorescence/staining. The pixel intensity (the area of each pixel was 0.4 μm²) and distance to the nearest vessel for all pixels within the selected region of interest above threshold were measured with a customized algorithm.

Doxorubicin intensity (I) was averaged over all pixels at a given distance (x) from the nearest vessel and plotted as a function of distance to the nearest vessel. Linear regression was performed to correlate the average doxorubicin fluorescence intensity with distance from the
nearest blood vessel; the slope of the linear regression was statistically compared between treatment groups using ANOVA and subsequent t-tests. All linear regressions were statistically significant and residual plots showed no consistent patterns.

A model comparing doxorubicin distribution in PgP overexpressing tumours pre-treated with either saline or a PgP inhibitor was generated using the mean slope and y-intercept from the linear regressions for both the murine and xenograft tumour types.

3.3.6 Growth delay studies

Mice bearing EMT6 or AR1 tumours were divided into six groups of 4-5 mice each and treated with either saline, doxorubicin alone (8mg/kg i.v., verapamil alone (25mg/kg i.p.), PSC 833 alone (25mg/kg), verapamil + doxorubicin (25mg/kg + 8 mg/kg) or PSC 833 + doxorubicin (25mg/kg + 8 mg/kg). In the latter two groups mice were treated with the PgP inhibitor 2 hours prior to doxorubicin treatment. Every 2-3 days the length and width of the tumours were measured using calipers and tumour volume was calculated. Measurements were taken until tumours reached their maximum limit in size. The body weight of the mice was also measured.

3.4 RESULTS

3.4.1 Blood vessel density

Blood vessel density within areas of interest was measured and found to be within a small range. Murine tumours are significantly more vascular than the xenografts (Table 3.1). Within a given tumour section there are small areas with high blood vessel density where no gradients of decreasing doxorubicin fluorescence are observed, but the majority of the tumour section is composed of areas of moderate blood vessel density with gradients of decreasing doxorubicin fluorescence with increasing distance from vessels.
**Table 3.1** Characteristics of wild-type (EMT6, MCF-7) and PgP overexpressing tumours (AR1, BC19) at 10 minutes after treatment with doxorubicin (DOX) or with pre-treatment 2 hours earlier with inhibitors of PgP.

<table>
<thead>
<tr>
<th>Tumour Type</th>
<th>PgP vs. Wild-type</th>
<th>Treatment</th>
<th>Blood Vessel Density (# of BV)</th>
<th>DOX uptake at distance from the nearest blood vessel (intensity units)</th>
<th>Gradient of Decreasing DOX Intensity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMT6</td>
<td>Wild Type</td>
<td>DOX</td>
<td>4.7 ± 1.2</td>
<td>29.4 ± 6.1&lt;sup&gt;a&lt;/sup&gt; 15.6 ± 1.9 11.3 ± 2.4</td>
<td>-0.23 ± 0.08&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>AR1</td>
<td>Overexpress PgP</td>
<td>DOX</td>
<td>6.0 ± 1.4</td>
<td>17.1 ± 2.9&lt;sup&gt;ab&lt;/sup&gt; 10.5 ± 2.8&lt;sup&gt;c&lt;/sup&gt; 7.4 ± 1.6</td>
<td>-0.11 ± 0.03&lt;sup&gt;cde&lt;/sup&gt;</td>
</tr>
<tr>
<td>AR1</td>
<td>Overexpress PgP</td>
<td>Verapamil + DOX</td>
<td>4.4 ± 1.1</td>
<td>24.7 ± 8.3 13.7 ± 4.2 9.2 ± 4.0</td>
<td>-0.18 ± 0.06&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>AR1</td>
<td>Overexpress PgP</td>
<td>PSC 833 + DOX</td>
<td>4.7 ± 1.3</td>
<td>34.0 ± 4.3&lt;sup&gt;b&lt;/sup&gt; 16.4 ± 2.3&lt;sup&gt;c&lt;/sup&gt; 12.1 ± 3.7</td>
<td>-0.27 ± 0.04&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Wild Type</td>
<td>DOX</td>
<td>2.6 ± 0.7</td>
<td>23.8 ± 4.2&lt;sup&gt;f&lt;/sup&gt; 13.7 ± 2.5 10.4 ± 2.3</td>
<td>-0.16 ± 0.05&lt;sup&gt;h&lt;/sup&gt;</td>
</tr>
<tr>
<td>BC19</td>
<td>Overexpress PgP</td>
<td>DOX</td>
<td>2.7 ± 0.4</td>
<td>13.0 ± 4.3&lt;sup&gt;g&lt;/sup&gt; 9.6 ± 2.8 8.7 ± 3.5</td>
<td>-0.05 ± 0.03&lt;sup&gt;hij&lt;/sup&gt;</td>
</tr>
<tr>
<td>BC19</td>
<td>Overexpress PgP</td>
<td>Verapamil + DOX</td>
<td>3.6 ± 0.8</td>
<td>22.1 ± 5.9 13.9 ± 3.7 9.2 ± 1.1</td>
<td>-0.14 ± 0.04&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td>BC19</td>
<td>Overexpress PgP</td>
<td>PSC 833 + DOX</td>
<td>2.9 ± 0.7</td>
<td>24.2 ± 4.1&lt;sup&gt;g&lt;/sup&gt; 14.4 ± 2.5 10.9 ± 3.6</td>
<td>-0.17 ± 0.05&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Top and bottom panels were tested separately for significant differences using ANOVA and subsequent t-tests. Within the top panel, a-e represent the pairs of data that are statistically significant (p < 0.05). Within the bottom panel, f-j represent the pairs of data that are statistically significant (p < 0.05). *Gradient represents the slopes of regression lines of decreasing doxorubicin fluorescence in relation to distance to the nearest blood vessel.
3.4.2 PgP overexpression and doxorubicin distribution

Representative composite images showing the distribution of doxorubicin 10 minutes after administration in relation to blood vessels of wild-type EMT6 tumours, and in tumours derived from cells that over-express PgP are shown in Figures 3.1a-b; similar images for MCF7/BC19 xenografts are shown in Figures 3.1c-d. A summary of data obtained from these sections is provided in Table 1. In PgP overexpressing tumours, a more homogeneous distribution of doxorubicin is observed as compared to wild-type tumours of both murine and human origin (Figure 3.1). The gradient of decreasing doxorubicin fluorescence intensity is significantly greater in wild-type tumours that have low levels of PgP expression (Table 3.1). Whereas wild-type tumours show an exponential decrease in doxorubicin fluorescence with distance from blood vessels, PgP overexpressing tumours show a more linear decrease (Figure 3.2). Close to blood vessels (i.e. in the first 10 µm), doxorubicin uptake is significantly lower in tumours that overexpress PgP, but at 50-60 µm from blood vessels, the difference in doxorubicin uptake is less and by 110-120 µm, there is no significant difference (Table 3.1 and Figure 3.2).

3.4.3 PgP inhibitors and doxorubicin penetration

The effects of verapamil and PSC 833 on distribution of doxorubicin in PgP-overexpressing AR1 tumours are shown in Figures 3A and 3B, and for corresponding BC19 xenografts in Figures 3.3c-d. Both PgP inhibitors lead to an increase in uptake of doxorubicin by cells close to blood vessels, but increase the gradient of decreasing intensity so that the distribution is more heterogeneous and similar to that of wild-type tumours (Table 3.1). Figure 3.4 shows the distribution of doxorubicin in PgP overexpressing tumours with or without pretreatment with PgP inhibitors. Doxorubicin fluorescence intensity in the first 10 µm from blood vessels is significantly greater in PgP overexpressing tumours that were pretreated with
verapamil and PSC 833 in the murine tumour model, and with PSC 833 in the xenograft model. At further distances (110-120μm), no significant difference is observed in doxorubicin uptake between control tumours and tumours pretreated with PgP inhibitors (Table 3.1 and Figure 3.4).

At distances greater than about 90 - 100μm from blood vessels estimates of doxorubicin fluorescence are subject to noise, most likely due to the influence of closer blood vessels out of the plane of the sections. While in some areas of the tumours, neighboring vessels may contribute doxorubicin fluorescence in distal cells, in other areas this may not be the case and there may be a sharp decrease in doxorubicin. We have shown the average doxorubicin distribution taken from several areas of interest. Modeling from this data by removing noise from neighboring vessels suggests that the use of potent PgP inhibitors, such as PSC 833 might lead to a paradoxical decrease in the uptake of doxorubicin in tumour cells situated more distant from tumour blood vessels (Figure 3.4c). According to the model, at around 90-100μm in the murine model and 100-115μm in the xenograft model doxorubicin fluorescence intensity is greater in the PgP overexpressing tumours that are treated with doxorubicin alone compared to those pretreated with a PgP inhibitor.

3.4.4 Growth delay and toxicity

Doxorubicin led to modest delay in growth of wild-type EMT6 tumours (Figure 3.5a) but had no significant effect on PgP overexpressing AR1 tumours. The PgP inhibitors did not modify growth delay for either type of tumour. Body weight was used as an indication of toxicity. Most treatments were well tolerated and caused no significant loss of body weight after treatment. The combination of doxorubicin and PSC 833 was toxic to mice and caused a 20-25%
reduction in body weight within the first 3-5 days. Mice in this group were killed after 5 days because of this toxicity.

3.5 DISCUSSION

In order for chemotherapy to have an optimal effect against solid tumours, there must be adequate distribution of drugs, such that there is accumulation of drug within all cancer cell populations that can regenerate the tumour. Repopulation of surviving tumour cells between courses of chemotherapy is an important mechanism of drug resistance [24-28], and viable cells distal from blood vessels that do not receive cytotoxic concentrations of drug might be an important source of such repopulation [29].

Drug distribution may be influenced in tumour tissue by the presence of high interstitial fluid pressure, an extensive extracellular matrix, cell-cell contact and the expression of efflux pumps such as PgP on the cell surface. It has been proposed that since PgP and other drug transporters reduce cellular accumulation of their substrates, they might assist in transporting the drugs to neighboring cells farther away from blood vessels, resulting in a net increase in tissue penetration of chemotherapeutic agents [2]. This hypothesis has been supported by the observation of increased penetration of $^{14}$C doxorubicin through drug-resistant MCCs (PgP overexpressing) compared to drug-sensitive MCCs (wild-type) [20]; inhibitors of PgP stimulated cellular accumulation of drugs, but resulted in reduced penetration through tumour tissue in this model [20]. While using MCCs provides insight into the penetration of drugs through solid tissue, they lack the 3-dimensional heterogeneity of solid tumours. In studies based on multicellular spheroids, verapamil showed limited reversal of resistance by doxorubicin likely due to its small diameter (approx. 100µm) and acidic microenvironmental affects on verapamil...
activity [30, 31]. When a wide array of PgP reversal agents including cyclosporin A, verapamil, quinidine, and sodium orthovanadate were used in larger spheroids an increase in doxorubicin fluorescence as far as 80µm was observed. However, doxorubicin retention at these depths was correlated with an increase in PgP expression, therefore changes in doxorubicin distribution patterns throughout the spheroid tissue due to PgP overexpression and inhibitors could not be assessed [32]. Multicellular spheroids allow good insight into drug fluorescence however, they lack vasculature and certain microenvironmental conditions that lead to specific phenotypes such as multidrug resistance and tumour acidity.

Here, we have investigated the effects of PgP overexpression and PgP inhibitors on doxorubicin distribution in solid tumours grown in mice. The distribution of doxorubicin is quite variable within tumour tissue. In well-vascularized areas drug distribution is relatively uniform; however in most areas of the tumour where there are few blood vessels, distribution to cells distal from blood vessels is limited so that many viable tumour cells have minimal exposure to drug, allowing them to survive and proliferate. For this reason, we selected areas of interest within a tumour section that had low vascular density. We examined doxorubicin distribution 10 minutes after intravenous administration because previous studies in our laboratory have shown no difference in doxorubicin distribution in relation to the nearest blood vessel beyond this time point [7].

Similar to data for MCCs, our results show an increase in doxorubicin distribution in the PgP overexpressing tumours AR1 and MCF-7/ADR compared to their wild-type variants EMT6 and MCF-7, respectively. There is rapid cellular uptake of doxorubicin in wild-type cells and this is largely due to its ability to bind tightly to DNA [33], and in tumours derived from them, large amounts of drug accumulate in cells closest to blood vessels. Doxorubicin is a PgP
substrate, that is efficiently pumped out of tumour cells where PgP is highly expressed [16]. Thus, in PgP overexpressing tumours there is less uptake and binding into proximal cells, resulting in a shallower gradient of decreasing doxorubicin fluorescence with increasing distance from tumour blood vessels. These results do not imply that PgP overexpressing tumours allow for more effective drug treatment, since cellular accumulation of drug is essential for cytotoxicity, and PgP expression will decrease relative uptake of substrate drugs in all tumour cells. However, our results demonstrate that PgP efflux can significantly alter the biodistribution of chemotherapeutic agents, such that decreased proximal uptake of drug may be counter-balanced by greater amounts of doxorubicin available to distal cells in solid tumours.

Methods of inhibiting PgP have been studied extensively for over two decades. Many agents that modulate PgP transport such as verapamil and cyclosporin were identified in the 1980s, and were evaluated as chemosensitizing agents. These agents produced disappointing results in clinical trials in part because their low binding affinities necessitated the use of high doses, resulting in unacceptable toxicities [15]. In our studies, a relatively low dose of verapamil (25mg/kg) was used to determine its effect on drug distribution. While toxicity is likely to be the primary cause of the ineffectiveness of verapamil to improve the sensitivity of tumours to substrate drugs, our studies show that modification of doxorubicin distribution in tumours might be an additional cause of their failure to improve tumour response to chemotherapy.

Second generation PgP modulators include valsodar (PSC 833), biricodar, and dexniguldipine. These agents are more potent and specific than first generation inhibitors [16]. Valspodar has been studied in clinical trials in combination with cytotoxic agents [14, 34]. A study by Coley et al [35], that used fresh tumour material from patients with soft-tissue sarcomas indicated that valspodar at 1nM had a modest effect to increase anthracycline accumulation (by ~
20%) in PgP positive samples. In a study of women with epithelial ovarian cancer, the effect was of a similar magnitude; these limited effects might explain in part the disappointing results of clinical trials. Some second generation PgP inhibitors are also, like cytotoxic agents, substrates of cytochrome P450 3A4, an important enzyme involved in metabolism, and the competition between cytotoxic drugs and PgP inhibitors for cytochrome P450 activity has resulted in unpredictable pharmacokinetic interactions. Here we found substantial toxicity when a low dose of PSC 833 was combined with doxorubicin. Marked pharmacologic interactions have been observed in patients treated with this combination, leading to substantial increases in hematologic toxicity [34], and requiring use of lower doses of anticancer drugs when used in combination.

Although cytotoxicity and pharmacokinetic interactions explain partially why PgP inhibitors have not been very effective in clinical trials, a clear explanation for the minimal effects to increase mean cellular accumulation of drug has yet to be provided. Studies to determine cellular accumulation often involve homogenizing tumour tissue and conducting high-performance liquid chromatography (HPLC) [36, 37]. This method is limiting, because it does not give an indication of the distribution of drug.

Our study uses a non-orthotopic tumour mouse model to show that tumour cells in areas far from blood vessels where PgP inhibitors have no effect (or possibly even a negative effect) due to changes in distribution of doxorubicin, might counterbalance areas close to blood vessels where uptake of doxorubicin into tumour cells is increased, thus limiting the effectiveness of these inhibitors. While tumour dynamics, vasculature and heterogeneity affect drug distribution, this non-orthotopic mouse model is still limited in fully representing the conditions in human patients.
Computer-simulated mathematical models have shown that adequate drug distribution is a crucial factor in determining drug effectiveness [38]. One model has demonstrated that drug efflux from cells, enhanced by PgP, will result in a longer diffusion length [39]. These mathematical models are powerful tools that can provide important insights about drug distribution, because they can take into account drug pharmacodynamics [40], the spatio-temporal accumulation of drug [41], the link between multiscale approaches [42, 43], and the effects of local drug, oxygen and nutrient gradients on tumour growth and response [44]. Future studies integrating our data with computer-simulated mathematical models may be a powerful tool in determining drug distribution and its association with drug effectiveness in human patients.

3.6 CONCLUSIONS

We have shown that PgP expression and inhibitors of PgP function can influence doxorubicin distribution in some solid tumours. The distribution of doxorubicin is more heterogeneous in wild-type tumours, than in those where constituent cells express PgP: drug uptake is higher in cells close to blood vessels in wild-type tumours, but there are minimal differences in drug uptake by more distal cells. Both the first-generation inhibitor verapamil and the second-generation inhibitor valspodar alter drug penetration in both a murine tumour and a human xenograft, leading to improved uptake of doxorubicin only in tumour cells within a restricted radius around functional blood vessels. Whether PgP overexpressing tumours are treated with a PgP inhibitor or not, cells distal to blood vessels at approximately 50-120μm, show no significant difference in doxorubicin fluorescence (Table 1). Our modeling suggests the possibility of a paradoxical effect of PgP inhibitors to cause PgP overexpressing tumours to have
increased uptake of doxorubicin in proximal cells, minimal or no effect on drug uptake at intermediate distances from blood vessels but decreased drug uptake in more distal cells (Figure 4c). These results emphasize that while limited cellular accumulation of a drug is an important mechanism of drug resistance, if there is a trade-off between uptake into proximal cells and penetration to distal cells, therapeutic effectiveness may be limited, especially for drugs like doxorubicin that have a short half-life in the circulation. Repopulation of tumour cells has been shown to occur in regions far from blood vessels [29], and it is important to consider factors that affect the distribution of chemotherapeutic agents in solid tumours. Numerous clinical trials evaluating the effects of verapamil or valspodar in combination with chemotherapeutic agents such as doxorubicin, vincristine, dexamethasone, cyclophosphamide, paclitaxel have shown that patients given concurrent administration of PgP inhibitors with chemotherapeutic agents have an increased toxicity and show modest or no increase in survival [45-49]. These results provide insight into an important limitation of PgP inhibitors and this principle is likely to be applicable to other membrane-based drug efflux proteins such as multiple drug resistance protein-1 (MRP-1) and suggest the importance of considering drug distribution in the design and development of novel treatment strategies.

**Competing interests**
The author has no competing interests.

**Authors' contributions**
KJP participated in designing, planning and carrying out in vivo drug distribution studies, imaging, analysis and growth delay studies as well as drafting and revising the manuscript. IFT conceived the concepts underlying the study, designed the experiments, revised the manuscript and provided funding, grant support and oversight.

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3.7 FIGURES
Figure 3.1 Distribution of doxorubicin in solid tumours. Murine tumours EMT6 (A) and its PgP overexpressing subline AR1 (B) and MCF-7 human breast cancer xenograft (C) and its PgP overexpressing subline BC19 (D) were resected from Balb/C and nude mice, respectively. Doxorubicin is shown in blue and blood vessels are shown in red. Note more uniform distribution of doxorubicin in the PgP overexpressing tumours. (Scale bars = 100µm).
Figure 3.2 The gradient of doxorubicin fluorescence intensity in relation to distance from the nearest blood vessel. Mice-bearing either EMT6 or AR1 tumours (A) (n = 6 tumours each) or MCF-7 or BC19 xenografts (B) (n = 11 and 5 tumours, respectively) were treated with doxorubicin and their tumours were resected, sectioned and imaged. Image analysis was undertaken using customized algorithms. (Data represents mean ± SE).
Figure 3.3  Distribution of doxorubicin in solid tumours. Murine AR1 tumours were treated with either doxorubicin (A) or PSC 833 and doxorubicin (C). Similarly, BC19 xenografts were treated with either doxorubicin (B) or PSC 833 and doxorubicin (D). Doxorubicin is shown in blue and blood vessels are shown in red. (Scale bars = 100 µm).
Figure 3.4 The gradient of doxorubicin fluorescence intensity in relation to distance from the nearest blood vessel and a model of doxorubicin distribution in solid tumours. Mice-bearing AR1 tumours (A) or BC19 xenografts (B) were treated with either doxorubicin alone, or pretreated with verapamil or PSC 833 and doxorubicin. Tumours were resected, sectioned and imaged. Image analysis was undertaken using customized algorithms (data represents mean ± SE). In panel A, 6, 10 and 9 tumours were analyzed, respectively. In panel B, 6, 7 and 6 tumours were analyzed, respectively. (C) represents a model of doxorubicin distribution without fluorescence interference from neighboring out-of-section blood vessels.
Figure 3.5 Tumour growth delay and toxicity studies. Mice-bearing EMT6 tumours (A) or AR1 tumours (B) were treated with either saline, doxorubicin alone, verapamil alone, PSC 833 alone or pretreated with verapamil or PSC 833 in combination with doxorubicin. Tumour volume and body weight was measured every 2-3 days. (Data represents mean ± SE; n = 5).
3.8 REFERENCES


CHAPTER 4

INFLUENCE OF PANTOPRAZOLE ON DISTRIBUTION AND ACTIVITY OF DOXORUBICIN IN CELL CULTURE AND IN SOLID TUMOURS

Krupa J. Patel and Ian F. Tannock
4.1 ABSTRACT

**Background:** Limited drug distribution within solid tumours is an important cause of drug resistance. Basic drugs (e.g. doxorubicin) may be sequestered in acidic organelles thereby limiting drug distribution to distal cells and diverting drugs from their target DNA. Here we investigate the effects of pantoprazole, a proton pump inhibitor, on endosomal pH, doxorubicin uptake, and doxorubicin penetration and activity using in vitro and murine models.

**Methods:** Wild-type EMT-6 and MCF-7, and their P-glycoprotein (PgP) over-expressing variant cells were treated with pantoprazole to evaluate changes in endosomal pH using fluorescence spectroscopy, and uptake of doxorubicin using flow cytometry. Effects of pantoprazole on tissue penetration of doxorubicin were evaluated in multilayered cell cultures (MCCs), and in solid tumours using immunohistochemistry. Effects of pantoprazole to influence tumour growth delay and toxicity due to doxorubicin were evaluated in mice.

**Results:** Pantoprazole (>200μM) increased endosomal pH in all cells, and increased nuclear uptake of doxorubicin in three of four cell lines. Pretreatment with pantoprazole increased penetration of doxorubicin in both wild-type and PgP-overexpressing MCCs. Pantoprazole increased doxorubicin distribution and tumour growth delay in wild-type but not PgP expressing NCI solid tumours, without apparent increase in toxicity.

**Conclusions:** Use of pantoprazole to enhance the distribution and cytotoxicity of anticancer drugs in solid tumours might be a novel treatment strategy to improve their therapeutic index.
4.2 INTRODUCTION

Initial or acquired drug resistance limits treatment of cancer by chemotherapy. Studies of drug resistance have focused on cellular and molecular mechanisms, but there is increasing evidence that the tumour microenvironment plays an important role in resistance to chemotherapy. For a tumour to respond to chemotherapy a drug must leave tumour blood vessels efficiently and distribute throughout tumour tissue to reach all cancer cells in concentrations that will lead to cytotoxicity (1, 2). The distribution of anticancer drugs such as doxorubicin is limited in solid tumours and this limited distribution may be an important mechanism of drug resistance (3-7).

Many solid tumours develop regions of extracellular acidity due to production and poor clearance of carbonic and lactic acid (8, 9). The pH gradient between an acidic extracellular environment and a neutral-alkaline intracellular pH may influence drug uptake and activity. Also, cells may contain acidic organelles such as lysosomes and endosomes (10-12). Since many chemotherapeutic drugs such as anthracyclines and vinca alkaloids are weak lipophilic bases, they readily enter acidic organelles where they are protonated and sequestered (13).

Vacuolar-H⁺-ATPases (V-H⁺-ATPases) are the major mechanism for regulation of endosomal pH (14). V-H⁺-ATPases pump H⁺ ions across the membranes of a wide array of intracellular compartments. Agents that disrupt the pH gradient between the cytoplasm and endosomes in tumours might decrease the sequestration of basic anticancer drugs and render cells more sensitive to the drug. A class of H⁺-ATPase inhibitors, called proton pump inhibitors (PPIs), inhibit acidification of cells in the wall of the stomach and are used clinically for treating patients with peptic ulcer disease. These agents also inhibit V-H⁺-ATPase albeit at somewhat higher concentration than is required to inhibit acidification in the stomach (15). PPIs
accumulate selectively in acidic spaces and with the inhibition of V-H\(^+\)-ATPase activity, increase both extracellular pH and the pH of acidic organelles (16). Pretreatment with a PPI might alter intracellular drug distribution by inhibiting drug sequestration. This would allow more drug to be available both to enter the nucleus and cause cytotoxicity, and to exit the cell and be taken up by cells distant from blood vessels. We hypothesized that preventing sequestration of drug in acidic endosomes would improve extracellular drug distribution. In support of this hypothesis modest improvements in drug distribution have been observed in multilayered cell cultures (MCCs) pretreated with chloroquine and the PPI, omeprazole (17).

There is evidence that PPIs such as omeprazole and pantoprazole are substrates and inhibitors of the drug export pump P-glycoprotein (PgP) (18). P-glycoprotein belongs to the ATP-binding cassette (ABC) family of transporter proteins that can export anticancer drugs (e.g. doxorubicin, paclitaxel, vincristine and many others) from cells and thereby cause multidrug resistance (MDR) (19, 20). Studies in our laboratory suggest that PgP overexpressing tumours have better distribution of doxorubicin to cells distal to blood vessels as compared to wild-type tumours (21). A possible interaction between PPIs and PgP activity might influence drug distribution and activity in solid tumours.

Here we describe and evaluate several agents that have been reported (or might be expected) to increase endosomal pH for their effects on uptake of doxorubicin into tumour cells, and its distribution within them. We then describe more extensive experiments to characterize the effects of pantoprazole to modify the distribution and activity of doxorubicin in solid tumours derived from wild-type and PgP overexpressing cells.

The goals of the present study were (i) to determine whether inhibiting sequestration of drug in endosomes using the PPI pantoprazole would improve drug distribution in MCCs and in
solid tumours, and (ii) to distinguish effects of pantoprazole on wild type and PgP overexpressing MCCs and solid tumours.

4.3 MATERIALS AND METHODS

4.3.1 Drugs and reagents

Doxorubicin (Pharmacia, Mississauga, Canada) was purchased from the hospital pharmacy as a solution at a concentration of 2 mg/mL. Purified rat anti-mouse CD31 (platelet/endothelial adhesion molecule 1) monoclonal antibody was purchased from BD PharmMingen (Mississauga, Canada), and Cy3-conjugated goat anti-rat IgG secondary antibody was purchased from Jackson ImmunoResearch Laboratories, Inc. (Pennsylvania, USA). Pantoprazole (Nycomed, Oakville, Canada) was purchased from the hospital pharmacy as a lyophilized powder and dissolved in 0.9% saline. Radiolabeled doxorubicin was obtained from Amersham Life Sciences (Buckinghamshire, United Kingdom). Lansoprazole was purchased from Sigma (St. Louis, MO) and dissolved in ethanol. Tamoxifen was obtained from Sigma (St. Louis, MO) and dissolved in ethanol.

4.3.2 Cell lines

The mouse mammary sarcoma EMT6, and its PgP-upregulated variant AR1, were provided originally by Dr Peter Twentyman, Cambridge UK. The human breast cancer cell line MCF-7 was obtained from the American Type Culture Collection (ATCC; Virginia, USA), and its PgP upregulated variant NCI-ADR was purchased from the National Cancer Institute (Bethesda, Maryland, United States of America). The human vulvar epidermoid carcinoma cell line A431 was purchased from ATCC.
EMT6 and MCF-7 cells were maintained as monolayers in α-MEM media, supplemented with 10% fetal bovine serum, (FBS; Hyclone, Logan, UT). The AR1 and NCI cell lines were maintained in similar conditions to the parental lines with the exception that the media contained 10µg/mL doxorubicin. NCI cells were maintained in RPMI media. A431 cells were maintained in Dulbecco’s Modified Eagle’s Medium supplemented with 10% FBS. All media were obtained from the hospital media facility. Cells were grown in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. Routine tests to exclude mycoplasma were performed.

Tumours were generated by subcutaneous injection of 1 – 5 x 10⁶ exponentially growing cells into the left and right flank regions of female athymic nude mice (MCF7) or syngeneic Balb/C mice (EMT6). Estrogen pellets were implanted into the nude mice one day prior to injection of MCF-7 tumour cells. All procedures were carried out following approval of the Institutional Animal Care Committee.

4.3.3 Measurement of endosomal pH

To measure endosomal pH, cells (10⁶/ml) were treated with varying concentrations of lansoprazole, pantoprazole, or tamoxifen (included because of prior reports of effects to raise endosomal pH, (22)). They were incubated for 3 hours with dextran-fluorescein-tetramethylrhodamine 10,000 MW, anionic (FITC/TMR-dextran, Molecular Probes, Inc. Eugene, OR) which is taken up into endosomes, followed by exposure to media for 2 hours. Fluorescence was measured using a Coulter Epics Elite flow cytometer (Beckman Coulter, Miami, FL) equipped with an argon laser emitting at 488nm. The argon laser was used to excite FITC and TMR with emission evaluated at 525nm (pH-dependent) and 575nm (pH-independent) respectively. Calibration of fluorescence measurements was performed using the ionophore nigericin (Sigma, St. Louis, MO) in buffers of known pH (23).
4.3.4 Doxorubicin uptake

Uptake of radiolabeled doxorubicin

The uptake into cells of radiolabeled \(^{14}\text{C}-\text{doxorubicin} (\text{Amersham, Buckinghamshire, United Kingdom}) was studied using a spin-through-oil technique (24, 25). Stirred suspensions of single cells, in the presence or absence of modifiers, were exposed to the radiolabeled drugs. Aliquots (100 µl) were removed as a function of time and layered on top of a mixture of dibutylphthalate and corn oil (4:1) in microcentrifuge tubes. The tubes were then spun at 14,000 rpm for 5 minutes, and the cells were pelleted at the bottom of the tube. The medium and then the oil were aspirated and the cell-associated radioactivity was determined by liquid scintillation counting.

Cells attached to a chambered cover glass were pre-treated with pantoprazole (1mM) for 2 hours and then incubated in media containing 2µg/ml doxorubicin for 1 hour. The drugs were washed out and the fluorescence signal was recorded with excitation at 514nm and emission at 488nm using a Zeiss Axiovert 200M fluorescence inverted microscope and the fluorescence signals were captured with a Roper Scientific CoolSnap HQ CCD camera. To visualize endosomes, the cells were exposed to the pH-sensitive endosomal dye, LysoSensor Yellow/Blue DND-160 (Molecular Probe, Inc. Eugene, OR) at a concentration of 5µM for 15 min. The fluorescent signal was measured with excitation at 360nm and emission at 420nm.

Flow cytometry

For each of the four cell lines, \(10^6\) cells were treated in vials with either saline or pantoprazole (1mM). After 2 hours, doxorubicin was added to each vial and incubated for 1 hour and then washed twice with PBS. Mean doxorubicin fluorescence was measured using the
Becton Dickinson FACScan (Franklin Lakes, NJ), and Cell Quest software. The FL1 filter (530nm) was used to detect doxorubicin fluorescence.

4.3.5 Evaluation of doxorubicin penetration in multilayered cell cultures

Drug penetration was evaluated in an in vitro tumour-like environment using multilayered cell cultures (26, 27). Approximately 2 x 10^5 cells were seeded on collagen-coated microporous teflon membranes (Millipore, Bedford, MA) and given 4-6 hours to attach. The membranes were immersed in α-MEM media in a large vessel with continuous stirring and allowed to incubate at 37°C for approximately 6-8 days; this led to the formation of MCCs containing approximately 5 x 10^6 cells. The MCCs were incubated in either media alone or media containing 1mM pantoprazole for 2 hours. The penetration experiments were performed at 37°C in an atmosphere of 95% air and 5% CO₂. Solutions containing 10μmol/L radiolabeled doxorubicin were prepared in 2 x α-MEM (without FBS) and mixed in a 1:1 ratio with 1% agar solution; the agar is added to prevent convection. A volume of 0.5mL of this mixture was added to one side of the MCC. The membranes were then floated in glass polyshell vials containing 18mL of α-MEM media. A cell-free membrane insert was included in all experiments as a control. The drug, after penetrating through the MCC, appeared in the compartment below and 150μL samples were taken from this compartment over time and assessed by liquid scintillation counting. ^3H-sucrose was used as an internal standard at a concentration of 2μmol/L to ensure minimal interexperimental variations of the MCC thickness.

Fluorescent micrographs of MCCs were generated after exposure to doxorubicin. After 1 hour of incubation, MCCs were removed and frozen, 10μm sections were imaged using an Olympus Upright BX50 microscope with a 100W HBO mercury light source equipped with 530
to 560 nm excitation and 573 to 647 nm emission filter sets. Images were pseudo-coloured using Image Pro Plus software.

4.3.6 Plasma concentrations of pantoprazole in mice

At various times after Balb/C mice were treated with pantoprazole (200mg/kg), blood was collected using cardiac puncture in heparin-coated tubes on ice, and the mice were killed. Whole blood was spun at 16000 RPM for 10 minutes and the plasma was isolated and frozen. Pantoprazole concentration was determined using high-performance liquid chromatography (HPLC). Stock solutions of pantoprazole were prepared in methanol–water (50:50 v/v) at concentrations of 1mg/mL. Calibration curves of pantoprazole were prepared by spiking the blank plasma at concentrations of 2.5, 5, 10, 20, 50, 100, 200, 1000 and 2000 ng/ml. The frozen plasma samples were thawed and centrifuged for 5 minutes at 2550 g at 4°C to precipitate solids. Fifty microliters of the internal standard solution were added to a 200-μl aliquot of plasma. The tubes were briefly mixed with a vortex and the compounds of interest were extracted with 4 ml of a mixture of diethyl-ether/dichloromethane (70:30; v/v). The mixture was mixed for approximately 40 s, and the organic phase was evaporated under N₂ at 37°C. The dry residues were reconstituted to 200-μl with a solution of CH₃CH and H₂O (90:10; v/v) containing 2.5mM of ammonia and mixed with a vortex for 15 s. The solutions were then transferred to auto-injector microvials. Shimadzu SIL-20-AC autoinjector and Shimadzu-20AD pumps were used for HPLC-MS/MS analysis and Applied Biosystem MDS Sciex (Concord, Ontario, Canada) API3200 tandem mass spectrometry equipped with a TurboIonSpray interface was used subsequently. The data were processed using Analyst 1.4.2 software (Applied Biosystem MDS Sciex).
4.3.7 Evaluation of doxorubicin distribution in solid tumours

Mice bearing EMT-6, AR1, MCF-7 or NCI subcutaneous tumours in the left and right flank regions were divided randomly into groups of five and were treated when the mean tumour diameter was in the range of 8-12 mm. Animals were treated with phosphate-buffered saline containing CaCl₂ and MgCl₂, doxorubicin alone, or pantoprazole prior to doxorubicin. Doxorubicin was given intravenously at a dose of 25 mg/kg to facilitate detection and quantification of drug auto-fluorescence. Pantoprazole was administered intraperitoneally two hours prior to doxorubicin treatment at a dose of 200 mg/kg. To detect hypoxia and functional blood vessels, EF5 was injected intraperitoneally approximately two hours prior to killing the mice (0.2 mL of a 10 mM stock per mouse), and the perfusion marker DiOC7 (1 mg/kg) was injected and intravenously 1 minute prior to killing the mice. Mice were killed 10 minutes after doxorubicin injection and the tumours were excised. The tissues were embedded immediately in OCT compound, frozen in liquid nitrogen, and stored at -70°C. Cryostat sections 10 µm thick were cut at 3 levels approximately 100 µm apart from each tumour, mounted on glass slides.

Fluorescence imaging

Doxorubicin fluorescence was detected utilizing an Olympus Upright BX50 microscope with a 100W HBO mercury light source equipped with 530-560 nm excitation/573-647 nm emission filter sets. Tissue sections were imaged with a Photometrics CoolSNAP HQ2 (monochrome for fluorescence imaging) camera and tiled using a motorized stage so that the distribution of doxorubicin was obtained for the entire tissue section. All images were captured in 8-bit signal depth and subsequently pseudo-colored.

Tumour sections were first imaged for doxorubicin and the perfusion marker DiOC7 using a TRITC and FITC filter set, respectively. Sections were then stained for blood vessels
using antibodies specific for the endothelial cell marker CD31 [rat anti-CD31 primary antibody (1:100); BD Biosciences, and C73-conjugated goat anti-rat IgG secondary antibody (1:400)], hypoxic regions were identified using a Cy5-conjugated mouse anti-EF5 antibody (1:50). Tumour sections were imaged for CD31 using the Cy3 (530-560 nm excitation/573-647 nm emission) filter set, and EF5 using the Cy5 far-red filter set.

Image analysis and quantification

Composite images of doxorubicin and CD31 were generated utilizing Media Cybernetics Image Pro PLUS software (version 5.0). Images displaying anti-CD31 staining were converted to a black and white binary image, and small white objects were removed as artifacts based on conservative estimation of minimal capillary diameter (28). The resultant image was overlaid with the corresponding field of view displaying doxorubicin fluorescence resulting in an 8 bit black and white image with blood vessels identified by pixels with intensities between 250-255 (white) and doxorubicin ranging from 0-249. Several regions, 1.6 mm² in area, with moderate blood vessel density were selected from each tissue section. Areas of necrosis and staining artifact were excluded. To minimize noise from tissue auto-fluorescence a minimum signal level just below threshold for detection of doxorubicin was set for each tissue section; this was based on an average background reading from regions without nuclear fluorescence/staining. The pixel intensity (the area of each pixel was 0.4 μm²) and distance to the nearest vessel for all pixels within the selected region of interest above threshold were measured with a customized algorithm. Doxorubicin intensity (I) was averaged over all pixels at a given distance (x) from the nearest vessel and plotted as a function of distance to the nearest vessel. Linear regression was performed to correlate the average doxorubicin fluorescence intensity with distance from the nearest blood vessel.
Quantification of blood vessels and hypoxia

Tumour vasculature and hypoxia were quantified using Media Cybernetics Image Pro PLUS Software. The total number of blood vessels was measured by setting a threshold for CD31 positive pixel intensity and minimum blood vessel area (62 μm²) and counting the number of objects within these settings. Objects above this threshold range but below the minimum area were removed as artifacts (28). The tumour area was recorded and areas of necrosis and artifact were excluded. The mean number of total blood vessels per tumour area was determined. The number of functional blood vessels per tumour area was calculated in a similar manner using DiOC7 positive pixels. Hypoxic regions were quantified by calculating the area of pixels with anti-EF5 fluorescence above a set threshold and dividing this region by the area of the entire tumour section.

4.3.8 Growth delay studies

Tumours were impanted on both flanks of mice as described above. Two perpendicular diameters were measured with a caliper and treatment began once tumours reached a diameter of 5-8 mm. Tumour volume was estimated using the formula: 0.5(ab²), where a is the longest diameter, and b is the shortest diameter.

To determine the effects of pantoprazole, mice were divided into four groups of 4-5 mice each and treated with either saline, doxorubicin alone (8mg/kg) administered intravenously, pantoprazole alone (200mg/kg) administered intraperitoneally or pantoprazole 2 hours prior to doxorubicin (200mg/kg + 8 mg/kg). Every 2-3 days the tumour volume and body weight were measured. Measurements were taken until tumours reached a maximum diameter of 1.2cm or began to ulcerate, when mice were killed humanely. All mice were ear tagged and randomized to avoid bias with measurements.
4.3.9 Statistical analysis

A one-way ANOVA, followed by a post hoc t-test were performed to determine statistical differences between treatment groups, and P<0.05 was used to indicate statistical significance. For drug uptake using flow cytometry and quantification of functional tumour vasculature and hypoxia, t-tests were performed to determine significant differences between treatment groups. P<0.05 was used to indicate statistical significance.

4.4 RESULTS

4.4.1 Proton pump inhibitors increase endosomal pH in tumour cells

Pantoprazole, lansoprazole or tamoxifen led to concentration-dependent increases in endosomal pH in EMT-6 cells; increases in endosomal pH were observed with concentrations of tamoxifen above 10μM, and with lansoprazole and pantoprazole at concentrations above 200μM (Figure 4.1a). Although basal pH levels differed slightly between the four cell lines, the effects of pantoprazole were consistent in increasing endosomal pH at concentrations above 200μM (Figure 4.1b).

4.4.2 Pantoprazole pre-treatment alters doxorubicin uptake in tumour cells in culture

Uptake of radiolabeled doxorubicin increased approximately 2-3 fold when cells were pretreated with either pantoprazole, lansoprazole or tamoxifen compared to cells treated with doxorubicin alone (Figure 4.2a). Photomicrographs of fluorescence in EMT-6 cells exposed to doxorubicin alone, show the drug to be present in the nucleus and in punctuate compartments within the cytoplasm (Figure 4.2a, panel i); and co-staining of cells with doxorubicin and
lysosensor gives evidence of endosomal sequestration of doxorubicin (Figure 4.2b, panel ii).

Treatment with 1mM pantoprazole reduces the amount of doxorubicin fluorescence in the cytoplasm while it is retained within the nucleus (Figure 4.2b, panel iii).

Doxorubicin uptake within cells pre-treated with either saline or pantoprazole (1mM) was measured using flow cytometry. Uptake of doxorubicin under control conditions was higher in MCF-7 cells compared to EMT-6, AR1 or NCI cells (p<0.001). In PgP-overexpressing cells, doxorubicin fluorescence increased by approximately 2-fold in pantoprazole-pre-treated samples (p<0.05), consistent with an effect of pantoprazole to inhibit the function of PgP. In MCF-7 cells doxorubicin fluorescence decreased 1.2-fold with pantoprazole pre-treatment (Figure 4.3a; p=0.06). When cells were treated with a lower concentration of pantoprazole (100µM), similar patterns in the changes in uptake of doxorubicin were observed, where EMT-6, AR1 and MCF-7 cells showed a 1.3-1.7-fold increase in doxorubicin fluorescence after pantoprazole pre-treatment and a 1.2-fold decrease in MCF-7 cells, but these results were not significant (Figure 4.3b). The effect of the PgP inhibitor verapamil on doxorubicin uptake was determined to compare and contrast PgP activity between cell lines. Verapamil (100µM) increased doxorubicin uptake in EMT-6, AR1 and NCI cells by 2-3 fold (p<0.05), but in MCF-7 cells, verapamil pre-treatment decreased doxorubicin uptake by one-third (p<0.05) (Figure 4.3b).
4.4.3 Doxorubicin penetration increases in multilayered cell cultures pre-treated with pantoprazole

The penetration of doxorubicin alone through MCCs derived from EMT-6 and AR1 cells was similar. In contrast, the penetration of doxorubicin through MCCs derived from MCF-7 cells was slower than those MCCs derived from the PgP-overexpressing variant NCI cells (p<0.05). When MCCs were pre-treated with 1mM pantoprazole there was a greater than 2-fold increase in doxorubicin penetration for those grown from EMT-6 and AR1 cells (p<0.05) and ~1.3-fold increase through those grown from NCI and MCF-7 cells (p<0.02) (Figure 4.4a-d). Internal standards indicated by 3H-sucrose penetration were used to control for variations in thickness of the cell cultures, and showed < 10% variation.

Photomicrographs of MCCs indicate an increase in doxorubicin fluorescence in cells more distal from the source of drug that are pre-treated with pantoprazole compared to control MCCs at 2 hours after the start of doxorubicin exposure (Figure 4.4e-f).

4.4.4 Plasma concentration of pantoprazole and toxicity in mice

After intraperitoneal administration of 200mg/kg pantoprazole in Balb/C mice, the peak plasma concentration was ~300μM within the first hour after administration, and ~150 μM after 2 hours. After 5 hours the concentration in the plasma decreased to less than 1% of the maximum concentration in the blood and by 24 hours less than 0.01 μM of pantoprazole was detectable (Figure 4.5).

There was no significant change in body weight of mice after a single treatment with pantoprazole (200mg/kg) or of doxorubicin (8mg/kg), but a combination of the two drugs consistently showed a temporary decrease in body weight (~15%) within the first 5-8 days after treatment. After 8 days, mice quickly regained their original body weight (Figure 4.6).
4.4.5 Effect of pantoprazole on doxorubicin penetration in MCF-7 xenografts

The distribution of doxorubicin was quantified in EMT-6, AR1, MCF-7 and NCI tumours in mice at 10 minutes after injection. Fluorescence intensity of doxorubicin was determined in relation to distance to the nearest blood vessel within areas of interest (AOIs). Blood vessel densities between AOIs in different treatment groups were not significantly different from each other. In all tumours there were steep gradients of decreasing fluorescence due to doxorubicin in relation to distance from the nearest functional blood vessel in the section. In EMT-6 tumours and PgP-expressing AR1 and NCI tumours, there was no significant difference in drug distribution between treatment groups (Figure 4.7a,d; 4.8d). In MCF-7 tumours, there was a shallower gradient of decrease in doxorubicin distribution in the tumours pre-treated with pantoprazole compared to the tumours treated with doxorubicin alone (p<0.05) (Figure 4.8a). Photomicrographs taken from MCF-7 tumours confirm substantial increases in doxorubicin fluorescence in tumours in mice pre-treated with pantoprazole, compared to mice treated with doxorubicin alone (Figure 4.8b-c).

4.4.6 Effect of pantoprazole on vasculature and hypoxia in solid tumours

As expected, there was no significant difference in the total number of blood vessels per unit area of sections in tumours treated with doxorubicin alone, or with doxorubicin plus pantoprazole, in any of the five tumours that were studied (Figures 4.9-4.11). However, the concentration of functional blood vessels per unit area was higher in EMT-6 tumours treated with pantoprazole + doxorubicin (as compared with those treated with doxorubicin alone) by approximately two-fold (p<0.01) (Figure 4.9b), whereas in MCF-7 xenografts the number of functional blood vessels per tumour area decreased by one-third (p<0.05) (Figure 4.10b). The
concentration of functional blood vessels in NCI and A431 tumours treated with pantoprazole did not change significantly (Figures 4.10b; 4.11b). Photomicrographs show DiOC7 fluorescence in perfused blood vessels. Due to non-specific staining, perfused vessels appear much thicker than blood vessels stained with CD31.

The area of hypoxic regions was calculated as a percent of the total tumour area. In EMT-6 and AR1 tumours, the percent of hypoxia decreased by a quarter (p<0.02) and a third (p<0.01), respectively, in mice that had received pantoprazole as well as doxorubicin (Figure 4.9c). In contrast, there was a minimal hypoxia in MCF-7 tumours and there was not much change in pantoprazole-treated tumours. A431 xenografts showed an increase in hypoxia in the pantoprazole-treated group compared to the control group (Figure 4.10c; 4.11c).

4.4.7 Influence of pantoprazole pre-treatment on growth delay

In the EMT-6 and AR1 groups, tumours treated with saline or pantoprazole alone showed rapid tumour growth. Tumours treated with doxorubicin with and without pretreatment with pantoprazole showed growth delay, but there was no significant difference between these treatment groups (Figure 4.12a-b).

Doxorubicin alone led to substantial growth delay of MCF-7 tumours (p<0.05), and there was no significant effect of pantoprazole alone (Figure 4.12c). Mice treated with the combination had increased growth delay compared with the other treatment groups (p<0.05), with minimal tumour growth out to 42 days, but due to ulceration, mice then had to be killed. In mice bearing NCI tumours, both pantoprazole alone and doxorubicin alone had modest effects to delay growth compared to the control, but there was no significant increase in growth delay with the combination (Figure 4.12d). NCI xenografts were relatively slow-growing compared to
MCF-7 xenografts with higher chances of ulceration, requiring the experiments to be terminated with smaller tumour volumes.

Growth delay studies were also carried out in xenografts derived from an epidermoid carcinoma cell line A431 (Figure 4.12e). Tumours treated with pantoprazole and doxorubicin were found to have increased growth delay compared to groups treated with the control (p=0.06) or doxorubicin alone (p=0.05).

4.5 DISCUSSION

The acidic tumour microenvironment in solid tumours has been shown to have a role in resistance to chemotherapy, proliferation and metastasis (29-31). The production of metabolic acids (lactate and/or CO₂) exported from cancer cells into the interstitial fluid is an important contributor to low pH within tumours. There is poor clearance of H⁺ ions due to disorganized vasculature of tumours, poor lymphatic drainage and elevated interstitial pressure (32), resulting in an extracellular pH that is more acidic than that of normal tissues (33, 34). The intracellular pH of cancer cells is maintained through up-regulation of membrane-based ion exchange mechanisms including the sodium-proton exchanger and the sodium-dependent chloride-bicarbonate exchanger (35, 36), leading to an increased pH gradient across the cell membrane. The pH gradient between the cytoplasm and the intracellular organelles may also be modified in cancer cells and this may lead to the sequestration of basic drugs (12, 37). Proton pump inhibitors, which block the action of the V-H⁺-ATPase have been found to increase the pH in acidic vesicles such as endosomes (38), inhibiting the accumulation of basic cytotoxic agents in acidic organelles (39) and may even lead to apoptosis of cancer cells (40).
In the present study, we investigated the effect of tamoxifen and the PPIs, lansoprazole, and pantoprazole on endosomal pH of cancer cells. Each of these agents was observed to increase endosomal pH by blocking acidification in these intracellular compartments. We observed the affects of pantoprazole on different cancer lines with varying PgP expression and found increased endosomal pH in both sensitive and resistant cell lines at pantoprazole concentrations of 500μM or higher. Cultured PgP-expressing cell lines have been observed to display abnormally high cytoplasmic pH values indicating complex cellular mechanisms that contribute to the resistant phenotype (41); these mechanisms may include effects of multidrug transporters to cause alkalinization of the cytosol, causing larger pH gradients between acidic vesicles and cytosol in resistant cells (41-43).

We observed increased uptake of doxorubicin in PgP overexpressing cells after pantoprazole pretreatment. Although EMT-6 cells were studied as a lower PgP-expressing variant compared to AR1 cells, the overall levels of PgP expressed in EMT6 cells were considerably higher than in MCF-7 cells. This is evident from the similarity in patterns of doxorubicin uptake in EMT-6 and AR1 cells treated with PgP inhibitors in contrast to that in MCF-7 cells.

Previous studies comparing MCF-7 cells and their doxorubicin-resistant variants show increased acidification of intracellular vesicles in the cytoplasm in resistant cell lines (42, 44). Chemotherapeutic agents are basic and in their uncharged, hydrophobic form, can easily diffuse across the lipid bilayers. Once they enter acidic compartments they become protonated and sequestered (42, 44). Drug sequestration as a mechanism of multidrug resistance has been well characterized and drugs have been observed to accumulate in endosomes, lysosomes (42, 45, 46) and vesicles within the Golgi compartment (42, 47, 48), all of which are acidic organelles.
Sequestration of drugs may be increased within the larger volume of the endosome/lysosome system that is often observed in resistant cells (45, 49, 50). Increased vesicular acidification in resistant cells may occur due to the overexpression of endosome/lysosome proton pumps (51). However, studies have shown that vesicular sequestration of drugs is not a necessary characteristic of multidrug resistant cells and does occur in wild-type drug sensitive cells (52).

Proton pump inhibitors have been shown to increase the sensitivity of resistant tumour cells to the cytotoxic effects of several drugs, including cisplatin, 5-flourouracil and vinblastine, and increase the cytotoxicity of these agents in drug-sensitive cells (32). Our data indicate that there are differences in the effects of PPIs between drug-sensitive and drug-resistant cells. Pantoprazole is likely to increase drug uptake in drug-resistant cells for the reasons outlined above. In the MCF-7 drug-sensitive cells we observed consistently decreased doxorubicin uptake with pantoprazole pretreatment. In these cells, the nucleus may be saturated with drug and excess concentrations are likely stored in acidic vesicles, while in PPI treated cells this sequestration will be reduced leading to a decreased net uptake of drug. With decreased uptake of drug, there is more drug available to penetrate to cells distal from blood vessels. Based on our results, MCF-7 cells were expected to have greater drug penetration than EMT-6, ARI and NCI cells because of the decreased drug uptake. Our in vitro data show an increase in drug penetration in MCCs of all four cell lines. This may be due to both high concentrations of pantoprazole (1mM) and easy access of drug to cells, which is not representative of conditions in solid tumours where there is limited drug delivery by the imperfect vasculature. This easily accessible drug may lead to saturation in cells close to the source of drug in MCCs, allowing unsequestered drug to penetrate to distal cells.
In MCF-7 xenografts we observed a significant increase in drug distribution in relation to the nearest blood vessel, but this effect was not observed in EMT-6 tumours that may express higher levels of PgP and in drug-resistant NCI and ARI tumours. It has been proposed that pantoprazole may act as a PgP inhibitor in drug-resistant cell lines (18). Previous studies in our laboratory have shown that PgP inhibitors may decrease doxorubicin distribution in solid tumours derived from PgP-expressing cells, most likely because they increase drug uptake in cells close to blood vessels, such that there is less drug available to penetrate to distal cells (21, 53). An effect to inhibit sequestration in endosomes would have the opposite effect, such that if pantoprazole decreases net drug uptake in cells close to blood vessels, there is likely to be increased doxorubicin distribution to more distant cells distal and the gradient of decreasing doxorubicin intensity will be significantly shallower. Improved drug distribution in MCF-7 xenografts might be due partly to changes in functional vasculature, however, the effect of pantoprazole to decrease the proportion of functional blood vessels in MCF-7 and A431 xenografts at short intervals after drug administration suggests that this inhibitor may actually have some antiangiogenic activity. The mitogen-activated protein kinase, extracellular signal-regulated kinase (MAPK ERK 1/2) has been shown to contribute to the expression of pro-angiogenic factors such as vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8) in cancer cell lines (54). The potential involvement of PPIs in anti-angiogenesis has been reported previously where pantoprazole effectively inhibited phosphorylation of MAPK ERK1/2 leading to reduced expression of VEGF (55). A decrease in functional blood vessels was coupled with an increase in hypoxia in pantoprazole treated MCF-7 and A431 xenografts, whereas in the murine tumours, functional blood vessels increased with PPI treatment and the extent of hypoxic regions decreased. Future studies on the effect of pantoprazole on functional blood vessels and
pro-angiogenic factors such as VEGF and IL-8 in the tumour models used in this study may shed light on its role as a potential antiangiogenic agent.

Causes of drug resistance in tumours are both multifactorial and interconnected, so that overcoming one mechanism may not improve drug resistance. An effective strategy is likely to influence several processes that contribute to resistance to give an overall therapeutic benefit. The effect of pantoprazole in MCF-7 cells and xenografts may improve therapeutic efficacy by influencing the net effect of different mechanisms of resistance.

Our HPLC data indicate maximum levels of pantoprazole in the plasma of mice at concentrations that are 5-fold lower than concentrations of pantoprazole administered in some of the \textit{in vitro} experiments. High concentrations of pantoprazole may account for increases in doxorubicin penetration in MCCs, but in drug-sensitive xenografts, lower \textit{in vivo} concentrations of pantoprazole in combination with doxorubicin can still significantly alter drug distribution. After 2 hours, plasma concentrations of pantoprazole are around 200\textmu M. In drug uptake experiments using flow cytometry, we observed that pantoprazole alters doxorubicin uptake into cells at 1mM and at 100 \textmu M, albeit to a lesser extent. The finding that lower doses of pantoprazole are effective in vivo might be due to the role of the microenvironment and its effects on ion gradients between intracellular and extracellular pH. Within the tumour microenvironment strong ion gradients are likely to occur between acidic vesicles and the cytoplasm, and changes in pH due to lower doses of pantoprazole might be more likely to produce significant alterations in sequestration of drug.

It has been suggested that modification of pH gradients across the cell membrane, and between the cytoplasm and intracellular vesicles, in multi-drug resistant cell lines might restore sensitivity in these cells and may be an important treatment strategy to consider in drug
development (18, 41, 44, 56-58). However, drug resistance is multifactorial and restoring sensitivity using this strategy may not effectively overcome drug resistance due to other factors such as limited distribution of drugs in solid tumours. Our studies of tumour growth delay do not indicate a therapeutic advantage of using pantoprazole in combination with doxorubicin in PgP-overexpressing tumours, whereas in wild-type MCF-7 xenografts there is a substantial increase in growth delay. These experiments were repeated in another low-PgP expressing xenograft model using A431 tumours. Pantoprazole combined with doxorubicin showed a similar pattern to MCF-7 tumours of increased tumour growth delay, although the study was limited by the formation of ulcerations characteristic of A431 xenografts.

Our data, and that of others (38), suggest that pretreatment with pantoprazole may be an effective strategy to improve the therapeutic efficacy of doxorubicin in some solid tumours. Our studies show that while drug export pumps such as PgP lead to drug resistance, other mechanisms such as increased drug sequestration and limited drug distribution may also have important roles in multifactorial drug resistance and must be considered when developing novel therapeutics and novel treatment strategies. The strategy of administering pantoprazole prior to doxorubicin is now being evaluated in a phase I trial supported by the Komen Breast Cancer Foundation.

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I would like to acknowledge Carol Lee for her collaboration in the endosomal pH and radiolabeled drug uptake experiments. As well, I would like to acknowledge Dr. Eric Chen for providing support with the HPLC analysis.
4.6 FIGURES
Figure 4.1  Endosomal pH measurements in cancer cells. EMT-6 cells exposed to varying concentrations of lansoprazole (■), pantoprazole (◆), and tamoxifen (▲) (A) and EMT6, AR1, MCF-7, and NCI cells exposed to varying concentrations of pantoprazole (B). (Data represents mean ± SE; n=3)
Figure 4.2 Doxorubicin uptake in tumour cells in culture. (A) EMT-6 cells were incubated with either doxorubicin (DOX) alone or in combination with pantoprazole (PTP), lansoprazole (LPZ), or tamoxifen (TAM), and fluorescence was measured after 5 hours using an uptake assay of radiolabeled DOX. (Data represents mean ± SE; n=3). (B) Fluorescent micrographs show alterations in intracellular DOX distribution in cancer cells in culture with PTP pretreatment. EMT-6 cells were treated with (i) DOX, (ii) lysosensor or (iii) PTP prior to DOX.
Figure 4.3 Doxorubicin fluorescence in cells pretreated with either saline or 1 mM pantoprazole measured using flow cytometry. EMT-6, AR1, MCF-7 and NCI cells were pretreated with either A) DOX alone or 1mM PTP prior to DOX or B) DOX alone, 100µM PTP or 100µM verapamil prior to DOX treatment. (Data represents mean ± SEM; n=3).
Figure 4.4 The penetration of doxorubicin in multilayered cell cultures (MCCs). MCCs derived from EMT-6 (A) and MCF-7 (C) cells, and PgP-overexpressing AR1 (A) and NCI (D) cells as a function of time. MCCs were pretreated with either saline or pantoprazole (1mM) followed by 14C-DOX (data represent mean ± SEM; n=4). Photomicrographs of fluorescent DOX in MCCs derived from EMT-6 cells. MCCs were pretreated with either saline (E) or PTP prior to DOX treatment (F). (Scale bars represent 100µm)
Figure 4.5  Plasma concentration of pantoprazole in nude mice at various time points after administration of 200mg/kg to Balb/C mice. Plasma levels of pantoprazole were determined by a validated HPLC method. (Data represents mean ± SEM; n=4)
Figure 4.6 Toxicity measurements of doxorubicin and pantoprazole treatments in nude mice. Mice were treated with either saline, DOX alone, PTP alone or a combination treatment and their body weight was measured every 2-3 days (Data represents mean ± SEM; n=4)
Figure 4.7 The distribution of doxorubicin fluorescence intensity in relation to the nearest blood vessel in murine tumours. EMT-6 (A) and AR1 tumours (D) in Balb/C mice pretreated with either saline or pantoprazole prior to DOX. (Data represents mean ± SEM; n=6). Photomicrographs of EMT-6 tumours treated with either DOX (B) or PTP and DOX (C) and AR1 tumours treated with either DOX (E) or PTP and DOX (F). Blood vessels (red), hypoxic regions (green) and DOX (blue). (Scale bar represents 100μm)
Figure 4.8 The distribution of doxorubicin fluorescence intensity in relation to the nearest blood vessel in xenografts. MCF-7 (A) and PgP overexpressing NCI xenografts (D) in nude mice (data represent mean ± SEM; n=6). Photomicrographs of MCF-7 tumours treated with either DOX (B) or PTP and DOX (C) and NCI tumours treated with either DOX (E) or PTP and DOX (F). Blood vessels (red), functional blood vessels (yellow), hypoxic regions (green) and DOX (blue). (Scale bar represents 100μm)
Figure 4.9 The distribution of blood vessels and hypoxia following treatment of EMT6 and AR1 tumours. Tumour-bearing mice were pretreated with either saline or pantoprazole prior to doxorubicin. (A) total blood vessels (B) functional blood vessels (C) hypoxia per area tumour) (Data represent mean ± SEM; EMT6: n=11; AR1: n=12).
Figure 4.10 The distribution of blood vessels and hypoxia following treatment of MCF-7 and NCI tumours. Tumour-bearing mice were pretreated with either saline or pantoprazole prior to doxorubicin. (A) total blood vessels (B) functional blood vessels (C) hypoxia per area tumour). (Data represent mean ± SEM; MCF-7: n=11; NCI: n= 9). Photomicrographs of blood vessel (red) and hypoxia (green) distribution is shown in MCF-7 (D, E) xenografts treated with either DOX or PTP and DOX, respectively. (Scale bar represents 100μm)
Figure 4.11 The distribution of blood vessels and hypoxia following treatment of MCF-7 and NCI tumours. Tumour-bearing mice were pretreated with either saline or pantoprazole prior to doxorubicin. (A) total blood vessels (B) functional blood vessels (C) hypoxia per area tumour. (Data represent mean ± SEM; n=7). Photomicrographs of blood vessel (red) and hypoxia (green) distribution is shown in A431 (D, E) xenografts treated with either DOX or PTP and DOX, respectively. (Scale bar represents 100μm)
Figure 4.12 Effect of pantoprazole and doxorubicin treatment on tumour growth in mice. Mice bearing EMT6 (A), AR1 (B), MCF-7 (C), NCI (D), or A431 (E) tumours were treated with either saline, DOX, PTP, or PTP pretreatment prior to DOX. Tumour volume in mice was measured every 2-4 days. (Data represent mean ± SEM; n=8)
4.7 REFERENCES

CHAPTER 5

CONCLUSIONS & FUTURE DIRECTIONS
5.1 SUMMARY OF FINDINGS

Chemotherapy is the most widely used treatment for many types of cancer. Chemotherapy is also used to prevent disease recurrence after treatment by eradicating micro-metastases and improving local control of the primary tumour. However, the effectiveness of chemotherapy is greatly limited by drug resistance. In addition to cellular and molecular causes, mechanisms of drug resistance that relate to the tumour microenvironment are also likely to have a profound impact on the sensitivity of tumours to chemotherapeutic agents. Drug distribution is likely to be an important microenvironmental factor that limits the drug’s ability to reach all viable cancer cells in sufficiently cytotoxic concentrations. By focusing our research efforts on understanding drug distribution and determining whether it is a modifiable phenomenon, we can determine whether improving drug distribution can improve chemotherapeutic efficacy in patients.

This thesis focused on various aspects of drug distribution; in particular: (1) the comparisons of drug distribution between normal and tumour tissue, and the change in drug distribution over time in solid tumours; (2) the effects of drug efflux pumps and their inhibitors on drug distribution in solid tumours, and whether these changes are detectable and quantifiable; and, (3) the effects of inhibiting drug sequestration within cellular organelles, through the use of proton pump inhibitors, on drug distribution in solid tumours.
5.2 DISTRIBUTION OF ANTICANCER DRUGS IN NORMAL AND TUMOUR TISSUES

5.2.1 Summary of major findings

In Chapter 2, I examined the distribution over time of three anti-cancer drugs (doxorubicin, mitoxantrone and topotecan) in human breast xenografts, and compared drug distribution in the tumour with that in the liver, kidney, heart and brain. These anti-cancer drugs are commonly used in the clinic as part of chemotherapy regimens in the treatment of various types of cancer, including lymphomas, leukemias, breast and prostate cancer, and ovarian and small cell lung cancer (1-6). Various HPLC-based pharmacokinetic studies of doxorubicin, mitoxantrone and topotecan have yielded valuable information such as total drug concentrations in normal tissues and tumour tissues (7-9). Additionally, however, it is important to gain an understanding about the spatial distributions of anticancer drugs and how they are affected by time and tissue because it will broaden our understanding of drug distribution as a mechanism of drug resistance in tumours and as an additional indicator of normal tissue toxicity.

The autofluorescent properties of the three drugs examined in this chapter allowed for the determination of drug distribution gradients in relation to distance to the nearest blood vessel. We hypothesized that drug distribution in the heart, liver and kidney would be far more uniform than that in the tumour; however, the brain was likely to show the least amount of drug. My data showed that, even 10 minutes after doxorubicin administration, the perivascular doxorubicin fluorescence in tumour tissue was substantially lower than in the heart, liver and kidney. As well, after 3 hours, the fluorescence of all three drugs in the heart, liver and kidney decreased, and after 24 hours
this trend was more pronounced. Due to the organized nature of the vasculature in normal tissues, distances between neighboring vessels are significantly less in normal tissues than in tumours. In contrast to the heart, liver and kidney, drug distribution in the brain was very low, due to the presence of the blood-brain barrier.

Within tumour tissue, the distance at which drug fluorescence intensity falls to half is between 50-60μm amongst four types of tumour lines. Doxorubicin, mitoxantrone and topotecan all showed a time-dependent decrease in distribution in tumours from 10 minutes to 24 hours. At 24 hours, the gradient of decreasing fluorescence in relation to distance to the nearest blood vessel is lower; however, the overall fluorescence is also significantly less. Doxorubicin treatment appeared to decrease tumour vasculature 24 hours after treatment and cause a transient increase in hypoxia after 3 hours followed by a significant decrease after 24 hours.

5.2.2 Implications of study

The majority of pharmacokinetic studies on anticancer drugs focus on using HPLC as the sole determinant of drug concentration in various tissues. These studies are good indicators of drug concentration; however, we showed that the distribution profiles of anti-cancer drugs in various tissues can also provide valuable information. The delivery of drugs to tissues involves three processes: distribution through vascular space, transport across the microvessel walls, and diffusion and/or convection through tissue (10). In normal tissues, drug delivery is efficient within these three processes, and differences in fluorescence distribution profiles between drugs is likely predominantly due to physiochemical properties of the drugs (e.g., binding affinities of drugs,
diffusivity). In contrast, limited drug delivery in solid tumours imply that drug
distribution profiles are likely dependent predominantly on biological properties of a
tumour (e.g., blood flow, interstitial fluid pressure, angiogenesis, vessel distribution, cell
density) in addition to physicochemical properties of the drugs. Thus, agents given in
conjunction with chemotherapy that alter the biological properties of a tumour, may not
alter drug distribution or have the same degree of cytotoxic effects in normal tissues.
Although this is a generalization, it proposes a promising area of drug development that
enhances tumour specificity and reduces normal tissue toxicity.

5.3 THE INFLUENCE OF PgP EXPRESSION AND ITS INHIBITORS ON
DOXORUBICIN DISTRIBUTION

5.3.1 Summary of major findings

From the data in Chapter 2, in addition to work done by others, it is evident that
limited drug distribution in solid tumours is likely to play a major role in multifactorial
drug resistance (11-15). Since drug distribution involves the movement of drug from the
vascular space into the interstitium, and finally through the tissue, there are a multitude of
factors that are likely to affect this movement. In Chapter 3, I proposed a set of
experiments to determine whether drug distribution was in fact a modifiable phenomenon
and whether the solid tumour model system was sensitive enough to detect these changes.
Previous work in our lab using multilayered cell cultures showed that drug efflux pumps
such as PgP can alter drug penetration in this in vitro system (16). In this chapter, I
applied this model of drug efflux to alter drug distribution in solid tumours. From the
observation that doxorubicin remains highly restricted to areas proximal to blood vessels,
we hypothesized that, in tumours that overexpress PgP, the pump, while preventing
cellular accumulation of drug within cells, may actively work to distribute drug to areas distal to blood vessels and improve drug distribution. If this held true, then the corollary would be that inhibitors of PgP would keep drug within cells and restrict its distribution to cells further away.

In Chapter 3, my data show that PgP overexpression alters the gradient of drug distribution such that drug uptake in cells proximal to blood vessels is significantly less and there is a shallower gradient of decreasing doxorubicin fluorescence with increasing distance from the nearest blood vessel compared to wild type tumours. I showed that the first and second generation inhibitors of PgP, verapamil and PSC 833, led to an increased uptake of doxorubicin by cells close to blood vessels, but also increased the gradient of doxorubicin intensity so that distribution was more heterogenous and similar to that of wild-type tumours. Growth delay studies showed that there was no significant effect of doxorubicin on the growth of both wild-type and PgP overexpressing tumours. The combination of verapamil or PSC 833 with doxorubicin showed no increase in tumour growth delay; however, these combinations exerted toxic side effects.

5.3.2 Implications of study

These data indicate that drug efflux pumps can alter drug distribution within a solid tumour model. PgP overexpression is very common in certain types of cancer such as liver, pancreas, kidney, ovary and breast (17, 18). Attempts to circumvent this type of drug resistance have led to the development of three generations of inhibitors; however, clinical trial data continues to provide disappointing results (19-25). While we have shown that PgP may increase drug distribution in solid tumours, this does not suggest that
PgP is conducive to improved drug effects. In fact, increased drug distribution is at the cost of limited cellular accumulation, and therefore this cannot lead to enhanced chemotherapeutic effects. The data in this chapter have two major implications: (1) in addition to toxic side effects and pharmacokinetic interactions, the inability of PgP inhibitors to successfully enhance cytotoxicity of chemotherapeutic agents maybe also be due to the fact that they limit drug distribution, such that the drug remains predominantly in areas peripheral to blood vessels, thus preventing sufficient concentrations of drugs to be available to viable cells more distally located; and, (2) drug distribution is a modifiable phenomenon, which is detectable and quantifiable using our subcutaneous solid tumour model system.

5.4 THE INFLUENCE OF PANTOPRAZOLE ON DOXORUBICIN DISTRIBUTION AND ACTIVITY IN CANCER

5.4.1 Summary of major findings

In the previous two chapters, I demonstrated that drug distribution was limited in solid tumours, that it was modifiable, and that we could detect and quantify these changes. In Chapter 4, I proposed a strategy to improve drug distribution and ultimately improve therapeutic efficacy of doxorubicin. Tumour acidity was proposed as a mechanism of drug resistance in part because of it leads to the sequestration of basic drugs such as doxorubicin in acidic endosomes within tumour cells. Increased acidification of organelles within tumour cells is facilitated by the expression of vacuolar-H⁺-ATPases. Agents that disrupt the pH gradient between the cytoplasm and the endosomes in tumours might decrease the sequestration of basic anticancer drugs. We hypothesize that this may make cells and tumours more sensitive to drug because more
drug is able to enter the nucleus and cause cell death, and more drug is available to penetrate to cells distal from blood vessels. In the data in Chapter 4, I show that the proton pump inhibitor pantoprazole increases endosomal pH in tumour cells and that pantoprazole pretreatment of cells in culture alters the uptake and intracellular distribution of doxorubicin, so that it is predominantly located in the nucleus, as opposed to diffusely present in cytoplasmic compartments. In wild-type, pantoprazole pretreated, cells, doxorubicin uptake decreased slightly, whereas in pretreated PgP overexpressing cells, doxorubicin uptake increased significantly. From this observation, we proposed that reduced doxorubicin uptake in pantoprazole-pretreated cells may lead to improved drug distribution compared to pretreated PgP overexpressing tumours. The MCC data showed that pantoprazole pretreatment led to the enhanced drug distribution in both wild-type and PgP-overexpressing tumour tissue. In solid tumours, however, a shallower gradient, and thus, improved drug distribution was observed in only PgP wild-type human xenografts. Growth delay studies indicate that only the PgP wild-type human xenografts pretreated with pantoprazole and then doxorubicin showed a significant increase in tumour growth delay, whereas wild-type murine and PgP overexpressing murine and xenografts showed only modest improvements in growth delay.

5.4.2 Implications of study

The data in Chapter 4 suggest that agents that alter the pH gradients in tumour tissue may influence both drug distribution and tumour growth delay. However, the data involving PgP overexpression also suggests that there are more factors at play. For example, pantoprazole may have an important interaction with PgP on the cell membrane that
alters doxorubicin activity, or the presence of PgP may lead to an altered pH profile than in wildtype variants (26, 27). The results from the PgP tumours demonstrate the complexity of multifactorial drug resistance. Though not universally applicable, the results from the wild-type tumours are encouraging and suggest a possible strategy to overcome multiple factors of drug resistance (ie. drug sequestration, intracellular accumulation and extracellular distribution).

5.5 LIMITATIONS AND FUTURE DIRECTIONS

5.5.1 Drug fluorescence intensity and drug concentration

Our studies of drug distribution give fluorescence intensity profiles of drugs in relation to distance from the nearest blood vessel. While changes in fluorescence intensity reflect changes in drug concentration, it is difficult to determine the ranges of fluorescent intensity that correlate with concentrations that lead to cytotoxicity. One limitation of our in vivo studies is that we cannot correlation the drug distribution gradients with concentration gradients. This correlation could provide additional information, such as: the fraction of cells within a tumour section that receive sufficient concentrations of drug to cause cell death; the fraction and location of cells that are likely to develop intrinsic or extrinsic drug resistance because of exposure to low levels of drug, and the fraction of cells that are likely to contribute to repopulation of the tumour because of minimal exposure to drug.

One potential strategy that may be used to establish this correlation could involve submerging small 2 mm tumour tissue pieces in various known concentrations of fluorescent drug in vitro, in order to establish calibration curves for concentration vs.
fluorescence intensity gradients. After a given time, tissue pieces would be frozen, sectioned and imaged. In this way, a range of fluorescence intensities may be correlated with a range of drug concentrations, with values that are comparable to intensity values obtained from tumours extracted from mice that were treated with the drug. This strategy has been developed and used to associate patterns in hypoxia (using an EF5 marker) with oxygen gradients within tumour tissue in an effective method referred to as cube reference binding (CRB) (28, 29). It would be difficult to generate exact correlations between drug fluorescence intensity and concentration using CRB due to factors such as tissue thickness and method of drug exposure to the tissue; however, it may allow us to develop a reference ‘ladder’ of decreasing fluorescence relating to decreasing concentration, which can then be translated into the in vivo tumour model system with the appropriate corrections. A similar concept can be applied to correlate drug fluorescence with occurrence of cytotoxicity (through markers such as cleaved caspase-3 or gamma-histone 2AX (γH2AX).

5.5.2 Quantification of drug intensity

Our analysis of drug distribution in tumour sections is limited to 2-dimensional analysis, as representative of a 3-dimensional tumour. As a result of this, there is a bias toward overestimating the distance of a pixel to its nearest blood vessel, particularly at large distances, due to the presence of out-of-plane vessels. This bias leads to a more uniform distribution profile than what may actually exist and implies that a 3-dimensional analysis would indicate gradients of doxorubicin distribution that are steeper than what have been represented in this thesis. To minimize the effect of this bias, and to reduce
the influence of ‘noise’ from non-specific fluorescence, we fitted data only to distances between 100-120μm from blood vessels for most distributions.

The wide-field image analysis method was used in our studies to generate drug distribution profiles. This method quantifies fluorescent pixel intensity due to drug, and its distance to the nearest blood vessel, taking into account all pixels in a given region of the tumour section. This is a robust and objective technique, because it takes into consideration the distribution of drug relative to all vessels within the image. This method of quantification generates a data matrix displaying the range of pixel intensities for all pixels at a specific distance to the nearest blood vessel; the matrix is then plotted as a 3-dimensional histogram, showing the distribution of pixel intensities for each distance from the nearest blood vessel (Figure 5.1). The mean intensity for each histogram (i.e. mean intensity at a given distance) can be plotted as a function of distance to the nearest blood vessel. These are the distribution profiles that were used in our studies. A limiting factor of this technique is that it is susceptible to artefact from background autofluorescence of the tissue, drug signal due to distribution from out-of-section blood vessels, or drug with no corresponding CD31 staining despite the fact that the lumen of the vessel can be readily seen. The algorithm cannot distinguish signal from artefact from that due to drug. Recognition of these limitations allows for their identification such that images that are relatively void of such artefacts can be selected. The algorithm computes pixel intensity and its distance to the nearest vessel and assumes, sometimes incorrectly, that the nearest vessel is the source of the drug fluorescence. While this is largely correct, the nearest vessel may be non-perfused and the detected fluorescence may be from a more distal vessel. Use of a perfusion marker, such as
DiOC7, may circumvent this limitation; however the diffuse staining of non-binding perfusion markers may overlay with drug fluorescence in the first few layers of cells and alter distribution gradients in proximal regions. By averaging the mean intensities at a given distance from several areas of interest within a tumour section and within tumours of the same treatment group, the effects of fluorescence from artefacts and out-of-section vessels do not strongly influence the overall distribution profiles.

Another limitation of this quantification method is that by taking the mean intensities at a given distance from the nearest blood vessel, a large amount of the 3-dimensional data matrix is not incorporated. Thus, our mean intensity distributions are likely to be underestimated. Strategies to incorporate the entire data matrix by averaging entire 3-dimensional histograms have been explored; however, there are challenges in spatially averaging each pixel intensity and distance between numerous areas of interest. Analysis programs that allow us to average multiple 3-dimensional data matrices while correcting for user-defined specified factors depending on the area of interest within the tumour, would create more accurate drug distribution profiles that may be even more sensitive and allow us to detect even small changes in drug distribution between treatment groups.
Figure 5.1 The 3-dimensional histogram displaying distribution of pixel intensities for a given distance to the nearest blood vessel. For each distance to the nearest blood vessel there exists a frequency of pixels with a range of intensities. These values are generated using a wide-field analysis algorithm for given areas of interest within a tumour section.
5.5.3 The influence of pH on drug fluorescence

In our studies of drug distribution, we examined changes in fluorescence intensity in relation to distance to the nearest blood vessels. While changes in fluorescence intensity implied changes in drug concentration at various distances, there are other factors that can influence drug fluorescence intensity, and therefore pose as potential limitations to these studies. In chapter 4, we showed that basic drugs such as doxorubicin can become sequestered in acidic organelles. The altered pH in the tumour microenvironment and the potential sequestration and protonation of such drugs in acidic organelles could alter the fluorescence of these drugs. Changes in fluorescence intensity due to factors such as pH can thus alter distribution profiles. It is important to know how such factors can affect drug distribution, so that when changes in fluorescence are detected, they can be attributed appropriately, either to physical and environmental properties of drug and tumour, or to changes in distribution. One way to detect whether factors such as pH affect drug fluorescence is to determine the emission spectra of the drugs across a range of pHs. Preliminary studies measuring the emission fluorescence intensities of doxorubicin, mitoxantrone and topotecan within their specified excitation wavelength filter sets were done for the drugs in buffer solutions ranging in pH from 4-8. This study showed that doxorubicin and mitoxantrone fluorescence are only slightly affected by pH, however, topotecan fluorescence changes quite dramatically across the pH range tested (Figure 5.2).
Figure 5.2 Fluorescence emission of drugs at various pHs. The fluorescence intensities integrated over excitation wavelengths specific to each drug were determined for doxorubicin (A), mitoxantrone (B) and topotecan (C) in buffer solutions with pHs ranging from 4-8.
These studies demonstrated that changes in drug fluorescence can be due to physical and microenvironmental factors such as pH. Other factors such as: the type of treatment strategy employed, bound drug versus free drug, and location of drug within the cytoplasm, drug target or interstitium may also alter drug fluorescence intensity. Future studies incorporating spectroscopic analyses of drug fluorescence are important to ensure drug distribution profiles accurately reflect drug intensity within the appropriate excitation and emission wavelengths.

5.5.4 Distribution of non-fluorescent drugs

We have shown that it is possible to detect and quantify the distribution of fluorescent anticancer drugs within tissue sections from solid tumours. Future studies will extend this technique to investigate distribution profiles for non-fluorescent drugs and the degree to which they may be amenable to modification. Studies to evaluate cisplatin distribution or the distribution of alkylating agents have been undertaken by examining fluorescent antibodies targeting DNA-drug adducts as a surrogate marker of drug distribution (30). Related techniques can identify additional surrogate markers of drug distribution such as apoptotic and/or cell cycle markers. For example, it was shown that cells located distal to the vasculature in human tumour xenografts had less disturbance of the cell cycle than proximal cells following treatment with gemcitabine, most likely due to limited distribution of the drug; the distal cells commenced cycling sooner than proximal cells and were largely responsible for repopulating the tumour (31).

As demonstration of the utility of this surrogate marker approach, ongoing studies in our laboratory have shown a positive correlation between doxorubicin fluorescence
intensity in relation to distance to the nearest blood vessel and the distribution γH2AX, a marker of DNA double-strand breaks, at 10 minutes after drug administration. Correlations between doxorubicin fluorescence gradients and markers for apoptosis (ex. cleaved caspase-3) are also apparent, however only at longer time points beyond 3 hours. Melphalan distribution, determined through the use of DNA-adducts and fluorescence gradients, also correlated well with γH2AX distribution gradients.

The use of surrogate markers to evaluate distribution in non-fluorescent anti-cancer drugs can be a powerful tool to determine whether limited drug distribution is contributing to drug resistance and can further lead to strategies to overcome resistance by tackling this microenvironmental mechanism.

5.5.5 Tumour model as a preclinical model

The present study examined drug distribution through the use of frozen tumour tissue taken from tumours grown subcutaneously in mice. Some studies suggest that the vasculature within spontaneous or orthotopic tumours is different from vessels in transplanted subcutaneous tumours (32, 33). Therefore, due to the pivotal role tumour blood vessels play in drug delivery and ultimately drug distribution, it will be important to study drug distribution within the tumour microenvironments of other tumour models. Future studies to determine drug distribution profiles in spontaneous or orthotopic tumours, or in primary patient samples (if available), would broaden our understanding of factors that affect drug distribution and how amenable they may be to modification.
As well, conditions in the tumour microenvironment change over time and continue to change following drug treatment and this fluidity of the tumour microenvironment is difficult to relay using the current methodology. In addition to using a 3-dimensional model, using a real time in vivo model would be beneficial. With advancements in imaging techniques it might now be feasible to measure changes vasculature, hypoxia and drug distribution in solid tumours following chemotherapy in live animals.

An example of one technique is the window-chamber model system. This system allows for accessible imaging of changes in tumour cells and vasculature as well as the movement of drug within a solid tumour; furthermore, since this technique is minimally invasive and animals recover well from anesthesia used during imaging, animals could be imaged at multiple time points following drug treatment in a continuous long-term study (34, 35). Other imaging modalities, such as Doppler Optical Coherence Tomography (DOCT), have been used to detect changes in blood vessel function in solid tissues grown in the window-chamber model by measuring the flow velocity with a resolution of <10\(\mu\)m (36). A potential disadvantage of this system is that a window-chamber system provides an artificial environment for tumour growth, and tissue growth is limited (35).

Bioluminescence imaging is a potential tool for measuring changes in the tumour in its innate environment. However, to date, the resolution of bioluminescence imaging is not sensitive enough to detect changes at the cellular level (i.e. micrometer range). Therefore, bioluminescence imaging might be used to determine how drugs are distributed overall within tumour tissue; however, it is presently not feasible to evaluate
the spatial distribution of changes in fluorescence in relation to blood vessels using this imaging modality.

5.5.6 Alternative strategies that may influence drug distribution

One avenue of improving the distribution of chemotherapeutic agents involves the manner in which drugs are administered; specifically dose, schedule and duration of therapy. In Chapter 4, we showed an improvement in drug distribution with a single dose administration of pantoprazole prior to a single dose of doxorubicin. As well, this treatment showed increased growth delay in a PgP wild-type xenograft model. One strategy that may enhance tumour growth delay to a greater degree would be to treat tumour-bearing mice with a multiple-dose schedule over a few weeks. This could potentially reduce repopulation of viable cancer cells that do not receive a sufficient concentration of drug to cause cytotoxicity from the initial single dose. As well, with the death of cells proximal to blood vessels from the first treatment, there may more access to drugs from the second treatment by cells distal to blood vessels. This type of treatment regimen would better reflect the dosing schedules utilized in the clinical setting.

Preliminary studies undertaken in our lab using a multiple dose treatment schedule of pantoprazole in combination with doxorubicin indicate improved growth delay in PgP wild-type xenografts. Further studies exploring different treatment schedules, doses and tumour models will help elucidate the whether this is a promising strategy that can translate into the clinic.

Other strategies to this effect are continuous infusion and metronomic chemotherapy. Continuous infusion chemotherapy may be described as a prolonged
infusion of drug for 5 days or more (37). The rationale behind continuous infusion chemotherapy is that if exposure to the cytotoxic agent can be prolonged over the duration of the cell cycle, drugs that are cell-cycle specific should have improved efficacy; by continuously exposed the tumour to drugs the tumour cells are more likely to be killed. In contrast, administering drugs by bolus injection may lead to the majority of tumour cells not being killed because they are not in their vulnerable phase (i.e. S or M phase of the cell cycle). Also, with respect to improved drug distribution, continuous infusion could lead to a more constant and prolonged pressure head that may enhance drug distribution in tumour tissue versus the rapid rise and fall off in serum drug concentrations that occurs with a single bolus injection.

The most frequently used drug for continuous infusion is 5-fluorouracil (5-FU) (37-39). In a phase III study comparing 5-FU continuous infusion with 5-FU bolus injection in colorectal cancer, the continuous infusion group showed a 44% response rate versus 22% in the bolus injection group with a complete lack of haematological toxicity in the continuous infusion group (39). However, while there seems to be a definite trend toward increased response rate in these studies, no clear survival advantage has been established (38-41).

Another drug administration strategy that may affect drug distribution is metronomic therapy. This is a method of drug delivery in which low, well below the maximum tolerated dose, of chemotherapeutic agents are given at frequent or even daily intervals (42). The initial rationale for this type of therapy stems from evidence that administering comparatively low doses of chemotherapy on a frequent or continuous schedule, with no extended interruptions, has anti-angiogenic effects (42). In addition to
reduced acute toxicity, the efficacy of metronomic therapy seems to increase when administered in combination with specific anti-angiogenic drugs, likely due to the combined normalizing effect of low dose, steady chemotherapy treatment and anti-angiogenic therapy.

The data presented in this thesis suggest that some chemotherapeutic agents such as doxorubicin have poor drug distribution. This is due, in part, to increased consumption by perivascular cells, which limits the availability of drug to cells further from blood vessels. While metronomic therapy may not improve drug distribution, it could lead to more efficient cell killing per dose of chemotherapy; this may progressively kill cell layers proximal to blood vessels on a continuous basis and circumvent the issue of poor drug distribution that can occur with single maximum tolerated doses of drugs.

5.6 CONCLUSIONS

In summary, work completed in this thesis has added knowledge to the field of cancer biology by demonstrating that: drug distribution profiles in tumours are highly limited compared to normal tissue; drug distribution can be modified in solid tumours; and, improving drug distribution may be an effective strategy to overcome drug resistance as well as enhance the therapeutic index of chemotherapy agents. I have established that the spatial distribution of three anticancer drugs, doxorubicin, mitoxantrone and topotecan is time-dependent and tissue-dependent, when comparing tumour, heart, kidney, liver and brain tissue. Using P-glycoprotein expressing tumour models and PgP inhibitors, I showed that there is a significant trade-off between high uptake of drugs in cells proximal to blood vessels and distribution of drugs in cells distal to blood vessels. As well, with
this model, I demonstrated that drug distribution is modifiable in an *in vivo* system and that these changes are detectable. Lastly, I studied a strategy to improve doxorubicin distribution with the use of the proton pump inhibitor, pantoprazole. Pretreatment with pantoprazole was shown to alter both intracellular and extracellular distribution of doxorubicin within human tumour cells and xenografts. In addition to increased drug distribution, there was also improved tumour growth delay suggesting that this may be an effective treatment strategy in the treatment of solid tumours.

The work in this thesis highlights the complexity of the solid tumour microenvironment, and illustrates the importance of examining mechanisms of drug resistance that relate to it. The distribution of anticancer drugs can have a profound impact on the effectiveness of the treatments. By broadening our understanding of factors that can influence drug distribution and exploring various ways to improve it, there is potential for developing optimal treatment strategies that may have promising results in the clinic.
5.7 REFERENCES

Wherever there is God's grace and wherever there are human efforts
That is where victory will be
~ Shrimad Bhagwad Gita 18:78 ~